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Declaration and Permission

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Abstract

**Introduction:** Neonatal encephalopathy (NE) is a clinically defined syndrome of disturbed neurologic function, in the earliest days of life. NE has a multifactorial aetiology and causes significant mortality and neurological morbidity. Therapeutic hypothermia (TH) is the only available treatment, but morbidity and mortality rates remain high. There is an urgent need for adjunctive therapies to improve neurodevelopmental outcomes. Persistent systemic inflammation has been implicated in the pathogenesis of NE. Identification of novel inflammatory biomarker combinations helped to determine the aetiology of NE and in the development of new adjunctive therapies.

**Methods:** Infants with NE and age matched controls were included to explore their inflammatory phenotype and mitochondrial function to assess immune dysregulation at baseline and following LPS stimulation. This detailed inflammatory phenotype was then correlated with their clinical outcome. Alongside the translational work, a systematic review of biomarkers in NE to predict outcome and a number of clinical management improvement initiatives were prepared.

**Results:** Infants with NE had a dysregulated inflammatory response and their whole blood hypo-responsive to endotoxin stimulation in contrast to the robust innate immune response from term control infants. The inflammatory phenotype correlated with NE clinical outcomes. Management guidelines and two animations on TH were developed in collaboration with parents and best evidenced based review.

**Conclusion:** Systemic inflammation is important in the pathogenesis of NE. Improving the understanding of this inflammatory signature has aided in revealing the pathophysiology, identified relevant biomarkers of severity and shows promise towards new adjunctive therapies.
Abbreviations

- Adenosine triphosphate (ATP)
- Acute kidney injury (AKI)
- American Academy of Pediatrics (AAP)
- Activated partial thromboplastin time (APTT)
- AIM2 (Absent in melanoma 2)
- Amiel-Tison Neurologic Assessment at Term (ATNAT)
- American Academy of Pediatrics (AAP)
- Amiel-Tison Neurologic Assessment at Term (ATNAT)
- Aspartate aminotransferase (AST)
- Alanine aminotransferase (ALT)
- Antibiotics (Abx)
- Apoptosis-associated speck-like protein containing a CARD (ASC)
- Arterial Blood gas (ABG)
- Brain and Muscle ARNT-Like 1 (BMAL1)
- Blood brain barrier (BBB)
- Basal ganglia (BG)
- Brain natriuretic peptide (BNP)
- Cardiotocography (CTG)
- Central nervous system (CNS)
- Cerebrospinal fluid (CSF)
- Cerebral function analysing monitor (CFAM)
- Circadian loco- motor output cycles kaput (CLOCK)
- Coombe Women and Infant's University Hospital (CWIUH)
- Cork University Maternity Hospital (CUMH)
- Cranial ultrasound scans (Cruss)
- Creatinine Kinase (CK)
- Cryptochrome (CRY)
- Complementary DNA (cdna)
- Cycle time (Ct)
- Cardiovascular (CVS)
- Creatinine-kinase muscle/brain (CK MB)
• Damage-associated molecular pattern molecules (damps)
• Diffusion weighted imaging (DWI)
• Direct Coombs Test (DCT)
• Echocardiography (ECHO)
• Enzyme-linked immunosorbent assay (ELISA)
• Erythropoietin (Epo)
• Fluorescence Minus One (FMO)
• Forward scatter (FSC)
• Full blood count (FBC)
• Glial fibrillary acidic protein (GFAP)
• Granulocyte – macrophage colony stimulating factor (GMCSF)
• Glucose infusion rate (GIR)
• Group B Streptococcal (GBS)
• Hypoxia inducible factor (HIF)-1α
• Herpes simplex virus (HSV)
• Interleukin-1 (IL-1)
• Interleukin-1β (IL-1β)
• Interleukin-6 (IL-6)
• Interferon gamma (IFN-γ)
• Ischaemic reperfusion (IR)
• Intravenous (IV)
• Intramuscular (IM)
• Interleukin–1 Receptor Associated Kinase 4 (IRA4)
• Inhaled Nitric Oxide (iNO)
• Kidney Disease Improving Global Outcomes (KDIGO)
• Knock out (KO)
• Lumbar puncture (LP)
• Lipopolysaccharide (LPS)
• Liver function test (LFT)
• Lactate dehydrogenase (LDH)
• Magnetic Resonance Imaging (MRI)
• Magnetic resonance spectroscopy (MRS)
• Magnetic Resonance Biomarkers in Neonatal Encephalopathy (MARBLE)
• Mitotracker Green (MTG)
• Myeloid differentiation primary response (myd88)
• Mean Arterial Pressure (MAP)
• NADPH Oxidase (Nox)
• NLRC4 (NOD-, LRR- and CARD-containing 4)
• NLRP3 (NOD-, LRR- and pyrin domain-containing 3)
• NLRP1 (NOD-, LRR- and pyrin domain-containing 1)
• NLRP6 (NOD-, LRR- and pyrin domain-containing 6)
• National Maternity Hospital (NMH)
• Number Needed To Treat (NNT)
• Nucleotide-binding domain, leucine-rich repeat (NLR) proteins
• Neuron specific enolase (NSE)
• Neonatal Resuscitation Programme (NRP)
• Neonatal Intensive Care (NICU)
• Near-infrared spectroscopy (NIRS)
• N-acetyl aspartate (NAA)
• Neutrophil gelatinase-associated lipocalin (NGAL)
• National Neonatal Transport Programme (NNTP)
• Pathogen-associated molecular pattern molecules (pamps)
• Period (PER)
• P2X purinoceptor 7 (P2X7)
• Peripherally inserted central catheter (PICC)
• Polymerase chain reaction (PCR)
• Patient Information Leaflet (PIL)
• Phosphate buffer solution (PBS)
• Persistent pulmonary hypertension (PPHN)
  • Partial pressure of arterial carbon dioxide (Paco₂)
• Phosphate buffer acetate (PBA)
• Posterior limb of the internal capsule (PLIC)
• Pulmonary vascular resistance (PVR)/systemic vascular resistance (SVR) ratio
• P2X purinoceptor 7 (P2X7)
• Prothrombin time (PT)
• Real time-PCR (RT-PCR)
• Red cell concentrate (RCC)
• Randomized control trials (RCT)
• RANTES (regulated on activation, normal T-cell expressed and secreted)
• Reactive oxygen species (ROS)
• Status epilepticus (SE)
• Standard Operating Procedure (SOP)
• Side scatter (Ssc)
• Sodium Chloride (NACL)
• Syndrome of inappropriate anti diuretic hormone (SIADH)
• Tumor necrosis factor-alpha (TNF-α)
• Toll like receptor (TLR)
• T1 weighted (T1W)
• TIR-domain-containing adapter-inducing interferon-β (TRIF)
• Total Body Hypothermia (TOBY)
• Total Parenteral Nutrition (TPN)
• Transient hyperammonaemia of the newborn (THAN)
• Urea and Electrolytes (U+E)
• Umbilical venous line (UV)
• Vascular endothelial growth factor (VEGF)
# Table Of Contents

Chapter One Introduction 1
  1.1. Neonatal Brain Injury 1
  1.2. Epidemiology Of Neonatal Encephalopathy 1
  1.3. Risk Factors For NE 3
  1.4. Diagnosis Of Neonatal Encephalopathy And Hypoxic Ischaemic Encephalopathy 6
  1.5. Neonatal Neurological Assessments 8
  1.6. Therapeutic Hypothermia 9
  1.7. Parent Experience Of Neonatal Encephalopathy And Therapeutic Hypothermia 11
  1.8. Pathophysiology Of Brain Injury In Neonatal Encephalopathy 12
  1.9 Conclusion Of Introduction 19
  1.10 Hypothesis 19
  1.11 Aims 19

Chapter 2 Materials And Methods 22
  2.1 Location And Dates Of Research 22
  2.2 Study Population 22
  2.3 Consent 25
  2.4 Patient Information Collection 25
  2.5 Phlebotomy 27
  2.6 Equipment And Re-Agents 28
  2.7 Sample Processing 28
  2.8 Biobanking For Enzyme Linked Immunosorbent Assay (ELISA) 28
  2.9 Flow Cytometry 30
  2.10 Adenosine 41
  2.11 Polymerase Chain Reaction 41
  2.12 Systematic Review 48
  2.13 Patient Participation In Research 51
  2.14 Statistics 51

Chapter 3 - Systematic Review; Biomarkers Of Neonatal Encephalopathy To Predict Outcome 53
  3.1 Introduction 53
  3.2 Aim 54
  3.3 Hypothesis 54
  3.4 Results 54
List of Tables and Figures

Table 1.1 Incidence of NE globally from a literature review 2
Table 1.2 Eligibility Criteria for TH 3
Table 1.3 Criteria to define Hypoxic Ischaemic Encephalopathy, 1996, The American Academy of Pediatrics and American College of Obstetrics and Gynaecology 6
Table 1.4 3 Essential Criteria to Diagnose HIE, International Cerebral Palsy Task Force Essential Criteria to define HIE, 1999 6
Table 1.5 5 additional criteria to Diagnose HIE, International Cerebral Palsy Task Force Additional Criteria to define HIE, 1999 6
Table 1.6 4 essential criteria of American College of Obstetrics and Gynaecology Essential criteria to define HIE 7
Table 1.7 5 additional criteria, American College of Obstetrics and Gynaecology Additional criteria to define HIE 7
Table 1.8: Modified Sarnat Staging, Neonatal Encephalopathy following foetal distress 8
Table 2.1 Demographics of Infants with NE 23
Table 2.2 Demographics of Healthy Term controls 24
Table 2.3: Required blood volumes for experiments 27
Figure 2.1 Flow Diagram of blood processing with samples post phlebotomy 28
Table 2.4 Antibody cocktail preparation for Panel A 31
Table 2.5 Compensation Bead set up for Panel A 32
Table 2.6 Antibody cocktail for Mitochondrial Fluorescence and Reactive Oxygen Species Experiment 34
Figure 2.3: Endogenous Control of examining FITC and PE fluorescence. 37
Figure 2.4 Gating Strategy for Mitochondrial Fluorescence and Reactive Oxygen Species Experiment: Monocyte Gating Strategy 38
Figure 2.5 Gating Strategy for Mitochondrial Fluorescence and Reactive Oxygen Species Experiment Granulocyte Gating Strategy 40
Table 2.7 Mastermix cocktail 45
Table 2.8: Table displaying the dilution Volumes of RNA and RNA-ase free water in making cDNA. 46
Table 2.9 Mastermix for PCR 47
Figure 3.1 Prisma Flow Diagram of Systematic Review 56
Table 3.1 Meta-Analysis results for short term biomarkers to predict outcome. 57
Figure: 3.2 IL-1beta forest plot to predict short term outcome in NE. 58
Figure 3.3: Troponin forest plot to predict short term outcome. 59
Figure 3.4: Lactate dehydrogenase forest plot to predict short term outcome. 60
Figure 3.5: White cell count forest plot to predict short term outcome.

Figure 3.6: Serum lactate forest plot to predict short term outcome.

Figure 3.7: Nucleated red blood cells forest plot to predict short term outcome.

Figure 3.8: Ionized calcium forest plot to predict short term outcome.

Figure 3.9: Serum glucose forest plot to predict short term outcome.

Figure 3.10: IL-6 forest plot to predict short term outcome.

Figure 3.11: TNF-alpha forest plot to predict short term outcome.

Figure 3.12: S100 forest plot to predict short term outcome.

Figure 3.13: IL-1 beta to predict long term outcome.

Figure 3.14: Serum IL-6 to predict long term outcome.

Figure 3.15: NSE to predict long term outcome.

Figure 3.16: TNF-alpha to predict long term outcome.

Figure 3.17: S100 to predict long term outcome.

Figure 3.18: CSF TNF-alpha to predict long term outcome.

Figure 3.19: Meta-analysis of MRI brain to predict long term neurodevelopmental outcome & death.

Figure 3.20: Risk of bias table for lactate dehydrogenase to predict short term outcome.

Table 3.2 Serum biomarkers to predict long-term outcome in NE.

Table 3.3 CSF biomarkers to predict long term outcome.

Table 3.4 MRI as biomarker to predict long term outcome in NE.

Figure 4.1 Baseline cytokine levels in healthy term control patients (Con) and in Neonatal Encephalopathy (NE) Days 1-2 and Days 3-4 of life.

Tables 4.2 (a+b) Neonatal controls and NE following LPS stimulation on Days 1+2, then 3+4 respectively.

Table 4.3 LPS responsiveness in Controls versus NE Days 1-2 and Days 3-4 life.

Figure 4.2: Comparison of Healthy Control Infants (Con) and infants with Neonatal Encephalopathy (NE) Days 1-2 & Days 3-4 life at baseline & post LPS.

Tables 4.4 Comparison of Cytokine Medians Infants with NE, divided into infants with NE II and NE III, at baseline and following LPS stimulation.

Table 4.5 Comparison of Cytokine Medians Infants with NE, divided into infants with and without seizures, at baseline and following LPS stimulation.

Table 4.6 Comparison of Cytokine Medians in NE divided into infants with normal and abnormal MRI brain imaging, at baseline and following LPS stimulation.

Figure 5.1 Neutrophil (a) and Monocyte CD11b (b) expression in healthy term controls and in Neonatal Encephalopathy.
Figure 5.2 Neutrophil and Monocyte Toll-like receptor-4 (TLR-4) expression in healthy term controls versus Neonatal Encephalopathy

Figure 5.3 Neutrophil and Monocyte Nox expression in healthy term controls and in Neonatal Encephalopathy.

Figure 5.4 Neutrophil and Monocyte Mitochondrial mass in healthy term controls compared to NE

Figure 5.5 Mitochondrial mass in Monocyte subsets: classical, intermediate and non-classical

Figure 5.6. Mitochondrial ROS from Neutrophils and Monocytes in Controls versus NE

Figure 5.7 Mitochondrial ROS from Monocyte subsets in controls versus NE

Figure 5.8 TRIF, IRAK-4 MyD88 in Term Controls and infants with Neonatal Encephalopathy.

Figure 5.9 HIF 1-alpha in healthy term controls compared with Neonatal Encephalopathy (NE) on Days 1 and 3 of life.

Figure 5.10 Epo concentration compared to HIF 1-alpha in Neonatal Encephalopathy

Figure 5.11 VEGF concentration and HIF 1-alpha in Neonatal Encephalopathy

Figure 5.12: IL-1β in term controls and Neonatal Encephalopathy on Days one and three of life

Figure 5.13 ASC expression in term controls and Neonatal Encephalopathy on Days 1 and 3 of life

Figure 5.14 NLRP3 expression in term controls and Neonatal Encephalopathy on Days 1 and 3

Figure 5.15 Concentration of Inflammasome related cytokines IL-18, IL-1ra and Epo in term controls and in Neonatal Encephalopathy on Days 1 and 3

Figure 5.16 Adenosine concentration as measured by rapid point of care testing in Neonatal Mice and in Human Neonates

Figure 5.17: P2X7 plasma levels in healthy term control and in Neonatal Encephalopathy

Figure 5.18: BMAL, CRY, CLOCK and REV-ERBβ in term controls and Neonatal Encephalopathy on Days 1 and 3 of life

Figure 5.19. Correlation of circadian gene expression (Rev-Erbβ, CLOCK and BMAL) to serum cytokine levels of IFN-γ, IL-6 and IL-2.

Table 6.1: Comparison of definitions of Multi-Organ Dysfunction in NE

Figure 6.1 National Management Guideline for Neonatal Encephalopathy Management
Chapter One Introduction

1.1. Neonatal Brain Injury

The neonatal period is the most vulnerable time for a child’s survival. Forty four percent of deaths in the under-five population occur during this time \(^1\), with twenty nine percent of these deaths being attributed to neonatal encephalopathy (NE). This period is the highest risk for brain injury during the lifespan. In 2010, the estimated global burden of NE was 1.15 million. Ninety six percent of infants with NE are born in low- and middle-income countries\(^2\).

After a normal pregnancy, infants can be born with evidence of brain injury and develop NE. NE is a clinically defined syndrome of disturbed neurologic function, in the earliest days of life in a term new-born, manifesting by a subnormal level of consciousness or seizures, and often accompanied by difficulty with initiating and maintaining respiration with depression of tone and reflexes \(^3\).

Hypoxia ischaemia is responsible for a subgroup of infants with NE but there is no gold standard diagnostic test to determine that a hypoxic or ischaemic event occurred causing NE \(^4\). Hypoxic Ischaemic Encephalopathy (HIE) refers to a subgroup of infants with NE that have had an interruption to oxygen delivery during birth. Many neurological sequelae once attributed to HIE, can be attributed in part to foetal compromise during the prenatal period that manifests as NE postnatally.

The term NE is preferable to HIE as it describes central nervous system dysfunction in neonates from all causes. The American College of Obstetricians and Gynaecologists’ Task Force on Neonatal Encephalopathy recommends that the term NE be used rather than HIE as it doesn’t confer causation \(^5\), and in many situations the aetiology is multi factorial. There remains a poor understanding of the full aetiology of NE, precluding a definitive test that accurately identifies the condition and predicts outcome for the infant.

1.2. Epidemiology of Neonatal Encephalopathy

Badawi et al discuss the lack of consensus on agreed definitions of HIE and NE contributing to difficulties in calculating the epidemiology of HIE \(^6\). NE is a heterogenous condition and the literature refers to it with different terminology including HIE, perinatal asphyxia, birth asphyxia
and perinatal acidaemia. Within the above terms, there are a varying number of definitions that use different clinical and biochemical parameters to diagnose NE.

The incidence of NE in population-based studies varies from 1-6/1000 live births in developed countries. The global estimated incidence from a systematic review in 2010 is 8.5 per 1000 live births\(^7\). The percentage of NE attributed to HIE varies between 20-66% in the literature. The incidence of HIE is 2.5 per 1000 in a systematic review of live born term infants with HIE across 10 studies dating from 1959 to 1997\(^7\). The authors acknowledge the incidence of HIE varies based on the definition of intrapartum hypoxia-ischemia used\(^7\). The National Institute of Child Health and Human Development (NICHD) in USA estimate the incidence at 2-3 per 1000 live term births\(^8\), UK studies estimate incidence at up to 6 per 1000 live term births, with 2/1000 of these on the severe end of the spectrum\(^9\). There is a significant difference in the incidence of NE between well-resourced and poorly developed healthcare systems.

**Table 1.1 Incidence of NE globally from a literature review**

<table>
<thead>
<tr>
<th>Country</th>
<th>Incidence per 1000 live term births</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK , 1985(^9)</td>
<td>6</td>
</tr>
<tr>
<td>Saudi Arabia(^10), 2003</td>
<td>5.5</td>
</tr>
<tr>
<td>Netherlands(^11), 2010</td>
<td>8.5 , 1.6 % severe (perinatal asphyxia is definition used)</td>
</tr>
<tr>
<td>South Africa(^12), 1996</td>
<td>4.6</td>
</tr>
<tr>
<td>Nigeria(^13), 1991</td>
<td>6.5</td>
</tr>
<tr>
<td>Malaysia(^14), 2012</td>
<td>2.5</td>
</tr>
<tr>
<td>Uganda(^15), 2018</td>
<td>30.6</td>
</tr>
<tr>
<td>France(^16), 2005</td>
<td>0.86</td>
</tr>
<tr>
<td>Sweden, 2018(^17)</td>
<td>0.67 (moderate to severe)</td>
</tr>
<tr>
<td>Spain, 2017(^18)</td>
<td>0.77</td>
</tr>
<tr>
<td>USA, 2018(^8)</td>
<td>1 (4.4 with neonatal acidaemia)</td>
</tr>
<tr>
<td>Australia, 1995(^19)</td>
<td>3.8 (moderate to severe)</td>
</tr>
<tr>
<td>Japan, 2008(^20)</td>
<td>0.37 (moderate to severe)</td>
</tr>
</tbody>
</table>
Therapeutic Hypothermia (TH), has been demonstrated to be protective for moderate to severe NE and is the gold standard of care these infants who meet standardised inclusion criteria. The National Institute of Child Health and Human Development (NICHD) and Committee of the Fetus and Newborn of the American Academy of Pediatrics (AAP) published a framework to ensure the appropriate use of TH, and recommend infants should meet inclusion criteria outlined below;

**Table 1.2 Eligibility Criteria for TH**

<table>
<thead>
<tr>
<th>Eligibility Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational Age ≥36 weeks, Age &lt;6 hours</td>
</tr>
<tr>
<td>pH of ≤7.0 or a base deficit of ≥16 mmol/L in of umbilical cord blood or blood obtained during the first hour after birth</td>
</tr>
<tr>
<td>History of an acute perinatal event</td>
</tr>
<tr>
<td>10 minute Apgar score of &lt;5 or assisted ventilation at birth and for 10+ minutes</td>
</tr>
<tr>
<td>Neurologic examination demonstrating moderate to severe encephalopathy</td>
</tr>
</tbody>
</table>

The incidence of TH treatment administration is easier to measure then the incidence of NE. There are currently no figures for national incidence of NE in Ireland but there is published data on the incidence of infants receiving TH for moderate to severe NE.

### 1.3. Risk Factors for NE

The risk factors for NE include antenatal, intra partum and postnatal risk factors. Badawi et al carried out a population-based study of NE in causative factors for NE. They identified that 69% of infants had antepartum risk factors, 25% had both antepartum and intrapartum risk factors, 4% intrapartum alone and 2% had no identifiable risk factors. Understanding the risk factors ante-natal and intrapartum for NE is important in primary prevention.

#### 1.3.1. Antenatal Risk Factors

The Vermont Oxford Network (VON), in a review of over 4000 infants with NE, identified that 46% of pregnancies resulting in NE had a pre identified maternal or foetal condition diagnosed prior to labour. 16% of mothers had hypertension and 10% had diabetes. 16% of babies were small for gestational age. A Western Australia study showed an increase in NE with increasing maternal age and a decrease with increased parity. The maternal age in an Irish review of infants treated with TH for NE showed that the overall maternal age distribution reflected that of
the general maternal age profile giving birth in Ireland, apart from a higher incidence of infants requiring TH the maternal age group of 20-24 \(^{24}\). A Swedish review showed no age difference in maternal ages between infants with NE and control groups \(^{25}\). High maternal BMI was a risk factor in the Swedish study. Incidence of maternal thyroid disease and fertility treatment were higher in all studies reviewed, as were pregnancy complications of severe preeclampsia, intrauterine growth restriction and gestational diabetes \(^{19,24,25}\). Increasing gestational age and a higher rate of induction of labour were higher NE compared to control \(^{25}\).

Abnormal placental pathology is common, with foetal vascular malperfusion increasing the risk of NE \(^{26}\). 63% of cases of NE not attributed to sentinel events, in one study, displayed abnormal placental pathologies \(^{27}\). NE is increasingly recognised as a sexually dimorphic condition, with male infants having a higher incidence of NE and a worse neurodevelopmental outcome \(^{28-31}\).

### 1.3.2. Intrapartum Risk Factors

Not all infants with NE will have had evidence of foetal compromise during labour, and similarly not all infants with evidence of foetal compromise will develop NE. Sentinel obstetric events of placental abruption, cord prolapse and uterine abruption are much higher in all studies of NE \(^{25}\), the rate of sentinel events in the VON review was 15%, with antepartum haemorrhage being the most common sentinel event. Cowan et al, identified 7 intrapartum risk factors for in 405 infants with NE, including prolonged membrane rupture, abnormal cardiotocography (CTG), thick meconium, sentinel events, shoulder dystocia, tight nuchal cord and failed vacuum delivery \(^{32}\). Lundgren \(^{25}\) et al, in Sweden, found an association between NE and abnormal CTG on admission, instrumental deliveries, emergency caesarean sections, acute obstetrical events and longer second stage. Grobman et al, in USA, found a higher rate of NE with prolonged second stage of >2 hours, in a large observational study (n=53,000) \(^{33}\)

The introduction of CTG was expected to decrease the incidence of NE but this was not the case in the majority of cases. A Cochrane review did not find any difference in perinatal mortality or cerebral palsy with use of CTG but detected a small decrease in neonatal seizures with its use \(^{34}\). Foetal bradycardia prior to delivery was present in 34% of cases in the VON review \(^{23}\). CTG has a high sensitivity and high negative predicative value in predicting the absence of foetal acidosis. The specificity and the positive predictive value, however, are very low.

A systematic review examined the outcome of infants with arterial cord pH of under 7, 17.2% of infants had a neurological morbidity, 16.3% had seizures an 5.9% died during the neonatal period \(^{7}\). In the VON review, a majority of cord bloods tested (54%) were not severely acidotic, 46%
did not have a base deficit of greater than 12. Chorioamnionitis is associated with over a four-fold increase in NE\textsuperscript{35,36}.  

Maternal pyrexia is consistently associated with NE across all studies. Maternal intrapartum pyrexia was documented in 24\% in The VON Register\textsuperscript{23}. The incidence of meconium stained liquor is higher in NE\textsuperscript{25}. Caesarean section is the most common method of delivery in cases of NE. In Ireland only 22\% of infants requiring TH were born by a normal vaginal delivery in comparison to 52\% of the general population\textsuperscript{22}.

1.3.3. Post Natal Risk

NE can occur as a consequence of postnatal events including intracranial haemorrhage, hypoglycaemia, kernicterus, metabolic disorders, neonatal seizure disorders and sepsis.
1.4. Diagnosis of Neonatal Encephalopathy and Hypoxic Ischaemic Encephalopathy

The diagnosis of NE/HIE is based on a number of clinical criteria, including, evidence of foetal distress during or prior to delivery, neonatal metabolic acidosis and need for neonatal resuscitation after delivery. Having a standard definition enables recognition, prompt management and prognostication. It also allows studies to be compared to each other.

The American Academy of Pediatrics and American College of Obstetrics and Gynaecology published the first statement to define HIE in 1996, that included the following criteria.

Table 1.3 Criteria to define Hypoxic Ischaemic Encephalopathy, 1996, The American Academy of Pediatrics and American College of Obstetrics and Gynaecology

<table>
<thead>
<tr>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Profound metabolic acidosis (pH &lt; 7.0) in umbilical artery blood</td>
</tr>
<tr>
<td>Apgar score ≤ 3 for longer than 5 minutes</td>
</tr>
<tr>
<td>Neonatal Encephalopathy</td>
</tr>
<tr>
<td>Multi system organ dysfunction</td>
</tr>
</tbody>
</table>

This was approved by the International Cerebral Palsy Task Force in 1999 and included three essential criteria and five additional criteria.

Table 1.4 3 Essential Criteria to Diagnose HIE, International Cerebral Palsy Task Force

**Essential Criteria to define HIE, 1999**

<table>
<thead>
<tr>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolic acidosis in early neonatal blood sample (pH &lt; 7.0 and base deficit ≥ 12 mmol/L)</td>
</tr>
<tr>
<td>Moderate or severe encephalopathy</td>
</tr>
<tr>
<td>Cerebral palsy of spastic quadriplegia, dyskinetic or mixed type</td>
</tr>
</tbody>
</table>

The 5 additional criteria were

Table 1.5 5 additional criteria to Diagnose HIE, International Cerebral Palsy Task Force

**Additional Criteria to define HIE, 1999**

<table>
<thead>
<tr>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sentinel event</td>
</tr>
<tr>
<td>Severe changes in fetal heart rate</td>
</tr>
<tr>
<td>Apgar score &lt; 6 beyond 5 min</td>
</tr>
<tr>
<td>Multi-system involvement</td>
</tr>
<tr>
<td>Early imaging evidence</td>
</tr>
</tbody>
</table>
**Table 1.6 4 essential criteria of American College of Obstetrics and Gynaecology**

<table>
<thead>
<tr>
<th>Essential criteria to define HIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolic acidosis (pH &lt; 7.0 and base deficit ≥ 12 mmol/L) in umbilical artery sample</td>
</tr>
<tr>
<td>Moderate or severe encephalopathy</td>
</tr>
<tr>
<td>Cerebral palsy of spastic quadriplegia or dyskinetic type</td>
</tr>
<tr>
<td>Exclusion of other aetiologies.</td>
</tr>
</tbody>
</table>

**Table 1.7 5 additional criteria, American College of Obstetrics and Gynaecology**

<table>
<thead>
<tr>
<th>Additional criteria to define HIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sentinel event</td>
</tr>
<tr>
<td>Abrupt changes in fetal heart rate</td>
</tr>
<tr>
<td>Apgar score ≤ 3 beyond 5 min</td>
</tr>
<tr>
<td>Multi-system failure within 72 h of life</td>
</tr>
<tr>
<td>Early imaging evidence</td>
</tr>
</tbody>
</table>
1.5. Neonatal Neurological Assessments

The neonatal neurological assessments to diagnose NE clinically rely on standardised scoring systems that have been correlated to clinical outcome and identify infants that may benefit from TH. Sarnat and Sarnat described the clinical manifestations of NE in 1976, dividing the stages of encephalopathy into mild, moderate and severe. The score is derived from a study of 21 infants with NE who were graded for their level of encephalopathy and correlated to clinical outcome. Sarnat concluded that persistence of moderate encephalopathy for more than 7 days was associated with poor neurologic outcome or death.

Table 1.8: Modified Sarnat Staging, Neonatal Encephalopathy following foetal distress

<table>
<thead>
<tr>
<th>Sarnat Stage</th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level Of Consciousness</td>
<td>Hyperalert</td>
<td>Lethargic/Obtunded</td>
<td>Stupor/ Coma</td>
</tr>
<tr>
<td>Activity</td>
<td>Normal</td>
<td>Decreased</td>
<td>Absent</td>
</tr>
<tr>
<td>Neuromuscular Control</td>
<td>Muscle Tone</td>
<td>Normal</td>
<td>Mild Hypotonia</td>
</tr>
<tr>
<td></td>
<td>Posture</td>
<td>Mild Distal Flexion</td>
<td>Strong Distal Flexion</td>
</tr>
<tr>
<td></td>
<td>Stretch Reflexes</td>
<td>Overactive</td>
<td>Overactive</td>
</tr>
<tr>
<td>Primitive Reflexes</td>
<td>Suck</td>
<td>Weak</td>
<td>Weak or Absent</td>
</tr>
<tr>
<td></td>
<td>Moro</td>
<td>Strong, low threshold</td>
<td>Weak/Incomplete, High Threshold</td>
</tr>
<tr>
<td></td>
<td>Atonic Neck Reflex</td>
<td>Slight</td>
<td>Strong</td>
</tr>
<tr>
<td>Autonomic Function</td>
<td>Pupils</td>
<td>Mydriasis</td>
<td>Miosis</td>
</tr>
<tr>
<td></td>
<td>Heart Rate</td>
<td>Tachycardia</td>
<td>Bradycardia</td>
</tr>
<tr>
<td>Seizures</td>
<td>None</td>
<td>Common</td>
<td>Uncommon, excluding decerebration</td>
</tr>
</tbody>
</table>

The degree of encephalopathy has been correlated with neurological impairment in a number of other standardised neurological assessments including Miller, Thompson and Amiel Tielson scoring. The Amiel-Tison Neurologic Assessment at Term (ATNAT) was developed to provide a framework for observing the development of cortical control in infants at term and has been shown to predict the occurrence of cerebral palsy after birth asphyxia. Amess et al examined term infants (n=28) with NE with the ATNAT at 8 hours and at 7 days, in comparison to healthy controls and concluded that both early and late neurological examination can be used as a reliable indicator for a favourable outcome at 1 year, having negative predictive values of 100% and 91% respectively. The ATNAT was compared to Prechtl's qualitative assessment of general movement in a group of 45 preterm infants with risk factors for brain injury and found to correlate better with neurodevelopmental outcome. Murray et al, used the ATNAT serially over the first three days of life in 57 infants with NE as the neurological signs observed in NE continue to evolve over the first 72 hours with a significant correlation between ATNAT and neurological...
outcome at 2 years on day 1, 2 and 3 of life. A normal early assessment predicted a normal outcome and the infants with persistent neurological abnormality on day 3 were more likely to have a neuro-disability. The risk was 100% in those with a severely abnormal ATNAT rating, and 41% in those with a moderately abnormal rating.

The Thompson score is a clinical tool that assesses central nervous system dysfunction in encephalopathic infants, based on the longitudinal clinical assessment of 9 signs. A prospective study showed its sensitivity and specificity were highest on day 3 and 4 life, and on day 7 to predict neurodevelopmental outcome at one year. Kothapali et al showed that Thompson accurately predicts morbidity and mortality on day one of life in a study of 145 infants with NE, (p =0.024 and p =0.001). Mendler et al examined the Thompson score in 36 infants undergoing TH in the first three days, and showed a strong association between Thompson score and adverse long-term outcome by means of a low Wechsler Preschool and Primary Scale of intelligence III test at a mean age of 53 months. Almost all surviving infants with a maximal Thompson score ≤10 had a normal IQ and almost all infants who showed an impaired IQ (<85) had a Thompson Score ≥11.

1.6. Therapeutic Hypothermia

1.6.1 History of Therapeutic Hypothermia

Therapeutic Hypothermia (TH) as a treatment dates back to five thousand years ago during ancient Egyptian times. Hippocrates recommended ice to control haemorrhage and observed that infants exposed to cold during winter months had a survival advantage. William Edwards in 1832 discussed TH in his book On the Influence of Physical Agents on Life and Claude Bernard, in 1865, a pioneer in the study of experimental medicine, described therapeutic hypothermia’s physiological impacts on human and animal models.

During the following century TH was researched ex vivo, in animal model and in human studies for therapeutic use post cardiac arrest. Following extensive work in laboratory studies and animal model studies, Gunn et al, in 1998, discussed the hypothesis that changes in post-ischaemic cerebral temperature can modulate processes which start during the primary energy failure phase of hypoxia-ischaemia and continue into the secondary phase of cerebral injury in a seminal paper and recommended randomized control trials (RCTs) to explore this evidence. Several small studies established the safety and feasibility safety of TH following this.

Six RCTs exploring the clinical efficacy of TH in NE were published from 2005 to 2011. The trials included infants with NE at a minimum of 35 weeks’ gestation, and had TH initiated within
6 hours of birth. Infants had their temperature lowered to 33.5 to 34.5 degrees Celsius for 72 hours. There were small differences between entry criteria to select infants into the trials, but all used a combination of intrapartum criteria, biochemical parameters, clinical criteria and some in addition used electrophysiological data. The infants had whole body cooling or selective head cooling. The primary outcome of death and neuro disability at 18 months to 22 months of age was the same in all studies. Four of the six studies’ primary outcome was statistically significantly in favour of TH.

A Cochrane review of the six studies showed a beneficial effect of number needed to treat of 6 for infants with moderate NE and 7 for those with severe NE to prevent one infant dying or having significant neuro-disability \(^{53}\). An updated Cochrane review of 1505 infants with NE including 11 RCTs \(^{54}\) found the reduction of death or major neurodevelopmental disability was 25% and infants with moderate NE appeared to have greater benefit from TH than infants with severe NE.

Despite the beneficial effects of TH, neonates with moderate to severe NE (Sarnat Stage II and II) still have significant morbidity and mortality. A systematic review looking at seven RCT’s including 1214 neonates with NE undergoing TH concluded a mortality of 28%, with a range of 24 to 38%. \(^{53}\) The percentage of cognitive impairment is 24%, the incidence of cerebral palsy is 22%, epilepsy is 19% and cortical visual impairment 6%. The overall figure of incidence of combined death or moderate/severe disability is 48%.

There has been significant progress over the past two decades in offering TH as gold standard of care. There remains a gap in the full understanding of the pathophysiology of NE, precluding the development of adjunctive treatments in NE to improve neurodevelopmental outcomes in NE. This thesis aims to further the understanding of the inflammatory phenotype in NE to contribute further towards this progress.

1.6.2 Mechanism of Action of Therapeutic Hypothermia

Laptook \(^{55}\) et al demonstrated that hypothermia reduces cerebral metabolism by demonstrating a linear relationship between brain temperature and brain energy utilisation rate in a piglet model of experiment. Fujishima \(^{56}\) et al examined effect of TH on ischaemic neurons in the hippocampus in a rat model experiment. Excitatory glutamate and aspartate were lower in the TH groups compared to normothermia. Thoresen et al showed that TH decreased secondary energy failure \(^{57}\), decreased nitric oxide and decreased excitatory amino acid release post hypoxia ischaemia \(^{58}\). Baumann \(^{59}\) et al demonstrated that TH reduced disruption of blood brain barrier post ischaemia. Gunn et al demonstrated that TH reduces secondary cytotoxic oedema, and reduces neuronal and white matter injury in sheep model post hypoxia ischaemia \(^{60}\). They showed the TH was
associated with reduced expression of activated caspase-3 and reactive microglia \(^{61}\). TH did not affect necrosis, but decreased apoptotic cell death in a piglet model \(^{62}\) and has been shown to maintain mitochondrial integrity by attenuating the release of cytochrome C \(^{63}\). This has been shown similarly in adult animal model of TH post cardiac arrest, with recovery of mitochondrial membrane potential and respiration \(^{64}\).

TH supresses the inflammatory response. \textit{In–vitro} animal model showed supressed micro glial activation and consequently its downstream effects of neuronal damage\(^{65}\). TH supresses the inflammatory transcription factor NFkB, and subsequently its target genes, inducible nitric oxide synthase and TNF-\(\alpha\) \(^{63}\). Diestel et al \(^{66}\) stimulated ex vivo microglial cells with endotoxin stimulation and showed that TH, followed by rewarming activate microglial cells, increase phagocytosis and increase anti- inflammatory IL-10 and IL1-receptor antagonist.

1.7. Parent Experience of Neonatal Encephalopathy and Therapeutic Hypothermia

NE is a devastating and unexpected outcome of an apparently healthy term pregnancy. Parents are unprepared for their baby's admission to the neonatal intensive care unit and have the burden learning of their child’s illness and its impact on the future of their child and family unit as a whole. TH makes parent contact and holding their baby more complex. Parents have highlighted that attachment and bonding with their baby is a concern.

A case control study of mothers with infants who had been treated with TH were evaluated for post-natal depression with the Edinburgh Postnatal Depression Scale. A trend towards higher depression scores was found in mothers with infants treated with TH and correlated with severity of encephalopathy \(^{67}\). A small study examined feasibility of parental holding during TH. The study reported no adverse events, stable vitals, stable hypothermic temperature and good parental and nursing feedback \(^{68}\). Parents of infants with NE had their emotional experience evaluated with semi structured interviews. Emotional experiences, trauma, loss of normality and separation were all described. Parents reported trauma in relation to the birth and in observing their infant shivering. They experience loss of normality from delayed feeding and holding. Parental satisfaction was improved when they participated in ward rounds and with participation in routine new-born care. Parents expressed a wish for further peer involvement with other parents whom experienced similar situations \(^{69}\).

Promoting parent attachment with their child and empowering them in the NICU through patient participation in research will be a key theme explored through the thesis.
1.8. Pathophysiology of Brain Injury in Neonatal Encephalopathy

NE is not a single cellular nor molecular event, but an evolving cascade triggered by an initial insult. Experimental evidence indicates that pathophysiological changes in NE can be divided into phases, the primary energy failure phase, the latent phase, the secondary energy failure phase and the tertiary phase. The central nervous system (CNS) needs constant perfusion for energy and oxygen to maintain homeostasis and aerobic metabolism. The most prominent feature in the pathophysiology behind neonatal brain injury is impaired cerebral perfusion. 

Primary energy failure occurs when there is a period of decreased cerebral blood supply. During this period, cerebral metabolism decreases, cells convert from aerobic to anaerobic metabolism leading to decreased energy production, decreased glucose stores and high lactate production. The low energy production (low ATP, adenosine triphosphate) leads to diminished cellular integrity, causing altered sodium and potassium ion levels. The altered ion balance causes neuron depolarization, resulting in glutamate release and allowing intracellular influx of calcium and sodium ions. The sodium and calcium ions cause cytotoxic oedema, and the excessive glutamate triggers a cascade of damaging pathways leading to a final common pathway of cell injury and cell death. Neuronal death from excessive and prolonged activation is termed excitotoxicity. Fatty acids, from increased membrane phospholipid turnover, are peroxidised by oxygen free radicals. Calcium accumulation causes nitric oxide production.

When cerebral perfusion is re-established, the latent phase follows, involving a period of normal cerebral metabolism and improved oxygenation. The latent phase is believed to be shorter in the most severe NE. Reperfusion post resuscitation triggers a cascade of secondary injury, occurring approximately 6 to 72 h later. The mechanism of secondary energy failure involves extension of the primary insults, depletion of adenosine triphosphate (ATP) reserves, and production of lactate and reactive oxygen species (ROS). Mitochondrial dysfunction causes further production of free radicals, above the level that endogenous anti-oxidants and free radical scavengers can destroy. During this phase the majority of cell death occurs. Clinically, seizures are most associated with this phase.

The tertiary phase of injury involves persistent inflammation and epigenetic changes that prevent regeneration and exacerbate brain damage over the months and years following a brain injury. There is less known about the tertiary phase of injury and repair, as so far treatments trialled have all focused on the primary and latent phases of brain injury, but experimentally late administration of erythropoietin and mesenchymal stem cells during the tertiary phase are areas of interest. Lin et al demonstrated persistent inflammation in the tertiary phase by showing an altered
neuroinflammatory profile in ex preterm infants age 7 with cerebral palsy in comparison to healthy controls.

1.8.1 Inflammatory Mediators and Innate Immune Cells

The immune response in brain is complex and highly regulated. Microglia are amongst the first innate immune cells to be activated in NE. Microglial cells are a specialised population of macrophages in the CNS. Astrocytes are glial cells of the CNS, that support and repair the CNS and modulate glutamate synthesis and release. Together they produce cytokines, growth factors and chemo attractants.

Cytokines are peptides secreted that communicate between cells. Mediators released by activated microglia include both pro and anti-inflammatory cytokines. Astrocytes, microglia and proteoglycans form a glial scar, a barrier at the site of inflammation. The astrocytes and microglia promote further injury at the site of injury by secreting pro inflammatory cytokines, enhancing further microglial activation and promoting an inflammatory response.

Systemic inflammation in NE is not confined to the brain. Endotoxin has been demonstrated to sensitise the vulnerability of the immature brain to damage in multiple animal models of experiment, which ties in with the fact that infants exposed to in utero inflammation via infection such as chorioamnionitis, have higher risk of NE, suggesting the inflammation sensitises the brain to injury and allows the hypoxic ischaemic damage at a much lower threshold, that alone may cause little or no injury.

Dammann et al, noted the relationship of inflammation and the immature brain in a seminal paper from 2008. Animal models of intrauterine exposure to non-CNS inflammatory stimuli leads to brain damage in the immature animal without the endotoxin gaining access to the brain. There are synergistic pathways between hypoxemia and inflammation which potentiate the evolution of brain damage in NE.

1.8.2 Cytokines, Inflammation and Neonatal Encephalopathy

Pro and anti-inflammatory cytokines are both neurotropic and detrimental in their role in NE. Dammann et al, discuss that in the study of cytokines, there is “a high degree of overlap in source, target cell, and function”, with some cytokines having both pro and anti-inflammatory effects. Cytokines modulate inflammation and repair after inflammation-related brain damage. The cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and Tumor necrosis factor-alpha (TNF-α) are
felt to be influential in the initiation of inflammation, recruitment of other cytokines and white cells. TNF-α, IL-1, IL-6, and IL-8 both attract and stimulate leucocyte adhesion, which cause immune reaction and activate phagocytosis.

The evidence thus far supports the idea that inflammatory cytokines play an important role in NE. Elevated serum glial fibrillary acidic protein (GFAP), IL-1, IL-6, IL-8, TNF α, interferon gamma (IFN-γ) and vascular endothelial growth factor (VEGF) at 6-24 hours were associated with abnormal neurological outcomes in infants with NE ⁸⁴. Raised serum IL-1β, IL-6, IL-8, and decreased IL-12 were associated with abnormal neurodevelopment (n=54) ⁸⁵. IL-1, IL-6, IL-8, IL-9, IL-11, IL-13, TNF-α and RANTES(regulated on activation, normal T-cell expressed and secreted) were higher in infants with cerebral palsy ⁸⁶⁻⁸⁷. Raised granulocyte – macrophage colony stimulating factor (GMCSF), IFN-γ, IL-10, IL-8 and TNF-α in the first 24 hours correlated with mortality ⁸⁸. Raised cerebrospinal fluid (CSF) Il-6 and IL-8, IL-1beta and TNF-α correlate with NE severity and adverse outcome ⁸⁹⁻⁹⁰⁻⁹¹. Abnormal outcome was best predicted by CSF IL-18 in a study of study of CSF cytokines ⁹². Infants with NE and seizures, who were exposed to clinical chorioamnionitis had higher IL-6, IL-8 and RANTES than controls ⁹³. IFN-γ was higher in term infants with spastic diplegic cerebral palsy in comparison to healthy controls ⁸⁶. Raised IL-1β, IL-6, IL-8 and TNF-α (n=42) were associated with lactate/choline peak in the deep grey nuclei on magnetic resonance spectroscopy (MRS) ⁸⁵, and low VEGF was associated with abnormal neuroimaging ⁸⁸.

Persistence of this inflammatory response has been described in school age children with cerebral palsy. Lin et al. described significantly higher monocyte TNF-α and Toll like receptor (TLR)-4 expression in these children compared to controls ⁷⁸. This demonstrates evidence of the tertiary phase of injury, that the sensitisation to inflammation continues long after the secondary phase on injury has recovered, indicating that immunomodulatory pathways could be targeted in the longer term to modulate this chronic inflammation ⁹⁴.

1.8.3 Leucocytes, Inflammation and Neonatal Encephalopathy

Leucocytes, otherwise termed as white blood cells, are nucleated cells derived from multipotent haemopoietic stem cells and are involved in protecting the body against infectious diseases. Hypoxia has been demonstrated to increase the maturation and production of haemopoietic cells ⁹⁵, especially neutrophils. Neutrophils have been demonstrated as the earliest leucocyte to contribute to brain injury, and are closely followed by monocytes. Neutrophil activation is key in the inflammatory response in NE. The neutrophil functions by producing an oxidative burst, cell migration, and degranulation ⁹⁶. Morkos et al, found that infants with NE who had a higher white cell count and neutrophil count at day one of life had a worse neurodevelopmental outcome,
and that the level of white cell count could be correlated to brain injury on MRI ⁹⁷. Hudome et al, depleted neutrophils in a rat model of hypoxia-ischaemia, resulting in a reduction in brain swelling suggesting neutrophils play a role in HI brain swelling ⁹⁸, and his co-author Palmer identified that the timing of the neutrophils’ contribution to vascular dysfunction is in the early phases of injury during the hypoxic ischaemic insult or in the early hours post injury (less than 8 hours) ⁹⁹.

Molloy et al demonstrated that granulocyte CD11b was increased in neonates requiring resuscitation at birth and were hypo-responsive to endotoxin stimulation. Neonates with severe NE had increased neutrophil apoptosis and decreased CD11b expression, suggesting a tendency towards immunosuppression ¹⁰⁰. O’Hare et al, demonstrated that neonates with moderate to severe NE produced higher granulocyte CD11b levels and monocyte CD11b expression and that delayed granulocyte apoptosis with increased CD18/CD11b expression ¹⁰¹. They found that the exposure of hypoxia and heat shock on neonatal cord blood resulted in endotoxin hypo-responsiveness in vitro in comparison to adult healthy controls, suggesting that during sepsis and hypoxia the normal neonatal neutrophil has decreased anti-bacterial capacity but this may potentially induce less tissue damage ¹⁰².

The challenge of immunomodulating the leucocyte physiological inflammatory process is the concern of immunosuppression and exposure to infection, thus exploring the phenotype of the leucocyte and in particular the neutrophil in NE in this thesis is key to developing safe and effective therapies in the future.

1.8.4 Mitochondria, Inflammation and Neonatal Encephalopathy

Mitochondria are membrane bound organelle that generate energy and store calcium for cell signalling activity and mediate cell growth and death. Ischaemic reperfusion (IR) injury occurs post hypoxia causing oxidative damage, cellular apoptosis and atypical immune responses through the production of reactive oxygen species (ROS). ROS are oxygen containing reactive species that are formed by aerobic metabolism upon oxygen utilisation, and are known to cause harmful oxidative stress when their formation is increased or there is decreased capacity of anti-oxidant species ¹⁰³.

Mitochondria are a target for ROS and vulnerable in NE as their capacity to scavenge is overcome with high levels of ROS, initiating mitochondrial permeability transition and potentiating oxidative stress ¹⁰⁴ ¹⁰⁵.
Insufficiency of mitochondrial energy and decreased ATP production are implicated in neurological injury in NE\textsuperscript{106}. Oxidative stress causes mitochondrial cytochrome C release which in turn causes apoptosis. Anaerobic glycolysis decreases ATP production in mitochondria. Subsequent phosphate, lactate and hydrogen ion increases cause acidosis and calcium influx. The acidic environment and lack of ATP impairs the ATP dependent channels that can control the intracellular calcium levels\textsuperscript{107}. Subsequent glutamate production causes loss of mitochondrial membrane potential, activating lipases, proteases and causing free radical formation. Mitochondria are both the cause of much of the free radical production and the vulnerable target for free radical production related damage\textsuperscript{108}. Following LPS stimulation \textit{ex-vivo}, neonates with severe brain injury produce significantly greater amounts of systemic neutrophil ROS compared to mildly affected neonates (NE 0/I) at 72-96 hours and day 7 of life\textsuperscript{109}. These ROS can cause significant tissue damage and may be mediated by mitochondrial dysfunction and HIF-1α up regulation. ROS play an important role in the damage caused by cerebral ischaemic reperfusion and neonatal meningitis\textsuperscript{110}.

NADPH Oxidase (Nox) contributes to the generation of ROS. Inducible NOX is a well-established source of NOX during CNS inflammation. Nox 2 is present in inflammatory cells and contributes to brain injury in adult animal models though its contribution in apoptotic pathways\textsuperscript{111}.

Succinate, an intermediate of the Kreb’s cycle, accumulates during ischaemia due to a reversal of the enzyme succinate dehydrogenase\textsuperscript{112}. Upon reperfusion, the accumulated succinate is rapidly oxidized and drives ROS production at mitochondrial complex I. Succinate dehydrogenase drives macrophage mitochondria from ATP synthesis to ROS production, resulting in a pro-inflammatory state\textsuperscript{113}. Succinate production from glutamine results in stabilization of the transcription factor hypoxia inducible factor (HIF)-1α, a key transcriptional factor in the hypoxic response associated with persistent inflammation\textsuperscript{112}. Reinke et al identified that raised succinate and glycerol in cord blood was associated with severe encephalopathy and low voltage EEG\textsuperscript{114}. Understanding how and when mitochondria become dysfunctional and contribute to apoptosis is crucial to develop treatment strategies and for prevention of brain injury.

\textit{1.8.5 The Inflammasome, Inflammation and Neonatal Encephalopathy}

The inflammasome is a multi-protein complex of the innate immune system, mediating defence against pathogens, with domains including AIM2 (Absent in melanoma 2), NLRC4 (NOD-, LRR- and CARD-containing 4), NLRP1 (NOD-, LRR- and pyrin domain-containing 1), NLRP3 (NOD-, LRR- and pyrin domain-containing-3), NLRP6 (NOD-, LRR- and pyrin domain-containing-6)
and pyrin. The difference in receptor sensor between inflammasome and the Toll-like receptor (TLR) family, is that TLR's sense danger on the cell surface or in endosomes, but the inflammasome recognises pathogens in the host cytosol.

The adaptor protein ASC, (apoptosis-associated speck-like protein containing a CARD) mediates a critical step in innate immune signalling by bridging the interaction between the pathogen recognition receptors and caspase 1 in inflammasome complexes. Many inflammasomes require the assembly of the adaptor protein ASC for activation. The inflammasome sensor overall responds to a loss of cellular homeostasis, with sensors of pathogen-associated molecular pattern molecules (PAMPs) and damage-associated molecular pattern molecules (DAMPs) that they respond to. The sensors oligomerize into a multiprotein complex that activates the maturity of highly pro-inflammatory interleukin-1β (IL-1β) and IL-18, and the inflammatory pathway of programmed cell death, via caspase-1 cleavage. The inflammasome allows neutrophil, natural killer cell activation and local inflammatory reaction. NLRP3 has a range of signals that can activate it including viruses, bacteria, ROS and damaged mitochondria. From a sepsis point of view, group B Streptococcus has been demonstrated to be a potent NLRP3 stimulator.

The inflammasome is implicated in a number of inflammatory disorders and there are therapeutics that target inflammasome activity. Deregulated inflammasome activity has been linked to several sterile inflammatory diseases, including Alzheimers, atherosclerosis, familial cold autoinflammatory syndrome and Muckle-Wells syndrome. The Inflammasome has been explored in animal models of NE but not human model. Exploration of the inflammasome in NE will further understand the inflammatory phenotype of the infant, with a view to therapeutic manipulation.

### 1.8.6 Chronobiology, Inflammation and Neonatal Encephalopathy

The immune system demonstrates changes with time of day and disruption of circadian rhythms has been linked to inflammatory disorders in human and animal disease models. The molecular clock has been characterised in all cells including immune cells. Diurnal variation has been demonstrated in endotoxin sensitivity and host defence. Dysregulation of the circadian rhythm is linked to conditions such as obesity, auto-immune disorders and neuro degeneration, and there is a higher rate of the metabolic syndrome in shift workers. The molecular clock is controlled by a series of positive and negative feedback regulator genes, alongside control by the suprachiasmatic nucleus and light. The transcription factors circadian loco-motor output cycles kaput, (CLOCK) and Brain and Muscle ARNT-Like 1 (BMAL1) are central to the positive transcriptional loop, binding to the clock controlled genes, period (PER) and Cryptochrome.
(CRY). PER and CRY are negative transcription activators. Nuclear receptors REV-ERBα and β regulate the molecular clock and suppress the expression of genes of the positive limb of the clock including CLOCK, BMAL1, and neuronal PAS domain protein 2 (NPAS2). Their interaction causes oscillation of the circadian gene expression. Melatonin, a hormone involved in the regulation of circadian rhythms, has shown improved outcomes in NE in a RCT as an adjunctive therapy in combination with TH. Melatonin has the capacity to cross the blood brain barrier, a good safety profile, and it has been demonstrated pharmacologically to increase anti-oxidant gene expression and increase anti-oxidant activity. In NE, the earlier acquisition of a normal sleep-wake cycle is associated with better neurodevelopmental outcomes. Improved understanding of chronobiology in NE and its correlation to the inflammatory profile in this thesis may lead to identification of therapeutic ways to manipulate the circadian rhythm endogenously via light dark exposure, and exogenously through melatonin administration.

1.8.7 Adenosine, Inflammation and Neonatal Encephalopathy

Adenosine is an endogenous purine metabolite with immunomodulatory properties, acting through its four receptor subtypes. Adenosine triphosphate (ATP) and its metabolite adenosine are important endogenous signalling molecules in inflammation. The conversion of ATP to adenosine generates an anti – inflammatory metabolite. Under physiological conditions, adenosine is present in low concentrations in extracellular fluid but it is known to rise dramatically in stress, hypoxia, inflammation and ischaemia. High ATP production can act as a danger signal, is known to activate the NLRP3 component of the Inflammasome and to bind to P2X purinoceptor 7 (P2X7) to activate IL-1β. Adenosine has a regulatory function in limiting tissue damage post inflammation, with adenosine receptor stimulation some TLR-4 mediated responses.

Levy et al discuss the adenosine role with regard to potential manipulation of adenosine to alter the innate immune response. They demonstrated that neonates have a relatively high adenosine concentrations in comparison to adult blood, and that their cells have heightened sensitivity to adenosine’s actions. They showed that upon adenosine receptor antagonism, TNF-alpha production was dramatically increased, demonstrating adenosines immunomodulatory properties in regulating the inflammatory response. There is increasing interest in understanding the role of adenosine in immune mediated disease with regard to potential immunomodulatory targets going forward.
1.9 Conclusion of Introduction

Neonatal brain injury can have devastating consequences for the baby and their family and may result in long term disability. The current gold standard of treatment is incompletely neuroprotective. Many children continue to die and to survive with severe neurological disability. Expanding the evidence and understanding of the inflammatory profile in these infants will ultimately improve care of these patients and their families.

1.10 Hypothesis

The abnormal persistent systemic inflammation seen in NE is associated with altered immune cell, metabolic and mitochondrial function. Correlation of such data with MRI and clinical outcomes will identify novel biomarkers of prognosis and also identify key targets which may be amenable to immunomodulation.

1.11 Aims

1.11.1 Aim 1: Persistent Inflammation and Neonatal Brain Imaging

Rationale

Persistent inflammation is implicated in neonatal brain injury and may be a potential therapeutic target. Using Infants with NE and age matched controls the evolution of inflammation can be studied.

Objectives

To examine cytokines, the inflammasome and circadian rhythm in new-borns with NE and age-matched controls.

Deliverables

The relationship between these serial biomarkers, neuroimaging and neurodevelopment will be used to establish cut-off values to predict clinical outcomes.

1.11.2 Aim 2: Mechanism: Metabolic and Immune cell function in hypoxia
Rationale

Explore mechanisms causing persistent inflammatory changes in systemic immune cells. Reactive oxygen species (ROS) can cause significant tissue damage and may be mediated by mitochondrial dysfunction and HIF1α stabilisation.

Objectives

To explore metabolic and mitochondrial function and chronobiology in immune cells from infants with NE and evaluate the potential of immunomodulation. Results will be correlated clinically.

Deliverables

(i) Examine HIF1α expression in NE (ii) Mitochondrial Function: mitochondrial ROS and mitochondrial mass, will be examined in whole blood from infants with NE in comparison to healthy neonatal control(iii) Clinical metabolic outcomes including MRS will be correlated.

1.11.3 Management of Neonatal Encephalopathy and Clinical Outcomes

Rationale

Parent group representatives, clinicians and research groups benefit and need close collaborative links to advance research.

Objectives

To translate these findings and outcomes into clinical practice by disseminating results and clinical guidelines.

Deliverables

(i) Presentations to the National Neonatal Advisory Group, Royal College of Physicians of Ireland and the Irish Neonatal Health Alliance. (ii) Systematic Review of Biomarkers in NE (iii) NE Management Guidelines.
Chapter 2 Materials and Methods

2.1 Location and Dates of Research

This research involved collaboration and recruitment from the three maternity hospitals in Dublin: the National Maternity Hospital (NMH), Coombe Women and Infant's University Hospital (CWIUH) and The Rotunda Hospital. These 3 hospitals have almost 9,000 deliveries each per annum and are National Centres for Therapeutic Hypothermia. There are four centres in the Republic of Ireland for administration of Therapeutic Hypothermia, the remaining centre is Cork University Maternity Hospital (CUMH). Infants requiring Therapeutic Hypothermia born in other centres in the Republic of Ireland are transported by the Neonatal Transport Service to one of the Dublin Units or CUMH.

The blood processing and laboratory work was completed in the Paediatric Laboratory of Professor Eleanor Molloy in the Trinity Translational Medicine Institute, St James’ Hospital Campus. The polymerase chain reaction (PCR) work and point of care Adenosine testing was completed in the Trinity College Laboratory in CWIUH. The SECTOR Image plate reader to complete the enzyme-linked immunosorbent assay (ELISA) analysis was completed in Trinity Centre for Neuro Sciences, Trinity College Campus. Each hospital’s ethical committee approved the study prior to commencement. The study began in August 2016 in CWIUH and NMH, and commenced in the Rotunda Hospital in September 2017. The recruitment was completed in January 2019. The copies of the Ethical approval from each hospital is included. (Appendix I)

2.2 Study Population

The study included infants with Neonatal Encephalopathy. The control group was healthy term control infants (TC). Standard operating techniques were confirmed using healthy adult donations of blood to ensure optimisation of experiments prior to the use on neonatal blood.

2.2.1 Infants with NE

The inclusion criteria were defined by criteria of Huang et al\textsuperscript{136}; Abnormal neurological signs, such as hypotonia or seizures in the immediate postnatal period, and/or other organ dysfunction (kidneys, liver, lung, heart, haematological) and at least two of the following three criteria:
• Evidence or suspicion of hypoxic-ischemic injury based on a history of foetal distress i.e. type II dips, loss of beat-to-beat variability on CTG and/or abnormal scalp pH;

• Need for resuscitation after birth i.e. bag and mask ventilation;

• Base deficit of > 15mmol/l or pH< 7.2 in cord blood or admission arterial sample.

Additional Infants with early brain injury: Infants postnatally diagnosed with brain injury on cranial ultrasound or with neonatal encephalopathy in the first 48 hours of life. Infants were subsequently subdivided by Sarnat Staging\textsuperscript{37}, a standardised assessment of severity of NE (Appendix II). The exclusion criteria are infants with major congenital abnormalities and maternal substance misuse.

Table 2.1 Demographics of Infants with NE

<table>
<thead>
<tr>
<th>Variables</th>
<th>NE-I (n=2)</th>
<th>NE-II (n=40)</th>
<th>NE-III (n=11)</th>
<th>p-value (95% CI) NE-II vs III</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA (wks)\textsuperscript{a}</td>
<td>40.65</td>
<td>39.7 +/-1.68</td>
<td>40.86 +/-2</td>
<td>0.21</td>
</tr>
<tr>
<td>BW (kgs)\textsuperscript{*}</td>
<td>2.99 +/-0.62</td>
<td>3.3 +/-0.59</td>
<td>3.2 +/-1.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Gender, male, n (%)\textsuperscript{b}</td>
<td>1 (50)</td>
<td>29 (72.5)</td>
<td>5 (45.5)</td>
<td>0.14</td>
</tr>
<tr>
<td>Mode of delivery n (%)\textsuperscript{b}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LSCS</td>
<td>0 (0)</td>
<td>23 (59)</td>
<td>6 (54.5)</td>
<td>1</td>
</tr>
<tr>
<td>SVD</td>
<td>1 (50)</td>
<td>7 (18)</td>
<td>3 (27.3)</td>
<td>0.67</td>
</tr>
<tr>
<td>Inst</td>
<td>1 (50)</td>
<td>9 (23)</td>
<td>2 (18.2)</td>
<td>1</td>
</tr>
<tr>
<td>Apgar @1 min \textsuperscript{a}</td>
<td>3 (2)</td>
<td>2 (8)</td>
<td>1 (6)</td>
<td>0.33</td>
</tr>
<tr>
<td>Apgar@5 mins \textsuperscript{a}</td>
<td>8 (2)</td>
<td>5 (8)</td>
<td>4 (7)</td>
<td>0.09</td>
</tr>
<tr>
<td>Apgar@10 mins \textsuperscript{a}</td>
<td>9 (NA)</td>
<td>7 (7)</td>
<td>5 (9)</td>
<td>0.02</td>
</tr>
<tr>
<td>TH, n (%)\textsuperscript{b}</td>
<td>1 (50)</td>
<td>40 (100)</td>
<td>11 (100)</td>
<td>1</td>
</tr>
<tr>
<td>Seizures, n (%)\textsuperscript{b}</td>
<td>N/A</td>
<td>20 (51.3)</td>
<td>9 (81.8)</td>
<td>0.09</td>
</tr>
<tr>
<td>MRI- Abnormal, n (%)\textsuperscript{b}</td>
<td>1 (50)</td>
<td>23 (59)</td>
<td>7 (63.6)</td>
<td>1</td>
</tr>
<tr>
<td>Cord Arterial pH \textsuperscript{a}</td>
<td>6.96 +/-0.05</td>
<td>7.03 +/-0.18</td>
<td>6.93 +/-0.20</td>
<td>0.21</td>
</tr>
<tr>
<td>Cord Arterial BE \textsuperscript{a}</td>
<td>-14.55 +/-2.5</td>
<td>-10.81 +/-6.2</td>
<td>-16.96 +/-6.3</td>
<td>0.06</td>
</tr>
<tr>
<td>CPR, yes (%)\textsuperscript{b}</td>
<td>0 (0)</td>
<td>12 (30.8)</td>
<td>4 (36)</td>
<td>0.73</td>
</tr>
<tr>
<td>Intubated, yes (%)\textsuperscript{b}</td>
<td>0(0)</td>
<td>25 (64.1)</td>
<td>9 (81.8)</td>
<td>0.3</td>
</tr>
</tbody>
</table>
2.2.2 Healthy Term Control Infants

Asymptomatic infants undergoing routine phlebotomy for maternal risk factors for sepsis and jaundice will be sampled. Subsequently any infants requiring NICU admission were be excluded from the control group.

Table 2.2 Demographics of Healthy Term controls

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control (n=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA (wks) a</td>
<td>39.21 +/- 1.58</td>
</tr>
<tr>
<td>BW (kgs) a</td>
<td>3.28 +/- 0.50</td>
</tr>
<tr>
<td>Gender, male, n (%) b</td>
<td>29%</td>
</tr>
<tr>
<td>Mode of delivery (%)</td>
<td></td>
</tr>
<tr>
<td>LSCS</td>
<td>41.2</td>
</tr>
<tr>
<td>SVD</td>
<td>47</td>
</tr>
<tr>
<td>Inst</td>
<td>11.8</td>
</tr>
<tr>
<td>Apgar @1 min a</td>
<td>9</td>
</tr>
<tr>
<td>Apgar@5 mins a</td>
<td>10</td>
</tr>
<tr>
<td>Apgar@10 mins a</td>
<td>N/A</td>
</tr>
<tr>
<td>TH, n (%) b</td>
<td>N/A</td>
</tr>
<tr>
<td>Seizures, n (%) b</td>
<td>N/A</td>
</tr>
<tr>
<td>MRI- Abnormal, n (%) b</td>
<td>N/A</td>
</tr>
<tr>
<td>Cord Arterial pH a</td>
<td>N/A</td>
</tr>
<tr>
<td>Cord Arterial BE a</td>
<td>N/A</td>
</tr>
<tr>
<td>CPR, yes (%) b</td>
<td>N/A</td>
</tr>
<tr>
<td>Intubated, yes (%) b</td>
<td>N/A</td>
</tr>
</tbody>
</table>

2.2.3 Adult Controls

Male and female adult controls donated 10-20 ml of blood as per Standard Operating Procedure (SOP) in the lab. Adult controls acted as internal controls to ensure consistency in laboratory techniques and reagents.
2.3 Consent

Parents were approached for informed consent within a timely manner if their infants had a diagnosis of NE. The parents had verbal information relayed to them and were given written information in a Patient Information Leaflet (PIL). The aspects they were approached to consent for include anonymised data collection of their infants clinical course and investigation results, and an extra 1-1.4 ml of blood to be taken at time of routine phlebotomy on one to two occasions. Consent was given in the form of written signature of parent or guardian on the consent form (Appendix III).

2.4 Patient information Collection

Following recruitment into the study, a standardised anonymised proforma of clinical information was filled in from the patient clinical notes. The information taken involved maternal pregnancy history, antepartum and labour details, initial resuscitation, stabilisation, neurological assessment for Therapeutic Hypothermia eligibility, all clinical examinations, vital signs, multi-organ assessments from birth until discharge or death. Any radiological data and neurophysiological monitoring were also recorded. This information was entered onto a proforma attached in appendix VII, and then inputted to a database made on Microsoft Excel for statistical analysis.

2.4.1 Clinical Information

Study infants underwent a daily clinical neurological examination and Sarnat staging. Their overall assigned Sarnat grade equated to the worst Sarnat grade they reached over the course of their admission. All study infants laboratory and neuro-imaging results over the course of their admission had these results collected. Baseline clinical demographics were collected on each control infant. This included gestational age, antenatal complications, maternal history, time of birth, birth weight, type of delivery, delivery complications, Apgar scores, need for resuscitation at birth, stabilisation and multi – organ evaluation. All of this data on clinical parameters was collected from each study infant and inputted into an encrypted password protected Excel database. The presence of a normal MRI brain scan, normal clinical examination and Sarnat Stage were used as surrogate short-term markers of neurodevelopmental status. Neurodevelopmental at 2 years of age for infants is planned, however these results are not available yet and form part of another study.
2.4.2 Neuro Imaging

Cranial Ultrasound

Cranial ultrasound scanning of infants with NE in first few days of life is standard practice. The results do not usually allow a precise diagnosis or prognosis but they do provide very useful information on the presence structural abnormalities or evidence of long-standing damage. Cranial ultrasound (US) provide useful adjuncts to MRI in early diagnostic imaging \(^{137,138}\). Cranial ultrasound scans (CrUSS) were carried out and clinically reported over the first week of life on nearly all infants in the study group by a consultant paediatric radiologist.

Magnetic Resonance Imaging

MRI helps to understand the patterns of brain injury sustained and provides an accurate method of timing of the insult. MRI is the gold standard technique to detect patterns of cerebral damage in NE and provide a reliable guide to prognosis \(^{139}\). Conventional MRI with T1 and T2 weighted images has been demonstrated to have good diagnostic ability to see brain damage at the end of the first week of life, however with the advancement of diffusion weighted imaging (DWI) \(^{140}\) and magnetic resonance spectroscopy (MRS) lesions can be visualised within the first few days.

Mild to moderate injury in NE involves parasagittal watershed infarcts between anterior/middle cerebral artery and middle/posterior cerebral artery, with both cortical and subcortical involvement. Severe NE results in injury to metabolically active tissues such as the basal ganglia, thalami, putamen, hippocampi, brainstem and corticospinal tracts \(^{141}\). Abnormalities in the signal of the posterior limb of the internal capsule, basal ganglia (BG) and thalami have been demonstrated to have the greatest predictive value of poor neurodevelopmental outcome \(^{142,143}\).

Magnetic Resonance Imaging (MRI) of the brain was performed on all infants with NE (with a few exceptions) between postnatal day 5 and 12 using a 1.5 Tesla scanner in the radiology departments of the Children’s Hospital Ireland at Temple St and Crumlin and National Maternity Hospital, Holles St, Dublin.

During the scanning procedure a number of different sequences were acquired. T1 weighted (T1W) were performed. They differentiate fat from water (water appears darker and fat brighter) and provide good grey matter/white matter contrast. T1 FLAIR: Fluid Attenuated Inversion Recovery is used to null signal from fluids. In brain imaging it is used to suppress cerebrospinal fluid and thereby brings out periventricular hyperintense lesions. T2-weighted (T2W) differentiates fat from water however fat appears darker and water brighter. These scans are particularly suited to imaging oedema. Spectroscopy was performed as a non-invasive examination on biochemical brain biomarkers. Diffusion Weighted Imaging (DWI) measured the
diffusion of water molecules in biological tissues. Scans were scored and reported independently by paediatric radiologists (Dr Gabrielle Colleran and Dr Angela Byrne) who were blinded to the clinical results and outcome, using the well validated Barkovich scoring system. The Barkovich scoring system involves examining the basal ganglia, the watershed score, the combination basal ganglia/watershed score and the summation of the basal ganglia and watershed score. The score was well correlated to clinical outcome and has been validated in a number of studies since.

2.5 Phlebotomy

Whole blood sampling was performed between day one and day seven life, following informed parental consent. Sampling was performed using aseptic technique via central and peripheral arterial lines and via venous sampling at times of routine patient phlebotomy. The blood was collected in sodium citrate tubes, transferred on ice to TTMI and processed immediately. The volume taken was 1 -1.4mls which was approved by the Ethics committee, as a volume that would not affect the infant’s haemodynamic status. The maximum sampling taken from any infant was two timepoints. The blood was split between different experiments, depending on volume, timeline during thesis and laboratory availability. These were the minimal volumes that were required to perform each experiment.

Table 2.3: Required blood volumes for experiments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocyte and Monocyte Activation markers</td>
<td>300 μL</td>
</tr>
<tr>
<td>Mitochondrial Mass and Reactive Oxygen Species</td>
<td>700 μL of blood</td>
</tr>
<tr>
<td>PCR</td>
<td>700 μL of blood</td>
</tr>
<tr>
<td>Adenosine</td>
<td>10 μL of blood</td>
</tr>
<tr>
<td>Serum ELISA</td>
<td>minimum 300 μL of blood</td>
</tr>
</tbody>
</table>
2.6 Equipment and Re-agents

Appendix IV

2.7 Sample Processing

![Flow Diagram of blood processing with samples post phlebotomy](image)

Figure 2.1 Flow Diagram of blood processing with samples post phlebotomy

2.8 Multiplex-Enzyme-Linked-Immunosorbent-Assay (ELISA)

The principles of the multiplex ELISA assay involved examining cytokines via a sandwich immunoassay format, where capture antibodies were coated in a patterned array on the bottom of the wells of a plate to which the serum sample was added. The multi-spot array allowed simultaneous measurement of multiple analytes in the same well, therefore conserving sample volume. The plate was then analysed on the SECTOR Imager, via electrochemiluminescence detection technology.
Whole blood was processed immediately on arrival to laboratory, always within four hours of phlebotomy. The minimum volume of 300μL was split into two 150μL volumes. The whole blood was incubated with Endotoxin (Lipopolysaccharide (LPS)) or Phosphate buffer solution (PBS). LPS 1μL/mL was added to the first sample to make a final concentration of 10 nanograms/ml in blood. PBS was added at a concentration of 1 μL/mL. The incubation was performed for one hour at 37°C. The whole blood was then centrifuged at 1500 rpm for 10 minutes at temperature of 4°C. The plasma was then separated and stored at minus 80°C for later batch processing. The plasma was defrosted and analysed in two different batches for Enzyme Linked Immunosorbent Assay (ELISA).

A 14 spot Elisa Multiplex Cytokine Array with Meso Scale Delivery was performed. (www.meso-scale.com). Cytokines were analysed using a multi-spot 96-well. There was a 5-spot and a 10-spot human serum plasma plate customised for the study..

Interferon-gamma (IFNγ), interleukin (IL)-1β, IL-1α, IL-2, IL-6, IL-8, granulocyte-macrophage colony stimulating factor (GM-CSF), tumour necrosis factor (TNF)-α, TNF-β, IL-18, vascular endothelial growth factor (VEGF), IL-10, IL-1ra, and erythropoietin (Epo) were studied.

Firstly, the individual U-plex-Coupled antibody solutions were prepared. The U-plex plate was prepared by creating the U-plex Coupled Antibody Solutions. 200μL of each individual biotinylated antibody was coupled to 300 μL of a unique linker. The appropriate linker was used for each antibody. This mixture was vortexed and incubated at room temperature for 30 minutes. 200 μL of Stop Solution was then added, vortexed and incubated at room temperature for 30 minutes. The multi-plex coating solution was prepared by adding 600μL of each U-Plex coupled antibody solution into a single tube and vortexed. Up to 10 antibodies were pooled. The antibody solution with the same linker were not combined. 50μL of multiplex coating solution was added to each well. The plate was sealed with an adhesive plate seal and incubated with shaking at room temperature for an hour. The plates were washed 3 times with 150μL/well of 1xMeso Scale Discovery (MSD) wash buffer solution (https://www.mesoscale.com). The plate was then coated and ready to use. Calibrator Standard solutions plus a zero-calibrator standard were prepared. Each vial of calibrator was reconstituted by adding 250μL of Diluent 43 to the glass vial and inverted 3 times. The reconstituted solution was left at room temperature for 15-30 mins and vortexed briefly. The Calibrator Standard 1 was prepared by adding 50μL of the calibrator to Diluent 43 to make up a final volume of 250μL. The Calibrator Standard 2 was prepared by adding 75μL of Calibrator Standard 1 to 225μL of Diluent 43, following which 4-fold serial dilutions were repeated to generate a total of 7 Calibrator standards. They were mixed well by vortexing between each sample dilution. To prepare the detection antibody solution, diluent 3 was added to bring the final volume from 60μL to 6mL from the stock solution. To prepare the Read buffer 10mL of the Read Buffer was combined with 10mL of deionised water.
To prepare the assay, 25µL of Diluent 43 was added to each well. The plate was tapped gently on all sides. 25µL of the prepared Calibrator solution was added to the calibration wells and 25µL of serum samples were added to each serum well. The plate was sealed by an adhesive seal and incubated at room temperature with shaking for 1 hour. The plate was washed 3 times with MSD Wash Buffer. 50µL of detection antibody solution was added to each well. The plate was sealed by an adhesive seal and incubated at room temperature with shaking for 1 hour. Read Buffer was added to each well. The plate was analysed on an MSD instrument. The results were displayed in picograms/ml, and analysed on graphpad prism software (https://www.graphpad.com).

2.9 Flow Cytometry

Flow cytometry involves the detection and measurement of certain characteristics of a cell population of interest. The sample containing cells of interest is suspended in fluid, taken up by the instrument and the fluidics system organises the suspension into a single stream of cells. This liquid stream is passed through a laser light beam, and the interaction with the light is measured by an electronic component as light scatter and fluorescence intensity. If a fluorescent label is bound to a cell, the fluorescence intensity represents the amount of that cell or cell component.

There were two flow cytometry experiments completed during the thesis. The first, PANEL A, examined neutrophil and monocyte activation markers of Toll-like receptor 4 (TLR-4), CD11b and Nitrogen Oxide (Nox). The second experiment was a mechanistic experiment looking at mitochondrial mass and mitochondrial reactive oxygen species in monocytes and neutrophils.

2.9.1 Flow Preparation, Panel A

The expression of CD11b, Toll-like receptor 4 (TLR-4) and Nitrogen Oxide (Nox) antigens on the surface of neutrophils and monocytes were measured by flow cytometry. Whole blood was divided into three eppendorfs of 100uL each. The blood was incubated at 37°C for one hour, one eppendorf with LPS at a concentration of 10 nanograms/mL, one eppendorf with PBS and one with no added reagent (unstained). An anti-body cocktail to identify leucocyte subsets of granulocytes and monocytes was added, alongside CD11b, TLR-4 and NOX at a volume per 100µL of blood.
Table 2.4 Antibody cocktail preparation for Panel A

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Tag</th>
<th>1x (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>PerCP</td>
<td>5</td>
</tr>
<tr>
<td>CD15</td>
<td>PECy7</td>
<td>5</td>
</tr>
<tr>
<td>NOX</td>
<td>FITC</td>
<td>5</td>
</tr>
<tr>
<td>CD66B</td>
<td>Pacific Blue</td>
<td>5</td>
</tr>
<tr>
<td>TLR4</td>
<td>APC</td>
<td>5</td>
</tr>
<tr>
<td>CD11b</td>
<td>PE</td>
<td>20</td>
</tr>
</tbody>
</table>

45µL of this antibody cocktail was added to each the Vehicle and LPS eppendorf and incubated at room temperature for 30 minutes in the dark. 1mL FACS lyse was added to each eppendorf. They were vortexed and then incubated in dark for 15mins, with a brief vortex half way through. They were then centrifuged at 1500 RPM for 7 minutes. The supernatant was discarded by tapping out and phosphate buffer acetate (PBA) was added to the pellet. They were then centrifuged at 1500 RPM for 7 minutes, and this step of discarding supernatant, washing with PBA and tapping out supernatant was repeated. The remaining volume was transferred to FACS tubes for analysis and resuspended in a small volume of PBA. The samples were analysed immediately on the BD FACSCANTO.

Compensation controls were prepared during the experiment comprising of a positive and negative control of One Comp beads, 5µL of the corresponding fluorophore-labelled antibody and 500µL of PBA in a FACS tube.
2.9.2 *BD FACS CANTO PROTOCOL for Panel A*

The fluorescence intensity was assessed by flow cytometry and expressed as mean channel fluorescence (MCF). Mean channel fluorescence is the average intensity of fluorescence emitted by all cells chosen for measurement, and is comparable to the relative number of receptors present on the surface of each cell. A minimum of 5000 events was collected and analysed. All measurements were performed under the same instrument settings.

The FACSCanto II was set up as per standardised protocol on each occasion. Upon starting up the machine, the start-up involved assessing tank fluid levels of sheath fluid, waste, shutdown solution and cleaning solution, and priming tanks if necessary. Fluids start-up was then performed. The cytometer performance was checked, with mandatory running of CS+T beads if they had not been run in a week or system summary during start up alerted that it required attention. When the machine was ready for analysis the experiment was opened, with a blank template on each occasion. The compensation set up was performed with compensation controls. Compensation beads singly stained with every fluorophore channel utilised were acquired to adjust for spectral overlap. The threshold was set at 5000 and a channel chosen for each fluorophore.

### Table 2.5 Compensation Bead set up for Panel A

<table>
<thead>
<tr>
<th>Antigen/Measurement</th>
<th>Fluorophore</th>
<th>Channel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nox</td>
<td>FITC</td>
<td>FL1 (FITC)</td>
</tr>
<tr>
<td>CD11b</td>
<td>PE</td>
<td>FL2 (PE)</td>
</tr>
<tr>
<td>CD14</td>
<td>PerCP</td>
<td>FL4 (PerCP)</td>
</tr>
<tr>
<td>CD15</td>
<td>PECy7</td>
<td>FL5 (PECy7)</td>
</tr>
<tr>
<td>CD66b</td>
<td>Pacific Blue</td>
<td>FL6 (Pacific Blue)</td>
</tr>
<tr>
<td>TLR4</td>
<td>APC</td>
<td>FL8 (Alexa Fluor 647)</td>
</tr>
</tbody>
</table>
After recording and calculating all of the compensation controls, the samples of Unstained, Vehicle and LPS were acquired. The FSC was set to 50,000. Granulocytes and monocytes were identified on the basis of their size and granularity (forward scatter; FSc, and side scatter; SSc, respectively), followed by labelling with CD66b positive for granulocytes, and CD66b negative and CD14 positive for monocytes. The gating strategy was performed as per referenced framework by Prabhu et al. The data was exported via FCS files. The machine was cleaned and shutdown was performed. Results were analysed with FloJo LLC software.

2.9.3 Mitochondrial Fluorescence and Reactive Oxygen Species Experiment

This experiment involved examining the mitochondrial mass fluorescence and mitochondrial reactive oxygen species. Prior to the final protocol being established, the experiment was optimised with adult blood. The optimisation protocol included 16 FACS tubes which were prepared to optimise monocyte and granulocyte identification by selecting the most suitable antibodies and dosages. The Fluorescence Minus One (FMO) Control is used to identify and gate cells in the context of data spread due to the multiple fluorophores in a given panel. Each FMO control contains all the fluorophores in a panel, except for the one for which the gates are being set. The FMO control ensured that the any spread of the fluorophores into the channel of interest was properly identified and allowed setting of gates accurately. FMOs were prepared for CD15, CD66b, Dead cell stain, CD14, Mitotracker Green (MTG) and Mitosox red. The concentrations of MitoSox and MTG were optimised by titration of dosages. The dose response range of Mitosox examined was from 1µM to 15µM. The Mitotracker Green (MTG) dose response range was from 200nM to 700nM. The final concentration of Mitosox chosen was 1.4µM and the MTG dosage was 15µM.

The final protocol involved seven tubes and 700 µL of blood. The tubes were labelled MTG, MitoSox Red, Unstained, CD14 FMO, CD16 FMO, Veh control and LPS. 100µL whole blood was added to each FACS tube. 1µL LPS (@100ng/mL) was added to the LPS-labelled tube. 1µL PBS was added to the Veh Control tube. Mitotracker Green (MTG) and MitoSOX dilutions were made up as follows just prior to use. MTG: (final conc = 1.4µM). Frozen stock aliquots of 2mM were diluted 1 in 143 with PBS to working solution of 14µM by adding 2µL MTG to 284µl PBS. MitoSOX: (final conc = 15µM). Frozen stock aliquots of 5mM were diluted 1 in 33.3 with PBS to working solution of 150µM by adding 2.2µL MTG to 71.1µL PBS.

For the mitochondrial stains, 12µL MTG was added to tubes 1, 4, 5, 6 and 7. 12µL MitoSOX was added to tubes 2, 4, 5, 6 and 7. 12µL PBS was added to tubes 1 and 2. 24µL PBS was added
to Tube 3. All samples were mixed on the vortex for 5-10 secs. Samples were all placed on the heat block at 37°C for 1 hour. During the incubation period an antibody cocktail was prepared.

**Table 2.6 Antibody cocktail for Mitochondrial Fluorescence and Reactive Oxygen Species Experiment**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Tag</th>
<th>1x (µL)</th>
<th>2.5x(µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD15</td>
<td>PeCy7</td>
<td>5</td>
<td>12.5</td>
</tr>
<tr>
<td>CD66b</td>
<td>PacificBlue</td>
<td>5</td>
<td>12.5</td>
</tr>
<tr>
<td>CD14</td>
<td>APC</td>
<td>5</td>
<td>12.5</td>
</tr>
<tr>
<td>CD16</td>
<td>V500</td>
<td>5</td>
<td>12.5</td>
</tr>
<tr>
<td>PBS</td>
<td>-</td>
<td>20</td>
<td>50</td>
</tr>
</tbody>
</table>

After the hour incubation the following antibodies were added: 40µL PBS to Tubes 1, 2 and 3. 5µL CD15-PECy7 + 5µL CD66b-PacificBlue + 5µL CD16-V500 + 25µL PBS were added to tube 4. 5µL CD15-PECy7 + 5µL CD66b-PacificBlue + 5µL CD14-APC + 25µL PBS were added to tube 5. 40µL Mitochondrial panel antibody cocktail were added to tubes 6 & 7. The samples were vortexed briefly and stained in the dark for 30 minutes. 1mL FACS was added to all tubes and they were vortexed twice during a 15 minute incubation in the dark. The tubes were centrifuged @ 1500rpm for 7 mins at room temperature. The supernatant was discarded by tapping the liquid out onto tissue. 1mL PBA was added to each tube to wash the cells. The tubes were then centrifuged @ 1500rpm for 7 mins at room temperature. The supernatant was again discarded and the step of washing with PBA, centrifuging and discarding supernatant was repeated. The remaining volume was resuspended and transferred to pre-labelled FACS tubes. A small volume of PBA was added using a pastette to bring the volume to the first ridge on tube on the FACS tube. The samples were immediately analysed on the BD FACS CANTO.

**2.9.4 Analysis on FACS Canto II**

The FACS Canto II was set up as per standardised protocol on each occasion. Upon starting up the machine, the start-up involved assessing tank fluid levels of sheath fluid, waste, shutdown solution and cleaning solution, and priming tanks if necessary. Fluids start-up was then performed. The cytometer performance was checked, with mandatory running of CS+T beads if
they hadn’t been run in a week or system summary during start up alerted that it required attention. When the machine was ready for analysis a standardised experiment template was opened that was duplicated for each experiment.

The compensation set up was performed with compensation controls. Compensation beads singly stained with every fluorophore channel utilised were acquired to adjust for spectral overlap. The threshold was set at 5000 and a channel chosen for each fluorophore. The compensation beads for FITC and PE were live compensation beads (unlike other compensation beads that involved OneComp beads and an addition of fluorophore-labelled antibody at dilution used in the experiment, these were made up fresh on each occasion with the patients’ blood, and PE and FITC fluorophores as Mitosox and MTG, so the the FSC threshold was set higher as there was other particles to be excluded from the sample, so it was set at 50,000 rather than 5,000). The duplicated template was opened and the CS& T beads were run as an endogenous control to ensure the FITC and PE peaks were within a narrow range between set gates. The voltage of PE and FITC were adjusted slightly to ensure the experiment was standardised on each occasion if the peaks did not lie within the gates. This standardisation meant that fluorescence intensities could be compared across experiments. The Fluorescence Minus One (FMO) controls were used to identify and gate cells in the context of data spread due to the multiple fluorophores in the panel. Each FMO control contains all the fluorophores in the panel, except for the one for which gates are being set. The FMO control ensured that any spread of the fluorophores into the channel of interest is properly identified and allowed the gates to be accordingly set. The FMO allowed identification of CD14 on non-classical monocytes which have low expression and CD16 on monocytes where the fluorescence is a continuum and ensured that the gates were set appropriately.

The samples of Unstained, Vehicle and LPS were then acquired. The FSC threshold was set to 50,000. A minimum of 100,000 events were recorded on each occasion. After all FACS tubes were run, the experiment was exported to a FCS file USB for further analysis with Flow Jo software. The FACS canto machine was cleaned as per standardised protocol to shut down the machine.

2.9.5 Flow Jo Analysis

The samples were analysed via FlowJo software. The initial steps involved to investigating and quantifying granulocytes and monocytes. They were identified on the basis of their size and granularity (forward scatter; FSc, and side scatter; SSc, respectively). The light scatter patterns of granulocytes and monocytes allow them to be distinguished from debris and dead cells. This was followed by labelling with CD66b positive for granulocytes, and CD66b negative and
CD14 positive for monocytes. The CD 14 and CD16 FMO ensured standardised way of dividing monocytes into their distinct populations in the context of the data spread due to the multiple fluorochromes in the panel.

This gating strategy was performed as per referenced framework by Prabhu et al. and in the figures below. After positive identification of monocyte and granulocyte populations, the median channel fluorescence of MitoSox and Mitotracker green was calculated for each.
Figure 2.3: Endogenous Control of examining FITC and PE fluorescence.

FITC and PE were run as an endogenous control to ensure their fluorescence was standardised on each occasion so that mean fluorescence intensities could be compared across experiments.
Figure 2.4 Gating Strategy for Mitochondrial Fluorescence and Reactive Oxygen Species Experiment: Monocyte Gating Strategy

The figures are the gating strategies performed to isolated monocytes. (i) CD14 (APC) FMO Sample All Cells, removing lymphocytes based on forward scatter and side scatter. (ii) Based on side scatter and CD66B (Pacific Blue), CD66b (Pacific Blue) negative cells gated on, to exclude granulocytes. (iii) CD16 (V500) was plotted on the Y axis and CD 14 (APC) on the X axis. A vertical gate was included to allow for <1% of cells in Q3, to adjust for spectral overlap. These gates were then cut and pasted into the CD16 FMO. (iv) The gates from CD14 FMO were pasted into this sample, gating on all cells, removing lymphocytes based on forward scatter and side scatter. (v) Based on side scatter and CD66B (Pacific Blue), CD 66b (Pacific Blue) negative cells were gated on, to exclude granulocytes. (vi) Based on cd14/cd16 axes, a horizontal gate was
drawn to allow for <1% of cells in Q1&2 in total, to adjust for spectral overlap. These gates were then cut and pasted into the Vehicle and LPS treated FCS files. (vii) The Gates from the CD16 FMO were then cut and pasted into the Vehicle and LPS FCS files. This example is the vehicle file. All cells were included and lymphocytes were excluded based on FSC SSC. (viii) CD66b (Pacific Blue) negative cells were taken to exclude granulocytes. (ix) The monocytes were gated on and divided into classical, non-classical and intermediate monocytes based on cd14 cd 16 from the gates made in the above CD 14 and CD 16 FMO. Following this, the mean channel fluorescence of Mitosox and Mitotracker green were evaluated for each population of monocytes; an example shown below:

<table>
<thead>
<tr>
<th><strong>Non Classical Monocytes MCF</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MTG  62971</td>
<td></td>
</tr>
<tr>
<td>MitoSox 11938</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Intermediate Monocytes MCF</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>MTG126122</td>
</tr>
<tr>
<td>MitoSox 37619</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Classical Monocytes MCF</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>MTG  57999</td>
</tr>
<tr>
<td>MitoSox 12674</td>
</tr>
</tbody>
</table>
Figure 2.5 Gating Strategy for Mitochondrial Fluorescence and Reactive Oxygen Species Experiment Granulocyte Gating Strategy

(i) SSc-A plotted against FSC-A, with lymphocytes removed. (ii) SSc-A plotted against CD66b, CD66b+ cells selected as Granulocytes. Following this, the MCF of Nox, TLR4 and CD11b of the granulocytes was evaluated.
2.10 Purinergic Measurement in NE

The adenosine experiment involved a novel enzyme-based amperometric biosensor (SMARTchip) that can detect a summed concentration of the purine nucleoside, adenosine. The experiment involves a gel matrix anchored to a gold plated electrode contains a layer of enzymes, which catalyze an iterative sequence of reactions, leading to the formation of electroactive hydrogen peroxide proportional to the concentration of adenosine in the blood. This, in turn, is converted into an electrical signal. The raw data is subsequently calibrated to a 10 µM adenosine buffer solution. Measurements were taken from whole and placed onto the gel-matrix-coated electrode and results were produced immediately.

The Potentiostat software and laptop were set up. The SMARTChip was calibrated with blank buffer followed by calibration buffer, using blotting paper in between. 10µL of fresh whole blood was applied to the SMARTChip immediately following calibration. Due to the very short half-life of adenosine and the equipment required, this experiment was done on a small number of patients in the CWIUH. Collaborators in RCSI, Dr Tobias Engel completed point of care Adenosine measurements in the mice population, in healthy mice and in mice exposed to hypoxia146. Results were expressed in micromolar per litre, and as the average of four readings.

2.11 Polymerase Chain Reaction (PCR)

PCR is a technique to amplify small segments of DNA or RNA. Real time-PCR (RT-PCR), used in this thesis, couples the conversion of RNA into complementary DNA (cDNA) by viral reverse transcriptases with the amplification of the cDNA by thermostable polymerases. The greater the initial concentration of target sequences in the reaction mixture the fewer the number of cycles required to achieve a particular yield of amplified product.

The PCR reaction exploits the 5´ nuclease activity of AmpliTaq Gold® DNA Polymerase to cleave a TaqMan® probe during PCR. The TaqMan® probe contains a reporter dye at the 5´ end of the probe and a quencher dye at the 3´ end of the probe. During the reaction, cleavage of the probe separates the reporter dye and the quencher dye, which results in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye.
To achieve these results, RNA is first isolated and characterised for quantity and integrity, then cDNA is synthesised and used as a PCR template. The steps on the real time PCR machine involves four phases of the PCR amplification curve; the linear ground phase, early exponential phase, the exponential phase, and plateau phase.

To calculate the results, the ΔΔCt method allows assessment of relative gene expression by comparing the gene expression of experimental samples to the mean of control samples, rather than quantifying the exact copy number of the target gene. The fold-difference (increase or decrease) can be compared between experimental and control samples. The fold-difference was assessed using the cycle time (Ct) difference between samples (i.e. based on the principle that samples in which the target gene amplifies at an earlier cycle time compared with another given sample, have greater quantities of the target gene to begin with).

The PCR assays were inventoried assays from Applied Biosystems. The genes examined were the circadian rhythm proteins, Brain and Muscle Arnt – like protein, (BMAL-1) CLOCK, REV-ERB β and cryptochrome circadian clock (CRY). Inflammasome proteins, Interleukin-1Beta, nucleotide- binding domain, leucine- rich repeat (NLR) proteins (NLRP3) and Apoptosis – associated speck like protein (ASC), Hypoxia inducible Factor (HIF)-1α, Tir-domain containing adapter-inducing-interferon (TRIF), MyD88 Innate Immune Signal Transduction Adaptor (MyD88) and Interleukin–1 Receptor Associated Kinase 4 (IRAK 4).

2.11.1 RNA Preparation

The laboratory bench was cleaned with RNAse Zap followed by 70% Ethanol. A minimum of 700µL of whole blood was split between vehicle and LPS RNA-ase free tubes. 1µL of 10ng/mL LPS and 1µL of PBS was added to the respective tubes. The blood was incubated on a heat block for an hour at 37°C. Following incubation, RNAlater® was added to each “RNA” tube (1.3mL per 300uL blood). RNA Stabilization Solution is an aqueous tissue storage reagent that rapidly permeates, stabilizes and protect RNA in blood. It eliminates the need to immediately process or freeze samples and allowed the samples to biobanked and stored for analysis at a later date. The eppendorfs were inverted and biobanked at -80ºC.
2.11.2 RNA Isolation

The RNA was isolated using the Ribopure Blood Kit Protocol for Samples in RNAlater Solution. The initial step involved cell lysis and initial purification. Samples were centrifuged for 1 minute at 13 000 rpm at room temperature. The supernatant was discarded by aspirating all the liquid above the pellet. 800μL of lysis solution and 50μL sodium acetate solution were added to the cell pellet. The sample was vortexed and inverted to ensure homogenisation. In the fume hood, 400μL of Acid-Phenol: Chloroform was withdrawn (from beneath the overlying aqueous buffer) and added to the sample. This combination was stored at room temperature for 5 minutes.

During this step, elution solution is added to an RNAse-free microtube. Each sample required 100μL of elution solution. For each sample, a filter cartridge was placed into one of the collection tubes supplied in the kit. The lids of the resulting filter cartridge assembly were labelled and as was a second collection tube for each sample, these were be used to elute the RNA from the glass fibre filter. The elution solution was heated to ~75°C in an RNAase-free tube.

The sample was centrifuged at room temp for 1 min to separate the aqueous and organic phases at 13 000 rpm. Using a p200, the aqueous phase containing the RNA was transferred to a new 2 mL tube, this volume was approximately 1mL. 500μL of 100% ethanol was added to each aqueous phase in the new 2mL tube. 700μL of the sample was applied to a filter cartridge assembly and centrifuged for 10 seconds to pass the liquid through the filter. The flow through was discarded from the collection tube. The filter cartridge was replaced in the same collection tube. The next ~700 μL of sample was passed onto the filter and centrifuged for 10 seconds to pass the sample through. The flow through was again discarded. This was repeated until all the sample was filtered. 700μL of Wash Solution 1 was applied to the filter cartridge assembly and centrifuged for 10 sec to pass the solution through the filter. The flow-through was discarded from the collection tube, and the filter cartridge was replaced into the same collection tube. 700μL of Wash Solution 2/3 was applied (working solution mixed with ethanol) to the filter cartridge assembly and centrifuged for 10 sec to pass the solution through the filter. This was repeated with a further 700μL aliquot of Wash Solution 2/3. After discarding the flow-through from the last wash, the filter cartridge was replaced in the same collection tube and centrifuged for 1 min at 13 000 RPM to remove residual fluid from the filter. The filter cartridge was transferred into a labelled collection tube. 50μL of preheated elution solution was applied to the centre of the filter, and the cap was closed. This was left at room temp for ~20 sec, and then centrifuged for 25 sec at maximum speed to recover the RNA. The second ~50μL of elution solution was added to the filter and centrifuged for 1 min to recover all of the elution solution in the collection tube. The filter was discarded and the eluted RNA was stored at –20°C.
2.11.3  Final Purification and DNAse digestion Step

DNAse buffer, enzyme and DNAse inactivation agents were taken from the -20°C to thaw. DNAse digestion removes contaminating genomic DNA from the eluted RNA. Given the RNA was eluted in 100 μL Elution Solution, 5 μL of 20X DNase Buffer and 1 μL DNase I (8 U/μL) were added to the eluted RNA and mixed gently. They were incubated for 30 min at 37°C. 20 μL of DNAse Inactivation Reagent was added to the sample, mixed gently and vortexed. This was vortexed twice during a two-minute incubation. The sample was then centrifuged for one minute to pellet the DNAse Inactivation Reagent, then the RNA solution was transferred to a new RNase-free tube.

2.11.4  RNA Quantification

Quantification of RNA and assessment of RNA purity and concentration were determined by using the NanoDrop ND-100 and analysed using ND-1000 Ver.3.1.2 software. The Nanodrop spectrophotometer was set at 260nm absorbance to determine RNA purity. The measurement surface was cleaned. 2μL RNase-free water was loaded onto the lower measurement pedestal and read as the “blank” measurement. The surfaces were cleaned to prevent sample carryover and the test samples were measured by loading 2μL of sample onto the pedestal followed by reading the RNA concentration and quality.

RNA purity from the samples was estimated by calculating the ratio between the absorbance values at 260 and 280 nm. For a pure RNA sample, a ratio of ≥1.6 (for RNA suspended in water) was considered acceptably pure. Ratios of 1.8 or greater are regarded as good quality RNA. A ratio of <1.6 for the RNA solution indicated possible contamination.

2.11.5  Synthesis of cDNA from template RNA

Real time-PCR (RT-PCR) couples the conversion of RNA into complementary DNA (cDNA) by viral reverse transcriptases with the amplification of the cDNA by thermostable polymerases. An appropriate amount of RNA was added to RNAse-free water depending on the RNA concentration to produce a final concentration of 1ug RNA in a volume of 20μls as shown. The samples were cooled on ice. The following 2X mastermix was prepared to include 1 reaction excess.
Table 2.7 Mastermix cocktail

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume per sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-MLV buffer (µL)</td>
<td>4 µL</td>
</tr>
<tr>
<td>dNTPs</td>
<td>1.6 µL</td>
</tr>
<tr>
<td>Primers</td>
<td>4 µL</td>
</tr>
<tr>
<td>Enzyme</td>
<td>2 µL</td>
</tr>
<tr>
<td>Water DEPC</td>
<td>8.4 µL</td>
</tr>
<tr>
<td>Total (µL)</td>
<td>20 µL</td>
</tr>
</tbody>
</table>

20µL of this mastermix was added to each sample tube and mixed briefly, making a final concentration of 40 µL. The samples were place on the thermocycler using the rt-cDNA program, 10 mins at 25°C and 120 mins at 37°C, 85°C for 5min, then hold at 4°C. The samples were then frozen at -80°C. The final concentration of the resulting cDNA, assuming 1µg of RNA was 50ng/µL.
Table 2.8: Table displaying the dilution Volumes of RNA and RNA-ase free water in making cDNA.

<table>
<thead>
<tr>
<th>RNA(conc) ng/μL</th>
<th>[RNA] ug/μL</th>
<th>Vol (μL) to give 1ug RNA</th>
<th>RNAse-free H₂O</th>
<th>final vol</th>
</tr>
</thead>
<tbody>
<tr>
<td>67.5</td>
<td>0.0675</td>
<td>14.8</td>
<td>5.2</td>
<td>20.0</td>
</tr>
<tr>
<td>67.5</td>
<td>0.0675</td>
<td>14.8</td>
<td>5.2</td>
<td>20.0</td>
</tr>
<tr>
<td>84.8</td>
<td>0.0848</td>
<td>11.8</td>
<td>8.2</td>
<td>20.0</td>
</tr>
<tr>
<td>89.2</td>
<td>0.0892</td>
<td>11.2</td>
<td>8.8</td>
<td>20.0</td>
</tr>
<tr>
<td>105</td>
<td>0.105</td>
<td>9.5</td>
<td>10.5</td>
<td>20.0</td>
</tr>
<tr>
<td>133.8</td>
<td>0.1338</td>
<td>7.5</td>
<td>12.5</td>
<td>20.0</td>
</tr>
<tr>
<td>123.1</td>
<td>0.1231</td>
<td>8.1</td>
<td>11.9</td>
<td>20.0</td>
</tr>
<tr>
<td>178.3</td>
<td>0.1783</td>
<td>5.6</td>
<td>14.4</td>
<td>20.0</td>
</tr>
<tr>
<td>207.3</td>
<td>0.2073</td>
<td>4.8</td>
<td>15.2</td>
<td>20.0</td>
</tr>
<tr>
<td>68.5</td>
<td>0.0685</td>
<td>14.6</td>
<td>5.4</td>
<td>20.0</td>
</tr>
<tr>
<td>153.8</td>
<td>0.1538</td>
<td>6.5</td>
<td>13.5</td>
<td>20.0</td>
</tr>
<tr>
<td>67.9</td>
<td>0.0679</td>
<td>14.7</td>
<td>5.3</td>
<td>20.0</td>
</tr>
<tr>
<td>128.1</td>
<td>0.1281</td>
<td>7.8</td>
<td>12.2</td>
<td>20.0</td>
</tr>
<tr>
<td>226.2</td>
<td>0.2262</td>
<td>4.4</td>
<td>15.6</td>
<td>20.0</td>
</tr>
</tbody>
</table>

The final volume is 20ul so the appropriate amount of RNA-se free water is added to 1ug of RNA for each sample.
2.11.6 RT PCR (Real-time Polymerase Chain Reaction)

The following genes were examined, circadian rhythm proteins, Brain and Muscle Arnt–like protein, (BMAL-1) CLOCK, REV-ERB β and cryptochrome circadian clock (CRY). Inflammasome proteins, Interleukin-1Beta, nucleotide-binding domain, leucine-rich repeat (NLR) proteins (NLRP3) and Apoptosis—associated speck like protein (ASC), Hypoxia-inducible Factor (HIF)-1α, Tir-domain containing adapter-inducing-interferon (TRIF), MyD88 Innate Immune Signal Transduction Adaptor (MyD88) and Interleukin–1 Receptor Associated Kinase 4 (IRAK 4).

The PCR was completed using TaqMan® Real time PCR. cDNA was diluted with RNAase free water to give final concentration of 5ng/µL. Each sample was measured in triplicate so for each gene to be measured, 6µL of sample at 5ng/µL was required (2µL per well in to give 10ng/well). The following master mix was prepared in the hood.

<table>
<thead>
<tr>
<th>Table 2.9 Mastermix for PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>For each Target gene</td>
</tr>
<tr>
<td>TaqMan Universal PCR buffer mix</td>
</tr>
<tr>
<td>Gene specific probe</td>
</tr>
<tr>
<td>RNase-free water</td>
</tr>
<tr>
<td>Total vol</td>
</tr>
</tbody>
</table>

The 96 well TaqMan plate was loaded with 2µL of sample or control in triplicate to each well. The gene specific probes for each gene, were added to the individual master mix. 8µL of endogenous control gene master mix were added to rows A and B in each well. In rows C and D, 8µLs of gene 1 master mix were added to each well. In rows E and F 8µL of gene 2 master mix were added to each well. In rows G and H, 8µL of gene 3 master mix were added to each well. The plate was covered with a seal and the plate was centrifuged for one minute at 1800 RPM. The real time PCR reaction was performed using an ABI 7500 sequence detection system for 2
minutes at 50°C, for 10 minutes at 95°C and 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. The 96 well Taqman plate is included in the Appendix (V).

2.11.7 PCR Analysis

The ΔΔCt method (Applied Biosystems 7300 RQ software) was used to assess gene expression for all RT-PCR analysis. This method was used to assess relative gene expression by comparing the gene expression of experimental samples to the mean of control samples, rather than quantifying the exact copy number of the target gene. The fold-difference (increase or decrease) can be compared between experimental and control samples. The fold-difference was assessed using the cycle time (Ct) difference between samples (i.e. based on the principle that samples in which the target gene amplifies at an earlier cycle time compared with another given sample, have greater quantities of the target gene to begin with).

A cycle difference of 1 (ΔCt=1) between 2 samples means a two-fold difference in gene expression. Cycle times were visualised in a linear amplification plot, and from this, an optimal baseline Ct was established. Each sample acts as its own control, meaning each samples own endogenous Ct value is subtracted from its Ct value for the given target gene. This accounts for discrepancies in cDNA quantity on the plate and is referred to as the ΔCt value. The average difference in cycle time (mean ΔCt) of control values is subtracted from the ΔCt value and this is referred to as the ΔΔCt value (i.e. the cycle time difference, corrected for β-actin and relative to the control group). To get the fold-difference in gene expression from control, the following formula was applied to all samples (2^(-ΔΔCt)), giving the fold-difference as a relative quantity (RQ).

2.12 Systematic Review

2.12.1 Methodology

The systematic review “Biomarkers In Neonatal Encephalopathy to Predict Outcome” was registered with Prospero. Prospero is an international prospective register of Systematic Reviews, where key features from the review protocol are recorded and maintained as a permanent record. The protocol was published on the Prospero website. The protocol is included in the appendix (VI). http://www.crd.york.ac.uk/PROSPERO/display_record.php?ID=CRD42017056763.
The search terms were decided on and tested amongst the databases until the search was completed in duplicate by two independent reviewers at the same time. The population of interest was Term Infants with Neonatal Encephalopathy. The indicator of interest was Systemic Biomarkers and the outcomes of interest included, Sarnat Grade of Neonatal Encephalopathy, death in the neonatal period and neurodevelopmental outcome.

2.12.2 The Search Strategy

This is the search strategy of four major databases, EMBASE, PubMed, World of Science and the Cochrane Library. A comprehensive search with these relevant search terms of online databases was performed.

**Embase**

1. 'biological marker'/exp OR 'cytokine'/exp
2. (Biomarker* OR ‘biological indicator’ OR ‘biological marker’ OR ‘serum marker’ OR ‘Immunologic Marker’ OR Cytokine*):ti,ab
3. #1 OR #2
4. 'hypoxic ischemic encephalopathy'/exp
5. (encephalopathy OR ‘Ischemia-Hypoxia’ OR ‘perinatal asphyxia’ OR ‘fetal anoxia’):ti,ab
6. #4 OR #5
7. 'newborn'/exp
8. (Neonat* OR newborn OR infant* OR perinatal):ti,ab
9. #7 OR #8
10. #3 AND #6 AND #9

**PubMed**

1. "Biomarkers"[Mesh] OR "Cytokines"[Mesh]
3. #1 OR #2
4. "Hypoxia-Ischemia, Brain"[Mesh]
6. #4 OR #5
7. "Infant, Newborn"[Mesh]
9. #7 OR #8
10. #3 AND #6 AND #9

**World of Science**

1. TS=(Biomarker* OR ‘biological indicator’ OR ‘biological marker’ OR ‘serum marker’ OR ‘Immunologic Marker’ OR Cytokine*)
2. TS=(encephalopathy OR ‘Ischemia-Hypoxia’ OR ‘perinatal asphyxia’ OR ‘fetal anoxia’)
3. TS=(Neonat* OR newborn OR infant* OR perinatal)
4. #1 OR #2 #3

**Cochrane Library**
1. [mh “Biomarkers”] OR [mh “Cytokines”]
2. (Biomarker* OR ‘biological indicator’ OR ‘biological marker’ OR ‘serum marker’ OR ‘Immunologic Marker’ OR Cytokine*):ti,ab,kw
3. #1 OR #2
4. [mh “Hypoxia-Ischemia, Brain”]
5. (encephalopathy or ‘Ischemia-Hypoxia’ or ‘Ischemia Hypoxia’ or ‘perinatal asphyxia’ or ‘fetal anoxia’):ti,ab,kw
6. #4 OR #5
7. [mh “Infant, Newborn”]
8. (Neonat* OR newborn OR infant* OR perinatal):ti,ab,kw
9. #7 OR #8
10. #3 and #6 and #9

Two independent reviewers used Covidence software (https://www.covidence.org/home) to select the studies. Covidence is the primary screening and data extraction tool for systematic reviews. Two independent reviewers applied a double screening on titles and abstracts. The included papers after screening were read in full while applying inclusion criteria. Discrepancies with the authors were resolved by a third reviewer. The two reviewers independently completed the full data extraction form and a risk of bias assessment. Quality assessment was performed using Cochrane Collaboration Tool for Risk of Bias Assessment and quantitative analysis was completed using Revman software (5.3).

The results of the systematic review are presented in tables, figures and visually in forest plots. Each figure represents one meta-analysis, The first column shows the included studies. Studies are represented by the name of the first author and the year of publication. The next columns include the mean and standard deviations of the biomarker in normal and abnormal outcome groups. The next column visually displays the study results. The boxes show the effect estimates from the single studies, while the diamond shows the pooled result. The horizontal lines through the boxes illustrate the length of the confidence interval. The longer the lines, the wider the confidence interval meaning the less reliable the study results. The width of the diamond serves the same purpose. The vertical line is the line of no effect, if the diamond touches the vertical line, the overall (combined) result is not statistically significant. The p-value indicates the level of statistical significance and if the diamond shape does not touch the line of no effect, the difference found between the two groups was statistically significant. The I^2 indicates the level of of heterogeneity, from 0% to 100%.
2.13 Patient Participation in Research

To involve and engage parents in this study, apart from recruitment and communication opportunistically on those occasions, this studied involved creating a parent focus group. There was a number of meetings exploring parental perspectives on their infants Neonatal Intensive Care Unit (NICU) course and their experience of their infant having therapeutic hypothermia (TH). Following this a decision was made to disseminate information with via science communication of two short animations. The first animation was an introduction to the NICU and the second animation explained therapeutic hypothermia.

2.14 Statistics

The software used for statistical support was Microsoft Excel version, GraphPad prism version and SPSS. Student’s t test was used to compare mean measurements between two independent groups. The cytokine data in Chapter 4 was not normally distributed, therefore median, interquartile and minimum and maximum values is how the values were represented. For analysis, Wilcoxon rank test analysed the differences between baseline and LPS stimulated levels. Mann Whitney test was used to analyse differences between healthy controls and infants with NE. The Friedman test was used to analyse differences between the groups post stimulation with LPS. In analysis of most results, a p value threshold of 0.05 was considered statistically significant. In Chapter 4, the Bonferroni correction test was used to prevent Type one errors of accepting a false positive result. The adjusted p value of $\leq 0.0035$ was deemed statistically significant in the cytokine Chapter due to the multiple comparisons being done. This was calculated from the analysis of 14 different cytokines, so the typical p value of 0.05 was divided by 14 giving a p value of $p=\leq 0.0035$. This was done on the advice of Centre for support and training in analysis and research (CSTAR, http://www.cstar.ie) following consultation.
Chapter 3 - Systematic Review; Biomarkers of Neonatal Encephalopathy to predict outcome

Introduction

Neonatal Encephalopathy (NE) describes central nervous system dysfunction from all causes and has a multifactorial aetiology. NE is difficult to diagnose, to treat and to predict outcome. In recent years there has been extensive research in blood, urine and cerebrospinal fluid (CSF) biomarkers in NE. The biomarkers may help identify infants at high risk of NE, objectively classify severity of NE, predict response to treatment and prognosticate outcome. Despite this research, there is no gold standard biomarker known at present and predicting outcomes in particular remains a significant challenge. Identifying prognostic biomarkers has implications for counselling parents on expected neurodevelopmental outcomes, future adjunctive therapies and guiding research.

The methodology of this systematic review are discussed in detail in the methods chapter, Chapter 2. In summary, the population studied is term neonates with NE. The indicator is systemic biomarkers. The short term outcome studied includes death in neonatal period, severity of NE by standardised neonatal scoring systems and MRI in neonatal period. The long term outcome studied was death and standardised developmental assessment after 6 months of age. The systematic review was performed as per PRISMA guidelines, (http://www.prisma-statement.org).

The review was registered and published with PROSPERO, http://www.crd.york.ac.uk/PROSPERO/display_record.php?ID=CRD42017056763), an international prospective register of systematic reviews, The studies have been presented as random effects model. The option of random effects model (REM) was chosen over fixed effects model as not all the studies could be located firstly (small number) and then not all studies were included due to heterogeneity of statistics reported so the meta-analysis represent a pooled sample of studies.
3.2 Aim

To identify biomarkers that may predict both short term outcome and long term outcome in NE.

3.3 Hypothesis

We hypothesized that through a detailed systematic review of available evidence, a series of biomarkers will be established to predict short and long term outcome in NE and may guide future research in larger cohort studies.

3.4 Results

1613 papers were identified after duplicates were excluded. Following title and abstract screening there were 314 studies suitable for full text screening. The exclusion reasons included wrong population; animal model, adult population, preterm neonate population, no indicator or biomarker referenced and no correlation to neonatal outcome. Following full text review, 107 papers were included to examine serum biomarkers to predict short term outcome. The exclusion at full text review was mainly due to statistical reporting making data inclusion in the meta-analyses. 14 meta-analyses were initially planned but there was insufficient data to perform meta-analyses on IL-8, GFAP and S100. 71 papers were included for systemic biomarker to predict long term outcome. 7 meta-analyses were performed on long term outcomes (Figure 3.1).

3.4.1 Blood biomarkers short term outcomes

Serum levels of IL-1 Beta (Figure 3.2), troponin (Figure 3.3), lactate dehydrogenase (Figure 3.4), white cell count (Figure 3.5), lactate (Figure 3.6), nucleated red blood cells (Figure 3.7), ionized calcium, glucose (Figure 3.8), interleukin 6 (IL-6) (Figure 3.9), tumour necrosis factor alpha (TNF-alpha) (Figure 3.10), interleukin-8 (IL-8), glial fibrillary acidic protein: (GFAP), S100 (Figure 3.12) and neuron specific enolase (NSE) were all examined via meta-analyses for their abilities to predict short term outcome. Between 2 and 16 studies were included in each analysis. Raised mean serum levels of IL-6 (p value 0.04, 95% CI -44.5 to -0.66) and lactate (p value <0.001, 95% CI -6.19 to -2.81) were associated with worse short term outcome in NE (Table 3.1).
3.4.2 Long Term Outcome

71 papers were included to examine biomarkers to predict long term outcome. These 71 papers reported outcomes for over 20 different biomarkers. There was sufficient data to complete meta-analysis for 6 serum biomarkers (Table 3.2) and on MRI as an individual biomarker (Table 3.4). There was not sufficient data available to do analysis on cerebrospinal fluid biomarkers. (Table 3.3), apart from CSF-TNF-alpha which was examined as a long term biomarker (Figure 3.18).

IL-1 beta (Figure 3.13), IL-6 (Figure 3.14), NSE (Figure 3.15), TNF-alpha (Figure 3.16) S100 (Figure 3.17) were examined as serum biomarkers. Raised serum Interleukin-6 (p value <0.01, effect estimate 141, 95% CI 32 to 252) and neuron specific enolase (p value <0.01, effect estimate 43.7, 95% CI 5 to 83) are associated with adverse long term outcome in NE.

MRI brain was the best biomarker to predict outcome. The meta-analysis included 938 patients from 23 studies. Abnormal MRI brain was predictive of adverse outcome with an odds ratio of 18.78 (95% CI 12.53 to 28.14), (Table 3.4) and (Figure 3.19).
Records identified through database searching

Additional records identified through other sources

Records after duplicates removed (n = 1613)

Records screened (n = 1324)

Records excluded (n = 1299)

Full-text articles assessed for eligibility (n = 314)

Full-text articles excluded, (n = 207)

Studies included in quantitative synthesis (meta-analysis)
(n = 107 in short term
n = 71 in long term)

**Figure 3.1 Prisma Flow Diagram of Systematic Review**
### Table 3.1 Meta-Analysis results for short term biomarkers to predict outcome.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Studies</th>
<th>Participants</th>
<th>Effect Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1 beta</td>
<td>3</td>
<td>44</td>
<td>-15.94 [-49.63, 17.76]</td>
</tr>
<tr>
<td>Troponin</td>
<td>3</td>
<td>191</td>
<td>-1.29 [-2.84, 0.26]</td>
</tr>
<tr>
<td>Lactate Dehydrogenase</td>
<td>5</td>
<td>290</td>
<td>-947.66 [-2071.96, 176.65]</td>
</tr>
<tr>
<td>White Cell Count</td>
<td>2</td>
<td>122</td>
<td>2.04 [-0.91, 5.00]</td>
</tr>
<tr>
<td>Lactate</td>
<td>5</td>
<td>267</td>
<td>-4.50 [-6.19, -2.81]</td>
</tr>
<tr>
<td>Nucleated red blood cells</td>
<td>2</td>
<td>149</td>
<td>-13157.05 [-35873.07, 9558.97]</td>
</tr>
<tr>
<td>Ionized calcium</td>
<td>3</td>
<td>83</td>
<td>0.05 [-0.08, 0.18]</td>
</tr>
<tr>
<td>Glucose</td>
<td>4</td>
<td>149</td>
<td>0.05 [-0.84, 0.95]</td>
</tr>
<tr>
<td>IL-6</td>
<td>16</td>
<td>172</td>
<td>-22.57 [-44.49, -0.66]</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>9</td>
<td>170</td>
<td>-14.27 [-42.60, 14.05]</td>
</tr>
<tr>
<td>IL-8</td>
<td>11</td>
<td>0</td>
<td>Not estimable</td>
</tr>
<tr>
<td>GFAP</td>
<td>6</td>
<td>27</td>
<td>-0.07 [-0.10, -0.03]</td>
</tr>
<tr>
<td>S100</td>
<td>8</td>
<td>105</td>
<td>-10.65 [-22.78, 1.48]</td>
</tr>
<tr>
<td>NSE</td>
<td>8</td>
<td>31</td>
<td>-0.02 [-1.34, 1.30]</td>
</tr>
</tbody>
</table>

Table Abbreviations: IL-6: Interleukin 6, TNF-alpha: tumour necrosis factor alpha, IL-8: Interleukin 8, GFAP: Glial fibrillary acidic protein, S100, NSE: Neuron specific enolase.
3.5. Meta-analysis results of serum biomarkers in NE to predict outcome

The total number of patients included was 44. The confidence interval was -49.63 to 17.76 and p value 0.35
Figure 3.3: Troponin forest plot to predict short-term outcome.

Three studies had sufficient data to include in the troponin meta-analysis. 191 patients were included in this meta-analysis. The confidence interval was 2.84 to 0.26, p value 0.1.

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Normal</th>
<th>Abnormal</th>
<th>Mean Difference IV, Random, 95% CI</th>
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<tbody>
<tr>
<td>Agrawal 2012</td>
<td>0</td>
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<tr>
<td>Alima 2017</td>
<td>0</td>
<td>0</td>
<td>Not estimable</td>
</tr>
<tr>
<td>Boo 2005</td>
<td>0</td>
<td>0</td>
<td>Not estimable</td>
</tr>
<tr>
<td>Chalak 2014</td>
<td>0</td>
<td>0</td>
<td>Not estimable</td>
</tr>
<tr>
<td>Liu 2013</td>
<td>0</td>
<td>0</td>
<td>Not estimable</td>
</tr>
<tr>
<td>Montalbo 2014</td>
<td>0.075</td>
<td>0.207</td>
<td>31.7% -2.17 [-3.16, -1.17]</td>
</tr>
<tr>
<td>Shastry 2012</td>
<td>0.0475</td>
<td>0.2066</td>
<td>31.9% -1.81 [-2.78, -0.85]</td>
</tr>
<tr>
<td>Simovic 2009</td>
<td>0</td>
<td>0</td>
<td>Not estimable</td>
</tr>
<tr>
<td>Simovic 2012</td>
<td>0.015</td>
<td>0.0717</td>
<td>36.4% -0.07 [-0.12, -0.02]</td>
</tr>
<tr>
<td>Simovic 2014</td>
<td>0</td>
<td>0</td>
<td>Not estimable</td>
</tr>
</tbody>
</table>

Total (95% CI): 50

141 100.0% -1.29 [-2.84, 0.26]

Heterogeneity: Tau² = 1.72; Chi² = 29.49, df = 2 (p < 0.00001); I² = 93%

Test for overall effect: Z = 1.63 (p = 0.10)
Figure 3.4: Lactate dehydrogenase forest plot to predict short term outcome.

The confidence interval was -207.96 to 176.65, p value of 0.1.

<table>
<thead>
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<th>Study or Subgroup</th>
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<th>Abnormal</th>
<th></th>
<th>Mean Difference</th>
<th>IV, Random, 95% CI</th>
<th>Mean Difference</th>
<th>IV, Random, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baken 2014</td>
<td>701</td>
<td>583</td>
<td>29</td>
<td>2,833.7538</td>
<td>4,722.5959</td>
<td>65</td>
<td>21.7%</td>
<td>-129.75 [-3297.28, -962.23]</td>
</tr>
<tr>
<td>Karlsson 2010</td>
<td>1,358</td>
<td>612</td>
<td>11</td>
<td>3.110</td>
<td>2,349</td>
<td>28</td>
<td>23.6%</td>
<td>-1952.00 [-2894.24, -1009.76]</td>
</tr>
<tr>
<td>Liu 2013a</td>
<td>1,319</td>
<td>571</td>
<td>16</td>
<td>1,541</td>
<td>1,016</td>
<td>12</td>
<td>25.9%</td>
<td>-222.00 [-861.32, 417.32]</td>
</tr>
<tr>
<td>Thoresen 2012</td>
<td>2,304.5455</td>
<td>2,615.6405</td>
<td>33</td>
<td>7.066</td>
<td>10,163</td>
<td>4</td>
<td>1.2%</td>
<td>-4761.45 [-24760.93, 5238.00]</td>
</tr>
<tr>
<td>Yum 2017</td>
<td>1,241</td>
<td>1,340</td>
<td>71</td>
<td>1.014</td>
<td>340</td>
<td>21</td>
<td>27.6%</td>
<td>327.00 [-16.94, 670.94]</td>
</tr>
</tbody>
</table>

Total (95% CI)     | 160 | 130 | 100.0% | 947.66 [-2071.96, 176.65] | 4 |

Heterogeneity: Tau² = 1162728.54; Chi² = 33.32, df = 4 (P < 0.00001); I² = 88%
Test for overall effect: Z = 1.65 (P = 0.10)
122 patients were included in the meta-analysis. The confidence interval was -0.91 to 5.0. p value
267 patients were included in the meta-analysis. The confidence interval was -6.19 to -2.81, p value < 0.00001.

Figure 3.6: Serum lactate forest plot to predict short term outcome.

<table>
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<th>Study or Subgroup</th>
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<th>Mean Difference</th>
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<td></td>
<td>Mean</td>
<td>SD</td>
<td>Total</td>
</tr>
<tr>
<td>Bekken 2014</td>
<td>7.36</td>
<td>10.9</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O’Hare 2016</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shah 2004</td>
<td>7.1</td>
<td>4.7</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simovic 2014</td>
<td>7.54</td>
<td>4.07</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Total (95% CI)    | 134    | 133      | 100.0%         | -4.50  | -6.19   | -2.81         |

Heterogeneity: Tau^2 = 0.00; Chi^2 = 2.88; df = 3 (P = 0.41); I^2 = 0%

Test for overall effect: Z = 5.23 (P < 0.00001)
149 patients were included in the meta-analysis. Confidence interval: 35873 to 9958, p value

<table>
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<tr>
<th>Study or Subgroup</th>
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<th>Normal SD</th>
<th>Normal Total</th>
<th>Abnormal Mean</th>
<th>Abnormal SD</th>
<th>Abnormal Total</th>
<th>Weight</th>
<th>IV, Random, 95% CI</th>
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<tbody>
<tr>
<td>Boskabadi 2017</td>
<td>697.28</td>
<td>519.3</td>
<td>32</td>
<td>25,674</td>
<td>18,557</td>
<td>31</td>
<td>49.0%</td>
<td>-24976.72 [-31511.63, -18441.81]</td>
</tr>
<tr>
<td>Walsh 2013</td>
<td>2,017.2439</td>
<td>1,943.4048</td>
<td>41</td>
<td>3,809.4667</td>
<td>4,644.7269</td>
<td>45</td>
<td>51.0%</td>
<td>-1792.22 [-3273.95, -310.50]</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>73</td>
<td>76</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100.0%</td>
<td>-13157.05 [-35873.07, 9558.97]</td>
</tr>
</tbody>
</table>

Heterogeneity: $\tau^2 = 262915247.52; \chi^2 = 45.99; df = 1 (p < 0.00001); I^2 = 98\

Test for overall effect: $Z = 1.14 (p = 0.26)$.
Figure 3.8: Ionized calcium forest plot to predict short term outcome.

83 patients were included in the meta-analysis. The confidence interval is -0.08 to 0.18, p value 0.43.
149 patients were included in the meta-analysis. The confidence interval is 0.84 to 0.95, p value 0.9.
172 patients were included in the meta-analysis. The confidence interval is 4.5 to 0.66, p value 0.04.

Figure 3.10: IL-6 forest plot to predict short term outcome.

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Normal</th>
<th>Abnormal</th>
<th>Mean Difference IV, Random, 95% CI</th>
<th>Mean Difference IV, Random, 95% CI</th>
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<td>Study or Subgroup</td>
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<td>Mean</td>
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<tr>
<td>Ay 2006</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bartha 2004</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bharathi 2015</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>Bodabadi 2016</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>Calkovor 2011</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chaiak 2015</td>
<td>37.62</td>
<td>51.07</td>
<td>7</td>
<td>131.7333</td>
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<tr>
<td>Chaiak 2014</td>
<td>17.62</td>
<td>13.78</td>
<td>7</td>
<td>1,656.8</td>
</tr>
<tr>
<td>Foster-Barber 2001</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<td>Jenkins 2012</td>
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<td>0</td>
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<td>Jiang 2014</td>
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<td>O’Hare 2012</td>
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<td>0</td>
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<td>Orzick 2016</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Shang 2014</td>
<td>19.59</td>
<td>2.94</td>
<td>32</td>
<td>37.75</td>
</tr>
<tr>
<td>Shewra 2003</td>
<td>118.96</td>
<td>152.82</td>
<td>10</td>
<td>149.32</td>
</tr>
<tr>
<td>Tekgul 2004</td>
<td>2.67</td>
<td>4.17</td>
<td>12</td>
<td>23.31</td>
</tr>
</tbody>
</table>

Total (95% CI)       68 104 100.0% -22.57 [-44.49, -0.66]
Figure 3.11: TNF-alpha forest plot to predict short term outcome. 170 patients were included in the meta-analysis. The confidence interval is -42.6 to 140.5, p value 0.32.

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Normal</th>
<th></th>
<th></th>
<th>Abnormal</th>
<th></th>
<th></th>
<th>Weight</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Total</td>
<td>Mean</td>
<td>SD</td>
<td>Total</td>
<td>IV, Random, 95% CI</td>
</tr>
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<td>Barna 2004</td>
<td>C</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Not estimable</td>
</tr>
<tr>
<td>Bharathi 2015</td>
<td>C</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Not estimable</td>
</tr>
<tr>
<td>Celik 2015</td>
<td>19.65</td>
<td>10.75</td>
<td>7</td>
<td>20.37</td>
<td>17.95</td>
<td>18</td>
<td>24</td>
</tr>
<tr>
<td>Cholak 2014</td>
<td>4.4</td>
<td>8.27</td>
<td>7</td>
<td>13.24</td>
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<td>20</td>
<td>20</td>
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<td>O'Hare 2012</td>
<td>C</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Not estimable</td>
</tr>
<tr>
<td>O'Hare 2017</td>
<td>C</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Not estimable</td>
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<tr>
<td>Oygur 1996</td>
<td>39.1</td>
<td>56.27</td>
<td>2</td>
<td>55.83</td>
<td>234.82</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Shang 2014</td>
<td>44.32</td>
<td>4.64</td>
<td>32</td>
<td>90.23</td>
<td>7.37</td>
<td>42</td>
<td>24.4%</td>
</tr>
<tr>
<td>Silveira 2003</td>
<td>5.35</td>
<td>1.55</td>
<td>10</td>
<td>6.27</td>
<td>4.73</td>
<td>9</td>
<td>24.4%</td>
</tr>
</tbody>
</table>

Total (95% CI) 58 112 100.0% -14.27 [-42.60, 14.05]

Heterogeneity Tau^2: 852.97, Chi^2: 460.83, df = 4 (p < 0.00001), I^2: 99%
Test for overall effect: Z = 0.99 (p = 0.32)
Figure 3.12: S100 forest plot to predict short term outcome. 105 patients were included in the meta-analysis. The confidence interval is -2.70 to 1.48, p-value 0.09.

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Normal Mean</th>
<th>SD</th>
<th>Total</th>
<th>Abnormal Mean</th>
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<td>10.4</td>
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<td>7</td>
<td>33.0175</td>
<td>54.71</td>
<td>24</td>
<td>19.2%</td>
<td>-22.62 [-50.26, 5.03]</td>
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<td>0</td>
<td>Not estimable</td>
<td></td>
</tr>
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<td>Nagyovics 2001</td>
<td>3.15</td>
<td>2.3</td>
<td>22</td>
<td>18.25</td>
<td>28.38</td>
<td>7</td>
<td>33.2%</td>
<td>-15.10 [-36.15, 5.95]</td>
</tr>
<tr>
<td>Nagyovics 2003</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Not estimable</td>
<td></td>
</tr>
<tr>
<td>Pei 2014</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Not estimable</td>
<td></td>
</tr>
<tr>
<td>Ruka 2012</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Not estimable</td>
<td></td>
</tr>
<tr>
<td>Thongrung-lerneck 2004</td>
<td>32.83</td>
<td>29.15</td>
<td>16</td>
<td>25.5259</td>
<td>28.2206</td>
<td>29</td>
<td>47.5%</td>
<td>-2.70 [-20.29, 14.90]</td>
</tr>
</tbody>
</table>

Total (95% CI) 45 60 100.0% -10.65 [-22.78, 1.48]

Heterogeneity: Tau² = 0.00, Chi² = 1.68, df = 2 (P = 0.43), I² = 0%

Test for overall effect: Z = 1.72 (P = 0.09)
### Table 3.2 Serum biomarkers to predict long-term outcome in NE.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Studies</th>
<th>Participants</th>
<th>Effect Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1B</td>
<td>2</td>
<td>61</td>
<td>-0.69 [-2.35, 0.97]</td>
</tr>
<tr>
<td>IL-6</td>
<td>2</td>
<td>53</td>
<td>-142.48 [-252.21, -32.75]</td>
</tr>
<tr>
<td>IL-8</td>
<td>2</td>
<td>0</td>
<td>Not estimable</td>
</tr>
<tr>
<td>NSE</td>
<td>2</td>
<td>54</td>
<td>-43.69 [-82.72, -4.65]</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>2</td>
<td>61</td>
<td>-2.42 [-5.95, 1.12]</td>
</tr>
<tr>
<td>S100</td>
<td>3</td>
<td>120</td>
<td>0.63 [-6.14, 7.39]</td>
</tr>
<tr>
<td>S100B</td>
<td>1</td>
<td>24</td>
<td>-22.62 [-36.26, -8.98]</td>
</tr>
</tbody>
</table>

### Table 3.3 CSF biomarkers to predict long term outcome.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Studies</th>
<th>Participants</th>
<th>Effect Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-alpha</td>
<td>2</td>
<td>41</td>
<td>-16.74 [-69.28, 35.81]</td>
</tr>
<tr>
<td>S100</td>
<td>1</td>
<td>23</td>
<td>Not estimable</td>
</tr>
<tr>
<td>NSE</td>
<td>1</td>
<td>23</td>
<td>-0.39 [-0.77, -0.01]</td>
</tr>
</tbody>
</table>

### Table 3.4 MRI as biomarker to predict long term outcome in NE.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Studies</th>
<th>Participants</th>
<th>Statistical Method</th>
<th>Effect Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRI</td>
<td>23</td>
<td>938</td>
<td>Odds Ratio (M-H, Random, 95% CI)</td>
<td>18.78 [12.53, 28.14]</td>
</tr>
</tbody>
</table>
Patients were included in the meta-analysis. The odds ratio was 0.69, confidence interval 2.35 to 0.97, p value 0.42.

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Normal</th>
<th>Mean</th>
<th>SD</th>
<th>Total</th>
<th>Abnormal</th>
<th>Mean</th>
<th>SD</th>
<th>Total</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellik 2015</td>
<td>2.27</td>
<td>0.25</td>
<td>13</td>
<td>2.3</td>
<td>0.1</td>
<td>18</td>
<td>62.5%</td>
<td></td>
<td>-0.03 [-0.17, 0.11]</td>
</tr>
<tr>
<td>Cogur 1998</td>
<td>1.16</td>
<td>1.9236</td>
<td>11</td>
<td>2.94</td>
<td>2.8769</td>
<td>19</td>
<td>37.5%</td>
<td></td>
<td>-1.78 [-3.50, -0.06]</td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td>24</td>
<td>37</td>
<td>100.0%</td>
<td>-0.69 [-2.35, 0.97]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Heterogeneity: $\tau^2 = 1.14$, $\chi^2 = 3.94$, df = 1 ($p = 0.05$), $I^2 = 75$

Test for overall effect: $Z = 0.81$ ($p = 0.42$)
80 patients were included in the meta-analysis. The odds ratio was 142.48, confidence interval 25.21 to 32.75, p value is 0.01.

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Normal Mean</th>
<th>SD</th>
<th>Total</th>
<th>Mean</th>
<th>SD</th>
<th>Total</th>
<th>Weight</th>
<th>Mean Difference IV, Random, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Celik 2015</td>
<td>36.2</td>
<td>40.4</td>
<td>13</td>
<td>165.6</td>
<td>243.6</td>
<td>18</td>
<td>91.6%</td>
<td>-129.40 [-244.06, -14.74]</td>
</tr>
<tr>
<td>Chalak 2014</td>
<td>62</td>
<td>232.3</td>
<td>10</td>
<td>204</td>
<td>255,815.5898</td>
<td>5</td>
<td>0.0%</td>
<td>-5342.00 [-232,199.72, 221515.72]</td>
</tr>
<tr>
<td>Jenkins 2012</td>
<td>54.7</td>
<td>88.5</td>
<td>10</td>
<td>339.6</td>
<td>661.6</td>
<td>12</td>
<td>8.4%</td>
<td>-284.90 [-663.23, 93.43]</td>
</tr>
</tbody>
</table>

Total (95% CI) 45 35 100.0% -142.48 [-252.21, -32.75]

Heterogeneity: Tau² = 0.00; Chi² = 0.60, df = 2 (P = 0.74); I² = 0%
Test for overall effect: Z = 2.54 (P = 0.01)
Figure 3.15: NSE to predict long term outcome.

82.72 to 4.65, p value 0.03.

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Normal Mean</th>
<th>Normal SD</th>
<th>Normal Total</th>
<th>Abnormal Mean</th>
<th>Abnormal SD</th>
<th>Abnormal Total</th>
<th>Mean Difference IV, Random, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roka 2012</td>
<td>53.76</td>
<td>22.83</td>
<td>18</td>
<td>76.91</td>
<td>28.77</td>
<td>6</td>
<td>-23.15 [-48.47, 2.17]</td>
</tr>
<tr>
<td>Yerdu 2001</td>
<td>9.9</td>
<td>4.4</td>
<td>19</td>
<td>72.9</td>
<td>35.9</td>
<td>11</td>
<td>-63.00 [-84.31, -41.69]</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>37</td>
<td></td>
<td>17</td>
<td>100.0%</td>
<td></td>
<td>-43.69 [-82.72, -4.65]</td>
<td></td>
</tr>
</tbody>
</table>

Heterogeneity: Tau^2 = 65.147; Chi^2 = 5.57, df = 1 (P = 0.02); I^2 = 82% Test for overall effect: Z = 2.19 (P = 0.03)
61 patients were included in the meta-analysis. The odds ratio was 2.42, confidence interval 5.95 to 12.12, p-value 0.18.

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Normal</th>
<th>Abnormal</th>
<th>Study or Subgroup</th>
<th>Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Total</td>
<td>Mean</td>
</tr>
<tr>
<td>Cekic 2015 (1)</td>
<td>16.87</td>
<td>6.39</td>
<td>13</td>
<td>17.61</td>
</tr>
<tr>
<td>Yesur 1998</td>
<td>4.55</td>
<td>4.8091</td>
<td>11</td>
<td>5.79</td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td>24</td>
<td>37</td>
<td>100.0%</td>
<td></td>
</tr>
</tbody>
</table>

Heterogeneity: Tau^2 = 0.00; Chi^2 = 0.05, df = 1 (p = 0.82); I^2 = 0%  
Test for overall effect: Z = 1.34 (p = 0.18)

Footnotes
1. ln pg/L
120 neonates were included in the meta-analysis. The odds ratio was 0.63, confidence interval: 6.14 to 7.39.

### Table 3.17: S100 to predict long term outcome

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Normal Mean</th>
<th>Normal SD</th>
<th>Normal Total</th>
<th>Abnormal Mean</th>
<th>Abnormal SD</th>
<th>Abnormal Total</th>
<th>Weight</th>
<th>Mean Difference IV, Fixed, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Celik 2015</td>
<td>56.112</td>
<td>13</td>
<td>13</td>
<td>17.20.2</td>
<td>18</td>
<td>1.2%</td>
<td>39.00</td>
<td>[-22.59, 100.59]</td>
</tr>
<tr>
<td>Nagdyman 2003</td>
<td>4.74</td>
<td>5</td>
<td>16</td>
<td>7.82 12.9</td>
<td>11</td>
<td>71.4%</td>
<td>-3.08</td>
<td>[-11.09, 4.93]</td>
</tr>
<tr>
<td>Thorngren-Jameck 2004</td>
<td>32.9</td>
<td>34.4</td>
<td>43</td>
<td>24.3 17.4</td>
<td>19</td>
<td>27.4%</td>
<td>8.60</td>
<td>[-4.32, 21.52]</td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td><strong>72</strong></td>
<td><strong>48</strong></td>
<td><strong>100.0%</strong></td>
<td><strong>0.63</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>[-6.14, 7.39]</strong></td>
</tr>
</tbody>
</table>

Heterogeneity: Chi² = 3.78, df = 2 (P = 0.15); I² = 47%
Test for overall effect: Z = 0.18 (P = 0.86)
41 patients were included in the meta-analysis. The odds ratio was 16.74, confidence interval was 69.28 to 35.81.
Figure 3.19: Meta-analysis of MRI brain to predict long term neurodevelopmental outcome & death.

The MRI meta-analysis included 938 patients from 23 studies. Abnormal MRI brain was predictive of adverse outcome with an odds ratio of 18.78 (95% CI 12.53 to 28.14).
3.5 Risk of Bias Assessment

The ascertainment of bias was performed and a risk of bias table was done for each study. The headings included: Study participation; Study Attrition; Prognostic Factor Measurement; Outcome Measurement; Study confounding factors; Statistical Analysis and Reporting. A sample risk of bias table for lactate dehydrogenase to predict short term outcome is shown below, (Figure 3.20). The majority of the studies were prospective observational and cohort studies rather than randomised control trials. The two main bias ascertained were firstly that in the long term studies there was large loss to follow up in some studies. The second was in confounding factors. As NE is a heterogenous condition the included population have mixed aetiologies that can confound results, for example infants with co-existing sepsis may have altered inflammatory profile, and as TH is known to delay the peak of inflammation in NE, TH may also confound results.
Figure 3.20: Risk of bias table for lactate dehydrogenase to predict short term outcome

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Normal</th>
<th>Mean</th>
<th>SD</th>
<th>Total</th>
<th>Abnormal</th>
<th>Mean</th>
<th>SD</th>
<th>Total</th>
<th>Mean Difference IV, Random, 95% CI</th>
<th>Mean Difference IV, Random, 95% CI</th>
<th>Risk of Bias</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bakken 2014</td>
<td>701</td>
<td>583</td>
<td>26</td>
<td>2,830.7538</td>
<td>4,722.5989</td>
<td>65</td>
<td>21.7%</td>
<td>-2129.75 [-3297.28, -962.23]</td>
<td>-2129.75 [-3297.28, -962.23]</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Karlsson 2010</td>
<td>1,158</td>
<td>612</td>
<td>11</td>
<td>3,120</td>
<td>2,349</td>
<td>28</td>
<td>23.6%</td>
<td>-1952.00 [-2894.24, -1009.76]</td>
<td>-1952.00 [-2894.24, -1009.76]</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Liu 2013a</td>
<td>1,319</td>
<td>571</td>
<td>16</td>
<td>1,541</td>
<td>1,016</td>
<td>12</td>
<td>25.9%</td>
<td>-222.00 [-861.32, 417.32]</td>
<td>-222.00 [-861.32, 417.32]</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Thorson 2012</td>
<td>2,304.5455</td>
<td>2,615.6405</td>
<td>33</td>
<td>7,066</td>
<td>10,162</td>
<td>4</td>
<td>12%</td>
<td>-4761.45 [-14760.91, 5238.00]</td>
<td>-4761.45 [-14760.91, 5238.00]</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Yung 2017</td>
<td>1,341</td>
<td>1,340</td>
<td>71</td>
<td>1,014</td>
<td>340</td>
<td>21</td>
<td>27.6%</td>
<td>327.00 [-16.94, 670.94]</td>
<td>327.00 [-16.94, 670.94]</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td></td>
<td>160</td>
<td></td>
<td>130</td>
<td></td>
<td><strong>-947.66 [-2071.96, 176.65]</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Heterogeneity: TAU² = 11.6272854; CHI² = 33.32; df = 4 (P < 0.00001); I² = 88%
Test for overall effect: Z = 1.65 (P = 0.10)

Risk of bias legend
(A) Study Participation
(B) Study Attrition
(C) Prognostic Factor Measurement
(D) Outcome Measurement
(E) Study Confounding
(F) Statistical Analysis and Reporting
3.6 Discussion

Raised mean serum levels of IL-6 and lactate were associated with worse short-term outcome in NE. The large confidence intervals, (95% CI -44.5 to -0.66) and (95% CI -6.19 to -2.81) respectively, however, reflect the small number of studies included and consequently the small population cohorts. The multiple other meta-analyses of serum troponin, S100, TNF-alpha, glucose, ionised calcium, nucleated red bloods cells, lactate dehydrogenase, II-β, , and white cell count were not statistically significant.

Raised serum IL-6 and neuron specific enolase (NSE) were associated with adverse long-term outcome in NE, however there were small patient numbers in both studies. Ramaswamy et al\textsuperscript{149} was only other similar systematic review showing that serum IL-1β, serum IL-6, cerebrospinal fluid neuron-specific enolase and cerebrospinal fluid IL-1β were indicators of abnormal outcomes in NE. This systematic review was performed in 2009 pre the TH era. Our review included papers before TH and since the introduction of TH as standard of care.

Early MRI brain provided the best early prognostic information on long-term developmental outcome for infants with NE. Early prognostic information to predict long term outcome is important to initiate early intervention of therapies, to counsel parents and for resource planning. NAA, N-acetylaspartate a proton marker in Magnetic resonance spectroscopy (MRS) has been demonstrated to discriminate neonates with abnormal outcome in a systematic review of MRS to predict outcome\textsuperscript{150}.

The limitations of the study include the substantial heterogeneity across the studies. A large proportion of studies were excluded from meta-analyses due to the variety of measured outcomes and methods of data reporting. Authors were contacted for clarity to enable inclusion of their published research and a time frame was given of one month to await reply, however no replies were received. Another limitation includes that all studies of MRS may not have been detected with our search terms.

This systematic review research question involved searching for consistency of biomarkers to predict short- and long-term outcome in NE across studies, with a view for the assessment to guide future research in NE. The data collection involved assessment of valid studies and data extraction which included a large amount of studies on biomarkers however when the statistical methods of the meta-analysis was performed to combine the data, many planned meta-analyses were impossible due to methodology of presenting the figures.
The completion of this study coincides with the publication of Inconsistent outcome reporting in large neonatal trials: a systematic review, by the COIN steering committee\textsuperscript{151}. Their review reports inconsistent outcome reporting with clinical trials in neonatology. Although our review primarily looked at observational studies rather than RCT the variable outcomes measured reported by the COIN steering committee amounted to 216 different outcomes with 889 different outcome measures, with 639 outcome measures were only reported in a single trial. Both these systematic reviews highlight the need for both core outcomes and standardised reporting in neonatology so outcomes of different studies can be compared and pooled for valuable meta-analyses to guide future research and improve clinical practice. The core outcome set is an agreed minimum set of outcomes that should be measured and reported in all studies and trials, with researchers being given guidance via “Core Outcome measurement instrument sets” to measure the outcomes in a Core Outcome Set\textsuperscript{152}.

\section*{3.7 Conclusion}

In summary, a number of promising biomarkers to predict outcome have been identified and have been replicated in a small number of studies. More studies are needed to both validate and correlate these biomarkers. MRI is the single best biomarker to predict long term outcome in NE. MRI is currently part of standard of care post TH in NE. For future direction MRI could be recommended as the CORE outcome reported in studies of NE.

Raised IL-6 is consistent in predicting adverse short and long term outcome in NE and shows promise future use in combination with clinical assessments and perhaps may play a role in future monitoring response to treatment.
Chapter 4 - Cytokine Responses in Neonatal Encephalopathy

4.1 Introduction

In NE, injury is not confined to the brain. Babies have multiorgan injury and systemic inflammation. Many studies have investigated the ability of biomarkers in the serum of infants with NE to predict brain injury. There is no single marker that is adequately sensitive and specific to diagnose NE nor reliably predict outcome in these infants at present.

The different families of cytokines include tumor necrosis factor (TNF), colony-stimulating factors (CSF), Interferons (IFN), chemokines and interleukins (IL)\textsuperscript{153}. Cytokines play a key role in the common pathways in NE that are induced by hypoxia-ischemia, reperfusion, metabolic derangements, inflammation and infection. This activates the inflammatory cells of the innate immune response including granulocytes, macrophages and microglia, leading to cell death and dysfunction.

We have previously shown that neonates exposed to perinatal asphyxia had higher Epo, and that Epo was significantly raised in moderate to severe NE in comparison to milder asphyxia, and correlated with grade of MRI abnormality. Lower VEGF was associated with severe asphyxia and mortality\textsuperscript{154}. O’Hare et al showed that raised GM-CSF, IL-8 and IL-10 were higher and that TNF-\textgreek{a} and VEGF were lower in infants with abnormal neuroimaging\textsuperscript{88}. Jenkins et al demonstrated higher early IL-6, IL-8, and IL-10 in NE treated with TH at 24 hours in comparison to NE treated with normothermia, and downregulation of IL-6, IL-8 and IL-10 at 36 hours in infants with better outcomes\textsuperscript{148}. They concluded that the 36 hour time period is when the trend reverses in the TH patients with a better outcome meaning perhaps TH shortens the time to initiate repair. The significance of this is that these biomarkers may allow individualised targeted therapy to where the neonate is in their injury-repair process of inflammation.

The use of lipopolysaccharide (LPS) challenges the innate immune system and examines its’ response ex-vivo following an endotoxin encounter. Prior exposure of innate immune cells to endotoxin causes them to become refractory to subsequent endotoxin challenge is termed “endotoxin tolerance”\textsuperscript{155}. This previous endotoxin exposure inducing LPS tolerance is known as a form of innate immune memory. This may result in reduced response to inflammatory stimulus, reducing the inflammatory cytokine output causing a relative immunosuppression\textsuperscript{156}. The reduced inflammatory response may be beneficial in preventing excessive inflammation which may cause tissue damage and be detrimental.
Understanding the responses of pro and anti-inflammatory cytokines in NE may facilitate the development of strategies to modulate the inflammatory response and decrease brain injury. Further research is required in the area of serum biomarkers in term neonates at risk of brain injury to ensure that a sensitive, specific, reliable marker of brain injury is identified, to help predict outcome as part of a larger biomarker panel.

4.2 Hypothesis

Infants with NE have a dysregulated cytokine inflammatory response in comparison to healthy term controls.

4.3 Aims

- To investigate the relationship between systemic cytokines in infants with NE in comparison to healthy term controls in response to LPS stimulation
- To examine the relationship of the inflammatory profile to short term outcomes in the infants with NE

4.4 Results

4.4.1 Infants with NE versus controls at baseline

The baseline cytokine profile in NE and in healthy term controls (TC) was investigated and examined for differences. This was examined with the hypothesis that the infants with NE would have a dysregulated inflammatory response in comparison to TC. On days 1-2 life, plasma levels of Epo and IL-6 were higher in NE than in control infants at baseline, (both \( p \leq 0.001 \)). VEGF was lower in NE than Control infants (\( p = 0.0013 \)). GM-CSF, IL-18 and IL-1ra were higher in NE than control infants (\( p = 0.03, p = 0.02 \) and \( p = 0.005 \) respectively) and IL-2 was higher in controls (\( p = 0.02 \)) than NE on days 1-2 but this \( p \) value was not considered significant when the Bonferroni correction applied (\( p \leq 0.0035 \)), (Table 4.1).
As explained in the methodology section, the value taken for statistical significance following the Bonferroni correction due to the multiple comparisons was $p \leq 0.0035$. IFN-γ, IL-10, IL-1α, IL-1β, IL-8, TNF-α and TNF-β medians did not differ between NE days 1-2 and controls (all $p \geq 0.0035$).

On days 3-4 Epo was higher in NE than controls, ($p \leq 0.0001$). IL-2 and VEGF were higher in controls than NE ($p=0.0094$ and $p=0.0008$ respectively). IL1-ra and IL-6 were higher in NE than controls but not statistically significant with the Bonferroni correction (both $p=0.01$). (All displayed in table 4.1 and figure 4.1)
The table represents the median and upper and lower range respectively. The p values highlighted in bold are statistically significant following the Bonferroni correction. Mann-Whitney test was used to calculate the difference between the medians for statistical significance. The (n) in controls was 28 and the (n) in NE D1-2 was 40 and NE D3-4 was 31.
Figure 4.1 Baseline cytokine levels in healthy term control patients (Con) and in Neonatal Encephalopathy (NE) Days 1-2 and Days 3-4 of life

Columns represent median values and error bars represent interquartile ranges. * represents $p \leq 0.0035$ when median cytokine levels were examined for differences between Con and NE by Mann-Whitney test. The (n) in controls was 28 and the (n) in NE D1-2 was 40 and NE D3-4 was 31.
4.4.2 Neonatal controls and NE following LPS stimulation

The cytokine profile in NE and in healthy (TC) was investigated in response to whole blood LPS stimulation ex-vivo. Previously this experiment had been carried out with cord blood as the comparator group to NE rather than TC. In healthy TC infants all cytokines were increased with LPS stimulation, (Table 4.2). The increases in Epo, IL-1α and TNF-β with endotoxin were not statistically significant (p=0.0211, 0.1475 and 0.0814 respectively). The increases in GM-CSF, IFN-γ, IL-10, IL-18, IL-1ra, IL-1β, IL-2, IL-6, IL-8, TNF-α, and VEGF were all statistically significant, with significance deemed as p≤0.0035.

Infants with NE on days 1-2 of life had an increase in all cytokines with LPS stimulation. The increase in Epo and IL-1α were not statistically significant, (p=0.04 and p=0.04 respectively). GM-CSF, IFN-γ, IL-10, IL-18, IL-1ra, IL-1β, IL-2, IL-6, IL-8, TNF-α, TNF-β and VEGF increases were all statistically significant (p≤0.0035).

On days 3-4 of life in infants with NE, IFN-γ, IL-18, IL-1ra, IL-1β, IL-2, IL-6 IL-8, TNF-α and VEGF are all increased with LPS (all p≤0.0035). Epo, GM-CSF, IL-10, IL-1α and TNF-β did not change significantly with LPS stimulation after using the Bonferroni correction.
Tables 4.2 (a+b) Neonatal controls and NE following LPS stimulation on Days 1+2, then 3+4 respectively

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Con</th>
<th>Con post LPS</th>
<th>p value</th>
<th>NE D1-2</th>
<th>NE D1+2 Post LPS</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epo</td>
<td>27.26 (6.9–308.0)</td>
<td>32.96 (0.87-503.7)</td>
<td>0.02</td>
<td>123 (8.51-5952)</td>
<td>154 (40.3-18402)</td>
<td>0.05</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0.22 (0.03-32.6)</td>
<td>3.34 (0.24-10.14)</td>
<td>&lt;0.0001</td>
<td>0.38 (0.05-3.69)</td>
<td>1.01 (0.43-19.5)</td>
<td>0.02</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>6.42 (0.58-499.6)</td>
<td>48.43 (2.43-1068)</td>
<td>&lt;0.0001</td>
<td>5.86 (1.69-31.3)</td>
<td>25.7 (2.82-172)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.8782 (0.19-19.68)</td>
<td>2.3 (0.68-60.01)</td>
<td>&lt;0.0001</td>
<td>1.15 (0.06-5.11)</td>
<td>1.69 (0.15-7.03)</td>
<td>0.02</td>
</tr>
<tr>
<td>IL-18</td>
<td>219.3 (60.45-464.5)</td>
<td>422 (29.29-781.5)</td>
<td>&lt;0.0001</td>
<td>295 (78.9-762)</td>
<td>500 (220-2020)</td>
<td>0.0004</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>657.2 (207-7830)</td>
<td>2789 (40.93-11001)</td>
<td>0.0004</td>
<td>1580 (172-8228)</td>
<td>5432 (675-25144)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-1α</td>
<td>5.86 (0.03-23.26)</td>
<td>13.53 (0.38-63.43)</td>
<td>0.15</td>
<td>2.09 (0.03-48.9)</td>
<td>8.97 (0.68-59.2)</td>
<td>0.03</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.95 (0.11-20.02)</td>
<td>15.83 (0.43-326.6)</td>
<td>&lt;0.0001</td>
<td>1.1 (0.06-6.48)</td>
<td>3.25 (0.93-32.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-2</td>
<td>3.85 (0.1-23.92)</td>
<td>24.86 (2.79-123.4)</td>
<td>&lt;0.0001</td>
<td>0.543 (0.02-3.36)</td>
<td>5.42 (0.69-19.5)</td>
<td>0.0001</td>
</tr>
<tr>
<td>IL-6</td>
<td>2.47 (0.63-160.2)</td>
<td>57.76 (0.63-455.5)</td>
<td>&lt;0.0001</td>
<td>6.72 (1.14-124)</td>
<td>35.3 (5.94-133.8)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-8</td>
<td>58.06 (15.45-1111)</td>
<td>726.5 (57.86-12615)</td>
<td>&lt;0.0001</td>
<td>66.6 (6.98-1029)</td>
<td>347 (59.4-1942)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TNF- α</td>
<td>4.22 (1.38-374)</td>
<td>800.8 (5.56-2039)</td>
<td>&lt;0.0001</td>
<td>4.21 (1.7-31.7)</td>
<td>260 (5.12-1969)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TNF- β</td>
<td>26.85 (2.38-198.4)</td>
<td>57.54 (2.11-351)</td>
<td>0.08</td>
<td>32.4 (0.08-168)</td>
<td>62.2 (0.6-395)</td>
<td>0.01</td>
</tr>
<tr>
<td>VEGF</td>
<td>175.2 (52.68-592.2)</td>
<td>368.6 (51.59-1514)</td>
<td>&lt;0.0001</td>
<td>76.8 (11.8-498)</td>
<td>224 (17.9-527)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

(a) The table represents the median and upper and lower range respectively. Wilcoxon rank test was used for examining for differences between baseline and stimulated levels. The p values highlighted in bold are statistically significant following the Bonferroni correction. The (n) in controls was 28 and the (n) in NE D1-2 was 40.
### Table 4.2 (b) Neonatal controls and NE following LPS stimulation on 3+4

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Con</th>
<th>Con post LPS</th>
<th>p value</th>
<th>NE D3-4</th>
<th>NE D3+4 Post LPS</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epo</td>
<td>27.26 (6.9-308.0)</td>
<td>32.96 (0.87-503.7)</td>
<td>0.02</td>
<td>123 (8.51-5952)</td>
<td>154 (40.3-18402)</td>
<td>0.05</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0.22 (0.03-32.6)</td>
<td>3.34 (0.24-10.14)</td>
<td>&lt;0.0001</td>
<td>0.38 (0.05-3.69)</td>
<td>1.01 (0.43-19.5)</td>
<td>0.02</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>6.42 (0.58-499.6)</td>
<td>48.43 (2.43-1068)</td>
<td>&lt;0.0001</td>
<td>5.86 (1.69-31.3)</td>
<td>25.7 (2.82-172)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.8782 (0.19-19.68)</td>
<td>2.3 (0.68-60.01)</td>
<td>&lt;0.0001</td>
<td>1.15 (0.06-5.11)</td>
<td>1.69 (0.15-7.03)</td>
<td>0.02</td>
</tr>
<tr>
<td>IL-18</td>
<td>219.3 (60.45-464.5)</td>
<td>422 (29.29-781.5)</td>
<td>&lt;0.0001</td>
<td>295 (78.9-762)</td>
<td>500 (220-2020)</td>
<td>0.0004</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>657.2 (207-7830)</td>
<td>2789 (40.93-11001)</td>
<td>0.0004</td>
<td>1580 (172-8228)</td>
<td>5432 (675-25144)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-1α</td>
<td>5.86 (0.03-23.26)</td>
<td>13.53 (0.38-63.43)</td>
<td>0.15</td>
<td>2.09 (0.03-48.9)</td>
<td>8.97 (0.68-59.2)</td>
<td>0.03</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.95 (0.11-20.02)</td>
<td>15.83 (0.43-326.6)</td>
<td>&lt;0.0001</td>
<td>1.1 (0.06-6.48)</td>
<td>3.25 (0.93-32.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-2</td>
<td>3.85 (0.1-23.92)</td>
<td>24.86 (2.79-123.4)</td>
<td>&lt;0.0001</td>
<td>0.543 (0.02-3.36)</td>
<td>5.42 (0.69-19.5)</td>
<td>0.0001</td>
</tr>
<tr>
<td>IL-6</td>
<td>2.47 (0.63-160.2)</td>
<td>57.76 (0.63-455.5)</td>
<td>&lt;0.0001</td>
<td>6.72 (1.14-124)</td>
<td>35.3 (5.94-133.8)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-8</td>
<td>58.06 (15.45-1111)</td>
<td>726.5 (57.86-12615)</td>
<td>&lt;0.0001</td>
<td>66.6 (6.98-1029)</td>
<td>347 (59.4-1942)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TNF-α</td>
<td>4.22 (1.38-374)</td>
<td>800.8 (5.56-2039)</td>
<td>&lt;0.0001</td>
<td>4.21 (1.7-31.7)</td>
<td>260 (5.12-1969)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TNF-β</td>
<td>26.85 (2.38-198.4)</td>
<td>57.54 (2.11-351)</td>
<td>0.08</td>
<td>32.4 (0.08-168)</td>
<td>62.2 (0.6-395)</td>
<td>0.01</td>
</tr>
<tr>
<td>VEGF</td>
<td>175.2 (52.68-592.2)</td>
<td>368.6 (51.59-1514)</td>
<td>&lt;0.0001</td>
<td>76.8 (11.8-498)</td>
<td>224 (17.9-527)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

(b) The table represents the median and upper and lower range respectively. Wilcoxon rank test was used for examining for differences between baseline and stimulated levels. The p values highlighted in bold are statistically significant following the Bonferroni correction. The (n) in controls was 28 and the (n) NE D3-4 was 31.
4.4.3 Neonatal controls compared to NE following LPS stimulation

The cytokine responses to LPS in NE and in healthy TC was examined for differences, to see if the innate immune response differed between the groups. Epo, IL-18 and IL-1ra levels post LPS stimulation were higher in infants with NE on days 1-2 than controls, \((p \leq 0.0035)\), when examined with the Friedman test. (Table 4.3 and Figure 4.2). IL-1β, IL-1α, IL-2 and TNF-α were higher in Control infants than those with NE, although this did not reach statistical significance after Bonferroni correction. TNF-β was higher in infants with NE with LPS stimulation but did not reach statistical significance upon Bonferroni correction.

On D3-4, GM-CSF and IL-2 were higher in Control infants than NE with LPS stimulation, \((p \leq 0.0035)\). Epo was higher in NE than control infants but did not reach statistical significance after Bonferroni correction. IL-1β, TNF-α and VEGF were higher in control infants than those with NE, but did not reach statistical significance after Bonferroni correction.
Table 4.3  LPS responsiveness in Controls versus NE Days 1-2 and Days 3-4 life

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Con</th>
<th>NE D1-2</th>
<th>P Value</th>
<th>Con</th>
<th>NE D3-4</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epo</td>
<td>32.96 (0.87-503.7)</td>
<td>234 (21.99-64141)</td>
<td>&lt;0.01</td>
<td>32.96 (0.87-503.7)</td>
<td>154 (40.3-18402)</td>
<td>0.004</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>3.34 (0.24-10.14)</td>
<td>2.04 (0.04-190.6)</td>
<td>0.41</td>
<td>3.34 (0.24-10.14)</td>
<td>1.01 (0.43-19.5)</td>
<td>0.001</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>48.43 (2.42-1068)</td>
<td>32.8 (0.08-233.6)</td>
<td>1</td>
<td>48.43 (2.42-1068)</td>
<td>25.7 (2.82-172)</td>
<td>0.43</td>
</tr>
<tr>
<td>IL-10</td>
<td>2.3 (0.67-60.01)</td>
<td>2.27 (0.21-97.85)</td>
<td>0.71</td>
<td>2.3 (0.67-60.01)</td>
<td>1.69 (0.15-7.03)</td>
<td>0.45</td>
</tr>
<tr>
<td>IL-18</td>
<td>422 (29.29-781.5)</td>
<td>467 (95.72-2576)</td>
<td>0.001</td>
<td>422 (29.29-781.5)</td>
<td>500 (220-2020)</td>
<td>0.11</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>2789 (40.93-11001)</td>
<td>7172 (345.8-45125)</td>
<td>0.001</td>
<td>2789 (40.93-11001)</td>
<td>5432 (675-25144)</td>
<td>0.28</td>
</tr>
<tr>
<td>IL-1α</td>
<td>13.53 (0.38-63.43)</td>
<td>5.58 (0.08-88.29)</td>
<td>0.36</td>
<td>13.53 (0.38-63.43)</td>
<td>8.97 (0.68-59.2)</td>
<td>0.76</td>
</tr>
<tr>
<td>IL-1β</td>
<td>15.83 (0.43-326.6)</td>
<td>9.15 (0.21-471.4)</td>
<td>0.02</td>
<td>15.83 (0.43-326.6)</td>
<td>3.25 (0.93-32.6)</td>
<td>0.02</td>
</tr>
<tr>
<td>IL-2</td>
<td>24.86 (2.78-123.4)</td>
<td>7.48 (0.49-109.1)</td>
<td>0.01</td>
<td>24.86 (2.78-123.4)</td>
<td>5.42 (0.69-19.5)</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-6</td>
<td>57.76 (0.63-455.5)</td>
<td>42 (5.919-23073)</td>
<td>0.7</td>
<td>57.76 (0.63-455.5)</td>
<td>35.3 (5.94-133.8)</td>
<td>0.26</td>
</tr>
<tr>
<td>IL-8</td>
<td>726.5 (57.86-12615)</td>
<td>84.7 (48.19-23596)</td>
<td>0.45</td>
<td>726.5 (57.86-12615)</td>
<td>347 (59.4-1942)</td>
<td>0.01</td>
</tr>
<tr>
<td>TNF-α</td>
<td>800.8 (5.56-2039)</td>
<td>294 (4.78-3090)</td>
<td>0.01</td>
<td>800.8 (5.56-2039)</td>
<td>260 (5.12-1969)</td>
<td>0.02</td>
</tr>
<tr>
<td>TNF-β</td>
<td>57.54 (2.1-351)</td>
<td>128 (1.0-835.5)</td>
<td>0.01</td>
<td>57.54 (2.1-351)</td>
<td>62.2 (0.6-395)</td>
<td>0.35</td>
</tr>
<tr>
<td>VEGF</td>
<td>368.6 (51.59-1514)</td>
<td>222 (26.18-1132)</td>
<td>0.06</td>
<td>368.6 (51.59-1514)</td>
<td>224 (17.9-527)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

The values represent median post LPS stimulation. The units were picograms per ml. To compare differences between stimulated levels between groups Friedman test was performed. The p-values highlighted in bold are significant following the correction. The (n) in controls was 28 and the (n) in NE D1-2 was 40 and NE D3-4 was 31.
Figure 4.2: Comparison of Healthy Control Infants (Con) and infants with Neonatal Encephalopathy (NE) Days 1-2 & Days 3-4 life at baseline & post LPS.

Columns represent median and error bars represent interquartile range. Friedman test was used to test for significant differences between the groups, * represents that the difference between the medians of Baseline and LPS stimulation was ≤0.0035, ** represents the difference between Con and NE medians was ≤0.0035.
4.4.4 Cytokine Correlations to Short Term Clinical Outcomes in NE

The cytokine profile within the NE infants was examined with regard to differences that might prognosticate their clinical outcome; the severity of NE; as per Sarnat Staging, (Table 4.4) normal compared to abnormal neuro-imaging (Table 4.6) and differences between infants with or without seizures (Table 4.5).

The differences in the baseline cytokine profiles between NE II and III from days 1-2 of life were examined, (Table 4.4) TNF-α was higher as the severity of NE increased (p≤0.05). The results of the patients with Sarnat Stage I NE were omitted as per advise from CSTAR statistical support as there were only 2 patients in this group so the statistical comparisons would not be valid. Anti-inflammatory IL-10, decreased with severity, and IL-1ra increased with severity. There were no differences detected in the cytokine profiles between the NE stages on days 3-4 life.

Following stimulation with LPS, IFN-γ decreased with increasing severity of NE, with the lowest IFN-γ being associated with NE III on Day 1-2. On days 3-4, TNF-β following LPS stimulation was significantly higher in infants with NE II in comparison to NE III. There were no differences detected upon LPS stimulation in the rest of the cytokines after subdivision for NE stage. There were no differences detected in cytokine profiles at baseline nor with LPS stimulation between NE infants with or without seizures using the Mann Whitney test, (Table 4.5)

The cytokines medians were examined for differences between NE infants with normal and abnormal MRI brain imaging using the Mann Whitney test, (Table 4.6). Baseline GM-CSF and IFN-γ, IL-1β, IL-1ra and VEGF were higher on Day 1-2 in infants with abnormal neuroimaging but were not significant with Bonferroni correction. Epo, IL-10, IL-18, IL-1α, IL-2, IL-6, IL-8, TNF-α and TNF-β did not differ at baseline between infants with NE and normal versus abnormal neuroimaging. GM-CSF, IFN-γ and TNF-α levels with LPS stimulation were between normal and abnormal neuroimaging but were not significant with the Bonferroni correction for multiple comparisons.
Table 4.4 Comparison of Cytokine Medians Infants with NE, divided into infants with NE II, (n)= 40 in NE II, (n)= 11 in NE III.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>NE II D1-2</th>
<th>NE II D3-4</th>
<th>NE III D1-2</th>
<th>NE III D3-4</th>
<th>P value</th>
<th>NE II LPS D1-2</th>
<th>NE II LPS D3-4</th>
<th>P value</th>
<th>NE III LPS D1-2</th>
<th>NE III LPS D3-4</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>1.15 (0.083-1.10)</td>
<td>3.59 (0.44-11.27)</td>
<td>0.08</td>
<td>0.8 (0.06-23.1)</td>
<td>2.29 (2.6-4.68)</td>
<td>0.87</td>
<td>7.5 (0.21-32.14)</td>
<td>5.31 (2.2-60.81)</td>
<td>0.56</td>
<td>3.15 (0.05-32.58)</td>
<td>10.07 (0.03-28.47)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.50 (0.043-10.39)</td>
<td>1.29 (0.794-1.99)</td>
<td>0.14</td>
<td>0.46 (0.02-8.41)</td>
<td>0.75 (0.04-4.11)</td>
<td>0.87</td>
<td>4.85 (0.05-14.1)</td>
<td>6.15 (0.55-27.3)</td>
<td>0.27</td>
<td>4.96 (0.12-7.97)</td>
<td>1.65 (0.9-9.99)</td>
</tr>
<tr>
<td>IL-6</td>
<td>11.76 (2.44-0.076)</td>
<td>127.65 (0.07-28.44)</td>
<td>0.06</td>
<td>8.06 (1.44-52.8)</td>
<td>6.43</td>
<td>0.4</td>
<td>41.95 (5.9-2107)</td>
<td>17.38 (6.82-72.24)</td>
<td>0.06</td>
<td>23.6 (5.94-91.55)</td>
<td>21.23 (10.38-122.5)</td>
</tr>
<tr>
<td>IL-8</td>
<td>75.32 (11.7-3291)</td>
<td>213.6 (12.52-458.9)</td>
<td>0.48</td>
<td>44.36 (69.7-168)</td>
<td>121.3 (7.8-123.8)</td>
<td>0.52</td>
<td>406.7 (94.19-2506)</td>
<td>392.6 (26.5-2580)</td>
<td>0.23</td>
<td>264.9 (39.7-1241)</td>
<td>43.7 (5.54-985)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>4.31 (1.8-54.5)</td>
<td>6.54 (2.56-456)</td>
<td>0.05</td>
<td>3.89 (1.8-948)</td>
<td>8.45 (1.69-31.71)</td>
<td>0.88</td>
<td>254 (4.78-1166)</td>
<td>204.8 (7.83-1379)</td>
<td>0.07</td>
<td>201 (5.51-1969)</td>
<td>224.4 (3.18-682.2)</td>
</tr>
<tr>
<td>TNF-β</td>
<td>33.96 (0.18)</td>
<td>72.19 (0.18-335.5)</td>
<td>0.28</td>
<td>47.43 (0.07-190.5)</td>
<td>0.43 (0.11-165.5)</td>
<td>0.28</td>
<td>26.4 (0.35-346.5)</td>
<td>2.57 (0.52-544.4)</td>
<td>0.04</td>
<td>8.85 (0.18-99.2)</td>
<td>268.9 (0.76-197.4)</td>
</tr>
<tr>
<td>VEGF</td>
<td>108 (4.39-298)</td>
<td>136 (0.33-276.3)</td>
<td>0.25</td>
<td>80.42 (11.84-477.4)</td>
<td>171.2 (19.03-498.4)</td>
<td>0.13</td>
<td>213.8 (7.98-1132)</td>
<td>226.9 (95.38-1045)</td>
<td>0.89</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

p<0.0025 was deemed statistically significant, highlighted in (n)=40 in NE II, (n)= 11 in NE III.

Kruskal-Wallis test was used to examine differences.
Table 4.5 Comparison of Cytokine Medians Infants with NE, divided into infants with and without seizures, at baseline and following LPS stimulation.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Median (range)</th>
<th>P value</th>
<th>n SEizures, NE</th>
<th>n No SEizures, NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>4.17 (0.86-26.97)</td>
<td>0.19</td>
<td>18 (22)</td>
<td>10 (2)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>11.16 (2.7-12.3)</td>
<td>0.02</td>
<td>17 (22)</td>
<td>16 (2)</td>
</tr>
<tr>
<td>IL-1α</td>
<td>115.5 (10.8-129)</td>
<td>0.04</td>
<td>17 (22)</td>
<td>16 (2)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>115.5 (10.8-129)</td>
<td>0.04</td>
<td>17 (22)</td>
<td>16 (2)</td>
</tr>
<tr>
<td>IL-6</td>
<td>115.5 (10.8-129)</td>
<td>0.04</td>
<td>17 (22)</td>
<td>16 (2)</td>
</tr>
<tr>
<td>TNF-β</td>
<td>115.5 (10.8-129)</td>
<td>0.04</td>
<td>17 (22)</td>
<td>16 (2)</td>
</tr>
</tbody>
</table>

Table representing median, range and p values. p=≤0.0035 was statistically significant. (n)=29 with seizures (n)=22 without.
Table 4.6 Comparison of Cytokine Medians in NE divided into infants with normal and abnormal MRI brain imaging, at baseline and following LPS stimulation

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Normal MRI</th>
<th>Abnormal MRI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>91.5 (24.0-107.7)</td>
<td>20.1 (3.7-160.0)</td>
<td>0.01</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>122.1 (32.0-286.6)</td>
<td>63.4 (12.0-176.4)</td>
<td>0.04</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.12 (0.03-0.14)</td>
<td>0.25 (0.02-0.37)</td>
<td>0.04</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.12 (0.03-0.14)</td>
<td>0.25 (0.02-0.37)</td>
<td>0.04</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.12 (0.03-0.14)</td>
<td>0.25 (0.02-0.37)</td>
<td>0.04</td>
</tr>
<tr>
<td>EPO</td>
<td>6.6 (0.9-65.3)</td>
<td>0.1 (0.01-0.2)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table represents median, range and p values. (n)=21 with normal MRI brain, (n)=30 with abnormal MRI brain.
4.5 Discussion

These results display a comprehensive overview of the innate immune in profiling the differences between infants with NE and healthy term controls in terms of their baseline cytokine profiles and their response to LPS stimulation. The relative distinctive response of infants with NE and the robust response of healthy term controls are particularly noteworthy.

LPS was used in this study to examine the inflammatory response of infants with NE and how this inflammatory response may differ from healthy term infants. Previous work by Molloy et al demonstrated an altered endotoxin phenotype in neonates pre-exposed to inflammation. Maternal neutrophils and those from healthy neonates with septic risk factors had increased neutrophil apoptosis in comparison to control. Neonates with sepsis were LPS hypo-responsive\textsuperscript{157}. They demonstrated that upon ex-vivo hypoxia or heat shock exposure, neonates do not upregulate their LPS response\textsuperscript{102}. In infants requiring resuscitation, they demonstrated a hypo-responsiveness to LPS stimulation on cord blood assessment, with mildly affected infants displaying a proinflammatory neutrophil response and the moderate to severe NE infants displaying a tendency toward immunosuppression\textsuperscript{100}.

Munoz et al demonstrated a reduced LPS-induced response of cytokines of IL-1\alpha, IL-1\beta, IL-6 and TNF-\alpha in patients with gram-negative infections. The patients who survived had a capacity to produce normal amounts of cytokines upon LPS stimulation compared to non survivors\textsuperscript{158}.

Neonates are vulnerable to infection, and this has been attributed to immaturity of their innate and adaptive immune function\textsuperscript{159}. Their minimal exposure in utero to antigens to induce adaptive immunity resulting in deficiencies in their adaptive cellular and anti-body mediated defences have been demonstrated, and means they heavily rely on their innate immune system which remains immature\textsuperscript{159}. In terms of profiling the innate immune system, a lot of the research has been done using cord blood, demonstrating a relative hypo-responsiveness to LPS stimulation compared to adult blood\textsuperscript{160}. Li et al demonstrated deficiencies in neonatal innate immunity cytokine responses following LPS stimulation but the neonatal group were based on cord blood and compared to mature adults. Olin et al demonstrated that neonatal cord blood phenotype did not predict post-natal immunity\textsuperscript{161}, so our choice of healthy term infants is a more realistic comparator.

In healthy term control infants all cytokines, of both a pro and anti-inflammatory nature, were increased with LPS stimulation. The LPS responsiveness indicates a robust innate immune response. Infants with NE also had an increase in all cytokines with LPS stimulation. When examining the different responses of infants with NE compared to healthy term controls, infants with NE at baseline had higher epo, IL-6, GM-CSF, IL-18 and IL-1ra than in controls. VEGF and IL-2 were higher in controls. Infants with NE were relatively hypo-responsive to LPS.
stimulation in comparison to healthy controls with regard to IL-1β, IL-1α, IL-2, GM-CSF, VEGF and TNF-α were higher in control infants than those with NE, indicating a potential immune dysfunction when exposed to infectious threats. With LPS stimulation epo, IL-18, TNF-β and IL-1ra were higher in NE than controls. The difference in NE cytokine profiles between 1-2 and days 3-4 may be explained by a combination of both the evolving inflammatory response and TH modulating this response. Differentiation of these two theories however is difficult to draw as there isn’t a control group of NE treated with normothermia, nor at this stage would it be ethical to have that in any study as TH is the standard of care.

Epo was higher in infants with NE than controls in this study at baseline, and following LPS stimulation. Sweetman et al similarly demonstrated that epo was higher in NE then controls, and that higher levels of epo were associated with abnormal neuro-imaging. Hagag et al examined differences in cord blood epo in NE and controls and found higher epo levels in NE cord blood, and within this group the level correlated to severity of NE. Epo has been extensively studied in NE as a potential therapeutic agent. Epo decreases leucocyte infiltration and prevents IL-1β rise post exogenous administration in an animal model of brain injury. Currently the High Dose Erythropoietin for Asphyxia and Encephalopathy (HEAL) trial (NCT-02811263) is underway, with the aim of recruiting 500 patients. The study is exploring whether high dose epo reduces the combined outcome of death or neurodevelopmental disability when given in conjunction with hypothermia to newborns with moderate/severe NE. Epo receptors are upregulated in NE. Without an increase in endogenous epo production to bind to these receptors, cells are prone to apoptosis, hence the rationale to administer exogenous epo, however it may be better to measure endogenous epo levels prior to exogenous administration. If endogenous epo levels are already high in NE may indicate that exogenous administration may not be beneficial on a widespread basis but perhaps only in selected patients who lack an endogenous increase in production.

Anti-inflammatory IL-1ra was raised in infants with NE at baseline and also in response to LPS stimulation. IL-1ra was higher in infants with NE and abnormal neuroimaging. IL-1 receptor antagonists have demonstrated reduced injury and acceptable safety profile in adult stroke. IL-10, also an anti-inflammatory cytokine, was increased following LPS stimulation in both infant controls and those with NE. LPS-stimulated IL-10 was significantly higher in infants with increased severity of NE in comparison to baseline levels. O’Hare et al found higher baseline IL-10 correlated with severity of NE and that IL-10 was elevated in non-survivors. Okazaki et al also found IL-10 was higher in NE then controls, and correlated with the degree of asphyxia. IL-10 when co-administered with endotoxin in animal model of brain injury counteracted the acute effects of LPS on cerebral metabolism. IL-10 agonism has also been demonstrated
to reduce white matter damage in neonatal rats post maternal sepsis suggesting a neuroprotective effect

GM-CSF was relatively LPS hyporesponsive in infants with NE compared to controls. GM-CSF was higher with LPS stimulation in infants with NE Sarnat III compared with II. Lower GM-CSF was associated with abnormal neuroimaging. GM-CSF is known to stimulate granulocyte, monocyte and eosinophil production. O’Hare et al found infants with NE and abnormal neuroimaging had significantly elevated GM-CSF at 0-24h and an association with death in NE, however this was pre TH. Savman et al, found no differences between CSF GM-CSF controls and infants with NE. Higher GM-CSF levels have been demonstrated to expand myeloid cell expansion, inflammation and reactive oxygen species leading to CNS injury independent of T-cells causing the injury.

IFN-γ median levels did not differ between NE and controls at baseline nor with LPS stimulation. Both groups demonstrated an increase with LPS stimulation. IFN-γ was decreased in more severe grades of NE and on days 3-4 in NE. IFN-γ was higher at baseline in infants with an abnormal MRI brain. IFN-γ interacts with brain resident cells to induce neurotrophins and provide a peripheral immune response by increasing neutrophil and monocyte function. IFN-γ has been demonstrated to produce neuro protective factors for myelin sheaths and protect against inflammation. Okazaki found the concentration of IFN-γ in the asphyxiated neonates was lower than in the normal neonates, however Massaro et al demonstrated a higher level of IFN-γ on day 1 was associated with abnormal neuroimaging in NE.

IL-18 was higher at baseline in NE then in controls. IL-18 was higher in NE with LPS stimulation in comparison to controls. The level was higher with increasing severity of NE when stimulated with LPS. Hagberg et al found increased IL-18 expression in microglia post hypoxia in an animal model and decreased brain injury in an IL-18 knock out model. Perrone et al reported an increase in IL-18 at 24 hours in NE during hypothermia compared to controls. Liu et al reported that TH progressively decreases IL-18 in NE compared to infants with NE managed at normothermia. IL-18 secretion and activation are associated with the inflammasome component NLRP3 which is a potential area for immunomodulation.

IL-1β was increased following LPS in Controls and NE. The increase with LPS was higher in controls in comparison to NE. IL-1β was higher in NE with abnormal neuroimaging in comparison to normal neuro-imaging. Massaro et al also found increased IL-1β was associated with abnormal neuro-imaging in NE but this rise was not associated with developmental outcome at 1 year. Bharathi et al found significantly higher IL-1β in cord blood in perinatal asphyxia and that the level correlated with the severity of asphyxia. The cord IL-1β had a significant negative correlation with developmental score at 6 months.
IL-6 was higher in NE than controls, and increased with increasing severity of NE. Okazaki et al similarly found that IL-6 was higher in NE than controls. O’Hare et al showed that IL-6 was higher in NE at 24-48 hours with abnormal neuroimaging. Massaro et al found increased IL-6 was associated with abnormal neuroimaging in NE but was not associated with developmental outcome at 1 year. Bharathi et al found significantly higher cord IL-6 in perinatal asphyxia and that the level correlated with the severity of asphyxia. The cord levels of IL-6 had a significant negative correlation with developmental score at 6 months. Foster-Barber et al found higher levels of IL-6 in neonatal period differentiated infants with CP at follow-up, but did not find a correlation between IL-6 and severity of initial NE grade.

IL-8, a chemokine involved in neutrophil recruitment, was increased with LPS stimulation in NE and in controls but there were no differences between the groups baseline medians nor LPS stimulated medians. Okazaki et al found IL-8 was higher in NE than control. O’Hare et al found IL-8 was significantly elevated at birth in infants with severe NE and abnormal neuroimaging. Massaro et al found increased IL-8 was associated with abnormal neuro-imaging in NE but not associated with developmental outcome at 1 year. Foster-Barber et al found higher levels of IL-8 in neonatal period differentiated infants with cerebral palsy at follow-up, but did not find a correlation between IL-8 and severity of initial grade of NE.

Baseline IL-2 levels were similar in NE and control. Both NE and control were increased with LPS, however IL-2 was relatively hypo-responsive to LPS in NE in comparison to control. IL-2 increased as the severity of NE increased. Bharathi and Okazaki found no differences in cord blood IL-2 levels between NE and controls. IL-2 is a potent immunomodulator, activating immune cells. IL-2 receptor antagonists are already used therapeutically in anti-inflammatory and anti-rejection treatment so may have a future in immunomodulation in NE.

Baseline TNF-α levels were similar in controls and NE and both increased with LPS. TNF-α was relatively hypo-responsive to LPS in NE in comparison to control. Within the NE group, TNF-α was decreased in NE with abnormal MRI brain imaging. O’Hare found that TNF-α was lower at 72-96 hours in NE with abnormal neuroimaging. Bharathi found no differences in cord blood TNF-α levels between NE and controls. TNF-α is known to impair the blood-brain-barrier permeability after hypoxia, and anti-TNF agents are in common therapeutic use for autoimmune and inflammatory disorders, such as rheumatoid arthritis and inflammatory bowel disease.

TNF-β was significantly increased with LPS stimulation in NE but not in controls. The median was higher in NE with LPS stimulation but did not reach statistical significance upon Bonferroni correction. TNF-β was higher in infants with NE III in comparison to NE II following LPS
stimulation. Modulation of TNF-β has been demonstrated to both decrease areas of cerebral ischaemia and also decrease hippocampal regeneration post injury.

VEGF is a growth factor activated by hypoxia. VEGF was lower in infants with NE then in controls. Within the NE group, VEGF was higher in infants with abnormal neuroimaging. Sweetman et al demonstrated lower serum VEGF was associated with abnormal neuroimaging and was predictive of death in NE. Aly et al demonstrated increased cord blood VEGF in perinatal asphyxia and a further elevated in infants who developed NE, however this study was done with cord blood rather than neonatal blood.

The strengths of this study include the large sample size, the use of healthy term infants as age matched controls, and in the examination of LPS responses. This is in contrast to other studies that use cord blood which is relatively LPS tolerant and is not reflective of postnatal immune responses. Olin et al have demonstrated that cord blood is not representative of post-natal immunity, from a longitudinal analysis of 100 neonates over the first three months of life. Since this study explores the immune phenotype, neonatal blood is a much better comparator to use, albeit difficult to obtain and difficult to do complex experiments on the small volumes that can be ethically obtained in comparison to the abundant availability of cord blood in the maternity hospitals. Olin et al found that cord blood immune cell correlation to neonatal blood demonstrated only 6 out of 21 measurements correlated to neonatal blood. They also demonstrated that the neonatal blood changes rapidly over the first few days of life making it even harder for more inert cord blood to predict a neonatal immunophenotype. They reported that the cell composition, plasma protein concentration and cell phenotypes are all different between the cord and neonates.

The weaknesses of the study include relatively small patient numbers on subdivision of the NE cohort for short term outcomes, in particular the inability to do detailed statistical analysis in NE I patients with only 2 infants included. The difficulty lies in obtaining phlebotomy in these patients as many do not have many bloods performed. The neuro-imaging results however displayed some differences in inflammatory profiles, especially in response to LPS stimulation. This work complements the systematic review in Chapter 3 recommending that core outcomes be established so research such as this study can be presented and pooled similarly. The extensive presentation of this statistical work will allow it to be added with other work within our own group and pooled in larger meta-analyses.
4.6 Conclusion

The exploration and analysis of the inflammatory phenotype in NE and in healthy term controls has shown a distinct inflammatory phenotype and innate immune response in NE. The infants with NE display an altered LPS responsiveness in comparison to the healthy term control infants. Understanding this balance of pro and anti-inflammatory cytokines ultimately helps in understanding the pathophysiology and identifying ways of modulating the harmful effects and potentiating the beneficial effects.
Chapter 5 - Immunophenotype in Neonatal Encephalopathy

5.1 Introduction

Although TH has improved neurodevelopmental outcome and mortality of NE, 50% of infants still have disability\(^1\). Therefore new adjunctive therapies are under investigation. Immune dysfunction is well-described in NE\(^2\). However prior to developing new immunomodulatory therapies, understanding the inflammatory phenotype of infants with NE is crucial. NE produces an immune response on different levels, in terms of both the adaptive and innate immune systems, in the cerebrospinal fluid, brain tissue and in the systemic circulation\(^3\). This inflammatory response has been demonstrated to both predispose the neonate to injury and act in synergy with hypoxia-ischaemia to cause injury\(^4\). Altered levels of both pro and anti-inflammatory cytokines and a dysregulated inflammatory response to endotoxin have been demonstrated in NE\(^5\)\(^6\)\(^7\)\(^8\)\(^9\). Different immunomodulators have been postulated to target inflammation\(^10\), excitotoxicity and oxidative stress\(^11\) in NE. This chapter explores some of these target pathways to gain a better understanding of the mechanistic role of the role of mitochondria in granulocytes and monocytes and the expression of a number of inflammation related genes such as the NLRP3 inflammasome components, Hypoxia-inducible factor (HIF) 1-alpha and circadian rhythm genes.

The blood brain barrier (BBB) plays an important role in injury sustained in NE. An altered blood brain barrier allows leukocyte recruitment into the brain. Reperfusion post-ischaemia causes leukocyte activation and reactive oxygen species (ROS) release that further exacerbates the injury. Dammann\(^12\) et al hypothesized that the leukocytes that contribute to brain injury are derived from outside the brain, and that by modulating leukocyte migration brain injury may be reduced. Cytokines are known to activate leukocytes peripherally which cross the BBB and produce more cytokines\(^13\). Adhesion molecules allow adherence of leukocytes to vessel walls, and facilitate leukocyte release. This has prompted exploration of modulators that reduce leukocyte activation\(^14\). Hypoxia increases the maturation and production of neutrophils\(^15\), which upon activation produce an oxidative burst, cause cell migration, and degranulation\(^16\). Neutrophil knock out animal model resulted in a reduction in brain swelling suggesting neutrophils play a role in HI brain swelling\(^17\). Palmer et al identified that the timing of the neutrophils’ contribution to vascular dysfunction is in the early phases of injury during the hypoxic ischaemic insult or in the early hours post injury (less than 8 hours)\(^18\).

Myeloid differentiation primary response (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) are adaptors that bind to the intracellular domains of the Toll-Like-Receptor
and Interleukin-1-receptor families, linking them to IL-1R-associated kinase (IRAK)\textsuperscript{199}. Activation of IRAK activates a number of downstream inflammatory pathways; leading to the expression of pro-inflammatory cytokines such as TNF-\(\alpha\), IL-1\(\beta\), IL-6 and IFN-\(\gamma\)\textsuperscript{200}.

TRIF knock out neonatal mice are susceptible to \(E\ Coli\) septicemia due to decreased innate immune function and recruitment\textsuperscript{201}, the mice had decreased recruitment of peritoneal neutrophils and macrophages, decreased neutrophil and macrophage ROS production with increased peritoneal and blood bacterial counts. MyD88 signalling to recruit neutrophils via CXCL1 neutrophil recruiting chemokine has been demonstrated with a defective immune response in MyD88 negative animal models. In addition, children with MyD88 mutations are prone to recurrent bacterial infections\textsuperscript{202} demonstrating a crucial role in signalling\textsuperscript{203}.

The NLRP3 inflammasome mediates innate immune defence against pathogens via recognition in the host cytosol\textsuperscript{115}, becoming active upon a loss of cellular homeostasis, activating the inflammatory pathway of programmed cell death, via caspase-1 cleavage. This allows neutrophil activation, natural killer cell activation and local inflammatory reactions\textsuperscript{115}. NLRP3 has a range of activating signals including viruses, bacteria, ROS and damaged mitochondria\textsuperscript{116}.

Adenosine triphosphate (ATP) and it’s metabolite adenosine are important endogenous signalling molecules in inflammation\textsuperscript{131}. Under physiological conditions, adenosine is present in low concentrations in extracellular fluid but it is known to rise dramatically in stress, hypoxia, inflammation and ischaemia\textsuperscript{133}. High ATP production can act as a danger signal, is known to activate the NLRP3 component of the inflammasome and binds to P2X purinoceptor 7 (P2X7) to activate IL-1\(\beta\). Levy et al demonstrated that neonates have a relatively high adenosine concentrations in comparison to adults\textsuperscript{133}. They showed that upon adenosine receptor antagonism, TNF-alpha production was dramatically increased, demonstrating adenosines immunomodulatory properties in regulating the inflammatory response and may be involved in the relative immune hypo-responsiveness of umbilical cord blood\textsuperscript{135}.

Diurnal variation has been demonstrated in endotoxin sensitivity and in host defence\textsuperscript{121}. The molecular clock is controlled by a series of positive and negative feedback regulator genes, the supra-chiasmic nucleus and light. The transcription factors circadian loco-motor output cycles kaput, (CLOCK) and Brain and Muscle ARNT-Like 1 (BMAL1) are central to the positive transcriptional loop, binding to the clock controlled genes, period (PER) and Cryptochrome (CRY)\textsuperscript{122,123,124,125}. Their interaction causes oscillation of the circadian gene expression\textsuperscript{122,123,124,125}.

Understanding the role of the innate immune response in brain injury is important including examining responses at a cellular level, receptor activation and mechanisms involved in the altered immune-phenotype.
5.2 Aims

- To explore the leucocyte phenotype in infants with NE in response to LPS and compare these responses to healthy term controls.
- To describe and understand the mechanisms underlying the immune phenotype and responses in NE.
- To examine target pathways of inflammatory gene expression, excitotoxicity and oxidative stress, including the mechanistic role of mitochondria in granulocytes and monocytes, and the expression of inflammation related genes: the Inflammasome, Hypoxia-inducible factor 1-alpha and the circadian rhythm.

5.3 Hypothesis

There is an altered innate immune phenotype in babies with NE in comparison to healthy term control infants in response to LPS which may be associated with inflammasome and circadian rhythm gene alterations.

5.4 Results

5.4.1 Leucocyte cell surface markers

Neutrophil and monocyte phenotypes were explored by flow cytometry in infants with compared to controls. Neutrophil and monocyte CD11b did not differ between healthy infant controls (Con) and infants with Neonatal Encephalopathy (NE) in unstimulated cells (Figure 5.1). Neutrophil CD11b expression was increased following LPS stimulation in Con (p \( \leq 0.05 \)) but not in NE. Monocyte CD11b did not change with LPS stimulation in NE or Con (Figure 5.1).

Neutrophil TLR-4 expression was significantly lower in NE at baseline than controls and had a significantly lower response to LPS stimulation in NE (all p \( \leq 0.05 \); Figure 5.2). Monocyte TLR-4 expression did not differ in NE compared to controls or with LPS stimulation (Figure 5.2).

Neither neutrophil nor monocyte Nox expression differed between NE and controls at baseline, nor did Nox increase with LPS stimulation in NE (Figure 5.3). However, Nox expression was significantly increased with LPS in controls (p \( \leq 0.05 \); Figure 5.3). Therefore, infants with NE had
relative LPS hyporesponsiveness in neutrophil CD11b, TLR4 and Nox expression indicating an altered inflammatory response compared to controls.

Figure 5.1 Neutrophil (a) and Monocyte CD11b (b) expression in healthy term controls and in Neonatal Encephalopathy

Results denote Mean Channel Fluorescence (MCF) and the error bars represent standard error of the mean (SEM) of whole blood neutrophil and monocyte CD11b expression in healthy term controls (n=11) and Neonatal Encephalopathy (n=19). Neutrophils and monocytes were identified on the basis of their size and granularity (forward scatter; FSc, and side scatter; SSc, respectively), followed by labelling with CD66b positive for granulocytes, and CD66b negative and CD14 positivity for monocytes. * equates to p≤0.05 versus respective vehicle (Veh).
Figure 5.2 Neutrophil and Monocyte Toll-like receptor-4 (TLR-4) expression in healthy term controls versus Neonatal Encephalopathy

Results denoted as Mean Channel Fluorescence (MCF) and standard error of the mean (SEM) neutrophil and monocyte TLR-4 expression in healthy term controls (n=11) and in Neonatal Encephalopathy (n=19). Neutrophils and monocytes were identified on the basis of their size and granularity (forward scatter; FSc, and side scatter; SSc, respectively), followed by labelling with CD66b positive for granulocytes, and CD66b negative and CD14 positive for monocytes. * equates to p≤0.05 versus respective controls.
Figure 5.3 Neutrophil and Monocyte Nox expression in healthy term controls and in Neonatal Encephalopathy.

Results are denoted as Mean Channel Fluorescence (MCF) and standard error of the mean (SEM) for neutrophil and monocyte Nox expression in healthy term controls (n=11) and in Neonatal Encephalopathy (n=19). Neutrophils and monocytes were identified on the basis of their size and granularity (forward scatter; FSc, and side scatter; SSc, respectively), followed by labelling with CD66b positive for granulocytes, and CD66b negative and CD14 positive for monocytes. * equates to p ≤ 0.05 versus respective control.
5.4.2 Mitochondrial mass, mitochondrial ROS and MyD88

Neutrophil and monocyte mitochondrial mass were explored via flow cytometry with the fluorophore Mitotracker Green (MTG) representing mitochondrial mass. Mitotracker green assesses the mitochondrial mass regardless of the mitochondrial transmembrane potential. There were no differences in baseline MTG levels between NE and controls (Figure 5.4). Neutrophils and monocytes had a higher mitochondrial mass in NE than controls following LPS stimulation, (p<0.05). Monocytes were subdivided into classical, intermediate and non-classical subtypes and similarly demonstrated that mitochondrial mass was higher in NE post-LPS in comparison to control LPS responses (p≤0.05; Figure 5.5).

Superoxide production from mitochondria (MitoSox Red) in neutrophils in controls and NE did not differ at baseline or after LPS stimulation. Similarly, mitochondrial superoxide production in monocytes in controls and NE was similar and did not change after LPS stimulation (Figure 5.6). Monocytes were subdivided into classical, intermediate and non-classical subtypes and no differences were detected in mitochondrial superoxide production level, (Figure 5.7).

Gene expression of TIR-domain-containing adapter-inducing interferon- beta (TRIF), Interleukin-1 receptor-associated kinase 4 (IRAK-4) and Myeloid differentiation primary response 88 (MyD88) were examined using PCR. TRIF expression was similar between NE and controls and post-LPS stimulation. IRAK-4 expression was lower in NE at baseline then in controls (p≤0.05) but there were no differences detected upon LPS stimulation. MyD88 expression did not change between controls and NE or with LPS stimulation (Figure 5.8).

In summary, monocyte mitochondrial mass was higher in NE in response to LPS in comparison to control, and IRAK-4 gene expression was lower in NE at baseline in comparison to control.
Figure 5.4 Neutrophil and Monocyte Mitochondrial mass in healthy term controls compared to NE

These results denote Mean Channel Fluorescence (MCF) and standard error of the mean (SEM) of Mitotracker (MTG) Green to examine mitochondrial mass in neutrophils (a) and monocytes (b) in healthy term controls (n=4) compared to infants with NE (n=6). ** p=≤0.01 of NE post-LPS versus respective Control post-LPS. Monocytes and neutrophils were identified on the basis of their size and granularity (forward scatter; FSc, and side scatter; SSc, respectively). This was followed by labelling with CD66b positive for granulocytes, and CD66b negative and CD14 positive for monocytes. CD14 and CD16 FMO ensured standardised division of monocytes into their distinct populations in the context of the data spread due to the multiple fluorochromes in the panel.
Results denote Mean Channel Fluorescence (MCF) and standard error of the mean (SEM) of Mitotracker (MTG) Green on Monocyte subsets, classical (a), intermediate (b) and non-classical (c) in controls (n=4) versus NE (n=6). ** p≤0.01 of NE LPS versus respective Control LPS. The monocytes were identified on the basis of their size and granularity (forward scatter; FSc, and side scatter; SSc, respectively). This was followed by labelling CD66b negative and CD14 positive. CD14 and CD16 FMO ensured standardised way of dividing monocytes into distinct populations in the context of the data spread due to the multiple fluorochromes in the panel. Monocytes were divided into classical, non-classical and intermediate monocytes.
Figure 5.6. Mitochondrial ROS from Neutrophils and Monocytes in Controls versus NE

Results are denoted as Mean Channel Fluorescence (MCF) and standard error of the mean (SEM) of MitoSox on Neutrophils and Monocytes to assess mitochondrial ROS in controls (n=4) versus NE (n=6). The monocytes and neutrophils were identified on the basis of their size and granularity (forward scatter; FSc, and side scatter; SSc, respectively). This was followed by labelling with CD66b positive for neutrophils, and CD66b negative and CD14 positive for monocytes. CD 14 and CD16 FMO ensured a standardised way of dividing monocytes into distinct populations in the context of the data spread due to the multiple fluorochromes in the panel.
Results are denoted as Mean Channel Fluorescence (MCF) and standard error of the mean (SEM) of MitoSox, representing mitochondrial ROS on Monocyte subsets, classical, intermediate and non-classical in controls (n=4) versus NE (n=6). The monocytes were identified on the basis of their size and granularity (forward scatter; FSc, and side scatter; SSc, respectively). This was followed by labelling CD66b negative and CD14 positive. CD14 and CD16 FMO ensured standardised way of dividing monocytes into distinct populations in the context of the data spread due to the multiple fluorochromes in the panel.
Figure 5.8  TRIF, IRAK-4 MyD88 in Term Controls and infants with Neonatal Encephalopathy.

Gene expression was measured by polymerase chain reaction (PCR) of TIR-domain-containing adapter-inducing interferon-β (TRIF), Interleukin-1 receptor-associated kinase 4 (IRAK-4) and Myeloid differentiation primary response 88 (MyD88) in Term Control (n=5) and Neonatal Encephalopathy (n=5). The results represent fold change when the control vehicle results were normalised to one. *=p≤0.05 in NE Veh versus respective Control Veh.
5.4.3 Hypoxia Inducible Factor-1 Alpha and Correlations

The gene expression of Hypoxia Inducible Factor-1 Alpha (HIF-1α) was explored via PCR analysis. There were no significant differences detected on analysis between controls and NE at baseline or with LPS stimulation.

HIF-1α is known to be a powerful stimulator of transcription of many genes, including erythropoietin (Epo) in hypoxia-affected cells. Over-expression of HIF-1α can promote angiogenesis via stimulation of Vascular endothelial growth factor (VEGF). The relationship between HIF-1α and Epo and VEGF were therefore explored via linear regression. The values of Epo and VEGF in NE at baseline nor with LPS stimulation did not predict the HIF-1-alpha expression in NE.

Figure 5.9 HIF 1-alpha in healthy term controls compared with Neonatal Encephalopathy (NE) on Days 1 and 3 of life.

The gene expression of Hypoxia-inducible factor 1-alpha (HIF 1-alpha) in Healthy term controls (n=10) and in Neonatal Encephalopathy (n=10) on Days 1 and 3 of life. The results are displayed at fold change expression when con veh is normalised to one.
Figure 5.10 Epo concentration compared to HIF 1-alpha in Neonatal Encephalopathy

Linear Regression of Epo concentration to HIF 1-alpha vehicle and HIF 1-alpha LPS in Neonatal Encephalopathy (n=10), p value non-significant.

Figure 5.11 VEGF concentration and HIF 1-alpha in Neonatal Encephalopathy

Linear Regression of VEGF concentration to HIF 1-alpha vehicle and HIF 1-alpha LPS in Neonatal Encephalopathy (n=10), p value non-significant.
5.4.4 *Inflammasome*

The inflammasome is a key regulator of pro-inflammatory responses and a potential immunomodulatory target without compromising antibacterial immunity completely. LPS can act as a priming agent, sending a pathogen associated molecular pattern (PAMP) to activate pro-IL1β and upregulate NLRP3, and cause assembly of the inflammasome complex. The cytokines secreted following inflammasome activation, include IL-1 and IL-18 and regulate cells of both the innate and adaptive immune system, guiding the subsequent immune responses.

IL-1β gene expression was upregulated with LPS stimulation in controls and NE, (p≤0.05; Figure 5.12). The IL-1 β in NE was relatively hypo responsive to LPS in comparison to controls (p≤0.05). The expression of ASC was similar in controls and NE and did not change with LPS stimulation (Figure 5.13). NLRP3 was increased with LPS in NE on Day 3 (D3) in comparison to baseline levels (p≤0.05; Figure 5.14).

Inflammasome related cytokines IL-18 and IL-1β, regulate cells of both the innate and adaptive immune system, guiding the subsequent immune responses, and were examined by ELISA. IL-18 was raised with LPS stimulation in controls and on days 3-4 in NE (p≤0.05) (Figure 5.15). IL-1ra was upregulated with LPS stimulation in controls and in NE D1-2 and D3-4 life (p≤0.05). IL-1β was upregulated with LPS stimulation but the infants with NE were relatively hypo-responsive to LPS in comparison to the healthy term controls.
Figure 5.12: IL-1β in term controls and Neonatal Encephalopathy on Days one and three of life

Real Time PCR gene expression of IL-1 β in Healthy Term control (n=10) and in Neonatal Encephalopathy (n=10) on Days 1 and 3 of life. The results are displayed at fold change expression when control vehicle is normalised to one. The error bars represent standard error of the mean (SEM). * represents p≤0.05 versus respective vehicle.
Real Time PCR gene expression of Adaptor Protein Apoptosis-Associated Speck-Like Protein Containing CARD (ASC) in Term control (n=10) and in Neonatal Encephalopathy (n=10) on Days 1 and 3. The results are displayed at fold change expression when control vehicle is normalised to one. The error bars represent standard error of the mean.
Figure 5.14  NLRP3 expression in term controls and Neonatal Encephalopathy on Days 1 and 3

The results denote Real Time PCR gene expression of nucleotide-binding domain and leucine-rich repeat containing protein 3 (NLRP3) in term controls (n=10) and in Neonatal Encephalopathy (n=10) on Days 1 and 3 of life. The results are displayed at fold change expression when control vehicle is normalised to one. They are represented as fold change at baseline and in response to endotoxin stimulation. The error bars represent standard error of the mean. * represents p≤0.05 versus respective control.
Figure 5.15  Concentration of Inflammasome related cytokines IL-18, IL-1ra and Epo in term controls and in Neonatal Encephalopathy on Days 1 and 3

Median Concentration of Inflammasome related cytokines done by ELISA (a) Interleukin-18 (IL-18), (b) Interleukin-1 Receptor Antagonist (IL-1ra), and (c) Interleukin-1 Beta (IL-1β) in Term Controls (Con), (n=35) and Neonatal Encephalopathy (n=55) on Days 1 and 3. Error bars represent inter-quartile ranges. * represents that the difference between the medians of Baseline and LPS stimulation was ≤0.0035, ** represents the difference between Con and NE medians was ≤0.0035.
5.4.5 *Purinergic pathways in NE*

In view of alterations in inflammasome activation in NE we examined the P2X7 receptor, as it is a key gatekeeper of inflammasome activation. Purinergic signalling play an important role in brain injury, with modulation of P2X7 receptor or modulating the downstream adenosine response being postulated as potential molecular targets to consider in NE.

Karmakar et al demonstrated in human and murine neutrophils that leucocytes release ATP, which activates the P2X receptors signalling pathways leading to purinergic signalling and activates the inflammatory response. The P2X7 receptor is known to activate the inflammasome and downstream IL-18 and IL-1β release. They showed clinical relevance of this finding using a murine model of corneal infection demonstrating P2X7 receptors on neutrophils that were recruited to *Streptococcus pneumoniae* infected corneas. Modulation of the P2X7 receptor has been demonstrated to reduce downstream ATP excitotoxicity in animal model of brain injury thus potentially being a future molecular target in brain injury.

We hypothesised that adenosine would be a suitable point of care measurement in babies with NE. Adenosine at point of care level was examined in NE and controls and also in the mouse animal model controls and hypoxia model. The adenosine level was higher in NE than in healthy neonatal controls (p=0.03). Similarly, in the mice neonatal population the adenosine level was higher in hypoxia then in control mice (p=0.003). P2X7 levels were evaluated in babies with NE and controls and the median was higher in NE then controls but the numbers were too small to perform statistical analysis on (n=3, n=3).
Figure 5.16 Adenosine concentration as measured by rapid point of care testing in Neonatal Mice and in Human Neonates

The concentration of adenosine as measured by rapid point of care testing in Neonatal Mice (n=17) and in Human Neonates (n=18). The two groups represent control mice (control) and human infants Controls and Mice with hypoxia and neonates with NE. The unit measurement is micrometre. * represents p≤0.05, ** represents p≤0.01.
Figure 5.17: P2X7 plasma levels in healthy term control and in Neonatal Encephalopathy

Figure represents P2X7 plasma level in healthy term controls (control) and in Neonatal Encephalopathy (NE). The numbers included were n=3 in each group.
5.4.6 Circadian Rhythm in Neonatal Encephalopathy

The circadian rhythm genes Brain and Muscle ARNT-Like 1 (BMAL1), circadian loco-motor output cycles kaput (CLOCK), REV-ERBβ and Cryptochrome (CRY) were explored via PCR in NE and in controls. BMAL and CLOCK are central to the positive transcriptional loop, binding to the clock-controlled genes, period (PER) and CRY. PER and CRY are negative transcription activators. Nuclear receptors REV-ERBα and β regulate the molecular clock and suppress the expression of genes of the positive limb of the clock including CLOCK, BMAL1, and neuronal PAS domain protein 2 (NPAS2)\textsuperscript{120}.

BMAL expression was similar in Control and in NE at baseline. On Day 3 NE BMAL expression was decreased with LPS stimulation in comparison to vehicle (p≤0.05; Figure 5.17). Baseline levels of CLOCK, REV-ERBβ and CRY were similar in NE and controls (Figure 5.17). CLOCK and REV-ERBβ were hyporesponsive to LPS stimulation in NE in comparison to their respective vehicle (p≤0.05). The expression of CRY did not change with LPS stimulation (Figure 5.17).

The circadian rhythm is known to be linked to the innate immune system\textsuperscript{120}. The gene expression was therefore correlated to the cytokine profile of the neonates. There was a positive correlation between Rev-erbβ and IFN-γ (R value=0.75, R\textsuperscript{2}=0.57, p-value=0.0119 and confidence interval (CI) 0.24 to 0.94; Figure 5.17). There was a positive correlation found between Rev-erbβ and IL-6 (R value =0.63, R\textsuperscript{2} = 0.4, p value= 0.036, CI 0.053 to 0.89; Figure 5.19). There was a positive correlation between CLOCK and IL-2 (R value =0.73, R\textsuperscript{2} =0.53, p value= 0.02 and CI 0.18 to 0.93; Figure 5.19). There was a negative correlation between BMAL and IL-2 (R value= -0.67, R\textsuperscript{2} s=0.44, p value= 0.03 and CI -0.91 to -0.07; Figure 5.19).
Figure 5.18: BMAL, CRY, CLOCK and REV-ERBβ in term controls and Neonatal Encephalopathy on Days 1 and 3 of life

Gene expression of circadian rhythm proteins, BMAL, CRY, CLOCK) and REV-ERB β in term controls (n=10) and in Neonatal Encephalopathy (n=10) on Days 1 and 3 of life. The results are displayed at fold change expression when Control was normalised to one. They are represented as fold change at baseline and in response to LPS stimulation. The error bars represent standard error of the mean. * represents p≤0.05 versus respective vehicle, ** represents p≤0.01 versus respective vehicle.
Figure 5.19. Correlation of circadian gene expression (Rev-Erbβ, CLOCK and BMAL) to serum cytokine levels of IFN-γ, IL-6 and IL-2.

Correlation of circadian gene expression of Rev-Erbβ, CLOCK and BMAL to serum cytokine levels of IFN-γ, IL-6 and IL-2.
5.5 Discussion

Systemic hypoxia has been demonstrated to alter the neutrophil phenotype\textsuperscript{100}. Neutrophils are key responders of the innate immune response and the earliest leucocyte to initiate the inflammatory response. Hypoxia can delay their apoptosis causing a persistent inflammatory response, increased reactive oxygen species production and CD11b activation. This thesis demonstrated that neutrophil CD11b expression did not differ between controls and infants with NE. Neutrophil CD11b expression was increased with LPS stimulation in healthy term controls (p=≤0.05) but not in NE. Monocyte CD11b did not change with LPS stimulation in NE or in controls. Molloy et al demonstrated that infants with NE II/III had decreased neutrophil CD11b and increased neutrophil apoptosis compared to controls, whereas infants with NE I had decreased apoptosis compared to controls\textsuperscript{100}. This however was in umbilical cord blood which is not representative of postnatal samples and is more LPS hypo-responsive and this study used isolated neutrophils which may be more activated than whole blood analysis\textsuperscript{161}. O’Hare et al showed a higher monocyte and neutrophil CD11b in NE in comparison to adult blood at baseline and in response to LPS stimulation. The different comparator group makes it difficult to draw significant comparisons between the studies\textsuperscript{101}. Hassan et al examined leucocyte CD11b response to LPS in paediatric patients in PICU and showed a decreased CD11b LPS mediated response compared to healthy adult and paediatric controls\textsuperscript{208}.

Toll-like receptor 4 (TLR4), is an immune cell surface receptor involved in recognising endotoxin. Neonates requiring resuscitation at delivery had higher leucocyte CD11b and TLR4 in comparison to controls. The level of rise was correlated with severity of NE, with TLR4 expression associated with increased mortality\textsuperscript{101}. Neutrophil TLR-4 expression was significantly lower in NE at baseline than in controls, (p≤0.05) and had a significantly lower in response to LPS stimulation in NE, (p≤0.05). Monocyte TLR4 expression did not differ in NE compared to controls nor with LPS stimulation.

TLR-4 has been demonstrated to be upregulated with endotoxin in brain injury in \textit{in vivo} models\textsuperscript{209}. TLR-4 knockout mice have lower infarct size in stroke model compared to wild type mice\textsuperscript{210}, suggesting that antagonising TLR-4 may have a role in neuroprotection\textsuperscript{211}. O’Hare et al demonstrated an increase in neutrophil TLR4 upon LPS stimulation in NE who did not survive however the control samples used were cord blood\textsuperscript{101}. The comparator group in that study of cord blood is likely relatively inert and not representative of the immune phenotype of neonatal postnatal control samples\textsuperscript{161}. The unique aspect of this study is displaying the robust immune response to LPS stimulation of healthy term controls as the comparator group to NE which has
really exposed the poor innate immune response in NE. The importance of using the correct control group is one of the key results of this study.

Stimulated neutrophils are known to be the biggest producer of superoxide radicals via activation of the NADPH Oxidase complex Nox. Neutrophils contain mitochondria that rely on anaerobic glycolysis and are well adapted to working under hypoxic conditions. This adaptability under hypoxia has also been demonstrated with delayed neutrophil apoptosis. NADPH oxidase increases upon hypoxia ischaemia and contributes to brain injury by production of reactive oxygen species. Following LPS stimulation, neonates with severe brain injury produce significantly greater amounts of systemic neutrophil ROS compared to mildly affected neonates (NE 0/I) at 72-96 hours and day 7 of life. These ROS can cause significant tissue damage and may be mediated by mitochondrial dysfunction and HIF-1α up regulation. Mitochondria are a target for ROS and are vulnerable in NE as their capacity to scavenge is overcome with high levels of ROS, initiating mitochondrial permeability transition and potentiating oxidative stress. Nox expression in neutrophils and monocytes did not differ between NE and controls at baseline. Nox expression increased with LPS stimulation in controls, but did not increase with LPS stimulation in NE.

Another source of cellular ROS was examined by exploring mitochondrial ROS. Mitochondrial ROS production via neutrophils and monocytes was similar in NE and controls at baseline and with LPS stimulation. Neutrophil mitochondrial ROS production has not previously been studied in NE but was investigated and shown to be increased in paediatric patients with Systemic lupus erythematosus, an inflammatory condition, in comparison to healthy paediatric controls. Mitochondrial autophagy, the ability of mitochondria to maintain homeostasis and adapt to stress, may explain the relatively few differences at baseline in NE and control.

The mitochondrial density differences in neutrophils and monocytes between infants with NE and controls were examined with the hypothesis that the mitochondrial mass might differ upon systemic hypoxic-ischaemic changes in NE. Although there were no differences at baseline in mitochondrial mass between controls and NE, the mitochondrial mass expression was higher in NE with LPS stimulation in comparison to controls. The monocyte subtypes all similarly demonstrated that mitochondrial mass expression was higher in NE with LPS in comparison to controls.

MyD88, TRIF and IRAK are amongst 5 adaptor molecules that activate downstream kinases and transcription factors that regulate inflammatory responses following TLR activation. Hagberg et al demonstrated the essential role MyD88 has in LPS sensitised hypoxic-ischaemic brain injury. MyD88 knock out (KO) mice have decreased LPS sensitivity to hypoxic ischaemic brain injury, when in contrast the wild type mice demonstrated an increase in brain injury when
pre-treated with LPS. MyD88 KO mice demonstrated decreased downstream pro-inflammatory cytokine response in comparison to wild type, providing evidence of the interaction of MyD88 with LPS. TRIF mediated signalling was demonstrated to be protective in stroke by reducing ischaemic injury by in animal model of stroke by use of a TRIF ligand, Poly I:C which mediates signalling exclusively via a TRIF dependent pathway. Pre-treatment with this ligand reduced the cell death in comparison to control. IRAK-4 has not extensively been studied in NE, however IRAK inhibition has been demonstrated to be protective in Necrotising enterocolitis animal model by inhibiting TLR downstream signally, preventing the rise of pro-inflammatory cytokines and reducing intestinal damage in neonatal rats. TRIF and MyD88 in NE and Controls was similar and did not change in response to LPS stimulation. IRAK-4 expression was lower in NE at baseline than in Controls and did not change with LPS stimulation.

HIF-1α plays a key role in modifying transcriptional responses to hypoxia. HIF-1 and its downstream effects allows the body respond to hypoxia through transcriptional upregulation of genes to maintain homeostasis via anaerobic metabolism and enhancing tissue perfusion. HIF-1α is maintained at very low levels under normoxic conditions, but during hypoxia the transcriptional activity of HIF increases, which further regulates a number of hypoxia responsive genes including Epo and VEGF. HIF-1α regulates the transcription of genes involved in angiogenesis, erythropoiesis, glycolysis, iron metabolism, and cell survival. We have previously demonstrated in an NE cohort significantly elevated EPO and decreased VEGF levels compared with age-matched controls. There were no significant differences detected on analysis of HIF-1α between Controls and NE at baseline or with LPS stimulation. Epo and VEGF at baseline and with LPS stimulation did not predict the HIF-1-alpha expression in NE. HIF-1α signalling was dysregulated between NE (n=12) and controls (n=6). They used next-generation sequencing and found two genes associated with the HIF-1α signalling pathway, MALAT and RICTOR were differentially expressed between NE and control.

The inflammasome plays an important role in the innate immune response in the central nervous system, with promising results demonstrating improved outcomes following its manipulation in traumatic brain injury. There are safe and licenced immunomodulators manipulating the inflammasome in immune mediated diseases so understanding its role in NE could lead to a therapeutic target. The inflammasome components IL-1β, NLRP3 and ASC were altered in infants with NE. In NE, IL-1β by PCR and ELISA was increased at baseline and relatively hypo-responsive to LPS stimulation in comparison to Controls. NLRP3 was increased with LPS in NE on Day 3 in comparison to baseline levels. There were no significant difference in ASC expression between controls and NE. The inflammasome-related cytokines IL-18 and IL1-ra were upregulated in response to LPS in NE and also controls.
Saugstad et al. demonstrated that ASC deficiency is neuroprotective in neonatal HI brain damage in mice, while NLRP3 deficiency increases brain damage, indicating future potential immunomodulatory role of manipulating inflammasome components. In an animal model of traumatic brain injury, NLRP3 inflammasome complex assembly increases, as well as increased ASC expression and IL-1β. NLRP3 deficient mice were examined in comparison to controls, with no difference in the cerebral infarct size at 24 hours. However when examined at a later timepoint of 7 days, NLRP3 deficient mice had larger infarct volumes in comparison to control, and ASC deficient mice had smaller infarct sizes. The results of serum IL-1β and IL-18 did not change between knock out and control mice. This suggests that NLRP3 deficiency is detrimental in brain injury whereas ASC deficiency is neuroprotective, suggesting that future manipulation of ASC could be postulated as a target immunomodulatory therapy in NE.

Adenosine levels have previously been shown to be raised in hypoxia in animal model experiment. This is the first time it has been displayed in NE. Adenosine tri phosphate, (ATP) is an important neurotransmitter. In status epilepticus (SE) high extracellular ATP levels activate P2X receptors at excitatory synapses. P2X receptors are hypothesised to be increased in SE and cause neurological impairment via glutamate release and mediate neuronal death. Human and murine neutrophil P2X7 receptor activation has demonstrated ATP induced downstream NLRP3 activation and IL-1β secretion. Modulation of the P2X receptors has demonstrated a reduction in neuronal death and reduced inflammatory IL-1β production and seizure termination, demonstrating a neuroprotective role, suggesting that modulation of these P2X7 receptors could be a future inflammatory target in NE. The number of patients included in this Adenosine and P2X experiment with NE is small but represents an area of future potential exploration and reflects the animal model literature.

NE disrupts the circadian rhythm and a delay in development of a normal sleep wake cycle in NE is associated with brain injury and a worse outcome. The altered circadian rhythm demonstrated in NE at a gene expression level demonstrates another potential immunomodulatory target with melatonin or alterations in light dark cycling. Melatonin is an endogenous hormone with a number of properties, including energy metabolism via mitochondrial integrity, sleep wake cycle regulation, anti-oxidant and anti-inflammatory roles. Melatonin has been demonstrated to be neuroprotective in animal model experiments of HIE. Aly et al. demonstrated in a pilot small human neonatal RCT (n=30) short and long term benefits of melatonin in NE. Fulia et al demonstrated the anti-oxidant role of melatonin in NE and a possible impact on mortality, however the study involved small numbers (n=20).

The molecular clock gene expression was explored knowing that it was linked to their immune function. Curtis et al demonstrated that the innate immune system is variably responsive to challenges depending on the molecular clock.
BMAL, the positive regulator of the molecular clock, expression with LPS stimulation was decreased in comparison to baseline in NE. Positive regulator CLOCK and transcriptional repressor Rev-erbβ were hypo-responsive to endotoxin stimulation in NE in comparison to their respective vehicle gene expression. The gene expression was correlated to the cytokine profile, with a positive correlation between the negative regulator Rev-erbβ and pro-inflammatory IFN-γ and Rev-erbβ and pro-inflammatory IL-6. The positive regulators CLOCK and BMAL were examined with pro-inflammatory IL-2, CLOCK had a positive correlation with IL-2, and BMAL had a negative correlation with IL-2.

Chronic disruption of the components of the molecular clock in animal model has been shown to lead to enhanced LPS sepsis236, thus understanding the pattern of the molecular clock in NE both helps to understand the LPS hypo-responsiveness compared to term controls and also identifies a target for potential immunomodulation.

5.6 Conclusion

This work provides important new findings in both describing the role of the innate immune system and provides insights in the mechanisms that may provide new approaches to immunomodulation in NE. The chapter examines the immune responses at a cellular level, responses at a receptor level and helps in understanding the mechanism behind the altered immuno-phenotype.

The novel findings in this chapter that have not been explored in NE to date, include demonstrating neutrophil nox hypo-responsive response to LPS stimulation in NE, the increase in monocyte mitochondrial mass in NE and the dysregulated circadian rhythm at a gene expression level. The adenosine and P2X7 receptor were explored for the first time in NE and demonstrated differences between NE and healthy TC. This chapter demonstrates the significant innate immune response to LPS stimulation in the healthy TC compared to in infants with NE, displaying a healthy control robust immune response and a relative immunodeficiency in the infants with NE. The findings show potential biological plausibility’s of manipulating the circadian rhythm, by melatonin or dark exposure, and in adenosine as a bedside biomarker of NE.
Chapter 6 - Clinical Management in Neonatal Encephalopathy

6.1 Introduction

An important aspect of research is engaging stakeholders to push forward the research question with the ultimate goal of improving the care and outcome of neonates with NE. This involved publication of animations following parental input and the preparation for publication of a national guideline to streamline the management of patients with NE and a submission of a paper to an international Paediatric journal on the evidenced based management of NE.

In terms of management of an infant with NE, the use of TH has an excellent evidence basis, with a number needed to treat of between 7-8 depending on the severity of NE to improve the morbidity and mortality of one infant. The concurrent multi-organ dysfunction management during NE has limited evidence to guide therapies regarding morbidities and mortality. The evidence was explored via an extensive literature review and recommendations based on best available evidence and highlighted areas requiring further research to establish best evidence-based practice.

6.2 Hypothesis

To improve the care of neonates with Neonatal Encephalopathy through improved Management guidance and parent participation in research.

6.3 Aim

To improve the care internationally by producing a management paper and identifying deficits in evidence-based management of NE.

To improve the care nationally with standardised clinical management guideline

To improve the care of infants with NE with parent’s participation in this research
6.4 Results

The results of this chapter are displayed as three publications; the management paper (6.4.1) the management guideline (6.4.2) and the animations (6.4.3).

6.4.1 Management paper

Management of Multi Organ Dysfunction in Neonatal Encephalopathy – this paper was submitted to Frontiers in Pediatrics in August 2019.

Introduction

Neonatal encephalopathy is a clinically defined syndrome of disturbed neurologic function in the earliest days of life in an infant born at or beyond 35 weeks of gestation, manifested by a subnormal level of consciousness or seizures, and often accompanied by difficulty with initiating and maintaining respiration and depression of tone and reflexes.

Hypoxia ischaemia is implicated in NE and in up to 50% of cases the exact underlying cause is unknown and is commonly a combination of factors. The terms neonatal encephalopathy and hypoxic-ischaemic encephalopathy are used interchangeably in the literature but as NE is more comprehensive and is not just associated with sentinel events it is the term used in this management review.

The neonatal period is the highest risk for brain injury during the lifespan. In 2010, the estimated global burden of NE was 1.15 million, with 96% of these infants being born in low and middle income countries. The global estimated incidence from a systematic review in 2010 is 8.5 per 1000 live births, with an estimated incidence of 1-3 per 1000 births in high income countries. There is no single gold standard diagnostic test to determine aetiology, severity or prognosis.

Therapeutic hypothermia (TH) is known to be neuroprotective by addressing the cascade of injurious events that follow a hypoxic-ischemic insult in NE. Randomized controlled trials demonstrated the safety and the efficacy of TH by demonstrating a reduction in death and major neuro-disability for infants with moderate to severe NE when their clinical history, laboratory criteria and neurological exam meets agreed standardised criteria. Despite TH, the incidence of death or moderate/severe disability remains high at 48%. A systematic review including seven randomised controlled trials (RCT) with 1214 neonates with NE undergoing TH concluded an overall mortality of 28%, with a range of 24 to 38%, with the following incidences of...
neurological impairment; cognitive impairment 24%, cerebral palsy 22%, epilepsy 19%, and cortical visual impairment 6%. These high morbidity and mortality rates leave a window of opportunity to improve outcomes by further optimising TH candidate selection, improving timeliness of treatment initiation, increasing the use of brain monitoring for the identification and treatment of seizures, improving multi-organ management during TH, and identifying biomarkers to offer individualised adjunctive therapies during TH.

Neurological dysfunction is only part of the spectrum of injury in NE following hypoxic ischemic insult, as these infants could have co-existing multi-organ dysfunction which contributes to subsequent morbidities and mortality. The pathophysiology underlying the brain injury in NE affects the immune, respiratory, endocrine, renal, hepatic and cardiac functioning. In addition, exposing these infants to TH has its own impact on multi-organ function. Optimisation of multi-organ monitoring and support during TH has the potential to prevent injury progression and enhance the neuroprotective effects of TH.

TH is the standard of care for moderate and severe NE. Major randomised clinical trials have demonstrated a reduction in death and disability with TH. A Cochrane meta-analysis of these trials concluded that in infants over 35 weeks and less then 6 hours of age with moderate or severe NE (n=638), TH to 33.5 to 34.5°C for 72 hours, reduced the mortality and disability at 18 months of age with a typical risk reduction (RR) of 0.75 and number needed to treat (NNT) of 7. The National Institute of Child Health and Human Development (NICHD) and Committee of the Fetus and Newborn of the American Academy of Pediatrics (AAP) subsequently published a framework to ensure the appropriate use of TH, and recommended that infants should meet inclusion criteria outlined in clinical trials as follows: gestational age >36 weeks; age <6 hours; pH of ≤7.0 or a base deficit of ≥16 mmol/L in of umbilical cord blood or blood obtained during the first hour after birth; history of an acute perinatal event; 10 minute Apgar score of <5 or assisted ventilation at birth and for 10+ minutes; neurologic examination demonstrating moderate to severe encephalopathy.

Evidence from animal studies suggests that earlier TH initiation increases neuroprotection. However, the Neonatal Resuscitation Programme (NRP) notes that there is a paucity of evidence regarding commencement of hypothermia during resuscitation as passive hypothermia without core temperature monitoring during resuscitation may result in overcooling with serious adverse effects. Thoresen et al, showed significantly improved motor development scores at 18 months when TH is initiated < 3h (n=35) compared to >3h of age (n=30). This suggests that once indicated, TH should be initiated immediately after resuscitation.
Two further randomized controlled trials examined modifications from the original TH protocols. Laptook et al\(^{244}\) examined late initiation of TH in NE at between 6 and 24 hours of age. The trial was a multicentre RCT (n=164) comparing late initiation of TH with normothermia. Although there was no statistically significant difference in death and disability between groups, the authors used a pre-specified Bayesian analysis to demonstrate that there was an increased probability of reduction in death or disability with minimal adverse events. Shankaran\(^{245}\) et al examined TH to a lower temperature of 32°C and/or for a longer duration of 120 hours in a multi-centre RCT. The trial was stopped at 50% (n=364) of the planned recruitment due to adverse events including anuria, arrhythmia, increased inhaled nitric oxide and extra corporeal membrane oxygenation (ECMO) use, more days of oxygen and a higher incidence of bradycardia as well as trend towards increased mortality. There was no difference between treatment groups in the primary outcome of death or neurodisability at 18-22 months of age.

The initial TH protocols included infants with moderate to severe NE. Despite recommendations from organizations like the AAP, there is evidence of therapeutic drift with TH being provided to neonates with mild NE. A national survey in the UK reported that 75% of centres offered TH to infants with mild NE\(^{246}\). The Prospective Research on Infants with Mild Encephalopathy (PRIME) Study found that 52% of infants with mild NE had an abnormal early aEEG or seizures, abnormal brain MRI, or neurological exam at discharge\(^{247}\). When this population was followed at a mean of 19 months, 16% had disability and 40% had Bayley III scores < 85\(^{248}\). Murray et al noted that children with mild NE, not treated with TH, had cognitive outcomes similar to that of children with moderate NE, who were treated with TH\(^{249}\). A systematic review examining outcome of infants with mild NE found that 25% (n=341) had abnormal neurodevelopment outcome\(^{250}\). There is an ongoing phase two RCT by Thayyil et al, Optimising the duration of Cooling in Mild Neonatal Encephalopathy (COMET Study; NCT03409770), examining the feasibility and duration of TH in mild NE in the UK. Interpretation of research and smaller cohort studies of neonates with mild NE is limited by a lack of consistent definition of mild NE across study groups. Although a beneficial effect of TH in this population is plausible, to date this benefit has not been demonstrated in an RCT\(^{251}\).

A detailed neurological assessment is required to diagnose NE and ideally before the administration of sedating medications that may alter the neurological examination. The Modified Sarnat Examination\(^ {37}\) is used in the evaluation of neurological status for initiation of TH. This was used in the main TH RCTs\(^ {252}\) apart from the Total Body Hypothermia (TOBY) trial and the Cool Cap Study both of which included amplitude integrated electroencephalogram (aEEG) as an entry criterion\(^ {253}\). The Sarnat Score assesses three stages of NE: mild (NE I), moderate (NE II) and severe (NE III) which were correlated with clinical outcome. The score is derived from a study in 1976 of 21 infants with NE graded according to their level of encephalopathy and EEG.
findings over the first week of life. Sarnat et al concluded that persistence of moderate encephalopathy for more than 7 days was associated with poor neurologic outcome or death\textsuperscript{37}. A systematic review of newborn assessment to predict neurological outcome including 12 studies concluded that the risk of neonatal death was 24 fold higher in a patient with Sarnat III than Sarnat II, and 171 times higher in Sarnat III than Sarnat I\textsuperscript{254}.

The Amiel-Tison Neurologic Assessment at Term (ATNAT)\textsuperscript{38} was developed to provide a framework for observing the development of cortical control in infants at term and has been shown to predict the occurrence of cerebral palsy after birth asphyxia. Amess et al examined term infants (n=28) with NE with the ATNAT at 8 hours and at 7 days in comparison to healthy controls. Both early and late neurological examinations were reliable indicators of a favourable outcome at 1 year, having negative predictive values of 100\% and 91\% respectively\textsuperscript{39}. Murray et al, used the ATNAT serially over the first three days of life in 57 infants with NE, and found a significant correlation between ATNAT and neurological outcome at 2 years\textsuperscript{41}. A normal early assessment predicted a normal outcome and the infants with persistent neurological abnormality on day 3 were more likely to have a neurodisability. The risk was 100\% in those with a severely abnormal ATNAT rating, and 41\% in those with a moderately abnormal rating.

The Thompson score is a clinical tool assessing central nervous system dysfunction in NE, based on the longitudinal clinical assessment of 9 signs, including tone, level of consciousness, seizures, posture, Moro, grasp, suck respiratory function and anterior fontanelle tension\textsuperscript{42}. Kothapali et al showed that the Thompson score has a good short-term predictive capacity and correlated on day one with neonatal morbidity and mortality in infants with NE (n=145)\textsuperscript{43}. Mendler et al showed a strong association between Thompson score and adverse long-term outcome using the Wechsler preschool and primary scale of intelligence III test at a mean age of 53 months in infants with NE (n=36)\textsuperscript{44}. Almost all surviving infants with a maximal Thompson score ≤10 had a normal IQ and almost all infants with an impaired IQ (<85) had a Thompson Score ≥11.

Prechtl’s assessment of general movements assesses spontaneous motor activity and has proven sensitive in the prediction of cerebral palsy\textsuperscript{255}. General movements assesses for two distinct patterns of normal movements, writhing movements at 6 to 9 weeks post term, and fidgety movements at 6 to 20 weeks. Abnormal movements include poor repertoire, cramped-synchronized and absent fidgety general movements. General movements were highly correlated to grey matter injury in infants with NE\textsuperscript{256}. All infants with severely abnormal general movements had grey matter injury and poor motor outcome. Infants with predominantly white matter, cortical lesions or mild basal ganglia or thalamic injury had normal or transiently abnormal general movements and normal or mild motor impairment. The ATNAT was compared to Prechtl's
qualitative assessment of general movement in a group of 45 preterm infants with risk factors for brain injury and correlated better with neurodevelopmental outcome 40.

Near-infrared spectroscopy (NIRS) is a tool to monitor regional cerebral oxygen saturation, via a calculation based on the absorption spectra of oxygenated and deoxygenated haemoglobin. The measurement of regional cerebral oxygen saturation represented mixed oxygenation of both arterial, venous and capillary readings257. A systematic review of the use of NIRS in NE showed an association between impaired cerebral autoregulation and cardio-respiratory injury, abnormal MRI and long-term outcome258.

The incidence of seizures in NE is approximately 50%259. The presence of seizures increases the incidence of neurodevelopmental impairment and a cumulative seizure burden of 40 minutes increases neurodevelopmental impairment by nine-fold, independent of grade of encephalopathy and TH260. TH reduces the seizure burden, especially in moderate NE261. Seizures can be difficult to diagnose in neonates and approximately 50% do not have obvious clinical signs262263.

Availability of continuous electroencephalography (EEG) with real-time interpretation by trained staff is ideal but most NICUs use the modified form of amplitude integrated EEG (aEEG), a single or double lead EEG recording from two parietal electrodes, or a combination of EEG and aEEG. aEEG is useful to monitor baseline brain activity and to detect seizures264. The combination of early neurological examination and aEEG, in comparison to each individually enhances the ability to identify infants with NE265. Svenningsen et al demonstrated that aEEG background activity over the first six hours of life accurately predicted neurological outcome266. The limitations of aEEG include in short low voltage seizure detection267 and inaccurate readings due to artefact compared to EEG. aEEG has been demonstrated to be less predictive of outcome at early time points in infants treated with TH compared to normothermia; infants with good outcome had normalized background pattern by 24 hours when treated with normothermia and by 48 hours when treated with hypothermia268. Various training modalities are available such as from the Total Body Hypothermia Register website. (https://www.npeu.ox.ac.uk/downloads/files/toby/TOBY-CFM-Manual.pdf)

The treatment of seizures in NE remains a therapeutic challenge269. Phenobarbitone is used as first line agent270, however its efficacy is limited. Phenobarbitone has been demonstrated to reduce both the amplitude and propagation of seizures271 which may result in electroclinical uncoupling of seizures, which refers to electrographic seizure activity that is not clinically manifest and may make seizure detection more difficult on aEEG monitoring. There are concerns about adverse cognitive effects of phenobarbitone on the neonatal developing brain272. Animal models have demonstrated that early phenobarbitone exposure causes adverse neurological outcomes later in life273 and most of the pharmacological data on phenobarbitone is extrapolated
from adult data. The ‘efficacy of intravenous levetiracetam in neonatal seizures’ trial (NEOLEV2; NCT01720667) examined the efficacy of using levetiracetam first line in comparison to phenobarbitone for neonatal seizures from all causes. The primary outcome was to determine the efficacy of intravenous levetiracetam in terminating neonatal seizures (from all causes) when given as first line therapy compared to phenobarbitone. The provisional results demonstrated that phenobarbitone was much more effective then levetiracetam with 80% remaining seizure free for 24 hours compared to 28%.

Sharpe et al provided continuous monitoring for patients in the NEOLEV2 trial with real-time response to seizure detection and reported that automatic seizure detection algorithm was useful but not accurate enough to replace human review and that placement of EEG monitors after hours was problematic. Levetiracetam pharmacokinetic studies in term newborns found a higher than expected renal clearance that increased significantly over the first week of life requiring increased interval dosing.

Second line agents to treat neonatal seizures include phenytoin, levetiracetam, topiramate, lidocaine and midazolam. Phenytoin was found to be 57% effective as a combination therapy with phenobarbitone to achieve seizure control in an RCT of neonates with seizures from all causes (n=59). Bumetanide was evaluated as a second line therapy for neonatal seizures via the NEMO (Treatment of Neonatal seizures with Medication Off-patent: evaluation of efficacy and safety of bumetanide) open label feasibility trial. The trial had safety concerns regarding ototoxicity and was stopped prematurely (n=14). Bumetanide did not show signs of clinical efficacy for the treatment of neonatal seizures in the 14 infants that were studied prior to the premature cessation.

A survey of 55 paediatric neurologists predominantly based in USA showed a high off label use of levetiracetam and topiramate as second line for neonatal seizures. No side effects in the levetiracetam group were reported in the survey and the treatment was reported as beneficial in over half of cases. There was no consensus on the dosage to use and a wide range of doses were reported (10–30mg/kg). Midazolam has been shown to have a good response rate in seizure termination in a group of neonates with seizures refractory to phenobarbitone and phenytoin.

A retrospective review of neonatal seizure management reported that lidocaine demonstrated effectiveness in 50% of neonatal seizures as second line therapy but caution is warranted in view of cardiac toxicity, with bradycardia reported in 3% of patients. These studies demonstrate that there is an urgent need for clinical trials to determine safe and effective treatment for neonatal seizures.

In summary, real time full EEG is the best tool for neonatal seizure detection with phenobarbitone remaining the first line agent with demonstrated clinical efficacy but some overall safety concerns. A global working group of experts established a consensus for protocols for new RCTs in neonatal seizures using evidence based neonatal seizure treatment.
MRI is the gold standard technique to detect patterns of cerebral damage in NE and provides a reliable guide to prognosis. However, cranial ultrasound (US) as well as Doppler sonography might have useful adjuncts in early diagnostic imaging. Conventional MRI with T1 and T2 weighted images has been demonstrated to have good diagnostic ability to detect brain injury at the end of the first week of life. However with the advancement of diffusion weighted imaging (DWI) and magnetic resonance spectroscopy (MRS), lesions can be visualised within the first few days. The limitation of DWI is that the changes normalise within the first week. A feasibility study examined the timing of MRI brain in 12 infants with NE on day one, day 2-3, day 8-13 and at one month of age. In cases of severe NE, DWI changes were subtle on day 1 and became more apparent on days 2-3. MRS displayed an elevation of lactate in the injured areas within the first 24 hours. All brain injuries were already visible on early MRI scans, the later MRIs did not show any new lesions. These scans were performed by 3 Tesla (T) MRI, which is a higher field strength MRI that improves image quality and diagnostic accuracy in comparison to a conventional 1.5 T MRI. 3T MRI has a good safety profile and is safe for the developing brain but is not universally available.

MRI changes in NE may commonly involve parasagittal watershed infarcts between anterior/middle cerebral artery and middle/posterior cerebral artery, with both cortical and subcortical involvement, and injury to metabolically active tissues such as the basal ganglia (BG), thalami, putamen, hippocampi, brainstem and corticospinal tracts. Abnormalities in the signal of the posterior limb of the internal capsule (PLIC), BG and thalami have been demonstrated to have the greatest predictive value of poor neurodevelopmental outcome, in particular for motor outcome. Severe BG and thalami lesions predictive accuracy for severe motor impairment was 0.89 in one study, with abnormal PLIC signal intensity predicting the inability to walk independently by 2 years with a positive predictive value of 0.88. In contrast, Infants with white matter damage and no BG or thalamic injury had a good prognosis for independent walking by two years of age in infants with NE (n=270). TH reduced BG, thalamic, white matter and PLIC signal abnormalities. TH may be better at reducing the severity of BG and thalamic injury as these regions are selectively vulnerable to acute hypoxia-ischemia compared to parasagittal areas which are associated with partial prolonged injury. The accuracy of MRI as a biomarker to predict their outcome is not altered by TH. There are limitations to the predictive value of MRIs as a normal MRI does not guarantee a normal neurodevelopmental outcome. In one study 32% of infants who had a normal MRI brain in the neonatal period post TH had abnormal development at follow up.

Magnetic resonance spectroscopy (MRS) provides a non-invasive examination of biochemical brain biomarkers. The Magnetic Resonance Biomarkers in Neonatal Encephalopathy (MARBLE) Study found that thalamic N-acetyl aspartate (NAA) concentration had the highest...
sensitivity (100%) and specificity (97%) to predict neurodevelopmental outcome at 2 years (n=223). A meta-analysis on MR biomarkers identified MRS deep grey matter Lactate/NAA as the most accurate biomarker on MR to predict neonatal outcome and commented that MRS scoring systems can increase prognostic objectivity.  

Diffusion tensor MRI is more sensitive than conventional MRI to explore brain development and white matter fibres density and maturation. Tractography provides a reliable method for early assessment of perinatal brain injury and provides information on microstructural brain injury. Massaro et al studied infants with NE (n=52) using MR tractography and found that impaired microstructural organization of the corpus callosum and corticospinal tract on Day 7-10 of life post TH predicted poorer cognitive and motor performance at 15-21 months of age, respectively.

The development of Neonatal Neurocritical care has shown clinical improvement in babies with NE. Neurocritical care is an evolving field of tertiary intensive care units through collaboration of neonatologists, neurologists, nurse specialists and allied health professionals who coordinate care for neurologically ill neonates. Neurocritical care aims to address the needs of neonates at risk of neurological injury. Neurocritical care provides consistency in diagnostic and management strategies which can lead to improved neurological outcome. A retrospective review of infants with moderate to severe NE compared the brain MRIs of infants cared for in their normal NICU (n=109) to brain MRIs of infants cared for after their introduction of a neurocritical ICU (n=107). Following the introduction of their specialised Neurocritical care the review demonstrated a reduction in abnormalities on MRI brain after adjusting for confounding factors (odds ratio 0.3, CI 0.15 to 0.57, p <0.001). The changes implemented in that Neuro NICU included the introduction of a new multi-disciplinary team, full EEG monitoring done for duration of hypothermia and rewarming, neuroprotection protocols, quality improvement practices, alongside implementation of a long-term follow-up program.

The spectrum of cardiovascular (CVS) dysfunction in NE ranges in severity and may be attributed to hypoxia or be secondary to ischemia, metabolic acidosis and multiorgan injury. Myocardial contractility, cardiac output and blood pressure are all negatively impacted and co-existing pulmonary hypertension is common. CVS dysfunction may be evaluated from a number of modalities including vital signs, biochemical parameters, echocardiography and other haemodynamic assessments.

TH affects haemodynamic functioning by causing bradycardia, peripheral vasoconstriction and decreasing cardiac output. TH increases the QTc interval and increases the risk of cardiac arrhythmias. The initial TH RCTs were not adequately powered to examine cardiovascular benefit however creatinine-kinase muscle/brain (CK MB) and brain natriuretic peptide (BNP)
decrease with TH suggesting a cardioprotective effect and animal model studies have demonstrated an improvement in cardiac ischaemia with TH.

Both troponin-T and troponin-I have been demonstrated to be sensitive markers of cardiac dysfunction in NE. Gunes et al measured serial troponin-T, creatinine Kinase, (CK-MB) in 45 infants with NE. CK-MB levels were significantly higher in moderate and severe NE then in mild NE and in healthy infants. Boo et al found the sensitivity of serum troponin-T in detecting myocardial injury in NE presenting with heart failure was 72.7% and the specificity was 35.9%. Serial serum troponin levels during the first 48 hours of life have been found to be significantly higher in infants with NE who died.

Hypotension is observed in up to 62% of patients and may cause secondary multiorgan ischaemic injury. There is no consensus on the ideal target mean systolic, diastolic nor pulse pressure during TH nor on the best pharmacologic agents to maintain it. Long term clinical outcomes of inotropic and chronotropic support lack evidence, with dopamine, dobutamine and adrenaline commonly chosen with short term haemodynamic improvements in BP. Vasopressor use warrants caution due to potential pulmonary and systemic vasoconstriction. McNamara et al recommended that inotropes administration on an individual patient basis as depending on their echocardiography (ECHO) and clinical status. Echo is the best diagnostic tool available to assess cardiovascular function and guide inotropic, chronotropic and fluid management. Medications used to treat systemic hypotension include dopamine, dobutamine, adrenaline and noradrenaline, depending on coexisting myocardial dysfunction. Adrenaline may be the most appropriate inotrope due to its action on α1, α2, β1, and β2 receptors and a favourable impact on pulmonary vascular resistance (PVR)/systemic vascular resistance (SVR) ratio. The action of dobutamine via α and β receptors decreasing SVR may have advantages as an inotrope in the context of persistent pulmonary hypertension of the newborn and myocardial dysfunction but has not been subject to controlled trials. Dopamine is predominantly a vasopressor and in neonatal animal studies has been shown to increase PVR and SVR, which has the potential to increase afterload, decrease left-to-right shunting and compromise systemic oxygen delivery.

McNamara et al recommended dobutamine use in NE to improve cardiac contractility and heart rate. Milrinone needs to be used cautiously due to altered pharmacokinetics during TH affecting its clearance and also caution with noradrenaline administration as there is little evidence of benefit and it has not been subject to RCT for follow up data. Dopamine is the most widely studied and the most commonly prescribed inotrope in neonatology. However studies of developmental outcome favour dobutamine use over dopamine in the preterm population and there are no comparative RCTs in NE.
A sustained difference of >5-10% between continuous pre- and post ductal saturation monitoring may indicate PPHN, which can be confirmed on echocardiography. Echocardiography helps to quantify the degree of PPHN and guides treatment including choice of inotropic support. The development of serial functional echocardiography in the NICU allows tracking of the dynamic changes occurring over the course of PPHN.

The management of pulmonary hypertension involves reducing the cardiac afterload and maintaining high preductal mean blood pressures. Milrinone has been shown to improve oxygenation index in term neonates with severe PPHN without compromising systemic blood pressure. A Cochrane review concluded that the efficacy and safety of milrinone in the treatment of PPHN are not known and recommended that use is restricted to RCTs. Milrinone metabolism is known to be decreased by hypothermia and in an animal model study the inotropic effect was abolished at temperatures of between 31 and 34°C. Sildenafil is increasingly used in PPHN. A Cochrane review of sildenafil for PPHN found a significant reduction in mortality in the sildenafil group versus the control group with a number needed to treat of 3. The review concluded that sildenafil has significant benefits especially in resource-limited settings and recommended a large-scale randomized control trial comparing sildenafil to the currently used vasodilator inhaled Nitric Oxide (iNO). A Cochrane Review of iNO for respiratory failure in near term or term infants found that iNO improved the outcome in hypoxaemic term infants by reducing the incidence of the combined endpoint of death or need for extra-corporeal membrane oxygenation (ECMO). Oxygenation improved in approximately 50% of infants receiving iNO. Long-term follow up studies have found no increase in neurodevelopmental impairment with its use.

In summary, cardiovascular dysfunction in NE can be negatively impacted by TH. Echo is the best tool to guide management and other physiological parameter thresholds have not been well-defined. Inotropic and chronotropic medications have altered pharmacokinetics during TH and the choice of agent is best chosen on an individual neonatal basis depending on haemodynamics.

Respiratory Management in NE

The incidence of respiratory dysfunction in babies with NE varies from 23-86% in NE. The spectrum of injury ranges from transient oxygen requirement to severe persistent pulmonary hypertension (PPHN). The pathogenesis of pulmonary dysfunction is complex, although hypoxia is a major component via disruption of the normal physiological fall in pulmonary vascular resistance.

The Neonatal Resuscitation Program® (NRP), 7th edition and European Resuscitation Council Guidelines advise on initial management and resuscitation for a non-vigorous term infant.
NRP recommends resuscitation using 21% fraction of inspired oxygen concentration and titrating the oxygen to maintain oxygen saturations within a standardised oxygen centile range depending on minutes of life. The exception to this is if a neonate requires cardiopulmonary resuscitation to titrate the fraction of oxygen to 100%. Neonates are at risk of hyperoxia when exposed to high oxygen concentration, after coming from a relatively hypoxic environment in utero and their free radical scavenger systems are underdeveloped. Infants with perinatal stress (n=609) were enrolled in a multi-centre RCT comparing resuscitation at 100% fraction of inspired oxygen to 21%. The infants with higher oxygen exposure had more oxidative stress but no differences were found in mortality or short-term morbidity. This study was limited by the fact that the infants who were resuscitated were relatively well, as all had oxygen saturations of over 90% at 2 minutes and less then 2% of them required supplementary oxygen after resuscitation. Expert opinion from this study recommended restoring normoxia as quickly as possible during resuscitation and that a properly powered RCT to establish correct fraction of inspired oxygen would need to recruit 7000 neonates.

Respiratory support in NE aims to maintain a pH over 7.25 and a normal to high partial pressure of arterial carbon dioxide (PaCO$_2$ 5-7 kPa, 37.5-52.5 mmHg). Hypocarbia has detrimental effects on cerebral perfusion in an already compromised infant and is associated with neurosensory hearing impairment and abnormal neurodevelopment. Both isolated low PaCO$_2$ levels and cumulative PaCO$_2$ less than 4.6 kPa (35 mmHg) were associated with death and disability.

Hypothermia is known to effect pH, PaCO$_2$ and PaO$_2$. The partial pressure of oxygen and carbon dioxide decrease and pH rises, as the temperature decreases. The temperature corrected value can be obtained by inputting the temperature to the blood gas analyser. Dassios et al reported a tendency of fraction of inspired oxygen, mean airway pressure, oxygenation index and alveolar-arterial gradient decreased during induction of hypothermia and increased during rewarming on neonates with NE (n=31). Minute ventilation increased with TH and decreased upon rewarming. The inspiratory time, respiratory rate and positive end expiratory pressure were unaffected. Eicher reported a higher iNO requirement with TH, with 5/35 patients undergoing TH required iNO compared to 1/30 at normothermia, (p<0.01), however a Cochrane meta-analysis of four TH trials showed no significant effect of hypothermia on PPHN of the newborn.

In summary TH impacts respiratory function in neonates and requires altered blood gas interpretation. Tight control of carbon dioxide and avoidance of hypoxia is essential to prevent adding to the neurological burden in NE. There is expert evidence on the use of iNO in PPHN however trials to cement the evidence of milrinone and sildenafil use are required.
Renal dysfunction resulting in Acute kidney injury (AKI) varies from 22-70% in NE. Selewski reported that infants with NE and co-existing AKI had both a longer length of stay even after controlling for other confounders of NE severity and an increased incidence of abnormal MRI brain. TH has not been associated with a reduction in AKI in NE.

The current Kidney Disease Improving Global Outcomes (KDIGO) guidelines for AKI use a rise in creatinine as part of its definition. Creatinine is not an ideal biomarker of neonatal AKI as it peaks late, only rises when 50% of renal function is impaired, may reflect maternal creatinine level and reflects kidney function rather than injury. The optimal biomarker would diagnose AKI earlier so active management can be initiated. Cystatin C is a better indicator of glomerular filtration rate than creatinine and correlates with NE severity. Neutrophil gelatinase-associated lipocalin (NGAL) correlates to severity of NE and can predict a later creatinine rise. Neonates with moderate to severe NE had significantly elevated urinary levels of cystatin-C, neutrophil gelatinase-associated lipocalin and lower epidermal growth factor in comparison to mildly affected infants.

Electrolyte abnormalities were seen in 50% of infants, with hyponatraemia, hypokalaemia and hypocalcaemia being the most common. Renal profile, fluid balance, urine electrolytes and acid-base balance need regularly monitoring in NE. Urinary catheterisation may be necessary as morphine can cause urinary retention via anti-cholinergic effects.

Oliguria is common in NE. There is a significant risk of fluid retention and hyponatremia due to a poor capacity to produce urine. Fluid intake is frequently restricted in NE during TH due to concerns regarding cerebral oedema. A Cochrane review however found no RCT evidence to support this practice and recommended further studies. A subsequent RCT of infants with NE undergoing TH randomized infants to a restricted fluid intake of two thirds of normal (n=40); at 40, 55, 65 and 80mls per kilogram per day on days one to four of life respectively versus normal fluid intake (n=40); of 60, 80, 100 and 120 mls per kilogram per day respectively for the first four days of life. The fluid composition was 10% dextrose in the first 48 hours of life with sodium and potassium added to the dextrose over the next 48 hours. Restricted fluid did not reduce death or major neuro-disability at 6 months of age and was associated with a trend towards more hypoglycaemia. Hyponatraemia can result from kidney injury causing fluid retention, the syndrome of inappropriate anti diuretic hormone (SIADH) and tubular dysfunction. The Bartter and Schwartz criteria define SIADH as hyponatremia (serum Na+ < 135 mmol/L) with corresponding serum hypoosmolality (<280 mOsm/kg), and continued renal excretion of Na+ (>40 mEq/L) in the absence of clinical evidence of volume and of other causes of hyponatremia. Water restriction is necessary to manage the SIADH safely.
Nephrotoxic medications, such as acyclovir, aminoglycosides, non-steroidal anti-inflammatory drugs and vancomycin administration are recommended at renal doses and require therapeutic monitoring. Cefotaxime can be substituted for gentamicin as it has similar coverage to gentamicin without nephrotoxicity. Renal replacement therapy for severe AKI refractory to medical therapy is not a frequently used therapy, but when indicated peritoneal dialysis is preferred over continuous renal replacement therapy.

There is a lack of data with long term renal follow up of neonates from a renal perspective post AKI, despite the knowledge that AKI from all causes carries the risk of chronic kidney disease. Mammen et al, in a study of 126 neonates and children post AKI from all causes found that 10.3% of children had chronic kidney disease in the following 1–3 years. Ashkenazi and Kent et al recommend the need for post AKI long term follow up on a three monthly basis with urinalysis and blood pressure measurement identify those children who will go on to develop chronic kidney disease.

The definition of AKI is less suitable in neonatal AKI. Electrolyte disturbance and SIADH are common in NE and require monitoring. There is no consensus on AKI management in NE nor recommendation for long term follow up which is essential post AKI. The optimal fluid management was subject to a small RCT on whether to restrict fluids with no difference in outcome.

Gastrointestinal, Liver and Nutrition

Enteral feeds were held in the initial TH RCTs, although the risk of necrotizing enterocolitis was similar in TH and non-TH infants. Some TH centers are now implementing trophic enteral feeds of expressed breast milk. A pilot retrospective review of 17 patients who received minimal enteral nutrition compared to no enteral feed during TH (n=17) with the aim of determining if minimal enteral nutrition was associated with reduced duration of parenteral nutrition, time to full oral feeds, and length of stay, but not be associated with increased systemic inflammation or feeding complications. The enteral feeding group was associated with a reduced length of stay and time to full feeds, and did not increase feeding complications nor systemic inflammation.

An important finding on the review of available evidence and literature is that no trials have examined the optimal type of fluids to be used nor the rate of administration. There are no recommendations on whether TPN or dextrose plus electrolytes is the optimal fluids. In anuric renal failure, losses (30ml/kg/day) plus urine output replacement are recommended for infusion volume. There is no evidence to support the use of frusemide in fluid overload in NE.
Glycaemic control is critical in NE as glycogen stores are metabolized via anaerobic metabolism commonly resulting in hypoglycaemia. Initial hypoglycaemia and subsequent hyperglycaemia are associated with poor neurological outcome. Optimal timing and intervals of glucose monitoring is unknown, however, one evidenced based recommendation is to initiate glucose infusion rate (GIR) of 6 to 8 mg/kg/min, with 2mg/kg/min increases in GIR if hypoglycaemia occurs. Glucose monitoring recommendation is every 30-60 minutes until the glucose is over 2.8 mmol/L (50mg/dl) and subsequently every 4-6 hours.

Shah et al defined hepatic involvement in NE as an elevated aspartate aminotransferase (AST) or alanine aminotransferase (ALT) to >100 IU/ during the first week after birth. Transaminitis, defined as 1.5 times the upper limit of normal was reported in 80% of babies with NE by Hankins et al. Hankins et al suggested that elevated lactate dehydrogenase, ALT and AST to 1.5 times the upper normal level indicates liver involvement in NE. Severity of NE is associated with higher ALT and AST. Abnormalities in markers of hepatic synthetic function such as albumin and prothrombin have not been shown to correlate with severity of NE. Management of liver dysfunction in NE remains supportive in nature, with platelet, plasma and albumin infusions as necessary and vitamin K administration. Caution is warranted with use of hepatotoxic medications (paracetamol ampicillin and gentamicin). Ensuring normalisation of liver function testing in the neonatal periods avoids missing underlying metabolic disorders.

In summary, glycaemic control is critical and can contribute to neurological morbidities. There is a lack of evidence from RCTs regarding enteral nutrition during TH, and optimal fluid volume and type to be infused parenterally. Liver dysfunction requires monitoring alongside caution with hepatotoxic medications.

Haematological Assessment and Monitoring

NE is associated with elevated nucleated red blood cells, thrombocytopenia and prolonged coagulation profile. Coagulopathy is caused by blood loss, hypoxia-ischaemia and disseminated intravascular coagulation. The incidence of coagulopathy causing major or life threatening bleeding reported in the initial TH RCTs ranged from 3-12%. Coagulopathy was reported in 18% in the NICHD study, 19% in the Cool CAP study and 40% in the TOBY study. There is limited data regarding recommended levels to currently transfuse neonates to overcome coagulopathic or anaemic states. One study reported that 57% of NE infants required a blood product transfusion in the first 12 hours.

Assessment of coagulation is commonly monitoring of activated partial thromboplastin time (APTT), prothrombin time (PT), fibrinogen and platelets. Foreman et al established a higher incidence of clinically significant bleeding in infants with NE with platelets below 130x10^9/litre,
fibrinogen under 1.5 g/L and international normalised ratio (INR) over 2 \textsuperscript{380}. Patel et al define haemostatic dysfunction as prothrombin time (PT) $\geq 18$ s, platelet count $< 100 \times 10^9$/litre and/or fibrinogen $< 150$ mg/dl\textsuperscript{379}. Hankins\textsuperscript{376} et al defined haematological injury as the development of early thrombocytopenia ($< 100 \times 10^9$ per litre) in the absence of other causes, or an increase in nucleated red blood cell count to $\geq 26$ per 100 white blood cells. A number of guidelines recommend discontinuation of TH in the case of life threatening haemorrhage and ILCOR recommend platelet monitoring but do not specify intervals nor thresholds to intervene at\textsuperscript{381}.

TH is known to slow the production of enzymes involved in the coagulation cascade\textsuperscript{382}, but has not been demonstrated to cause an increase in any major haemorrhage\textsuperscript{383}. Severe hypoxia has been shown to decrease the platelet lifespan\textsuperscript{384}, whereas hyperoxia exposure has been demonstrated to worsen platelet aggregatory response\textsuperscript{385}. Protein C, protein S, and antithrombin III were increased in 100% of infants with NE demonstrating a potential to have an increase in thromboembolic events\textsuperscript{386}. Fetal thrombotic vasculopathy is a common finding on placental pathology in NE\textsuperscript{387}.

Neonatal stroke was implicated in 4.8% of cases of NE in one study, all neonates had seizures and all had a significantly worse neurodevelopmental outcome at 30 months\textsuperscript{388}. Ferrero et al compared the outcome of 15 infants with NE secondary to perinatal stroke to a matched cohort of NE from other causes. The stroke group had worse neurodevelopmental scores at follow up however in a separate analysis only 5 of the infants received TH. None of the TH group had seizures in comparison to 7 out of 10 of the other group suggesting a TH neuroprotective effect in perinatal stroke\textsuperscript{389}. Bashir et al reported subdural haemorrhage in 34% of patients with NE, with the incidence increasing with NE severity however their study found no correlation between haemorrhage and the degree of thrombocytopenia or coagulopathy\textsuperscript{390}.

Leukocytosis has been shown to correlate to abnormal neurodevelopmental outcome\textsuperscript{391, 392}. Morkos reported that elevated neutrophil best predicted adverse neurological outcome at 1 year.

Infection

Infection and inflammation are implicated in the aetiology of NE\textsuperscript{3}. Maternal chorioamnionitis is known to be a risk factor for NE, with up to one third of placentas in NE displaying histological chorioamnionitis\textsuperscript{378}. The Vermont Oxford Network reported that 24% of cases of NE have an associated antenatal inflammatory finding\textsuperscript{23}.

TH has not been associated with a higher incidence of culture positive sepsis\textsuperscript{252}. However Robertson et al postulated that the higher mortality in a pilot RCT in Uganda in infants receiving
TH may have been related to a higher incidence of sepsis but the infrastructure was lacking to make a definitive laboratory diagnosis. In this study 33% died in the TH group in comparison to 7% in the normothermia group. Broad spectrum antibiotic therapy covering gram positive, negative and anaerobes is common practice until sepsis has been excluded, with negative blood cultures and normal infection markers of white cell count and C-reactive protein (CRP). Caution with CRP interpretation is advised as the peak value is delayed by TH. Group B Streptococcal (GBS) sepsis was implicated in 0.58% of cases of NE from a systematic review. The NE mortality was higher in cases of GBS associated NE at 21% compared to NE mortality not complicated by GBS at 13.7%. The systematic review identified that infants with NE have a tenfold higher risk of GBS in comparison to term infants without NE.

A small pilot study of 16 infants with NE screened extensively for neurotropic viruses, bacteria and protozoa, by performing bacterial cultures in blood and cerebrospinal fluid (CSF) before antibiotic treatment, and viral CSF, polymerase chain reaction (PCR) for cytomegalovirus, herpes simplex 1 and 2, Epstein-Barr virus, enterovirus and human parechovirus. One case of blood culture positive bacterial sepsis and four cases of clinical sepsis were diagnosed, with no PCR positive results.

Neonatal herpes simplex virus (HSV) central nervous system (CNS) infection may be associated with neonatal seizures and present as with similar signs to NE. Clinical signs of HSV infection include irritability, lethargy, temperature instability and focal seizures, CSF pleocytosis, and transaminitis. Maternal primary infection of HSV during the third trimester or concerning maternal mucocutaneous or genital lesions raises suspicion and warrants investigation and empiric treatment in the neonate. Mucocutaneous infection has an absence of clinical lesions in 20% of cases and in 80% of neonatal HSV infection there are no known maternal risk factors. In cases of clinical suspicion of HSV starting acyclovir 20mg/kg 8 hourly is recommended with a rapid HSV Swab PCR testing and viral culture. The American Academy of Paediatrics (AAP) advise the areas of skin swabbing to include the anus, conjunctivae, mouth, nasopharynx and any suspected vesicles. Renal function monitoring and adequate hydration are important in view of nephrotoxicity associated with acyclovir.

In summary, sepsis evaluation and broad spectrum antibiotics are routine in NE but there is no expert or evidence-based consensus on indication to sample cerebrospinal fluid (CSF) nor viral and bacterial PCR screening. The incidence of HSV and GBS is higher and warrant prompt investigation and management if any clinical suspicion.

Skin
Regular full skin observation is suggested in view of the potential complications during TH such as subcutaneous fat necrosis is a benign condition characterized by inflammation and necrosis of subcutaneous fat and cold panniculitis which is an acute, nodular, erythematous eruption. In the TOBY RCT 1% of infants had subcutaneous fat necrosis. Hypercalcaemia is a rare but dangerous complication of subcutaneous fat necrosis which may require hyperhydration and diuretic treatment. Sclerema Neonatorum is a diffuse hardening of the subcutaneous tissue and is a rare complication of TH that usually self resolves. The use of a gradient variable mode of temperature control has less adverse skin events than automatic servo-controlled mode.

In summary, daily skin examination for complications and calcium monitoring during TH is recommended.

Metabolic & Endocrine Systems

Many metabolic disorders present with NE, including urea cycle defects, amino acid and organic acid disorders due to early accumulation of toxic metabolites in the CNS. Persistent acidoses, hyperlactataemia and refractory hypoglycaemia may indicate a metabolic cause underlying the encephalopathy. Burton et al, recommend serum investigations of blood gas, electrolytes, glucose, ammonia, amino acids and lactate as well as urinalysis for reducing substances, ketones, amino acids and organic acids as first line investigations. Moderately high ammonia levels can be seen in NE without metabolic causes, with mean levels of 222 μg/dl in one review of infants with NE. Isolated higher ammonia levels may be attributed to THAN (transient hyperammonaemia of the newborn) or urea cycle defects. Congenital malformations, dysplasias and dysmorphic features raise suspicion of inherited metabolic disorders in an infant with NE. Hypertonia, opisthotonus and myoclonic jerks may distinguish metabolic encephalopathies from other causes. EEG findings typical of metabolic encephalopathies include varied degrees of slowing, assorted mixtures of epileptic discharges and high incidence of triphasic waves.

Recommendation: persistent acidosis with high lactate and hypoglycaemia may indicate a metabolic and require prompt investigation and management.

Multi-Organ Scoring in NE

In view of the multiorgan involvement on NE several groups have developed scoring systems to evaluate organ involvement (Table 2). Shah et al described multi organ scoring dysfunction in infants with severe NE (n=144). They included renal, pulmonary, cardiovascular and hepatic parameters. All infants had minimum of one end organ dysfunction. Renal, cardiovascular, pulmonary, and hepatic dysfunction were found to present in 70%, 62%, 86%, and 85% of infants respectively. They concluded that multi organ dysfunction may be included to support a diagnosis of NE, but that multi-organ dysfunction did not correlate with adverse neurodevelopmental
outcomes or death. Martin-Ancel et al examined multi organ dysfunction in 72 infants with NE and found the following distribution: pulmonary (26%), cardiac (29%), gastrointestinal (29%), renal (15%) and respiratory (19%). They included infants from mild to severe NE but only 35% of their included infants required NICU admission, suggesting many of the included infants had milder NE. Apgar score was the only perinatal factor that correlated with the degree of multi-organ dysfunction in their review. Hankins et al reported liver injury in 80%, cardiac involvement in 78% and renal injury in 72% in a prospective review (n=46). These scoring systems allow the evaluation of organ dysfunction but have not yet been assessed in conjunction with longer-term neurodevelopmental or multiorgan follow-up in childhood.
### Table 6.1: Comparison of definitions of Multi-Organ Dysfunction in NE

<table>
<thead>
<tr>
<th></th>
<th>Shah et al&lt;sup&gt;234&lt;/sup&gt;</th>
<th>Hankins et al&lt;sup&gt;276&lt;/sup&gt;</th>
<th>Martin-Ancel et al&lt;sup&gt;239&lt;/sup&gt;</th>
<th>Perlman et al&lt;sup&gt;407&lt;/sup&gt;</th>
<th>Alsina et al&lt;sup&gt;408&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CVS</strong></td>
<td>Hypotension treated with an inotrope for &gt; 24 hours to maintain normal BP ECG evidence of transient myocardial ischaemia</td>
<td>Need for inotropic support beyond 2 hours of life Elevated CK-MB isoenzyme</td>
<td>Systolic and or diastolic BP &lt; 5&lt;sup&gt;th&lt;/sup&gt; percentile for age &amp; sex ECG abnormalities ECHO abnormalities</td>
<td>ECG or ECHO abnormalities (values &gt; 2 SDs from the mean normal values)</td>
<td>Troponin &gt;1ug/L Need for vasoactive drugs</td>
</tr>
<tr>
<td><strong>Hepatic</strong></td>
<td>AST or ALT &gt; 100 IU/l during week one Elevation of AST, ALT, LDH to 1.5 x upper normal level</td>
<td>Not included</td>
<td>Not included</td>
<td>Not included</td>
<td>GOT or GPT &gt;100 Prothrombin activity &lt;60%</td>
</tr>
<tr>
<td><strong>Renal</strong></td>
<td>Anuria or oliguria for ≥ 24 hrs and a SCr &gt; 100 mmol/l Anuria/oliguria for &gt; 36 hrs Any SCr &gt; 125 mmol/l Serial SCr increasing postnatally</td>
<td>Elevation of SCr to ≥ 88.4 mmol/l Oliguria &gt; 24 hrs Persistent haematuria or proteinuria</td>
<td>Oliguria for &gt; 24 hrs ≥ 2&lt;sup&gt;+&lt;/sup&gt; of proteinuria Azotaemia = BUN &gt; 7mmol/l</td>
<td>Creatinine &gt;1 mg/dl Diuresis &lt;0.99 ml/kg/hr Need for replacement therapy</td>
<td></td>
</tr>
<tr>
<td><strong>Neuro</strong></td>
<td>Not included</td>
<td>Clinical evidence of NE EEG abnormalities Neuroimaging abnormalities</td>
<td>Abnormal Neurological exam EEG abnormalities Neuroimaging abnormalities</td>
<td>Abnormal neurological exam Cranial ultrasound abnormalities</td>
<td>Not included</td>
</tr>
<tr>
<td><strong>Resp</strong></td>
<td>Need for ventilatory support with 40% oxygen for at least the first 4 hrs after birth</td>
<td>Not included</td>
<td>Abnormal Silverman Score Need for O&lt;sub&gt;2&lt;/sub&gt; supplementation Need for Mechanical Ventilation</td>
<td>Requirement for intubation and Mechanical Ventilation &gt; 48 hrs</td>
<td>Need for resp support due to causes other than central apnoea or pharmacological effects</td>
</tr>
<tr>
<td><strong>GI</strong></td>
<td>Not included</td>
<td>Not included</td>
<td>Gastric residuals, vomiting, abdominal distension/tenderness and GI bleeding</td>
<td>Evidence of NEC</td>
<td></td>
</tr>
<tr>
<td><strong>Haem</strong></td>
<td>Not included</td>
<td>Not included</td>
<td>Not included</td>
<td>Not included</td>
<td>Leucocyte &lt;4.5 or ≥30 mm&lt;sup&gt;3&lt;/sup&gt; Platelet &lt;149 APTT &gt;45 sec Platelet or FFP</td>
</tr>
</tbody>
</table>

Scoring systems by Shah, Hankins, Martin-Ancel, Perlman and Alsina assess systems of Cardiovascular, Hepatic, Renal, Neurological, Gastrointestinal and Haematological Dysfunction.

Abbreviations; CV<sup>S</sup> = cardiovascular; Neuro = neurological; resp = respiratory; GI= gastrointestinal; Haem= haematological; BP = Blood Pressure; AST = Aspartate Amino Transferase; ALT = Alanine Amino Transferase; SCr = Serum Creatinine; CK-MB = Creatinine Kinase Muscle-Brain-Type Isoenzyme; ECG = Electrocardiographic; ECHO = Echocardiographic; SDs = Standard Deviations; NE = Neonatal Encephalopathy; EEG = 154
Electroencephalographic; BUN = Blood Urea Nitrogen; $O_2$ = Oxygen; GI = Gastrointestinal; MV = Mechanical Ventilation; NEC = Necrotising Enterocolitis; nRBCs = Nucleated Red Blood Cells; WBCs = White Blood Cell; Glutamic oxaloacetic transaminase (GOT) Glutamic pyruvic transaminase (GPT).

Conclusion

There has been significant progress over the past two decades in neuroprotective strategies and with the establishment of TH as the standard of care in NE. There remains a gap in the full understanding of the optimal management of the infants during TH, and evidenced based multi-organ support. Ongoing RCTs and systematic reviews to gather information to recommend evidence based best practices is essential to establish the most appropriate practices for management of neonatal seizures, fluid status, inotropic support, and respiratory support. Establishing evidenced based guidelines for managing multi-organ dysfunction in NE during TH can reduce practice variation, optimize management and contribute to better outcomes. New adjunctive therapies for NE efficacy may be dependent on the adequate functioning of specific end organs. Dysfunction of these end organs may negatively impact on the efficacy of new treatments and conversely new treatments may have possible adverse side effects on already impaired organ functioning. The introduction of specialised neurocritical care units shows promise in non-pharmacological advancements of management of NE. Development of a predictive model which includes multiorgan dysfunction as part of its criteria is vital to furthering our understanding of NE, and will help in the long-term follow up and care of survivors of NE.
6.3 Neonatal Encephalopathy National Management Guideline

This guideline was prepared for the Royal College of Physicians Therapeutic Hypothermia Committee and intended for publication at a national level to guide Irish physicians on the initial management of NE. There are similar guidelines available in a one- or two-page format that are in use to guide physicians nationally on initial management on various neonatal morbidities.

The guideline publication is subject to approval of the National Clinical Programme for Paediatrics and Neonatology, which has a quality aim of developing guidelines to help standardise clinical care nationally.

The rationale for preparing the guideline was the paucity of standardised management of neonates owing to the lack of evidenced based guidelines to follow.
**Post Resuscitation Stabilisation**

- Resuscitation as per Neonatal Resuscitation Programme, see link\(^1\)
- Transfer to NICU
- Weigh infant
- Airway Secured & Ventilation Settings
- Pre and Post Ductal Saturation Monitoring
- Central Venous (PICC OR UV) and Arterial Access
- Bloods for ABG / FBC / DCT / Glucose / Blood Culture / CRP / U+E / LFT / Troponin/ CK / LDH / Coagulation/
- IV fluids / Bolus / Blood Products
- IV Empiric Antibiotics +/- Anti Viral therapy
- IM Vitamin K
- Apply CFAM
- Detailed Neurological Examination & Sarnat Staging
- Therapeutic Hypothermia Checklist; see link\(^2\)
- Start Active cooling if meets clinical criteria
- NNTP referral if outside Tertiary Unit
- Request Placental Pathology

**Fluids**

- Consider Fluid Bolus 10ml/kg 0.9% NaCl if hypovolaemia or hypoperfusion
- Start TPN or 10% Dextrose
- Fluid restrict 40 - 70mls/kg/ day
- May need to increase GIR to maintain normoglycaemia
- Monitor Urine Output +/- catheterisation
- Renal Impairment dosing of nephrotoxic medication
- Minimal trophic feeds of maternal expressed breast milk
- Monitor skin for Jaundice and subcutaneous fat necrosis
- Daily weight

**Respiratory**

- Adjust ventilation as per ABG if ventilated,
- Keep PaCO\(_2\) 5.3 to 7.3; PaO\(_2\) 6.6 to 13 kPa
- Avoid Hypocapnia & Avoid Hyperoxia
- Pre & Post Ductal Saturation Monitoring, Monitor for PPHN
Neurological
- Daily Neuro Examination & Sarnat Staging
- Start CFAM & 6 hourly documentation,
- Morphine at low dose (5-10mcg/kg/hr) & titrate for comfort & sedation
- Monitor for & treat seizures
- Cranial US
- Arrange formal EEG
- MRI with DWI & MRS at 7-11 days

Cardiovascular
- Avoid hypotension & BP fluctuation
- Maintain MAP > 40 - 60mmHG, higher if PPHN
- ECHO to guide Inotropic/Pressor Support and ischaemic changes

Haematological
- Monitor FBC, Coagulation and Fibrinogen
- RCC / Platelet / Fibrinogen transfusion
- +/- Maternal Kleihauer

Infectious Disease
- Empiric ABx as per Local Guideline
- Blood Culture / Urinalysis
- Placenta Swabs & Maternal HVS
- Bacterial PCR, see link \(^3\) & Viral PCR, see link \(^4\)
- +/- Aciclovir
- LP when clinically stable

Metabolic
- Ensure lactates normalize
- Consider Metabolic Evaluation

Family
- Update family and provide support
- Provide written and verbal information on Therapeutic Hypothermia; animation on TH
- Encourage and support expressing of colostrum and breast milk
Follow Up

- Safely Oral Feeding
- Ensure all bloods normalized
- Serial Hearing Assessments
- 6 week check and Neuro Developmental Plan

Links

1. http://pediatrics.aappublications.org/content/pediatrics/136/Supplement_2/S196/F1_large.jpg
6.2.1 Abbreviations

- Neonatal Intensive Care NICU
- Peripherally inserted central catheter (PICC)
- Umbilical venous line (UV)
- Arterial Blood gas (ABG)
- Full blood count (FBC)
- Direct Coombs Test (DCT)
- C-reactive protein (CRP)
- Urea and Electrolytes (U+E)
- Liver function test (LFT)
- Creatinine Kinase (CK)
- Lactate dehydrogenase (LDH)
- Intravenous (IV)
- Intramuscular (IM)
- Cerebral function analysing monitor (CFAM)
- National Neonatal Transport Programme (NNTP)
- Sodium Chloride (NACL)
- Total Parenteral Nutrition (TPN)
- Glucose infusion rate (GIR)
- Persistent Pulmonary Hypertension of the newborn (PPHN)
- Magnetic Resonance Spectroscopy (MRS)
- Diffusion weighted imaging (DWI)
- Mean Arterial Pressure (MAP)
- Red cell concentrate (RCC)
- Antibiotics (Abx)
- Polymerase chain reaction (PCR)
- Lumbar puncture (LP)

6.4 Patient participation of Research

There is a paucity of research on parents’ experience of having a baby with NE. The nature of the treatment of NE, therapeutic hypothermia (TH) requires admission to the NICU for a minimum of four days. Neonatal admission to the NICU can make parents feel excluded. TH is not offered in all neonatal units so the baby may be transferred externally. During TH, maintaining a cooler body temperature of 33-34°C can preclude parents from holding and bonding with their new-born. Clear communication to parents during this timeframe is perceived by parents as an important caring attribute that reassures their confidence in professionals’ competence. A study from Sweden interviewed 26 parents of infants born with NE and highlighted the initial experience of a traumatic birth followed by the processing of the news that the infant might not survive or would survive with morbidity. The parents reported bonding...
difficulties from infant-parent separation and the challenge of adapting to life upon discharge being improved by family centred support during the admission and after discharge. Lemmon et al presented three findings following 20 interviews with parents who’s infants had NE; parents described grief and loss of developmental milestones, parents struggled to balance needs of their newborn with the maternal and rest of family needs, and finally that parents evolved into advocates for their babies\textsuperscript{412}.

Patients’ and public involvement in research involves the engagement of non-health care professionals in research with the aim of addressing patient and public needs through research questions, and gaining a better understanding of patient priorities through their involvement. Involving patients in research ensures patients are informed about research that is relevant to them and helps clinicians understand their needs and jointly improve the design of important clinical research questions and dissemination of results to make a different to patient health outcomes.

Molloy et al discuss that patient participation in research is becoming more integrated in children’s research with the movement towards family-centred model of care, especially in neonatal care.\textsuperscript{413} The United Nations Convention on the Rights of the Child (UNCRC) recognises children’s rights to participate and benefit from research\textsuperscript{414}. The Royal College of Paediatric and Child Health highlighted the need for promoting active involvement of children’s participation in research in 2012 in their “Turning the tide report”\textsuperscript{415}.

Parents of children whom had NE, alongside health care professionals met and decided on priorities of a video introducing the Neonatal Intensive care unit (NICU) and Therapeutic Hypothermia (TH) to parents. These meetings were helpful for all in discovering the concerns of parents and providing a framework for the planned animations. We discussed the motivation for all participants to be involved and what we hoped to achieve from discovery to clinical guideline implementation and family information which resulted in the successful completion of two animations being published.

http://nbci.ie/2018/12/17/you-your-baby-and-the-nicu/


6.5 Discussion

The management paper highlights importance areas of evidenced based management of multi organ dysfunction in NE. The majority of research in NE is directed to reducing brain injury and not therapeutic strategies to reduce the end organ effects. There is a paucity of evidenced based management in the management of TH and identifies therapeutic creep of extrapolating practices 161
from adult based ICU practice. The management paper highlights areas of evidenced based care and areas where evidence is lacking to encourage further research.

The national guideline was produced in a similar format to Royal College of Physicians Ireland Guidelines on neonatal management. There are 60 to 70 neonates in Ireland with NE requiring TH annually, with forty percent of these born outside tertiary NICU’s. The guideline will be helpful as a quick reference guideline especially in the 15 maternity hospitals that do not provide TH, that is accessible with issues such as placental pathology and maternal Keilhauer test that will contribute to determining the aetiology of the NE.

The animations are an important aspect of engaging in both patient participation in research and of science communication. In the production of the animations, the complex NICU environment and process of TH were made accessible to non-health care professionals following identification of priorities of family centred outcomes. The feedback to date on the animations has been very positive from families since their launch in November 2018. The limitations to the study include the recruitment of families to give feedback for the videos means that the experience of all families were not captured in the data.

The strength of the chapter is the overall aim of improving neonatal outcome by producing evidences based management guidelines alongside tying in family centred care and patient participation in research to produce outcomes that are important to families.
Chapter 7 Conclusion

7.1 Introduction

There has been significant progress over the past two decades in management of NE offering TH as the gold standard of care. This therapy is incompletely neuroprotective, with a combined mortality or moderate to severe disability of 48% in infants post TH\textsuperscript{53}. There remains a gap in the full understanding of the pathophysiology of NE, precluding the development of adjunctive treatments in NE to improve the neurodevelopmental outcomes in NE. This lack of full understanding in the pathogenesis makes prognostication and communication to parents challenging. This thesis aimed to further the understanding of the inflammatory phenotype in NE to contribute further towards this progress and improve the overall management of NE.

NE is a heterogenous condition. It is not a single cellular nor molecular event, but an evolving cascade triggered by an initial insult. There are synergistic pathways between hypoxia and inflammation which potentiate the evolution of brain injury in NE. The evidence thus far supports the idea that inflammatory cytokines alongside leucocyte activation play an important role in NE, both in a detrimental and reparative way. The challenge of immunomodulating the inflammatory process is the concern of immunosuppression and exposure to infection, thus exploring the phenotype of the innate immune system in NE in this thesis may contribute to developing safe and effective therapies in the future.

7.2 Systematic Review

Chapter three involved an in depth systemic review to examine biomarkers in NE to predict outcome. There is a broad spectrum of outcomes possible in NE, from intact survival, to neurodisability and death. It is difficult to prognosticate the outcome, as there is no single gold standard biomarker. At present defining the prognosis involves a combination of clinical examination, neuroimaging and developmental follow up. Improved prognostic ability would enable clinicians to initiate therapies earlier and most importantly communicate likely outcomes to parents. The second aim of the systematic review involved searching for promising biomarkers to guide research, identifying IL-6 as the most promising cytokine for further evaluation.

The results concluded that raised mean serum levels of IL-6 and lactate were associated with worse short term outcome in NE. Meta-analyses of serum troponin, s100b, TNF-\textgreek{z}, glucose, ionised calcium, nucleated red bloods cells, lactate dehydrogenase, IL-1\textbeta and white cell count.
were not statistically significant. These studies had substantial heterogeneity and a large proportion of studies were excluded from meta-analyses due to insufficient information published to analyse, variety of measured outcomes and methods of data reporting.

Raised serum Interleukin-6 and neuron specific enolase were associated with adverse long-term outcome in NE. However, there were small patient numbers in both studies. Early MRI brain appeared to provide the best early prognostic information on long-term developmental outcome for infants with NE.

This systematic review involved searching for consistency of biomarkers to predict short- and long-term outcome in NE with a view for the assessment to guide future research in NE. The data collection involved assessment of valid studies and data extraction which included a large amount of studies on biomarkers however when the statistical methods of the meta-analysis was performed to combine the data, many planned meta-analyses were impossible due to methodology of presenting the figures. The Core outcomes in Neonatology (COIN) steering committee recently highlighted the inconsistent outcome reporting in neonatal clinical trials making comparisons across studies difficult, and although we primarily looked at observational studies rather than randomised controlled trials our conclusion was similar.

A small number of promising biomarkers to predict outcome have been identified but more studies are needed to both validate and correlate these biomarkers with outcomes in different populations. This systematic reviews highlighted the need for COIN and CROWN recommendations to be realised; for standardised reporting in neonatology so outcomes of different studies can be compared and pooled for valuable meta-analyses to guide future research and improve clinical practice.

### 7.3 Cytokine Exploration

The inflammatory cytokine profile in a large cohort of healthy term controls and infants with NE was examined for the baseline differences and then response to LPS stimulation. Previous cytokine studies may not have included term postnatal controls instead using cord blood or adult blood as the controls. In addition only baseline cytokine levels have been measured and we studied response to LPS to assess the immune regulation following a stimulus.

Healthy term infants were responsive to LPS stimulation, demonstrating a robust innate immune response. Infants with NE had a dysregulated response to LPS stimulation, indicating a potential immune deficiency when exposed to infectious threats.
Epo and IL-6 were higher in NE and VEGF was lower than in control infants at baseline. These findings agree with previously published literature showing higher Epo and lower VEGF as the severity increases in NE\(^8\) \(^{154}\) \(^{173}\). Epo has been suggested as an adjunctive therapy in NE\(^4\)\(^{17}\), however this study and previously published data display that endogenous Epo is already high in NE so perhaps exogenous Epo may only be useful as a tailored therapy in selected NE patients with low Epo measurements done prior to therapy, indeed in our patient cohort and previously published data, all patients with severe NE had high endogenous Epo levels perhaps highlighted a limited therapeutic benefit. VEGF plays a role in endogenous neuroprotection reducing excitotoxic brain damage\(^4\)\(^{18}\). The reduction of VEGF in NE perhaps may allow for more brain injury and worse outcome\(^4\)\(^{19}\).

More severe grades of encephalopathy were most significantly associated with elevation of pro-inflammatory TNF-\(\alpha\) in keeping with our group’s previous findings\(^8\). Interestingly anti-inflammatory cytokines, IL-10 baseline levels decreased with severity which is in contrast to our group’s previously published literature\(^8\) although the previously reported data is from prior to the TH era so TH may explain the decrease in anti-inflammatory response, and IL-1ra baseline levels increased with severity. Upon stimulation with LPS, IFN-\(\gamma\) decreased as the severity of NE increased. Increased GM-CSF and IFN-\(\gamma\), IL-1 \(\beta\), IL-1ra and VEGF on Days 1-2 in NE were associated with abnormal neuroimaging. In addition, following LPS GM-CSF, IFN-\(\gamma\) and TNF-\(\alpha\) levels similarly higher with abnormal neuroimaging.

In the future, cytokine cut off values that might predict clinical outcome will be established by pooled data from a larger cohort of babies with NE (n=300). The cytokine profile will be correlated with the neonatal neurodevelopmental outcome at 2 years of age. In addition, immunomodulators may be assessed in NE ex-vivo by evaluating cytokine response patterns.

### 7.4 Immunophenotype

In this chapter the immunophenotype of an infant with NE was explored. Key components of inflammation, including immune cells, oxidative stress and inflammatory gene expression were profiled in NE and healthy infants, and the differences between the stimulation responses were examined with LPS stimulation.

Inflammatory responses at a cellular and receptor level and the mechanism of immune-phenotype alteration was examined. The potential for immunomodulation by environmental exposure was explored by evaluation of the circadian rhythm genes. The most immediate clinical output was the point of care measurement of Adenosine, to show the biological plausibility.
Healthy infants displayed a robust response with neutrophil CD11b upregulation when stimulated with LPS in comparison to infants with NE who were unable to mount a significant response to LPS stimulation. The TLR family, which provides the critical link between immune stimulants and initiation of the innate immune response, was examined and the neutrophil TLR-4 expression was significantly lower in NE at baseline than controls, and had a significantly lower response to LPS stimulation in NE. The adaptor proteins involved in TLR activation; IRAK-4, MyD88 and TIRFF were examined at a gene expression level. IRAK-4 expression was lower in NE at baseline then in controls however there were no differences detected amongst the other genes.

Reactive oxygen species (ROS) contributes to brain injury and two sources of ROS were explored during the thesis. Neutrophil Nox as a source of ROS in NE was evaluated as well as neutrophil and monocyte mitochondrial ROS production. Nox expression on neutrophils was significantly increased with LPS in controls but again relatively hyporesponsive in NE. The differences in levels of neutrophil and monocyte mitochondrial superoxide production as a source of ROS was measured but no significant differences were found between groups or with LPS stimulation. This experiment may have been limited by small numbers.

Monocyte mitochondrial mass was examined as mitochondrial dysfunction is involved in the pathogenesis of NE. Mitochondrial mass was higher in NE with LPS in comparison to control LPS responses. These results were replicated in monocytes subtypes. This LPS hyporesponsiveness in NE implies a dysregulated inflammatory response and potentially means increased susceptibility to infection and impaired responses to environmental stress. In the future, mitochondrial glycolytic gene expression and succinate can be examined to further understand immunometabolism in NE.

HIF-1α, which has a crucial role in adapting downstream targets to hypoxia, did not show any differences in gene expression level between NE and healthy controls nor with LPS stimulation. No significant relationship was found between HIF-1α and Epo and VEGF. Future directions include examining gene expression of downstream responses to HIF-1α that may be potential targets for examining the action of HIF-1α.

The Inflammasome-related gene expression IL-1β increased with in LPS in NE and controls, but IL-1β was relatively hyporesponsive in NE. The expression of ASC was similar in controls and NE. NLRP3 was increased with LPS in NE on Day 3 in comparison to baseline levels. Abnormal inflammasome gene expression may suggest a future target for immunomodulation with therapies already having an acceptable safety profile such as anakinra, an IL1-ra antagonist.
The most immediate translational result was point of care adenosine, which was significantly higher in NE then in healthy term control. Adenosine has potential as a bedside biomarker of hypoxia and seizures and further validation is ongoing.

In terms of potential for environmental manipulation, the abnormal circadian rhythm was described at a gene expression level, with decreased BMAL, CLOCK and REV-ERBβ expression upon LPS stimulation in NE. The relationship between positive regulators of clock gene expression and pro-inflammatory cytokines was demonstrated. The manipulation endogenously and exogenously of the chronobiology has great potential in future adjunctive therapies in NE, with exposure of the infant to increased darkness to augment endogenous melatonin and exogenous melatonin administration to normalise the chronobiology. Currently ex-vivo treatment of immune cells with melatonin in a similar cohort of patients with NE is underway and may improve understanding of this immunomodulator as a potential therapy in NE.

### 7.5 Clinical Outputs

This work focused on improving the clinical outcome in NE. Firstly, a parent focus group of parents of children whom had NE was set up via the Irish Neonatal Health Alliance. Family centred care and parent involvement in neonatal intensive care is both crucial and challenging to integrate. Parents often have no time for antenatal preparation including NICU visits as NE is of sudden onset. The 72-hour duration of TH makes parent contact and holding their baby more complex. Parents have highlighted that attachment and bonding with their baby is a concern. We developed and published animations aimed to inform and expand parents' knowledge about the NICU environment and also about therapeutic hypothermia. This project allowed family engagement, empowerment and implementation of the project. Deficits in communication were highlighted as a priority and animations on TH and demystifying the NICU were produced by the focus to improve the science communication. The animations are publicly available online, are shown to parents of infants in the NICU and have received positive feedback to date.

There is a strong evidence basis for TH in the management of infants with NE. Although NE is associated with increased risk of multiorgan injury, there is a paucity of evidence-based management of multi-organ dysfunction in NE. Each organ system of the body is at risk of cell injury and death when subjected to hypoxia, with certain organs more susceptible than others. A management paper following review of best available literature has been submitted to Frontiers in Pediatrics to improve the multiorgan management of NE. The paper identified areas of
management that have a strong evidence base and advised on other areas where randomized controlled trials are required to improve neonatal management. In parallel with the management paper, a clinical guideline was prepared for a new national guideline for publication with the Royal College of Physicians Ireland to ensure standardisation of management of NE in Ireland.

### 7.6 Limitations to the study

In the recruitment of neonates into the study, there were a number of infants with severe encephalopathy and although their parents’ approved consent for clinical and multiorgan data collection they did not consent to phlebotomy. In addition, some infants died or had redirection of care to palliation prior to availability of blood samples potentially skewing the study population to more infants with moderate encephalopathy.

The pilot studies in Chapter 5 had smaller numbers included in each individual experiment so scientifically and clinically meaningful results are more difficult to draw conclusions from. The blood volume reserved in the study was 1-1.4 mLs whole blood per infant included, and many of these experiments required nearly this full blood volume.

Another limitation was access to serial blood samples. The bloods following informed consent were taken at times of routine phlebotomy across three different maternity hospitals. Clinical staff kindly helped in the phlebotomy, but in the busy clinical environment frequently the repeat research bloods were not included. As the study continued the access to bloods improved with staff familiarity with the project, with frequent presentations and updates to clinical staff and in comparison to many published studies, the recruitment of over eighty babies with NE and the control group of forty babies is still relatively large numbers for examining their immune function.

### 7.7 Conclusion and Future Directions

Neonatal Encephalopathy is a devastating outcome often after an apparently healthy term pregnancy. The overall aim of this thesis was to improve the outcome of infants with NE, from a translational perspective with clinical correlations and also advancing clinical management guidelines.

This thesis demonstrated that innate immune dysfunction occurs in NE. Novel findings of the thesis include LPS hypo-responsiveness of infants with NE and dysregulated immune responses
in comparison to age-matched controls. Our research group has several complementary projects that will have data combined together to expand the numbers of the research and enable further subdivision of the aetiology of NE for analysis and allow the development of a panel of biomarkers to correlate with clinical and MRI outcomes. In addition to understanding the intracellular mechanisms involved, this will assist in modulating these responses in order to assess therapies that could be used by testing them on immune cell function, such as melatonin, with hypothermia to improve outcome. No single clinical, radiological nor biochemical marker is likely to be sufficiently powerful to accurately predict long-term outcome. However a carefully chosen panel of serum biomarkers, neuroimaging findings and biochemical data may possess the ability to predict outcome more precisely and therefore guide prognostication and management decisions in the acute phase. In the future, it is important that these biomarkers are correlated to long-term neurodevelopmental outcome, which is beyond the timeframe of the thesis but is currently underway with neurodevelopmental follow up at 2 years of age.
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173


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178


179


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192


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9. Appendix

Appendix I
Ethical Approval from Rotunda
Ethical Approval National Maternity Hospital
Ethical Approval Coombe Women and Infants University Hospital
Appendix II Sarnat scoring system\textsuperscript{37}
Appendix III Patient Information Leaflet and consent for study
Appendix IV Equipment and Re-agents
Appendix V PCR Plate Layout
Appendix VI Prospero
Appendix VII Proforma of Neonatal Clinical information
Appendix I Ethical Approval from Rotunda

29th June, 2017.

Prof. Eleanor Molloy
Dept. of Paediatrics, Trinity College Dublin,
Trinity Centre for Health Sciences,
National Children’s Hospital, AMNCH,
Tallaght,
Dublin 24.

Our ref: REC-2016-028 (please quote this reference on all correspondence)
Re: Neonatal Inflammation and Multiorgan dysfunction and Brain injury research group (NIMBUS group)

Dear Eleanor,

Many thanks for the amended documentation received in relation to the above research. I am pleased to advise that the requirements set out by the Committee in respect of your study have now been met. This being the case, ethical approval for the research is granted and it may now commence.

You are requested to submit a progress report to the Committee in twelve months, and annually thereafter as applicable. We would also like to know when and where you publish or present your results. Please be aware of your responsibilities with respect to the Hospital’s good research practice policies and guidelines.

Kind regards.

Yours sincerely,

[Signature]

Dr. Maeve Eogan,
Acting Chairman,
Research Ethics Committee.
PRIVATE AND CONFIDENTIAL

Professor Eleanor Molloy,
Neonatology Consultant &
Professor of Paediatrics,
Trinity College,
Dublin 2,

27th June 2016.

Re: Neonatal Inflammation and Multiorgan dysfunction and Brain injury research group.
(EC 1/4.2016)

Dear Eleanor,

I am happy to inform you that the above study has now received ethical approval.

Kind regards,
Yours sincerely,

[Signature]

Dr. John Murphy
Chairman,
Ethics Research Committee
Ethical Approval Coombe Women and Infants University Hospital

20 June 2016

Prof. Allison Molloy,
Consultant Neurologist,
SMRCC.

Re: Study No. 12 – 2016 – Neuroinflammation and Multiorgan dysfunction and Brain injury research group (NUMBIRG)

Dear Prof. Molloy,

Thank you for kindly attending the research ethics committee meeting and explaining this study to us. It was understood that the blood samples in this study will be done only if indicated by clinical reasons and also MRI will be done only for clinical reasons. Your study was approved by the committee, however, this approval does not include performing MRI on a 2 year old and does not include MRI outside clinical indications.

Yours sincerely,

[Signature]

Dr. Jill Mhic Mhaol
Chairman

Copy: Prof. John O’Loughlin, Professor of Pathology, Dr. Ian Milder, Director of Paediatrics,
Dr. Orla Franklin, Consultant Paediatric & Perinatal Cardiologist, CUH/UL, Dr. David Ryan,
Consultant Paediatric Radiologist, CUH/UL and Prof. Maria White, Consultant Neurologist, CUH/UL.
Appendix II Sarnat scoring system\textsuperscript{37}

<table>
<thead>
<tr>
<th>Sarnat Stage</th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
</tr>
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<tbody>
<tr>
<td>Level Of Consciousness</td>
<td>Hyperalert</td>
<td>Lethargic/Obtunded</td>
<td>Stupor/ Coma</td>
</tr>
<tr>
<td>Activity</td>
<td>Normal</td>
<td>Decreased</td>
<td>Absent</td>
</tr>
<tr>
<td>Neuromuscular Control</td>
<td>Normal</td>
<td>Mild Hypotonia</td>
<td>Flaccid.</td>
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<tr>
<td>-Muscle Tone</td>
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<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>-Posture</td>
<td>Mild Distal Flexion</td>
<td>Strong Distal Flexion</td>
<td>Intermittent decerebration(extension)</td>
</tr>
<tr>
<td>-Stretch Reflexes</td>
<td>Overactive</td>
<td>Overactive</td>
<td>Decreased or absent</td>
</tr>
<tr>
<td>Primitive Reflexes</td>
<td>Weak</td>
<td>Weak or Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Suck</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>Moro</td>
<td>Strong, low threshold</td>
<td>Weak/Incomplete, High Threshold</td>
<td>Absent</td>
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<tr>
<td>Atonic Neck Reflex</td>
<td>Slight</td>
<td>Strong</td>
<td>Absent</td>
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<td>Autonomic Function-Pupils</td>
<td>.Mydriasis</td>
<td>.Miosis</td>
<td>.Variable, unequal, poor light reflex, fixed dilated</td>
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<tr>
<td>-Pupils</td>
<td>&quot;</td>
<td>&quot;</td>
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<tr>
<td>Heart Rate</td>
<td>Tachycardia</td>
<td>Bradycardia</td>
<td>Variable</td>
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<td>Seizures</td>
<td>None</td>
<td>Common</td>
<td>Uncommon, excluding decerebration</td>
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Appendix III Patient Information Leaflet and consent for study

Consent: NICU

Title of Study: Neonatal Inflammation and Multiorgan dysfunction and Brain injury research group (NIMBUS group)

Short Title: Inflammation and the Newborn Brain

Investigators:

Pl: Professor Eleanor Molloy; Co-investigators in CWIUH: Prof J Miletin, Prof M White, Dr D Rea

Your baby is being invited to take part in a study as he/she admitted to the neonatal intensive care unit. However, before you decide whether or not to take part, it is important that you fully understand what the research is about and what you will be asked to do. It is important that you read the following information in order to make an informed decision and if you have any questions about any aspects of the study that are not clear to you, do not hesitate to ask me. Please make sure that you are satisfied before you decide to take part or not. Thank you for your time and consideration of this invitation

Purpose of the Research Study

This study is about finding out more about the brain health and wellbeing of babies admitted to the unit under our care. We are studying big babies who are off form and not feeding well after birth along with well babies who have not required any help after birth.

Why as a Participant/Respondent have I been asked to take part in this study?

You have been asked to include your child in this study as he/she has been admitted to the neonatal intensive Care Unit. This study will be done in the intensive care unit at the Rotunda Hospital, the Coombe Women and Infants University Hospital and the National Maternity Hospital.

The voluntary nature of participation:

Taking part in this research study is entirely up to you and if you do decide for your baby to take part you will be provided with an information leaflet to take with you. Additionally, you will be required to sign a consent form. However, if you do not wish to take part and if you change your mind at any time (prior to publication), you can withdraw your baby from the Research Study without giving a reason.

During the Study

The study essentially involves all aspects of routine care. We would be grateful if you would allow the study group to use the data from some of the following tests if they are required as part of routine care for your baby:

1. Examining the baby’s brain waves over the first 72 hours of life using a routine technique called EEG (Electroencephalogram) whereby electrical brain wave activity can be measured from your baby and the tracing is...
recorded on a machine. This test is painless and will not cause any discomfort to your baby. It is part of routine care for many babies in the Neonatal Intensive Care Unit.

2. Your baby may have an MRI brain scan if required as part of their routine care and this does not involve radiation.

3. We also make sure we have checked all the parts of the body such as the kidneys, lungs and heart using ultrasound techniques such as echocardiography.

4. The other case information and tests that are routinely performed on the babies in the unit (e.g urine sample collection) and the placenta will also be assessed. For example, if a spinal fluid sample is taken as part of routine care, a small amount of this sample will be set aside for the study.

5. We will follow your baby as they grow and mature to see how their development is progressing.

In addition we would ask to examine blood samples at a few different times. The first sample is from blood taken from the placenta/afterbirth. This would normally be discarded after birth. Baby blood samples would not be additional samples as a portion of the blood sample taken as part of the baby’s routine care will be set aside for the study, using international guidelines to ensure this does not affect your baby.

Maternal sample: In order to understand the effects on inflammation on both mother and baby we request a sample of blood from you/your partner.

If having considered the study you decide not to participate, we assure you that in no way will your baby’s care be affected. You may also decide to participate in some parts of the study and not all.

**Potential harms/risks**

There are no potential/harms or risks to your baby from these tests which are in routine practice. Blood samples will only be taken with other bloods if needed as a routine part of your baby’s day-to-day care and this will be decided by the consultant oncall who is caring for your baby.

**Potential Benefits**

To individual babies: The benefit to your baby would be to assess the way the organs in the body function. Although many of the tests are part of routine care this study involves more detailed analysis of the results. We also measure inflammation as a way of predicting outcome.

To Society: Having a better understanding of inflammation and individual organ function will improve the treatments and tests available. New therapies to target inflammation could reduce complications for babies admitted to the NICU. By understanding inflammation in these babies we can target new treatments to add to cooling therapy to protect their brain and improve outcome.

**Confidentiality**

We will respect you and your infant’s privacy. No information about who your infant is will be given to anyone or be published. The data produced from this study will be stored in a secure, locked location. Only members of the research team will have access to the data. Following completion of the research study the data will be kept as long as required by the Hospital policy. The data will then be destroyed according to this same policy.

**At the End of the Study**

As this is just a monitoring study, there will be change regarding treatment, which continues as necessary. We will retain the information obtained from this study (which is anonymised) for a period of 5 years. We will also use the summarised data of all the babies enrolled to plan a
future study. Only summarised data of all the infants grouped together will be used for the purpose of future planning. We will not use individual baby data for any future planning or publications. We plan to publish the results of the study in a medical journal.

Other considerations
The study team may require access to your medical records and those of your baby. Again, any information collected will be completely anonymised.

Contact Details
If you have any questions about the study, call Prof Eleanor Molloy, consultant neonatologist, through the hospital Switch board.
Neonatal Inflammation and Multiorgan dysfunction and Brain injury research group (NIMBUS group)

CONSENT FORM: NE

Date: ________________  Subject Number: __________________

☐ I consent for my baby to take part in the above study.

☐ I consent to having my medical notes and my baby’s medical notes examined as part of this study.

☐ I confirm that I have read and understood the patient information leaflet.

☐ I am happy that my questions have been answered regarding the study.

☐ I understand that I may freely withdraw from the study at any time without my care or the care of my baby being affected.

☐ I consent to having an extra blood sample saved if/when routine blood sampling is being performed.

☐ I consent to having a urine sample collected from my baby.

☐ I consent for my placenta/afterbirth to be examined.

Name of Parent  Date  Signature of Parent

Name of Doctor  Date  Signature of Doctor
Appendix IV Equipment and Re-agents

1. Sodium citrate tubes
2. Sterile needle
3. Alcohol Wipes
4. T-piece connector for phlebotomy
5. P1000, P200, P100, P20 and P10 pipettes and Tips
6. RNAse/DNAse free eppendorfs
7. RNAse/DNAse free pipette tips
8. RNAse/DNAse PCR Tubes
9. 1.5ml microtubes
10. FACS tubes
11. Tube rack
12. Heat Block
13. Gilson pipettes (P10, P20, P200 etc)
14. PCR workstation
15. Microcentrifuge
16. Centrifuge
17. Thermocycler
18. BD FACS Canto II
19. Potentiostat
20. Laptop with DF69_QuadPotentiostat Software
21. USB connecting cable
22. Sarissa SMARTChips
23. Blotting paper cut into triangles
24. Tool for dispensing blood
25. Gilson pipettes (P10, P20, P200 etc)
26. RNAse-free microtubes 1.5ml (AM12425)
27. RNAse/DNAse free pipette tips
28. 0.2ml PCR tubes (Molecular Bioproducts P/N 3412/3412A)
29. PCR workstation
30. Microamp® Optical 96 well reaction plates/Optical caps (P/N 403012)
31. Microamp® Optical Adhesive Covers (P/N 4360954)
32. ABI Prism 7500 Sequence Detector (or 7900 with 384 well plates)
33. Plate and tube rack
34. 10-spot-96 well U-Plex Plate
35. 5-spot-96 well U-Plex Plate

Reagents

1. 10 µM Adenosine in PBS
2. Taqman Universal PCR mastermix (Applied Biosystems P/N 4304437)
3. RNAse free water (Commercial or DEPC treated and autoclaved water (0.1%))
4. 70% EtOH
5. RNAse Zap
6. RNAse-free water
7. High Capacity cDNA reverse transcriptase kit (Applied Biosystems 4368814)
8. dNTPs
9. Random primers
10. M-MLV Reverse Transcriptase
11. Reverse Transcriptase buffer
12. LPS – Sigma # L2630, Aliquots stored at various stock concentrations @ -20°C
   a. Reconstituted; 10ml PBS to 10mg LPS to give 1mg/ml stock. Make 1 in
      1000 dilution to get 1000ng/ml stock (5µl in 5mls PBS).
13. PBS - Sigma # P4417. To make 1L of 1X:Add 5 tablets to 1L dH2O and stir on
    stir-block until dissolved.
14. FACS lyse – BD # 349202
   a. To make 50mls of 1X:Add 5ml stock to 45mls de ionized water. Stored
      @ 4°C
15. 10% Sodium Azide – Sigma # S2889. To make 50mls:5g sodium azide
    a. Made up to 50mls with 1x PBS. Stir on stir-block until dissolved
    b. Store @ 4°C
16. Phosphate Buffer Azide –PBA
    a. To make 500mls:499mls PBS. 1ml 10% sodium azide. Stir briefly on
       stir-block
    b. Store @ 4°C
17. Antibodies –
   a. Anti-CD15-PECy7 – BioLegend # 323030
   b. Anti-CD66b-Pacific Blue – BioLegend # 305112
   c. Anti-CD16-V500 – BD # 561393
   d. Anti-CD14-APC – Beckman Coulter # IM2580
   e. Anti-CD14-PerCP -Biolegend #
   f. Anti-NOX-FITC -BD BioSciences #
   g. Anti-TLR4-APC -Biolegend #
   h. Anti-CD11b-PE - Biolegend #
18. MitoTracker Green (MTG) – Thermo Fischer Scientific #M7514 (20 vials of
   50µg)
   Aliquots stored at 2mM stock. To reconstitute new vial: Add 37.2µl DMSO to
   1x50µg vial and ensure dissolved to give 2mM stock. Aliquoted into 10 x 0.5ml
   microtubes (3.5µl/tube). Frozen @ -20°C
19. MitoSOX Red - Thermo Fischer Scientific #M36008 (10 vials of 50µg)
   Aliquots stored at 5mM stock. To reconstitute new vial; Add 13.2µl DMSO to
   1x 50µg vial and ensure dissolved to give 5mM stock. Aliquoted into 4 x 0.5ml
   microtubes (3µl/tube). Frozen @ -20°C
20. BD FACSDiva CS&T Research beads – BD # 655050 (For set up of machine
    and adjustment of voltages).Add 1 drop to 350µl PBA in FACS tube. Stored@
    4°C
21. OneComp Beads – eBiosciences # 01-1111-42
22. (For compensation controls). Add 1 drop to 500µl PBA in FACS tube, then add
    fluorophore-labelled antibody at dilution used in experiment. Stored @ 4°C
23. Linkers
24. U-Plex Antibody Sets
25. Calibrators
26. Diluent 43
27. Diluent 3
28. Stop Solutions
29. Read Buffer T
30. MDS wash Buffer

Appendix V PCR Plate Layout

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</table>

Table 1: 96 well Taqman colour coding

- **Gene 1**: endogenous control
- **Gene 2**: target 1
- **Gene 3**: target 2,
- **Gene 4**: target 3
- **NTC**: (no template /no RNA) control
- **NAC**: (no amplification/ no RT) control
Appendix VI Prospero

Systematic review of systemic biomarkers in neonatal encephalopathy to predict outcome

Citation

Review question
Systematic review of systemic biomarkers in neonatal encephalopathy to predict outcome
Population: Term newborns with Neonatal Encephalopathy
Indicator: Systemic Biomarker
Outcome: 1. Death in neonatal period
2. MRI in neonatal period
3. Sarnat Score / Miller Standardised Clinical Score
4. Standardised Developmental Outcome at 1 to 2 years (BSID etc.)

Searches
Sources to be searched:
• MEDLINE (1966-2016),
• Ovid MEDLINE In-Process and Other Non-Indexed Citations (up to November 2016),
• Cochrane Central Register of Controlled Trials (1950-2016),
• EMBASE (1988-2016),
• BIOSIS Previews (via Web of Science; 1969-2016),
• Science Citation Index Expanded (via Web of Science; 1900-2016).

Search strategy
https://www.crd.york.ac.uk/PROSPEROFILES/56763_STRATEGY_20170115.pdf

Types of study to be included
Retrospective and prospective observational studies.

Condition or domain being studied
Neonatal encephalopathy is a clinically defined syndrome of disturbed neurologic function, in the earliest days of life in an infant born at or beyond 35 weeks of gestation, manifesting a subnormal level of consciousness or seizures, and often accompanied by difficulty with initiating and maintaining respiration with depression of tone and reflexes. Neonatal encephalopathy describes central nervous system dysfunction in neonates from all causes, including hypoxic ischaemic encephalopathy, and therefore is challenging to classify infants into definite groups and define their prognosis. Cerebral and multi organ involvement is associated with the release of organ specific biomarkers in cerebrospinal fluid, urine and blood. These biomarkers may have a role in the assessment of the severity of asphyxia and long-term prognosis in neonates with neonatal encephalopathy.

Participants/population
Babies with neonatal encephalopathy, (including patients with hypoxic ischaemic encephalopathy (HIE), a clinically defined syndrome of disturbed neurologic function, in the earliest days of life in an infant born at or beyond 35 weeks of gestation, manifesting a subnormal level of consciousness or seizures, and often accompanied by difficulty with initiating and maintaining respiration with depression of tone and reflexes. Measurement of Molecular Biomarker in Blood, Urine & CSF within the neonatal period. Exclusion:
Infants with major congenital abnormalities and maternal substance misuse.
Infants less than 35 weeks.
Non-human studies.

Intervention(s), exposure(s)
Infants with neonatal encephalopathy.
Comparator(s)/control
Not applicable.

Context

Main outcome(s)
1. Death in neonatal period
2. MRI in neonatal period
3. Thompson / Samrat Score / Miller Standardised Clinical Score
4. Standardised Developmental Outcome at 1 to 2 years (BSID etc).

Additional outcome(s)
None.

Data extraction (selection and coding)
Two independent reviewers will apply a double screening on titles and abstracts. The included papers after screening will be read in full while applying inclusion criteria. Any discrepancies with the authors will be resolved by a third reviewer. The two reviewers will independently complete the full data extraction form and a risk of bias assessment. Both authors will have to agree otherwise the third reviewer will aid. Covidence software, https://www.covidence.org, will be used.

Risk of bias (quality) assessment
We will use the Cochrane Collaboration Tool for Risk of Bias Assessment.

Strategy for data synthesis
Quantitative analysis will be carried out, using Revman 5 software (http://community.cochrane.org/tools/review-production-tools/revman-5) and the Simmonds binomial model for meta analysis.

Analysis of subgroups or subsets
Babies undergoing therapeutic hypothermia compared to babies managed without therapeutic hypothermia.

Contact details for further information
Mary O’Dea
odeam@tcd.ie

Organisational affiliation of the review
Trinity College Dublin

Review team members and their organisational affiliations
Dr Mary O’Dea. TCD

Collaborators
Professor Eleanor Molloy. TCD
Dr Bob Phillips. University of York
Dr Kasper Kyng. University of Aarhus
Mr David Mocker. TCD

Type and method of review
Systematic review

Anticipated or actual start date
13 February 2017

Anticipated completion date
06 December 2017

Funding sources/sponsors
None
Conflicts of interest
None known

Language
English

Country
Ireland

Stage of review
Review Ongoing

Subject index terms status
Subject indexing assigned by CRD

Subject index terms
Biomarkers; Brain Diseases; Humans; Infant, Newborn; Infant, Newborn, Diseases

Date of registration in PROSPERO
15 February 2017

Date of publication of this version
15 February 2017

Details of any existing review of the same topic by the same authors

Stage of review at time of this submission
The review has not started

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<tr>
<td>Piloting of the study selection process</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Formal screening of search results against eligibility criteria</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Data extraction</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Risk of bias (quality) assessment</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Data analysis</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Versions
15 February 2017

PROSPERO
This information has been provided by the named contact for this review. CRD has accepted this information in good faith and registered the review in PROSPERO. The registrant confirms that the information supplied for this submission is accurate and complete. CRD bears no responsibility or liability for the content of this registration record, any associated files or external websites.
### Appendix VII Proforma of Neonatal Clinical Information

**NIMBUS DATA COLLECTION**

**NE NUMBER:**

### ANTENATAL DETAILS:

| Maternal Age: | | |
| Maternal Parity: | | |
| Pregnancy Complications: | | |
| None | IVDU | Placenta Praevia |
| IDDM | Gestational DM | Hypertension |
| Pre-eclampsia | Eclampsia | Primary |
| Epilepsy | Thyroid Disorder | UTI |
| Other | | |

| Maternal Infection: | Chorio suspected: |
| Pyrexia in Labour: Y/N | Temp: |
| Foul smelling Liquor: Y/N | |
| PROM >18hrs/ GBS status | |
| Maternal Antibiotic in Labour: Y/N | No. Of antibiotics /No of doses: |

Neonatal Temp:

### BIRTH DETAILS:

| Gestational age: | |
| Induction: Y/N | |
| Sex: M/F | |
| Birth Weight: | |
| OFC (cms): | |
| Length (cms): | |
| Apgar Scores: | |
| 1st gasp at (mins): | |
| 1st Heart Rate: | |
| 1st Intubated: | |

Cord Sample:
Infant blood gas result (first sample)

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Source</th>
<th>pH</th>
<th>Po2</th>
<th>PCO2</th>
<th>Base excess</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Venous</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>Cord</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mode of Delivery:

<table>
<thead>
<tr>
<th>Pre labour CS</th>
<th>In Labour CS</th>
<th>El. CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVD cephalic</td>
<td>SVD breech</td>
<td>Forceps</td>
</tr>
<tr>
<td>Suction</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Complications:

<table>
<thead>
<tr>
<th>Variable / late foetal HR decelerations</th>
<th>Prolapsed / ruptured / tight nuchal cord</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uterine Rupture</td>
<td>Maternal haemorrhage / placental abruption</td>
</tr>
<tr>
<td>Maternal trauma (eg. vehicle accident)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
</tr>
</tbody>
</table>

Foetal Distress:

<table>
<thead>
<tr>
<th>Unreassuring CTG:</th>
<th>Scalp pH:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration:</td>
<td>1st stage:</td>
</tr>
<tr>
<td>Oxytocin: Y/N</td>
<td>Duration:</td>
</tr>
</tbody>
</table>

Resuscitation:

<table>
<thead>
<tr>
<th>BMV/Neopuff: Mins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intubated: Y/N</td>
</tr>
<tr>
<td>Chest Compressions</td>
</tr>
<tr>
<td>Access UVC / Peripheral</td>
</tr>
<tr>
<td>Adrenaline: Y/N</td>
</tr>
<tr>
<td>ETT/IV:</td>
</tr>
<tr>
<td>Fluid Bolus:</td>
</tr>
</tbody>
</table>
Blood:
- O2 Concentration used:
- Congenital abnormalities noted at birth: Y/N

Transport:
- Was infant Transferred to another Hospital:
- Time transport called:
- Age (hrs/mins) on departure from referral hospital:

Admission to NICU:
- Encephalopathy Dx:
- CFAM:
  - Seizures: Y/N
- Initial Sarnat Score:
- Time baby started Passive Cooling:
- Time Of active Cooling:
- Time baby reached target temperature:

INVESTIGATIONS:

<table>
<thead>
<tr>
<th>Day 1 (0-24h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFAM/ Amplitude integrated EEG</td>
</tr>
<tr>
<td>Age (hrs from birth)</td>
</tr>
<tr>
<td>Lower limit ........µV</td>
</tr>
<tr>
<td>Electrical seizures Yes / No</td>
</tr>
<tr>
<td>Background activity</td>
</tr>
<tr>
<td>Sleep wake cycling</td>
</tr>
</tbody>
</table>

Fluid Management

<table>
<thead>
<tr>
<th>Fluid Input</th>
<th>mls/kg</th>
</tr>
</thead>
</table>
During these 24 hours were these conditions noted or treated?

<table>
<thead>
<tr>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seizures</td>
</tr>
<tr>
<td>Clinical or subclinical</td>
</tr>
<tr>
<td>Hypotension Mean BP&lt;40mmHg</td>
</tr>
<tr>
<td>Sepsis Antibiotic Rx and culture positive</td>
</tr>
<tr>
<td>Coagulopathy any needing Rx</td>
</tr>
<tr>
<td>Hypoglycaemia &lt;2.6mmol/l</td>
</tr>
<tr>
<td>Anhythmia Sinus bradycardia&lt;80bpm/other abn</td>
</tr>
</tbody>
</table>

**Respiratory support**

- Mechanical ventilation:
  - CPAP:
  - FiO2:
  - NO:
  - Other:

**Modified Sarnat Stage**

<table>
<thead>
<tr>
<th>Stage**</th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
<th>Normal</th>
<th>Undocumented</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level of Consciousness</td>
<td>Hypalgesic</td>
<td>Lethargy or obtunded</td>
<td>Stupor or coma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activity</td>
<td>Normal</td>
<td>Decreased</td>
<td>Absent</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Neuromuscular Control**

- Muscle Tone: Normal, Mild hypotonia, Flaccid
- Posture: Normal, Distal flexion, Strong, Distal flexion, Intermittent, Decombration
- Stretch Reflexes: Overactive, Inactive, Decreased or absent

**Complex Reflexes**

- Suck: Normal, Absent
- Moro (startle): Strong, Low threshold, Weak, Incomplete, High threshold, Absent
- Tonic neck: Normal, Absent

**Autonomic Function**

- Pupils: Miosis, Mydriasis, Variable (equal, unequal), Poor light reflex, Fixed, Dilated
- Heart Rate: Tachycardia, Bradycardia
- Seizures: Common, Uncommon, Uncommon

*Sarnat H.B, Sarnat M.S., Neonatal encephalopathy following fetal distress, Arch Neurol. 30:839-705, 1970
** Stage 0 = Normal
**Day 2 (24-48)**

**CFAM/ AEEG**

<table>
<thead>
<tr>
<th>Age (hrs from birth)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower limit ..........µV</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Electrical seizures</th>
<th>Yes / No</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of seizures identified</td>
<td>..........</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Background activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sleep wake cycling</td>
</tr>
</tbody>
</table>

**Fluid Management**

<table>
<thead>
<tr>
<th>Fluid Input</th>
<th>mls/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine Output</td>
<td>mls/kg/h</td>
</tr>
</tbody>
</table>

**During these 24 hours were these conditions noted or treated?**

<table>
<thead>
<tr>
<th>Seizures</th>
<th>Clinical or subclinical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypotension</td>
<td>Mean BP&lt;40mmHg</td>
</tr>
<tr>
<td>Sepsis Antibiotic Rx and culture positive</td>
<td></td>
</tr>
<tr>
<td>Coagulopathy</td>
<td>any needing Rx</td>
</tr>
<tr>
<td>Hypoglycaemia</td>
<td>&lt;2.6mmol/l</td>
</tr>
<tr>
<td>Arrhythmia Sinus bradycardia&lt;80bpm/other abn</td>
<td></td>
</tr>
</tbody>
</table>

**Respiratory support**

<table>
<thead>
<tr>
<th>Mechanical ventilation:</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPAP:</td>
</tr>
<tr>
<td>FI02:</td>
</tr>
<tr>
<td>NO:</td>
</tr>
<tr>
<td>Other:</td>
</tr>
</tbody>
</table>
Day 3 (48–72)

**CFAM/a EEG**

<table>
<thead>
<tr>
<th>Age (hrs from birth)</th>
<th>Lower limit ……μV</th>
<th>Upper limit…….μV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrical seizures</td>
<td>Yes / No</td>
<td>No. of seizures identified……….</td>
</tr>
<tr>
<td>Background activity</td>
<td>Sleep wake cycling</td>
<td>present / absent</td>
</tr>
</tbody>
</table>

**Fluid Management**

<table>
<thead>
<tr>
<th>Fluid Input</th>
<th>mls/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine Output</td>
<td>mls/kg/h</td>
</tr>
</tbody>
</table>

**During these 24 hours were these conditions noted or treated?**

- **Seizures** Clinical or subclinical
- **Hypotension** Mean BP<40mmHg
- **Sepsis** Antibiotic Rx and culture positive
- **Coagulopathy** any needing Rx
- **Hypoglycaemia** <2.6mmol/l
- **Arrhythmia** Sinus bradycardia<80bpm/other abn

**Respiratory support**

| Mechanical ventilation: |

---

---
### Modified Sarnat Stage*

<table>
<thead>
<tr>
<th>Stage**</th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
<th>Normal</th>
<th>Undocumented</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level of Consciousness</td>
<td>Hyperalert</td>
<td>Lethargic or obtunded</td>
<td>Stupor or coma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activity</td>
<td>Normal</td>
<td>Decreased</td>
<td>Absent</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Neuromuscular Control

<table>
<thead>
<tr>
<th>Muscle Tone</th>
<th>Normal</th>
<th>Mild hypotonia</th>
<th>Flaccid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Posture</td>
<td>Mild distal flexion</td>
<td>Strong distal flexion</td>
<td>Intermittent decerebration</td>
</tr>
<tr>
<td>Stretch Reflexes</td>
<td>Overactive</td>
<td>Overactive</td>
<td>Decreased or absent</td>
</tr>
</tbody>
</table>

#### Complex Reflexes

<table>
<thead>
<tr>
<th>Suck</th>
<th>Weak</th>
<th>Weak or absent</th>
<th>Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moro (startle)</td>
<td>Strong; low threshold</td>
<td>Weak; incomplete high threshold</td>
<td>Absent</td>
</tr>
<tr>
<td>Tonic Neck</td>
<td>Slight</td>
<td>Strong</td>
<td>Absent</td>
</tr>
</tbody>
</table>

#### Autonomic Function

<table>
<thead>
<tr>
<th>Pupils</th>
<th>Mydriasis</th>
<th>Miosis</th>
<th>Variable; often unequal, poor light reflex, fixed, dilated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Rate</td>
<td>Tachycardia</td>
<td>Bradycardia</td>
<td>Variable</td>
</tr>
</tbody>
</table>

#### Seizures

| none | Common; focal or multifocal | Uncommon (excluding decerebration) |

---

**Sarnat H.B, Sarnat M.S.: Neonatal encephalopathy following fetal distress. Arch Neurol. 35:808-705. 1976**

** Stage 0 = Normal**

---

### POST COOLING

#### MULTIORGAN OUTCOMES

### RESPIRATORY:

- IPPV at birth
- Intubation at birth
- Duration of Intubation (days):
  - HFOV
  - N.O.
- CPAP
- O2
- Max. FiO2 (%)
- PNTX/ Chest drain
- Pulmonary haemorrhage
- Pneumonia
Meconium aspiration

**CARDIOVASCULAR:**

- Inotropes: Y/N
  - Adrenaline
  - Noradrenaline
  - Dopamine
  - Dobutamine
  - Milrinone
  - Hydrocortisone
  - Sildenafil
  - Bosentan
- ECMO: Y/N
- Bradycardia: Y/N
- Lowest Heart Rate Recorded:
  - ECHO: Y/N
  - Report:
  - PPHN: Y/N
  - NO: Y/N

**RENAL**

- Oliguria<1ml/kg/h: Y/N
- Max Creatinine:
- Max urea:
- Abn Renal USS:

**COAGULATION**

- Max PT: Max APTT:
  - Min Fibrinogen:
- Min Platelets:
  - Blood products:
  - FFP
  - Fibrinogen
  - Cryoprecipitate
  - Platelet
  - RCC

**GASTROINTESTINAL**

- GER: Medications:
- Necrotising enterocolitis:
- Full sucking feeds at discharge: Y/N
  - If yes age in days:
<table>
<thead>
<tr>
<th><strong>NEUROLOGY</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>MRI: Y/N</td>
</tr>
<tr>
<td>DOL:</td>
</tr>
<tr>
<td>Results:</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Cranial Ultrasound: Y/N</td>
</tr>
<tr>
<td>DOL:</td>
</tr>
<tr>
<td>Results:</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Neurology Consult</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Anticonvulsants: Y/N</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Phenobarbitone</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>EEG: Y/N</td>
</tr>
<tr>
<td>Results:</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Meningitis: Y/N</td>
</tr>
<tr>
<td>CSF WCC:</td>
</tr>
<tr>
<td></td>
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<tr>
<td>Neuro Examination on DC</td>
</tr>
<tr>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>OTHERS</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombophilia screen if indicated eg neonatal stroke</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Placental Histology</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Late onset sepsis&gt;72h on blood or CSF culture</td>
</tr>
<tr>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Outcome</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Discharged home:</td>
</tr>
<tr>
<td>Age/days:</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Transferred to another hospital:</td>
</tr>
<tr>
<td>Age/days: Where:</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Died:</td>
</tr>
<tr>
<td>Age/days:</td>
</tr>
<tr>
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<tr>
<td>PM planned: Y/N</td>
</tr>
<tr>
<td>Performed: Y/N</td>
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<tr>
<td></td>
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<tr>
<td>Audiology:</td>
</tr>
<tr>
<td>Ophthalmology:</td>
</tr>
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<td></td>
</tr>
</tbody>
</table>
### Blood Results:

1st line in labour ward and on admission

#### Haematology:

<table>
<thead>
<tr>
<th>Day</th>
<th>Time Sample taken</th>
<th>Hgb</th>
<th>HCT</th>
<th>Total WCC</th>
<th>Neuts Count</th>
<th>Platelets</th>
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<tbody>
<tr>
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</tbody>
</table>

#### Coagulation:

<table>
<thead>
<tr>
<th>Day</th>
<th>Time Sample Taken</th>
<th>PT (secs)</th>
<th>PTT (secs)</th>
<th>Fibrinogen</th>
</tr>
</thead>
<tbody>
<tr>
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</table>

#### Biochemistry:

<table>
<thead>
<tr>
<th>Day</th>
<th>Urea</th>
<th>NA</th>
<th>K+</th>
<th>Creat</th>
<th>LDH</th>
<th>AST</th>
<th>CK</th>
<th>ALT</th>
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<tbody>
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</tbody>
</table>

#### Blood Gas:

<table>
<thead>
<tr>
<th>Day</th>
<th>Time since</th>
<th>Time samp</th>
<th>Sourc e of sampl e</th>
<th>pH</th>
<th>pCO2</th>
<th>pO2</th>
<th>BE</th>
<th>La ct</th>
<th>Gl uc</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>admission</td>
<td>le taken</td>
<td>(Cap, art, ven)</td>
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</tbody>
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### Metabolic Screen:

<table>
<thead>
<tr>
<th>Day</th>
<th>Ammonia</th>
<th>Amino Acids</th>
<th>Amino &amp; Organic Acids (Urine)</th>
<th>Ketones &amp; Reducing Substances (Urine)</th>
<th>Others</th>
</tr>
</thead>
</table>

### Micro:

<table>
<thead>
<tr>
<th>Blood CX</th>
<th>LP</th>
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</thead>
</table>

<table>
<thead>
<tr>
<th>WCC</th>
<th>Glucose</th>
<th>Protein</th>
<th>Viral Screen</th>
</tr>
</thead>
</table>
