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Altered Cord Blood Biomarkers in Neonatal Brain Injury

Thesis presented by

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Declaration

This is to certify that the work submitted is the candidates own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Signed: _____

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Publications, Conference Presentations and Dissemination

Year	Title
Peer-reviewed publications	
Published manuscripts	
2018	**O'Sullivan MP**, Sikora KM, Ahearne C, Twomey DM, Finder M, Boylan GB, Hallberg B, Murray DM. Validation of raised cord blood Interleukin 16 in Perinatal Asphyxia and neonatal Hypoxic-Ischaemic Encephalopathy in the BiHIVE2 cohort. Developmental Neuroscience 2018. DOI: 10.1159/000491386.
2018	Looney, AM, **O'Sullivan MP**, Ahearne CE, Felderhoff-Musser U, Boylan GB, Hallberg B, Murray DM. Altered expression of umbilical cord blood levels of miR-181b and its downstream target mUCH-L1 in infants with moderate and severe neonatal hypoxic-ischaemic encephalopathy. Molecular Neurobiology 2018. DOI: 10.1007/s12035-018-1321-4.
2018	**O'Sullivan MP**, Looney AM, Moloney GM, Finder M, Hallberg B, Clarke G, Boylan GB, Murray DM. Validation of altered umbilical cord blood microRNA expression in neonatal hypoxic-ischaemic encephalopathy. JAMA Neurology 2018. DOI:10.1001/jamaneurol.2018.4182.
Manuscripts prepared for submission	
2019	**O'Sullivan MP**, Ní Chaoimh C, Clarke C, Blanco CE, Hanson M Stalling R, Murray DM. Dysregulated Circulating microRNA Expression Profiling in the Umbilical Cord Plasma at Birth Predates Obesity at 5-years.
2019	**O'Sullivan MP**, Casey S, Finder M, Ahearne CE, Twomey DM, Clarke G, Hallberg B, Boylan GB, Murray DM. Alterations in umbilical cord blood messenger RNA expression in neonatal hypoxic-ischaemic encephalopathy and long-term outcome.
2019	**O'Sullivan MP**, Denihan N, Sikora K, Finder M, Ahearne CE, Twomey DM, Clarke G, Hallberg B, Boylan GB, Murray DM. Umbilical cord activin A and mACVR2B are not reliable biomarkers of mild or moderate neonatal hypoxic-ischaemic encephalopathy.
2019	**O'Sullivan MP**, Clarke G, Murray DM. Cellular blood-based and circulating cell-free microRNA as functional clinical biomarkers: A clinicians review.

Year	Title
Conference Presentations	
Oral presentations	
2019	Pavel AM, O'Riordan M, **O'Sullivan MP**, Panaite L, Culliney L, Nabialek T, De Meulemeester J, Caulfield F, Stanescu A, Yusuf Z, Doolan A. Exploring the impact of gestational diabetes mellitus on infant's body composition *Pediatric Academic Societies (PAS) 2019 Meeting, April 27-30, in Baltimore, Maryland*
2017	**O'Sullivan MP**, Looney AM, Moloney GM, Finder M, Hallberg B, Clarke G, Boylan GB, Murray DM. MicroRNA Panel Validation in Hypoxic-Ischemic Encephalopathy. *Second Congress of joint European Neonatal Societies- jENS, Venice, Italy*
2017	**O'Sullivan MP**, Looney AM, Moloney GM, Finder M, Hallberg B, Clarke G, Boylan GB, Murray DM. Downstream mRNA Targets in Hypoxic-Ischemic Encephalopathy *10th International Conference on Brain Monitoring and Neuroprotection in the Newborn, Ireland*
2016	**O'Sullivan MP**, Sikora KM, Ahearne C, Twomey DM, Finder M, Boylan GB, Hallberg B, Murray DM. Validation of Raised Cord Interleukin 16 in Neonatal Hypoxic-Ischemic Encephalopathy *6th Congress of the European Academy of Paediatric Societies- EAPS, Geneva, Switzerland*
Poster presentations	
2018	**O'Sullivan MP**, Looney AM, Ní Chaoimh, Clarke C, Stallings R, Murray DM. Cord Blood microRNA alterations at birth predate persistent childhood obesity. *7th Congress of the European Academy of Paediatric Societies- EAPS, Paris, France*
2017	**O'Sullivan MP**, Looney AM, Moloney G, Hallberg B, Clarke G, Boylan GB, Murray DM. MicroRNA Panel Validation in Hypoxic Ischemic Encephalopathy. *NAI and Irish Brain Council Joint Conference, Science Gallery, Trinity College Dublin*
2016	**O'Sullivan MP**, Looney AM, Ahearne CE, Moloney G, Clarke G, Boylan GB, Murray DM. Early Detection of Hypoxic Ischemic Encephalopathy Using a Combined MicroRNA Panel. *School of Medicine New Horizons Research Conference, UCC*
2016	**O'Sullivan MP**, Finn D, Livingstone V, Stevenson N, O'Toole J, Murray DM, Boylan. Relationship of Serial Recorded Temperature with HIE Severity and Electrographic Seizure Burden in Infants with Hypoxic-Ischemic Encephalopathy. *Infant Research Day, UCC*

Year	Title
Modules and Training	
2018	Good Clinical Practice (GCP) Training
2018	Liquid Biopsy Course, European Molecular Biology Lab (EMBL), Heidelberg
2017	Cochrane Ireland 2-day systematic review training
2016	Good Clinical Practice (GCP) Training
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2016	ML6005 Biotechniques (5 credits)
2015	ST6013 Statistics and Data Analysis for Postgraduate Research Students (10 credits)
2015	Writing for Publication Workshop (RCSI) with Dr Dan Soule
2015	Techniques & Strategies in Molecular Medicine - Molecular Medicine Ireland, University College Dublin
2014	Good Clinical Practise (GCP) Training
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2017	1st Prize for Best Oral Presentation at the 10th International Conference on Brain *Monitoring and Neuroprotection in the Newborn, Ireland*
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2016-2017	Founder and Chairperson *SPEAK Public Speaking Workshop, College of Medicine and Health, UCC*
2015-2017	Chairperson *Postgraduate Student Committee, College of Medicine and Health, UCC*

Abstract

Introduction

Alterations in RNA and proteins observed at birth may have long-term effects on early life. Perinatal asphyxia (PA) accounts for a quarter of all global neonatal deaths. Early intervention is critical for developing strategies to improve long-term outcome. Early identifiers for infants at risk of HIE is prone to subjective-bias or poorly objective biochemical measurements. The umbilical cord blood (UCB) can give a snapshot of both the newborn and the in-utero environment. It is non-invasive to both mother and infant and contains microRNA (miRNA), messenger RNA (mRNA) and circulating proteins that can exist stably in the circulation. This thesis aims to progress further the knowledge of UCB biomarkers using low-throughput techniques in both RNA and protein analyses, and to explore and validate proposed and potential biomarkers in HIE.

Methods

This thesis utilised three distinct, well-defined cohorts throughout, the BiHIVE1 cohort (2009-2011), the BiHiVE2 cohort (2012-1015), and the BASELINE longitudinal birth cohort (2008-2016). The BiHIVE1 cohort was recruited in Cork, Ireland (7500 live births per annum), this study included full-term infants with PA enrolled at birth. This cohort recruited 112 cases (40 developed HIE - 24 mild, 6 moderate and 10 severe), of these 52 had neurodevelopmental follow-up, and 7 died in infancy. For this cohort, healthy controls were recruited from the BASELINE cohort. The BiHiVE2 validation cohort was recruited in Cork, Ireland and Karolinska Huddinge, Sweden (4400 live births per annum), this study included infants with PA along with healthy control infants. This cohort recruited 353 cases (48 developed HIE - 32 mild, 14 moderate and 2 severe) and 289 controls, 449 had neurodevelopmental follow-up, and no deaths occurred in infancy.

Infants with perinatal asphyxia had matched inclusion criteria across both BiHiVE1 and BiHiVE2. Infants were assigned a modified Sarnat score at 24 hours and were

followed-up with neurological assessment and neurodevelopmental outcome at 18-36 months of age. Umbilical cord serum, plasma and whole blood were processed and biobanked across all cohorts at delivery and stored at -80°C.

Techniques employed throughout the thesis include blood processing, RNA isolations of whole blood miRNA and mRNA, and subsequent cDNA synthesis and quantitative real-time polymerase chain reaction (qPCR). Protein expression measurements were conducted with sandwich enzyme-linked immunosorbent assays (ELISA) on cord serum samples. All statistical analyses were conducted using IBM SPSS Statistics 24, graphical representation of data was generated using GraphPad Prism 8.

Results

miRNA Panel: One-hundred and sixty infants had miRNA qPCR analyses across both BiHiVE1 and BiHiVE2. In BiHIVE1, 12 candidate miRNAs were identified, and 7 of these miRNAs were chosen for validation in BiHIVE2. The BiHIVE2 cohort showed consistent alteration of 3 miRNAs; miR-374a-5p was decreased in infants diagnosed as having HIE compared with healthy control infants ($P = 0.009$), miR-376c-3p was decreased in infants with PA compared with healthy control infants ($P = 0.004$), and miR-181b-5p was decreased in infants eligible for therapeutic hypothermia (TH) ($P = 0.02$).

Predicted mRNA Targets: One-hundred and twenty-six infants had mRNA analyses. In the grade of HIE severity, the level of mFZD4 was increased in severe HIE vs mild HIE ($P = 0.004$), and severe HIE vs moderate HIE ($P = 0.003$). Fifty-six infants were included in the neurodevelopmental outcome analysis; Levels of mNFAT5 were increased in severely abnormal vs normal outcome ($P = 0.036$), and in severely abnormal vs mildly abnormal outcome ($P = 0.013$). Levels of mFZD4 were increased in severely abnormal vs normal outcome ($P = 0.004$), and in severely abnormal vs mildly abnormal outcome ($P = 0.026$).

Interleukin-16: One-hundred and thirteen infants were included in the final analyses. Cord blood-based IL-16 was increased in infants with perinatal asphyxia and HIE relative to controls ($P = 0.025$). IL-16 was also increased in the HIE group relative to

controls (*P* = 0.042). There was no significant difference in IL-16 across grades of HIE or in those with abnormal outcomes at 2-years of age.

Activin A and mACVR2B: Serum activin A analyses included 101 infants. No differences were observed across the groups (*P* = 0.693). The HIE group included 23 infants; a combination of mild and moderate HIE, without infants with a severe grade. No differences were observed across the grades of HIE (*P* = 0.115). Whole blood mACVR2B analyses included 68 infants, and no differences were observed across the groups (*P* = 0.746). The HIE group included 22 infants, and no differences were observed across the grades of HIE (mild and moderate only) (*P* = 0.468). No differences were observed in infants followed up to 18-36 months in serum activin A or in whole blood mACVR2B, (*P* = 0.550) and (*P* = 0.881) respectively.

Conclusions

This thesis has identified and validated the decreased expression of UCB miRNA (miR-181b, 374a and miR-376c), increased expression of UCB mRNA (mNFAT5 and mFZD4) and increased UCB cytokine expression (IL-16) across two cohorts in HIE. It validates previous miRNA whole blood biomarkers of PA (miR-376c) and HIE (miR-374a) and proposes novel whole blood mRNA biomarkers as assessors for both HIE severity (mFZD4) and long-term neurodevelopmental outcome (mNFAT5 and mFZD4), which can outperform current measurements. These miRNA and mRNA could aid current measures as objective diagnostic and prognostic markers of HIE. It has further explored the strengths of cytokines and proteins and confirmed their inability as biomarkers across HIE groups and grades. This work has an important role in further developing UCB-based biomarkers in early life.

Abbreviations

Acvr	Activin receptor
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BASELINE	Babies after SCOPE: Evaluating Longitudinal Impact using Neurological & Nutritional Endpoints
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BiHIVE	Biomarkers in Hypoxic-Ischemic Encephalopathy
BMI	Body-mass-index
BSID-III	Bayley Scales of Infant and Toddler Development- third edition
cDNA	complementary DNA
CD	Cluster of differentiation
CI	Confidence interval
CP	Cerebral palsy
CSF	Cerebrospinal fluid
CT	Cycle threshold
CVD	Cardiovascular disease
DIANA	DNA Intelligent Analysis
DNA	Deoxyribonucleic acid
EAA	Excitatory amino acids
EEG	Electroencephalogram
ELISA	Enzyme-linked immunosorbent assay
FDR	False discovery rate
FPR	False Positive rate
Fzd	Frizzled
GFAP	Glial fibrillary acidic protein
HDL	High-density lipoprotein
HIE	Hypoxic-ischaemic encephalopathy
IFN	Interferon
IL	Interleukin
IQR	Interquartile range
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LDL	Low-density lipoprotein
LMS	Lambda-mu-sigma
Log	Logarithm
MCP-1	Monocyte chemoattract protein 1
MIP-1α	Macrophage inflammatory protein 1-alpha
MIP-2	Macrophage inflammatory protein 2
miRNA	microRNA
MRI	Magnetic resonance imaging
mRNA	messenger RNA

Nfat	Nuclear factor of activated T-cells
NGS	Next-generation sequencing
NICU	Neonatal intensive care unit
NMDA	N-methyl-D-aspartate
NOS	Nitric oxide synthase
NPV	Negative predictive value
NSE	Neuron-specific enolase
PA	Perinatal asphyxia
PLS-DA	Partial least-squares discriminant analysis
PPV	Positive predictive value
QC	Quality control
qPCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RQ	Relative quantification
RWG	Rapid weight gain
SDS	Standard deviation score
T2D	Type 2 diabetes mellitus
TH	Therapeutic hypothermia
TNF	Tumour necrosis factor
TOBY	Total Body Hypothermia for Neonatal Encephalopathy
TPR	True positive rate
UCB	Umbilical cord blood
UCH-L1	Ubiquitin-C terminal hydrolase L1
VEGF	Vascular endothelial growth factor
VIP	Variable importance in projection
WFL	Weight-for-length
WHO	World Health Organisation
Wnt	Wingless and NT-1
Units of Measurement	
D	Day
°C	Degrees Celsius
g	Grams
h	Hour
L	Litre
µl	MicroLitres
mL	Millilitres
mmol	Millimolar
min	Minutes
mo	Month
ng	Nanograms
%	Percentage
pg	Picograms
wk	Week

Chapter 1

Introduction

1.1 Overview

Early life is a period of profound change, and potentially altered transcript expression may lead to long-term alterations in growth and development. This thesis will examine the ability of microRNA (miRNA) and messenger RNA (mRNA) expression in umbilical cord blood (UCB) to predict the occurrence of hypoxic-ischaemic encephalopathy (HIE) in infants.

Perinatal asphyxia (PA) occurs when the fetal blood flow through the umbilical cord is impaired reducing the supply of oxygen and glucose supply to the neonate; if this impairment is prolonged the newborn will develop an injury to the brain called HIE. Therapeutic hypothermia (TH) is the only treatment for HIE to date that can improve outcome, but only if initiated within the first 6 hours of delivery (1-4). Accurate HIE diagnosis within this limited window is difficult, and current biochemical and clinical identifiers are poor positive predictors (5-8). More specific objective biomarkers need to be explored and validated urgently.

In recent years, biomarker research in HIE has explored small non-coding molecules including miRNAs that are present in peripheral blood cells and stable in the bloodstream by transportation within extracellular vesicles including membrane fragments, exosomes, microvesicles, and microparticles (9-11). Conducting analyses in UCB allows us to examine miRNA transcripts and mRNA expression and answer questions about conditions where the prediction of outcome is important. Furthermore, in HIE, reliable biomarkers would have the potential to change management and improve outcome. This thesis uses state of the art approaches to examine the ability of alterations in UCB miRNA and mRNA as biomarkers for early detection to aid in addressing these problems.

1.2 Human Brain Development

Insults and injuries in the neonatal period are unique due to the rapid development of the brain occurring simultaneously, there is a high likelihood that these injuries may cause significant alterations to long-term neurodevelopment. The major milestones of brain development begin at week 3 of gestation and finishes in the adult life. The major developmental events begin with primary neurulation, and subsequent to this the development of the prosencephalon, the development of the posterior fossa structures, neuronal proliferation, neuronal migration, organisation, and myelination (12, 13).

The formation of the neural tube, together with the development of the prosencephalon constitute the embryonic aspects of neural development. The development of the prosencephalon occurs predominantly during months 2-3 of gestation, particularly around weeks 5-6, and occurs across 3 events, and the most important molecular pathways in prosencephalic development are the Sonic hedgehog signalling pathway and the nodal pathway (13, 14).

The development of the posterior fossa structures begins with brain flexure development in the anterior neural tube at gestational week 5, which has an important role in defining the region of the future rhombencephalon, the pontine flexure separates the future fourth ventricular roof, and the mesencephalic flexure appears at the border between the mesencephalon and rhombencephalon, which will become the future midbrain-hindbrain junction. The mid-hind brain area is where patterning events define cerebellar and related structures, these events are followed by the development of the fourth ventricle roof and the development of the cerebellar hemispheres (13, 15).

Neuronal proliferation occurs primarily between 3-4 months at the ventricular and subventricular zones at the subependymal location across the developing nervous system, where neuronal units are produced by progenitor cells. Succeeding this, in months 3-5 primarily, neuronal migration occurs, where the neurons migrate from the ventricular and subventricular zones to their endpoints in the brain. Two primary

forms of migration occur called radial and tangential migration, along with axophilic and multipolar migration (13, 16).

Organisational events in the brain occur from 5 months gestation until the postnatal years, and include the formation and differentiation of subplate neurons, alignment, orientation, and lamination of cortical neurons, gyral development, expansion of dendritic and axonal ramifications, formation of synaptic contacts, cell death and selective elimination of neuronal processes and synapses (13, 15).

Finally, myelination occurs within the first 2 years postnatally, separated into oligodendroglial proliferation and differentiation, and myelin deposition around axons. The oligodendrocytes develop in waves from distinct brain regions from oligodendroglial progenitors to pre-oligodendrocyte to the immature oligodendrocyte and finally to mature oligodendrocytes, with the changes from pre-oligodendrocyte to immature and mature oligodendrocyte occurring predominantly after 28 weeks gestation with transitions continuing through postnatal life (13, 17).

1.3 Hypoxic-Ischaemic Encephalopathy

1.3.1 Overview

PA is the deprivation of oxygen to the newborn and can have profound effects on the majority of the infant's organs, i.e. brain, heart, lungs, liver, gut, and kidneys. Brain injury in PA can have profound and irreparable effects in the newborn, from mild cognitive impairment to severe disability or death, and when this occurs, the injury is termed HIE (18). HIE may result in a focal, diffuse or both focal and diffuse brain injury (19). Central gray matter damage, such as basal ganglia and thalami (BGT) lesions are considered the hallmark of HIE in newborns, with severe injury associated with lesions in the posterior limb of the internal capsule (PLIC), brainstem, hippocampal region, and cortex (20). Both BGT and PLIC lesions from HIE can be predictive of motor impairments and cerebral palsy (CP). Severity of BGT lesions are associated with presence and severity of motor impairments and PLIC signal intensity is associated with walking ability, both of which occur in infants with CP; and injury to the brainstem is associated with death (21). Basal ganglia/thalamus patterns of injury have been shown to be closely associated with the most severe motor and cognitive long-term damage (22). Prolonged partial asphyxia can cause white matter injury in HIE which has been linked to cognitive, visual, language, behavioural, and seizure problems(23). HIE and neonatal stroke share common mechanisms of injury; it can be difficult to differentiate HIE from neonatal stroke, as both present similarly, and are two of the most common injuries associated with seizures in the neonatal period (13, 24-26).

Neonatal resuscitation is one of the earliest steps of intervention for PA infants with impaired breathing at birth, neonatal resuscitation has been separated into steps and standardised across many countries with the neonatal resuscitation programme, which uses an evidence-based approach to newborn care. Effective resuscitation and support at birth can significantly reduce neonatal deaths (27, 28). Improvements in early neonatal care for HIE has been made in high-income countries, including the introduction of electrophysiological monitoring and diagnostic imaging techniques, such as multi-channel continuous electroencephalogram (cEEG) and magnetic

resonance imaging (MRI) respectively (22, 29). These techniques can both act as powerful tools with high performance in the identification of injury severity, and operate in tandem with neurological evaluation for aiding in prognoses; both fall short as they carry requirements for expert training in application and interpretation, and fail to be cost-effective and time-sensitive for early intervention.

A significant advance in HIE was made two decades ago, following successful HIE sheep models, when pilot studies treating HIE infants with therapeutic hypothermia (TH) were delivered into the clinical setting, and these were subsequently followed by randomised controlled trials (30-32). A few years later, meta-analyses of these studies, demonstrated TH was associated with reduced neurological impairments in outcome and decreased death rates in infants with HIE; these findings progressed TH into standard care of infants with moderate-severe grade of HIE (33). However, cooling needs to be implemented rapidly after diagnosis as timing of cooling beyond 6 hours has shown to be ineffective and needs to commenced within 3 hours of delivery to ensure improvement in outcome (1-4).

Although there is an understanding of the origin of HIE, and the intricate pathophysiology that follows; knowledge gaps still exist leaving a number of open questions, including timing of injury onset, understanding the balance between injury and recovery, and creating more effective treatments for HIE infants, particularly those that go on to develop long-term disabilities. Development of robust and reliable biomarkers would create minimally invasive, cost-effective diagnostic and prognostic markers that may give insight into the underlying pathophysiology and aid current markers in improving the standard of care.

1.3.2 Incidence

The majority of child deaths are attributed to the neonatal period, and PA accounts for roughly a quarter of all global neonatal deaths with most of these deaths occurring in low- and middle-income settings (34, 35). Further to this, although global neonatal mortality has declined in the past 50 years, the fear is that this decline is coming from late neonatal deaths, and early neonatal deaths have failed to drop at the same rapid pace (34).

The incidence of HIE specifically is estimated at 1.5 per 1000 live births in the developed world, with risk factors including antecedent events, growth restriction, infection and management during labour and delivery (36). Although the past 15 years have seen mortality decreasing in neonatal encephalopathy, it has the highest global increase in years lived with disability of all 'communicable, maternal, neonatal, and nutritional diseases'. This would indicate that improvements in care and treatments are preventing death, but the surviving infants are left with lifelong disability (37).

1.3.3 Pathophysiology

HIE is a complex process that begins at injury and can continue to develop for days postpartum. The initial insult occurs when the brain deprived of blood, is starved of oxygen, glucose and other essential nutrients; arising during a critical period in early brain development (figure 1.1).

Cerebral blood flow sends oxygen and glucose across from the placenta to the fetal brain. This blood flow helps maintain homeostasis and meet cellular energy demands in the brain. Hypoxic events cause a reduction in fetal cardiac output, which reduces cerebral blood flow. If the decrease in cerebral blood flow is moderate, the cerebral arteries push blood flow from the anterior circulation to the posterior circulation to maintain sufficient perfusion to vital brain regions, i.e. the brainstem, cerebellum, and basal ganglia. This restricts the damage to the cerebral cortex and watershed areas of the cerebral hemispheres; with acute hypoxic events injuring the basal ganglia/thalamus (38).

1.3.3.1 Primary Injury

The progression of injury that develops after the initial asphyxial event involves decreased cerebral blood flow which reduces the delivery of oxygen and glucose to the brain, leading to anaerobic metabolism. As a result, production of adenosine triphosphate (ATP) decreases causing failure of energy-dependent mechanisms of homeostasis, including Na^+/K^+ ATP dependent pump to fail and for the production of lactic acid increases. Following pump failure, neuronal depolarisation occurs, causing a transcellular transport reduction and leads to intracellular accumulation of sodium

and water, the cell releases the excitatory amino acid (EAA) glutamate, and calcium flows into the cell via *N*-methyl-D-aspartate (NMDA)-gated channels, resulting in cytotoxic oedema. The generation of oxygen free radicals leads to the peroxidation of free fatty acids and DNA/RNA fragmentation (39, 40). This culmination of energy failure, acidosis, glutamate release, lipid peroxidation, and the toxic effects of nitric oxide leads to cell death through both necrosis and apoptosis (41, 42).

Different neuronal cells are affected by different cell death pathways post insult. In immature rodent models, the forebrain cortical and striatal neurons exhibit both apoptotic and necrotic cell death; and in the postnatal rodent model, layer-specific cell death pathways occur in pyramidal and nonpyramidal neurons, these pathways can be dependent on brain maturity (43, 44). In thalamic neurons, apoptosis appears to act as the central form of cell death independent of brain maturity (45). Rodent and porcine models have shown NMDA receptor excitoxicity to cause apoptosis in O4 oligodendrocyte precursors and HI and excitotoxity cause necrosis in astrocytes (46, 47). The post-injury release of cellular content due to necrosis causing inflammation, leading to an influx of microglia that release inflammatory messengers; this inflammation can lead to white matter injury via apoptosis (48). Death of immature oligodendrocytes occurs during proliferation, migration and maturation, prior to the onset of myelination causing long-term damage (17, 49).

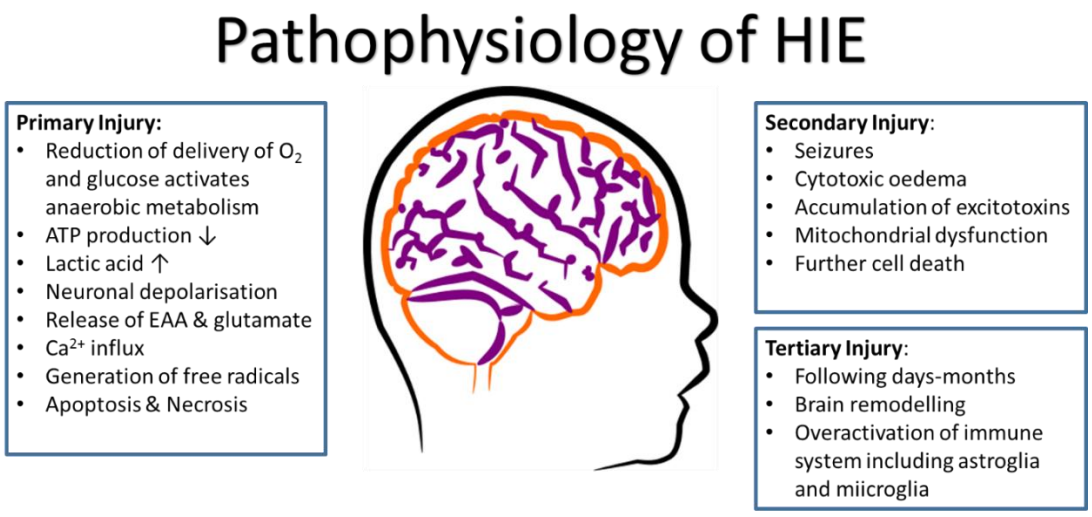

Figure 1.1. Pathophysiology of Hypoxic-Ischaemic Encephalopathy. Three stages of injury.

1.3.3.2 Recovery Phase and Secondary Injury

Depending on the timing of injury and the degree of medical intervention, a partial recovery of the cerebral oxidative metabolism, cytotoxic oedema, and accumulation of EAAs occurs over 30-60 minutes, and is followed by a transient latent phase that sees the return of high energy phosphates, and hypoperfusion with reduced cerebral metabolism and improved tissue oxygenation (50). The latent phase is proceeded by a secondary phase of injury (second hit) that is typically associated with seizures, cytotoxic oedema, accumulation of excitotoxins, failure of cerebral mitochondrial activity and further cell death.

1.3.3.3 Tertiary Injury

A tertiary phase occurs during the days, weeks and possibly months after the acute insult and involves slow cell death, remodelling of the injured brain, and overactivation of astroglia and microglia (51). This may potentially expand to neutrophils and lymphocytes, which appear to be unable to mount an immune response during the initial insult, but lymphocytes may be implicated in the injury days later (52, 53).

1.3.3.4 Inflammatory Response in HIE

Inflammation plays a vital role in healthy development, but overactivation can have profound effects on the immature brain due to developmental plasticity. Innate and adaptive immunity both play an exclusive role in HIE, with a number of components involved in the crosstalk between the two, i.e. immune cells, adhesion molecules, cytokines, chemokines and oxidative stress (54).

Attempts to understand the immune system is an ever-evolving research area. Further to this, the progression of research in 'inflammatory responses to HIE' is dependent on our understanding that an inflammatory response may have differing effects in the underdeveloped nervous system and developing brain. The inflammatory response for HIE occurs within minutes post insult in the neonate, compared to within hours in the adult (55, 56).

Microglia, as resident immune cells of the brain, are the first to respond, followed by infiltration of circulating leukocytes, i.e. neutrophils, monocytes and T-cells (57). The

activation and aggregation of microglia are typical in HIE, particularly in the dentate gyrus, periventricular and subcortical white matter where they produce inflammatory cytokines, glutamate, NO and ROS, causing oligodendrocyte cell death, axonal degeneration and blood-brain barrier (BBB) disruption (58, 59). Resting microglia extend and retract the ramified processes while surveying the brain; following ischaemic insult the microglia become activated and macrophage-like, i.e. phagocytotic, produce inflammatory and anti-inflammatory cytokines, antigen presentation and release of matrix metalloproteinases causing breakdown of the BBB (60).

Astrocytes become reactive in the last trimester of pregnancy and have a hypertrophic response in HIE. Their activation can be both beneficial and detrimental in HIE, by both supporting neurons, but also by producing pro-inflammatory cytokines, including interferon (IFN)-γ, interleukin (IL)-1α, IL-1β, IL-6, tumour necrosis factor(TNF)-α, which cause cell death in injured cells (61-64).

Cytokines are produced by neurons, microglia and astroglia, and act as critical inflammatory mediators of the immune response. They provide both pro-inflammatory and anti-inflammatory effects, and studies have seen a rise in cytokines in response to HIE (65, 66). Due to the rapid onset of the inflammatory response after HIE, cytokine expression levels can be studied at birth using enzyme-linked immunosorbent assays (ELISAs) across the cerebrospinal fluid, serum and plasma, making these an ideal biomarker for diagnosis and injury severity (65-68).

Sex-differences also appear to play a role in the inflammatory response according to the Rice-Vanucci mouse model where females exhibiting a more robust anti-inflammatory response were linked to fractalkine, a transmembrane protein and chemokine that plays a role in adhesion and migration of leukocytes (69, 70). Also, a few days' post-HIE females were seen to have a smaller infarct size and fewer seizures, along with less brain tissue loss and behavioural deficits during the chronic stage of HIE. Males have been shown to exhibit a higher infiltration of peripheral lymphocytes and higher levels of TNF-α and IL-10 (69, 70).

1.3.4 Diagnosis, Prognosis and Treatment

Diagnosis of HIE is not an easy task, as the timing, severity, and progression of the injury occurring over time can have profound effects on the infant. The timing of injury sustained can be antepartum or intrapartum, as the neonate enters into a disrupted asphyxia state with an obstruction to placental or pulmonary gas exchange. The PA events are caused by any decrease in blood flow including placenta praevia, vasa praevia, placental abruption, fetal-maternal transfusion, uterine rupture, cord prolapse, and nuchal cord (71, 72), determining the exact cause is difficult, but placental analysis may aid in this (38).

1.3.4.1 Identification and Grading

Study identification of infants with PA typically involves fulfilment of one or more of the following criteria (29, 32, 66, 73):

- Umbilical cord pH < 7.1 (acidemia)
- 5-minute Apgar ≤ 6
- Base deficit ≥ 16 mmol/L
- Resuscitation in the delivery room

The low blood pH is due to increased production of hydrogen ions and is normally caused by metabolic acidosis; this occurs when the body increases production of metabolic acids, i.e. lactic acid, or when the kidneys are unable to excrete acids, i.e. accumulation of urea and creatinine. A base deficit is another indicator for the blood acidosis.

Virginia Apgar created the Apgar score in 1952, to evaluate the infant immediately post-delivery. It's a five criteria score, from 0-2 in each category, with a maximum total score of 10 (optimal health) and a minimum total score of 0 (requires medical attention). Apgar examines skin colour, pulse rate, tone, activity, and respiratory effort; it was not intended for long-term outcome, and although there are correlations between poor Apgar score and neurological impairment, CP and death, it is considered by many to be a poor clinical predictor of long-term outcome (8, 74). In low-resource settings, a low Apgar score at 1-min. and 5-min. indicate adverse

short-term outcomes in more than 50% of cases, including HIE (75). In a developed country randomised infants with Apgar scores, less than 4 at 5-min. were attributed to HIE or hypoxic death in 70% of the cases (76).

Although pH, Apgar and resuscitation can provide an initial assessment of infants with PA that may develop HIE, they are poor indicators of insult severity and the progression of the HI injury. Timely and costly diagnostic techniques following the criteria mentioned above currently accompany neurological examination and Sarnat score to determine the infants' HIE severity and prognoses, such as cEEG and MRI both of which require specialised equipment and interpretation. Other biomarkers for HIE severity and prognosis are discussed in 1.4.4.

The Sarnat score is a classification grading scale that group's infant into Grade I mild, Grade II moderate, and Grade III severe. The mild HIE infants are expected to have a healthy outcome, with moderate HIE expected to have neurological impairments and severe HIE infants expected to have a high disability and mortality rate (77). Although recent studies may indicate a poorer long-term outcome associated with mild HIE then previously suspected (78, 79).

Neonatal cEEG acts as a typically non-invasive electrophysiological monitoring biomarker; it examines the brains spontaneous electrical activity over a given period, and it's the current gold standard for detecting electrical seizures (80, 81). In HIE, the neonatal EEG can be used as an accurate identifier of HIE severity, long-term outcome, and can aid in clinical decision making to commence TH (29, 82, 83). Normal and delayed EEGs are associated with normal evolutions; and low-voltage EEGs, inactivity, and suppression of activity are associated with severe abnormal outcomes. The neonatal cEEG can be used to identify HIE severity by classification according to background EEG activity, where grades 0 and 1 indicate continuous background patterns with either normal physiologic features or slightly abnormal activity respectively, grades 2 and 3 indicate discontinuous activity with short and long inter burst intervals (IBI) respectively, and grade 4 indicates minimal background activity and severe discontinuity with further extended IBIs. To determine HIE outcome, one-hour EEG recordings have been classified at different timepoints i.e.

6, 12, 24 and 48 hours, and it's been demonstrated that classification at 6 hours can be used to predict outcome (29, 83).

MRI is a robust magnetic imaging technique but has a high running cost, so equipment is only available in larger centres and high-resource settings. MRI is commonly applied to understand the heterogeneity of brain injury in HIE; previous MRI studies have highlighted the vascular watershed damage to the white matter associated with prolonged partial asphyxia, and the basal ganglia/thalamus grey matter damage associated with severe injury, seizures and impaired long-term motor and cognitive outcome (18, 22, 84).

1.3.4.2 Treatment

For a long time, treatment options for infants with HIE were quite limited and were focused on systemic, respiratory and cardiovascular baseline support immediately post-injury, this was until the introduction of TH as standard care. TH is administered using either whole body cooling or selective head cooling and involves dropping the infant's core temperature to 33.5°C for 72 hours (73). TH reduces injury in both basal ganglia/thalamus and white matter injury; it has also been shown to reduce seizure burden and disabling neurodevelopmental sequelae in HIE, except in the most severe infants (85-88). Although the benefits of TH have been demonstrated, it's also important to recognise the significance of undergoing TH in the neonatal period, adverse effects of hypothermia include both sinus bradycardia and thrombocytopenia (89).

Anticonvulsants continue to be used for infants being treated for electrographic and clinical seizures in HIE; but questions remain regarding their efficacy and long-term effects on the newborn brain in early development (90, 91).

Emerging therapies for HIE include xenon, erythropoietin, melatonin, and stem cell therapy which are currently being tested in combination with TH to improve outcome (92-95).

1.4 Biomarkers

1.4.1 Overview

To improve clinical research, there is a need for the use of accurate clinical measurement tools to explore disease pathology and human development, i.e. a particular biological state. This includes the use of an extensive range of analytical tools that can measure biological parameters; these are referred to as biomarkers, which the National Institute of Health working group defined as (96):

"A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention."

These biomarkers have been used across *in vitro* cell culture, *in vivo* and clinical research, and can be used as:

1. A diagnostic tool for identification, staging, classification
2. A prognostic indicator
3. Monitoring intervention response
4. A clinical endpoint surrogate

Biomarkers are readily used in the hospital setting today including blood counts, blood glucose, blood pressure, serum low-density lipoproteins (LDL) cholesterol, and p53 gene, amongst numerous others (97-99). The earliest use of the term 'biological marker' dates back to studies in the 1950s, using sex chromatin which was more prominent in female tissue. Sex chromatin was used to examine success rates and proliferation rates in corneal transplants and bone marrow injections (100, 101). The use of biomarkers has increased dramatically over the past decade as 'omic' tools using high-throughput machines have become more cost efficient (figure 1.2) (102).

To move novel biomarkers to clinical practice, they need to be readily available and efficient with minimal training prerequisites to allow medical staff to implement rapidly. They need to discriminate against the disease of interest from the healthy patient and possibly provide novel information about severity, progression, regression, pathophysiology and treatment outcomes. Finally, in best-case scenarios,

the biomarker should be both non-invasive with acquisition being non-deleterious to the patient. In cases where a single biomarker can assist with the diagnosis, but more information is required, it may be more useful to move towards a combined biomarker approach. With this, the use of multiple biomarkers that individually represent various areas of disease pathways may improve the exploration of diagnosis, staging and prognosis (103). Studying complex conditions means controlling for and separating out conditions based on the disease and sample-specific heterogeneity. To develop this area further, the clinical scientist needs to work closely with biostatisticians and bioinformaticians, to explore traditional statistical models and novel machine learning algorithms respectively (104-107).

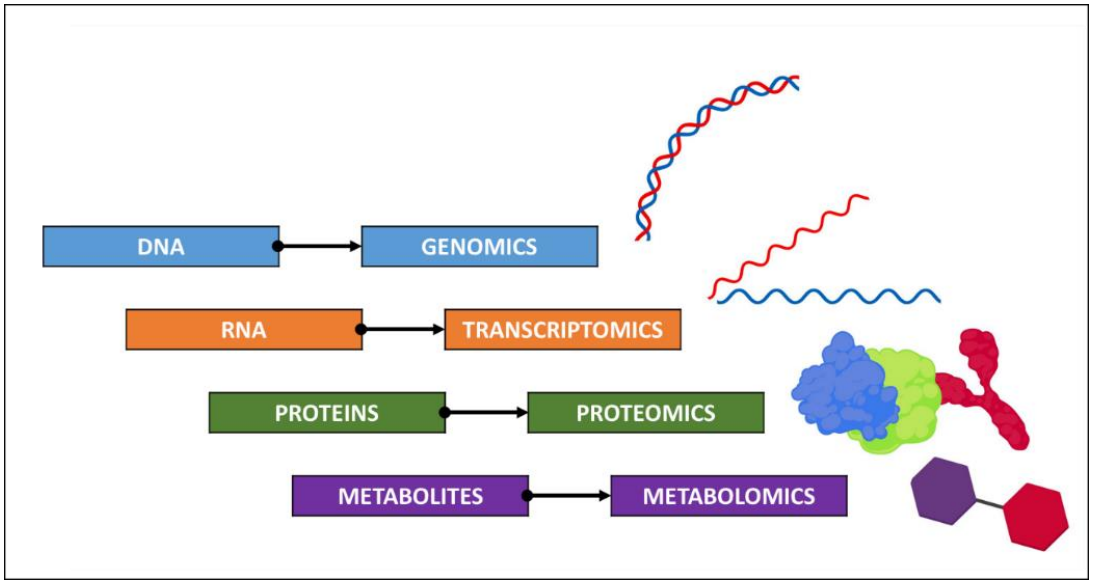

Figure 1.2. Omics. The 'omic' approaches to biomarker discovery across DNA, RNA, proteins, and metabolites.

High-throughput machines are driving the discovery of large quantities of novel biomarkers in multiple areas, but many problems prevent these biomarkers from moving from bench to bedside. High-throughput experiments can create large volumes of unique data; however, these outputs can be interpreted differently, and the clinical scientist and bioinformatician need to optimise the production by carefully choosing the method of normalisation, i.e. size factor normalisation vs global mean normalisation, and also the introduction of false discovery corrections, i.e. Benjamini Hochberg Procedure (108, 109). Following high-throughput discovery, these biomarkers need careful validation across independent cohorts, but ever-changing parameters, techniques, and low-throughput assays, used for testing these

biomarkers, represent a bottleneck in their progress. Changing conditions between laboratories and methods have proven to have significant implications for the end-product. Ideally, biomarkers should be reproducible across labs and with this the development of standardised protocols, methodologies and assays should be put in place. As we move closer towards more molecular and proteomic-based discoveries that exist on a significantly smaller platform we need to understand the considerable impact minor changes may have on the discovery and validation process.

1.4.2 Diagnostic Modelling

Diagnostic modelling is implicit before a diagnostic marker can aid in clinical decision-making, and the last century has seen significant developments in statistical tools for identifying robust biomarkers. It is clear merely demonstrating a statistical difference between case and control may not benefit the identification process for a viable biomarker. One prediction tool commonly used in diagnostic testing and seen throughout this thesis is the receiver-operating-characteristic (ROC), which receives two characteristics and plots them, both the true positive rate (TPR) against the false positive rate (FPR), at various thresholds. Identifying the area under the ROC (AUROC) can provide an optimal value of a model for diagnostic decision-making (104).

Although AUROC can give part of the picture, this can be expanded further by indicating the sensitivity (TPR) and specificity (FPR) values, which are measurements for ‘diseases correctly identified’ and ‘healthy controls correctly identified’ respectively. Following this, further testing can be conducted by establishing the positive predictive value (PPV) and negative predictive value (NPV), which are measurements of tests positive accuracy and tests negative accuracy respectively (110). These similar terms can be easily misclassified, and it is best to think of sensitivity and specificity as a measurement of accurate disease classification, and PPV and NPV as a measurement of test accuracy (figure 1.3).

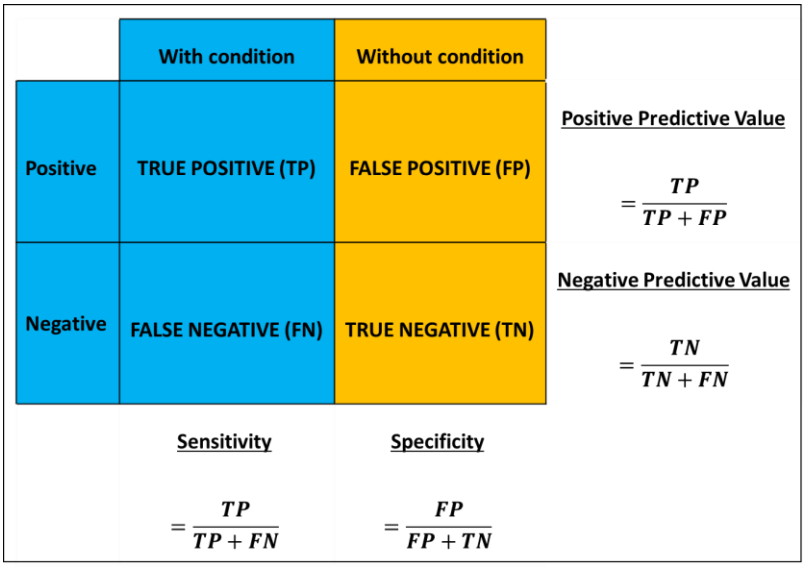

Figure 1.3. Diagnostic predictions. Identification of sensitivity and specificity, positive predictive value (PPV) and negative predictive value (NPV) in diagnostic testing.

1.4.3 Collection, Processing and Storage

Biomarker research aims to establish straightforward cost-effective, minimally invasive clinical tests with high specificity and sensitivity for early detection. Good practice in sample collection, processing, and storage is vital for 'gold standard' biomarker discovery. Unfortunately, ever-changing methodologies can impede these efforts; as significant advances in the field are also accompanied by novel techniques. Access to high-quality samples that are collected and handled in an approach that minimises potential confounding factors is crucial. Defining and testing standard operating procedures during study design and prior to study commencement is critical, as minor alterations to sample processing can have profound effects in analytical reliability and reproducibility (111, 112).

Best practice guidelines are needed before commencing the sample collection stage of early biomarker work. It is worth the time required to carefully decide on the biomarker source as it can have significant implications on the study, as specific biomarker sources can introduce a bias before the study begins, i.e. clotting cascades effect on cytokines (113, 114).

The majority of biobanking in clinical research involves the collection of blood over other common fluids, i.e. cerebrospinal fluid (CSF), urine, saliva, semen, breast milk;

other sources are well-recognised. However some of the biobanking required for these areas are in their infancy (115, 116). Although CSF is a powerful tool with a close relationship to the brain, it falls short of blood due to its invasive nature, often meaning that CSF biomarker studies are inadequately powered, and extensive collaborative efforts are needed for this field to expand further (117).

As changes can occur across laboratory-based methods, staffing, collection, storage, transport, and analysis, there is a need to develop robust and consistent procedures at all stages of biomarker discovery (118). Problems during biofluid collection of blood are spread across temperature, processing times, storage, freeze-thaw cycles, and haemolysis, all which can affect the biomarker being explored.

1.4.3.1 Temperature Monitoring

Temperature monitoring is essential in maintaining good sample quality, with changes in temperature affecting samples at any point from collection to long-term storage, including sample stability which can be both temperature-dependent and temperature-sensitive. For this, the use of both ice and cold storage, and quick and efficient processing will minimise sample degradation. The long-term sample storage temperature for blood at -80°C has been recommended, although short-term storage of DNA and RNA at 4°C and -20°C may be permissible (116, 119, 120). Plasma and whole blood-based miRNA stored at -80°C appear stable during long-term storage, but unlike plasma whole blood risks degradation after 9 months (121). Alternatively from -80°C biobanking, the use of dehydrated samples, dried blood spots, and stabilising reagents are all being considered. The storage of dehydrated DNA and RNA samples at room temperature is promising, but problems can arise as they can be prone to air contamination (122), and dried blood spots currently have issues with viscosity and haematocrit levels which can lead to high variability in sample quality (123, 124).

1.4.3.2 Blood Processing

Choices in collection type between serum and plasma continue to be debated in miRNA, some groups see high correlations between the two mediums, while others have cautioned the effect the coagulation process may have on the miRNA profile (125 , 126). Blood serum, is blood plasma after clotting, and so does not contain fibrinogen or clotting factors, but does contain albumins, globulins, regulatory proteins, electrolytes, antibodies, antigens, hormones and exogenous proteins. The cells and clotting factors must be removed from the sample by allowing adequate clotting time, i.e. 30-60 minutes, without this there's an increased risk of fibrin clots and other cellular contents being present; on the other hand, extended clotting time will result in cellular lysis, and may cause the contamination of sample with additional cellular content (112, 127). Blood plasma contains the same components as serum but also contains fibrinogen and clotting factors. It differs from serum in its collection method as plasma collection tubes include anticoagulants, i.e. EDTA, heparin, and sodium citrate, which prevent the clotting cascade (112). Centrifugation may be required twice to ensure platelet-poor plasma is collected as remaining platelets may predominate or mask extracellular RNA-based profiling; centrifugation speed may also affect the quantity of miRNA (128-130).

1.4.3.3 Haemolysis

Haemolysis is an additional factor that needs to be considered for blood-based sampling, as the release of cellular material into the blood due to haemolysis may affect the downstream analysis (131). Haemolysis and altered blood cell counts can manipulate miRNA biomarker levels (132). For example, in miRNA research, a common reference gene, miR-16, can be significantly altered following haemolysis due to the high levels of miR-16 present in red blood cells. However miR-16 continues to act as a consistent reference gene in non-haemolysed samples, best practice for this is confirmed via free haemoglobin measurement (133). The optimal method to prevent haemolysis is careful venepuncture at the collection, followed by delicate sample handling during processing (134).

Overall, pre-analytical factors such as sample processing, collection, and storage, have far-reaching effects, and careful observation of these promotes good scientific

practice, reduce ‘type I errors’ in biomarker discovery, and enhance the likelihood of future biomarker reproducibility.

1.4.4 Current biomarkers of Hypoxic-Ischaemic Encephalopathy

Multiple biomarkers are currently used to aid in the identification of HIE infants, that detect neonatal seizures, brain function, brain lesions, structural injury, metabolic acidosis and anaerobic metabolism; these include both non-biofluid based and biofluid-based biomarkers, these are used in tandem with the Apgar score to try and evaluate the severity of HIE. The most commonly used biomarkers in clinical practise are cEEG, MRI, cord pH, base-deficit and lactate; although these biomarkers have advantages, they also have disadvantages which prevent them from acting as the perfect robust and reliable biomarker for the diagnosis of HIE (135, 136).

For non-biofluid-based biomarkers the cEEG is useful as its the gold-standard tool for detecting neonatal seizures and can predict the outcome of infants with HIE at 6 hours of age, it can also be used as a classification tool for the EEG grade of HIE; but the tool is costly, requires training for application of electrodes, requires a trained neurophysiologist for interpretation (137-139). The MRI is also a powerful tool for identifying the pattern of injury in HIE and is predictive of long-term injury (22, 140). However similar to the cEEG is costly and requires specialist training for interpretation, further to this it the neonates may not be stationary for the full duration of the MRI affecting image quality (135, 141).

For biofluid-based biomarkers, a number of useful biomarkers are collected from the cord blood gas analyser at birth these are all particularly useful due to the short-time needed for processing and analysis of the sample; cord pH <7.0 is used to predict pathological acidosis, although this can identify acidosis additional information such as base deficit >12 mmol/l is needed to confirm metabolic acidosis (142). Although both are useful for identifying infants with perinatal asphyxia at risk of developing HIE and can act as strong predictors of an adverse outcome, both have difficulty predicting severity of HIE or the long-term outcome (143-145). Finally, cord lactate, which is an end-product of anaerobic metabolism, and a good indicator of tissue hypoxia, can differentiate no HIE/mild HIE vs moderate-to-severe HIE, it can also

complement MRI in identifying infants with poor long-term outcome (146, 147). Limitations to lactate are its gestational age-dependent values can prevent suitable reference ranges and cut-offs, and its inability similar to differentiate mild HIE from moderate HIE (148).

Pilot biomarker studies, see 1.4.5, have demonstrated the potential of cytokines, miRNA, and neuronal-specific molecular markers that could complement current biomarkers and aid in the clinical diagnosis, however to date most research has failed to recruit a large-scale well-defined validation cohort to confirm the novel findings.

1.4.5 Biofluid-based Markers of Hypoxic-Ischaemic Encephalopathy

HIE biomarkers have the potential to give the clinician an early diagnosis, prognosis, and insight into the severity of the newborns injury. For the past two decades, the evolving understanding of HIE pathophysiology has given researchers a focus for candidate biomarkers associated with the injury. This research has examined different aspects of the injury as potential avenues for biomarker discovery, including:

Breakdown of neuronal cells

Necrosis and apoptosis occur throughout the various stages of HIE in a “continuum” (149). These processes lead to the breakdown of the neuronal cells, causing the release of neuronal-specific materials post injury (150). i.e. Brain-derived neurotrophic factor (BDNF), Fas ligand, Neuron-specific enolase (NSE), NR2, and Ubiquitin-C terminal hydrolase L1 (UCH-L1) (65, 151-154). Breakdown of oligodendrocytes can be identified in HIE by measuring myelin basic protein (MBP) which can leak into the CSF following acute injury (155). TNF-α when released from astrocytes and microglia has also been shown to damage oligodendrocyte progenitor cells (OPC) in vitro (156). NMDA receptor excitotoxity can cause apoptosis in OPC and both hypoxic insults and excitotoxity will induce necrosis in astrocytes in animal models of HIE (44, 46, 47).

Excitotoxic effects of excitatory amino acids (EAAs)

EAAs act as neurotransmitters and are critical for the generation of action potentials. Overactivity of EAAs glutamate, aspartate and EAA agonists can bring an excitotoxic effect to the immature brain (157). Multiple EAA receptors in neurons have an increased likelihood to be perturbed during HIE. Early life is a critical period for receptor development and EAA uptake, and when HIE occurs during this period the organisational development and synapse formation may be affected. Additionally, increased intracellular calcium through the activation of the NMDA receptor, and mitochondrial dysfunction leads to EAA-mediated injury (157, 158).

Free radicals and Oxidative stress

Free radicals are atoms with an unpaired electron, i.e. nitric oxide, and reactive oxygen species (ROS), are free radicals directly related to oxygen species, i.e. superoxide anions, and hydroxyl radicals. Free radicals are generated both intracellularly and extracellularly, with complex III mitochondrial ROS production acting as the primary intracellular source (159). The release of free radicals can cause damage to all components of the cell and is one of the hallmarks of HI, such as nitric oxide synthase (NOS) which is a significant source of brain ROS during HIE (160, 161).

Release of cytokines and inflammatory markers

Cytokines are proteins secreted by immune cells, endothelial cells and fibroblasts; cytokines are categorised as pro-inflammatory, anti-inflammatory and growth factors, but all function in intercellular communication and promote recruitment across cells. i.e. C-reactive protein, IL-1β, -6, -8, -10, -16, and TNF-α.

The immature brain is more susceptible to inflammation; Adinolfi and Leviton have described the damage caused by cytokine production, such as TNF-α, following maternal infection, and how this damage can contribute to both white matter injury and preterm birth (162-164). Cytokines can be produced in the placenta, the fetal circulation; and in the fetal brain by both microglia and astrocytes; cytokines have the ability to cross the placenta and the BBB, and subsequently disrupt BBB function

and permeability, which may have long-term consequences on brain development (58, 165-168).

Inflammation occurs temporally across neonatal brain injury and differs from adult injury due the plasticity and development of the immature brain (54). Inflammation in the perinatal period can have long-term effects on neurodevelopmental outcome; and the inflammatory processes triggered can continue over the postnatal weeks, and progress across from proinflammatory, to anti-inflammatory, to reparative and resolution/chronic inflammation (54, 169, 170).

A wide-body of cytokine-related HIE publications began in the 1990s that demonstrated the presence of cytokines in both the brain tissue of HIE rodent models and in biofluids of neonates with HIE and white matter injury, this research together with Leviton's adapted hypothesis has pushed this field ahead although the understanding of newborn inflammation remains incomplete (171). Rodent models demonstrated increased expression of both chemokines and cytokines, IL-1β, IL-6, MCP-1, MIP-1α, MIP-2 and TNF-α mRNA in hypoxic-ischemic brain tissue (52, 172, 173). Models have also demonstrated rapid activation of microglia and mast cells and the accumulation of leukocytes in areas of injury prior to the initiation of secondary injury; innate immune activation is followed by inflammatory injury via the adaptive immune response (54).

Animal models are needed to understand biological processes particularly at the molecular levels however, a bioinformatic-based study on neuroinflammation comparing humans to mice, demonstrated that only 27% of cytokine-cytokine pathway interactions are similar across species; however at the cellular level a higher similarity of 62% of interactions was present (174). Animal models of HIE can commonly use the endotoxin lipopolysaccharide to induce inflammation but it remains difficult to have a faithful animal model of inflammation due to varying development of glia, neurons and oligodendrocytes across species; further to this current literature is contradictory in the use of mouse models in inflammatory diseases (12, 175-177).

Although animal models have continued to display evidence supporting inflammation in HIE, largescale clinical validation of the inflammatory process is still ongoing. Neonatal biofluid studies in HIE and white matter injury described the presence of IL-1β, IL-6, IL-8 TNF-α in amniotic fluid, umbilical cord plasma, and CSF (52, 178-183). Dried blood spots have demonstrated elevated Il-1β, IL-6 and IL-8 at 30 months in HIE infants, cord serum IL-6 was also increased in HIE at 2-year outcome (184, 185). More recent publications such as Bartha and Walsh have progressed cytokine-related biofluid/biomarker research into larger scale studies using chromatography and the Luminex multiplex assay respectively, both of which have shown encouraging results and potential, but subsequent validation research is needed (66, 185, 186).

Multiple neuroinflammation therapies have been tested in animal models of HIE, i.e. minocycline, melatonin and erythropoietin. The effect size of these treatments has been low, and not translated consistently between animal and clinical studies; this can be particularly difficult due to negative effective effects on early brain development.

Biomarker pilot studies to date are primarily focused on the first four days of life and vary between mediums with most focused-on serum and plasma. Although these studies have been conducted temporally throughout the postnatal period, the umbilical cord remains an essential resource as a minimally invasive method for blood collection, where larger volumes can be collected to allow biomarker discovery (table 1.1).

Multiple biomarker studies have looked beyond the biomarkers used in the initial diagnosis and explored its use in long-term prognosis for infants with HIE. These studies look at the candidate biomarkers ability to predict normal vs abnormal outcomes based on death, development of CP, epilepsy and in less severe cases developmental delay. Developmental assessment tools, which screen infant and toddler development, i.e. Bayley Scales of Infant and Toddler Development-III (BSID-III), the Griffiths Developmental Scales and the Denver Developmental Screening Test II, are used to aid in developmental assessment (187, 188). A number of biomarkers

have shown to be increased in infants with poor long-term outcome including copeptin, NSE, S100β, IL-1β, -6, -8, -16, TNF-α, INF-γ, GFAP, and BDNF (189-193).

Table 1.1. Clinical biofluid-based biomarker studies in identification and severity of Hypoxic-Ischaemic Encephalopathy.

Biomarker	Description	Effect	Time point	Medium	References
Activin A	*Homodimeric TGF-β superfamily cytokine that signals primarily through SMAD proteins, to regulate multiple functions.*	**Increase**	0-24 hours (h)	Cerebrospinal fluid (CSF)	(194)
			0 h, 12 h, 24 h, 48 h, 72 h	Urine	(195)
Alanine aminotransferase	*Transaminase enzyme, the transamination reaction it participates in produces pyruvate and L-glutamate. Found predominantly in the liver.*		0-12 h	Plasma	(196)
			0-6 h	Serum	(197)
Amino acids	*Taurine, alanine, tyrosine, methionine, valine, phenylalanine, leucine, isoleucine.*		18 h (cases), 66 h-4.4 D (controls)	CSF	(157)
	Taurine.		16 h (cases) 24 h (controls)	CSF	(198)
Aspartate aminotransferase	*Transaminase enzyme, the transamination reaction it participates in produces oxaloacetate and glutamate. Found predominantly in the liver, cardiac muscle, skeletal muscle, kidneys, brain and erythrocytes.*		0-12 h	Plasma	(196)
Brain-derived neurotrophic factor	*Protein; member of the neurotrophin family of growth factors, related to canonical nerve growth.*		Cord	Plasma	(152)
Cardiac troponin I	*Cardiac regulatory protein controlling calcium-mediated interaction between actin and myosin.*		0-36 h	Serum	(199)
C-reactive protein	*Pentameric plasma protein produced by the liver that increases in response to systemic inflammation.*		Unknown	Serum	(200)
Creatinine kinase muscle-brain fraction	*Enzyme converting creatine to phosphocreatine.*		Cord, 2 h, 6 h, 12 h, 24 h	Serum	(201)
			0-6 h, 48 h	Serum	(202)
			4 h, 10 h	Serum	(203)
			Cord, 6-10 h, 24-30 h, 72-80 h	Serum	(204)
			6 h	Plasma	(205)

Excitatory amino acids	*Aspartate, glutamate.*	**Increase**	18 h (cases), 66 h-4.4 D (controls)	CSF	(157)
			16 h (cases), 24 h (controls)	CSF	(198)
Fas-ligand	*Type II transmembrane protein. Binding with its receptor induces apoptosis.*		0-3 days (D)	CSF	(206)
Glial fibrillary acidic protein	*Cytoskeleton intermediate filament protein specific to astrocytes.*		Cord, 6-24 h, 48-72 h, 78-96 h	Serum	(65)
			0-6 h, 24 h, 72 h, 4 D	Serum	(207)
Interleukin-1β	*Macrophage produced cytokine that's act as an important mediator of the inflammatory response.*		0-24 h	Serum, CSF	(208)
			0-24 h	CSF	(178)
			Cord, D 1, D 3	Plasma	(209)
Interleukin-6	*Pro-inflammatory cytokine that actives multiple inflammatory pathways by inducing transcription factors.*		6-24 h	Serum	(65)
			0-3 D	CSF	(206)
			Cord	Plasma	(66)
			Cord	Serum	(184)
			0-24 h	Serum, CSF	(208)
			Unknown	Serum	(200)
			0-48 h	Plasma, CSF	(210)
Interleukin-8	*Inflammatory chemoattractant cytokine that targets neutrophil recruitment and degranulation. Increased by oxidative stress.*		6-24 h, 48-72 h	Serum	(65)
			0-24 h, 48-72 h	Serum	(67)*
			Cord, D 1	Plasma	(209)
Interleukin-10	*Anti-inflammatory cytokine*		48-72 h	Serum	(67)*
Interleukin-16	*Pleiotropic cytokine functioning as chemoattractant and modulator of T-cell activation. Processed by caspase-3 and has neuronal-specific isoform.*		Cord	Plasma	(66)
Lactate: creatinine			0-6 h, 48-72 h	Urine	(211)

	Lactate Intermediary metabolite, produced in anaerobic metabolism, excreted by the kidney. Creatinine Breakdown product of creatinine phosphate in muscle.	**Increase**	0-6 h, 24 h, 48 h	Urine	(202)
Lactate dehydrogenase	*Enzyme converting lactate into pyruvate.*		0-12 h	Plasma	(196)
Malondialdehyde	*Product of lipid peroxidation.*		0 h, 48 h	Serum	(212)
miR-374a-5p	*miRNA involved in post-transcriptional modification.*		Cord	Whole blood	(213)
Neuron-specific enolase (NSE)	*Dominant enolase-isoenzyme in neuronal and neuroendocrine tissues.*		12 h, 24 h	Serum	(151)
			4-48 h, 5-7 D	Serum	(214)
			D 1	Plasma	(154)
Neuronal nitric oxide synthase (NOS1)	*Synthase that catalyses production of nitric oxide from L-arginine.*		Cord	Plasma	(215)
			0-24 h	CSF	(216)
NR2	*Binding site on NMDA receptor of neurotransmitter glutamate.*		D 1	Plasma	(154)
Nucleated red blood cells (NRBC)	*Progenitors to mature erythrocytes. Present at low levels in infancy.*		0-24 h	Red blood cells	(66)
Phosphorylated axonal neurofilament heavy chain (pNF-H)	*Major subunit of neurofilaments, the main component of axonal cytoskeleton.*		0-6 h	Serum	(217)
Protein carbonyl	*Oxidative stress marker.*		0 h	Serum	(212)
Protein S-100β	*Calcium-binding protein component of cytosol. Predominant in astrocytes and Schwann cells.*		Cord, 2 h, 6 h, 12 h, 24 h	Serum	(151)
			Cord	Serum	(218)
			Cord	Serum	(219)
			0 h	Saliva	(220)
Soluble intercellular adhesion molecule 1 (sICAM-1)	*Protein involved in trans-endothelial migration of leukocytes during inflammation.*		D 3	Plasma	(154)
Troponin T	*Part of troponin complex expressed in skeletal and cardiac myocytes*		0-6 h	Serum	(197)
Tumour necrosis factor-α	*A major pro-inflammatory cytokine involved in early inflammatory events. Triggers series of inflammatory molecules.*		0-24 h	Serum, CSF	(208)
			Unknown	Serum	(200)
			0-24 h	CSF	(178)
			0-48 h	CSF	(210)

		Increase	Cord, D 1	Plasma	(209)
Ubiquitin C-terminal hydrolase L1 (UCH-L1	*Neuronal and neuroendocrine-specific deubiquitinating enzyme. Concentrated in neuronal perikarya and dendrites.*		Cord	Serum	(221)
			Cord	Serum	(65)
			0-6 h	Serum	(217)
Vascular endothelial growth factor (VEGF)	*Inflammatory signal protein associated with increased permeability in the blood-brain barrier.*		48-72 h	Serum	(65)
			0-24 h	CSF	(216)
Secretoneurin	*Highly conserved polypeptide present in large dense-core vesicles of several endocrine, neuroendocrine and neuronal cells.*	**Increase** [1], **Decrease** [2]	Cord [1], 48 h [2]	Serum	(222)
Total serum bilirubin (TSB)	*Compound produced from breakdown of heme.*	**Decrease**	2-5 D	Serum	(223)

*HIE with seizures.

1.5 microRNA

1.5.1 Overview

Francis Crick's central dogma of molecular biology saw DNA transcribed into RNA, and this RNA translated into protein, but this began to change in 1970 when a Nature article by an unassigned author wrote, "The central dogma, enunciated by Crick in 1958 and the keystone of molecular biology ever since, is likely to prove a considerable over-simplification" (224). Already researchers were beginning to find differences in Crick's original vision, and discoveries including reverse transcription of viral RNA into DNA, identification of small RNA molecules and RNA splicing, which continued over the next 20 years (225-227). Following this, in the early 1990s, the first miRNA and small interfering RNA (siRNA) was discovered, at this point, it became clear that Crick's central dogma needed to be expanded as these non-coding RNAs were acting to fine-tune gene expression and adding new layers to the complexity of gene regulation (228, 229).

The first miRNA discovered was in the *Caenorhabditis elegans*, a roundworm model for neuronal development (230). It was named *lin-4* and mediated temporal control of postembryonic development by repressing the *lin-14* protein (228, 231). A few years later both *lin-4* and *let-7* in *C. elegans,* consisting of 22- and 21-nucleotides respectively were found to be conserved across a wide range of species, including humans, implicating these small RNAs as effectors of human development (230). Over the following years, miRNAs were shown to have critical roles as fine-tuning gene regulators in both development and physiology, including cell proliferation, cell death, fat metabolism, hematopoiesis, circadian rhythm and nervous system development (232-235).

It was initially predicted that over one-third of human regulatory genes were conserved targets of miRNA; it's now believed that over 60% of protein-coding genes are targeted (236, 237). In 2004, it was suggested that the total number of human miRNAs was over 800, including ~400-500 conserved miRNAs (238), as of 2018 that number has been far exceeded with registered human mature miRNA now at 2,654 according to the miRBase repository (239).

As research continued in miRNA, researchers began to explore miRNA in human cancers, first described in 2002 (240). Cancer research found miRNA genes were located at both fragile sites and genomic regions involved in cancers, and a general downregulation of miRNA across cancer tissues when compared with healthy tissue. From this, the researchers could classify tumours using miRNA expression profiles, a task that could not be completed with mRNA expression profiles, suggesting a novel role for miRNA profiling in diagnostics (240, 241). At this time, researchers also proposed miRNA had links with genetic diseases, insulin regulation, adipocyte differentiation, and viral infection; and in the last two decades, this has expanded further into immunity, nervous system disorders, psychiatric disorders and metabolic disorders (242-248).

Although significant work was conducted in miRNA profiling of disease-specific tissue, it wasn't until 2008 when researchers described miRNAs present in serum that are stable, reproducible, and consistent in humans that the true diagnostic potential of miRNA was revealed (249). This research went on to describe serum-specific miRNA expressions unique to several cancers, and diabetes, and with this, a novel class of biomarkers were identified (249). In the last decade, this has created an ever-expanding reservoir of miRNA-based publications and patents trying to establish a robust and reliable biomarker that can perform a minimally invasive 'liquid biopsy' for screening, diagnosis and prognosis across thousands of diseases and disorders.

1.5.2 Biogenesis and Mechanism of Action

The mature miRNA sequence that degrades or represses the target mRNA is a short non-coding RNA (ncRNA), approximately 21-25 nucleotides in length; but biogenesis of the mature miRNA begins in the genome. The miRNA genes need several proteins for processing, the main players being RNA polymerase II, RNase III endonucleases (Drosha and Dicer), exportin 5 (EXP5) and Argonaute (AGO) proteins (250). These proteins progress the miRNA gene from its passive state in the nucleus, to being actively involved in the post-transcriptional modification of mRNA targets. The importance of some of these proteins, i.e. Drosha, Dicer, and AGO has been displayed

in embryonic mice models, which showed lethality when deficient of these proteins during embryogenesis due to abnormal placental development (251-254).

The biogenesis process begins when RNA polymerase II transcribes the miRNA gene from introns (or exons occasionally), creating a primary miRNA (pri-miRNA) stem-loop structure, typically over 1 kb (255). This imperfect stem-loop (hairpin) structure is a partially double-stranded RNA molecule with single-stranded overhangs at both ends, with each side of the upper portion of double-stranded RNA containing both the 5' and 3' miRNA end product (250, 256). The next step of processing involves the cleavage of the pri-miRNA by the RNase III endonuclease Drosha that removes the single-stranded RNA 5' and 3' overhangs and part of the double-stranded RNA lower stem, creating a pre-miRNA complex (257). The pre-miRNA made up of the remaining upper portion of the stem-loop structure approx. 70bp is exported from the nucleus by the exportin 5 (XPO5) protein; interestingly export of pre-miRNA is increased following DNA damage (250, 258, 259).

Once the pre-miRNA has left the nucleus, it is cleaved by another RNase III endonuclease called Dicer, which removes the loop structure forming a small RNA duplex, which is loaded on the AGO protein, in an ATP-dependent step, to create a complex called the RNA-induced silencing complex (RISC) (260). After loading, the guide strand (be it the 5' or 3' miRNA strand) is retained in RISC, and the passenger strand is released and quickly degraded, leaving a mature miRNA RISC complex. Strand selection between the 5' and 3' miRNA can vary, and the less thermodynamically stable miRNA is generally chosen as the guide strand (261, 262).

For mRNA target degradation to occur, the miRNA binds to the 3'UTR of the mRNA target with perfect complementarity. However, this is not the standard mechanism in mammals. In most cases, human mature miRNA complexes regulate gene expression in a sequence-specific manner by binding to the target 3' untranslated region (3'UTR) with imperfect complementarity and function as translational repressors; this involves perfect base pairing of nucleotides in the 'seed region', i.e. nucleotides 2-8 of the miRNA. Additionally, good pairing in miRNA nucleotides 13-16 will further assist translational repression, along with additional factors (263-265) However, the exact mechanism for mRNA post-transcriptional repression by miRNAs

is still unclear (266). Although the functional connection is not clear, it's been shown that the mature RISC complex and translationally repressed mRNA targets are localised to processing bodies (P-bodies) for further mRNA degradation (figure 1.4) (267).

miRNA can act on and repress multiple mRNA targets, and multiple miRNAs can act on and repress an individual mRNA target (268). In addition, in some cases, miRNA have been shown to upregulate their mRNA targets (269). It is important to recognise that miRNAs are suspected to regulate the majority of cellular processes, and change in response to injury, developmental or disease processes; miRNA are under complex regulation and exist in a tissue-specific and cell type-specific manner (243, 270, 271). It is also important to be aware that many unknowns appear across the miRNA field in biogenesis, regulation, and function. This is an exciting area of research where each answer seems to be loaded with multiple new questions (268, 272).

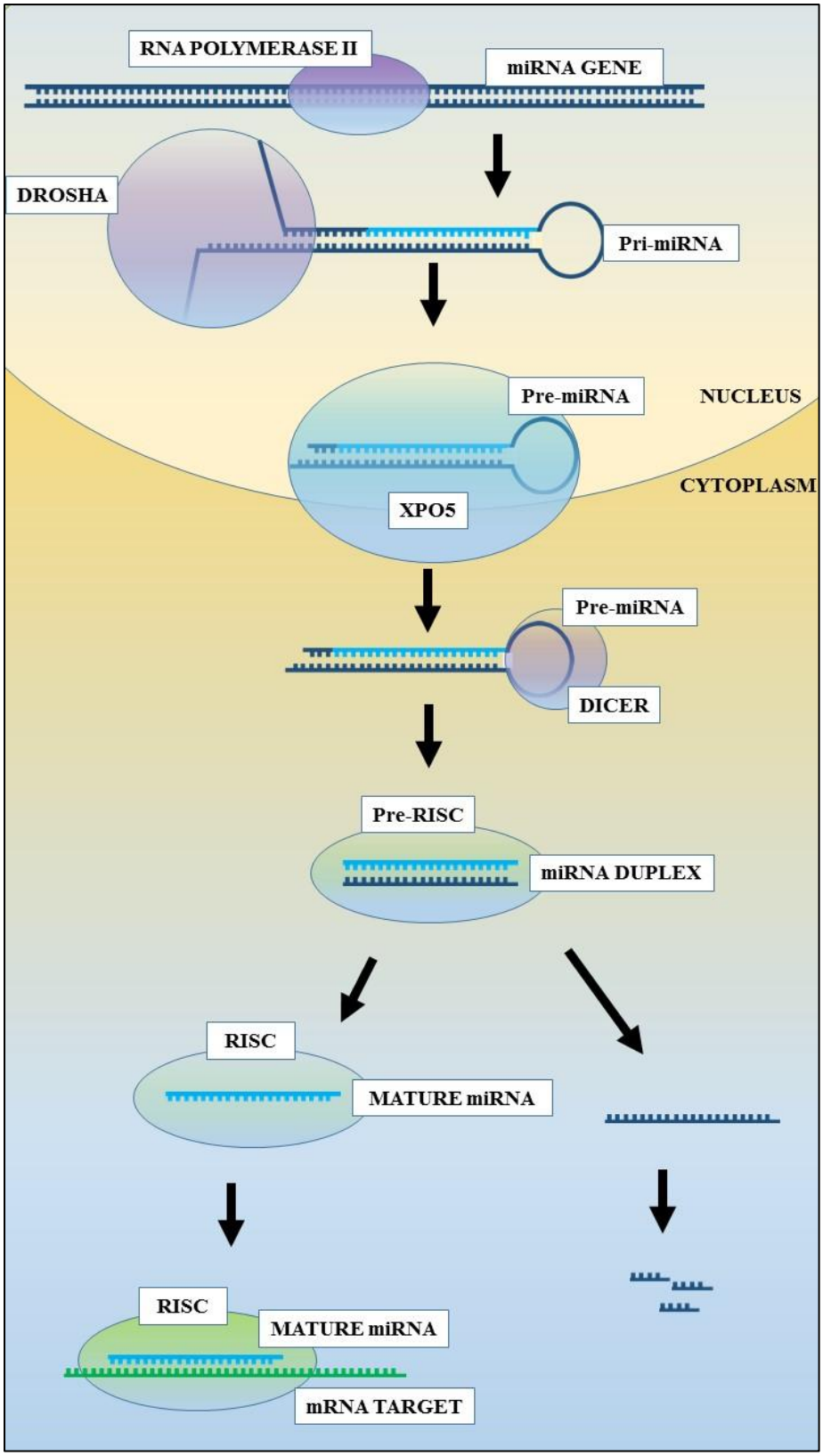

Figure 1.4. microRNA biogenesis. RNA polymerase II processes the microRNA (miRNA) gene. The RNA Polymerase II enzyme Drosha cleaves the pri-miRNA overhang. The pre-miRNA is exported from the nucleus by the exportin-5 (XPO5). The stem-loop is cleaved from the pre-miRNA by the RNA polymerase II enzyme Dicer. The miRNA duplex is loaded into the RNA-induced silencing complex (RISC). The guide strand (mature miRNA) is maintained in the RISC complex for target mRNA repression or degradation.

1.5.3 Circulating microRNA and mRNA as Biomarkers

When miRNA are explored beyond the cellular scope in biofluids, we begin to appreciate further complexities in the miRNA field. miRNA not only appear to be present in biofluids but appear ubiquitous and stable across all fluids, i.e. plasma, cerebrospinal fluid, urine, breast milk, colostrum, seminal fluid, tears, saliva, amniotic fluid, peritoneal fluid and bronchial lavage (273). Whole blood-based cellular miRNA have also been shown to be stable (274).

Circulating miRNA has been shown to be protected from RNase degradation through either protein-binding or extracellular vesicles. Protein-bound miRNAs are the predominant form of extracellular miRNA. However a smaller portion of miRNA are transported in extracellular vesicles (275, 276). Protein-bound miRNA include those bound to AGO proteins, high-density lipoproteins and low-density lipoproteins. Extracellular vesicles that contain miRNA include high molecular weight complexes, membrane fragments, exosomes, microvesicles, and microparticles (figure 1.5) (9, 277). Terms for these extracellular vesicles can be confused and merged under one umbrella term but they exist in their own right under restrictive ‘size’, ‘marker’ and ‘function’ definitions (9-11). The extracellular vesicles have been suggested as specific cell-to-cell RNA delivery systems (278, 279). Some open questions regarding the specific role of circulating miRNA remain: are miRNA actively or passively released from the cells? Do miRNA appear in the biofluids due to apoptotic budding? Are miRNA involved in a form of intercellular communication?

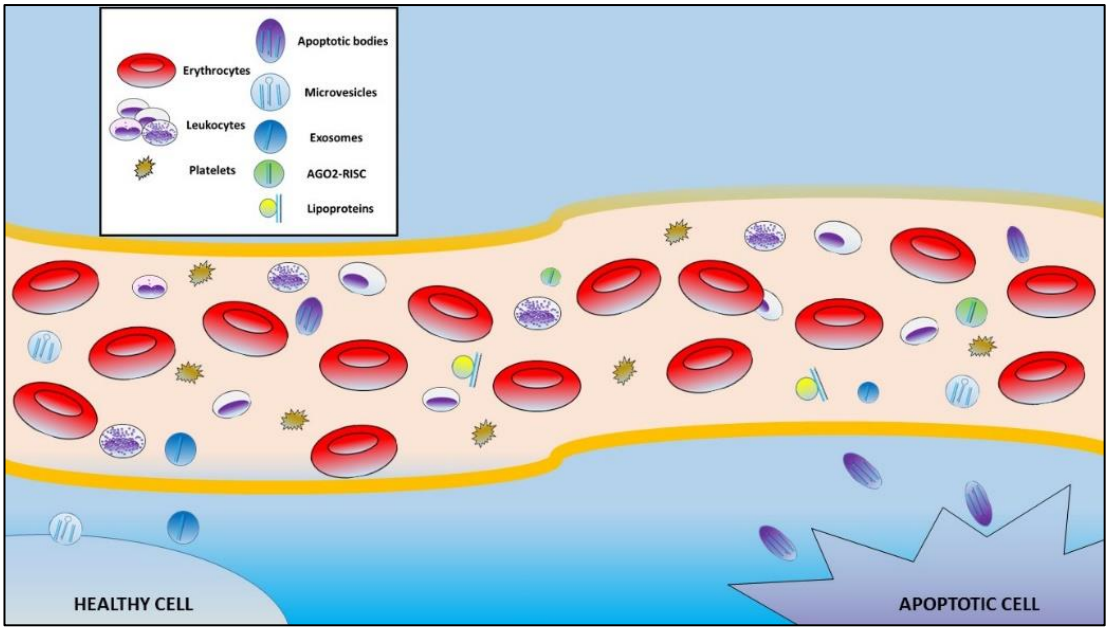

Figure 1.5. Circulating microRNAs. The various sources of circulating microRNAs.

Beyond the potential miRNA mechanisms of transport and circulation, it is their stability in biofluids that make miRNA particularly attractive as detectable and quantifiable biomarkers for the disease process. In 2008, several groups brought this idea forward proposing circulating miRNA may be a viable biofluid biomarker for various cancer types (249, 280, 281). Following this, the circulating miRNA biomarker field exploded, with the volume of publications increasing each year. Circulating miRNA biomarkers have been explored across cancer, fetal programming, birth injury, neuropsychiatric disorders, neurodegenerative diseases and non-communicable diseases (213, 277, 282-284).

1.5.3.1 Circulating mRNA as Blood-based Biomarkers

Alongside miRNA, circulating mRNA have also been explored as promising biomarkers of injury (285, 286). Both miRNA and mRNA may exchange transcellular genetic information via exosome-mediated genetic exchange across cells (278, 287). Exosomal mRNA is fragmented making it smaller compared to normal mammalian mRNA but longer than both pre- and mature miRNA (288). Whole blood-based mRNA, similar to miRNA, includes mRNA from erythrocytes, leukocytes, platelets, and circulating via exosomes, meaning mRNA -based biomarker discovery may be another rich source of information during a critical event.

1.5.4 Techniques and Tools for miRNA Research

Multiple techniques and tools exist for the study of circulating miRNA profiling, in most cases for detection and quantification the miRNA must be isolated from the blood sample and go through high-throughput discovery, i.e. next-generation sequencing (NGS) and microarray hybridisation. After mass quantities of data are produced, these methods require computational and statistical expertise to normalise and correct for the large data output. This data output could give insight into the altered miRNA expression across a large panel of miRNA, something that can prove difficult with low-throughput methods. Low-throughput validation involves the qPCR method, this is the more common approach for studying miRNA across laboratories, as it is more cost effective, and can bypass discovery work based on potential biological significance and previous literature in the field. For terminologies and definitions used across miRNA profiling studies (table 1.2).

Table 1.2. Terminologies and definitions applied in blood-based microRNA profiling.

Name	**Description**
microRNA (miRNA)	*Small non-coding 21-25 RNA involved in the post-transcriptional modification of mRNA.*
miRNA precursor (pre-miRNA)	*Stem-loop miRNA includes two arms, both -5p and -3p, and is cleaved by the Dicer enzyme to produce mature miRNA.*
miR-1-5p, miR-1-3p	*-5p, -3p indicate sense and antisense arms respectively of miRNA precursor before an arm is processed into the mature miRNA; normally one arm is processed, but in certain situations, both can be processed and functional.*
miR-1a, miR-1b, miR-1c	*Letters following the number indicate same 'miRNA family'.*
iso-miR-1	*Minor variations of established pre-existing annotated miRNAs.*
Seed region	*Nucleotides 2-8 from 5' end of the miRNA; seed region is critical for target recognition; matching seed regions between miRNA indicate 'miRNA family'.*
miRNA gene clusters	*miRNA genes clustered in tandem within the genome; may collectively contribute to the regulation of a set of mRNA targets; normally co-transcribed*
messenger RNA (mRNA)	*A large family of RNA molecules that supply ribosomes with genetic information from DNA to produce protein synthesis.*
3'-untranslated region (UTR)	*A region of the mRNA molecule near the poly(A) tail, it's not translated into a protein and can contain regulatory regions that post-transcriptionally regulate gene expression.*
Microvesicles	*Large 50-1000 nanometres (nm) extracellular vesicles in the eukaryotic fluid that contain both mRNA, miRNA and proteins.*
Exosomes	*Small 30-100 nm cell-derived vesicles in the eukaryotic fluid that can contain both mRNA, miRNA and proteins.*
Lipoproteins	*Lipoproteins that transport lipids around the body; these can also contain and transport mRNA and miRNA.*
Serum	*The component of blood remaining following clotting post-centrifugation after removal of erythrocytes and leukocytes and activated clotting factors.*
Fibrin clot	*A clot formed in blood serum following incomplete clotting prior to experiment or storage of serum sample.*
Plasma	*The component of blood remaining post-centrifugation after removal of erythrocytes and leukocytes (buffy coat); it contains an anticoagulant, and so plasma contains inactivated clotting factors.*
Buffy coat	*The fraction of blood containing leukocytes in a plasma sample post-centrifugation; resting between plasma and erythrocytes.*
Whole blood	*All components of blood including erythrocytes, leukocytes, plasma, and clotting factors.*
Haemolysis	*Rupturing of erythrocytes during serum, plasma or whole blood collection, due to incorrect need, tubing, mixing, filling, or excessive forces, this can affect experimental testing.*
High-throughput	*Large data acquisition, usually from a small sample set; generally used in the biomarker discovery stage, as it is both costly and may contain a large volume of false positives, i.e. next-generation sequencing and microarray.*
Low-throughput	*Small data acquisition, usually from a large sample set; generally used in the biomarker validation stage as it both cheaper and more accurate, i.e. qPCR.*
RNA extraction	*Purification of RNA from biological cells, tissues and fluids.*

Spectrophotometer	*Used for absorbance measurements of a molecule, such as nucleotides, RNA and DNA.*
RNase contamination	*Environmental and biological ubiquitous ribonucleases that degrade RNA samples during RNA extractions.*
260/280 and 260/230	*The ratio of absorbance at 260 nm and 280 nm, and at 260 nm and 230 nm respectively, are used as the primary (260/280) and secondary (260/230) measures of nucleic acid purity in DNA and RNA, following DNA and RNA extractions.*
RNA integrity number (RIN)	*The RIN is an algorithm for assigning integrity values to RNA measurements during electrophoresis to determine the quality of the RNA.*
Quantitative Real-Time polymerase chain reaction (qPCR)	*The low-throughput method that monitors the amplification of a targeted complementary DNA (cDNA) or DNA molecule in real-time.*
TaqMan method	*Hydrolysis probes designed to increase the specificity of quantitative PCR.*
SYBR Green method	*A dye for the quantification of double-stranded DNA in quantitative PCR.*
Absolute quantification	*Quantification using the exact number of target RNA molecules by comparison with RNA standards using a calibration curve.*
Relative quantification	*Quantification using reference genes to determine fold-change differences in expression of the target gene.*
Housekeeper/reference gene	*Cellular and extracellular genes with consistent expression under normal and pathophysiological conditions.*
RNA spike-in control	*Adding a transcript of known sequence and quantity during RNA isolation or cDNA step, the spike-in is measured during qPCR to ensure normal expression across the sample set.*
Microarray	*A high-throughput method where RNA is converted to fluorescently labelled cDNA and is hybridised with multiple probes, measurement of the hybridised chemiluminescent reaction allows quantification of the nucleic acid abundance.*
Next-generation sequencing	*A high-throughput method where RNA sample is converted to cDNA, fragmented, and ligated with an adaptor sequence. Modified cDNA is added to a chip with multiple probes and clusters are generated. Next, primers and modified fluorescent nucleotides are added. After each round of synthesis, the fluorescence is recorded using wavelength to identify bases on each parallel sequence. The process is repeated until the sample is fully sequenced.*
False discovery rate	*A statistical method for correcting false positives on large datasets, but is less stringent than more common methods, i.e. Bonferroni correction.*
Fold change	*Increase or decrease of times miRNA of interest in the case group compared to the control group, i.e. '2-fold increase in miR-X' is equivalent to 'miR-X is 2 times higher in cases than controls'.*
Bioinformatics	*Methods and software tools for understanding biological data using biology, computer science, mathematics and statistics.*
Gene ontology (GO)	*Bioinformatics initiative to conglomerate gene and gene product data across all species, including cellular components, molecular function, and biological process.*

1.5.4.1 Isolation of RNA

miRNA extraction and isolation are performed immediately after blood processing, or following thawing of the frozen sample. Four universal techniques can accomplish the RNA isolation: filter-columns, magnetic beads, organic extractions, and the cell-lysis method. In some situations, these techniques may be amalgamated to optimise and purify the RNA isolate, i.e. chemical extraction followed by filter-columns. These techniques each come with strength and weaknesses, for example, the organic extraction method can be difficult to automate, and the direct lysis method, once correctly optimised may be preferred for NGS, but this sample will not be applicable for qPCR as it is reliant on concentration and will not produce a spectrophotometric yield (289, 290). Comparisons across quality of different kits can be difficult as numerous confounders exist due to events prior to isolation and potential laboratory bias. Previous research has suggested that Exiqon miRCURY Biofluids Kit (Exiqon, Denmark, following the acquisition; Qiagen, Germany) and Qiagen Serum/Plasma kit (Qiagen, Germany) both perform superior to other isolation methods; but other groups have drawn alternate conclusions, finding the Macherey-Nagel kit superior (Macherey-Nagel, Germany) (128, 291).

Following RNA isolation RNA quantity and quality is assessed across two mediums, both the NanoDrop spectrophotometer for RNA quantity (NanoDrop Technologies, Germany) and the Bioanalyser chip for RNA quality, this is completed using an RNA integrity number (RIN) value (Agilent, USA). The NanoDrop measures the RNA yield but also takes primary and secondary measures of the absorbance ratio at 260 nm/280 nm, and 260 nm/230 nm for assessing RNA purity(292). The Agilent Bioanalyzer is the recommended method for RNA quality which creates a RIN value from 1-10, with 10 implying intact and non-fragmented RNA and 0 implying poor and fragmented RNA (293)

1.5.4.2 Microarray and Next-Generation Sequencing

When exploring high-throughput methods, it becomes clear that two methods are competing for the researcher's attention. Both microarray hybridisation and NGS provide the researcher with vast quantities of data that would have taken years to

produce previously. These methods have significantly more implications across diagnostic/clinical setting over a molecular biology lab, they give the clinical researcher new insight into the disease process, although this opaque view will need low-throughput validation, to clarify and cement these discovery platforms.

The microarray is based on detecting complementarity between nucleic acids in the sample and pre-designed oligonucleotide probes. One of the original issues with miRNA hybridisation was finding an optimal temperature as variations were present across miRNA, some microarrays have dealt with this problem by using locked nucleic acid (LNA) technology, which has removes the temperature bias due to its higher melting point; however an issue that remains is cross-hybridisation between similarly annotated miRNA (294, 295). The microarray platform may seem outdated compared to NGS but has been readily and stably used across labs for two decades as NGS technologies have developed in parallel (296). Microarrays involve less time for sample preparation, a more straightforward data analysis process, and remains more cost-effective, particularly when it comes to large sample groups. Groups have used microarray data to create blood-borne miRNomes' of human diseases, by creating disease profiles across multiple disease processes, and linking these back to disease-specific genetic variants (297).

The NGS platforms have been in constant development for the past few years between multiple competing companies, including Illumina, Life Technologies and Roche 454 (298, 299). NGS is a form of massively parallel sequencing that can sequence the entire genome, transcriptome, and miRNOME. The sequencing is performed across four steps: library preparation, cluster generation, sequencing, and data analysis. The NGS performance is superior to a microarray for novel miRNA discovery as it will the sequence the entire miRNome, unlike microarray which is reliant on its probe-specific design, making it impossible for microarray to discover novel miRNA, albeit the annotated miRNA may be novel to the disease process (300).

1.5.4.3 Computational and Bioinformatic Approaches

Expertise in bioinformatics and computational predictive algorithms are required to comprehend the vast quantities of data produced by the high-throughput workflow,

decipher potential miRNA-mRNA interactions, and investigate the gene enrichment datasets. The miRNA biomarker of interest can be explored *in silico* to interpret the miRNA gene locus, cell-specificity, and potential downstream targets (301). Findings in this area may assist in linking the miRNA back to the disease or injury process.

Although many databases and tools exist to date, specific miRNA databases and predictive tools stand out due to their high specificity, comprehensive design, and regular maintenance and upgrades, including miRBase, DIANA Tools, TargetScan, and miRWalk (302 , 303). An outline of current tools is given below:

- miRBase (http://www.mirbase.org/) is an annotation tool for high confidence miRNAs including ID, sequence, miRNA loci across species, precursor miRNA sequences, links to potential targets and references. It is managed by the University of Manchester, UK, and was updated in 2018 (239, 301).
- DIANA Tools (http://www.microrna.gr/) provides algorithms, databases and software for interpreting data and it's separated into microT-CDS and microT v4 (miRNA target prediction), TarBase v7.0 (Experimentally supported miRNA targets), and mirPath (Incorporating miRNA in pathways). It is managed by the University of Thessaly, Greece, and was updated in 2018 (304).
- TargetScan (http://www.targetscan.org/) predicts miRNA targets by searching conserved 8mer, 7mer and 6mer sites on mRNA that match the miRNA seed region. It is managed by the Whitehead Institute, Massachusetts Institute of Technology, USA and was updated in 2018 (236).
- miRWalk (http://mirwalk.umm.uni-heidelberg.de/) is. It is managed by the Medical Research Center, University of Heidelberg, Germany and was updated in 2018 (305).

Gene set enrichment, over-representation, and network topology-based analysis is essential for investigating target gene ontology (biological process, cellular component, and molecular function) and KEGG pathways (knowledge of molecular interaction, reaction and relation). DAVID and WebGestalt are both functional gene enrichment tools that use gene lists to generate a biological interpretation of high-

throughput data to gain biological insights. Both sites are managed in the USA and have been updated in 2018 and 2017 respectively (306, 307).

Careful consideration and caution are required when exploring predictive web tools as well-established tools and databases can be shut down or fail to be regularly maintained leading to downstream errors (308, 309). Another consideration is errors in gene names from *in silico* database outputs; this has been seen across 20% of publications within supplementary lists (310). *A* limitation of *in silico* work is that most predictions will need low-throughput experimental validation to confirm expression, this is important for both miRNA and the respective mRNA targets (264).

1.5.4.4 Quantitative Real-Time Polymerase Chain Reaction

qPCR is the universal low-throughput validation method for studying miRNA expression. Although some studies can use the rather labour intensive Northern blot method which can present pre-miRNA amounts and ratios, miRNA variability and modifications, most clinical labs prefer the use of qPCR as it's a time-efficient measure of gene expression with high sensitivity and low sample input (295). There are five chemistries for qPCR amplicon detection: DNA-binding fluorophores, linear oligoprobes, 5' nuclease oligoprobes, hairpin oligoprobes, and self-fluorescing amplicon; from these only two chemistries, have been carried forward into miRNA detection (311, 312). The DNA-binding fluorophores chemistry uses the SYBR Green method and the 5' nuclease oligoprobes chemistry uses the TaqMan method. The SYBR Green method uses a fluorescent dye that fluoresces upon dsDNA binding and so the fluorescence increases after each cycle allowing a measure for amplification, although this method can be prone to SYBR-green binding to primer-dimers or non-specific products. The TaqMan method uses a fluorescent reporter and quencher resting on adjacent nucleotides, as Taq polymerase cleaves in a 5'→ 3' direction the fluorescent probes is separated from the quencher allowing fluorescence which measures the amount of product produced, this method does not generate fluorescence from primer-dimers or non-specific products(312).

Quantification of extracted RNA is completed using a spectrophotometer as described in section 2.2.4.1; this is essential to ensure the same quantities of RNA

(ng/µl) are used across samples, this along with absolute consistency in both cDNA and PCR kits across the study helps ensure precision (313). Careful control measures can be put in place from RNA to cDNA to PCR step to ensure best practice, including assaying samples in triplicate to verify precision and expression stability, cDNA spike-in to ensure consistency from cDNA to qPCR across samples, negative enzyme control (NEC) to assess DNA contamination, and negative template control (NTC) to evaluate primer-dimer contamination. All of which can help for quality control the workflow of the qPCR process for best results.

Good quality qPCR results are dependent on stable and reliable normalisation methods. Although global, quantile and size factor specific normalisation is used for high-throughput miRNA profiling, the most well-recognised form of qPCR normalisation is relative quantification using $2^{-\Delta\Delta CT}$, which creates values based on reference genes (housekeeping genes) being used as internal controls (314, 315). However, differences can arise for reference genes across different tissue-types and blood-based mediums, and so careful pre-testing or previous validation of stability is necessary for all reference genes in miRNA. Some common miRNA reference genes include miR-16, miR-223, and SN48 (213, 316-318). RNA isolation kits may consist of a synthetic spiked-in cel-miR-39 RNA, which can be useful for sample recovery but cannot be used as a type of normalisation method in qPCR steps, although some groups continue to do so (128).

1.5.4.5 Exploring miRNA and miRNA Target Activity

Cell culture modelling is required to investigate the activity of the miRNA and its respective mRNA target. A common way of monitoring a miRNA-mRNA interaction involves the use of reporters, such as dual-luciferase reporters (295). These typically involve two fluorescent reporters, one, which can be used as a normalisation control, and the other, which acts in place of a specific miRNA 3'UTR binding site. Expression of the specific miRNA that targets the 3'UTR binding site would cause binding at the aforementioned binding site preventing the reporter from fluorescing but would not affect the normalisation control, providing evidence of complementary binding (319). It is crucial to note complimentary binding *in vitro*, does not necessarily implicate *in vivo* binding.

Further studies beyond proof of miRNA-mRNA interactions can be conducted with artificial short-interfering RNA (mimics), short-hairpin RNA (shRNA), and miRNA inhibitors, these can be investigated *in vitro* and *in vivo* and include overexpression, miRNA inhibition and the blocking of the entire pathway. The RNA interference (RNAi) is currently being assessed as part of the drug discovery pipeline, including for the treatment of Duchenne muscular dystrophy (figure 1.6) (320, 321).

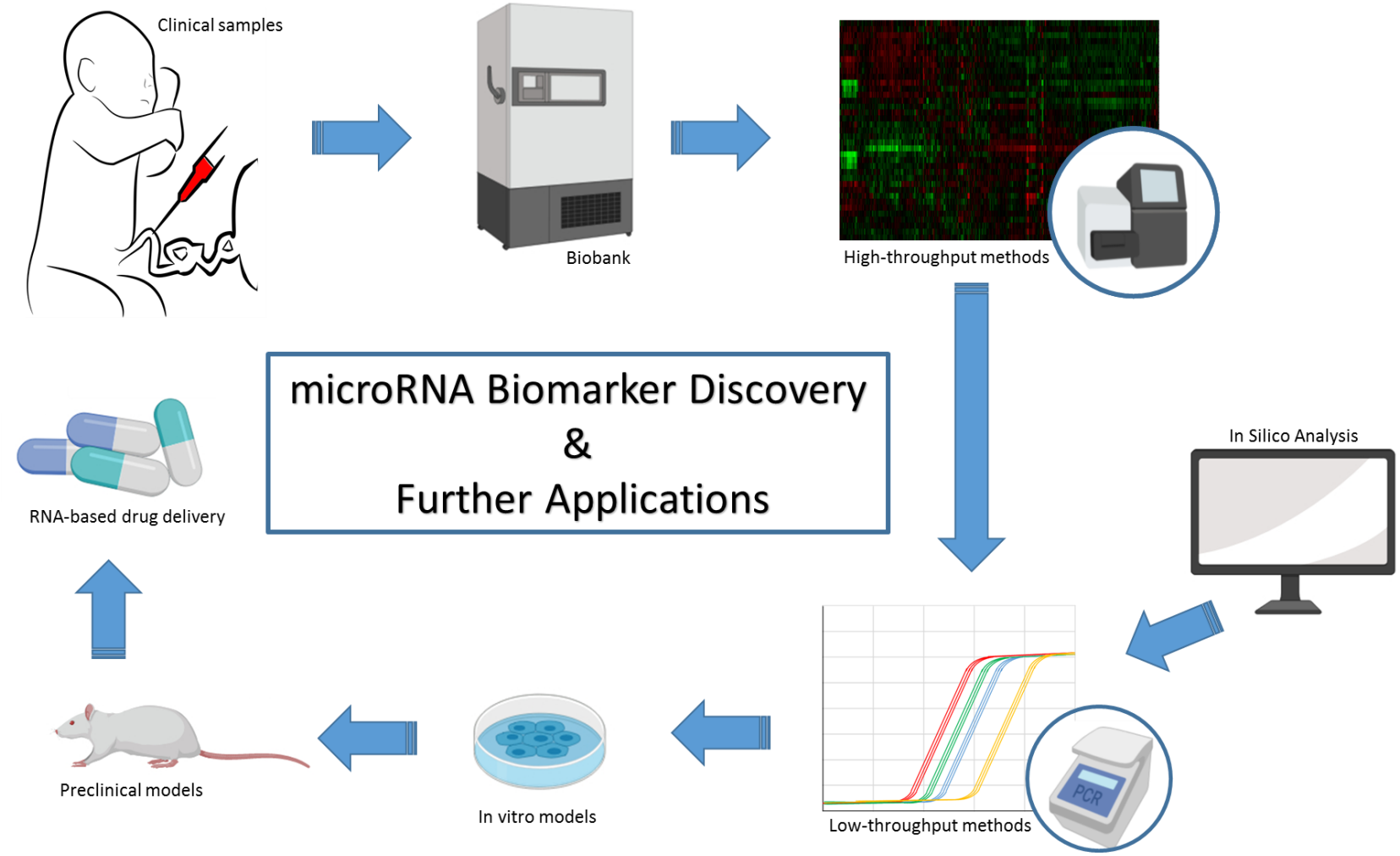

Figure 1.6. microRNA biomarker discovery and further applications.

1.5.4 microRNA in Hypoxic-Ischaemic Encephalopathy

miRNA function at the molecular level by affecting mRNA, with downstream effects on protein pathways during development and recovery following injury. A number of miRNAs have been previously reported in acute cellular hypoxia (table 1.3).

Although miR-210, which is coined “the master hypoxamir”, appears as the most studied miRNA in HIE, the studies have focused primarily on the neonatal rat models and cell culture (322). miR-210 is considered of particular interest as a miR-210 mimic has reduced the expression of junction protein occlusion and β-catenin in HI rat pup brains, which shows the ability of miR-210 to disrupt blood-brain barrier integrity and potentially contribute to cerebral oedema associated with HIE (323). To our knowledge miR-374a appears to be the only miRNA to date reported to be altered in clinical studies. Studies of miR-210 were inconclusive, and most reports have been in acute tissue hypoxia post-myocardial infarction or within necrotic malignant tissue (213, 324). miR-374a has been consistent across both a clinical and porcine model of HIE; however, miR-374a studies have observed alterations in the bloodstream, but not in brain tissue which may implicate miR-374a as a biofluid-specific marker of HIE.

Alterations in miR-374a are of particular interest due to its association with Wingless and NT-1 (Wnt)/β-catenin signalling in cancer metastasis, where miR-374a suppresses the negative signalling regulators Wnt inhibitors factor, phosphate and tensin homolog, and WNT5A (325). Wnt signalling is a major signalling system involved in regulating embryonic growth and development, cell differentiation, proliferation, migration and polarity (326). Wnt activity regulates adult neurogenesis, and neurogenesis is stimulated post HIE injury (327-329). Wnt/β-catenin signalling has been described in human neonatal tissue where high Wnt activity prevents degradation of beta-catenin and can inhibit pre-oligodendrocytes from differentiating into mature oligodendrocytes (330).

Table 1.3. microRNA in Hypoxic-Ischaemic related brain injury.

miRNA	**Associated Target(s)**	**Model(s)**	**References**
Let-7f	NDRG	Cell culture model; clinical biofluids	(324, 331)
miR-124	-	Murine model	(332)
miR-132	*FOXO3; Bax; Cytochrome c; caspase-9; Bcl-2*	Cell culture model; murine model	(332, 333)
miR-136	*TIMP3*	Murine model; cell culture model	(334)
miR-137	*Notch1*	Murine model; cell culture model	(335)
miR-17-5p	*TXNIP; NLRP3; caspase-1; IL-1β*	Murine model	(336)
miR-181b/c	*TLR4; NF-κB; TNF-α; IL-1β; iNOS*	Murine model; cell culture model	(337, 338)
miR-203	*MyD88*	Cell culture model	(339)
miR-210	*Bax; Bcl-2; Caspase-3; Caspase-9:GR; BDNF; TrkB*	Murine model; cell culture model	(324, 340-343)
miR-21	*Cyclin D1*	Cell culture model	(344)
miR-23a/b	*Apaf-1*	Murine model; cell culture model	(345)
miR-24	-	Cell culture model	(346)
miR-27a/b	*Apaf-1*	Murine model; cell culture model	(345, 347)
miR-30d-5p	-	Murine model	(348)
miR-325-3p	*Aanat*	Murine model	(349)
miR-337-3p	-	Murine model	(350)
miR-374a-5p	-	Clinical biofluids; porcine model	(213, 324, 351)
miR-376b-5p	*HIF-α; VEGFA; Notch1*	Murine model	(352)
miR-519	*HIF-α*	Murine model	(353)

To date, miR-374 has shown to be a promising HIE biomarker but needs to be validated in an independent cohort. It remains unclear if more cord whole blood-based miRNA are significantly altered in HIE. Furthermore, the specific function these miRNA play in the cord remains unclear; it also remains unknown if predicted downstream mRNA targets may be altered in cord whole blood. Both validation and downstream exploration are critical to provide a reliable, validated, and quantifiable biomarker that could support both clinical decision-making and assist in early-targeted intervention. Understanding of both miRNA and predicted downstream targets could provide insight into injury development and potential mechanisms of action.

1.6 Aims of Thesis

The specific aims of this thesis are:

1) To identify novel UCB miRNA biomarkers and potential downstream mRNA targets of perinatal asphyxia, HIE and long-term outcome.

2) To validate previously identified UCB inflammatory biomarkers and miRNA biomarkers in perinatal asphyxia, HIE, and long-term outcome.

Chapter 2

Validation of Altered Umbilical Cord Blood microRNA Expression in Neonatal Hypoxic-Ischemic Encephalopathy

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2.1 Abstract

Background

Neonatal hypoxic-ischemic encephalopathy (HIE) remains a significant cause of neurologic disability. Identifying infants suitable for therapeutic hypothermia (TH) within a narrow therapeutic time is difficult. No single robust biochemical marker is available to clinicians. To assess the ability of a panel of candidate miRNA to evaluate the development and severity of encephalopathy following perinatal asphyxia (PA).

Methodology

This validation study included 2 cohorts. For the discovery cohort, full-term infants with PA were enrolled at birth to the BiHIVE1 study (2009-2011) in Cork, Ireland. Encephalopathy grade was defined using early electroencephalogram and Sarnat score (n = 68). The BiHIVE1 cohort also enrolled healthy control infants (n = 22). For the validation cohort, the BiHIVE2 multicenter study (2013-2015), based in Cork, Ireland (7500 live births per annum), and Karolinska Huddinge, Sweden (4400 live births per annum), recruited infants with PA along with healthy control infants to validate findings from BiHIVE1 using identical recruitment criteria (n = 80). The experimental design was formulated prior to recruitment, and analysis was conducted from June 2016 to March 2017.

Results

From 170 neonates, 160 had qPCR analysis. The BiHIVE1 cohort included 87 infants (21 control infants, 39 infants with PA, and 27 infants with HIE), and BiHIVE2 included 73 infants (control [n = 22], PA [n = 26], and HIE [n = 25]). The BiHIVE1 and BiHIVE2 had a median age of 40 weeks (interquartile range [IQR], 39-41 weeks) and 40 weeks (IQR, 39-41 weeks), respectively, and included 56 boys and 31 girls, and 45 boys and 28 girls, respectively. In BiHIVE1, 12 candidate miRNAs were identified, and 7 of these miRNAs were chosen for validation in BiHIVE2. The BiHIVE2 cohort showed consistent alteration of 3 miRNAs; miR-374a-5p was decreased in infants diagnosed as having HIE compared with healthy control infants (median relative quantification, 0.38; IQR, 0.17-0.77 vs 0.95; IQR, 0.68-1.19; P = .009), miR-376c-3p was decreased in

infants with PA compared with healthy control infants (median, 0.42; IQR, 0.21-0.61 vs 0.90; IQR, 0.70-1.30; P = .004), and mir-181b-5p was decreased in infants eligible for TH (median, 0.27; IQR, 0.14-1.41) vs 1.18; IQR, 0.70-2.05; P = .02).

Conclusion

Altered miRNA expression was detected in UCB of neonates with PA and HIE. These miRNA could assist diagnostic markers for early detection of HIE and PA at birth.

2.2 Introduction

Neonatal hypoxic-ischemic encephalopathy (HIE) remains a significant cause of long-term neurologic disability and death in newborns and occurs in 1 to 6 per 1000 live full-term births. (354) Hypoxic-ischemic encephalopathy is a multifactorial evolving triphasic encephalopathy, involving both the primary injury, a latent window of reoxygenation, and secondary injury(355, 356).

To date, the only proven treatment for HIE is therapeutic hypothermia (TH), which can improve outcome if introduced within the first 6 postnatal hours (357). However, accurate and timely diagnosis of HIE is difficult, and these infants are currently identified using biochemical and clinical measurements including Apgar scores, initial lactate, and base deficit, which individually have poor positive predictive values (6, 358-360). The Sarnat grading scale of HIE with electroencephalogram grading is accurate when assessed at 24 hours postpartum, but this is beyond the limited therapeutic window(138, 361). A reliable, validated, and quantifiable biomarker would support clinical decision making and early targeted intervention.

Aberrant miRNA expression has been linked to many disease states(362, 363) and appears stable in a cell-free state in various biologic fluids (364). Therefore, miRNAs offer potential as reliable and robust blood-based biomarkers in molecular diagnostics(249). Research on miRNA in HIE remains limited. Past studies have focused on cellular ex vivo work, and animal studies focused on regulation of apoptosis(341, 342, 347, 365). Our group has previously published what is, to our knowledge, the first description of miRNA profiling in a human neonatal cohort and reported downregulation of cord blood miR-374a-5p in infants with HIE(213).

This study aimed to expand on our previous work in the Biomarkers in Hypoxic-Ischemic Encephalopathy (BiHIVE1) study using a separate validation cohort and to explore further additional candidate miRNAs in UCB following HI injury. We wished to explore whether miRNAs could reliably assess the development and severity of HIE in infants born with clinical and biochemical signs of perinatal asphyxia (PA).

2.3 Methodology

2.3.1 Patient Cohorts

Discovery Cohort: The BiHIVE Study

Infants with a gestation greater than 36 weeks were recruited from May 2009 through June 2011 using 1 or more of the following inclusion criteria: umbilical cord pH less than 7.1, 5-minute Apgar score 6 or less, and need for intubation or cardiopulmonary resuscitation at age 10 minutes. Recruitment procedures have been previously described(213). Infants with suspected or confirmed sepsis or coexisting congenital abnormalities were excluded from analysis. The healthy control population was recruited from the baseline longitudinal birth cohort(366); control infants were all healthy full-term infants, with uneventful deliveries, normal neonatal examinations, and without admission to the neonatal intensive care unit. For the study, infants were matched for age, sex, and gestation.

All cases had a continuous multichannel electroencephalogram recorded, as previously described(66). Clinical HIE grade of encephalopathy, if any, was assigned at 24 hours post partum using the modified Sarnat score.9 Standardized neurologic assessment was performed on day 3 and at discharge. Case infants were divided into those with mild, moderate, or severe grade of HIE (HIE group), and those with biochemical or clinical signs of PA but without clinical encephalopathy (PA group).

Validation Cohort: The BiHIVE2 Study

A second multicenter cohort study, the BiHIVE2 validation cohort, was recruited from March 2013 through June 2015 at Cork University Maternity Hospital, Cork, Ireland, with 7500 deliveries per annum, and Karolinska University Hospital Huddinge, Stockholm, Sweden, with 4400 deliveries per annum. The study was designed to validate and expand on findings from the BiHIVE1 cohort and used identical recruitment criteria.19 The BiHIVE2 cohort also recruited study-specific control infants with uneventful deliveries, normal neonatal examination, and 5-minute Apgar scores greater than 8. The BiHIVE2 study is registered (NCT02019147).

2.3.2 Ethical Approval

Both studies were approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals, and the BiHIVE2 cohort was also approved by the regional ethical review board in Stockholm, Sweden. Written informed consent was obtained from parents of all study participants.

2.3.3 Sample Collection

UCB of newborns in all cohorts was collected immediately following delivery of placenta and processed within 3 hours of delivery. Three milliliters of cord blood was placed directly into Tempus Blood RNA tubes (Applied Biosystems) and biobanked at −80°C.

2.3.4 Detection and Quantification of miRNA

Total RNA was isolated from the Tempus system using MagMAX for Stabilized Blood Tubes RNA Isolation Kit (Ambion, Life Technologies) and quantified using a NanoDrop 8000 Spectrophotometer (ThermoScientific NanoDrop). Additionally, RNA quality from the BiHIVE1 study was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc) to ensure sample quality from a random subgroup, as previously described(213). Isolated RNA was subsequently stored at −80°C before quantitative real-time polymerase chain reaction (qRT-PCR). Five nanograms per microliter of isolated RNA was used for reverse transcription to synthesize complementary DNA using Universal cDNA synthesis kit II (Exiqon), followed by an amplification step using the ExiLENT SYBR Green master mix (Exiqon). The qRT-PCR amplifications were performed in triplicate using LNA PCR Primer sets (Exiqon) on the StepOnePlus (Applied Biosystems). Data was analyzed according to the 2-ΔΔCT method(314).

The panel of 12 miRNAs was chosen based on our previously published microarray data(213) and previous reports from the literature indicating potential alterations in miR-146a, miR-155, and miR-181b (367-369) (supplementary table 2.1). These miRNAs were analyzed using qRT-PCR in the BiHIVE1 cohort, and if the altered expression was confirmed, further validation was performed in the BiHIVE2 cohort. The qRT-PCR was performed using the miRCURY LNA Universal RT microRNA PCR

system (Exiqon) using SN48(370, 371) and miR-223-3p as reference genes. SN48 was tested for stability before qRT-PCR, and miR-223-3p had previously been found to be stable using these methods(213). We also included UniSp6 as a spike-in for complementary DNA synthesis quality control.

2.3.5 Evaluation and Functional Classification of miRNA Gene Targets

Possible downstream targets of the miRNAs altered across both cohorts were identified using the online resource miRWalk 2.0 atlas database. Four prediction databases were included in the retrieval (miRWalk, miRanda, miRDB, and TargetScan), and predicted target genes and their conserved miRNA binding sites were annotated. The criteria used involved a minimal seed length of 7 base pairs and a P value less than .05; for gene ontology, predicted targets needed to be present in 3 or more predictive algorithms. Predicted gene targets of the miRNA were analyzed for fold enrichment in both gene ontology terms and Kyoto Encyclopedia of Genes and Genomes pathways using the DAVID 6.8 bioinformatics database (https://david.ncifcrf.gov/).

2.3.6 Statistical Analysis

Means and standard deviations and medians and interquartile ranges (IQRs) were used for normal and nonnormal distributions respectively. For groupwise comparisons, analysis of variance, Kruskal-Wallis test (n groups), t test, or Mann-Whitney test (2 groups) were used as appropriate. Both cohorts were merged for predictability analysis; this is by dividing the mean relative quantification (RQ) of the control group in the BiHIVE discovery cohort across all groups of the discovery cohort. This step was repeated in the BiHIVE2 validation cohort by dividing the mean RQ of the control group in the validation cohort across all groups of the validation cohort. The predictive ability of the individual markers for HIE was assessed using positive and negative predictive values (NPVs) and area under the receiver operator curves. Correlation of data was performed using the Spearman ρ correlation coefficient. Binomial logistic regression was used to examine combinations of significant markers for associations with current eligibility for therapeutic hypothermia based on a moderate/severe HIE grade. In all cases, a P value of less than .05 was considered

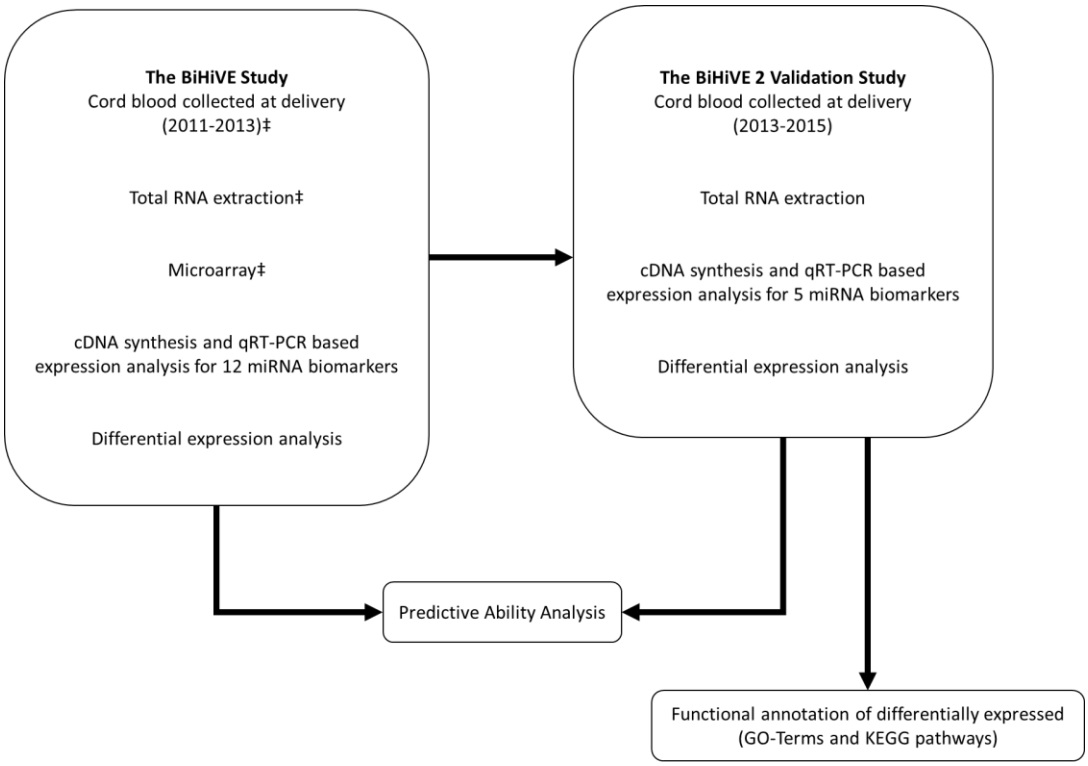
statistically significant, and all P values were 2-sided. In silico analyses used an adjusted P value corrected for multiple testing using false discovery rate based on the Benjamini-Hochberg procedure(371), and an adjusted P value was considered significant at less than .10(108). Statistical analysis was carried out using SPSS Statistics, version 22 (IBM) and GraphPad Prism 5.

2.4 Results

2.4.1 Patient Characteristics

In the BiHIVE1 discovery cohort, 68 infants had UCB collected at delivery, and 66 (39 with PA and 27 with HIE) were included in the final analysis. Healthy control infants (*n* = 22) were recruited from the BASELINE cohort, and 21 were included in the final analysis.

In the BiHIVE2 validation cohort, 80 infants, including healthy control infants, had umbilical whole blood collected at delivery, and 73 infants (22 control infants, 26 with PA, and 25 with HIE) were included in the final analysis. Ten samples were lost during extraction. The clinical characteristics of both populations are summarized in Table 2.1.



Supplementary Figure 2.1. Flowchart of recruitment, experimental procedure, statistical and in silico analysis.‡ = Work performed prior to the current study.

Table 2.1. Clinical demographics.

	BiHIVE Discovery Cohort				BiHIVE2 Validation Cohort			
	Control	PA	HIE	***P*-Value**	Control	PA	HIE	***P*-Value**
	n = 21	*n* = 39	*n* = 27		*n* = 22	*n* = 26	*n* = 25	
Gestation (wk)	-	40 (39-41)	40 (39-41)	0.565	40 (39-41)	40 (39-41)	40 (39-41)	0.929
Birthweight (g)	3420 (3200-3685)	3560 (3150-4055)	3530 (3300-4040)	0.501	3895 (3497.5-4090)	3735 (3470-4020.25)	3840 (3358.5-4149)	0.767
Sex (M/F)	11/10	25/14	20/7	0.302	14/8	16/10	15/10	0.968
1 min. Apgar ¥	9 (9-9)	5 (3.25-7)	3 (1-4.25)	**<0.001**	9 (9-9)	6.5 (5-9)	2 (1-3)	**<0.001**
5 min. Apgar ¥	10 (9.75-10)	9 (8-9)	5 (3-7)	**<0.001**	10 (9-10)	9 (9-10)	4 (4-6)	**<0.001**
Cord pH *	-	7.035 (6.95-7.09)	7 (6.94-7.1)	0.379	7.265 (7.205-7.315)	7.03 (7-7.08)	6.975 (6.895-7.11)	**<0.001**
Initial Lactate ¥	-	5.9 (4.875-10.1)	9.95 (7.15-12.55)	**0.012**	-	5.6 (3.275-9.575)	11.05 (8.775-12.1)	**0.006**
Initial Base Deficit ¥	-	5.7 (2.875-9.675)	11.7 (9.15-16.625)	**<0.001**	-	5.2 (4.55-11.35)	14.75 (10.025-18.275)	**0.002**
Sarnat score								
Mild/Moderate/Severe			16/5/6				16/8/1	
Mode of Delivery *				0.524				**0.035**
SVD	-	6 (19.5%)	6 (22%)		13 (59%)	9 (34.5%)	4 (16%)	
Instrumental	-	18 (56.5%)	15 (56%)		7 (32%)	14 (54%)	17 (68%)	
LSCS	-	7 (22%)	6 (22%)		2 (9%)	3 (11.5%)	4 (16%)	
Unknown	-	1 (0.5%)	-		-	-	-	

Clinical Demographics in the BiHIVE and BiHIVE2 Validation Cohorts. Median (IQR) or n (%). Wk = week, g = grams, M = Male, F = Female, SVD = Spontaneous vaginal delivery, LSCS = Lower segment caesarean section. * Significantly altered across one cohort, *P* <0.05. ¥ Significantly altered across both cohorts, *P* <0.05.

2.4.2 Candidate Whole Blood miRNA in the BiHIVE Discovery Cohort

In the BiHIVE1 discovery cohort, 12 different miRNAs were analyzed. Relative expression levels (2-ΔΔCT) of miRNAs across 3 groups were compared between healthy control infants (n = 21), infants with PA (n = 39), and infants with HIE (n = 27) (workflow in supplementary figure 2.1). Two miRNAs were reduced in the HIE group compared with healthy controls; miR-199a (median RQ, 0.60; IQR, 0.39-0.79 vs median, 0.87; IQR, 0.56-1.40, P = .04) and miR-374a (median, 0.24; IQR, 0.13-0.40 vs median, 0.73; IQR, 0.22-1.46; P = .01). Three miRNAs were reduced in the PA group compared with healthy control individuals: miR-374a (median, 0.21; IQR, 0.07-1.19 vs median, 0.73; IQR, 0.22-1.46; P = .04), miR-376c (median, 0.28; IQR, 0.14-0.55 vs median, 0.55; IQR, 0.41-1.40; P = .01), and miR-410 (median, 0.65; IQR, 0.20-0.78 vs median, 0.99; IQR, 0.43-1.23; P = 0.04) (Figure, 2.1A; Table 2.2).

Supplementary Table 2.1. List of microRNA included in the BiHIVE qRT-PCR panel

miRNA Name	Accession ID	Sequence	Reference
hsa-miR-18a-5p	MIMAT0000072	UAAGGUGCAUCUAGUGCAGAUAG	Microarray
hsa-miR-27b-3p	MIMAT0000419	UUCACAGUGGCUAAGUUCUGC	Microarray
hsa-miR-101-3p	MIMAT0000099	UACAGUACUGUGAUAACUGAA	Microarray
hsa-miR-128-3p	MIMAT0000424	UCACAGUGAACCGGUCUCUUU	Microarray
hsa-miR-146a-5p	MIMAT0000449	CCUCUGAAAUUCAGUUCUUCAG	(369)
hsa-miR-155-5p	MIMAT0000646	UUAAUGCUAAUCGUGAUAGGGGU	(368)
hsa-miR-181b-5p	MIMAT0000257	AACAUUCAUUGCUGUCGGUGGGU	(372)
hsa-miR-199a-5p	MIMAT0000231	CCCAGUGUUCAGACUACCUGUUC	Microarray
hsa-miR-374a-5p	MIMAT0000727	UUAUAAUACAACCUGAUAAGUG	Microarray
hsa-miR-376a-3p	MIMAT0000729	AUCAUAGAGGAAAAUCCACGU	Microarray
hsa-miR-376c-3p	MIMAT0000720	AACAUAGAGGAAAUUCCACGU	Microarray
hsa-miR-410-3p	MIMAT0002171	AAUAUAACACAGAUGGCCUGU	Microarray

The names, accession numbers and sequences of all miRNA used as targets in this study. The microarray is from previously published work (213).

We next focused within the HIE group (n = 27) and looked at the differences between the mild (n = 16), moderate (n = 5), and severe (n = 6) grade of HIE. miR-181b was significantly reduced in the severe group compared with the mild group (median, 0.08; IQR, 0.04-0.21 vs median, 0.82; IQR, 0.36-1.38; P = .01).

To examine the ability of miRNAs to aid in clinical decision-making, we compared infants eligible for TH (11 with moderate or severe encephalopathy) with those ineligible (healthy control infants, those with PA, and those with mild HIE [n = 76]). This analysis revealed reduced expression of 2 miRNAs for infants eligible for TH, miR-

199a (median, 0.49; IQR, 0.33-0.60 vs median, 0.75; IQR, 0.43-1.08, P = .049) and miR-181b (median, 0.25; IQR, 0.16-0.32 vs median, 0.61; IQR, 0.26-1.39; P = .03).

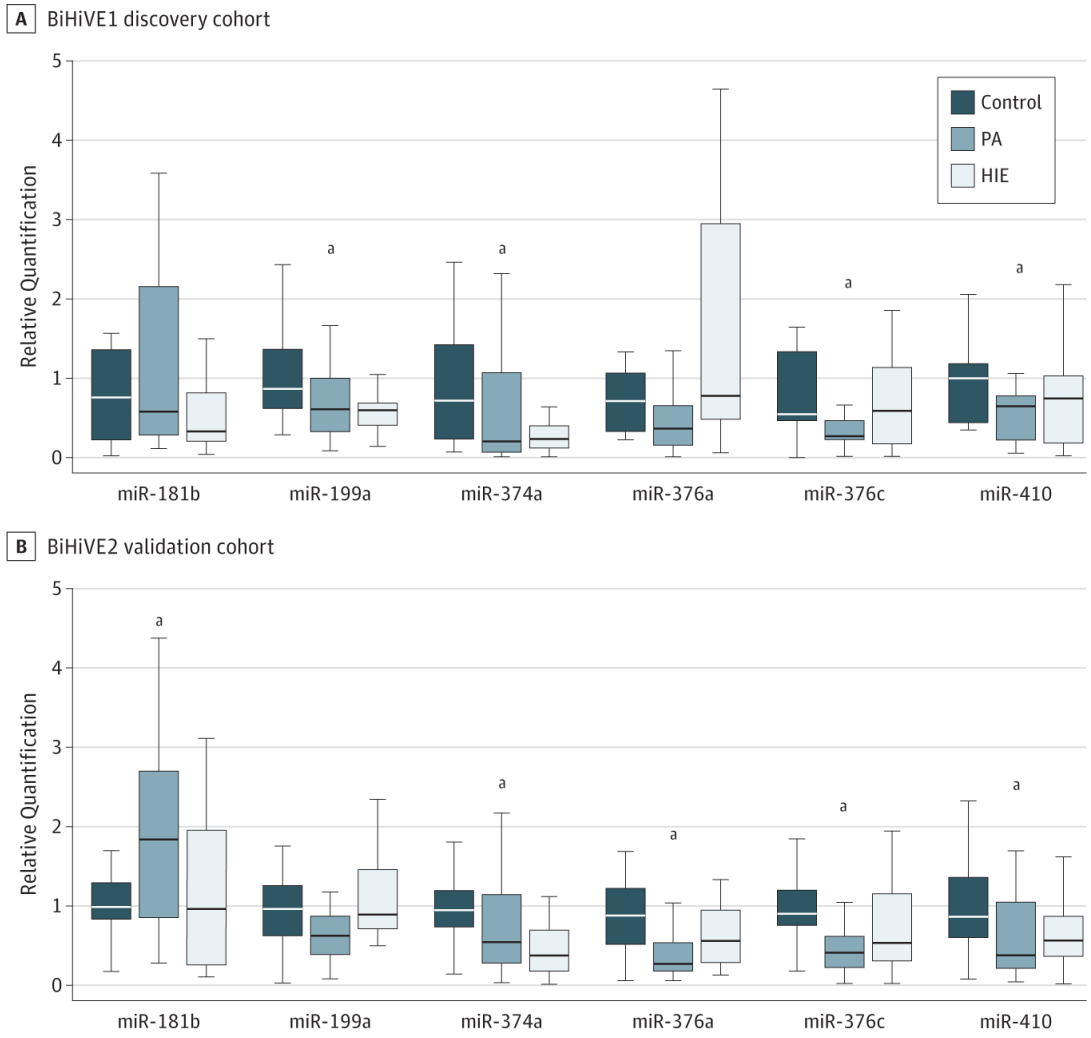


Figure 2.1. Altered relative expression levels in miRNA from the BiHIVE discovery cohort and BiHIVE2 validation cohort across groups. **A.** In the BiHIVE discovery cohort there were significant differences in miR-199a-5p and in miR-374a-5p between the control (*n* = 21) and hypoxic-ischemic encephalopathy (HIE) (*n* = 27) groups and differences in miR-374a-5p, miR-376c-3p, and miR-410 between the control and perinatal asphyxia (*n* = 39) groups. **B.** In the BiHIVE2 validation cohort there were significant differences in miR-374a-5p and miR-410 across the control (*n* = 22) vs HIE (*n* = 25) groups. In miR-181b-5p, miR-376a-3p and miR-376c-3p there were significant differences across the control vs PA (*n* = 26). Finally, in miR-181b-5p, miR-199a-5p and miR-376a-3p there were significant differences across the PA vs HIE groups. Data in boxplots represent median and interquartile range (IQR). * Significant at *P* < 0.05.

2.4.3 Validation of Candidate Whole Blood miRNA in BiHIVE2 Cohort

From the 12 candidate miRNAs, 5 miRNAs showed consistent significance (miR-374a, miR-376c, miR-410, miR-181b, and miR-199a), and 2 additional miRNA were included: miR-376a, with a strong trend in differentiating PA vs HIE (median, 0.37; IQR, 0.15-0.78 vs median, 0.78; IQR, 0.44-3.06; P = .06), and miR-155 owing to its

suggested role in immune response and microglial activation (368). The 5 miRNAs with no significant and consistent changes were not tested further in BiHIVE2.

Of the 7 candidate miRNAs tested, 3 miRNAs showed consistent altered expression levels in the BiHIVE2 cohort. miR-374a was significantly reduced in the HIE group vs control group (median, 0.38; IQR, 0.17-0.77 vs median, 0.95; IQR, 0.68-1.19; P = .009), and miR-376c was significantly reduced in the PA group compared with the control group (median, 0.42; IQR, 0.21-0.61 vs median, 0.90; IQR, 0.70-1.30; P = .004) (Figure, 2.1B; Table 2.2). There was no difference between grades of HIE in the validation cohort.

As in our discovery cohort, we separated infants in the validation cohort into 2 groups, infants eligible for TH (n = 9) and infants ineligible for TH (n = 64), and found miR-181b to be significantly reduced in the group eligible for TH compared with infants not eligible (median, 0.27; IQR, 0.14-1.41 vs median, 1.18; IQR, 0.70-2.05; P = .02) as previously described (373).

Table 2.2. Altered miRNA expression in the BiHIVE discovery cohort and BiHIVE2 validation cohort.

BiHIVE miRNA	181b	199a	374a	376a	376c	410
Control vs Hypoxic Ischemic Encephalopathy	0.76 (0.22-1.36) vs 0.34 (0.21-0.88)	* 0.87 (0.56-1.40) vs 0.60 (0.39-0.79)	¥ *0.73 (0.22-1.46) vs 0.24 (0.13-0.40)*	0.72 (0.30-1.32) vs 0.78 (0.44-3.06)	0.55 (0.41-1.40) vs 0.59 (0.15-1.22)	0.99 (0.43-1.23) vs 0.75 (0.17-1.10)
Control vs Perinatal Asphyxia	0.76 (0.22-1.36) vs 0.58 (0.27-2.20)	0.87 (0.56-1.40) vs 0.61 (0.29-1.03)	* 0.73 (0.22-1.46) vs 0.21 (0.07-1.19)	0.72 (0.30-1.32) vs 0.37 (0.15-0.78)	¥ *0.55 (0.41-1.40) vs 0.28 (0.14-0.55)*	* 0.99 (0.43-1.23) vs 0.65 (0.20-0.78)
Perinatal Asphyxia vs Hypoxic Ischemic Encephalopathy	0.58 (0.27-2.20) vs 0.34 (0.21-0.88)	0.61 (0.29-1.03) vs 0.60 (0.39-0.79)	0.21 (0.07-1.19) vs 0.24 (0.13-0.40)	0.37 (0.15-0.78) vs 0.78 (0.44-3.06)	0.28 (0.14-0.55) vs 0.59 (0.15-1.22)	0.65 (0.20-0.78) vs 0.75 (0.17-1.10)
Ineligible for cooling vs eligible for cooling	¥ *0.61 (0.26-1.39) vs 0.25 (0.16-0.32)*	* 0.75 (0.43-1.08) vs 0.49 (0.33-0.60)	0.24 (0.08-1.09) vs 0.37 (0.24-0.53)	0.59 (0.25-1.20) vs 0.61 (0.39-2.35)	0.49 (0.27-1.02) vs 0.46 (0.14-1.20)	0.75 (0.36-1.08) vs 0.60 (0.15-0.85)

BiHIVE2 miRNA Validation	181b	199a	374a	376a	376c	410
Control vs Hypoxic Ischemic Encephalopathy	0.98 (0.82-1.30) vs 0.96 (0.25-2.12)	0.96 (0.57-1.26) vs 0.90 (0.70-1.53)	¥ *0.95 (0.68-1.19) vs 0.38 (0.17-0.77)*	0.88 (0.51-1.29) vs 0.56 (0.25-1.03)	0.90 (0.70-1.30) vs 0.54 (0.27-1.23)	* 0.86 (0.60-1.37) vs 0.57 (0.37-0.95)
Control vs Perinatal Asphyxia	* 0.98 (0.82-1.30) vs 1.85 (0.85-2.77)	0.96 (0.57-1.26) vs 0.63 (0.39-0.92)	0.95 (0.68-1.19) vs 0.55 (0.28-1.19)	* 0.88 (0.51-1.29) vs 0.27 (0.17-0.72)	¥ *0.90 (0.70-1.30) vs 0.42 (0.21-0.61)*	0.86 (0.60-1.37) vs 0.38 (0.22-1.06)
Perinatal Asphyxia vs Hypoxic Ischemic Encephalopathy	* 1.85 (0.85-2.77) vs 0.96 (0.25-2.12)	* 0.63 (0.39-0.92) vs 0.90 (0.70-1.53)	0.55 (0.28-1.19) vs 0.38 (0.17-0.77)	* 0.27 (0.17-0.72) vs 0.56 (0.25-1.03)	0.42 (0.21-0.61) vs 0.54 (0.27-1.23)	0.38 (0.22-1.06) vs 0.57 (0.37-0.95)
Ineligible for cooling vs eligible for cooling	¥ *1.18 (0.70-2.05) vs 0.27 (0.14-1.41)*	0.73 (0.51-1.20) vs 0.95 (0.79-2.05)	0.70 (0.28-1.17) vs 0.39 (0.22-0.70)	0.53 (0.24-1.15) vs 0.37 (0.24-0.70)	0.53 (0.24-1.04) vs 0.69 (0.35-1.27)	0.64 (0.27-1.16) vs 0.46 (0.32-0.92)

Data represents median relative quantification (RQ) and interquartile range (IQR). * Significantly altered, $P < 0.05$ (blue). ¥ Significantly altered, $P < 0.05$, in both studies (green).

Table 2.3. Spearman's Rho correlation between miRNA relative quantifications and current markers.

	miR-155	miR-181b	miR-199a	miR-374a	miR-376a	miR-376c	miR-410	Cord pH	Initial Lactate	Initial Base Deficit	1 min. Apgar	5 min. Apgar	10 min. Apgar
miR-155	**1.00**	-0.05	0.30†	0.23*	0.45†	0.57†	0.41†	0.08	-0.22	-0.14	-0.09	-0.05	-0.27*
miR-181b	-0.05	**1.00**	0.04	0.32†	0.04	0.02	0.06	-0.08	-0.17	-0.36†	0.14	0.12	0.33†
miR-199a	0.30†	0.04	**1.00**	0.10	0.26*	0.31†	0.22*	-0.03	0.03	-0.07	0.01	-0.04	-0.05
miR-374a	0.23*	0.32†	0.10	**1.00**	0.17	0.19	0.16	0.09	-0.19	-0.12	0.18*	0.14	0.08
miR-376a	0.45†	0.04	0.26*	0.17	**1.00**	0.87†	0.82†	-0.02	-0.08	0.15	-0.03	-0.01	-0.27*
miR-376c	0.57†	0.02	0.31†	0.19	0.87†	**1.00**	0.83†	0.08	-0.10	0.00	0.05	0.00	-0.24
miR-410	0.41†	0.06	0.22*	0.16	0.82†	0.83†	**1.00**	0.18	-0.04	-0.01	0.14	0.15	-0.18
Cord pH	0.08	-0.08	-0.03	0.09	-0.02	0.08	0.18	**1.00**	-0.39†	-0.15	0.30†	0.26†	0.12
Initial Lactate	-0.22	-0.17	0.03	-0.19	-0.08	-0.10	-0.04	-0.39†	**1.00**	0.57†	-0.19	-0.15	-0.38*
Initial Base Deficit	-0.14	-0.36†	-0.07	-0.12	0.15	0.00	-0.01	-0.15	0.57†	**1.00**	-0.29*	-0.53†	-0.50†
1 min. Apgar	-0.09	0.14	0.01	0.18*	-0.03	0.05	0.14	0.30†	-0.19	-0.29*	**1.00**	0.83†	0.67†
5 min. Apgar	-0.05	0.12	-0.04	0.14	-0.01	0.00	0.15	0.26†	-0.15	-0.53†	0.83†	**1.00**	0.83†
10 min. Apgar	-0.27*	0.33†	-0.05	0.08	-0.27*	-0.24	-0.18	0.12	-0.38*	-0.50†	0.67†	0.83†	**1.00**

Spearman's Rho correlation with correlation coefficients describing associations across miRNAs and current markers. * Correlation is significant at $P < 0.05$ (blue). † Correlation is significant at $P < 0.01$ (yellow).

2.4.4 Ability to Predict Eligibility for Therapeutic Hypothermia in the BiHIVE and BiHIVE2 Cohorts

We merged both cohorts to examine the ability of miR-181b expression to detect moderate/severe HIE. We compared it with current early biochemical and clinical markers, using the standard cutoffs used in previous trials of TH (374). Associations across miRNA and current markers can be seen in Table 2.3.

miR-181b had an area under the curve of 0.752 (95% CI, 0.610-0.893) for the prediction of moderate-severe HIE. miR-181b performed similar to current biochemical markers and had the highest NPV of 99% (Table 2.4). Clinical Apgar scores performed better than biochemical markers and miR-181b (Table 2.4). Logistic regression was performed to assess the ability of a combination of miR-181b and Apgar at 5 minutes to predict moderate-severe HIE. The model was statistically significant at $\chi 2 = 42.576$; $P < .001$. The model explained 60% (Nagelkerke R2) of the variance in the eligibility for TH and correctly classified 93% of cases. However, the combined marker did not make significant improvements to Apgar at 5 minutes alone.

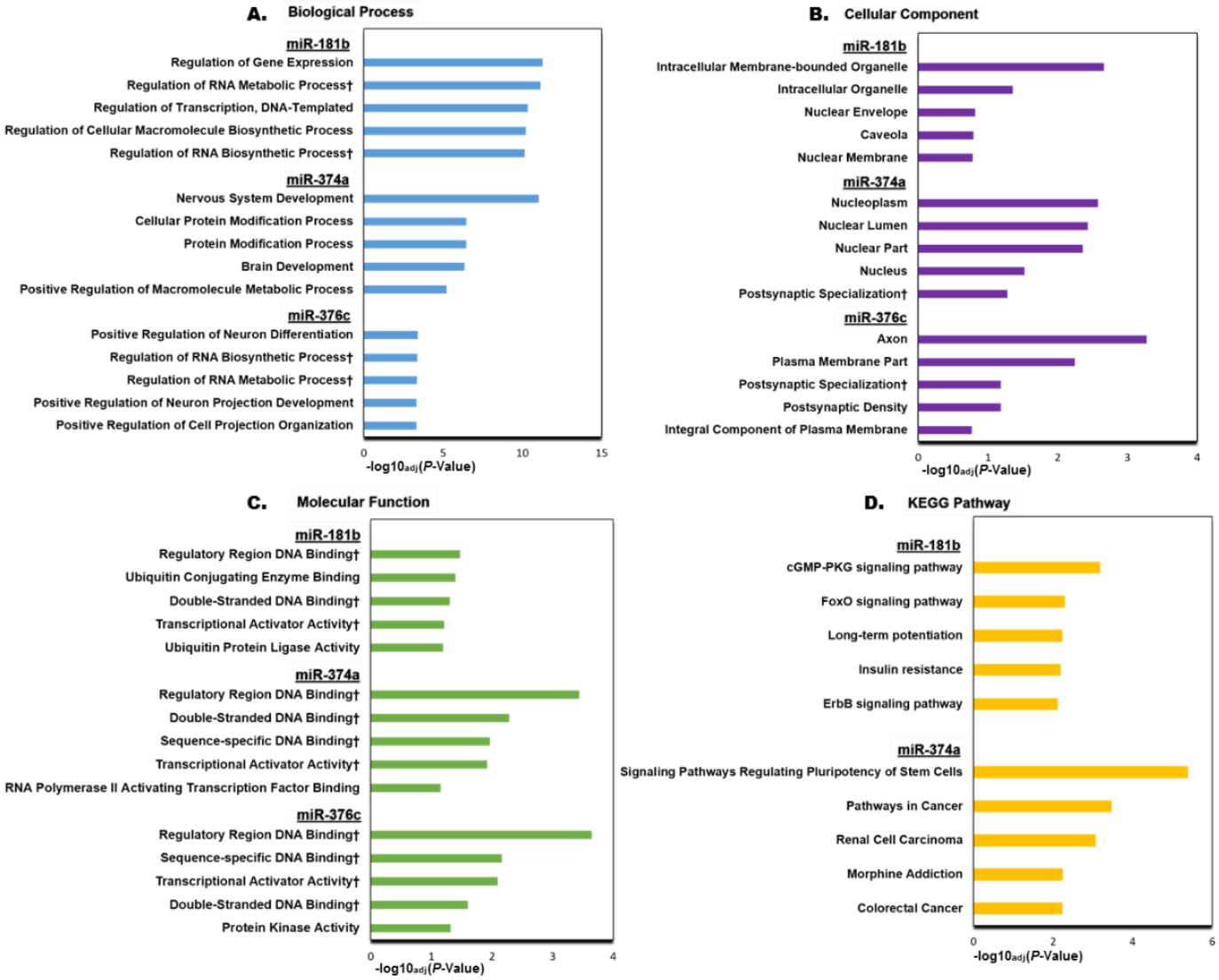
Table 2.4. Biochemical and clinical markers of infants' eligible for therapeutic hypothermia.

	PPV (%)	NPV (%)	AUC (CI)	P-Value
Biochemical markers				
pH *	48	85	0.758 (0.638-0.879)	0.001
Lactate *	33	91	0.759 (0.617-0.902)	0.001
Base Deficit †	69	86	0.833 (0.729-0.936)	<0.001
miR-181b *	25	99	0.752 (0.610-0.893)	0.002
Clinical markers				
Apgar at 1min. †	55	97	0.867 (0.790-0.944)	<0.001
Apgar at 5min. †	90	92	0.918 (0.854-0.981)	<0.001
Apgar at 10min. †	31	93	0.926 (0.847-1.000)	<0.001
Combined marker				
miR-181b + Apgar at 5min. †	83	95	0.918 (0.826-1.000)	<0.001

Biochemical and clinical markers of infants' eligible for therapeutic hypothermia at birth, across both cohorts. PPV = positive predictive value. NPV = negative predictive value. AUC = area under the curve. Min. = minute. The optimum cut-off value for miR-181b is RQ = 0.492, with a sensitivity = 79% and specificity = 60%. Standard cut-off values for hypothermia were used for Apgar scores (≤ 5), pH (≤ 7.0) and base deficit (≥ 16 mmol/L), based on the Toby criteria (375), and lactate (≥ 7.0). * $P < 0.05$. † $P < 0.001$.

2.4.5 Targets and Roles of miRNA

A list of potential miRNA-gene targets were generated for each of the 3 miRNAs significantly altered across both cohorts: miR-376c, miR-374a, and miR-181b. For enrichment of biologic processes, there was an overlap between miR-376c and miR-181b targets for regulation of RNA metabolic process and regulation of RNA biosynthesis processes (Supplementary figure 2.2). For enrichment of the cellular component, there was an overlap in miR-374a and miR-376c for postsynaptic specialization (Supplementary figure 2.2). Finally, for the enrichment of molecular function, there was an overlap across all 3 miRNAs for regulatory region DNA binding and transcriptional activator activity. For miR-181b and miR-374a, there was an overlap for double-stranded DNA binding, and in miR-374a and miR-376c there was an overlap for sequence-specific DNA binding (Supplementary figure 2.2).



Supplementary Figure 2.2. Functional Enrichment Analysis of GO and KEGG. **A.** Biological Process. **B.** Cellular Function. **C.** Molecular Function. **D.** KEGG. Data graphs represent fold enrichment from list of miRNA target genes. Adjusted *P*-value corrected for multiple testing using false discovery rate (FDR) based on Benjamini-Hochberg procedure was considered significant. † = Occurred in more than one miRNA-gene target list.

2.5 Discussion

We have validated 3 miRNAs in neonatal PA and HIE by using 2 well-defined, independent cohorts. From the 12 candidate miRNAs, 5 miRNAs were differentially expressed in cord blood in the BiHIVE1 discovery cohort. Following this, expression patterns of 3 of 5 miRNAs were validated as consistently altered in the BiHIVE2 multicenter validation cohort. The miRNA biomarkers assessed have shown utility in distinguishing perinatal asphyxia and HIE from healthy control infants and may help identify newborns suitable for TH. In both the BiHIVE1 and BiHIVE2 cohorts, miR-374a, previously identified (213) and validated in this study, was found to be expressed at lower levels in the UCB of HIE infants compared with the healthy control infants. We also found a consistent downregulation of miR-376c in perinatal asphyxia compared with healthy controls and previously reported downregulation of miR-181b in infants with moderate and severe HIE compared with healthy control infants (373).

Research in miRNA is still quite novel, and most studies of miR-374a and miR-376c have been focused on cancer research. MiR-374a was first described in colon cancer (376) and miR-376c in ovarian cancer (377) These studies linked them to dysregulation of the Wnt/β-catenin pathway (325, 378) by targeting associated genes in the pathway via functional assays, including WIF1, PTEN, WNT5A, and LRH-1, respectively. Other pathways explored in miR-374a include PI3K/AKT via CCND1 (379) and for miR-376c include TGF-β signaling via ALK7 (380). Our miR-374a in silico analysis found signaling pathways regulating pluripotency of stem cells as a predicted Kyoto Encyclopedia of Genes and Genomes pathway, which encompasses a number of pathways including TGFβ/Smad, PI3K-Akt, and the Wnt/β-catenin signaling pathway. The Wnt/β-catenin signaling described in both miR-374a and miR-376c has also been described in a HIE cell modeling, where high Wnt activity prevents degradation of β-catenin and can inhibit preoligodendrocytes from differentiating into mature oligodendrocytes (330). ALK7, a functional target of miR-376c, has also been shown to impair oligodendrocyte differentiation and myelination, where ALK7 is highly expressed in nonrepairing lesions (377).

Few studies to date have examined miR-374a in neonatal HIE. A piglet model reported temporal changes in expression after injury (351) and a 2017 clinical HIE study confirmed downregulation in UCB in neonatal HIE (324).

This study also reported the downregulation of miR-181b in infants with moderate and severe HIE and compared the predictive ability of miR-181b with biochemical and clinical markers in HIE (Table 2.4). The strong NPV of 99% from miR-181b could make this miRNA a useful tool for the clinical setting because it is quantifiable and not subjective. The Apgar score is reliant on subjective observation and can differ in interobserver variability, and past studies have called for more objective and precise measures (381). Additionally, instrumental deliveries were more prevalent in the PA and HIE groups but were not associated with our validated miRNAs.

While miR-199a, miR-376a, and miR-410 were not validated as significantly altered in both cohorts, it is essential to recognize the distribution of Sarnat grades across BiHIVE2 (Table 2.1). Although recruitment went on for a similar period across 2 different centers, the number of severe-grade HIE infants recruited to the BiHIVE2 validation cohort was significantly lower. Thus, validation with an equal distribution of grades of HIE may highlight further miRNAs altered in more severe grades of HIE in future studies.

Our study has focused on whole blood instead of either serum or plasma; this approach has given a higher yield of miRNA and allowed us to explore both cellular and extracellular miRNA expressed in the blood. This may have masked altered expression seen in extracellular miRNA owing to abundant levels of cellular miRNA in whole blood (274). Previous studies on circulating miRNA have proposed that cellular damage caused by injury may release cellular miRNA into the serum or plasma. This may be comparable to how cellular enzymes are released after apoptosis and necrosis or possibly through a cell-specific transport that releases specific miRNAs (382). We do not know how these decreased circulating expression levels might relate to altered expression within tissues, particularly within the brain. Studies in peripheral tissues would help better understand the mechanism of this altered expression. Future studies will need to explore potential downstream targets of

these miRNA to assess whether they are innocent bystanders or play an active role in the pathophysiology of hypoxic injury.

The strength of our study lies in the fact that we have studied the miRNA in 2 completely separate cohorts, recruited using identical and strict recruitment criteria. The BiHIVE2 validation cohort was recruited across 2 different countries, indicating that our findings may be applicable across distinct geographical areas. The altered expression of UCB miRNAs immediately after birth implies that changes in miRNA expression may occur during injury. Additional research is required to demonstrate whether these changes persist during the subsequent progression of HI injury.

In conclusion, we have validated the decreased expression of 2 miRNAs, miR-374a and miR-376c, in whole blood from the umbilical cord of newborns with PA and HIE in 2 independent cohorts. We have also expanded on our previous research in miR-181b; its strong NPV of may bring this miRNA forward as a quantifiable non-invasive biomarker with a reliable NPV to aid in identifying those infants who will not require intervention.

Chapter 3

Alterations in Umbilical Cord Blood messenger RNA Expression in Neonatal Hypoxic-Ischaemic Encephalopathy and Long-Term Outcome

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3.1 Abstract

Background

Hypoxic-ischaemic encephalopathy (HIE) remains an important cause of neonatal death and long-term neurological disability. It remains challenging to classify infants eligible for therapeutic hypothermia (TH) within the short postnatal therapeutic time window of 6 hours. No objective and robust biological marker is available in the clinical setting. The purpose of this study was to explore two predicted downstream mRNA targets, nuclear factor of activated T-cells 5 (Nfat5) and Frizzled-4 (Fzd4), of the previously validated microRNAs, miR-181b, miR-374a, and miR-376c, in whole blood across our two independent BiHiVE cohorts, and to assess their ability to predict grade of HIE and neurodevelopmental outcome.

Methodology

This study included two cohorts. The discovery cohort recruited full-term infants with PA at birth to the Biomarkers in Hypoxic-Ischemic Encephalopathy (BiHiVE1) study in Cork, Ireland (2009-2011). Encephalopathy grade was defined using early electroencephalogram and Sarnat score (n = 68). Healthy control infants were recruited from the BASELINE birth cohort (2008-2016) (n = 22). The validation cohort recruited full-term infants in Cork, Ireland, and Karolinska Huddinge, Sweden to the BiHiVE2 multi-centre study (2013-2015), the validation study recruited infants with PA along with healthy control infants to validate findings from BiHiVE1 using identical recruitment criteria (n = 80). UCB was processed and biobanked at delivery. Infants were assigned a modified Sarnat score at 24 hours. Infants were followed-up at 18-36 months of age. Candidate mNFAT5 and mFZD4 were measured using quantitative real-time polymerase chain reaction.

Results

From 170 neonates, 126 were included in the analyses. The BiHiVE1 cohort included 55 infants (control infants [*n* = 16], infants with PA [*n* = 19], and infants with HIE [*n* = 20]), and BiHiVE2 included 71 infants (control [*n* = 22], PA [*n* = 25], and HIE [*n* = 24]). The two cohorts had a mean age of 40 weeks [interquartile range (IQR) = 39-41

weeks] and included 82 males and 44 females. In grade of HIE severity, the level of mFZD4 was increased in severe HIE (median relative quantification (RQ) = 2.98 (IQR = 2.23-3.68)) vs mild HIE (0.88 (0.46-1.37)), *P* = 0.004, and in severe HIE (2.98 (2.23-3.68)) vs moderate HIE (1.05 (0.81-1.20)), *P* = 0.003. In eligibility for therapeutic hypothermia, the level of mFZD4 was increased in infants eligible for TH (1.20 (0.93-2.37)) vs infants' ineligible for TH (0.81 (0.46-1.53)), *P* = 0.017. Neurodevelopmental outcome was available in 56 infants. Levels of mNFAT5 were increased in severely abnormal (1.26 (1.17-1.39)) vs normal outcome infants (0.97 (0.83-1.24)), *P* = 0.036, and in severely abnormal (1.26 (1.17-1.39)) vs mildly abnormal outcomes infants (0.96 (0.80-1.06)), *P* = 0.013. Levels of mFZD4 were increased in severely abnormal (2.51 (1.60-3.56)) vs normal outcome infants (0.74 (0.48-1.49)), *P* = 0.004, and in severely abnormal (2.51 (1.60-3.56)) vs mildly abnormal outcome infants (0.97 (0.75-1.34)), *P* = 0.026.

Conclusions

Increased mFZD4 expression was observed in umbilical cord whole blood of neonates with severe HIE; both mNFAT5 and mFZD4 expression was increased in infants with severely abnormal long-term outcome. These mRNA could aid current measures as early objective prognostic markers of HIE severity at delivery.

3.2 Introduction

HIE is the most prevalent form of brain injury in the newborn and affects 1.5 per 1000 live births in the developed world (36). Rates are significantly higher in the developing world, with significant risk of long-term disability (383). Currently, the only treatment for the moderate/severe grade of HIE is therapeutic hypothermia, which must be implemented within the first 6-hours of life (384, 385). Current clinical and biochemical markers can be inconclusive in forming a definitive diagnosis and seem to be poor markers for long-term neurodevelopmental outcome (153). Novel biomarkers continue to be explored in pilot studies, but no clear molecular marker has been developed as of yet to compete with current markers.

Gene transcripts have been used for diagnosis and prognosis across various conditions, and the mRNA explored could also give insight into the underlying biological mechanism (386, 387); miRNA are small non-coding RNA nucleotides involved in the post-transcriptional modification of mRNA. Both may be dysregulated in the tissue and blood in many disorders of cell growth and differentiation (249, 362, 364). Cellular miRNA and mRNA are present in erythrocytes, leukocytes and platelets; and extracellular miRNA and mRNA are present in exosomes, microvesicles, and bound to proteins (278, 388, 389). Our group has previously reported a panel of whole blood miRNA (miR-374a, miR-376c and miR-181b), with the ability to differentiate HIE, PA, and infants eligible for therapeutic hypothermia (390). This study aimed to expand on our previous work using predicted downstream mRNA targets of previously validated miRNA in the UCB following HIE.

The downstream mRNA targets focused on two mRNA associated with the Wnt pathway both mFZD4 and mNFAT5. The canonical and non-canonical Wnt signalling pathways are known to affect gene transcription, cytoskeletal regulation and calcium regulation (391). Wnt signalling has been studied across both cancer and embryonic development, with a recent comparative study highlighting pathological levels of Wnt in oligodendrocyte progenitor cells of neonatal HIE tissue (330). Fzd4 acts as a cell surface receptor for the Wnt ligand; and Nfat5 regulates cell adhesion, migration and tissue separation in non-canonical Wnt/Ca^{2+} signalling and can repress canonical

Wnt signalling via inhibition of β-catenin acetylation (392-395). The study explored if mRNA could assess the severity in HIE grade and long-term outcome in HIE.

3.3 Methods

3.3.1 Patient Populations

The BiHiVE1 Discovery Cohort

Infants with a gestation > 36 weeks were recruited from May 2009 to June 2011 using one or more of the following inclusion criteria: cord pH < 7.1, 5-minute Apgar score ≤ 6, need for intubation or cardiopulmonary resuscitation at 10 minutes of age. Recruitment and data collection procedures have been previously described (213). Infants with suspected or confirmed sepsis, or co-existing congenital abnormalities were excluded from analysis. The healthy control population was recruited from the BASELINE longitudinal birth cohort (396); controls were all healthy full-term infants, with uneventful deliveries, normal neonatal examinations and without admission to the neonatal intensive care unit. All case infants had a continuous EEG recorded, which commenced shortly after delivery, as previously described (66). Clinical HIE grade of encephalopathy, if any, was assigned at 24 hours postpartum using the modified Sarnat score (77). Standardised neurologic assessment was additionally performed on day 3 and at discharge. Case infants were divided into those with a mild, moderate, or severe grade of HIE (HIE group), and those with biochemical or clinical signs of perinatal asphyxia, but without clinical encephalopathy (PA group).

The BiHiVE2 Validation Cohort

The second multi-centre study, the validation cohort was recruited from March 2013 to June 2015 at Cork University Maternity Hospital, with 7,500 deliveries per annum, and Karolinska University Hospital Huddinge, with 4,400 deliveries per annum. The study was designed to validate the findings of the BiHiVE1 discovery cohort and used identical recruitment criteria to the BiHiVE1 cohort (213). The BiHiVE2 cohort, unlike BiHiVE1, recruited additional control infants; these were infants with uneventful deliveries, normal neonatal examination and a 5-minute Apgar scores > 8. Infants were matched for gestational age, sex, and birthweight. The clinicaltrials.gov identifier for the BiHiVE2 study is **NCT02019147**. The Clinical Research Ethics Committee of the Cork Teaching Hospitals approved both studies, and the Regional

Ethical Review Board in Stockholm also approved the multi-centre validation cohort. Written informed consent was obtained from parents of all study participants.

3.3.2 Sample Collection

UCB was collected following the delivery of the placenta and processed within 3 hours of birth. 3 mL of cord blood was placed directly into Tempus Blood RNA tubes (Applied Biosystems, USA) and biobanked at -80°C in both Cork, Ireland, and Karolinska, Sweden.

3.3.3 Prediction of mRNA Targets

A list of previously validated miRNAs, miR-181b, miR-374a, and miR-376c (390) were entered into miRecords (http://c1.accurascience.com/miRecords/) (397) which generated a list of predicted downstream target genes in Homo Sapiens. Search criteria were set to compile results from at least 3 target prediction programs including miRanda, miRTarget2, picTar, PITA, RNAhybrid and Targetscan. The generated list of target genes were subsequently compared with the Wnt signalling pathway using the KEGG pathway targets generated from WebGestalt, the Web-based Gene Set Analysis Toolkit (http://www.webgestalt.org/webgestalt_2013/) (330, 398).

3.3.4 Detection and Quantification of miRNA and mRNA Expression

Total RNA was isolated from the Tempus system using the MagMAX for Stabilized Blood Tubes RNA Isolation Kit (Ambion, Life Technologies, USA) and quantified using a NanoDrop 8000 Spectrophotometer (ThermoScientific NanoDrop, USA). RNA quality from the BiHiVE1 study was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc, Agilent Laboratories, California) to ensure sample quality from a random subgroup of the samples, as previously described (390). Isolated RNA were stored at -80°C before quantitative real-time PCR (qPCR, polymerase chain reaction). 5 ng/µl of isolated RNA was used for reverse transcription to synthesise cDNA (complementary DNA) using Universal cDNA synthesis kit II (Exiqon, Denmark, acquired by Qiagen, Netherlands). The expression levels of mNFAT5 and mFZD4 were analysed using the TaqMan® Gene Expression Assays (Applied Biosystems, Life

Technologies, Paisley, UK); 18s was used as a reference gene, see table 3.1 (399). Samples were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute on the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Life Technologies, Paisley, UK). All samples were run in triplicate, cycle threshold (CT) values were recorded, and expression levels were calculated relative to controls using the $2^{-\Delta\Delta CT}$ method (400). Both cohorts were merged by dividing the average relative quantification (RQ) of the control group in the BiHiVE discovery cohort across all groups of the discovery cohort. This step was repeated in the BiHiVE2 validation cohort by dividing the average RQ of the control group in the validation cohort across all groups of the validation cohort.

Table 3.1. TaqMan® Gene Expression Assays.

Gene symbol	**TaqMan® Assay ID**
NFAT5	Hs00232437_m1
FZD4	Mm00487538_m1
18s	Mm02745649_m1

3.3.5 Neurodevelopmental Assessment

The neurodevelopmental assessment was carried out by a trained clinician or psychologist when the child was aged between 18 and 36 months of age and consisted of the BSID-III (401). The BSID-III is an individually administered, gold-standard neurodevelopmental assessment designed to measure the developmental functioning of both infants and toddlers (1 to 42 months) and includes Cognitive, Language (Receptive and Expressive), Motor (Fine and Gross), Social-Emotional, and Adaptive Behaviour Scales. The child's outcome as measured by the BSID-III is determined using the composite scores of the administered subscales. Each composite score has a standardised mean of 100 and a standard deviation (SD) of 15 such that a value below 85 is conventionally used to indicate neurodevelopmental delay (402). For this study, the outcome was designated as normal if the child scored > 85 across all 3 BSID-III subscales. Children were excluded if they were tested in a language that was not their first language. The infant was designated mildly abnormal if 1 or more subscales scored ≤ 85 but > 70, and severely abnormal

outcome if 2-3 subscales scored ≤ 70 or the infant suffered CP or death, as previously described (189).

3.3.6 Statistical Analyses

Data were analysed using the statistical software package IBM SPSS version 24.0 (USA) and GraphPad Prism version 8. Descriptive analysis was carried out using mean and standard deviation (SD), and median and interquartile ranges (IQRs) for parametric and non-parametric data respectively for continuous variables and percentages for categorical variables. For group-wise comparisons, ANOVA, Kruskal-Wallis test (n groups), Student's t-test or Mann-Whitney test (2-groups) were used as appropriate for continuous variables. Chi-square tests were performed to assess categorical demographics. Correlation of data was performed using Pearson's correlation coefficient. The predictive ability of the individual markers for the severely abnormal outcome was assessed using Positive and Negative Predictive Values (PPVs and NPVs), and area under the curve of the Receiver Operating Characteristic (AUCs).

3.4 Results

3.4.1 Patient Populations

One-hundred and twenty-six infants (mean (SD) gestational age in weeks = 39.8 (1.2); 82 [65%] male) from both the BIHiVE1 and BiHiVE2 cohort were included in the downstream mRNA expression analysis. The patient populations are summarised in Table 3.2.

Table 3.2. Patient populations.

	BiHIVE1 Discovery Cohort				BiHIVE2 Validation Cohort			
	Control *n* = 16	PA *n* = 19	HIE *n* = 20	***P*-Value**	Control *n* = 22	PA *n* = 25	HIE *n* = 24	***P*-Value**
Gestation (wk)	40.25 (0.86)	39.7 (1.1)	40.0 (1.2)	0.573	40 (39-41)	40 (39-41)	40 (39-41)	0.736
Birthweight (g)	3483 (412)	3688 (618)	3583 (555)	0.539	3803 (420)	3732 (464)	3723 (447)	0.806
Sex – M/F	9/7	12/7	16/4	0.286	14/8	16/9	15/9	0.994
1 min. Apgar [a]	N/A	6 (4-8)	3 (1-4)	<0.001	9 (9-9)	6 (5-8)	1 (2-4)	<0.001
5 min. Apgar [a]	10 (9.5-10)	9 (7-10)	5 (3-7)	<0.001	10 (9-10)	9 (9-9)	4 (4-6)	<0.001
Cord pH	N/A	7.04 (0.10)	6.99 (0.11)	0.0147	7.26 (0.08)	7.04 (0.06)	6.99 (0.14)	<0.001
Initial Lactate [a]	N/A	7.2 (2.9)	8.9 (4.6)	0.043	N/A	6.2 (3.1)	10.7 (3.0)	0.001
Initial Base Deficit [a]	N/A	7.1 (3.8)	12.7 (5.6)	0.012	N/A	6.7 (4.6)	14.7 (4.9)	<0.001
Sarnat Score Mild/Moderate/Severe			10/5/5				15/8/1	
Mode of Delivery, No. (%)				0.493				0.054
SVD	N/A	2 (10)	5 (25)		13 (59)	8 (32)	4 (17)	
Instrumental	N/A	14 (74)	12 (60)		7 (32)	14 (56)	16 (67)	
LSCS	N/A	3 (16)	3 (15)		2 (9)	3 (67)	4 (17)	

Population Demographics a-cross both cohorts. Mean (SD), Median (IQR) or n (%). Wk = week, g = grams, M = Male, SVD = Spontaneous vaginal delivery, LSCS = Lower segment caesarean section. [a] = $P < 0.05$ across both cohorts.

3.4.2 mRNA Expression Across Groups and HIE Grade

Two genes were predicted in the Wnt signalling pathway as downstream targets of all three of the previously validated miRNAs (390). Both mNFAT5 and mFZD4 expression were subsequently analysed using qPCR. The final groups consisted of 124 infants; including healthy controls, *n* = 37, infants with PA (without encephalopathy), *n* = 43, and infants with HIE, *n* = 44. The mRNA expression analysis showed no altered expression across the healthy control vs PA vs HIE groups.

The mRNA expression analysis across grades within the HIE group included 44 infants. The HIE group included mild HIE grade, *n* = 25, moderate HIE grade, *n* = 13, and severe HIE grade infants, *n* = 6. Altered levels of mFZD4 were observed across the grades of HIE, *P* = 0.008; increased levels of mFZD4 were observed in severe HIE (median relative quantification (RQ) = 2.98 (IQR = 2.23-3.68)) vs mild HIE (0.88 (0.46-1.37)), *P* = 0.004, and in severe HIE (2.98 (2.23-3.68)) vs moderate HIE (1.05 (0.81-1.20)), *P* = 0.003. No differences were determined between mild and moderate HIE infants (figure 3.1A). No altered levels of mNFAT5 were observed across grades, *P* = 0.568.

The mRNA expression analysis of ability to detect therapeutic eligibility (TH) grouped the total population into infant's ineligible for TH (healthy controls, PA, and mild HIE), *n* = 105, and infants eligible for TH (moderate HIE and severe HIE), *n* = 19. Increased levels of mFZD4 were observed in infants eligible for TH (1.20 (0.93-2.37)) vs infants' ineligible for TH (0.81 (0.46-1.53)), *P* = 0.017 (figure 3.1B). No altered levels of mNFAT5 were observed between the two eligibility groups, *P* = 0.093.

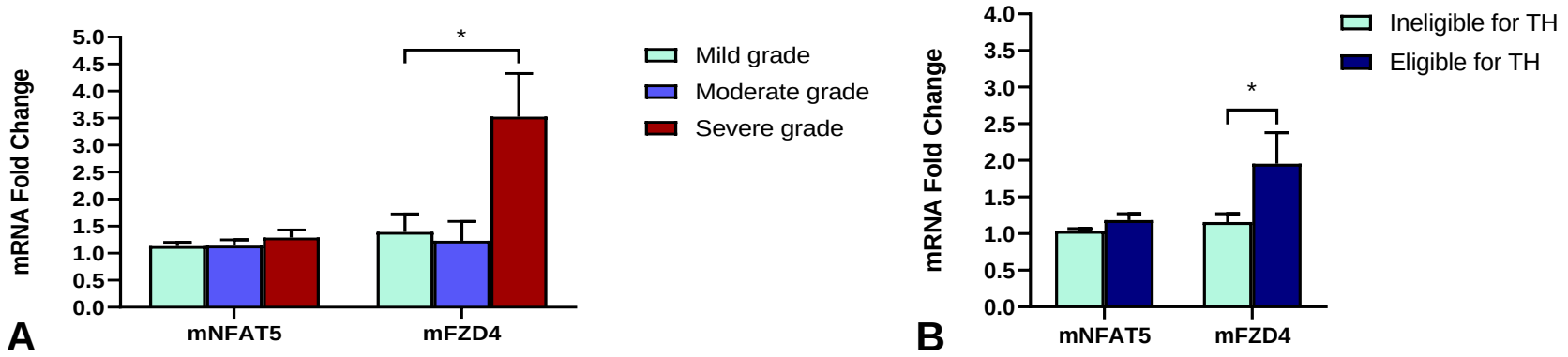

Figure 3.1. Relative quantification levels in messenger RNA from the BiHiVE studies. **A. Across population groups.** The hypoxic ischaemic encephalopathy (HIE) group included infants with mild HIE grade, *n* = 25, moderate HIE grade, *n* = 13, and severe HIE grade, *n* = 6. Increased RQ levels of mFZD4 were observed in infants with severe HIE grade vs mild HIE grade, and in severe grade vs mild grade. **B**. **Assessing eligibility for TH.** The groups included infants ineligible for TH (healthy controls, PA, and mild HIE), *n* = 105, and infants eligible for TH (moderate HIE and severe HIE), *n* = 19. Increased RQ levels of mFZD4 were observed in infants eligible for TH vs infants ineligible for TH. **P* < 0.05. Bar graphs represent fold change of RQ, and error bars represent standard error of the mean. RQ = relative quantification.

3.4.3 mRNA Expression in Neurodevelopmental Outcome

Neurodevelopmental outcome was available in sixty-three infants from both the BIHiVE1 and BiHiVE2 cohort, from this group; seven infants were excluded as they were tested in a language that was not their first language. The final mRNA expression analysis of the neurodevelopmental outcome group included 56 infants including a normal outcome, *n* = 38, a mildly abnormal outcome, *n* = 11, and a severely abnormal outcome, *n* = 7. Altered levels of mNFAT5 were observed across the outcome groups, *P* = 0.051; increased levels of mNFAT5 were observed in severely abnormal (1.26 (1.17-1.39)) vs normal outcome infants (0.97 (0.83-1.24)), *P* = 0.036, and in severely abnormal (1.26 (1.17-1.39)) vs mildly abnormal outcomes infants (0.96 (0.80-1.06)), *P* = 0.012. Altered levels of mFZD4 were observed across the outcome groups, *P* = 0.012; Increased levels of mFZD4 were observed in severely abnormal (2.51 (1.60-3.56)) vs normal outcome infants (0.74 (0.48-1.49)), *P* = 0.004, and in severely abnormal 0.74 (2.51 (1.60-3.56)) vs mildly abnormal outcome infants 0.97 (0.75-1.34)), *P* = 0.026 (figure 3.2). No differences were determined between normal and mildly abnormal outcome infants across each mRNA.

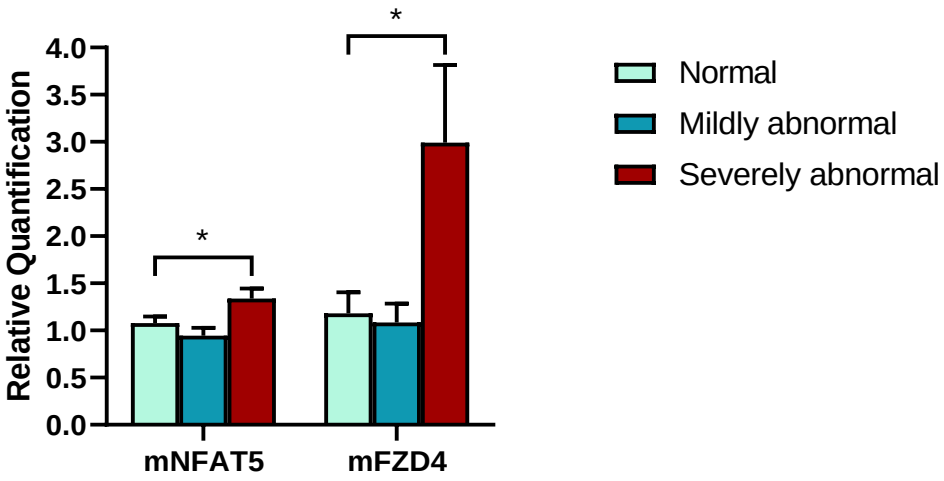

Figure 3.2. Relative quantification levels in messenger RNA from the BiHiVE studies assessing neurodevelopmental outcome. The analysis included infants with a normal outcome, *n* = 38, infants with a mildly abnormal outcome, *n* = 11, and infants with a severely abnormal outcome, *n* = 7. Increased RQ levels of mNFAT5 and mFZD4 were observed in the severely abnormal outcome group vs normal and moderately abnormal outcome. **P* < 0.05. Bar graphs represent fold change of RQ mean, and error bars represent standard error of the mean. RQ = relative quantification.

3.4.4 Ability to Assess Eligibility for TH and Neurodevelopmental Outcome

In our final analysis we compared mNFAT5 and mFZD4 with current early biochemical and clinical markers, using the standard cut-offs used in previous trials of TH (374), these were determined for both eligibility for TH and neurodevelopmental outcome independently. Infants with normal/mildly abnormal outcome, *n* = 49, were grouped against infants with severely abnormal outcome, *n* = 7. Associations between mNFAT5 and mFZD4 and current standard of care markers can be seen in Table 3.3. Both mNFAT5 and mFZD4 had a high NPV compared to current markers in assessing eligibility for therapeutic hypothermia. In predicting severely abnormal neurodevelopmental outcome mFZD4 had a higher AUC and PPV than all current biochemical and clinical measurements at birth when compared with standard cut-offs (374).

Table 3.3. Comparison of biochemical and clinical markers.

Marker	PPV (%)	NPV (%)	AUC (95% CI)	*P*-Value
Eligibility for therapeutic hypothermia				
Biochemical markers				
Cord pH	24	88	0.613 (0.445-0.781)	0.156
Lactate *	39	43	0.778 (0.640-0.915)	0.001
Base deficit *	67	82	0.792 (0.669-0.914)	<0.001
mNFAT5	22	90	0.622 (0.495-0.748)	0.093
mFZD4 *	26	93	0.673 (0.542-0.805)	0.017
Clinical markers				
Apgar at 1 min. *	31	98	0.869 (0.792-0.946)	<0.001
Apgar at 5 min. *	58	96	0.912 (0.846-0.978)	<0.001
Severely abnormal neurodevelopmental outcome				
Biochemical markers				
Cord pH	6	92	0.309 (0.060-0.558)	0.274
Lactate	22	100	0.656 (0.372-0.940)	0.278
Base deficit	44	91	0.727 (0.470-0.983)	0.089
mNFAT5 *	29	95	0.776 (0.651-0.900)	0.019
mFZD4 *	50	96	0.843 (0.692-0.993)	0.004
Clinical markers				
Apgar at 1 min. *	20	96	0.800 (0.550-1.000)	0.011
Apgar at 5 min. *	29	95	0.780 (0.563-0.997)	0.017

The optimum cut-off value in mNFAT5 and mFZD4 in eligibility for TH was an RQ = 1.0674 and 1.0038; and in neurodevelopmental outcome was an RQ = 1.2279 and 1.9896 respectively. Standard cut-off values for PPV and NPV were based on the TOBY criteria (374) for Apgar scores ≤ 5, pH ≤ 7.0 and base deficit ≥ 16 mmol/L; and lactate ≥ 7.0. * $P < 0.05$. AUC = area under the curve; CI = confidence interval; PPV = positive predictive value; NPV = negative predictive value.

3.5 Discussion

This is the first clinical study to use two independent cohorts to measure mRNA targets of previously validated miRNA in the whole blood of infants with HIE. The study identified increases in mFZD4 in the severe grade of HIE, increases in mFZD4 in infants eligible for TH; and mNFAT5 and mFZD4 were both increased in infants that went on to have a severely abnormal outcome. These mRNAs show the potential to use cord blood as a potential biomarker to distinguish infants that will develop a severely abnormal neurodevelopmental outcome from the normal and mildly abnormal follow-up population.

Molecular diagnostics is becoming an increasingly important clinical tool; and now molecular material in circulating biofluids can be measured for both diagnostic and prognostic purposes (286, 403, 404). Whole blood contains mRNA from various sites of origin including leukocytes, erythrocytes, platelets, microvesicles and exosomes and potential apoptotic budding. mRNA in vesicles is believed to be primarily short fragments of mRNA (288, 405). Previous reports have shown that circulating exosomal mRNA can be translated into proteins in target mast cells and can alter protein production in these cells (278, 288).

Pathological states of Wnt have been previously identified in neonatal brain injury, and this high Wnt activity has been proposed as an explanation for failed myelination in white matter lesions at birth; the two mRNA in this study are both associated with the Wnt pathway, mNFAT5 and mFZD4, and were chosen as they were predicted downstream targets of the previously validated miRNAs, miR-181b, miR-374a and miR-376c, and all three miRNAs were downregulated in HIE (330, 390). Wnt signalling plays an essential role in embryonic development, including neural development, axon guidance and synaptogenesis; and controls self-renewal across multiple adult tissues (326, 406, 407). In relation to the central nervous system, its role has been described in inflammation, axon regeneration, myelination, prevention of neural death, astrocyte generation, glial cell proliferation and blood-brain barrier integrity (408-410). Wnt signalling operates via the canonical Wnt/β-catenin pathway and the non-canonical Wnt/Ca^{2+} pathway and the planar cell polarity pathway (411, 412).

Wnt signalling has previously been described in neonatal white matter injury where oligodendrocyte progenitor cells in lesions exhibited high levels of Wnt activity (330). Hippocampal neuronal models of oxygen-glucose deprivation/reoxygenation-induced injury can suppress the Wnt/β-catenin pathway which can be reactivated by exposing the neurons to mild hypothermia (413).

The mFZD4 codes for the Fzd4 receptor, a member of the frizzled/smoothened superfamily of cell-surface proteins that function as receptors for the Wnt ligands; and can activate both canonical and non-canonical Wnt pathways (414). The Fzd4 mRNA is 7.7 kb in size and is detectable across most normal human tissues with larger quantities in fetal kidneys, and adult hearts, skeletal muscles, and ovaries (415). High expression of Fzd4 is seen during early hematopoiesis and may suggest a role in hematopoietic regulation. Mice Fzd4 knockout models develop cerebellar and auditory dysfunction (416, 417). The study was unable to identify the source of whole blood mFZD4 and with this it is unclear if the increased expression is directly or indirectly linked to the brain or another source, one proposed explanation is that FZD4 has previously been implicated in angiogenesis during retinal vasculature formation in the brain; and loss of FZD4 can reduce endothelial proliferation and lead to leakiness of the blood-retinal barrier and the blood-brain barrier, which may permit the release of mFZD4 into the peripheral circulation (418-420).

NFAT5 was the first discovered of the NFAT family; this family of proteins are expressed almost ubiquitously across every tissue, including the placenta, and are known as transcriptional regulators in cytokine and immune cell expression (421-423). NFAT5 is widely expressed in the kidneys and is an essential regulator of osmotic stress; both the fetal and adult brain have a high expression of NFAT5, with 10-fold higher levels in fetal brains, with highest expression in the cerebellum (424-426). Recent studies have reported that NFAT5 is positively regulated by hypoxia; renal ischaemic studies have proposed that NFAT5 functions as a molecular switch towards cell survival following ischaemic injury (427-429). NFAT5 has also been described to act as a regulator in Wnt/β-catenin signalling by inhibiting β-catenin acetylation (393).

For this study, we have conducted a subgroup analysis of infants with long-term outcome and have observed significant alterations for infants at risk of severely abnormal outcome with both mNFAT5 and mFZD4 performing better than current clinical markers at birth. These infants had an extremely poor outcome despite hypothermia. This could be used in clinical practise by collecting cord blood in Tempus tubes for infants who meet the criteria for perinatal asphyxia (see recruitment criteria in methodology) at birth, the samples could then have the whole blood RNA isolated and a qPCR performed during the first day of life, the results of the analysis could then be useful to guide treatment decisions during the subsequent postnatal days and identify those newborns who should be offered more experimental adjunct therapies to optimise developmental outcomes.

Limitations to this study include the use of whole blood instead of serum or plasma, making it more difficult to distinguish if the differences we are detecting are cellular blood-based mRNA or extracellular mRNA. If the alterations are extracellular-based mRNA this could imply a form of active transport, apoptosis, or removal of excess cellular mRNA (430-432). Although this study analyses mRNA targets of previously validated miRNA biomarkers, the alterations we have observed must only be considered as mRNA biofluid alterations and cannot be construed as a cause and effect miRNA-mRNA interaction. However, this study does promote further investigation into the potential role of the putative miRNA-mRNA interactions and how they may affect the related pathways, such as Wnt, in the HIE process. This study also highlights a theme that is not new to HIE biomarker studies, where it remains easier to establish differences between infants with a severe grade of HIE against other groups. It remains difficult to determine differences between the moderate grade of HIE and other groups.

The strengths of our study include using two individual cohorts we were able to increase the sample size in a population that remains difficult to recruit in the developed world, with both studies requiring strict identical recruitment criteria across all centres. The BiHiVE2 cohort was a multi-centre study recruited across two countries, and both cohorts included EEG monitoring interpreted by trained neurophysiologists; and neurodevelopment follow-ups conducted by trained

psychologists. Finally, the umbilical cord was used for sample collection to gain insight into the neonate's condition at birth, this was the optimal choice, as it remains non-invasive to both the mother and infant and contains a large blood volume, which can be difficult to acquire from the infant during the early postnatal period. Blood was collected using the Tempus^{TM} blood RNA system, a reliable method for maintaining high quality and quantity of mRNA and required a minimum of 3mL of whole blood (433).

In conclusion, we have observed increased umbilical cord whole blood mRNA expression in infants with severe HIE (mFZD4), in infants eligible for TH (mFZD4) and in infants with severely abnormal long-term outcome (mNFAT5 and mFZD4). These mRNA combined with current diagnostic tools may have potential as early objective diagnostic and prognostic quantitative biomarkers of HIE at delivery.

Chapter 4

Validation of Raised Cord Blood Interleukin 16 in Perinatal Asphyxia and Neonatal Hypoxic-Ischaemic Encephalopathy in the BiHIVE2 Cohort

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4.1 Abstract

Background

The role of inflammation is an important factor in the progression of hypoxic-ischaemic encephalopathy. We have previously shown that (interleukin-16) IL-16 is increased in infants with moderate and severe HIE and relates to poor neurodevelopmental outcomes. We aimed to validate IL-16 as a cord blood-based biomarker of HIE and to examine its relationship with long-term outcomes.

Methodology

The study sample consisted of 105 full-term infants who experienced perinatal asphyxia (with and without an encephalopathy) along with healthy, gestational age-matched newborn controls. Umbilical cord serum was processed and biobanked at delivery. Infants were assigned a modified Sarnat score at 24 hours. Analysis of IL-16 cytokine cord blood levels was performed using the sandwich-based ELISA technique.

Results

Cord blood-based IL-16 was increased in infants with perinatal asphyxia and HIE relative to controls, $P = 0.025$. IL-16 was also increased in the HIE group relative to controls, $P = 0.042$. There was no significant difference in IL-16 across grades of HIE or in those with abnormal outcomes at 2-years of age.

Conclusion

This study validates findings that cord blood-based IL-16 levels are increased in infants with perinatal asphyxia, including those that go on to develop HIE.

4.2 Introduction

Hypoxic-Ischaemic Encephalopathy (HIE) is an important cause of brain injury in the newborn, with an evolving inflammatory response (18, 434). Early classification of HIE remains difficult, as the extent of injury increases over the first few hours of life, along with the potential for long-term neurological disability (435, 436). Therapeutic hypothermia (TH) can attenuate oxygen and metabolic demand, reduce inflammation, and may supress the evolution of apoptotic and necrotic cell death (437-439). To achieve these benefits TH must be initiated within the first 6 hours of life. Within this narrow time window, we must correctly identify those infants that would benefit the most from intervention(32, 73, 440). A robust and reliable early biochemical marker would greatly aid in clinical decision-making.

Currently, acid-base balance, along with clinical examination, are relied upon to guide treatment, but individually, these can have low predictive values (8, 441) (442). The exact extent of injury may not be apparent without using electroencephalogram (EEG) or magnetic resonance imaging (MRI). However, both methods require specialised equipment and expertise and are often unavailable within the 6-hour therapeutic window(22, 29).

The potential of biomarkers has been explored in a variety of biological measurements both invasive and non-invasive including serum, plasma, whole blood, cerebrospinal fluid, saliva, and urine (153, 443, 444). Many biomarkers have been considered a success in early screening, but no one HIE biomarker has been validated in a second cohort (153). Interleukin 16 (IL-16) is a pro-inflammatory cytokine stored and released from CD8+ cytotoxic T-cells, and upon release can generate an inflammatory response by using its chemotactic properties for attracting cells with the CD4+ surface molecule such as CD4+ helper T-cells (445). Before activation, this cytokine can exist within a neuronal precursor, NIL-16, found in neurons in both the cerebellum and hippocampus, or a non-neuronal precursor, pro-IL-16. Both precursors are cleaved by the apoptosis-related caspase-3 to produce mature IL-16 (446, 447). We have previously shown that IL-16 was a measurable cytokine, raised in UCB, and is predictive of severe neurological outcome at three

years of age (66, 189). We aimed to validate raised cord blood IL-16 as a marker for HIE in a second independent cohort.

4.3 Methodology

4.3.1 BiHIVE2 Study

This multi-centre cohort study (clinicaltrials.gov ID: NCT02019147) recruited cases of perinatal asphyxia and healthy controls from Cork University Maternity Hospital and Karolinska University Hospital from 2013 to 2015. The study was designed to validate findings from the original Biomarkers in Hypoxic-Ischaemic Encephalopathy (BiHIVE) study (a biomarker discovery cohort), identical inclusion and exclusion criteria were applied in the two studies (66). All cases recruited to the study were inborn and recruited if they had: gestational age of greater than or equal to 36 weeks and one or more of the following: cord pH less than 7.1, 5-minute Apgar score ≤6 or had been intubated or resuscitated at birth. All cases were observed for the duration of the perinatal period to detect the development of HIE and infants that did not go on to develop HIE were grouped as perinatal asphyxia at discharge. Multi-channel EEG at 24 hours was used to corroborate the clinical Sarnat grade of HIE. Healthy controls were recruited concurrently. Controls were full-term infants with uneventful deliveries, normal Apgar scores, and normal neonatal examinations and without admission to the neonatal intensive care unit. All HIE cases had continuous multi-channel EEG applied, and recordings were started as soon as possible after birth and graded as previously described (29). Clinical HIE grade was assigned at 24 h postpartum using a modified Sarnat score (448). Infants in this cohort went on to be cooled according to the entry criteria and guidelines set by the UK Total Body Hypothermia for Neonatal Encephalopathy (TOBY) cooling registry (from the UK TOBY Cooling Register Clinician's Handbook, section 2.1, http://www.npeu.ox.ac.uk/toby). The Clinical Ethics Committee of the Cork Teaching Hospitals and the Stockholm Ethical Review Board approved these studies in respective countries. Written informed consent was obtained from the parents.

4.3.2 Sample Collection

UCB was drawn and biobanked in all infants. The sampling, processing, and storage of samples was conducted with reference to strict standard operating procedures. The cord blood was collected at delivery, placed in a serum vacutainer, and was given

30 minutes to clot. Following this, the serum was centrifuged at 2,400 ×g for 10 minutes at 4°C. It was then pipetted into a sterile second spin tube and centrifuged at 3,000 xg for a further 10 minutes at 4°C, and aliquoted into 250 µl microtubes before storage at −80°C.

4.3.3 Serum Cytokine Quantification

IL-16 serum levels were measured with a sandwich ELISA using the DuoSet ELISA development kit (R&D Systems, Minneapolis, MN, USA). The kit used a mouse anti-human IL-16 capture antibody, biotinylated goat anti-human IL-16 detection antibody and recombinant human IL-16 standard. Samples were diluted 1:1 and run in duplicates. The range of cytokine detection was from 15.6-1,000 pg/mL and a CV cut off < 20% was used.

4.3.4 Assessment of Outcomes

The neurodevelopmental assessment was carried out when the child was aged between 18 and 36 months of age. The assessment consisted of the BSID-III in addition to three parental report questionnaires: a developmental screening questionnaire, the Child Behaviour Checklist for children 1½ -5 years and the Social-Emotional and Adaptive Behaviour Scale of the BSID-III (449). For the present study, only the BSID-III will be described. The BSID-III is an individually administered, gold-standard neurodevelopmental assessment designed to measure the developmental functioning of infants and toddlers (1 to 42 months). It includes Cognitive, Language (Receptive and Expressive), Motor (Fine and Gross), Social-Emotional, and Adaptive Behaviour Scales. The child's outcome as measured by the BSID-III is determined using the composite scores of the administered subscales. Each composite score has a standardised mean of 100 and a standard deviation (SD) of 15 such that a value below 85 is conventionally used to indicate neurodevelopmental delay (450). In this study, to adjust for the differences between Irish and Swedish populations, a population-based Z-score was created in place of the standardised mean and standard deviation. Both countries had a mean calculated. The two-year outcome was considered normal if it did not fall one standard deviation below the calculated control group mean in all three composite categories (cognitive, language and motor). The outcome was considered abnormal if it fell one standard deviation below

the calculated control group mean in one or more of the three composite categories and severely abnormal if it fell two standard deviations below the mean, the child suffered from severe autism or if the infant was diagnosed with CP or suffered neonatal death.

4.3.5 Statistical Analysis

Statistical analysis was carried out using IBM SPSS Statistics 22 (USA). For group-wise comparisons, ANOVA or the Kruskal-Wallis test (n groups), or Student's t-test or Mann-Whitney test (2-groups) were used as appropriate. The predictive ability of the individual markers for HIE was assessed using positive and negative predictive values (PPVs and NPVs), sensitivity and specificity, and area under the receiver operator curves (AUROC). IL-16 was compared with the current standard markers for HIE including pH, lactate, base deficit and 1, 5, and 10-minute Apgar. The threshold for statistical significance was set at a *P*-value of 0.05.

4.4 Results

4.4.1 Patient Characteristics

A total of 113 infants recruited to the BiHIVE2 study were included in the analyses for the present study. The sample consisted of 27 neonates who experienced perinatal asphyxia without HIE, 26 who experienced HIE (15 mild, 10 moderate and 1 severe) and 60 healthy, sex and age-matched controls. In the HIE infants, 8 moderate and 1 severe, went on to receive TH according to TOBY criteria and guidelines. Eight infants were excluded from the analyses, as their IL-16 levels were undetectable. The clinical characteristics of the study sample are summarised in Table 4.1.

4.4.2 Validation Results

There was a significant increase in IL-16 in infants with perinatal asphyxia, including those that went on to develop HIE, median (interquartile range (IQR)) = 298.61 (204.26-374.25) pg/mL, relative to the healthy sex and age-matched control group, median (IQR) = 204.89 (128.82-338.9) pg/mL, P = 0.024 (figure 4.1A). IL-16 was further increased in the HIE group, median (IQR) = 324.12 (185.03-391.31) pg/mL relative to the control group, median (IQR) = 204.89 (128.82-338.9) pg/mL, P = 0.041 (figure 4.1B). No significant differences in IL-16 were observed in infants considered eligible for TH (moderate/severe HIE, n = 11) when compared with infants not eligible for TH (control/PA/mild HIE, n = 94), P = 0.198. There was no significant difference in IL-16 between infants with normal and abnormal outcome at two years of age, P = 0.397. There was no differences in IL-16 between genders.

Table 4.1. Characteristics of study sample as a function of clinical and non-clinical groups.

BIHIVE2 Study	Control	PA	HIE	P-Value
***n* = 113**	*n* = 60	*n* = 27	*n* = 26	
Gestation (wk)	40 (39-41)	40 (39-41)	40 (39-41)	0.782
Sex (M/F)	34/26	16/11	14/12	0.925
Birth Weight (g)	3737.5 (549.1)	3610.0 (510.8)	3680.0 (495.6)	0.098
Admitted to NNU (Y/N)†	3/57	8/19	26/0	<0.001
Mode of Delivery†				<0.001
SVD	47 (78.3%)	12 (44.4%)	5 (19.2%)	
Instrumental	8 (13.3)	11 (40.7%)	18 (69.2%)	
LSCS	5 (8.3%)	4 (14.4%)	3 (11.5%)	
1min Apgar†	9 (9-9)	7 (5-8)	3 (1-4)	<0.001
5min Apgar†	10 (10-10)	9 (8-10)	5 (4-6)	<0.001
10min Apgar†	10 (10-10)	10 (10-10)	7 (6-8)	<0.001
First Cord pH*	7.4 (7.3-7.4)	7.2 (7.2-7.3)	7.0 (6.9-7.1)	0.001
First Base Deficit (-) *	6.1 (3.6-8.5)	5.0 (4.6-9.0)	14.7 (12.5-17.3)	0.003
First Lactate*	4.8 (2.7-6.8)	4.4 (3.6-7.9)	11.0 (9.6-12.1)	0.004

Mean (Standard Deviation) [one-way analysis of variance to compare groups], Median (Interquartile Range) or n (%) [Kruskal-Wallis H test used to compare groups]. M = Male, F = Female, NNU = Neonatal Unit, SVD = Spontaneous vaginal delivery, LSCS = Lower segment caesarean section, MRI = Magnetic Resonance Imaging. *P <0.05. P <0.001.

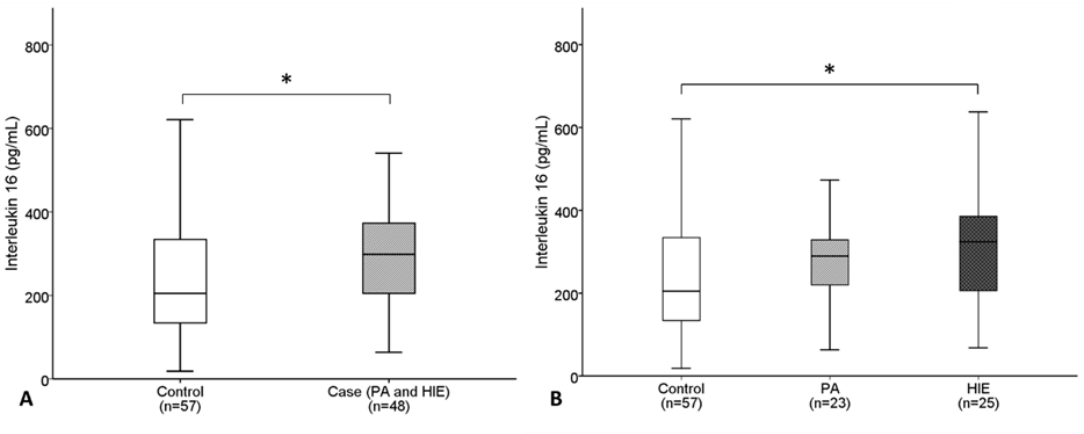

Figure 4.1. IL-16 assay levels in umbilical cord blood serum. Represented by box plots across A. Two groups where there was an increase in IL-16 in the cases (PA and HIE) compared to the controls, *P* = 0.025. B. There was a significant increase in IL-16 in the HIE group compared to the control group, *P* = 0.041.

4.4.3 Predictability Compared with Current Markers for Cooling Eligibility

Assessors of predictability were used to compare the current biochemical (pH, lactate, base deficit) and clinical markers (Apgar) for identifying infants eligible for TH with our candidate marker IL-16 (table 4.2). IL-16 had a high negative predictive value (NPV) of 95% but had a low positive predictive value (PPV) of 16% compared to the current clinical and biochemical markers using a cut-off of > 256 pg/mL. The values for current markers were set using standard cut-off values used to determine eligibility for TH (375).

Table 4.2. Current standard parameters and IL-16, to predict moderate-severe Sarnat score at 24hr for infants with HIE.

	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	AUC (CI)	P-Value
Biochemical Markers						
pH*	47	82	64	77	0.740 (0.579-0.90)	0.023
Lactate*	40	89	91	34.8	0.763 (0.592-0.933)	0.014
Base Deficit*	67	84	60	92	0.844 (0.704-0.984)	0.002
Clinical Markers						
1min. Apgar†	29	99	73	85	0.864 (0.782-0.946)	<0.001
5min. Apgar†	47	96	55	93	0.906 (0.840-0.973)	<0.001
10min. Apgar†	80	93	18	100	0.920 (0.812-1.0)	<0.001
Candidate Marker						
IL-16	16	95	73	55	0.619 (0.450-0.788)	0.198

PPV = positive predictive value, NPV = negative predictive value, AUC = area under the curve, CI = Confidence Internal. For calculating PPV and NPV standard cut off values for cooling eligibility were used for pH (≤7.0), lactate (>7), base deficit (≥16 mmol/L) and Apgar 1,5 and 10-minute (≤5). The optimum cut off values for IL-16 was calculated to be >256 pg/mL with 73% sensitivity and 55% specificity. *P <0.05. †P <0.001.

4.5 Discussion

This validation study echoes the findings of our initial study (189) where we demonstrated that the levels of IL-16 in cord blood were raised in infants with HIE. These results suggest that an early inflammatory response occurs in perinatal asphyxia but that it is more pronounced in infants that go on to develop HIE.

The immune response of the body is an active process that continues to change during early development. This response can cause massive disruptions following perinatal injury (54). Cytokines are produced by immune cells, including microglia and astrocytes, which act as regulators of inflammatory interactions, while also changing with length and severity of injury (434, 451). The role of cytokines can be either pro-inflammatory or anti-inflammatory. In the context of ischaemic insults in both adult and newborns, the exact role of the inflammatory response remains controversial (434).

Changing cytokine levels have been described in HIE, and several studies have focused on the pro-inflammatory cytokines IL-1β, IL-6 and tumour necrosis factor (TNF-α) as potential markers for this condition (184, 208, 210, 452). Results of these studies have varied around viable markers in cerebrospinal fluid and cord blood, but to our knowledge none of these possible biomarkers have been validated in an independent cohort (184, 208, 452).

This study explores the pro-inflammatory cytokine, IL-16, that may act as a viable biomarker for HIE. A series of biomarker studies have demonstrated an increase in IL-16 levels in stroke, ischaemic cerebrovascular dementia and HIE (66, 453, 454). Mature IL-16 is produced following caspase-3 cleavage from either its precursor proteins pro-IL-16 or NIL-16 (446, 447). IL-16 is recognised as a mediator of CD4+ T-cells, to promote the production of the TNF-α, IL-1β and IL-6, and may act as a regulator of apoptosis (455, 456).

Regarding the clinical utility of serum IL-16 levels for predicting eligibility for TH, it did not give better prediction than existing clinical and biochemical markers (table 4.2). Within the cohort, the most substantial PPV value for a biochemical marker was

67% for the base deficit, and it had an NPV of 84%. The most substantial clinical marker was Apgar at 10 minutes with the highest PPV of 80% and NPV of 93%. Apgar at 10 min < 5 is a frequent but not absolute marker for a Moderate/Severe HIE grade (5), and as seen in this study, it can have low sensitivity = 18% (table 4.2).

Although this study aimed to mirror the original study as much as possible, there were some critical differences between the two (66). For example, the present study only included one infant with severe HIE whereas the original study, included seven infants with severe HIE. It is likely that this reduction in severe HIE cases is driven by improvements in the obstetric care provided at the two recruiting hospitals.

R&D human IL-16 DuoSet ELISA development kit was used to measure the IL-16 cytokine in this study, and the previous study was performed with multiplex assays using Luminex technology (Rules Based Medicine, Austin, TX; acquired by Myriad Genetics, Utah, USA). Use of different assays for measuring cytokines have been known to correlate well, but it is unclear if identical antibody pairs, diluents and blockers were used and these may be needed to produce similar quantitation values, therefore, this must be highlighted for future studies (457).

In the present study, umbilical cord serum was used due to a limited number of plasma samples. Changing between serum and plasma have been suggested to affect cytokine measurements (458, 459), including measurements of IL-16, which is shown to yield lower levels in serum compared to plasma (460). This lower yield is consistent in our study where the median of both control and HIE group were lower compared to the original study. However, the trend towards increased IL-16 levels in HIE remains.

Our group previously described alterations in IL-16 with a level above 514 pg/mL using the Luminex assay, which predicted severe disability or death at three years. However, we did not observe similar outcomes in this study (189). Here, only three infants had the severely abnormal outcome (including autism), and these were spread across control, PA and moderate HIE groups. There were no incidents of CP or death in the present sample.

This study was performed with a large cohort of 113 infants recruited independently from the original study. We continued to see an increase in cord blood-based IL-16 levels in asphyxiated and HIE infants compared to healthy controls. Changes in the ELISA kit, the medium (serum vs plasma), and the number of infants with a severe grade of HIE, prevented us from comparing our absolute ranges of IL-16 with those observed in the original study.

IL-16 is raised in the UCB of infants with perinatal asphyxia and HIE. Although this suggests that asphyxia produces an early inflammatory response, IL-16 alone is not more predictive than current markers, and so, the search for a reliable and robust biomarker in HIE continues (461).

Chapter 5

Umbilical Cord Activin A and mACVR2B are Not Reliable Biomarkers of Mild or Moderate Neonatal Hypoxic-Ischaemic Encephalopathy

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5.1 Abstract

Background

The Activin A protein and its receptor mACVR2B have both been considered viable biomarkers for the diagnosis of hypoxic-ischaemic encephalopathy; mACVR2B is considered a downstream target of the previously validated miR-374a (chapter 3). The aim of this study was to assess levels of serum Activin A and whole blood mACVR2B as early biomarkers of perinatal asphyxia, severity of hypoxic-ischaemic encephalopathy, and long-term neurodevelopmental outcome.

Methodology

One-hundred and twenty-six infants were included in these analyses of the BiHIVE2 validation cohort selected from the total population of the BiHIVE2 cohort, a multi-centre independent study, recruited in both Cork University Hospital, and Karolinska Huddinge, Sweden from 2013-2015. Serum Activin A was measured using ELISA, and whole blood mACVR2B was measured using quantitative real-time polymerase chain reaction.

Results

From 126 infants included in the analyses, the serum Activin A analysis included 101 infants (controls = 50, PA = 28, HIE = 23). No differences were observed across the groups, $P = 0.693$. The HIE group included 23 infants (mild = 14, moderate = 9), and no differences were observed across the grades of HIE, $P = 0.115$. The whole blood mACVR2B analyses included 67 infants (controls = 22, PA = 23, and HIE = 22), and no differences were observed across the groups, $P = 0.746$. The HIE group included 22 infants (mild = 15, moderate = 7) and no differences were observed across the grades of HIE, $P = 0.468$. No differences were observed in infants followed up to 18-36 months in serum Activin A or in whole blood mACVR2B, $P = 0.550$ and $P = 0.881$ respectively.

Conclusion

Umbilical cord serum Activin A and whole blood mACVR2B are not effective biomarkers of infants with moderate HIE; they are unable to distinguish across the grades of mild to moderate HIE and long-term neurodevelopmental outcomes.

5.2 Introduction

Novel biomarkers in neonatal hypoxic-ischaemic encephalopathy (HIE) are critical to improving efficiency in early diagnosis. Currently, a combination of biochemical and clinical measurements are used to identify the presence of the encephalopathy and the grade of injury, but these measurements are inadequate (6, 8, 441, 442). Early identification is critical as therapeutic hypothermia within the first 6 hours of life can improve long-term outcome in the moderate and severe HIE infants. Both early electroencephalogram (EEG) and magnetic resonance imaging (MRI) have been proven as accurate measures for early diagnosis, but both require specialist training for interpretation (22, 29). Multiple biomarkers have been explored across pilot studies, but unfortunately, large-scale independent study validation is scarce across the field (135). Our group has previously found alterations in umbilical cord whole blood levels of mACVR2B, an activin receptor, along with altered trends in umbilical cord serum levels of activin A specific to severe HIE(462). The mACVR2B receptor has been identified as a predicted target of miR-374a, this miRNA is decreased in cord whole blood of infants with HIE (390, 462).

Activins are circulating proteins that origin from various tissue sources and are involved in regulating stimulation and secretion of the follicle stimulating hormone (463). Activin A is the most biologically active form of activin, and is a dimeric growth factor containing two βA subunits, and its biological activity is mediated via two type 1 receptors, ACVR1A and ACVR1B, and two type 2 receptors, ACVR2A and ACVR2B (464, 465). Activin receptors and the activin subunit mRNA are highly expressed in neuronal cells (466, 467). The receptors are part of the TGF-β receptor family and control ligands that signal through the TGF-β pathway (468). The activin receptors are involved in growth, differentiation, regulation and apoptosis (469).

Specific roles in activin signalling include muscle growth, reproduction and early development, with overactivation related to cancer (470-472). Activin has previously displayed neuroprotective properties, and its levels have varied across the brain (473). Activin mRNA expression has been noted in a wide variety of tissues including the reproductive system, placenta and brain in the cerebral cortex (474). Activin A is

of particular interest in HIE as it has been shown to enhance oligodendrocyte differentiation (475), and its receptor ACVR2B appeared to be downregulated during oligodendrocyte remyelination and increased specifically in the non-repairing lesions (476). The pattern of distribution of activin mRNA has been observed around microvessels in the days following HIE in the rat brain (477). Activin A has been studied in clinical HIE and found to be correlated with both magnetic resonance imaging diffusion co-efficient value and severity of HIE grade, and changes in activin A have been observed across the first days of life (194, 195, 478, 479).

This study aimed to assess if alterations in mild or moderate HIE could be detected both cord whole blood mACVR2B and serum cord blood levels of activin A in an independent multi-centre cohort study recruited across two countries. Further to this, we wanted to assess if alterations in these cord blood biomarkers was associated with the long-term neurodevelopmental outcome of these infants.

5.3 Methods

5.3.1 Patient Population

The BiHIVE2 multi-centre cohort was recruited from March 2013 to June 2015 at Cork University Maternity Hospital, with 7,500 deliveries per annum, and Karolinska University Hospital Huddinge, with 4,400 deliveries per annum. The study was designed to validate and expand on findings from the original BiHIVE (Biomarkers in Hypoxic-Ischaemic Encephalopathy) study and used identical recruitment criteria to the BiHIVE1 cohort (213). All cases recruited to this study were inborn and fulfilled the following inclusion criteria: gestational age ≥36 weeks and one or more of the following: cord pH <7.1, 5 min. Apgar score ≤6, and intubation or resuscitation at birth. All cases were observed for the duration of the perinatal period to detect the development of HIE, and infants who did not go on to develop HIE were grouped as PA at discharge. All HIE cases had continuous multi-channel EEG applied, and recordings were started as soon as possible after birth and graded as previously described (66). A clinical HIE grade was assigned at 24 h postpartum using a modified Sarnat score (77). Full-term healthy control infants were concurrently recruited with uneventful deliveries, both normal Apgar scores and neonatal examinations, and without admission to the neonatal intensive care unit. Infants were matched for gestational age, sex, and birthweight. The clinicaltrials.gov identifier for the BiHIVE2 study is **NCT02019147**. The Clinical Research Ethics Committee of the Cork Teaching Hospitals and the Regional Ethical Review Board in Stockholm approved the BiHIVE2 multi-centre validation cohort. Written informed consent was obtained from parents of all study participants.

5.3.2 Sample Collection

UCB was collected immediately following the delivery of the placenta and processed within 3 hours. The 3 mL cord blood used for gene expression analyses was placed directly into Tempus Blood RNA tubes (Applied Biosystems, USA) and biobanked at -80°C. The cord blood serum was placed in a serum vacutainer and allowed 30 minutes to clot. Following this, the serum was centrifuged at 2,400 x g for 10 min at 4°C. It was then pipetted into a sterile second spin tube and centrifuged at 3,000 x g

for a further 10 min at 4 °C and aliquoted into 250-µL microtubes before storage at −80 °C

5.3.3 Serum Activin A Quantification

Activin A serum levels were measured with a sandwich ELISA using the DuoSet ELISA development kit (R&D Systems, Minneapolis, MN, USA). The kit used a mouse anti-human activin A capture antibody, biotinylated mouse anti-human activin A detection antibody and recombinant human activin A standard. Samples were diluted 1:1 and run in duplicates. The range of cytokine detection was from 125-8,000 pg/mL, and a coefficient of variation (CV) cut off < 20% was used between duplicates.

5.3.4 Detection and Quantification of messenger RNA Expression

Total RNA was isolated from the Tempus system using the MagMAX for Stabilized Blood Tubes RNA Isolation Kit (Ambion, Life Technologies, USA) and quantified using a NanoDrop 8000 Spectrophotometer (ThermoScientific NanoDrop, USA). Isolated RNA was stored at -80°C before quantitative real-time PCR (qRT-PCR, polymerase chain reaction). Five ng/µl of isolated RNA was used for reverse transcription to synthesise cDNA (complementary DNA) and the mACVR2B expression was analysed using ExiLERATE LNA^{TM} qPCR (Exiqon, Denmark, acquired by Qiagen, Netherlands), with SN48 as a reference gene (370). All samples were run in triplicate, cycle threshold (CT) values were recorded, and expression levels were calculated relative to controls using the $2^{-\Delta\Delta CT}$ method (400).

5.3.5 Neurodevelopmental Assessment

The neurodevelopmental assessment was performed by a trained clinician or psychologist when the child was aged between 18 and 36 months of age and consisted of the BSID-III (401). The BSID-III is an individually administered, gold-standard neurodevelopmental assessment designed to measure the developmental functioning of both infants and toddlers (1 to 42 months) and includes Cognitive, Language (Receptive and Expressive), Motor (Fine and Gross), Social-Emotional, and Adaptive Behaviour Scales. The child's outcome as measured by the BSID-III is determined using the composite scores of the administered subscales. Each composite score has a standardised mean of 100 and a standard deviation (SD) of 15

such that a value below 85 is conventionally used to indicate neurodevelopmental delay (402). In this study, to adjust for the differences between Irish and Swedish populations, a population-based Z-score was created in place of the standardised mean and standard deviation (480). The two-year outcome was considered normal if it did not fall one SD below the calculated control group mean in all three composite categories (cognitive, language and motor). The outcome was considered abnormal if it fell one SD below the calculated control group mean in one or more of the three composite categories and severely abnormal if it fell two standard deviations below the mean in one or more group, or if the child had severe autism, CP or neonatal death.

5.3.6 Statistical Analyses

Descriptive analyses were conducted using mean and standard deviation (SD), and median and interquartile ranges (IQRs) for parametric and non-parametric data respectively, and continuous variables and percentages were used for categorical variables. For group-wise comparisons, ANOVA, Kruskal-Wallis test (*n* groups), Student's t-test or Mann-Whitney test (2-groups) were used as appropriate for continuous variables. Chi-square tests were performed to assess categorical demographics. Data were analysed using the statistical software package IBM SPSS version 24.0 (USA) and GraphPad Prism version 8.

5.4 Results

5.4.1 Patient Population

One-hundred and twenty-six infants were included in these analyses of the BiHIVE2 validation cohort. The patient population is summarised in Table 5.1.

Table 5.1. BiHIVE2 cohort patient population.

	Control *n* = 57	PA *n* = 35	HIE *n* = 31	***P*-Value**
Gestation (wk)	40 (39-41)	40 (39-41)	40 (39-41)	0.680
Birthweight (g)	3789 (599)	3712 (500)	3641 (484)	0.471
Sex - M (%)	33 (58%)	20 (57%)	18 (58%)	0.997
1 min. Apgar*	9 (9-9)	7 (5-9)	3 (1-4)	**<0.001**
5 min. Apgar*	10 (10-10)	9 (9-10)	4 (4-6)	**<0.001**
Initial Lactate*	2.7 (2.6-4.75)	4.4 (2.9-6.8)	10.95 (9.6-12)	**<0.001**
Initial Base Deficit*	3.6 (2.9-6.05)	4.85 (2.5-7.3)	14.7 (12.5-17.3)	**<0.001**
Sarnat Score				
Mild/Moderate/Severe			18/12/1	
Mode of Delivery*				**<0.001**
SVD	43 (75%)	15 (43%)	7 (7%)	
Instrumental	10 (18%)	15 (43%)	20 (14%)	
LSCS	4 (7%)	5 (14%)	4 (13%)	

Clinical Demographics in the BiHIVE2 Validation Cohorts. Mean (SD), Median (IQR) or n (%). Wk = week, g = grams, M = Male, SVD = Spontaneous vaginal delivery, LSCS = Lower segment caesarean section. * = $P < 0.05$.

5.4.2 Activin A and mACVR2B Across Groups and HIE Grades

The serum activin A analysis included one-hundred and one infants, including controls, *n* = 50, PA, *n* = 28, and HIE, *n* = 23 (14 mild and 9 moderate), nineteen serum samples were excluded due to haemolysis, fibrin clots, and high CV levels. On analysis of ELISA results, there was no statistically significant difference observed between controls (median pg/ml = 614.88 (interquartile range (IQR) = 409.63-1217.54)), *n* = 50, and cases (847.81 (378.89-1402.68)), *n* = 51, *P* = 0.392; or across the patient groups, controls (614.88 (409.63-1217.54)), PA (1016.05 (329.58-1796.91)), and HIE (779.69 (498.47-1226.72)), *P* = 0.693 (figure 5.1A). There was also no differences

observed across grades of HIE, mild (739.46 (321.08-1141.89)) and moderate (1219.73 (795.37-1421.15)), *P* = 0.115 (figure 5.1B).

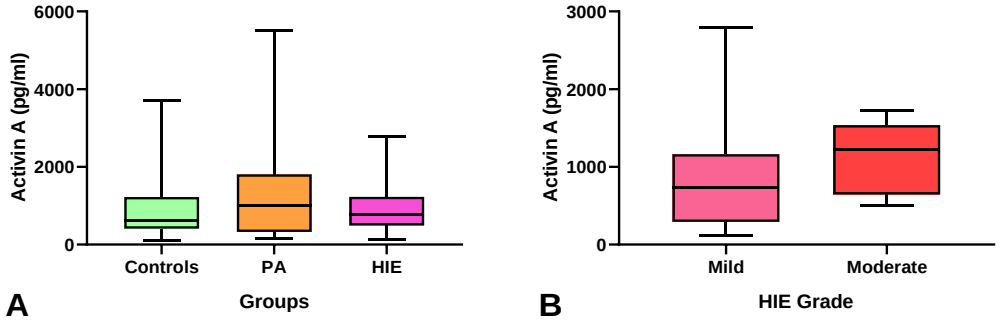

Figure 5.1. Activin A in serum. Analysis across **A**. Patient groups, no significant difference across groups, *P* = 0.693. **B.** Grades of HIE, no significant difference across grades of HIE, *P* = 0.115. Boxplots represent median, IQR and min-max.

The mACVR2B expression analysis were carried out on a subgroup of the population due to limited cord whole blood samples and included sixty-seven infants, including controls, *n* = 22, PA, *n* = 23, and HIE, *n* = 22 (15 mild and 7 moderate). In the gene expression analysis there was no significant difference observed between controls (median relative quantification (RQ) = 0.92 (IQR = 0.61-1.15)), *n* = 22, and cases (0.85 (0.58-1.18)), *n* = 46, *P* = 0.689, or across the patient groups, controls (0.92 (0.61-1.15)), PA (0.79 (0.56-1.11)) and HIE (0.89 (0.58-1.31)), *P* = 0.746 (figure 5.2A). There were also no differences observed across grades of HIE, mild (mean RQ = 1.05 (standard deviation = 0.53)) and moderate (0.88 (0.45)), *P* = 0.468 (figure 5.2B).

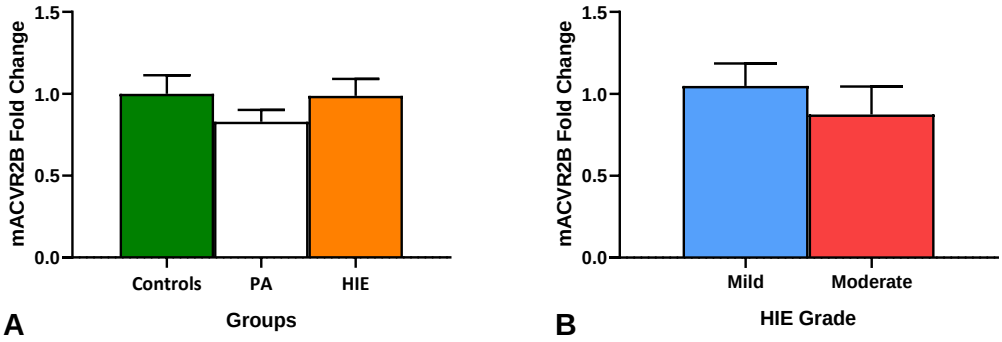

Figure 5.2. Fold change mACVR2B expression (RQ). Analysis across **A**. Patient groups, no significant difference across groups, *P* = 0.746. **B.** Grades of HIE, no significant difference across grades of HIE, *P* = 0.468. Bar graphs represent fold change of RQ mean, and error bars represent standard error of the mean. RQ =relative quantification.

5.4.3 Activin A and mACR2B in Neurodevelopmental Outcome

This patient population included 70 infants followed-up with neurodevelopmental assessment. The activin A outcome analysis had follow-up on fifty-six infants, including normal, *n* = 43, abnormal, *n* = 10, and severely abnormal outcome, *n* = 3; on analysis of ELISA results there was no statistically significant difference observed across the normal (median pg/ml = 795.37 (IQR = 431.45-1358.98)), abnormal (608.57 (333.32-1074.25)) and severely abnormal outcome groups (433.87 (322.49-1042.04)), *P* = 0.550 (figure 5.3A).

The mACVR2B expression analysis included follow-up on thirty-one infants, including infants with a normal, *n* = 25 and abnormal outcome, *n* = 6; there was no statistically significant difference observed across the normal (mean RQ = 0.91 (SD = 0.43) and abnormal outcome infants (1.05 (0.77)), *P* = 0.881 (figure 5.3B).

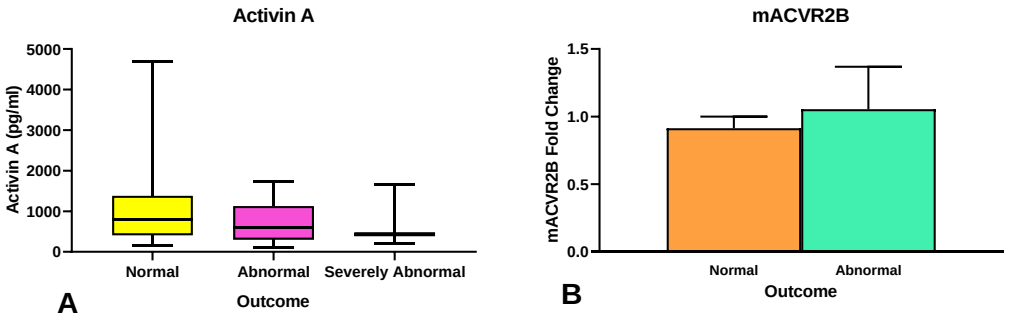

Figure 5.3. Neurodevelopmental outcome in Activin A and mACVR2B. Analysis in **A.** Activin A expression observed no differences across the outcome groups, *P* = 0.550. **B.** mACVR2B expression (RQ) observed no differences across the outcome groups, *P* = 0.881. Boxplots represent median, IQR and min-max. Bar graphs represent fold change of RQ mean, and error bars represent standard error of the mean. RQ = relative quantification.

5.5 Discussion

Activin A has been considered a potential biomarker of acute neuronal damage as it has shown to be increased in CSF following traumatic brain injury and increased across the cortical and hippocampal tissue of HIE rodent models (481, 482). Increased levels of activin A have also previously been reported in multiple clinical PA studies of umbilical cord plasma levels at birth, urinary levels in the first days of life, and in cerebrospinal fluid in the first day of life (194, 195, 478, 479).

This study was part of a more extensive validation study performed to determine if previous findings from our BiHIVE1 cohort for severe HIE generalised to mild and moderate HIE (462). The original study identified mACVR2B as a predicted downstream target of the previously validated miR-374a, and demonstrated increased levels of umbilical cord whole blood mACVR2B in severe HIE; the original study also observed higher levels of umbilical cord serum activin A as the grade of HIE severity increased, but these trends were not significant. The current study, however, was unable to find any significant alterations across the groups.

Activins signal via their receptors at the plasma membrane; and the ACVR2B receptors are considered constitutively active kinases. Activin type II receptors, ACVR2A and ACVR2B, are required for ligand-binding and for the expression of type I receptors, ALK4 and ALK5, which together form a stable complex, and activate downstream SMAD2 and SMAD3 molecules which control apoptosis, proliferation and differentiation (483, 484). Decreased ACVR2B expression has been associated with reduced neuronal apoptosis, where knockdown of expression deregulation programmed cell death in an avian model of embryonic neuronal development (485).

The study has some limitations; although this study was recruited across two countries, it includes only mild and moderate HIE. Although previous studies have recognised changes in moderate HIE, animal models of HIE indicate that activin A is only altered in a severe HI insult, with no alterations observed in the moderate HIE model (486, 487). Future validations should include severe HIE although it remains challenging to recruit large numbers of infants with a severe grade of HIE. Operational difficulties can sometime preclude the collection of these samples and

we have seen aninability to recruit a large number of infants with a severe grade of HIE is related to amalgamation of maternity services and the development of in-house senior obstetric call. Most of the centre's cases of severe HIE are thus outborn and so collection of UCB is not routinely possible. The use of mRNA as blood-based biomarkers is a continuously expanding field and the ability to detect accurately precise changes continues to be a challenge (286, 488). This study used the Tempus system for blood collection, which should contain both cellular transcripts from erythrocytes, leukocytes and platelets and extracellular transcripts from mRNA bound stably to both exosomes, microvesicles and proteins (278, 489-491). Therefore, it is difficult to decipher the original source of origin for mACVR2B, although highest levels of mACVR2B appear to originate from both the testis and brain, followed by the ovary and placenta (492) .

The Activin A receptor mACVR2B appears to be another biochemical marker of severe encephalopathy only, which limits its ability to aid clinical decision-making. This study highlights the fact that although many studies report the ability of candidate biomarkers to predict moderate-severe encephalopathy, these findings may be driven by the cases with severe HIE who show a very different clinical and biochemical course (373, 493).

The strengths of this validation multi-centre study involve the ability to recruit and analyse a large well-described infant population across two countries. The study also collected samples at delivery, all of which were processed according to strict standard operating procedures and stored in an ISO compatible biobank. This study used UCB, which was minimally invasive to both mother and infant and allowed a clear snapshot of placental-fetal circulation.

This study focused on the umbilical cord serum activin A and umbilical cord whole blood mACVR2B as an early non-invasive biomarker that could be collected at birth, but neither biomarker could distinguish between mild and moderate grades of HIE; or predict mild or moderately abnormal developmental outcome. Other studies, have focused on urine and CSF in the postnatal days of life, these two biomarkers of Activin A should be validated in a large-scale study (194, 195). Future biomarker

studies should not consider umbilical cord serum levels of Activin A or mACVR2B as viable markers of HIE.

Chapter 6

Concluding Summary

6.1 Description of Main Findings

This research was conducted with the overarching aim of making novel contributions to the field of UCB-based biomarkers in neonatal brain injury. This thesis has also endeavoured to explore and validate a number of neonatal brain injury biomarkers using two independent cohorts and explore both diagnostic and prognostic potential.

In Chapter 2, we identified UCB-based miRNA alterations in PA. The panel of miRNA explored in the discovery cohort consisted of both miRNA altered in a previous high -throughput microarray conducted by our group (see supplementary table 2.1 (213)), and also consisted of a selection of miRNA previously described in the literature focused on ischaemia, apoptosis, and microglial activation, all implicit in HIE injury (58, 434, 494). This allowed the study to conduct both the discovery vs validation approach and explore miRNA that may have gone undetected in the microarray (495). This was achieved with qPCR across two independent cohorts; with novel low-throughput decreased expression of miR-376c in infants with PA in the BiHIVE1 cohort and validated in the BiHiVE2 cohort. We also validated the decreased expression of miR-374a in infants with HIE compared to control infants across the BiHiVE2 cohort and explored the diagnostic potential of the decreased expression of miR-181b, across the two cohorts where it performed well compared to current markers of eligibility for TH. To date, altered expression of the three miRNAs have not been described in maternal or fetal circulation in the literature, but the three miRNAs are not-conserved to umbilical cord blood and have been described in the child and adult circulation previously; miR-374a and miR-181b have both been observed in the high abundance in the brain, when compared to placenta, testis, thymus, and prostate (496-498).

In chapter 3, we explored the predicted downstream targets of miR-376c, miR-374a, and miR-181b associated with the Wnt pathway, mNFAT5 and mFZD4, with qPCR. The Wnt pathway was of interest due to previous research in neonatal brain injury (330). mFZD4 was significantly increased in infants with severe HIE, and in infants eligible for TH, and both mNFAT5 and mFZD4 were significantly increased in infants with a severely abnormal outcome across a combined cohort of BiHIVE1 and BiHIVE2.

The mRNAs performed better than other biomarkers measured at birth as an indicator of poor neurodevelopmental outcome.

In chapter 4, increased levels of IL-16 were observed in both PA and HIE in BiHiVE2 validating previous findings from the original BiHiVE study. However, it was not reliable enough to differentiate between infants eligible for TH or constitute as a strong predictor of long-term outcome. This cytokine was not as reliable as current biochemical measurements at birth. The original BiHiVE study was the first to explore IL-16 in PA and HIE, prior to this, alterations have been seen in stroke, traumatic brain injury, ischaemic cerebrovascular dementia, astrocytic brain tumours and multiple sclerosis lesions, and IL-16 is expressed by microglial cells (454, 499-501).

Finally, in chapter 5, we were unable to determine differences between the receptor, mACVR2B, a predicted target of miR-374a, in infants with moderate/severe HIE, and we were unable to determine these differences in the protein activin A, most likely due to the absence of severe HIE in the BiHiVE2 cohort. In addition, a direct correlation between miRNA and mRNA was not consistently seen when comparing miRNAs and their putative mRNA targets, such as mACVR2B and miR-374a, this may be due to the fragmented nature in which mRNA travels within the circulation, meaning that the mRNA may be more difficult to detect compared to the mature miRNAs which can exist within exosomes and microvesicles (278); additionally, it appears that although miRNA can be detected in PA (miR-376c) and HIE (miR-374a), and those eligible for TH (miR-181b), the mRNA are only detectable in those eligible for TH and infants that develop a severely abnormal long-term outcomes (mNFAT and mFZD4), see chapter 3.

The purpose of the explored miRNA and mRNA in the circulation remains unclear, whether they have a mechanistic purpose or are being filtered from the blood as a form of clearance following apoptosis. One proposed mechanistic purpose could be that during a critical event, miRNAs are actively transported from the circulation into the cell to perform targeted mRNA repression - leading to a downregulation of miRNA in the blood. In the cell, the increased number of miRNAs may bind ‘imperfectly’ to the target mRNA leading to translational repression of mRNA. Instead of degradation in the cell, the repressed mRNA may be transported out of the cell to

be degraded via exosome-mediated degradation - leading to an upregulation of mRNA in the blood. Beyond this, miRNA and mRNA can exist stably in extracellular vesicles, bound to proteins, and cellular based in the circulation, however, the origin of the miRNA and mRNA we have studied is beyond the scope of this thesis.

6.2 Clinical Implications

This thesis expands the field of biomarker research in neonatal brain injury, analysing miRNA, mRNA and proteins. It has elucidated novel UCB biomarkers of PA and has validated and failed to validate a number of proposed biomarkers associated with HIE.

The miRNAs miR-374a, miR-376c have shown consistent decreased expression across high-throughput analysis (previously published microarray data (213)) and low-throughput validation putting these candidates forward as altered miRNA. However, their potential as blood-based biomarkers remains difficult to discern; miR-181b, which is decreased in HIE infants eligible for TH, has shown promise with an extremely high NPV compared to current biomarkers but Apgar at 5 minutes continues to be a strong marker of infants eligible for TH. Further studies with a larger sample size would be necessary if this were to be considered further as a combined biomarker of HIE. However, rapid testing in qPCR is currently challenging with an approximate lead-time of 5 hours for RNA extraction and two-step qPCR, which is quite limited as a diagnosis of moderate/severe HIE is needed within 6 hours of delivery so TH can be implemented. Although decreased expression of miR-374a for HIE and decreased expression of miR-376c in PA have been observed, their future potential as point of care blood-based biomarkers is currently unclear. However, if altered levels of either miRNA have functional implications in future HIE studies, these could have a profound impact on how we approach the injury.

Current diagnosis of the grade of HIE is subjective based on the modified Sarnat score, and it remains difficult to align subjective scores and objective biomarkers (502). HIE has been described as an evolving injury, and it may not be feasible to have a comprehensive biological snapshot of the injury prior to its full development which could be days or weeks after injury. It could also be proposed that if the injury has occurred at an early stage in utero, it may be too late for specific biomarkers to detect alterations.

The mRNA, mFZD4, could distinguish infants with eligible for TH, similar to miR-181b, but miR-181b had a higher negative predictive value. The BiHIVE1 and BiHIVE2 cohort

were merged to explore mRNA in a larger number of infants with long-term neurodevelopmental follow-up. mFZD4 performed better in PPV than current measurements at birth at distinguishing infants with poor neurodevelopmental outcome, and both mNFAT5 and mFZD4 performed well in NPV. These findings are of particular interest in the clinical setting, as the importance of rapid identification of these infants is not a necessity, as infants ineligible for cooling, i.e. PA or mild HIE could still experience a poor long-term outcome, and identification of infants with poor neurodevelopmental outcome may aid in clinical decision making over the first week of life. Further studies would need to be conducted with a larger sample size if this would be considered for potential clinical use of it at the cot-side.

6.3 Thesis Strengths

The main strength of this thesis is the well-defined cohorts; and the uniqueness of the population. Alongside this, BiHiVE2 was conducted as a multi-centre study allowing recruitment and analysis of a large well-described infant population across two countries.

The well-defined cohorts included demographical, biochemical, physiological, and clinical information. BiHiVE specifically included grading of HIE using a modified Sarnat score at 24 hours, and confirmed encephalopathy using continuous multichannel EEG which was interpreted by a trained and experienced neurophysiologist; it also included long-term neurodevelopmental follow-up at 18-36 months with the validated BSID-III conducted by either a trained clinician or psychologist.

UCB is used throughout as the medium for biomarker discovery, and it is an optimal source as it is non-invasive to both mother and infant; allowing simple collection of large volumes postpartum. All samples were processed according to strict standard operating procedures within 3-hours post-delivery, scanned and stored in a monitored ISO compatible biobank. Cases and controls were age-sex and birth weight matched across all studies to reduce biological variation. MIQE guidelines were followed for best practice in qPCR (313); miRNA and mRNA analyses were conducted in triplicate, including no enzyme controls, no template controls, spike-in controls to ensure optimal cDNA reverse transcription, monitoring melt curves, ensuring low CVs across samples, and samples were repeated when feasible to ensure accuracy. The miRNA, 374a and 376c, altered in chapter 2 of our study have been previously reported as altered using a high-throughput method (microarray (213)).

Many questions arise around differences particularly in miRNA and mRNA across the blood-based mediums such as whole blood, serum, plasma, and buffy coat (126, 132). For our HIE studies, we have focused on whole blood in Tempus tubes as they can be collected and stored rapidly without processing, and whole blood yields higher quantities of RNA than both serum and plasma. However, the origin of our

miRNA and mRNA in whole blood remains unclear compared to serum or plasma as whole blood includes cellular-based miRNA and mRNA.

6.4 Thesis Limitations

The main difficulty to control for in this thesis is heterogeneity across patients, i.e. each neonatal brain injury can differ in timing and severity, meaning no injury is the

No *a priori* sample size calculations were performed prior to recruitment of the BiHiVE cohorts, further to this a power calculation was completed *post-hoc* and demonstrated that the BiHiVE1 and BiHiVE2 cohort were powered at 0.78 and 0.75 respectively indicating that both studies were adequately powered, although the subgroup analyses for miR-181b would require a larger moderate/severe HIE sample size for further validation, n = 21 (503). Recruitment of moderate/severe HIE was difficult across both studies, as recruitment remains difficult in middle- to high-income countries, particularly in infants with severe HIE in BiHiVE2 which had *n* = 1 UCB samples available; further to this larger sample sizes would be required before any biomarker could be readily translated to the cot side.

Across the BiHiVE, BiHiVE2 and BASELINE cohorts mixed arterial/venous blood was collected with the aim of simplifying the collection process. However, this means it is unclear if the release or uptake of our biomarkers is reliant on venous or arterial blood, it is also unclear if mixed UCB is sufficient as a source for reflecting what's occurring in the neonatal brain.

In the thesis the techniques used for miRNA expression analysis were not analysed within the 6 hour window needed to identify infants eligible for TH, the RNA isolation needed approx. 1.5 hours, the cDNA synthesis needed approx. 1.5 hours, and qRT-PCR needed approx. 3 hours, followed by the statistical analysis. However, novel molecular biology techniques have been developed over the past 4 years such as one-step qPCR and fast qPCR products that could reduce the turnaround time to within 3 hours, so miR-181b expression would need to be tested with these novel procedures (504).

Pre-analytical variables played a significant role across this thesis, as both fibrin clots and haemolysis can have negative impacts on both RNA and protein downstream applications (505). Although these could be prevented during the lab-processing step

if the sample became haemolysed due to collection technique at delivery, these samples could not be included in our analysis and needed to be excluded. Most whole blood RNA samples extracted had high quantities of RNA present after extraction from the Tempus tube, but some samples were unsuitable for extraction due to the failure of adequate mixing of the stabilising reagent with the blood at the collection. Limitations due to cost and time of experiments prevented further validation and exploration of functional interactions across our studies.

6.5 Future Work

Chapter 2 revealed decreased expression of 3 miRNAs, in PA (miR-376c), HIE (miR-374c) and infants eligible for TH (miR-181b), putting forward several compelling questions:

i. What are the proposed downstream targets of miR-181b, miR-374a, miR-376c; and are these alterations observed in the circulation?

Chapter 3, aided in answering part of this question, it focused on two mRNA targets associated with the Wnt pathway (mNFAT5 and mFZD4), but miRNA have hundreds of downstream mRNA targets and it's unclear if further mRNA may be related to the miRNAs altered in chapter 2 (268, 506). Future work should consider, examining alternative pathways including mRNA associated with cell death pathways, which have been previously described in HIE, such as caspase signalling, as miRNA and mRNA may enter the circulation following apoptotic budding and crossing the BBB (44, 507). This work could be conducted by exploring predicted and validated downstream mRNA targets using miRBase, miRWalk, TargetScan and DIANA, see section 1.5.4.3.2.

Additionally, in the cellular enrichment analysis of chapter 2, both miR-374a and miR-376c were considered to play a role in postsynaptic specialisation; this is of interest as synaptic dysfunction has been considered in PA previously; which is occurring during development, formation, and maturation of the synapses, a process regulated by Wnt Signalling (51, 508-510). PA models have demonstrated a decrease in neuronal branching and synaptogenesis in the rat hippocampus, along with multiple studies describing thickening of postsynaptic densities, protein misfolding and ubiquitination post HIE injury (511, 512). One potential explanation is the miRNA are released via apoptotic budding or exosomal-mediated transport after microglial activation in the hippocampus during BBB leakage post-injury (513-515).

ii. Are alterations seen in the cord blood reflected in the neonatal brain; and if so, what effects do the miRNA have on neuronal function?

This can be explored with both *in vitro* and *in vivo* animal models. The *in vitro* models could aid in understanding if the miRNAs are expressed in neuronal cells, i.e. rodent primary neurons, immortalised fetal brain cells, SH-SY5Y cells, and OPCs. These cells would be treated with hypoxic treatment, either cobalt chloride, oxygen glucose deprivation, or a hypoxic chamber (516, 517). Altered expression in the 3 miRNAs, and additionally the 2 mRNAs from chapter 3 (mNFAT5 and mFZD4), could be measured with qPCR and the functional effects of these miRNA-mRNA interactions could then be confirmed via luciferase assays (518).

Further to this, a good *in vivo* animal model has the potential to give further insight into areas of the brain where the miRNAs may be present, and by extracting both brain tissue and blood from the *in vivo* models a direct comparison between miRNAs in the circulation and miRNAs in the brain post-hypoxia can be considered.

Multiple animal models have been considered in HIE, with smaller models used for mechanistic understanding of the injury and larger animals for translational studies, and include rodents, piglets, lamb, rabbit and non-human primates (1, 519). The postnatal P7 rat is used to correspond with term newborn maturity, and uses unilateral carotid artery ligation combined with a hypoxic chamber, i.e. Rice-Vanucci model, or the more recent modified P3 rat which has a higher impact on cortical development (519, 520). The rodent model has been considered useful due to the large sample size with rodents compared to larger animals and the ability to conduct subsequent neurobehavioral studies; however, caveats in this model include failure to reproduce oxygenation-reperfusion and representative global HIE and systemic organ failure (521). Another consistently used model is the piglet model, which is considered optimal due to its human similarities in both the cerebrovascular system and the cardiovascular system, and its larger size compared to the rodent model allows it to be monitored at birth similar to the clinical condition (522-524). Unfortunately, although the piglet would be considered the optimal model it can be costly due to animal handling and instrumentation preventing it from largescale use across centres (521). Finally, the lamb model has also been widely studied and played a critical role in the development of TH, the insult is conducted with either compression of the uterine arteries or umbilical occlusion; the lamb model is a good

representation of the insult and injury, but the neural maturity is considered beyond the term newborn making it difficult to make a comparison with neuronal tissue (1, 2, 525).

iii. Finally, can the miRNAs be manipulated to develop an RNA-drug delivery?

Following detection and mechanistic understanding of miRNA function in the brain the use of *in vitro* and *in vivo* models can be used to explore miRNA manipulation using miRNA mimics and miRNA inhibitors to examine if these alterations can have positive effects on neuronal tissue post-injury; this will allow the potential for the development of miRNA-based drug delivery systems (526).

In chapter 4, a cytokine biomarker was explored, and the alterations observed do not appear to detect clinically meaningful changes in IL-16 with the inability to differentiate between PA and HIE, or across HIE grades. In future work, it may be more useful to focus primarily on either the umbilical arteries (fetal cytokine release) or the umbilical vein (placental cytokine release) as this may provide novel information (527, 528). However, postnatal alterations or temporal alterations may also be promising as cytokine alterations develop and evolve over the first 72 hrs post injury.

Currently, multiple pilot studies have been conducted in cytokine biomarkers, which have seen elevated expression of cytokines (IL-1β, IL-6, IL-8, IL-10, IL-13, IL-16, TNF-α) across multiples mediums of biofluids (amniotic fluid, UCB, postnatal blood, CSF) with blood samples being both serum and plasma, postnatal bloods have also been explored temporally across the first days of life; the cytokine biomarkers have been correlated with brain injury in relation to MRI, graded injury, and studies have conducted long-term follow-up to examine neurodevelopmental outcome at 18-30 months (52, 178-183, 208). Consistent elevation of these biomarkers across these smaller pilot studies warrant further validation studies; further to this as high sensitivity ELISA assays are developed these may provide more accurate detection of inconsistent results (153, 529, 530).

The ideal animal model for studying cytokines would be a piglet HIE model due to its close relationship with the neonatal brain, the study could be a large comparison

study which involves the collection of piglet plasma cord blood, temporal plasma postnatal bloods and CSF samples and postnatal piglet brain MRI and subsequent collection of postnatal brain tissue (531, 532). The bloods and CSF could be tested using a high-throughput cytokine panel that includes all cytokines in clinical studies to date; this study could be conducted in tandem with a large-scale clinical cohort, which would collect plasma cord blood and temporal postnatal plasma bloods and CSF samples and the infants would have MRI and long-term follow-up, and the bloods and CSF could be tested using a high-throughput cytokine panel that includes all cytokines in clinical HIE studies to date (533). This study would allow the researcher to compare piglet cytokine levels across the perinatal period with postnatal MRI, grade of injury and correlate cytokine levels with postnatal brain tissue; the study would also allow the measurement of infant cytokine levels across the perinatal period along with postnatal MRI and long-term follow-up; a well-powered clinical study would allow the researcher to move potential biomarkers to clinical practise and further to this it would allow the researcher to compare inflammatory differences between an *in vivo* model of HIE and a clinical study.

Sex differences were not explored in the thesis as samples were matched for biological sex within the study, but currently HIE occurs more in males than females, and males go on to experience poorer long-term outcomes, this is considered to be a consequence of both endocrinology and genetics (534). Sex-differences in HIE have also been associated with microglial activation, one of the primary immune responses in HIE; previous research describes males exhibiting a higher cytokine response, and less neurogenesis compared to females, with these differences appearing later in the injury (70). Females also exhibit a more significant anti-inflammatory response compared to males (535). Future studies could consider exploring sex-specific temporal inflammatory biomarkers in HIE.

Throughout the thesis, the test that would be most beneficial based on all studies would be a rapidly available miRNA and mRNA combination of miR-181b and mFZD4, which could highlight both if the infant did not require TH and if the infant was not at high risk of poor neurodevelopmental outcome. These miRNA and mRNA both have high NPV so could aid the clinician in detecting the unnecessary application of

TH and inform parents that the child is not in the high-risk of severely abnormal outcome group. These miRNA and mRNA could both be analysed simultaneously as they can both be analysed using RNA isolated from whole cord blood, the timeframe would need to be reduced to allow rapid detection for infants eligible for TH, this may be possible with one-step qPCR or more novel point of care technologies (536).

To design an ideal biomarker study in the future would involve the recruitment of a large multi-centre cohort study, across multiple developed and rapidly developing countries, to allow for population differences, and it should recruit sufficient infants so all subgroup analyses remain adequately powered throughout, i.e. 150 HIE infants, consisting of 50 severe HIE, 50 moderate HIE, and 50 mild HIE, 50 PA infants without encephalopathy, and 200 healthy age-sex matched controls. This would allow the cohort to be split 50:50 into a discovery cohort (n = 200), and validation cohort (n = 200) and allow a 20% drop-off rate for the long-term outcome analysis.

Umbilical cord blood would be collected arterially to ensure its an accurate reflection of the infant's condition. The cord blood would be collected using Tempus tubes at birth, and postnatal platelet-poor plasma would be collected also on the first 3 days of life in the infant, plasma would be used as Tempus would be incompatible as it would require too much blood for a newborn. Following recruitment, the miRNA would be isolated, and quality tested and miRNA-seq (NGS) would be performed on 75 HIE (25 mild, 25 moderate, 25 severe), 25 PA and 25 controls, to identify novel miRNAs which may have been discovered (n >2000) since the original microarray in 2012 (n = 866) (213).

Bioinformatic analysis would be conducted in DESeq2 and corrected for FDR (108). The top 40 miRNA would be validated on the discovery dataset with rapid one-step qPCR to test minimum time taken to analyse samples. Further validation of consistently altered miRNAs would be conducted on the validation-half of cohort. Temporal miRNA alterations would be tested on postnatal samples to identify if altered expression continues into the postnatal period, and if changes persist across different grades of HIE (351). Additionally, downstream mRNA targets would be identified and novel Olink proteomic technologies would be used to test a custom high-throughput panel of downstream proteins related to the putative miRNA-mRNA

targets on postnatal samples to examine temporal alterations in circulating proteins post-injury, this would allow for a more functional understanding of changes occurring in the circulation, as altered mRNA may not correspond perfectly with altered protein function (537, 538). *In vitro* work could model the miRNA-mRNA interactions. The study would then analyse how the miRNAs and proteins correspond to long-term outcomes, and finish by the potential development of a robust minimally invasive point-of-care biomarker or panel of biomarkers for HIE.

6.6 Concluding Remarks

This thesis highlights both the complexity and the potential of UCB-based diagnosis and prognosis in early infancy. Globally perinatal asphyxia is one of the primary causes of neonatal death with approximately 920,000 deaths each year. Early identification is critical to implement early intervention and for future treatments (34). To date no robust and reliable biomarker can rapidly distinguish HIE, grade of HIE, or long-term neurodevelopmental outcome; failure for early diagnosis means infants may miss early intervention strategies. Early and accurate prognosis would allow long-term treatment plans to be implemented to ensure best quality of life. This thesis has shown that 3 miRNAs are consistently decreased in the cord blood infants with PA (miR-376c), HIE (miR-374a) and infants eligible for TH (miR-181b); it has shown that downstream target mRNAs of the miRNAs altered are increased in infants with a severely abnormal outcome (mNFAT5 and mFZD4) at 18-36 months; and finally, it has validated (IL-16) a circulating cytokine increased in HIE, and invalidated a circulating protein marker (Activin-A) and its mRNA receptor (mACVR2B) that were originally observed as increased in HIE.

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Appendix A. List of Figures

Appendix B. List of Tables

Appendix C. Cohort Identification

C1.1 BiHIVE Discovery Cohort (2009-2011)

Prof Murray was the Principal Investigator, and Prof. Boylan was the co-investigator steering this project. The study was established to identify a panel of biomarkers measurable in umbilical cord blood at birth that could predict severity of hypoxic-ischaemic injury and predict neurodevelopmental outcome in a cohort of infants with signs of perinatal asphyxia at delivery. Promising biomarkers were to be identified using a multi-level approach with targeted and untargeted analyses across transcriptomics, metabolomics, and proteomics. It was proposed that an optimal panel of biomarkers determined by bioinformatics techniques would be developed into a point-of-care device to be used in the delivery suite, and this would contribute to the clinical decision-making process, treatment management, and prognosis. The study was conducted at Cork University Maternity Hospital and recruited between September 2009 and June 2011. Informed parental consent for UCB analysis, data collection, and neurodevelopmental assessment was a requirement in all cases, and the Cork Research Ethics Committee granted ethical approval for this study.

Cases were identified if they met the following criteria: -

- Infants having a gestational age ≥36 weeks and one or more of the following:
    - Apgar score ≤6 at 5 mins of life
    - Cord pH < 7.1
    - Required CPR or IPPV > 10 mins

If the infant met the inclusion criteria, the attending midwives drew UCB, and filled 2 serum vacutainers, 1 EDTA vacutainer and 1 Tempus blood RNA tube provided by a prepared study pack which were stored in a designated research fridge on the labour ward. Midwives then contacted the research team via an on-call phone number available 24/7 to inform them that blood had been collected. A designated researcher than processed the blood according to strict standard operating procedures within 3-hours of delivery and biobanked at -80°C.

All infants had demographic and clinical data collected during their in-patient stay. All infants were assessed neurologically using the Thompson score on day 1, day 2, and day 3 of life, and at discharge depending on the length of in-patient stay. All infants requiring admission to the neonatal unit who developed hypoxic-ischaemic encephalopathy had a modified Sarnat score performed at 24 hours of life. Continuous multi-channel EEG application and therapeutic hypothermia were provided based on clinical requirement as determined by the consultant on duty.

Neurodevelopmental outcome assessment was conducted between 18-36 months of age. Assessment consisted of the cognitive, language and motor scales of the Bayley Scales of Infant and Toddler Development (Edition 3). The parents were also asked to complete the social-emotional and adaptive behavioural Checklist (1.5 – 5 years).

The BiHiVE1 cohort recruited 112 cases, of these 40 developed HIE. The grade of HIE severity was broken down into 24 mild, 6 moderate and 10 severe. Fifty-two infants had neurodevelopmental follow-up, and 7 died in infancy.

C2.1 BiHIVE2 Validation Cohort (2013-2015)

Prof Murray was the Principal Investigator, and Prof Boylan and Dr Hallberg were the co-investigators steering this project, named BiHIVE2, a validation study of the original findings from the BiHiVE1 study. Informed parental consent was a requirement in all cases, and the local ethics boards in both countries granted ethical approval for this study. The study was a multi-centre study across two countries recruited across both Cork University Maternity Hospital, Ireland and Karolinska Huddinge, Sweden between March 2013 and June 2015. Informed parental consent for UCB analysis, data collection, and neurodevelopmental assessment was a requirement in all cases, and the local research ethics committees for each country in this study granted respective ethical approval.

The inclusion criteria of the cases, methods for processing and neurodevelopmental follow-up were performed as in the BiHiVE1 study, described in full in D1.1. In the BiHiVE2 study, a prospective control population was recruited simultaneously with the cases. The parents were approached early in labour regarding their infants participating in the healthy control group. Infants were eligible as control infants if they met none of the cases inclusion criteria.

The BiHiVE2 cohort recruited 353 cases, of these 48 developed HIE. The grade of HIE severity was broken down into 32 mild, 14 moderate and 2 severe. The healthy control population included 289 controls. Four-hundred and forty-nine infants had neurodevelopmental follow-up, and no deaths occurred in infancy.

C2.2 BiHIVE2 Patient Information Leaflet

BiHiVE 2 study

The Investigation and Validation of Predictive Biomarkers in Hypoxic-ischaemic Encephalopathy

Information Leaflet for Parents

What is the BiHiVE study?

Some baby's require resuscitation when they don't cry or move after delivery. This may occur when a baby has had a difficult or stressful time during labour, or before delivery. Sometimes this can even lead to long term injury to the baby. The injury which we worry about the most is newborn brain injury. This type of brain injury is called hypoxic-ischaemic encephalopathy or HIE. As your baby has required resuscitation they may need careful monitoring for the next few days. We have no good way of knowing whether your baby has had a significant injury, and usually can't tell for sure until at least 24 hours after birth. The BiHiVE study hopes to find chemical and protein markers

in the blood which can tell us very soon after birth which babies have suffered significant injury so that we can intervene and improve their outcome. At the moment we have no good reliable early marker which can give us this information straight after birth. We hope that in the future a simple blood test at delivery will be able to give doctors and parents more information within hours of a baby's birth.

Because your baby has needed resuscitation at birth, blood has been drawn from the umbilical cord and placenta after the baby was born. This is the standard care in our hospital and many other modern maternity hospitals. This blood is used to measure acid levels in the blood. We have also drawn extra blood and stored it in 3-4 tubes. If you wish your baby to take part in the study we will store this blood to use for research into newborn brain injury. If you do not wish to take part in the study these samples will be destroyed.

If your baby is admitted to Neonatal Intensive Care Unit, and, if they are having a blood test as part of their routine care, we will ask for an extra 1.5 milliliters of blood on the first, second and third day of life. We will not disturb your baby an extra time for this sample, and if your baby does not require a blood test for other reasons, we will not take this sample. These samples will be frozen and stored for analysis.

Our current best way of looking at those who might be at risk of brain injury is to carry out a recording of their brain waves using an EEG machine. This is routinely done on the neonatal unit. We want to be able to compare what is currently our best method, to the simpler blood test which we hope to develop, and therefore all babies in the study will also have an EEG if they have signs of brain irritation (HIE).

In order to help babies who have difficulties after birth, it is also important for us to study babies who have had normal, uneventful deliveries. These babies act as controls so that we can know that the changes we find in the unwell babies are genuine. If your baby is a control infant, blood is taken from the umbilical cord and stored at birth. We will follow up your baby with an examination the day after birth. This takes about 5 minutes and will not upset your baby in any way. It can be carried out when they are still asleep in many cases. In addition we would like to contact you at 2 years of age to look at the growth and development of your child. The researchers will be very clear with you to reassure you that your baby is a control, or well baby.

What will the study involve?

If you are happy for the research team to follow your baby after they are born, the extra samples of blood taken from your baby's umbilical cord will be frozen, stored and analyzed later when we have collected enough samples. An additional 1.5 milliliters of

blood will be taken if the baby is having other blood samples on day 1, 2 and 3, these will be analyzed once enough samples are collected. We will look at many different chemicals and proteins which may change in babies with significant brain injury. We will compare the levels to those of babies who did not need resuscitation. Your baby's progress after birth will be recorded. If your baby needs any extra medical care the details of this care will be collected from your baby's medical notes. This information will be anonymous, and will be identified only using a study number, not your baby's name or address.

We will also ask you to meet us again at 24 months of age so that we can record the progress and development of your baby. You will be contacted and an appointment will be made for a follow up assessment at a time convenient for you and your family. This will happen in the Cork University Hospital. In these follow up appointments, your baby's development will be assessed and discussed with you. Your baby will not need any further samples to be taken after the initial sample from the placenta for this study.

What are my options?

Being part of the BiHiVE study is completely voluntary. You or your child can withdraw from this study at any stage. Not being part of this study will not affect any part of the medical care of you or your baby.
The BiHiVE study will be managed by the staff of the Department of Paediatrics and Child Health, University College Cork. We will publish our results in international medical journals. Once a year we will organize a meeting to tell families about how the study is progressing.

Where can I get more information?

If you have any questions regarding the BiHiVE study, please contact the research coordinators

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BiHiVE 2 study

The Investigation and Validation of Predictive Biomarkers in Hypoxic-ischaemic Encephalopathy

Information Leaflet for Parents Control Group (well babies)

What is the BiHiVE study?

The BiHiVE study is a research study currently taking place in your hospital looking at infants who have needed resuscitation after birth. This happens in about 2% of deliveries. Some of these infants may be at risk of long term problems and need intervention in the first few days of life. At the moment we have no good reliable early marker which can give us this information straight after birth. Through this study we hope to find chemical and protein markers in the umbilical cord blood which can tell us very soon after birth which babies have suffered significant injury so that we can intervene and improve their outcome. We hope that in the future a simple blood test at delivery will be able to give doctors and parents more information within hours of a baby's birth.

In order to help babies who have difficulties after birth, it is also important for us to study babies who have had normal, uneventful deliveries. These babies act as **controls (well babies)** so that we compare them to infants who are unwell. We are asking for your baby to be a control infant for the study. If your baby is a control infant, blood is taken from the umbilical cord and stored at birth. We will follow up your baby with an examination the day after birth. This takes about 5 minutes and will not upset your baby in any way. It can be carried out when they are still asleep in many cases. In addition we would like to contact you at 2 years of age to look at the growth and development of your child. The researchers will be very clear with you to reassure you that your baby is a control (well baby).

What will the study involve?

If you are happy for the research team to follow your baby after they are born, the extra samples of blood taken from your baby's umbilical cord will be frozen, stored and analyzed later when we have collected enough samples. We will look at many different chemicals and proteins and we will compare their levels to those of babies who needed resuscitation. Your baby's progress after birth will be recorded. If your baby needs any extra medical care the details of this care will be collected from your baby's medical notes. This information will be anonymous, and will be identified only using a study number, not your baby's name or address.
We will also ask you to meet us again at 24 months of age so that we can record the progress and development of your baby. You will be contacted and an appointment will be made for a follow up assessment at a time convenient for you and your family. This

will happen in the Cork University Hospital. In these follow up appointments, your baby's development will be assessed and discussed with you.

What are my options?

Being part of the BiHiVE study is completely voluntary. You or your child can withdraw from this study at any stage. Not being part of this study will not affect any part of the medical care of you or your baby.
The BiHiVE study will be managed by the staff of the Department of Paediatrics and Child Health, University College Cork. We will publish our results in international medical journals. Once a year we will organize a meeting to tell families about how the study is progressing.

Where can I get more information?

If you have any questions regarding the BiHiVE study, please contact the research coordinator.

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C2.3 BiHIVE2 Consent Form

Ospideal Maithreachais na h'Oiliscoile Corcaigh
Cork University Maternity Hospital

HRB Health Research Board

UCC

BiHIVE 2 STUDY

The Investigation and Validation of Predictive Biomarkers in Hypoxic-Ischaemic Encephalopathy

Information and Consent form

Information and consent leaflet:

Introduction

We would ask you to read the information leaflet given to you again now, and give you the opportunity to ask any questions you may have.

Do I hawe to take part in this study? It is entirely your choice. If you agree we will ask you to sign a consent form, and you will be given a copy of this to keep. You are free to change your mind and withdraw your ba by from the study at any time, without giving a reason. The care that you or your baby receive now or in the future will not be affected in any way by your decision whether or not to take part.

What will happen to my baby if I agree to take part? Because your baby has needed resuscitation at birth, blood has been drawn from the umbilical cord and placenta after the baby was born. This is part of routine care in our hospital and most other modern maternity hospitals. This blood is used to measure acid levels in the blood. We have also drawn extra blood and stored it in 3-4 extra tubes. If you agree to take part in the study this extra blood will be analysed at a later period to measure levels of other chemicals in the blood. This blood is otherwise discarded and if you do not wish to take part in the study we will discard this stored blood immediately. We are collecting these samples because we hope to identify which chemicals and proteins can best predict how babies cope with stressful deliveries and resuscitation. We are looking for markers in the blood which can predict which infants may have suffered significant stress and who may need treatment in the first few hours after birth. Most importantly we want to improve our ability to predict a baby's development over the first few years of life.

Ospideal Maithreachais na h'Oilscoile Corcaigh
Cork University Maternity Hospital

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We would also like to see if these chemicals change over the first few days of life. Therefore if your baby is having a blood test as part of their routine care, we will take an extra 1.5 milliliters of blood on the first, second and third day of life or if your baby develops seizures (convulsions). This is unlikely, but if it happens seizures can also affect a baby's develop and this is important for us to study. We will only take the sample if your baby requires a blood test or intravenous line for other reasons as part of their medical care.

After your baby is born, we will be able to gather information about your baby's birth, and treatment from your maternity notes and the baby's notes.

To accurately measure any effect on your baby's brain we wish to record an EEG (electroencephalograph) and other vital signs such as heart rate, breathing rate and oxygen saturation levels. This is part of routine monitoring in our hospital and carrying out in all babies who have signs of brain irritation or injury. This is usually carried out for up to 24 hours. However as part of the study we may ask you to allow us to monitor your baby's brain waves for longer, so that we can gather extra information.

Lastly as part of the study we will ask to meet you and your baby again between 18 months and 2 years of age to monitor their growth and development. This will involve short questionnaires and a one hour developmental assessment.

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Cork University Maternity Hospital

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BiHIVE INFORMED CONSENT FORM

Principal Investigator: Dr. Deirdre Murray **Phone Number:** +353 21 4901271

EEG ID Number_____ **Patient ID Number for this study**_____

Y/N

- I have read the information leaflet about this research and have been given a copy to keep. The information has been fully explained to me and I have been able to ask questions. I understand why the research is being done and any risks involved. ☐
- I agree to donate a sample of my baby's umbilical cord blood for this research project. I understand that giving a sample for this research is voluntary and that I am free to withdraw my approval at any time without my medical treatment being affected. ☐
- I agree to donate a sample of DNA from my baby's umbilical cord blood for storage and research related to the project ☐
- I agree to donate a sample of my baby's blood for this research project on day 1, 2 or 3 of life, or if my baby develops seizures. I understand that giving a sample for this research is voluntary and that I am free to withdraw my approval at any time without my medical treatment being affected. ☐
- I agree to allow my baby to take part in the follow up visits required at 18-24 months of age ☐
- I give permission for research personnel to look at my baby's medical records to obtain information about my baby's progress after birth, and the records of my labour and delivery. I have been assured that this information will be kept confidential ☐
- I agree to donate a sample that can be used now or in the future for commercial collaborative research to develop screening tests for brain injury in children. I understand that I will not benefit financially if this research leads to a development of a new treatment or medical test. ☐
- I give permission for my baby's sample and information collected about my baby to be stored for possible future research related to this study *without my further consent being required* and subject to approval by a research ethics committee. ☐

Role	Print Name	Signature	Date
Parent/legal guardian 1			
Parent/legal guardian 2			
Investigator			
Witness			

Insert Hospital Addressogram Label Here

Local Principal Investigator: Dr Deirdre Murray

Ospideal Maithreachais na h'Ollscoile Corcaigh
Cork University Maternity Hospital

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BiHIVE INFORMATION LEAFLET AND CONSENT

BiHIVE 2 STUDY

The Investigation and Validation of Predictive Biomarkers in Hypoxic-Ischaemic Encephalopathy

Consent Form Control Group (Well babies)

Information and consent leaflet:

Introduction

We would ask you to read the information leaflet given to you again now, and give you the opportunity to ask any questions you may have.

Do I have to take part in this study? It is entirely your choice. If you agree we will ask you to sign a consent form, and you will be given a copy of this to keep. You are free to change your mind and withdraw your baby from the study at any time, without giving a reason. The care that you or your baby receive now or in the future will not be affected in any way by your decision whether or not to take part.

What will happen to my baby if I agree to take part? In order to study differences in normal healthy births and those that needed resuscitation at birth we need to study a healthy control group. Your baby had an uneventful normal delivery and this is why we are asking your permission to include your baby as a **control** (well baby) in this study. We are asking if you would allow us to draw blood from the umbilical cord and placenta after your baby is born. This blood is used to measure acid levels in the blood. We store it and it will be analysed at a later period to measure levels of other chemicals in the blood. We are collecting these samples because we hope to identify which chemicals and proteins can best predict how babies cope with stressful deliveries and resuscitation. We will be looking at normal levels in the control group and comparing them to the study group of resuscitated babies. We are looking for markers in the blood which can predict which infants may have suffered significant stress and who may need treatment in the first few hours after birth. Most importantly we want to improve our ability to predict a baby's development over the first few years of life.

After your baby is born, we will be able to gather information about your baby's birth, and treatment from your maternity notes and the baby's notes.

Lastly as part of the study we will ask to meet you and your baby again between 18 months and 2 years of age to monitor their growth and development. This will involve short questionnaires and a one hour developmental assessment.

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Cork University Maternity Hospital

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BiHIVE INFORMED CONSENT FORM

Principal Investigator: Dr. Deirdre Murray **Phone Number:** +353 21 4901271

EEG ID Number_____ **Patient ID Number for this study**_____

	Y/N
• I have read the information leaflet about this research and have been given a copy to keep. The information has been fully explained to me and I have been able to ask questions. I understand why the research is being done and any risks involved.	☐
• I agree to donate a sample of my baby's umbilical cord blood for this research project. I understand that giving a sample for this research is voluntary and that I am free to withdraw my approval at any time without my medical treatment being affected.	☐
• I agree to donate a sample of DNA from my baby's umbilical cord blood for storage and research related to the project.	☐
• I agree to allow my baby to take part in the follow up visits required at 18-24 months of age	☐
• I give permission for research personnel to look at my baby's medical records to obtain information about my baby's progress after birth, and the records of my labour and delivery. I have been assured that this information will be kept confidential	☐
• I agree to donate a sample that can be used now or in the future for commercial collaborative research to develop screening tests for brain injury in children. I understand that I will not benefit financially if this research leads to a development of a new treatment or medical test.	☐
• I give permission for my baby's sample and information collected about my baby to be stored for possible future research related to this study *without my further consent being required* and subject to approval by a research ethics committee.	☐

Role	Print Name	Signature	Date
Parent/legal guardian 1			
Parent/legal guardian 2			
Investigator			
Witness			

Insert Hospital Addressogram Label Here	**Local Principal Investigator: Dr Deirdre Murray**

C3.1 BASELINE Longitudinal Birth Cohort (2008-2016)

Prof Murray was the Principal Investigator, and Prof. Jonathan Hourihane, Prof. Mairead Kiely, Prof. Alan Irvine, and Prof. Louise Kenny were the co-investigators steering this project, The Cork BASELINE Birth Cohort Study (Babies After SCOPE: Evaluating the Longitudinal Impact on Neurological and Nutritional Endpoints) recruited infants from 2008 to 2016 in Cork University Maternity Hospital. The SCOPE Ireland pregnancy cohort formed the basis of recruitment of infants to BASELINE, and additional infants were recruited post-delivery. Cork Research Ethics Committee granted ethical approval.

BASELINE had three main aims: to investigate the effects of intrauterine growth restriction and early nutrition on metabolic health and neurodevelopment; to ascertain the incidence and determinants of food allergy and eczema in early childhood; and to describe early infant feeding, supplementation and nutritional status and their effects on physical and neurological growth and health outcomes. Infants were assessed on day 2 and at 2, 6, 12, and 24 months, and at 5-years of age(366). Blood samples were collected at birth, 24 months, and 5 years of age, processed according to strict standard operating procedures and biobanked at -80°C.

The Baseline cohort recruited 1537 infants from SCOPE and an additional 600 infants on the postnatal ward. From the 2137 infants recruited 1954 continued after the initial assessment, 1537 infants were assessed at 2-years of age, and 759 infants were evaluated at 5-years of age. At the 2-year weight status 56 infants were obese (BMI > 98th percentile), 138 infants were overweight (BMI > 91st percentile), and 809 infants were not overweight or obese. At the 5-year weight status 18 infants were obese (BMI > 98th percentile), 60 infants were overweight (BMI > 91st percentile), and 681 infants were not overweight or obese.

C3.2 BASELINE Patient Information Leaflet

BASELINE information leaflet Version 6

The Baseline Study

Babies **A**fter **S**cope: **E**valuating the **L**ongitudinal **I**mpact using **N**eurological and **N**utritional **E**ndpoints

Information Leaflet for Parents

BASELINE information leaflet Version 6

What is the Baseline Study?

Your doctors and midwives have been watching your baby grow during your pregnancy. We know that many things which may effect your baby's growth in the womb may also effect their growth and health in early childhood. We hope to continue to meet you and your child after the birth to look at how they grow and develop. The Baseline study will be focused on your baby's growth, health and development over the next two years and beyond.

We will collect information about common childhood illnesses like asthma, eczema and food allergy. We will look at each child's growth over the first two years of life and compare this to the growth patterns before birth when they were in your womb. Using simple questionnaires and growth measurements we will be able to gain very important information which may help us to find out why some children develop common childhood illnesses and some don't.

If your baby has poor growth (growth restriction) during your pregnancy, they will be followed even more closely for their growth after birth. Many babies "catch up" in weight and length quickly after birth, but some don't. Those babies who catch up too quickly may be at risk of obesity and diabetes in later life. We need more information before we can give parents accurate advice about this important issue. We will also be looking more closely at these children at two years of age, and will carry out detailed assessments of their development and their sugar handling.

What will the study involve?

If you are happy for the Baseline research team to follow your baby after they are born, a sample of saliva will be taken on a cotton swab from your baby's mouth. Your baby's weight, length and head circumference will be measured. Your baby's body fat will be measured using a PEAPOD machine, which like a small, warm incubator where your baby will be placed for 1-2 minutes. The water loss through your baby's skin will be measured at birth and at 2 and 6 months of age. Your baby's progress after birth will be recorded. If your baby needs any extra medical care the details of this care will be collected from your baby's medical notes. This information will be anonymous and will be identified only using a study number, not your baby's name or address.

There will be routine appointments at 2, 6, 12 and 24 months, when you will be contacted and an appointment at a time convenient for you and your family. This will happen in the Children's Day Unit and Children's Research Centre of the Cork University Hospital. You will be asked to straightforward questions about your child's health and diet. One of our research team will measure your baby's height, weight, head size and body fat. You may be asked to give a sample of stool at the 2 and 6 month visits. At 2 months your baby's body fat will be measured again using the same PEAPOD machine. At each visit your baby will be checked for eczema and will have skin tests for common allergies at 12 and 24 months. You may also be asked to consent to your child giving a single blood test at 2 years of age which will measure their Vitamin D levels and their sugar handling. Once again consent and participation is voluntary. Babies who show problems such as

eczema, diabetes or have problems with development will be monitored more closely by the study team. If your baby has unrelated illnesses such as colds and chest infections, you should see your family doctor first.

If your baby has poor growth (growth restriction) before they are born, we will also arrange a more detailed assessment of their development. This will be carried out by an educational psychologist at 2 years of age.

What are my options?

Being part of the Baseline study is completely voluntary. You or your child can withdraw from the Baseline study at any stage. Not being part of the Baseline study will not affect any part of the medical care of you or your baby.

The Baseline study is managed by staff of the Department of Paediatrics and Child Health and Department of Nutrition at University College Cork. We will publish our results in international medical journals. Once a year we will organise a meeting to tell families about how the study is progressing. With your permission we will keep your family doctor up to date with any relevant health issues that arise for you and your baby during the study.

Where can I get more information?

If you have any questions regarding the Baseline study, please contact the research coordinators:

Dr Deirdre Murray or Prof Jonathan Hourihane
Dept of Paediatrics and Child Health
Clinical Investigations Unit
Cork University Hospital
Tel: +353 21 4901271
Fax: +353 21 434 5217
Email: d.murray@ucc.ie

C3.3 BASELINE Consent Form

Consent to BASELINE version 6

Participant's Consent Form

The Baseline Study

Babies After SCOPE: Evaluating the Longitudinal Impact using Neurological and Nutritional Endpoints

If you wish to take part in this research project having read the information leaflet please read and sign this form.

Participant name:_____
Participant DOB:_____

BASELINE study number:_____

Project title: BASELINE study
Principal investigator: Dr Deirdre Murray
Contact Details: 021 4901271/086 8461941

Please tick the appropriate boxes

	Yes	No
1. I have read the information leaflet about this research and have been given a copy to keep. The information has been fully explained to me and I have been able to answer questions. I understand why the research is being done and any risks involved.	☐	☐
2. I agree to donate a sample of my baby's saliva for this research project and a stool sample from my baby at 2 and 6 months of age. I understand that giving a sample for this research is voluntary and that I am free to withdraw my approval at any time without my medical treatment being affected.	☐	☐
3. I give permission for research personnel to look at my baby's medical records to obtain information about my baby's progress after birth. I have been assured that information about my child will be kept confidential	☐	☐
4. I agree allow my baby to take part in the follow up visits required at 2, 6,12 and 24 months of age. I give permission for my baby's stool sample to be stored for future research into children's nutrition and health.	☐	☐
5. I agree to donate a sample that can be used now or in the future for commercial purposes. I understand that I will not benefit financially if this research leads to a development of a new treatment or medical test.	☐	☐
6. I give permission for my baby's sample and information collected about my baby to be stored for possible future research <u>unrelated</u> to this study (including DNA studies) *without my further consent being required* and subject to approval by a research ethics committee.	☐	☐

Signed:_____ Date:_____
Name of research participant

Signed:_____ Date:_____
Name of person taking consent

BASELINE consent form version 6

The CORK
BASELINE
Birth Cohort

UCC

NATIONAL CHILDREN'S RESEARCH CENTRE

- I have read the information leaflet/letter about this research and have been given a copy to keep. The information has been fully explained to me and I have been able to answer questions. I understand why the research is being done and any risks involved. ☐
- I agree to allow my child to take part in the follow up visits required at 5 years of age to measure their growth, health and development. These will involve parental questionnaires, measurements of growth and health. ☐
- I agree to repeat skin prick testing to look for allergies to food and dust borne allergens. ☐
- I agree to the measurement of my child's bone strength and body fat using a DEXA scanner. ☐
- I agree for my child to donate a sample of blood (6mls) that can be used now or in the future for commercial purposes. I understand that I will not benefit financially if this research leads to a development of a new treatment or medical test. ☐
- I give permission for my child's sample and information collected about my child to be stored for possible future research unrelated to this study (including DNA studies) without my further consent being required and subject to approval by a research ethics committee. ☐

BASELINE study number_____ **Participant signature**_____

Date consent signed _____

Consent taken by _____

Appendix D. Extended Methodology

Recruitment

The PhD candidate was involved in the final recruitment stage for the BiHIVE2 cohort; this involved partaking in a 72-hour on call rota for processing and biobanking cases. Recruitment took place in two large single maternity hospitals, Cork University Maternity Hospital, Ireland and Karolinska Maternity Hospital, Sweden between March 2013 and July 2015.

Sample Processing

- (Adapted from BiHIVE Standard Operating Procedure)

Once a case or control was born in BiHIVE2, cord blood was collected across four separate tubes in the delivery room; and transported to the research fridge in the NICU and the BiHIVE processing team was alerted, blood tubes were processed in the laboratory within 3 hours, and the four tubes were:

- 6 ml red capped serum tube
- 3 ml blue Tempus RNA tube
- 6 ml lavender plasma EDTA tube
- 6 ml additional red capped serum tube

Serum

Serum was allowed at least 30 minutes from collection for clot formation prior to processing. Samples were checked for clotting prior to centrifugation. Serums were centrifuged at 2,400 xg for 10 minutes at 4°C. Samples were pipetted to second spin tubes with Pasteur pipette, labelled with patient ID, and marked in red to represent serum and centrifuged at 3,000 xg for 10 min. at 4°C. Samples were then transferred in 250 µL aliquots into red-capped barcoded microtubules; scanned into the BiHIVE2 database and stored in -80°C freezer.

EDTA Plasma

Plasma was centrifuged immediately at 2,400 xg for 10 minutes at 4°C. Samples were then transferred in 250 µL aliquots into lavender-capped barcoded microtubules; scanned into the BiHIVE2 database and stored in -80°C freezer.

RNA Whole Blood

Three ml of blood was transferred directly into TEMPUS blood tube (Applied Biosystems, U.S.A.) until it reached the black mark on tube. Immediately after the TEMPUS tube was filled, the blood was stabilised with tubes reagents by shaking tube vigorously, then barcoded, scanned into the BiHIVE2 database, and stored in -80°C freezer.

RNA Isolation

Whole blood

Total RNA was isolated from the Tempus system with the MagMAX™ for Stabilized Blood Tubes RNA Isolation Kit (Ambion, Life Technologies, U.S.A.), as per the manufacturer's instructions. In summary, stabilised blood samples were thawed on ice, resuspended with phosphate buffered saline (PBS), centrifuged and washed to pellet crude RNA. Following resuspension and addition of TURBO DNase, the RNA was purified and eluted using RNA magnetic binding beads. The RNA concentration was quantified using Nanodrop 8000 spectrophotometer, and isolated RNA was labelled and subsequently stored in -80°C freezer.

qPCR

mRNA SYBR Green qPCR

In summary, total isolated RNA and reagents were thawed, total isolated RNA was diluted to 20 ng/µL using nuclease-free water to form template RNA. Template RNA was prepared per sample using the Universal cDNA Synthesis Kit (Exiqon, Denmark; acquired by Qiagen, Germany), as per the manufacturer's instructions. The cDNA was acquired with 2 µL of 5x Reaction buffer + 4.5 µL of nuclease-free water + 1 µL of enzyme mix + 0.5 µL of synthetic RNA spike in (UniSp6) + 2 µL of template RNA. The cDNA was synthesised by an incubation step at 60 min. at 42°C, followed by heat-

inactivation of the reverse transcriptase for 5 min. at 95°C and the cDNA template was stored in the freezer at -20°C.

The cDNA template and reagents were thawed, and the template cDNA was diluted at 40x. The PCR working solution was acquired using ExiLERATE LNA qPCR, for mRNA and long non-coding RNA (Exiqon, Denmark; acquired by Qiagen, Germany), as per the manufacturer's instructions. This was conducted with 5 µL of Exilent SYBR Green master mix + 1 µL of PCR primer set + 4 µL of diluted cDNA template. The PCR working solution was mixed thoroughly, and real-time PCR amplification was performed by a 10 min. at 95°C polymerase activation/denaturation, followed by 40 amplification cycles for 10 sec. at 95°C, followed by 1 min. at 60°C, with optical read and subsequent melt curve analysis. Negative enzyme controls were performed, and negative template controls were performed across all primers.

mRNA TaqMan qPCR

In summary, total isolated RNA and reagents were thawed, total isolated RNA was diluted to 20 ng/µL using nuclease-free water to form template RNA. Template RNA was prepared per sample using the Universal cDNA Synthesis Kit (Exiqon, Denmark; acquired by Qiagen, Germany), as per the manufacturer's instructions. The cDNA was acquired with 4 µL of 5x Reaction buffer + 8 µL of nuclease-free water + 2 µL of enzyme mix + 2 µL of template RNA. The cDNA was synthesised by an incubation step at 60 min. at 42°C, followed by heat-inactivation of the reverse transcriptase for 5 min. at 95°C and the cDNA template was stored in the freezer at -20°C.

The cDNA template and reagents were thawed, and the template cDNA was diluted at 20x. The PCR working solution was acquired using TaqMan Gene Expression Master Mix (Thermo Fisher Scientific, U.S.A.), as per the manufacturer's instructions. This was conducted with 10 µL of TaqMan Gene Expression Master mix (including ROX dye) + 1 µL of TaqMan Gene Expression Assay + 9 µL of diluted cDNA template. The PCR working solution was mixed thoroughly, and real-time PCR amplification was performed by a 10 min. at 95°C polymerase activation/denaturation, followed by 40 amplification cycles for 10 sec. at 95°C, followed by 1 min. at 60°C, with optical read

and subsequent melt curve analysis. Negative enzyme controls were performed, and negative template controls were performed across all primers.

Protein Expression

Sandwich ELISA - Activin A

Human activin A was quantified with the DuoSet ELISA Development System (R&D Systems, U.S.A.), as per the manufacturer's instructions. In summary, 100 µL of the diluted mouse anti-human activin A capture antibody was added to each well, this was sealed and incubated overnight. The next morning the plate was aspirated and washed; 300 µL of diluted reagent diluent was added to each well, the plate was sealed and incubated for 1 hr; this was followed by a subsequent aspiration and wash step. The recombinant human activin A standard was made up to 25.8 µL of stock and diluted with a seven-point standard curve using 2-fold serial dilutions in reagent diluent. Then 100 µL of samples and standards were added to the plate, followed by 100 µL of 1M urea in PBS, the plate was sealed and incubated for 2 hrs. The wells were aspirated and washed and 100 µL of the biotinylated mouse anti-human activin A detection antibody was added to each well, the plate was sealed and incubated for 2 hrs. The wells were aspirated and washed, and 100 µL of diluted streptavidin-conjugated to horseradish-peroxidase was added to each well, the plate was sealed and incubated for 20 mins. The wells were aspirated and washed, and 100 µL of substrate solution was added to each well, the plate was sealed and incubated for 20 mins. Finally, 50 µL of stop solution was added to each well, and the plate was read.

Sandwich ELISA - IL-16

IL-16 was quantified with the DuoSet ELISA Development System (R&D Systems, U.S.A.), as per the manufacturer's instructions. In summary, 100 µL of the diluted mouse anti-human IL-16 capture antibody was added to each well, this was sealed and incubated overnight. The next morning the plate was aspirated and washed; 300 µL of diluted reagent diluent was added to each well, the plate was sealed and incubated for 1 hr; this was followed by a subsequent aspiration and wash step. The recombinant human IL-16 standard was made up to 25.8 µL of stock and diluted with a seven-point standard curve using 2-fold serial dilutions in reagent diluent. Then 100

µL of samples and standards were added to the plate, followed by 100 µL of 1M urea in PBS, the plate was sealed and incubated for 2 hrs. The wells were aspirated and washed, and 100 µL of the biotinylated goat anti-human IL-16 detection antibody was added to each well, the plate was sealed and incubated for 2 hrs. The wells were aspirated and washed, and 100 µL of diluted streptavidin-conjugated to horseradish-peroxidase was added to each well, the plate was sealed and incubated for 20 mins. The wells were aspirated and washed, and 100 µL of substrate solution was added to each well, the plate was sealed and incubated for 20 mins. Finally, 50 µL of stop solution was added to each well, and the plate was read.

Appendix E. Systematic Review

Mild Hypoxic-Ischaemic Encephalopathy: A Systematic Review

Methods

A systematic review of the literature was undertaken in June 2015 to assess the outcome of mild grade HIE infants after 18 months.

Relevant literature reporting the outcome of mild HIE infants after 18 months that was published from 1980 was searched for in two electronic bibliographic databases:- Medline and ScienceDirect. The last search date was 22nd June 2015. Searches of the databases were conducted using the following strategies:

i. Medline:

(((((outcome) AND hypoxic ischemic encephalopathy) OR hypoxic-ischaemic encephalopathy) OR HIE) OR perinatal asphyxia) OR neonatal encephalopathy

The search was restricted with the following “filters”: Journal articles only; Published in English; Human only.

ii. ScienceDirect:

"outcome" AND "hypoxic ischemic encephalopathy" OR "hypoxic-ischaemic encephalopathy" OR "HIE" OR "neonatal encephalopathy" OR "perinatal asphyxia”.

The search was restricted with the following “limits”: Journal articles only.

Eligibility criteria are outlined in figure E1.

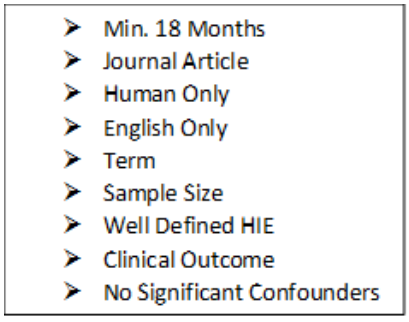

Figure E1. Eligibility criteria.

Process for Selecting Studies:

A flow chart of the study selection process is shown in Figure E2.

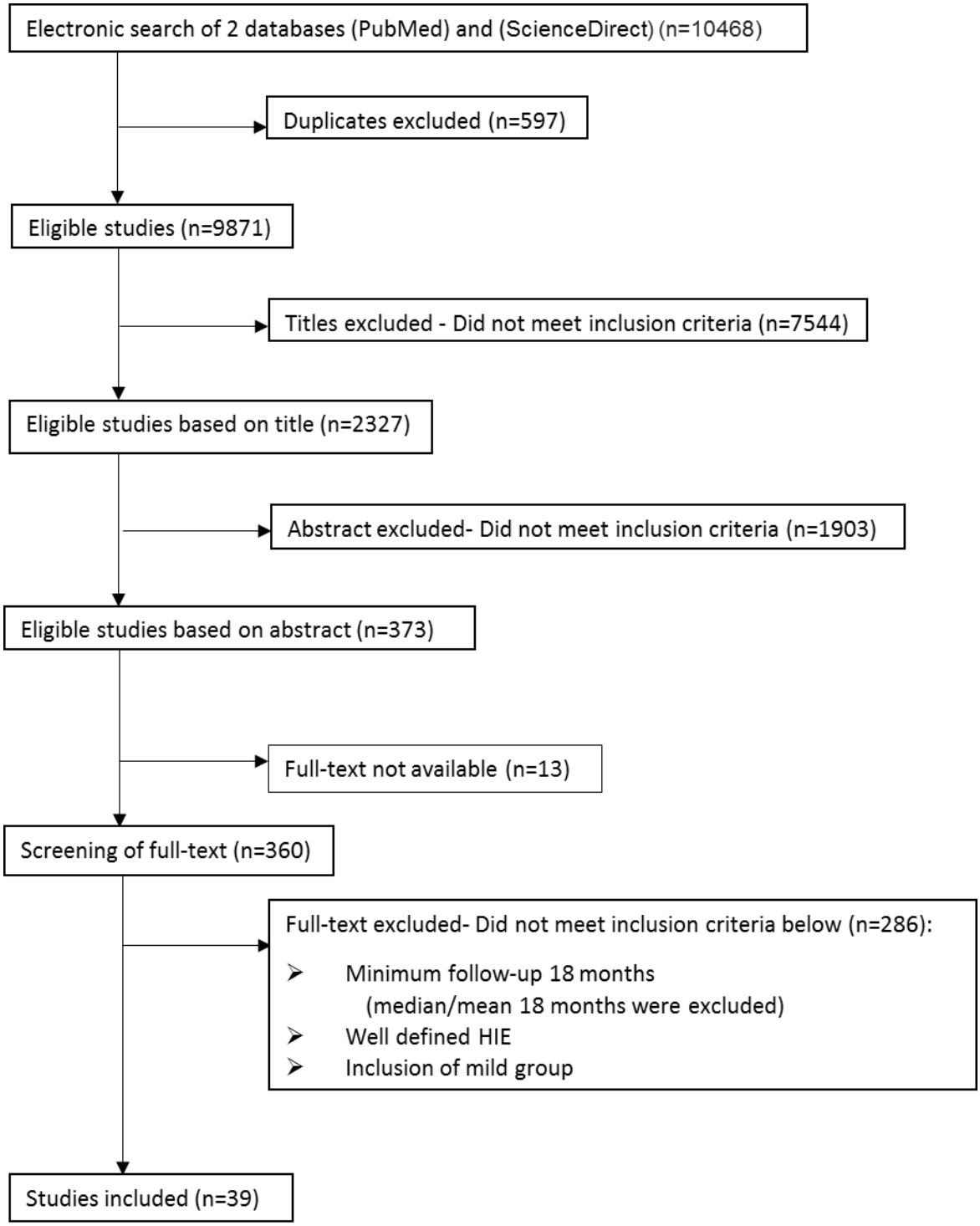

Figure E2. Flowchart of study selection.

Results

Table E1. Mild outcome in HIE studies.

	Author	No. of Patients	No. of Mild HIE	Outcome of Mild HIE Infants	Follow-up
1	Alderliesten (2011)	81	2	0 Adverse outcome (Griffiths Mental Developmental Scale)	18-46 mo
2	Barnett (2002)	88	21	1 Mild hemiplegia/dystonia, (Touwen Examination (1979))	5.5-6.5 yrs
3	Battin (2001)	40	11	1 Moderate hypotonic, developmental delay	18 mo
4	Belet (2004)	24	2	0 Adverse outcome	4 yrs
5	Biagioni (2001)	25	8	1 Moderate outcome, mild quadriplegia	24 mo
6	Bjork (2013)	35	8	0 Adverse outcome	18 mo
7	*El-Ayouty (2007)	34	3	0 Adverse outcome	
8	Ferrari (2011)	34	9	2 Dystonic CP	24 mo
9	Finer (1981)*				
10	Finer (1983)*				
11	Flisberg (2011)	21	1	0 Adverse outcome	18 mo
12	Gardiner (2014)	65	13	2 Mild disability	24 mo
13	George (2009)*				
14	Goergen (2014)	19	3	1 Below average BSID-III	24 mo
15	Haiju (2008)*				
16	Hallioglu (2001)	47	17	0 Adverse outcome	24 mo
17	Ilves (2004)	140	42 Mild/ Moderate	7 Mild impairments, 3 severe disability	18 mo
18	Ishikawa (1987)				
19	Kontio (2013)	56	17	9 Moderate injury on MRI	9-10 yrs
20	Lally (2014)	16	8	1 Abnormal vision	3.5 yrs
21	Leijser (2007)*				
22	Maneru (2001)	56	6	0 Adverse outcome	8 yrs
23	Maneru (2003)	42	6	Worst memory functioning then control ($p<0.047$)	15 yrs
24	McCulloch (1991)*				
25	Mercuri (1999)	29	9	0 Adverse outcome	24 mo
26	Murray (2010)	22	3	1 Motor delay	24 mo
27	Murray (2009)	44	18	1 Hypotonic, gross motor delay, 1 global developmental delay	24 mo
28	Murray (2009)	35	17	2 Abnormal outcome	18-24 mo
29	Muttitt (1991)*				
30	Nagdyman (2003)	49	15	2 Mild retardation, 1 severe	20 mo
31	Perez (2013)	68	30	0 Adverse outcome	11 yrs
32	Pisani (2009)	92	27	0 Adverse outcome	24 mo
33	Polat (2013)	25	11	1 Adverse outcome	44-48 mo
34	Precht (1993)*				
35	Robertson (1985)*				
36	Robertson (1988)	127	56	0 Adverse outcome	5.5 yrs

37	Robertson (1989)				
38	Scalais (1998)	40	5	0 Adverse outcome	24 mo
39	Shany (2006)	39	16	1 mildly abnormal, 1 severely abnormal	31-67 mo
40	Suppiej (2010)	59	21	0 Adverse outcomes	18-36 mo
41	Ter Horst (2004)	30	4	1 mildly abnormal	24 mo
42	Thornberg (1995)*				
43	Thorngren-Jerneck (2004)	62	21	0 Adverse outcomes	18 mo
44	Thorngren-Jerneck (2001)	20	6	0 Adverse outcomes	24 mo
45	Van Handel (2010)	126	33	3 TRF above clinical cut-off, 6 CBCL above clinical cut-off	9-10 yrs
46	Van Kooij (2008)	71	32	11 abnormal corpus callosum shape	9-10 yrs
47	Van Kooij (2010)	80	6	3 Moderate/severe injury	9-10 yrs
48	Van Schie (2010)	32	7	1 Poor mental outcome	24 mo
49	Van Schie (2015)	25	6	1 Mild motor impairments, borderline CBCL	6-8 yrs
50	Vermeulen (2008)	46	10	0 Adverse outcomes	24 mo
51	Walsh (2011)	44	19	0 Adverse outcomes	24 mo
52	Wertheim (1994)	37	10	1 Died	18 mo

*Full-text unavailable.

Concluding Summary

This systematic review identified a number of mild infants with poor neurodevelopmental outcome at 18 months. Following this systematic review, these search terms and results were used as a validation aid for Conway et al., 2018 (78).

Appendix F. Outcome in mUCHL

Introduction

This study explores mRNA of UCH-L1, as UCHL1 has previously been described as a target of miR-181b (350, 374). UCH-L1 is a highly neuronal and neuroendocrine-specific enzyme involved in regulating protein degradation (375, 376).

Methods

As described in chapter 3.

Results

The patient population is as described in chapter 3.

mRNA Expression in Neurodevelopmental Outcome

From the population, the neurodevelopmental outcome was available in sixty-three infants from both the BiHIVE1 and BiHIVE2 cohort, from this group seven infants were excluded as they were tested in a language that was not their first language. The neurodevelopmental outcome group included infants with a normal outcome, *n* = 38, a mildly abnormal outcome, *n* = 11, and a severely abnormal outcome, *n* = 7. Increased levels of mUCHL1 were observed in severely abnormal (median RQ = 2.27 (IQR = 1.32-2.63)) vs normal outcome infants (0.75 (0.47-1.57)), *P* = 0.009, and in severely abnormal (2.27 (1.32-2.63)) vs mildly abnormal outcome infants (0.78 (0.45-1.19)), *P* = 0.008, figure F1.

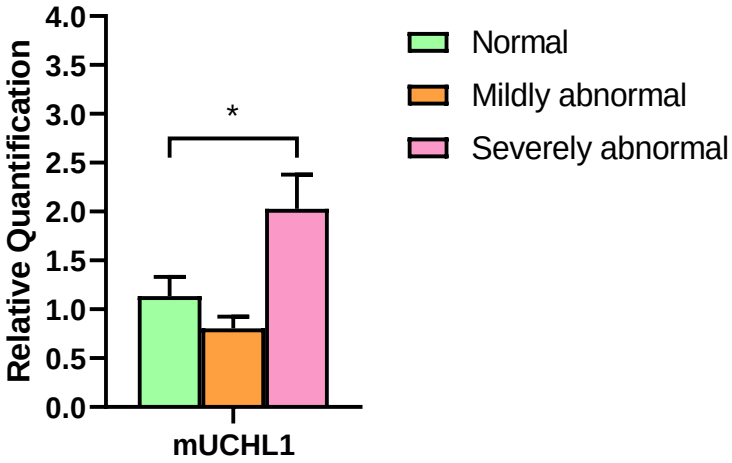

Figure F1. RQ levels in messenger RNA assessing neurodevelopmental outcome. The analysis included normal outcome, n = 38, mildly abnormal outcome, n = 11, and severely abnormal outcome, n = 7. *P < 0.05. Bar graphs represent fold change of RQ mean, and error bars represent standard error of the mean. RQ = relative quantification.

Discussion

UCH-L1 is a highly abundant and specific neuronal and neuroendocrine deubiquitinating enzyme (410). UCH-L1 acts as a marker of prior activation of memory T-cells and a determinant of cell survival maintaining axonal integrity after brain injury (411, 412). Increased levels of blood-based UCH-L1 is a recognised marker for severity of brain injury, with highest levels seen in those patients who do not survive (413, 414); it has been described in a number of studies as a possible HIE biomarker (415, 416). UCH-L1 can be affected by both long non-coding RNAs and miRNA and has been further defined in studies of miR-181b (374, 417-419). Increased mUCHL1 is increased in infants with severely abnormal long-term outcome.