CHILDHOOD ASSESSMENT OF MULTI-ORGAN DYSFUNCTION POST NEONATAL ENCEPHALOPATHY (CHAMPION STUDY)

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A thesis submitted to the school of Postgraduate Studies,

Faculty of Medicine and Health Sciences,

Trinity College Dublin, in fulfilment of the degree of

Doctorate of Philosophy

2019

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at Trinity College, University of Dublin or any other University.

This thesis is entirely my own work.

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Acknowledgement

I would like to express my huge thanks and gratitude to my supervisor Professor Eleanor Molloy for her full support and encouragement, generosity with her time over the course of my research and PhD writing. I would never have reached this point in my research without her encouragement, and full dedication to the project.

I also wish to wholeheartedly thank my co-supervisor Dr Denise Mc Donald and Dr Deirdre Sweetman for their advice and support over the course of my thesis.
I would like to extend my deepest thanks to the National Children's Hospital Funding Committee for their funding support over the duration of my PhD thesis. Many thanks to the other consultants and collaborators in our research project. I would especially like to thank Dr Afif El Khuffash, Dr Ciara Mc Donnell and Dr David Webb for their ongoing support of our research project and constructive advice.

I also extend my huge thanks to all the nursing staff at the National Children Hospital, Tallaght, and the National Maternity Hospital, Holles Street who were always very supportive of my research project and helped me in many ways. I am very grateful to the administrative staff in the department of Neonatology and Medical Records in Holles Street for their help in data collection and chart tracking.
Sincere thanks to the laboratory staff at National Children Hospital, Tallaght and at National Maternity Hospital, Holles Street, for sample processing and with laboratory data acquisition and analysis. I also wish to thank the research scientist Dr Victoria Mc Eneaney and Dr Lynn Kelly for helping with the processing and analysis of the samples.
I also wish to thank Dr Derek Doherty and his research team members at Institute of Molecular Medicine, Trinity Centre of Health Sciences, for laboratory services and expertise during the analysis of our biomarker samples. I wish to thank Mr. John Butler for providing his services and help with the cytokine analysis.

I wish to thank Mr Ricardo and Ms Olivia Mason from C-STAR in UCD for their help and practical advice during the statistical analysis of our data. I am very grateful to Ms Strickland for her great help with cytokine data analysis.
I also wish to thank my research colleagues, Dr Murwan Omer, Dr. Mary Isabel O Dee, Tammy Strickland and Dr Nawal Taher who always supported and helped me over the course of my PhD project.

Finally, I could not have completed this body of work without the help, unfailing support and love of my family especially my husband who always provided me lots of encouragement and moral support and friends who were always there for me when I needed it most and gave me the encouragement and strength to strive on.

Abstract

Neonatal brain injury is an important cause of neonatal death and disability such as cerebral palsy. Perinatal global hypoxic ischaemia associated with neonatal encephalopathy results in multi-organ dysfunction which may persists in later childhood.

The aim of our study was to examine multi-organ dysfunction in childhood in children with neonatal encephalopathy (NE). Detailed multi organ dysfunction (MOD) was completed in infants with NE, quantified organ outcomes including serum, urine and cerebrospinal fluid (CSF) biomarkers. We followed-up this cohort at school-age measuring multiorgan outcomes at school-age compared to age matched controls and children with Cerebral palsy (CP). We also assessed the sleep pattern, quality of life (QOL) in children with NE. Persistent or sustained inflammation has been implicated in neonatal brain injury, therefore we examined the innate immune response including expression of the inflammasome, HIF-1α, CD11b and Toll-like receptor-4 expression on the surface of neutrophils and cytokine response in children with NE.

We recruited children who had NE (n=55), 65 age-matched controls (n=65) and with CP and complex needs (n=26). Blood & urine samples, questionnaires (developmental, sleep and QOL) and clinical examination were performed.

Increased incidence of sleep disorders and low QOL scores were seen in children with NE in comparison to children in the control group. Persistently deranged renal function including elevated urea and creatinine were found in children with NE. Persistently high white cell and neutrophil count were noted in children with NE and a correlation between low cognitive score at 2 years and elevated neonatal white cell count was noted.

Increased expression of inflammasome NLRP3, HIF-1α and circadian rhythm genes were noted, increased with LPS and subsided by melatonin. Elevated cytokine response of pro and anti-inflammatory cytokines was also noted in children with NE at school age suggesting persistent altered inflammation.

This study demonstrated the extent of multiorgan dysfunction (MOD) including neurological, renal, haematological involvement and sleep disorders in infants and in children who had NE. Quantifying multiorgan dysfunction in the neonatal period to ensure appropriate follow-up of all organs is merited. This would help in advanced clinical planning and long term follow up. Understanding, the immune response in these children with NE and exploring systemic inflammation holds promise for future development of immunomodulatory adjunctive therapies.

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Abbreviations

ADC = Apparent Diffusion Coefficient

ADP = Adenosine Diphosphate

AEDs = Anti-Epileptic Drugs

aEEG = amplitude integrated Electroencephalogram

AKI = Acute Kidney Injury

AKIN = Acute Kidney Injury Network

ALI = Acute Lung Injury

ALT = Alanine Aminotransferase

AP = Apnoea

APC= Allophycocyanin

APTT = Activated Partial Thromoplastin Time

ARDS = Acute Respiratory Distress Syndrome

AST = Aspartate Aminotransferase

ASQ-3 =Ages & Stages Questionnaire

ATN = Acute Tubular Necrosis

ATP = Adenosine Triphosphate

AUROC = Area Under Receiver Operating Characteristic

B2M = Beta-2 Microglobulin

BAPs = Biological-Antioxidant-Potentials

BBB = Blood Brain Barrier

BG score = Basal Ganglia score

BG/W = Combined Basal Ganglia/Watershed score

BMD= Bone mineral density

BNP = B-Type Natriuretic Peptide

BP = Blood Pressure

BSID-III = Bayley's Scale of Infant Development Version 3

BSID-III = Bayley's Scale of Infant Development-III

BUN = Blood Urea Nitrogen

cAMP = Cyclic Adenosine Monophosphate

CAT = Catalase

cEEG = Continuous Electroencephalogram

CK = Creatinine Kinase

CK-BB = Brain-Specific Creatine Kinase

CKD = Chronic Kidney Disease

CK-MB = Creatinine Kinase Muscle-Brain-Type Isoenzyme

CLOCK= Circadian Locomotor Output Cycles protein Kaput

CNS = Central Nervous System

CO = Cardiac Output

Coag = Coagulation Screen

CP = Cerebral Palsy

CPAP = Continuous Positive Airway Pressure

CPB = Cardioplumonary Bypass

CRIB = Clinical Risk Index for Babies

CRP = C-Reactive Protein

CrUSS = Cranial Ultrasound Scan

CRY= Cryptochrome1

CSF = Cerebrospinal Fluid

CSHQ= Child Sleep Habit Questionnaire

CSFs = Colony Stimulating Factors

CVS = Cardiovascular System

Cys C = Cystatin C

Desat = Desaturation

DIC = Disseminated Intravascular Coagulation

DOL = Day Of Life

ECG = Electrocardiogram

ECHO = Echocardiogram

EEG = Electroencephalogram

EF = Ejection Fraction

EGF = Epidermal Growth Factor

EGF/Cr = Ratio of urinary Epidermal Growth Factor to urinary Creatinine

EGFR = Epidermal Growth Factor Receptor

Epo = Erythropoietin

EpoR = Erythropoietin Receptor

ES = Encephalopathy Score

FBC = Full Blood Count

GBS = Group-B Streptococcus

GER = Gastro-Esophageal Reflux

GFR = Glomerular Filtration Rate

GI = Gastrointestinal

GI = Gastrointestinal

GIT = Gastrointestinal

GM-CSF = granulocyte macrophage colony-stimulating factor

GMFCS= Gross motor function classification system

GPX = Glutathione Peroxidase

GRAN = Granulocyte

GSH/GSSG Ratio = Reduced Glutathione/Oxidised Glutathione Ratio

HAEM = Haematological

Hb = Haemoglobin

HFOV = High Frequency Oscillatory Ventilation

HIE = Hypoxic Ischaemic Encephalopathy

HLA = Human Leukocyte Antigen

HR = Heart Rate

IFN-γ = Interferon-γ

IL-10 = Interleukin-10

IL-12 = Interleukin-12

IL-18 = Interleukin-18

IL-1β = Interleukin-1β

IL-4 = Interleukin-4

IL-6 = Interleukin-6

IL-7 = Interleukin-7

IL-8 = Interleukin-8

IL-α = Interleukin-1α

INR = International Normalised Ratio

IQR = Interquartile Range

IVS = Intraventricular Septum

KDIGO = Kidney Disease Improving Global Outcomes

KIM-1 = Kidney Injury Molecule-1

kPa = Kilopascals

LDH = Lactate Dehydrogenase

L-FABP = Liver-type Fatty Acid Binding Protein

LFTs = Liver Function Tests

LP = Lumbar Puncture

LPS=Lipopolysaccharide

LV = Left Ventricle

MEL= Melatonin

MODS = Multi-Organ Dysfunction Score

MRI = Magnetic Resonance Imaging

MTP = Mitochondrial Permeability Transition

MV = Mechanical Ventilation

NE = Neonatal Encephalopathy

NEURO = Neurological

Neut = Neutrophil

NGAL = Neutrophil Gelatinase-Associated Lipocalin

NGT = Nasogastric Tube

NICHD = National Institute of Child Health and Human Development

NICU = Neonatal Intensive Care Unit

NLRP3= Node like Receptor Protein

NMH = National Maternity Hospital

02 = Oxygen

OPN = Osteopontin

OS = Oxidative Stress

P = p-value

PA = Perinatal Asphyxia

PaCO2 = Partial Pressure of Carbon Dioxide

PaO2 = Partial Pressure of Oxygen

Plt= Platelet count

PPV = Positive Predictive Value

pRIFLE = Paediatric RIFLE

PT = Prothrombin Time

PVL = Periventricular Leukomalacia

PVR = Pulmonary Vascular Resistance

QOL= Quality of Life

RCT = Randomised Controlled Trial

REQ = Requirement

RESP = Respiratory

RIFLE = Risk Injury Failure Loss End-stage kidney disease

RNS = Reactive Nitrogen Species

ROC = Receiver Operating Characteristic

ROS = Reactive Oxygen Species

SCr = Serum Creatinine

sCys C = Serum Cystatin C

SDs = Standard deviations

TDI = Tissue Doppler Imaging

TH = Therapeutic Hypothermia

TH = Total hydroperoxide

TH1 = Helper T cell 1

TH2 = Helper T cell 2

TNF-α = Tumour Necrosis Factor-α

TOBY trial = Whole Body Hypothermia for the Treatment of Perinatal Asphyxial Encephalopathy

U & Es = Urea, Creatinine & Electrolytes

UCC = University College Cork

uCys C = Urinary Cystatin C

UMOD/UM = Uromodulin

uNGAL = Urinary Neutrophil Gelatinase-Associated Lipocalin

VEGF = Vascular Endothelial Growth Factor

W score = Watershed score

WBC = White Blood Cell

WBCs = White Blood Cell.

Chapter 1

INTRODUCTION

1. Introduction

1.2. General Overview

Neonatal brain injury is an important cause of neonatal death and disability such as cerebral palsy. Perinatal global hypoxia-ischaemia is associated with neonatal encephalopathy (NE) and can result in brain injury in both term and preterm infants. Cerebral palsy (CP) has an incidence of 13.5% in infants who had NE (Badawi et al., 2005). There are many heterogeneous causes of CP and NE is the one of the important precursors.

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. NE describes central nervous system (CNS) dysfunction in new-born from all causes including hypoxic ischaemic encephalopathy (HIE) and birth asphyxia (Nelson and Leviton, 1991). HIE is a term applied to only a small proportion of infants with NE suffering from an intrapartum hypoxic ischaemic insult. Neonatal asphyxia induces global hypoxia-ischaemia resulting in multi-organ injury (Shah et al., 2004) and cardiac, renal, hepatic and haematological dysfunction are well described. Therefore, multi-organ as well as neurodevelopmental follow up studies may be required to ensure complete resolution and avoid complications in later childhood or adulthood.

Inflammation combined with hypoxic ischemia (HI) also plays an important pathophysiological role in NE. Diffuse activation of microglia in the neonatal brain occurs during inflammation which increases the injury process by expressing inflammatory mediators and pro-inflammatory cytokines. (Leviton et al., 2005, Berger et al., 2012). Inflammation is recognised as one of major causes of long term injury (Berger et al., 2012).

Although therapeutic hypothermia has been proven to be effective, the mortality rate and rate of moderate to severe disability remains high, at 46% in infants with moderate to severe NE after therapeutic hypothermia (Edwards et al., 2010). Therefore, there is a need for alternative therapeutic agents such as melatonin,

erythropoietin or topiramate and additional therapies for management of infants with neonatal encephalopathy to improve neurodevelopmental outcome in childhood.

In addition, children with mild NE were traditionally assumed to have good prognosis but recent studies have demonstrated otherwise. A recent systemic review including twenty studies on children with mild NE reported abnormal outcome including death and neurodisability in 25% children with mild NE (Conway et al., 2018a). Thus, children with mild NE should be followed up for early detection of development delay.

1.3. Multi-organ Dysfunction

1.3.1 General Overview

There is an increased risk of multi-organ injury associated with perinatal ischaemia (Shah et al., 2004, Hankins et al., 2002, Martin-Ancel et al., 1995, Perlman, 1989). Multiorgan Dysfunction is defined as the dysfunction involving two or more organs or systems including neurological, cardiovascular, respiratory, hepatic, renal, gastrointestinal and haematological. Acute interruption of placental blood flow enough to result in NE commonly results in ischaemic injury to multiple organs, including the heart, kidney and liver and not solely the brain (Perlman, 2011). There is risk of cell injury and death to multiple organs when subjected to global hypoxia secondary to interruption of the placental vascular supply. The extent to which each organ is affected depends on the severity and duration of the initial insult, circulatory responses to interruption of placental blood flow, and resistance of each specific organ to hypoxia/ischaemia. Following restoration of normal circulation, the extent of each organ involvement depends on the degree of reperfusion injury and on treatment interventions (Perlman, 2011). Certain organs are more susceptible to injury than others, and organs with a longer ischaemic time including the liver, kidneys, heart and lungs are exposed to hypoxaemia and the conversion to anaerobic metabolism causes cell injury. There are several proposed theories to explain the underlying cause of cell damage, which may be secondary to a mixture of reperfusion injury, direct reactive oxidative stress injury and cytokine injury.

1.3.2 Multiorgan Dysfunction Scoring

Multiorgan dysfunction syndrome (MODS) is a syndrome which is diagnosed based on clinical and laboratory criteria. Different scoring systems have been developed in neonates, children and adults to assess morbidity and to predict outcome.

1.3.2 Neonatal Multiorgan dysfunction (MODS)

The concept behind multiorgan dysfunction (MODS) has been associated with the "diving reflex" which involves conservation of blood flow to vital organs at the cost of non-vital organs. Multiorgan dysfunction in infants post NE has been defined in a variety of ways. Thus, the rates of individual organ dysfunction also vary across the literature probably due to non-uniformity of the definitions used. For instance, few studies report a rate of cardiac involvement in 78% of the term infants with NE (Hankins et al., 2002) whereas other studies have reported a rate of 50% (Shankaran et al., 1991). and 62% of term infants (Shah et al., 2004).

Similarly rate of renal involvement varied in different studies. Shah et al reported involvement in 70% cases compared to 40% by Perlman et al. However, despite using different criteria for lung dysfunction, 86% incidence of lung involvement was seen in studies by both Shah et al and Shankaran et al. (Shah et al., 2004, Shankaran et al., 1991).

In addition, scoring systems have been developed to help to quantify specific organ system injury following NE e.g. Sepsis (Haematological Scoring System = HSS) (Narasimha and Harendra Kumar, 2011). Another scoring system was developed to quantify neonatal seizures in term infants (Garfinkle and Shevell, 2011). Recent studies have demonstrated a significant association between MODS and mortality and emphasised the need for global management.

In premature infants, a neonatal multiorgan dysfunction (NEOMOD) scoring system was developed to enable accurate and early diagnosis of MODS (Janota et al., 2001). This provides information on organ functions influencing mortality and predicts mortality in the first 28 days of life. Assessment of the central nervous system, coagulation, respiratory, gastrointestinal, cardiovascular, renal system, and acid-base balance at 24h intervals during the first 28 days of life were carried out and combined

creating a scoring system and a score of 9 or above was associated with 100% mortality (Cetinkaya et al., 2012).

1.2.2b Paediatric MODS: In critically ill children, several scoring systems for measuring paediatric MODS have been described in the literature. These include the Multiple Organ System Failure score (Wilkinson et al, 1986, the Paediatric Multiple Organ Dysfunction Score, the Paediatric Logistic Organ Dysfunction score (Leteurtre et al., 1999, Leteurtre et al., 2015), and the Paediatric MODS (Graciano et al., 2005).

In critically ill children admitted to PICU, the PEdiatric Logistic Organ Dysfunction-2 score (PELOD-2) and the daily dPELOD-2 scores have been found to be good estimates in describing clinical outcome of critically ill children throughout their stay in PICU. The most abnormal value of each variable observed during each of these time points is considered to calculate the PELOD-2 score. Following the PELOD score, same authors developed the daily dPELOD score proposing that daily scores would provide more information. The progression of the severity of organ dysfunctions may be evaluated by measuring the dPELOD-2 scores during a specified set of days in PICU (admission and days 2, 5, 8, 12, 16 and 18). Only the Paediatric Logistic Organ Dysfunction score has been validated in a multicentre study.

The daily PELOD -2 score evaluates five organ functions using ten items: neurologic (Glasgow coma score and pupillary reaction), cardiovascular (serum lactate, mean arterial pressure), renal (creatinine), respiratory (PaO2/FiO2 ratio, PaCO2, invasive ventilation), and hematologic (white blood cell count and platelets). Using the daily PELODS score the incidence of multiorgan dysfunction and mortality rate have been demonstrated to be higher among neonates compared to older children. (Bestati et al., 2010). Significant contributors to neonatal mortality were neurological, cardiovascular, and hepatic dysfunctions.

Goldstein et al modified the adult Systemic Inflammatory Response Syndrome (SIRS) for the paediatric population with the goal of creating first-generation paediatric definitions and criteria of multi-organ dysfunction associated with sepsis (Goldstein et al., 2005). However, they advised that this should be considered as “work in progress”, this definition and scoring system will require continuous adjustment in the future as additional biomarkers of sepsis and newer dysfunctions will emerge.

Though attempts have been made to define and quantify multiorgan dysfunction in neonates and a neonatal MODS scoring is currently being validated but MODS has not been quantified beyond the neonatal period and no scoring system has been developed and validated in children post neonatal encephalopathy. There is a need for a scoring system for multiorgan assessment and long term follow up in children with NE to prevent further complications in childhood.

Table 1-1: PELODS Scoring System

Adapted from (Leteurtre et al., 1999).

Organ system and variable	Points assigned			
	0	1	10	20
Neurologic*				
Glasgow coma score	12–15	7–11	4–6	3
	and		*or*	
Pupillary reaction	Both reactive		Both fixed	
Cardiovascular				
Heart rate, beats/min				
< 12 years	≤ 195		> 195	
≥ 12 years	≤ 150		> 150	
	and		*or*	
Systolic blood pressure, mm Hg				
< 1 mo	> 65		35–65	< 35
≥ 1 mo–< 1 yr	> 75		35–75	< 35
≥ 1 yr–< 12 yr	> 85		45–85	< 45
≥ 12 yr	> 95		55–95	< 55
Renal				
Creatinine, μmol/L (mg/dL)				
< 7 d	< 140 (< 1.59)		≥ 140 (≥ 1.59)	
≥ 7 d–< 1 yr	< 55 (< 0.62)		≥ 55 (≥ 0.62)	
≥ 1 yr–< 12 yr	< 100 (< 1.13)		≥ 100 (≥ 1.13)	
≥ 12 yr	< 140 (< 1.59)		≥ 140 (≥ 1.59)	
Respiratory				
PaO_2:FiO_2 ratio, mm Hg	> 70		≤ 70	
	and		*or*	
$PaCO_2$, mm Hg (kPa)	≤ 90 (≤ 11.7)		> 90 (> 11.7)	
	and			
Mechanical ventilation†	No ventilation	Ventilation		
Hematologic				
Leukocyte count, × 10^9/L	≥ 4.5	1.5–4.4	< 1.5	
	and	*or*		
Platelet count, × 10^9/L	≥ 35	< 35		
Hepatic				
Glutamic oxaloacetic transaminase, IU/L	< 950	≥ 950		
	and	*or*		
Prothrombin time, % of standard (international normalized ratio)	> 60 (< 1.40)	≤ 60 (≥ 1.40)		

Note: FiO_2 = fraction of inspired oxygen, $PaCO_2$ = partial pressure of carbon dioxide in arterial blood, PaO_2 = partial pressure of oxygen in arterial blood.
*For the Glasgow coma score, use the lowest value. If the patient is sedated, record the estimated coma score before sedation. Assess the patient only with known or suspected acute central nervous system disease. For pupillary reactions, nonreactive pupils must be > 3 mm; do not assess after iatrogenic pupillary dilatation.
†The use of mask ventilation is not considered to be mechanical ventilation.

1.4. Neurological & Developmental

1.4.1 General Overview

CP has an incidence of 13.5% (Badawi et al., 2005) in infants who had NE. Neuropsychological & cognitive function are commonly impaired in children with CP. Therapeutic hypothermia initiated at 6 hours of age in infants of more than 35 weeks of gestation, with NE has been shown to reduce the risk of death and disability and increased the rate of survival without disability at 18 to 24 months of age (Gluckman et al., 2005, Shankaran et al., 2005, Eicher et al., 2005, Azzopardi, 2010, Jacobs et al., 2011, Simbruner et al., 2010, Zhou et al., 2010). In a systemic Cochrane review which included evidence from 11 randomised controlled trials comprising 1505 infants, therapeutic hypothermia was found to be beneficial in both term and late preterm infants with NE and hypothermia improved survival and development at 18-24 months (Jacobs et al., 2013).

A randomized, controlled trial of whole-body hypothermia for neonatal NE, reported rate of death or moderate to severe disability at 18 to 24 months of age of 62% in the control group versus 44% in the therapeutic hypothermia (TH) group (p value 0.001) and mortality of 37% and 24% respectively. A follow up study at 6-7 years of age however, showed no significant between-group differences in the level of disability among all survivors. The rates of cerebral palsy were 17% and 29%, respectively, the rates of blindness were 1% and 4%, and the rates of hearing impairment were 5% and 2% (p value >0.05, for all comparisons) (Shankaran et al., 2005).

The periventricular or “watershed” area is the most affected area following a hypoxic ischaemic insult, in an immature brain, whereas at full term damage is to the basal ganglia. Thus, disruption to blood flow and hypoxic insult pre or post term leads to different clinical patterns and different physical impairment (Fairhurst, 2012).

Several perinatal factors including hypoxic ischaemic insult, maternal inflammation/ infection, maternal stress and nutrition, affect the foetal brain structure causing alteration in the size and shape of the grey matter structures (hippocampus & amygdala) and also alter the functional connectivity leading to increased risk of

neurodevelopmental and neuropsychiatric disorders (Buss et al., 2012). Brain growth and connectivity continue to increase rapidly during the first 2 years of postnatal life. (Gilmore et al., 2007, Gao et al., 2009). Foetal exposure of increased maternal levels of cytokine IL-8 have been linked with changes in the brain (decreased volume of left cortex, right caudate and putamen) and associated with increased risk of schizophrenia in later childhood and adult life (Ellman et al., 2010).

Children born at term who develop CP following NE, have a poorer prognosis than those with CP who were not encephalopathic in the first week of life (Badawi et al., 2005). Badawi et al also demonstrated that these infants are more likely to develop epilepsy, cognitive impairment, severe disability and die early in the period ranging from the onset of diagnosis to 6 years of age. At 5 years of age various studies have reported disability in 6 to 21% of children with moderate encephalopathy after perinatal asphyxia and in 42 to 100% in severe encephalopathy. (Marlow and Budge, 2005, Gonzalez and Miller, 2006, de Vries and Groenendaal, 2010). In children who had no disability there were reports of delay in speech and language, memory, arithmetic and sensorimotor perception scores at 5 years of age. (Marlow and Budge, 2005, Gonzalez and Miller, 2006).

In terms of general intellectual disability children with mild NE performed in a range equal to the healthy comparison group whereas children with moderate NE were weaker in performance. They had obvious difficulties in reading, spelling and mathematics (Barnett et al., 2004). Van Handel et al showed that children with a history of NE had more behaviour problems including anxiety, depression, attention regulation and thought problems. Therefore, all children with NE require long term follow up of cognitive & behaviour difficulties, especially children with moderate NE require detailed neuropsychological examination (van Handel et al., 2007). Children in the mild NE group, which include 39% of the total children affected with NE, do not qualify for therapeutic hypothermia and are not routinely followed. But recent studies report increased incidence of abnormal MRI finding and disability in 16-25% in infants with mild NE. (Chalak et al., 2018, Conway et al., 2018b) .

1.4.2. Predictors of long-term neurological outcome post NE

1.4.2.1 Electroencephalograph (EEG)

Clinically neonatal seizures may be difficult to recognize as they are subtle and not accompanied by any identifiable clinical symptoms. Electro-clinical dissociation is common in neonatal seizures, clinically seizure activity ceases while electrographic seizures may persist. Thus, neonatal seizures should be ideally evaluated using electroencephalogram (EEG) monitoring.

EEG, particularly amplitude-integrated continuous EEG (aEEG) is commonly used in infants with NE (Boylan et al., 2013), and can help detect seizures. An EEG is a very useful monitoring tool in infants with NE allowing quantification of the amplitude of cerebral activity and sleep-wake cycling. aEEG abnormalities including seizures and a suppressed background pattern have high predictive value towards an adverse outcome after perinatal asphyxia (Toet et al., 1999, van Laerhoven et al., 2013). This predictive ability occurs in both normothermic infants as well as during therapeutic hypothermia (Thoresen et al., 2010). However, aEEG use has certain disadvantages as it is not very specific or sensitive in detection of focal seizures (Shah, 2008, Rennie et al., 2004) and not very informative about frequency, synchrony, symmetry and inter burst interval measurements. Moreover, its ability to predict long-term outcome may be weakened after the infant receives therapeutic hypothermia (Azzopardi et al., 2014).

A normal or mildly abnormal EEG at 6 hours had 100% positive predictive value for a normal outcome at 2 years. Both EEG features (e.g., interictal pattern) and the aetiology of seizure (e.g., global cerebral hypoxia-ischemia) are important factors in determining outcome and prognosis. Features of aEEG including background pattern, cyclicity, seizure activity in addition to seizure aetiology are significantly associated with the outcome. The background pattern was the most informative predictor for the neurological outcome at one year. Zhang et al developed a clinically applicable scoring system based on aetiology and suggested that three aEEG indices would enable paediatricians to assess the risk for neurodevelopmental impairment and allow early intervention (Zhang et al., 2013).

1.4.2.2 MRI in NE and Long-term outcome

Magnetic resonance imaging (MRI) is one of the principal investigations to assess cerebral injury following NE. MRI is particularly useful for imaging the posterior fossa, where ultrasound scanning is more challenging. MRI should be ideally performed on all infants with moderate to severe neonatal encephalopathy, between day 5 to 10 of life. However, following TH, the ideal timing for imaging is either on day 5 or after 14 days due to concerns that images taken earlier may underestimate brain injury post TH due to the process of pseudo-normalisation (Winter et al., 2007, Bednarek et al., 2012).

On MRI two basic patterns of brain injury have been described in infants with NE secondary to hypoxia-ischaemia: i) Basal ganglia-thalamus predominant pattern which is consistent with an acute and profound asphyxia insult and ii) Watershed-predominant pattern which involves the white matter in the vascular watershed areas between the territory of the major cerebral arteries and this is consistent with a subacute, more longstanding insult. (Miller et al., 2005). Children with the basal ganglia thalamic (BGT) pattern of injury may have severe disability due to dyskinetic cerebral palsy (CP). A follow up study at mean age of 9 years (range 4 to 13 years) of children (n=48) with dyskinetic CP mostly secondary to BGT injury on MRI, demonstrated that most children had severe motor disability and the rate of learning disability and epilepsy increased with the severity of motor impairment (Himmelmann et al., 2007).

In infants with mild NE, magnetic resonance imaging (MRI) abnormalities have been demonstrated in 20-40% of cases suggestive of a predominant watershed pattern of injury occurring during the perinatal period. Patterns of injury seen on MRI in infants with mild NE have been associated with long-term neuro-developmental impairment (Walsh and Inder, 2018).

1.4.2.3 MRI Scoring

Several studies have demonstrated MRI to be one of the best predictors of outcome (Cheong et al., 2012, Weeke et al., 2016). To facilitate the assessment of severity of injury and help in determining the prognosis following neonatal encephalopathy, Barkovich created a MRI scoring system (Table1.2) (Barkovich, 1998). High points on the scoring system correlated with neuromotor and cognitive deficits in early childhood. This scoring system was expanded as some infants with NE may have

focal white matter injury in the absence of basal ganglia (BT)/thalamic (T) lesions and this gives rise to tissue atrophy and cognitive impairment in childhood (Rutherford et al., 2010). The significance of focal white matter injury has received comparatively less emphasis than BG/T and watershed injury in both in non-cooled and cooled neonates with NE.

Table 1-2 MRI Scoring system: Term Neonatal encephalopathy*

Table Legend 1.2
*(Barkovich, 1998)

Score	Finding
	Basal Ganglia
0	Normal or isolated focal cortical infarct
1	Abnormal signal in thalamus
2	Abnormal signal in thalamus and lentiform nucleus
3	Abnormal signal in thalamus, lentiform nucleus and peri Rolandic cortex
4	More extensive involvement
	Watershed (W)
0	Normal
1	Single focal infarction
2	Abnormal signal in anterior or posterior watershed white matter
3	Abnormal signal in anterior or posterior watershed cortex and white matter
4	Abnormal signal in both anterior and posterior watershed zones
5	More extensive cortical involvement
	Basal Ganglia/Watershed (BG/W)
0	Normal
1	Abnormal signal in basal ganglia or thalamus
2	Abnormal signal in cortex
3	Abnormal signal in cortex and basal nuclei (basal ganglia or thalamus)
4	Abnormal signal in entire cortex and basal nuclei
	Summation (S)
	Arithmetic sum of Basal Ganglia and Watershed Scores
	Enhancement (E)
0	No enhancement
1	Enhancement in white matter only
2	Enhancement in deep grey matter nuclei
3	Enhancement in cerebral cortex
4	Enhancement in cortex and deep grey matter or white matter

Diffusion weighted (DW) images and ADC

The conventional T1 and T2 sequences may show changes in affected areas, but for early visualisation of hypoxic ischemic injury, in the first week following the hypoxic insult, diffusion-weighted (DW) imaging should be acquired (Alderliesten et al., 2011, Bednarek et al., 2012). The water diffusion in the brain tissue is represented in DW images and this is known to be reduced in ischemic regions in the first week following hypoxic insult in neonates with NE. As DW imaging (DWI) is affected by T2 shine and many other factors thus an apparent diffusion coefficient (ADC) map is recommended, which is a ratio between a diffusion-weighted and a non-diffusion-weighted image. MRI abnormalities including DWI and ADC have a high predictive value for an abnormal outcome following perinatal asphyxia (Alderliesten et al., 2011, Rutherford et al., 2010).

Cowan et al found that mild white matter injury was associated with either a normal outcome at 2 years or mild language delay and “moderate” injury with cognitive delays and behaviour problems but not with debilitating neuromotor problems. “Severe” injury was associated with more severe neuromotor problems and a higher incidence of seizures and behavioural problems (Cowan et al., 2003). As mild degrees of brain injury may be associated with developmental disabilities in early childhood caution should be taken when counselling parents about prognosis in babies with NE after TH, especially when perinatal MRI show seemingly mild degrees of focal white matter and cortical injury. However, various studies comparing MRI patterns of injury and neurodevelopmental outcome demonstrated that severe watershed predominant pattern seen in survivors of NE without functional motor deficits at 4 years of age is associated with more impaired language-related skills (Steinman et al., 2009).

Advanced MRI techniques including MR spectroscopic imaging (MRSI) and diffusion tensor imaging (DTI) have the ability to predict early brain injury in neonates with NE. Proton MR spectroscopy of the basal ganglia and thalamus are of high predictive value for an abnormal neurodevelopmental outcome (Alderliesten et al., 2011). A meta- analysis showed that basal ganglia or thalamic lactate/N-acetyl aspartate is a highly accurate marker for the prediction of adverse long-term neurodevelopmental outcome or death (Thayyil et al., 2017). In addition, other markers including N-acetyl aspartate choline, myo-inositol on MRS and whole brain white matter water diffusion

fractional anistrophy (FA) has been analysed in neonates with NE. Reduced whole brain white matter FA was associated with reduced MRS N-acetyl aspartate/ choline, moderate or severe basal ganglia/thalamic or cortical injury and adverse neurodevelopmental outcome at 3.5 years of age assessed by Bayley Scale of Infant Development (BSID-III) (Lally et al., 2014).

In newborns with NE, quantative MRI or diffusion imaging can detect brain injury on day 1and 3 before qualitative MRI (Gano et al., 2013).

Functional MRI:

Resting state functional MRI (rsfMRI) is a novel mechanism used to identify and assess early functional cerebral development in normal infants and infants at risk. It can be used alongwith the above investigations including DTI. It can also identify networks with spontaneous neuronal activity termed resting state networks. Research is being carried out to establish fMRI as a biomarker as prediction of neurodevelopmental outcome in children following brain injury. (Smyser D et al 2015).

1.4.3 Hearing & Vision

Hearing loss has been reported to be as high as 17.1% in those children who also had other persistent neurological deficits following (Jiang et al., 2005). Persistent pulmonary hypertension of the new-born along with the diagnosis of NE places the child at risk for late-onset sensorineural hearing loss and repeated testing in early childhood is indicated (Robertson and Perlman, 2006). Hearing impairment occurs in approximately 12% of children with CP. This is commonly seen if etiological factors include severe NE, kernicterus or neonatal meningitis.

Up to 41% of infants with a diagnosis of NE in the first year of life present with an abnormality in some element of visual function and this rises to 100% when it is associated with moderate to severe basal ganglia and severe white matter changes on MRI (Mercuri et al., 2004b). A follow up study by the same group demonstrated a correlation between the severity of basal ganglia lesions and the severity of visual impairment children of school-age. There was approximately 90% concordance between assessments performed in the first year of life and at school going age (Mercuri

et al., 2004a). Visual impairment is commonly seen in children with CP (28%). There is increased incidence of associated ocular abnormalities like strabismus, amblyopia, nystagmus and refractory errors. Lesions involving the basal ganglia have been reported to correlate with impaired visual function in the first year of life (Mercuri et al., 2004a).

1.5. Cardiovascular system

1.5.1 Cardiovascular dysfunction and NE

Ancel et al showed that 29% of neonates with perinatal asphyxia had cardiac dysfunction consistent with myocardial ischaemia (Martin-Ancel et al., 1995). The rate of cardiovascular system involvement in infants with post asphyxial hypoxic ischaemic encephalopathy was reported as high as 62% by Shah et al (Shah et al., 2004), 50% by Shankaran et al and a 78% was reported by Hankins et al. This variation may be due to the difference in criteria used for involvement of cardiac dysfunction and severity of NE in the above studies. However, there is paucity in the literature on the follow up of cardiac dysfunction in childhood.

Troponins are protein molecules that are part of cardiac and skeletal muscle, they are the biomarker of choice for the detection of cardiac injury. Cardiac troponins play a major role in the screening and diagnosis of myocardial ischaemia in adults and children. In the paediatric population, troponins demonstrate a good correlation with the extent of myocardial damage following cardiac surgery and cardio toxic medication and can be used as predictors of subsequent cardiac recovery and degree of cardiac injury in the first week of life in perinatally asphyxiated infants. Cardiac troponin-T had higher sensitivity and specificity compared to CK-MB levels. Moreover, C troponin-T levels correlated well with severity and outcome in babies with perinatal asphyxia.

A reduction in left ventricular function, reduced LV strain and systolic strain rate has been demonstrated in several studies in infants with NE with or without therapeutic hypothermia when compared to controls (Nestaas et al., 2014). By measuring the severity of LV dysfunction using deformation, mortality can be predicted in this population (Sehgal et al., 2013).

A novel insight into LV myocardial function has been obtained in several studies by measuring the LV rotational mechanics and torsion deformation in these infants. Rotational mechanics can be assessed using 2 D speckle tracking echocardiography. A decrease in both the deformation and rotational mechanics has been shown in infants NE most probably occurring due to ischaemic insult to the heart and the impact of TH (Breatnach et al., 2017). However, there is paucity of data on LV rotational mechanics in children. Assessment with Echocardiography using tissue Doppler and myocardial velocity imaging combined with serum biomarkers including troponin and pro BNP may allow more accurate evaluation of cardiac dysfunction (Sweetman et al., 2012, Armstrong et al., 2012)

1.5.2 Cardiovascular Dysfunction in children with CP

Life expectancy is reduced in children with Cerebral Palsy and the most common cause of death are cardiovascular and respiratory disorders. Persistent low-grade inflammation in children with cerebral palsy is considered to play a major role in pathogenesis of atherosclerosis. Increased carotid intima media thickness (CIMT), a predictor of atherosclerosis was demonstrated in children with cerebral palsy when compared to control, using B-mode ultrasound, suggesting that children with CP are at increased risk of atherosclerosis and coronary artery disease. Thus, follow up studies of these children in later childhood and adulthood are required for prompt diagnosis and management (Cece et al., 2012)

1.6. Renal System

1.6.1 General Overview

Acute kidney injury (AKI) is a common complication of perinatal asphyxia, occurring in up to 56% of these infants (Durkan and Alexander, 2011) It is difficult to exactly assess the incidence as there is a lack of consensus on definitions of acute kidney injury (AKI) in neonates. 30-40% of AKI in the new-born period is associated with hypoxic ischaemia. (Agras et al., 2004, Mortazavi et al., 2009) Renal involvement in the neonatal period also seems to be associated with severity of long term neurological injury (Perlman and Tack, 1988). The prognosis and recovery from AKI are dependent upon

the underlying cause of the injury and is much worse in neonates who suffered multi-organ failure. This suggests that new-born with Acute Renal Failure (ARF) need life-long monitoring of renal function, blood pressure and urinalysis. (Andreoli, 2004). Acute kidney injury (AKI) has historically been described as an abrupt decline in renal function. AKI and have relied frequently upon the identification of a variable combination of rising serum creatinine, oliguria and rising blood urea (BUN).

Acute kidney injury in neonates has been described using markers including elevated serum creatinine (> 132.6 µmol/L), lowered GFR and reduced urinary output. Serum creatinine may not be a good marker of renal dysfunction especially in the neonate Firstly, the creatinine concentration reflects the maternal level for up to 72 hours after birth, thus probably rendering it unhelpful in the assessment of the neonate in the immediate postnatal period (Drukker and Guignard, 2002). Secondly, there are significant variations in neonatal glomerular filtration rate or creatinine values, which change rapidly in the immediate postnatal period as the infant adapts to extra-uterine life. These factors suggest that any interpretation of serum creatinine alone in the immediate postnatal period should be cautiously treated.

Oliguria is another clinical sign associated with AKI. However, in neonates, renal failure can occur, in more than 50% of cases, in the absence of oliguria (Andreoli, 2009). Thus, for comparing studies and for improved ability in predicting clinical outcomes, more specific criteria for AKI are needed.

1.6.2 Definitions of AKI

Recently three definitions of AKI have been derived in adults: Kidney Disease Improving Global Outcomes (KDIGO) Guidelines (Khwaja, 2012). Risk Injury Failure Loss End-Stage Kidney Disease (RIFLE) criteria (Basu et al., 2011) and Acute Kidney Injury Network (AKIN) (Basu et al., 2011) staging. These classification systems have been validated in a neonatal populations: AKIN (Kaur et al., 2011, Morgan et al., 2013) and RIFLE (Gadepalli et al., 2011).

AKIN criteria were used by Kaur et al to stage AKI in 36 term neonates with birth asphyxia. AKI was demonstrated to be significantly more common in the severe asphyxia

group (12/25-56%), compared to the moderate asphyxia group (1/11; 9.1%). The overall incidence of AKI using AKIN criteria was described to be 41.7%; in 15/36 neonates. This study also showed that if the diagnosis of AKI had been based solely on serum creatinine (SCr > 132.6 μmol/L), then only (10/36) 27.8% of the infants would have met the diagnostic criteria for AKI. Thus, highlighting that an absolute serum creatinine cut off value is an insensitive marker of AKI.

Highly significant associations were demonstrated between elevated urinary albumin, Cystatin C, NGAL, osteopontin and AKI in infants with NE (Sweetman and Molloy, 2013). In contrast urinary EGF were shown to be decreased in infants with AKI compared to those without AKI (Askenazi et al., 2012, Sweetman and Molloy, 2013)

1.6.3 AKI in Children

The Acute Dialysis Quality Initiative group created the RIFLE criteria in 2004; Risk Injury Failure Loss End-Stage Kidney Disease (RIFLE) criteria (Basu et al., 2011) which has been modified to p-RIFLE for use in children. Similarly, other classifications including AKIN and KIDGO have been used to define AKI in the paediatric population.

Paediatric RIFLE (p-RIFLE) subdivides AKI into three severity stages (risk, injury, and failure) and into two outcomes (loss and ESRD), whereas both AKIN and KDIGO use only three severity stages (stage 1, stage 2, and stage 3). Here stage 1 corresponds to risk, stage 2 corresponds to injury, and stage 3 corresponds to failure. AKI is quite common, occurring in one third of hospitalized children causing high mortality and morbidity. Inaccurate and inconsistent definition of AKI delay the staging and the management leading to poor outcome and prognosis.

1.6.4 Long term follow-up of Renal dysfunction

Hyperfiltration of the surviving nephron following AKI in the neonatal period leads to imbalance in the tubular function and GFR causing chronic kidney disease (CKD); (Subramanian et al., 2008, Brenner et al., 1996). Typically, the late development of CKD will first become evident with the development of hypertension, proteinuria and eventually elevation of blood urea nitrogen (BUN) and serum creatinine (Andreoli,

2004). Studies of acute kidney injury (AKI) biomarkers in adults and children have shown that urine and serum biomarkers can improve early diagnosis of AKI. Biomarkers like serum and urinary neutrophil gelatinase associated lipocalin (NGAL), interleukin 18, kidney injury molecule1, liver type fatty acid binding protein may have a good ability to predict AKI (Wheeler et al., 2008, Sweetman and Molloy, 2013) Infants who have sustained AKI in the neonatal period with regular lifelong measurement of growth and nutrition, serum creatinine, blood pressure and urinalysis for proteinuria and albumin/creatinine ratio is indicated (Andreoli, 2004, Moghal and Embleton, 2006, Subramanian et al., 2008)

Biomarkers may help in providing an accurate diagnosis and guide in the management of AKI following perinatal hypoxic ischaemia. Long-term renal prognosis for infants with perinatal asphyxia experiencing acute renal impairment is not certain, requiring follow-up in childhood and in adult life because it is possible that a significant number of them will have renal dysfunction later in life (Andreoli, 2009).

1.7. Gastrointestinal and Hepatic System

1.7.1 Hepatic dysfunction post NE

Hepatic dysfunction was reported in 84% in children post NE using the following: aspartate aminotransferase (AST) of >100 IU/l or alanine aminotransferase (ALT) of >100 IU/l at any time during the first week after birth (Shah et al., 2004). Hankins et al found an incidence of 80% and 23% by Phelan et al using almost similar criteria. These differences in liver involvement may be due to the differences in timing of the measurements, as values normalise in most infants after few days of an insult (Shah et al., 2004). Serum markers of liver and muscle injury like Lactate dehydrogenase (LDH), AST & ALT measured within 96 hrs of birth were found to be of borderline significance (p=0.046) when used to predict an abnormal outcome post NE (Hayes et al., 2013). Karlsson et al carried out further studies on the ability of LDH, ALT and AST to predict NE and adverse neurodevelopmental outcome in new-born term infants with intrapartum signs of foetal distress (Karlsson et al., 2010). They studied 246 infants studied, and 41 infants had NE. A significant relationship between Apgar

score at 5 minutes, severity of NE and LDH and ALT was found by linear regression analysis. The LDH, ALT and AST levels were significantly higher ($p<0.0001$) in the NE group compared with the non-NE group. LDH was also found to be the most suitable marker for prediction of adverse neurodevelopmental outcome as measured by neurological assessment at 18 months.

Similarly, Hayes et al reviewed the predictive value of LDH, creatinine kinase (CK) and transaminase (AST & ALT) concentrations in the first 96 hours of life for grade of encephalopathy and neurodevelopmental outcome (Hayes et al., 2013). The liver function tests of 146 infants were analysed using ROC curve analysis. They demonstrated that the predictive value of LDH, AST and ALT was significantly associated with NE III ($p=0.046$). Their results were similar to that of Karlsson et al group, demonstrating that LDH (5000 U/L) was the best predictor of abnormal outcome (sensitivity 50%, specificity 85%) AST (100 U/L) was the next most valuable predictor (sensitivity 78%, specificity 59%) and CK was neither sensitive nor specific for predicting abnormal outcome.

1.7.2 Gastro-intestinal and Hepatic Dysfunction in children with CP

Children with neurodevelopmental disabilities are at an increased risk of Gastro-oesophageal reflux (GER) because of numerous factors including: low tone in the lower oesophageal sphincter, poor posture, scoliosis, recurrent seizures and several medications (Trinick et al., 2012) Constipation is a common problem in children with developmental disabilities. Associated abdominal distension increases the risk of reflux and cramping may interfere with appetite, positioning and sleep. Overly loose stools are also a problem and may be related to diet or dietary supplements.

Children with cerebral palsy face nutritional challenges. Oral motor involvement in children with CP often has a detrimental impact on feeding and nutrition (Dahlseng et al., 2012). In one study 90% of children had significant oromotor dysfunction and 60% were dependent on caregivers for feeding (Reilly et al., 1996). Most of the children have poor weight gain (Tuzun et al., 2013). Factors contributing to this are numerous and include prolonged feeding time due to oromotor, pharyngeal

or oesophageal dysphagia; reduced appetite or discomfort associated with GER or constipation, increased energy expenditure due to spasticity or dystonia.

It is possible that nutritional deficits may exacerbate the effects of the primary brain injury in very young children with CP. Poor head growth in ex-premature babies at 2 years is strongly associated with poor neurodevelopmental outcome (Kuban et al., 2009, Cheong et al., 2008) and cerebral palsy (Cheong et al., 2008). Campanozzi et al showed that 6 months of nutritional rehabilitation resulted in improved motor skills in a cohort of children with cerebral palsy (Campanozzi et al., 2007).

Hepatic dysfunction is seen in children with cerebral palsy secondary to prolonged anticonvulsant drugs especially use of sodium valproate and carbamazepine. Using the right anti-epileptic drug can be difficult as many medications are metabolised through the liver. Awareness of the serious hepatotoxicity associated with certain antiepileptic drugs and monitoring liver function tests in children with CP are indicated.

1.8. Haematological system

1.8.1 Leucocytes and NE

Neonatal brain injury induced by NE is associated with inflammation. Cerebral ischaemia in neonates induces an immediate innate immune response within minutes of the insult (Algra et al., 2013). Following the insult, microglia are activated and develop macrophage like properties, phagocytosis and the release of pro and anti-inflammatory cytokines leading to breakdown of the blood brain barrier. This results in infiltration of the brain by circulating leucocytes, neutrophils, monocytes and T cells exacerbating the inflammatory response and brain damage (Zheng and Yenari, 2004, Liu and McCullough, 2013)

An elevated peripheral leucocyte count is associated with a high risk of mortality and neurological disability in infants with NE. There were significant differences in white cell count (WCC) and neutrophil count in the first few hours of life between survivors and non-survivors following NE. However, no correlation was noted between the high WCC and Bayley score in the survivors. Association between poor neurodevelopmental

outcome in neonates with NE and significantly higher WCC and ANC levels as early as 12 hours to 96 hours after birth have been demonstrated (Morkos et al., 2007).

In addition, it has been demonstrated in clinical trials that treatment with hypothermia results in chemokine associated immunosuppression resulting in persistently low leucocyte counts (Jenkins et al., 2013)

Neutrophils and Monocytes and NE

Hypoxic ischaemic insult at birth may also alter the neutrophil phenotype in neonates. Neonates resuscitated at birth demonstrated decreased neutrophil apoptosis. Pro-inflammatory neutrophil response was demonstrated in neonates with mild encephalopathy whereas infants with moderate or severe NE showed tendency towards immunosuppression (Molloy et al., 2007). Neonates resuscitated at delivery with abnormal neuroimaging and /or severe grade of encephalopathy were demonstrated to have increased CD11b, reactive oxygen intermediates (ROI) and TLR-4. Increased polymorphonuclear leukocytes TLR-4 expression was associated with increased mortality in infants with NE (O'Hare et al., 2016).

1.8.3 Coagulopathy and NE

Coagulopathy is defined as a prolonged activated partial thromboplastin time (APTT), prolonged prothrombin time (PT) or a decreased fibrinogen level. Hypoxic injury leads to a process of consumptive coagulopathy associated with high levels of fibrin degradation products (FDPs), prolongation of the PT and decreased platelet counts. Coagulopathy is a common issue in infants with NE, especially those treated with therapeutic hypothermia (TH) are at increased risk. The enzymatic activity of the coagulation cascade is slowed by hypothermia leading to prolongation of routinely tested coagulation studies (Forman et al., 2014). Perinatal asphyxia tends to alter the haemostatic balance between bleeding and clotting leading to increased abnormalities of bleeding and thrombosis in these asphyxiated neonates.

High levels of fibrinogen, fibrin degradation products, D-dimers, and thrombin-anti-thrombin complexes, are also reported in the asphyxiated term infant correlating with disseminated intravascular coagulation (Fichera et al., 1989, Suzuki and Morishita, 1998).

1.8.4 Coagulation and CP

Cerebral infarction secondary to pre/ perinatal cerebral occlusion occurs in 13-37% children with hemiplegic CP and the infarction occurs most probably due to a coagulation disorder. Children with perinatal stroke following large branch infarction of a unilateral middle cerebral artery were predictive of later cerebral palsy (Golomb et al., 2008). Hemiplegic cerebral palsy has been reported to result from perinatal stroke in 60% cases (Wu et al., 2006).

1.8.5 Thrombocytopenia and NE

Thrombocytopenia is defined as platelet count of < 150 x 10^9/L. However, a platelet count < 20 x 10^9/L without clinical signs of active bleeding or a platelet count < 50 x 10^9/L with signs of active bleeding are considered clinically significant and may require treatment with a platelet transfusion. Thrombophilia may be associated with other disorders like foeto-placental vasculopathy and neonatal stroke. The role of routine neonatal thrombophilia work-up is highly debated and may have a limited role given the large amount of blood needed and the lack of normative data regarding levels of anticoagulant proteins(Roach et al., 2008, Boutaybi et al., 2014)

In a study of neonates (n=91) with neonatal arterial-ischaemic stroke 68% had at least one pro-thrombotic tendency as did 24% of control (Gunther et al., 2000). Coagulation proteins also may contribute to brain damage through the production of oxygen free radicals secondary to inflammation, as well as by direct means.

1.8.6 Anaemia and NE

Severe anaemia can impair cerebral blood supply and result in NE. There is a significant increase in rates of anaemia in hypoxic-ischaemic term new-borns compared

to healthy controls. Neurodevelopmental outcome at 2 years of age in children who with severe neonatal anaemia showed white matter changes and lesions in the basal ganglia and thalami were seen on MRI brain (Zonnenberg et al., 2016). However, the early neurodevelopmental outcome in these children at 2 years with severe neonatal anaemia was within the normal range.

1.9. Endocrine system

1.9.1 Endocrine disorders in CP

Endocrine disorders found in children with disabilities include hypothalamic pituitary disorders, bone health and in girls particularly menstrual problems. Pubertal problems are also seen including central precocious puberty, associated with midline defects, premature adrenarche with neural tube defects, and delayed puberty.

Osteoporosis is common in children with cerebral palsy. Osteoporosis is a condition characterized by reductions in bone strength, leading to an increased risk of fractures. Multiple factors contribute to decreased bone mass including poor nutrition, anticonvulsant, reduced mobility [9, 10], vitamin D deficiency and increased cytokine activity. These factors subsequently impair growth and directly affect bone health.

Eighty to 90% of children with severe cerebral palsy have low bone mineral density (BMD) and an increased risk of fragility fractures. Many of those who sustain a fracture will sustain repeated fractures (Henderson et al., 2005). The most common site of fracture is the femur. Prevalence of fracture femur have been variably estimated at 20% in non-ambulant children and young adults with cerebral palsy (Henderson et al., 2005, Stevenson et al., 2006). The incidence of fracture in children with severe cerebral palsy has been reported as 7% to 9.7% per year (Fehlings et al., 2012).

Assessment of BMD can be difficult in children with disability, but BMD measurement of the lumbar spine may be possible however the child needs to be positioned properly. It is necessary to use sites other than the femoral neck because the child cannot be positioned properly, for measuring BMD in the appendicular skeleton. Other sites commonly used are the lower femur or radius. Peripheral quantitative computed tomography (pQCT) provides a measurement of cortical

thickness and quality and thus is valuable in assessment of bone health in a child with a disability. In children with CP the marked reduction in the total cross-sectional area and the cortical cross-sectional area although the cortical BMD remains unaffected. Low pQCT measurement have been shown to predict increased risk of fractures. The small and thin bones especially in a prepubertal child are at increased risk of fracture. Regular DEXA scanning has been proposed depending on risk factors such as children with GMFCS IV-V on anticonvulsant therapy and previous history of significant fracture (Stevenson et al., 2006)

Treatment involves conservative measures, including optimising the management of underlying conditions, maintaining appropriate calcium and vitamin D intake, encouraging weight-bearing physical activity, and medications such as bisphosphonate. Puberty increases bone mass by nearly 50% in both sexes. However, disordered puberty, particularly delayed puberty, is extremely commonly seen in children with CP.

Guidelines for the management of bone health in this population have been published following systematic literature review, reporting probable evidence for using bisphosphonate, possible evidence for vitamin D and calcium use, and not enough evidence for weight-bearing activities as effective interventions to improve BMD (Fehlings et al., 2012).

1.10. Quality of Life

Cerebral palsy (CP) is one of the most common chronic disabling condition of childhood. Cerebral palsy can have a strong impact on the child's ability to carry out activities of daily living (ADL); hence affecting the QOL of the child. QOL is a multidimensional construct and is an overall assessment of health in all areas.

It is important to assess the quality of life of children with CP to inform and evaluate their individual care plans, service planning, and interventions (Gilson et al., 2014). QOL was reported to decrease considerably as the level of physical disability increased. Associated co-morbidities including epilepsy, visual impairment, hearing deficit and intellectual disability greatly impact the QOL of these children. Several other factors including pain, parental stress and socioeconomic factors also affect QOL of a child.

Leigh et al found that individuals with CP experience a QOL that was 63% lower than that experienced by the UK population in general. QOL was reported to decrease considerably as the level of ambulatory disability increased, for those with GMFCS 1, QOL largely resembled the general population but for those with GMFCS 2, 3, and 4, reductions in QoL was considerable, 25, 43, and 92%, respectively (Leigh et al., 2014). Studies on QOL are quite subjective in nature and have many limitations thus a combination of well valid parent based, and child-based questionnaire and generic tools is required. There are several paediatric QOL questionnaires available including the Child health questionnaire (CHQ), Paediatric quality of life inventory (PedQOL), DISABKIDS, but few are available for children with CP. The commonly used QOL questionnaire in children with CP are the CP QOL, DISABKIDS cerebral palsy module or Peds QL cerebral palsy module.

1.11. Sleep and circadian rhythm

1.11.1 Sleep Disorders in CP

Children with Cerebral Palsy (CP) have a high incidence of sleep problem including sleep disordered breathing, parasomnias and excessive daytime sleepiness when compared to the sleep pattern of children with normal development. In the general population prevalence of sleep disorders in schoolchildren varies from 10% to more than 40%.

Children with cerebral palsy (CP) are more prone to sleep disturbances compared to children with no chronic illness. Prevalence of sleep disorders in school age children with CP is 40-48%, around six to eight-fold increase compared to controls (Newman et al., 2006) (Romeo et al., 2014). Various factors contributing to sleep disorders in children with CP include epilepsy, intellectual disability, visual impairment, anti-epileptic medications, obstructive sleep apnoea, and decreased ability to move due to contractures. Cognitive impairment is frequently seen in children with total body involvement CP and is highly associated with sleep problems, especially sleeplessness.

Epilepsy in children with CP, has been shown to result in increased total sleep disturbance and increased daytime sleepiness (Newman et al., 2006) Various antiepileptic drugs cause daytime sleepiness. On the other hand, sleep loss or disturbed sleep may lead to an increase in frequency of seizures. Total body involvement,

including spastic quadriplegia and dyskinetic subtypes of CP, is strongly associated with disorders of initiation and maintenance of sleep. In addition, other frequently identified sleep disorders in children with CP are sleep-wake transition (15-19%), sleep related breathing disorders (12-15%) and excessive daytime sleepiness in 12-13% (Newman et al., 2006) (Romeo et al., 2014, Atmawidjaja et al., 2014)

Severe visual impairment seen in many children with CP, can affect the timing and maintenance of sleep secondary to their effect on melatonin secretion and the lack of light perception. Light is a synchroniser for circadian rhythm and visual stimuli help the child to differentiate between day and night (Lockley et al., 1997)**.**

Other environmental factors also contribute to sleep problems, children with single parent are prone to total sleep disorder and sleep arousal, probably nightmares induced by psychological stress of parental separation leads to disturbed sleep and sleep arousal. In addition, habit of bed-sharing with a parent is associated with an increase disorders of initiation and maintenance of sleep, and sleep–wake transition disorders (Newman et al., 2006)

Management of sleep disorder can be by behaviour intervention, use of sleeping system and by pharmacological treatment. Use of Melatonin in children CP mostly those with sleep maintenance problems, showed consistent improvements in night waking time, sleep latency and in some patients, improved total sleep time. (Galland et al., 2012). Melatonin has also been found to be effective in treatment of sleep disorders in children with other conditions including autism, attention deficit hyperactivity disorder, Rett's syndrome and Tuberous sclerosis.

1.11.2 Melatonin and Neonatal Encephalopathy

In recent years, melatonin has emerged as an attractive choice of neuroprotective agent in decreasing the neurological sequelae from hypoxic-ischemic brain injury. After ischemic brain injury or stroke in different mammalian species, melatonin reduces infarct volume and inhibits neuronal cell death (Carloni et al., 2008). This was partially due to its capacity to cross physiological barriers including the blood brain barrier, placenta and its safety and efficacy. Melatonin also have

immunomodulatory properties; it reduces both chronic and acute inflammation. It acts on the immune system by regulating cytokine production of immunocompetent cells.

(Robertson et al., 2013) demonstrated that melatonin improves outcome as an adjunct to therapeutic hypothermia in an animal model of neonatal hypoxia-ischemia. Melatonin was also able to decrease sensorimotor asymmetry and learning deficits thus protecting the experimental pups from the long-term consequences of neonatal asphyxia.

In asphyxiated term neonates, melatonin administration may ameliorate brain injury, as demonstrated in a randomised control study. (Aly et al., 2015) The infants in the melatonin/hypothermia group demonstrated decreased seizure activity on follow-up EEG and less white matter abnormalities on MRI, compared to the hypothermia group. On follow up at 6 months of age the melatonin/hypothermia group showed improved survival rate without neurological or developmental abnormalities ($p<0.001$).

1.11.3 Melatonin and CP

Melatonin is used widely used in the treatment of sleep disorder in children with CP mostly those with sleep maintenance problems. It showed consistent improvements in night waking time, sleep latency and in some patients, improved total sleep time. (Galland et al., 2012) Use of melatonin in children with CP with different motor impairment and sleep disorders, has been assessed using questionnaires completed by parents and where possible by children. An improvement of quality and quantity of sleep has noted in majority of children, especially in sleep latency and night-time sleep duration with a dose ranging from 0.5 to 12 mg administered 30-60 minutes before the child's bedtime. Melatonin is well tolerated with minimal side effects even in those children with active epilepsy.

1.11.4 Circadian rhythm and Inflammation

Circadian rhythm (latin *circa diem*, means 'for about a day') was first described by Halberg as endogenous oscillations observed in organisms in close association with the earth's daily rotation cycle. The main components of the immune system in the

blood including hematopoietic cells, lymphocytes along with hormones and cytokines exhibit circadian rhythms (Scheiermann et al., 2013).

The immune cellular and humoral components in blood display opposite rhythms: the hematopoietic stem and progenitor cells (HSPCs) and majority of leukocytes (except the effector $CD8^+$ T cell) increase during the resting phase (during the night for humans and during the day for rodents) and decrease during the active period. (Dimitrov et al., 2009)

1.11.5 Clock proteins and immune cell function

Research studies have delineated the immune response to circadian rhythm and have highlighted the regulatory genes involved. (Curtis et al., 2014). The core clock proteins BMAL1, CLOCK and REV-ERBα, control fundamental aspects of the immune response. These proteins can affect the immune cell function, host response and inflammation. The time of the day is crucial to ascertain the type and extent of the immune response. During the daytime and during increased level of activity there is an increased risk of infection/ injury causing increase in number of leukocytes and high sensitivity of immune cells to infectious agents. Whereas, during the night in the resting state when the risk of infection and injury is less, tissue repair and resolution of inflammation occurs.

The clock protein BMAL is a central component of the circadian cycle, it functions as an anti-inflammatory molecule in immune cells such as monocytes. Its absence from myeloid cells leads to excessive inflammation and sepsis. The expression of BMAL1 m RNA alters significantly during the day in macrophages.

Similarly, the BMAL1 binding partner CLOCK also has an impact on the immune system. CLOCK is found in protein complexes with the NFKB unit. The embryonic fibroblasts (MEFs) and bone marrow derived macrophages (BMDMs) (Bellet et al., 2013) of CLOCK mutant mice are less responsive to Lipopolysaccharides (LPS) or Tumour necrosis factor alpha (TNF-α) in terms of NFKB activation. Inflammation and infection disrupt the oscillation in the above clock genes and affect the expression of the core clock proteins and clock-controlled genes (Bellet et al., 2013). In addition, animal studies have shown that LPS transiently suppresses clock gene expression and oscillation in the suprachiasmatic nucleus (SCN) of the brain (Okada et al., 2008).

1.12. Immunity and Inflammation

1.12.1 General Overview

Injury to the brain in the perinatal period is one of the leading causes of cerebral palsy, epilepsy, cognitive and sensory impairment. During inflammation, diffuse activation of microglia in the neonatal brain occurs which increases the injury process by expressing inflammatory mediators and pro-inflammatory cytokines. The pro-inflammatory cytokines activate cytotoxic T cells, natural killer cells, which enhance cellular and tissue damage. This leads to cell proliferation, differentiation and cell death causing white matter damage and long-term neurological damage. The inflammatory effects triggered by pro-inflammatory cytokines, prostaglandins, or LPS on the developing CNS of premature infants may have long-term consequences on their ability to cope with environmental exposures during childhood and adulthood (Hagberg et al., 2005).

1.12.2 Cytokines and NE

Inflammation combined with Hypoxia-ischemia (HI) plays an important pathophysiological role in NE. Pro-inflammatory cytokine expression within the brain, especially of Interleukin-1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α), has been demonstrated following perinatal brain damage by pathogen triggers and HI both in experimental model and the human new born brain (Dammann and Leviton, 2004). Following endotoxin (Lipopolysaccharide: LPS) and/or HI exposures, predominant pro-inflammatory IL-1β response within the brains of preterm and term neonates may occur, causing patchy areas of white matter damage in preterm and major cortico-subcortical infarcts associated with BBB disruption and leukocyte infiltration in terms neonate. Anti-inflammatory cytokines, such as IL-1 receptor antagonist (IL-1ra), Interleukin-6 (IL-6), Interleukin 10 (IL-10), and TGF-β1, have already been shown in animal studies to promote neuronal survival and brain development (Savard et al., 2013) Increased levels of several cytokines in the neonatal blood at term may correlate strongly with the likelihood of cerebral palsy (Nelson et al., 1998).

1.12.2 Cytokines and Cerebral Palsy

Inflammation is closely associated with CP. (Hua Di et al 2016). There are several studies demonstrating high levels of proinflammatory cytokines (e.g. TNFα, IL-6, IL-8) in the plasma, cord blood and amniotic fluid are associated with development of CP and periventricular leukomalacia.(Hagberg et al., 2012)

Perinatal inflammation is also associated with many neuropsychiatric and neuropsychological disorders and it is suggested that inflammation has long term consequences on the brain during childhood (Hagberg et al., 2012). Gressens et al suggest that the injury processes can persist for months and years and propose a tertiary mechanism of damage, which includes inflammation and epigenetic changes. (Hagberg et al., 2012) Understanding, this tertiary mechanism could lead to safe and effective therapies to treat a developmentally disrupted brain long after the insult.

1.12.3 Inflammasomes

The inflammasomes are complex of proteins found in macrophages and neutrophils, plays a fundamental role in the production of inflammation in innate immunity. They are innate immune system receptors and sensors that induces inflammation in response to microbes and sterile stressors. The inflammasome is activated during brain injury and that NLRP1 and NLRP3 (NLRs) are detected in adult neurons and macrophage/microglia, respectively.

Inflammasomes are formed by activation of nucleotide-binding oligomerization domain like receptor family proteins (NLRP). NLRP3 is most widely studied inflammasome, expressed in cytosol of monocytes, neutrophils, lymphocytes & dendritic cells. Microbial or pathogen associated molecules such as bacterial lipopolysaccharide (LPS) and fungal organism can activate the NLRP3 inflammasome and induce IL-1β secretion in the presence of ATP. In addition, reactive oxygen species (ROS) plays an important role in the activation of the NLRP3 inflammasome. The inflammasomes caspases are activated leading to the processing and secretion of pro-inflammatory cytokines—e.g., IL-1 β, IL-18 and IL-33 (Figure 1).

Hypoxic ischaemia induces an inflammatory reaction in the immature brain of the neonate leading to activation of the cells of the innate immune system including

macrophages, polymorphonuclear cells, lymphocytes, NK-cells and mast cells. The accumulation of these cells is associated with the activation of receptors including Toll like receptors (TLR) and Nod like receptors (NLR), cytokines, ROS and receptor agonist including TNF which contribute towards cell death. Studies have shown that inflammasomes and the NOD like receptors are also involved, as IL 1β production is seen to be increased following hypoxic ischaemic injury and the injury process may be reduced by IL-receptor antagonist (IL-IRA) or by caspase 1 (Hagberg et al., 2016).

Melatonin also plays an important role in inhibition of NLRP3 inflammasome activation. It has been proven to be effective in Acute Lung Injury (ALI). The mechanism by which melatonin directly blocks activation of the NLRP3 inflammasome in ALI remains unclear. Inhibition of NLRP3 may occur by suppressing the release of extracellular histones and directly blocking histone-induced NLRP3 inflammasome activation (Zhang et al., 2013).

Melatonin treatment also attenuates early brain injury in mice, following subarachnoid haemorrhage (SAH) by inhibiting NLRP3 inflammasome-associated apoptosis (Dong et al., 2016). Thus, melatonin holds promise as a neuroprotective agent, but further studies and clinical trials are required.

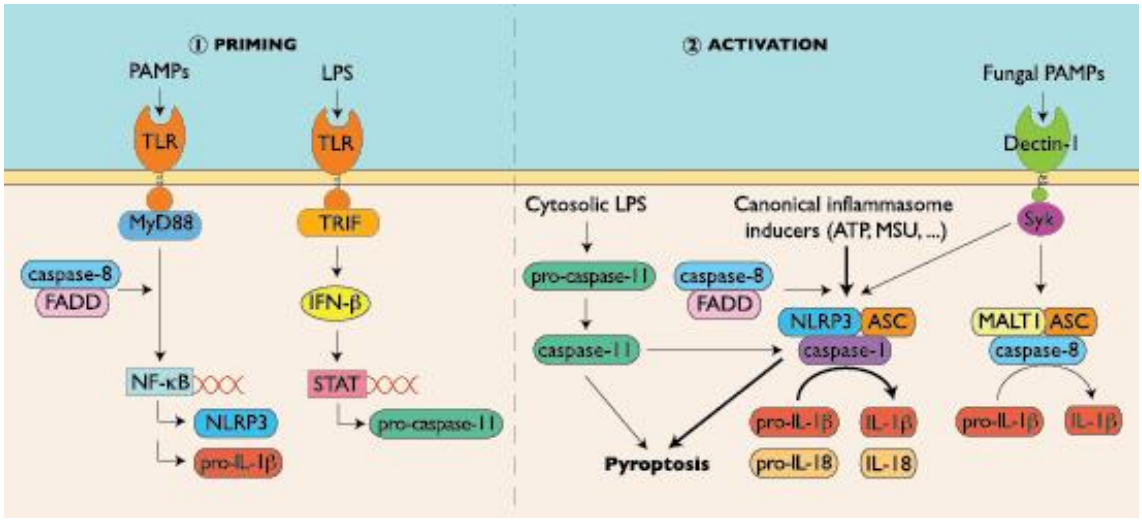


Figure 1-1 Inflammasome activation and release of cytokines IL-1β and IL18

Activation of Inflammasome gene NLRP3, ASC. NLRP3= Nod Like Receptor Protein, ASC= Apoptosis associated speck like protein, MALT1= Mucosa associated lymphoid tissue, TLR= Toll like receptor, LPS= Lipopolysaccharide, IL= Interleukin, IFN-β= Interferon beta

1.13. Hypothesis

Early multiorgan dysfunction in neonates with brain injury may persist in later childhood.

1.14. Aims

1. **To examine Multiorgan function in childhood in children with neonatal brain injury:** Detailed multi organ dysfunction (MOD) had been analysed in infants with NE and organ outcomes quantified, including serum, urine and cerebrospinal fluid (CSF) biomarkers. We assessed & correlated multiorgan outcomes from this cohort at school-age and compared them to age matched controls and children with CP.
2. **To assess Sleep pattern and Quality of life in children with NE:** We evaluated sleep problems and QOL of children with NE in comparison to children with normal development. We also evaluated the response of circadian rhythm genes in these children.
3. **To examine innate immune function in children with brain injury compared to controls:** Persistent or sustained inflammation has been implicated in neonatal brain injury. In addition, children with CP have altered immune function compared to age-matched controls. We were therefore interested in detailing innate immune function including Inflammasome activation & CLOCK proteins in these cohorts. We examined pro and anti- inflammatory responses to endotoxin and explored monocyte and neutrophil function.

Chapter 2
Materials and Methods

2. Materials and Methods

2.1. Ethical Approval

Ethical approval for the CHAMPION (Childhood Assessment of Multiorgan Dysfunction Post Neonatal Encephalopathy) study was received from the Ethics Committee of the National Maternity Hospital (NMH), Holles Street, Dublin, The National Children Hospital (NCH) Tallaght, Dublin and The Children University Hospital, Temple Street, Dublin.

Approval from NCH, Tallaght was obtained in September 2014 and recruitment was started in January 2015. Approval was also obtained from the Ethics Committee of NMH, Holles Street in Oct 2015 and recruitment commenced in January 2016.

Approval was obtained from the Ethics Committee of CUH, Temple Street in April 2017 and recruitment was commenced in June 2017 for cardiology assessment including Echocardiography and speckle tracking. Informed written consent was obtained by Dr Zunera Zareen (PhD student) prior to recruitment to the study in all cases.

2.1.1 National Children Hospital, (NCH) Tallaght, Dublin

Tallaght Hospital, incorporating the National Children's Hospital, is one of Ireland's largest acute teaching hospitals and is one of two main teaching hospitals of Trinity College Dublin - specialising in the training and professional development of staff in areas such as nursing, emergency medicine and surgery, amongst many others. Tallaght Hospital is part of the Dublin Midlands Hospital Group which serves a population of over 1.2 million across seven counties.

Children with Neurodisability including Cerebral palsy, Neuromuscular disorders, Complex Epilepsy and Metabolic syndromes attend the Neurodisability/ Complex Needs Clinic at NCH Tallaght. These children have complex needs and co morbidities including Epilepsy, learning difficulties, hearing and visual impairment, respiratory problems, feeding difficulties, sleep and behaviour problems.

2.1.2 National Maternity Hospital, (NMH), Holles Street, Dublin

The Neonatal intensive care unit (NICU) in the National Maternity Hospital (NMH) is a tertiary neonatal centre with 10,000 deliveries in 2011 and has a 24-bed intensive care/high dependency unit with ~150 very low birth weight infants admitted per annum. The NMH is also a tertiary referral unit, accepting transfers (preterm and term infants) from regional hospitals all over the island of Ireland. The NMH is one of the four referral centres for therapeutic hypothermia in Ireland.

2.1.3 Children's University Hospital, Temple Street (CUH)

The Children's University Hospital is a tertiary referral hospital in Dublin with various multi-speciality departments including Respiratory, Cardiology, Neurology, Neuro-disability, General Paediatrics, Neonatology, Endocrinology, Metabolic Medicine, Dermatology, Surgery, Orthopaedics, Ophthalmology, Radiology, Psychiatry, and Paediatric Intensive care unit (ICU). The cardiology assessment of group of patients from our cohort of school age children with NE was done here. The assessment included Echocardiography including Tissue Doppler and speckle tracking.

2.2. Study Population

2.2.1 Control Group

The control group included age and sex-matched children attending the day ward at The National Children Hospital (NCH), Tallaght undergoing phlebotomy as part of a day case procedure were included (n=65). They fulfilled the following criteria: (i) Healthy children born at full term, had no problems at birth and in neonatal period ii) have no underlying co-morbidities. Following informed parental consent, children who fitted these criteria were recruited to the study as controls.

2.2.2 Study Groups

Neonatal Encephalopathy Group: All infants born with NE between 2006-present have been prospectively included in a database on computerised register at the National Maternity Hospital (n=150).

Infants born between 2011 and 2012 with NE were included in the CHAMPION study (Childhood Assessment of Multi-organ dysfunction Post Neonatal encephalopathy). These infants had serial urine and serum inflammatory biomarkers measured along with detailed measurement of multi-organ outcomes. (Sweetman and Molloy, 2013). These children with NE at age of 4-6 years were included in the CHAMPION study (n=55). They were age-matched with controls and children from the complex needs group. These infants were categorised as mild (Sarnat grade 0/I), moderate (Sarnat Grade II) and Severe (Sarnat Grade III) encephalopathy at birth.

Mild encephalopathy (NE I): hyper alertness, decreased spontaneous motor activity, activation of sympathetic functions lasting less than 24 hours with a normal EEG (Sarnat and Sarnat, 1976). There were total 20 children from this group (NE 0=3 and NE I =17) who were followed in childhood.

Moderate/Severe encephalopathy (NE II/III): NE Stage II: generalised muscular hypotonia, strong distal flexion, multifocal seizures and/or pathological EEG.
NE Stage III: stupor level of consciousness, flaccid muscle tone, suppression of brain stem and autonomic functions, severely pathological EEG with a periodic pattern and isopotential phases (Sarnat and Sarnat, 1976). This group included 35 children (NE II=32 and NE III=3). Out of the 35 children in group 24 children had underwent therapeutic hypothermia (TH) and 20 had abnormal imaging (MRI). Cerebral Palsy was diagnosed in 8 out of 35 children with NEII/III.

2.2.3 Complex Needs group

This group included children attending the Complex Neuro-disability clinic at the National Children's Hospital, Tallaght (n=26). Out of a total of twenty-six children, 15 children had heterogeneous diagnoses, including infections, metabolic disorders, and epileptic encephalopathy and remaining 11 had hypoxic ischaemic encephalopathy. We included the 15 children with CP due to a cause other than NE, to compare the MODS and innate immune function with the NE group. Nine out of the 15 children with complex needs had GMFCS V and 6/15 had GMFCS II-III. They have co morbidities and complex medical needs including Epilepsy, Physical & Intellectual

Disability, Visual Impairment, Deafness and Feeding Difficulties (Gastrostomy feeds). Parents were sent the information leaflet with details about the study 4-6 weeks in advance by post.

Exclusion Criteria: Children with severe congenital malformations and history of maternal substance misuse were not included.

METHODS AND MATERIALS

2.3. Clinical Evaluation

At the clinic visit detailed clinical assessment, questionnaire and investigations were carried out by the PhD student Dr Zareen. The neonatal database also was reviewed to gather information about the neurological examination findings and investigation carried in the neonatal period.

Database: As this is a follow up study of the cohort of infants with neonatal encephalopathy, all infants included on a prospective clinical database from 2011 onwards were asked to participate. We examined a cohort of patients (n=55) with brain injury to clearly follow the pathogenesis of this disorder. These included full term infants > 36 weeks gestation at risk of neonatal brain injury who were admitted to the NICU in NMH and fulfilled the following criteria:

a) Infants who demonstrated abnormal neurological signs, such as hypotonia or seizures in the immediate postnatal period, and/or other organ dysfunction (kidneys, liver, lung, heart, haematological) and demonstrated at least 2 of the following 3 criteria:

i) Evidence or suspicion of hypoxic-ischaemic injury based on a history of fetal distress i.e. type II dips, loss of beat-to-beat variability on cardiotocograph and/or abnormal scalp pH.

ii) Need for resuscitation after birth i.e. bag valve mask ventilation

iii) Base deficit of > 14 mmol/L or pH < 7.2 in cord blood or admission arterial blood gas.

b) Infants who were well at birth and did not require resuscitation but who subsequently developed NE (hypotonia/seizures) within the first 48 hours of life were also enrolled in the study.

Neonatal Clinical Examination and Neurological Scoring Systems

The importance of a full neurological assessment of any infant with suspected NE cannot be underestimated. A large amount of information about the neurological state of the infant can be gleaned by observing the infant closely in his/her incubator, before even laying a hand on the infant. The oldest and still the most widely used score for assessing infants with neonatal encephalopathy is called the Sarnat Score (Sarnat and Sarnat, 1976). Although this score was developed based on the observations of only 21 term newborns who suffered perinatal asphyxia, it remains the gold standard for classification of severity of NE. Three clinical stages were designated ranging from mild stage 1 to severe stage 3. Stage 1 was characterized by hyper alertness, uninhibited Moro and stretch reflexes, sympathetic effects lasting less than 24 hours and a normal electroencephalogram. Stage 2 was distinguished by obtundation, hypotonia, strong distal flexion, and multifocal seizures with electroencephalographic changes showing a periodic pattern sometimes preceded by continuous delta wave activity. Stage 3 described infants who were stuporous and flaccid with suppressed brain stem and autonomic functions. The electroencephalogram (EEG) was isopotential or showed infrequent periodic discharges (Table 2.1).

A paediatric neurologist with a special interest in neonatal encephalopathy (Dr Bryan Lynch) was a collaborator in this project and was consulted on the neurological status of the infants with brain injury. Cerebral imaging using serial cranial ultrasounds was carried out by a consultant paediatric radiologist with a special interest in neonatal brain injury (Dr Veronica Donoghue) and study infants also had an MRI brain scan performed on postnatal day 5-7.

Neonatal Neuroimaging Grading System

Cranial Ultrasound

Cranial ultrasound scans (CrUSS) were carried out serially over the first week of life (generally day 1 and 3) on all infants in the study group by a consultant paediatric

radiologist with a special interest in neonatal brain injury (Dr Veronica Donoghue). CrUSS was used to confirm normal cerebral anatomy, quantify cerebral oedema and echogenicity of the basal ganglia and in addition haemorrhage was graded according to the Papile classification system (Papile et al., 1978).

Neonatal Magnetic Resonance Imaging (MRI)

Magnetic Resonance Imaging (MRI) of the brain was performed on all infants with NE between postnatal day 5 and 10 using a 1.5 Tesla scanner in the radiology department of the Children's University Hospital, Temple Street, Dublin. Scans were scored and reported independently by a paediatric radiologist (Dr Veronica Donoghue) who was blinded to the clinical results and outcome, using the well validated Barkovich scoring system (Barkovich, 1998) which employs a combination score including components of both basal ganglia and watershed patterns of injury. Different sequences applied were T1 weighted imaging (T1WI), T2 weighted imaging (T2WI), T2 flair, Spectroscopy and Diffusion weighted imaging.,

Continuous Neonatal EEG

Continuous multichannel EEG was recorded for as many study infants as possible from as soon as possible after birth (<6 hours) and for 72 hours in keeping with the current protocol of the collaborators, the Neonatal Brain Research Group at Cork University Maternity Hospital, Cork City, Ireland. The evolution of EEG findings over the recording period of approximately 72 hours after birth was examined, with particular focus on features that were shown previously to be associated with poor outcomes, including background amplitude, presence of discontinuity, length of EEG activity burst and inter burst interval, return of sleep-wake cycling (SWC), and presence or absence of seizures.

The EEGs were visually analyzed by a neurophysiologist who is experienced in the analysis of neonatal EEG recordings (Professor G Boylan).

This study was a follow up of these children at 3-6 years of age. Detailed antenatal, birth, resuscitation, oxygen requirements and saturations throughout inpatient stay and detailed neonatal intensive care management were collected. In addition, details of therapeutic hypothermia treatment including initiation, duration and clinical examination, investigations including cranial USS, MRI, EEG and placental histology analysis performed as mentioned above in the neonatal period were

recorded. The database also included analysis of the original multi-organ neonatal data on the infants with NE including novel renal and serum biomarkers and neurodevelopmental outcome.

2.4. Multiorgan evaluation

1) Neurological & Neurodevelopmental

- History taking: Seizures, medication, hospitalisations, EEG reports
- Bulbar function: History of dysphagia, chewing difficulties, nasal regurgitation, slurred speech, choking on fluids, dysphonia, dysarthria, dysphasia;
  Investigations: Video fluoroscopy; Micro-laryngoscopy and Bronchoscopy (MLB).
- MRI brain: Neonatal and follow up MRI results at 2 years of age were collected where available/ applicable.
- Developmental Outcome: Ages and stages Questionnaire-3, Gross Motor Function Classification System – Extended & Revised (GMFCS-E&R), Manual Ability Classification System (MACS) and Bayley Scale of Infant Development (BSID) scores at 2 years.
- Speech delay, Hearing and Visual Impairment: Speech and Language delay, Cortical visual impairment and Deafness.
- Education: Special School, Special needs assistant (SNA), Resource teaching.

2. Gastrointestinal system

- Medications: Proton pump inhibitors, antacids, laxatives, prokinetics: Dose and response.
- Feeding History: volume and type- Tube fed or gastrostomy feeds
- Hepatology: Liver function tests, Albumin; Jaundice history
- Nutritional status: Weight, height and BMI

3. **Cardiac**

    - Echocardiography: Ejection fraction, Tissue Doppler, speckle tracking where available.
    - Bloods: Troponin I, B-type natriuretic peptide (BNP) or N-terminal pro b-type natriuretic peptide (NT-pro BNP).

4. **Respiratory/ ENT**

    - History of hospitalisations; Pneumonia and Asthma
    - History of Obstructive sleep apnoea; ear infections
    - History of Respiratory support -Continuous positive air pressure (CPAP/BiPAP)
    - Medications: Prophylactic antibiotics and metered dose inhalers.

5. **Renal/ Genitourinary:**

    - Urea & Electrolytes (U+E), Creatinine
    - History of renal stones, Urinary tract infections (UTI)
    - History of prophylactic antibiotics.

6. **Haematology**

    - Full blood count, differential count and coagulation screen
    - Thrombophilia screen (if needed as per advice of Consultant Haematologist)
    - Haematology history- easy bruising, bleeding
    - Family history of early stroke, early Myocardial Infarction (MI).

7. **Metabolic**

    - Metabolic study as per advice of Metabolic consultant
    - May include:
        - Urinary Organic Acids and serum amino acids
        - Chloride to calculate an anion gap
        - Lactate

8. **Endocrine**

    - Weight and height- calculate BMI
    - History of calcium and vitamin D intake and supplements.

- Biochemistry- calcium, phosphorous, alkaline phosphate, Vitamin D and parathyroid hormone.

9. Immune Function:

- Serum pro and anti-inflammatory cytokines.
- Whole blood endotoxin responses.
- Flow cytometry evaluation of peripheral mononuclear cells (PMN) and monocyte response and function.

10. Infections

- History of more than two infections in the previous year. Details of hospitalisations for infections.
- Vaccine titres, Immunoglobulin subsets if indicated
- Urine CMV results if done

11. Genetics

- Dysmorphic features
- Karyotype: result +/- cytogenetics and microarray if done
- Consultant Genetics referral as indicated.

12. Audiology

- Results of formal audiology assessment
- History from parents of screen for hearing deficit.
- Family History of hearing loss.

13. Vision

- Documentation of any formal Ophthalmology assessment will be obtained and recorded. Hx of cortical visual impairment.
- Examination of visual acuity at clinic and of visual fields.

2.5. Questionnaire

2.5.1 Ages and Stages Questionnaires (ASQ-3)

Development was measured using the Ages and Stages Questionnaire which is one of the most commonly used developmental tools in this age group.

ASQ-3 screens and assesses the developmental performance of children in the areas of communication, gross motor skills, fine motor skills, problem solving, and personal-social skills.

he Ages and Stages Questionnaire (ASQ3) is a parent-completed questionnaire that may be used as a general developmental screening tool. The ASQ-3 is a parent reported initial level developmental screening instrument consisting of 21 intervals, each with 30 items in five areas: (i) personal social, (ii) gross motor, (iii) fine motor, (iv) problem solving, and (v) communication for children from 2-66 months. There are different versions for different age groups. It is a universal, most widely used development assessment tool with high sensitivity and specificity for detecting developmental delay (Schonhaut et al., 2013)

2.5.2 Sleep Questionnaire

Sleep was measured on all children using the CSHQ (Child Sleep Habit Questionnaire). Sleep disturbance is a well-known issue in children with Cerebral palsy and Intellectual disability. The CSHQ is a 45-item, parent-rated questionnaire that assesses the frequency of behaviours associated with common paediatric sleep difficulties. The total score is combined to create eight subscales that relate to common sleep problems in children: Bedtime Resistance, Sleep Onset Delay, Sleep Duration, Sleep Anxiety, Night waking, Parasomnias, Sleep Disordered Breathing, and Daytime Sleepiness. Finally, all the scores are summed to create a Total Sleep Disturbances index, for which a score of over 41 indicates a paediatric sleep disorder.

2.5.3 Quality of Life Questionnaire

Quality of life was assessed using the Cerebral Palsy Quality of Life for Children questionnaire which is the first health condition specific questionnaire designed for measuring quality of life in children with CP. (Chen et al., 2014, Davis et al., 2010) have shown its validity in this group. It is designed in accordance with International Classification of Function (ICF) and the definition of quality of life by the World Health Organization (Waters et al., 2007). CP QOL questionnaire also assesses the child's emotional and social wellbeing apart from assessing their physical health. It is essential to identify and manage the emotional and social problems that the children with CP

cope as early as possible, to promote their health and well-being. We used this questionnaire to assess QOL in our group of children with CP.

The PED QL questionnaire was used to assess quality of life in children with normal development. The PedsQL Measurement Model is a tool to measuring health related quality of life (HRQOL) in healthy children and adolescents and those with acute and chronic health conditions including CP. (Varni et al., 2005). We used this questionnaire to assess QOL in children with mild to moderate NE in comparison to typically developed children in the control group.

2.6. Developmental Assessment

2.6.1 Bayley scale of Infant Development (BSID)

The Bayley Scales of Infant Development (BSID) measure the mental and motor development and test the behaviour of infants from one to 42 months of age. Children are assessed in the five key developmental domains of cognition, language, social-emotional, motor and adaptive behaviour. Each of these five subscales is given a raw score based on the number of items the child has achieved. From raw scores scaled scores are calculated; normal (8-14), 1 SD below or above average (7-13) and 2 SD from the mean (4-16). High scores indicate mature development and low scores a delay. These raw scores can be used to calculate composite scores, percentile ranks, confidence interval and developmental age. In addition, adaptive behaviour was measured by using Behaviour Observation Inventory (BOI).

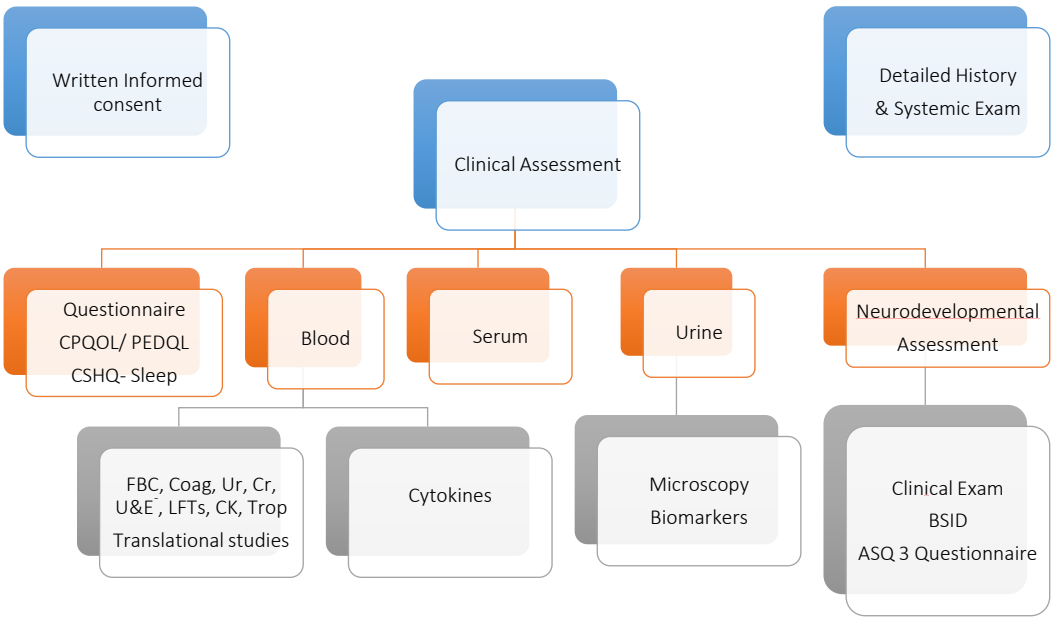


Figure 2-1 Methods and Materials

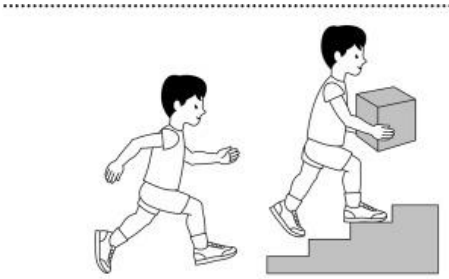
CPQOL= Cerebral Palsy Quality of Life questionnaire, PEDQL= paediatric quality of life questionnaire, CSHQ=Child Sleep Habit Questionnaire,ASQ 3= Ages and Stages Questionnaire 3rd Edition, BSID=Bayley Scale Infant Development, FBC=Full blood count, Ur=Urea, Cr=Creatinine, U&E= Urea & Electrolyte, LFT= Liver function test, CK=Creatinine kinase, Trop= Troponin

Table 2-1 Sarnat Staging; Distinguishing features of the three clinical stages of Hypoxic Ischaemic Encephalopathy in the Full-Term Newborn Infant*

EEG = Electroencephalogram
* = (Sarnat and Sarnat, 1976)

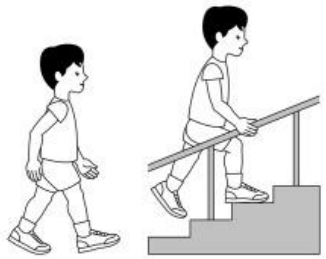
	Stage1	Stage 2	Stage 3
Level of consciousness	**Hyperalert**	**Lethargic or obtunded**	**Stuporous**
Neuromuscular control			
Muscle tone	Normal	Mild hypotonia	Flaccid
Posture	Mild distal flexion	Strong distal flexion	Intermittent decerebration
Stretch reflexes	Overactive	Overactive	Decreased or absent
Segmental myoclonus	Present	Present	Absent
Complex reflexes			
Suck	Weak	Weak or absent	Absent
Moro	Strong; low threshold	Weak; incomplete; high threshold	Absent
Oculovestibular	Normal	Overactive	Weak or absent
Tonic neck	Slight	Strong	Absent
Autonomic function	Generalized sympathetic	Generalized parasympathetic	Both systems depressed
Pupils	Mydriasis	Miosis	Variable; often unequal; poor light reflex
Heart rate	Tachycardia	Bradycardia	Variable
Bronchial and salivary secretions	Sparse	Profuse	Variable
Gastrointestinal Motility	Normal or decreased	Increased; diarrhoea	Variable
Seizures	None	Common: focal or multifocal	Uncommon (excluding decerebration)
EEG Findings	Normal (awake)	Early: low-voltage continuous delta and theta. Later: periodic pattern (awake). Seizures: focal 1-to 1.5 Hz spike-and-wave	Early: periodic pattern with isopotential phases. Later: totally isopotential
Duration	< 24 hours	2-14 days	Hours to weeks

Figure 2-2 GMFCS Score



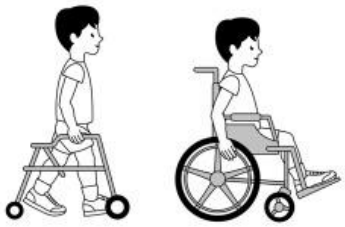
GMFCS Level I

Children walk at home, school, outdoors and in the community. They can climb stairs without the use of a railing. Children perform gross motor skills such as running and jumping, but speed, balance and coordination are limited



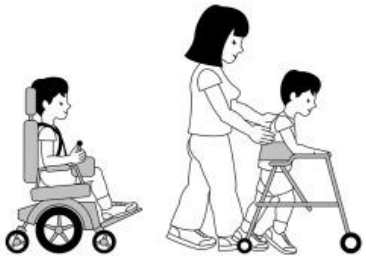
GMFCS Level II

Children walk in most settings and climb stairs holding onto a railing. They may experience difficulty walking long distances and balancing on uneven terrain, inclines, in crowded areas or confined spaces. Children may walk with physical assistance, a hand-held mobility device or used wheeled mobility over long distances. Children have only minimal ability to perform gross motor skills such as running and jumping.



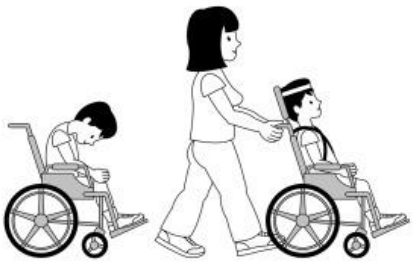
GMFCS Level III

Children walk using a hand-held mobility device in most indoor settings. They may climb stairs holding onto a railing with supervision or assistance. Children use wheeled mobility when traveling long distances and may self-propel for shorter distances.



GMFCS Level IV

Children use methods of mobility that require physical assistance or powered mobility in most settings. They may walk for short distances at home with physical assistance or use powered mobility or a body support walker when positioned. At school, outdoors and in the community children are transported in a manual wheelchair or use powered mobility.



GMFCS Level V

Children are transported in a manual wheelchair in all settings. Children are limited in their ability to maintain antigravity head and trunk postures and control leg and arm movements.

GMFCS descriptors: Palisano et al. (1997) Dev Med Child Neurol 39:214-23
CanChild: www.canchild.ca

Illustrations copyright © Kerr Graham, Bill Reid and Adrienne Harvey,
The Royal Children's Hospital, Melbourne

2.7. Blood Sampling

Paediatric Controls

Blood samples were collected from control paediatric patients (following parental and child consent/ agreement) who came for day case surgeries at the day ward during routine phlebotomy. 2mls of blood was obtained at each time point: 1 ml for whole blood neutrophil and monocytes function testing was collected in a sodium citrate anti-coagulated blood bottle and 1ml of blood collected for full blood count, and biochemical profiles including bone, renal and liver function tests.

Paediatric Patients

Blood samples were collected from paediatric patients (following parental and child consent/ agreement) who attended the review clinic at NMH, Holles Street and Complex Needs clinic at NCH, Tallaght. 2mls of blood was obtained at each time point: 1 ml was collected in a sodium citrate anti-coagulated blood bottle and 1ml of blood collected for full blood count, and biochemical profiles including bone, renal, liver function tests, albumin, Vitamin D levels and coagulation test if indicated.

2.8. Blood Analysis

Blood samples were transported on ice to the Trinity Transitional Medicine Institute and processed for analysis within 90 minutes of sample collection in all cases.

2.8.1 Standard Procedure in Processing Whole blood for RNA extraction

Blood samples were received in the lab and aliquoted for exposure to treatments, LPS and Melatonin for 1 hr at 37°C. Initially the whole blood sample collected was split into different microtubes for serum and RNA isolation and for flow cytometry. The heat block was then turned to 37°C. The bench was prepared with all materials required. Microtubes were labelled for each treatment as: 1) control, 2)

Lipopolysaccharide (LPS), 3) Melatonin (Mel), 4) LPS +Mel**.** The whole blood was split across each treatment tube, leaving an extra 50ul in the Vehicle control tube as the FACS unstained control**.** 1µl LPS (10ng/ml) was added to the LPS-labelled tubes (ensuring correct LPS concentration) and appropriate volume of Mel (42uM) was added to the Mel-labelled tubes.

1µl PBS/0.15% EtOH was added to the Vehicle control-labelled tube, these were then gently mixed on the vortex for 5-10secs. They were then placed on the heat block for 1hour. The tubes were labelled as FACS tubes, RNAse-free tubes for RNA storage and serum tubes for serum storage. The tubes were labelled clearly and correctly, stating project, sample number, sample type and the date (only for serum and RNA tubes for freezer storage)

During this incubation period, the antibody cocktail *(description in flow cytometry section)* was prepared for Flow cytometry. Once the incubation was completed the heat block was turned off and the samples were removed. Each sample was split into 50ul for RNA (into RNAse-free tubes) and 50ul for FACS. Then 50ul from control sample was added to the "unstained" FACS tube

Processing blood for RNA isolation: In the RNA labelled tubes, 1ml of Trizol reagent was added, this was then mixed well and stored at -80°C for analysis later.

Blood for serum extraction: The serum labelled tubes were spun in the microcentrifuge tube for 10mins @ 1,500rpm. The serum layer was identified in each tube and holding to the light, the serum was removed using a low volume pipette and expelled into the corresponding labelled serum tube and stored at -80°C.

2.8.2 RNA Extraction using Trizol method.

TRIzol Reagent is a complete, ready-to-use reagent for the isolation of high-quality total RNA or the simultaneous isolation of RNA, DNA, and protein from a variety of biological samples. This monophasic solution of phenol and guanidine isothiocyanate is designed to isolate separate fractions of RNA, DNA, and proteins from cell and tissue samples of human, animal, plant, yeast, or bacterial origin. TRIzol Reagent allows

sequential precipitation of RNA, DNA, and proteins from a single sample. Reagents used included, Chloroform, Isopropyl alcohol, 75% Ethanol in DEPC treated water, RNAse free H20 and RNA Zap.

Procedure

In the RNA labelled tubes 50 ul of whole blood was taken, to this 1ml of Trizol reagent was added, this was then mixed well and stored at -80°C. Cells were stimulated with or without LPS (1µg/ml), with or without Melatonin (1.2ul/50ul). Subsequently, cells were centrifuged at 1,200 rpm for 10 minutes and the resultant pellet was resuspended in 1ml Trizol Reagent (Molecular Research Centre, Inc., Ohio, USA). Samples were allowed to stand at room temperature for 5 minutes after which samples were processed or stored at -80^{o}C freezer.

To process the sample, 200µl of Chloroform per 1ml of Trizol was added, (Figure 2.3) the sample was vortexed for 15 seconds and was incubated at room temperature for no longer than 2- 3 minutes. This was then micro-centrifuged at 4^{0}C for 15 minutes at 12,000 rpm to separate the RNA (aqueous phase) from DNA and protein (organic phase). The aqueous phase was then transferred to a fresh sterile RNase-free tube and 500 µl of Isopropyl alcohol/1ml Trizol added. After the third room temperature incubation step, for 10 minutes, sample was then centrifuged at 4^{o}C at 12,000 rpm for 10 minutes to pellet the RNA. The RNA pellet was washed with 75% Ethanol (minimum of 1ml/ml of Trizol used) and vortexed to dissolve, after which sample was micro-centrifuged at 4^{o}C for 5 minutes at 7,500 rpm. Ethanol was aspirated without disturbing the RNA pellet which was then allowed to air-dry for about 5 minutes to remove the last traces of Ethanol. The pellet was finally re-dissolved in 30 µl RNase-free DEPC water (3% Diethyl-pyro carbonate) and heated at 65^{o}C for 10 minutes. At this point, the sample was assessed for RNA purity using the Nanodrop as described below.

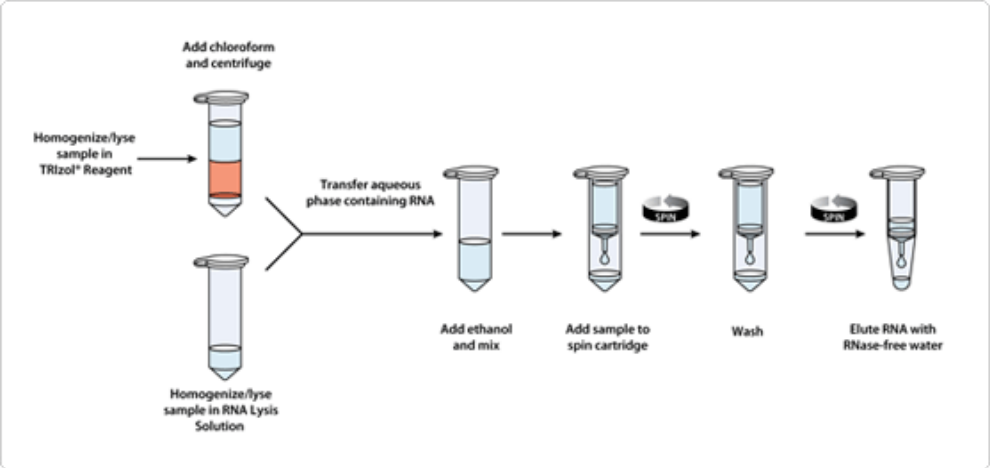


Figure 2-3 RNA extraction using Trizol

Steps involved in extraction of RNA using Trizol reagent.

2.8.3 Quantification of RNA and Assessment of RNA purity

RNA purity and concentration were determined by using the Nanodrop ND-100 Spectrophotometer (supplied and serviced by Labtech International) and analysed using ND-1000 Ver.3.1.2 software. This software generates an absorbance curve for the sample (Figure 2.4) 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, OD_{260nm}/OD_{280nm}=2.0. A ratio of ≥1.6 (for RNA suspended in water) was considered acceptably pure. A ratio of <1.6 for the RNA solution indicates possible contamination with protein or phenol. RNA samples were stored at -80 ^{o}C freezer.

2.8.4 Synthesis of cDNA from template RNA

cDNA synthesis with High Capacity Reverse Transcriptase kit

Reverse transcription-PCR (RT-PCR) couples the conversion of RNA into complementary DNA (cDNA) by viral reverse transcriptase with the amplification of the cDNA by thermostable polymerases.

Reagents used

- RNAse-free water
- High Capacity cDNA reverse transcriptase kit (Applied Biosystems 4368814)
    - dNTPs 25X concentration (100mM)
    - Random primers 10X concentration
    - M-MLV Reverse Transcriptase 50 U/µl as delivered
    - Reverse Transcriptase buffer 10 times concentration

Precaution prior to commencing the procedure

The whole procedure was carried out in a clean environment. Aseptic techniques were observed. The bench areas, pipettes /plastic ware were sprayed with RNAse Zap. Only RNAse free sterile H_2O was used and gloves were frequently changed. The master mix volume was calculated based on number of samples per run. Ideally up to 2µg of RNA is reverse transcribed, concentrations higher than this were adjusted with water.

Procedure

An appropriate amount of RNA was added to DEPC/RNAse-free water to produce a final concentration of 1ug RNA in a volume of 20µls as shown in example below:

Figure 2.3.1 Master mix preparation

Example:

Sample	RNA (ng/ul)	RNA (ug/ul)	RNA (ul)	H2O (uls)	Total volume (ul)
1	100	0.1	10.0	10.0	20
2	56	0.056	17.9	2.1	20

An NTC- (no template /no RNA) and NAC (no amplification/ no RT) control were also included. The samples were kept cooled on ice.

The following 2X **master mix** was prepared by adding M-MLV buffer (4ul), dNTPs (1.6 ul) primers (4ul) enzyme (2ul), water DEPC (8.4ul) adding up to a total volume of 20uls.

The master mix was then added to each sample tube containing the RNA and mixed briefly. The samples were then placed on the thermocycler (Gene Amp PCR System 9700) using the RT-cDNA program (10 mins at 25°C, 120 mins at 37°C, 85°C for 5min and then held at 4°C). The volume was to 40µl. The cDNA was stored at -20°C until assay. Concentration of the resulting cDNA (assuming 1µg of RNA) The concentration of the sample was 1µg/20µls H20 = 50ng/µl.

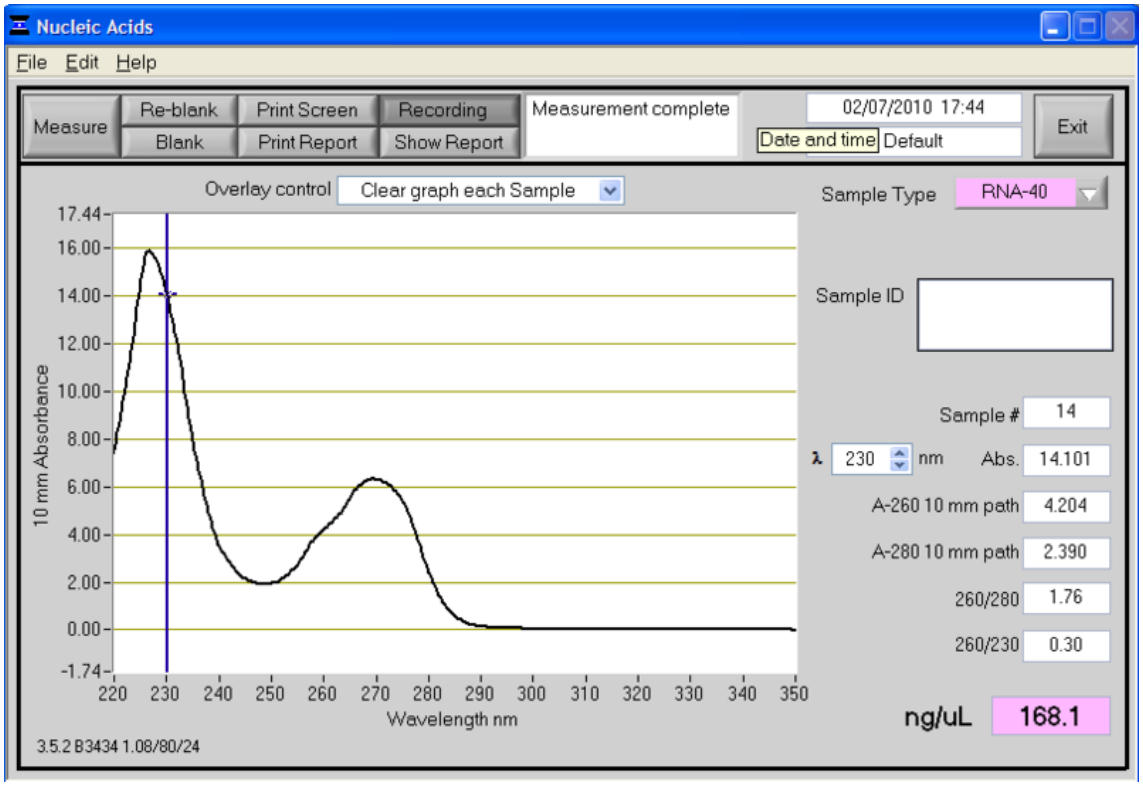


Figure 2-4 Example of mRNA Nanodrop absorbance curve

mRNA reading on the Nanodrop Spectrophotometer in pink in ng/ul. A 260/280 ratio of ~2 considered “pure” for RNA.

2.9. PCR Analysis

TaqMan® Real time PCR

Real time PCR is used to quantify gene expression; amplified DNA is quantified during the exponential phase of the PCR, allowing the concentration of a target DNA or RNA relative to a standard to be quantified. The concentration of the target sequence is determined by interpolation real time PCR using commercially available fluorescence – detecting thermocyclers.

The greater the initial concentration of target sequences in the reaction mixture the fewer the number of cycles required to achieve a yield of amplified product. Taqman® probes are oligonucleotides that have fluorescent reporter dyes attached to the 5` ends and a quencher moiety coupled to the 3` ends. These probes are designed to hybridise to an internal region of a PCR product. In the unhybridized state, the proximity of the fluorescents and the quench molecules prevents the detection of fluorescent signal from the probe. During PCR, when the polymerase replicates a template on which a Taqman probe is bound, the 5' nuclease activity of the polymerase cleaves the probe. This decouples the fluorescent and quenching dyes and FRET no. Thus, fluorescents increase in each cycle, proportional to the amount of probe cleavage.

Reagents used

- Taqman Universal PCR master mix (Applied Biosystems P/N 4304437)
- RNAse free water (Commercial or DEPC treated and autoclaved water (0.1%))
- 70% EtOH

2.9.1 Procedure

Aseptic techniques were observed while working in the hood, no templates were ever allowed in the hood. All bench areas, pipettes /plastic ware were sprayed with RNAse Zap®. Only RNAse free sterile H_2O. was used and gloves were changed frequently. cDNA was used as sample; the total volume was diluted in RNAse-free water to give final concentration of 5ng/µl. For each gene to be measured, 6µls of sample at 5ng/µl were required (2µl per well in triplicate to give 10ng/well).

The maximum number of samples to fit on a plate depended on how many genes/assays done. For 4 genes (including endogenous control) 5 samples were fit in triplicate along with a NAC control, +ve control and –RT control on a 96 well plate.

For each Target Gene: The master mix was prepared (in the hood) as follows in a total volume of 8ul by adding 5 ul of TaqMan Universal PCR buffer mix ,0.5ul of Gene specific probe and 2.5ul of RNAase-free water. *If using 24 wells, 25x master mix was prepared.

Table 2.3.2: Loading the 96well TaqMan plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample1	Sample1	Sample1	Sample2	Sample2	Sample2	Sample3	Sample3	Sample3	Sample4	Sample4	Sample4
B	Sample5	Sample5	Sample5	+ve	+ve	+ve	NAC	NAC	NAC	NTC	NTC	NTC
C	Sample1	Sample1	Sample1	Sample2	Sample2	Sample2	Sample3	Sample3	Sample3	Sample4	Sample4	Samp 4
D	Sample5	Sample5	Sample5	+ve	+ve	+ve	NAC	NAC	NAC	NTC	NTC	NTC
E	Sample1	Sample1	Sample1	Sample2	Sample2	Sample2	Sample3	Sample3	Sample3	Sample4	Sample4	Sample4
F	Sample5	Sample5	Sample5	+ve	+ve	+ve	NAC	NAC	NAC	NTC	NTC	NTC
G	Sample1	Sample1	Sample1	Sample2	Sample2	Sample2	Sample3	Sample3	Sample3	Sample4	Sample4	Sample4
H	Sample5	Sample5	Sample5	+ve	+ve	+ve	NAC	NAC	NAC	NTC	NTC	NTC

Gene 1 -rows A & B endogenous control master mix to each well

Gene 2 target 1rows C & D gene 1mastermix to each well

Gene 3 target 2 rows E & F gene 2 mastermix to each well

Gene 4 target 3 rows G &H gene 3 mastermix to each well

NAC= No Amplification Control; NTC=No Template Control; +ve= positive control

2.9.2 Loading the 96well TaqMan plate

Using the template above 2µl of sample or control was added in triplicate to each well. Samples were loaded first followed by the three controls.

To rows A and B, 8µl of endogenous control gene mastermix were added to each well, and to rows C and D, 8µl of gene 1 mastermix were added. To rows E and F, 8µl of gene 2 mastermix were added to each well and to rows G and H, 8µl of gene 3 mastermix were added. The plate was then covered with plate seal provided, ensuring that the surface of the plate is un-touched, and this was then centrifuge briefly for 1minute at 1800 rpm.

The real-time PCR reaction was performed using an ABI 7500 sequence detection system using the following thermocycler conditions: 2 minutes at 50°c and 10 minutes at 95°c, 40 cycles of 95°c for 15 seconds followed by 60°c for 1 minute. (Figure 2.5).

2.9.3 Analysis of results

When the program was complete, the whole file was saved onto the disk drive before proceeding. Show analysis is clicked and wells were displayed for each dye and wells labelled which were not in use as such. Next to analyse, the results from the instrument are taken and the plot was displayed. The y axis of the plot was changed from log to linear. The cycle number was selected at which the amplification begins and 2 was subtracted from this and the cycle number was entered in the option at the bottom right. If changes were made from the default, then it was clicked as update calculations. Going back into the log scale of the plot, the threshold was altered until it sits approximately halfway up the linear part of the curve. The calculations were updated if settings were changed. The display of Ct versus well number were checked to view if negative results (other than controls) are altering results. Following this the results were saved to the memory stick. The results were analysed on excel using $2^{-\Delta\Delta Ct}$ method.

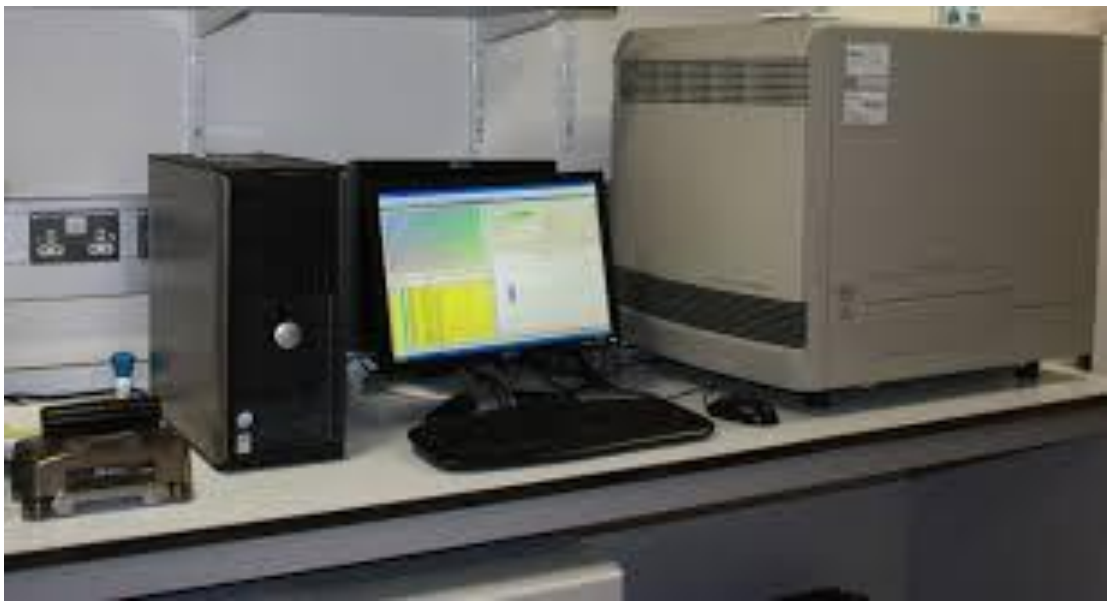


Figure 2-5 ABI 7900 Real time PCR Machine

2.10. Flow cytometry

Whole blood flow cytometry was performed to assess expression of neutrophil monocyte and lymphocyte markers of function: CD11b for neutrophil activation, Toll-like receptor 4 (TLR4) for pathogen recognition.

Quantification of cell surface antigen expression

The expression of cell surface antigens including CD4, CD8, CD11b and Toll-like receptor-4 (TLR-4) antigens on the surface of lymphocytes, neutrophils and monocytes was measured by flow cytometry. Two antibody panel cocktails; Monocyte panel (Panel A) and Lymphocyte panel (Panel B) were prepared:

Table 2.5.1: Antibody panel cocktails
Panel A: Monocyte Panel; Panel B: Lymphocyte Panel

Panel A	**Tag**	**1X**	**4X**	**Panel B**	**Tag**	**1X**	**4X**
CD14	PerCp	2.5ul	10ul	**CD3**	PB	2.5ul	10ul
CD15	PeCy7	2.5ul	10ul	**CD4**	PeCy7	10ul	40ul
CD16	FITC	2.5ul	10ul	**CD8**	PerCp	2.5ul	10ul
CD66b	PB	2.5ul	10ul	**CD19**	APC	2.5ul	10ul
TLR4	APC	2.5ul	10ul	**CD56**	APC/Cy7	2.5ul	10ul
CD11b	PE	10ul	40ul	**Vdelta1**	FITC	2.5ul	10ul
				Vdelta2	PE	2.5ul	10ul
PBS		27.5ul	110ul	**PBS**		25ul	100ul

Fifty microliters (50µL) of whole blood was treated with 10ul of dead cell stain (from Bio Legend bv500 amcyan stain) and 40 ul of antibody cocktail (separate tubes for Panel A and Panel B) and incubated in dark at 4°C for 20 minutes. Then FACS 1X (1ml) was added and incubated in dark for 10 minutes at room temperature, to lyse red blood cells. The samples were centrifuged at 252 G-force for 7 mins at room temperature. The supernatant was removed, and the pellet was suspended twice in 1 ml PBA (phosphate

buffer azide) to wash the cells. Then 500ul 1% PFA (Paraformaldehyde) is added to fix the cells. Then incubated for 15 mins in the dark at room temperature. Then centrifuge at 252 g-force for 7 mins at room temperature. The supernatant is discarded ensuring to remove all the PFA solution. The sample is then resuspended in 100ul PBA and stored at 4^{o} C before analysis by flow cytometry.

The fluorescence intensity was denoted by mean channel fluorescence (Mnx), which 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.(Molloy et al., 2004). The flow cytometer used was FACS Canto II and a minimum of 50000 events were collected and analysed. All measurements were performed under the same instrument settings.

The analysis of CD11b and TLR4 expression were performed on Neutrophil and Monocyte populations. CD11b was labelled with Phycoerythrin (PE) which is excited by a 488nm wavelength laser. TLR4 was labelled with APC which is excited by a 633nm laser. This facilitated the quantification of CD11b and TLR4 expression in the same sample aliquot.

2.10.1 Flow cytometry analysis using FACS Canto Flow cytometer

Samples were analysed using a BD FACS Canto II flow cytometer with a BD FACSDiva™ clinical software. Leukocyte populations were selected based on their scatter profiles, forward scatter (FSC) and side scatter (SSC). BD FACSDiva™ II system is an easy-to-use benchtop analyser that delivers proven performance, accuracy, and high-quality results. The BD FACSDiva II can be configured with two or three lasers to detect up to eight colours. It features many innovations, including a true fixed alignment flow cell to minimize start up time and improve reproducibility. The optical system maximizes signal detection and increases sensitivity and resolution for each colour in a multicolour assay. (Fig 2.5)

2.10.2 Use of FACSDiva

Procedure Steps

The CS&T beads were first taken out of the fridge to allow them to come to room temperature. (CS&T beads: 350µL of PBA and 1 drop of the beads, vortex the bead vial before adding the drop to the tube). The FACS machine and the computer were turned on. The computer was then connected to the cytometer. The tank levels were checked, the fluids were primed. The three filters in the fluidics cart were checked for bubbles. If present, they were bled by unscrewing the caps. The cytometer menu was selected on the computer, then cleaning mode selected. After degassing of the filter cells and cleaning the filter the Fluidics set up was selected, following this the system starts up which takes 7 mins.

From cytometer menu the CST beads were then selected on the screen. The tube of CS&T beads was then inserted and run the sample. When it gets passed, the system gets connected to FACS Diva page on exit.

Setting up experiment template and voltages

A new experiment was selected on the screen, and a new specimen was created. One specimen for each set of fluorochromes was selected. This automatically creates a new tube, which was then selected along with the parameters needed e.g.: FSC, SSC, FITC, PE etc. The height was selected for FSC and SSC and log & area for Fluorochromes. Then on the experiment menu each tube fluorochromes were selected with the antibody e.g.: CD3, CD56 etc. The voltages were then changed to FSC: 330 and SSC: 440 for the lymphocytes.

Compensation set up

The compensation set up was selected and compensation controls were created. The specimen called compensation controls was selected – contains unstained and single stained tubes for each parameter. The unstained tube was first selected, then setting the flow rate to medium data was recorded. The threshold was turned off as the beads are smaller than the cells. Then the stained tube (e.g.; FITC) was selected and data acquired. When all the compensation tubes were acquired and recorded, the compensation set up was selected on the experiment menu and the compensations were calculated. The compensation set up was done using BD compensation beads labelled

with the panel of antibodies. We created the label specific controls and began with the unstained control. After setting FSC, SSC, threshold values, we measured our p1 (negative) and copied this to all compensations. Following this, when running our antibody labelled controls, we adjusted the p2 (positive) gate to fit the positive populations. Once compensation was calculated we began the experiment samples.

Acquiring samples

The tube to be acquired was selected on the browser. The desired plot was made which was applied to all the tubes. The FSC threshold was turned back to 50,000. The sample tube was selected, and data was acquired. This was repeated for all the tubes.

Exporting data

The experiment is highlighted on the browser and the File selected which is exported on to a folder. FCS 3.0 is selected to analyse the file on ***Flow Jo*** before saving on the folder.

Cleaning

A tube was inserted and FACS clean was acquired for 5 mins. On the Cytometer menu cleaning mode was selected, a tube of FACS Clean and clean flow cell was performed 4 time. Then a tube of ddH2O was inserted and clean flow cell performed 2 times. The cytometer and the computer were turned off. The waste container was detached and decontaminated using a Virkon tablet

2.10.3 Flow Jo Analysis

Flow Jo software was used for the analysis of flow cytometry data. The following steps were involved in analysing one basic sample experiment.

- Loading sample into the workspace.
- Analysing the control sample in detail (gate subsets and adding statistics).
- Copying gates and statistics to all samples.
- Verifying gates on all samples.
- Generating a graphical report including all samples.
- Generating a table of statistics from all samples.

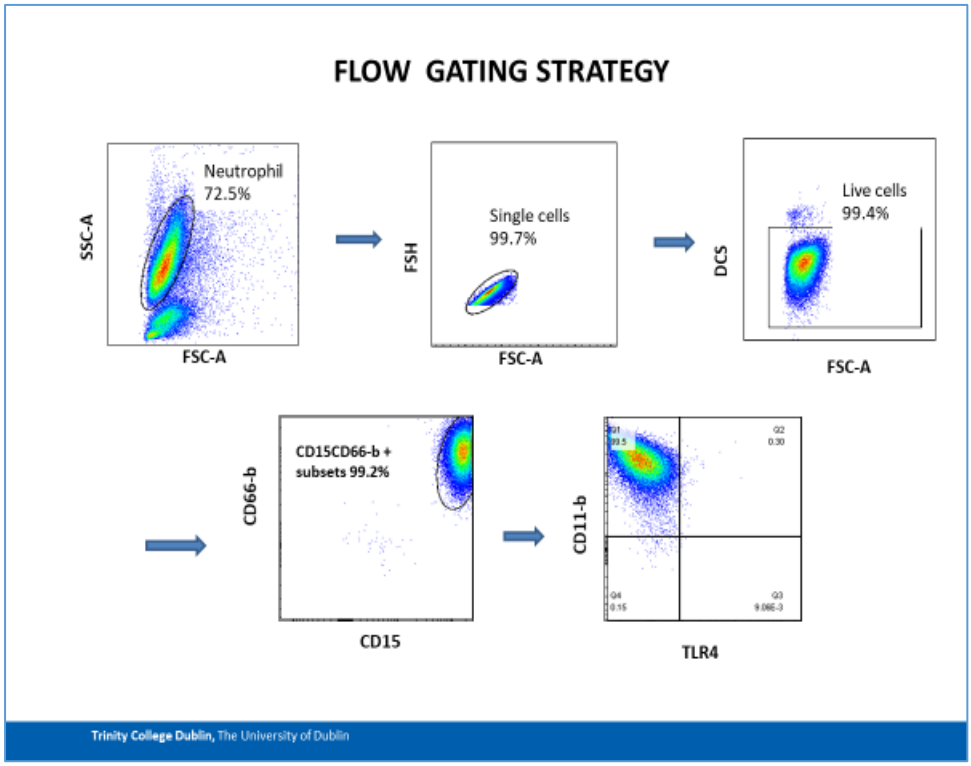


Figure 2-6 Flow Gating Strategy

Granulocytes identified & gated first, single cells plotted on scatter plots of FSH and FSC-A, identified as live cells on FSC-A and DCS. Live cells then gated & antibodies CD15b+ and CD66b+ (markers of neutrophils) are gated & plotted to quantify population of TLR4 and CD11b.

SSCA: side scatter area, FSC-A: Forward scatter area FSH: forward scatter height, DCS: dead cell stain.

Figure 2-7 BD FACS Canto II

2.11. Cytokine Analysis

Serum cytokines were analysed using the U Plex biomarker group 1 multiplex assay. The 96-well MULTI-SPOT plates quantify up to 10 compatible analytes simultaneously using no more than 25 µL of sample. The assays employed a sandwich immunoassay format where capture antibodies were coated in a patterned array on the bottom of the wells of the plate.

2.11.1 Reagents

A 10 Spot 96 well U Plex Plate, Linkers, U plex Antibody sets, Calibrators, Diluent 43, Diluent 3, Stop Solution, Read Buffer T (4X), MSD Wash Buffer, Phosphate 4 Buffer Saline (PBS).

2.11.2 Procedure

Preparing the UPLEX Plate

Step 1 Creating the Individual U-plex Coupled Antibody Solutions:

Around 200 UL of biotinylated antibody was added to 300 UL of the assigned Linker. A different Linker was used for each biotinylated antibody, this was mixed by vortex and incubated at room temperature for 30 minutes. Then 200UL of Stop Solution was added, vortex and incubated at room temperature for 30 minutes.

Step 2 Preparing the Multiplex Coating Solution

Around 600ul of each U Plex coupled antibody solution was added into a single tube and vortex. Up to 10 antibodies were pooled. The antibody solution with the same liner were not combined.

Step 3 Coating the U Plex Plate

50 ul of multiplex coating solution was added to each well. The Plate was sealed with an adhesive plate seal and incubated with shaking at RT for I hour or at 2-8C overnight.

The plates were then washed 3 times with at least 150ul /well of 1xMSD wash buffer solution or PBS-T (PBS plus 0.05% Tween -20). The plate was then coated and ready to use.

2.11.3. Preparation of Calibrator Standards

A 7 Calibrator Standard solution plus a zero-calibrator standard was prepared for 6 replicates. Each vial of calibrator was reconstituted by adding 250ul of Diluent 43 to the glass vial and inverted 3 times. The reconstituted solution is equilibrated at RT for 15-30 mins and then vortex briefly. The calibrator was then ready to use. The Calibrator Standard 1 was prepared by adding 50ul of the calibrator to Diluent 43 to make up a final volume of 250ul and mixed well by vortex. The Calibrator Standard 2 was prepared by adding 75ul of Calibrator Standard 1 to 225ul of Diluent 43 and mixed well by vortex. The 4-fold seral dilutions were repeated 5 additional times to generate a total of 7 Calibrator standards. They were mixed well by vortexing between each sample dilutions.

Sample Dilution

Depending on the sample set, a dilution may be necessary. We used Diluent 43 for sample dilution. 20ul of 1 M HCl was added to 100ul of the sample and vortexed briefly. The sample was incubated at RT for 10 mins. The sample was then neutralised by adding 14ul of 1.2 M NaOH/0.5M HEPES per 100ul of sample volume and mixed by vortex.

2.11.4 Preparation of Detection Antibody Solution:

The detection antibody was provided as a 100X stock solution. For each plate, to 60ul of each detection antibody, Diluent 3 was added to bring the total volume to 6ml.

2.11.5 Preparation of 2X Read Buffer T

For one plate, 10ml of Read Buffer T 94x) was combined with 10 ml of deionised water.

Assay Protocol:

Step 1 Adding Samples and Calibrators: 25ul of Diluent 43 was added to each well. The plate was tapped gently on all sides. Then 25 ul of the prepared Calibrator solution was added to sample in each well. The plate was sealed by an adhesive seal and incubated at RT with shaking for 1 hour.

Step 2 Washing and adding Detection Antibody Solution: The plate was washed 3 times with at least 150ul of 1XMSD Wash Buffer of PBS-T. Then 50 ul of detection antibody

solution was added to each well. The plate was sealed by an adhesive seal and incubated at RT with shaking for 1 hour.

Step 3 Washing and Reading: The plate was washed 3 times with at least 150ul of 1XMSD Wash Buffer of PBS-T and then 150ul of 2x Read Buffer was added to each well. The plate was analysed on a MSD instrument.

2.12. Statistical Analysis

The sample size estimation and statistical analysis plan for the project were designed with the statistical support of Centre for Support and Training in Analysis and Research (C-STAR), University College Dublin (CSTAR). Since 2009 we have studied >150 infants with neonatal encephalopathy in a single institution. Using the rule of thumb of 15 patients/predictor this would allow us to evaluate 10 predictors.

In our study we recruited 146 children: Controls (n=65), Neonatal Encephalopathy (n=55): complex needs (n=26). Statistical analysis was carried out using the PASW statistical package version 24 (www.ibm.com/SPSS_Statistics).

For comparisons of continuous variables between groups, the approximate normal distribution was verified by inspection of histograms. Continuous normally distributed data was displayed as means and standard deviations (SDs) and comparisons were made using the independent student-t test. For continuous non-normally distributed data, medians and interquartile ranges were used and comparisons were made using the Mann-Whitney U Test or the Kruskal-Wallis test with Monte Carlo significance if small numbers were present. To compare categorical variables, Chi-square test was applied to test equality of proportions. Pearson and Spearman's rank correlation was employed to assess for correlation between different variables.

To compare data between two groups we used Paired t- tests including Wilcoxon Signed rank test or Mc Nemar paired Ch-square test were used. Receiver operator characteristic curve analysis was employed to assess the ability of variables to predict outcomes of interest. To compare 3 groups, we used the Omnibus tests, to compare means One Way Analysis of Variance (ANOVA) or the Kruskal -Wallis test was used.
Statistical analysis was carried out using ANOVA analysis. ANOVA one-way analysis of variance was used to detect CD11b and TLR4 Fold Increase and Staining Index

parameters. We also used ANOVA-one-way analysis of variance to compare cytokine results in the different groups of patients. Two-way ANOVA analysis of variance was carried out to compare 2 independent variables; cytokine baseline results and endotoxin (LPS) stimulated sample results in each group.

Chapter 3

Sleep and Quality of life

3. Sleep disorders in children with Neonatal Encephalopathy (NE) and Cerebral Palsy (CP)

3.1. Introduction

Prevalence of sleep disorders in school children varies from 10% to more than 40% as per different epidemiological studies. (Blunden, Kahn and Stein 2004). Children with cerebral palsy (CP) are more prone to sleep disturbances compared to children with normal development (Molloy et al., 2007, Newman et al., 2006) Six to eight-fold increase in sleep disorders are seen in children with CP (Romeo et al., 2014, Atmawidjaja et al., 2014). Various factors contribute to sleep disorders in children with CP including epilepsy, visual impairment, anti-epileptic medications, obstructive sleep apnoea, and decreased ability to move due to reduced strength. Disturbed sleep occurs in these children due to dystonia and pain. Compared to the sleep pattern of children with normal development, children with CP have a high incidence of sleep problems including initiation and maintenance of sleep (22-37%) sleep disordered breathing (12-15%), parasomnias and excessive somnolence in 12-13% (Newman et al., 2006, Romeo et al., 2014, Atmawidjaja et al., 2014)

Management of sleep disorder involves maintaining good sleep hygiene, behaviour therapy, and medications. Melatonin has been widely used in the treatment of sleep disorder in children with neurodisability including CP, Intellectual disability (ID) genetic syndromes, neuromuscular disorders and Autism. Melatonin showed consistent improvements in night waking time, sleep latency and in some patients, improved total sleep time (Galland et al., 2012). Sleep disorders have huge impact on the quality of life of children and their families thus need to be monitored and managed appropriately.

Inflammation combined with Hypoxia-ischemia (HI) plays an important pathophysiological role in neonatal encephalopathy (NE). Inflammation is also closely associated with CP. (Hua Di et al 2016). The main components of the immune system in the blood including hematopoietic cells, lymphocytes along with hormones and cytokines exhibit circadian rhythms (Haus and Smolensky, 2013, Scheiermann et al., 2013)

Circadian rhythm or our body clock regulates our periods of wakefulness and sleep. They are the daily changes or oscillations in physiology and are due to numerous

genes whose expression peaks and troughs approximately 12 hours apart. The control on the genome by the molecular clock was revealed by Zhang et al (Zhang et al., 2013)The core clock proteins BMAL1, CLOCK and REV-ERBα, control fundamental aspects of the immune response (O'Neill et al. 2013). Inflammation and infection, disrupts the oscillation and affects the expression of the core clock proteins and clock control genes (Bellet et al., 2013). Melatonin also have immunomodulatory properties; it reduces both chronic and acute inflammation. It acts on the immune system by regulating cytokine production of immunocompetent cells.

There are several studies describing sleep disorders in children with Cerebral Palsy but not much is found in the literature regarding the sleep pattern and disorders seen in school age children with mild to moderate neonatal encephalopathy and their response of circadian rhythm genes BMAL, CRY, CLOCK and REV ERb, in comparison to age matched controls.

3.2. Hypothesis

Children with NE have increased sleep disturbance compared to age-matched controls and altered response in circadian related genes.

3.3. Aims

1. To evaluate the sleep pattern in children who had mild to moderate neonatal encephalopathy (NE), in comparison to children with normal development and children with cerebral palsy (CP) using validated sleep questionnaire; Child Sleep Habit Questionnaire (CSHQ).
2. To evaluate the response of circadian rhythm genes or clock protein BMAL, CRY, CLOCK and REV ERb in children with NE in comparison to normal children.
3. To assess the role of melatonin in altering circadian rhythm genes in children with NE compared to controls.

3.4. Clinical Characteristics

Sleep pattern of 45 school age children who had mild to moderate neonatal encephalopathy (NE) were assessed and compared with 55 children with normal development, and 15 children with severe cerebral palsy using the children sleep habit questionnaire (CSHQ). The assessment included history taking, filling the sleep questionnaire and carrying a detailed clinical examination. Eight sub-scales, including (bedtime resistance, sleep-onset delay, sleep duration, sleep anxiety, night waking, parasomnias, sleep-disordered breathing and daytime sleepiness) were measured which together, generated a total sleep disturbance score.

3.4.1 Children with normal/ typical development

Children in the control group were in the age range of 4-12 years. They had typical development with no intellectual impairment, and no prior neuropsychiatric illness or mental health problems. In addition, the participants in the control group had no chronic medical or neurological condition.

3.4.2 Children with mild to moderate Neonatal Encephalopathy (NE)

In the NE group (n=45), children were in the age range of 4-6 years. They were classified as per grade of encephalopathy in the neonatal period as mild (NE 0/I; n=15) and moderate encephalopathy (NE II/III; n=30) as per Sarnat Staging (Sarnat and Sarnat, 1976). Seven children had motor impairment, as per GMFCS scale, two had GMFCS V and remaining 5 had GMFCS II/III. Epilepsy was diagnosed in 3 children with NE and 2 children had visual impairment.

3.4.3 Children with Severe Cerebral Palsy

Assessment of sleep disorders was also performed in fifteen school age children (age range 4-12 years) with severe CP

who had heterogenous underlying diagnoses including congenital infections, metabolic disorders, epileptic encephalopathy and Rett's Syndrome. Sleep assessment was done using the CSHQ and the scores were compared to scores of typically developing children of the same age group. Children with severe CP were classified

based on their gross motor function using GMFCS scale. Nine children had GMFCS level V, and six children had GMFCS level II/III. Epilepsy was diagnosed in nine children and they were on anti-epileptic medications. Visual impairment was noted in two children and nine children were diagnosed to have intellectual disability. Only three children with CP were on Melatonin.

3.5. Results

Sleep assessment was performed on all the 3 groups of children using the CSHQ during their clinic visit. The CSHQ was filled and scored by the clinician according to the answers to the 33 questions provided by the parents. In our study, we used the total sleep score of ≥41 as a high score and to classify a child as a poor sleeper as suggested in previous studies. (Owens, 2011).

High pathological sleep score was seen in 30 out of 52 children (57%) with NE when compared to children in the control group, mean score of 43.8 vs 40.2; (p value 0.001). Children with NE were noted to have increased prevalence of sleep problems including bedtime resistance (*P* value 0.028) and sleep anxiety (p value 0.01) compared to school age controls.

Comparing children according to grade of encephalopathy, children with moderate to severe NE had high total sleep scores compared to children with mild NE; mean total CSHQ score of 44.5 vs 42.3; (p-value 0.4). Children with severe NE and motor impairment had very high total scores ranging from 49-66. Most common sleep problem noted in children with moderate NE was sleep onset delay (SOD). Children with moderate to severe NE had significantly high SOD scores compared to children with mild NE (p- value 0.04). Sub scale scores for bedtime resistance, sleep duration, sleep anxiety, night waking, parasomnias, sleep-disordered breathing and daytime sleepiness, were also high in children with moderate NE in comparison to subscale scores of children with mild NE but failed to reach statistical significance. (Table 3.1).

High pathological total sleep scores were seen in children with CP, mean total score of 44.9 when compared with children with normal development with mean total score 40.36 (p-value <0.001). Sleep disorders noted in majority of children with CP were excessive daytime sleepiness (EDS) in all 16 children (mean subscale score of 10.6 vs

score of 9.6 in children with normal development p-value <0.04), bedtime resistance (high mean score 9.6), sleep onset delay (SOD) and parasomnias in 9 children. Children with CP demonstrated high total sleep score and high subscale score for bedtime resistance, SOD, parasomnias and excessive day time sleepiness in compared to children with normal/typical development and in comparison, to children with mild to moderate NE.

3.6. Circadian rhythm and Inflammation

The circadian rhythm has been proven to be involved in various physiological processes, including sleep and wakefulness, regulation of body temperature, hormone secretion, proliferation of cells and inflammation. The CLOCK genes are important components of the circadian clock, they interact with each other in generating oscillations/ cycles of gene expression. The underlying principle is successive gene activation in the form of a circle. In humans, the circadian clock is made up of 2 interlocking feedback loops. In the first loop the 2 transcriptional activators BMAL & CLOCK bind to other cytochrome genes (CRY1 & 2) and activate their expression. Similarly, in the second loop BMAL contribute to other gene regulations. The CLOCK genes apart from generation of the circadian rhythm also respond to pathways that connect the biochemical organisation of the organisms to changes in the environment. (Albrecht and Ripperger, 2018). In our study we looked at the response of the core CLOCK proteins BMAL1, CLOCK, CRY and REV-ERBα which control the fundamental aspects of the immune response and inflammation. The expression of circadian rhythm genes, BMAL1, CRY, CLOCK and REV-ERBα in children with NE compared to controls, in response to in vitro stimulation with endotoxin (LPS) and Melatonin, using RT PCR in whole blood was assessed.

Results

Increased expression of BMAL1 and REV-ERB, was noted in children with NE on LPS stimulation compared to baseline response, this was noted to suppress on stimulation with Melatonin. However, the response was not significantly different in

comparison to the control group. The expression of CRY protein increased in both groups; children with NE and control group, on LPS stimulation but reduced on stimulation with melatonin only in the control group. (Fig 3.1-3.4). Thus, we demonstrated here a dysregulated response in circadian rhythm genes in children with NE on in vitro stimulation with an endotoxin (LPS) which was reduced by melatonin probably due to its immunomodulatory action.

3.7. Discussion

Sleep disorders in childhood can be treated and should be identified in clinical practice. Child Sleep Habits Questionnaire (CSHQ), is a screening tool for school-aged children based on their clinical symptoms of common sleep disorders (Wayte et al., 2012). A cut-off total CSHQ score of 42 identifies children with a clinical sleep problem with a sensitivity of 0.80 and specificity of 0.72 (Owens et al., 2000) This is the first description of sleep disorders in school age children post NE, especially in those classified to have mild to moderate encephalopathy (Grade I/II). However, the gold standard for measurement of sleep is polysomnography and actigraphy. Assessment of the validity of CSHQ with polysomnography and actigraphy demonstrated low sensitivity and high specificity (Markovich et al., 2014). Thus, CSHQ should not be used as a sole assessment tool to diagnose sleep problem in children.

We have demonstrated in our study high rate of sleep disorders in children with CP and those with mild to moderate NE in comparison to children with normal development. Males outnumbered female as expected, male being at increased risk of CP and NE. High pathological total sleep scores were noted in children with CP and the NE group compared to the control group. Children with mild NE also had higher total sleep scores compared to children in the control group. Disorders of initiation and maintenance of sleep or sleep onset delay was the most common sleep disorder seen. Total body involvement, including spastic quadriplegia and dystonia were strongly associated with disorders of initiation and maintenance of sleep. Other sleep disorders commonly noted in children with CP were sleep disordered breathing and excessive daytime sleepiness.

Epilepsy was the major factor associated with sleep disturbance in children with CP. Epilepsy in children with CP, has been shown to result in increased total sleep

disturbance and increased daytime sleepiness (Wayte et al., 2012). In addition, various antiepileptic drugs cause daytime sleepiness. On the other hand, sleep loss or disturbed sleep may lead to an increase in frequency of seizures. (Frucht et al., 2000). We found children with CP and epilepsy had excessive daytime sleepiness compared to children with mild NE and children with normal development, children with CP and visual impairment in our study had problems with initiation and maintenance of sleep.

We found an altered expression of the CLOCK proteins in response to endotoxin (LPS) in school age children with NE. The clock protein BMAL is a central component of the circadian cycle and functions as an anti-inflammatory molecule in monocytes. BMAL was increased in both groups; children with NE and control but did not find a significant difference between both the groups. Similarly, expression of CRY was increased in children with NE compared to control group, but the response was not more than 2-fold. The other genes CLOCK and Rev Erb also demonstrated a similar increased expression on stimulation with LPS but the change was not significant. Further studies are required, and probably larger sample size would be helpful in demonstrating a difference in the response.

Sleep problems in children post neonatal encephalopathy (NE) have not been assessed previously. We found high rates of sleep problems in school children with both mild and moderate NE, thus assessment of sleep problems in children especially in those at high risk could be incorporated into routine practice. Treatment of sleep problems in children should be prioritised with the possibility of improving not only the quality of life and well-being and of the child but also of the parents or carer.

Table 3-1 CSHQ score of children with Mild NE (NE0/I) vs Moderate to Severe NE(NEII/III)

CSHQ Score	Mild NE (n=20)	Mod- Severe NE (n=20)	p-value
Sleep score (Total)	[1]42.65 (8.4)	44.53 (7.8)	0.40
Bed-time resistance	8.11 (2.6)	9.26 93.2)	0.17
Sleep Onset Delay (SOD)	1.21 (0.4)	1.52 (0.6)	*0.04
Sleep Duration (SD)	3.47 (1.3)	3.42 (1.1)	0.80
Sleep Anxiety	5.89 (2.3)	6.32 (2.2)	0.50
Night wakening	4.42 (1.8)	4.94 (1.6)	0.34
Parasomnias	8.95 (1.7)	9.39 (2.4)	0.50
Sleep disordered breathing (SDB)	3.79 (1.1)	3.52 (1.1)	0.40
Daytime sleepiness	9.84 (2.8)	9.45 (2.5)	0.60

[1] Mean (SD) calculated for Sleep Total and sub scores -Student t-test, *p value<0.05 as significant

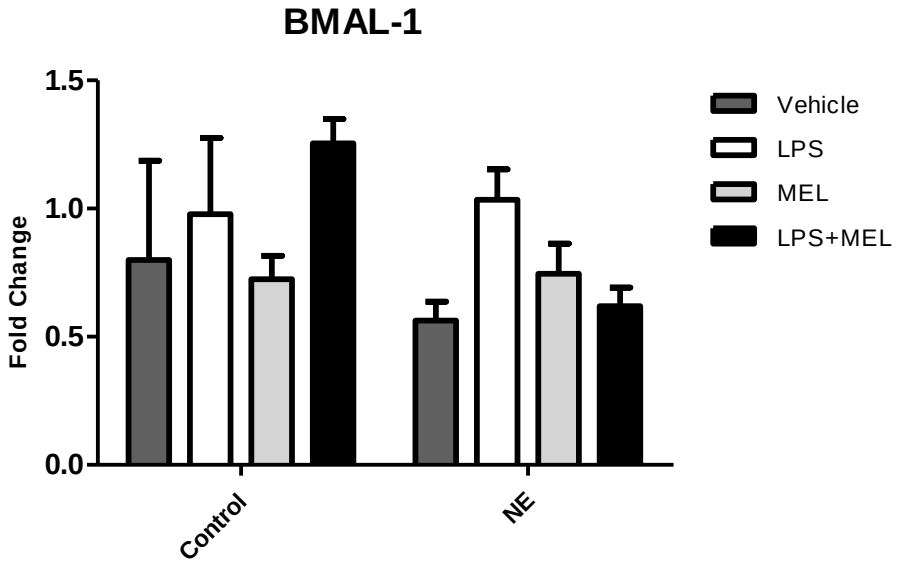


Figure 3-1 Expression of Circadian gene BMAL in children with NE vs Control on in vitro stimulation of LPS and Melatonin, using r-PCR

Whole blood from children with NE (n=5) and control group (n=5) were treated with 1uL (10ng/ml) of lipopolysaccharide (LPS), & 42 uM of Melatonin (Mel) and both (LPS & Mel) using Reverse polymerase chain reaction (rPCR). Results show fold changes in mRNA levels at baseline and after each treatment.

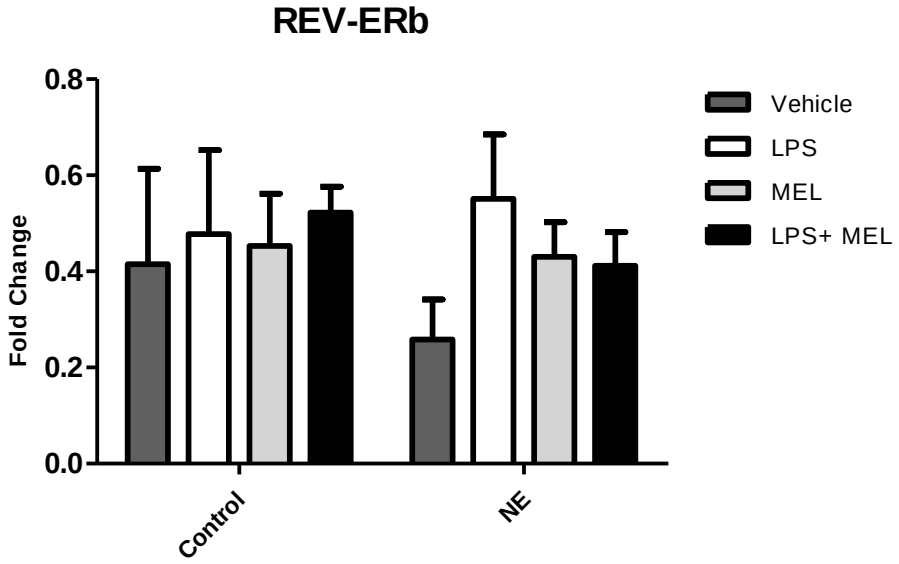


Figure 3-2 Expression of Circadian gene rev-ERb in children with NE vs Control on in-vitro stimulation of LPS and Melatonin, using r-PCR

Whole blood from children with NE (n=5) and control group (n=5) were treated with 1uL (10ng/ml) lipopolysaccharide (LPS) & 42 uM of Melatonin (Mel) and both (LPS & Mel) using Reverse polymerase chain reaction (rPCR). Results show fold changes in mRNA levels at baseline (vehicle) and after each treatment.

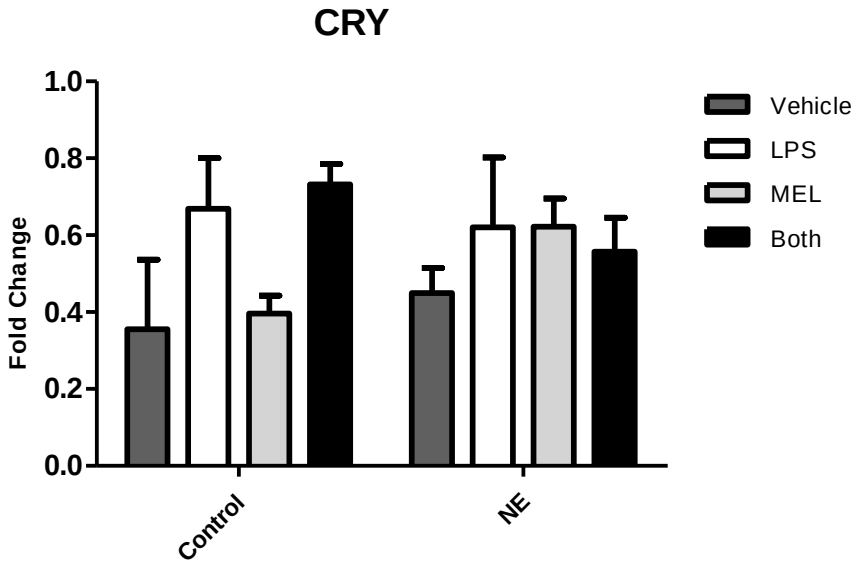


Figure 3-3 Expression of Circadian gene CRY in children with NE vs Control on in-vitro stimulation of LPS and Melatonin, using rPCR

Whole blood from children with NE (n=5) and control group (n=5) were treated with 1uL (10ng/ml) of lipopolysaccharide (LPS), & 42 uM of Melatonin (Mel) and both (LPS & Mel) using Reverse polymerase chain reaction (rPCR). Results show fold changes in mRNA levels at baseline and after each treatment.

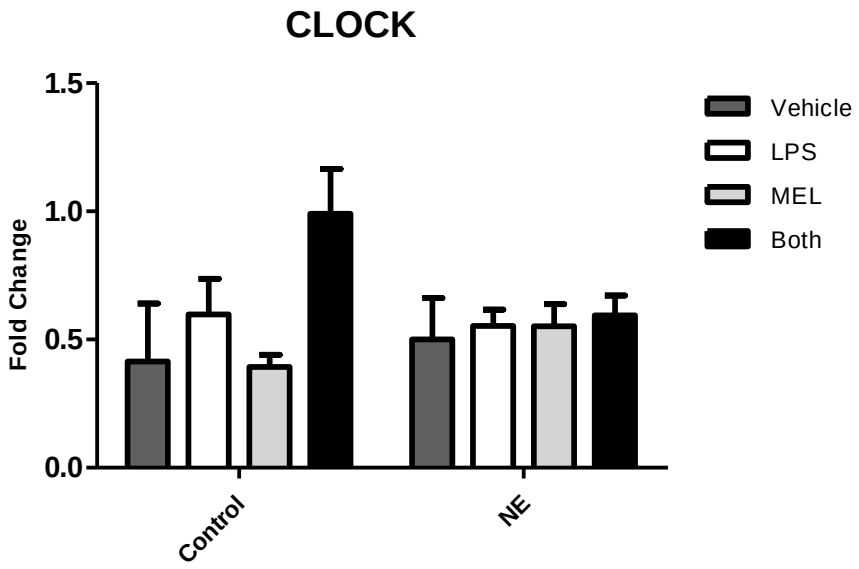


Figure 3-4 Expression of Circadian gene CLOCK in children with NE vs Control on in-vitro stimulation of LPS and Melatonin, using rPCR

Whole blood from children with NE (n=5) and control group (n=5) were treated with 1uL (10ng/ml) of lipopolysaccharide (LPS) & 42 uM of Melatonin (Mel) and both (LPS & Mel) using Reverse polymerase chain reaction (rPCR). Results show fold changes in mRNA levels at baseline and after each treatment.

3.8. Quality of Life in Children post Neonatal Encephalopathy

3.8.1 Introduction

Neonatal Encephalopathy leads to several complications which may affect the cognitive, motor, sensory abilities and behaviour outcome. These complications affect the quality of life of the child, and neurological outcome following NE remains the main predictor for the child's QOL.

"Quality of life (QoL) is a person's subjective well-being and physical health, material well-being, interpersonal relationships within and outside the family, work and other activities in the community, personal development and fulfilment."(Niemi et al., 1988) QOL is a multidimensional construct and is an overall assessment of health in all areas. It represents an important factor in therapeutic evaluation in children with CP. Quality of life is also an important outcome measure of interventions such as ambulation training in physiotherapy for children with CP (Bjornson and McLaughlin, 2001). The concept of QOL evaluation is not clearly integrated in ICF (International Classification of Functional disability & Health). ICF have concepts of activity and participation but they are all multidimensional concepts and do not explore the same domain (Morris et al., 2005).

Increase in degree of physical disability, low cognitive levels and low communication skills are some of the factors affecting QOL in children with CP (Mohammed et al., 2016) Several other factors including pain, parental stress and socioeconomic factors also affect QOL of a child (SPARKLE Study 2008). The common challenge faced by researchers in the field of cerebral palsy is the inability of most children to communicate themselves thus, the need to rely on the caregiver for information regarding this aspect of health care. Studies have shown that answers from different responders do not correlate perfectly (Upton et al., 2008)**.** Several studies have shown that parents usually report lower QOL for their children in all aspects, the children themselves usually rate their QOL in emotional and social domains equal to that of the typically developed children in the general population.

The Cerebral Palsy Quality of Life for Children (CPQOL–Child) is the first quality of life questionnaire designed for children with CP. It is designed in accordance with International Classification of Function (ICF) and the definition of quality of life by the World Health Organization (Waters et al., 2007). CP QOL questionnaire also assesses the child's emotional and social wellbeing apart from assessing their physical health. It is essential to identify and manage the emotional and social problems that the children with CP cope as early as possible, to promote their health and well-being (Davis and

Gavidia-Payne, 2009, Davis et al., 2010). We used this questionnaire to assess QOL in our group of children with CP.

The PedsQL Measurement Model is a tool to measuring health related quality of life (HRQOL) in healthy children and adolescents and those with acute and chronic health conditions including CP (Varni et al., 2005). We used this questionnaire to assess QOL in children with mild to moderate NE in comparison to typically developed children in the control group.

3.8.2 Hypothesis

Mild hypoxic ischaemic insult may lead to minor developmental problems which may manifest only later in childhood with cognitive demands and motor abilities affecting the child's quality of life (QOL).

3.8.3 Aims

1. To assess the quality of life of children who had mild to moderate Neonatal encephalopathy (NE), in comparison to children with normal development and children with CP using age appropriate Cerebral Palsy (CPQOL) and Paediatric Quality of Life (Peds QL)
2. To corelate the quality of life (QOL) with various characteristics including mobility (GMFCS scale), hearing impairment, speech delay, visual defect and behaviour problems.

3.8.4 Results

Clinical Characteristics

Quality of life (QOL) of 45 school age children who had mild to moderate neonatal encephalopathy (NE) were assessed and compared with 55 children with normal development, and 15 children with CP and complex needs. The assessment included history taking, filling the QOL questionnaire and carrying a detailed clinical examination. We used age specific PedQL inventory questionnaire for children in the control group and for children with mild NE. Ped QL CP module was used for children with NE who developed CP. For children in the CP group (who had underlying cause other than NE) the CP QOL questionnaire was used.

Children in the control group (n=55) were in the age range of 4-12 years. They had typical development with no intellectual impairment, and no prior neuropsychiatric illness or mental health problems. In addition, the participants in the control group had no chronic medical or neurological condition.

In the NE group (n=45), children were in the age range of 4-6 years (mean age). They were classified as per grade of encephalopathy in the neonatal period as mild (NE 0/I; n=15) and moderate encephalopathy (NE II/III; n=30) as per Sarnat Staging. (Sarnat and Sarnat, 1976). Eight children were diagnosed to have CP, and out of these two had GMFCS V and remaining 6 had GMFCS II/III. Epilepsy was diagnosed in 3 children and 2 children had visual impairment.

Children in the complex needs group (n=15) had heterogenous underlying diagnoses including congenital infections, metabolic disorders, and epileptic encephalopathy. Nine children had GMFCS level V, and six children had GMFCS level II/III. Epilepsy was diagnosed in nine children and they were on anti-epileptic medications. Visual impairment was noted in two children and nine children were diagnosed to have intellectual disability. CPQOL questionnaire was used in this group of children with CP and complex needs, to assess QOL and the scores were compared to scores of typically developing children of the same age group.

3.8.5 QOL scores in children with NE vs Control Group

Children in the NE group were noted to have significantly low total QOL scores in comparison to children in the control group; mean score 82.5 vs 95.8 (p value <0.01). Children with NE had comparatively low scores in all domains of QOL, especially in emotional, social functioning and school activities.

Comparing children with mild NE with the control group, QOL total scores were significantly low in children with mild NE compared to control group (mean total QOL score 90.03 vs 95.8 p value 0.003). Children with mild NE had low scores in emotional, social functioning and school activities. (Table 3.2)

On comparison between mild and moderate NE, children with mild NE had higher total QOL scores compared to children with moderate to severe NE (mean 90 vs 78.3 p value 0.007). Children with moderate to severe NE had significantly low scores in domains of physical ability, and in school activities compared to children with mild NE (p-value 0.021 and 0.02).

Children in the NE group who were diagnosed to have CP (n=8) had very low total QOL scores as assessed on the CP module of Ped QL questionnaire (mean score 44.1).

There was strong correlation of low QOL scores with high GMFCS level, children with GMFCS level I/II had high total QOL scores compared to children with GMFCS level III/IV; mean score 89 vs 44 (p-value 0.001). Low scores were also associated with co-morbidities including epilepsy, intellectual disability and visual impairment.

Correlation of QOL scores with Sleep Scores: We also found strong correlation of low QOL with high total sleep scores. As the data was not normally distributed spearman correlation was used for analysis and correlation was noted (Rho 0.339, p value 0.014).

3.8.6 QOL in children with Cerebral Palsy and complex needs

QOL was assessed in children with CP and complex needs (n=15) using the CP QOL questionnaire. The domains included in the questionnaire were social well-being and acceptance, feelings about functioning, participation and physical health, emotional well-being, access to services, pain and impact of disability, and family health.

Children in the CP group demonstrated very low QOL scores compared to children in the control group (mean score 47.3 vs 95.8 p value <0.01). Majority of children in CP group (9/15) had GMFCS of IV/V and had associated co-morbidities or complex needs including epilepsy, visual impairment, speech delay, feeding difficulties and sleep disturbances. Very low scores were noted in domains of functional ability, participation and health and high pain scores. Parent/ primary caregiver filled the questionnaire in majority of cases as the children to their disability including cognitive visual, speech or hearing impairment were unable to do so, except few filled by children themselves. There was difference noted between the scores put in by the child and those reported by parents. Several studies report that the agreement depends on the domain assessed. Generally, there was good agreement (correlations > 0.5) between parents and children for domains reflecting physical activity, functioning, and symptoms but poorer agreement (correlations <0.30) for domains which reflected social or emotional domain.

3.9. Discussion

Quality of life (QOL) is an important determinant of health. QOL explores a child psychosocial wellbeing, school environment, social acceptance, self-perception, parental relations and pain or discomfort experienced by the child.

We measured QOL in 45 children with mild and moderate to severe NE and found very low total QOL scores in children with NE compared to children in the control group. Low scores were noted in all domains of QOL, especially in emotional, social functioning and school activities. QOL total scores were significantly low in children with mild NE compared to control group (p value0.003). However, they were higher compared to children with moderate to severe NE. Children with moderate to severe NE had significantly low scores in domains of physical ability, and in school activities compared to children with mild NE (p-value 0.021 and 0.02).

We demonstrated a strong correlation of low QOL scores with high GMFCS level, children with GMFCS level I/II had high total QOL scores compared to children with GMFCS level III/IV; mean score 89 vs 44 (p-value 0.001). Low scores were also associated with co- morbidities including epilepsy, intellectual disability and visual impairment. Physical impairment has been reported to be associated with low QOL scores, but a child with mild impairment can have low QOL scores in domains of social acceptance and school activities.(White-Koning et al., 2008). We also found a correlation with low QOL scores and high sleep scores suggesting that sleep disorders impact on the child's QOL.

Pain is the most important factor contributing to QOL. Children who report pain have very low QOL scores especially in terms of emotional and social acceptance. It is important to find out the source of the pain and manage as it has an impact on the child QOL and emotional well-being. We found high pain scores in children with moderated to severe NE.

We also assessed the QOL in group of children with CP and complex needs, using CPQOL questionnaire. We found very low QOL scores in children with CP and complex needs compared to children in the control group (p value <0.01). Several factors including GMFCS level, intellectual disability, epilepsy and visual impairment affected their QOL. The questionnaire was filled by the parent or carer and we noted a difference between the scores put in by the child and those reported by the parents. Lower scores

were reported by the parent compared to the child, this has been reported in other studies, but reports vary.(Arnaud et al., 2008). Parents with high stress level also report low scores in all domains.

Thus, these factors if identified and managed appropriately can improve the QOL of the child and the parent. This is probably the first study looking at QOL in children with mild to moderate NE, it is an important determinant of a child's physical and emotional well-being and should be assessed routinely in clinical practice.

Table 3-2 QOL scores in children with mild NE in comparison to control.

[1] QOL SCORE	CONTROL (n=46)	MILD NE (n=20)	P -value
TOTAL QOL	[2]95.8 (3.8)	90 (7.4)	*0.003
PHYSICAL	97.2 (4.4)	89.6 (8.3)	*0.01
EMOTIONAL	88.3 (3.6)	85.8 (6.7)	0.05
SOCIAL	99.1 (4.1)	95.5 (8.2)	0.07
SCHOOL	97.8 (4.1)	90 (12)	*0.016

[1]Quality of life (QOL), [2] Mean (SD), *p value <0.05

Chapter 4

NEUROLOGICAL SYSTEM

Neurodevelopmental Progress of school age children with NE

4. Neurological System

Neurodevelopmental Progress of school age children with NE

4.1. Introduction

Neonatal Encephalopathy (NE) describes neurological dysfunction in new-born from all causes including hypoxic-ischaemic encephalopathy (HIE) and birth asphyxia. Cerebral Palsy (CP) has an incidence of 13.5% (Badawi et al., 2005) in infants who had NE. The risk of developing CP increases three-fold if the child had neonatal seizures. Following NE, the commonest subtypes of CP are Dyskinetic and bilateral spastic CP. (Rennie et al., 2004). Children born at term who develop CP following NE, have a poorer prognosis than those with CP who were not encephalopathic in the first week of life (Badawi et al., 2005). These infants are also more likely to develop epilepsy, cognitive impairment, severe disability and die early in the period ranging from the onset of diagnosis to 6 years of age. Attention and executive function impairment, memory problems and poor educational attainment has been reported in children with moderate to severe NE (Pappas et al., 2015)**.** Neuropsychiatric problems including autism spectrum disorder and schizophrenia are also seen in older children with NE.

Therapeutic hypothermia (TH) has been reported to be effective in neonates with moderate to severe encephalopathy. Following the Total Body hypothermia for Neonatal Encephalopathy Trial (TOBY), neonates with NE who received TH had improved neurological outcome at 18 months of age compared to those who did not (Azzopardi et al., 2009). Improved survival rates with normal IQ and development were also reported in children who received TH at 6-7 years of age by the same authors. The risk of CP among survivors was reported to be significantly reduced (21 vs. 36%, p=0.03) along with a reduction in risk of moderate to severe disability (22 vs. 37%, p=0.03) (Azzopardi et al., 2014)

Outcome has been predicted in these infants using scoring system based on clinical indicators and investigations including electroencephalography and neuroimaging (Miller et al., 2002). Neurodevelopmental assessment tools and questionnaires including Bayley Scale of Infant & Toddler Development (BSID III) and Ages and Stages Questionnaires are also widely used to identify developmental delay.

BSID III has been used globally to assess and predict developmental delay both in term and preterm infants.

4.2. Hypothesis

Long term neurological outcome in children following NE may be predicted by neonatal clinical examination and MRI.

4.3. Aims

1. To assess the neurodevelopmental progress of children with NE at school age in compared to age-matched controls.
2. To examine the results of neonatal examination, neuroimaging and developmental assessment (Bayley's Scale of Infant Development) in relation to the long-term neurodevelopmental outcome.
3. To assess the neurodevelopmental outcome in children with NE who received therapeutic intervention with therapeutic hypothermia in comparison to those with normothermia.

4.4. 4.4 Results

4.4.1 Clinical Characteristics

Fifty-five children with NE in our study had follow up at school age. A detailed neurodevelopmental assessment including history and a developmental questionnaire (ASQ 3) and neurological examination was performed. They were classified by the severity of encephalopathy according to Sarnat Scores (Sarnat and Sarnat, 1976) as follows: NE 0 (required only resuscitation; n=3); mild NE I (n=17); moderate NE II (n=32); and severe NE III (n=3) (Table 2.5).

From the total 55 children, 24 children (43%) received therapeutic hypothermia (TH) for neonatal encephalopathy using TOBY criteria (Azzopardi et al 2009). Forty-seven infants had MRI brain imaging, of which 27 were normal and 20 abnormal and eight infants did not undergo MRI imaging.

There were no significant differences within the NE group with regards to gestational age, birth weight, or gender. Infants with NE were significantly more likely to be delivered by lower section caesarean section (LSCS) or instrumental delivery and had significantly lower Apgar scores at 1 and 5 minutes compared to controls. Children in the control group were born by normal delivery, had normal Apgar scores and development milestones with no medical problems (Table 4.1).

MRI Brain scan was performed in total 47 children, out of which 20 (40%) were abnormal. Nineteen children with abnormal MRI brain scan were classified to have NE grade II and remaining one infant was classified as NE Grade I encephalopathy.

Cerebral Palsy (CP) was diagnosed in 8 children out of the 35 children (23%) with moderate to severe NE. Four children had unilateral spastic CP and 3 had bilateral spastic CP and one child had dyskinetic CP. Children diagnosed with Cerebral Palsy were assigned NE Grade III at birth and the remaining 6 were classified to have NE grade II. As per the GMFCS, 2 children with CP in the NE group had GMFCS V and remaining 6 had GMFCS II-III. Other neurodevelopmental problems noted were speech delay in 15 children (27%), visual impairment in 5 (9%), hearing impairment in two children with NE. Epilepsy was diagnosed in 3 children, febrile seizures were noted in another 3 children and developmental co-ordination disorder (DCD)- functional motor or co-ordination problems was diagnosed in 3 children. (Figure 4.1).

4.4.2 Neonatal Neurological Examination

Overall the neurological examination that was performed assessed each infant's level of consciousness, central and peripheral tone, reaction to stimulus, primitive reflexes and deep tendon reflexes. Each infant was assigned a Sarnat grade for the day of examination. The outcome of the neurological examination between day 2-8 of life and abnormal MRI result were compared using Chi squared statistical testing. An abnormal neonatal neurological examination between day 2 and 8 of life (p values <0.04) was significantly correlated with MRI brain abnormality (Table 4.2).

We also found an association between low BSID scores and abnormal neurological examination in the neonatal period. Children who were noted to have an

abnormal neurological exam performed poorly on Bayley assessment compared to children with a normal neurological exam. The composite motor score was significantly higher in children with normal exam compared to those with abnormal exam (mean 107 vs 93; p value=0.03). Similarly, the language and cognitive scores were higher in children who had normal neurological exam in comparison to those with an abnormal exam in the neonatal period (mean score 104 vs 96; p value= 0.4 for language and mean score 105 vs 96; p value= 0.1 for cognitive skills respectively). (Table 4.3)

4.4.3 Neonatal MRI

An MRI Brain was performed in the first 10 days of life in 47 out of the total 55 children with NE enrolled in the study. Infants with NE who received TH, had an MRI scan performed at a median age of 7 days MRI brain scans were scored and reported independently by a paediatric radiologist who was blinded to the clinical results and outcome, using the well validated Barkovich scoring system (Barkovich, 1998) which employs a combination score including components of both basal ganglia and watershed patterns of injury (Table 1.3)**.**

There were 20 abnormal MRI scans from the 47 infants who had an MRI. MRI scan were abnormal in 19/35 (51%) children who were diagnosed to have NE II/III and only 1 scan was abnormal out of the 10 MRIs performed in children with NE I. Not all children who received TH had abnormal MRI scans, but all who had abnormal neuroimaging received TH.

4.4.4 Neonatal MRI and Neurodevelopmental Outcome

An association was found between low BSID scores and abnormal MRI in the neonatal period. Children who were noted to have an abnormal neonatal MRI performed poorly on Bayley assessment compared to children with a normal neonatal MRI. As expected, the Bayley composite motor score was significantly higher in children

with normal neonatal MRI compared to those with abnormal MRI scans (mean 109.4 vs 96.2; p value=0.03). (Table 4.4)

At school age, 20 children who were classified as NE Grade I & II had abnormal MRI. Nine children with an abnormal MRI in the neonatal period had completely normal developmental assessment on ASQ-3 when reviewed in the clinic at 4-6 years of age. The 11 remaining children with an abnormal MRI had the following abnormal outcome: CP (n=8); speech delay (n=3); epilepsy (n=1); developmental discoordination disorder (n=1). However, 9 children with normal MRI scans in the neonatal period went on to have developmental problems including speech delay (n=7), hearing impairment (n=1), and delay in personal & social skills (n=1). Thus, abnormal neuroimaging alone did not predict poor neurodevelopmental outcome.

4.4.5 Developmental Assessment

Neurodevelopmental outcomes using the Bayley's Scale of Infant Development-III (BSID-III) was carried out by a clinical psychologist (M Slevin) experienced in assessing infants with brain injury and NE, at 2 years of age for the children enrolled in the study. Development assessment using BSID was carried out on total 45 children at 2 years, out of these 35 were classified as NE II/III and 10 children had NE 0/I.

4.4.6 Bayley's Scores in children with Mild NE vs Moderate to Severe NE

Forty-five children out of the total 55 (82%) who were noted to be at risk of developmental delay (NE II/III) had BSID assessment and 16 children of the total 45 assessed (35%) were found to be to be delayed in one of the developmental domains including cognition, speech and language, motor and adaptive behaviour. As expected, all children with NE 0/I performed better in all developmental areas compared to children with NE II/III. The mean composite scores were better in all developmental domains in children with mild NE compared to children with moderate NE, though did not reach statistical significance; mean cognitive score 100 vs 104 (p value=0.3), mean language score 103 vs 102 (p value=0.8) and a motor score (mean 108 vs 101; p value=0.1). (Table 4.3a). Only one out of the 16 children, was classified as NE Grade I

and the rest were classified to have NE II/III in the neonatal period. All of them had very low scaled scores between 4-7 (i.e. 1-2 SD below the mean).

Cognitive delay as per the scaled scores was noted 9 children (20 %), speech and language delay were noted in 12 children (26%), delay in gross motor skills in 5 children and in fine motor skills in 6 children. The composite scores were also low and using a cut off score of 85 as previously described in other studies to define delay the following was found (total n=45): cognitive delay (n=6); language delay (n=7); motor delay (n=5) as per the composite scores. (Table 4.4)

4.4.7 Bayley Scores in children with Therapeutic Hypothermia

BSID assessment was performed in 45 children with NE, out of these 23 children underwent therapeutic hypothermia (TH) and 22 children did not. We compared the BSID composite scores between the two groups and found children in the hypothermia group scored better in all areas of development compared to the children who did not receive TH, but interestingly no statistical difference was noted. The composite cognitive mean score was high 104 vs 98; p value=0.4, similarly the motor and language scores were high, (mean score 108 vs 98; p value=0.1 and mean score 103 vs 100; p value=0.6 respectively). (Table 4.5)

4.5. Developmental Questionnaire

The age-appropriate ASQ-3 was completed by the parents of school age children in both the NE group (n=55) and the control group (n=65) enrolled in the study. The age of the children was in the range of 4-6 years. All domains of development including gross motor, fine motor, problem solving, communication and personal social skills were examined. The parents completed the questionnaire at the clinic visit and any parental concerns were addressed. It took around 10-15 mins for the parent to complete the questionnaire. A detailed history was taken, and neurological examination was carried out at the same visit.

Development Ages & Stages Questionnaire (ASQ-3) scores were significantly lower in children with NE in all domains compared to controls (Table 4.5). Twelve children had developmental delay using the ASQ scores and 7 children scored very low in gross motor and problem-solving skills. Cerebral Palsy was diagnosed in 8 children with NE and three out of these had severe form of CP with co-morbidities including cortical visual impairment, hearing defect, speech delay and epilepsy. All these eight children had NE II/III had abnormal neuroimaging in the neonatal period. One child who was classified as NE Grade III and who received hypothermia had normal cognitive function and speech but had weakness on the left side affecting gross motor and fine motor skills.

Speech delay was noted in 15 children with NE and 2 had hearing impairment. One child with NE who had neonatal seizures but had normal developmental milestones developed seizures in childhood and was diagnosed with Epilepsy. Neuropsychiatric problems like Autism, behaviour problems & ADHD were also seen in 5 children with NE.

Correlation of ASQ-3 scores with Bayley III scores

Correlation between the BSID and ASQ scores was analysed using the non-parametric Spearman test as the variable were not normally distributed. Correlation was noted between the gross motor Bayley score and ASQ score. (Rho; 0.35 and p value=0.014).

4.6. Discussion

Detection of developmental delay in children at risk can be achieved by follow up development assessment (BSID) at 2 years of age and later by surveillance programmes including developmental tools such as the Ages and Stages Questionnaire (ASQ), Parental Evaluation of Developmental Status (PEDS) and the Child Development Inventories (CDI). Assessment with Bayley's Scale of Development at 2 years of age detected development delay in 35 % children in either cognitive development, communication or motor skills. A total of 45 children were assessed and 35 were classified as NE II/III and 10 children had NE 0/I.

Developmental delay was noted in 21% (12/55) children with NE who had an assessment done at 4-6 years of age using Ages and Stages Questionnaire (ASQ3) and BSID. Children with NE 0/I scored better than the children who had NE II/III, but less than the children in the control group in all developmental domains. (Table4.4). Thus, children with mild NE need follow up assessment for detection of neurodevelopmental delay.

There was a correlation noted between the gross motor BSID and the ASQ-3 scores (Rho 0.36 and p-value 0.014). Majority of the children defined to have developmental delay following the BSID, demonstrated similar difficulties in performing developmental tasks when assessed at 4-6 years of age using ASQ 3. The ASQ scores were consistent with BSID scores. This shows that ASQ3 could be used as a reliable screening tool in children who had NE. The validity of the Ages and Stages Questionnaire (ASQ2) has been correlated with the Bayley's Scales of Infant Development (BSID II). The sensitivity of 100% and a specificity of 87% was reported for Ages and Stages Questionnaire for children of 24 months of age. (Gollenberg et al., 2010).Recently, the ASQ, Third Edition (ASQ3) was compared with BSID III in a mixed population of term-born and preterm-born children at different age groups and demonstrated adequate sensitivity and specificity (Schonhaut et al., 2013)

Abnormal neuroimaging in the neonatal period was associated with poor neurodevelopment outcome in 57% (11/19) children with NE on developmental assessment. MRI may provide added information to predict neuromotor outcomes in children. Several studies have looked at the motor outcome in children with NE and their relationship with neonatal MRI in prediction of outcome (Perez et al., 2013) (Ahearne et al., 2016). The use of diffusion-weighted imaging as an additional sequence, on MRI scan adds to the predictive value for motor outcome in infants with NE (de Vries and Groenendaal, 2010). Significant motor impairment was noted in 6 children with abnormal MRI scans in our study. However, 40% children with abnormal MRI scans made good developmental progress (BSID) and had normal development at school age (ASQ). Thus, an abnormal neonatal neurological examination or abnormal neuroimaging alone in infants with NE did not predict outcome in childhood. However long-term neurological outcome in children at risk for developmental problems caused by prematurity or NE may be predicted by a combination of indicators including

clinical examination, developmental assessment tools including BSID and ASQ3, and neuroimaging.

Majority of children with developmental delay in the study had been referred appropriately at 2-3 years of age following assessment by clinicians to the respective therapist and were linked in with the services when assessed at 4-6 years of age. There has been concerns and questions raised in the literature regarding criteria and appropriate time of referral (Adams et al., 2013). Early detection of neurodevelopmental impairment is one of the key recommendations by the American Academy of Paediatrics. (AAP 2006).

In addition, we noted that children with mild NE had significant low developmental scores in comparison to the control group. These children with mild NE were traditionally assumed to have good prognosis but recent studies have demonstrated otherwise. A recent systemic review including twenty studies on children with mild NE reported abnormal outcome including death and neurodisability in 25% children with mild NE (Conway et al., 2018a). Thus, children with mild NE should be followed up for early detection of development delay. Early recognition provides chances for early intervention and opportunity for better developmental outcome.

Table 4-1 Demographics of controls versus infants with NE, NE grade 0/I versus NE grade II/III.

Variables		Control (n=65)	NE 0/1 (n=20)	NE- II/III (n=35)	p- value (NE 0/I vs NE II/III)
*GA (wks.)		40 (0.9)	39.7 (1.43)	40.5 (1.21)	0.04
BW (kgs)		3.6 (0.5)	3.47 (0.58)	3.61 (0.61)	0.44
Gender, male, n (%)		45 (69%)	14 (70%)	22 (62%)	0.59
Mode of delivery n (%)	LSCS	3	8 (40%)	16 (46%)	0.9
	SVD	57	6 (30%)	10 (28%)	0.9
	Inst	5	6 (30%)	9 (26%)	0.9
*Apgar @1 min Median (IQR)		9 (9-9)	5(3-6)	2(1-5)	0.001
Apgar@5 mins Median (IQR)		9 (9-9)	7(5.2-8)	4(2-7)	<0.001
Apgar@10 mins Median (IQR)		N/A	7.5 (6-9)	5(3-7)	<0.001
TH, n (%)		N/A	1	23 (65%)	<0.001
Seizures, n (%)		N/A	0	24 (68.5 %)	<0.001
MRI- Total, (n= 47) Abnormal, (n=20 (36%)		N/A	12 1	35 19 (54%)	0.005

* For normally distributed data, mean +/- SD expressed and independent student-t test used for comparison, p value compares NE0/I vs NEII/ III and $p< 0.05$ is significant with 95% Confidence Intervals (CI); π = For skewed data, medians and IQRs expressed and Mann-Whitney U Test used for comparison; α = For binary variables, chi-squared test used and when cells had expected counts < 5, Monte Carlo exact test (2-sided significance) used for comparison; GA = Gestational Age; BW = Birth Weight; LSCS = Lower Section Caesarean Section; SVD = Spontaneous Vaginal Delivery; Inst = Instrumental delivery;

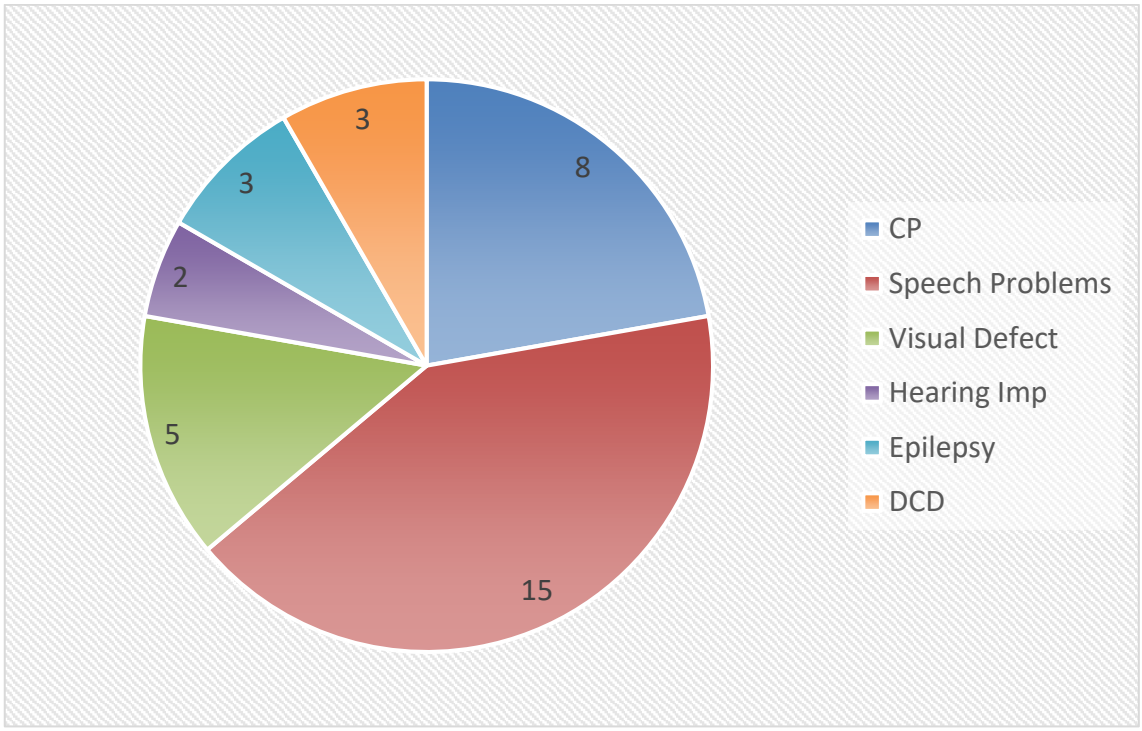


Figure 4-1 Neurodevelopmental outcome in children with NE

On follow up assessment at school age, children with NE had a diagnosis of Cerebral palsy (CP) (n=8), speech problems (n=15), visual defect (n=5), epilepsy (n=3), Developmental co-ordination disorder (DCD) (n=3), and Hearing Impairment (n=3).

Table 4-2 Correlation between normality of neurological exam on day 1 – 8 and MRI brain result

Neuro Exam Day	Normal	Abnormal	*MRI-Brain (χ^2Statistic)	p value
1	6	58	0.21	0.65
2	11	56	6.78	0.009
3	16	51	5.76	0.02
4	22	46	4.40	0.04
7	38	26	8.79	0.003
8	43	17	4.41	0.04

***** Chi-squared statistical test was used to compare these dichotomous outcomes and degrees of freedom (df) was = 1 for all analysis.

Table 4-3 Comparison of Bayley's Scores in children with normal neonatal neurological examination vs children with abnormal examination

BSID Score	Normal Examination (n=33)	Abnormal Examination (n=12)	p-value
Motor	107.36 (14.07)	93.33 (27)	***0.03**
[1]Comp Cognitive	103.18 (15.03)	95.8(24.7)	0.2
Language	104.48 (17.56)	96.75 (20)	0.2

[1]Comp Cognitive- Composite cognitive score, Language scores, and motor scores Independent t-test used to compare the means (SD). *p-value<0.05 significant.

Table 4-4 Comparison of Bayley's Scores in children with mild NE vs moderate to severe NE

BSID Score	Mild (NE0/I) (n=12)	Mod-Severe (NEII/III) (n=33)	p-value
[1]Comp Cognitive	104.17 (4.6)	100.15 (20.9)	0.3
Language	103.08 (14)	102.18 (24)	0.8
Motor	108.25 (7)	101.94 (22)	0.1

Table 4-5 Comparison of Bayley's Scores in children who received TH vs children with normothermia

BSID Score	Therapeutic Hypothermia (TH) (n=23)	Normothermia (n=22)	p-value
[1]Comp Cognitive	103.48 (17.6)	98.8(18.7)	0.4
Language	103.96 (23.1)	100.82 (20)	0.6
Motor	108.17 (17.6)	98.86 (20)	0.1

Table 4-6 Comparison of Bayley's Scores in children with abnormal neonatal MRI vs children with normal neonatal MRI

BSID Score	Normal Neonatal MRI (n=20)	Abnormal Neonatal MRI (n=20)	p-value
[1]Comp Cognitive	105.00 (13.5)	96.5 (23)	0.16
Language	103.65 (17)	102.20 (26)	0.81
Motor	109.45 (7)	96.25 (25)	***0.03**

4.4-4.6

[1]Comp Cognitive- Composite cognitive score, Language scores, and motor scores Independent t-test used to compare the means (SD). *p-value<0.05 significant.

Table 4-7 Ages and Stages Questionnaire Scores (ASQ-3) in children with NE vs Control

Variable ASQ Score	Control (n= 60)	NE 0/I (n= 19)	NE II/III (n =33)	p-value p (a)	p-value p (b)
Communication	55.6 (4.9)	53.9 (7.9)	51.1 (8.4)	*0.04	0.37
Gross Motor	57.3 (3.7)	49.25 (8.7)	48 (9.1)	*0.03	0.8
Fine Motor	58.3 (2.4)	48.6 (12.1)	39.7 (14.6)	*0.03	*0.025
Problem Solving	58.3 (3)	55.5 (5.7)	48.6 (12)	*0.04	*0.036
Personal & Social	59 (2)	56.05 (8.5)	52.8 (13)	*0.04	0.31

Mild NE- Grade NE0/1, Moderate to severe NE- NEII/III. Data are means (+/-SD), p (a)- p value NE I/0 vs Control and p (b) p value NE II/III vs NE I/II, *p-value <0.05 taken as significant.

Chapter 5

Renal function in children with NE in comparison to control and children with severe CP

5. Renal Dysfunction

5.1. Introduction

Perinatal ischaemia leads to acute kidney injury in around 50-70% of term infants with hypoxic ischaemic encephalopathy. (Gupta et al., 2016) (Askenazi et al., 2012). Infants with severe asphyxia have a higher incidence of renal dysfunction compared to those with moderate asphyxia (Aggarwal et al 2005). The prognosis and recovery from AKI are dependent upon the underlying cause of the injury and is much worse in neonates who suffered multi-organ failure. Several older studies in the literature have suggested an association between renal dysfunction following perinatal hypoxia-ischaemia and worsening long-term neurological outcome (Karlowicz and Adelman, 1995, Perlman and Tack, 1988). However, these studies did not have a control group for comparison and were small, therefore the findings need to be interpreted by caution.

Acute kidney injury (AKI) or renal dysfunction is usually diagnosed in infants and children based on a high creatinine level and a falling urine output but currently there is no uniform definition for AKI in adult or paediatric population. In neonates AKI has been described using the AKIN criteria. (Kaur et al., 2011) (Morgan et al., 2013) and RIFLE (Gadepalli et al., 2011). AKIN criteria were used by Kaur et al to stage AKI in 36 term neonates with birth asphyxia. AKI was demonstrated in a higher percentage of children with severe asphyxia (56%) compared to moderate asphyxia (9.1%).

Recently the RIFLE criteria (*R* risk for renal dysfunction, *I* injury to the kidney, *F* failure of kidney function, *L* loss of kidney function, and *E* end-stage renal disease) has been proposed as a standardized classification system for acute kidney injury in adult and has been adapted for children. (Bellomo et al., 2004). The paediatric RIFLE criteria have been found to be promising in classifying paediatric AKI better and reflecting on the course. pRIFLE criteria are based on a decrease in estimated creatinine clearance (eCCl) and in urine output (Uop) based on weight. (Akcan-Arikan et al., 2007). pRIFLE score has been reported to be a useful instrument to identify paediatric AKI in critically ill children. (Soler YA et al 2013). Multi-organ dysfunction occurs following NE and

kidneys being the most common organ involved Following NE, renal dysfunction has been reported in 60-70% neonates with post asphyxia HIE. (Shah et al., 2007, Shankaran et al., 1991, Hankins et al., 2002)

Recent studies have investigated role of serum and urinary biomarkers including NGAL, Cystatin C and KIM-1, in diagnosing acute kidney injury or renal dysfunction. (Devarajan, 2007, Sweetman et al., 2016). These are more sensitive and specific and contribute towards an early diagnosis and outcome prediction. (Sarafidis et al., 2012, Li et al., 2012).

5.2. Hypothesis

Renal dysfunction following neonatal encephalopathy (NE) remains persistently altered at school age and may be associated with poor neurological outcome.

5.3. Aims

1. To assess renal dysfunction in school aged children with mild to moderate neonatal encephalopathy (NE), in comparison with children with normal birth and development and with a group of children with severe cerebral palsy (CP).
2. To compare the renal dysfunction and lab parameters in the neonatal period with school age.
3. To assess if there is any correlation with persistently high creatinine levels and poor neurodevelopmental outcome.

5.4. Results

5.4.1 Clinical Characteristics

Renal parameters of fifty-five school aged children with NE were compared to 60 children born by normal delivery who had normal Apgar scores and normal development and 17 children with severe CP. Children with NE had been classified according to the grade of encephalopathy as NE 0 (n=3), NE I (n=17), NE II (n=32) and NEIII (n=3) using Sarnat scores (Sarnat and Sarnat, 1976). From the total 55 children, 24 children (43%) received therapeutic hypothermia (TH)

for neonatal encephalopathy using TOBY criteria (Azzopardi et al., 2009). Forty-eight infants had MRI brain imaging, of which 27 were normal and 20 abnormal and eight infants did not undergo MRI imaging.

There were no significant differences between controls and NE cases with regards to gestational age, birth weight, or gender. Infants with NE were significantly more likely to be delivered by lower section caesarean section (LSCS) or instrumental delivery and had significantly lower Apgar scores at 1 and 5 minutes compared to controls. Children in the control group were born by normal delivery, had normal Apgar scores and development milestones with no medical problems (Table 4.1).

Acute kidney injury (AKI) was diagnosed in 36% term infants with NE. Oliguria which was defined as a urinary output of < 1 ml/kg/hr per 24-hour period was seen in 59% of infants with NE.

Renal ultrasounds were requested by the treating clinician if deemed necessary for the management of the neonate. There was a significant association seen between performing a renal ultrasound and therapeutic hypothermia and having an abnormal neurological examination at discharge. Renal ultrasound scans were performed in 14% of children with NE and were abnormal in 3 cases.

5.4.2 Laboratory Results

5.4.3 Neonatal period

The results of urea, creatinine and electrolytes of the infants with NE in the neonatal period taken on Day 1, 3 and 7 were examined. The values of sodium, potassium and chloride values, calcium, magnesium and phosphate were normally distributed at all time points and therefore their means and standard deviations were recorded. However, the values of creatinine and urea were not normally distributed across all time points and demonstrated a skewed distribution. Therefore, medians and interquartile ranges were reported and non-parametric tests (Mann-Whitney U test) were used.

Infants with moderate to severe grade of encephalopathy had a significantly lower sodium level on day 2 of life and lower calcium levels on day

3 compared to infants with mild NE (mean: 131.7 vs 134.3mmol/l; p-value 0.04). Infants who received TH had low sodium levels on day 3, low calcium levels on day 1,2,3, high magnesium and phosphate levels on day 1. In addition, low sodium and chloride levels on Day 1 and high potassium levels on Day 3, were associated with clinical seizures. There was no significant association found between abnormal neurological examination at discharge and sodium, potassium, chloride, calcium, magnesium or phosphate levels at any time point. An abnormal MRI brain scan result was significantly associated with higher potassium levels on day 2 and 3 (Table 5.1).

Oliguria was significantly associated with therapeutic hypothermia, seizure development and abnormal NE grade II/III (p-values < 0.001, 0.006, < 0.001), but there was no association between oliguria and an abnormal MRI brain result.

There was no strong association between renal outcome measures such as proteinuria/ haematuria and serum urea and creatinine levels. However, oliguria was associated with higher urea levels on day 2 of life (mean rank 35.38 vs 25.14mmol/l, p-value = 0.048) and higher creatinine levels on day 7 (mean rank 14.09 vs 5.00 umol/l, p-value = 0.045). Oliguria was also significantly associated with lower sodium levels on day 2 (131.52 vs 134.89 mmol/l, p-value = 0.009), lower calcium levels on day 2 (1.95 vs 2.12, p-value = 0.04), lower magnesium levels on day 2 (0.66 vs 0.72, p-value = 0.02) and higher phosphate levels on day 7 (1.95 vs 0.94, p-value = 0.003).

Urea and creatinine were separately analysed using the non-parametric test, Mann-Whitney U test. Therapeutic Hypothermia was significantly associated with higher urea levels on day 1,2,3,7 and higher creatinine levels on day 7. The occurrence of seizures was significantly associated with higher urea levels on day 2 and higher creatinine levels on day 2 and 3. Moderate/severe NE was significantly associated with higher urea levels on day 1,2 and higher creatinine levels on day 1,2 and 3. The presence of an abnormal MRI brain result was significantly associated with higher serum creatinine levels on day 2 and 3 but not with urea levels at any of the time points measured.

5.4.4 Urinary Biomarkers

Evaluation of seven previously studied urinary biomarkers; albumin (Alb), beta-2 microglobulin (β2M), Cystatin C, epidermal growth factor (EGF), neutrophil gelatinase associated lipocalin (NGAL), osteopontin (OPN), and uromodulin (UMOD) were done in term infants with NE. Infants with AKI had higher levels of urinary albumin (Alb) on (day 2, 3 and 6-8; p-values <0.001, 0.008, 0.02), significantly higher levels of Beta-2-Microglobulin (B2M) on (day 6-8 p-value 0.01), CysC on (day 1,2,3 and 6-8;p-values 0.001, <0.001, 0.001, <0.001), NGAL on day 2,3 and 6-8;p-values <0.001, <0.001, 0.001) and osteopontin (OPN) on (day 2, 3, 4-5 and 6-8; p-values <0.001, 0.001, 0.03, <0.001). NE Grade II/III infants had significantly elevated levels of urinary biomarkers NGAL, Cystatin C, and decreased EGF levels compared to NE Grade 0/I.

5.5. Renal function at school age and clinical outcome

At school age, altered renal function was noted in children with NE. Significantly high urea and creatinine levels were seen in school age children who had NE compared to children in the control group (NE v controls: mean urea level 5.2 (1.2) versus 4.4 (1.1) mmol/l (p-value 0.001). Similarly, higher creatinine levels were seen in school age children compared to controls (mean 45.4 vs 40.8 umol/l; p value 0.025) (Table 5.2). However, there was no significant difference noted between the urea and creatinine levels of children with mild NE and the children with moderate neonatal encephalopathy at school age (Table 5.3).

Comparing between values of urea and creatinine levels in the neonatal period and school age, infants with NE had significantly higher creatinine in compared to school age (Mean creatinine 106 (27) vs 45.4 (5.4) umol/l; p=value<0.01).

A strong association was noted between persistently altered urea and creatinine levels and poor neurological outcome. Children with seizures, and who underwent therapeutic hypothermia (TH) were noted to have high urea and

creatinine levels in the neonatal period and at school age. (Table 5.5) Children diagnosed with CP in the NE group had higher urea and creatinine levels in the absence of oliguria. There was no strong association seen between high urea and creatinine levels in neonatal period and poor renal outcome.

Electrolytes at school age

The values of sodium, potassium, calcium, phosphate, were normally distributed therefore their means and standard deviation were calculated. School age children with NE had significantly low sodium levels and high calcium levels compared to the control group (p-value= 0.001 and 0.01 respectively) There was no significant difference between values of potassium and phosphate (Table 5.4).

These values were compared with the values in the neonatal period and no significant difference were seen between them. There was no association between the values of sodium, potassium and calcium levels at school age and the severity of neonatal encephalopathy (Table 5.5). Children, with NE diagnosed with Cerebral Palsy and with other neurodevelopmental problems including sleep delay, visual impairment and behaviour problems had normal sodium, calcium and potassium levels at school age.

5.6. Renal Function of children NE Group vs children in Cerebral Palsy Group

The renal function of children with NE (n=55) was compared with children with normal development (n=50) and children with CP (n=17) due to an underlying cause other than NE (i.e. Infection, metabolic disorder, epileptic encephalopathy, and Rett's Syndrome). The values of urea and creatinine of school age children with NE were found to be higher compared to children in the control group (p value= 0.001, 0.013). The older children in the CP group had very low levels of creatinine compared to children in the control group and in NE group (35 vs 41 & 45 umol; p value 0.01) (Table 5.6).

5.7. Discussion

Oliguria and serum creatinine are the two main manifestations of renal dysfunction or acute kidney injury following perinatal ischaemia. In the cohort of 55 infants with NE, AKI was diagnosed in 36% infants and they were classified as those with AKI and those without according to the serum creatinine based, Kidney Disease Improving Global Outcomes (KDIGO) criteria. (Jetton JG et al 2012).

Oliguria was noted in 60% of the infants with AKI. Persistently altered and high levels of urea and serum creatinine in the absence of oliguria were noted at school age children with NE in comparison to school age children in the control group. Low sodium levels noted in infants with NE on Day 2 of life and associated with oliguria, had normalised in school age children with NE. Children with NE who had high urea and creatinine levels and low sodium at school age were non-oliguric. Oliguria is not a sensitive marker of AKI and if serum creatinine measurements are not monitored, AKI may not be recognised. As reported in previous studies (Aggarwal et al., 2005, Gupta et al., 2005) 60-78% of post asphyxial AKI is non-oliguric.

Hyper filtration of the surviving nephron following AKI in the neonatal period leads to imbalance in the tubular function and GFR causing Chronic kidney disease (CKD); (Subramanian et al., 2008). The development of CKD usually manifests with hypertension, proteinuria and elevation of BUN and serum creatinine (Andreoli, 2004). Thus, it is sensible to follow up infants who have sustained AKI in the neonatal period with regular lifelong measurement of growth and nutrition, serum creatinine, blood pressure and urinalysis (Andreoli, 2004, Moghal and Embleton, 2006, Subramanian et al., 2008).

Majority of children in our study with moderate to severe NE had high serum creatinine in the neonatal period and at school age. Though the value were within high range of normal, they were higher in comparison to the serum creatinine of children of the same age in the control group. However lower than normal levels of serum creatinine were noted in children with severe CP with

GMFCS IV/V. Creatinine is a product of muscle creatinine-phosphate metabolism, and its level reflects muscle mass.

We demonstrated that moderate to severe neonatal encephalopathy and poor neurological outcome was associated with high urea and creatinine level in the neonatal period and at school age. In addition, urinary biomarkers NGAL, Cystatin C and osteopontin (OPN) were noted to be significantly elevated across all time points in infants with moderate to severe NE (Grade II/III) and with AKI in comparison to infants without AKI and mild NE. This association of AKI and brain injury has been described (Gupta et al., 2005, Perlman and Tack, 1988) but the underlying cause remains unclear.

Oliguria and severity of AKI is associated with severity of the HIE. Therapeutic hypothermia (TH) is not a confounder, rather it has protective effect on the kidneys. Haemodynamics occurring during TH are not associated with oliguria or AKI (Stavel M et al;2015)

The infants diagnosed with AKI are usually critically unwell with high mortality and morbidity and therefore are at risk of brain injury and poor neurological outcome. Renal dysfunction may be part of multiorgan damage following perinatal asphyxia or there may be close link between AKI and inflammation as described in previous animal studies. It was noted that mice who had AKI were found to have increased activation of proinflammatory chemokines, disruption of blood brain barrier and brain inflammation compared to mice without AKI (Liu et al., 2008a). Persistent inflammation thus may be the cause of altered renal dysfunction seen at school age in these children with NE. However broader understanding of this mechanism and further studies are required for validation.

The prognosis and recovery from AKI are dependent upon the underlying cause of the injury and is much worse in neonates who suffered multi-organ failure. It is suggested that infants with Acute Renal Failure (ARF) need life-long monitoring of renal function, blood pressure and urinalysis (Andreoli, 2004). Urinary biomarkers may prove to be helpful in predicting renal outcome following NE and in directing clinical care. Analysing a panel of certain biomarkers; NGAL, Cystatin C, EGF and OPN which were useful in predicting AKI

and demonstrated an association with the severity of encephalopathy, may strengthen outcome prediction (Sweetman et al., 2016). Further studies and broader understanding of the role of urinary biomarkers and AKI are required. Follow up of renal function and validation of the urinary biomarkers may guide in prompt diagnosis and management of subtle chronic kidney dysfunction in children post neonatal encephalopathy.

Table 5-1 Comparison between maximum daily electrolyte levels (Na, K & Cl) and general treatment/outcome measures of neonates with NE

	TH		Seizures		NE Grade		MRI Brain		Mortality	
Day	**Mean**	**p**	**Mean**	**p**	**Mean**	**p**	**Mean**	**p**	**Mean**	**p**
[1]Na 1	132.3 (4.4), 132.5 (4)	0.86	131 (4.9), 133.5 (3.3)	**0.01**	133.5 (3.2), 131.7 (4.6)	0.09	132.5 (3.5), 131.1 (5.5)	0.25	134.0 (2.6), 132.3 (4.3)	0.51
[1]D2	131.5 (4.4), 133.6 (4.8)	0.07	131.3 (4.7), 133.8 (4.4)	**0.03**	134.3 (5.0) 131.7 (4.4)	**0.04**	132.3 (4.3), 132.3 (5.1)	0.98	129.3 (1.2), 132.6 (4.7)	0.24
D3	132.1 (4.6), 134.7 (2.8)	**0.02**	133.6 (4.4), 132.4 (3.7)	0.30	134.4 (2.6), 132.8 (4.4)	0.29	132.9 (4.0), 133.1 (4.5)	0.85	128.0 (4.2), 133.3 (4.1)	0.08
K1	4.3(0.8) 4.5(0.6)	0.37	4.3(0.8), 4.4(0.7)	0.54	4.4(0.5), 4.4(0.9)	0.85	4.2(0.6), 4.3(0.8)	0.61	4.9(1.8) 4.3(0.7)	0.17
D2	4.0(0.8) 4.2(0.6)	0.42	4.2(0.8), 3.9(0.6)	0.17	4.1(0.6), 4.1(0.8)	0.95	3.7(0.4), 4.4(0.8)	**<0.001**	5.7(0.3) 4.0(0.6)	**<0.001**
[1]D3	4.2(0.8) 4.2(0.7)	0.94	4.4(0.8), 3.9(0.6)	**0.02**	3.8(0.8), 4.3(0.7)	0.09	3.9(0.6), 4.5(0.8)	**0.004**	5.0(1.0) 4.2(0.7)	0.13
[1]Cl D1	100.3 (5.1), 99.9 (4.4)	0.74	98.7 (4.4), 101.2 (4.8)	**0.03**	101.2 (4.5), 99.5 (4.9)	0.15	100.2 (5.4), 99.5 (4.9)	0.65	99.7 (2.5), 100.1 (4.9)	0.87
D3	101.5 (6.2), 102.7 (3.9)	0.42	101.8 (5.6), 102.1 (5.4)	0.84	103.1 (3.9), 101.7 (5.7)	0.49	103 (6.3), 100.9 (4.7)	0.18	93.5 (5.0), 102.3 (5.3)	**0.02**

[1]Correlation of low sodium on Day 1 (D1) and (D2), high potassium on D3 and low chloride with increased seizure activity. Means (SDs) of cooled(n=39) vs non-cooled, seizure(n=39) vs non-seizure infants, normal(n=32) vs abnormal(n=53) grade of NE, normal(n=37) vs abnormal(n=31) MRI brain, death(n=4) vs survival(n=81) listed in each column for each electrolyte followed by p-value (p)(Independent samples student-t test). Na = Sodium (mmol/L); K = Potassium (mmol/L); Cl = Chloride (mmol/L).

Table 5-2 Renal function of school age children who had NE vs Controls

Variable	NE (n=42)	Control (n=54)	P-value
Urea	5.2 (1.2)	4.4 (1.12)	***0.001**
Creatinine	45.40 (5.4)	40.8 (11.9)	***0.025**

Means (SDs) of urea and creatinine levels of children who had NE vs age matched control, with *p values<0.04 as significant, (independent sample student-t-test).

Table 5-3 Renal function in children with mild to moderate NE

Variable	NE 0/I (n=14)	NE II/III (n=28)	P-value
Urea	5.62 (1.0)	5.08 (1.2)	0.17
Creatinine	45.3 (3.4)	45.4 (6.2)	0.96

Means (SDs) of urea and creatinine levels of children with Mild NE (NE0/I) vs Moderate to Severe NE (NEII/III) with *p values<0.04 as significant, (independent sample student-t-test).

Table 5-4 Electrolyte levels in children with NE in comparison to controls

Variable	NE (n=42)	Control (n=54)	P-value
Sodium	136.6 (2.2)	139.7 (2.3)	***<0.001**
Potassium	3.97 (0.25)	3.98 (0.33)	0.78
Calcium	2.34 (0.89)	2.29(0.78)	***0.01**
Phosphorus	1.5 (0.12)	1.4 (0.17)	0.90

Means (SDs) of electrolytes of children who had NE (n=42) vs age matched control (n=54), with *p values<0.04 as significant, (independent sample student-t-test).

Table 5-5 Renal function and electrolytes values in children with NE

Variable	Control (n=54)	NE (n=42)	CP (n=17)	P-value (a)	P-value (b)
Urea	4.4 (1.12)	5.2 (1.2)	3.7 (1.4)	***0.001**	***0.01**
Creatinine	40.8 (11.9)	45.40 (5.4)	35 (10)	***0.025**	***0.03**
Sodium	139.7 (2.3)	136.6(2.2)	140 (1.5)	***0.001**	0.09
Potassium	3.98 (0.33)	3.97 (0.25)	4.1 (0.36)	0.78	0.07
Calcium	2.24(0.78)	2.34 (0.89)	2.3 (0.8)	0.08	0.08
Phosphorus	1.4 (0.17)	1.5 (0.12)	1.5 (0.12)	0.90	0.09

Means (SDs) of urea (mmol/l), creatinine (umol/l) & electrolytes (mmol/l) of children with NE (n=42) vs Control (n=54) vs children with CP and complex needs (n=17) *p (a) p value NE vs Control and *p (b) p value CP with complex needs vs Control group.

Chapter 6

HAEMATOLOGY

6. Haematological System

6.1. Introduction

Haematological abnormalities including anaemia, thrombocytopenia, leucocytosis and coagulation abnormalities in infants may result as a part of multi-organ dysfunction following ischaemic injury in the perinatal period (Hankins et al., 2002). Cerebral ischaemia in neonates induces an immediate innate immune response within minutes of the insult (Algra et al., 2013). Following the insult, there is proliferation of pro and anti-inflammatory cytokines leading to breakdown of the blood brain barrier resulting in infiltration of the brain by circulating leucocytes, neutrophils, monocytes and T cells exacerbating the inflammatory response and brain damage. (Liu and McCullough, 2013).

In the pre therapeutic hypothermia era, elevated total circulating white blood cells and an absolute neutrophil count in term newborns with Neonatal Encephalopathy (NE) was associated with a higher risk of adverse long-term neurological outcome compared to similar infants with normal leukocyte counts (Morkos et al., 2007). Elevated peripheral leucocyte count is associated with high risk of mortality and neurological disability in infants with NE. In a study on infants with ischaemic encephalopathy, significant difference in white cell count (WCC) and neutrophil count were noted in the first few hours of life between survivors and non-survivors. There are not many studies looking at the haematological abnormalities in school age children post NE.

Therapeutic hypothermia (TH) in term infants with NE have been associated with higher incidence of leukopenia, decreased leukocyte mobility and phagocytosis with a potential increased risk of infection (Chakkarapani et al., 2014, Jenkins et al., 2013). White blood count (WCC) and leucocyte count have also been noted to be significantly higher during first 3 days of life in neonates who received TH and had abnormal MRI compared to those who were not cooled and had normal MRI (Li et al., 2014).

Changes in absolute neutrophil count, lymphocyte count and neutrophil-to-lymphocyte ratio have also been demonstrated in neonates with HIE during the first 60 hours after birth suggesting a modulation in neutrophil/lymphocyte response. It was also revealed that the absolute lymphocyte count changes occurring at 0–12 h in neonates with HIE are likely due to disease progression, rather than therapeutic hypothermia (Povroznik et al., 2018).

6.2: Hypothesis

Haematological abnormalities in children with neonatal encephalopathy (NE) may be associated with neurodevelopmental outcome.

6.3 Aims

1. To evaluate haematological abnormalities in school age children with NE and compare the results with the abnormalities in the neonatal period.
2. To assess haematological abnormalities in school aged children with mild to moderate neonatal encephalopathy (NE), in comparison with children with normal development and children with severe cerebral palsy (CP).
3. To evaluate any correlation with persistent abnormal leucocyte count and poor neurological outcome.

6.4 Results

6.4.1. Clinical characteristics

Fifty- five school aged children who had NE were compared to 60 children born by normal delivery with normal Apgar scores and normal development. Infants with NE were classified by the severity of encephalopathy as NE 0 (n=3), NE I (n=17), NE II (n=32) and NE III (n=3) using Sarnat staging scores (Sarnat and Sarnat, 1976). Twenty-four children (43%) underwent therapeutic hypothermia (TH) for neonatal encephalopathy as per TOBY criteria (Azzopardi et al 2009). Forty-eight infants had MRI brain imaging, of which 27 were normal and 21 abnormal.

6.4.2 Haematological Parameters

Neonatal Period

The neonatal data was reviewed retrospectively. Comparison between the means (SDs) of all the haematological variables and TH, grades of encephalopathy, neurological examination, occurrence of seizures, and MRI brain result were made using the student t-test.

Infants who underwent TH had significantly lower white cell counts, and platelet counts compared to non-cooled infants. Low platelet counts were significantly associated with TH on day 1 – 7 (p-value < 0.001 – 0.03) and lower platelet counts on day 2, 3 and 6 were associated with NE grade II/III (p value = 0.02, 0.001, 0.006 and 0.02 respectively). Abnormal neurological examination at discharge was significantly associated with lower mean haemoglobin counts on day 3 and 5 (p values = 0.04, 0.005 respectively) and higher mean neutrophil counts on day 4 of life (p value = 0.003). High WCC count and neutrophil count on day 2,3,4 and 5 of life were significantly associated with mortality. (p value= 0.006, 0.003, 0.006 and 0.003 respectively for WCC and p-values 0.009, <0.001, <0.001, <0.001 respectively for neutrophil count) (Table 6.1 & 6.2).

6.4.3 Haematological Parameters at school age

Lab parameters including haemoglobin (Hb), platelet count (Plt), white blood cell count (WCC) and neutrophil (Neut) count were tested in school age children with NE and their values were compared to children with normal development. The values by histograms confirmed a normal distribution of the data and therefore means, standard deviations and parametric tests were used in the statistical analysis.

The WCC count in school age children with NE was found to significantly higher than children in the control group (mean WC of $9.1x10^9$ /L vs mean WC $8.1x10^9$ /L; p value=<0.02). However, no significant difference was seen between the neutrophil count, Hb and platelet values between the two groups (p value= >0.04). (Table 6.3)

The WCC and neutrophil count were significantly higher in children with NE II/III compared to children with NE 0/I (mean WCC $9.4x10^9$/L vs mean WCC $8.5x\ 10^9$/L; p value=0.07 and mean neutrophil count $4.3\ x\ 10^9$/L vs $3.3x\ 10^9$/L, p value 0.007). On comparing the haematological values between children with mild NE (NE 0/I) and children with moderate to severe NE (NE II/III), no significant difference was noted between the haemoglobin (Hb) or platelet level at school age (mean Hb 11.94g/dL in NE 0/I vs mean Hb 12.26g/dL in NE II/III , p value=0.6 and platelet count of $293\ x\ 10^9$/L vs $311\ x\ 10^9$/L, p value=0.4) Similarly, on comparison of children with NE who received (TH) versus those who did not , no difference in the haematological values were noted at school age (Table 6.4).

6.5 Correlation of haematological parameters with outcome

A strong association was noted between infants with moderate to severe NE (NE II/III) and poor neurodevelopmental outcome as detailed below. Comparison between the means (SDs) of all the haematological variables, grades of NE, therapeutic hypothermia (TH) MRI brain result, and outcome was done using the student t-test. Infants who underwent TH had significantly lower white cell count and platelet counts (WCC on day 2 (13.0g/dL (5.2) versus 15.6g/dL (3.8), p value = 0.03) and mean platelet count on day 1 to 7 (p-value < 0.001 – 0.03) in comparison to non-cooled infants. Significantly lower mean (SD) haemoglobin counts on day 5 and 6 of life were seen in infants who developed seizures (13.5g/dL (3.4) versus 17.0g/dL (2.6); 14.6g/dL(2.7) versus 17.4g/dL (1.9), p-values = 0.047, 0.036 respectively) along with significantly higher mean (SD) neutrophil counts on day 1 of life ($14.5x10^9$/L (5.2) versus $12.0x10^9$/L (3.8), p value = 0.018).

We also correlated the cognitive score assessed on Bayley scale of infant development (BSID III) done at 2 years of age, with WCC and neutrophil count of children with NE, done in the neonatal period and at school age. Statistical analysis was done using bivariate Spearmann correlation. There was significant association with maximum WCC and neutrophil count in the neonatal period and low Bayley's Composite Cognitive Score (rho = 0.400, p value=0.007) for WCC and (rho=0.310, p=0.04) for neutrophil count. However, there was no correlation with the BSID scores and WCC at school age in children with NE. (Table 6.6 and Figure 6.1). Non-parametric tests i.e. the Mann-Whitney U test were used to compare the cognitive, motor and language scores with grades of NE, seizures and abnormal neurodevelopmental outcome (Cerebral palsy, speech delay and epilepsy). Bayley's low motor scores and communication scores were associated with poor outcome (p value= 0.02). A strong correlation was also seen between the grades of NE and BSID; low motor and communication scores were seen in children with moderate to severe NE compared to mild NE (p value=0.04 and 0.02 respectively) (Table 6.5).

6.6. Discussion

Infants with NE had elevated WCC and neutrophil counts and this was associated with abnormal neurological examination at discharge and mortality. In the pre-TH era Morkos et al also corroborated this finding (Morkos AA et a., 2007). They used the Paediatric Cerebral Performance Category Scale (PCPCS) score to measure long-term neurologic outcome at 18 months of age retrospectively in 30 term neonates with HIE and the outcomes dichotomized as either good or poor. They compared between the two PCPCS groups the white blood cell and ANC levels done during first four days of life and magnetic resonance imaging (MRI) performed within one month of life. They reported a significantly higher WCC and ANC levels in the first 96 hours after birth in neonates with poor outcomes compared to those with good outcomes.

However, to our knowledge there are very few follow up studies looking at the haematological abnormalities in children with NE at school age. We have demonstrated that school age children with NE have elevated WBC in comparison to school age children with normal birth and development. In addition, the elevated WBC count seen in infants with NE was associated with low composite cognitive scores on Bayley's scale of Infant Development. (BSID). Hypothermia has been associated with increased incidence of leukopenia, thrombocytopenia and coagulation disturbances. TH can lead to a decrease in the activation of the inflammatory cascade and subsequently reduce cytokine release (Drury et al., 2010, Chatzipanteli et al., 2000). This in turn may cause neurotoxic effect leading to long-term damage to the brain.

Increased inflammatory reaction following a hypoxic ischaemic insult result in infiltration of the brain by peripheral leucocytes, aggravating brain injury. This process may manifest with elevated WCC and neutrophilia. (Liu and McCullough, 2013). We demonstrated a persistently elevated white cell count at school age in children with NE compared to controls. We also demonstrated that an elevated white cell count in neonatal period may be associated with poor cognitive performance and neurological outcome. The findings described in our study demonstrate that monitoring haematological parameters especially white cell count and neutrophil count are important in children who have neonatal encephalopathy as these abnormalities can help in prediction of long-term neurological outcome.

Table 6-1 Maximum daily values and associations between haematological indices and, TH and outcome in neonates with NE

Haem Indice	TH n=39		Seizures n=39		NE Grade II/III (n=52)		Abn MRI Result (n=31)		Death (n=4)	
	Day	**p**	**Day**	**p**	**Day**	**P**	**Day**	**p**	**Day**	***P on Day 2-5**
[1]WCC	2	0.031	N/S	N/S	N/S	N/S	N/S	N/S	2-5	0.006, 0.003, 0.006, 0.003
Neut	N/S	N/S	1	0.18	N/S	N/S	N/S	N/S	2-5	0.009, <0.001, <0.001, <0.001
Plat	1-7	<0.001 -0.03	N/S	N/S	2,3,& 6	<0.001 0.011, 0.019,	N/S	N/S	N/S	N/S

Day represents the time point when there was a significant association found between for e.g. maximum daily WCC & neutrophil count and death (Independent student-t test), followed by a p-values (p) on Day 2-5, significance taken as < 0.05; NE = Neonatal Encephalopathy; N/S = Not significant; TH = Therapeutic Hypothermia; Abn = Abnormal, WCC=white cell count, Neut= neutrophil, Plat= Platelets

Table 6-2 Daily values of Platelet count in Neonates with NE and TH.

[1]Platelet	TH	Number	Mean	Std. Deviation	P value
D1	Yes	26	184.12	67.12	***0.010**
	No	25	229.96	56.67	
D2	Yes	25	147.96	48.91	***0.001**
	No	21	208.95	67.92	
D3	Yes	23	140.52	52.34	***0.001**
	No	13	221.38	81.60	
D4	Yes	14	141.36	71.12	0.20
	No	4	194.50	87.65	
D5	Yes	6	73.50	35.43	***0.007**
	No	5	234.80	107.88	
D6	Yes	7	127.14	51.81	0.09
	No	6	209.83	104.67	
D7	Yes	12	197.50	76.15	0.16
	No	5	277.60	153.05	

Daily Platelet count of infants with NE with TH vs those without TH Plt= Platelet count, D1 =Day1 of life, NE = Neonatal Encephalopathy; N/S = Not significant; TH = Therapeutic Hypothermia; Independent student-t test), SD = Std Deviation followed by a p-value (p), significance taken as * p value < 0.05.

Table 6-3 Haematological values of WCC, Neutrophil, Hb and Platelet count

Haem indice	Group	N	Mean	SD	p- value
WBC	NE	48	9.15	1.88	***0.023**
	Control	52	8.16	2.35	
NEUT	NE	48	4.04	1.19	0.50
	Control	52	3.86	1.61	
Plt	NE	47	305	70.31	0.50
	Control	53	314	72.45	
Hb	NE	48	12.15	0.73	0.20
	Control	52	12.36	1.14	

Hb = Haemoglobin (g/dL); Plt = Platelet count (x 10^9/L); WBC = White blood cell count (x 10^9/L); Neut = Neutrophil count (x 10^9/L). N= number of children, SD=Standard deviation; p-value (p), significance taken as < 0.05

Table 6-4 Haematological values of school age children who had mild grade (NE 0/I) and moderate to severe (NE II/III)

Haem indice	Grade NE	N	Mean	SD	p- value
WCC	**NE 0/I**	15	8.54	1.26	0.07
	NE II/III	33	9.42	2.06	
Neut	**NE 0/I**	15	3.37	0.92	***.007**
	NE II/III	33	4.34	1.18	
Hb	**NE 0/I**	15	11.9	0.67	0.28
	NE II/III	33	12.2	0.75	
Plt	**NE 0/I**	15	283.6	63.94	0.15
	NE II/III	32	315.3	71.82	

Hb = Haemoglobin (g/dL); Plt = Platelet count (x 10^9/L); WBC = White blood cell count (x 10^9/L); Neut = Neutrophil count (x 10^9/L). N= number of children, SD=Standard deviation; p-value (p), significance taken as < 0.05

Table 6-5 Correlation of BSID Cognitive score with Neonatal haematological parameters

Variable	**Spearmann Coefficient (rho)**	**P value**
	Bayley Cognitive score	
Bayley Cog score	1	-
[1]Max_ WCC	0.40	***0.007**
Max_Neut	0.31	***0.04**

Significant association of maximum WCC & Neutrophil with BSID cognitive scores. Bayley Cog Score= Bayley's Cognitive score, Max WCC= Maximum white cell count, Hb= Haemoglobin, Plt= Platelet, rho= Spearmann correlation coefficient, *significant p-value <0.05

Chapter 7
INFLAMMATION

7. Inflammation

7.1. Introduction

Inflammation plays an important pathophysiological role in perinatal brain injury and neonatal encephalopathy (NE). Inflammation can precede NE or occur during or after injury to the brain. In the perinatal period Injury to the brain is one of the leading causes of cerebral palsy, epilepsy, cognitive and sensory impairment. Diffuse activation of microglia in the neonatal brain occurs during inflammation which increases the injury process by expressing inflammatory mediators and pro-inflammatory cytokines. (Leviton et al., 2005, Berger et al., 2012). The proinflammatory cytokine can cause further damage by activating the natural killer cells, T-cells and lymphokine activated killer cells (LAK cells). This leads to cell proliferation, cell damage, white matter changes and long term neurological damage (Leviton et al., 2005, Khwaja and Volpe, 2008).

A hypoxic ischaemic insult at birth may also alter the neutrophil phenotype in neonates. Decreased neutrophil apoptosis was demonstrated in neonates resuscitated at birth. Pro-inflammatory neutrophil response was demonstrated in neonates with mild encephalopathy whereas infants with moderate or severe NE showed tendency towards immunosuppression (Molloy et al., 2007). Neonates resuscitated at delivery with abnormal neuroimaging were demonstrated to have increased CD11b, reactive oxygen intermediates (ROI) and Toll like receptor (TLR-4). Increased polymorphonuclear leukocytes TLR-4 expression was associated with increased mortality in infants with NE (O'Hare et al., 2016).

Inflammasome are innate immune system receptors and sensors that induces inflammation in response to microbes & sterile stressors. NLRP3 is a most widely studied inflammasome, expressed in cytosol of monocytes, neutrophils, lymphocytes & dendritic cells. It has been implicated in the pathogenesis of a wide variety of diseases, including genetically inherited auto inflammatory conditions & chronic diseases, such as inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis etc in which NLRP3 is abnormally activated (Ozaki et al., 2015). It also recognizes a wide variety of microbes, including *Staphylococcus aureus*, *Escherichia coli*, *Influenza A* virus, and *Candida albicans* (Hise et al., 2009).

Inflammasome and hypoxia inducible factor-1α (HIF1α) pathways are important mediators of inflammation and brain injury. The inflammatory effects triggered by proinflammatory cytokines, or LPS on the developing brain of neonates may have long term consequences on their ability to cope with infection.

Therapeutic hypothermia has proven to be effective in reducing mortality in preterm and term infants with NE. A Cochrane review with evidence from 11 randomised controlled trial has proven it to be beneficial (Jacobs et al., 2013). Melatonin has also been reported to be neuroprotective, by its immunomodulatory properties. It decreases the production of CD11b in neutrophils, which is a marker of neutrophil activation and migration and may depress the augmented systemic inflammatory response seen in infants with neonatal encephalopathy.

Perinatal inflammation is also associated with many neuropsychiatric and neuropsychological disorders and it is suggested that inflammation has long term consequences on the brain during childhood (Hagberg et al., 2012). Studies have suggested that the injury processes can persist for months and years and a tertiary mechanism of damage has been proposed which includes inflammation and epigenetic changes (Hagberg et al., 2012)

7.2. Hypothesis

Altered inflammatory response persists at school age in children with Neonatal Encephalopathy (NE)

7.3. Aims

1. To describe the altered inflammatory response in children with NE including the activation of neutrophils and monocytes in response to endotoxin (LPS).
2. To examine the response of genes for Hypoxia inducible factor- 1 α (HIF1α) and inflammasome in children with NE.
3. To assess the inflammatory response in children with mild to moderate NE in comparison to children with normal development and children with severe CP.

7.4. Results

In our study we examined systemic expression of the genes for Hypoxia inducible factor- 1 α (HIF1α) and inflammasome as well as monocyte and neutrophil activation in response to endotoxin (LPS) and melatonin in children with Neonatal Encephalopathy (NE) at school age compared to children with normal development and children with severe cerebral palsy (CP). We looked at 3 groups of patients; school age children with moderate NE, children with normal development and children with severe Cerebral Palsy (CP) due to an underlying cause other than NE.

7.4.1 Clinical Characteristics

Children in the NE group (n=5) were classified to have NE Grade II according to Sarnat and Sarnat Staging, and had underwent therapeutic hypothermia (TH), in accordance with the total body hypothermia criteria for neonatal encephalopathy (Azzopardi et al., 2009). They were in the age range of 4-6 years and were all males. They were compared with children with normal development, in the age range 4-14 years (n=5). There were no differences in gender distribution, birth weight and mode of delivery between the children with NE and control group. Children in the severe CP group had complex needs including epilepsy, feeding difficulty, cortical visual impairment and hearing defect. (n=5).

7.4.2 Inflammasome gene expression in children with NE

The mRNA expression of inflammasome genes; (NLRP3) NOD like receptor containing pyrin domain, ASC (Adaptor protein apoptosis associated speckle like protein) and IL1β (Interleukin 1β) in whole blood, before and after treatment in vitro with Lipopolysaccharide (LPS) (10ng/ml) and Melatonin (42μM) using real time polymerase chain reaction (RTPCR), were measured. We also looked at the expression of HIF1α in the above group of patients.

We found elevation in the expression of NLRP3 gene in children with NE in comparison to control and children with severe CP at baseline and significant increased response on treatment with LPS in vitro. We noted a peak in the expression of NLRP3 which was seen to be reduced on treatment with Melatonin in vitro with > 2-fold change noted between children with NE and children in the control group (p value <0.04; Figure 7.1). The expression of gene ASC (Adaptor protein apoptosis Speck like containing CARD) and Interleukin 1β (IL1β) were also noted to be increased in children with NE, both at baseline and in response to LPS stimulation, though not significant, compared to children with normal development and children with CP and complex needs (Fig 7.2 & 7.3)

HIF1α was significantly increased in CP group compared to the control group both at baseline and on treatment with LPS. There was also a significant increase in the expression of HIF1α following melatonin treatment alone in the NE group versus the controls (p<0.03). (Figure 7.4).

7.4.3 Surface expression of CD11b, TLR4 and NE

Flow cytometry was used to measure the expression of neutrophil and monocyte markers of function CD11b (neutrophil activation) and Toll like receptor (TLR)-4 (endotoxin recognition) in whole blood before and after treatment with lipopolysaccharide (LPS) (10ng/ml) and melatonin (42μM) in vitro, in children with NE (n=5)

The expression of neutrophil CD11b was significantly increased in children with NE (p=0.04) at baseline and after LPS stimulation in comparison to children in the control group. This was reduced with in vitro treatment with melatonin (probably due its immunomodulatory effect). A similar response was seen in children with CP, expression of neutrophil stimulated CD11b was significantly increased compared to control group but the response was less compared to that seen in children with NE (Figure 7.5)

There was a significant increase in LPS-stimulated neutrophil TLR-4 in school age children with NE compared to age and sex matched controls. Similarly, expression of TLR4 in neutrophil was seen to be increased in children with CP especially after LPS stimulation in vitro. (Figure 7.6).

7.5. Discussion

We have shown in our study that school age children with NE demonstrated vigorous systemic innate immune response compared to children with normal development. Neutrophil activation, HIF1α and NLRP3 inflammasome expression is elevated in school age children with CP and post-NE.

We have demonstrated increase expression of CD11b and TLR4 on the surface of neutrophils at baseline and following LPS stimulation, in school age children with NE in comparison to children with normal development. Previous studies have described increased CD18/CD11b expression along with delayed peripheral mononuclear cells (PMN) apoptosis in neonates suggesting their role in neonatal inflammation (Nguyen et al., 2010, Koenig et al., 2005). It has been demonstrated that higher neutrophil and monocyte CD11b and TLR-4 expression has been seen in neonates with abnormal neuroimaging with or without severe NE and increased TLR4 expression was associated with increased mortality in neonates with NE (O'Hare et al., 2016).

We demonstrated a correlation between altered and increased expression of TLR4 and severity of NE. Previous studies have demonstrated high TLR4 levels corelates with severity of acute cerebral infarction in adults (Yang QW et al; 2008). Animal studies also report an association of low TLR 4 levels and improved neurological outcome in mice who suffered from hypoxia ischaemic injury and intracerebral haemorrhage. (Samsing LH et al; 2011). Similarly, increased CD11b expression with delayed peripheral mononuclear cells (PMN) apoptosis has been described to be associated with neonatal inflammation. We have described similar results in neonates and children with NE(O'Hare et al., 2016).

Our study also demonstrates significantly high expression of HIF-1α and inflammasome genes NLRP3, ASC and IL1β in children with NE, and severe CP in comparison to age matched control group children, suggesting an altered inflammatory response in children with NE. We have shown that the expression of NLRP3 gene was significantly increased in these children with NE ($p=0.04$) after LPS stimulation in comparison to children in CP and control groups. This was reduced with in vitro treatment with melatonin (probably due its immunomodulatory effect).

We also noted that the HIF1α could be measured within these groups of children at the mRNA level from whole blood and that there was a significant difference in the hypoxic response on treatment with LPS and melatonin in the CP group (Figure 7-4).

Persistent inflammation may be associated with persistent activation of hypoxia-inducible factor (HIF) 1 alpha (Anand et al., 2007). HIF-1α upregulation is also associated with enhanced anti-bactericidal activity, phagocytosis and persistent neutrophilic inflammation (McGovern et al., 2011).

Periventricular leukomalacia (PVL) or related inflammation events or both during the perinatal and postnatal period may have a programming effect, causing altered inflammatory responses in preterm children with CP (Lin et al., 2010). In their study they demonstrated that preterm school-age children with periventricular leukomalacia (PVL) induced (CP) had significantly higher levels of tumour necrosis factor (TNFα) and elevated TLR4 mRNA in peripheral blood mononuclear cells (PBMC's), in comparison to preterm term school-age control group children.

Melatonin has several actions; several studies shows that it is an important anti-oxidant and has anti-inflammatory action (Mauriz et al., 2013, Dong et al., 2016, Agil et al., 2013).Melatonin through its anti-inflammatory action regulates pro and anti-inflammatory cytokines in different pathophysiological disorders. It plays a protective role against activation of NLRP3 inflammasome (Favero et al., 2017).

Our study suggests that dysregulated inflammation seen in new-born with NE may persist into childhood and is amenable to immunomodulation with melatonin.

To our knowledge, there are no studies published to date of TLR-4, CD11b and HIF 1α and inflammasome expression in school age children with NE. Neonatal Encephalopathy remains a major cause of mortality and long-term morbidity. There are universally accepted markers to identify infants with NE at risk of poor neurodevelopmental outcome. Further studies are required to validate these findings which may help in development of early tests or markers to predict outcome in children with NE. Targeting specific immune pathways may be a therapeutic option in persistent inflammation in children with brain injury.

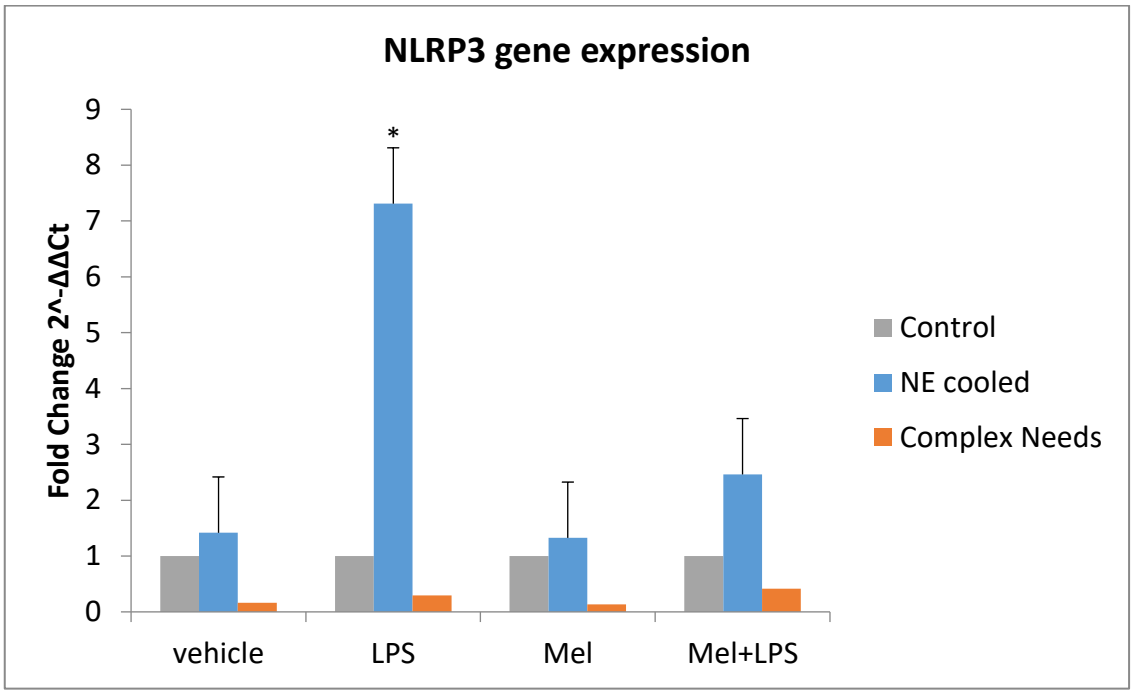


Figure 7-1 NLRP3 gene expression in children with NE

Whole blood from children with NE (n=5) and control group (n=5) was treated with 1uL (10ng/ml) of lipopolysaccharide (LPS), & 42 uM of Melatonin (Mel) and both (LPS & Mel) using Reverse polymerase chain reaction (rPCR). Results show fold changes in mRNA levels at baseline and after each treatment. *p value <0.04 seen in Baseline (Veh) NE vs NE LPS

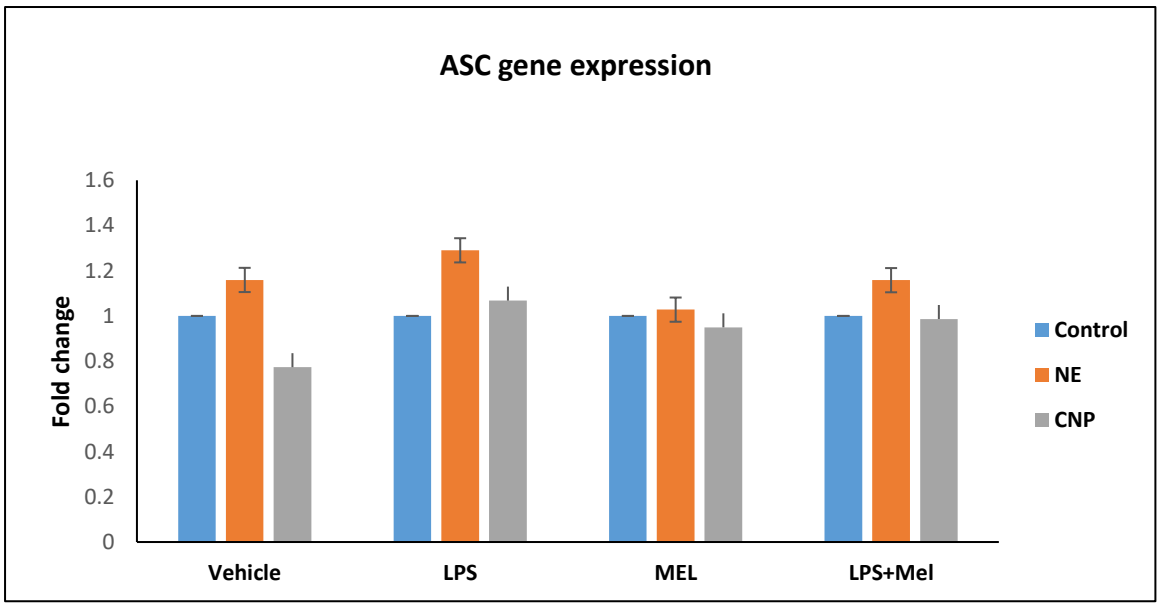


Figure 7-2 ASC expression in children with NE in comparison to control and children with complex needs.

Whole blood from children with NE (n=5) and control group (n=5) was treated with 1uL (10ng/ml) of lipopolysaccharide (LPS), & 42 uM of Melatonin (Mel) and both (LPS & Mel) using Reverse polymerase chain reaction (r-PCR). Results show fold changes in mRNA levels at baseline and after each treatment. ASC=Adaptor protein apoptosis Speck like containing CARD,

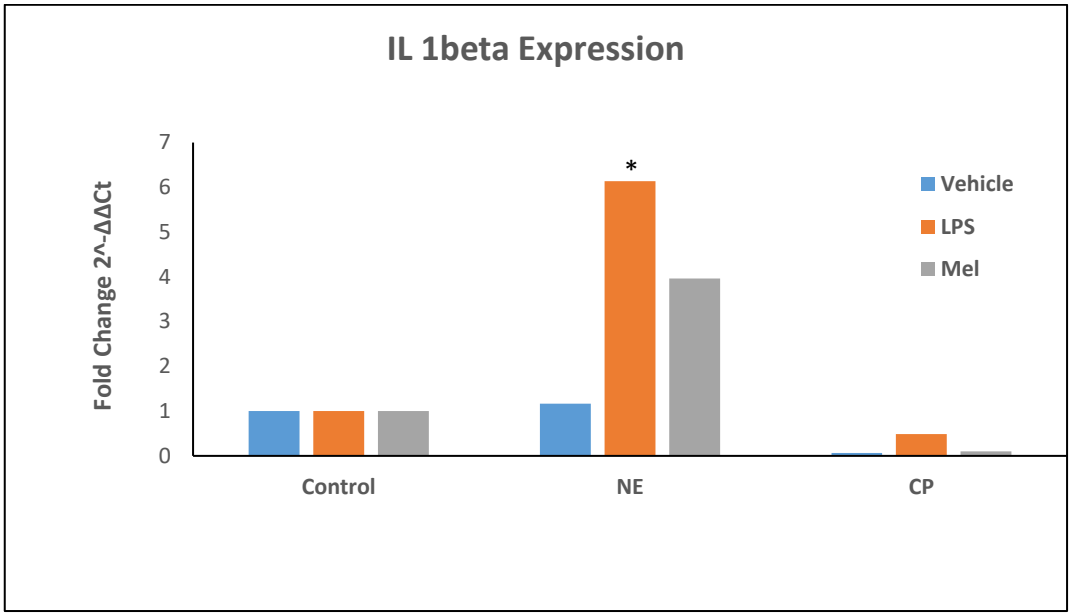


Figure 7-3 Interleukin 1β (IL1β) expression in children with NE in comparison to control and children with complex needs.

Whole blood from children with NE (n=5) and control group (n=5) was treated with 1uL (10ng/ml) of lipopolysaccharide (LPS), & 42 uM of Melatonin (Mel) and both (LPS & Mel) using Reverse polymerase chain reaction (r-PCR). Results show fold changes in mRNA levels at baseline and after each treatment. *p value <0.04 IL 1β in NE vs control

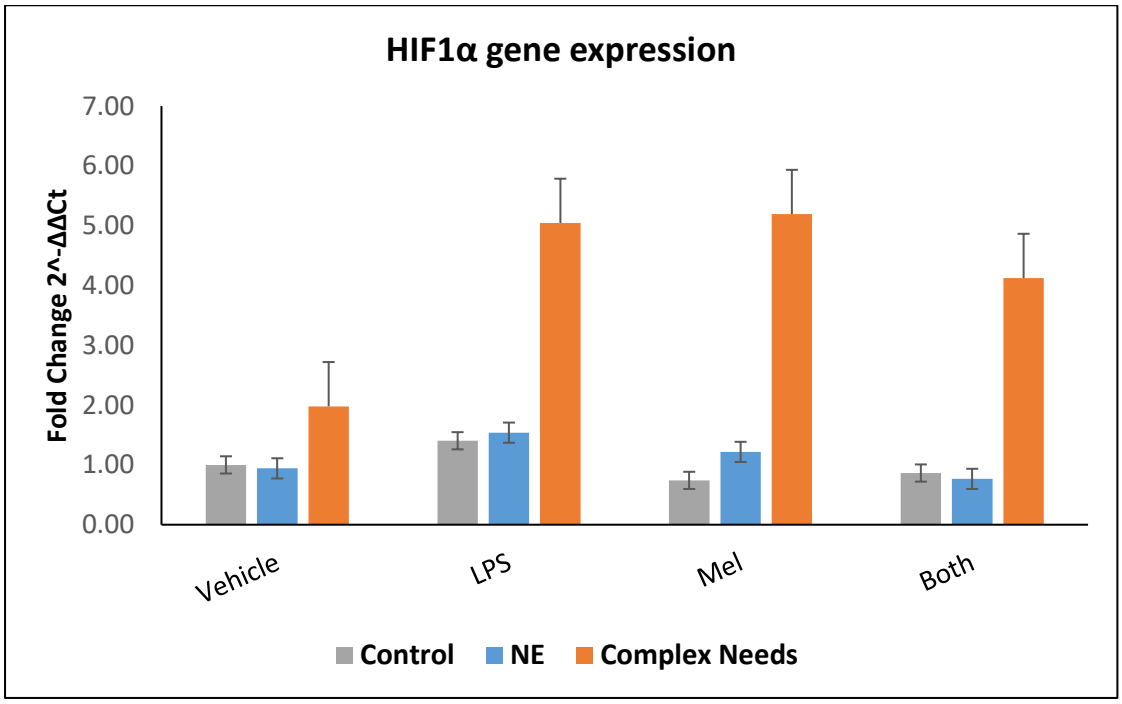


Figure 7-4 HIF 1α expression in children with NE

Whole blood from children with NE (n=5) and control group (n=5) was treated with 1uL (10ng/ml) of lipopolysaccharide (LPS), & 42 uM of Melatonin (Mel) and both (LPS & Mel) using Reverse polymerase chain reaction (r-PCR). Results show fold changes in mRNA levels at baseline and after each treatment. *p value <0.04 in children with CP on LPS stimulation vs children with NE & control.

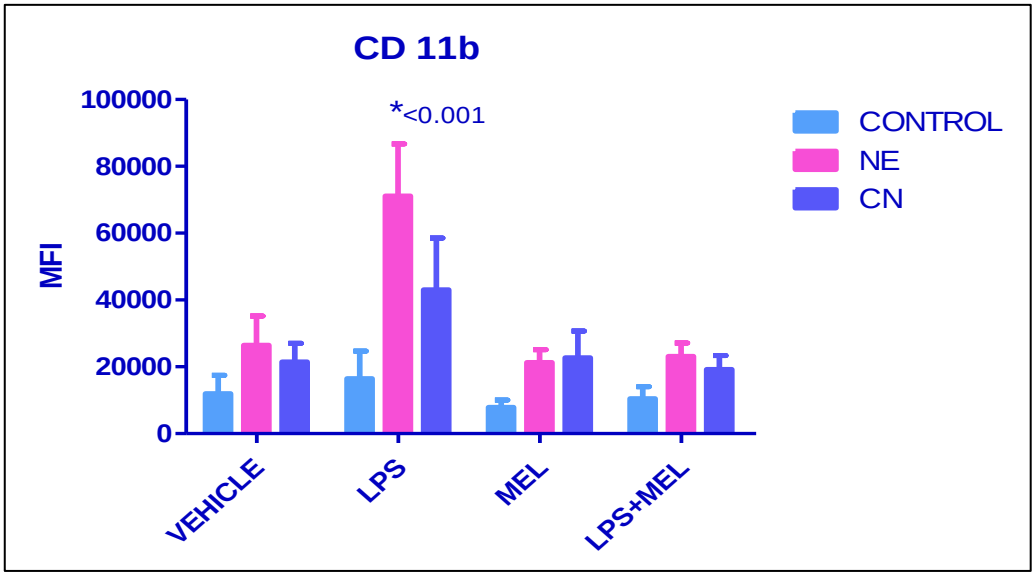


Figure 7-5 CD11b Expression in school age children with NE

Whole blood from children with NE (n=5), with CP and complex needs (CN) (n=5) and control group (n=5) was treated with 1uL (10ng/ml) of lipopolysaccharide (LPS), & 42 uM of Melatonin (Mel) and both (LPS & Mel) and expression of surface antigen CD 11b was analysed using flow cytometry. Results show mean fluorescence intensity (MFI) at baseline and after each treatment. *p value= <0.001 in NE on LPS stimulation vs NE baseline.

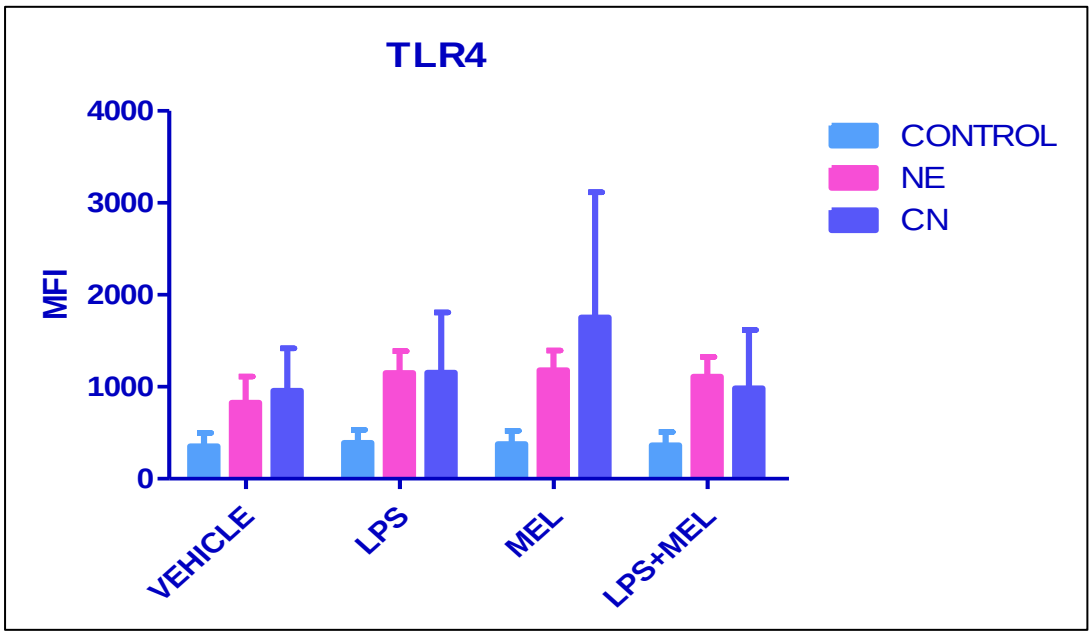


Figure 7-6 Expression of TLR4 in school age children with NE

Whole blood from children with NE (n=5), with CP and complex needs (CN) (n=5) and control group (n=5) was treated with 1uL (10ng/ml) of lipopolysaccharide (LPS), & 42 uM of Melatonin (Mel) and both (LPS & Mel) and expression of surface antigen TLR4 was analysed using flow cytometry. Results show mean fluorescence intensity (MFI) at baseline and after each treatment. *p value= <0.001 in NE on LPS stimulation vs NE baseline.

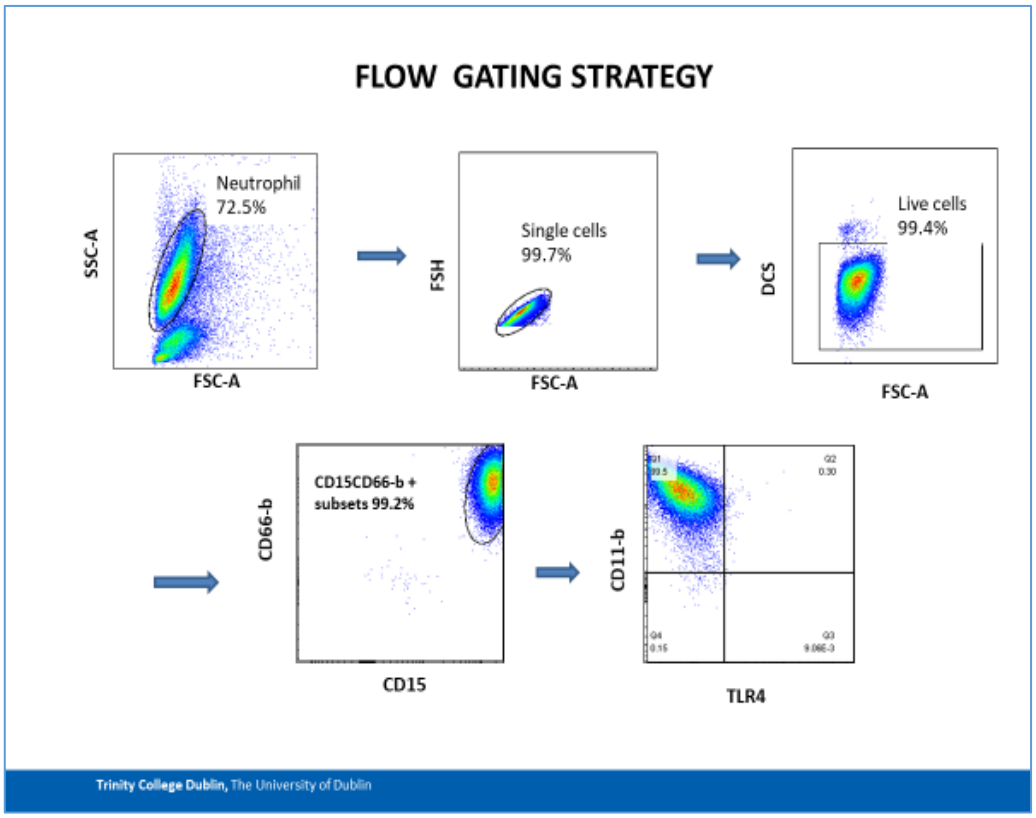


Figure 7.7 1: Dot plot of CD11b and TLR4 expression

Granulocytes identified & gated first, single cells plotted on scatter plots of FSH and FSC-A, identified as live cells on FSC-A and DCS. Live cells then gated & antibodies CD15b+ and CD66b+ (markers of neutrophils) are gated & plotted to quantify population of TLR4 and CD11b.
SSCA: side scatter area, FSC-A: Forward scatter area FSH: forward scatter height, DCS: dead cell stain.

Chapter 8

Cytokine response in children with Neonatal Encephalopathy

8. Cytokines

8.1. Introduction

Perinatal global hypoxia-ischaemia is associated with neonatal encephalopathy (NE) and can result in brain injury in both term and preterm infants. Neonatal brain injury is an important cause of neonatal death and disability such as cerebral palsy.

Inflammation combined with Hypoxia-ischemia (HI) plays an important pathophysiological role in neonatal encephalopathy (NE). Pro-inflammatory cytokine expression within the brain, especially of IL-1β and TNF-α, has been demonstrated following perinatal brain damage by pathogen triggers and hypoxic injury both in experimental model and the human new born brain (Dammann and Leviton, 2004).

The pro-inflammatory cytokines activate cytotoxic T cells, natural killer cells, by binding to specific cell surface receptors, which induce intracellular reaction resulting in cellular and tissue damage. This leads to cell proliferation, differentiation and cell death causing white matter damage and long-term neurological damage.

Previous studies suggest that the injury processes can persist for months and years and propose a tertiary mechanism of damage, which includes inflammation and epigenetic changes. (Hagberg et al., 2012). Infants with neonatal encephalopathy have been reported to have persistent inflammatory response over the first week of life correlating with the degree of brain injury. (Sweetman et al., 2017, O'Hare et al., 2016) Elevated levels of cytokines including elevated IL-6 noted in first few hours after birth in infants with NE who received TH were found to be associated with death and abnormal neurodevelopmental outcome at 12 months of age (Jenkins DD et al, 2012). We hypothesise that the inflammatory response persists in childhood and aimed to examine the cytokine profile of school aged children with neonatal encephalopathy in comparison to children with normal neonatal course. Understanding this persistent inflammatory mechanism could lead to safe and effective therapies to treat a developmentally disrupted brain long after the insult.

8.2.Hypothesis

Altered cytokine response persists in school age children with Neonatal Encephalopathy (NE).

8.3.Aims

1. To examine cytokine response in children with mild to moderate NE in comparison to children with normal development and children with Cerebral Palsy (CP).
2. To assess the response cytokine in children with NE who underwent Therapeutic Hypothermia in comparison to those who did not.
3. To compare the cytokine response in children with NE at school age with the cytokine response in the neonatal period.

8.4. Results

Clinical Characteristics

Cytokine analysis was performed on the serum of total 77 school age children (age range 4-7 years), including children who had NE (n= 37) and controls (n=40). We also assessed the cytokine profile of older children with severe CP (n=12) age range of (8-16 years) in comparison to age matched controls.

Cytokine analysis was done on 37 out of total 55 children with NE. These children were classified into different grades as per the Sarnat scores as infants (Sarnat and Sarnat, 1976). Cytokine analysis done on Infants exposed to asphyxia but with no neurological signs (designated as grade 0 for study purposes, (n =2); mild NE (grade I, n =11); moderate NE (grade II, n =22); and severe NE (grade III, n =2).

Out of the 24 children with NE II/III, 15 (62%) underwent treatment with therapeutic hypothermia (TH), 17 (70%) developed seizures in the neonatal period and 13 (54%) had abnormal MRI of the brain. However, there were no significant differences between the NE groups 0/I and II/III regarding gestational age, birth weight and gender, etc. (Table 4.1).

8.4.2 Cytokine Results in children with NE vs Control

Cytokines including GM-CSF, IL-2, IL-6, IL-8, and IL-18 were significantly higher (p value <0.05) in children with NE compared to controls.

A significant rise of serum cytokines GM-CSF, EPO, IL-6, IL-8, IL-1β, IFN Ý and TNF β were seen in children with NE in response to LPS stimulation (p value <0.05). IL-6 & IL-8 and TNFα were significantly increased in both groups (p<0.01). Increased concentrations of all pro-inflammatory cytokines were seen in both NE and controls following endotoxin stimulation. There was no statistically significant difference in IL-IRA, IL 2, and IL-18 on stimulation with LPS (Fig 8.5-8.6).

8.4.3 Cytokine Results in neonates with NE vs at school age

We compared the levels of serum cytokines associated with hypoxia inducible factor-1 (HIF-1) pathway EPO and VEGF, and cytokines IL-8 and GM-CSF in the neonatal period with the cytokine levels at school age. IL-8 was noted to rise at Day 2-3 of life and the response appeared to subside by day 5- 7, but at school age significantly high levels of IL-8 were noted compared to children with normal development. A similar response was noted with GM-CSF, it increased on Day 3 then decreased by Day 7 and again was noted to rise at school age in comparison to children in the control group. (Figure 8.1 & 8.2). However, EPO and VEGF did not show similar response, EPO increased at Day 1 in infants with NE, then the response subsided by Day 7 of life and did not rise at school age, similarly VEGF started to rise on Day 1 of life and remain elevated by Day 7. However, there was no change in response in VEGF at school age in comparison to control children (Figures 8.3 & 8.4). This result warrants the need to monitor cytokine levels at different time points. This also suggest a persistently altered inflammatory response at school age in children with NE.

8.4.4 Cytokine results in children with Mild NE vs Moderate to Severe NE

We also compared the results of different NE sub groups according to grades of encephalopathy IL-2 was significantly raised in children with NE II/III compared to NE0/I. Proinflammatory cytokines IL 18, IL-8, IL-6, IL-1α and GM-CSF were seen to be elevated in children who were classified in group NE II/III compared to children who were NE I, but the results were not statistically significant (p value >0.05). There was no difference in levels of IL-1β, TNFβ, IL-10 and EPO. (Fig 8.7 & 8.8).

8.4.5 Cytokine results in Children who underwent TH vs Normothermia

The children with NE who received therapeutic hypothermia at birth were also examined and their cytokine results were compared with children who were normothermic. The cytokine results showed significantly low levels of TNFβ ($p < 0.05$) and IL-6 ($p < 0.06$) in children who underwent therapeutic hypothermia in comparison to children who did not. Levels of EPO, GM-CSF, IL-18 and TNF-β were comparatively high in children who underwent hypothermia than in children who did not, but the values were not statistically significant. There was no significant difference seen between the groups in the levels proinflammatory cytokine, TNF α, IL-8, IL-6, IL-1α, VEGF and anti-inflammatory IL-10 and IL-1RA. (Fig 8.9 & 8.10)

8.4.6 Cytokine results in children with NE compared to children with CP

We compared cytokine levels in older children with severe CP (n=12) in age range of (8-16 years) with age matched controls. EPO was noted to be significantly high in older children with CP compared to age matched controls (p value 0.02). Other cytokines though were elevated in children with CP compared to control but did not reach statistical significance. (Fig 8.11-8.14)

8.5. Neurodevelopmental Outcome in children with NE

In 24 children with moderate to severe NE, 5 (20%) developed Cerebral Palsy, I child developed epilepsy, 5 suffered from speech problems, 2 had hearing deficit and 3 children had features suggestive of Developmental Co-ordination Disorder (DCD). Behaviour problems were also seen in 5 children who had NE. Abnormal neuroimaging in the neonatal period was seen in 14 children in the NE group, out of these 13 were classified to have NE II/III grade and one child with mild NE (NE0/I) had abnormal neuroimaging. Children with NE II/III with abnormal neuroimaging and underwent TH had significantly high level of TNF β, IL-6 and IL-1α (p value <0.05).

8.6. Discussion

We found a persistently abnormal cytokine response in school aged children who had neonatal encephalopathy. GM-CSF, TNF-β, IL-2 IL-6, IL-8, IL-10, IL-18, and EPO, were significantly higher in children with NE compared to controls. We noted significant rise of GM-CSF, IL-2, IL-6, IL-8 & IL-18 in children with NE in response to LPS stimulation. (p-value <0.05).

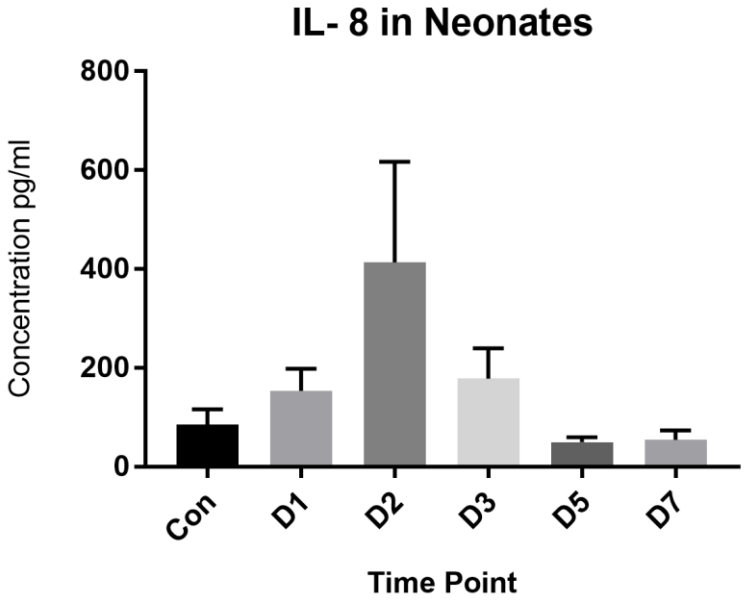
IL-8 was noted to be significantly elevated in school children with NE when compared with controls, and after in vitro treatment with an endotoxin (LPS) the response was seen to be very high in children with NE (p value=0.007). Previous studies have described significantly high level of serum IL-8 in neonates with perinatal asphyxia compared to normal healthy new-borns on day one of life. (Ozaki et al., 2004). Elevated IL-8 levels in the group of children with NE compared to normal children demonstrates a persistently altered immune response at school age. Proinflammatory cytokines (TNFα, IL-1β and IL-6) were demonstrated to be significantly increased in children with NE compared to control (Chaparro-Huerta et al., 2017).

IL-6 was noted be significantly high on LPS stimulation in children with NEII/III who received TH and had abnormal neurodevelopmental outcome (p value 0.005). Similar results have been described by Jenkins et al, elevated IL-6 noted in children with NE who received TH was reported to be associated with death and abnormal neurodevelopmental outcome at 12 months of age (Jenkins et al., 2013).

GM-CSF was also noted to be significantly elevated in children with NE compared to the controls (p value 0.025). However, there was no significant difference in children with NE who received therapeutic hypothermia at birth compared to those who did not. We demonstrated no significant difference in level of GM-CSF in NE group of children classified in NEII/III and had abnormal neuroimaging with poor neurological outcome compared with those who had a normal outcome. This could be since GM-CSF crosses the blood–brain barrier and may be neuroprotective. Previous experimental studies on stroke have shown that GM-CSF reduces damage (Schabitz et al., 2008).

We demonstrated that the anti-inflammatory cytokine IL-10 was comparatively high in children classified in NE II/ II and who had abnormal neuroimaging compared to those in NE I group. Correlation of high levels of IL-10 with multiorgan dysfunction & mortality was demonstrated in PICU patients. Patients with septic shock also demonstrated high levels of IL-10. (Marchant et al., 1995)

Neonatal Encephalopathy remains an important cause of mortality and long-term neurodevelopmental impairment including Cerebral Palsy. Hypoxic ischaemic injury induces an inflammatory response involving excessive cytokine production. We demonstrated significant alteration in cytokines in children with NE at school age compared to normal children which supports our hypothesis of a persistent inflammatory response years after hypoxic ischaemic injury. Further studies in a larger cohort are required to validate these findings.



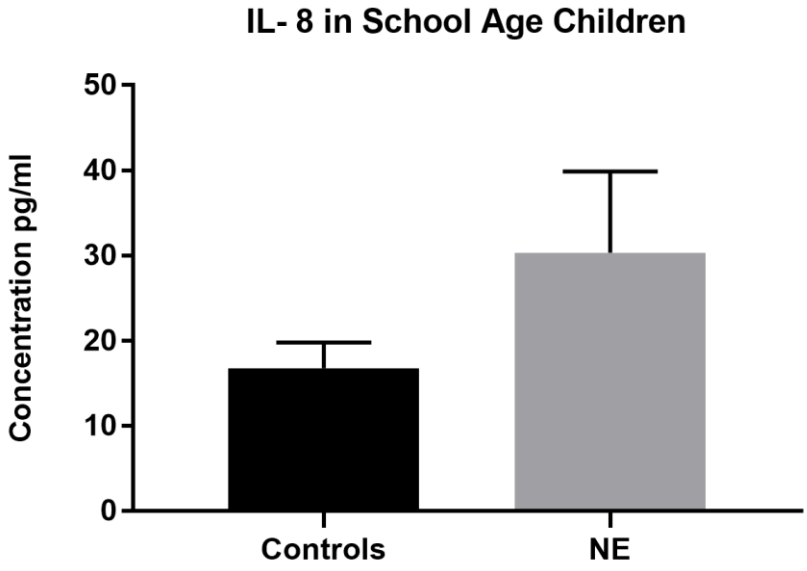


Figure 8-1 Cytokine response of IL-8 in neonates with NE and at school age, compared to age matched control children.

Serum of neonates at D1 of life (n=82) and school age children with NE (n=37) and neonates(n=12) and control group (n=40) were analysed using cytokine multiplex assay. Result of cytokine Interleukin-8 (IL-8) response was expressed as pg/ml, D1= Day of life1

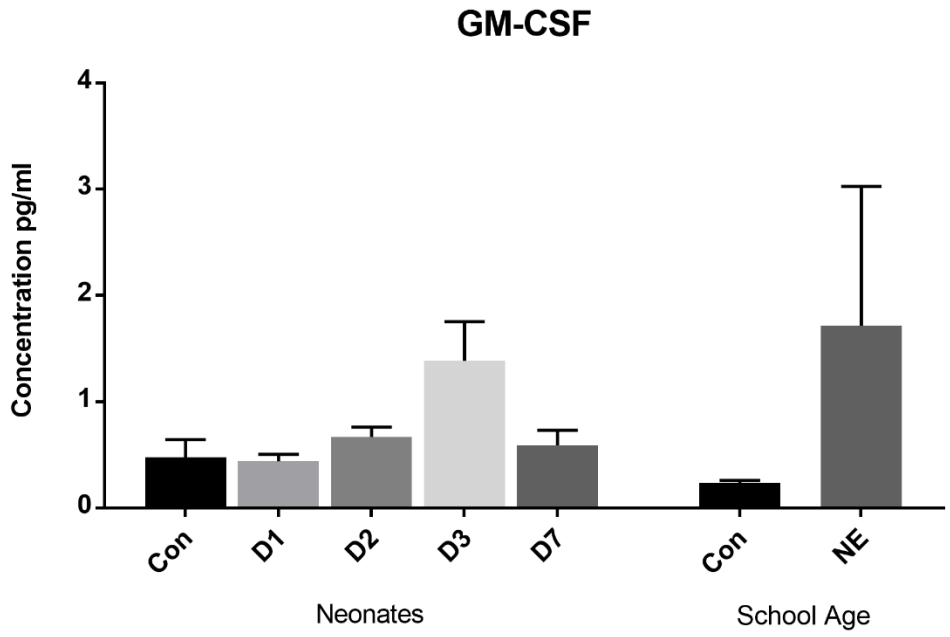


Figure 8-2: Cytokine response of GM-CSF in neonates with NE and at school age, compared to age matched control children.

Serum of neonates at D1 of life and school age children with NE and neonates and control group were analysed using cytokine multiplex assay**.**GM-CSF response expressed as pg/ml, in neonates with neonatal encephalopathy -NE (n=82) and children with NE (n=37), Control= age matched neonates (n=12) & control children (n=40). D1= Day of life1

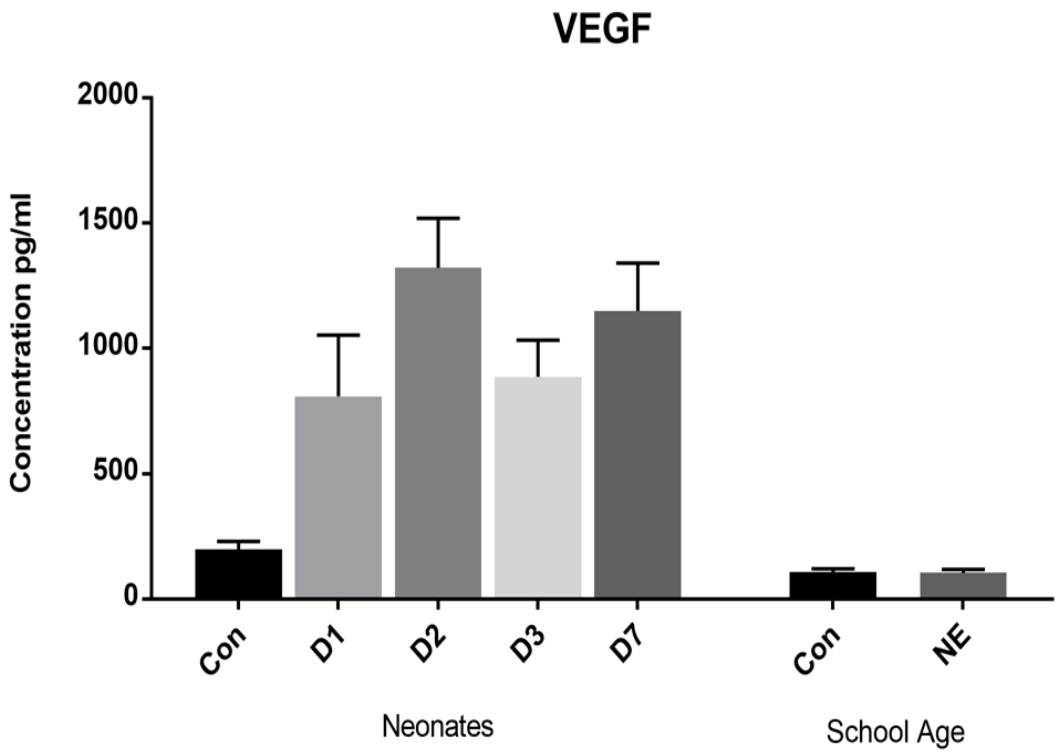


Figure 8-3 Cytokine response of VEGF in neonates with NE and at school age, compared to age matched control children.

Serum of neonates at D1 of life and school age children with NE and neonates and control group were analysed using cytokine multiplex assay. VEGF response expressed as pg/ml, NE= Neonatal Encephalopathy neonates with NE (n=82) and children with NE (n=37), Control= age matched neonates (n=12) & control children (n=40). D1= Day of life1

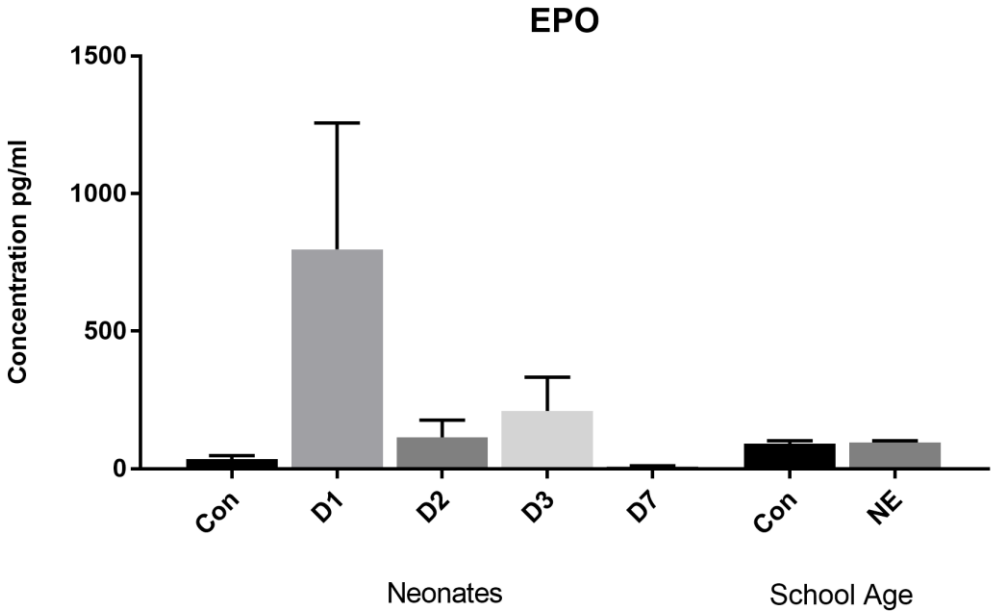


Figure 8-4 EPO Response in NE versus Control at Birth and in School Age Children

Serum of neonates at D1 of life and school age children with NE and neonates and control group were analysed using cytokine multiplex assay. EPO response expressed as pg/ml, NE= Neonatal Encephalopathy neonates with NE (n=82) and children with NE (n=37), Control= age matched neonates (n=12) & control children (n=40). D1= Day of life1

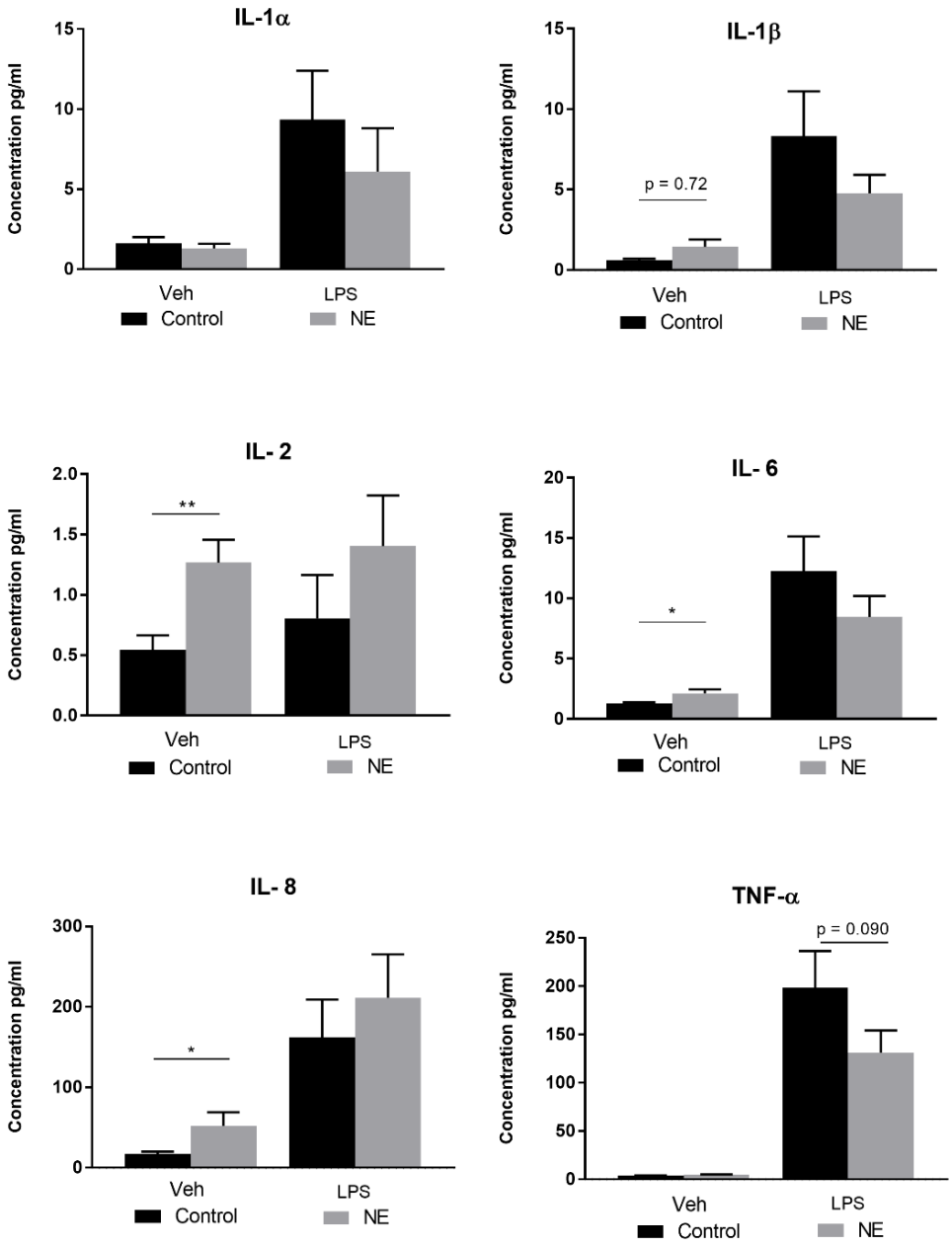


Figure 8-5 Cytokine response in school age children with NE compared to control at baseline and on LPS stimulation.

Whole blood was treated with 1ul (10ng/ml) of lipopolysaccharides (LPS). Cytokines were then measured in the serum of children with NE and control group using cytokine multiplex assay. Results of cytokine concentration expressed in pg/ml, TNFα= Tumor necrosis factor alpha, IL= Interleukin, IL-1β= Interleukin 1beta, Veh= Vehicle or baseline, * Significant star show difference between Control and NE groups.

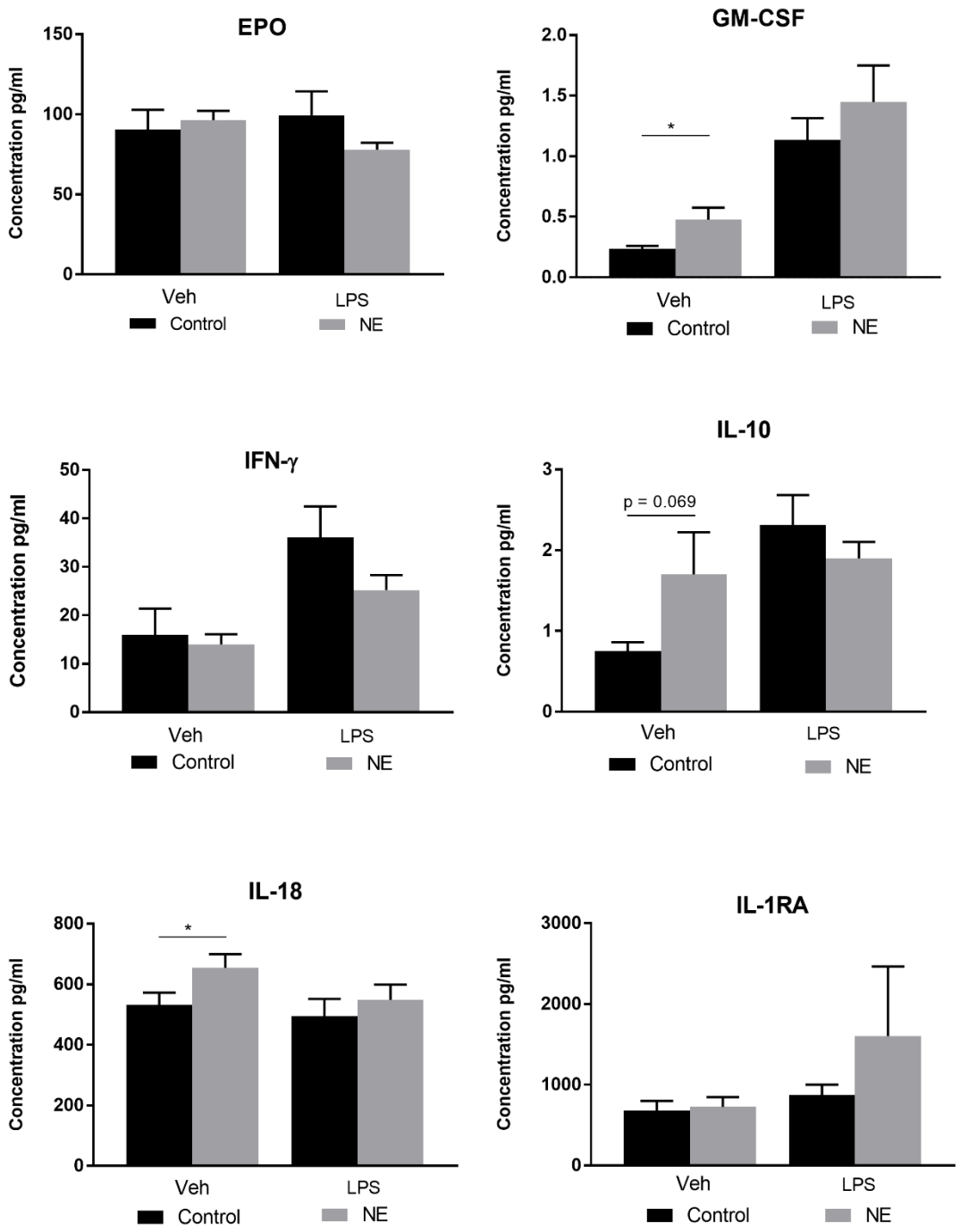


Figure 8-6 Cytokine response in school age children with NE compared to control at baseline and on LPS stimulation.

Whole blood was treated with 1ul (10ng/ml) of lipopolysaccharides (LPS). Cytokines were then measured in the serum of children with NE and control group using cytokine multiplex assay. Cytokine concentration expressed in pg/ml, GM-CSF= Granulocyte macrophage colony stimulating factor, IL-1RA= Interleukin1-Receptor antagonist, IL-1β= Interleukin 1beta, IFN Ý= interferon gamma, Veh= Vehicle or baseline, * Significant star show difference between Control and NE groups.

Table 8-1 Cytokine Levels in children with NE compared to controls

Cytokines	Control (n=40)	NE (n=37)	p value
IL2	[1]0.54	1.27	**0.002**
IL6	1.27	2.13	***0.01**
IL8	16.77	51.93	***0.04**
TNF α	3.63	4.73	0.09
GMCSF	0.24	0.47	***0.025**
IFN Y	15.98	13.96	0.72
EPO	90.45	96.36	0.66
IL10	0.75	1.7	0.08
IL 18	532.57	654.47	***0.023**
IL -IRA	681.91	725.96	0.79
IL-1α	1.60	1.29	0.35
IL-1β	0.62	1.45	0.08
TNF β	1.29	2.98	**0.01**
VEGF	106.65	131.81	0.36

[1]Means and p value using Student t-test. IL= Interleukin, TNF= Tumor necrosis factor, IFN= Interferon, VEGF= Vascular endothelial growth factor, EPO= Erythropoietin, GMCSF= Granulocyte colony stimulating factor. *p value <0.05 as significant.

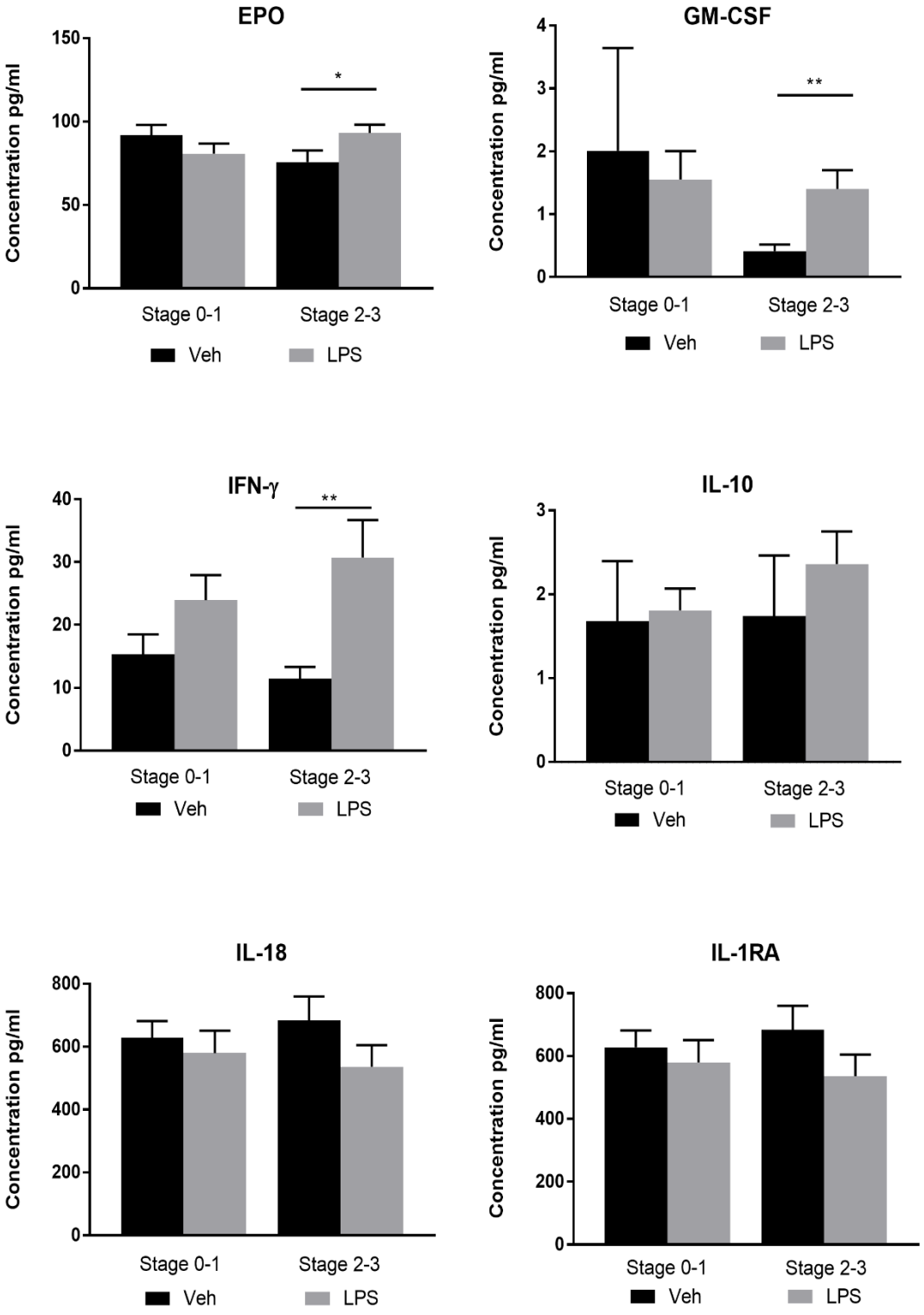


Figure 8-7 Cytokine response in children with NE as per stage of Encephalopathy

Whole blood was treated with 1ul (10ng/ml) of lipopolysaccharides (LPS). Cytokines were measured in the serum of children with mild vs moderate NE using cytokine multiplex assay. Cytokine concentration expressed in pg/ml, Stage 0-1 Mild NE, Stage 2-3= Moderate to severe NE, GM-CSF= Granulocyte macrophage colony stimulating factor, IL-1RA= Interleukin1-Receptor antagonist, EPO= Erythropoietin, IFN Ý= interferon gamma. * Significant star show difference between baseline and on LPS stimulation.

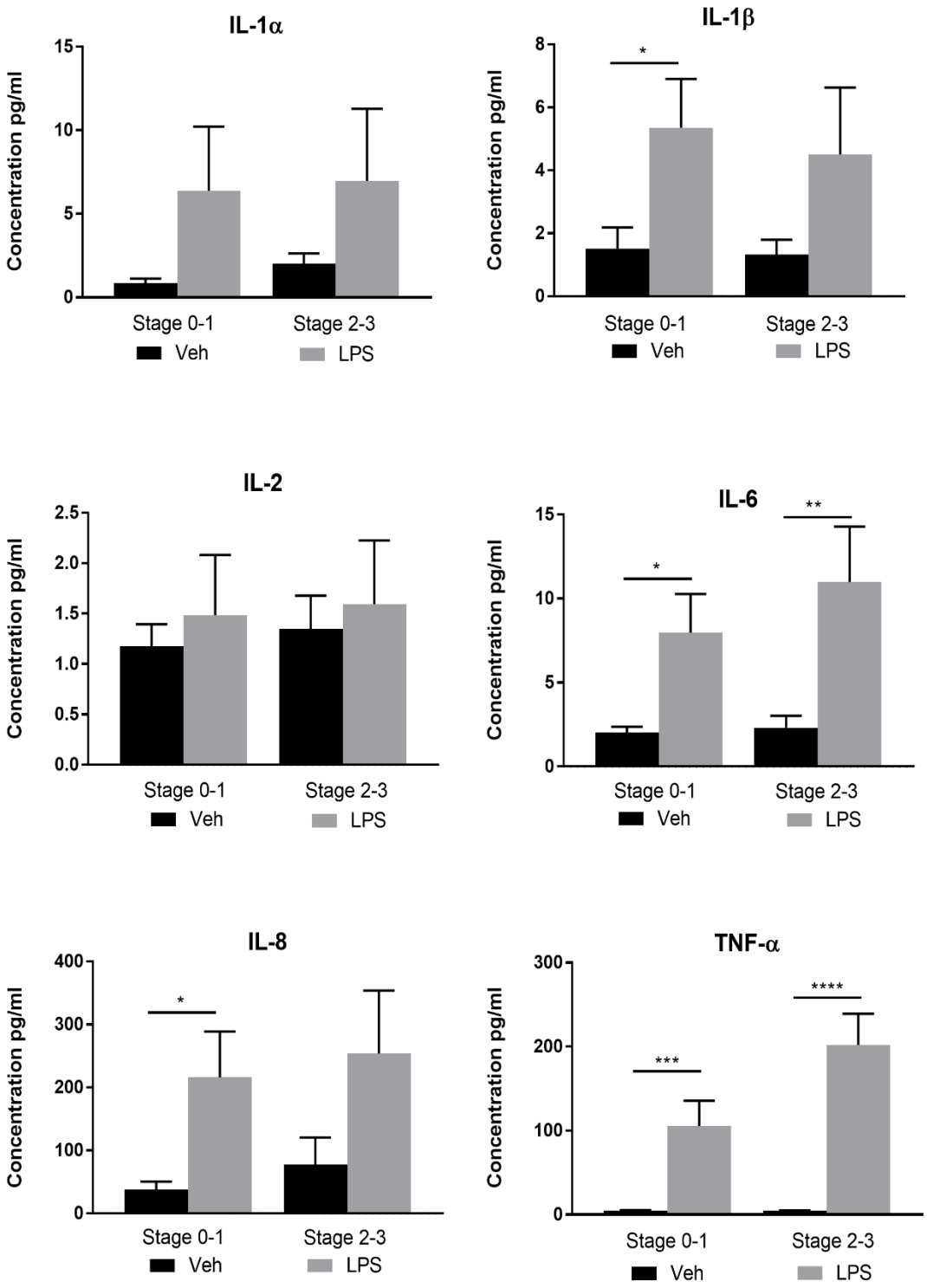


Figure 8-8 Cytokine response in children with NE as per stage of Encephalopathy

Whole blood was treated with 1ul (10ng/ml) of lipopolysaccharides (LPS). Cytokines were then measured in the serum of children with mild NE vs moderate/severe NE using cytokine multiplex assay. Cytokine concentration expressed in pg/ml, Stage 0-1 Mild NE, Stage 2-3= Moderate to severe NE, IL= Interleukin, TNF-α= Tumor Necrosis factor alpha* Significant star show difference between baseline and on LPS stimulation.

Table 8-2 Cytokines levels in control group, NE0/I and NE II/III

Cytokines	Control (n=40) (Mean)	NE (0/I) (Mean)	NE (II/III) (Mean)	p value (a)	P value (b)
IL2	[1]0.54	1.18	1.35	0.02	**0.04**
IL6	1.27	2.03	2.31	**0.05**	0.73
IL8	16.77	37.99	77.67	0.11	0.39
TNF α	3.63	4.74	4.69	0.17	0.95
GMCSF	0.24	2.01	0.41	0.29	0.34
IFN Y	15.98	15.33	11.44	0.92	0.29
EPO	90.45	98.07	93.22	0.62	0.63
IL10	0.75	1.68	1.74	0.21	0.95
IL 18	532.57	628.01	683.14	0.16	0.56
IL -IRA	681.91	767.10	649.44	0.68	0.61
IL-1α	1.60	0.85	2.01	0.12	0.11
IL-1β	0.62	1.52	1.32	0.20	0.81
TNF β	1.29	3.17	2.63	**0.01**	0.42
VEGF	106.65	122.57	148.57	0.50	0.68

[1] Means and p value using Student t-test. IL= Interleukin, TNF= Tumor necrosis factor, IFN= Interferon, VEGF= Vascular endothelial growth factor, EPO= Erythropoietin, GMCSF= Granulocyte colony stimulating factor. p (a)-p value Control vs NE 0/I and p (b) p value NE0/I vs NEII/III.

Table 8-3 Cytokines levels in control group, NE+ TH and NE- Normothermia

Cytokines	Control (n=40) (Mean)	NE + TH (Mean)	NE Normothermia (Mean)	p value (a)	p value (b)
IL2	0.54	1.32	1.12	**0.01**	0.62
IL6	1.27	2.32	1.84	**0.04**	0.47
IL8	16.77	76.61	15.73	**0.04**	**0.04**
TNF α	3.63	4.64	4.85	0.09	0.85
GMCSF	0.23	0.43	2.94	**0.02**	0.36
IFN Y	15.98	12.98	15.40	0.60	0.63
EPO	90.45	90.33	105.21	0.99	0.29
IL10	0.75	1.39	2.16	0.17	0.54
IL 18	532.57	678.37	601.93	0.51	0.67
IL -IRA	681.91	794.79	624.51	0.56	0.50
IL-1α	1.60	1.68	0.62	0.89	**0.05**
IL-1β	0.62	1.34	1.61	0.06	0.81
TNF β	1.29	2.68	3.42	**0.001**	0.26
VEGF	106.65	146.48	110.03	0.32	0.40

[1]Mean and p values using Student t-test. VEGF= Vascular endothelial growth factor, EPO= Erythropoietin, GMCSF= Granulocyte colony stimulating factor. p (a)-p value Control vs NE +TH and p (b) p value NE+TH vs NE- Normothermia.

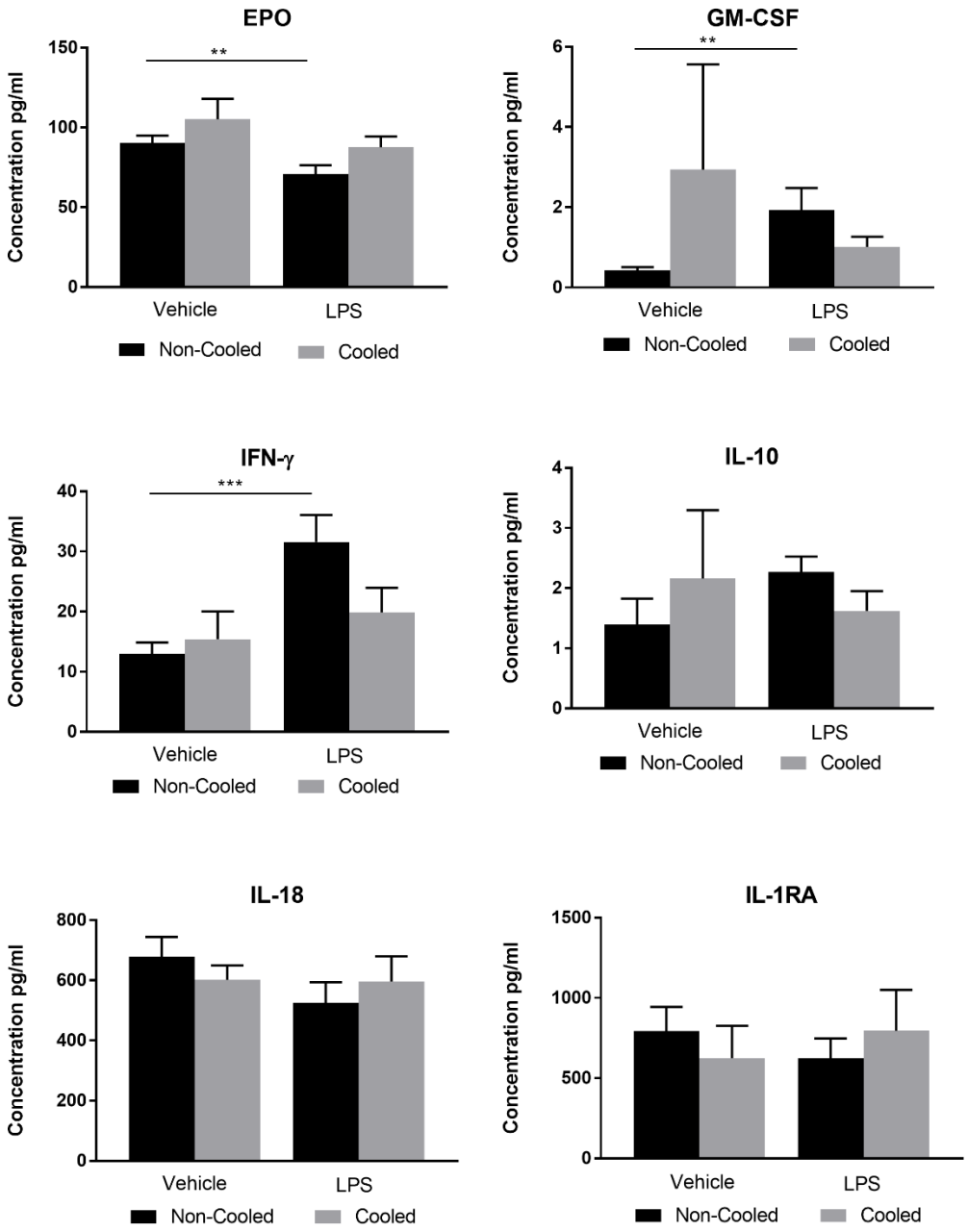


Figure 8-9 Cytokine response in children with NE who underwent TH (cooled) vs NE without TH (Non-cooled) at baseline and on LPS stimulation.

Whole blood was treated with 1ul (10ng/ml) of lipopolysaccharides (LPS). Cytokines were then measured in the serum of children with NE with TH and those without TH, using multiplex assay. Cytokine concentration expressed in pg/ml EPO= Erythropoietin, GM-CSF= Granulocyte Macrophage Colony stimulating factor, IL= Interleukin, IFNÝ= Interferon gamma, IL-1RA= Interleukin 1 Receptor antagonist, Veh= Vehicle or baseline, LPS= Lipopolysaccharide. * Significant star show difference between Control and NE groups.

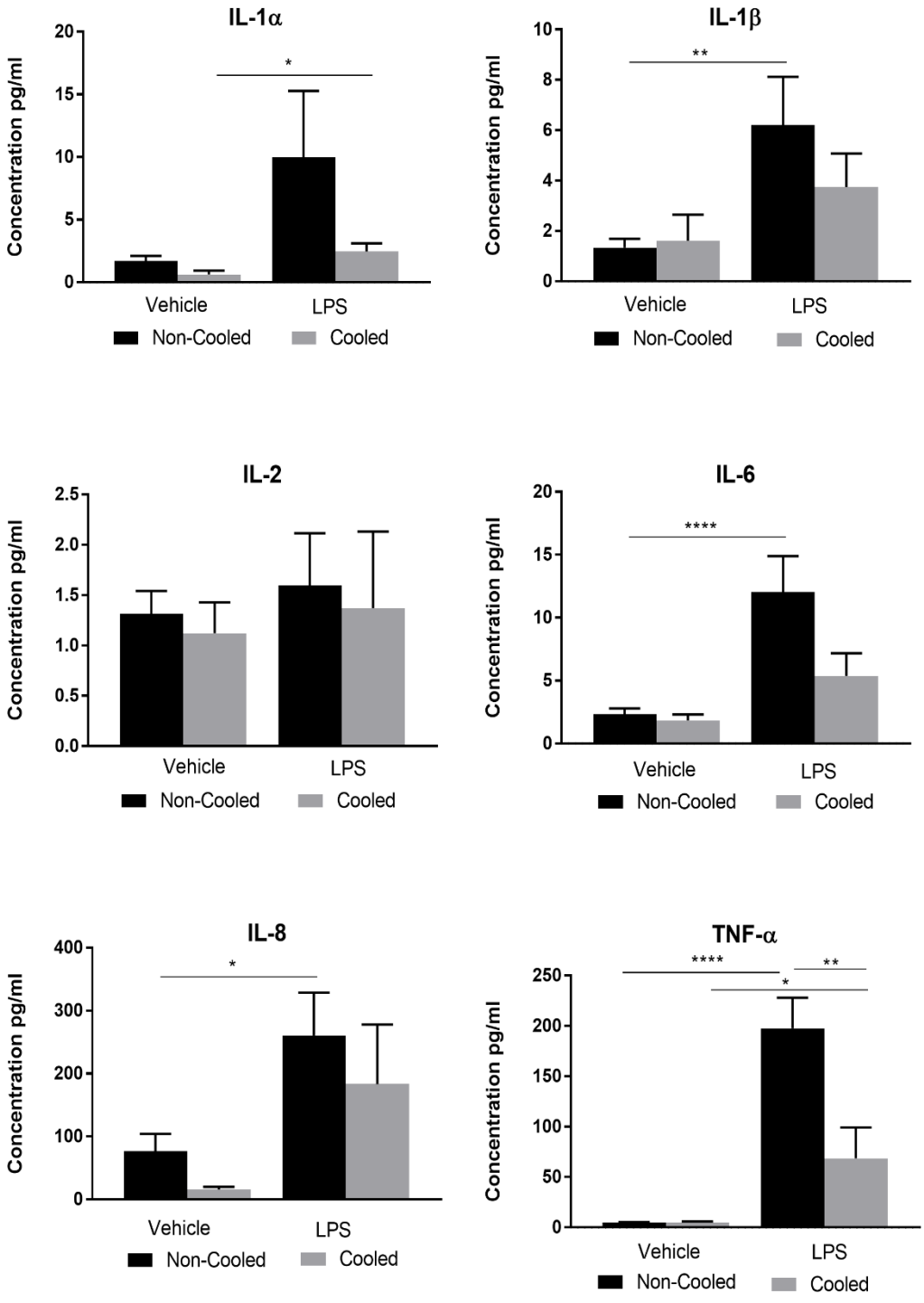


Figure 8-10 Cytokine response in children with NE who underwent TH (cooled) vs NE without TH (Non-cooled) at baseline and on LPS stimulation

Cytokine concentration in serum analysed using multiplex assay, expressed in pg/ml, Cooled+ NE with TH, Non-cooled = NE without TH, IL= Interleukin, TNF-α= Tumor Necrosis factor alpha. * Significant star show difference between Control and NE groups.

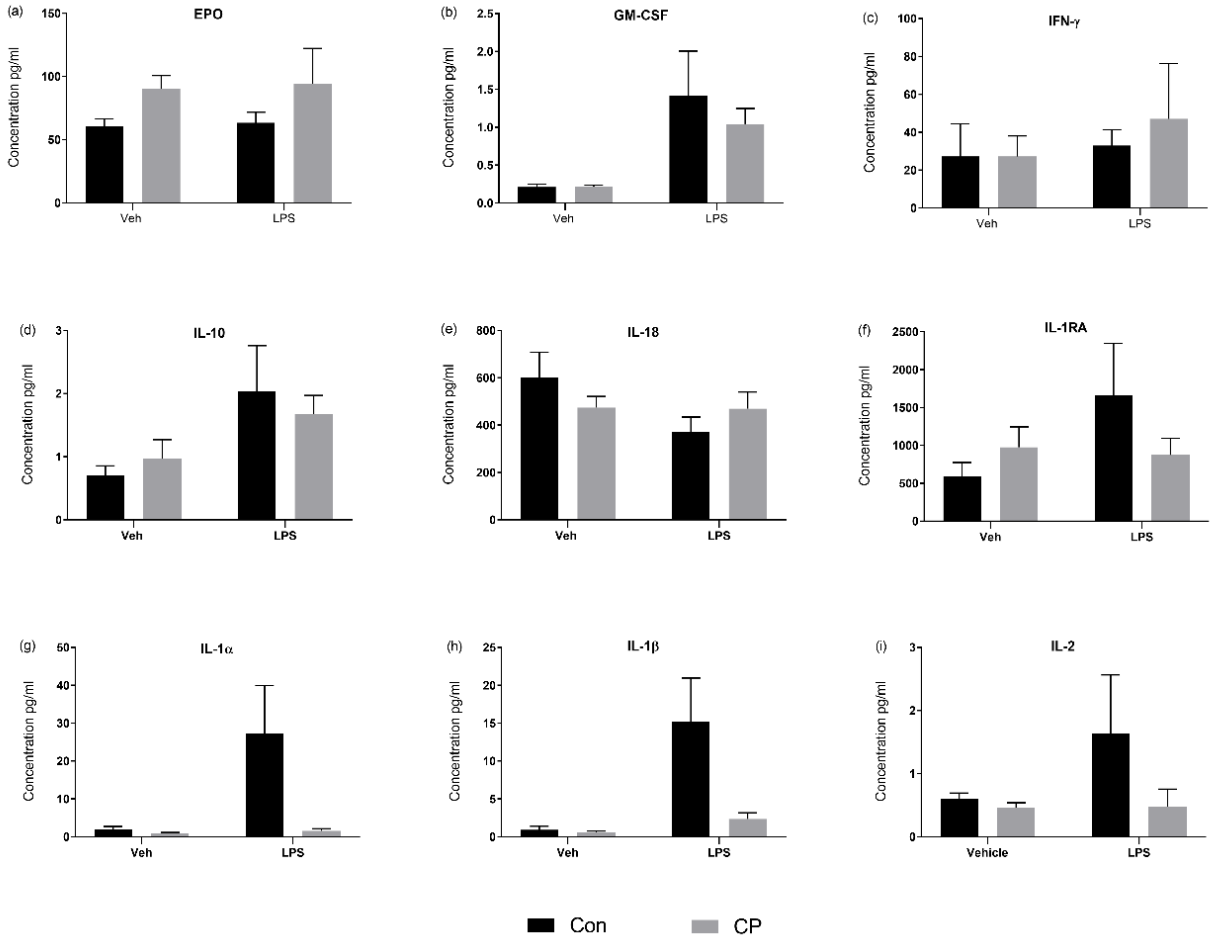


Figure 8-11 Cytokine response in children with CP and complex needs vs Age matched control.

Whole blood was treated with 1ul (10ng/ml) of lipopolysaccharides (LPS). Cytokines were then measured in the serum of children with CP and control group using cytokine multiplex assay. Cytokine concentration expressed in pg/ml in Complex Needs = CP with Complex needs (n=12), Control (n=12) EPO= Erythropoietin, GM-CSF= Granulocyte Macrophage Colony stimulating factor, IL= Interleukin, IFNÝ= Interferon gamma, IL-1RA= Interleukin 1 Receptor antagonist, Veh= Vehicle or baseline, LPS= Lipopolysaccharide.

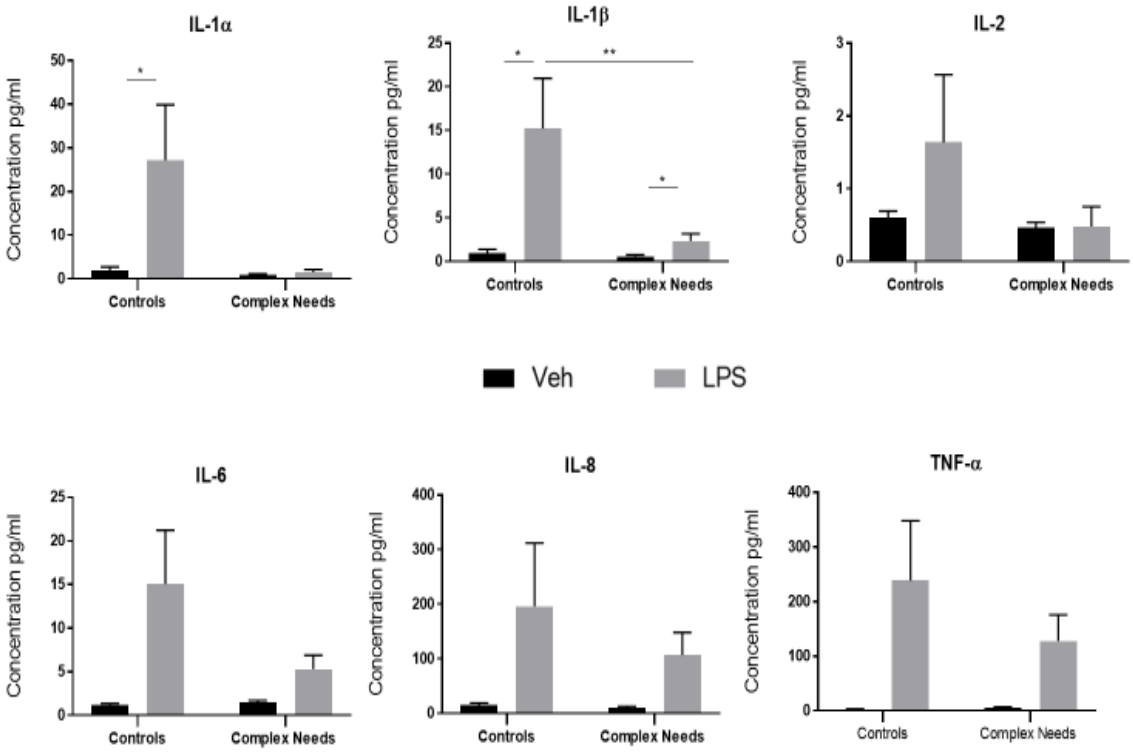


Figure 8-12 Cytokine response in children with CP and complex needs vs Age matched control.

Whole blood was treated with 1ul (10ng/ml) of lipopolysaccharides (LPS). Cytokines were then measured in the serum of children with CP & complex needs and control group using cytokine multiplex assay. Cytokine concentration in serum analysed using multiplex assay, expressed in pg/ml, in Complex needs = CP with Complex needs (n=12) and Control= Age matched controls (n=12), IL= Interleukin, TNF-α= Tumor Necrosis factor alpha. * Significant star show difference between Control and NE groups.

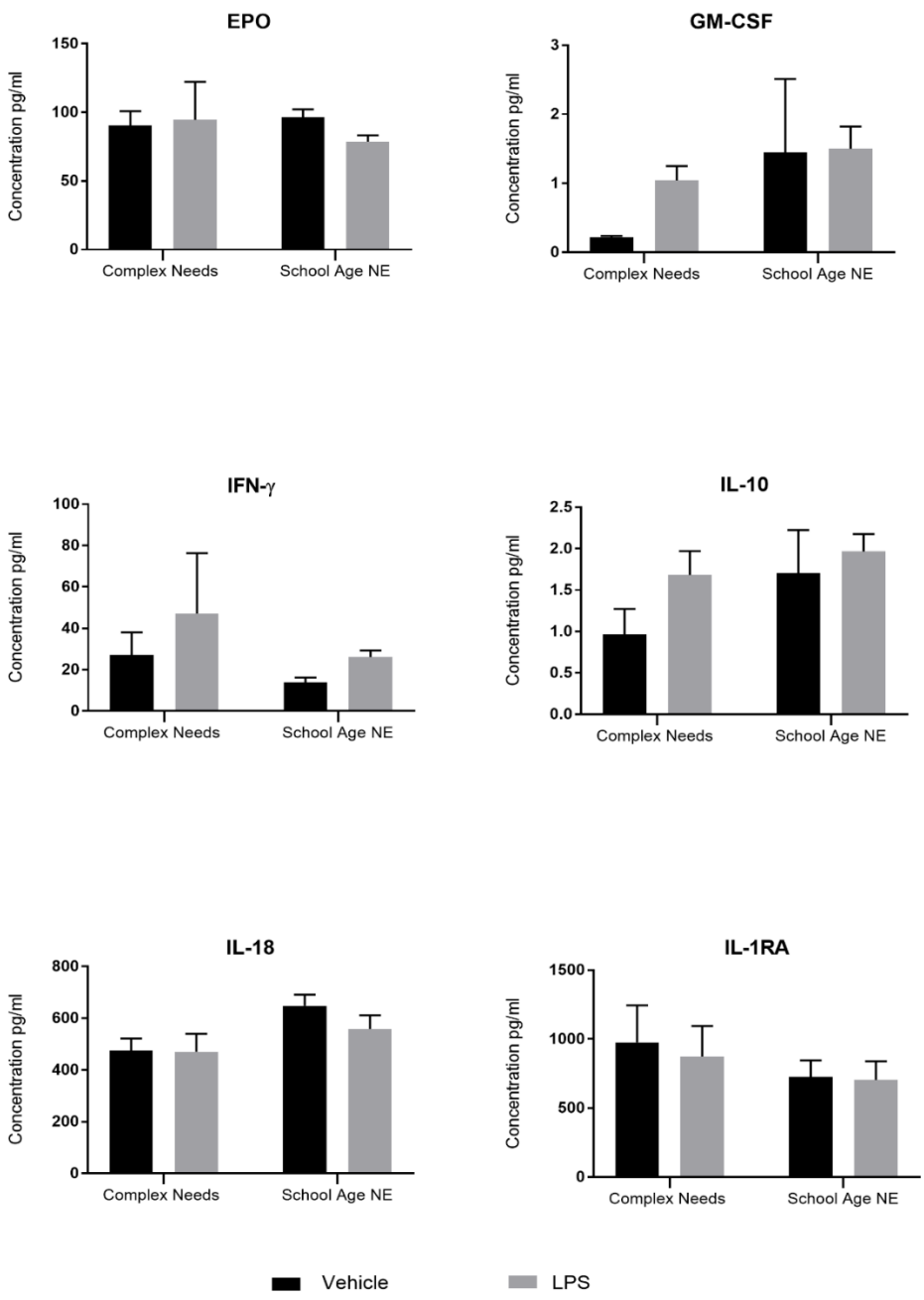


Figure 8-13 Cytokine response in children with CP & complex needs VS school age children with NE

Cytokine level in serum expressed as pg/ml, in children with Complex Needs= CP with complex needs (n=12) vs children with NE (n=37) .EPO= Erythropoietin, GM-CSF= Granulocyte Macrophage Colony stimulating factor, IL= Interleukin, IFNÝ= Interferon gamma, IL-1RA= Interleukin 1 Receptor antagonist, Veh= Vehicle or baseline, LPS= Lipopolysaccharide.

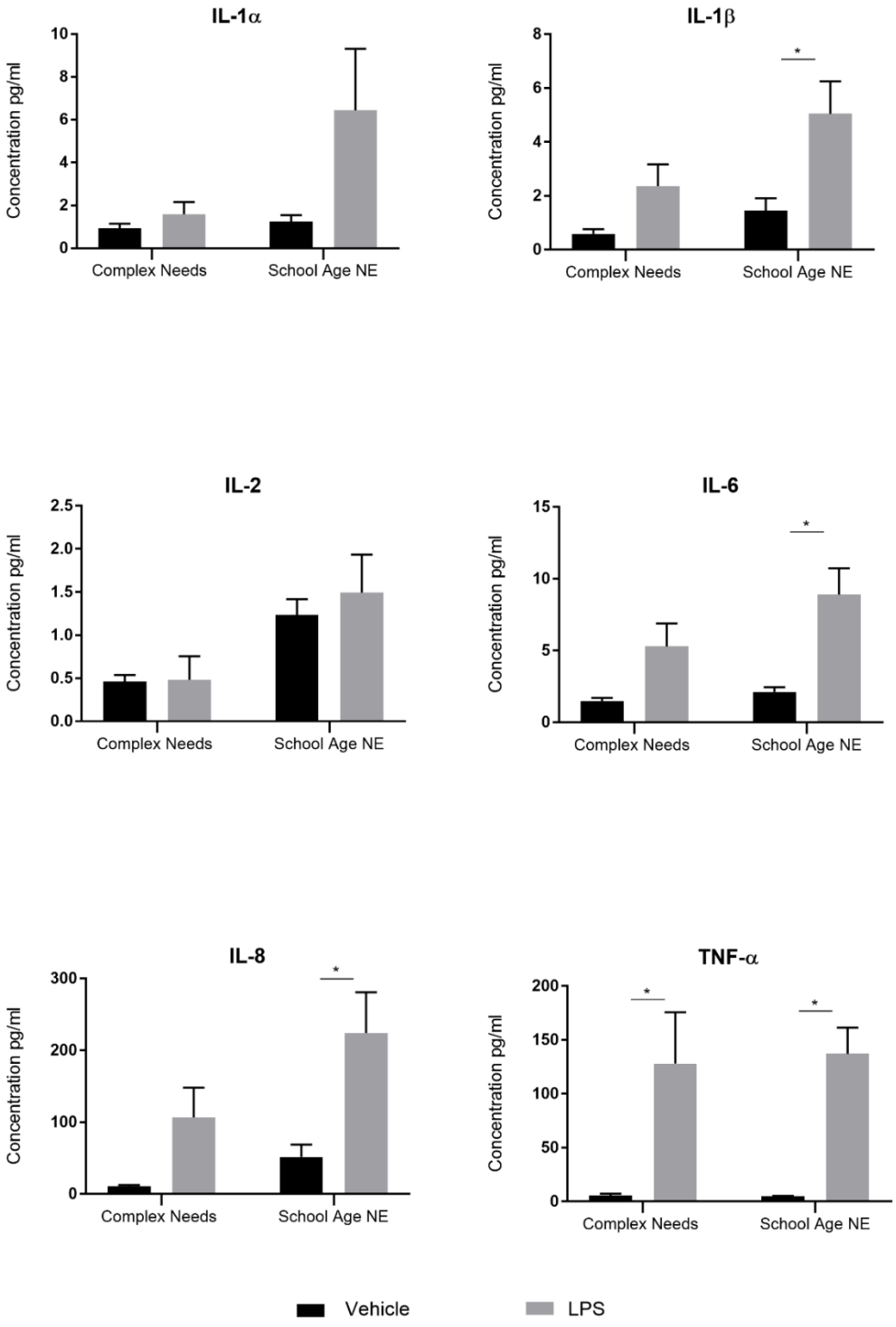


Figure 8-14 Cytokine response in children with CP & complex needs VS school age children with NE

Whole blood was treated with 1ul (10ng/ml) of lipopolysaccharides (LPS). Cytokines were then measured in the serum of children with CP with complex needs and control group using cytokine multiplex assay. Cytokine level in serum expressed as pg/ml, in children with CP & Complex Needs (n=12) vs children with NE (n=37) at baseline and after 1 ul (10 ng/ml) of LPS. IL= Interleukin, TNF-α= Tumor necrosis factor alpha.

Chapter 9

DISCUSSION

9. Discussion

9.1. Introduction

Perinatal global hypoxia-ischaemia is associated with neonatal encephalopathy (NE) which is an important cause of permanent neurological impairment in the term new-born infant. NE can result in death, cerebral palsy, epilepsy, significant cognitive impairments, developmental and behavioural problems.

There is an increased risk of multi-organ injury associated with perinatal ischaemia (Shah et al., 2004, Hankins et al., 2002, Martin-Ancel et al., 1995, Perlman, 1989). An interruption of placental blood flow enough to result in NE commonly involves multiple organs, including the heart, kidney and liver and not solely the brain Extent and severity of damage to each organ depends on the severity of initial insult. Following restoration of normal circulation, the extent of recovery of each organ depends on the degree of reperfusion injury and on treatment interventions (Perlman, 2011). The long-term outcome of infants with multi-organ dysfunction following NE has not to date been fully evaluated.

In our study we have attempted to evaluate the outcome of infants who experienced multiorgan dysfunction in NE. We examined multiorgan function including innate immune response at school age in children with neonatal brain injury.

9.2. Sleep and Quality of Life Results

Sleep pattern of 45 school age children who had mild to moderate neonatal encephalopathy (NE) were assessed and compared with 55 children with normal development, and 15 children with cerebral palsy and complex needs, using the children sleep habit questionnaire (CSHQ). High pathological sleep score was seen in 30 out of 52 children (57%) with NE when compared to children in the control group (mean score of 43.8 vs 40.2) (p value 0.001). Children with NE were noted to have increased prevalence of sleep problems including bedtime resistance (*P* value 0.028) and sleep anxiety (p value 0.01) compared to school age controls. Children with moderate to severe NE had high total sleep scores compared to children with mild NE; mean total CSHQ score of 44.5 vs 42.3; (p-value 0.4). Children with severe NE and motor

impairment had very high total scores ranging from 49-66. Children with moderate to severe NE had significantly high sleep onset delay (SOD) scores compared to children with mild NE (p- value 0.04). Children with mild NE also had higher total sleep scores compared to children in the control group. Disorders of initiation and maintenance of sleep or sleep onset delay was the most common sleep disorder seen in children with mild NE.

High rate of sleep disorders was also demonstrated in children with CP and complex needs. Males outnumbered female as expected, male being at increased risk of CP and NE (Jarvis et al., 2005, Wu et al., 2006). Sleep disorders noted in majority of children in the CP and complex needs group were excessive daytime sleepiness (EDS) in all 16 children (mean subscale score of 10.6 vs 9.6; p-value <0.04), bedtime resistance (high mean score 9.6), sleep onset delay (SOD) and parasomnias in 9 children. Total body involvement, including spastic quadriplegia and dystonia were strongly associated with disorders of initiation and maintenance of sleep. Other sleep disorders commonly noted in children with CP and complex needs, were sleep disordered breathing and excessive daytime sleepiness.

Epilepsy was the major factor associated with sleep disturbance in children with CP. Epilepsy in children with CP, has been shown to result in increased total sleep disturbance and increased daytime sleepiness (Wayte et al., 2012).

We also examined the response of circadian genes or CLOCK proteins, BMAL1, CRY, CLOCK and REV-ERBα in children with NE compared to controls. The circadian rhythm has been proven to be involved in various physiological processes, including sleep and wakefulness, hormone synthesis, proliferation of cells and inflammation. Increased expression of BMAL1, CRY, CLOCK & REV-ERBα was noted in children with NE compared to controls, on in vitro stimulation with an endotoxin (LPS). The response was noted to suppress on stimulation with melatonin, probably due its immune modulatory properties. However, the response did not reach statistical significance, further studies are required and probably a bigger sample size to demonstrate a significant response.

This is the first description of sleep disorders in school age children post NE, especially in those classified to have mild to moderate encephalopathy (Grade I/II).

Sleep disturbances in children with NE may have an impact on their behaviour, seizure activity, feeding problems and quality of life, thus require prompt management.

9.2 b Quality of Life (QOL) in children with NE in comparison to children with normal development and CP

Neonatal encephalopathy is associated with several complications which affect the cognitive, motor, sensory abilities and behaviour outcome. These may have a significant impact on the child's quality of life. We hypothesised that mild hypoxic ischaemic insult may lead to minor developmental problems which may manifest only later in childhood with cognitive demands and motor abilities affecting the child's quality of life (QOL). Thus, we assessed the QOL of children with NE compared to children with normal development and with children with severe CP and complex needs.

Children in the NE group were noted to have significantly low total QOL scores in comparison to children in the control group; mean score 82.5 vs 95.8 (p value <0.01). On comparison of children with mild NE with the control group, QOL scores were significantly low in children with mild NE compared to control group (mean total QOL score 90.03 vs 95.8 p value 0.003). However, children with mild NE had higher total QOL scores compared to children with moderate to severe NE (mean 90 vs 78.3 p value 0.007). Children with moderate to severe NE had significantly low scores in domains of physical ability, and school activities compared to children with mild NE (p-value 0.021 and 0.02).

Eight out of thirty-five children in the moderate to severe NE group were diagnosed to have CP. They had very low total QOL scores as assessed on the CP module of Ped QL questionnaire (mean score 44.1 vs 95.8 in control group; p value <0.01). We noted strong correlation of low QOL scores with high GMFCS level, children with GMFCS level I/II had high total QOL scores compared to children with GMFCS level III/IV; mean score 89 vs 44 (p-value 0.001). Low scores were also associated with co- morbidities including epilepsy, intellectual disability and visual impairment.

QOL in children in CP group with complex needs was assessed using the CPQOL questionnaire. They demonstrated very low QOL scores compared to children in the control group (mean score 47.3 vs 95.8 p value <0.01). Majority of children in CP and complex needs group (9/16) had GMFCS of IV/V and had associated co-morbidities or

complex needs including epilepsy, visual impairment, speech delay, feeding difficulties and sleep disturbances. Very low scores were noted in domains of functional ability, participation and health and high pain scores. Parent/ primary caregiver filled the questionnaire in majority of cases as the children with CP due to their disability were unable to do so. Few were filled by the children themselves. We noted that parent reported lower scores in terms of social and emotional well-being and high pain scores compared to the scores reported by the children. Several studies have reported that the level of agreement depends on the domain assessed. We also found strong correlation between high sleep scores and low QOL in this cohort.

QOL assessment provides broad insight of the child's psychological well-being, self-perception regarding disability, impact of the associated comorbidities and pain & discomfort on the QOL. Management of these factors can improve QOL of the child and of the family. Thus, QOL should be assessed routinely in practice in children with NE and children with CP.

9.3. Neurological Results

Fifty-five children with NE were reviewed at school age. A detailed neurodevelopmental assessment including history and a developmental questionnaire (ASQ 3) and neurological examination was performed. They were classified by the severity of encephalopathy according to Sarnat Scores (Sarnat and Sarnat, 1976) as follows: NE 0 (required only resuscitation; n=3); mild NE I (n=17); moderate NE II (n=32); and severe NE III (n=3). Cerebral Palsy (CP) was diagnosed in 8 children out of the 35 children (23%) with moderate to severe NE. Four children had unilateral spastic CP and 3 had bilateral spastic CP and one child had dyskinetic CP. Children diagnosed with severe Cerebral Palsy were assigned NE Grade III at birth and the remaining 6 were classified to have NE grade II. As per the GMFCS, children with NE had spastic quadriplegia with GMFCS V (n=2), spastic diplegia GMFCS II/III (n=2), hemiplegia GMFCS II/III (n=3) and weakness and spasticity in one limb with poor balance (n=1).

Speech delay was the most predominant problem noted in 15 children (27%), visual impairment in 5 (9%), hearing impairment in two children with NE. Epilepsy was diagnosed in 3 children, febrile seizures were noted in another 3 children and functional

motor and co-ordination problems were noted in 3 children. Neuropsychiatry problems including Autism was diagnosed in one child, two children were diagnosed with ADHD and behaviour problem were noted in 5 children with NE.

The database of these children including details of neonatal examination, neuroimaging reports and the Bayley's scale of infant development scores done at 2 years of age were examined. A strong association was noted between low BSID scores and abnormal neurological examination in the neonatal period. The composite motor score was significantly higher in children who had a normal exam compared to those with abnormal exam (mean 107 vs 93; p value=0.03). The language and cognitive scores were higher in children who had normal neurological exam in comparison to those with an abnormal exam in the neonatal period (mean score 104 vs 96; p value= 0.4 for language and mean score 105 vs 96; p value= 0.1 for cognitive skills respectively).

MRI scan were abnormal in 19/35 (51%) children who were diagnosed to have NE II/III and only 1 scan was abnormal out of the 10 MRIs performed in children with NE I. Not all children who received TH had abnormal MRI scans, but all who had abnormal neuroimaging received TH. Nine children with an abnormal MRI in the neonatal period had completely normal developmental assessment on ASQ-3 when reviewed in the clinic at 4-6 years of age. Whereas, another 9 children with normal MRI scans in the neonatal period went on to have developmental problems including speech delay (n=7), hearing impairment (n=1), and delay in personal & social skills (n=1). Thus, abnormal neuroimaging alone could not predict poor neurodevelopmental outcome.

Development Ages & Stages Questionnaire (ASQ-3) scores were significantly lower in children with NE in all domains compared to controls. On correlating with BSID scores, correlation was noted between the gross motor BSID and ASQ score. (Rho; 0.35 and p value=0.014). There was no correlation noted with the communication and fine motor scores. Children diagnosed to have developmental delay following the BSID, demonstrated similar difficulties in performing developmental tasks when assessed at 4-6 years of age using ASQ 3. The ASQ scores were consistent with BSID scores. This shows that ASQ3 could be used as a reliable screening tool in children who had NE. ASQ, Third Edition (ASQ3) when compared with BSID III in a mixed population of term-born and preterm-born children at different age groups demonstrated adequate sensitivity and specificity (Schonhaut et al., 2013).

Children with NE who were noted to have developmental delay in the study had been referred appropriately at 2-3 years of age following assessment by clinicians to the respective therapist and were linked in with the services when assessed at 4-6 years of age. Early detection is very important for early intervention and management.

We demonstrated that children with mild NE have significant low developmental scores in comparison to the control group Children with mild NE were traditionally assumed to have good prognosis but recent studies have reported abnormal outcome including death and neurodisability in 25% children with mild NE (Conway et al., 2018a). Thus, children with mild NE should be followed up for development assessment, as early recognition and intervention can lead to a better neurodevelopmental outcome in this group of children. Future research should involve in development of new biomarkers which would help in predicting outcome even in infants with mild NE. The use of 3T MRI to give more detailed neuroimaging combined with biomarkers to predict outcome and assist in tailoring therapy. In addition, ensuring neonatal neurological examination is incorporated in routine clinical care in view of the capacity to predict neurodevelopmental outcome using quality improvement and educational packages.

Therapeutic hypothermia (TH) is the only option available for the treatment of NE and has been proven to improve neurodevelopmental outcome and reduce mortality. But there is a need for adjunct therapies and several neuroprotective agents have been the focus of research in recent years including melatonin, erythropoietin, allopurinol, magnesium, inhaled xenon and stem cell therapy.

9.4. Renal results

In the group of children with NE enrolled in our study, Acute kidney injury (AKI) was diagnosed in the neonatal period in 36% infants. Oliguria which was defined as a urinary output of < 1 ml/kg/hr per 24-hour period was seen in 59% of infants with NE. Oliguria was significantly associated with therapeutic hypothermia, seizure development and abnormal NE grade II/III (p-values < 0.001, 0.006, < 0.001), but there was no association between oliguria and an abnormal MRI brain result.

There was no strong association between renal outcome measures such as proteinuria/ haematuria and serum urea and creatinine levels. Moderate/severe NE and

occurrence of seizures were significantly associated with higher urea levels on day 1,2 and higher creatinine levels on day 1,2 and 3. An abnormal MRI brain result was significantly associated with higher serum creatinine levels on day 2 and 3 but not with urea levels

Persistently high levels of urea and serum creatinine in the absence of oliguria were noted at school age children with NE in comparison to children in the control group. (mean urea level 5.2 (1.2) versus 4.4 (1.1) mmol/l (p-value 0.001) and mean creatinine 45.4 vs 40.8umol/l; p value 0.025). Oliguria is not a sensitive marker of AKI and if serum creatinine measurements are not monitored, AKI may not be recognised. Our findings are supported by previous studies which report that 60-78% of post asphyxial AKI is non-oliguric (Aggarwal et al., 2005, Gupta et al., 2005).

School age children with NE had significantly low sodium levels and high calcium levels compared to the control group (p-value= 0.001 and 0.01 respectively) (Table 5.4). There was no significant difference between values of potassium and phosphate. The older children in the CP group with low GMFCS IV/V had very low levels of creatinine compared to children in the control group and in NE group (35 umol vs 41 & 45 umol; p value 0.01). Creatinine is a product of muscle creatinine-phosphate metabolism, and its level reflects muscle mass.

A strong association was noted between persistently altered urea and creatinine levels and poor neurological outcome. Children who had neonatal seizures, and who underwent therapeutic hypothermia (TH) were noted to have high urea and creatinine levels both in the neonatal period and at school age (Table 5.5).

Renal dysfunction in children with NE may be part of multiorgan damage following perinatal asphyxia or there may be close link between AKI and inflammation as described in previous animal studies (Liu et al., 2008b). Persistent inflammation therefore may be the cause of altered renal dysfunction noted in school age children with NE. However broader understanding of this mechanism and further studies are required for validation.

9.5. Haematological results

Infants with NE who underwent TH had significantly lower white cell counts, and platelet counts compared to non-cooled infants. A strong association between

haematological parameters at neonatal age and outcome was noted. An abnormal neonatal neurological examination at discharge was significantly associated with lower mean haemoglobin counts on day 3 and 5 (p values = 0.04, 0.005 respectively) and higher mean neutrophil counts on day 4 of life (p value = 0.003). Mortality in the neonatal period was associated with high WCC count and neutrophil count on day 2,3,4 and 5 of life (p value= 0.006, 0.003, 0.006 and 0.003 respectively for WCC and p-values 0.009, <0.001, <0.001, <0.001 respectively for neutrophil count).

We demonstrated that at school age, children with NE had significantly higher WCC count compared to children with normal/ typical development (mean WCC of $9.1x10^9$ /L vs mean WC $8.1x10^9$ /L; p value=<0.02). On comparing the results as per the grade of encephalopathy, WCC and neutrophil count were significantly higher in children with NE II/III compared to children with NE 0/I (mean WCC $9.4x10^9$/L vs mean WCC 8.5x 10^9/L; p value=0.07 and mean neutrophil count 4.3 x 10^9/L vs 3.3x 10^9/L, p value 0.007). However, no significant difference was noted between the results haemoglobin (Hb) and platelet level.

We correlated the WCC and neutrophil count performed in the neonatal period in infant with NE and the Bayley's cognitive scores (BSID). We found a strong association between elevated WCC and neutrophil count and low composite cognitive scores (Rho 0.40, p value=0.007 and Rho 0.31 and p value 0.04, for WCC and neutrophil count respectively). Increased inflammatory reaction following a hypoxic ischaemic insult result in infiltration of the brain by peripheral leucocytes, aggravating brain injury. This process may manifest with elevated WCC and neutrophilia. (Liu and McCullough, 2013)

Studies in the pre-TH era have reported significantly higher WCC and ANC levels in the first 96 hours after birth in neonates with poor neurodevelopmental outcome at 18 months of age (Morkos et al., 2007). Hypothermia has been associated with increased incidence of leukopenia, thrombocytopenia and coagulation disturbances. However, to our knowledge there are very few follow up studies looking at the haematological abnormalities in children with NE at school age. We demonstrated a persistently elevated white cell count at school age in children with NE compared to controls. We also demonstrated that an elevated white cell count in neonatal period may be associated with poor cognitive performance and neurodevelopmental outcome.

These findings thus suggest that haematological abnormalities especially elevated WCC and neutrophil count in infants with NE can be used as predictors for long term neurological outcome. It would be worthwhile to look for any correlation between WCC and neutrophil count in children with NE and cognitive assessment scores including (Wechsler Preschool and Primary scale of Intelligence (WPPSI) performed in preschool children. This could validate further the importance of monitoring haematological abnormalities in children with NE.

9.6. Inflammation results

Inflammation has long term consequences on the brain during childhood and perinatal inflammation is associated with many neuropsychiatric and neuropsychological disorders. (Hagberg et al., 2012). Studies have suggested that the injury processes can persist for months and years and a tertiary mechanism of damage has been proposed which includes inflammation and epigenetic changes. (Hagberg et al., 2012).

We examined systemic expression of the genes for hypoxia inducible factor- 1 α (HIF1α) and inflammasome as well as monocyte and neutrophil activation in response to endotoxin (LPS) and melatonin in children with neonatal encephalopathy (NE) at school age compared to children with normal development and children with severe cerebral palsy (CP). We measured the mRNA expression of inflammasome genes; NLRP3, ASC and IL1 beta in whole blood, before and after treatment in vitro with Lipopolysaccharide (LPS) and Melatonin using real time polymerase chain reaction (RTPCR). Significant elevation in the expression of NLRP3 gene was noted in children with NE, in comparison to children in the control group and children with severe CP. The response was significant with > 2-fold change in children with NE at baseline and on treatment with LPS *in vitro* and was seen to decrease considerably on in vitro treatment with Melatonin (Figure 7.1). The expression of other inflammasome genes ASC and IL1β were also noted to be increased in children with NE, both at baseline and in response to LPS stimulation, compared to children with normal development and children with CP and complex needs (Figures 7.2 & 7.3).

HIF1α was significantly increased in CP group compared to the control group both at baseline and on treatment with LPS. Combining melatonin and LPS treatment slightly lowered this response. There was also a significant increase in the expression of HIF1α following melatonin treatment alone in the NE group versus the controls ($p<0.03$). (Figure 7.4).

The expression of neutrophil CD11b was significantly increased in children with NE ($p=0.04$) at baseline and after LPS stimulation in comparison to children in the control group. This was reduced with *in vitro* treatment with melatonin (probably due its immunomodulatory effect). A similar response was seen in children with CP, expression of neutrophil stimulated CD11b was significantly increased compared to control group but the response was less compared to that seen in children with NE.

There was a significant increase in LPS-stimulated neutrophil TLR-4 in school age children with NE compared to controls. Similar expression of TLR4 in neutrophil was seen to be increased in children with CP especially after LPS stimulation in vitro. Thus school-age children with NE demonstrated vigorous systemic innate immune response compared to children with normal development. Previous studies have described increased CD18/CD11b expression along with delayed peripheral mononuclear cells (PMN) apoptosis in neonates suggesting their role in neonatal inflammation (Nguyen et al., 2010, Koenig et al., 2005). In addition, high neutrophil and monocyte CD11b and TLR-4 expression has been reported to be associated with poor outcome in neonates with NE (O Hare F et al 2015). Preterm school-age children with periventricular leukomalacia (PVL) induced (CP) have been reported to have significantly higher levels of tumour necrosis factor (TNFα) and elevated TLR4 mRNA in peripheral blood mononuclear cells (PBMC's) compared to preterm children with normal imaging (Lin et al., 2010) suggesting a programming effect causing altered inflammatory responses in preterm children with CP. To our knowledge, there are no studies published to date of TLR-4, CD11b and HIF 1α and inflammasome expression in school age children with NE.

Persistent inflammation may be associated with persistent activation of hypoxia-inducible factor (HIF) 1α (Anand et al., 2007). We demonstrated significantly higher expression of HIF-1α and inflammasome genes NLRP3, ASC and IL1β in children with NE,

and severe CP in comparison to age matched control group children. We also showed that this response is is amenable to immunomodulation with melatonin.

Melatonin has several actions including anti-oxidant and has anti-inflammatory actions, it plays a protective role against activation of NLRP3 inflammasome (Favero et al., 2017). Targeting specific immune pathways may be a therapeutic option in persistent inflammation in children with brain injury.

9.7. Cytokine results

The pro-inflammatory cytokines activate cytotoxic T cells, natural killer cells, which enhance cellular and tissue damage. This leads to cell proliferation, differentiation and cell death causing white matter damage and long-term neurological damage.

We examined the cytokine response in children with mild to moderate NE in comparison to children with normal development and children with Cerebral Palsy (CP). We also compared the levels of cytokines in infants with NE in the first week of life and at school age. Cytokines including EPO, IL-8, GMCSF and VEGF analysed in infants with NE at different time points after birth (Day 1,3 & 7) and at school age. IL-8 was noted to remain persistently elevated both in the neonatal period and at school age, however EPO and VEGF levels had normalised at school age. There are hardly any studies comparing cytokine response in neonatal period with the response in childhood.

At school age we demonstrated that cytokines including GM-CSF, IL-2, IL-6, IL-8, and IL-18 were significantly increased (p value <0.05) in children with NE compared to controls. And on in-vitro stimulation with an endotoxin (LPS) several cytokines including GM-CSF, EPO, IL-6, IL-8, IL-1β, IFN Ý and TNF β were significantly increased (p value<0.05). Proinflammatory cytokines (TNFα, IL-1β and IL-6) have been reported to be significantly increased in children with NE compared to control (Chaparro-Huerta et al., 2017).

We demonstrated that IL-6 was noted be significantly high on LPS stimulation in children with NEII/III who received TH and had abnormal neurodevelopmental outcome (p value 0.005). Similar results have been described by Jenkins et al, elevated Il-6 noted in children with NE who received TH was reported to be associated with death and

abnormal neurodevelopmental outcome at 12 months of age (Jenkins et al., 2012). However, we also noted that children with mild NE demonstrated high IL-6 levels in comparison to children with normal development (p value 0.05).

We also compared the cytokine levels in children with NE who received TH with those who were normothermic. We found no significant difference in the cytokine levels between the two groups. Cytokine levels have been analysed in umbilical cord blood and after birth in neonates with moderate to severe NE and treatment with hypothermia and subsequent rewarming showed no effect on the levels (Chalak et al., 2014). The anti-inflammatory cytokine IL-10 was comparatively high in children classified in NE II/ II and who had abnormal neuroimaging compared to those in NE I group. Similarly, several studies have looked at anti-inflammatory cytokines levels as predictors of poor prognosis.

GM-CSF was also noted to significantly elevated in children with NE compared to the controls (p value 0.025). However, there was no significant difference in children with NE who received therapeutic hypothermia at birth compared to those who did not. In addition, no significant difference in level of GM-CSF was noted in NE group of children classified in NEII/III with abnormal neuroimaging and poor neurological outcome compared with those who had a normal outcome. GMCSF has been shown to reduce injury in stroke patients.

Cytokine analysis was also carried on a group of children with CP with complex needs as we wanted to compare the results between 3 groups; normal development, mild to moderate NE and severe CP. High levels of EPO and IFNÝ were seen to be the only cytokines elevated in children with severe CP and all other cytokine levels were noted to be low even on stimulation with an endotoxin (LPS).

Cytokines have been reported to be mediators of neonatal brain injury through neuro-inflammatory pathways (Jenkins et al., 2013). They play a vital role in implication of brain injury following a hypoxic ischaemic insult. Thus, they hold promise as biomarkers of ongoing brain injury and in prediction of neurodevelopmental outcome and we aim to assess their correlation with childhood cognitive scores including WPPSI.

9.8. Conclusion

Neonatal asphyxia induces global hypoxia-ischaemia resulting in multi-organ injury involving renal, cardiac, hepatic and haematological system. Multiorgan dysfunction has not been quantified beyond the neonatal period. We demonstrated in our study multiorgan dysfunction (MOD) including neurological, renal, haematological involvement and sleep disorders in children who had NE. We correlated their neonatal outcome with the outcome at school age. These finding could help in developing a MOD scoring system in children with NE which has already been developed in newborn with NE. This would help in advanced clinical planning and long-term follow.

In addition, we also demonstrated altered immune response in children with NE including HIF-1α and inflammasome expression in children with NE.

HIF expression is upregulated through pathways involving the key immune response regulator nuclear factor kappa B, highlighting an interdependence of the innate immune and hypoxic responses to infection and tissue damage. In a hypoxic environment, HIF accumulates exponentially. This is a result of oxygen sensing which shapes the immune response. We know that LPS induces HIF in a TLR4 dependant manner, what follows is the production of proinflammatory mediators such as VEGF and EPO. We have shown dysregulation in all three of these targets in neonates which continues into childhood.

We also found increased expression of CD11b and TLR4 on the surface of neutrophils at baseline and following LPS stimulation, and elevated cytokine response in children with NE suggesting persistently altered inflammation in childhood. Further research is required to validate these findings, understanding the immune response in these children with NE and exploring systemic inflammation holds promise for future development of immunomodulatory adjunctive therapies. Therapeutic hypothermia (TH) is currently the only treatment available for children with NE, but rate of disability is still high at 46% post TH, thus there is need for adjunct treatment modalities.

10. Presentations & Publications

"Early elevated leucocyte count predicts neurodevelopmental outcome in children with Neonatal Encephalopathy and remains altered at school-age". *at European Society of Paediatric Research (ESPR), Conference at Paris in October 2018*

Childhood Assessment of Multiorgan Dysfunction Post Neonatal Encephalopathy (CHAMPioN STUDY). *Invited Speaker, at Faculty of Paediatrics Spring Conference, Dublin, Ireland, May 2018.*

"Persistently altered Cytokine response in school age children with Neonatal encephalopathy"*, Paediatric Academy Societies Conference, Toronto, Canada May 2018*

"Neonatal Encephalopathy: Dysfunctional Inflammation at school age is responsive to Melatonin". *Poster Presentation, Irish Paediatric Association Conference, Dublin, December 2017*

"Persistent monocyte and neutrophil activation in school age children post Neonatal Encephalopathy". *Poster Presentation at European Society of Paediatric Research (ESPR), Conference at Venice, October 2017*

"Systemic alterations in hypoxia-inducible factor 1a expression, NLRP3 inflammasome, neutrophil and monocyte activation in Post Neonatal encephalopathy in school age children and response to melatonin in vitro" *Poster Presentation at Immunology Scientific Research Meeting, TBSI, Dublin, Sept 2017.*

"Melatonin alters inflammasome responses in school age children post Neonatal Encephalopathy." *Poster Presentation at* ***Paediatric Academics Society (PAS) Conference at San Francisco Feb 2017.***

Childhood Assessment of Multiorgan Dysfunction Post Neonatal Encephalopathy (CHAMPioN STUDY). *Oral Presentation at National Children Hospital Research Study Day, Tallaght, Dublin in December 2016.*

"Sleep Problems in Children with Cerebral Palsy and Neonatal Encephalopathy" *E Poster discussion* ***at European Academy of Paediatric Society (EAPS) Oct 2016 at Geneva, Switzerland.***

"Sleep Disorders in Children with Neonatal Encephalopathy" *Poster Presentation at Joint Irish Paediatric association and American Paediatric Society Meeting, Sept 2016 at Dublin, Ireland.*

Publications & Manuscripts

"*Bisphosphonates use in children with cerebral palsy*"

Zunera Zareen, Ciara McDonnell, Denise Mc Donald, Eleanor Molloy

Cochrane Draft Protocol First published: 28 August 2017

Editorial Group: ***Cochrane Developmental, Psychosocial and Learning Problems Group*** *DOI: 10.1002/14651858.CD012756*

"Persistently altered Cytokine response in school age children with Neonatal encephalopathy"*- Manuscript under preparation. Due for Submission*

"Early elevated leucocyte count predicts neurodevelopmental outcome in children with Neonatal Encephalopathy and remains altered at school-age". *Manuscript under preparation. Due for Submission*

"Multi Organ Dysfunction Follow up in Children after Neonatal Encephalopathy". *Review article, due for Submission.*

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12. Appendices

12.2. Child's Sleep Habits Questionnaire (pre-school and school-aged children)

The following statements are about your child's sleep habits and possible difficulties with sleep. Think about the past week in your child's life when answering the questions. If last week was unusual for a specific reason (such as your child had an ear infection and did not sleep well or the TV set was broken) choose the most recent typical week.

Answer USUALLY if something occurs **5 or more times** in a week.
Answer SOMETIMES if it occurs **2-4 times** in a week.
Answer RARELY if something occurs **never or 1 time** during a week.

Indicate whether or not the sleep habit is a problem by circling "Yes", "No," or "not applicable (N/A)".

Write in child's bedtime: _____ Write in child's usual wake time: _____
Child's usual amount of sleep each night (no naps): _____hours and _____minutes

Child's usual amount of sleep each day (naps): _____hours and _____minutes

	1 Usually (5-7)	2 Sometimes (2-4)	3 Rarely (0-1)	Problem?		
1. Child goes to bed at the same time at night	□	□	□	Yes	No	N/A
2. Child falls asleep alone in own bed	□	□	□	Yes	No	N/A
3. Child falls asleep within 20 minutes after going to bed	□	□	□	Yes	No	N/A
4. Child sleeps the right amount	□	□	□	Yes	No	N/A
5. Child sleeps about the same amount each day	□	□	□	Yes	No	N/A
6. Child wakes up by him/herself	□	□	□	Yes	No	N/A

Child has appeared very sleepy or fallen asleep during the following (check all that apply):

	0 Not Sleepy	1 Very Sleepy	2 Falls Asleep
7. Watching TV	□	□	□
8. Riding in a car	□	□	□

	3 Usually (5-7)	2 Sometimes (2-4)	1 Rarely (0-1)	Problem?		
9. Child falls asleep in parent's or sibling's bed	□	□	□	Yes	No	N/A
10. Child struggles at bedtime (cries, refuses to stay in bed, etc.)	□	□	□	Yes	No	N/A
11. Child needs parent in the room to fall asleep	□	□	□	Yes	No	N/A
12. Child is afraid of sleeping alone	□	□	□	Yes	No	N/A
13. Child sleeps too little	□	□	□	Yes	No	N/A
14. Child is afraid of sleeping in the dark	□	□	□	Yes	No	N/A
15. Child has trouble sleeping away from home (visiting relatives, vacation)	□	□	□	Yes	No	N/A
16. Child moves to someone else's bed during the night (parent, sibling, etc.)						
17. Child awakens once during the night	□	□	□	Yes	No	N/A
18. Child awakens more than once during the night	□	□	□	Yes	No	N/A
Write the number of minutes a night waking usually lasts: _____						
19. Child talks during sleep	□	□	□	Yes	No	N/A
20. Child is restless and moves a lot during sleep	□	□	□	Yes	No	N/A
21. Child sleepwalks during the night	□	□	□	Yes	No	N/A
22. Child wets the bed at night	□	□	□	Yes	No	N/A
23. Child grind teeth during sleep (your dentist may have told you this)	□	□	□	Yes	No	N/A
24. Child awakens alarmed by a frightening dream	□	□	□	Yes	No	N/A
25. Child awakens during night screaming, sweating, and inconsolable	□	□	□	Yes	No	N/A
26. Child snores loudly	□	□	□	Yes	No	N/A
27. Child seems to stop breathing during sleep	□	□	□	Yes	No	N/A
28. Child snorts and/or gasps during sleep	□	□	□	Yes	No	N/A
29. Child wakes up in a negative mood	□	□	□	Yes	No	N/A
30. Adults or siblings wake up child	□	□	□	Yes	No	N/A
31. Child has difficulty getting out of bed in the morning	□	□	□	Yes	No	N/A
32. Child takes a long time to become alert in the morning	□	□	□	Yes	No	N/A
33. Child seems tired in the morning	□	□	□	Yes	No	N/A

CPQOL
Cerebral Palsy Quality of Life

Version 2 July 2013

Quality of Life Questionnaire for Children (CP QOL-Child)

Primary Caregiver Questionnaire
(4-12 years)

ID# _____

Date:_____

PedsQL™

Pediatric Quality of Life Inventory

Version 4.0

PARENT REPORT for **CHILDREN** (ages **8-12**)

DIRECTIONS

On the following page is a list of things that might be a problem for **your child**. Please tell us **how much of a problem** each one has been for **your child** during the **past ONE month** by circling:

0 if it is **never** a problem
1 if it is **almost never** a problem
2 if it is **sometimes** a problem
3 if it is **often** a problem
4 if it is **almost always** a problem

There are no right or wrong answers.
If you do not understand a question, please ask for help.