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BIOMARKERS AND OUTCOME IN CHILDREN WITH HYPOXIC ISCHEMIC ENCEPHALOPATHY

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Biomarkers and Outcome in Children with Hypoxic Ischemic Encephalopathy
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my large and loving family, but especially to Ellie

POPULAR SCIENCE SUMMARY OF THE THESIS

Perinatal asphyxia is the condition that occurs in the fetus if blood flow or oxygen supply is interrupted or impaired during delivery. Perinatal asphyxia is the third leading cause of child mortality, causing around 8-13% of all child mortality and 23-24% of all neonatal deaths. Approximately 1-6 per 1000 born infants will develop signs of brain injury called hypoxic-ischemic encephalopathy (HIE). Some of the signs of HIE are affected level of consciousness, affected muscle tone, reflexes, pupillary reaction and heart rate. Many of the survivors of HIE develop secondary neurological disabilities including cerebral palsy, cognitive delay, seizure disorders, sensory-neural deafness, and visual loss. For the past 45 years, severity of HIE has been stratified into mild, moderate, and severe. Seizures are common in moderate to severe cases of HIE. There is evidence that neonatal seizures are harmful, especially after perinatal asphyxia and current clinical consensus is that neonatal seizures should be identified early and treated. Apart from treatment of seizures and supportive care, there is only one neuroprotective treatment currently available for infants with moderate-severe HIE, which has been proven to protect against death and neurocognitive disabilities. This treatment is therapeutic hypothermia and consists of wrapping the infant in an infant-sized blanket that is cooled through a servo-controlled water circulation system connected to a central temperature probe. The infant's core temperature is decreased to 33.5°C for 72 hours after which the temperature is increased slowly by 0.5°C every hour until the infant's temperature is 36.5°C. Shorter time from insult to start of treatment has been shown to improve outcome, which is why early detection of infants eligible for treatment has been the focus of many research groups for the last decade. Early studies demonstrated that none of the children with mild HIE died or suffered major disabilities including cerebral palsy. Hence, infants with mild HIE were never intentionally included in the trials of neuroprotective treatments due to a perceived good prognosis.

In Study I, we studied two umbilical cord blood biomarkers of neuronal injury, miRNA-181b and mUCH-L1, which have been suggested to play a role in neuroprotection in animal models, to see if they could improve detection of children in need of neuroprotective treatment. miRNA-181b showed potential as a predictor of moderate-severe HIE, however much uncertainty remains. Levels of mUCH-L1 was significantly higher in children who developed HIE, but that difference was mainly caused by high levels in children with severe HIE.

In Study II, we studied the addition of an automated seizure detection algorithm (ANSeR) to see if this algorithm would improve recognition of infants with seizures and improve detection of seizures when they were repetitive. The algorithm analyzed weak electric signals measured at the scalp (electroencephalography – EEG) in real-time and alarmed if any seizures were suspected. The addition of ANSeR to the regular EEG did not improve recognition of infants with neonatal seizures in a clinical setting. However, we did observe

an increased sensitivity for individual hours where seizures occurred, with the largest difference in sensitivity observed during weekends.

In Study III, we studied how children with mild HIE performed in a cognitive test situation at two years of age compared to children with and without a history of asphyxia at birth and across all grades of HIE. We demonstrated that children with a history of mild HIE at birth had a lower mean cognitive score than their healthy peers. The mean cognitive score of children with mild HIE was not significantly different from that of survivors of moderate HIE treated with therapeutic hypothermia.

In Study IV, we compared how well the tests used in Study III could predict cognitive performance measured at 6-8 years of age and how well they could predict neurological impairments at school age. We showed that the tests might detect children at risk of later poor outcome but is insufficient to predict neurocognitive trajectory and to rule out need for later interventions. We concluded that it is of great importance that studies of outcome in HIE, but also clinical follow-up, continue into school age.

ABSTRACT

Approximately 1-6 per 1000 born infants will develop hypoxic-ischemic encephalopathy (HIE) with significant associated mortality and morbidity. For the past 45 years, severity of HIE has been stratified into mild, moderate, and severe. Seizures are common in moderate to severe cases of HIE. There is evidence that neonatal seizures are harmful, especially after perinatal asphyxia, and current clinical consensus is that neonatal seizures should be identified early and treated promptly. Therapeutic hypothermia has been shown to reduce risk of death and disability among newborns with moderate-severe HIE. There is evidence that early cooling improves outcome, which makes timely identification of infants with HIE important.

The overall aim of this thesis was to improve treatment of children with HIE through studies of early identification of infants in need of neuroprotective treatment, and to assess outcome in children with mild HIE.

In two separate cohorts, umbilical cord blood miRNA-181b showed potential as a predictor of moderate-severe HIE. Levels of mUCH-L1 were significantly higher in children who developed HIE, but that difference was mainly caused by higher levels in children with severe HIE.

In a multicenter randomized controlled trial (RCT) including patients recruited from eight European NICUs, the addition of an automated seizure detection algorithm (ANSeR) did not improve sensitivity in identification of infants with seizures in a clinical setting. We did however observe an increased sensitivity for individual seizure hours, with the largest difference in sensitivity observed during weekends.

Using data from four prospective cohorts from Cork, Ireland and Stockholm, Sweden, children with a history of mild HIE at birth were shown to have lower cognitive composite scores measured with BSITD-III at two years of age compared to a healthy control group. The cognitive composite scores of children with mild HIE were not significantly different from that of survivors of moderate HIE treated with therapeutic hypothermia.

In a population-based, longitudinal study of all children treated with therapeutic hypothermia at birth between 2007-2009 in Stockholm, Sweden, BSITD-III was shown to detect children at risk of later poor outcome but was insufficient to predict neurocognitive trajectory. We concluded that it is important that studies of outcome in HIE, but also clinical follow-up, continue into school age.

LIST OF SCIENTIFIC PAPERS

I. Looney AM, O'Sullivan MP, Ahearne CE, **Finder M**, Felderhoff-Mueser U, Boylan GB, et al. 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. 2019;56(5):3657-63.

II. Pavel AM; Rennie JM; de Vries LS; Blennow M; Foran A; Shah DK; Pressler RM; Kapellou O; Dempsey EM; Mathieson SR; Pavlidis E; van Huffelen AC; Livingstone V; Toet MC; Weeke LC; **Finder M**; Mitra S; Murray DM; Marnane WP; Boylan GB. A machine-learning algorithm for neonatal seizure recognition: a multicentre, randomised, controlled trial. The Lancet Child & Adolescent Health. 2020;4(10):740-9.

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I. 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;40(3):271-277

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CONTENTS

LIST OF ABBREVIATIONS

aEEG	Amplitude-integrated Electroencephalography
ANSeR	Algorithm for Neonatal Seizure Recognition
ASM	Anti-Seizure Medication
AUROC	Area Under Receiver Operating Characteristic curve
BiHiVE	Biomarkers in Hypoxic-Ischaemic Encephalopathy
BiHiVE2	Validation of Biomarkers in Hypoxic-Ischaemic Encephalopathy
BSITD-II	Bayley Scales of Infant and Toddler Development, 2nd edition
BSITD-III	Bayley Scales of Infant and Toddler Development, 3rd edition
CB-III	Combined BSITD-III-score
CI	Confidence Interval
CNS	Central Nervous System
CP	Cerebral Palsy
CPR	Cardiopulmonary Resuscitation
GCP	Good Clinical Practice
EEG	Electroencephalography
FSIQ	Full-Scale IQ
HI	Hypoxic Ischemic
HIE	Hypoxic Ischemic Encephalopathy
miRNA	Micro Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
MTA	Material Transfer Agreement
mUCH-L1	UCH-L1 messenger RNA
NICU	Neonatal Intensive Care Unit
NMDA	N-methyl-D-aspartate
PA	Perinatal Asphyxia
PPHN	Persistent Pulmonary Hypertension in the Newborn
PRIME	Prospective Research in Infants with Mild Encephalopathy Study
RCT	Randomized Controlled Trial
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristic

RQ	Relative Quantification
qRT-PCR	quantitative Reverse-Transcription Polymerase Chain Reaction
SE	Standard Error
SOP	Standard Operating Procedure
WISC-IV	Wechsler Intelligence Scale for Children, 4th edition
WPPSI-III	Wechsler Preschool and Primary Scale of Intelligence, 3rd edition

1 INTRODUCTION

1.1 PERINATAL ASPHYXIA IN THE FULL-TERM INFANT

1.1.1 Epidemiology

Over the past 20 years, the world has made remarkable progress in reducing child mortality. However, every day 15 000 children die before the age of five. Today, perinatal asphyxia (PA) is the third leading cause of child mortality, causing around 8-13% of all child mortality and 23-24% of all neonatal deaths [1, 2]. Approximately 1-6 per 1000 liveborn infants will develop hypoxic-ischemic encephalopathy (HIE) [3-7]. For the survivors of HIE, there is significant associated morbidity, including cerebral palsy (CP), cognitive delay, seizure disorders, sensory-neural deafness, and visual loss [8, 9].

1.1.2 Causes

Birth asphyxia can occur due to processes before, during or after delivery. Risk factors described for birth asphyxia include vaginal breech delivery, high birth weight, second born twin, primiparity, increased maternal age, chronic maternal illness, lower maternal socioeconomic status, smoking, post-date pregnancy, epidural analgesia, male gender and being born at night [4, 7, 10]. Selected causes of perinatal asphyxia are listed in Table 1.

Intrauterine	Perinatal	Neonatal
Placental insufficiency	Placental abruption	Airway abnormalities
Hypertension	Uterine rupture	Neurologic disorders
Preeclampsia	Uterine hyperstimulation	Medication effect
Diabetes	Umbilical cord compression	Severe cardiopulmonary disease
Maternal chronic disease	Prolapse	Severe circulatory compromise
Maternal hypoxemia	Nuchal cord	Hemorrhage
Maternal illness	Knotted cord	Infection
Fetal anemia	Hemorrhage	
Immunization	Vasa previa	
Infection	Velamentous insertion	
Fetomaternal transfusion	Fetomaternal hemorrhage	
Twin-to-twin transfusion syndrome	Dystocia	
Affected fetal cardiac output	Shoulder dystocia	
Congenital heart disorder	Infection	
Twin-to-twin transfusion syndrome	Premature rupture of membranes	
Fetal arrythmia		
Maternal environmental factors		
Substance abuse		
Smoking		
Infection		

Table 1: Selected risk factors for and causes of perinatal asphyxia.

1.1.3 Mechanisms of Brain Damage in HIE

Birth asphyxia involves an affected gas exchange, due to causes and risk factors mentioned above, leading to hypoxia, hypercarbia and acidosis depending on the degree and duration of the disruption [11]. The presence of fetal acidemia on its own is not a sufficient prognostic marker of brain injury as the infant's brain has a resistance to asphyxia [5, 12, 13]. The fetus has several compensatory mechanisms protecting blood flow and oxygen supply to the more vital organs (brain, heart and adrenal glands), such as using a catecholamine surge to redistribute blood from less vital organs (kidneys, intestine, skin, muscle and liver) [11]. When compensatory mechanisms are insufficient, the fetal brain can be affected through two mechanisms: *hypoxemia*, meaning a reduced level of oxygen in the blood; and *ischemia*, meaning a less-than-optimal blood-supply [14]. If a cerebral hypoxic-ischemic (HI) insult is moderate, blood is shunted to the posterior circulation from the anterior in order to preserve perfusion of the brain stem, basal ganglia and cerebellum. As a result, injury would be reduced to the parasagittal watershed areas of the cerebrum. More severe and prolonged insults result in diffuse and cortical-deep nuclear neuronal injury. If the HI insult is more acute or abrupt, autoregulation of cerebral blood flow can be insufficient, thus resulting in basal ganglia and brain stem injuries [11, 14, 15].

Reduced energy supply through hypoxia-ischemia (HI) with depletion of energy reserves and subsequent failure of oxidative metabolism is recognized as the *primary insult*. This acute phase is characterized by anaerobic metabolism, depletion of adenosine triphosphate and a subsequent decrease in transcellular transport of ions resulting in an accumulation of intracellular sodium, water, and calcium. This leads to a cellular release of the excitatory amino acid glutamate and inflow of calcium through the N-methyl-D-aspartate (NMDA) gated channels inducing lipid peroxidation, production of nitric oxide and oxidative stress resulting in necrosis and activation of apoptotic cascades [16-19]. This process, called *excitotoxicity*, is usually followed by a transient period of recovery after delivery and resuscitation and thereby cessation of the HI insult. During this *latent phase* of injury, cerebral perfusion and oxygenation is restored and neural metabolism and activity is suppressed [11, 19]. This phase may last up to 6 hours and has been called the therapeutic window [20] before the *secondary phase* of brain injury. During this phase, delayed neuronal death encompasses a complex cascade of events including secondary energy failure, cytotoxic edema, mitochondrial dysfunction, calcium influx, caspase release, oxygen and nitrosative stress leading to apoptosis and necroptosis [16, 19, 21-27]. This process triggers the innate immune response including astrocyte and microglial activation and production of inflammatory cytokines and chemokines [28, 29]. This secondary injury is often associated with clinical deterioration and encephalopathy due to brain swelling and seizure activity [25, 30]. The clinical state of encephalopathy after a HI insult is commonly referred to as hypoxic-ischemic encephalopathy (HIE) [5].

Findings in animal as well as clinical studies are consistent with the hypothesis that a neuro-inflammatory process caused by a HI insult proceeds long after the neonatal period [31]. Inflammatory responses such as microglial and astrocyte over-activation, chemokine

and cytokine release and epigenetic changes have been considered as part of the active process, that prevents regeneration or exacerbates brain damage. This process that is thought to sensitize the patient to further injury, has been proposed as the *tertiary phase* of brain injury [19, 21]. There is evidence that preterm children with cerebral palsy have increased peripheral cytokines as a marker for an ongoing cerebral inflammation lasting at least seven years after the initial neonatal insult [32]. Recognizing and identifying such mechanisms can even be of therapeutic value if the persistent inflammation can be inhibited or modulated [25, 33].

1.2 HIE – DIAGNOSIS AND DIAGNOSTICS

1.2.1 Staging of HIE

	Stage 1	Stage 2	Stage 3
Level of consciousness	Hyperalert	Lethargic or obtunded	Stuporous
Neuromuscular control			
Muscle tone	Normal	Mild hypotonia	Flaccid
Posture	Mild distal flexion	Strong distal flexion	Intermittent decerebration
Stretch reflexes	Overactive	Overactive	Decreased or absent
Segmental myoclonus	Present	Present	Absent
Complex reflexes			
Suck	Weak	Weak or absent	Absent
Moro	Strong; low threshold	Weak; incomplete high threshold	Absent
Oculovestibular	Normal	Overactive	Weak or absent
Tonic neck	Slight	Strong	Absent
Autonomic function			
	Generalized sympathetic	Generalized parasympathetic	Both systems depressed
Pupils	Mydriasis	Miosis	Variable; often unequal; poor light reflex
Heart rate	Tachycardia	Bradycardia	Variable
Bronchial and salivary secretions	Sparse	Profuse	Variable
Gastrointestinal motility	Normal or decreased	Increased; diarrhea	Variable
Seizures	None	Common: focal or multifocal	Uncommon (excluding decerebration)
Electroencephalogram findings	Normal (awake)	Early: low-voltage continuous delta and theta. Later: periodic pattern (awake). Seizures: focal 1- to 1½-Hz spike-and-wave	Early: periodic pattern with isopotential phases Later: totally isopotential
Duration	Less than 24 h	Two to 14 days	Hours to weeks

Table 2: Sarnat scoring system adapted from Sarnat & Sarnat [34]

In 1976, Harvey and Margaret Sarnat described clinical features of 21 term infants with a defined episode of fetal distress and an Apgar score of less than five at five minutes of age. They described three clinical stages of encephalopathy later defined as mild, moderate and severe grades of encephalopathy, seen in Table 2 [34]. The Sarnat scoring system is still the most widely used, even though several other scoring systems and adaptations of the Sarnat score have been developed without any consensus regarding the definitions of grades of HIE. Thompson et al developed a simpler scoring system for prediction of outcome seen in

Table 3 [35], but is generally not used for grading of encephalopathy. With the introduction of therapeutic hypothermia, the importance of clinical grading as a means of determining eligibility for treatment became increasingly important. Most current grading systems are based on the Sarnat staging system. As there is thought to be a continuum of severity [36] within each grade of HIE and a variation over time, there is a possibility that grading performed within the first six hours of life will subsequently progress [34, 37, 38].

Maximum Thompson Score	Children with cerebral palsy/total per score category	Mild HIE	Moderate HIE	Severe HIE
0-10	0/10	0/10	0/0	0/0
11-14	3/13	0/0	2/12	1/1
15-22	14/17	0/0	2/2	12/15
Children with cerebral palsy/total per Sarnat stage		0/10	4/14	13/16

Table 3: Thompson score related to Sarnat stage and cerebral palsy adapted from Thompson et al (1997) [35]

Infants with mild HIE have historically been considered to have a good prognosis, since no increased risk of mortality or severe disabilities were seen in this group [39]. Children with a history of severe HIE had a probability of 75-100% of suffering neurodevelopmental disabilities or death. With a history moderate HIE, outcome has been shown to be more variable with a risk for poor outcome or death of approximately 25% [40, 41]. However, even more subtle disabilities have been noted among survivors in late adolescence, where 71% of children without cerebral palsy had cognitive disabilities [42, 43].

1.2.2 Neurophysiology

1.2.2.1 Seizures

Seizures occur in about 1-3 per 1000 newborn children [44]. Seizures can be caused by several disorders such as HIE, sepsis/meningitis, stroke, intracranial hemorrhage, metabolic and genetic disorders. HIE has been found to account for most cases [45, 46], where seizures occur in about 30-60% of children with moderate-severe HIE [46]. In infants with HIE, seizures usually start 12-24 hours after the HI event [46, 47], why an earlier onset could indicate an antepartum HI insult [48].

The clinical classification of neonatal motor seizures by Volpe, summarized in Table 4, has remained unchanged since 1989 [49]. However, in a recent report by the International League Against Epilepsy (ILAE), a new classification is suggested, as seen in Table 4. With the new classification, division into focal and generalized seizures is considered redundant as seizures are considered to always be focal at onset. The other major difference is a clarification that seizures can occur with or without clinical manifestations, which is why a new class of electrographic-only seizures has been added [50]. Visual assessment of suspected seizures has poor sensitivity, specificity and interobserver agreement [51]. Hence, electroencephalography (EEG) is imperative in the diagnosis of neonatal seizures.

Volpe 1989	ILAE 2021
Multifocal clonic	Automatisms
Focal clonic	Clonic
Tonic	Epileptic spasms
Myoclonic	Myoclonic
Subtle seizures	Tonic
	Autonomic
	Behavioral arrest
	Sequential seizure
	Electrographic-only seizure
	Unclassified seizure type

Table 4: Classification of seizures according to Volpe 1989[49] and ILAE 2021[50]

Identification of infants with seizures is essential as the majority are secondary to underlying neurological conditions [52], which are the main causes of later morbidity and mortality. However, there is evidence that neonatal seizures are harmful on their own [53, 54], especially after perinatal asphyxia [55], why clinical consensus is that neonatal seizures should be treated. There is also emerging evidence that treatment may be time-critical [56]. Few randomized trials have investigated initial treatment strategies of neonatal seizures [57, 58] and there are a few studies investigating second-line treatment [59-63]. There is no consensus on treatment strategies of neonatal seizures [64], but the strongest evidence is for Phenobarbitone as first-line treatment [58].

1.2.2.2 Electroencephalography – EEG

EEG is resource demanding, time consuming and requires expertise to apply and interpret [65]. Amplitude-integrated EEG (aEEG) is a filtered, rectified, and time-compressed version, created in the 1960's for use in adults resuscitated after cardiac arrest [66], during open-heart surgery, for monitoring after brain damage or drug overdose and for testing anesthetic drugs [67]. aEEG requires less expertise to apply and interpret and is commonly used in neonatal intensive care units (NICU) for monitoring of background activity and for seizure detection. Background pattern on aEEG/EEG was shown to have a high predictive value of poor neurodevelopmental outcome in children with perinatal asphyxia [68, 69] and can be predictive even during the "window of opportunity" before 6 hours of age [68], but with a lower predictive value if the infant is treated with therapeutic hypothermia. Possible causes of the lower predictive value with therapeutic hypothermia can be partly explained by delay of normalization [70] and neuroprotective effect of cooling [71]. Hence, the time to recovery has been presented as an alternative prognostic marker in children with ongoing therapeutic hypothermia, where predictive values have been demonstrated to be highest at 48 hours of age [70, 72, 73] for children with moderate-severe HIE.

EEG remains the gold standard for detection of neonatal seizures [50]. Single channel aEEG without EEG has been shown to have a poor sensitivity ranging from around 12-38% of individual seizure detection [74] up to 55% with increased output speed [75]. With

access to single channel EEG, individual seizure detection is increased to 78% [76]. However, aEEG has been shown to provide almost negligible clinical information about neonatal seizures if the interpreter is inexperienced [77]. aEEG has thus been shown to be unreliable for diagnostics of neonatal seizures. As EEG availability varies across centers [64], and requires highly experienced staff for interpretation, many research groups have developed and investigated different seizure detection algorithms with different levels of performance that has been reviewed by Mathieson et al [78].

1.3 HIE - TREATMENT

1.3.1 Therapeutic Hypothermia

In both observational and animal studies, fever in conjunction with HI events has been shown to worsen the extent of injury [79, 80]. Hypothermia, on the other hand, reduces secondary energy failure [26]. Between 28°C to 41°C, energy utilization in the brain has a linear relationship with temperature [81]. The notion of therapeutic hypothermia has existed for centuries in some form as there are records of physicians lowering newborns in cold water to reanimate them after delivery [82]. Trials of therapeutic hypothermia were initiated by Westin et al in the 1950's [83]. However, after trials showing that that preterm infants had improved survival if kept warm [84], no babies, preterm or term, were allowed to get cold. In the former Soviet Union, therapeutic hypothermia was still regarded as successful strategy, but due to language and political barriers, the therapy did not spread to the West [82]. In the 1990's, the notion of the secondary energy failure [27] and the concept of the therapeutic window after HIE [20] renewed the interest of cooling in reanimation and neuroprotective strategies [82].

Experimental work in the preclinical setting led to clinical trials of hypothermia in the late 90's and the beginning of the 21st century. 11 randomized controlled trials (RCTs) have been included in the latest systematic Cochrane review from 2013 looking at the effect of therapeutic hypothermia [85]: NICHD and TOBY-trials were the largest studies testing whole body cooling and the CoolCap study being the largest testing selective head cooling [86-96]. Children were treated with induced hypothermia to a temperature of 33.0°C to 36.5°C for 48-72 hours starting before 5.5 to 6 hours of age depending on the study. Method of temperature measurement differed between the studies. The meta-analysis, encompassing 1505 children with signs of encephalopathy, concluded that therapeutic hypothermia reduces the risk for mortality or neurodevelopmental disability at 18-24 months with 25%. All of the large clinical trials of therapeutic hypothermia used a composite outcome of "death or disability" as primary outcome with varying definitions of disability between the trials [97]. In order to prevent one death or severe disability, 5-10 children need to be treated with therapeutic hypothermia [85]. Even though there is a substantial reduction of risk for death or disability, the risk for neurodevelopmental disabilities remains high among the survivors [97]. "Time is brain": early start of treatment is preferable as it is associated with better motor outcome [98]. There is no evidence that longer or deeper cooling is advantageous [99]. Treatment has been offered to infants

outside of the protocols used in the clinical trials to infants with post-natal collapse, late preterm infants, infants with intracranial hemorrhage and infants with cardiac and surgical diagnoses and to infants with start of cooling later than 6 hours after a HI event. There are only observational studies of these patient groups indicating that cooling appears to be feasible and may be beneficial except for children with ongoing hemorrhage [100].

There is a significantly increased incidence of sinus bradycardia, thrombocytopenia and leukopenia seen with therapeutic hypothermia. There is also a non-significant trend towards higher incidence of persistent pulmonary hypertension (PPHN) and reports of subcutaneous fat necrosis in about 1% of infants registered as treated with cooling [85].

1.3.2 Biomarkers in HIE

As treatment with therapeutic hypothermia needs to be initiated as soon as possible before six hours have passed after the HI injury, swift identification of children who will benefit from therapeutic hypothermia is essential. The optimal biomarker, or panel of biomarkers, would aid the clinician in identification of responders and non-responders to neuroprotective strategies [28]. Clinically, acid-base balance and Apgar score are still used as biomarkers for neonatal asphyxia.

There have been several studies looking at cerebrospinal fluid (CSF) biomarkers [101, 102]. However, with limited accessibility and usefulness of CSF biomarkers in the clinical setting of neonatal brain injury, more effort has been focused on more readily available biomarkers for identification, timing, and monitoring of the HI insult [103].

1.3.2.1 Apgar

Apgar score at 5 and 10 minutes of age has a strong association with adverse neonatal outcomes [104]. It is commonly used as a predictor of neurologic outcome after perinatal asphyxia in spite of its low sensitivity and positive predictive value [105]. A low Apgar score is subject to serious inter-observer variability [106]. A low Apgar score is associated with an increased risk for cerebral palsy, epilepsy [104], cognitive delay and death. With every point gained at 10 minutes of age, the risk for adverse outcomes is reduced by about 30 percent, but even with a 10-minute Apgar score of zero, 20.8% of term infants survive without disability [107].

1.3.2.2 pH

There is a strong association between low cord blood pH and adverse neonatal mortality, HIE and cerebral palsy [108]. However, sensitivity and positive predictive value of adverse neonatal outcome is poor [105]. With a pH of above 7.0, there is only a weak association between pH and adverse outcomes [109].

1.3.2.3 Lactate

A scalp lactate concentration of 4.8 mmol/L has been suggested as a cut-off value for fetal asphyxia, with a sensitivity of 66.7% and specificity of 75.7% for moderate to severe HIE [110]. There is an association between radial artery blood lactate levels of > 9 mmol/L and later moderate-severe HIE with a sensitivity of 84% and specificity of 67% [111]. Another study could not show that initial serum lactate levels predicted severity of HIE, but demonstrated an association between time to normalization with severity of HIE and seizure burden on EEG [112].

1.3.2.4 Inflammatory and Brain Specific Biomarkers

Inflammation appears to play an important role in the development of HI injury, where inflammation may both modify injury, response to treatment [113] and impact later neurodevelopmental impairment including cerebral palsy [25, 114, 115]. Many studies have investigated inflammatory biomarkers after hypoxia such as such as interleukins (IL) IL-1α, IL-1β [29], IL-6 [101], IL-8 [116], IL-16 [117] and brain specific biomarkers such as Glial Fibrillary Acidic Protein (GFAP) [102, 118, 119], Neuron Specific Enolase (NSE) [120], S100b [121] and Ubiquitin Carboxyl-terminal Hydrolase L1 (UCH-L1) [118, 119]. Although many biomarkers have shown promising results in small studies, no available biomarker is currently recommended for clinical use to predict outcome or response to neuroprotective strategies [28, 122].

1.3.2.5 Micro-Ribonucleic Acid – RNA

Micro-ribonucleic acid (miRNA) molecules were discovered some 20 years ago. They are small sequences of ribonucleic acid (RNA) of approximately 21-24 nucleotides in length that do not code for any proteins. The function of these tiny transcripts is to regulate the function of other RNAs [123, 124]. Why miRNA was not discovered earlier has been the cause of some speculations as it has been shown that miRNAs and their associated proteins appear to be among the more abundant RNA-complexes in the cell. However, as some miRNAs can be restricted to non-abundant cell types, computational approaches have been developed to complement experimental miRNA gene identification [125]. miRNAs control cell function by degrading messenger RNA (mRNA), repressing translation and transcription, subsequently repressing protein synthesis [124, 125]. Through this influence on protein synthesis, miRNAs have been shown to influence cellular processes such as cell proliferation, cell death, fat metabolism, neuronal patterning and modulation of hematopoietic differentiation in animal models [125].

miRNAs have been considered as potential biomarkers for brain injury, as levels of miRNAs have been shown to be affected in both brain and in blood samples after cerebral ischemia in animal models [126], as well as in peripheral blood samples in young stroke patients [127]. Certain miRNAs have also been shown to play a significant role in gene suppression related to stress response and hypoxia [128, 129]. Upstream miRNA response may precede mRNA and protein alterations, why miRNA response may be detected more

rapidly than protein and mRNA biomarkers [130]. miRNAs are also stable and reproducible within the same species and appear to be resistant to ribonuclease degradation in serum [131]. This provides the rationale for their role as blood biomarkers for cerebral hypoxic-ischemic injury.

1.3.2.6 UCH-L1 and miRNA-181b

Ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) is a neuron-specific protein found in abundance in the brain and is estimated to represent 5-10% of all cytoplasmic protein [132]. UCH-L1 has been found to be elevated in CSF after traumatic brain injury and seizures [133, 134] and in serum after traumatic brain injury [135]. UCH-L1 has been shown to be increased in cord blood in children who developed HIE compared to controls [116, 118, 136] and in cord blood in children with moderate-severe HIE compared to mild HIE, but no difference was seen in post-natal samples after initiation of therapeutic hypothermia [116].

miRNA-181b has been identified as a hypoxia regulated miRNA [128] and levels of miRNA-181b have been shown to be significantly reduced in a murine model of ischemic stroke. It is suggested that miRNA-181b downregulation provides neuroprotection since miRNA-181b negatively regulates UCH-L1 [137].

1.3.2.7 MRI

Currently, magnetic resonance imaging remains the gold standard in prediction of injury severity and outcome [138]. However, MRI is resource demanding, time consuming, requires expertise to interpret and infants might not tolerate transport or can be too unstable to withstand the duration of the scan [16, 65].

1.4 HIE - OUTCOME

1.4.1 Assessments of Neurodevelopmental Outcome

1.4.1.1 Bayley Scales of Infant and Toddler Development, 3rd edition (BSITD-III)

BSITD-III [139] is still considered the gold standard tool for assessing neurodevelopmental outcome in trials of neuroprotective strategies [28], but is also used in clinical settings. BSITD-III is used in children up to 42 months of age. Studies have compared BSITD-III with its predecessor Bayley Scales of Infant and Toddler Development 2nd edition (BSITD-II) [140-144] and BSITD-III scores of extremely preterm children have been compared with both the normative mean of BSITD-III and with BSITD-III scores of a healthy control group [145], after which concern has been raised that BSITD-III underestimates neurodevelopmental delay [141-145]. Studies have also investigated the ability of BSITD-III to estimate later cognitive abilities in children born preterm [146, 147] and in healthy children born at term [148] and results are inconsistent. Subtle cognitive problems are not always evident until later school age [42, 149] when cognitive demands increase.

1.4.1.2 WISC-IV

WISC-IV is widely used as a measurement of cognitive ability at age 6-16. Through ten subtests, it provides a measurement of four cognitive domains: Verbal Comprehension, Perceptual Reasoning, Working Memory, and Processing Speed as well as a full-scale IQ (FSIQ) that represents the sum of all scale scores [150].

1.4.2 Moderate-Severe HIE

1.4.2.1 Mortality

In the early description of stages of encephalopathy by Sarnat and Sarnat, 4 of the 21 patients died [34]. In a later study including children born between 1974 and 1979 (before the era of therapeutic hypothermia), 5% of children with moderate encephalopathy and 82% of infants with severe encephalopathy died during the first 8 years of life [151]. With the introduction of therapeutic hypothermia, incidence of death in the randomized trials has varied between 17% and 38% among children randomized to treatment with therapeutic hypothermia [152]. Bonifacio et al [153] compared children treated with therapeutic hypothermia according to standard protocol in 2010-2013 with those treated in the original NICHD-trial in 2000-2003 [88]. After implementation of therapeutic hypothermia as clinical practice, they observed reduced odds of death (with a reduced incidence from 24% to 9%) and of the composite outcome of "death or disability" (from 44% to 29%). However, the point estimates for odds-ratios had wider confidence intervals and non-significant p-values when adjusted for level of HIE and intra-center clustering [153]. It has been suggested that future trials of neuroprotective treatment for moderate-severe HIE should base sample-size calculations on an incidence of 29% of the combined outcome of death or disability [97].

1.4.2.2 Motor and other Neurodevelopmental Disabilities

Survivors of severe HIE often develop neurodevelopmental disabilities [151] including problems with feeding, speech and language, vision, cognition, and to some extent seizures [154]. Survivors of moderate HIE have more variable outcomes [152, 154].

In the Cochrane Review of trials of therapeutic hypothermia, 109 children of 475 (23%) treated with therapeutic hypothermia developed cerebral palsy [85]. With the implementation of therapeutic hypothermia, Bonifacio et al [153] observed a reduced incidence of death (see above) comparing the NICHD cooling trial [88] and the NICHD Optimizing Cooling Strategies trial [155], but no difference was seen in incidence of cerebral palsy, 19% vs 19%. Using two different editions of Bayley Scales of Infant and Toddler Development (2nd and 3rd edition) limited the ability to make comparisons of neurodevelopmental scores [153].

Children who have developed encephalopathy have been shown to have increased risk of lower working memory, reading accuracy and comprehension scores as well as increased risk of needing educational support in early school-age in comparison with children who

were healthy at birth. This association is weaker among children without cerebral palsy [156].

1.4.3 Mild HIE

Infants with mild HIE exhibit signs of hyperalertness (irritability) and sympathetic activation but have normal muscle tone and absence of seizures. Studies of long-term outcome in this group of patients have been rare. Early studies demonstrated that none of the children with mild HIE had died or suffered major disabilities including cerebral palsy. Hence, infants with mild HIE were never intentionally included in the RCTs of therapeutic hypothermia due to the supposed good prognosis [3, 38, 85, 100, 151, 157]. Of the randomized trials, only four reported treatment of any children with mild HIE [80, 90, 91, 158], with a total of 91 patients included in the latest systematic review on long term developmental outcome in mild HIE [159].

More recent studies have looked at more subtle differences later in life, reporting higher than expected rates of brain injury and disability in this patient group [37, 149, 159-165] with as many as 22-26% having mild to moderate disabilities at two years of age [159] and 35% having difficulties at 5 years of age [163]. The first prospective study in the era of therapeutic hypothermia, the Prospective Research in Infants with Mild Encephalopathy (PRIME) study, concluded that 52% of children with mild HIE had an abnormal short-term outcome measured on either early aEEG, MRI, or neurological exam at discharge [166]. 68% of the children enrolled completed BSITD-III at 18-22 months of age, of whom 40% had a Bayley score of ≤85 in any subscale and 16% had any disability defined as at least "cognitive score of 70–84 alone, or a cognitive score ≥85 and GMFCS level 1 or 2, seizure disorder (without anti-epileptic medication), or hearing deficit with ability to follow commands without amplification" [167]. The PRIME-study did not include a control group and suffered from a 32% loss-to-follow-up. It was possible to get a diagnosis of mild HIE even with seizures < 9 h [166].

It is not yet known if cooling in non-moderate-severe HIE is harmful, even if cooling appears to be feasible in other patient groups than the ones included in the strict protocol developed by Shankaran et al [88, 100]. Despite there being no evidence of benefit of treatment in this patient group, there is an ongoing therapeutic drift in many centers to treat these infants with therapeutic hypothermia [37, 38, 168, 169]. 75% of centers in the UK report that they offer cooling to infants with mild HIE [168].

2 RESEARCH AIMS

The overall aim of this thesis was to improve treatment of children with HIE through studies of early identification of infants in need of neuroprotective treatment, and to assess outcome in children with mild HIE.

2.1 SPECIFIC AIMS

2.1.1 Study I

The aim of this study was to assess miRNA-181b and its downstream target UCH-L1 mRNA (mUCH-L1) as a potential biomarker for all grades of HIE. This aim was a part of the overall aim of the BiHiVE (Biomarkers in Hypoxic-Ischaemic Encephalopathy) studies: to develop and validate early, objective, and reliable biomarkers of perinatal brain injury.

2.1.2 Study II

The aim of this study was to evaluate if an addition of an automated seizure detection algorithm (ANSeR) could improve diagnostic accuracy in identification of neonatal seizures.

2.1.3 Study III

The aim of Study III was to assess neurocognitive outcome of children with mild HIE compared to healthy controls.

2.1.4 Study IV

The aim of Study IV is to assess the predictive value of the Bayley Scales of Infant and Toddler Development, 3rd edition for later neurodevelopmental development in children treated with therapeutic hypothermia for HIE at birth.

3 MATERIALS AND METHODS

3.1 STUDY POPULATIONS AND CLINICAL DATA

3.1.1 BiHiVE (Study I & III)

The Biomarkers in Hypoxic-Ischaemic Encephalopathy (BiHiVE) cohort was recruited in Cork, Ireland between May 2009 and June 2011. The recruitment followed strict recruitment criteria of umbilical cord pH of <7.1, Apgar score of ≤6 at 5 minutes of age and/or intubation or cardiopulmonary resuscitation (CPR) at birth. Exclusion criteria were preterm birth < 36 weeks of gestation or co-existing morbidities such as sepsis, metabolic disease and/or CNS malformation. Written and oral consent were obtained from parents of all infants.

As healthy controls for the BiHiVE study (used in Study I), the Cork BASELINE study (www.baselinestudy.net) was used. The BASELINE study is an Irish birth cohort of healthy infants with normal uneventful deliveries following the same strict Standard Operating Procedure (SOP) used for the BiHiVE study. Relevant clinical data were uploaded to a pre-designed electronic Case Report Form (eCRF) (MedSciNet, Stockholm, Sweden) [170].

3.1.2 BiHiVE2 (Study I & III)

The Validation of Biomarkers in Hypoxic-Ischaemic Encephalopathy (BiHiVE2) study was a multicenter cohort recruited between March 2013 and June 2015 at Cork University Hospital and Karolinska University Hospital. The purpose of recruiting this cohort was to validate findings from the BiHiVE study, using the same recruitment criteria and SOP. BiHiVE2 also recruited healthy control infants with normal deliveries, normal neonatal examinations and normal 5-minute Apgar scores. The BIHVE2 study has been registered with clinicaltrials.gov as NCT02019147. Relevant clinical data and EEGs were uploaded to a pre-designed eCRF (MedSciNet, Stockholm, Sweden) [170].

3.1.3 ANSeR1 (Study III)

Algorithm for Neonatal Seizure Recognition, phase 1 (ANSeR1) was a multicenter cohort recruited from six NICUs across four European countries. All infants >36 weeks of gestation who were in clinical need of monitoring with EEG were eligible. Retrospective data was available from two centers from infants born between January 2011 and February 2014, while prospective data was collected between June 2013 and June 2015. Infants with less than 6 hours of EEG-recordings with sufficient quality were excluded. Clinical data and EEGs were uploaded to a pre-designed eCRF (MedSciNet, Stockholm, Sweden) [46]. In the Cork University Maternity Hospital arm of the study, follow-up data including BSITD-III was collected.

3.1.4 ANSeR2 (Study II)

Algorithm for Neonatal Seizure Recognition, phase 2 (ANSeR2) was designed as a multicenter randomized controlled trial in eight NICUs across Ireland, the Netherlands, Sweden, and the UK. Patients were recruited between February 2015 and February 2017. Any infant between 36- and 44-weeks gestational age admitted to any of the NICUs in need of EEG monitoring were eligible for the study. Relevant clinical data and EEGs were uploaded to a pre-designed eCRF (MedSciNet, Stockholm, Sweden).

3.1.5 NeoCool (Study III & IV)

NeoCool was a population-based cohort of all children >34 weeks gestational age treated with therapeutic hypothermia in the greater Stockholm region between January 1st 2007 and December 31st 2009. Exclusion criteria were any known genetic disorders and/or inborn errors of metabolism. Perinatal data was recorded prospectively after birth and was validated retrospectively January to May 2021. All data was managed using RedCap electronic data capture tools hosted at Karolinska Institutet [171, 172].

3.2 EXPOSURES, OUTCOMES AND STATISTICAL ANALYSIS

3.2.1 Study I (BiHiVE and BiHiVE2)

In Study I, the BiHiVE cohort was used as a discovery cohort, while the BiHiVE2 cohort was used for validation.

3.2.1.1 Exposure – miRNA-181b and mUCH-L1

3 ml of umbilical cord blood (UCB) was drawn in Tempus™ Blood RNA tubes (Applied Biosystems, Foster City, CA) from all infants at birth. The blood was stabilized by shaking the tube vigorously for ten seconds before storing at -80°C within 3 h of delivery.

Total RNA was isolated using MagMAX™ for Stabilized Blood Tubes RNA Isolation Kit (Ambion, Life Technologies, Austin, Tx). Isolated RNA was stored at -80°C until further processing. RNA concentration was quantified using a NanoDrop 8000 Spectrophotometer (ThermoScientific, NanoDrop, Wilmington, DE). Additionally, RNA quality was assessed with Agilent 2100 Bioanalyzer to ensure purity of samples.

Reverse transcription was achieved using miRCURY LNA™ Universal RT microRNA protocol (Exiqon A/S, Vedbaek, Denmark) using commercially available primers for hsa-miR-181b-5p (AACAUUCAUUGCUGUCGGUGGGU) and hsa-miR-223-3p (UGUCAGUUUGUCAAAUACCCCA) (Exiqon, Woburn, MA). Hsa-223-3p was used due to its stable expression in an initial micro-array and later validation using real time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) [173]. Levels of UCH-L1 mRNA (mUCH-L1) were analyzed using the TaqMan® Gene Expression Assay (Applied Biosystems, Life Technologies, Paisley, UK), using 18S ribosomal RNA (rRNA) as reference gene [174]. 4 µl RNA diluted to 10 ng/µl, RT reaction solutions, 2 µl 5x reaction buffer and 1 µl of enzyme mix were incubated for 60 min at 42°C followed by heat

inactivation for 5 min at 95°C, then stored at 4°C for less than 12 hours before qRT-PCR was performed. All samples were run in duplicates. Relative expression level of miRNA-181b and mUCH-L1 was calculated using the $2^{-\Delta\Delta Cycle\ Threshold}$ method [175].

3.2.1.2 Outcome – Sarnat Score

Grade of encephalopathy was assigned using a Sarnat score with a modified EEG background grading [176] for children monitored with aEEG or multichannel EEG. Infants who fulfilled the inclusion criteria, but who did not subsequently develop HIE were defined as having suffered perinatal asphyxia without HIE (PA).

3.2.1.3 Statistical Analysis

Statistical analysis in this paper was performed by co-authors Ann-Marie Looney and Marc O'Sullivan. Differences in expression levels were analyzed using Mann-Whitney U and Kruskal-Wallis tests. Spearman's rank correlation coefficients were calculated to test for dependence between miR-181b and mUCH-L1. Statistical analysis was executed using IBM SPSS Statistics 22 (SPSS Inc., USA).

3.2.2 Study II (ANSeR2)

3.2.2.1 Exposure – conventional EEG monitoring and ANSeR Algorithm

This study was designed as a randomized, two-arm, controlled study. Parents to infants eligible for EEG monitoring for clinical purposes were approached for oral and written consent before randomization to standard care with routine EEG monitoring or intervention using standard EEG monitoring with the addition of the ANSeR algorithm, an EEG-based seizure detection software that has been developed using repeated testing and offline training on neonatal EEGs from several cohorts [78, 177, 178]. Randomization to intervention or standard care was performed 1:1 using a block randomization in STATA's ralloc procedure, using blocks of varying size of two to four, stratified by hospital. Randomization was performed at time of registration of the infant in the eCRF system.

The ANSeR system was run on a laptop linked to a Nihon Kohden Neurofax monitor (EEG-1200, Tokyo, Japan) displaying a visual seizure probability trend, previously described by Mathiesen et al [78]. If the probability trend breached the predefined threshold of 0.5, the laptop would give of an audible alarm to alert the clinical staff.

Infants in the control group were monitored according to standard care with Nihon Kohden Neurofax monitor (EEG-1200, Tokyo, Japan) without the seizure algorithm active, or NicoletOne ICU Monitor (Natus, Middleton, WI, USA) or XLTek (Natus). Only the former two were used at Karolinska University Hospital. Identical montages were used for the intervention and control group.

3.2.2.2 Outcome – Seizure detection

The main outcome of the study was correct identification of a) babies with electrographic seizures and b) "seizure hours". All infants had a bedside seizure record form, where the clinical team would annotate per hour if a seizure had been recognized. If no seizure was noticed for an hour, the hour was marked as "no seizures recorded" or equivalent. If any seizure was marked on the seizure record form or if an anti-seizure medication (ASM) was administered, the hour was considered to have been identified as a "seizure hour" by the clinical team.

3.2.2.3 Statistical Analysis

For this multicenter study, power calculations and statistical analyses were performed by biostatistician and co-author Vicki Livingstone at the INFANT Research Centre, Cork, Ireland using Stata statistical software, version 15.0 (StataCorp LLC).

A bootstrap method was performed, where children were divided into 16 clusters based on allocation (algorithm vs non-algorithm) and by hospital. This was added to consider the within-infant clustering of infant-hours and the stratified randomization by site. It would produce point and interval estimates of the diagnostic accuracy, including differences between treatment groups, without having to resort to odds ratios.

3.2.3 Study III (ANSeR1, BiHiVE, BiHiVE2, NeoCool)

In this study, data was pooled from the four cohorts ANSeR1, BiHiVE, BiHiVE2 and NeoCool.

3.2.3.1 Exposure – HIE grade

In the two BiHiVE-studies, perinatal asphyxia (PA) was defined as children with one or more of the following: pH less than 7.1, 5-minute Apgar score ≤6, and intubation and/or CPR at birth. Any child with PA was assessed for development of HIE. For all studies, worst HIE grade during the first 24 hours was assigned by the treating clinician. A modified EEG background grading was used [176], but without EEG staging for mild HIE. At discharge, all children had been grouped into the five exposure categories: Healthy control, PA, mild HIE, moderate HIE and severe HIE. Infants with any other cause of encephalopathy were excluded. All infants with moderate-severe HIE were monitored with aEEG or multichannel EEG. All healthy controls for Study III were recruited as a part of the multicenter study BiHiVE2.

3.2.3.2 Outcome – BSITD-III

At 18-42 months of age, children recruited to their respective cohorts were assessed with BSITD-III. Children unable to partake in one or more of the BSITD-III categories due to severe motor dysfunction or severe autistic traits were assigned a score of -3 SD less than the mean of the control group. When Swedish or English was not the first language spoken at home, the language scores of that child was excluded from analysis.

A mean group difference of ≥5 points was regarded as clinically significant. A normal cognitive outcome was defined ≥1SD below the mean of the control group.

3.2.3.3 Statistical Analysis

Statistical analysis was performed by the author with biostatistical support from Professor Matteo Bottai and the Biostatistics Core Facility at Karolinska Institutet. Mean BSITD-III composite scores, mean differences and 95% confidence intervals (CI) were estimated using linear regression with robust estimates of the standard errors (SEs). For post-estimation comparison of composite scores in children with mild HIE and children with moderate HIE, an F-test was used.

All analyses were adjusted for country of birth. Stepwise linear regression was performed, where independent variables with a significance level $p<0.20$ were kept in a multivariable regression model. The predictor HIE-grade was not subject to the abovementioned selection criterion. Cohen's *d* with adjustment for unequal variances was used to estimate effect sizes. Significance level was set to a 2-sided $p<0.05$.

For missing covariates, predictive mean matching was used for continuous variables and logistic regression was used for binary variables. A chained imputation was performed using 20 imputed data sets, including neurodevelopmental outcome, HIE grade, country of birth, and an interaction term containing both exposure and outcome.

Statistical analyses were performed using Stata statistical software, version 14.2 (StataCorp LLC).

3.2.4 Study IV (NeoCool)

3.2.4.1 Exposure – BSITD-III

At two years of age, children from the NeoCool cohort were assessed with the Swedish version of BSITD-III. A combined BSITD-III-score (CB-III), similar to the BSITD-II's mental developmental index (MDI), equal to the mean of the cognitive composite and language composite score was calculated [144].

3.2.4.2 Outcome – WISC-IV

At 6-8 years of age, the Swedish version of the Wechsler Intelligence Scale for Children, 4th edition (WISC-IV) was administered to assess cognitive function. Poor outcome was defined as having a WISC-IV full-scale IQ (FSIQ) of <85 at 6-8 years of age or having any registered clinical diagnosis of epilepsy, developmental coordination disorder (DCD), cortical visual impairment (CVI), attention deficit hyperactivity disorder (ADHD), attention deficit disorder (ADD), autism spectrum disorder (ASD) and central hearing loss before follow-up at age 10-12 when the study period ended. Cerebral palsy was not included as a poor outcome in this analysis as this diagnosis was established before the BSITD-III follow-up [179].

3.2.4.3 Statistical Analysis

As both BSITD-III and WISC-IV composite scores are standardized to a mean (SD) of 100 (15), the scores from both scales were assumed to be comparable. We used Pearson's correlation coefficient to describe pairwise linear correlations between scores from both tests including CB-III. We assessed trajectory between each assessment using a mixed effects model. Any linear relationship between BSITD-III and WISC-IV FSIQ was assessed using linear regression models.

We assessed the predictive ability of BSITD-III to detect later poor outcome using a logistic regression and nonparametric receiver operating characteristic (ROC) analysis. To explore any non-linear relationship between BSITD-III scores and proportions of poor outcome, natural cubic splines were used [180].

3.3 ETHICAL CONSIDERATIONS

Studies in infants demand meticulous ethical consideration as the infants are not able to partake in consenting to the research but are represented by their parents.

For the BiHiVE studies (Study I, III), cord blood samples were drawn before parental consent could be obtained. If the parents decided not to participate, the blood samples were destroyed. Apart from umbilical cord blood sampling, no blood samples were drawn from any baby without parental oral and written consent. Biobanking studies require ethical credibility that procedures and tests performed must be according to ethical applications and especially the patient information leaflet. In biobanking studies, informed consent is required for collection of tissue samples, processing of personal data and for medical research. To secure a given consent in international and multicenter collaborations, material transfer agreements (MTA) must be used. In collaboration with KI Biobank, we scrutinized our ethical permits and patient information leaflets and prepared MTAs for our close collaboration with University College Cork.

For the Swedish recruitment to ANSeR1, retrospective data was available for the majority of the 60 patients recruited from Stockholm [46], where the researchers and the Regional Ethical Review Board in Stockholm agreed that refraining from asking the parents for permission to use already collected EEG material constituted a small invasion of privacy in comparison with the benefit of the research. The retrospective data from ANSeR1 was however not part of the studies included in this thesis.

ANSeR2 has been run as an RCT in accordance with Good Clinical Practice (GCP). The study did not require any blood sampling, treatment, or procedures apart from clinical practice. The intervention only required the added algorithm to the EEG-machine which would give off an alarm if the algorithm quantified the probability of a seizure to be higher than 50%. If the algorithm had increased clinical accuracy significantly, there would have been a potential risk of an ethical dilemma of continuing recruitment as the principle of equipoise would have been void. In order to prevent this from happening, an interim

analysis was performed that did not find a difference with significance superseding Haybittle-Peto's boundary for cessation of the trial [181, 182].

Recruitment for the cohorts BiHiVE, BiHiVE2, ANSeR1 and ANSeR2 (Study I, II, III) all involved approaching and collecting written consent from parents in crisis. Even though both written and oral consent was collected, it was made clear that a written consent is not a contract and could be withdrawn at any time for any reason. Only one parent from Sweden participating in ANSeR2 withdrew their consent without wanting to explain the reason for it, which must be and was respected due to the principle of autonomy and personal integrity.

In Study IV, the children had reached an age of 10-12 years of age at the latest follow-up. For the integrity of the data but also of ethical consideration, any procedure or exam performed at 6-8 years of age and at 10-12 years of age must be with the active consent and participation of the child, even though the written consent is collected from the parents. A concern with the protocol for NeoCool (Study III, IV) was that long-term follow-up could cause potential harm of parents reliving their traumatic experiences from the child's birth and time in the NICU. Our experience was quite the opposite. The parents were appreciative of further follow-up, and many demonstrated a need to talk about their experiences. The parental experience following treatment with therapeutic hypothermia has also been focus of study from our research group [183, 184]. All results were conveyed to the parents with the possibility of referral if deemed necessary for neuropsychiatric testing, psychological support, further follow-up etcetera.

The study protocols of BiHiVE2, ANSeR1, ANSeR2 and NeoCool were approved by the Regional Ethical Review Board in Stockholm. The study protocol for NeoCool 10-12-year follow-up was approved by the Swedish Ethical Review Authority as this department was started January 1st, 2019, and thus replaced the Regional Ethical Review Boards in Sweden. The study protocols for BiHiVE, BiHiVE2, ANSeR1 and ANSeR2 were approved by the Clinical Ethics Committee of the Cork Teaching Hospitals. ANSeR2 was also approved by the appropriate local ethics committees of all participating centers.

4 RESULTS

4.1 STUDY I

The aim of this study was to assess miRNA-181b and its downstream target UCH-L1 mRNA (mUCH-L1) as potential biomarkers for all grades of HIE.

A total of 131 children were included from the two cohorts: 59 from the discovery cohort, BiHiVE, and 72 from the validation cohort, BiHiVE2.

4.1.1 miRNA-181b

4.1.1.1 BiHiVE – discovery

57 children had measurable levels of miRNA-181b. There was no significant difference in relative quantification (RQ)-levels of miRNA-181b between controls, PA, or grades of HIE (Figure 1).

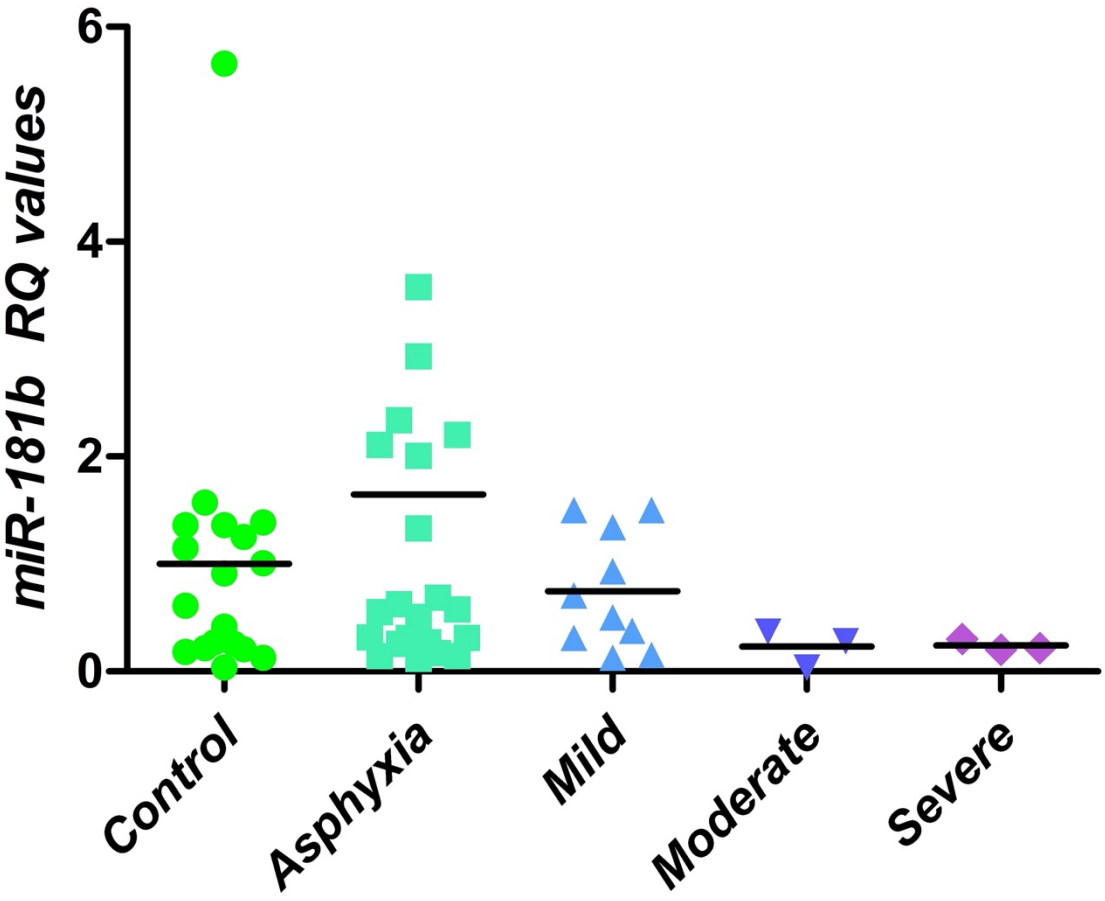

Figure 1: RQ-levels of miR-181b in the discovery cohort, BiHiVE, per patient group (healthy controls, PA, mild HIE, moderate HIE and severe HIE). Reprint from Looney et al, with permission [185].

When comparing children with moderate-severe HIE, which were eligible for therapeutic hypothermia according to current guidelines in Cork and in Stockholm, with those who did not meet current criteria for therapeutic hypothermia (healthy controls, PA and children with mild HIE) there was a significant difference in median RQ-levels, however with

overlapping confidence intervals: 0.25 (95% CI: 0.16-0.32) vs 0.61 (95% CI: 0.26–1.39) respectively, p=0.029 (Figure 2).

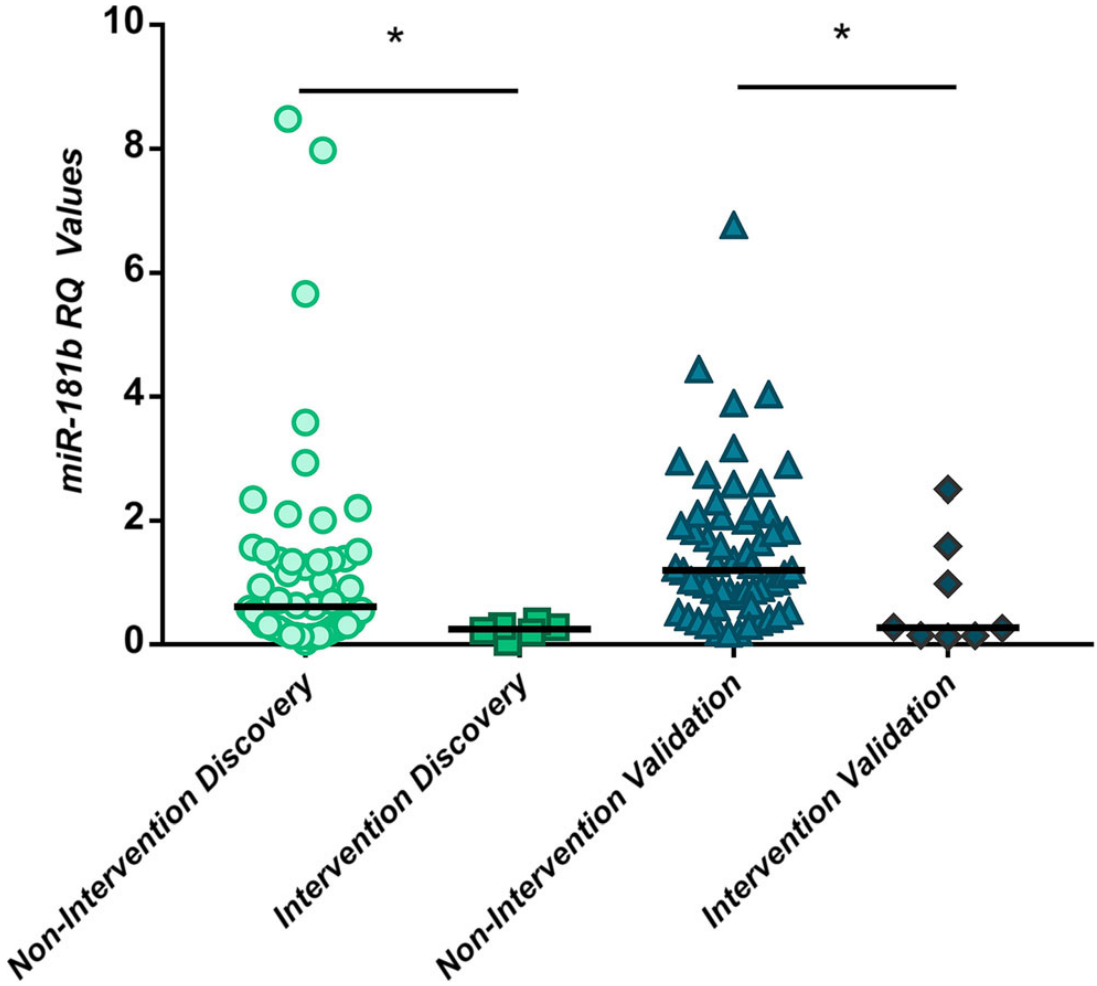

Figure 2: RQ-levels of miRNA-181b in children with moderate-severe HIE compared to children not eligible for therapeutic hypothermia according to current criteria (healthy controls, PA, mild HIE). Reprint from Looney et al, with permission [185].

4.1.1.2 BiHiVE2 – validation

65 children in the validation cohort had measurable levels of miRNA-181b. Similar findings were seen in the validation cohort with downregulated levels of miRNA-181b in children with moderate-severe HIE (n=8) 0.33 (95% CI: 0.15-1.78) compared with all other infants (n=57) 1.2 (0.71-2.09), p=0.035, again with overlapping confidence intervals.

4.1.2 mUCH-L1

4.1.2.1 BiHiVE – discovery

36 children in the discovery cohort had sufficient cord blood RNA remaining for analysis of mUCH-L1. There was a difference in expression of mUCH-L1 comparing controls (n=13) 0.71 (95% CI: 0.46-0.94), PA (n=15) 1.39 (95% CI: 0.62-1.73) and children with HIE (n=8) 1.23 (95% CI: 0.99-3.16), p=0.047. However, confidence intervals were overlapping and there was no biological gradient seen between PA and children with HIE,

nor within grades of HIE: mild HIE (n=4) 1.23 (95% CI: 1.14-1.63), moderate HIE (n=2) 0.78 (95% CI: 0.61-0.78) and severe HIE (n=2) 4.55 (3.63-4.55).

4.1.2.2 BiHiVE2 – validation

70 children in the validation cohort had measurable levels of mUCH-L1. The validation cohort was unable to validate any significant differences in expression of mUCH-L1 between controls (n=22) 0.75 (95 % CI: 0.61-1.32), PA (n=0.75) 0.75 (95% CI: 0.61-1.32) and children with HIE (n=8) 1.25 (95% CI: 1.15-1.96).

4.1.3 miRNA-181b /mUCH-L1

When comparing children with moderate-severe HIE with the remaining infants in both cohorts, a ratio of mRNA-181b/mUCH-L1 demonstrated similar results to what we had seen with miRNA-181b (Figure 2), with overlapping confidence intervals in the validation cohort.

4.1.3.1 BiHiVE – discovery

Moderate-severe HIE (n=4) 0.23 (95% CI: 0.06–0.44) vs all other children (n=30) 1.59 (95% CI: 0.46–2.54), p=0.01.

4.1.3.2 BiHiVE2 – validation

Moderate HIE (n=8) 0.41 (95% CI: 0.10–0.81) vs all other children (n=56) 1.38 (95% CI: 0.59–2.56), p=0.009.

4.2 STUDY II

The aim of this study was to evaluate if the addition of an automated seizure detection algorithm (ANSeR) could improve diagnostic accuracy in identification of neonatal seizures.

To each arm of the study, 132 infants were recruited. Six children were excluded, leaving 128 in the algorithm group and 130 in the non-algorithm group.

A comparison of detection of infants with seizures and of seizure hours is presented in Table 5. There was no significant difference in clinical detection of neonates with seizures. There was a non-significant difference in rate of false detection of infants with seizures with a higher rate in the algorithm-group, 36.6% (95% CI: 22.7-52.1) compared to 22.7% (95% CI: 11.6-35.9) in the non-algorithm group. There was a higher rate of seizure hours detected by the clinical team with the ANSeR-algorithm, with a difference in sensitivity of 20.8 percentage points (95% CI: 3.6 to 37.1) visualized in Figure 3. There was a non-significant trend towards more anti-seizure medication (ASM) being administered without seizures during the preceding 1-2 hours in the algorithm group (Table 5).

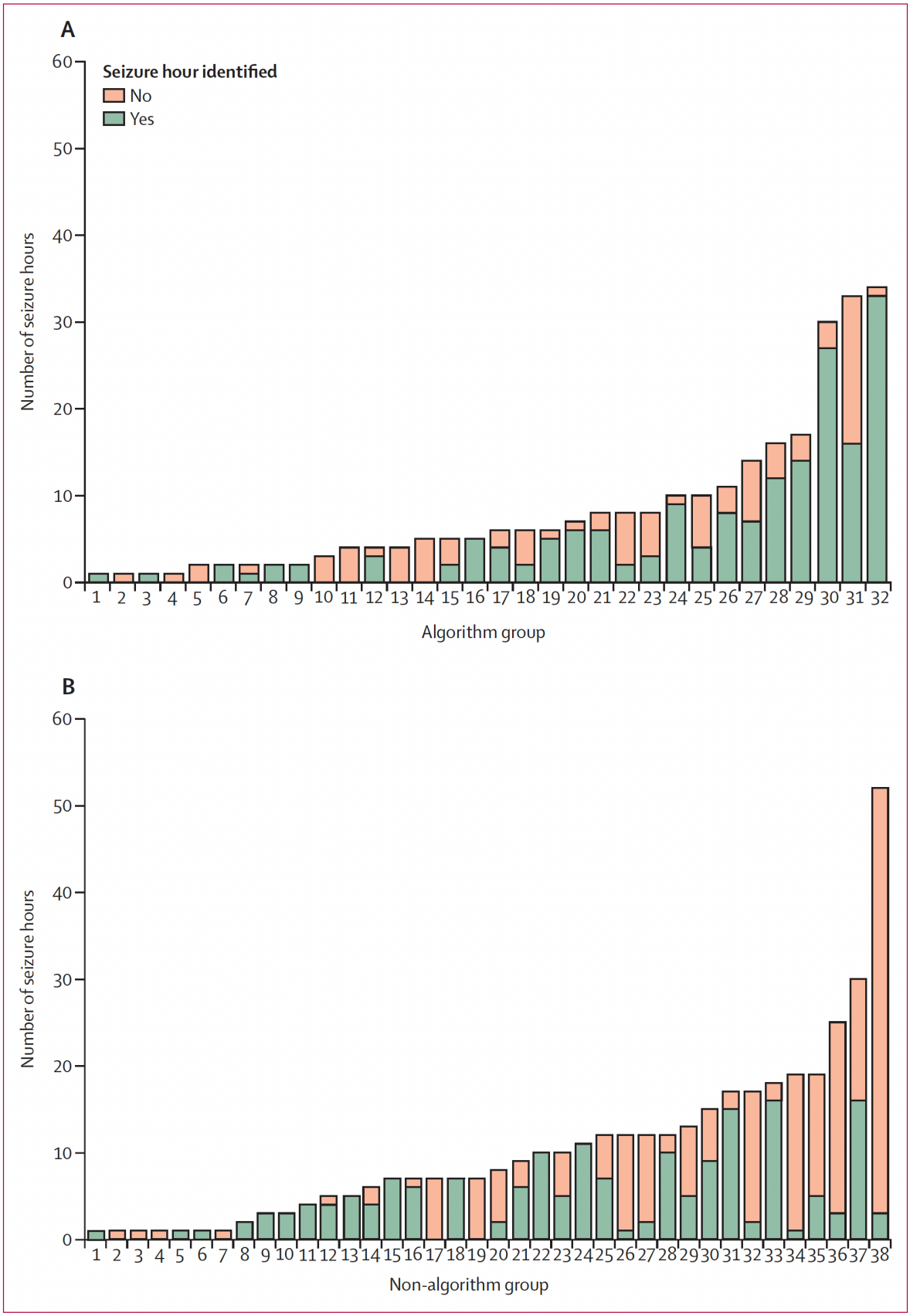

Figure 3 Detected (green) and undetected (orange) seizure hours per patient in A) ANSeR algorithm group and B) Non-algorithm group. From Pavel et al with permission [186].

	ANSeR	Non-algorithm	Difference, %-points
Neonates with seizures	32	38	
Sensitivity, % (95% CI)[a]	81.3 (66.7 to 93.3)	89.5 (78.4 to 97.5)	-8.2 (-25.0 to 7.7)
Specificity, % (95% CI)[b]	84.4 (76.9 to 91.0)	89.1 (82.5 to 94.7)	-4.8 (-14.1 to 4.6)
Individual seizure hours	268	391	
Sensitivity, % (95% CI)[c]	66.0 (53.8 to 77.3)	45.3 (34.5 to 58.3)	20.8 (3.6 to 37.1)
ASM administered without seizure, n (%)	10 (10.4)	4 (4.3)	6.1 (-0.3 to 13.5)

Abbreviations: ASM, Anti-Seizure Medication

[a]Percentage of seizing neonates correctly classified by clinical team.

[b]Percentage of non-seizing neonates correctly classified by clinical team.

[c]Percentage of seizure hours correctly classified by clinical team.

Table 5 Comparison between ANSeR and Non-algorithm for detection of neonates with seizures and hours with seizures. Adjusted from Pavel et al [186].

An effect modification was seen on weekends compared with weekdays with a significantly higher sensitivity for seizure hours seen during the weekend. There was no significant interaction seen between time of day (08.00-20.00 vs 20.00-08.00) and intervention group.

4.3 STUDY III

The aim of Study III was to assess neurocognitive outcome of children with mild HIE compared to healthy controls.

Of 741 children recruited to all four cohorts, 51 children were excluded. Of the remaining 690 eligible for the study, 471 children had available outcome data, of whom 449 had completed the BSITD-III cognitive scale. The children were grouped into five patient groups: healthy controls (n=152), PA (n=185), mild HIE (n=55), moderate HIE (n=56) and severe HIE (n=23). Country of birth varied significantly over patient groups. Mean cognitive composite scores for all patient groups are listed in Table 6.

Cognitive composite scores	*n*	Mean (SD)
Healthy Controls	152	103.6 (14.6)
PA	185	102.6 (15.7)
Mild HIE*	55	97.6 (11.9)
Moderate HIE	53	98.4 (18.1)
Severe HIE*	12	88.3 (19.0)

* Significantly different from healthy controls ($p<0.05$) using linear regression with robust estimates of standard errors.

Table 6 BSITD-III cognitive composite scores at two years of age. Adapted from Finder et al [187].

Mean cognitive composite score in children with mild HIE was 6 points lower than in healthy controls (95% CI, -9.9 to -2.1), with a Cohen *d* effect size of -0.43 (95% CI, -0.12 to -0.74). Adjusted for country of birth, the difference remained significant, but was less than 5 points: -4.9 (95% CI, -8.7 to -1.2). There was a non-significant difference between children with mild HIE and children with moderate HIE with -2.2 points in the mild HIE group (95% CI: -8.1 to 3.7).

We performed a stepwise regression, including country of birth, maternal age, maternal work status, maternal tertiary education, sex, and birth weight, keeping independent variables of $p<0.2$ for a multivariable regression analysis. 392 children had complete data and were included in the analysis. There was a significant difference in mean cognitive composite scores where children with mild HIE had 5.2 points lower scores than healthy controls (95% CI: -9.1 to -1.3). Differences in composite scores are explored in Table 7. After multiple imputation of missing covariates, the difference in mean cognitive composite score in the multivariable model was -4.5 (95% CI: -8.3 to -0.7). The distribution of cognitive composite scores in children with mild HIE not treated with therapeutic hypothermia compared to that of the healthy controls is visualized in Figure 4.

	Differences in composite scores (95% CI)[a]		
	Mean difference	Adjusted[b]	Multivariable model[c,d]
Cognitive Composite Score	-6.0 (-9.9 to -2.1)	-4.9 (-8.7 to -1.2)	-5.2 (-9.1 to -1.3)
Language Composite Score	-2.7 (-8.5 to 3.1)	-4.7 (-10.7 to 1.3)	-5.1 (-11.8 to 1.5)
Motor Composite Score	-1.4 (-6.4 to 3.7)	-2.1 (-7.4 to 3.1)	-2.1 (-7.2 to 2.9)

[a] Differences in means and *p*-values were obtained from linear regression analysis with robust estimates of SE.
[b] Adjusted for country of birth.
[c] Adjusted for country of birth, maternal work status, birth weight, sex.
[d] Complete data available for 374 children.

Table 7 Difference in BSITD-III scores in children with Mild HIE compared to healthy controls. Adapted from Finder et al [187].

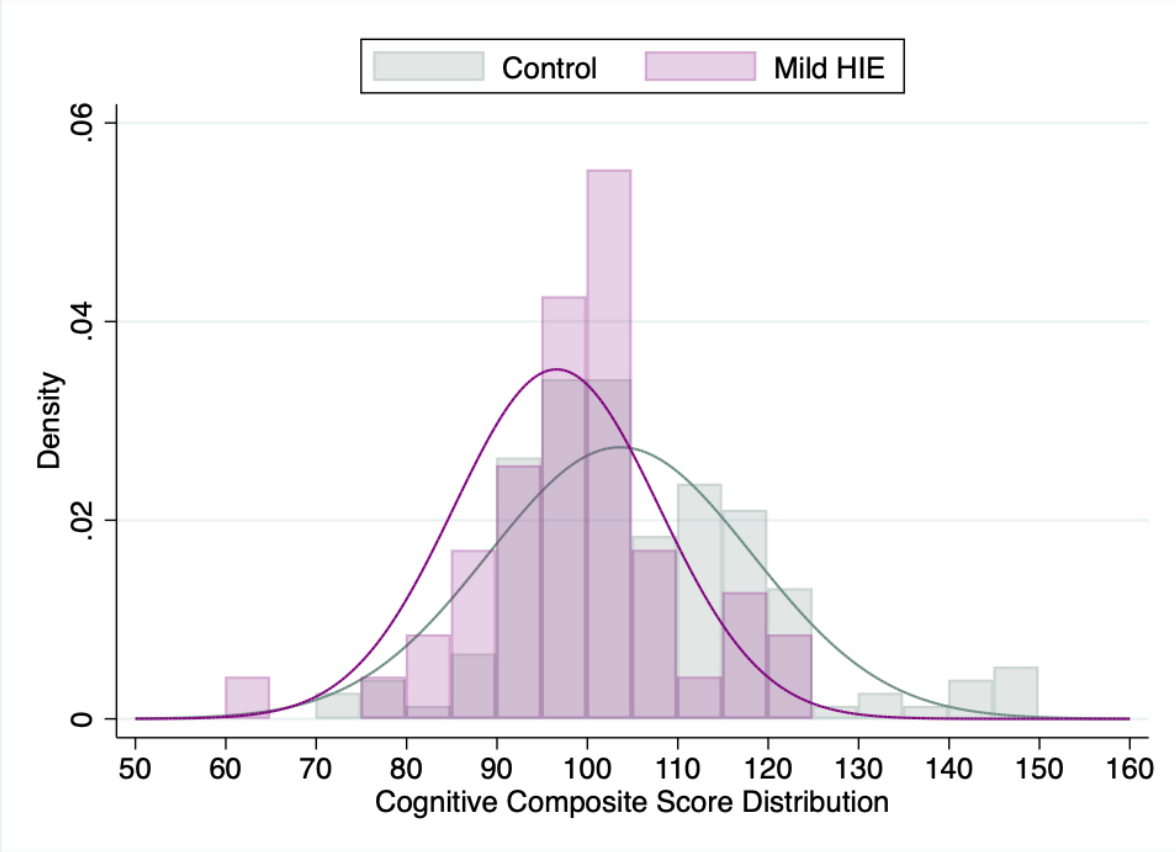

Figure 4: Cognitive Composite Scores of Children with Mild HIE not treated with Therapeutic Hypothermia and of Healthy Controls.

4.4 STUDY IV

Between January 2007 and December 2009, 66 children were treated with therapeutic hypothermia in Stockholm, Sweden. One child was excluded due to a genetic disorder. Of the remaining 65, 4 (6%) were graded as mild HIE, 49 (75%) as moderate HIE, and 12 (18%) as severe HIE. 44 (68%) children were assessed with both BSITD-III and WISC-IV.

Pairwise correlations between all subscales of BSITD-III and WISC-IV had very weak to moderate strength with the strongest correlation seen between BSITD-III cognitive composite score and WISC-IV working memory. A mixed effects regression model comparing BSITD-III cognitive composite score and WISC-IV FSIQ demonstrated a non-significant increase in score of 3.41 (95% CI: -0.86 to 7.69). The regression residuals had a correlation of 48% (95% CI: 22 to 74).

There was a significant decrease in odds for a poor outcome with every increase of BSITD-III composite scores. BSITD-III can work as a predictor of poor outcome excluding cerebral palsy (that was always diagnosed before BSITD-III-assessment) with an area under the ROC-curve (AUROC) of 0.76 (95% CI: 0.59 to 0.93) for the cognitive composite score, 0.76 (95% CI: 0.85 to 0.94) for the language composite score and 0.81 (95% CI: 0.63 to 0.96) for the motor composite score. Predictive ability for different cut-offs for BSITD-III is presented in Table 8.

BSITD-composite scores	Cut-off	Sensitivity	Specificity	Correctly Classified
Cognitive composite score	75	0.38	1.00	83%
AUROC = 0.76 (95 % CI 0.59 to 0.93)	90	0.46	0.91	79%
	95	0.69	0.71	71%
Language composite score	77	0.46	0.97	85%
AUROC = 0.76 (95% CI 0.85 to 0.94)	83	0.46	0.89	83%
	86	0.54	0.80	79%
Motor composite score	82	0.38	0.97	80%
AUROC = 0.81 (95% CI 0.67 to 0.96)	85	0.38	0.94	77%
	88	0.62	0.88	80%

Table 8 Ability of each BSITD-III composite score to predict a poor outcome per cut-off.

5 DISCUSSION

5.1 MAIN FINDINGS AND INTERPRETATIONS

5.1.1 miRNA-181b and mUCH-L1 as Biomarkers in HIE

We have shown that miRNA-181b shows potential as a predictor of moderate-severe HIE in children exposed to perinatal asphyxia and have validated the findings in a separate multicenter validation cohort. In a later study [170], we grouped children with measured levels of miRNA-181b from both cohorts (BiHiVE and BiHiVE2) into one group and showed that an RQ-level of 0.492 generated a sensitivity of 79% and specificity of 60% with an AUROC of 0.752 (95% CI: 0.61 to 0.89). Compared with current clinical biomarkers such as pH, lactate, base deficit or Apgar score, no improvement was seen with the addition of miRNA-181b. In a piglet model of HI injury, similar to our findings, altered miRNA-181b was found to be specific to moderate-severe HIE [188].

Levels of mUCH-L1 were significantly higher in children who developed HIE, but that difference was mainly caused by high levels in children with severe HIE. Other studies have also pointed out that infants with severe HIE differ in biomarker expression [189, 190] and response to treatment [87]. We were unable to demonstrate differences of mUCH-L1 between grades of HIE similar to the differences in serum UCH-L1 that have been demonstrated in other studies [116, 118, 191].

5.1.2 Algorithm for Neonatal Seizure Recognition

In this multicenter RCT of the ANSeR seizure algorithm compared with standard EEG monitoring, we were unable to demonstrate a significant difference in sensitivity of infants with seizures. Surprisingly, the point estimate of sensitivity was 8.2 percentage points lower (95% CI: -25.0 to 7.7) in the algorithm group, although with wide confidence intervals including zero. The lower point estimate of sensitivity in the ANSeR algorithm group could be due to random error (5.2.1) demonstrated by wide confidence intervals. All recruiting centers were experienced with multichannel EEG monitoring, which could explain the high sensitivity in the control group. It is also possible that the addition of an automated seizure detection algorithm can lower tendencies to review the EEG for seizures if there has been no alarm from the algorithm. The algorithm did however sound an alarm for four of the six neonates that were not identified as seizure infants, but the clinical team did not react for unknown reasons, most likely due to short seizure durations.

Detection of hours with seizures was significantly higher in the algorithm group with a difference in sensitivity of 20.8 percentage points (95% CI: 3.6 to 37.1). This was below the threshold set at the sample size calculation but can still be argued to be of clinical importance, as increased seizure burden is associated with abnormal outcome at 24-48 months of age [54]. The difference in sensitivity was higher during weekends (36.6 percentage points, 95% CI: 4.4 to 64.3) in comparison with weekdays (16.6 percentage points, 95% CI: 0.1 to 32.3). This could reflect on differences in experience or staff density

on weekdays. There is reason to suspect that the lower fraction of correctly identified seizure hours, as opposed to correctly identified neonates with seizures, in the non-algorithm group can be due to electro-clinical uncoupling [192]. After identification of an early seizure and administration of ASM and subsequent cessation of clinical symptoms, electrographic-only seizures can be more difficult to detect by less experienced interpreters [77]. However, this was not studied under the scope of Study II.

5.1.3 Cognitive Outcomes in Children with Mild HIE and Predictive Ability of BSITD-III

In Study III, we have demonstrated that children with mild HIE at two years of age have lower BSITD-III cognitive composite scores than a healthy control group, still within normal ranges, similar to the findings of Robertson and Finer from 1985 [39] and other studies adding to the mounting evidence that mild HIE might result in neurocognitive impairment [37, 38, 159, 162-164, 166, 167, 193]. Our findings remained significant after adjustment for potential confounders. We found no significant difference in mean cognitive composite scores comparing non-cooled children with a history mild HIE with children treated with therapeutic hypothermia for moderate HIE.

In Study IV, we assessed the ability of BSITD-III to predict neurocognitive impairments at school age. We saw decreased odds of a poor outcome with every increase of BSITD-composite score. Similar to the results of Månsson et al in healthy term children [148], we found very weak to moderate positive linear correlations between BSITD-III and WISC-IV composite scores. Our findings from Study III support the growing evidence that children with mild HIE do not follow the same developmental trajectory as their peers. However, the findings from Study IV suggest that longer follow-up is needed to assess school-age difficulties, since assessments at two years of age do not have strong correlations with later cognitive performance. In a recent meta-analysis of neurodevelopmental outcomes in hypoglycemia, no association was seen in early childhood between exposure to hypoglycemia and neurodevelopmental impairment. However, in mid-childhood there was a significant increased risk of neurodevelopmental impairment (OR 3.62, 95% CI: 1.05-12.42) [194], which implies that subtle disabilities after a cerebral insult might become more apparent with age [43, 149, 195].

A problem with comparison of results of outcome in mild HIE, or other grades of HIE for that matter, is that the definitions of exposure vary between studies. In the original Sarnat classification from 1976, EEG patterns were part of the classification and seizures did not occur in stage 1 encephalopathy [34]. In some large, well cited studies, such as the NICHD study of therapeutic hypothermia [88] and Robertson and Finer's assessment of cognitive outcome after a history of HIE [39], aEEG and occurrence of seizures were not part of the classification of grades of encephalopathy, why many studies allow occurrence of seizures in mild HIE. Also, studies of mild HIE have included seizures as outcome and not part of the exposure [162, 164, 166, 167, 193]. The PRIME study even included early abnormalities on EEG <9h of age as a short term outcome and not part of the HIE grading

[166], while the TOBY- and the CoolCap studies instead used similar aEEG criteria for stratification of HIE severity [87, 196]. Walsh et al performed a secondary analysis of their data and reclassified all children with mild HIE and seizure activity into the moderate HIE category, which did not alter their results [164]. In Study III and IV, any infant with clinical or electrographic seizures would have been graded as having moderate HIE, why children with a history of mild HIE in Study III and IV can be expected to have suffered from a less severe injury than, for example, those in the PRIME study.

To the author's knowledge, only one study has investigated predictive value of BSITD in a cohort treated with therapeutic hypothermia. Among the children included in the NICHD trial of therapeutic hypothermia, the predictive value of BSITD-II was assessed comparing BSITD-II with Wechsler Preschool and Primary Scale of Intelligence, 3rd edition (WPPSI-III) [197] for English speaking children and WISC-IV for Spanish speaking children seen beyond 7 years and 3,5 months of age. BSITD-II scores and WPPSI/WISC scores were significantly correlated, but by rank. Comparing BSITD-II MDI of 70-84 vs >84, there was a non-significant difference in FSIQ of -5.6 (95% CI: -12.4 to 1.3), but children with MDI <70 had on average 42 points lower FSIQ than those with an MDI of >84 (95% CI: -49.3 to -35.0). Predictive values were higher among children treated with normothermia than with hypothermia [198]. Predictive value of moderate or severe CP at 6-7 years of age for children with a diagnosis of CP at 18 months of age had an almost perfect sensitivity and specificity for both groups [179]. However, CP is almost exclusively diagnosed before BSITD assessment, why we in Study IV excluded CP as part of a poor outcome after BSITD-III had been performed.

5.2 METHODOLOGICAL CONSIDERATIONS

In Study I, the ambition was to recruit all children with signs of perinatal asphyxia during the study period to have a representative sample of cases. However, samples of umbilical cord blood were drawn before recruitment. Cases from whom we were unable to draw samples were never recruited. These infants might have been sicker than the ones we did recruit since a stressful delivery or severe hemorrhage might reduce the chance of successful umbilical cord blood collection. As we have not collected data on these infants, we are unable to analyze this potential selection bias.

In Study II, seizures were not independent events, but rather hierarchically clustered 1) within each infant with seizures and 2) within each hospital of randomization. To take dependency of data into account, a bootstrap approach was performed where infants were divided into 16 clusters based on allocation and hospital. For each iteration out of 100.000 performed, a bootstrap sample of infants from each cluster was generated through random sampling with replacement. Bootstrap samples where combined and sensitivity was calculated for the two allocation groups. An alternative approach would have been to perform a logistic regression with generalized estimating equations (GEE) that takes dependency of data into account. This would provide the mean and 95% CI of the logit, which could be back transformed into sensitivity scale [199].

Unfortunately, data from MRI and ultrasound exams were not available on children with mild HIE, why this was not included in our analysis in Study III. At the two tertiary centers, Karolinska University Hospital and Cork University Maternity Hospital, the decision of neuromonitoring and neuroimaging is guided by the clinical grade of HIE and children with mild HIE are not routinely examined with neuroimaging.

5.2.1 Random Errors

In epidemiologic studies, researchers categorize errors into random errors and systematic errors (5.2.2). Random errors consist of variability in data and sampling that cannot be explained. By increasing sample size, the risk of random errors is reduced, demonstrated statistically with a reduction of confidence intervals [200]. In Study I, III and IV, no sample size calculation was performed. HIE being a relatively rare occurrence [3-7] with more severe cases being even more rare, we were unable to recruit any severe cases of HIE during the BiHiVE2 study period, thus being unable to validate any results on that patient group from BiHiVE.

In Study II, being an RCT, any imbalances, or confounding (5.2.2.3), between the two exposure groups (algorithm vs non-algorithm) would be determined by the random assignment. With increasing sample size, imbalances by randomization will be reduced. Confounding by random assignment in an RCT will not replicate with a replicated study, even using the same participants, as there will be a new assignment by randomization. Thereby, confounding in an experimental study is an example of random error rather than systematic error [200].

5.2.2 Systematic Errors

Errors that are independent of sample size are systematic errors, also known as biases. Systematic biases will replicate if the study is replicated [200].

5.2.2.1 Selection Bias

Loss to follow-up is always a potential cause of selection bias in any longitudinal study Since the association of exposure and outcome in non-participants is unknown, we can only infer, rather than observe, selection bias [200].

In Study III, we were concerned that missing data could introduce bias. The loss to follow-up was 32% and in our multivariate model, data was missing in 14% of the surviving children. The PRIME study, with neurodevelopmental testing at 18-24 months, had drop-outs at a comparable rate [167]. To assess potential selection bias caused by loss to follow-up, we analyzed differences in maternal work status and tertiary education between children with available vs missing outcome data in the healthy control and mild HIE groups. There was no significant difference between missing and non-missing regarding maternal work status. There was however a significant difference in the maternal tertiary education profile between healthy controls who returned for follow-up compared to those who were lost to follow-up. The proportion of tertiary education was 60% in controls group and 73% in

children with mild HIE returning for follow-up. However, the difference in mean cognitive composite score between children with mild HIE and healthy controls remained significant after post-hoc adjustment for maternal tertiary education.

Eight children diagnosed with mild HIE were treated with therapeutic hypothermia. None of the centers had a policy of cooling children with mild HIE at the time of recruitment. Cooling of children with mild HIE was made inadvertently as it was in the original trials. As Oliviera et al has shown, there has been significant therapeutic drift in many centers, where many infants with mild are being offered therapeutic hypothermia [168, 201].

Study IV was a population-based study, where all cases of children treated with therapeutic hypothermia in Stockholm 2007-2009 were included. Thus, the risk of introducing selection bias at recruitment was minimal. However, 77% of all eligible surviving participants were assessed with both BSITD-III and WISC-IV. There is a possibility that the estimate of correlation between two assessments of neurocognitive outcome can been affected by loss to follow-up.

5.2.2.2 Information Bias

If bias is introduced by erroneous data from study subjects, such bias is referred to as information bias [200].

In Study I, we used miRNA-223 and 18S as reference genes. Reference genes or "housekeeping genes" as an internal control is the most common method for normalization of miRNA data as RNA can be degraded and bias the results. RNA molecules such as RNU6B, 18S, and miR-16 have previously been used to normalize target serum miRNA expression data [174], but there is no consensus on reference miRNAs for qRT-PCR [174, 202].

Our study group has focused on whole blood miRNA [170], instead of serum or plasma [130]. With this method, altered expression in plasma or serum may be masked by the abundance of intracellular miRNA [203]. Our group has previously demonstrated a significant downregulation of whole blood miRNA-374a in cord blood of infants with HIE compared to PA or healthy controls [173]. In contrast, Garberg et al demonstrated a relative upregulation of plasma miRNA-374a in a piglet model [130]. Much more research is needed in the field to establish comparable methods and understanding of biological function of these miRNA signals.

In Study I, we measured whole blood mUCH-L1 as a proxy of serum UCH-L1 levels. Earlier attempts to measure UCH-L1 protein levels had failed at an external lab, why mUCH-L1 was measured as a proxy in this study. This method has also been used by Lingam et al in an exploratory piglet model of inflammation sensitized hypoxic-ischemic injury [204]. Both our study and the aforementioned study suffer from the limitation that one cannot infer mechanisms of injury from transcript levels, as they are by themselves not

sufficient to predict protein levels [205], but have to be regarded as separate biomarkers altogether.

For both Study III and IV, different assessors administered BSITD-III and WISC-IV. Data was not available on individual assessor performance, which is of course a risk for both information bias and confounding (5.2.2.3). To minimize the potential of information bias and confounding caused by differences in performance, all assessors had been trained in administration of BSITD-III and WISC-IV prior to performing any actual study assessments. In BiHiVE, BiHiVE2 and ANSeR1, they were blinded to the medical history of the patients. However, this was not the case in the NeoCool cohort, where 4 children with mild HIE were included. In Study III, country of birth was used as a proxy for assessor performance.

5.2.2.3 Confounding

If the effect of the exposure is caused by or mixed with the effect of another variable, bias is introduced. This "confusion of effects" is called confounding [200].

In Study I, we were not investigating an effect, but miRNA-181b and mUCH-L1 as biomarkers for an outcome, why confounding would not be the primary bias to expect in such a study.

In Study II, being a randomized controlled trial, the potential confounding effects seen by random allocation should be considered a random error (5.2.1), but potential confounding is still presented under the confounding section. In Study II, the non-algorithm group did have a higher amount of seizure hours (391 vs 268), which could influence the detection rate. In a previous study, low seizure burden increased risk of not recognizing an infant with seizures [206]. There were also some differences regarding the underlying conditions causing seizures with almost twice as many children with severe HIE in the algorithm group, which also could influence detection rates.

In Study III, all adjusted analyses were adjusted for country of birth, as there was a large potential for introducing bias by any cultural, demographical, educational or language differences between Swedish and Irish children. We did not have data available for what time of day the tests were performed in either Study III or IV, which can have an effect on test performance [207].

In Study III, among the Swedish healthy controls, there were more children in the upper ranges of cognitive composite scores. This can be due to demographic factors or differences in psychologist evaluations, parenting factors, socioeconomic factors etc. Within the Cork data, where these high scoring children did not occur, the difference remained significant between children with mild HIE and healthy controls -5.11 (95 % CI: -9.48 to -.73), which increased our confidence in our findings that the differences were not only driven by the upper ranges in scores.

5.2.3 Strengths

A systematic meta-analysis of more than 100 biomarkers in neonatal encephalopathy concluded that none of the biomarkers were studied sufficiently to recommend for clinical use. They concluded that to recommend implementation of biomarkers, alone or combined, validation in independent cohorts is warranted [208]. In Study I, we have studied miRNA-181b and mUCH-L1 in to separate cohorts that were recruited using identical recruitment criteria, which is quite unique in the field of biomarker studies in neonatal encephalopathy. Research investigating miRNA as a biomarker for HIE at birth is still quite novel. There are still only a few human neonatal studies that have investigated associations between grades of HIE and miRNA [209].

Study II is, to the author's knowledge, the first study to evaluate a seizure detection algorithm in a clinical setting in the NICU to evaluate the effect on both clinical recognition of seizures and treatment thereof.

Study III is one of only a few studies after the introduction of therapeutic hypothermia, investigating long-term outcome of mild HIE. The pooled cohort of children is the largest cohort to date that has assessed cognitive outcome in HIE across all grades of HIE, as well as in healthy controls and in PA without HIE.

Study IV is to the author's knowledge the first study to investigate correlations between BSITD-III and later assessments of IQ in school age in children treated with therapeutic hypothermia for HIE. We have used a well investigated and described cohort that has been followed from birth until the age of 10-12 years of age [210-212].

5.2.4 Generalizability

Generalizability or external validity, is the extent to which the results of a study holds true in other settings [213]. Therapeutic hypothermia is among the most evidence-based treatments used in the NICU, with several pre-clinical and clinical trials demonstrating efficacy [18, 85]. Recent studies from low- and middle-income countries, demonstrated that cooling is feasible, but with a significant increase of death among children treated with therapeutic hypothermia [214, 215]. In the HELIX trial, children with encephalopathy had a different phenotype than what is generally seen in high-income countries: clinical seizures debuted within a few hours of birth, MRI demonstrated a higher-than-expected rate of white matter injuries, and the mortality rate was much higher, even though inclusion criteria were less strict than the trials performed in high-income countries [215]. Study I, II and III are multicenter studies from tertiary centers in Europe, where findings are most likely applicable to infants and children in most high-income countries. The results from Study I and III are most likely not directly applicable to the HIE population seen in low- and middle-income countries. Study IV is a population-based study based on a Swedish population, using a Swedish translation of BSITD-III without Swedish norms [148], why generalizability to other countries is difficult to assess. Thus, the results from Study IV need to be replicated in other countries.

6 CONCLUSIONS

The overall aim of this thesis was to improve treatment of children with HIE through studies of early identification of infants in need of neuroprotective treatment, and to assess outcome in children with mild HIE.

- Umbilical cord blood miRNA-181b showed potential as a predictor of moderate-severe HIE. Levels of mUCH-L1 were significantly higher in children who developed HIE, but that difference was mainly caused by higher levels in children with severe HIE.
- The addition of an automated seizure detection algorithm (ANSeR) did not improve sensitivity in identification of infants with neonatal seizures in a clinical setting. We did however observe an increased sensitivity for individual seizure hours, with the largest difference in sensitivity observed during weekends.
- At two years of age, children with a history of mild HIE at birth had lower cognitive composite scores measured with BSITD-III than their healthy peers. The cognitive composite scores of children with mild HIE were not significantly different from that of survivors of moderate HIE treated with therapeutic hypothermia.
- BSITD-III might detect children at risk of later poor outcome, but is insufficient to predict neurocognitive trajectory and to rule out a later need for interventions. It is of great importance that studies of outcome in HIE, but also clinical follow-up, continue into school age.

7 POINTS OF PERSPECTIVE

HIE is a clinical condition with many different etiologies, neuropathological varieties, with a spectrum of symptoms, which has altogether been trichotomized into three grades of HIE. The grading scale suggested by Sarnat and Sarnat in 1976 continues to be used world-wide with different modifications [216] even though one of its creators recently suggested revision with addition of olfactory response [217]. Two Sarnat Score point systems have been suggested [218, 219] as alternative HIE grading systems. They show potential in predicting which children with mild HIE will develop an abnormal outcome. Both score systems show good agreement and were superior to their respective grading systems in predicting MRI injury. Seizures do not automatically define the grade as at least moderate in either of the scoring systems [220]. A score rather than stratification of an injury spectrum into categorical grades is appealing but needs studies across all grades of HIE and especially in discrimination between children with PA and with HIE.

With the HELIX trial, earlier onset of encephalopathic symptoms and higher than expected prevalence of white matter injuries were noted, which could imply a high rate of antepartum injuries. If that is the case, priorities of future research should not be on how to implement therapeutic hypothermia in low resource settings, but rather how to improve antenatal care and prevention of perinatal asphyxia.

With the introduction of therapeutic hypothermia, a major focus has been directed to early recognition of infants that may benefit from therapy. The search for biomarkers in HIE has been active for more than a decade [221]. Still, no single biomarker is sufficient to detect which neonates will develop significant HIE, predict response to treatment, or predict outcome. Machine learning algorithms may combine biomarkers to improve identification [222]. More studies are needed as we have just started studying the immense potential of machine learning.

The author would like to acknowledge planned studies of therapeutic hypothermia in children with mild HIE:

- COMET 1+2 (clinicaltrials.gov: NCT03409770), recruiting. An RCT of 140 infants with mild HIE with two phases:
    - Phase 1: comparing therpeutic hypothermia for 72 hours with “usual care” (temperatures not specified) in 60 infants. Outcome will be measured as MR imaging and spectroscopy at 4-14 days of age.
    - Phase 2: comparing rewarming at 48 hours of age vs 72 hours of age in 80 infants treated with therapeutic hypothermia. Outcome will be measured with MR imaging and spectroscopy at 4-14 days of age.
- COOLPRIME (clinicaltrials.gov: NCT04621279), not yet recruiting. A multicenter RCT of 460 infants with mild HIE randomized to therapeutic hypothermia 33.0-34.0°C vs normothermia 36.0-37.0°C. Normothermia will be achieved by usual

care. Outcome will be measured as neurodevelopmental outcome at age two years of age.

- TIME (clinicaltrials.gov: NCT04176471), not yet recruiting. An RCT of 68 infants with mild HIE randomized to therapeutic hypothermia 33.0-34.0°C vs normothermia 36.5-37.3°C, where both temperature intervals achieved by using a servo-controlled temperature regulating blanket. Outcome will be measured as neurodevelopmental outcome at age 12-14 months.

The results from the planned studies of therapeutic hypothermia in mild HIE are much anticipated. With studies and implementation of new treatments, we have in Study IV demonstrated the importance of school-age follow-up to assess long term benefit and differences in developmental trajectories. Adult outcomes are yet to be studied.

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