

Imperial College London

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Genome-wide expression in neonatal encephalopathy

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DECLARATION OF ORIGINALITY

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ABSTRACT

Developing new treatments for babies with birth asphyxia related brain injury (neonatal encephalopathy) and preventing cerebral palsy is hampered by two major roadblocks. Firstly, different babies with birth asphyxia may appear clinically similar, but their brain injury may be due to different underlying reasons and they may have different outcomes. Secondly, it takes several years before the impact of brain injury becomes measurable through clinical testing.

In my thesis, I examine two key hypotheses (a) cerebral magnetic resonance biomarkers during the neonatal period can accurately predict neurodevelopmental outcomes at 2 years following therapeutic hypothermia for neonatal encephalopathy (b) neonatal encephalopathy is associated with a unique host gene expression profile at the time of birth.

THESIS DESIGN AND FINDINGS

To test the first hypothesis of my thesis, I examined 190 consecutive term and near-term infants with neonatal encephalopathy recruited as part of Magnetic Resonance Biomarkers in Neonatal Encephalopathy (MARBLE) study who had neurodevelopmental outcome assessments (Bayley III) at 2 years and conventional MR imaging within two weeks of birth. Basal ganglia/thalami MR imaging scoring had the best accuracy in predicting the long-term outcome, area under the receiver operating characteristic curve of 0.81 (95% CI 0.72-0.91).

In a subgroup of 47 babies with mild neonatal encephalopathy on neurological examination performed within 6 hours after birth, cooling therapy (n= 32) was associated with significantly higher levels of thalamic N-acetylaspartate/Creatine, N-acetylaspartate/Choline ($p<0.001$) and lower white matter injury scores than non-cooled (n=15) babies ($p=0.02$).

In the second part of the analysis, I examined the whole blood gene host gene expression of neonatal encephalopathy and showed that encephalopathic babies (n=12) have a different gene expression profile when compared to healthy controls (n=6) and babies with culture proven sepsis (n=10).

I then analysed the gene expression profile of 45 infants with moderate/severe neonatal encephalopathy recruited as part of Hypothermia for encephalopathy in low and middle-income countries (HELIX) trial and found an RNA signature of 320 genes that discriminated between the encephalopathic babies with good (n=22) from the ones with adverse outcome (n=23) (death or moderate/severe disability at 18-24 months).

In this thesis I provide evidence which indicates that the overall accuracy of conventional MRI for prediction of long-term outcome is suboptimal, and that babies with mild NE have less brain injury after whole-body cooling. This suggests that cooling may be neuroprotective in mild neonatal encephalopathy. In addition, I demonstrate that gene expression has the potential to become a promising disease stratification tool and a surrogate biomarker in neonatal encephalopathy.

ABBREVIATIONS AND ACRONYMS

BGT	Basal ganglia thalami
BNIP3	BCL2/adenovirus E1B interacting protein 3
CI	Confidential interval
Cr	Creatine
Cho	Choline
FBC	Full blood count
FDR	False discovery rate
GPI	Glucose phosphate isomerase
HELIX	Hypothermia for encephalopathy in low and middle-income countries
HIF-1A	Hypoxia-inducible factor 1A gene (Humans)
<i>Hif1A</i>	Hypoxia-inducible factor gene (Rats)
HIF-1 α	Hypoxia-inducible factor protein (Humans)
ICE	Infant Cooling Evaluation
IL7R	Interleukin 7 receptor
Lac	Lactate
NE	Neonatal Encephalopathy
MALAT1	Metastasis Associated Lung Adenocarcinoma Transcript 1
MARBLE	Magnetic Resonance Biomarkers in Neonatal Encephalopathy
mRNA	Messenger RNA
MR(I/S)	Magnetic Resonance (Imaging/Spectroscopy)
NF	Nuclear factor
NAA	N-acetylaspartate
PCA	Principal Component Analysis
PLIC	Posterior limb of internal capsule
PSMB2	Proteasome subunit beta 2
RGS1	Regulator of G-protein signalling 1
RNA	Ribonucleic acid
<i>Vegf</i>	Vascular endothelial growth factor gene (Rats)

THESIS CONTRIBUTIONS

I conducted all the work presented in this thesis apart from the following:

- T_1 - and T_2 -weighted MR imaging was reported first by Dr Sudhin Thayyil (Supervisor) and then by me blinded to Dr. Thayyil's score.
- Peter J. Lally developed the MR spectroscopy protocol and analysed the MR spectroscopy data by using software developed in-house and LCModel, which formed part of his thesis. I have not included any work that has been part of Lally's PhD in my thesis.
- Recruitment and blood sample collection of the encephalopathic babies in HELIX was undertaken by a number of dedicated research nurses in India, Bangladesh and Sri Lanka
- RNA extraction was performed by me and Professor Max Vento's lab staff in Valencia, Spain and by the Medgenome lab team in Chennai, India.
- Next Generation sequencing was performed by Prof Max Vento's lab in Valencia, Spain and by the Medgenome team lab in Chennai, India.

Research output

Peer reviewed publications

1. **Montaldo P**, Lally PJ, Oliveira V, Thayyil S (in press). *Hypothermic Neuroprotection for Neonatal Encephalopathy in Low and Middle-Income Countries: A New Approach to an Old Problem*. Neoreviews 2018.
2. Kariholu U., **Montaldo P.**, Markati T. et al. (in press). *Therapeutic hypothermia for mild neonatal encephalopathy: A systematic review and meta-analysis*. Arch Dis Child Fetal Neonatal Ed. 2018
3. *Lally PJ, ***Montaldo P**, Olivera V et al. *Magnetic resonance spectroscopy assessment of brain injury after moderate hypothermia in neonatal encephalopathy: a prospective multicentre study*. Lancet Neurol. 2018 Nov 14. pii: S1474-4422(18)30325-9.
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Platform presentations at major conferences

1. Transcriptomic signatures of adverse outcomes after neonatal encephalopathy. The Neonatal Society, London, UK, November 2018.
2. Gene expression profiling soon after birth reveals specific transcriptome profiles in full term babies with neonatal encephalopathy, Poster Symposium "Neonatal Brain Metabolism and Injury" (Pediatric Academic Societies 2018), Toronto, Canada, April 2018.
3. Cooling therapy is associated with improved MR biomarker measures after mild neonatal encephalopathy. The Neonatal Society, London, UK, March 2018.
4. Temporal changes in genome-wide expression in hypoxic ischaemic encephalopathy. Annual Meeting of the European Society of Paediatric and Neonatal Intensive Care (ESPNIC 2017), Lisbon, Portugal, June 2017.
5. Genome-wide expression in perinatal hypoxic ischaemic encephalopathy. The Neonatal Society, London, UK, November 2016.

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Chapter 1: Background

Neonatal Encephalopathy

Neonatal encephalopathy (NE) is presumed to be the end-point of an acute and unexpected reduction in blood flow and oxygen to the fetal brain, immediately prior to delivery. A few hours after birth the injury progresses to catastrophic secondary energy failure, leading to permanent brain damage.

NE is a broader term which reflects the clinical manifestation of a global cerebral dysfunction due to an intrapartum hypoxic insult or other causes¹. The term NE is just descriptive without any assumptions about either the pathogenesis or the aetiology. On the other hand, the term hypoxic-ischaemic encephalopathy assumes that the NE is due to a hypoxic ischaemic event and implies that the cause of the encephalopathy is known. Although at the moment there is still debate about which term to use^{2, 3}, in my thesis I decided to focus on the term NE since I examined different disease mechanisms and causes of NE with the aim of developing novel disease stratification biomarkers.

NE occurs in 2 to 4 per 1000 live births and is the most common cause of death and neurodisability in term babies⁴. The economic burden to the Treasury on support costs of cerebral palsy is extremely high (£4 billion per year), with a £3.1 billion annual litigation cost⁵. Unfortunately, there has been little progress in preventing and treating this condition over the past three decades, except for the introduction of cooling therapy, which is now used as a standard therapy in the NHS.

Clinical and animal studies showed that a primary phase of energy failure occurs during hypoxia-ischaemia, followed by a transient recovery of cerebral metabolism measured by MR spectroscopy, prior to the onset of secondary energy failure with rapid depletion of ATP and Phosphocreatine, during which majority of the cell death occurs⁶.

The efficacy of therapeutic hypothermia is highly dependent on the duration, depth and timing of the treatment. The current recommendations based on overwhelming pre-clinical data and

robust randomized trials show that a 72 hour-cooling duration at 33.5°C is required for neuroprotection⁷⁻⁹.

The initial pre-clinical evidence showed inconsistent results when a brief period of mild to moderate therapeutic hypothermia (0.5–3 hours) was tested in different animal models¹⁰⁻¹⁵. These data highlighted also a reduction in neuroprotection effect over the time and demonstrated that neuroprotection was lost when hypothermia treatment was delayed by as little as 15–45 min after the insult^{12, 16-18}.

The subsequent animal studies therefore focused on how to prevent the secondary phase of energy failure by extending the cooling period between 5 and 72 h. In an adult rat model of reversible middle cerebral artery occlusion, 21 hours but not 1 hour of mild hypothermia appeared to be effective in decreasing the area of infarction¹⁹. Further evidence in unanesthetized postnatal day 21 rats subjected to hypoxia-ischemia, highlighted that mild hypothermia (2° to 3°C reduction in body temperature) for 72 hours after the insult prevented cortical infarction, whereas cooling for 6 h did not²⁰. Large animal evidence showed that mild systemic hypothermia started after the hypoxic ischaemic insult and lasting 12 to 48 hours decreased neuronal death and prevented from the secondary energy failure phase^{21,22}. Finally, Gunn et al studied in a hypoxic model of near-term fetal sheep whether prolonged head cooling for 72 hours, delayed into the late post-insult period, improved outcome from severe ischemia. The authors demonstrated that therapeutic hypothermia maintained throughout the secondary phase of injury significantly decreased the extent of cortical infarction and neuronal loss^{23, 24}.

Altogether these preclinical studies led to the discovery of rescue hypothermic neuroprotection, which was able to reverse the injury if given during an initial window period. Although the mechanisms of this neuroprotection are not fully explained, it is considered to reduce cerebral metabolism, stabilise the blood-brain barrier, reduce inflammatory and excitatory amino acid responses, and suppress apoptosis.

Therapeutic hypothermia is now used as the standard therapy for NE in all industrialised countries and it reduces the apparent brain injury on MR imaging, improves the survival of babies, and increases their likelihood of having a normal neurological outcome. The pooled data from 7 large randomised trials have clearly shown that this therapy reduces the likelihood of death (Risk Ratio (RR) 0.75; 95% Confidence Intervals (CI) 0.64 to 0.88), and neurodisability (RR 0.77; 95% CI 0.63 to 0.94) after moderate or severe neonatal encephalopathy⁷. Since the introduction of therapeutic hypothermia, it has provided an estimated saving of approximately £125 million per year for the NHS²⁵.

However, in low and middle-income countries there is still conflicting evidence regarding the benefits of whole-body cooling, which may reflect the extreme heterogeneity in trial methodologies and their small sample sizes in these settings. Further concerns about the introduction of therapeutic hypothermia in low and middle-income countries are related to differences in population comorbidities, in the underlying brain injury after NE and the lack of healthcare infrastructure with optimal tertiary intensive care ²⁶. Despite the lack of data about the safety and efficacy of therapeutic hypothermia in these settings, many centres have now started offering whole-body cooling. We recently conducted a survey in India including 90 tertiary neonatal units and showed that over half of them offered whole body cooling even though, in most of cases, cooling practices were outside the criteria established in high-income countries²⁷. These data are particularly concerning given that the direct extrapolation of evidence from high-income countries has resulted in harm in different instances like the Fluid Expansion as Supportive Therapy (FEAST) trial²⁸. This study examined the efficacy of fluid bolus for septic shock in African children and was prematurely stopped because of the increased mortality in the interventional arm. Without this evidence, the use of fluid bolus would have potentially caused thousands of deaths in low and middle-income countries.

A large study, the Hypothermia for Encephalopathy in Low and middle- Income countries (HELIX) Trial is now being conducted and will recruit over 408 babies with moderate or severe NE randomised to therapeutic hypothermia or normothermia²⁹. This study will provide the most comprehensive data about the efficacy of therapeutic hypothermia in low and middle-income countries.

Magnetic resonance biomarkers and host gene expression in neonatal encephalopathy

DISEASE BURDEN AND UNMET CLINICAL NEEDS

Despite the widespread use of therapeutic hypothermia, up to 35-40% of these infants are still likely to have long-term adverse outcome with a cost to the NHS of £750,000 for each child⁴. The potential long-term morbidities following NE include cerebral palsy, cognitive impairments, epilepsy, blindness and hearing impairments.

This observation contrasts with the pre-clinical data, where therapeutic hypothermia has been uniformly effective, both in small and large animal models. The animal models are based on inducing an acute perinatal hypoxic event, either by occluding the umbilical cord (intra-uterine sheep models)²³, or by postnatal induction of acute hypoxia with or without transient occlusion of cerebral blood flow, in otherwise healthy animals²¹. Instead, in a clinical scenario, such acute events (typically placental abruption) only account for a small proportion of perinatal NE³⁰. NE is more likely to have a multifactorial aetiology with perinatal infection/inflammation considered as potential casual pathway^{31, 32}.

The presence of placental lesions is common in NE, suggesting that perinatal infection/inflammation might play a role in the pathogenesis of NE. McDonald *et al* in fact, compared placenta histology of 93 infants with NE with normal term controls (n=816) and random controls (n=387) and showed that chorioamnionitis and villitis were more frequent in NE³³. More recently Tann *et al.* studied the perinatal risk factors for NE in an unmatched case-control study in Uganda and found that neonatal bacteraemia and histological funisitis, but not chorioamnionitis were independent risk factors of NE³⁴. However, results of histology often are not in agreement with the clinical scenario and the success rate of pathogen isolation from the blood of neonates with suspected sepsis is as low as 6 %^{35, 36}. Therefore, the evidence so far, still cannot be considered conclusive.

Animal evidence suggests that perinatal infection increases the susceptibility of neonatal brain to hypoxic-ischaemic brain injury^{37, 38}. Moreover, the neuroprotective effect of therapeutic hypothermia seems to be highly pathogen-dependent. Falck *et al.* showed that therapeutic

hypothermia was ineffective in a rat model of inflammatory pre-sensitisation with lipopolysaccharide, whereas its neuroprotection was preserved in a model of Gram-positive infection^{39, 40}. These findings are extremely important given that over 90 % of infections in term infants appears to be caused by Gram-positive bacterial species in developed countries³⁵. On the contrary, in low and middle-income countries, where the incidence of gram-negative infections is much higher⁴¹, neuroprotective effect of therapeutic hypothermia is still debated.

Despite the complex pathophysiology due to the uncertain severity, timing, and patterns of the hypoxic insult, therapeutic hypothermia is still offered uniformly as a one size-fits-all strategy based on an early neurologic examination, which itself has a limited value in identifying all infants at risk of an adverse neurological outcome⁴². Thus, the lack of rescue neuroprotective effects in all babies is not surprising, considering the clinical heterogeneity. Furthermore, approximately 25% of babies who are considered to have milder encephalopathy soon after birth, who are thus not offered therapeutic hypothermia, will then go on to develop a long-term adverse neurological outcome^{43, 44}.

Currently, there is a concerted effort from the research community to develop additional therapeutic strategies for these babies, which will rely on an effective test at birth to identify babies at the highest risk of long-term neurodisability. In fact, despite many novel neuroprotectants showing promising results in preclinical studies, clinical translation is extremely challenging due to the lack of robust validated surrogate biomarkers. These difficulties have held back the development of novel neuroprotective approaches, which could be utilised in combination with therapeutic hypothermia.

In all the major trials in NE, outcome definition was dichotomous with abnormal outcome considered as death or moderate/severe disability⁷. The selection of the most appropriate outcome to evaluate the efficacy of an intervention is essential. However, rarely parents are involved in the decision process of the outcome definition. Sinha *et al.* systematically reviewed all the studies, which assessed the appropriate choice of outcomes for clinical research in paediatrics and found that only 3 groups out of 13, involved parents in selecting outcomes and none of them was in NE⁴⁵. In most of studies in NE the primary prognostic measure is the area under the curve (AUC). However, it might be that some parents prefer a result that is more

specific and therefore if positive, there is a high probability of having a disease or sensitive and therefore, if negative it is possible to rule out a disease.

Long-term neurodevelopmental outcome is certainly still considered the gold standard as an outcome measure. However, the gap of several years between the intervention and the outcome, and the large sample sizes required for such studies have led researchers worldwide to seek robust surrogate biomarkers which could improve the efficiency and power of trials. Over the past decade different biomarkers have enhanced our ability to identify, assess the onset and quantify the features of brain injury soon after birth. These tools may be valuable as surrogate outcome measures of the treatment effects of adjunct neuroprotective therapies and become powerful translational biomarkers. One potential source of effective biomarkers is magnetic resonance (MR) imaging and spectroscopy.

MAGNETIC RESONANCE IMAGING IN NEONATAL ENCEPHALOPATHY

MR imaging (MRI) has been used in NE since the 1980s, first as a research tool and subsequently, in the clinical field thanks to the development of dedicated MR acquisition protocols and MRI scoring systems for newborn infants after NE.

Neonatal MRI is currently the mostly widely used modality for the assessment of injury and prediction of neurodevelopmental outcome after neonatal encephalopathy. Other brain imaging modalities such as head ultrasound are less accurate for disease stratification and their role for prognostication is only marginal⁴⁶.

Different systematic reviews and meta-analyses have examined the diagnostic accuracy of MRI for prediction of adverse neurodevelopmental outcome after NE showing an overall sensitivity of 91% (95% CI: 87%–94%) and specificity of 51 % (95% CI: 45%–58%) with late MRI (day 8-30 after birth) showing a high sensitivity (97%)⁴⁷.

The American Academy of Neurology recommends a brain MRI between days 2 and 8 in term NE, both for diagnostic and prognostic purposes⁴⁸. The two most commonly used MR techniques to quantify macrostructural and metabolic injury in babies are T₁- and T₂-weighted imaging and single voxel proton MR spectroscopy (MRS), respectively.

Brain MRI shows specific patterns of brain injury on T₁- and T₂-weighted imaging after a hypoxic-ischaemic insult, depending on its timing, severity and duration. The commonest injury patterns involve either the watershed region or the deep grey nuclei of the basal ganglia and thalamus (BGT)⁴⁹. The watershed predominant pattern involves the parasagittal white matter in the vascular watershed areas, sometimes extending to the cortical grey matter if the insult is particularly severe. This pattern of brain injury is usually due to a partial prolonged asphyxia⁵⁰. On the contrary, a BGT injury pattern reflects an acute profound event and predominantly involves the deep grey nuclei. The vast majority of the NE babies have white matter injury (40–60% of the cases) whereas injury to the BGT occurs in approximately 25% of cases⁵¹. The duration, pattern and features of hypoxia-ischemic brain injury are a continuum and therefore MRI often has mixed appearances. Of note over 30% of MRI abnormalities show a mixed BGT/ white matter pattern⁴⁹. The presence of severe white matter injury in the

presence of deep central deep nuclei damage suggests a more-prolonged hypoxic-ischemic insult and these cases have a poor prognosis.

These distinctive MRI brain injury patterns, together with the involvement of specific brain structures (e.g. the posterior limb of the internal capsule, PLIC) can be used to predict specific neurodevelopmental outcomes^{47, 52}. Miller SP *et al.* showed that basal-ganglia brain injury pattern was associated with the most impaired motor and cognitive outcome at 30 months with over 50% of infants presenting with spastic quadriplegia⁴⁹. On the other hand, infants with white matter injury are more likely to have cognitive deficits⁵³.

Brain injury on conventional MRI appears to be more evident between the first and the second week of life. However, recent evidence has demonstrated the accuracy of early scans. Chakkarapani *et al.* reported a good agreement between early (days 3 to 6) and late scans (days 10 to 14) with regard to white matter injury $\kappa = 0.87$ and BGT injury $\kappa = 0.67$ ⁵⁴. This is consistent with Skranes *et al.* who found an equally good agreement ($\kappa = 0.85$) in the imaging findings between early and late scans⁵⁵.

Brain MRS acquires signals from the metabolites most present in the tissue and, in this way, can quantify them and give an insight into metabolic perturbations following NE. The strongest signals in the neonatal brain arise from N-acetylaspartate (NAA), creatine (Cr) choline (Cho) and lactate (Lac). NAA is the most abundant amino acid in neurons, where it is almost exclusively located, making it a useful marker of neuronal integrity. NAA reacts with glutamate to form N-acetylaspartylglutamate. Although on MRS these metabolites form 2 separate peaks at high magnetic fields, in clinical MRI scanners their peaks converge. Therefore, they are usually combined and referred to as 'tNAA'.

Cr and Phosphocreatine are contained in the neuronal and glial cells and play a crucial role in energy metabolism maintaining stable the levels of adenosine triphosphate. Due to the strong overlap between Cr and Phosphocreatine peaks, they are combined and referred to as 'tCr'. The different compounds containing Cho are the main constituents of the cell membranes and these give insight into membrane turnover and synthesis. Due to the difficulty in separating the peaks from the different compounds, they are typically summed and referred to as 'tCho'.

Lac is a product of anaerobic glycolysis. It is present only in low levels in healthy brain whereas its levels are elevated in case the brain energy substrate delivery is altered, as is the case after hypoxic ischaemia. Lac produces a prominent doublet at 1.31ppm, which overlaps with Threonine's doublet at 1.32ppm making them indistinguishable on clinical MRI systems. The sum is therefore quantified, and termed tLac. The levels of these metabolites can be reported as absolute concentrations, or more simply, as relative ratios of their signal intensities.

Numerous studies have evaluated the use of MRS in NE. Thayyil *et al.* conducted a large meta-analysis on the efficacy of different MR biomarkers and found that tLac/tNAA ratio in the basal ganglia had the highest accuracy, with a pooled sensitivity of 0.82 and specificity of 0.95 with a cut-off of tLac/tNAA>0.29⁴⁷. However, this was based on publications from single centre studies, meaning it is of limited use for large multi-centre trials. Ancora *et al.* subsequently performed a direct comparison of conventional MRI, diffusion-weighted MRI and ¹H MRS in a group of 20 infants with NE and showed that the tNAA/tCr ratio measured in the basal ganglia was the most effective at predicting adverse outcome at 2 years (predictive value of 1.0 and negative predictive value of 0.93)⁵⁶. More recently, MRS biomarkers have been validated in one of the largest prospective studies in NE, Magnetic Resonance Biomarkers in Neonatal Encephalopathy (MARBLE)⁵⁷. The MARBLE study involved 8 different neonatal intensive care units (3T MRI scanners) across the UK and USA and all of the MRI sequences were harmonised for use prior to the start of the study⁵⁷. This study tested the prognostic accuracy not only of different MRS biomarkers but also of diffusion weighted MRI, conventional T₁- and T₂-weighted MRI, as well as clinical examinations and amplitude integrated electroencephalography. The authors showed that the absolute concentration of thalamic NAA, measured in the thalamus within two weeks of birth, had a near perfect 97% specificity and 100% sensitivity in predicting an adverse neurological outcome at 2 years after NE⁵⁸.

Despite being highly accurate, MRI is held back by the inability to perform a scan as soon as possible after birth. Inevitably, clinicians have to make treatment decisions before this information is available. The use of blood biomarkers as an early diagnostic tool has been extensively investigated in NE – this includes biomarkers associated with brain, blood vessels, blood brain barrier, oxidative stress and inflammation. Although some of them have been shown to be promising, the pathophysiology of NE involves multiple contributory pathways

and therefore, a single “magic” biomarker is unlikely to be the solution. Gene expression profiling has the ability to interrogate the entire genome, providing an opportunity for de novo discoveries and for these reasons, it is now emerging as an early and powerful prognostic and diagnostic biomarker.

GENE EXPRESSION AFTER HYPOXIC ISCHAEMIA

Gene expression profiling measures the expression level (i.e. number of gene copies) of messenger RNAs (mRNA) in a cell population at a certain time. This approach enables researchers to study the expression of thousands of genes simultaneously and in this way, it is possible to identify a subset of genes whose expression is associated with a specific condition, clinical outcome or therapeutic response.

During the immediate period of hypoxia-ischaemia (the “primary” phase of the injury), high-energy metabolites are depleted, leading to the progressive depolarisation of cells, severe cytotoxic oedema, and the extracellular accumulation of excitatory amino acids due to a failure of reuptake by astrocytes. Although many neurons initially partially recover from the insult in a so called ‘latent’ phase, they then develop progressive dysfunction and die many hours, or days later (secondary phase)⁵⁹.

Cells experiencing hypoxia undergo different biological responses following these unfavourable conditions. Although hypoxia generally inhibits mRNA synthesis, the expression of a subset of genes is strongly upregulated⁶⁰. In particular, preclinical evidence showed increased mRNA levels of membrane receptors Notch1, glycoprotein 130 and epidermal growth factor receptor in the sub-ventricular zone of a perinatal hypoxia/ischemia rat model^{61, 62}. These changes in mRNA levels corresponded to an increased cell proliferation in the subventricular zone, suggesting a possible role for these membrane receptors in mediating neurogenesis after brain injury.

Preclinical studies have largely contributed to the understanding of the biological changes after hypoxic-ischemia showing that the master switch, orchestrating the cellular response to low oxygen levels, is the transcription factor termed hypoxia-inducible factor (HIF). Animal evidence showed that after unilateral occlusion of common carotid artery, there was an increase of the *Hif1A* levels in rat neurons^{63, 64}. Subsequent studies suggested a temporal change in the *Hif1A* expression after a hypoxic event with a peak after 3–4 hours and *Hif1A* levels persisted elevated for up to 24 hours after the insult. HIF-1 α expression also seems to be influenced by the presence of pure hypoxia or a combination of hypoxia and ischaemia⁶⁵.

HIF-1 α plays an important role in the brain development. Iyer *et al.* showed that *Hif1A* expression increases from day 8 to day 9 in mouse embryos⁶⁶. Meanwhile, *Hif1A* knockout mice had hydrocephalus and spatial memory impairment at 10 weeks of age. In a study with murine midbrain-derived neural precursor cells, 3% O₂ exposure for two weeks led to an impairment of neuron survival and proliferation, probably through the downregulation of *Vegf*⁶⁷.

Since HIF-1 α was first recognised as essential for the development of different mammalian organs, including the brain, different studies have targeted the HIF-1 α pathway. Rosenberger *et al.* inhibited prolyl-4-hydroxylase activity, which is responsible for HIF-1 α ubiquitination and destruction⁶⁸. In this way, there was a protective effect mediated by *Hif1A* upregulation in an animal model of hypoxic kidney injury⁶⁹. Schneider *et al.* instead, injected FG-4497 (a prolyl-4-hydroxylase activity inhibitor) intraperitoneally in P7 mice and observed a dramatic increase in brain levels of *Hif1A* together with upregulation of target genes like *Vegf* and erythropoietin⁷⁰. Jones *et al.* showed in a P6 rat model that after hypoxia pre-conditioning (3 hours at 8% O₂) there was a prolyl-4-hydroxylase activity inhibition, resulting in a significant increase of targeted *Hif1A*.

Preclinical evidence suggests that HIF-1 α is responsible for neural cell fate determination after NE. HIF-1 α activates genes, which are translated into both pro-apoptotic proteins such as NIP3, BCL2/adenovirus E1B interacting protein 3 (BNIP3), Noxa, and anti-apoptotic proteins like Mcl-1⁷¹⁻⁷³. This has led to the idea that the balance between pro- and anti-apoptotic factors has an essential role in the response to hypoxia and is dependent on the cell-type and severity of the hypoxic insult.

Chen *et al.* found in a cell culture model that HIF-1 α inhibited p53 ubiquitination and blocked the nuclear export of p53 during hypoxia, resulting into an increased expression of apoptosis-associated genes⁷⁴. These findings are in agreement with a neonatal rat model where *Hif1A* overexpression induced upregulation of *Bnip3* leading to an increased apoptosis in the neonatal rat brain⁷⁵.

On the other hand, emerging evidence suggests that HIF-1 α also has anti-apoptotic functions. In a rat model of chronic hypoxia (10% O₂ for 2 weeks) there was no apoptosis after a sustained increase of *Hif1A* levels⁷⁶. This bivalent role of HIF seems to be dependent on the duration and severity of the hypoxic event. In fact, after mild hypoxic insults HIF-1 α is phosphorylated and dimerised and HIF-1 β leads to upregulation of anti-apoptotic genes such as erythropoietin, whereas more severe hypoxic insults cause HIF-1 α dephosphorylation with an overall increase in p53 levels⁷⁷.

Other studies have showed a time-dependent effect of HIF. In a mouse ischemia model, HIF-1 α activation had biphasic effects after cerebral ischemia. In the first phase shortly after ischaemia, HIF-1 α activation led to upregulation of pro-apoptotic genes whereas from 48 hours until 8 days after the insult, there was an increase of anti-apoptotic genes⁷⁸. In this model, the HIF pathway mediated an overall beneficial action as indicated by increased cell death and brain tissue damage in mice with *Hif1A* neuron-specific knockdown subjected to transient focal cerebral stroke⁷⁸.

GENE EXPRESSION DATA FROM MIXED CELL POPULATIONS

In the last years gene expression has substantially contributed to the understanding of the biological mechanisms underlying different diseases⁷⁹⁻⁸². However, the heterogeneous nature of the samples analysed has been often underestimated. Gene expression analysis from heterogeneous samples (such as whole blood) often ignores cell type composition as an important confounding factor. This results in either a loss of signal from low abundance cell types or a false gene expression difference. Different individuals might have a different proportion of immune cells with different contributions to the overall gene expression profile. Therefore, a difference in gene expression might solely be due to different blood cell type composition rather than a genuinely different RNA signature.

Traditionally gene expression analysis has avoided this problem, limiting the conclusions that could be drawn from the experiments and more importantly the reproducibility of the findings.

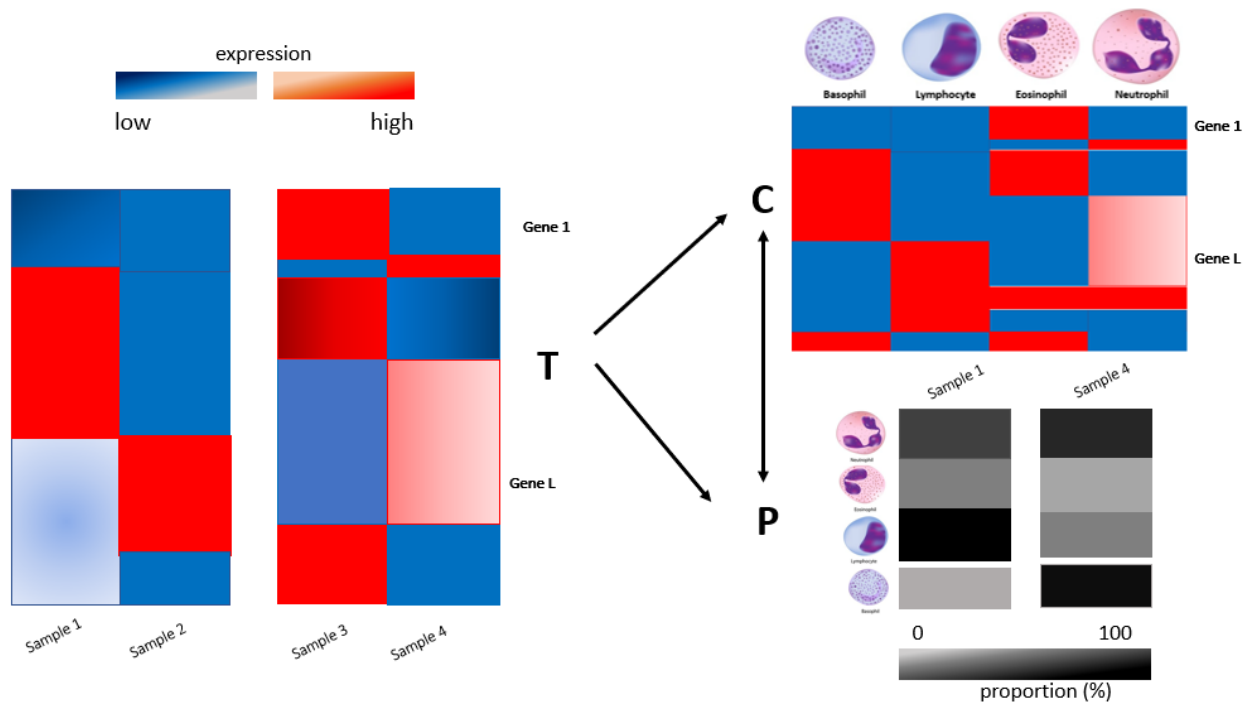
Whilst the field of single-cell genomics (gene expression derived from a single cell) has grown exponentially, different computational methods have come to the fore so that it is now possible to correct the gene expression profile according to cell type composition (deconvolution). One of the first deconvolution models was developed by Venet *et al.* in 2001⁸³. The authors showed that it was now possible 'to infer the gene expression profile of the various cellular types [. . .] directly from the measurements taken on the whole sample'⁸³.

All deconvolution algorithms are based on multiple linear regression models based on the following equation,

$$T = P \cdot C$$

where T is the overall gene expression data from heterogeneous samples and represents the gene expression data we want to deconvolve, P is the mixing proportion and represents the proportion of the different blood cell types we want to know. C is the cell type specific RNA signature and is considered the fingerprint for each blood cell type (Figure 1). There are different sources where it is possible to download cell type RNA signatures. One of the most used is GEO database where thousands of different experiments' gene expression data are accessible⁸⁴, including studies where different blood cell types have been isolated and sequenced so as to identify their specific signature^{88, 178-181}.

Figure 1. T is the matrix containing the measured gene expression values from heterogeneous samples; C is the matrix of cell type-specific RNA expression values; P is the matrix containing the mixing proportions. In this thesis, I have estimated P given T and C (adapted from Computational deconvolution of transcriptomics data from mixed cell populations, Bioinformatics 2018, 1;34:1969-1979).



There are different mathematical approaches to solve the deconvolution equation. The most commonly used are regression-based methodologies which include non-negative least squares, latent variable analysis and support vector regression.

Non-negative least squares is based on two main assumptions: 1) the proportions of the different cell-types must be positive between 0 and 1 ('non-negativity' constraint). This is important since a negative value of the proportion following deconvolution would be meaningless; 2) the sum of proportions within each sample is 1 ('sum-to-one' constraint)⁸⁵. However, this approach can fail when dealing with outliers resulting in high computational times and low rates of convergence. To overcome these problems Gong *et al.* have developed an alternative technique based on quadratic programming, which is now part of an R package (DeconRNASeq package)⁸⁶.

A different mathematical approach has been developed as part of the R package CellCODE. This model is based on a latent variable analysis where non-negative least squares optimisation

unknown cells type in the mixture. Supervised learning model has the advantage of having prior knowledge of what the output values for deconvolution should be. Therefore, the aim of supervised learning is to learn a function that, based on the data and desired outputs, best predicts the relationship between input and output observable in the data. This approach performs better in case of multicollinearity (presence of correlated cell types in the mixture), which is an important issue in whole blood gene expression.

Pollara *et al.* recently performed a meta-analysis of different transcriptional modules (cell-type specific RNA signatures)⁸⁹. In this meta-analysis the authors derived new modules by using a specific strategy. They compared multiple datasets from purified cells and identified which transcripts had >2-fold increased expression in the cell of interest compared to all other cells and were shared in the different datasets. Secondly, they found the genes which were in common amongst multiple datasets to identify co-correlated genes and assemble new modules. They also developed a modular discrimination index that assesses the expression of each module in the target cell type in comparison to other cells, which can be used to identify the best performing modules⁸⁹.

Whilst the deconvolution algorithms I have described here have been increasingly included in analyses of gene expression data in adults and children, there is no published data on the use and accuracy of these deconvolution methodologies in neonates.

GENE EXPRESSION IN NEONATAL ENCEPHALOPATHY

Gene expression profiling is well established in certain clinical areas. For example, it is employed in cancer research to predict clinical outcome, identify the presence of metastases, and develop personalised treatments⁹⁰. More recently, specific expression patterns and pathways have been described in several paediatric infectious diseases, including paediatric tuberculosis, allowing disease stratification⁹¹⁻⁹³.

However, at the time of writing, there are no clinical studies concerning gene expression in NE apart from what I present in this thesis and in a related publication⁹⁴. The evidence therefore,

is mainly obtained from animal and culture studies, which allow a better understanding of the biological mechanisms involved in brain injury after NE.

These data have revealed that cell death after NE manifests as an apoptotic-necrotic continuum with the presence of intermediate structural and biochemical forms such as autophagy and excitotoxicity⁹⁵. These findings showed that different factors affect the extent of brain injury including brain maturity, capacities for DNA repair and antioxidant status⁹⁵. To decide the cell fate, there is a complex interaction among different pathways, which involve proteins such as p53 and the Bcl-2 family. Neonatal rat models showed that within 24 hours of hypoxic-ischaemia, there was a rapid and significant upregulation of Bax prior to the activation of apoptosis-effector mechanisms following excitotoxic neonatal brain injury^{96, 97}. In support of these findings, inhibition of the Bax pathway resulted in increased neuroprotection⁹⁸.

Animal evidence demonstrates that hypoxic ischaemia exacerbates autophagic flux in neonatal rats and leads to learning and memory impairment⁹⁹. Furthermore, these data highlight that the inhibition of autophagy could offer potential protection, which is partly mediated by cAMP-response element-binding protein activation, an important protein, which controls synaptic plasticity¹⁰⁰. Autophagy is a crucial intracellular catabolic pathway that works in parallel with the ubiquitin-proteasome system. It represents a homeostatic mechanism, which is responsible for the intracellular bulk degradation of cellular constituents and which is over-activated after hypoxic-ischaemic injury¹⁰¹.

The concept of a cell death continuum is crucial to the development of new therapeutic interventions. It is now clear that any pharmacological interventions targeting only a single mechanism of injury, such as apoptosis or autophagy, are unlikely to be beneficial^{102, 103}. In fact, new evidence suggests that this might push cell degeneration from apoptosis to necrotic cell death, and hence worsen hypoxic-ischaemic brain injury¹⁰³.

It is now becoming apparent that neurogenic capacity is preserved or even increased after events such as stroke¹⁰⁴, seizures¹⁰⁵, or hypoxia–ischaemia¹⁰⁴. Previous microarray studies in neonatal hypoxic-ischaemic models have showed an up-regulation of genes involved in

neurogenesis such as Epidermal growth factor receptor, Glycoprotein 130, TGFb-1, membrane receptors Notch1 and its intracellular portion^{61, 62, 106}. These genes play an essential role in promoting the proliferation, migration, differentiation, and survival of neurons.

As discussed above, HIF-1 α is crucial in the delicate balance between apoptosis and anti-apoptosis. Many factors regulate HIF-1 α expression and stabilisation including cytokines and oxidative stress radicals. By using fetal astrocyte cultures, Argaw *et al.* showed that HIF-1 α and VEGF were upregulated 6 hours after cytokine IL-1 β treatment¹⁰⁷. The same increase of HIF was also found after TNF- α treatment whereas this upregulation was blocked by nuclear factor (NF)- κ B infusion¹⁰⁸. A cell culture study on murine hippocampal cells showed that N-acetylcysteine butylated hydroxyanisole infusion inhibited the pro-apoptotic effects of HIF-1 α overexpression through the BNIP3 pathway¹⁰⁹.

The evidence so far therefore, suggests that gene expression profiling may be the answer to the need for an effective disease stratification tool for the development and evaluation of neuroprotective therapies in NE. Gene expression is an extremely promising disease stratification tool in paediatric and neonatal infection as highlighted by several high-profile publications⁹¹⁻⁹³. However, its use in NE had not been tested before, and therefore whether the available gene expression algorithms are valid in this population is not known.

MRI biomarkers despite being effective in stratifying NE based on aetiology and as prognostic tools, are limited because MRI is less informative and far more challenging to undertake soon after birth. At the moment, there is a great interest into gaining a better insight into the pathophysiology of NE in order to develop a targeted neuroprotection approach. Studying the relationship between structural MRI and MRS biomarkers has the potential to add to our knowledge of NE by linking macroscopic brain injury to the correspondent brain metabolic perturbation.

In my thesis, my key aims are (a) to assess inter-rater robustness and accuracy of T₁- and T₂-weighted structural cerebral magnetic resonance biomarkers in predicting neurodevelopmental outcomes at 2 years and to study the relationship between MRI and MRS biomarkers (b) to examine if NE is associated with a unique host gene expression profile at the

time of birth and if gene expression differs between those who subsequently develop favourable or adverse long-term neurological outcomes.

PART A:

This part of my thesis examines my first hypothesis: that cerebral magnetic resonance biomarkers during the neonatal period can accurately predict the neurodevelopmental outcomes at 2 years following therapeutic hypothermia for neonatal encephalopathy. The most widely used prognostic tool is T₁- and T₂-weighted structural MRI, therefore I investigated its prognostic value, the inter-rater robustness and relationship between injury characteristics from conventional MRI and MRS in a large prospective study.

Chapter 2: Prognostic accuracy of conventional MR imaging and its relationship with MR spectroscopy

INTRODUCTION

Cerebral MR biomarkers have improved our ability to identify and quantify brain injury days or weeks after the initial insult and are more objectively quantifiable than any neurodevelopmental outcome measures. Conventional MRI (i.e. T₁- and T₂-weighted structural imaging) can give valuable information about macroscopic tissue structure whereas MR spectroscopy gives insight into neuronal metabolism. The latter has been shown to have an optimal prognostic accuracy in various single centre studies and in different meta-analyses^{47, 56}. However, most institutions currently lack the local support to acquire and interpret MR spectroscopy on a routine basis. In fact, the largest barriers to its routine use in the clinical setting are the prolonged acquisition time (30 minutes), and the need for specialist MR physics expertise for analysis.

By using conventional MRI, it is possible to identify different brain injury patterns, which in turn reflect different underlying disease mechanisms. Basal ganglia and cortical injury have been classically linked to acute hypoxic ischaemia. Grey matter neurons and early myelinating tissues within the immature brain have a higher metabolic rate than the surrounding white matter and are significantly more vulnerable to acute anoxia.

White matter injury has been traditionally linked to sub-acute, chronic hypoxic ischaemia. In animal models, white matter in the developing brain is particularly vulnerable to infective insults, due to the disruption of the developing tracts by neuro-inflammatory mediators, either directly through vasoactive mechanisms that involve hypoxia-ischaemia or by the damage to the oligodendrocytes and their progenitors. However, the clinical evidence has been more variable^{110, 111}.

Current available conventional MRI scoring systems include those developed by Barkovich, the National Institute of Child Health and Development (NICHD) and Rutherford¹¹²⁻¹¹⁴. However, whilst the Barkovich scoring system was developed in the pre-cooling era (i.e. before

therapeutic hypothermia became standard care), the others were tested for accuracy in a mixed group of cooled and non-cooled infants.

We recently reported the results of 223 infants with NE enrolled as part of the MARBLE study and showed that spectroscopy was highly predictive of neurodevelopmental outcomes, with an AUC for the concentrations of NAA of 0.99 (0.94–1.00), much higher prognostic accuracy than injury to the posterior limb of internal capsule assessed by conventional MRI, AUC 0.82 (0.76–0.87) ¹¹⁵. This represents one of the largest observational studies in NE and therefore these data represented a great opportunity to conduct a secondary analysis to further examine the accuracy of MRI scoring systems, the inter-rater robustness and the link between MRS and MRI biomarkers in the cooling era, which has never been done before. In particular, the combination of conventional MRI and MRS can help to identify which imaging features are most linked to metabolic abnormalities and has the potential to provide highly relevant complementary information to explore brain injury after NE.

Therefore, in this secondary analysis of the MARBLE study, I decided to assess the accuracy of conventional MR biomarkers in predicting adverse neurodevelopment after NE, to evaluate the inter-rater robustness and to study the relationship between injury characteristics from conventional MRI and MRS.

METHODS

The Magnetic Resonance Biomarkers in Neonatal Encephalopathy (MARBLE, NCT01309711) study is a prospective observational study, which involved 8 different neonatal intensive care units across the UK and USA. All the centres were tertiary neonatal units, cooling referral centres. My role in the study was as neurodevelopmental lead and I was involved in the recruitment at Queen Charlotte's and Chelsea Hospital and St. Mary Hospital.

Consecutive term and near-term infants (36–43 weeks gestation) with evidence of neonatal encephalopathy and treated with therapeutic hypothermia at the participating neonatal units were recruited as part of MARBLE study between January 2013 and June 2016^{57, 58}.

Exclusion criteria were life-threatening congenital malformations, syndromic disorders, neurometabolic diseases, or any alternative diagnoses for encephalopathy that were apparent within six hours of birth.

Upon recruitment at each site, the encephalopathy stage was assessed by using a standardised, validated, neurological examination, the NICHD neurological examination by a certified examiner^{43, 116}. This examination yielded an encephalopathy grade (none, mild, moderate, or severe) which reflected the severity of neurological symptoms.

All parents gave written informed consent and the study was approved by the North London Research Ethics Committee (13HH1843), and each of the participating clinical sites^{57, 58}.

We performed MR imaging between 4 and 14 days after birth (i.e. after completion of therapeutic hypothermia) in all infants using a 3 Tesla MRI scanner (Philips, Siemens or GE). MRI sequences were harmonised for use across all sites⁵⁷. Typical MRI and MRS parameters are shown in Appendix I.

Analysis of MR and MRS biomarkers

Dr Sudhin Thayyil and I systematically reported each image blinded to the clinical history, but not the gestational age or postnatal age at scan.

Based on the scoring system developed by Rutherford *et al.*, I scored brain tissue injury from 0 to 3 for basal ganglia/thalami, white matter and cortex (0=normal, 1=mild signal abnormality, 2=moderate signal abnormality, 3=severe signal abnormality) and I scored the posterior limb of internal capsule from 0 to 2 (0=normal, 1=equivocal, 2=abnormal). I also noted the presence of focal hemispheric lesions and their site ¹¹².

I also reported the scans according to the National Institutes for Child Health and Development scoring system, which is characterised by an increasing score with a greater involvement of brain injury: 0, normal; 1A, minimal cerebral lesions only; 1B, more extensive cerebral lesions without basal ganglia and thalamic involvement or infarction; 2A, lesions in the basal ganglia

and thalamic area and internal capsule; 2B, lesions in the BGT, internal capsule and cerebral areas; and 3, cerebral hemispheric devastation¹¹⁷.

The MR spectroscopy postprocessing and analysis was performed centrally by a single MR physicist blinded to the clinical history.

Neurodevelopmental outcome

I performed a standardised, validated and scorable neurological examination in 85 infants recruited at Queen Charlotte's and Chelsea Hospital, St. Mary Hospital and University Hospitals Coventry & Warwickshire where there was no local certified Bayley examiner. The remaining infants were examined by local trained examiners in the other sites. The presence and type of cerebral palsy was determined according to Gorter¹¹⁸ and severity classified using the five levels defined in the Gross Motor Function Classification System (GMFCS), from 1 (minimal impairment) to 5 (severe impairment with dependence on carers for most daily activities).

I assessed the neurodevelopmental outcome using the Bayley Scales of Infant Development (BSID-III), masked to the imaging information and neonatal data. I also performed vision and audiometric evaluations alongside the collection of anthropometric data (weight, height and head circumference) as part of the follow-up assessments.

I defined an adverse outcome as death or moderate/severe disability. The aim of the study was to assess the use of MRI biomarkers for prognostication in NE so that they could be used as surrogate biomarkers in future trials. Therefore, in the definition of the outcome I decided to use the same definition as what was used in the previous therapeutic hypothermia trials^{7, 116, 119, 120}. The disability was classified as severe if any one of the following criteria were met: Bayley III, both cognitive and language composite score <70; GMFCS level 3-5; hearing impairment requiring hearing aids; or blindness. Moderate disability was defined as both cognitive and language composite scores between 70-84 and one or more of the following: GMFCS level II; hearing impairment with no amplification; or a persistent seizure disorder⁵⁸.

Statistical analysis

I studied the relationship between different MRI injury patterns and the primary outcome (death or moderate/severe disability) by Linear-by-Linear Association test. The NICHD pattern of injury was used as a six-level variable (0, 1A, 1B, 2A, 2B, 3) whereas Rutherford's pattern of injury was used as a four-level variable (0-1-2-3) for the BGT, white matter, and cortex, and a three-level variable for the PLIC (0-1-2).

I used a logistic regression model to assess the relationship between each of the two different MRI scoring systems and the long-term neurological outcome (death or moderate/severe disability). I performed the analysis firstly unadjusted, and then adjusted for postnatal age at MRI scan. Due to its non-normal distribution, I analysed the peak-area ratio of lactate to NAA on the log scale.

I examined the prognostic accuracy of Rutherford's MRI scoring system (PLIC, BGT, white matter and cortex), the equivalent NICHD scoring system, and the neurological examination at discharge, measuring prognostic accuracy as the AUC. I obtained prognostic indices with 95% confidence intervals using the exact binomial method.

I assessed the relationship between MRS metabolite ratios/concentrations and the MRI scoring of PLIC, BGT, WM and cortex using Pearson correlation. I then used analysis of covariance (ANCOVA) analysis to assess the effect of age at MRI scan on the correlations between the MRS results and MRI scoring results.

I described inter-observer agreement in the MRI scoring between me and the other examiner using Bland-Altman plots. I also computed Cohen's κ to determine the degree of agreement with regards to the PLIC, BGT, WM and cortex scoring.

A p value < 0.05 was considered significant. I analysed all of these data using SPSS Statistics 24 (IBM Corp., Armonk, NY).

RESULTS

Overall, 223 patients with neonatal encephalopathy who underwent therapeutic hypothermia were recruited. Of these, 190 (85%) had neurodevelopmental outcome assessments and

conventional MRI for analysis. There was no significant difference in terms of baseline characteristics between the children lost at the follow-up and the others.

Thirty-one patients (16%) had an abnormal neurodevelopmental outcome at 2 years (death or moderate/severe disability) (Table 1). The mean postnatal age at the time of the MRI scan was 8.4 days (SD 4.3). There was no significant difference in terms of age at the time of the MRI scan between infants with normal and abnormal outcome.

Table 1. Baseline clinical characteristics (From Magnetic resonance spectroscopy assessment of brain injury after moderate hypothermia in neonatal encephalopathy: a prospective multicentre cohort study. Lancet Neurol. 2018 Nov 14. pii: S1474-4422(18)30325-9).

Category	Total recruits (n=223)	Normal outcome (n=159)	Abnormal outcome (n=31)	Lost to FU (n=33)
Primi-gravida	104 (47)	79 (50)	13 (42)	12 (38)
Reduced fetal movements	30 (17)	19 (12)	9 (29)	2 (8)
Prolonged rupture of membranes	36 (17)	25 (16)	6 (19)	5 (16)
Abnormal CTG	112 (82)	84 (53)	11 (36)	17 (89)
Ante-partum haemorrhage	15 (7)	9 (6)	4 (13)	2 (6)
Meconium stained liquor	76 (36)	59 (37)	8 (26)	9 (28)
Emergency LSCS	92 (51)	65 (41)	4 (13)	13 (46)
Delivery complications				
Uterine rupture	11 (5)	6 (4)	4 (13)	1 (3)
Cord prolapse	7 (3)	4 (3)	2 (7)	1 (3)
Shoulder dystocia	26 (12)	14 (0)	6 (19)	6 (21)
Obstructed labour	4 (2)	3 (2)	1 (3)	0
Birth and resuscitation				
Male sex	124 (56)	90 (57)	17 (55)	17 (52)
Birth weight, Kg	3.4 (0.5)	3.4 (0.5)	3.2 (0.6)	3.4 (0.6)
Gestation, Weeks	39.9 (1.5)	40.1 (1.4)	39.5 (1.5)	39.8 (1.6)

Cord pH	6.9 (1.9)	6.9 (1.8)	6.9 (2.3)	6.9 (1.4)
Apgar, 10 min	5.6 (2.2)	5.7 (2.2)	4.9 (2.3)	5.4 (2.3)
Intubation	68 (31)	49 (31)	8 (26)	11 (34)
Cardiac massage	53 (24)	39 (25)	10 (32)	4 (13)
Drugs (adrenaline, bicarbonate)	9 (4)	5 (3)	2 (7)	2 (6)
Admission encephalopathy stage				
None	1 (1)*	1 (1)*	0	0
Mild	36 (16)	29 (18)	1 (3)	6 (18)
Moderate	163 (73)	119 (75)	17 (55)	27 (82)
Severe	23 (10)	10 (6)	13 (42)	0
Neonatal course				
Coagulopathy	57 (27)	35 (22)	10 (32)	12 (38)
Positive blood culture	12 (6)	8 (5)	3 (10)	1 (3)
Pneumothorax	12 (6)	10 (6)	1 (3)	1 (3)
Hypotension	88 (40)	53 (33)	19 (61)	16 (48)
PPHN	18 (8)	14 (9)	0	4 (12)
Pulmonary Haemorrhage	3 (1)	2 (1.3)	0	1 (3)
Seizures on admission	115 (55)	78 (49)	21 (68)	16 (52)
Age at MRI scan, Days	8.4 (4.3)	8.3 (4.5)	8.0 (3.3)	7.5 (3.5)

Data are n (%) or mean (SD). CTG: Cardiotocogram; LSCS: Lower segment caesarean section; PPHN: Persistent pulmonary hypertension. MRI: Magnetic resonance imaging. Percentages are in terms of the number of mothers or neonates for whom data were available. *Progressed to moderate encephalopathy and hence cooled

I found good agreement in the scoring of the different items of Rutherford's MRI scoring system, PLIC $\kappa = 0.91$ (95% CI, .83 to .97), $p < 0.001$; BGT, $\kappa = 0.84$ (95% CI, .74 to .91), $p <$

0.001; WM κ = 0.63 (95% CI, .53 to .70), $p < 0.001$; Cortex κ = 0.84 (95% CI, .79 to .94), $p < 0.001$.

The Bland–Altman plots are presented in Figure 3. These revealed strong agreement in the PLIC scoring with limits of agreement of -0.43 and 0.37 and a bias (mean difference) of 0.00 . Good agreement was found in the BGT and cortex scoring whereas weaker correlation was displayed in the WM.

The MRI findings split according to normal and abnormal outcome groups are shown in Table 2. Any of the three NICHD patterns were associated with death or long-term adverse outcome (Linear-by-Linear Association test; $p < 0.001$). Regarding Rutherford's MRI scoring system, any abnormality in the PLIC, BGT, white matter or cortex was associated with long-term outcome (Linear-by-Linear Association test; $p < 0.001$, $p < 0.001$, $p < 0.001$ and $p = 0.03$ respectively).

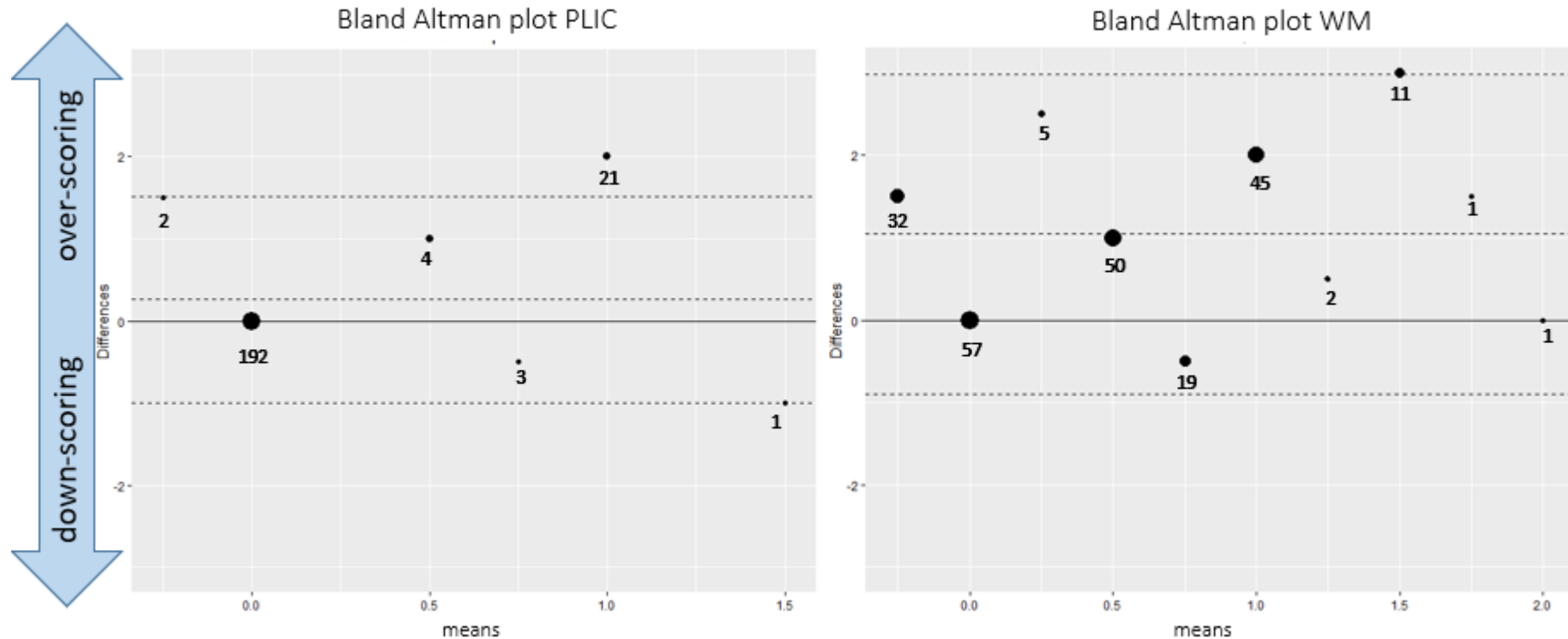
I further examined the relationship between the MRI scoring and primary outcome by logistic regression analysis while controlling for postnatal age at MRI (Table 3). This analysis revealed that each point increase in the severity of the PLIC pattern of injury was independently associated with a 6-fold increase in the odds of death or disability at 18–22 months of age, with an odds ratio (OR) of 6.6 (95% CI 3.4 to 12.8) after controlling for age at MRI.

BGT achieved the highest accuracy for prediction of abnormal outcome at 2 years (AUC 0.81 ; 95% CI 0.72 – 0.91) with 89% sensitivity (95% CI 83 – 93) and 74% specificity (95% CI 55 – 88). The positive predictive value (PPV) and negative predictive value (NPV) were 95% (95% CI 91 – 97) and 56% (95% CI 44 – 67) (Table 4).

I observed an MRI score of 0 (NICHD scoring system) in 118/190 patients; 112/118 (94%) had normal BSID-III, whereas 6/118 (6%) had moderate/severe developmental delay.

The receiver operating characteristic (ROC) curves of the different biomarkers for predicting a normal or abnormal outcome are shown in Figure 4.

Figure 3. Bland-Altman (modified) plots comparing my scoring and the gold standard examiner (ST). Dashed lines represent the 95% confidence intervals for the limits of agreement and mean difference (bias) in estimations. The dots have an increased size depending on the frequency of multiple overlapping values. Below each dot there is the number of overlapping values (WM= white matter, PLIC= Posterior limb of internal capsule, BGT= Basal ganglia thalami).



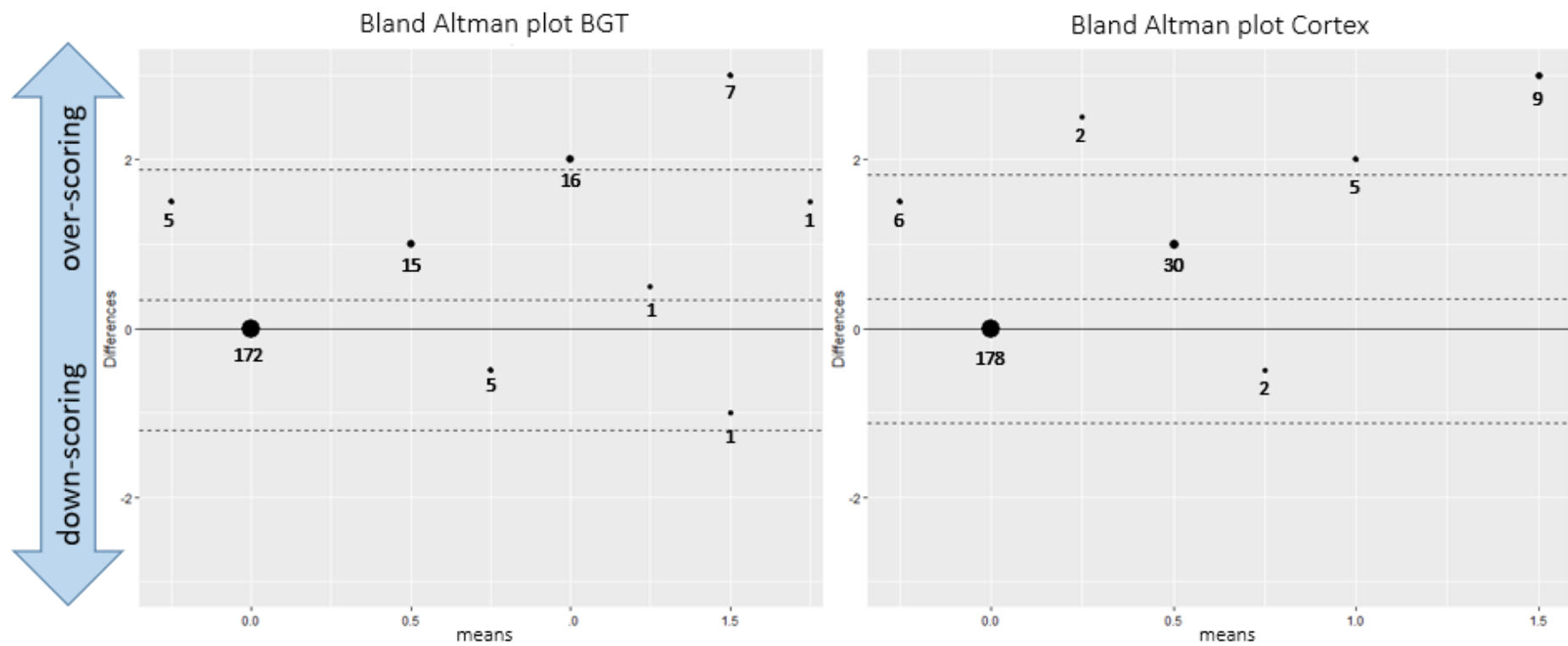


Table 2. MR imaging scores in neonatal encephalopathy babies with normal and abnormal outcome

		Normal outcome (N=159)	Abnormal outcome (N=31)
Rutherford MRI scoring system			
Basal ganglia and thalami*	Normal	141	8
	1	9	6
	2	8	10
	3	1	7
Posterior limb of internal capsule*	Normal	142	9
	1	13	7
	2	4	15
	3	0	0
White matter‡	Normal	64	9
	1	59	2
	2	31	12
	3	5	8
Cortex*	Normal	131	17
	1	23	6
	2	2	2
	3	3	6
NICHD MRI scoring system*			
0		112	6
1A		1	0
1B		24	3
2A		8	3
2B		11	9
3		3	10

Data are number of patients

Linear-by-Linear Association test, * p<0.001, ‡ p=0.03

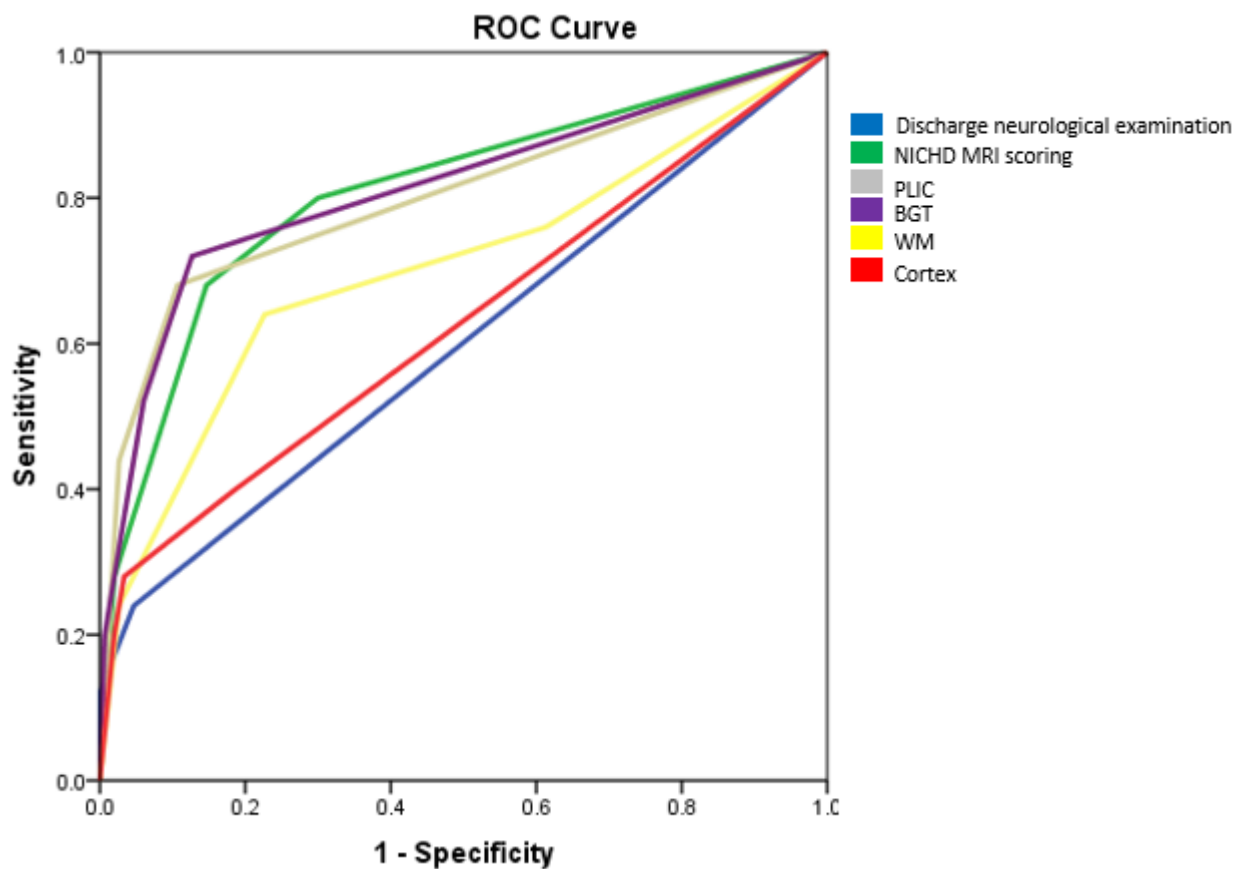
Table 3 Logistic regression analysis. Odds ratios (OR) and 95% confidence intervals (CI) of the MRI scoring systems for abnormal outcome unadjusted and after adjustment for postnatal age at MRI scan.

Variable		Unadjusted		Adjusted	
		OR (95%CI)	p value	OR (95%CI)	p value
NICHD score		3.8 (95% CI 2.4 to 5.9)	<0.001	3.8 (95% CI 2.3 to 6.1)	<0.001
Rutherford's score	PLIC	7.8 (95% CI 4.1 to 14.6)	<0.001	6.6 (95% CI 3.4 to 12.8)	<0.001
	BGT	4.9 (95% CI 3 to 8)	<0.001	4.7 (95% CI 2.7 to 8.1)	<0.001
	WM	2.3 (95% CI 1.5 to 3.5)	<0.001	2.3 (95% CI 1.5 to 3.6)	<0.001
	Cortex	2.5 (95% CI 1.6 to 3.8)	<0.001	2.6 (95% CI 1.6 to 4.3)	<0.001

Table 4. Predictive ability of the MRI scoring systems to differentiate between normal and abnormal outcome infants

	Sensitivity % (95% CI)	Specificity % (95% CI)	LR+ (95% CI)	LR- (95% CI)	PPV % (95% CI)	NPV % (95% CI)	AUC (95% CI)
NICHD score ≥ 1 B	80 (62-92)	70(63-77)	2.7(2.0-3.6)	0.27(0.1-0.6)	35(28-41)	95(90-97)	0.80 (0.7-0.9)
PLIC ≥ 1	70(52-86)	89(83-93)	6.6(4-11)	0.33(0.1-0.5)	56(44-68)	94(90-96)	0.80 (0.7-0.9)
BGT ≥ 1	74 (55-88)	89(83-93)	6.5 (4-10)	0.29(0.1-0.5)	56(44-67)	95(91-97)	0.81(0.7-0.9)
WM ≥ 1	71(52-86)	45(33-48)	1.19(0.9-1.5)	0.72(0.4-1.2)	19(15-23)	88(80-93)	0.70(0.5-0.8)
Cortex ≥ 1	45 (27-64)	82(76-88)	2.6(1.5-4.2)	0.67(0.4-0.9)	16.32(11-22)	88 (84-91)	0.63 (0.5-0.7)
Discharge neurological examination	26 (10-48)	95 (90-98)	5 (2-14)	0.8(0.6-1)	46(24-70)	88(85-90)	0.60 (0.4-0.7)

Figure 4. Receiver operating characteristic curves for MRI scoring systems.



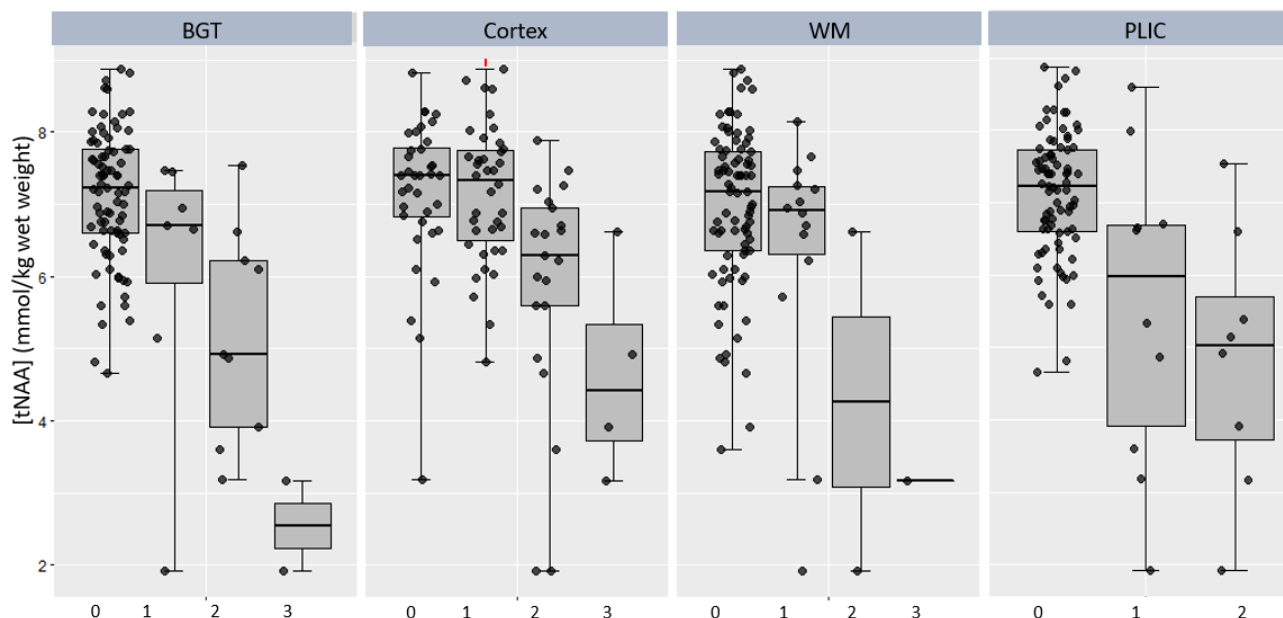
N-acetylaspartate concentration, [tNAA]

I found a significant inverse correlation between [tNAA] and PLIC ($r = -0.54$, 95% CI $-0.70 - -0.33$, $p < 0.001$), [tNAA] and BGT ($r = -0.61$, 95% CI $-0.75 - -0.38$, $p < 0.001$), [tNAA] and WM ($r = -0.39$, 95% CI $-0.58 - -0.25$, $p < 0.001$) and [tNAA] and cortex ($r = -0.38$, 95% CI $-0.60 - -0.29$, $p < 0.001$).

In order to assess whether the correlation between MRS metabolites and MRI scoring system was affected by the age at MRI scan, I performed a separate ANCOVA analysis. The results showed that [tNAA] results were significantly associated with MRI scoring system, after adjusting for age at MRI scan (Table 5).

There was significant difference in the [tNAA] between infants with normal and the ones with moderate/severe involvement of PLIC (score 1-2). There was also a significant difference in the [tNAA] between score 0 (normal) and 2-3 (moderate/severe) for BGT, WM and cortex (Figure 5).

Figure 5. Dotplots of various [tNAA] values (median, minimum, maximum and interquartile range) according to the different conventional MRI scores in basal ganglia thalami (BGT), cortex, white matter (WM) and posterior limb of internal capsule (PLIC).



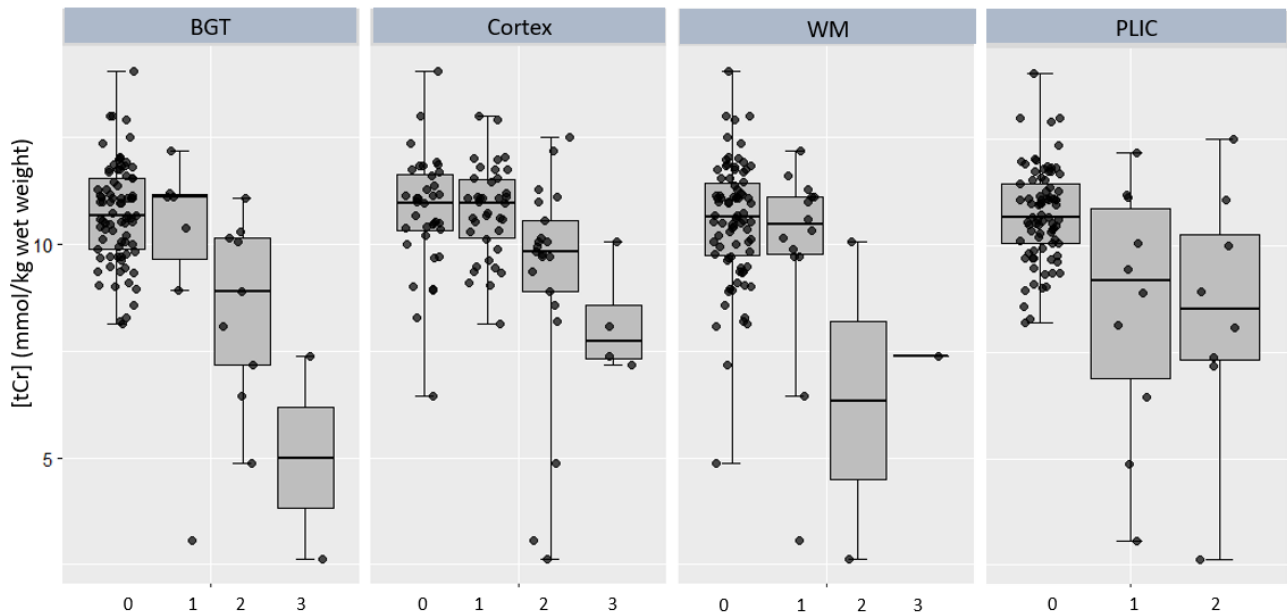
Creatine concentration [tCr]

I found a significant inverse correlation between [tCr] and PLIC ($r = -0.45$, 95% CI -0.64 – -0.20 , $p < 0.001$), [tCr] and BGT ($r = -0.54$, 95% CI -0.72 – -0.23 , $p < 0.001$), [tCr] and WM ($r = -0.37$, 95% CI -0.51 – -0.22 , $p < 0.001$) and [tCr] and cortex ($r = -0.34$, 95% CI -0.59 – -0.02 , $p < 0.001$).

After adjusting for age at MRI scan, [tCr] results were significantly associated with MRI scoring of PLIC, BGT, WM and cortex (ANCOVA analysis- Table 5).

Dotplots of the raw data display the clear relationship between [tCr] and injury scores across brain regions (Figure 6).

Figure 6. Dotplots of various [tCr] values (median, minimum, maximum and interquartile range) according to the different conventional MRI scores in basal ganglia thalami (BGT), cortex, white matter (WM) and posterior limb of internal capsule (PLIC).



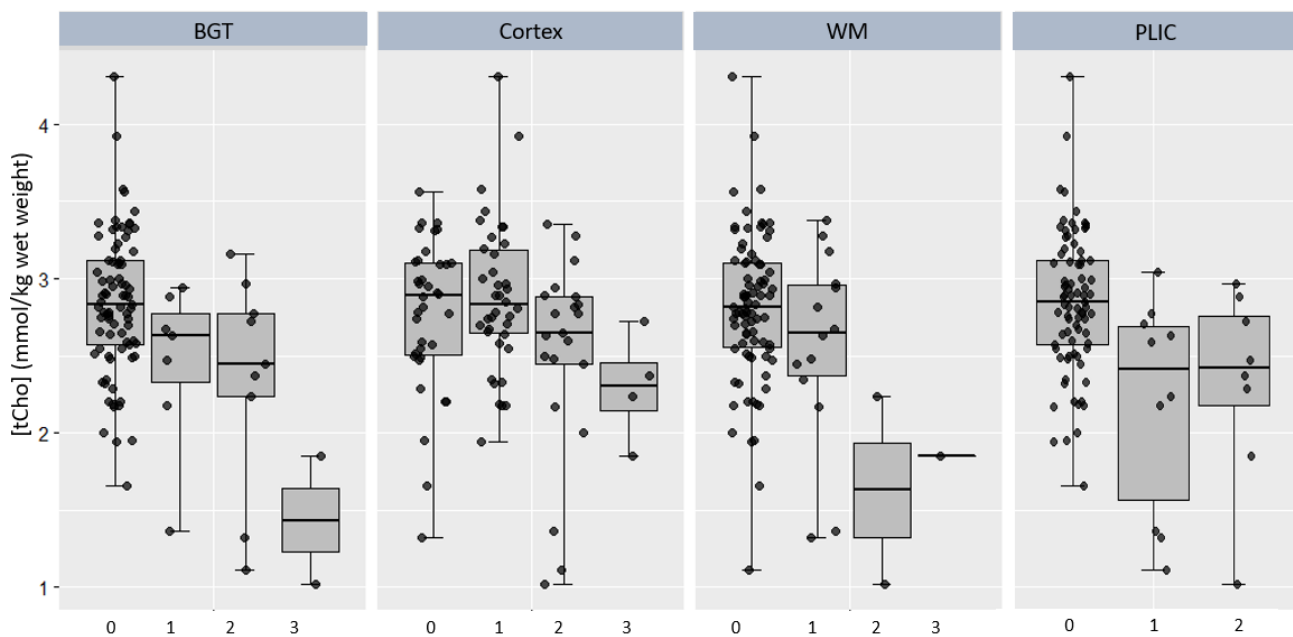
Choline concentration [tCho]

I found a significant inverse correlation between [tCho] and PLIC ($r = -0.38$, 95% CI -0.55 – -0.18 , $p < 0.001$), [tCho] and BGT ($r = -0.44$, 95% CI -0.63 – -0.18 , $p < 0.001$), [tCr] and WM ($r = -0.21$, 95% CI -0.39 – -0.14 , $p = 0.03$) and [tCho] and cortex ($r = -0.34$, 95% CI -0.54 – -0.08 , $p = 0.001$).

ANCOVA analysis showed that [tCho] results were significantly associated with MRI scoring of PLIC, BGT and cortex but not with WM after adjustment for age at MRI scan (Table 5).

Dotplots of the raw data display the clear relationship between [tCho] and injury scores across BGT, cortex, PLIC but not WM (Figure 7)

Figure 7. Dotplots of various [tCho] values (median, minimum, maximum and interquartile range) according to the different conventional MRI scores in basal ganglia thalami (BGT), cortex, white matter (WM) and posterior limb of internal capsule (PLIC).



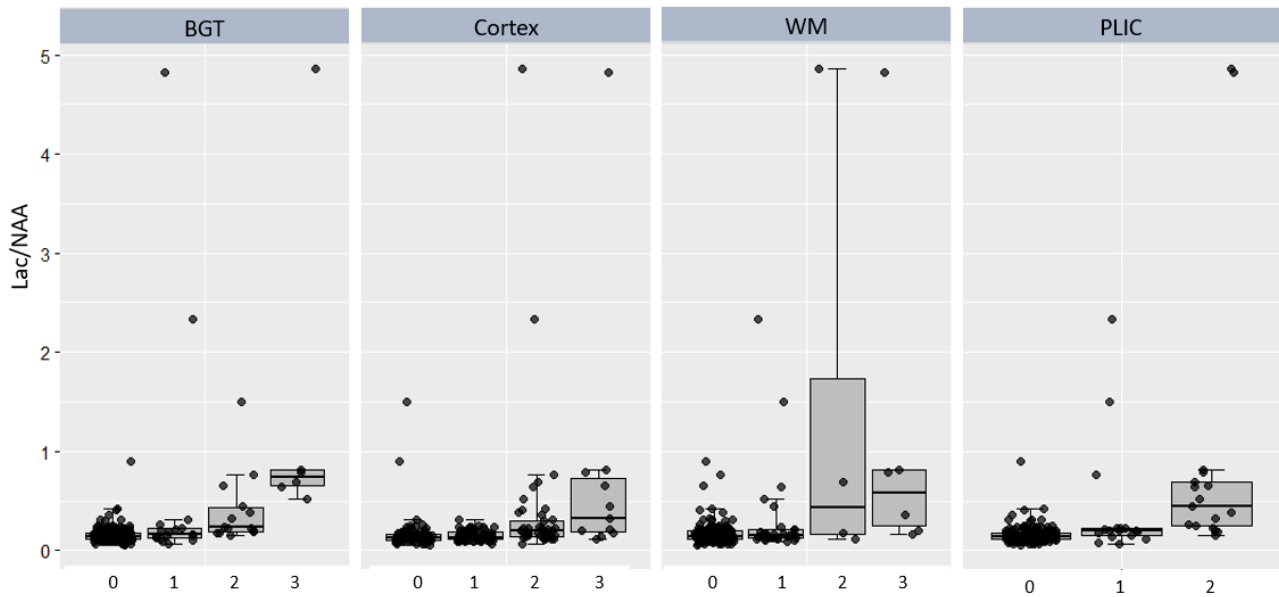
tLac/tNAA peak area ratio

I found a significant correlation between tLac/tNAA and PLIC ($r = 0.43$, 95% CI 0.30 – 0.50, $p < 0.001$), tLac/tNAA and BGT ($r = 0.40$, 95% CI 0.25–0.52, $p < 0.001$), tLac/tNAA and WM ($r = 0.37$, 95% CI 0.24–0.69, $p < 0.001$) and tLac/tNAA and cortex ($r = 0.22$, 95% CI 0.07–0.34, $p < 0.001$).

ANCOVA analysis showed that tLac/tNAA results were significantly associated with MRI scoring of PLIC, BGT, WM and cortex after adjustment for age at MRI scan (Table 5).

Dotplots of the raw data display the clear relationship between tLac/tNAA and injury scores across brain regions (Figure 8).

Figure 8. Dotplots of various tLac/tNAA values (median, minimum, maximum and interquartile range) according to the different conventional MRI scores in basal ganglia thalami (BGT), cortex, white matter (WM) and posterior limb of internal capsule (PLIC).



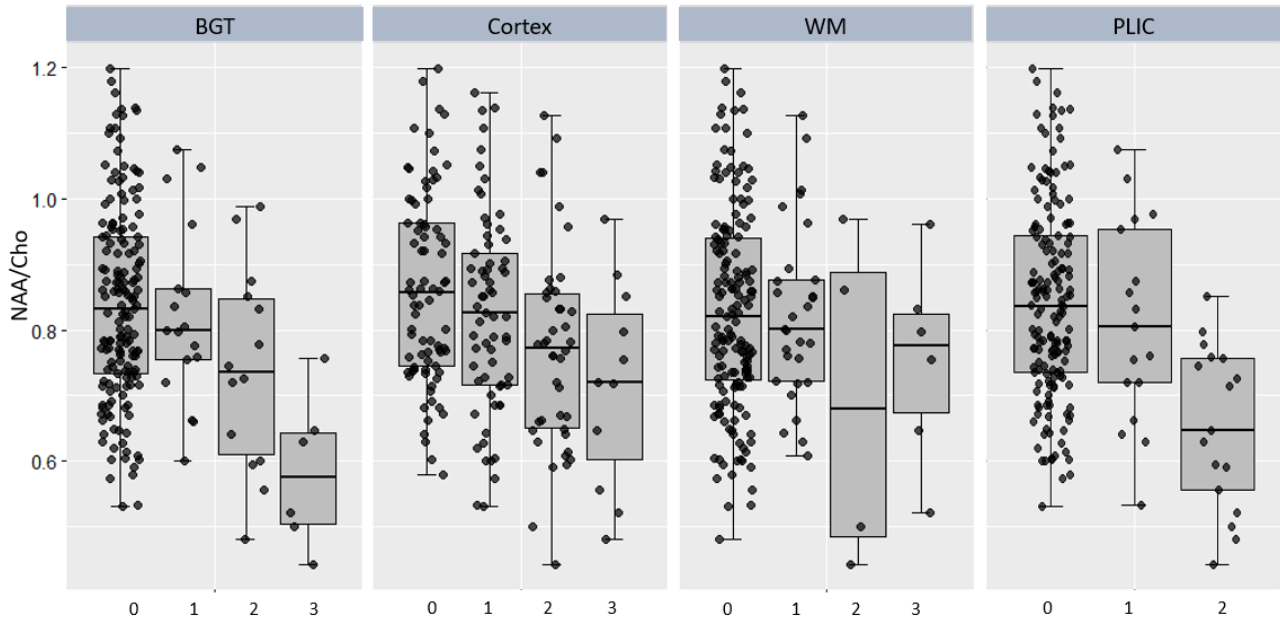
tNAA/tCho peak area ratio

I found a significant inverse correlation between tNAA/tCho and PLIC ($r = -0.32$, 95% CI $-0.44 - 0.19$, $p < 0.001$), tNAA/tCho and BGT ($r = -0.22$, 95% CI $-0.35 - 0.06$, $p = 0.002$), tNAA/tCho and WM ($r = -0.25$, 95% CI $-0.38 - 0.11$, $p < 0.001$) but not between tNAA/tCho and cortex ($r = -0.06$, 95% CI $-0.19 - 0.07$, $p = 0.36$).

ANCOVA analysis showed that tNAA/tCho results were significantly associated with MRI scoring of PLIC, BGT, WM but not cortex after adjustment for age at MRI scan (Table 5).

Dotplots of the raw data display the clear relationship between tLac/tNAA and injury scores across all the brain regions apart from cortex (Figure 9).

Figure 9. Dotplots of various tNAA/tCho values (median, minimum, maximum and interquartile range) according to the different conventional MRI scores in basal ganglia thalami (BGT), cortex, white matter (WM) and posterior limb of internal capsule (PLIC).



tNAA/tCr peak area ratio

I found a significant inverse correlation between tNAA/tCr and PLIC ($r = -0.38$, 95% CI $-0.51 - 0.24$, $p < 0.001$), tNAA/tCr and BGT ($r = -0.33$, 95% CI $-0.47 - 0.18$, $p < 0.001$), tNAA/tCr and WM ($r = -0.29$, 95% CI $-0.42 - 0.13$, $p < 0.001$) and between tNAA/tCr and cortex ($r = -0.23$, 95% CI $-0.42 - 0.04$, $p = 0.001$).

ANCOVA analysis showed that tNAA/tCr results were significantly associated with MRI scoring of PLIC, BGT, WM and cortex after adjustment for age at MRI scan (Table 5).

Dotplots of the raw data display the clear relationship between tNAA/tCr and injury scores across all the brain regions (Figure 10).

Figure 10. Dotplots of various tNAA/tCr values (median, minimum, maximum and interquartile range) according to the different conventional MRI scores in basal ganglia thalami (BGT), cortex, white matter (WM) and posterior limb of internal capsule (PLIC).

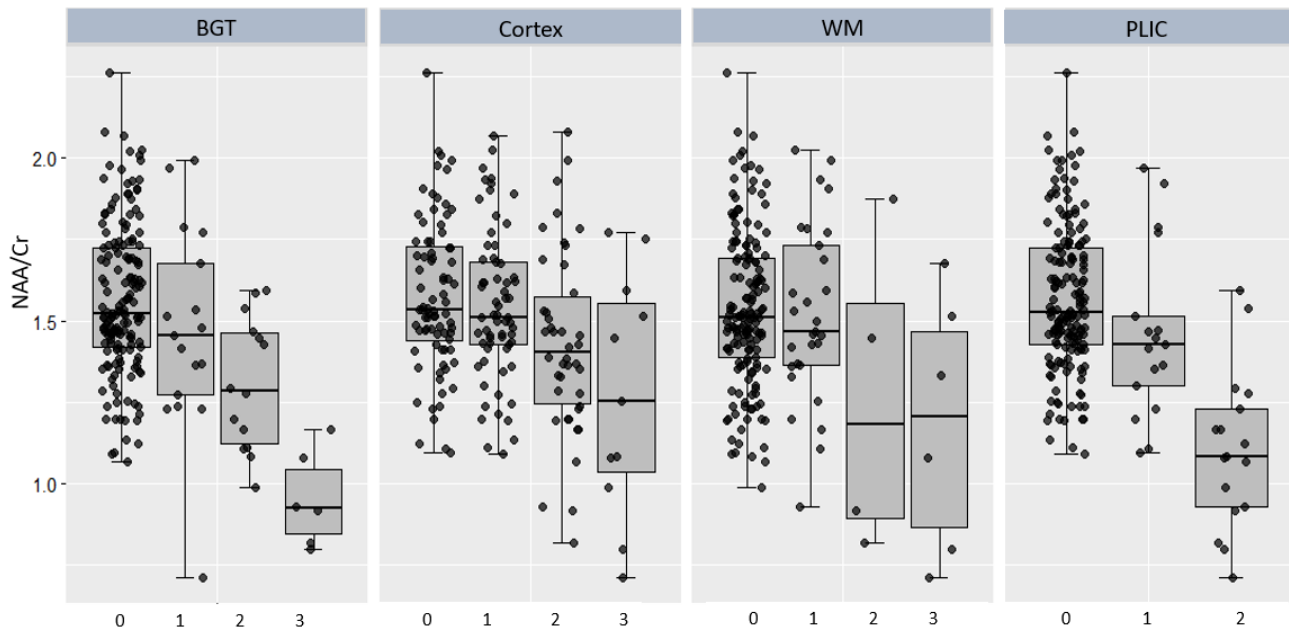


Table 5. Analysis of covariance correlations of [tNAA], [tCr], [tCho] and MRI score (Rutherford scoring system) after adjustment for postnatal age at MRI scan.

[tNAA]		
Variable	F ratio	p value
PLIC	14.48 (2-94)	<0.001
BGT	17.11(3-93)	<0.001
WM	8.446 (3-93)	<0.001
Cortex	6.578 (3-93)	<0.001
[tCr]		
Variable	F ratio	p value
PLIC	11.9 (2-94)	<0.001
BGT	13.33 (3-93)	<0.001
WM	6.33 (3-93)	0.01
Cortex	5.32 (3-93)	0.002
[tCho]		
Variable	F ratio	p value
PLIC	6.72 (2-94)	0.002
BGT	6.99 (3-93)	<0.001
WM	2.06 (3-93)	0.11
Cortex	4.99(3-93)	0.03
tLac/tNAA		
Variable	F ratio	p value
PLIC	20 (2-193)	<0.001
BGT	16 (3-192)	<0.001
WM	7 (3-192)	<0.001
Cortex	23 (3-192)	<0.001
tNAA/tCho		
Variable	F ratio	p value
PLIC	13 (1-194)	<0.001
BGT	7 (3-192)	<0.001
WM	5 (3-192)	0.002
Cortex	0.9 (3-192)	0.69
tNAA/tCr		
Variable	F ratio	p value
PLIC	28 (2-193)	<0.001
BGT	15 (2-193)	<0.001
WM	6 (2-192)	0.001
Cortex	5 (2-192)	0.002

DISCUSSION

This study represents the largest prospective observational study of conventional MRI and MRS, which includes different MR scanners widely available in the clinical setting and with thorough quality assurance and central blinded analysis. These data show that conventional MRI scoring systems have good agreement, but suboptimal accuracy in predicting long-term adverse outcome with the best performance achieved by BGT and PLIC scoring. When comparing MRI findings with thalamic metabolites measured using MRS, I found that metabolic alterations reflected macroscopic brain injury in other brain regions such as white matter and cortex, suggesting that BGT is invariably involved at a microscopic level regardless of the severity and timing of the hypoxic event. These findings are supported by preclinical evidence showing a high metabolic activity in thalamus at birth that spreads to other areas over time¹²¹ and highlight the pivotal role of BGT in the pathology of brain injury after NE.

Two characteristic patterns of brain injury have been traditionally identified in neonatal encephalopathy: deep nuclear grey matter and white matter injury. Deep nuclear grey matter represents the most metabolically active area at birth and is mainly involved in case of acute hypoxia⁵⁰. Previous research showed a strong association between moderate/severe basal ganglia lesions and cerebral palsy or adverse long-term outcome^{122, 123}. More recently, Shankaran *et al.* demonstrated that infants with perinatal sentinel events recruited in the NICHD Neonatal Research Network (NRN) Trial had more basal ganglia and thalamic lesions on conventional MRI but without any significant difference in the neurodevelopmental outcomes at 18 months of age compared with infants without perinatal sentinel events¹²⁴. Neonatal MRI findings had also a significant correlation with death or moderate/severe disability at childhood¹²⁴. However, these studies were affected by a smaller sample size, retrospective designs, and the inclusion of infants who did not undergo whole-body therapeutic hypothermia.

The present study shows that in case of BGT and PLIC injury, there is a four and seven-fold respectively higher risk of adverse outcome. Basal ganglia/thalamic injury had a sensitivity of 74% and a specificity of 88% and PLIC a sensitivity of 70% and a specificity of 89% for predicting death or moderate/severe disability. Previous studies evaluating the prognostic accuracy of conventional MRI reported variable results with sensitivities ranging from 60% to 90% and specificities ranging from 65% to 92%.⁴⁷ This likely reflects different timings of the MRI scans, the relatively small sizes of single

studies, and the different scoring systems used. My results are consistent with a previous meta-analysis conducted when whole-body therapeutic hypothermia was not yet standard treatment.⁴⁷

Since then, three sub-studies of large cooling trials have showed that whole-body therapeutic hypothermia reduces brain injury in infants with NE without changing the predictive value of conventional MRI ^{112, 125}.

Rutherford *et al.* assessed the accuracy of conventional MRI performed at a mean age of 8 days in predicting long-term neurological outcome in 131 of 325 infants who were recruited as part of the TOBY trial. The study showed that therapeutic hypothermia decreased the risk of injury to the posterior limb of the internal capsule (OR 0.38, 95% CI [0.17–0.85]), basal ganglia and thalamus (OR 0.36, 95 % CI [0.15–0.84]), and white matter (OR 0.30, 95 % CI [0.12–0.77]) in the infants who underwent cooling when compared with the non-cooled ones. Conventional MRI had a sensitivity of 88% and a specificity of 82% in cooled and a sensitivity of 94% and specificity of 62% in non-cooled babies to predict death or moderate/severe disability at 18 months¹¹².

Shankaran *et al.* studied the accuracy of conventional MRI performed at a mean age of 15 days in predicting long-term neurological outcome in 136 out of 208 NE infants recruited as part of the NICHD NRN trial¹¹⁷. They developed a specific scoring system with a higher score in case of greater injury. In the hypothermia group 38 out of 73 scans were normal whereas 22 out of 63 (35%) scans were normal in the normothermia group ($p = 0.06$). Infants in the hypothermia group were less likely to have areas of infarction (12%) when compared with the normothermia group (22%) ($p = 0.02$). They reported an overall sensitivity of 90% and specificity of 65% for conventional MRI ¹²⁵. The NICHD score was also tested for accuracy as marker of childhood outcomes. Death or IQ less than 70 were present in all the children with pattern 3 whereas only 4 out of 50 (8%) children with pattern 0 and 1 out of 6 (17%) with 1A had the primary outcome ($p < 0.001$). Therapeutic hypothermia did not change this association. When they evaluated the diagnostic indices, normal neonatal MRI had a sensitivity of 61% and a specificity of 92% for death or IQ <70. When 2B and 3 patterns were combined, there was a sensitivity of 81% and specificity of 78%¹¹³.

In the above studies, the sensitivity and specificity of conventional MRI was higher than I observed in this study. Despite using different conventional MRI scoring systems, the highest sensitivity was

achieved by the NICHD score (80, 95% CI [62-92]) and the highest specificity by PLIC and BGT in Rutherford's scoring system (89, 95% CI [83-93] in both cases). This difference might be due to the different NE severities of the patients recruited, since only 16% of the infants of this study had an abnormal neurodevelopmental outcome at 2 years, which is remarkably low. Although the target group of this observational study was term infants requiring therapeutic hypothermia, 30 infants (16%) with mild NE were recruited, which reflects the trend to cool babies with mild encephalopathy in UK and worldwide^{126, 127}. Recently, the Prospective Research in Infants with Mild Encephalopathy (PRIME) study (a multi-centre, prospective study of mild NE) reported the same rate of disability (16 %) in infants with mild NE who did not receive therapeutic hypothermia⁴³. These MRI scoring systems were originally developed for NE babies with moderate/severe NE and hence their validity in the mild NE population is unknown.

Cheong *et al.* assessed the prognostic utility of MRI for death and/or disability at 2 years in 127 infants recruited as part of the ICE (Infant Cooling Evaluation Collaboration) trial¹²⁸. They found that therapeutic hypothermia decreased the odds of WM abnormalities (OR 0.28, 95% CI [0.09–0.82]) and grey matter (0.41, 95% CI [0.17–1.00]) abnormalities and that all the MRI abnormalities in T₁- and T₂-weighted imaging were predictive of a long term adverse outcome. However, when they combined all the predictors into a single model, PLIC (OR 4.10) and moderate/severe BGT abnormalities (OR 10.09) were independent predictors of the 2-year outcome. In that study, PLIC had a sensitivity of 48% and a specificity of 93% whereas moderate/severe BGT had a sensitivity of 60% and a specificity of 92%¹²⁸. These data are consistent with my findings and are likely to reflect a similarity in the population of NE infants recruited. The ICE trial was a multi-centre, international RCT, recruiting term infants with evidence of hypoxic-ischaemia and moderate/severe NE who were randomly allocated to whole-body cooling or standard care. However, despite moderate to severe NE as necessary inclusion criteria, 42 infants (19%) classified as mild at ≤ 6hrs of life were included in the study¹²⁸.

A meta-analysis has recently been performed including 17 studies of neonatal encephalopathy, where subjects underwent brain MRI within 4 weeks of birth and had neurodevelopmental follow-up for at least 12 months. The authors evaluated the prognostic accuracy of brain MRI depending on NE clinical severity and showed that in babies moderate NE, the MRI had an odds ratio of 17.7 (95% CI [5.3-59.3]) whereas in severe NE, the odds ratio was 125.0 (95%CI [2.0-7917.1]) to predict adverse outcome. However, the relationship between MRI and adverse outcome was not statistically

significant in mild NE neonates.⁵² Unfortunately, the analysis was not corrected for time of neonatal brain MR and the outcome used was either 12 or 18-24 month-follow-up. Therefore, these results are not easily comparable with my findings.

Conventional MRI is commonly used in clinical settings for prognostication. However, the main limitations in its use include the subjectivity and semi-quantitative nature of the different scoring systems. Although in the present study there was an excellent inter-observer agreement, especially concerning the PLIC ($\kappa = 0.91$) and BGT ($\kappa = 0.84$), the higher accuracy of conventional MRI (defined as AUC of the receiver operating characteristic curve) was 0.80 for predicting long-term outcome. In routine clinical settings, where there are different neuro-radiologists reporting the scans, this accuracy is likely to be even lower.

We have recently showed with the MARBLE study that thalamic [tNAA], as measured by using MR spectroscopy, has an excellent accuracy in predicting neurodevelopmental outcome at two years after neonatal encephalopathy (Sensitivity=100%, Specificity =97%, AUC=0.99), in comparison to conventional MRI (AUC=0.81) and clinical examinations (AUC=0.71)⁵⁸. In this secondary analysis of the Marble data, the conventional MRI accuracy has not changed although the scans were reported by a different person and different validated MRI scoring systems were used.

My data show that clinical examination at the time of discharge does not have sufficient accuracy for long-term outcome prediction and could not accurately identify babies at risk of an adverse neurological outcome. Neurological examination is certainly challenging when performed within the first 6 hours of birth because it might be affected by different factors including maternal anaesthesia/analgesia, timing and the type of delivery. Moreover, the examination's results may change over the time reflecting a compensatory response to the hypoxic insult.

There are no previous studies analysing the correlation between conventional MRI scores and MR spectroscopy in babies with NE. My data highlighted that [tNAA] and [tCr] correlated well with PLIC and BGT scores ($r = -0.54$ and -0.61 respectively, $p < 0.001$ for [tNAA] and $r = -0.45$ and -0.54 respectively, $p < 0.001$ for [tCr]). Weaker but significant correlations were also observed between these metabolites and white matter and cortex. Animal evidence suggests that thalamic injury is present even after brief episodes of ischaemia and in case of mild hypoxic-ischaemic insults whereas

hippocampus and parasagittal cortex are invariably involved ¹²⁹. My findings are consistent with these observations and suggest that MRS thalamic metabolic perturbations might mirror the involvement of other brain areas like white matter and cortex. Although in the primary analysis of the Marble study the direct relationship between conventional MRI and MRS was not assessed, thalamic [NAA] was significantly associated with performance not only in the motor but also across cognitive and language domains at two years of age, which supports my results ⁵⁸.

CONCLUSION

This study demonstrates that conventional MRI scoring between 4-14 days of birth is associated with an outcome of death or disability at 18–22 months of age. While good inter-observer agreement was found in MRI scoring, the overall accuracy of conventional MRI for prediction of long-term outcome was suboptimal. There is now convincing evidence about the accuracy of MRS as predictor of neurodevelopmental outcome after NE. However, MRI is less useful than MRS to guide the neuroprotection strategy and therefore, this may limit its use as prognostication tool.

Specific biomarkers such as gene expression, that increase within the first hours after NE, may be the way forward for a prompt identification of which neonates qualify for neuroprotection. I will examine these in more detail in Part B of the thesis.

In the following chapter, I examine the characteristics of the so-called ‘mild’ NE infants in more detail.

Chapter 3: Magnetic resonance biomarker measures in mild neonatal encephalopathy

There is increasing evidence suggesting that babies with mild encephalopathy may have hypoxic-ischaemic brain injury detected on MRI and higher rates of disability at childhood. In this chapter I explore the features of babies with mild NE and examine differences in cerebral MR biomarkers and neurological outcomes at two years between such babies who underwent whole-body hypothermia and those who did not.

INTRODUCTION

Over the past decade major trials have provided definitive evidence regarding the efficacy and safety of hypothermia treatment in moderate and severe encephalopathy in high-income countries¹³⁰. However, babies with mild NE were excluded from most of these studies due to a perceived low risk of having an adverse long-term outcome.

This idea has now been challenged by recent reports showing that over 25% of mild NE infants still have long-term neurodisability. However, pooled data from 117 babies recruited in clinical trials fail to show any benefits of therapeutic hypothermia in these cases⁴⁴.

Despite this lack of evidence, therapeutic hypothermia has been creeping into clinical practice worldwide as a treatment for mild NE¹³¹. This might reflect the perception that therapeutic hypothermia can also be effective in mild NE, and the false reassurance from the good safety profile of therapeutic hypothermia in babies with moderate and severe encephalopathy.

In the first days following perinatal asphyxia, there are substantial changes in cerebral metabolism, which accurately predict whether an infant will follow a trajectory of normal or abnormal neurodevelopment – the equivalent information from clinical examinations would only be available years later. With proton magnetic resonance spectroscopy, these changes can be accurately quantified and interpreted¹³². The use of brain MRI in mild encephalopathy has been only recently studied showing that in the vast majority of mild NE cases, there is no basal ganglia injury visible by using conventional MRI¹³³. However, in animal models, some degree of apoptosis is present in the

thalami even with milder ischemic insults, probably reflecting the intense metabolic activity of this area at birth^{121, 129}. This also agrees with my observations in the previous chapter, identifying prevalent metabolic injury in the BGT despite various injury phenotypes on MRI.

My aim was to use cerebral MR biomarkers as a sensitive measure of mild injury in the neonatal brain, and relate this with neurological outcomes at two years, both in those who had and had not undergone whole body hypothermia.

METHODS

I included consecutive full term (≥ 36 weeks) infants with evidence of perinatal hypoxia and with mild NE in this nested study of MARBLE⁵⁷. I considered any of the following criteria as evidence of perinatal asphyxia: metabolic acidosis on cord/baby blood gas within one hour of birth; an acute intrapartum event (e.g. cord prolapse, abruption, antepartum bleed); a 10-minute Apgar score ≤ 5 ; or assisted ventilation initiated at birth and continued ≥ 10 min.

Babies who fulfilled these criteria were examined within six hours of birth by a certified examiner by using the modified Sarnat score as per the National Institute of Child Health and Human Development Neonatal Research Network (NICHD NRN) trial of hypothermia¹³⁴. This score is made up of six categories: level of consciousness, spontaneous activity, posture, tone, primitive reflexes (suck and Moro), and the autonomic nervous system (pupils, heart rate and respiration). Each category was scored as normal/mild, moderate or severe. The original system was designed only for moderate or severe NE classification. This was later modified to include also babies with mild NE¹³⁵.

I considered mild NE in case of ≥ 2 abnormal categories but with no evidence of moderate or severe NE (defined as moderate and/or severe abnormality in ≥ 3 categories).

Amplitude integrated electroencephalography (aEEG) was recorded for at least the first six hours after birth and was classified by voltage method as normal, moderately abnormal and severely abnormal trace according to the upper and lower margin of band of the aEEG activity¹³⁶.

I divided infants into two groups: i) those who received 72 hours of therapeutic hypothermia, and ii) those who did not receive any hypothermia or had hypothermia for less than 12 hours. The attending clinician decided whether to provide therapeutic hypothermia.

Magnetic resonance acquisition and analysis

Brain MRI scans were performed between 4 to 14 days of age on a 3 Tesla scanner as described in the previous chapter.

MRS analysis was performed by an MR physicist (PJL) with seven years of experience, using LCModel (v6.3–1J, LCModel, Oakville, Canada). A neonatal neurology consultant (ST), with ten years of neonatal neuroimaging experience, reported conventional MR images using the Rutherford MRI scoring system, and was masked to the clinical details.

Neurodevelopmental assessment

Once the infants reached two years of age, I performed a detailed neurological examination using Bayley-III. In cases where the severity of disability was so severe to make the Bayley-III assessment not possible, I used the British Association of Perinatal Medicine (BAPM)/Royal College of Paediatrics and Child Health (RCPCH) working group classification.

I considered an adverse outcome if mild/moderate/severe disability was present at 18-24 months of age as defined by the NICHD Neonatal Research Network ¹³⁷. I defined mild disability by a cognitive score of 70 to 84 alone, or a cognitive score ≥ 85 and: GMFCS Level 1 or 2; a seizure disorder (without anti-epileptic medication); or hearing deficit with ability to follow commands without amplification. I considered moderate disability in case of cognitive score from 70-84 and GMFCS Level 2; active seizure disorder (receiving anti-epileptic medication); or hearing deficit with the ability to follow commands after amplification. Finally, I classified severe disability by a cognitive score < 70 ; GMFCS Level 3-5; blindness; or hearing impairment with inability to follow commands in spite of amplification.

Statistical analysis

I used a two-sided Student's t-test for parametric continuous variables and a Mann-Whitney U-test for continuous non-parametric variables. I compared categorical variables using a chi-squared test or

Fisher's exact test. I performed analyses with SPSS Statistics 24 (IBM Corp., U.S.A.) and considered a p-value of <0.05 as statistically significant.

RESULTS

I included a total of 47 babies with mild NE in the study. Of these, 15 (32%) were not cooled (n=10) or were cooled for less than 12 hours (n=5, median duration of cooling 3 hours IQR 3-10), and 32 (68%) had 72 hours of cooling. There was no significant difference in the baseline characteristics between cooled and non-cooled babies apart from lower 10 min Apgar scores in the cooled babies (p=0.02, Table 6). Cooled babies had significantly longer hospitalisation (p=0.04). A moderately abnormal aEEG was found within six hours of age in eight (25%) of the cooled babies and one (6%) of the non-cooled babies.

Four babies (8.5%) had seizures after six hours of age. All of them had normal aEEG at the time of admission (Table 6). One baby (case 4) was subsequently cooled for 72 hours, starting from 10 hours of age.

I found that 29 out of the 47 (61%) infants had white matter injury on conventional MRI. Fully cooled babies had lower white matter injury scores (mean 0.5, SD 0.6, p= 0.01) when compared with non-cooled babies (mean 1.3, SD 0.7) (Table 7). White matter injury was present in 50% (16/32) of the cooled babies and 87% (13/15) of the non-cooled babies (p=0.01).

Only 3 infants had evidence of deep brain nuclei injury of whom two were cooled for 72 hours, while one was not cooled. In all of them it was mild. I observed no difference in injury scores between the two groups in the deep nuclei or cortex (Table 7).

Table 6. Baseline characteristics and neonatal course of the cooled and non-cooled babies. (From Therapeutic hypothermia initiated within 6 hours of birth is associated with reduced brain injury on MR biomarkers in mild hypoxic-ischaemic encephalopathy: a non-randomised cohort study. Arch Dis Child Fetal Neonatal Ed. 2018 Nov 13).

	No Cooling (n=15)	72 hours of cooling (n=32)
Antenatal history		
Maternal diabetes	2(13)	0
Maternal hypertension	1(6)	3
Thyroid disease	0	0
Reduced fetal movements	2(13)	2(6)
CTG bradycardia	2 (13)	10 (31)
Late decelerations	1(6)	2(6)
Variable decelerations	1(6)	1(3)
Prolonged rupture of membranes ¹	2(13)	6(18)
Birth and resuscitation		
Gestational age at birth (weeks)	39.0 (2.0)	40 (1.2)
Birth weight (kg)	3.2 (0.4)	3.6 (0.4)
Cord pH	7.1[0.5]	6.9 [0.2]
Cord BE	-9.2 [6.8]	-14.0 [6.7]
Temperature admission	36.0 (0.6)	36.0 (0.8)
Apgar score (1 minutes)	4 [4]	2 [3]
Apgar score (5 minutes)	6 [2]	5 [2]
Apgar score (10 minutes)	8 [2] †	6 [3] †
Neonatal course		
Abnormal aEEG	1(6)	8(25)
Invasive ventilation	7(46)	22(68)
Non-invasive ventilation	1(6)	3(9)
Hypotension requiring inotropes	2(13)	5(15)
Abnormal clotting	1(6)	6(19)
Decreased urine output	0	3(9)
Maximum creatinine levels during the first 72 hours of age	80(22)	78(17)
Maximum CRP levels during the first 72 hours of age	22(44)	23(23)
Blood culture positivity	0	1(3)
Seizures after 6h of age	4 (27)	0
Postnatal age at MRI (days)	6.5 [3.0]	7.0 [4.0]
Hospital stay (days)	5.5 [6.2] †	8.0 [2.0] †

Data are mean (SD), n (%), median [inter-quartile range], †p < 0.5 (Mann-Whitney U-test). ¹Suspected or confirmed rupture of membranes for more than 24h

Table 7. MR imaging and spectroscopy in cooled and non-cooled groups with mild encephalopathy. (From Therapeutic hypothermia initiated within 6 hours of birth is associated with reduced brain injury on MR biomarkers in mild hypoxic-ischaemic encephalopathy: a non-randomised cohort study. Arch Dis Child Fetal Neonatal Ed. 2018 Nov 13).

		No cooling (N=15)	72 hours of cooling (N=32)
Rutherford MRI scoring system			
Basal ganglia and thalami	Normal	14 (93.3)	30 (93.8)
	1	1 (6.7)	2 (6.3)
	2	0	0
	3	0	0
Posterior limb of internal capsule	Normal	15 (100)	32 (100)
	1	0	0
	2	0	0
	3	0	0
White matter	Normal	2 (13.3)	16 (50)
	1	9 (60)	13 (40.6)
	2	4 (26.7)	3 (9.4)
	3	0	0
Cortex	Normal	12 (80)	29 (90.6)
	1	3 (20)	3 (9.4)
	2	0	0
	3	0	0
		No cooling (N=14)	72 hour cooling (N=26)
MR spectroscopy			
tLac/tNAA		0.16 (0.06)	0.12 (0.03)
tNAA/tCho		0.67 (0.08) *	0.89 (0.11)*
tNAA/tCr		1.42 (0.11) *	1.64 (0.18)*

Data are number of patients or mean (SD), * p<0.001 (Student's t-test).

tNAA=N-acetylaspartate+N-acetylaspartylglutamate, tCho=total choline, tCr=total creatine, tLac=threonine + lactate.

Table 8. Neurodevelopmental outcomes at two years after mild encephalopathy. (From Therapeutic hypothermia initiated within 6 hours of birth is associated with reduced brain injury on MR biomarkers in mild hypoxic-ischaemic encephalopathy: a non-randomised cohort study. Arch Dis Child Fetal Neonatal Ed. 2018 Nov 13).

	No cooling (n=14)	72 hours of cooling (n=26)
Cognitive composite score	106 (13)	105 (15)
Language composite score	90 (12)	99 (25)
Motor composite score	100 (9)	100 (10)
Motor composite score < 85	1(7)	1(3)
Language composite score < 85	4(28)	2(8)
Cognitive composite score < 85	1(7)	1(3)
Cerebral palsy	1(7)	0
Autism/hearing difficulties	0	0

Data are number (%), mean (SD),

There was no statistically significant difference between the cooled and non-cooled groups.

I excluded six babies (one non-cooled and five cooled) from the MRS analysis due to poor quality data, or loss of data during transfer from the scanner. These six cases were recruited at 5 different centres and I did not find any significant difference in terms of baseline characteristics between the excluded cases and the remaining ones.

When I analysed the remaining MRS values, I found that the thalamic ratios of tNAA/tCr and tNAA/tCho were significantly higher in cooled babies than in non-cooled babies (Figure 11). The differences in brain injury between cooled and non-cooled babies persisted even after the exclusion of those infants who progressed to moderate NE. In particular, thalamic ratios of tNAA/tCr and tNAA/tCho were significantly higher in fully cooled babies and white matter injury scores significantly higher in the non-cooled group.

Two mild NE babies who did not undergo cooling had tLac/tNAA ratio ≥ 0.22 , which indicates a high risk of adverse neurodevelopmental outcome⁵⁸. The tLac/tNAA ratios were all below this threshold

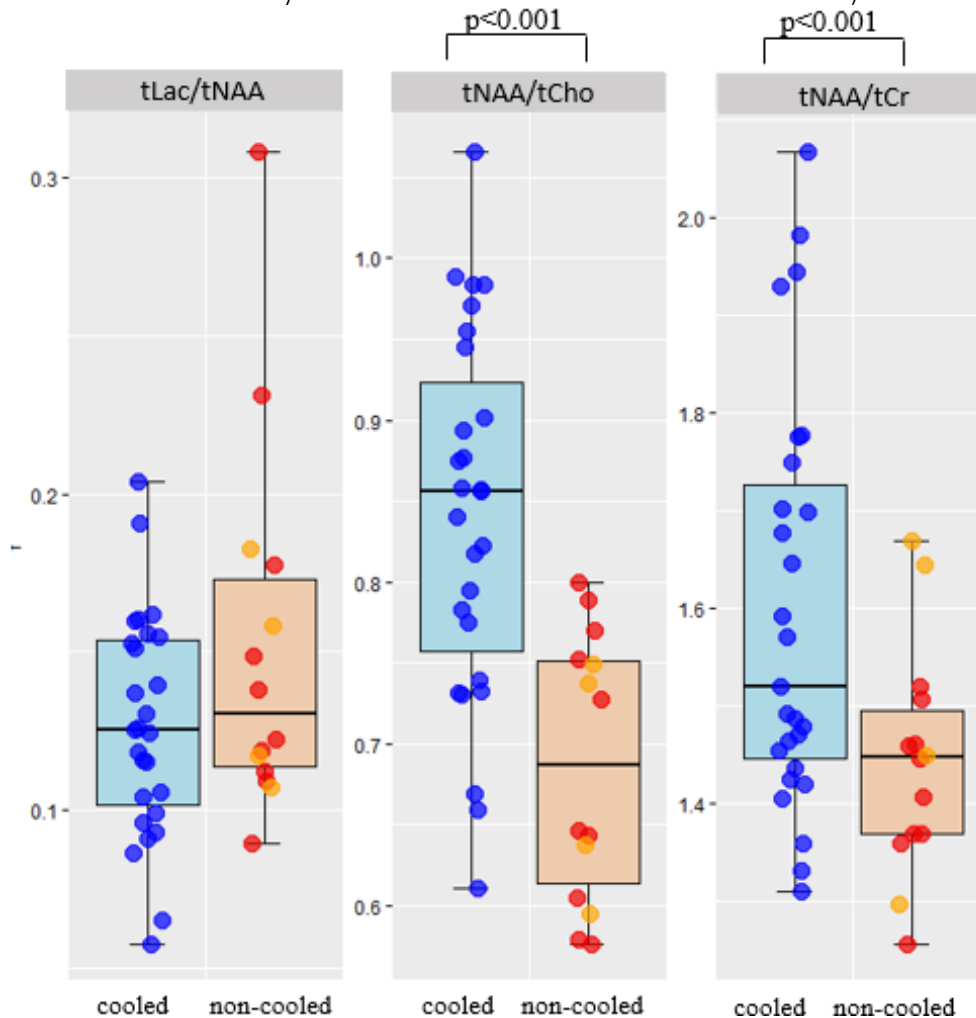
in the cooled babies. I did not find any significant differences in the thalamic ratios of tLac/tNAA between the two groups ($p=0.21$) (Figure 11).

Seven children were lost at follow-up and therefore a total of 40 infants (85%) had neurodevelopmental outcome assessments available for analysis (Table 8 and Figure 12). I did not find any significant difference with regards to the baseline characteristics between the children who were lost at the follow-up and the others.

Two patients had mild disability at two years and did not receive whole-body cooling. One of them had progression in his NE stage and developed seizures at 33 hours of age. He was later diagnosed with hyperekplexia, confirmed by a homozygous novel missense mutation SLC6A5. When I performed the neurological assessment (18 months of age) there was severe language delay and mild motor disability, but without cerebral palsy.

In the second case, when I performed the Bayley-III assessment at 21 months of age, I found a cognitive composite score of 80 (equivalent developmental age of 16 months), a language composite score of 74 (receptive communication equivalent developmental age of 16 months; expressive communication equivalent developmental age of 15 months) and a motor composite score of 82 (fine motor equivalent developmental age of 17 months; gross motor equivalent developmental age of 16 months). She had right arm monoplegia (Gross Motor Function Classification System=1).

Figure 11. Dot plots (median, interquartile range) for MRS measures in the cooled and non-cooled babies with mild encephalopathy. (Adapted from Therapeutic hypothermia initiated within 6 hours of birth is associated with reduced brain injury on MR biomarkers in mild hypoxic-ischaemic encephalopathy: a non-randomised cohort study. Arch Dis Child Fetal Neonatal Ed. 2018 Nov 13).



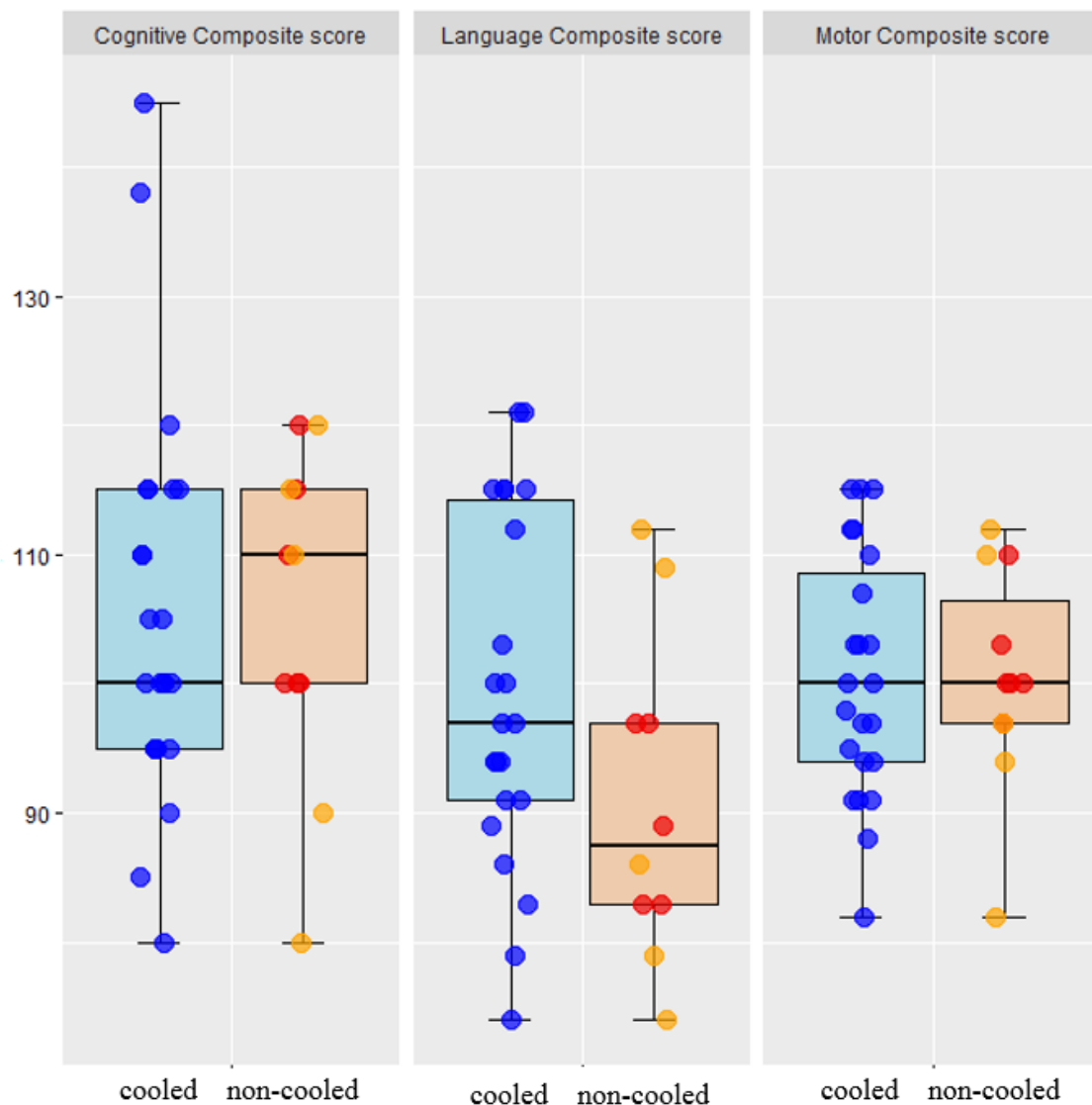
Blue dots indicate babies cooled for 72 hours, orange dots indicate babies cooled for less than 24 hours and red dots indicate non-cooled babies.

tNAA/tCho=N-acetylaspartate+N-acetylaspartylglutamate/total choline,

tNAA/tCr= N-acetylaspartate+N-acetylaspartylglutamate/total creatine,

tLac/tNAA=threonine + lactate/tNAA

Figure 12. Dot plots (median, interquartile range) of the neurodevelopmental outcome scores in the cooled and non-cooled babies with mild encephalopathy. (Adapted from Therapeutic hypothermia initiated within 6 hours of birth is associated with reduced brain injury on MR biomarkers in mild hypoxic-ischaemic encephalopathy: a non-randomised cohort study. Arch Dis Child Fetal Neonatal Ed. 2018 Nov 13).



Blue dots indicate babies cooled for 72 hours, orange dots indicate babies cooled for less than 24 hours and red dots indicate non-cooled babies.

DISCUSSION

In this observational study I demonstrate that babies with mild NE who did not undergo whole body cooling had worse metabolic indicators on MRS and significantly more white matter injury when compared with those who were cooled. Four out of the 15 non-cooled babies had seizures and showed a progression of their encephalopathic stage to moderate NE after six hours of age. However, the results did not change even after excluding these cases. There was no significant difference in the mean cognitive, motor and language scores at two years between cooled and non-cooled babies even though two out of the 14 (14.3%) non-cooled cases had mild disability whereas none of the cooled babies had an adverse outcome at two years.

Wisnowski JL. *et al.* previously studied the effect of cooling on brain metabolism in 31 babies with moderate and severe NE by using MRS. They found that hypothermia improved brain energy metabolism by decreasing the cellular energy demands and the availability of excitatory neurotransmitters. In the study the authors analysed brain metabolism in basal ganglia thalami, grey and white matter and found a significant increase of [NAA] and [Cr] whereas there was a significant reduction of Glutamate and GABA concentrations after whole body cooling¹³⁸.

However, similar evidence is lacking in mild NE where only few studies have assessed the nature of injury and the effect of cooling therapy on this. Previous reports showed that infants with NE have different brain MRI injury patterns according to the severity of NE, with mild NE infants having mainly white matter injury and infants with moderate or severe NE having BGT injury^{49, 133}. The explanation for this probably lies in the presence of different pathological mechanisms. White matter injury has been traditionally linked to sub-acute hypoxia whereas acute profound hypoxia has been associated with deep nuclei injury¹³⁹. However, those studies did not include babies who undergo whole body cooling, and therefore the effect of cooling in mild NE was not evaluated.

In the present report, more than 60% of mild NE infants had white matter injury and the encephalopathic infants who were cooled for 72 hours had lower white matter injury scores. These findings are in agreement with previous evidence showing less brain injury on MRI in

infants who received whole-body cooling (4/13 (31%)) when compared with those who were not cooled (20/50 (40%))¹⁴⁰. In this study, the authors observed that an abnormal signal in the cortex was present in both cooled and not cooled babies. Interestingly, preclinical evidence shows that therapeutic hypothermia preserves white matter after mild hypoxia-ischemia¹⁴¹.

Walsh *et al* conducted a retrospective study on 89 encephalopathic babies who underwent whole-body therapeutic hypothermia therapy and reported no significant difference in the incidence of watershed injury in infants with mild NE (18/48 (36%)) when compared with those with moderate (11/32 (32%)) or severe NE (3/6 (50%)). In this study, only two (4%) babies had thalamic or basal ganglia injury¹⁴². My data support these observations, since I found that none of the babies had significant thalamic injury on the conventional MRI, even though white matter injury was present to some extent in 50% (16/32) of the cooled babies and almost 87% (13/15) of the non-cooled babies. On the other hand, thalamic MRS markers were more favourable (higher tNAA/tCho and tNAA/tCr) in cooled babies with mild NE. However, despite this difference in MRS, there was no difference in the neurodevelopmental outcome at 18-24 months between cooled and non-cooled infants and neurodevelopmental outcome is currently the gold standard for measuring efficacy of cooling treatment. This lack of association with outcome may reflect that the study was underpowered to look at the neurodevelopmental outcome. The absolute concentration of [NAA] would have increased the power of the study, but it was available only in few patients. Another possible explanation is that babies with mild NE are more likely to develop mild disability, which is more easily identified at school age or later and not at 2 years¹⁴³.

One possible explanation is that conventional MR imaging cannot detect sub-macroscopic injury to the basal ganglia, whereas MRS might be more sensitive to these milder insults. In a NE fetal sheep model, William and colleagues showed that hippocampus and parasagittal cortex were the most commonly involved regions at all durations of ischemia (10, 20, 30 and 40 minutes), with more severe injury in case of longer duration of ischemia¹²⁹. Moreover, thalamic injury was present to some extent even in case of a short period (10 minutes) of ischaemia, even though thalamic injury at all durations was lower than hippocampal injury in this model, which is in agreement with my observation of thalamic metabolite alterations.

There is a paucity of MRS studies on mild NE infants and the limited data that are available are from the pre-cooling era¹⁴⁴. Hanrahan and colleagues found a lactate peak in over 80% of the babies with mild NE when scanned within 24 hours of birth. This is in contrast with my study, where only two babies had high tLac/tNAA ratios and both of them were part of the non-cooling group. However, this might reflect the different timing of MRS (24 hours versus 6.5 days in our study) with respect to the hypoxic-ischaemic event. A recent study, which described cerebral lactate concentration in neonatal NE in relation to time and severity of injury, also showed that cerebral lactate concentration was low for those with less severe injury across the first 2 weeks of life¹⁴⁵.

Twenty-four to 48 hours of cooling have been shown to be neuroprotective in animal models, unlike very short periods or very long periods of cooling¹⁴⁶. This study suggests that cooling for less than 24 hours is unlikely to be beneficial in mild NE, as shown by the lack of difference in the metabolite profiles between non-cooled and partially cooled babies. This is consistent with previous findings, which showed MRI evidence of residual brain injury in 50% of the babies with mild NE who were cooled for less than 24 hours, with 20% of them later developing adverse outcomes¹⁴⁷.

The progression of the severity of NE after six hours of age with the development of seizures is particularly worrying for clinicians. In this report only 4 infants (8.5%) with mild NE progressed to moderate NE after 6 hours and only one had an adverse neurodevelopmental outcome at two years. This patient was later diagnosed with hyperekplexia. These findings are consistent with what reported by Gagne-Loranger *et al.* who found progression of mild to moderate NE in 6% of babies (12% in the non-cooled group and 3% in the cooled group) and with the data from the PRIME (Prospective Research in Mild Encephalopathy) study, where a standardised neurological examination was used¹⁴⁸.

On the other hand, in the ICE trial there was a progression to moderate NE in a much higher percentage of babies with mild NE (50%). However, no standardised neurological examination was used and therefore it cannot be excluded that some babies were wrongly considered with mild NE at the admission neurological examination¹¹⁹. None of these data can be directly

compared with the seminal description of NE by Sarnat *et al.*, where mild NE was diagnosed retrospectively based on resolution of signs by 24 hours¹⁴⁹.

In this report, NE infants who were cooled had significantly lower 10-minute Apgar scores and higher rates of invasive and non-invasive ventilation, which might reflect that these patients were sicker when it was decided to offer cooling. The reasons behind the decision to cool these babies might be the expectation that these cases could benefit the most from cooling therapy, the uncertainty about the possible progression of the encephalopathy stage into moderate and the reassurance of the safety profile of therapeutic hypothermia. We recently conducted a survey including 54 of the 68 UK cooling centres and found that over 60% of these offer whole-body cooling for 72 hours to mild NE babies¹⁵⁰. The same has also been shown in Australia and in USA where almost half and 15% of the mild NE infants respectively, were cooled despite not fulfilling the criteria of moderate/severe NE at initiation of cooling therapy^{126, 127}.

This study suggests that cooling therapy might be beneficial in mild NE, however, considering the observational design and the sample size of this study, caution is needed before drawing any conclusions and a properly powered randomised controlled study is now clearly essential. Preclinical evidence has showed that cooling of the healthy brain induces apoptosis, and therefore the decision to cool encephalopathic infants should be taken seriously¹⁵¹.

This study has limitations. This is a small observational study and therefore was clearly underpowered to examine clinical outcomes. Nevertheless, I found a significant difference in the thalamic metabolite peak area ratios between cooled and non-cooled babies, confirming the need for further evaluation of therapeutic hypothermia in mild NE. The strengths of this study are the immediate clinical urgency to confirm that cooling is beneficial for infants with mild NE in the first 6 hours of life, the use of rigorous, well validated neurological examinations, central, blinded analysis of MRS and MRI, scanner harmonisation prior to the start of the study and 2-year neurodevelopmental follow-up.

CONCLUSION

In this study I demonstrate, using sensitive MR biomarkers, that babies with mild NE have less brain injury after whole-body cooling suggesting that cooling may be neuroprotective in mild

NE. These findings point out the need for a properly powered randomised trial to investigate the efficacy of therapeutic hypothermia in this population. However, there are some challenges to consider before starting a randomised controlled trial including the short time frame (6 hours after birth) to define mild NE and lack of agreement on a standardized definition of mild NE.

PART B:

Although MRS is effective in predicting long-term outcome in babies with NE, scanning them within few hours after birth is unrealistic. Gene expression has huge translational potential as a prognostic biomarker since it can be done immediately after birth to guide the best treatment strategy, it is objective and has strong potential to become a simple bedside test in the near-future.

This part of the thesis tests my second hypothesis concerning gene expression profiling. Namely, I examine whether neonatal encephalopathy is associated with a unique host gene expression profile at the time of birth. I then examine whether whole blood gene expression profiles in babies with neonatal encephalopathy differ between those who subsequently develop favourable or adverse long-term neurological outcomes.

Chapter 4: Whole blood gene expression in neonatal encephalopathy

INTRODUCTION

A number of serum biomarkers have been extensively studied for many years, although none have sufficient accuracy to influence treatment decisions.¹⁵² Therefore, there is an unmet need for an accurate bedside prognostication tool in babies with neonatal encephalopathy, soon after birth. This represents a major obstacle in the bench-to-bedside translation of over 15 novel highly promising neuroprotectants in NE, which have been proven to be effective in animal models and are awaiting evaluation in clinical trials.

Over the past years, increasing evidence has shown that infectious and inflammatory diseases elicit specific patterns of gene expression measured in blood⁹¹⁻⁹³. Specific transcriptomic profiles have been identified in a wide range of acute and chronic infections and a concerted effort from the research community has been employed to develop bedside diagnostic tests suitable for clinical practice¹⁵³.

Preclinical studies have shown that oxygen shapes gene expression at different levels and in this way, affects cell proliferation, apoptosis, differentiation, and vascularization/angiogenesis. Besides this, cell culture studies demonstrate that oxygen sensing also controls the immune response through increased neutrophil survival and T cell differentiation^{154, 155}.

My aim was to assess if whole blood host gene expression profiles in neonatal encephalopathy are different to those in either healthy or septic neonates, and to describe the underlying biological pathways of any key genes which differentiate these groups. I also compared the gene expression profile soon after birth and at 24 hours of age in term babies with NE undergoing cooling therapy.

METHODS

I recruited twenty full term infants who had therapeutic hypothermia for neonatal encephalopathy – 12 aged 5h to 10h (early group) and 8 aged 22h to 35 h (late group) and six matched (for time of blood sampling with the early group) healthy term control babies, as a

part of the MARBLE (Magnetic Resonance Biomarkers in Neonatal Encephalopathy) study, after informed parental consent (NIHR; Ethics: 11/H0717)⁵⁷.

All babies underwent a standardised neurological examination within six hours of age birth using the Sarnat score as modified by the NICHD NRN trial of hypothermia, by a certified examiner^{43, 134}. For mild NE infants I used the same classification as described in the previous chapter. However, the decision to offer therapeutic hypothermia was based on the attending clinician's preference.

I collected 0.5 ml of blood from all of the recruited infants and mixed this with 1.4 mL RNA stabilising solution (PreAnalytiX BD/QIAgen) keeping the same blood/additive ratio as per the manufacturer's instructions. In the encephalopathic group I also collected blood for blood culture, C-reactive protein and full blood count within 12 h of birth.

Exclusion Criteria

- Confirmed intrauterine infection (toxoplasmosis, rubella, cytomegalovirus, syphilis and herpes).
- Chromosomal abnormalities, major congenital malformations
- Blood stream positive perinatal sepsis (post-hoc)

RNA extraction and next generation sequencing

Samples were frozen at -80 °C and then transported to Prof Max Vento's lab in Valencia. Following an initial induction and training at this lab, I isolated RNA from each sample using a MagMAX RNA isolation kit (Ambion/Applied Biosystems, 51 stems) according to the manufacturer's instructions. I assessed RNA integrity and concentration by using Agilent RNA 6000 nano Kit with 2100 Bioanalyzer (Agilent, Palo Alto, CA) and Qubit RNA HS assay Kit with Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Carlsbad, CA).

I prepared DNA libraries for sequencing with the Ion AmpliSeq™ Transcriptome Human Gene Expression Kit (LifeTechnologies, Carlsbad, CA) according to the manufacturer's instructions. Libraries were quantified by real-time PCR using Ion Library TaqMan Quantitation Kit (LifeTechnologies, Carlsbad, CA) and normalised prior to sequencing using the Ion PI Chef™ Kit.

I also used this system to prepare enriched, template-positive Ion Sphere™ Particles for up to 200 base-read sequencing of libraries on the Ion Proton™ Sequencer I (LifeTechnologies, Carlsbad, CA). Sequencing was performed using an Ion PI Hi-Q Sequencing 2000 Kit with PI v3 chips (LifeTechnologies, Carlsbad, CA).

Data analysis

The read alignment was performed by a built-in plugin software part of Ion Proton™ Sequencer I. Reads were aligned to the hg19 human genome and then the expression abundance for each gene was acquired by the same software. I first explored the next generation sequencing data using principal component analysis (PCA). PCA summarises the dataset, and allows for visualisation despite the high dimensionality, to identify unwanted variance and outliers due to bias.

I estimated normalisation factors and negative binomial dispersions from the raw count data. With these estimates, I adjusted negative binomial generalised linear models for each gene by conducting likelihood tests. I corrected the p values for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) method to control for type I errors. I performed statistical analysis using R (version 3.3.1) and DESeq2 (v1.17.18) R-packages¹⁵⁶.

I used Ingenuity Pathway Analysis software (QIAGEN) to study the biological pathways enriched in the significantly differentially expressed genes. I performed the pathway analysis of the first 1000 most significant genes ordered by FDR with an absolute $\log_2$ fold change of >1.5 .

After I compared the gene expression profile of babies with NE of the early group and healthy controls, I used a previously published dataset of gene expression data of babies with bacterial neonatal sepsis (GSE25504) to find a separate set of differentially expressed genes in bacterial neonatal sepsis⁷⁹. The two datasets were not merged, and they were analysed separately. I compared the RNA expression data of babies with bacterial neonatal sepsis (blood culture positive sepsis) and age-matched healthy controls. Finally, I assessed the overlap between the two different gene sets (genes with $\text{FDR} < 0.05$ and absolute $\log_2$ fold changes >1) identified from the comparison of babies with NE versus healthy controls and babies with blood positive culture sepsis and controls.

To identify the significantly differentially expressed genes in the comparison of neonates with bacterial sepsis (n=10) to healthy controls (n=6), I used GEO2R analysis⁸⁴.

Statistical analysis

I described clinical data as number (%) or median (interquartile range) where appropriate. I performed statistical analysis by using Mann-Whitney U-test for continuous variables and Pearson chi-squared test or Fisher exact test in case of prevalences.

I performed analyses with SPSS Statistics 24 (IBM Corp.—U.S.A.) and a p value of <0.05 was considered statistically significant.

RESULTS

No significant demographic differences were found between early NE infants and controls apart from body temperature at the time of sample collection, spontaneous vaginal delivery, and the initial Apgar scores, as would be expected (Table 9).

Healthy control babies were normothermic (36.4°C) whereas encephalopathic babies had undergone therapeutic cooling therapy for a median of three hours at the time of blood collection.

PCA showed complete separation of the NE infants of the early, late and control groups (Figure 13).

Figure 13. 2D principal components analysis mean centering and scaling based on the complete genome. Patients are plotted according to their respective position along the 2 axes. A) Principal components analysis shows the 12 NE (early group) babies within red balloons, 6 controls within blue balloons and 8 NE (late group) babies within green balloons. B) 2D principal components analysis after excluding the 8 NE (late group) (Adapted from Whole Blood Gene Expression Reveals Specific Transcriptome Changes in Neonatal Encephalopathy. *Neonatology*. 2018;115(1):68-76).

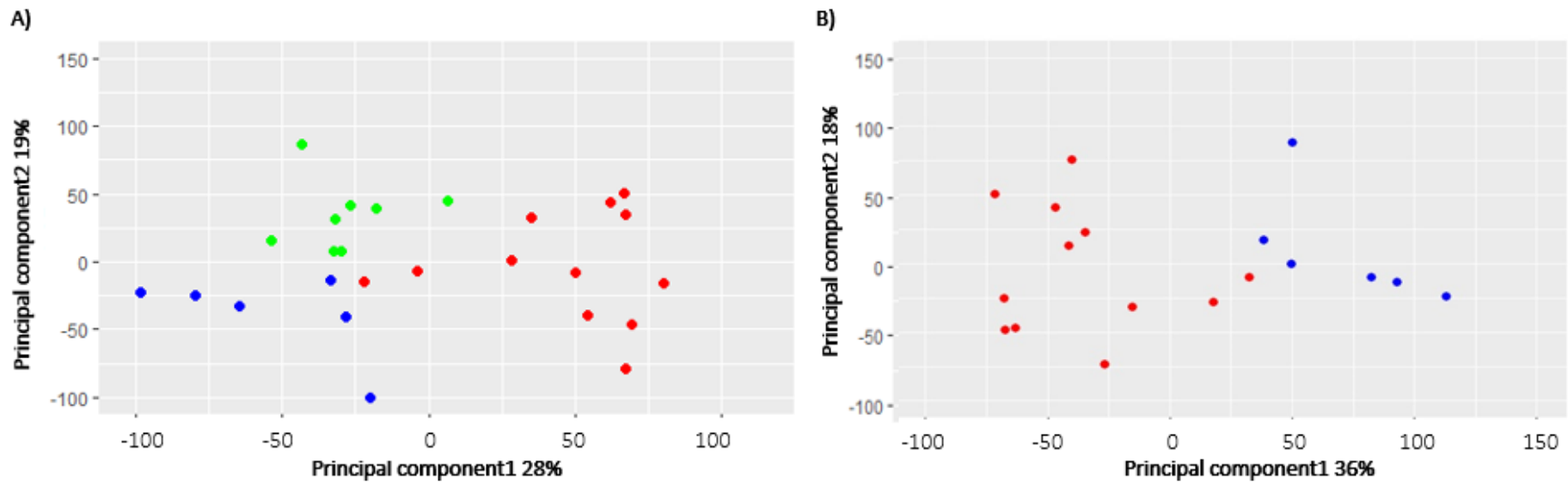
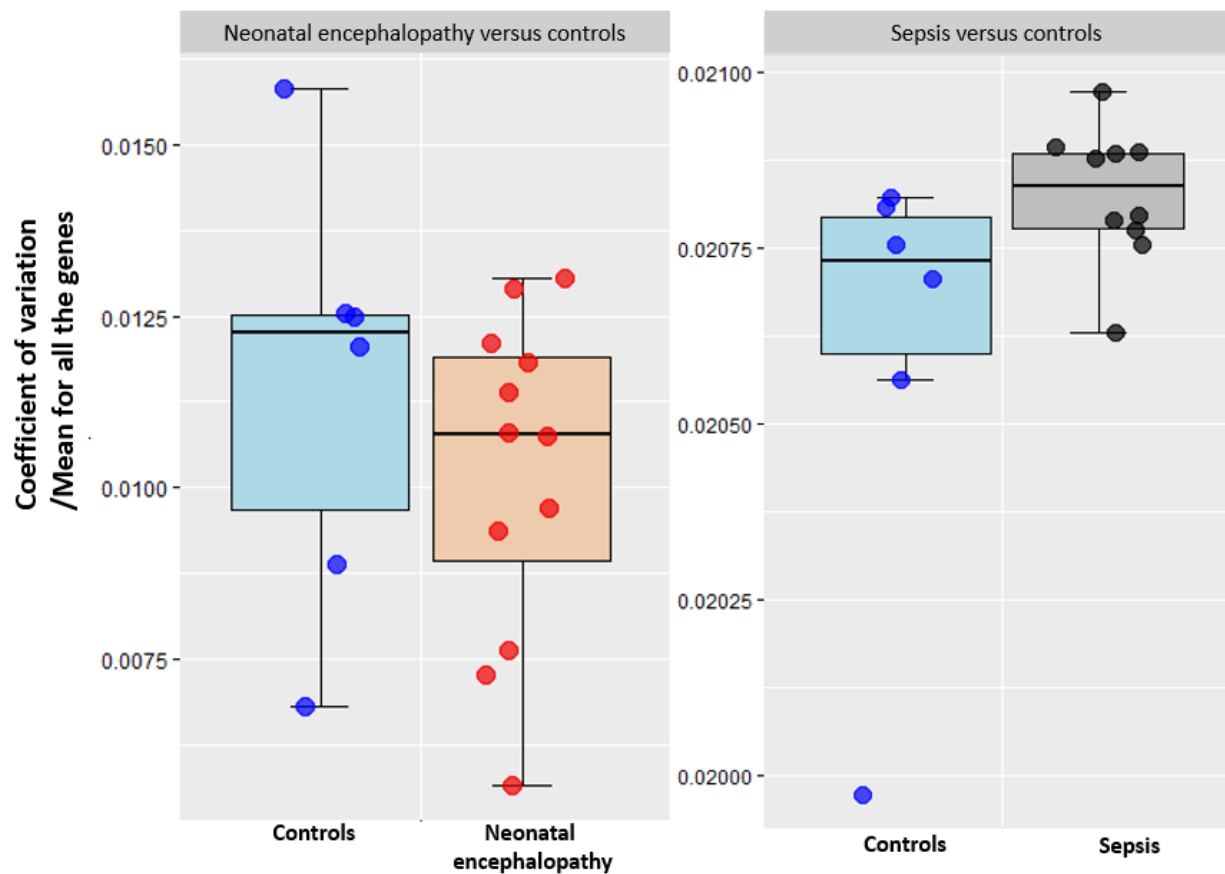


Table 9. Clinical characteristics of babies with neonatal encephalopathy (NE) (early group) and healthy control babies (From Whole Blood Gene Expression Reveals Specific Transcriptome Changes in Neonatal Encephalopathy. Neonatology. 2018;115(1):68-76).

Clinical Values	NE babies (n=12)	Healthy controls (n=6)	P
Gestational age, median [IQR], weeks	40.1 (39.3-40.7)	39.7 (39.1-40.2)	>0.05
Birth weight, median [IQR], g	3627 (3179-3731)	3743 (3247-3964)	>0.05
Caucasian ethnicity, no. (%)	12(100)	6 (100)	>0.05
Spontaneous vaginal delivery, no. (%)	5 (41)	6 (100)	0.02
Arterial cord pH, median [IQR]	6.9 (6.8-7.1)	Not done	-
Arterial cord Base Excess, median [IQR]	-10.2(-6.8 - 16.5)	Not done	-
Arterial cord Lactate, median [IQR], mmol/L	9 (6.9-12.8)	Not done	-
Apgar 1 min, median [IQR]	1.5 (0.2-2.7)	10	0.001
Apgar 5 min, median [IQR]	4.0 (1.5-4.7)	10	0.001
Encephalopathy grade (Mild/Moderate/Severe)	2/8/2	Nil	-
Resuscitation at birth, no. (%)			-
Bag and mask	2 (17%)	0	-
Intubation	4 (33%)	0	-
Intubation and external cardiac massage	6 (50%)	0	-
Death	1	0	-
Invasive ventilation, no. (%)	12 (100)	0	-
Age at sample collection, median [IQR], hours	6 (4-9)	7 (4-8)	>0.05
Hours of cooling at the time of blood collection, median [IQR]	3 (1-5.5)	Nil	-
Age cooling was started, median [IQR], hours	3 (0-4.5)	Nil	-
Body temperature at sample collection, median [IQR], °C	33.5 (33.2-35.4)	36.4 (36.4-36.7)	<0.001

I did not observe any significant difference in the coefficient of variation between neonatal encephalopathy and control groups and between sepsis and control groups (Figure 14)

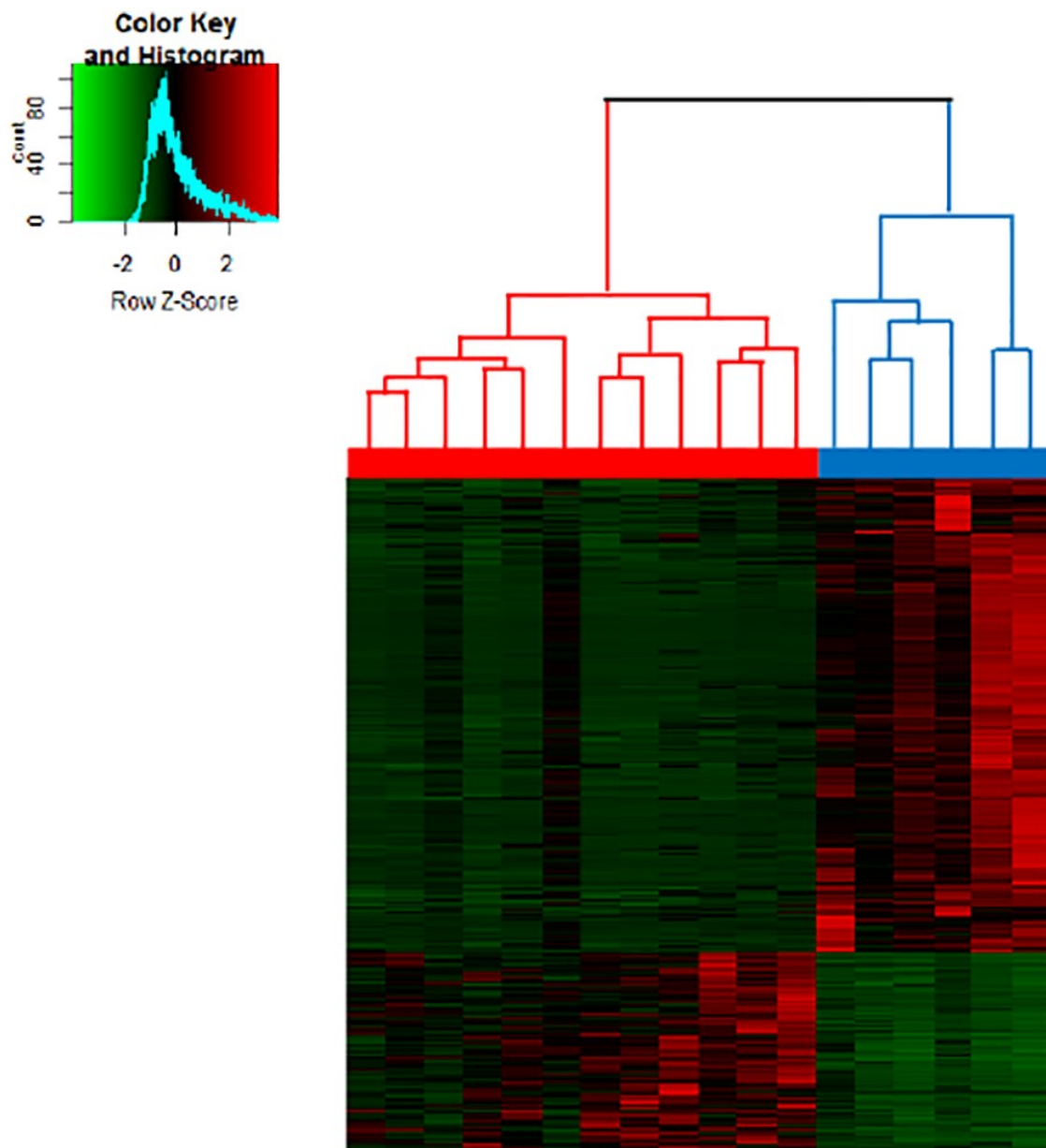
Figure 14. Dot plots of the coefficient of variation divided by the mean for all the genes in each sample of the neonatal encephalopathy (red), control (blue) and sepsis groups (black). (From Whole Blood Gene Expression Reveals Specific Transcriptome Changes in Neonatal Encephalopathy. Neonatology. 2018;115(1):68-76)



When I compared the early NE infants vs the matched controls, I found that 950 significant differentially expressed genes fulfilled an absolute $\log_2$ fold change > 1.5 (FDR value < 0.0001). Of these, 280 (29%) were overexpressed and 670 (71%) under-expressed. Unsupervised hierarchical clustering analysis based on the gene expression values of these genes completely discriminated cases from controls (Figure 15).

Figure 15. Unsupervised hierarchical clustering of 18 babies (12 babies with neonatal encephalopathy and 6 healthy babies, horizontal axis), with 950 genes derived from negative binomial likelihood tests. Each column represents a patient and each line a gene. Upregulated

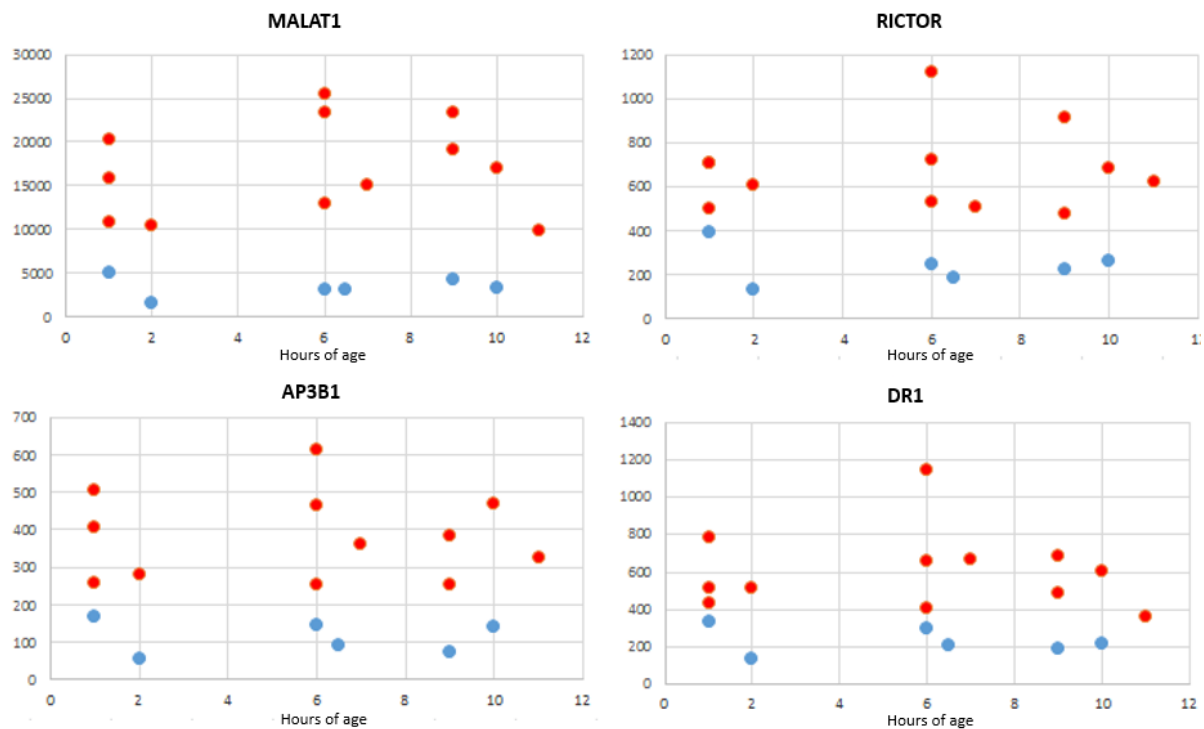
genes are represented in red and downregulated genes in green. The red bar represents neonatal encephalopathy (n = 12), and the blue bar represents healthy control babies (n = 6) (From Whole Blood Gene Expression Reveals Specific Transcriptome Changes in Neonatal Encephalopathy. *Neonatology*. 2018;115(1):68-76).



The top 4 significant genes identified in the comparison between babies with NE and healthy controls did not show any variation over the time in the 2 groups of patients (Figure 16).

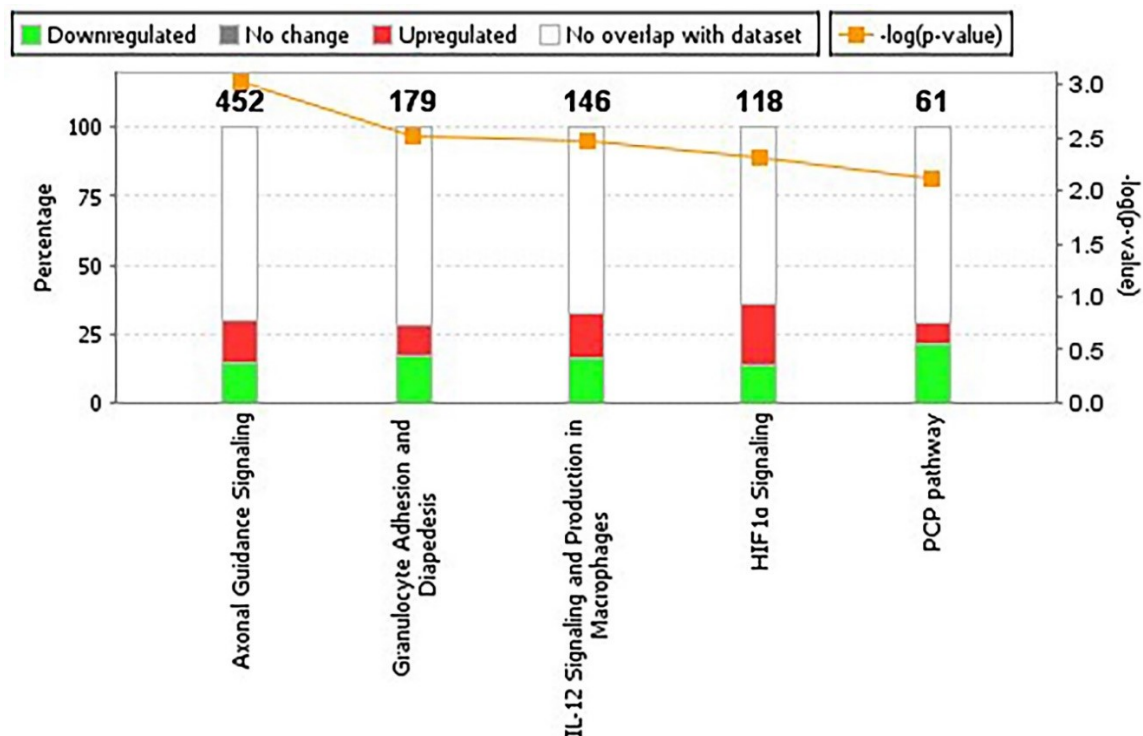
Figure 16. Scatter plot of the top 4 significant genes according to the timing of the blood sample collection (hours of age) of the neonatal encephalopathy (red) and control (blue) groups (From

Whole Blood Gene Expression Reveals Specific Transcriptome Changes in Neonatal Encephalopathy. *Neonatology*. 2018;115(1):68-76).



The most significant genes ordered by FDR were *MALAT1* (FDR <0.001, log2 Fold change 2.28) and *RICTOR* (FDR <0.001, log2 Fold change 1.48). Pathway analysis of the top 1000 most significant genes with a log2 fold change >1.5 in early NE vs controls revealed that the most significant pathways were axonal guidance signalling (p <0.001), granulocyte adhesion and diapedesis (p= 0.003), IL-12 signalling and production in macrophages (p=0.003) and hypoxia-inducible factor 1 α (HIF-1 α) signalling (p= 0.004) (Figure 17). The diseases/functions analysis showed an olfactory receptor response after hypoxic/ischemic insult (p<0.001).

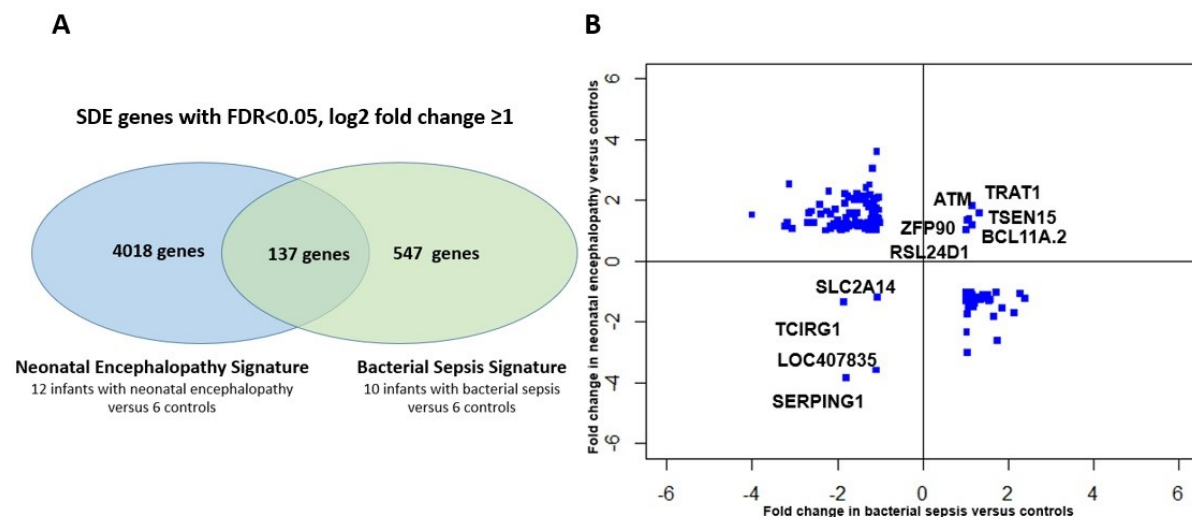
Figure 17. Canonical pathway analysis in terms of $-\log(p\text{-value})$ derived from the first 1000 significant genes with a $\log_2$ fold change >1.5 in the comparison of neonatal encephalopathy and healthy control babies. The upregulated genes are shown in red and the downregulated genes are shown in green. On the top of each bar there is the total number of genes, which each pathway is made of. (From Whole Blood Gene Expression Reveals Specific Transcriptome Changes in Neonatal Encephalopathy. *Neonatology*. 2018;115(1):68-76).



684 genes were differentially expressed between babies with bacterial sepsis and controls (FDR < 0.05 and $\log_2$ fold change >1) in the GSE25504 microarray gene expression dataset. When I assessed the overlap of the two lists of significantly differentially expressed genes (i.e. NE vs control, sepsis vs control), there were 137 genes in common (Figure 18 A). However, out of these 137 only 10 had a concordant fold change in expression relative to healthy controls (Figure 18 B).

In the comparison between early and late NE groups, there were 2470 significant differentially expressed genes with an absolute $\log_2$ fold change > 1.5 (FDR value <0.0001). Of these, 23 (1%) were under-expressed and 2447 (99%) were over-expressed. Pathway analysis of the top 1000 most significant genes with a $\log_2$ fold change >1.5 revealed that the most significant pathways were Agranulocyte Adhesion and Diapedesis ($p <0.01$) followed by Role of Cytokines in Mediating Communication between Immune Cells ($p <0.01$).

Figure 18. The GSE25504 microarray gene expression experiment. A) Venn diagram. 4155 genes were differentially expressed between neonatal encephalopathy and healthy controls; 684 genes were differentially expressed between bacterial sepsis and healthy controls ($FDR < 0.05$ and $\log_2$ fold change >1). There were 137 genes in common between neonatal encephalopathy and bacterial sepsis sets. B) Crossplot of the 137 shared genes ($FDR < 0.05$ and $\log_2$ fold change >1) shows that only 10 genes had the same fold change direction in the 2 datasets (From *Whole Blood Gene Expression Reveals Specific Transcriptome Changes in Neonatal Encephalopathy*. Neonatology. 2018;115(1):68-76).



DISCUSSION

This study is the first to examine whole blood gene expression in neonatal encephalopathy. I have showed that expression profile soon after birth in encephalopathic babies was markedly different from that in age matched healthy control infants. The main biological pathways involved after a hypoxic-ischaemic insult were axonal guidance signalling, granulocyte adhesion/diapedesis, IL-12 signalling and HIF-1 α signalling. There was only minimal overlap in the transcriptomic profiles of neonatal encephalopathy and bacterial infections suggesting differences in the underlying mechanisms.

After a hypoxic-ischaemic insult, cells respond to these unfavourable conditions by putting in place different biological responses including the switch to anaerobic metabolism. This study shows a striking differential expression in key genes of the hypoxic response such as: *HIF1A*, *MALAT1* and *RICTOR* (Figure 19). Cell culture studies have previously showed that HIF transcription factors are key regulators, which guide the cellular response to low oxygen levels¹⁵⁷. These preclinical models also highlighted an interaction between HIF-1 α and axon guidance genes, which is responsible for neuronal migration defects¹⁵⁸. Xiaowei *et al.*, reported that spinal cord injury is associated with upregulation of *HIF-1A* as a response to local

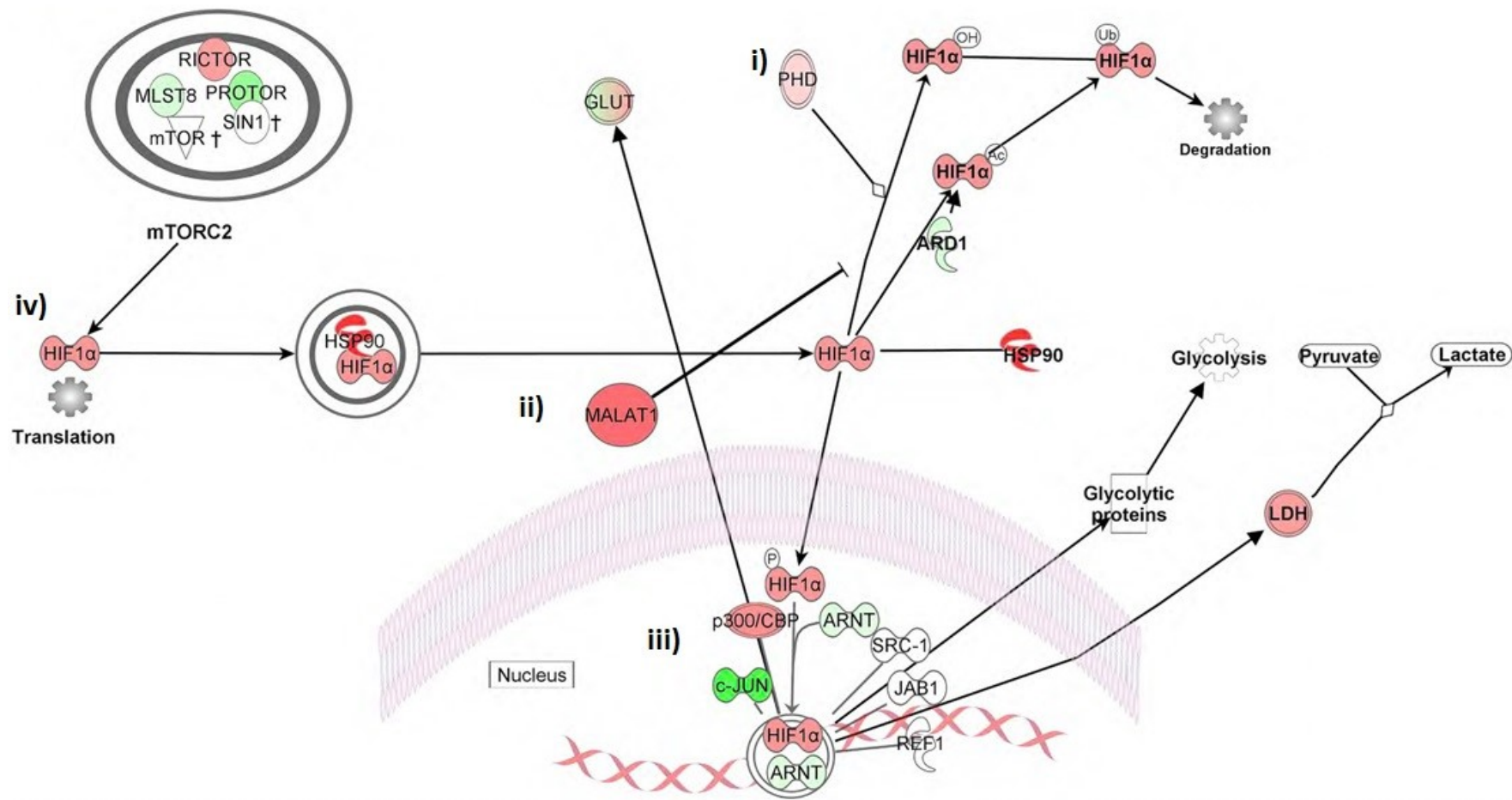
hypoxia caused by compression and vascular injury¹⁵⁹. Other preclinical evidence suggests that *HIF-1A* is responsible for the reappearance of axonal guidance receptors through the upregulation of the axonal guidance signalling¹⁵⁸. The data presented here confirm these findings, as I have identified an overexpression of *HIF1A* and genes involved in the axonal guidance pathway.

The most significantly over expressed gene in neonatal encephalopathy was Metastasis Associated Lung Adenocarcinoma Transcript 1 (*MALAT1*), a long non-coding RNA, which has been recently recognised as an oxygen-dependent regulator and potential tumour-hypoxia biomarker^{160, 161}. Emerging data in solid tumours has showed that *MALAT1* directly controls the HIF axis by decreasing HIF-1 α ubiquitination and in this way, potentiates its activity and increases anaerobic glycolysis¹⁶². In particular, in HeLa cells under hypoxic conditions, *MALAT1* interacts with the HIF-1 α axis with a positive feedback mechanism¹⁶³. More recently, Zhang J *et al.* studied the gene expression profile of long non-coding RNAs in a cellular model of ischemic stroke by using brain microvascular endothelial cells after oxygen-glucose deprivation and found that *MALAT1* protects against hypoxia-reperfusion induced apoptosis^{164, 165}. Together, this suggests that in babies with NE there is an upregulation of HIF1 α axis thorough the action of *MALAT1*, which might protect from the reperfusion induced apoptosis and brain injury.

RICTOR represented the second most significantly expressed gene. RICTOR is part of the mTOR complex, which takes part in HIF1 α regulation (Figure 19). Cell culture models showed that *RICTOR* inhibition causes downregulation of *HIF1A* and of tumour growth¹⁶⁶.

By using the Ingenuity Pathways Functional analysis, I identified an increased olfactory receptor response ($p < 0.001$) in babies with NE. This agrees with animal evidence, which recently showed that olfactory receptors play a key role in the acute phase of the hypoxia insult where they act as oxygen-deprivation sensors and lead to carotid body signalling and hyperventilation¹⁶⁷.

Figure 19. Coordination of the hypoxia response by HIF1A, MALAT1 and RICTOR. **(i)** In the presence of oxygen, prolyl hydroxylase (PHD) post-translationally modifies HIF1- α , allowing it to interact with E3 ubiquitin ligase, leading to ubiquitination and proteasomal degradation. **(ii)** Under hypoxic conditions, MALAT1 prevents the ubiquitination of HIF-1 α , leading to HIF-1 α accumulation and translocation to the nucleus, where it **(iii)** regulates transcription of hypoxia-induced genes. **(iv)** HIF- α mRNA translation is controlled by mTOR signalling pathways, which include RICTOR. The upregulated genes in our analysis are shown in red, while the downregulated genes are shown in green (From *Whole Blood Gene Expression Reveals Specific Transcriptome Changes in Neonatal Encephalopathy*. Neonatology. 2018;115(1):68-76).



Overall 137 out of the 4155 significantly differentially expressed genes in neonatal encephalopathy were shared with the genes found significantly differentially expressed in neonatal sepsis. Almost all of them had opposite fold change directions (Figure 18 B). This modest overlap is something unexpected because both neonatal encephalopathy and sepsis are usually characterised by an inflammatory response. Preclinical evidence suggests that hypoxia enhances T cell cytotoxic activity and differentiation as well as increasing neutrophil granule release¹⁵⁴. Moreover, a hypoxia-reperfusion insult leads to an upregulation of genes involved in the inflammatory response such as Nfkb1, Hmox1, Cxcl10 and downregulation of oxidative phosphorylation pathways¹⁶⁸.

My findings suggest that sepsis and neonatal encephalopathy activate inflammation via independent pathways. Animal models clearly showed that hypothermia driven neuroprotection is completely lost in case of coexistent Gram – infection, although the underlying mechanisms are still to be determined⁴⁰. This clear difference between neonatal encephalopathy and sepsis transcriptomic profiles might help to understand the reason of the loss of neuroprotection in these circumstances and lead to the development of more effective neuroprotective therapies. At the moment, there is much focus from the research community on how to promptly identify NE babies who have a coexistent perinatal infection and NE since they are less likely to respond to whole-body cooling. This clear difference in the gene expression profile between babies with NE and babies with sepsis highlights the translational potential of gene expression as diagnostic/prognostic biomarker.

In the babies of the late NE group (blood sample collection after 12 hours of age) there was a different gene expression profile when compared with the early group (blood sample collection within 12 hours of age) which is probably due to the effect of therapeutic hypothermia on the gene expression and highlights the importance of the timing in the blood sample collection in babies with NE undergoing whole body cooling. The pathway analysis of the most significant differentially expressed genes showed that the main biological pathways involved, were Agranulocyte Adhesion and Diapedesis and Role of Cytokines in Mediating Communication between Immune Cells. This is in agreement with previous studies where the temporal changes in cytokines were examined in babies undergoing therapeutic hypothermia

and demonstrated a decrease of cytokines over time with a significant change from 12 to 48 hours after birth^{169, 170}.

This study has some limitations. Firstly, encephalopathic infants at the time of blood sample collection had already been undergoing therapeutic hypothermia. Although animal and cell culture evidence has showed that the transcriptomic profile changes with hypothermia, the duration (median of three hours) and degree of hypothermia (2.5 to 3.5°C) in this report is unlikely to have played a significant role in the results of the comparison of the babies of the NE early group versus controls. Those studies suggested that a decrease of 6 to 10°C for more than 12 hours was necessary to affect the gene expression profile¹⁷¹⁻¹⁷⁵. Besides, I conducted further analysis of the differentially expressed genes identified in this study and I found that none of the hypothermia inducible genes was significantly different between neonatal encephalopathy and control babies¹⁷⁶. Therapeutic hypothermia is now considered standard treatment for neonatal encephalopathy and hence, an early disease stratification tool will have to be used in babies undergoing this. The encephalopathic babies in this study had been resuscitated at the time of birth. Therefore, their gene expression profiles may have been influenced by supplementary oxygen given at birth and by the resuscitation manoeuvres^{168, 177}.

Secondly, I used gene expression changes in blood as a reflection of brain injury. Neonatal encephalopathy represents a multisystem disease. The use of gene expression in blood as a surrogate of the injury to other organs has already been effective in different infectious and inflammatory conditions, since blood cells act as sentinels of the disease⁸¹. In fact, a comparison of the peripheral blood transcriptomic profile with genes expressed in nine different human tissue types has showed that over 80% expression was shared¹⁷⁸. The data I present here point out the translational potential for gene expression profiling in neonatal encephalopathy. Different systemic coexisting factors may affect the efficacy of therapeutic hypothermia and blood gene expression changes may reflect these.

Furthermore, changes in whole-blood gene expression after injury are not only caused by the differential regulation of genes within cells, but also the relative composition of cell types in

the blood. I have therefore made the assumption that the latter was consistent across subjects. However, in the following chapter I will explore the impact of this assumption in more detail.

Finally, my sample size is small and this study only provides preliminary data. However, the minimal overlap between gene expression profiles of bacterial infection and neonatal encephalopathy advocates powered studies to investigate the use of gene expression in disease stratification.

CONCLUSION

In summary, this is the first report to show that neonatal encephalopathy is associated with a unique gene expression signature soon after birth. These data provide a crucial insight into the neonatal response after a hypoxic-ischaemic insult. Further larger studies are required to examine the role of gene expression as a disease stratification tool in neonatal encephalopathy, and its potential to serve as surrogate biomarker. If successful, this could represent a paradigm shift toward the development of personalised neuroprotection.

Chapter 5: Examining cell-specific changes to gene expression in neonatal encephalopathy

In this chapter, I investigate whether further insight can be gained into neonatal gene expression using different deconvolution analysis methods.

INTRODUCTION

Each of the blood cell type expresses a distinct set of genes and therefore gives a different contribution to the overall RNA expression in whole blood. Therefore, the observed differences in gene expression sometimes can reflect the differences in the cell type composition instead of a change in the gene expression profile, which is the effect of interest. However, as explained in the background section of chapter 1, this problem can be addressed computationally using deconvolution methods. This method estimates the percentage of the different blood cells from the RNA expression, and in this way helps to dissect the gene expression signature into its cell-type-dependent and cell-type-independent components, allowing further investigations on them separately.

While this is a promising methodology, none of the available deconvolution algorithms have yet been tested in the neonatal population, and particularly in NE infants. This is particularly important in such patients since they have unique characteristics.

Study design

I employed different in silico deconvolution methods to acquire estimates of the cell counts in the samples based on lineage-specific transcripts present in the data set.

METHODS

I used the blood samples collected from twenty full term infants recruited as a part of the MARBLE study⁵⁷.

I compared the percentages of lymphocytes and neutrophils from the full blood counts (FBC, collected at the same time as the RNA expression samples) with estimates of the cell counts

based on lineage-specific transcripts present in different data sets, as estimated by deconvolution analysis.

I performed deconvolution analysis using and comparing the performance of the following published algorithms DeconRNASeq R package (v1.8.0), CellCODE and Cibersort⁸⁶⁻⁸⁸.

As explained in the background chapter, the deconvolution model is based on the equation:

$$T = C \cdot P$$

T is the overall gene expression data from heterogeneous samples, in my case the 20 samples

P is the mixing proportion which I want to calculate with the deconvolution methodologies

C is the cell type specific RNA signature, which was different in the different deconvolution methodologies I used.

i) For the DeconRNASeq package, I used the normalized count matrix of the previously published human immune cells RNAseq data (GSE64655)¹⁷⁹

ii) For Cibersort software, I used the default input matrix of LM22, with 547 genes distinguishing 11 leukocytes subtypes.

iii) For CellCODE, I selected 4 major immune cell populations from Immune Response in Silico data set: neutrophil, CD4+ T-cell, CD8+ T-cell, and B-cell¹⁸⁰. To find the most accurate surrogate proportion variables values for each cell-type, I used a heuristic approach with a cut-off value of 2 and a maximum of 60 marker genes, which resulted in the best discrimination between cell-type signatures as described in the CellCode package⁸⁷.

iv) I also tested 14 different previously published neutrophils' gene signatures^{89, 180-183} defined as modules⁸⁹. I calculated the module geometric mean in each of the samples sequenced and then plotted that number against the absolute number of neutrophils. There are no published modules for the B and T lymphocytes together and therefore I limited my analysis to the neutrophil count. To assess the contribution of reticulocytes to the whole gene expression profile, I regressed the whole transcriptome against the median expression of the reticulocyte module and I then repeated the computation of the correlation coefficient of the 14 neutrophil modules already tested.

Statistical analysis

I evaluated the accuracy of the deconvolution methods employed by using r and p values derived from Spearman rank test.

RESULTS

The clinical characteristics and full blood count results of NE babies whose samples were used for the analysis are shown in the

Table 10.

Median (IQR) or n (%)	NE infants (n=20)
Gestational age (weeks)	40.5 (39.3-40.7)
Birth weight (g)	3643 (3243-3751)
Caucasian ethnicity	20(100)
Spontaneous vaginal delivery	9 (45)
Cord pH	6.9 (6.8-7.1)
Apgar 1 min	2 (0.4-3)
Apgar 5 min	3.5(1.7-5)
NE grade (Mild/Moderate/ Severe)	2/14/4
Death	1
Blood stream infection	0
Invasive ventilation	19(95)
Age at sample collection (hours)	16(4-30)
Body temperature at sample collection (°C)	33.5 (33.2-35.4)
Neutrophils percentage	69 (53-76)
Lymphocytes percentage	18(17-32)
Monocytes percentage	4(3-7)
Basophils percentage	0

Table 10. Clinical and lab characteristics of the patients included into the study

When I performed statistical deconvolution using DeconRNASeq, mRNA-Seq yields did not accurately estimate blood cell fractions for monocytes ($r=-0.2$, $p=0.41$), but there was a stronger correlation for neutrophils ($r=0.5$, $p=0.02$) and lymphocytes ($r=0.47$, $p=0.03$). I observed that estimates were worse for the patients with the highest percentages of cells (Figure 20).

I assessed the accuracy of CellCODE estimation by using its pipeline (visualisation step), which represents the correlation of all the coefficients of the marker genes of interest with the

computed SPVs in a heatmap. By using the heuristic approach suggested in the manual, I identified 3 heatmaps (maximum number of marker genes 5, 11, 24), which had the best performance. However, in all the three cases the correlation with the actual cell counts was poor ($r=-0.24$) (Figure 21).

Figure 20. A). Plotting of estimated proportions (y axis) vs. actual proportions (x axis) shows weak congruity for Lymphocytes and Neutrophils. Each dot represents a sample. **B)** Scatter plot of the actual and estimated cell count percentages (blue=lymphocytes, red=monocytes, black=neutrophils)

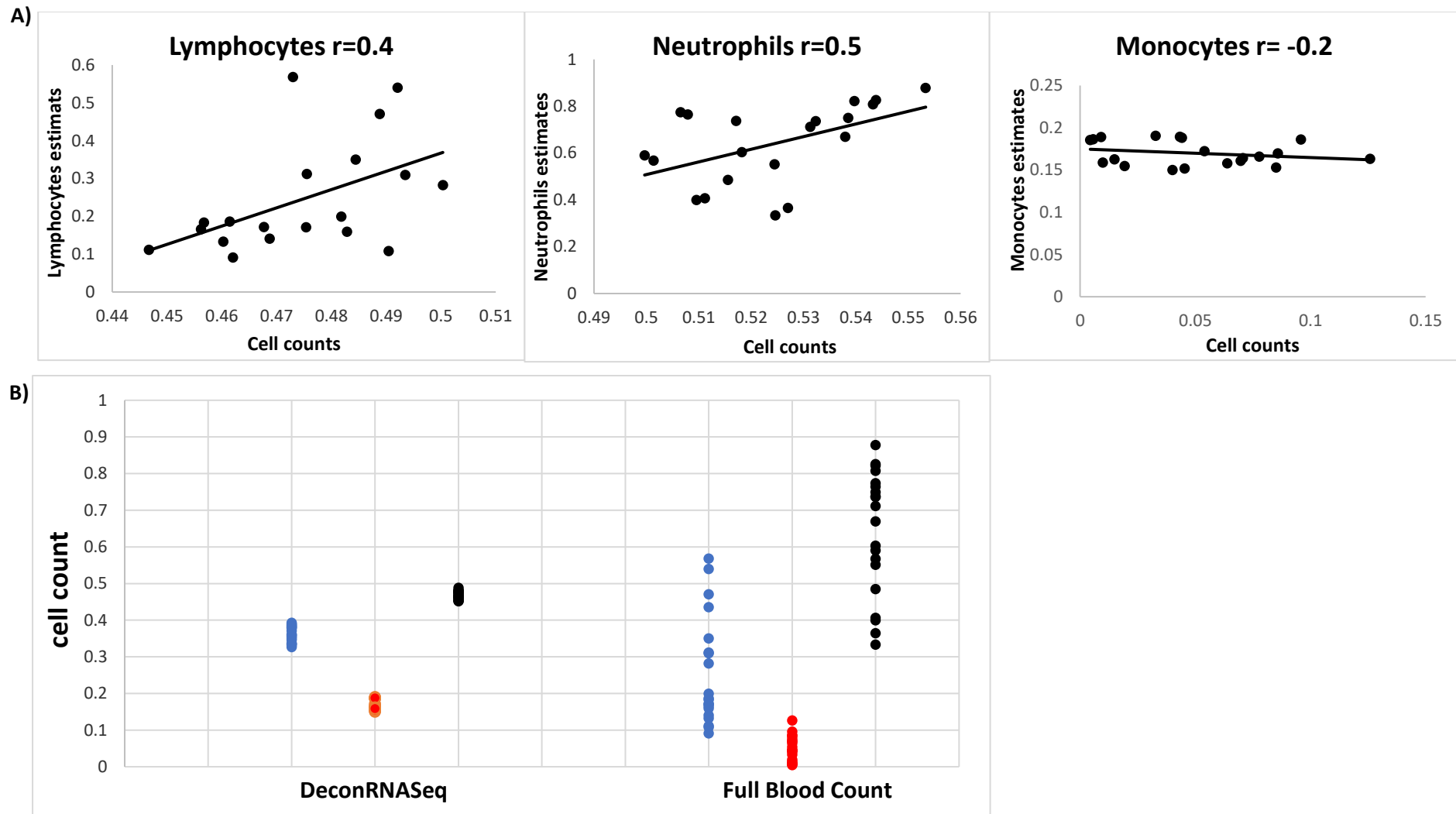
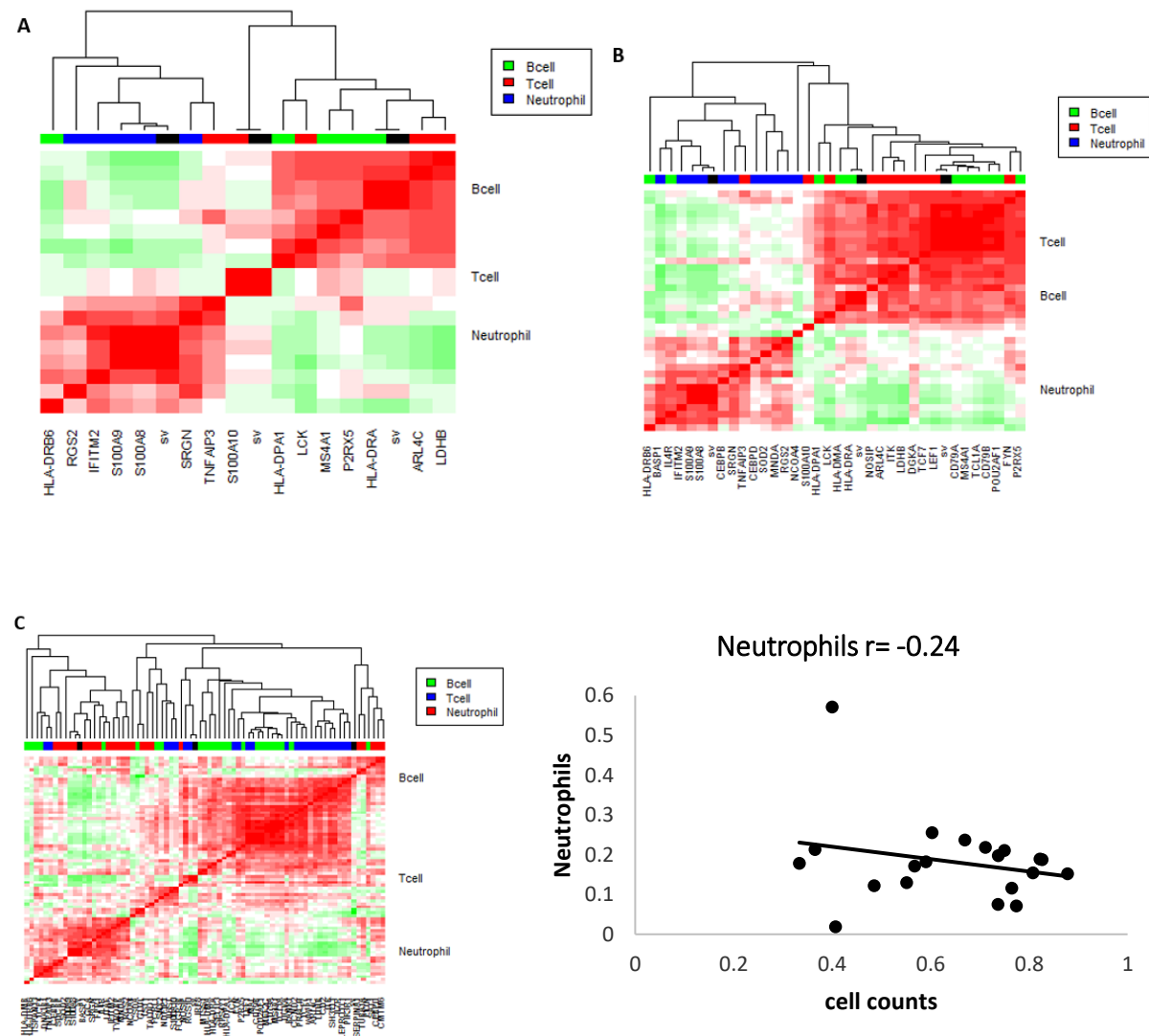


Figure 21. The 3 heatmaps (maximum number of marker genes A = 5, B= 11, C= 24) represent correlation coefficients between different marker genes (red =correlation and green = anti-correlation, while darker/lighter=greater/smaller magnitude of the correlation coefficient). Marker genes initially selected for a specific cell type are indicated by colours as shown in the key. The CellCODE surrogate proportion variables (indicated by black) are also included. On the right, plotting of estimated proportions (y axis) vs. actual proportions (x axis) shows weak congruity for Neutrophils.

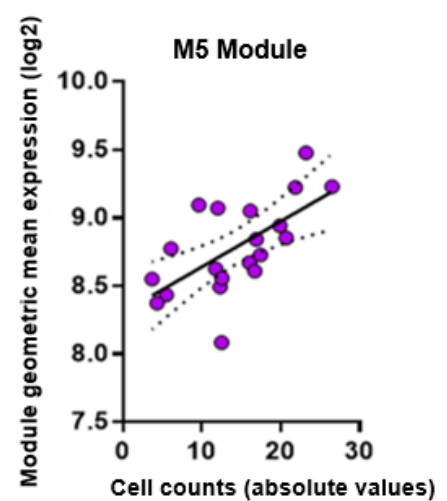
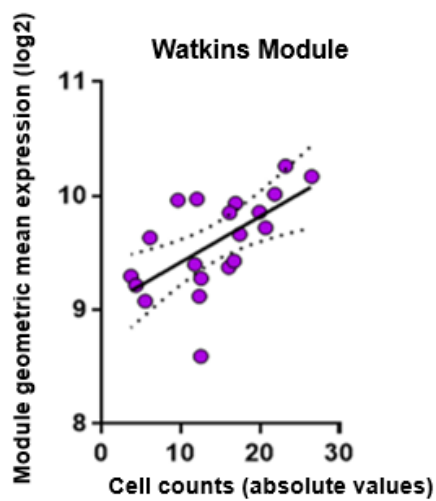


The accuracy of the different neutrophils modules is shown in Table 11. The best performance was achieved by Watkins and M5 modules with r 0.63 and $p = 0.0027$ in both cases. I observed that the validity of these modules remained for both low and high cell proportions (Figure 22).

Table 11. Immune cell modules used for the analysis with the relative r coefficient and p value

Module	r coefficient	p
Abbas Neutrophils	0.15	0.506
Pollara Neutrophils	0.59	0.005
M11	0.59	0.005
M132	0.53	0.015
M163	0.55	0.011
M37	0.56	0.009
M5	0.63	0.002
neut CL104	0.58	0.007
neut CL170	0.55	0.010
neut CL34	0.52	0.016
neut CL73	0.49	0.026
neut CL97	0.60	0.004
Neutrophil 2-fold	0.43	0.058
Watkins neutrophils	0.63	0.002

Figure 22. Relationship between the module geometric mean expression of the top two Neutrophils modules (y axis) and Neutrophils absolute cell counts from FBC (x axis). Data points represent data derived from individual modules.



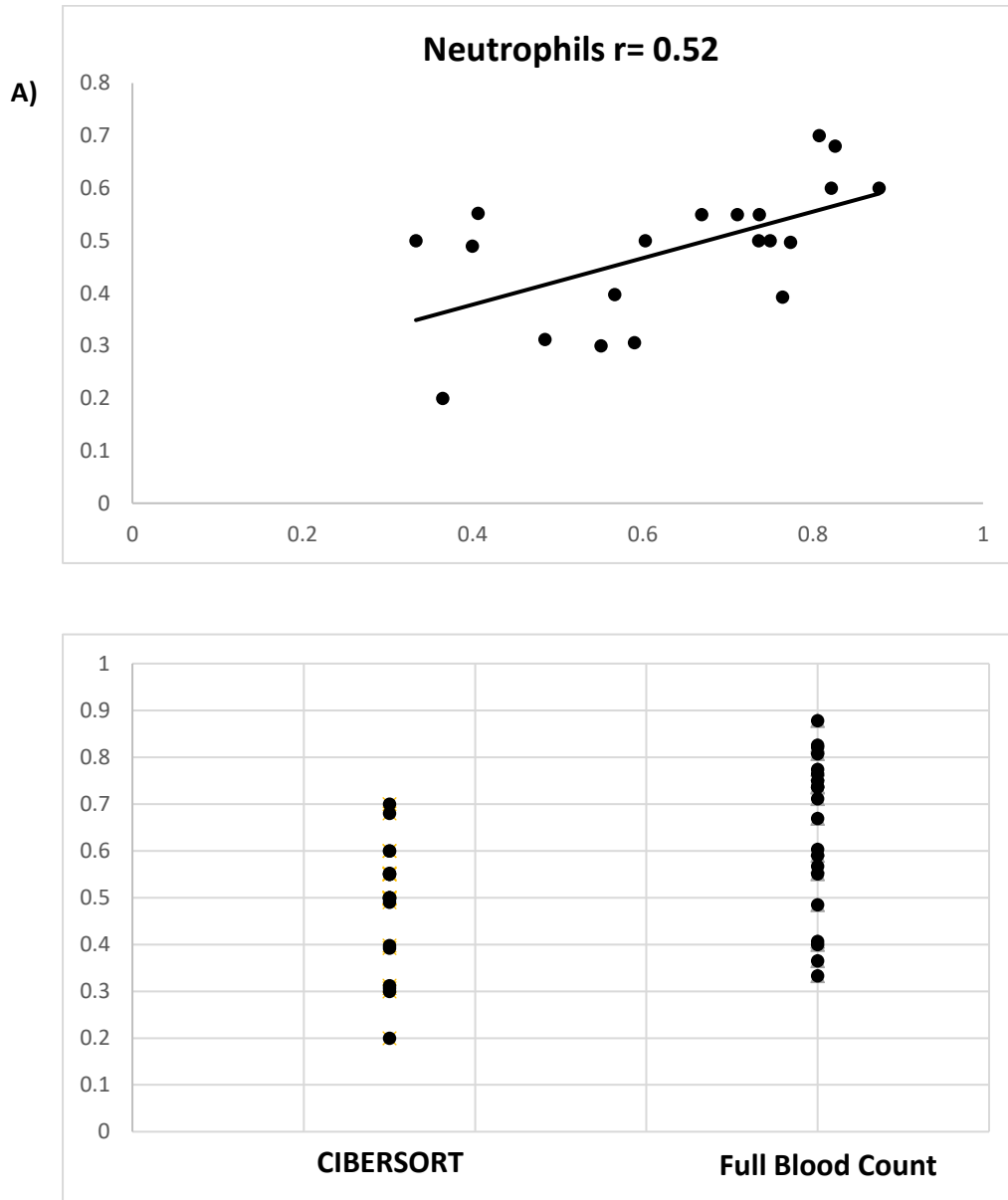
When I subsequently corrected my data for the median expression of the reticulocyte module in the different samples and I retested the accuracy of the neutrophils modules, there was no improvement. Interestingly, correction for reticulocytes shuffled Abbas, M11 modules around whereas modules with high r values remained unchanged (Table 12).

Table 12. Immune cell modules used for the analysis with the relative r coefficient before and after regression for reticulocytes

Module	r coefficient	r coefficient after regression for reticulocytes
Abbas Neutrophils	0.15	0.58
Pollara Neutrophils	0.59	0.56
M11	0.59	0.01
M132	0.53	0.59
M163	0.55	0.60
M37	0.56	0.53
M5	0.63	0.53
neut CL104	0.58	0.56
neut CL170	0.55	0.54
neut CL34	0.52	0.49
neut CL73	0.49	0.62
neut CL97	0.60	0.45
Neutrophil 2-fold	0.43	0.63
Watkins neutrophils	0.63	0.61

I also evaluated CIBERSORT performance by comparing its estimates of neutrophils against the actual neutrophils FBC proportion of the 20 patients analysed. There was a diversion from the original proportion as indicated by a regression coefficient of 0.52 (Figure 23).

Figure 23. A) Plotting of CIBERSORT cell estimated proportions (y axis) vs. actual proportions (x axis) for Neutrophils. **B)** Scatter plot of the actual and estimated cell count percentages (black=neutrophils).



DISCUSSION

Multiple computational methodologies have been used for extracting cell type-specific information from heterogeneous samples in the adult population. However, none of them had been tested in neonates before. My study shows that some of the available deconvolution algorithms perform poorly in neonates, with correlation with flow cytometry estimated cell type proportions as low as -0.24.

With the widespread use of whole blood gene expression to measure and interpret biological changes between specific conditions in neonates^{79, 94, 184}, the use of deconvolution is becoming increasingly important to gain a better insight into cell-subset specific information. The major risk is that the accuracy of deconvolution methods might be directly extrapolated from studies conducted in adults and children, and these approaches might not be tested before their use in neonates. In fact, I have demonstrated here that this is particularly important, as different signatures of the same cell type may vary significantly in their accuracy and give completely different cell type estimates. When I compared 14 different neutrophil modules, there was a correlation coefficient with flow cytometry estimated cell proportions varying between -0.24 and 0.63.

There are different points which need to be considered when deconvolution algorithms are employed. First, cytokines and innate immune system themselves trigger gene expression changes, which go beyond the cellular composition. Therefore, deconvolution must not quench the signal from the genuine perturbation. Secondly, cell-type RNA signatures are often derived from cell cultures where the cell cycle can be synchronised and chemically arrested whereas in the clinical field each sample has a mixture of cells in different cell cycle phases. This is particularly important in whole blood gene expression since blood represents a dynamic sensing system. Third, it is arbitrary to decide which cells to deconvolve against. I performed deconvolution for neutrophils and lymphocytes based on the assumption that these cell types were the most abundant. However, it is now debated whether to include less abundant cells or those that have not been completely analysed or sequenced yet.

This study highlights that the deconvolution methods DeconRNASeq and CellCODE may perform poorly in the case of high cell type proportions whereas other approaches may give

better results, such as the use of module geometric means and of CIBERSORT and hence, I decided to use the latter for deconvolution in the next chapter. Pollara *et al.* previously conducted a large meta-analysis showing significant heterogeneity in module gene content for specific cell types⁸⁹. Moreover, they found that unsupervised deconvolution analysis generated more heterogeneous modules with worse specificity for their annotated cell type whereas a supervised approach by incorporating significantly differently expressed genes and limiting a module's constituent genes to those identified in multiple datasets improved their accuracy⁸⁹.

In the present study, I used a guided approach by using matrices consisting of cell type-specific average expression values, even though none of the matrixes used had been specifically developed for neonates. A completely supervised approach with either neonatal cell-type-specific signatures or lymphocyte sub-populations would have been clearly preferable. However, now that gene expression is becoming increasingly popular, an unsupervised or partially guided approach is more likely to represent the commonest scenario in bioinformatics.

My study has strengths and limitations. First, I used different approaches for deconvolution including support vector regression, latent variable analysis and quadratic programming. Secondly, I used a validated gold standard, which is blood cell proportions estimated by flow cytometry. There is emerging evidence showing that after a hypoxic insult, there is an increase of the reticulocyte numbers¹⁸⁵. Therefore, I tried to improve the accuracy of deconvolution methods by also including reticulocytes. However, I did not observe any clear benefit after including reticulocytes in the deconvolution model. This might reflect low sample numbers, low noise from red cells in RNAseq data or the lack of specificity of the reticulocyte module used.

The main limitation of the present study is that the matrices used to deconvolve my data were not developed for neonates. Moreover, I could only assess the accuracy of deconvolution methods for neutrophils, lymphocytes and monocytes blood cells since full blood count did not include lymphocytes subpopulations. In addition to this, I had information about the blood cell type proportions, which do not necessarily correspond to the amount of messenger RNA present in the samples.

CONCLUSION

In summary, this is the first report on the use of deconvolution in neonates. These findings show that some of the deconvolution methods available have a suboptimal accuracy in neonatal encephalopathy. New training data are needed in neonates to develop specific deconvolution methodologies in this population. It is important that any deconvolution methods should be tested for accuracy before their use in neonates.

Chapter 6: Host gene expression profile for disease stratification in neonatal encephalopathy

INTRODUCTION

While I have showed that neonates with NE have a unique gene expression profile⁹⁴, there are no data about the use of transcriptomics as prognostication tool in NE.

Gene expression has the potential to overcome the major roadblocks in clinical translation of putative neuroprotectants and result into a paradigm shift in neonatal neuroprotection. A minimal host transcript signature for bacterial infections is currently being developed as a rapid diagnostic test based on polymerase chain reaction. Moreover, low-cost RNA sequencers are now booming, hence a point of care RNA extraction device is expected to be developed within the next five years⁸¹. This will have a major impact not only in high income countries, but also in low and middle-income countries where the burden of neonatal encephalopathy is particularly high.

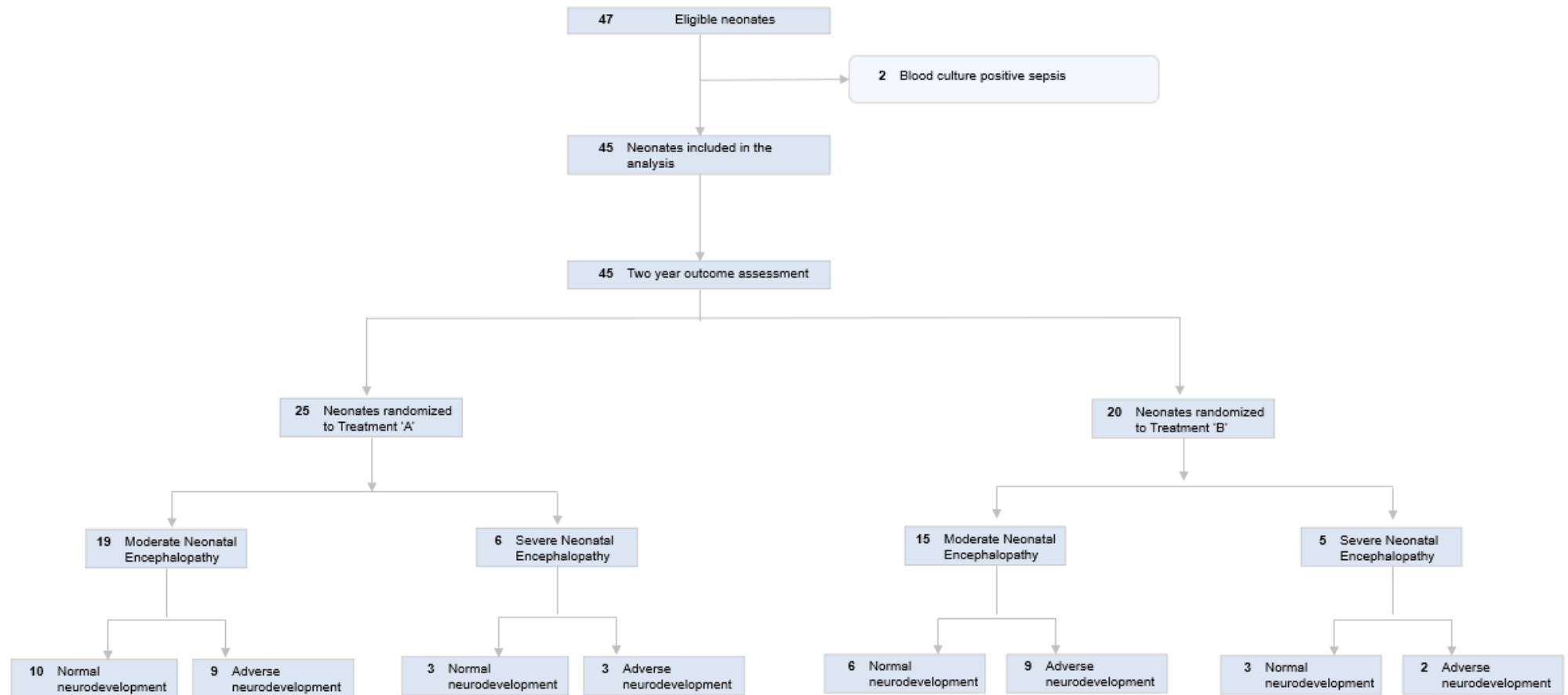
As explained in the previous chapter, after optimising the use of deconvolution in NE, gene expression profiling can unveil new biological pathways and hence, could guide novel neuroprotective strategies.

In this study I examined if babies who later develop adverse neurodevelopmental outcome after NE have a unique host gene expression profile at birth.

METHODS

Forty-seven infants with NE were recruited as a part of the HELIX (Hypothermia for Encephalopathy in Low and Middle-Income Countries) trial²⁹. I excluded babies with culture positive sepsis from the analysis. Flow-chart of the study is in Figure 24.

Figure 24. Flow-chart of the study.



As a part of the HELIX trial, all babies had a structured neurological examination based on the NICHD system by a certified examiner and an infection screen. 0.5ml of blood for gene expression was collected from all the recruited infants and mixed with 1.4 mL RNA stabilising solution (PreAnalytiX BD/QIAgen) at the time of admission and before the start of whole body cooling in case they had been randomised to therapeutic hypothermia.

At two years of age, neurodevelopmental evaluation was performed by local examiners using Bayley-III. I defined moderate/severe disability according to the NICHD Neonatal Research Network¹³⁷. I considered an adverse outcome as death or moderate/severe disability.

RNA extraction, alignment and next generation sequencing

Samples were frozen at -80 °C and then transported to MedGenome lab in Chennai, India in 2 batches. RNA was isolated from each sample using a MagMAX RNA isolation kit (Ambion/Applied Biosystems, 51 stems) according to the manufacturer's instructions. RNA integrity and concentration was assessed by using Agilent RNA 6000 nano Kit with 2100 Bioanalyzer (Agilent, Palo Alto, CA) and Qubit RNA HS assay Kit with Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Carlsbad, CA).

RNA purification was performed by using Ribo-Zero™ Magnetic Kit (Epicentre Biotechnologies, Madison, WI) following the manufacturer's procedure. Ribosomal RNA and globin mRNA was depleted from 4 micrograms of total RNA and both cytoplasmic and mitochondrial rRNA was removed. Following purification, the mRNA was fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments were copied into first strand cDNA using reverse transcriptase and random primers. Second strand cDNA synthesis was subsequently done by using DNA Polymerase I and RNase H. The products were then purified and enriched with PCR to create the final cDNA library for sequencing. Prepared libraries were sequenced on Illumina HiSeq2500 to generate 30M, 2x100 bp reads/sample.

Based on quality report of fastq files the reads containing high quality sequence ($Q \geq 30$) were retained for further analysis and the low-quality sequence reads were excluded from the analysis. The adapter trimming was performed using fastq-mcf program (version - 1.04.676) and cutadapt version 1.8dev. Contamination removal was performed using Bowtie2 (version

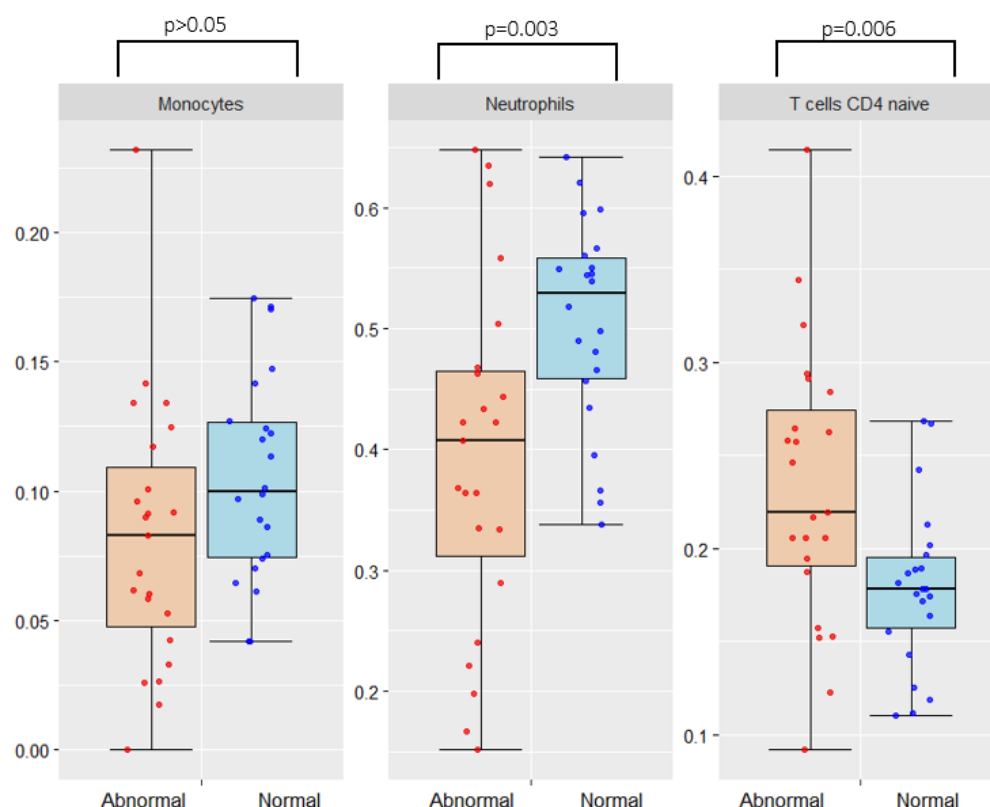
– 2.2.4) and the paired end reads were aligned to the reference human genome (GRCh37/hg19) by using “STAR-v2.4.1d”. Coverage analysis for genes was performed with “HTSeq-v0.6.1” (reference gtf: GRCh37 version 75) and “bedtools-v2.17.0”. The aligned reads were used for estimating expression of the genes and transcripts by using cufflinks program.

Data analysis

To assess the significant sources of variability and effect of batch in my dataset, I first performed PCA and assessed the distribution of the samples according to the number of batches (1st and 2nd batch) and outcome (good and adverse outcome). Second, I evaluated the median gene count for each patient and the median count of the Housekeeping genes across all samples.

I decided to study the difference in gene expression profile beyond what is due to inter-individual variation of different blood cell types' proportions. Therefore, I determined the contribution of each blood cell type to the RNA from whole blood by using CIBERSORT tool¹⁸⁸ and I then adjusted for the effect of cell-type proportion when analysing the association between gene expression and outcome. In the previous chapter I showed that CIBERSORT not only had the same or higher accuracy when compared with other deconvolution tools (e.g. DeconRNASeq and CellCODE), but it also gives the different lymphocyte subsets estimates, which is particularly important when analysing the biological underlying mechanisms of a disease. I adjusted the analysis for Neutrophils and Lymphocytes CD4+, which are the most abundant leucocytes subsets in neonates¹⁸⁶ and were significantly different between NE babies with adverse and good outcome (Figure 25).

Figure 25. Cell proportions of Monocytes, Neutrophils and Lymphocytes CD4+ estimated by using the CIBERSORT tool.



I fitted quasi-likelihood negative binomial generalized log-linear models to identify significantly differentially expressed genes in the comparison of babies with adverse and good outcomes. The analysis was performed first unadjusted and then adjusted for the randomisation allocation (masked as 'A' and 'B'), Neutrophils and CD4+ cell counts estimated by using CIBERSORT (deconvolution). The differentially expressed genes were then subjected to pathway analysis (Ingenuity Pathway Analysis). I also assessed whether there was any difference in the gene expression profile in the comparison of the infants with moderate and severe encephalopathy in the group of babies with adverse and good outcome.

I corrected all the p values for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) method to control for type I error. I performed statistical analysis using R (version 3.3.1) and DESeq2 (v1.17.18) R-packages¹⁵⁶.

I also tested the NE signature previously found, by using a novel computational method (disease risk score)⁹¹. I identified the NE signature after comparing infants with NE to healthy

controls and to babies with positive bacterial sepsis as described in the previous chapter⁹⁴. For each patient, I calculated the disease risk score by adding the total intensity at up-regulated transcripts and subtracting the total intensity at all down-regulated transcripts. This method translates the RNA signature into a single value that can be assigned to each individual, and in this way, can be the basis of a simple diagnostic test. I then compared the disease risk score in the babies with adverse to those with good outcome and in the babies with moderate and severe encephalopathy⁹¹.

Statistical analysis

I described clinical data as number (%) or median (interquartile range). I compared continuous variables by using Mann-Whitney U-test and prevalences by using Pearson chi-squared test or Fisher exact test.

I performed the statistical analysis with SPSS Statistics 24 (IBM Corp.—U.S.A.) and a p value of <0.05 was considered statistically significant.

RESULTS

Overall 47 infants with moderate or severe NE were recruited. Two infants with positive blood culture sepsis from *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were excluded and the remaining 45 patients were used for the analysis. Twenty-three infants had adverse and 22 good outcome at 2 years of age.

When I assessed whether any batch effects were present in my data, I found no significant difference in the median gene count (Figure 26) and in the expression level of the housekeeping genes, glucose phosphate isomerase (*GPI*) and proteasome subunit beta 2 (*PSMB2*) across all the different patients (Figure 27).

Figure 26. The median gene count across all samples (blue=NE babies with adverse outcome 1st batch; yellow= NE babies with adverse outcome 2nd batch; red= NE babies with good outcome 1st batch; grey= NE babies with good outcome 2nd batch).

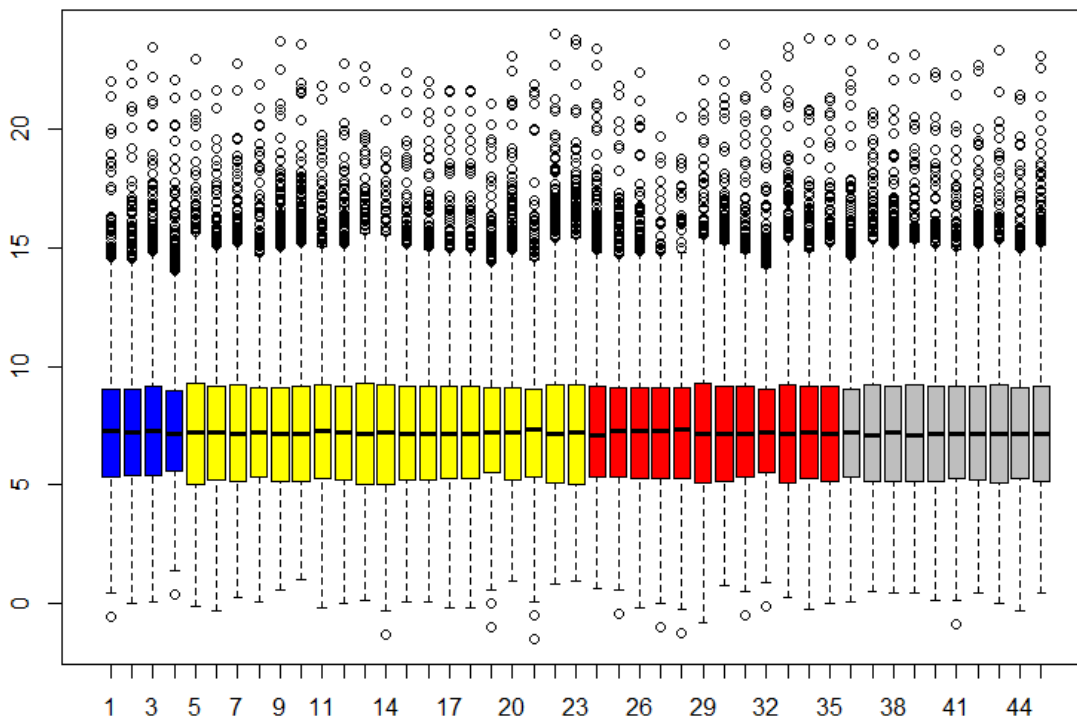
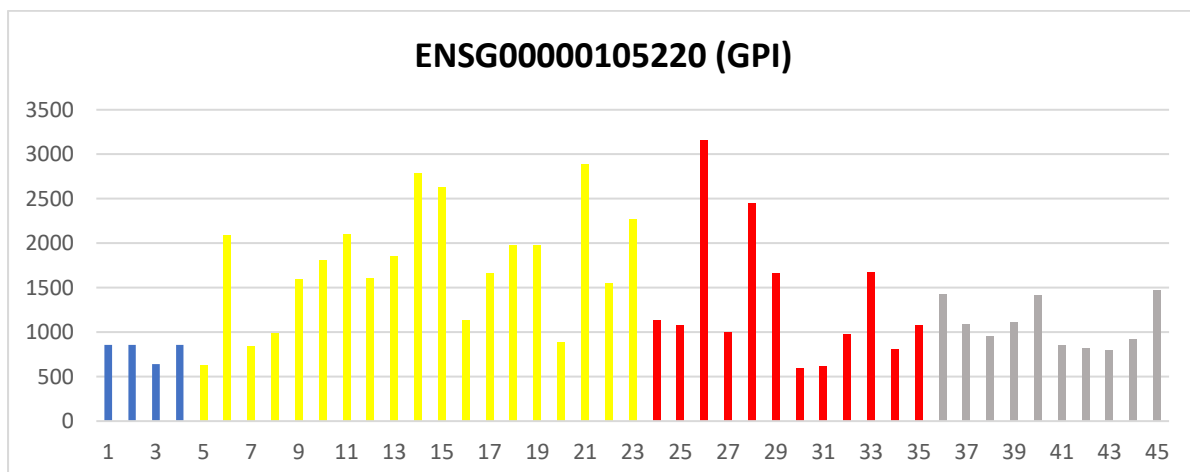
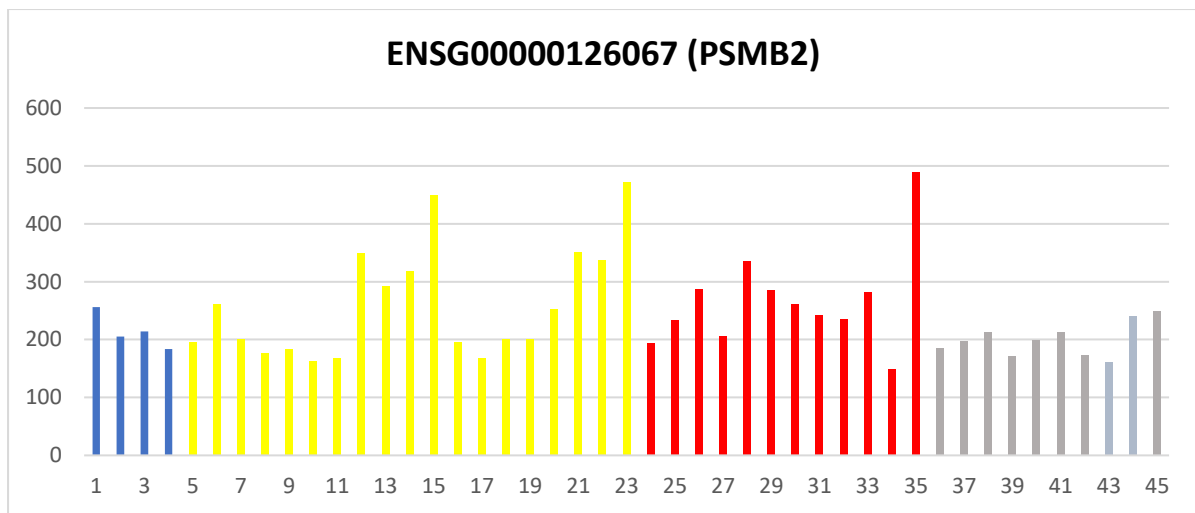


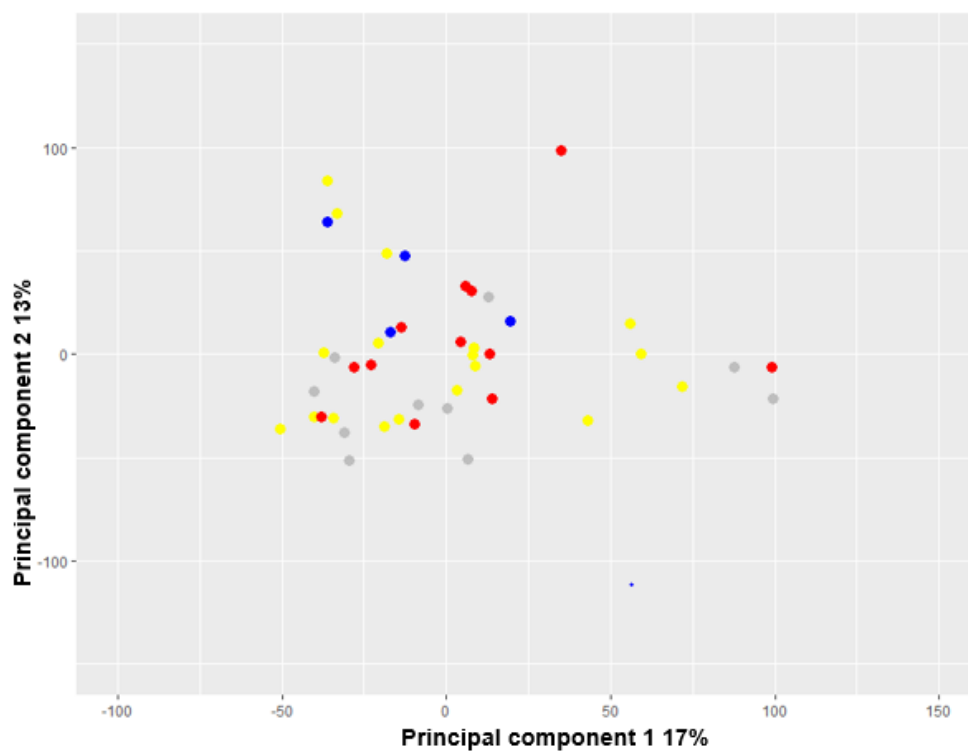
Figure 27. Expression level of the housekeeping genes *GPI* and *PSMB2* across all the different patients (blue=NE babies with adverse outcome 1st batch; yellow= NE babies with adverse outcome 2nd batch; red= NE babies with good outcome 1st batch; grey= NE babies with good outcome 2nd batch).





When I performed PCA, I did not observe any clusters according to whether the patients were sequenced in the first or second batch (Figure 28)

Figure 28. 2D principal components analysis mean centering and scaling based on the complete genome. Patients are color coded according to whether they were part of the first or second batch and according to their outcome (blue=NE babies with adverse outcome 1st batch; yellow= NE babies with adverse outcome 2nd batch; red= NE babies with good outcome 1st batch; grey= NE babies with good outcome 2nd batch).



No significant demographic differences were found between NE infants with adverse and good outcome (Table 13).

Table 13. Baseline clinical characteristics.

Median(IQR) or N (%)	Adverse outcome (n=23)	Good outcome (n=22)	P value
Birth weight, g	2757 (2550-3050)	2900 (2700-3250)	0.30
Gestation, weeks	38.5 (37-40)	40 (39-40)	0.30
Gender (Males)	15 (65)	14 (63)	0.99
Apgar 1 minute	2 (1-3)	3(2-4)	0.06
Apgar 5 minute	5 (4-5)	5(5-6)	0.25
Death	17 (73)	--	--
Resuscitation at birth, no. (%)			
Bag and mask	23 (100)	22 (95)	0.30
Intubation	16 (69)	7 (31)	0.004*
Intubation and external cardiac massage	2 (9)	0	0.16
Use of drugs in the delivery room	3 (13)	1 (4)	0.32
Persistent pulmonary Hypertension	6 (26)	3 (14)	0.04*
Hypotension requiring inotropes	11 (48)	4 (18)	0.03*
Abnormal clotting	3 (13)	1 (4)	0.42
Seizures	17 (73)	20 (90)	0.09

*p<0.05 , Pearson chi-squared test

The 2 groups were balanced in terms of randomisation allocation and severity stage of NE as shown in the confusion matrix (Table 14). There was no relationship between stage of neonatal encephalopathy at admission and outcome at 2 years (Figure 29)

Table 14. Confusion matrix of the good and adverse outcome groups showing the prevalence of infants randomised to treatment 'A' and 'B' and the NE stage severity.

Adverse Outcome n (%)	Total 23
Treatment 'A'	12 (52)
Treatment 'B'	11 (48)

Good Outcome n (%)	Total 22
Treatment 'A'	13 (59)
Treatment 'B'	10 (46)

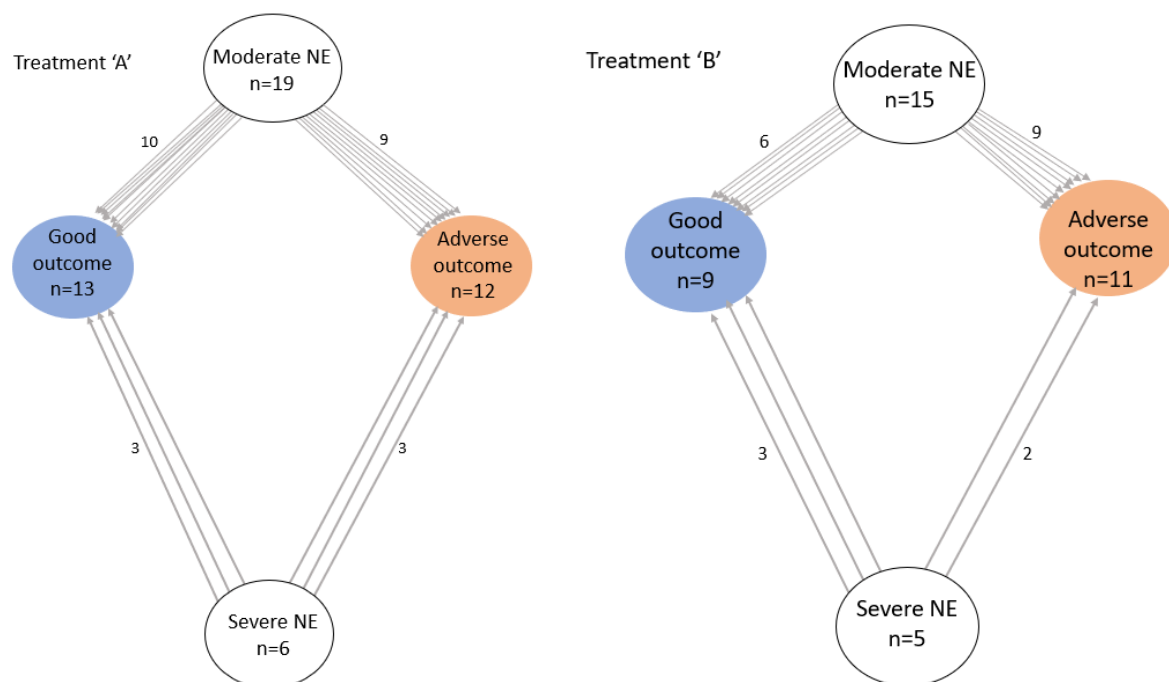
Adverse Outcome n (%)	Total 23
Moderate	18 (78)
Severe	5 (22)

Good Outcome n (%)	Total 22
Moderate	16 (73)
Severe	6 (27)

Moderate n (%)	Total 34
Treatment 'A'	19 (55)
Treatment 'B'	15 (44)

Severe n (%)	Total 11
Treatment 'A'	5 (45)
Treatment 'B'	6 (54)

Figure 29. Network Visualization of Relationships between outcome at 2 years and stage of neonatal encephalopathy according to the randomization allocation. Each arrow represents a patient.

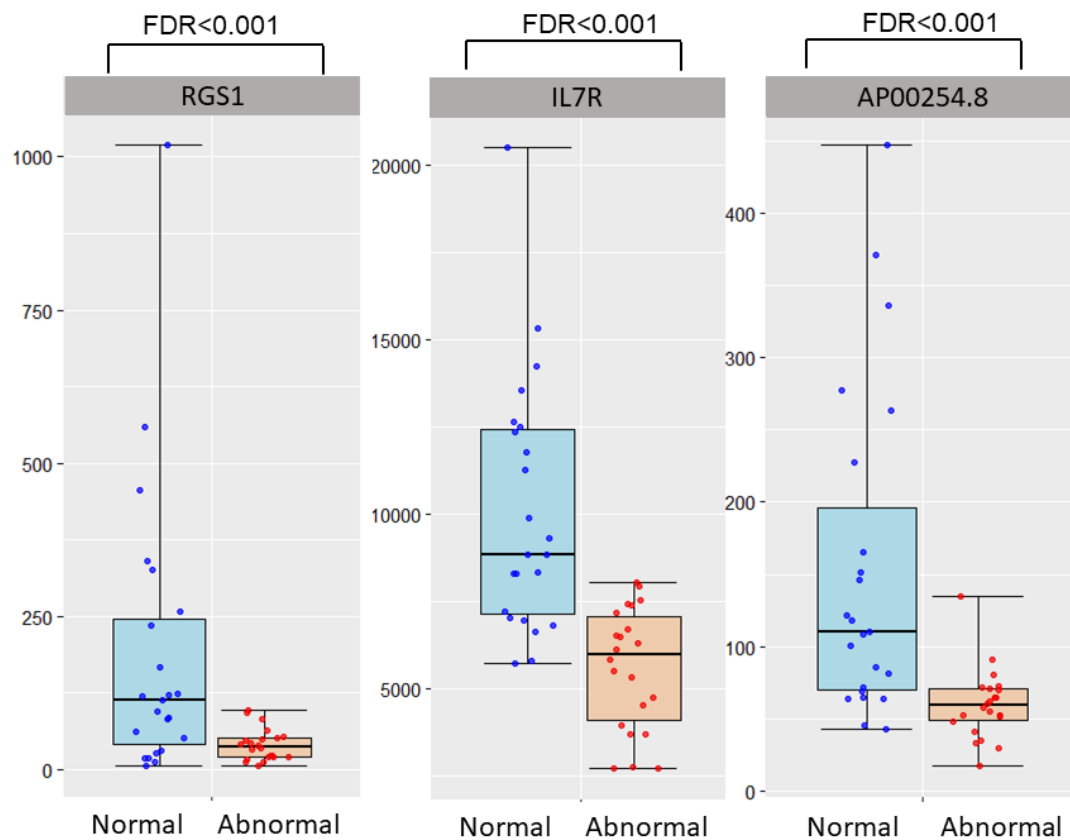


After I completed the data quality check, I explored the gene expression of whole genome by using PCA and found the presence of separate clusters in babies with adverse outcome (red) and those with good outcome (green). The separation of the 2 clusters improved after deconvolution adjustment (Figure 31 A-B).

I found 680 significant differentially expressed genes with an FDR value <0.05 in the comparison of NE babies with adverse against those with good outcome. Of these, 157 (23%) were overexpressed and 523 (77%) under-expressed in NE infants with adverse outcome as shown in the volcano plot (Figure 32). The most significant genes ordered by FDR were the regulator of G-protein signalling 1 (*RGS1*) (FDR value <0.001 , log2 fold change -2.23) followed by interleukin 7 receptor (*IL7R*) (FDR value <0.001 , log2 fold change -0.85) and *APOO254.8* (Figure 30).

I also examined if any of the specific neuronal injury markers was differentially expressed and I examined MDA receptor, NF-H, glial fibrillary acidic protein, ubiquitin C-terminal hydrolase–L1, NSE, MBP, tau and s100 β . I found that s100 β and NF-H were significantly higher ($p < 0.001$) in babies with adverse outcome. However, after correction for multiple comparison, none of these biomarkers was still significant (FDR value >0.05).

Figure 30. Box plots (median, IQR) of gene count values of the three most significant genes for children with normal compared with abnormal neurodevelopmental outcome at 2 years.



I performed the pathway analysis of the differentially expressed genes with an absolute $\log_2$ fold change >1 and I found that the most significant pathway was Protein Ubiquitination pathway ($p < 0.0001$).

When I adjusted the regression model for Neutrophils and CD4+ percentages, I identified 320 genes with a $FDR < 0.05$ and which discriminated between the NE babies with adverse from the ones with good outcome. After deconvolution the most significant genes remained *RGS1* ($FDR = 0.0006$) and *IL7R* ($FDR = 0.0002$) although their p values decreased (Figure 33). I used the 320 differentially expressed genes for the pathway analysis and I found that the main pathways in babies with adverse outcome were related to Melatonin ($p = 0.0024$) and T cell receptor signalling ($p = 0.003$) (Figure 34).

When I compared the NE infants separated by severity of their clinical presentation in the adverse outcome group, I found 2 differentially expressed genes, *MT-RNR2* ($FDR = 0.001$) and

MT-RNR1 (FDR=0.01) whereas in the good outcome group I found 1 differentially expressed gene *RNA5SP202* (FDR <0.0001).

Figure 31. 2D principal components analysis mean centering and scaling based on the complete genome. Patients are plotted according to their respective position along the principal component 1 and 2. Principal components analysis shows the 23 NE babies with adverse outcome within red balloons and 22 NE babies with good outcome within green balloons **A)** before Deconvolution and **B)** after Deconvolution.

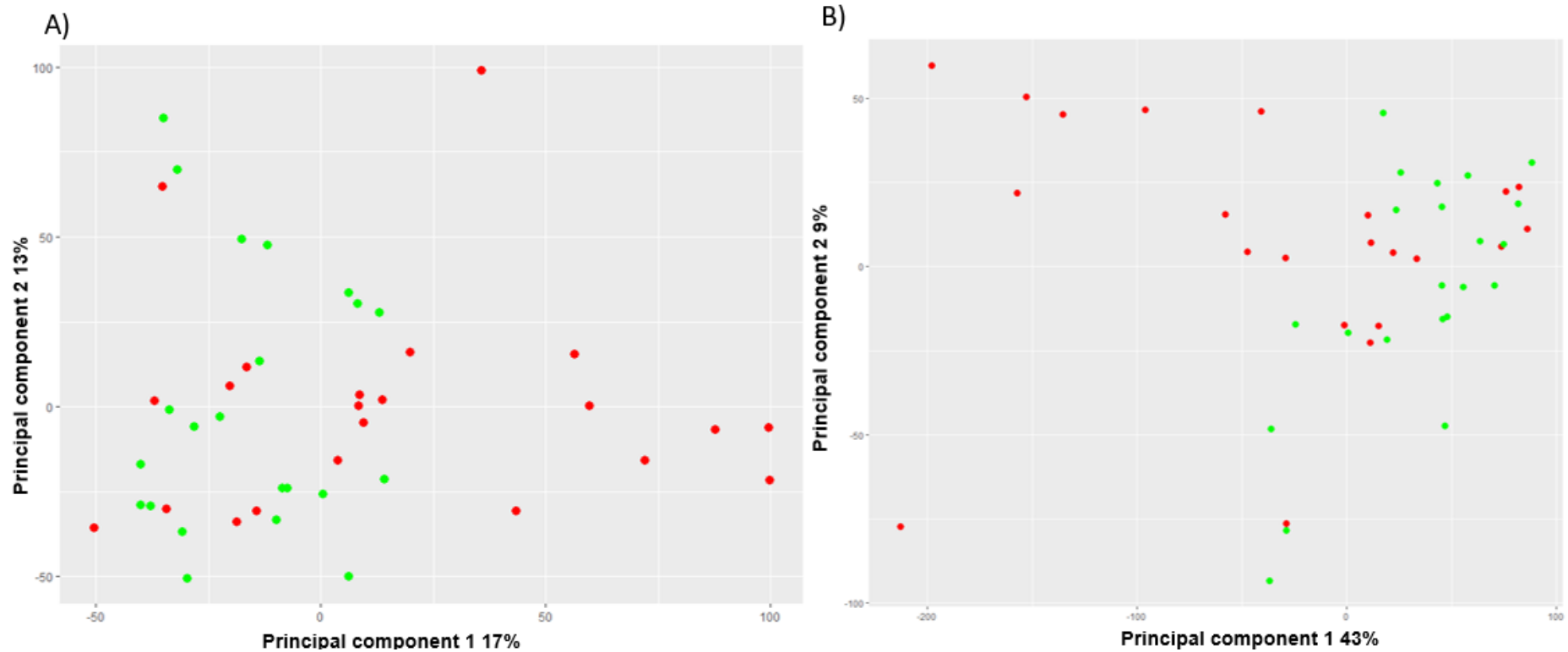


Figure 32. Volcano plot shows the 680 significant genes identified in the comparison of babies with adverse against those with good outcome, plotted according to their respective position along the x axis ($\log_2$ fold-change) and on the y axis ($\log_{10}$ p value). In green all the genes with $FDR < 0.01$ and in red, the gene with $FDR < 0.01$ and fold change > 1 . Fold change was calculated based on the comparison of abnormal versus normal outcome groups.

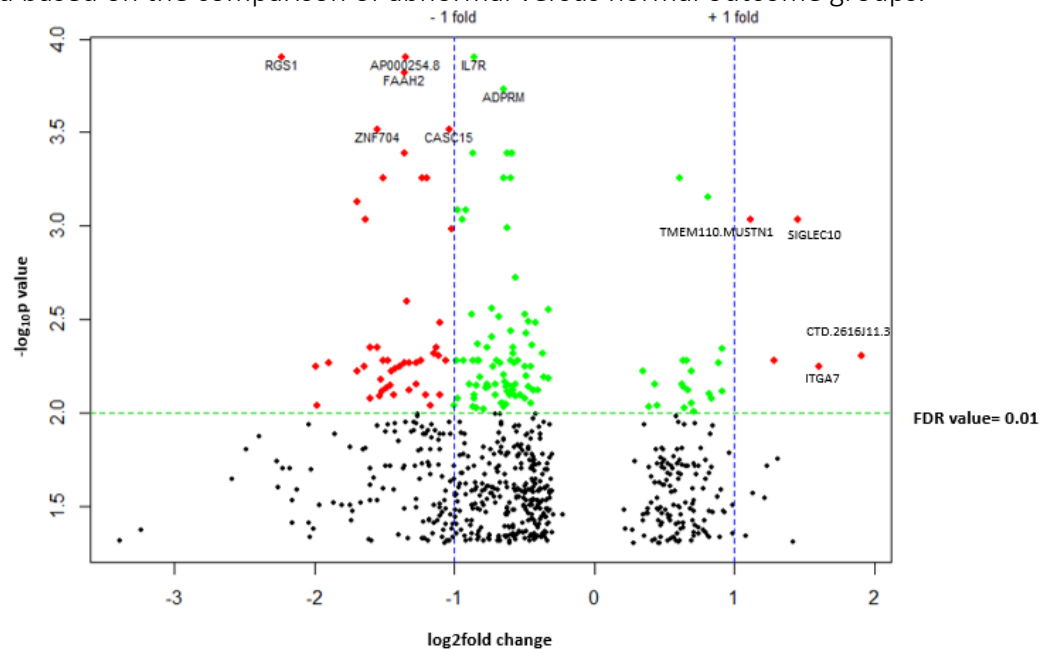


Figure 33. Volcano plot shows the significant genes identified without deconvolution (black dots) and the same ones after deconvolution (green dots) connected. They are plotted according to their respective position along the x axis ($\log_2$ fold-change) and on the y axis ($\log_{10}$ p value). In red the genes identified only after deconvolution. Fold change was calculated based on the comparison of abnormal versus normal outcome groups.

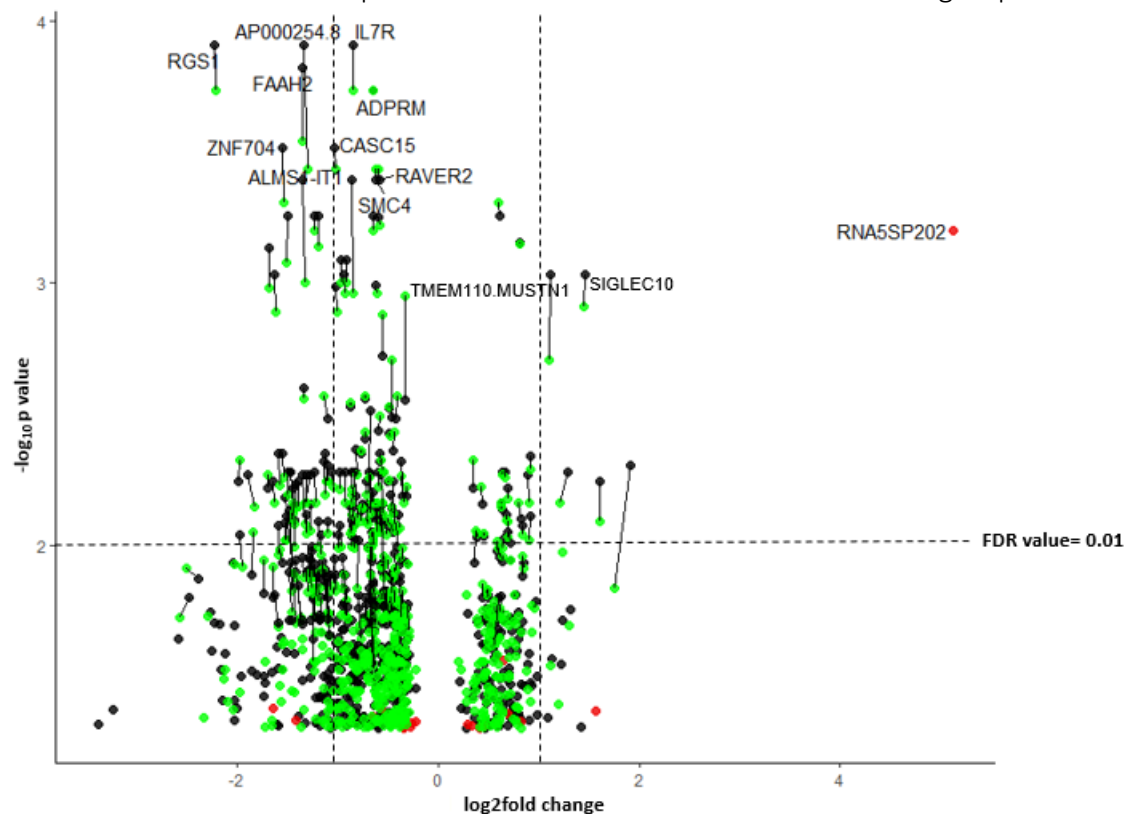
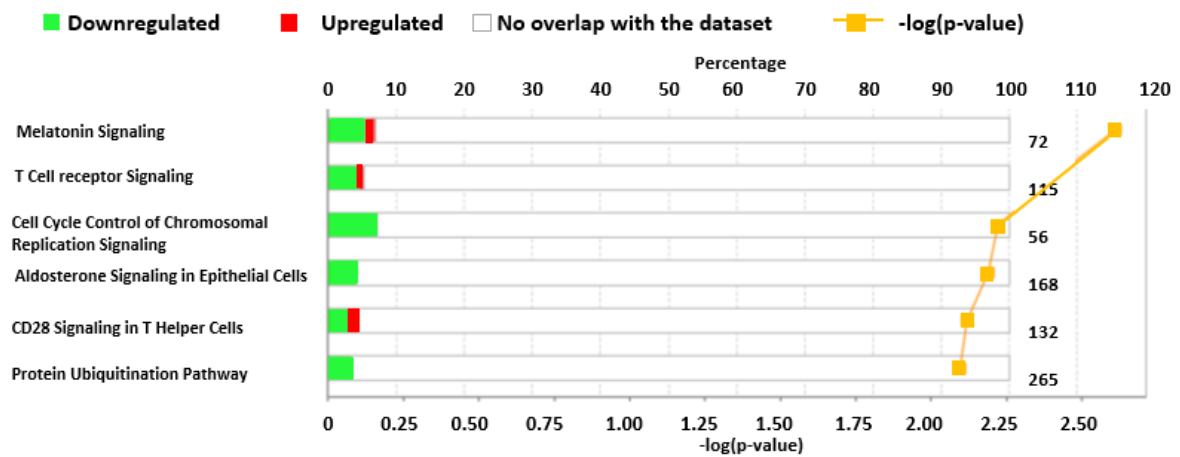
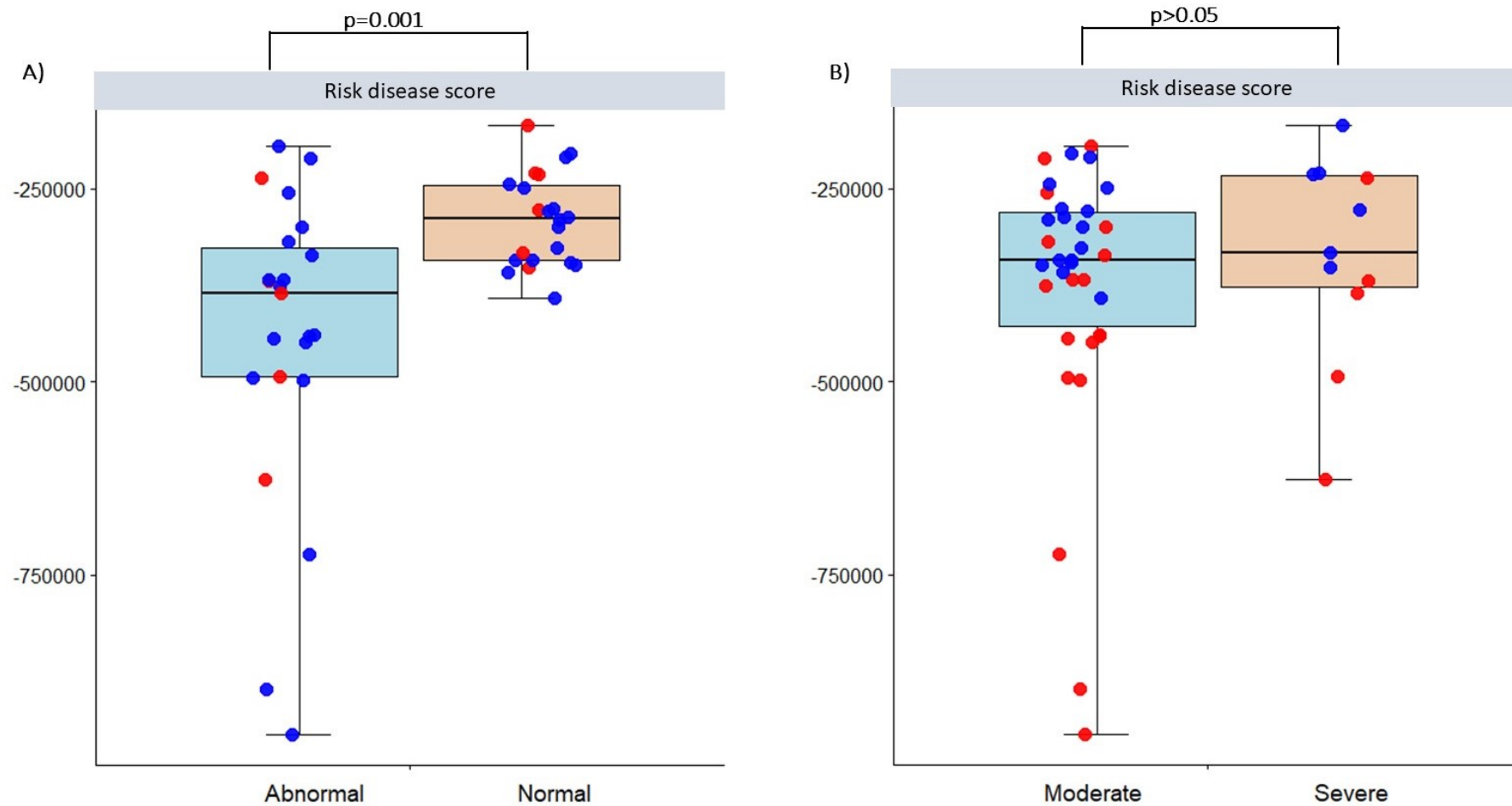


Figure 34. Canonical pathway analysis in terms of $-\log(p\text{-value})$ derived from the 320 differentially expressed genes in the comparison of babies with adverse outcome after neonatal encephalopathy and those with good outcome. On the top of each bar there is the total number of genes, which each pathway is made of. The upregulated genes are shown in red and the downregulated genes are shown in green.



There was no statistically significant difference in the risk disease score between babies with moderate and severe encephalopathy ($p > 0.05$) whereas babies with adverse outcome had a statistically significantly lower risk disease score when compared with those who had a good outcome ($p=0.001$) (Figure 35).

Figure 35. Box plots (median, IQR) for disease risk score values in the babies with abnormal and normal outcome at 2 years (A) and in the babies with moderate and severe neonatal encephalopathy (B). Blue dots indicate babies with moderate neonatal encephalopathy, red dots indicate babies with severe neonatal encephalopathy (A). Blue dots indicate babies with normal outcome and red dots indicate babies with abnormal outcome (B).



DISCUSSION

This is the first study to show that infants with adverse outcome after NE have a unique gene expression profile when compared to those who have good outcome. I found that the most significant pathway in these neonates was protein Ubiquitination signalling whereas after applying deconvolution, the main biological pathways were Melatonin and T cell receptor signalling.

These data echo the findings of my previous study where I demonstrated that babies with NE have a different gene expression profile when compared with healthy control babies, and babies with perinatal sepsis⁹⁴. Overall these results suggest that gene expression in babies with NE changes few hours after birth and therefore has the potential to become a promising diagnostic and prognostication tool.

It is now increasingly recognised that neuroinflammation and neurogenesis have a leading role in the neonatal brain damage following NE^{187, 188}. The inflammatory cascade is induced by the activation of peripheral and central immune system within minutes after the injury and can persist even for months after the insult.

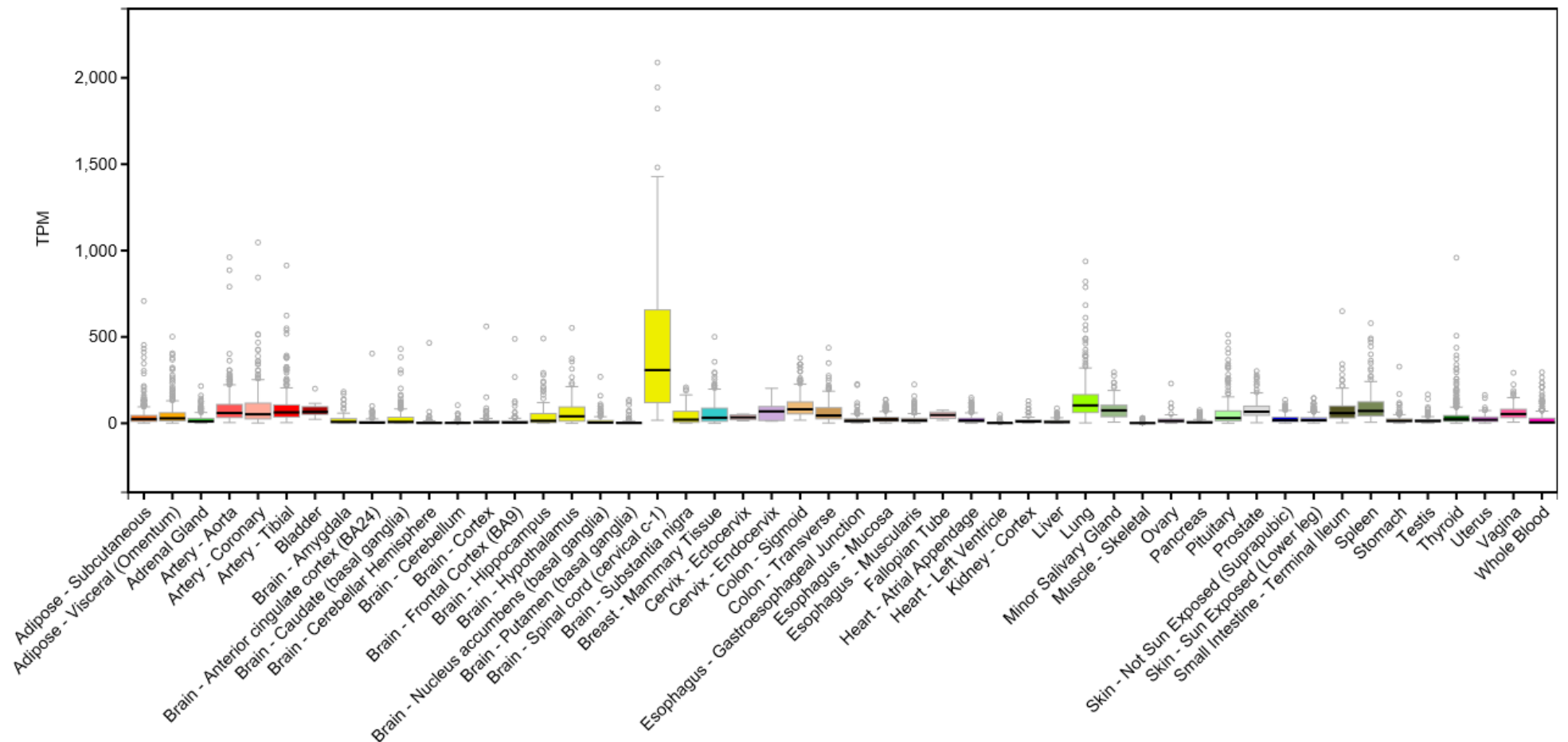
This study shows a marked differential expression in key genes of the neuroinflammation and immunomodulatory response such as *RGS1* and *IL7R*.

RGS proteins regulate chemokine-induced activation by enhancing the GTPase activity associated with the G-protein alpha subunit. *RGS1* plays a critical role in inflammatory microglial activation via negative regulation of NF- κ B signalling and strongly correlates with T cell pathogenicity. Hoppmann *et al.* recently studied the T cell transcriptomic changes in the brain tissue of a murine model for multiple sclerosis and found a differential expression of *RGS1*¹⁸⁹. Cell culture evidence suggests that *RGS1*-null microglia produced significantly higher levels of proinflammatory cytokines than wild-type upon LPS treatment¹⁹⁰. Interestingly, *RGS1* was also found upregulated in the blood of patients with multiple sclerosis after beta interferon therapy suggesting a pivotal role in regulating neuroinflammation and lymphocyte migration to the brain¹⁹¹.

Given that NE is a multiorgan disease, it is likely that blood gene expression is a proxy of the injury to other organs and other tissues' transcriptomic profile change as shown in different infectious diseases^{91, 92}. However, *RGS1* gene is only minimally expressed in the whole blood whereas it is mostly expressed in the brain and spinal cord (Figure 36). The intriguing hypothesis that this finding might reflect cell-free RNA content in peripheral blood is supported by recent evidence in patients with Non-Small Cell Lung Carcinoma¹⁹². Xin-Min Yu *et al.* studied a total of 111 patients with Non-Small Cell Lung Carcinoma who underwent curative surgery and found that the expression of four epithelial origin markers *CK7*, *ELF3*, *EGFR* and *EphB4* in cancer tissues and blood were both upregulated and their expression in the blood significantly correlated with clinical and histopathological type¹⁹². *CK7*, *ELF3*, *EGFR* and *EphB4* are not present in mononuclear cells and are specific for the detection of circulating epithelial cells¹⁹³. To further explore this hypothesis, I examined whether specific neuronal injury biomarkers were differentially expressed in babies with adverse outcome after NE. My results showed that s100 β and NF-H were significantly higher in the adverse outcome group, however after correction for multiple comparison the results were no longer significant. This may be the evidence of free RNA released from damaged cells, but in low abundance.

IL7 not only modulates B and T lymphocytes development and homeostasis, but has also an important role in cell survival and regeneration properties as shown in different tissues culture models¹⁹⁴⁻¹⁹⁶. Habib *et al.* found that IL7 overexpression after hypoxic stress enhanced regeneration potential of bone marrow mesenchymal stem cells¹⁹⁶. Michaelson MD *et al.* showed that cultures of embryonic brain treated with exogenous IL7 had an increased neuronal survival and growth suggesting that IL7 might work as neuronal growth factor during central nervous system development¹⁹⁵. Other evidence from hippocampal neuronal cultures is consistent with these results and suggests that interleukins 7 and 8 enhanced hippocampal neuronal survival and led to a marked increase of the number of astroglia and microglia. There is clear link between hypoxia and IL7 as shown by Lu *et al.*¹⁹⁷. The authors in fact, showed that hypoxia decreased IL7 expression in PC12 cells, a rat pheochromocytoma tumor cell line and reported that IL7 downregulation is greater with lower oxygen tensions and longer durations of exposure¹⁹⁷.

Figure 36. Box plots (median, IQR) of the gene expression levels of RGS1 in the different tissues from The Genotype-Tissue Expression project portal. On the X axis there are the different tissues and on the Y axis the transcripts per million (TPM).



Chesné *et al.* studied the whole blood gene expression profile of pre-transplant patients with cystic fibrosis, pulmonary hypertension, and chronic obstructive pulmonary disease and identified 3 gene signatures related to these chronic respiratory diseases¹⁹⁸. In their analysis they found that *T-Cell Factor 7* and *IL7R*, both responsible of T lymphocyte activation were downregulated. The authors also evaluated the variation of the expression levels of these genes in peripheral blood mononuclear cells from healthy controls incubated 12 hours under hypoxic or normoxic conditions and found no difference¹⁹⁸. These data may suggest that longer duration of hypoxia or more severe hypoxic insults might be required to induce these genes' downregulation. The *IL7R* downregulation in the present study therefore supports this preclinical evidence and may suggest that in case of unfavourable outcome, there is an underlying sub-acute or chronic hypoxic insult.

The pathway analysis of the differentially expressed genes before deconvolution showed that protein ubiquitination pathway represented the most significant biological pathway and was downregulated in case of adverse outcome. Ubiquitination represents a homeostatic mechanism, which is responsible for the intracellular bulk degradation of cellular constituents. Although it is generally expected that ubiquitination signal is increased after a hypoxic ischaemic insult¹⁹⁹, my findings might reflect that proteasomal degradation is impaired in case of adverse outcome. These results are consistent with my previous study that showed an upregulation of *MALAT1* after hypoxia⁹⁴. *MALAT1* in fact, inhibits HIF-1 α ubiquitination and in this way, enhances its activity and increases anaerobic glycolysis (Figure 19).

The importance of deconvolution to better understand the underlying pathological disease mechanisms is increasingly recognised. Hence, I decided to use this approach to unveil the biological pathways involved in brain injury following NE. In this analysis I found that Melatonin signalling was the main biological pathway and it was downregulated in the group of neonates with adverse outcome. These findings are supported by the recent study of Denihan *et al.*²⁰⁰. The authors studied untargeted metabolomics to examine early metabolic alterations in a population of infants with perinatal asphyxia, those who developed NE and healthy controls and showed that melatonin was 1.7 and 1.9 fold higher in the perinatal asphyxia and NE population respectively, when compared to matched controls.

Melatonin is a powerful free radical scavenger, preserves the integrity of the electron transport chain, increases ATP production and maintains glutathione balance. My data of a down-regulated melatonin pathway in those babies with NE who later develop adverse outcome may reflect an impaired endogenous melatonin response to a hypoxic ischaemic insult and therefore as consequence, an increased oxidative stress response in case of unfavourable outcome.

Different animal models and small clinical studies have shown that melatonin plus therapeutic hypothermia increases neuroprotection²⁰¹. These results further support the neuroprotectant potential of this drug in NE.

When I compared babies with moderate to those with severe encephalopathy, I found that only 3 genes were differentially expressed. This may reflect that gene expression profile was similar between babies with moderate and severe NE or alternatively just the challenges in differentiating these grades of encephalopathy within the first 6 hours after birth.

Disease risk score is a powerful bioinformatics tool to translate the RNA signature into diagnostic tools. This approach has been validated in previous papers, where the risk disease score has been employed for the detection of tuberculosis, Kawasaki and bacterial versus viral infection in febrile children⁹¹⁻⁹³. By using the risk disease score, it is possible to translate multiple transcript RNA signatures into a single value (disease risk score), and therefore, this can lead to a low cost, diagnostic test requiring basic laboratory infrastructures and minimal bioinformatics input.

When I used this bioinformatics approach, I showed that it was possible to differentiate babies with adverse from those with good outcome by using the NE RNA signature I found previously⁹⁴ and its performance was better than the clinical NE staging. Of note, these babies were recruited in a completely different setting and therefore this underlines its translational potential even more. However, the key aim of this study was to elucidate the biological pathways involved the most severe cases of NE and I did not examine specifically the predictive value of gene expression for which a bigger sample size and a validation cohort are needed.

This study has some limitations. First these patients were recruited in low-middle income countries and therefore the results might not be generalizable outside this context. Second the incidence of death in the adverse outcome group was much higher than expected, which might reflect an extremely high severity of NE in the adverse outcome group. Third, although babies with blood culture positive sepsis were excluded, other cases of sepsis with negative blood cultures might have been included in the analysis. However, Tann *et al.* reported an incidence of 8.9% of neonatal bacteremia in NE cases in Uganda by using in combination blood culture and PCR²⁰². This is not dissimilar from that reported in the ICE and NICHD trials, 8.1 % and 6% respectively^{116, 119}. Finally, I used for deconvolution CIBERSORT tool, which, as shown in chapter 5, had good performance in the neonatal population. However, specific deconvolution methodologies in neonates with higher accuracy are needed.

CONCLUSION

This is the first study to show that babies with adverse outcome after NE are characterised by a specific gene expression signature soon after birth and show the potential of gene expression as a disease stratification tool and as surrogate biomarker in neonatal encephalopathy. Further studies are required to validate these findings in other settings and if successful, this could represent the foundation toward the development of a targeted neuroprotection approach.

Chapter 7: SUMMARY

The thesis examines the prognostic utility of conventional MRI for predicting adverse outcomes and the relationship between thalamic MRS and WM injury as secondary analysis of the prospective multicentre study, MARBLE. Increasing evidence suggests that babies with mild NE are at risk for adverse neurodevelopmental outcomes and there is now need for confirming the risk: benefit ratio of therapeutic hypothermia in this group of patients. I therefore decided to study the cerebral MR biomarkers and neurological outcomes at two years between such babies who underwent whole-body hypothermia and those who did not.

Conventional MRI is considered the brain imaging modality of choice in the clinical field for diagnosis and prognostication as highlighted by the American Academy of Neurology recommendations⁴⁸. By using a combined approach of MRI and MRS I found that babies with predominant white matter injury had a metabolic perturbation in the basal ganglia thalami suggesting that thalamus is invariably damaged due to its high metabolic activity. These findings are novel and relevant given that over 40 % of babies with NE have predominant white matter injury⁴⁹ and can give further insight into the pathophysiology of NE. MRI is essential to determine the aetiology and to understand the nature of the brain injury. However, when I tested the prognostic accuracy of conventional MRI, my results were not different from the Marble study and they showed its suboptimal accuracy¹¹⁵. This outlines the need for bedside disease stratification tools in NE.

In April 2018, a group of experts met at the annual Neonatal Neurocritical Care Special Interest Group and assessed the evidence behind the use of whole-body hypothermia for mild NE²⁰³. The authors pointed out a therapeutic creep of whole-body hypothermia in mild NE and highlighted the need for a randomised controlled trial²⁰³. The results presented in this thesis showed improved MRI and MRS biomarkers in mild NE and are encouraging for potential benefit of therapeutic hypothermia in this population. However, the study was not controlled, and the sample was small. Therefore, caution is needed before drawing any conclusions and a powered randomised controlled study is required.

Currently the diagnosis and prognosis of NE is based on imaging, clinical and electrophysiological examinations. However, these take time and cannot always be performed bedside. Therefore, this may delay the best treatment time and lead to varying degrees of brain injury. The discovery of new accurate bedside disease stratification biomarkers is now considered the future research direction.

Gene expression specific expression patterns and pathways have been described in several serious infections, allowing disease stratification and researchers worldwide are now working on the translation from bench to bedside of these novel discoveries^{92, 93, 153}. I investigated for the first time the use of gene expression in babies with NE and I identified a unique gene expression profile in babies with NE and in those infants, who later develop adverse outcome. These findings set the foundation for future work where it will be essential to validate these results in larger cohorts of patients.

In my work I used gene expression mainly to understand the biological mechanisms involved in NE. For this purpose, it is essential to correct the gene expression data for the proportion of the different blood cells (deconvolution) to avoid false discoveries⁸⁵. However, when I assessed the accuracy of the available deconvolution algorithms in the NE population, I found that some of them had low accuracy in the neonatal population. These findings underline the importance to test the deconvolution algorithms before their use in this population. The relevance of the use of deconvolution is underscored by the last section of my thesis, where by using this approach I showed that melatonin pathway was downregulated in NE babies with adverse outcome. This is supported by recent evidence based on untargeted metabolomics analysis which showed perturbation of tryptophan metabolism in babies who develop NE²⁰⁰.

Perinatal brain research is at cross roads at present and what I present in this thesis shows a potential way forward by working at the interphase of different specialities including genomic medicine, bio-informaticians and neuroimaging. By using this approach, it is possible to offer a unique platform for neuroprotection trials and to develop new accurate biomarkers of adverse

neurologic outcomes in infants with NE. This can eventually lead to a paradigm shift in the way we identify and treat babies with perinatal asphyxia and can develop a new way of personalised medicine for babies at risk of long-term adverse neurological outcomes. I wish to further develop my career combining clinical neonatology, neonatal neuroscience and gene expression. My experience in all these areas enables me to communicate effectively with experts in these fields, and to bring in novel and collaborative ways of addressing important questions in neonatal brain research.

NEW FINDINGS

1. Conventional MRI Rutherford scoring system has 81 % accuracy and NICHD has 80% accuracy measured by the area under the curve in cooled babies.
2. MRS metabolic alteration in the BGT area correlate with macroscopic brain injury identified on conventional MRI not only in the BGT and PLIC areas, but also in white matter and cortex.
3. Cooling improves MR spectroscopy biomarkers and reduces white matter injury on MRI in mild encephalopathy.
4. Babies with neonatal encephalopathy have a unique gene expression profile when compared with healthy age-matched controls.
5. Babies with neonatal encephalopathy have a differential expression in key genes part of hypoxia inducible factor pathway.
6. Some of the available deconvolution algorithms perform poorly in neonates, with correlation with flow cytometry estimated cell type proportions as low as -0.24.
7. Babies with adverse outcome after neonatal encephalopathy have a specific gene expression profile soon after birth.
8. Babies with adverse outcome after neonatal encephalopathy have down-regulation of the biological pathways Melatonin and T cell receptor signalling.
9. The neonatal encephalopathy signature-based risk disease score successfully discriminates babies with adverse outcome from those who have a good outcome.
10. The most significant differentially expressed gene in babies with adverse outcome after neonatal encephalopathy is *RGS1* gene which is only minimally expressed in the whole blood whereas it is mostly expressed in the brain and spinal cord.

FUTURE WORK

These points represent suggestions for further work, based on the work presented in this thesis.

- Whole body cooling in mild neonatal encephalopathy

Whole-body cooling reduces cerebral metabolite perturbations and white matter brain injury seen on MRI in babies with mild NE. There is now an urgent need to assess the efficacy of whole-body cooling in mild NE infants in adequately powered randomised controlled trials.

-Temporal changes of gene expression profiles after neonatal encephalopathy

My data show that after an acute hypoxic insult, there is a massive upregulation of the 'hypoxia-inducible transcription factor', which regulates glycolysis, survival and angiogenesis and acts as master switch of the cellular adaptive response to limited oxygen availability. Studying the temporal changes of this pathway and the dynamic changes of differentially expressed genes associated with NE could help understand the response in the process of the brain injury and find new treatment strategies. Moreover, once these therapeutic pathways are discovered, they can be used for monitoring the efficacy of new neuroprotection interventions.

- Genomic Imaging in neonatal encephalopathy

Different patterns of brain injury on MRI can help stratify NE based on the aetiology and nature of insult. However, MR imaging soon after birth is far more challenging to undertake. The work presented in this thesis shows that gene expression profile changes soon after birth in babies with NE. To develop and validate the (aetiological) gene expression pathways associated with brain injury on MRI is a necessary step to use gene expression as disease stratification tool.

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Appendix I

T₁- and T₂-weighted MRI parameters

T₁-weighted 3D magnetisation-prepared rapid gradient-echo (sagittal, repetition time (TR)=17ms, echo time (TE)=4.6ms, flip angle=13°, inversion time=1465ms, 0.8x0.8x0.5mm³ voxels), T₂-weighted 2Dturbo spin echo (axial, TE=130ms, TR=8800ms, 0.5x0.5x3mm³voxels)⁵⁷.

MR spectroscopy protocol

Single-voxel point-resolved spectroscopy (PRESS), 15x15x15 mm³ cubic voxel in the left thalamus, TR=2288ms/TE=288ms (16 averages with 8 phase cycles, water suppressed) and TR=2060ms/TE=60ms (8 averages with 8 phase cycles, water suppressed). Metabolite peak-area ratios involving NAA, total choline, total creatine and lactate was derived.

Same thalamic voxel; additional PRESS scans (a) TE=60ms, TR=5000ms, (8 averages with 8 phase cycles, water suppressed) (b) TE=60, 124, 205, 316, 495, and 1000ms, each with TR=10s+TE, with one average of 8 phase cycles, and without water suppression. NAA concentration was derived from an assumed brain water concentration by comparing the T₂ corrected fully-relaxed NAA and brain-water signals⁵⁷.