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Serum Neuron-Specific Enolase as a prognostic predictor in newborns with Hypoxic-Ischemic Encephalopathy

Bachelor's thesis in Medicine

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Abbreviations

aEEG	amplitude-integrated electroencephalogram
ALT	alanine aminotransferase
aPTT	activated partial thromboplastin time
AST	aspartate aminotransferase
AUC	area under the ROC curve
BBB	blood-brain barrier
BD	base deficit
BGP	background pattern
BGT	basal ganglia and thalamus
BNP	B-type natriuretic peptide
BP	blood pressure
BS	burst suppression
BSID-III	Bayley Scales of Infant and Toddler Development, Third Edition
BUN	blood urea nitrogen
CBC	complete blood count
CBF	cerebral blood flow
CEIC	Comitè d'Ètica d'Investigació Clínica
CFM	cerebral function monitoring
CLV	continuous low voltage
CNS	central nervous system
CNV	continuous normal voltage
CO_2	carbon dioxide
CRP	C-reactive protein
CSF	cerebrospinal fluid
DALY	Disability-Adjusted Life Years
DC	discontinuous
DOR	diagnostic odds ratio
DWI	diffusion-weighted imaging
ECG	electrocardiogram
EEG	electroencephalogram
ESPNIC	European Society of Paediatric and Neonatal Intensive Care

FiO_2 fraction of inspired oxygen
FN false negative
FP false positive
FT flat tracing
GA gestational age
HIE hypoxic-ischemic encephalopathy
hol hours of life
HR heart rate
HSJD Hospital Sant Joan de Déu
HUGTP Hospital Universitari Germans Trias i Pujol
HUJ23 Hospital Universitari Joan XXIII
HUJT Hospital Universitari Josep Trueta
HUPT Hospital Universitari Parc Taulí
HUVH Hospital Universitari Vall d'Hebron
Idescat Institut d'Estadística de Catalunya
IDIBGI Institut d'Investigació Biomèdica de Girona
iNO inhaled nitric oxide
IQR interquartile range
LP lumbar puncture
LR likelihood ratio
MAP mean arterial pressure
MAS meconium aspiration syndrome
MRI magnetic resonance imaging
MRS magnetic resonance spectroscopy
NICU Neonatal Intensive Care Unit
NIRS near-infrared spectroscopy
NPV negative predictive value
NSE neuron-specific enolase
O_2 oxygen
pCO_2 partial pressure of CO_2
PLIC: posterior limb of the internal capsule
PN: parenteral nutrition
pO_2 partial pressure of O_2
PPHN persistent pulmonary hypertension of the newborn
PPV positive predictive value
PS100B S100B protein

PT	prothrombin time
REDCap	Research Electronic Data Capture
ROC	receiver operating characteristic
RR	respiratory rate
$rScO_2$	regional cerebral oxygen saturation
S	sensitivity
$SatO_2$	oxygen saturation
SCr	serum creatinine
SENEO	Sociedad Española de Neonatología
Sp	specificity
SVS	single voxel spectroscopy
SWC	sleep-wake cycling
TDS	total damage score
TH	therapeutic hypothermia
TN	true negative
TP	true positive
TW1	T1-weighted sequence
TW2	T2-weighted sequence
UCIN	Unitat de Cures Intensives Neonatals
US	ultrasound
WM	white matter
WMA	World Medical Association

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Abstract

TITLE: Serum Neuron-Specific Enolase as a prognostic predictor in newborns with Hypoxic-Ischemic Encephalopathy

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BACKGROUND: Hypoxic-ischemic encephalopathy (HIE) stands as an important cause of mortality and neurological morbidity in full-term newborns. While there are established techniques for HIE prognosis, such as clinical evaluation, amplitude-integrated electroencephalogram (aEEG), and magnetic resonance imaging (MRI), they are not clearly effective indicators for an early prognosis of neurodevelopment in newborns with HIE. Thus, this study aims to evaluate the predictive value of neuron-specific enolase (NSE), a biomarker of neuronal damage in the central nervous system (CNS), for the neurological outcomes in newborns with HIE.

OBJECTIVES: The main objective is to determine whether serum levels of NSE serve as a prognostic predictor for neurodevelopmental outcome in newborns with HIE.

DESIGN AND METHODS: Structured as a multicentric, prospective observational descriptive study, this is a prognostic tool accuracy study, which will be settled in the Neonatal Intensive Care Unit (NICU) of six hospitals of Catalunya. NSE serum values will be determined during the newborn's hospitalization period, and the obtained results will be compared to the neurodevelopmental outcome, assessed through the Bayley Scales of Infant and Toddler Development, Third Edition (BSID-III score) at 18 months from birth. The sensitivity of NSE serum values to predict a favorable or unfavorable outcome will be evaluated along with other measurements, such as specificity, positive and negative predictive values, likelihood ratio and diagnostic accuracy.
Also, NSE serum values will be associated with the well-established and routine prognostic techniques, including clinical assessment, aEEG and MRI.

STUDY PARTICIPANTS: 90 full-term newborns diagnosed with HIE within the first 6 hours of life will be consecutively recruited over an estimated period of 1 year from the NICU of Hospital Universitari Josep Trueta (HUJT), Hospital Sant Joan de Déu (HSJD), Hospital Universitari Vall d'Hebron (HUVH), Hospital Universitari Germans Trias i Pujol (HUGTP), Hospital Universitari Parc Taulí (HUPT) and Hospital Universitari Joan XXIII (HUJ23).

KEYWORDS: perinatal asphyxia; hypoxic-ischemic encephalopathy (HIE); Neuron-Specific-Enolase (NSE); neurodevelopmental outcome; prognostic predictor

Chapter 1

Introduction

1.1. Motivation

1.1.1. Incidence of HIE

The incidence of hypoxic-ischemic encephalopathy (HIE) varies significantly worldwide and depends on the resources available in different countries or geographic regions. The global incidence of HIE (mild, moderate, or severe) in developed countries ranges from 1 to 8 per 1,000 newborns. However, the rate depends on the type of study and especially on the operational definition of HIE [1,2].

A review that included 40 studies from 26 European and United States countries indicated an average incidence of 1.6 per 1,000 full-term newborns, with the proportion attributable to moderate-severe HIE accounting for 60% of the total cases [1]. In our country, the incidence is approximately 1 per 1,000 live newborns [3]. Regarding the distribution by autonomous communities, there is considerable homogeneity among them; it is similar across all of them [1].

1.1.2. Relevance of HIE

HIE stands as the primary cause of neonatal mortality in full-term newborns [1,2,4], leading to severe neurological morbidity in both full-term and nearly full-term newborns [5], as well as being a significant cause of neonatal convulsions. In a review of recent literature, it is noted that the overall rate of neonatal deaths attributable to HIE is approximately 16%, excluding deaths that occur within a few minutes or hours after birth [1].

Neonates with moderate HIE have a 3-10% risk of death, and among survivors, 20-45% will experience varying degrees of permanent disability. In contrast, newborns with severe HIE face a 50-75% risk of death, and nearly all survivors will have permanent disabilities [5,6]. HIE represents 20% of cerebral palsy cases in childhood and, following prematurity, is the second most significant neurological issue in terms of Disability-Adjusted Life Years (DALYs) estimated for childhood neurological problems [5].

The impact on juvenile health, as well as the consequences for families and society, along with economic and legal costs, define this condition as an important public health concern [3,7].

1.2. Asphyxia and hypoxic-ischemic encephalopathy

1.2.1. Definitions

Perinatal asphyxia is defined as a condition arising from a diminished oxygen supply (hypoxemia) and/or compromised blood perfusion (ischemia) during the perinatal period, occurring in close temporal proximity to labor (peripartum) and delivery (intrapartum). This asphyxia period manifests in the fetus or the newborn as hypoxemia, hypercapnia and lactic acidosis due to tissue hypoperfusion [5,8] and can lead to multi-organ failure, with brain involvement as the major organ of concern (referred to as hypoxic-ischemic encephalopathy) [2].

It should be considered in the case of every newborn with a history of non-reassuring fetal status, sentinel event or labor dystocia that requires advanced resuscitation during the birth period, and manifesting a 5-minute Apgar score ≤ 5 and/or umbilical cord pH ≤ 7.0 or base deficit (BD) ≥ 12 [1,2,5]. **Figure 1** below summarizes the criteria in order to establish a relationship between perinatal asphyxia and significant neurological sequelae. However, perinatal history alone does not establish a diagnosis; it merely defines a concerning or risky situation.

Non-reassuring fetal status (formerly referred to as *fetal distress* or *fetal suffe*ring) refers to the presence of abnormalities in the fetal heart rate monitoring and/or fetal acidosis.

Sentinel hypoxic event includes acute events around birth capable of causing neurological damage to an otherwise intact fetus. These events include premature placental detachment, uterine rupture, umbilical cord prolapse, amniotic fluid embolism, fetal exsanguination due to vasa previa, and fetal-maternal hemorrhage [2].

Hypoxic-ischemic encephalopathy (HIE) is defined as an acute central nervous system (CNS) dysfunction associated with a hypoxic-ischemic insult that is severe enough to damage the newborn's brain. HIE is typically attributable to intrapartum perinatal asphyxia in the first hours of life, either due to arterial hypoxemia, cerebral ischemia, or the combination of both situations [8]. It is characterized by difficulty in waking or maintaining wakefulness, difficulty in initiating or maintaining respiration, alteration of muscle tone and motor responses, reactivity and reflexes, feeding capacity, and often seizures [5,8]. The neurological examination allows establishing the presence or absence of acute encephalopathy [8]. In most cases of HIE, there is concomitant hypoxic-ischemic injury to other major organ systems, including the heart, kidney, lung, and/or liver [2].

Perinatal asphyxia as a cause of significant neurological sequelae.
Necessary criteria to establish this relationship

Essential criteria

1. Evidence of a intrapartum metabolic acidosis in fetal, umbilical arterial cord, or very early neonatal blood samples (pH < 7.0 and base deficit > 12 mmol/L)
2. Early onset of moderate to severe neonatal encephalopathy
3. Cerebral paralysis of the spastic quadriplegic or dyskinetic type

Criteria that together suggest an intrapartum timing but by themselves are non-specific

4. A sentinel hypoxic event occurring immediately before or during labor
5. A sudden, rapid, and sustained deterioration of the fetal heart rate pattern, usually after the hypoxic sentinel event where the pattern was previously normal
6. 5-minute Apgar score of 0-6
7. Early evidence of multisystem dysfunction
8. Early imaging evidence of acute cerebral abnormality

Figure 1. **Criteria to define an acute intrapartum hypoxic event**. Adapted from MacLennan A. A template for defining a causal relation between acute intrapartum events and cerebral palsy: international consensus statement. BMJ. 1999; 319(7216):1054–9 [6]

1.2.2. Physiopathological phases

Hypoxic-ischemic cerebral injury is a complex process initiated by an hypoxic-ischemic insult (due to any of the risk factors mentioned above) and continues during a recovery process, comprising sequential phases: *primary* or *acute phase*, *latent phase* and *secondary* or *delayed phase*. Furthermore, the newborn's brain is especially susceptible to hypoxic-ischemic insult because it has a high oxygen consumption, a greater amount of water, a lower concentration of antioxidants and less neuronal myelination, so that the damage is multifactorial, provoked by different mechanisms such as excitotoxicity, inflammatory response, oxidative stress, apoptosis, and subsequent neurogenesis [9].

Immediately after the insult, there is a reduction in oxidative metabolism, corresponding to the *primary phase* of the injury. However, just after reperfusion, there is a partial recovery of such metabolism. This period, known as the *latent phase*, corresponds to the "therapeutic window", which precedes the *secondary* or *delayed phase* of the injury. In this phase, a chain of several chemical, cellular and molecular reactions occurs, extending and worsening the damage over the following hours, leading to cerebral injury [10].

During the *latent phase*, the initiation of therapeutic intervention can prevent or slow down cerebral injury. The duration of this therapeutic window is not precisely defined, but it has been demonstrated to be no longer than 6-8 hours [8]. A crucial point is the temperature's impact on destructive processes. While hyperthermia increases early neurological deterioration, mortality and neurological morbidity, a temperature reduction of 3-4º initiated during the *reperfusion phase* or *latent phase* prevents or slows down cerebral injury. This is why therapeutic hypothermia is applied within the 6-hour window period as a neuroprotective treatment, which will be discussed later.

1.2.3. Temporal evolution during the first days

HIE is present from birth, with no period of time free from clinical symptoms. The evolving neurological profile in the first days or weeks allows differentiation between perinatal HIE and prenatal-origin encephalopathy. While the first one exhibits a dynamic or changing profile, the latter shows a stable one. Additionally, the temporal course is of great value to more accurately establish the prognosis.

In general, in mild to moderate HIE, the newborn may experience subtle neurological signs, including alterations in wakefulness and muscular tone abnormalities, and multifocal clonic seizures. However, there is a gradual clinical improvement after the first 72 hours of life.

In severe HIE, the newborn is stuporous or in a coma, intensely hypotonic, and may experience subtle and multifocal clonic seizures. Particularly between 24 and 72 hours of life, the ability to wake up seems to worsen, and often there is dysfunction of the brainstem, with some newborns showing signs of intracranial hypertension. It is during this critical period that the newborn typically succumbs. Those who survive undergo a gradual improvement in wakefulness, with the initial hypotonia progressing to dystonia or extensor hypertonia. A combination of bulbar and pseudobulbar paralysis may appear, causing feeding problems. The progression of neurological improvement is variable and difficult to predict, and it is believed that those who improve rapidly may have a better prognosis.

1.2.4. Multiorgan dysfunction

The initial physiological response to perinatal asphyxia involves the redistribution of blood flow from nonvital organs, such as the skin and splanchnic area, to vital organs like the brain and heart. In most infants with moderate to severe HIE, there is evidence of dysfunction in at least one other organ system. However, systemic effects of perinatal asphyxia may be present even in the absence of encephalopathy [2].

A significant portion of HIE patients manifests varying degrees of dysfunction across multiple organs and systems, representing a spectrum of alterations associated with the hypoxic-ischemic insult. This multiorganic dysfunction significantly impacts therapeutic management and can potentially exacerbate cerebral damage or neurological dysfunction.

The severity of HIE directly correlates with the extent of multiorganic involvement, with more severe cases affecting a greater number of organs and resulting in more pronounced dysfunction.

Principal organs or systems affected by multiorganic dysfunction in HIE patients, along with their clinical expression and corresponding control and treatment strategies are outlined below [2] and illustrated in **Figure 2.**

1) **Encephalopathy** or **HIE** is the primary concern following perinatal hypoxic-ischemic events.

2) **Respiratory failure** is common in severe cases, often resulting from underlying disorders such as sepsis, pneumonia, or meconium aspiration syndrome (MAS) [11]. Perinatal asphyxia is also associated with persistent pulmonary hypertension of the newborn (PPHN) [12–14]. After perinatal asphyxia, apnea or hypoventilation may occur due to HIE and seizures. Diagnostic tools such as chest radiography, pulse oximetry, and capillary blood gases are usually sufficient to assess pulmonary status.

3) **Cardiovascular manifestations** include reduced cardiac output and hypotension due to ventricular dysfunction and/or poor vascular tone [15]. Electrocardiogram (ECG) can demonstrate ischemic changes (like ST depression and T-wave inversion) and echocardiography may demonstrate ventricular dysfunction. Also, cardiac markers such as troponin and serum B-type natriuretic peptide (BNP) are often used to assess myocardial damage [16].

4) **Acute kidney injury** commonly presents as oliguria and is due to either reduced cardiac output or tubular necrosis. Elevated serum creatinine indicates impaired kidney function [12].

5) **Liver injury** is reflected in elevated liver enzymes and may manifest as direct hyperbilirubinemia, hypoalbuminemia, coagulopathy, and poor drug metabolism [12].

6) **Gastrointestinal manifestations** include feeding intolerance due to blood flow redistribution. Perinatal asphyxia increases the risk of necrotizing enterocolitis [17].

7) **Hematologic manifestations** include thrombocytopenia and abnormal coagulation due to disseminated intravascular coagulation, bone marrow suppression, and impaired hepatic production [18–20].

8) **Hypo**- and **hyperglycemia** are common, with initial stress-induced hyperglycemia followed by a sharp drop in blood glucose levels. Hypoglycemia is more frequent in severe cases of liver damage.

These multiorgan manifestations underscore the complex impact of perinatal asphyxia on various physiological systems, necessitating comprehensive monitoring and tailored therapeutic interventions.

Cardiovascular = 80% severe HIE = 30% moderate HIE	Respiratory = 90% severe HIE = 35% moderate HIE	Renal = 50% severe HIE = 20% moderate HIE	Gastrointestinal Hepatic	Coagulopathy = 30% severe HIE = 10% moderate HIE
Clinical spectrum				
· Hypotension · ↓ cardiac contractility · ↓ cardiac output · Bradycardia	· Respiratory failure – need for respiratory support · Persistent pulmonary hypertension (PPHT)	· ↓ diuresis/oliguria · Acute renal failure · Hyponatremia · SIADH · Tubular dysfunction	· Hypoglycemia · ↑ transaminases (>100 IU/L) · Gastric bleeding	· Thrombocytopenia · Coagulopathy · Active bleeding (especially in the lungs and GI tract)
Studies / Control				
· Troponin T and Troponin I and/or CK-MB (in blood) · ST segment depression and Q wave changes in the ECG · Blood pressure, $SatO_2$, pre- and postductal · Echocardiography: preload, contractility, afterload, cardiac output	· Monitoring: blood gases · Target: - pH > 7.3 - PCO_2 37.5-52.5 mmHg	· Fluid balance · Cystatin C · Creatinine · Renal ultrasound · Urine: - Urinalysis - Urinary ionogram - Density/osmolality - $\beta_2$mg/NAG	· Blood-tinged gastric residues · Gastric pH · Blood glucose · ALT and AST · LDH · Prothrombin · Albumin	· APTT · Prothrombin time · INR · Fibrinogen · Platelets · D-dimer
Treatment				
· Adrenaline · Dobutamine · Dopamine · Milrinone	· If PPTH: - Milrinone - Sildenafil · Nitric oxide (NO)	· If replacement therapy is needed for acute renal failure: peritoneal dialysis	· If necessary: administration of vit K, albumin infusion, or fresh frozen plasma	· Fresh frozen plasma if DIC · Cryoprecipitate (hypofibrinogenemia) · Platelets

Figure 2. **Multiorgan dysfunction in HIE**. Adapted from García-Alix A., Arnáez, J., Neurología Neonatal de un Vistazo, Ed Cabeza de Chorlito; 2022 [21]

1.2.5. Gravity scale

Various grading schemes have been designed to classify the gravity of HIE into different stages. These schemes reflect the fact that the greater the impairment of wakefulness and the ability to wake, the more severe the encephalopathy.

The gravity scale for hypoxic-ischemic encephalopathy is essential for a comprehensive evaluation and must be conducted as outlined below:

1) **Observation**: carefully observe the baby over an extended period, paying close attention to wakefulness state, position, spontaneous motor activity, shaking, or trembling.

2) **Stimulation**: if the baby is not awake, gently and gradually stimulate the baby to assess awakening capacity and determine the seconds the baby remains alert, paying close attention to motor activity (spontaneous and induced by stimulus), ocular opening and sobbing.

3) **Gravity scale utilization**: employ a gravity scale for HIE evaluation, either through categorical grading (such as the Sarnat severity scale, refer to **Table 1**, which is the most common approximation, although new, more comprehensive scales are being developed, see **Annex 1** for more detail), or by employing numerical gravity scales that stratify neurological dysfunction in points (see **Annex 1** for more detail).

Gravity			Mild	Moderate	Severe
Clinical signs	Alertness		Normal or hyperalert or irritable	Lethargy	Stupor or coma
	Muscular tone or position		Normal	Hypotonic	Flaccid
	Spontaneous activity		Normal or hiperexcitability	Diminished	Mostly absent or stereotyped
	Position		Slight distal flexion	Strong distal flexion	Decortication or decerebration
	Myotatic reflexes		Exaggerated	Exaggerated	Diminished or mostly absent
	Primitive reflexes	Moro	Normal or exaggerated	Diminished or incomplete	Absent
		Suction	Diminished	Diminished or absent	Absent
	Motor response		Normal or slightly diminished	Diminished, normal quality	
	Pupils		Mydriasis or reactive	Miosis	Asymmetrical, fix or dilated Poor luminescent reflex
	Clinical seizures		Absent	Frequent	Repetitive seizures or status epilepticus
aEEG items	Baseline tracing		Normal	Moderately altered	Seriously altered
Duration			< 24 hours	2 to 14 days	Hours to weeks

Table 1. **Sarnat severity scale for HIE** [22]. Adapted from Sarnat HB, Sarnat MS. Neonatal encephalopathy following fetal distress. A clinical and electroencephalographic study. Arch Neurol. 1976; 33(10):696–705..
*The severity degree of encephalopathy will be determined when the number of items is greater than three. If the number of items is the same for two degrees of encephalopathy, the severity will be defined by the level of alertness.

Adhering to this systematic approach ensures a comprehensive evaluation of the severity of HIE, allowing for precise categorization and, if needed, a more detailed stratification using points on a scale. This method is critical for accurate assessment and optimal management of infants with HIE.

The clinical characterization of the gravity of HIE serves as a sensitive indicator of the severity of the insult to the CNS and is of significant prognostic utility in the early days of life, as it closely correlates with the likelihood of neurological sequelae, such as cerebral palsy, seizures, hearing loss, blindness, learning disabilities, and behavioral disabilities [23].

1.2.6. Management and treatment

Clinical stabilization and evaluation

The aim of an initial evaluation during stabilization is to assess the extent of damage to vital organs, identify potential causes or associated conditions requiring specific treatment, such as therapeutic hypothermia, and establish a baseline for tracking changes in organ function over time. This comprehensive assessment is based on physical examinations, laboratory analyses, imaging studies, and monitoring of brain function [2].

The general evaluation involves assessing the newborn's respiratory condition (identifying signs of respiratory distress such as labored breathing or tachypnea and determining the need for respiratory support), cardiac condition (such as hypotension or the need for inotropic support to maintain blood pressure), neurological condition (detecting hypotonia and the presence of seizures), and basic laboratory analysis. Additionally, urinary output is closely observed.

Routine laboratory tests for neonates with perinatal asphyxia encompass the following [2]:

1) **Blood gas analysis**: arterial or venous blood gases are analyzed to assess gas exchange and identify acid-base abnormalities. Therapeutic hypothermia is considered if umbilical cord pH ≤ 7.0 or base deficit ≥ 16 mmol/L, along with evidence of hypoxic-ischemic encephalopathy.

2) **Cranial ultrasound** (US): performed to exclude subdural or intraventricular hemorrhage. Daily imaging with cranial US helps detect ischemic lesions, particularly in the deep gray matter, which may not be visible within the first 24 hours after the insult.

3) **Sepsis evaluation**: blood cultures are performed in all newborns with perinatal asphyxia due to the increased risk of serious infections (sepsis) [24].

4) **Respiratory evaluation**: in newborns with respiratory distress, chest radiography is performed to identify concomitant conditions such as pneumonia, MAS, pulmonary congestion, or PPHN.

5) **Cardiac evaluation**: ECG is conducted to identify myocardial ischemia. For newborns with evidence of cardiac injury (such as hypotension, need for inotropic support or abnormal ECG), echocardiography, troponin and serum BNP may be conducted.

6) **Kidney function studies**: kidney tests include serum creatinine (SCr), blood urea nitrogen (BUN), and electrolytes to detect acute kidney injury and associated electrolyte abnormalities.

7) **Liver function studies**: liver tests include total and conjugated bilirubin, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST). High levels of AST and ALT, together with hyperbilirubinemia, suggest hepatic damage associated with perinatal asphyxia.

8) **Complete blood count** (CBC): to identify anemia (which may have contributed to asphyxia), thrombocytopenia (increased bleeding risk), and elevated white count (suggestive of infection).

9) **Coagulation studies**: for infants showing signs of liver dysfunction or presenting overt bleeding symptoms, coagulation studies, including prothrombin time (PT) and activated partial thromboplastin time (aPTT), should be conducted.

10) **Glucose**: monitored due to the risk of abnormal glucose levels (hypoglycemia and hyperglycemia).

Therapeutic hypothermia

Therapeutic hypothermia (TH), an intentional temperature reduction of 3-4ºC with a diane temperature of 33-34ºC, is an effective and safe intervention when administered in Neonatal Intensive Care Units (NICU), representing the standard treatment for newborns with moderate to severe HIE [2,3,25].

TH should be initiated within the critical window period, specifically before 6 hours from birth, and should be maintained for 72 hours, to newborns with moderate to severe HIE [1]. TH is applied with the goal of preventing severe disability and death in newborns with HIE. Despite TH treatment, approximately 25% of newborns may die and, among the survivors, around 20% may exhibit a greater degree of disability.

Therapeutic hypothermia can have clinical effects on other major organ systems affected by perinatal asphyxia, such as kidney, heart, liver, gastrointestinal tract, lungs, as explained below [2,26]:

1) **Respiratory effects**: partial pressure of CO_2 (pCO_2) decreases by approximately 3-4% for every 1ºC drop in temperature below 37ºC. As body temperature is lowered, metabolism decreases and CO_2 production is reduced, resulting in lower CO_2 blood levels. In blood gas samples, there is a concomitant increase in pH with the lower pCO_2.

2) **Cardiovascular effects**: cardiac output falls due to a reduction in heart rate and stroke volume. However, there is no substantial increase in the risk of hypotension due to the initial perinatal asphyxia, which is likely attributed to peripheral vasoconstriction induced by the cooling process. A systematic review suggests that TH offers a cardioprotective effect [27].

3) **Metabolism and glucose**: the metabolic rate declines linearly with decreasing temperature. As the metabolic rate declines, glucose use, insulin release and insulin sensitivity may decrease, leading to increased glucose levels.

4) **Coagulopathy and thrombocytopenia**: it seems that there is no additional effect of hypothermia on the increased risk of coagulopathy associated with perinatal asphyxia, but cooling is related to an increased risk of thrombocytopenia [28]. Nevertheless, coagulopathy requiring intervention continues to be a frequent issue during TH. Therefore, continual monitoring of clotting factors, such as plasma fibrinogen, and liver function remains essential.

5) **Liver and kidney function**: it seems that therapeutic hypothermia does not increase the risk of liver and kidney injury. However, hypothermia decreases hepatic metabolism and function, altering drug dosing for medications that depend on hepatic metabolism and excretion.

6) **Intestinal function**: hypothermia does not seem to elevate the risk of necrotizing enterocolitis and, in fact, may offer benefits by potentially preventing additional ischemic intestinal injury.

Monitoring

The monitoring of newborns with perinatal asphyxia, especially those undergoing therapeutic hypothermia, involves three primary branches [2]:

1) **Clinical monitoring**:

    - Continuous heart rate (HR) and respiratory monitoring, including respiratory rate (RR) and pulse oximetry.
    - Blood pressure (BP) monitoring, using an intra-arterial catheter or frequent noninvasive measurements.
    - Central temperature monitoring, particularly in newborns undergoing therapeutic hypothermia
    - Continuous electroencephalogram (EEG), employing standard or amplitude- integrated EEG (aEEG).
    - Cerebral oximetry with near-infrared spectroscopy (NIRS), if available.
    - Daily cranial US

2) **Laboratory monitoring**:

    - Blood gas analysis, glucose, and CBC, obtained several times a day during the initial 3 days after resuscitation and subsequently on a daily basis until values normalize. For newborns not eligible for therapeutic hypothermia, most abnormalities typically normalize by the third day.
    - Daily monitoring of SCr, total and conjugated bilirubin, liver enzymes (ALT and AST) and C-reactive protein (CRP). Additionally, coagulation tests (PT and aPTT) should be monitored in newborns with liver dysfunction or overt bleeding.

3) **Drug monitoring and dosing**: elevated drug levels may occur due to perinatal asphyxia, altering drug pharmacokinetics through reduced kidney excretion and hepatic metabolism/excretion [29]. This effect may be aggravated by therapeutic hypothermia. Monitoring drug levels is generally advisable for drugs with potential harmful side effects (such as aminoglycosides, vancomycin, specific antiseizure medications) [30].

See **Annex 2** for more schematic and summarized information on the steps to monitor and manage newborns with HIE.

Supportive care

Supportive care measures are provided for all newborns with perinatal asphyxia, irrespective of whether therapeutic hypothermia is administered. These measures are applied both before, during, and after therapeutic hypothermia, as well as for newborns in whom cooling is not initiated [2].

1) **Neuroprotective management**: neuroprotective measures and seizure management.

2) **Respiratory support**: respiratory support is essential for most newborns with perinatal asphyxia and particularly crucial for those undergoing therapeutic hypothermia [13]. The primary objective goal of this intervention is to ensure adequate oxygenation and ventilation, especially during sedation required for TH, while avoiding changes in blood oxygen (O_2) and carbon dioxide (CO_2) levels. The target is to maintain a preductal oxygen saturation ($SatO_2$) above 95% and pCO_2 between 35 and 45 mmHg. In many cases, newborns undergoing TH will need intubation and mechanical ventilation for optimal respiratory support due to sedation. PPHN is a known complication associated with perinatal asphyxia. In such cases, inhaled nitric oxide (iNO) may be administered, which can also be done during TH.

3) **Cardiovascular support**: newborns with ventricular dysfunction and/or shock often require inotropic agents to support cardiac function and maintain perfusion. Sympathomimetic stimulation through catecholamine agents (like dopamine, dobutamine and milrinone) enhances myocardial contractility and may offer additional beneficial effects on peripheral vascular beds.

4) **Empiric antibiotic therapy**: empiric antibiotics are administered as a preventive measure until culture results are known, given that these patients are at an increased risk of serious infection.

5) **Fluid and electrolyte management**: adjustments in fluid and electrolyte management include regular assessments of net fluid intake, weight, and respiratory status, along with frequent evaluations of blood electrolytes. It is essential to maintain electrolyte levels within the normal range during the cooling process by modifying fluid therapy as needed

6) **Nutrition**: minimal enteral feeding, sometimes called "trophic feeds", and parenteral nutrition (PN) are provided for nutrition support.

7) **Coagulopathy and thrombocytopenia**: for newborns exhibiting significant coagulopathy, such as severely prolonged aPTT and PT times, or experiencing overt bleeding, fresh frozen plasma is provided in order to replace clotting factors that may either be consumed, as in the case of disseminated intravascular coagulation, or be low due to impaired hepatic function. In newborns with critical thrombocytopenia, platelet transfusions are administered when the platelet count drops below 50,000/microL.

Family centered care of the newborn with perinatal asphyxia or HIE

The birth and hospitalization of a newborn with perinatal asphyxia resulting in hypoxic-ischemic encephalopathy are an unforeseen, stressful and traumatic experience for parents.

Factors such as baby separation, exposure to NICU technology, fear of mortality and uncertainty about the future contribute to difficulties in physical interaction with the baby, disruption of parental bonds establishment and alteration of maternal-paternal roles, adversely impacting the emotional well-being of the family. Therapeutic communication stands as a pivotal tool in this context, facilitating the dissemination of family information, providing emotional support, accompanying parents through the process, and fostering a collaborative relationship.

1.2.7. Prognosis estimation

All newborns diagnosed with HIE should have access to tests with demonstrated diagnostic and prognostic capacities. The significance of prognosis establishment is profound, being crucial for both parents and medical professionals. Key points for establishing a prognosis are the following:

1) **Relevance of prognosis**: on the one hand, providing parents with a clear prognosis is not only their right but also an imperative aspect of ethical medical practice; on the other hand, doctors are ethically obligated to communicate prognosis accurately, considering the potential impact on decision-making and emotional well-being.

2) **Anticipation to clinical evolution**: estimating prognosis allows for anticipation of the clinical evolution of the newborn and potential challenges during childhood. Furthermore, it enables medical professionals to provide informed guidance to parents about the likely trajectory of the condition.

3) **Individualized planning**: prognosis estimation facilitates individualized planning for both short and long-term follow-up and helps to establish potential needs or rehabilitation interventions, creating a tailored plan for optimal outcomes.

4) **Decision making**: prognosis estimation is crucial for considering a reorientation from curative to palliative treatment, if considered appropriate based on the prognosis.

In conclusion, prognosis estimation in HIE is a fundamental aspect of medical care, with far-reaching implications for parents, medical professionals, and the overall well-being of the newborn. It serves as a guiding tool for planning, decision-making, and providing compassionate care throughout the journey of managing hypoxic-ischemic encephalopathy.

Several strategies are available for estimating the prognosis in newborns with HIE, as summarized in Figure 3 below. The significance of the studies is time-dependent concerning the moment of the acute insult. All newborns with HIE should have access to all studies with proven diagnostic and prognostic capabilities during the perinatal period.

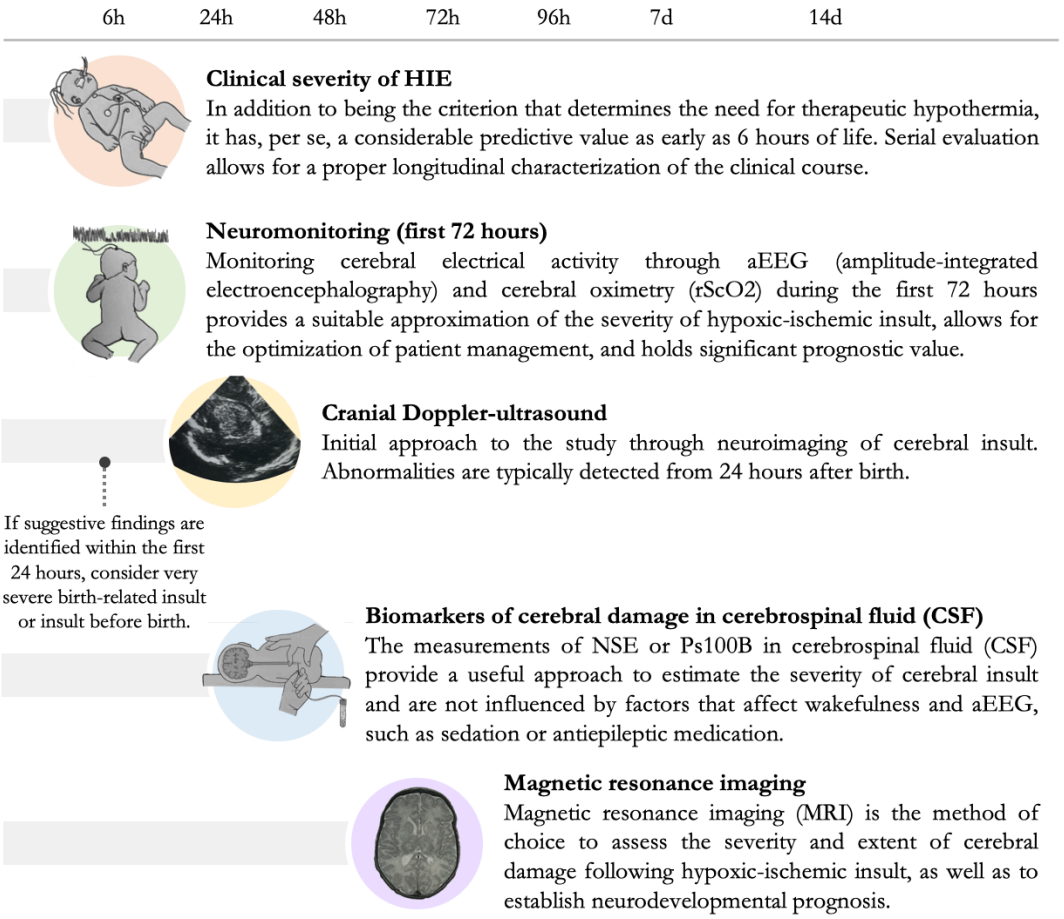


Figure 3. **Key studies for establishing the prognosis in HIE**. Adapted from García-Alix A., Arnáez, J., Neurología Neonatal de un Vistazo, Ed Cabeza de Chorlito; 2022 [21]

As illustrated in Figure 3, multiple techniques are employed for estimating HIE prognosis, including clinical assessment of HIE severity, neuromonitoring (using either aEEG or cerebral oximetry), cranial US, biomarkers of cerebral damage in CSF, and MRI. Nevertheless, the most commonly used and recommended techniques are clinical assessment, aEEG and MRI.

Clinical assessment

The clinical grading of HIE severity is conducted through severity scales, among which the most commonly used is the Sarnat severity scale, illustrated in **Table 1** above, which categorizes HIE as mild, moderate, or severe. Therefore, clinical grading of HIE severity is crucial for prognosis and is outlined as follows:

- **Moderate HIE**:
    - Moderate HIE involves a high degree of uncertain evolution.
    - A reliable prediction cannot only depend on the clinical grading of HIE.
- **Severe HIE**:
    - The majority of newborns with severe HIE either succumb or exhibit severe disability.
    - With TH, approximately 30% survive without severe disability.
    - The persistence of severe HIE at 72 hours predicts an adverse outcome.

However, some considerations should be taken into account when performing clinical grading of HIE:

1) The clinical grading of severity depends on the training and experience of the evaluator.
2) Classifications are not uniform, with significant differences between scales, particularly in distinguishing between mild and moderate HIE.
3) There are no universally accepted operational definitions to establish the severity of HIE, leading to potential variations in classification between centers and even within the same group.
4) Always consider the impact of confounding factors such as antiepileptic drugs or sedatives.
5) The clinical course in the first days reflects the severity of the insult, but information on its predictive value is limited.

Considering these points, it is essential to follow a consistent approach in each evaluation to avoid omissions of necessary information for severity grading. Additionally, the use of scales, either categorical (as illustrated in **Table 1** or **Annex 1**) or quantitative scales (see **Annex 1**), provides a standardized, systematic, and organized approach, can be performed in minutes, and allows for an adequate stratification of HIE severity points.

Neuromonitoring: aEEG and cerebral oximetry ($rScO_2$)

Neuromonitoring is primarily based on two techniques: amplitude-integrated electroencephalogram (aEEG) and cerebral oximetry ($rScO_2$).

Regarding aEEG, attention should be focused on the evolution of the background pattern over time, especially in the first 72 hours, and its behavior in different time intervals, as shown in **Table 2**.

Table 2 illustrates that the longer the aEEG pattern remains altered, the higher the risk of adverse neurological outcomes for the newborn. Additionally, it demonstrates how the application of therapeutic hypothermia reduces the risk of poor neurological outcomes [31].

Risk of adverse neurological outcome based on the age in hours of life during which the aEEG pattern remains highly altered					
Age in hours of life	**6**	**24**	**36**	**48**	**72**
Normothermia	88%	98%	98%	98%	94%
Hypothermia	59%	72%	84%	93%	96%

Table 2. **Risk of adverse neurological outcome based on the age in hol during which the aEEG pattern remains highly altered**. Adapted from Del Río R, et al., Amplitude Integrated Electroencephalogram as a Prognostic Tool in Neonates with Hypoxic-Ischemic Encephalopathy: A Systematic Review. PLOS ONE. 2016 [31]

The assessment and interpretation of aEEG are based on three pillars: the background pattern, sleep-wake cycles, and seizures (see **Annex 3** for more detail).

For aEEG interpretation and significance, the following considerations should be remarked [21]:

1) The evolution over time is more accurate for predicting outcomes than the background pattern in a given interval.
2) In neonates undergoing TH, a highly altered background pattern from 48 hours of life onward has a very high predictive capacity for adverse outcomes. Conversely, normalization of the pattern (normal or discontinuous) before 48 hours of life is associated with an outcome without major disability.
3) The recovery time of sleep-wake cycling (SWC) in the pattern is a good prognostic indicator. Non-recovery of SWC within 72 hours predicts adverse outcomes.
4) The presence of epileptic discharges, particularly status epilepticus, darkens the prognosis.
5) Do not rely solely on the aEEG pattern to establish an accurate prognosis.

Additionally, regarding seizures or convulsions, the following points should be considered [21]:

1) Newborns with HIE often experience a high burden of seizures, both in number and frequency, and status epilepticus is common.
2) Approximately 80% of seizures are subclinical, known as electrographic seizures.
3) Seizures that coincide with clinical expression may seemingly respond to antiepileptic treatment but persist in aEEG. Treatment exacerbates electroclinical dissociation.
4) Sedation or antiepileptic medication has the potential to impact the background pattern.
5) Treat electrographic seizures with the same approach as clinical seizures. Monitor the response to antiepileptic treatment with aEEG.
6) Subtle and tonic clinical seizures are associated with a worse outcome.

Regarding cerebral oximetry, it is a neuromonitoring technique that measures regional cerebral oxygen saturation ($rScO_2$), providing continuous and real-time information about:

- Cerebral oxygenation and oxygen supply.
- Oxygen demand and, consequently, cerebral oxygen consumption and metabolism.
- Surrogate of physiological stability and cerebral hemodynamics.

Therefore, changes in $rScO_2$ indicate alterations in brain tissue oxygenation or in the demand for cerebral oxygen consumption.

Continuous monitoring of $rScO_2$ in HIE provides:

1) Information about severity and prognosis.
2) Guidance for interventions aimed at correcting imbalances in cerebral oxygen balance, whether in supply or demand.
3) When rScO2 is assessed in relation to blood pressure and SatO2, it allows for non-invasive evaluation of cerebral autoregulation in critically ill neonates.
4) The combined use of aEEG and rScO2 better guides the timing and necessity of interventions in neonates with hemodynamic stability.

Therefore, to use $rScO_2$ as a prognostic tool, two variables should be considered:

A) $rScO_2$ values in the first 48 hours:
    - $rScO_2$ values increase in newborns with HIE in the first 48 hours. The values are higher in neonates with severe HIE, with or without TH.
    - Maintained values > 90% are associated with pathological MRI and adverse outcomes. On the one hand, they indicate luxury perfusion and vasoparesis, leading to increased cerebral perfusion. On the other hand, there is reduced oxygen utilization due to mitochondrial dysfunction or damage.
B) Variability of values over time:
    - Maintained variability <5% is associated with a poor prognosis.

Hence, as a summary, the combined use of aEEG and rScO2 during the first 72 hours enhances neurodevelopmental predictive capability. The combination of both neuromonitoring tools appears to be superior to each one alone.

Cranial Doppler-ultrasound

Cranial Doppler-ultrasonography is the initial imaging evaluation performed on newborns with HIE and serves as a complementary tool to MRI in characterizing cerebral damage.

It offers the advantages of being a non-invasive evaluation conducted at the baby's bedside, requiring no sedation, allowing for serial assessments, and having a lower cost compared to MRI. In many cases, it might be the only neuroimaging study available due to patient instability for transfer to the radiology room or the lack of MRI equipment in the facility.

Cranial ultrasound enables:

1) Identification of the pattern and severity of cerebral injury.
2) Definition of the evolution of findings from the acute to the chronic phase and identification of any complications.

The Annink scoring system (see **Annex 4** for more detail) grades the severity of findings observed in cranial Doppler-ultrasonography and correlates well with neurodevelopmental outcomes at 2 years [32].

The findings change during the first few days. Cerebral edema takes time to develop and may be very mild in the first 24 hours. If abnormalities are present in the first 24 hours, there is a high probability of adverse outcome, likely due to a very severe insult or injury before the onset of labor.

Additionally, it is advisable to perform early cranial Doppler-US to rule out pre-existing cerebral pathology before hypoxic-ischemic insult, such as hemorrhagic lesions or vascular malformations. Newborns with neurological pathology may be more prone to difficulties in the progression of labor, leading to hypoxic-ischemic events.

Magnetic resonance imaging

Magnetic Resonance Imaging (MRI) is the method of choice to assess the severity and extent of damage and to establish neurodevelopmental prognosis. Its prognostic value is not altered by TH. The systematic MRI evaluation must assess the following items: white matter, cortex, basal ganglia and thalamus, posterior limb of the internal capsule, cerebellum, brainstem, and the pattern of damage.

The MRI study should include T1-weighted (TW1) and T2-weighted (TW2) sequences, Diffusion Weighted Imaging (DWI), and Single Voxel Spectroscopy (SVS) if available, always in basal ganglia and thalamus and, if possible, another in white matter. The optimal timing of the examination is between 4 and 14 days of life.

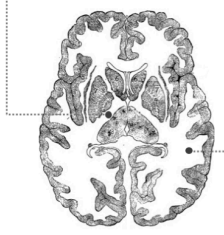
Findings change over time, and very early scans (before 2 days of life) may underestimate the injury. Without DWI, the optimal time is between 7 and 14 days of life. The most informative sequence in the first week of life is DWI. Between the 2nd and 5th days of life, DWI-enhanced MRI effectively detects the severity of hypoxic-ischemic brain injury and correlates well with findings observed in MRI after the 7th day of life. Therefore, MRI between the 2nd and 5th days of life is a useful prognostic tool, aiding in medical decisions for redirecting care.

Regarding the assessment of severity and extent of damage based on cualitative observation, various scales exist. The simplest is the Rutherford scale, represented in **Table 3**, which scores each structure separately: posterior limb of the internal capsule (PLIC), basal ganglia and thalamus (BGT), white matter (WM) and cortex. Each is scored from 0 (normal) to 3 (severe), except for PLIC, which is scored from 0 to 2 (where 0 is normal, 1 is equivocal signal, and 2 is absent signal). The Total Damage Score (TDS) is the sum of all points, ranging from 0 to 11. A higher TDS indicates more severe damage, greater extent, and a higher risk of adverse outcomes (death or major disability).

Posterior limb of the internal capsule (PLIC)	**Normal**	**Equivocal** (reduced or asymmetrical)		**Loss** (abnormal or inverted signal)
	0	1		2
Basal ganglia and thalamus (BGT)	**Normal**	**Mild** (focal)	**Moderate** (multifocal)	**Severe** (diffuse)
	0	1	2	3
White matter (WM)	**Normal**	**Mild** (periventricular)	**Moderate** (subcortical/ punctate lesions/ focal infarct)	**Severe** (significant loss of WM-GM differentiation, infarctions, hemorrhage)
	0	1	2	3
Cortex	**Normal**	**Mild** (1-2 regions)	**Moderate** (3 regions)	**Severe** (>3 regions)
	0	1	2	3

Table 3. **Rutherford scoring system**, based on the assessment of PLIC, BGT, WM and cortex. Adapted from Rutherford M et al., Assessment of brain tissue injury after moderate hypothermia in neonates with hypoxic–ischaemic encephalopathy: a nested substudy of a randomized controlled trial, Lancet Neurol. 2010 [33], and García-Alix A., Arnáez, J., Neurología Neonatal de un Vistazo, Ed Cabeza de Chorlito; 2022 [21]

BGT damage	M	C	L	F
Mild	· Normal PLIC: no cerebral palsy, walking at 2 years · Equivocal PLIC: 10-15%	80% ≥ 85	25% mild-moderate	10% mild
Moderate	· Equivocal PLIC: 60% (75% mild) · Pathological PLIC: 75% (40% severe)	90% > 70	Majority 25% severe	40-50% (10% gastrostomy)
Severe	· 98% (95% severe) · No walking at 2 years	35% < 70	95% Majority severe	90% gastrostomy



WM damage	M	C	L	B	E
Mild	No cerebral palsy	0	4%	3%	0
Moderate	3%	3% < 85	28%	30%	9%
Severe	18%	33% < 85	64%	68%	36%

Figure 4. **Neurodevelopmental damage at 2 years according to the affected region** (BGT or WM). Adapted from Martinez-Biarge M et al., Outcomes after central gray matter injury in term perinatal hypoxic-ischaemic encephalopathy, Early Hum Dev, 2010 [34], Martinez-Biarge M et al., White matter and cortical injury in hypoxic-ischemic encephalopathy: antecedent factors and 2-year outcome. J Pediatr. 2012[35], and García-Alix A., Arnáez, J., Neurología Neonatal de un Vistazo, Ed Cabeza de Chorlito; 2022 [21].

*B: behavioral disorder; C: cognitive disorder; E: epilepsy F: feeding disorder; L: language disorder; M: motor disorder or cerebral palsy.

In summary, magnetic resonance imaging systematically evaluates and grades the severity of damage by structures. As reflected in **Figure 4** above, the severity of BGT injury is a good predictive indicator of developmental disorders, and frank signal alteration in the PLIC is one of the best predictors of motor development and the ability to walk at 2 years. White matter disorders have less serious consequences than BGT injury. Severe white matter alteration can impact cognitive, language, and behavioral domains. But even with a score of 3, if there is no BGT injury, the evolution at 2 years can be favorable.

Biomarkers of cerebral damage in cerebrospinal fluid

The most commonly used biomarkers of damage are neuron-specific enolase (NSE), which will be more extensively discussed below, and S100B protein (PS100B). During both the ischemic and reperfusion phases, these proteins are released into the interstitial space and, then, into the CSF, where they become diluted. It is worth noting that CSF concentration is not influenced by sedation-analgesia or antiepileptic drugs, unlike other prognostic techniques such as aEEG. These proteins are measured by commercial kits, with good reproducibility, and results are available within a few hours, typically between 12 and 72 hours of life. Moreover, their CSF concentration depends on the severity of the insult and the time elapsed since it occurred.

Supporting the use of these cerebral damage biomarkers, NSE in CSF within the first 72 hours is a valuable indicator to estimate brain damage, as evidenced by its correlation with clinical grading of encephalopathy, aEEG, and MRI findings. Most importantly, NSE concentrations in CSF are a good predictive indicator at 2 years of age in neonates with HIE. On the flip side, CSF is easily accessible through lumbar puncture (LP), but this invasive procedure is contraindicated when neonates exhibit signs of increased intracranial pressure, cardiovascular or respiratory instability (such as hypotension or severe pulmonary hypertension), or significant coagulopathy.

Neuron-specific enolase

Biomarkers, which are specific molecules released by or associated with a particular organ, provide valuable insights into the physiological or pathological status of that specific organ. These biomarkers can be obtained from various bodily fluids, such as blood, urine, or CSF [36].

In newborns with brain injuries, biomarkers might have the potential to predict the extent and location of injury shortly after its occurrence [36].

Neuron-specific enolase, belonging to the enolase family, enzymes found in all tissues capable of glycolysis [36], is specific to central and peripheral neurons, as well as neuroendocrine cells [36,37]. NSE plays a unique role in the intracellular energy metabolism system shared by neurons [38,39], although it is also present in physiologically low concentrations in the blood, erythrocytes, platelets, plasma cells, lymphocytes, capillary walls, and myoepithelial cells [37].

After cell injury, NSE is released into extracellular fluids, the CNS, and the blood [37]. Studies have demonstrated increased NSE concentrations in cerebral circulation after experimentally induced cerebral lesions, correlating with focal neuronal loss in the ischemic area [40]. NSE is believed to be released into the blood due to a combination of brain injuries and blood-brain barrier disruption in infants with HIE resulting from ischemia or edema [36,41]. Consequently, elevated blood levels of NSE are associated with poor outcomes following cardiac arrest, stroke, and pediatric traumatic brain injury [36].

Despite lacking comprehensive studies on the relationship between NSE and HIE [38,39], some suggest that NSE could serve as a potential predictor of HIE severity [42]. The need for early, reliable, and specific biomarkers could enhance the effectiveness of therapeutic hypothermia, the only proven treatment for HIE so far. Recent studies have explored the combination of NSE levels, MRI and aEEG within the context of HIE outcomes, yet a profound study of the association of serum NSE and neurodevelopmental outcome remains unexplored [43]. One study proposed that the combination of cerebrospinal fluid NSE, MRI, and aEEG could predict HIE outcomes [44].

Chapter 2

Justification, hypothesis and objectives

2.1. Justification

Despite advancements in medical technology, including TH as the unique specific intervention proven to enhance neurodevelopmental outcomes, HIE remains a significant contributor to major worldwide perinatal mortality, morbidity, and long-term disability in full-term and near-term newborns [45].

The challenge of early and accurate assessment of the severity of brain damage after a perinatal hypoxic-ischemic event persists in neonatal care. Clinicians' ability to predict the outcome of newborns with HIE is not straightforward.

A variety of tools like clinical assessment, aEEG, biochemical markers and neuroimaging, and combinations of such, are used to assess the severity of HIE and predict long-term outcomes [46]. Although recommended by guidelines [47] and the literature [48], current methods for assessing brain injury risk in newborns have inherent limitations, particularly in the first hours of life, leading to uncertainty about ongoing brain damage severity and eventual neurological outcomes persists during this early period [49].

The clinical assessment, commonly assessed through the Sarnat grading system, relying on clinical criteria, categorizes newborns into mild, moderate or severe HIE, and measures the progression of the neurologic insult to predict the baby's progression. Nevertheless, its subjectivity and changes over time limit its effectiveness. It has to be reminded that the clinical grading of severity depends on the training and experience of the evaluator and, in addition, classifications are not uniform, with differences between scales, particularly in distinguishing between mild and moderate HIE. Moreover, there are no universally accepted operational definitions to establish the severity of HIE, leading to potential variations between centers and even in the same group. Furthermore, follow-up studies demonstrated a low predictive value of Sarnat scale for the outcome with HIE, especially moderate encephalopathy [50,51]. Last but not least, confounding factors such as drugs or sedatives have to be considered for the clinical severity assessment.

Another bedside tool, aEEG, may help stage the severity of injury and predict prognosis. however, do not rely solely on the aEEG pattern to establish a good prognosis. Nevertheless, aEEG and the Sarnat score prove less effective in predicting outcomes during hypothermia, lack information on the injury's timing, and can be affected by antiepileptic drugs or sedatives, which have to be taken as confounding factors.

Neuroimaging techniques, like cranial ultrasound and MRI are valuable tools to determine the extent of ischemia and brain damage, with MRI being the most sensitive modality for determining the patterns of brain injury, without the use of radiations. Particularly, brain MRI can help determine when the injury occurred, but obtaining an MRI is not possible in unstable patients and the optimal timing for performing the MRI is at one week from the insult, so that it does not provide an early prognosis.

The need for early and objective indicators for diagnosing and predicting HIE outcomes calls for biomarkers that could provide a biochemical index of brain injury, monitor disease progression, assess neuroprotective strategies' efficacy, and enhance the reliability of neurological sequelae predictions after hypoxic-ischemic brain injury.

While TH has evolved into standard care for moderate to severe HIE, the next step in neuroprotection involves identifying biomarkers that guide clinical decisions. These biomarkers would distinguish newborns responding to hypothermia from those requiring alternative neuroprotective interventions. As a key feature of future trials, biomarkers would play a crucial role in gauging the intervention's short and long term efficacy, aiming to identify injuries and predict long-term outcomes.

NSE stands out as a useful and reliable marker for neuronal damage and prognosis in various neurological disorders. However, limited studies focus on its values in diagnosing and prognosing HIE after perinatal asphyxia, mainly relying on CSF values of NSE as an indicator of neurological impairment [43]. The least invasive fluid source for biomarkers in critically ill newborns is crucial, emphasizing the need for alternatives to CSF sampling, because it implies more associated risks than blood sampling. Therefore, NSE could be determined in blood instead of CSF, as blood samples can be taken more easily and frequently and more independently of raised intracranial pressure than CSF samples, and can be performed in all conditions. Also, one study suggested that the combination of serum NSE levels, MRI and aEEG could predict outcomes within the context of HIE [23]. Nevertheless, the study of the potential predictive capacity of serum NSE for neurodevelopmental outcome in newborns with HIE remains unexplored.

Currently, clinicians do not routinely use biomarkers in newborns with perinatal asphyxia or HIE. This study seeks to examine a potential biomarker for bedside clinicians to improve the prognosis and treatment of newborns with HIE. The overall goal is to enhance the understanding of serum NSE concentrations as a marker of HIE severity and its predictive value, for short and long-term neurological outcomes in newborns with HIE.

2.2. Hypothesis

The assumptions leading to the start of this study are as follows:

2.2.1. Main hypothesis

The main hypothesis is that serum levels of Neuron-Specific Enolase (NSE) serve as a prognostic predictor for neurodevelopmental outcome in newborns diagnosed with Hypoxic-Ischemic Encephalopathy (HIE).

2.2.2. Secondary hypothesis

The secondary hypothesis of this study are as follows:

1) Serum levels of NSE are associated with the severity classification of HIE according to the Sarnat scale performed in the clinical assessment, categorizing it as mild, moderate, or severe.

2) Serum levels of NSE are associated with findings from reference prognostic techniques, such as abnormal patterns and seizures in the aEEG, and the level of injury in the MRI.

2.3. Objectives

2.3.1. Main objective

The main objective of this Final Degree Project is to determine whether serum levels of Neuron-Specific Enolase (NSE) serve as a prognostic predictor for neurodevelopmental outcome in Hypoxic-Ischemic Encephalopathy (HIE).

2.3.2. Secondary objectives

The secondary objectives of this study are as follows:

1) To determine the range of serum NSE values associated with the Sarnat severity scale for HIE performed in the clinical assessment, classifying it as mild, moderate or severe.

2) To determine the range of serum NSE values associated with the findings in the reference prognostic techniques, such as abnormal patterns and seizures in the aEEG and the level and regions of injury in the MRI.

3) To identify the most significant time points or time intervals for serum NSE determination and its prognostic correlation.

Chapter 3

Materials and methods

3.1. Study design

Structured as a prospective observational descriptive study, particularly, as a prognostic tool accuracy study, this project is primarily focused on analyzing the predictive value of serum NSE for neurodevelopmental outcome in newborns diagnosed with HIE. Furthermore, it also includes the association between NSE values and well-established prognostic techniques, including aEEG, MRI, and clinical assessment.

3.1.1. Study setting

For this study, the study patients are estimated to be recruited in 1 year. Furthermore, since there is a medium to long-term follow-up to assess the neurodevelopmental outcome at 18 months from the newborn's birth, this study will have an expected duration of approximately 2 to 3 years.

Therefore, this multicenter study will specifically take place in the Neonatal Intensive Care Unit of the following hospitals:

- Hospital Universitari Josep Trueta (HUJT, Girona)
- Hospital Sant Joan de Déu (HSJD, Barcelona)
- Hospital Universitari Vall d'Hebron (HUVH, Barcelona)
- Hospital Universitari Germans Trias i Pujol (HUGTP, Barcelona)
- Hospital Universitari Parc Taulí (HUPT, Barcelona)
- Hospital Universitari Joan XXIII (HUJ23, Tarragona).

3.1.2. Study population

To identify participants for this study, it is crucial to establish our target population. The study population will include full-term newborns diagnosed with HIE who are admitted to the afore-mentioned hospitals, meet the inclusion criteria and do not meet any of the exclusion criteria, and agree to participate by providing informed consent for their involvement in the study.

These patients will undergo management and treatment in accordance with clinical practice guidelines and protocols, ensuring that the study does not impact their clinical progression.

The data from newborns will include the following, collected in the data collection sheet (see **Annex 7**):

- General information: sex, date and time of birth, gestational age, and weight.
- Perinatal information indicative of potential perinatal asphyxia: mode of delivery, labor dystocia, sentinel event, non-reassuring fetal status, need for advanced resuscitation, 5-minute Apgar score, umbilical cord pH and base deficit.

3.1.3. Inclusion criteria

The inclusion criteria at the time of enrollment are as follows:

- Newborns with a gestational age of $\geq$ 37 and $<$ 42 weeks and a weight $\geq$ 1800 and $<$ 4000 grams, considered to have HIE if meeting the following three criteria:

    1) Evidence of intrapartum distress or potential hypoxic-ischemic insult, either with altered fetal heart rate in the cardiotocograph pattern (including late decelerations, absence of variability, or persistent bradycardia), sentinel event, or labor dystocia.

    2) Presence of 5-minute Apgar score $\leq$ 5, need for advanced resuscitation during the birth period (with positive pressure ventilation for more than 10 minutes after birth and/or laryngeal intubation), or evidence of metabolic acidosis (either with pH $\leq$ 7.0 and/or base deficit $\geq$ 16 mmol/L) determined in the umbilical cord or the most unfavorable blood gas analysis within the first 60 minutes of life.

    3) Evidence of early onset of neonatal encephalopathy, defined as a syndrome of neurological dysfunction within the first 6 hours from birth manifested either by a subnormal level of consciousness with or without seizures (corresponding to moderate to severe HIE according to Sarnat), or by palmary hyperexcitability, tremor, overactive myotatic reflexes, hypersensitivity to stimulation or startle responses (corresponding to mild HIE according to Sarnat).

3.1.4. Exclusion criteria

To clearly define the study population, the exclusion criteria at the time of enrollment are as follows:

- Newborns meeting any of the following conditions:

    1) Death at the time of birth.

    2) Congenital syndrome or abnormalities, including severe congenital malformations, metabolic disease, congenital heart disease or suspected neuromuscular disease.

    3) Other identifiable etiologies of neurological dysfunction such as infection or genetic disease.

- Refusal from the parents to give informed consent.

3.1.5. Withdrawal criteria

To conduct the study, every possible effort should be made, always within the bounds of safety and patient choice (or legal representatives, in this case), to ensure that each participant completes the study. Patients who initiate this study should continue to be monitored to analyze their progression unless there is a justified reason. Reasons for withdrawing a patient from the study follow-up include the following:

- Withdrawal of informed consent for the study conduct or follow-up: the patient (or legal representatives) request withdrawal from the study at any time for personal or uncontrollable reasons. This event must be documented, and the patient's data up to that point should be used in the analysis if possible.

- Severe adverse effect or unexpected medical condition of the patient during the study, which is unrelated to the study procedures but makes their participation unsafe.

To ensure proper documentation, the patient's abandonment or withdrawal, along with their documents and the reason for withdrawal, must be recorded. The data collected up to the point of withdrawal should be included in the study and, if feasible, in the analysis. Given the study's consecutive sampling approach to recruit patients, throughout the recruitment period, it will be possible to replace withdrawing patients with new ones. This ensures a consistently sufficient number of participants in the study for obtaining meaningful and representative results.

3.2. Sampling

3.2.1. Sample selection

In this study, a non-probabilistic consecutive sampling method will be employed. All newborns diagnosed with HIE who meet the inclusion criteria and none of the exclusion criteria will be offered to participate in the study over an estimated period of 1 year. This selection process will take place within the hospitals involved in this study.

Moreover, patients will receive information about the study's purpose and will be provided with the information sheet (see **Annex 5**) and the consent form (see **Annex 6**). It is essential to emphasize to the patients (or their legal representatives) the voluntary and confidential aspects of their participation, along with their right to withdraw from the study at any point. After the informed consent process, researchers will fill out the data collection sheet (see **Annex 7**).

3.2.2. Sample size

In accordance with various studies (23), the ratio of patients with a favorable prognosis (defined as normal neurodevelopment) to patients with an unfavorable prognosis (defined as mild, moderate, or severe delay) is approximately 1.5. Thus, using the Epidat calculator, assuming an expected sensitivity of 90%, a precision of 10%, and a confidence level of 95%, the estimated sample size is 88 patients.

Taking into account that there are approximately 60,000 annual births according to data provided by Idescat (Institut d'Estadística de Catalunya), and using an estimated incidence of HIE of approximately 1.6 per 1,000 newborns as a basis, we can estimate 96 patients with HIE in a year. In anticipation of the possibility of study dropout or withdrawal of 10%, this approach ensures that the intended sample size of about 90 patients can be achieved in 1 year.

3.3. Variables and measurement methods

The study variables, summarized in **Table 4** below, will be collected prospectively starting from the moment of recruitment in the study, which will be from the birth of the newborn diagnosed with HIE, and throughout the routine follow-up, management, and treatment in the NICU. Furthermore, the neurodevelopmental evaluation will be assessed at 18 months of age.

Given that this is a study evaluating the accuracy of a prognostic tool, the variables under investigation will be treated as *index test* and *reference standard*, rather than as typically defined, such as *independent variable* and *dependent variable*, since there is no causality between NSE values and neurodevelopmental outcomes.

Thus, the *index test* refers to the prognostic tool or test evaluated in a study to determine its validity or accuracy in predicting a specific condition. On the other hand, the *reference standard* is the test or method considered as the gold standard for predicting this particular condition. In this way, the *reference standard* is used as the standard measure against which the performance of the *index test* is compared. Comparing the results of the *index test* with the *reference standard* helps evaluate the sensitivity, specificity, and other performance metrics of the test in question.

3.3.1. Index test

This index test of study is the **serum NSE level**, defined as a continuous quantitative variable expressed as serum concentration.

3.3.2. Reference standard

The reference standard is the **neurodevelopmental outcome** of HIE, which will be assessed through the Bayley Scales of Infant and Toddler Development, Third Edition (BSID-III), which evaluates three individual developmental scores: cognitive composite score, language composite score (with receptive and expressive scores) and motor composite score (with gross and fine motor scores). Therefore, the neurodevelopmental outcome is categorized as an ordinal categorical qualitative variable, where a developmental score of ≥ 85 indicates no delay, a score of < 85 (or > 1 SD below the mean) indicates at least mild to moderate delay, and a score of < 70 (or > 2 SD below the mean) indicates severe delay.

3.3.3. Covariables

Since the prognosis is assessed through the three main branches —clinical assessment, electrophysiological studies, and neuroimaging studies— to complete the study and for a better understanding of the results, various covariables will be studied as follows:

1) **Clinical assessment** will be performed through HIE severity. As explained above, the clinical assessment of severity, besides being a sensitive indicator of the insult to the CNS, also holds significant prognostic utility in the early days of life, correlating with clinical evolution and the possibility of neurological sequelae. Thus, the severity of HIE will be assessed through the Sarnat severity scale, categorizing HIE into mild, moderate, or severe categories, characterizing it as an ordinal categorical qualitative variable.

2) **aEEG** will be assessed through the commonly used evaluation in routine clinical practice, by evaluating the following three items (see **Annex 3** for more detail) [43]:

    a) **Baseline tracing**, concerning the following patterns: continuous normal voltage (CNV), discontinuous (DC), burst-suppression (BS), continuous low voltage (CLV), or flat tracing (FT), where BS, CLV, and FT are defined as severely abnormal patterns.

For a simpler assessment of the results, the baseline tracing will be categorized as a dichotomous nominal qualitative variable, indicating the absence or presence of abnormal background pattern (BGP).

b) **Sleep-wake cycling** (SWC), assessed as present SWC, immature SWC or absent SWC. For a simpler evaluation, SWC will be categorized as a dichotomous nominal qualitative variable, indicating the presence or absence of SWC.

c) **Seizures**, assessed as the absence of seizures or presence of seizures (specifically single seizures, repetitive seizures, or status epilepticus). As well, for a simpler assessment, seizures will be categorized as a dichotomous nominal qualitative variable, indicating the absence or presence of seizures.

3) **MRI**, being less quantitative and more subjective, will be assessed by an experienced researcher as follows [43]:

a) **Rutherford scale**, which scores the severity of damage in the following brain regions: posterior limb of the internal capsule, basal ganglia and thalamus, white matter, and cortex. Based on the findings, each item will receive a score, and the Total Damage Score will be calculated, with points ranging from 0 to 11, resulting in a discrete quantitative variable.

b) **Moderate to severe injury**, defined as moderate to severe damage in any of the four regions. This item will be characterized as a dichotomous nominal cualitative variable, indicating the absence or presence of moderate to severe injury.

c) **Pattern of injury**, which groups MRI findings into four patterns of injury:

i) Normal.
ii) Basal ganglia and/or thalamus injury: includes basal ganglia and/or thalamus ± cortical injury without white matter injury.
iii) Watershed pattern: includes moderate to severe white matter injury without any other damage.
iv) Global injury: includes minimally basal ganglia and/or thalamus and white matter injury.

For further understanding, the pattern of injury will be defined as a dichotomous nominal qualitative variable, indicating the absence or presence of global injury.

There are other variables that could potentially influence the mentioned variables, but they are not objects of study of the current project. Since these variables could act as confounders, their collection and control will be implemented to enhance internal and external validity.

1) **Perinatal variables**:

    a) **Sentinel event**, including premature placental detachment, uterine rupture, umbilical cord prolapse, amniotic fluid embolism, fetal exsanguination due to vasa previa, and fetal-maternal hemorrhage. The sentinel event is defined as a dichotomous nominal qualitative variable, indicating the absence or presence of sentinel event.

    b) **Labor dystocia**, defined as a dichotomous nominal qualitative variable, indicating the absence or presence of labor dystocia.

    c) **Advanced resuscitation after birth period**, including positive pressure ventilation for more than 10 minutes or tracheal intubation. It is defined as a dichotomous nominal qualitative variable, indicating no need or need for advanced resuscitation.

    d) **5-minute Apgar score**, characterized as a discrete quantitative variable, with points ranging from 0 to 10.

    e) **pH**, assessed by umbilical cord pH at the time of birth or by blood gas analysis during the first days of life, characterized as a continuous quantitative variable.

2) **Newborn variables**:

    a) **Sex**, defined as a dichotomous nominal qualitative variable, being male or female.

    b) **Gestational age**, defined as a discrete quantitative variable, categorized as 37 weeks (between 37–37+6), 38 weeks (between 38–38+6), 39 weeks (between 39–39+6), 40 weeks (between 40–40+6), and 41 weeks (between 41–41+6).

    c) **Weight**, defined as a continuous quantitative variable.

3) **Treatment variables**:

    a) **Therapeutic hypothermia**, categorized as a dichotomous nominal qualitative variable, indicating no TH administration or TH administration.

4) **Clinical evolution variables**:

    a) **Adverse event**, including death or any other withdrawal event (such as infection, hemorrhage, clinical instability, etc.), defined as a dichotomous nominal qualitative variable, indicating the absence or presence of an adverse event.

5) **Study site**:

    a) **Hospital**, defined as a polytomous nominal qualitative variable, indicating the hospital in which the study has been conducted.

Index test					
Serum NSE level		Serum concentration measurement	Continuous quantitative	Serum [NSE] value	
Reference standard					
Outcome		BSID-III score	Ordinal categorical qualitative	No delay (>85)	Favorable
				Mild-moderate delay (70-85)	Unfavorable
				Severe delay (<70)	
Covariables					
Prognostic	**Clinical assessment**	Sarnat severity scale	Ordinal categorical qualitative	Mild (grade I)	
				Moderate (grade II)	
				Severe (grade III)	
	aEEG	Baseline	Dichotomous nominal qualitative	Absence of abnormal BGP	
				Presence of abnormal BGP	
		SWC	Dichotomous nominal qualitative	Presence of SWC	
				Absence of SWC	
		Seizures	Dichotomous nominal qualitative	Absence of seizures	
				Presence of seizures	
	MRI	Rutherford scale	Discrete quantitative	Score ranging from 0 to 11	
		Moderate-severe injury	Dichotomous nominal qualitative	Absence of moderate-to-severe injury	
				Presence of moderate-to-severe injury	
		Global injury	Dichotomous nominal qualitative	Absence of global injury	
				Presence of global injury	
Perinatal	**Sentinel event**		Dichotomous nominal qualitative	Absence of sentinel event	
				Presence of sentinel event	
	Labor dystocia		Dichotomous nominal qualitative	Absence of labor dystocia	
				Presence of labor dystocia	
	Advanced resuscitation		Dichotomous nominal qualitative	No need for advanced resuscitation	
				Need for advanced resuscitation	
	5-min Apgar score		Discrete quantitative	Score ranging from 0 to 10	
	pH		Continuous quantitative	pH value	
Newborn	**Sex**		Dichotomous nominal qualitative	Male	
				Female	
	Gestational age		Discrete quantitative	37, 38, 39, 40 or 41 weeks	
	Weight		Continuous quantitative	Weight value	
Treat-ment	**Therapeutic hypothermia**		Dichotomous nominal qualitative	No TH administration	
				TH administration	
Clinical evolution	**Adverse event**		Dichotomous nominal qualitative	Absence of adverse event	
				Presence of adverse event	
Study site	**Hospital**		Polytomous nominal qualitative	HUJT	HSJD	HUVH
				HUGTP	HUPT	HUJ23

Table 4. **Summary table of index test, reference standard, and covariables, and its characteristics**.

3.4. Study intervention and procedures

3.4.1. Enrollment and data collection

As mentioned earlier, all full-term newborns diagnosed with HIE who meet the inclusion criteria and none of the exclusion criteria will be informed about the study's purpose. Alongside this, they will receive an information sheet (see **Annex 5**) outlining the option to withdraw, along with details regarding the voluntary and confidential nature of the study. If they choose to participate, legal representatives will be required to sign the informed consent form (see **Annex 6**).

Following this, general and perinatal data (previously detailed) will be collected in the data collection sheet (see **Annex 7**) through reports from the obstetrics and gynecology, pediatrics and nursing teams.

All data collected during the study will be stored in the patient's clinical history, in the SAP system and in the Research Electronic Data Capture (REDCap), which is a secure platform database. Covariables and additional data from the data collection sheet will also be integrated into the clinical history. Additionally, all the management and treatment procedures, along with any other important data or information throughout the study process, will be documented in the database.

3.4.2. Procedure

Clinical assessment

The severity of HIE will be assessed immediately after admission, and always before starting therapeutic hypothermia, by one investigator. Encephalopathy will be classified as mild, moderate or severe according to the Sarnat severity scale. This neurological examination will be performed everyday during the hospitalization period.

As stated by protocols and guidelines of the hospital, newborns with moderate or severe HIE will receive therapeutic hypothermia (either by selective head cooling or total body cooling) with rectal temperature maintained at 33-34ºC for 72 hours, following which they will be slowly rewarmed at ≤ 0.5ºC per hour. All cooled infants will receive sedation with fentanyl infusion through the treatment.

Amplitude-integrated electroencephalography (aEEG)

aEEG recordings will be immediately started at admission in the NICU and maintained for at least 48 hours in both newborns with mild HIE and newborns with moderate to severe HIE throughout therapeutic hypothermia and rewarming. The aEEG recordings will be collected using each Cerebral Function Monitoring (CFM) available in each hospital and digitally scored. The tracing will be blindly assessed by one researcher, focusing on the background pattern, sleep-wake cycling, and seizure activity.

Neuron-specific enolase in serum

Following the guidelines and protocols for monitoring and managing HIE at each hospital, various blood samples will be collected, usually at 6, 12, 24, 48, 72, and 96 hours of birth.

Serum NSE values will be determined from routine blood samples, specifically those corresponding to the first (1-day NSE), second (2-day NSE), and third day (3-day NSE) of life. The concentration of NSE in serum will be assessed using the immunoassay equipment available at each hospital. Subsequently, NSE serum values will be evaluated blindly with respect to clinical data, including the HIE stage, as well as findings from aEEG and neuroimaging.

Magnetic resonance imaging (MRI)

MRI will be performed at 7 days of life using a 1.5 Tesla MRI unit, available in each hospital, equipped with a specific neonatal head coil. At least, TW1, TW2 and DWI will be obtained for all patients.

Two radiologists, masked to clinical data and serum NSE levels, will independently and simultaneously review MRIs. The images will be scored according to the Rutherford scale, as mentioned earlier, which categorizes the severity of damage in four brain regions (PLIC, BGT, WT, and cortex). Additionally, the evaluation will include an assessment of moderate to severe injury and global injury for a more comprehensive understanding.

3.4.3. Follow-up

Neurodevelopmental assessment will be made in all surviving patients at 18 months of age, at the hospital where they received previous care. These assessments will be performed by a researcher who is unaware of clinical and diagnostic data obtained up to that point.
The neurodevelopmental assessment will be performed using the BSID-III, by evaluating the three developmental scores: cognitive, language and motor composite scores, as detailed earlier.

3.4.4. Flow chart

The study enrollment, procedures and follow-up are summarized in a study flow chart illustrated in Figure 5 below.

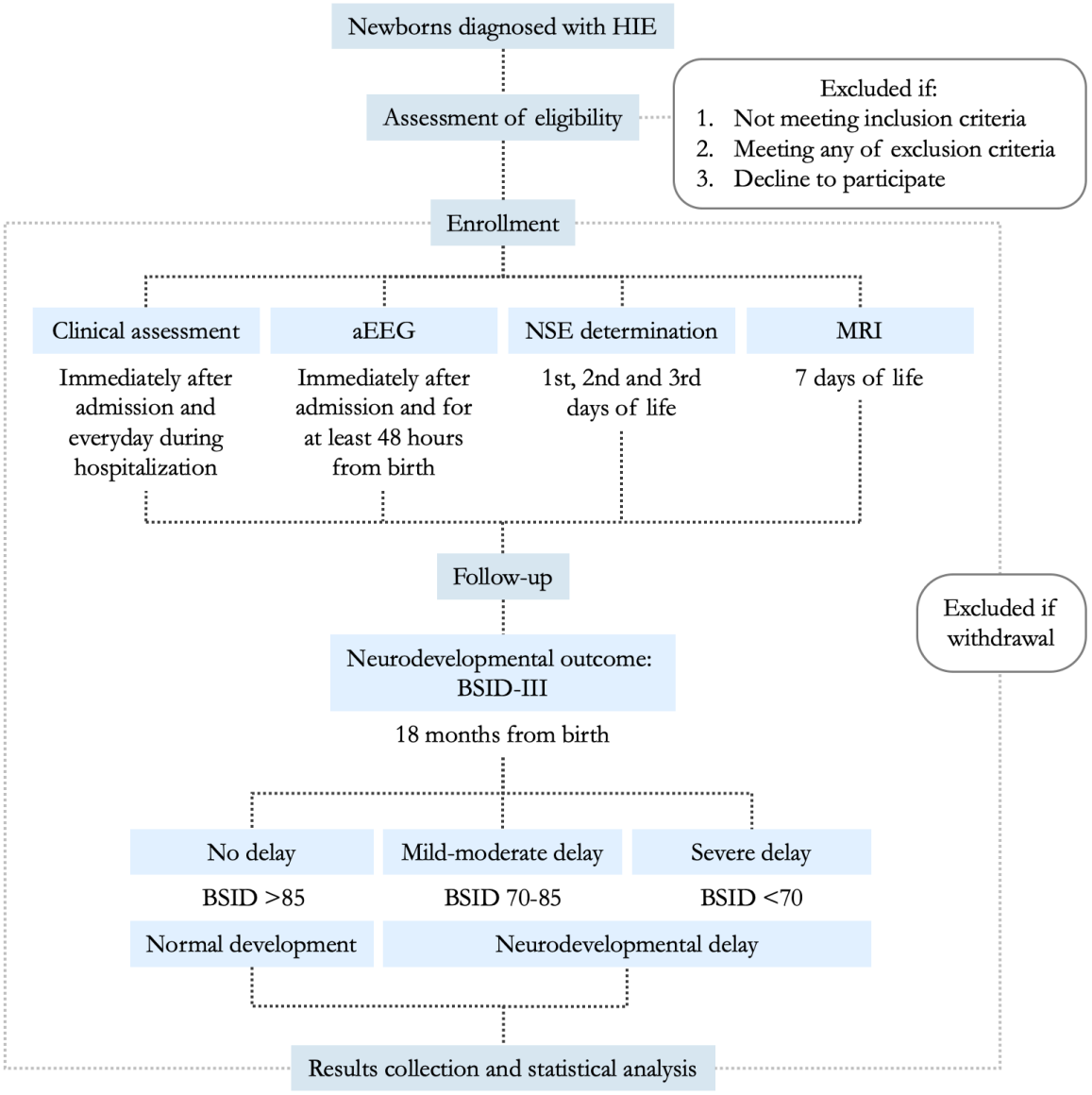


Figure 5. **Study flow chart**.

3.5. Statistical analysis

Statistical analyses will be conducted by a specialized statistician using the Statistical Package for Social Sciences (SPSS), version 29.0.1. All reported results will be considered to be statistically significant for P-values of < 0.05, and 95% confidence intervals (95% CIs) will be included.

3.5.1. Descriptive analysis

Firstly, a descriptive analysis will be performed and included in a table (see **Annex 8**), varying depending on whether they are qualitative or quantitative.

Regarding perinatal, neurological and outcome findings, variables will be summarized as follows. On the one hand, qualitative variables, including reference standard (neurodevelopmental outcome), prognostic covariables (clinical assessment, all the aEEG variables, MRI-moderate to severe injury and MRI-pattern of injury), and the rest of covariables, will be summarized with absolute and relative frequencies (i.e. proportions). On the other hand, quantitative variables will be summarized with median and interquartile range (IQR), when they follow an asymmetrical distribution (such as MRI-Rutherford scale), or with mean and standard deviation, when they follow a symmetrical distribution (such as NSE serum levels).

3.5.2. Bivariate inference

The difference of proportions of the qualitative variables (including neurodevelopmental outcome, clinical assessment, all aEEG variables, MRI-moderate to severe injury and MRI-pattern of injury) among groups defined by newborns with normal neurodevelopment, those with mild to moderate delay, and those with severe delay, will be tested using the Chi-squared test or Fisher's exact test (if the expected cases in a cell will be lower than 5).

The difference of medians of the quantitative discrete variables (such as MRI-Rutherford scale) and the difference of means of quantitative continuous variables (such as NSE serum levels) will be tested using the ANOVA test and the Kruskal-Wallis test.

Additionally, stratification by the covariables will be done and quantitative variables will be categorized in quartiles.

3.5.3. Cut-off and validity

In order to obtain a normal distribution and to achieve homogeneity of variance, serum NSE levels will be log-transformed. To establish the optimal cut-off value for serum NSE, defined as the highest point of sensitivity multiplied by specificity in predicting neurodevelopmental outcome, a receiver operating characteristic (ROC) curve analysis for serum NSE levels will be applied.

From this analysis, a cut-off point will be determined, corresponding to the serum NSE value that distinguishes between infants with neurodevelopmental delay and those with a normal neurodevelopment. Subsequently, a cross-tabulation table will be generated to compute the sensitivity and specificity of the serum NSE value in discerning the neurodevelopmental outcome, as seen in **Table 5** below.

For the following concepts, an unfavorable neurodevelopmental outcome will be understood either as mild, moderate or severe neurodevelopmental delay (indicating a BSID score < 85), whereas a favorable neurodevelopmental outcome will be understood as normal neurodevelopment (indicating a BSID score > 85).

- True positive (TP): subjects with higher NSE values presenting an unfavorable outcome.
- False positive (FP): subjects with higher NSE values presenting a favorable outcome.
- True negative (TN): subjects with lower NSE values presenting a favorable outcome.
- False negative (FN): subjects with higher NSE values presenting an unfavorable outcome.

		Neurodevelopmental outcome (BSID-III score)	
		Unfavorable outcome (delay)	Favorable outcome (normal)
NSE serum level	Higher NSE values	TP	FP
	Lower NSE values	FN	TN

Table 5. **Cross tabulation of main variables in 2x2**, showing results of neurodevelopmental outcome in columns measured through BSID-III (unfavorable or favorable outcome), and NSE serum level in rows (high or low NSE values).

Therefore, taking the previous table as a reference, the internal validity of this study is measured with sensitivity and specificity; on the contrary, external validity will be analyzed with positive predictive value and negative predictive value, defined as follows:

- Sensitivity (S): ability to predict an unfavorable outcome, defined as: **S = TP / (TP + FN)**.
- Specificity (Sp): ability to predict a favorable outcome, defined as **Sp = TN / (TN + FP)**.
- Positive predictive value (PPV): probability of an unfavorable outcome in subjects with higher NSE values, defined as: **PPV = [S x P] / [S x P + (1-Sp) x (1-P)]**, where P stands for prevalence.
- Negative predictive value (NPV): probability of a favorable outcome in subjects with lower NSE values, defined as: **NPV = [Sp x (1-P)] / [Sp x (1-P) + (1-S) x P]**, where P stands for prevalence.
- Area under the ROC curve (AUC): helps to estimate how high is the discriminative power of NSE values in the neurodevelopmental outcome. The AUC is represented through **(1-Sp)** in the X-axis and **(S)** in the Y-axis. It must be reminded that:
    - A perfect diagnostic test has an AUC of 1.0.
    - A non-discriminating test has an AUC of 0.5.

- Likelihood ratio (LR):
    - For a positive test (LR+): likelihood of having higher NSE values in subjects with an unfavorable outcome, defined as: **LR+ = S / (1 - Sp)**. It must be remembered that a good diagnostic test has a LR+ > 10.
    - For a negative test (LR-): likelihood of having lower NSE values in subjects with a favorable outcome, defined as: **LR- = (1-S) / Sp**. It must be remembered that a good diagnostic test has a LR- < 0.1.
- Diagnostic odds ratio (DOR): ratio of the odds of individuals with an unfavorable outcome (higher NSE values) relative to the odds of individuals with a favorable outcome (lower NSE values), defined as: **DOR = (TP/FN) / (FP/TN)**.
- Diagnostic efficiency (**accuracy**): proportion of correctly classified individuals among all individuals, defined as: **A = (TP+TN) / (TP+TN+FP+FN)**. It must be reminded that this parameter is affected by the disease prevalence.

Furthermore, to better understand the relationship between the main variables –the serum NSE level (a continuous quantitative variable) and the outcome (a categorical ordinal qualitative variable categorized as normal, mild-moderate delay, and severe delay)– they will be visually represented using two types of plots. Firstly, a box plot will be employed to illustrate the distribution of serum NSE levels across different outcome categories, providing insights into central tendency, spread and potential outliers. Additionally, a scatter plot will be utilized, with different shapes representing the various outcome categories, aiming to explore if there seems to be a discernible linear relationship between the serum NSE level and the neurodevelopmental outcome categories.

3.5.4. Multivariate analysis

Linear regression models, controlling for all the covariables, will be employed to assess the relationship between serum NSE levels and the following variables:

- HIE severity, categorized as mild, moderate, or severe.
- Time of serum NSE level determination, categorized as 24, 48, and 72 hours of life.

To control confounding, a Poisson regression will be carried out for the association of MRI-Rutherford scale with the neurodevelopmental development dichotomized.

Finally, logistic regressions, controlling for all the covariables (regarding perinatal, newborn, treatment, clinical evolution and hospital variables) for the association between dichotomous variables (such as clinical assessment, all the aEEG variables, MRI-moderate to severe injury and MRI-pattern of injury) and the dichotomized neurodevelopmental outcome, will be estimated.

3.6. Ethical and legal considerations

3.6.1. Ethical principles

The study will adhere to the requirements established by the World Medical Association (WMA) in the Declaration of Helsinki principles for Medical Research Involving Human Subjects (October 2013) and the Spanish law concerning medical investigation on observational studies "**Ley 14/2007, de 3 de Julio, de investigación biomédica**" [52].

The ethical principles of Beauchamp and Childress will be respected as follows:

1) Autonomy: patients' autonomy will be ensured by providing a written information sheet (see **Annex 5**) to the parents or legal representatives, and an informed consent form (see **Annex 6**) will be obtained upon admission of each infant. The explanation of the study and its implications will be provided before its onset, ensuring they understand, and are free to refuse entry, and can withdraw whenever they wish without prejudice. The decision to participate will be respected, in line with "**Ley 41/2002, de 14 de noviembre, básica reguladora de la autonomía del paciente y de derechos y obligaciones en materia de información y documentación clínica**" [53]. Only those who sign the consent form will be, and these documents will be submitted in advance for formal approval by the ethics committee .

2) Non-maleficence and beneficence: patients meeting exclusion criteria will be excluded from the study, as they will not benefit from the study procedure. In contrast, patients meeting inclusion criteria will be selected to participate in this study.

3) Justice: all eligible patients who meet inclusion criteria and none of the exclusion criteria, and who have signed the consent form, will be considered equally for participation. This ensures a homogeneous and non-discriminatory approach, promoting equality among individuals.

3.6.2. Privacy and confidentiality

The privacy and confidentiality of all study patients will be guaranteed through by complying with the following laws: "**Ley 41/2002, de 14 de noviembre, básica reguladora de la autonomía del paciente y de derechos y obligaciones en materia de información y documentación clínica**" [53], "**Ley Orgánica 3/2018, de 5 de diciembre, de Protección de Datos Personales y garantía de los derechos digitales**" [54] and "**Regulation (EU) 2016/679 of The European Parliament and of the Council of 27 April 2016 on the protection of natural persons with regard to the processing of personal data and on the free movement of such data**" [55].

To achieve this, all collected data from patients will be anonymous and confidential. Patients will be identified through a randomized code and placed on a secure network accessible only to the research team. This data will be used exclusively for research purposes.

3.6.3. Comitè d'Ètica d'Investigació Clínica

This preliminary study will be submitted to the Comitè d'Ètica d'Investigació Clínica (CEIC) of each hospital for evaluation and approval before the study begins. Any suggestions will be considered, and approval from the ethics committee is mandatory before commencing the study.

3.7. Work plan

3.7.1. Research team members

The study will be conducted in the following six hospitals: Hospital Universitari Josep Trueta (Girona), Hospital Sant Joan de Déu (Barcelona), Hospital Universitari Vall d'Hebron (Barcelona), Hospital Universitari Germans Trias i Pujol (Barcelona), Hospital Universitari Parc Taulí (Barcelona) and Hospital Universitari Joan XXIII (Tarragona). The study team will be consist of the following group of professionals, as summarized in **Figure 6** below:

1) **1 principal investigator**, responsible for protocol elaboration, study oversight, and typewriting of the article, information sheet, informed consent form, and data collection sheet. The principal investigator will also be responsible for data collection –clinical assessment, aEEG recording, NSE levels and MRI findings– and presenting it to the statistician.
2) **13 pediatricians**, divided in groups as follows:
    a) **6 pediatricians** (1 in each hospital's NICU), responsible for explaining the study's purpose to the newborn's legal representatives, providing the information sheet and informed consent form, and filling out the data collection sheet for recruited patients.
    b) **6 pediatricians** (1 pediatrician in each hospital's NICU), blind to history and clinical data, who will conduct clinical assessment from admission and daily during the patient's hospitalization and perform neurodevelopmental assessment at 18 months of age.
    c) **1 pediatrician** from HUJT, masked to history and clinical data, who will assess aEEG monitoring (regarding background pattern, sleep-wake cycling and seizures).
3) **6 laboratory technicians** (1 in each hospital), responsible for determining NSE serum levels through immunoassay equipment, blind to history and clinical data.
4) **2 radiologists** from HUJT, masked to history and clinical data, who will assess MRI images, regarding regions of interest (BGT, PLIC, WM and cortex) and the level of injury or damage.
5) **1 statistician** from Institut d'Investigació Biomèdica de Girona (IDIBGI), responsible for performing statistical analysis.

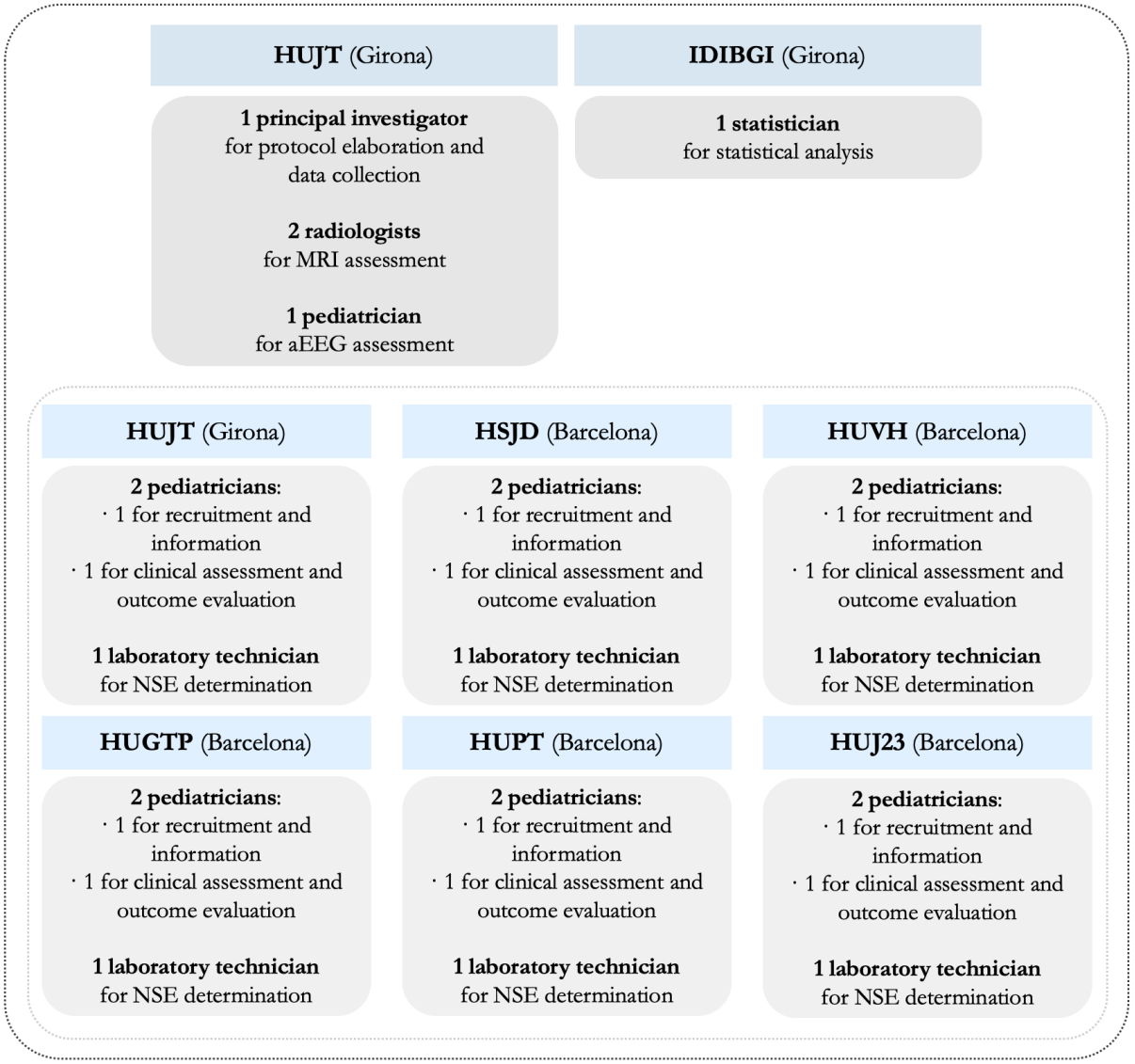


Figure 6. **Research team members**

3.7.2. Study stages

This study will last approximately 3 years, from November 2023 to May 2027, and will be divided into the following stages:

Stage 0: Protocol elaboration and study design (2 months, November 2023–December 2023)

This stage, primarily overseen by the principal investigator, will take approximately 2 to 3 months. It will involve an extensive literature review and bibliographic research, as well as the determination of the study's hypothesis and objectives for an adequate protocol design. Additionally, during this stage, the participating hospitals will be contacted and confirmed.

Stage 1: Ethical evaluation and approval (2 months, January 2024–February 2024)

Following protocol design, the protocol will undergo evaluation and acceptance by the CEIC of HUJT, HSJD, HUVH, HUGTP, HUPT, and HUJ23. During this period, any suggestions will be considered, and modifications will be made accordingly.

Stage 2: Coordination and training (1 month, March 2024)

Upon approval by the hospital's CEIC, training workshops will be conducted for all participating professionals, including pediatricians, radiologists and laboratory technicians, to standardize intervention and prevent bias.

Stage 3: Subject recruitment and data collection (1 year, April 2024–March 2025)

The patient recruitment and data collection period will last approximately 1 year.

During this period, newborns diagnosed with HIE admitted to each of the hospital's NICU participating in the current study, meeting the inclusion criteria and none of the exclusion criteria, will be informed and invited to participate by the corresponding pediatrician (referred to as Pediatrician 1). If they agree, an information sheet and an informed consent form will be provided to the legal representatives, ensuring that they are properly informed and decide voluntarily whether they want to participate. Only the newborns whose legal representatives have signed the informed consent form will be included; those without signed consent form will be excluded. After recruiting the study participants and ensuring they understand the study's purpose, the corresponding investigator will fulfill the data collection sheet.

Throughout hospitalization, patients will receive management and treatment following guidelines and protocols from each hospital. During this period, the corresponding pediatrician (referred to as Pediatrician 2), distinct from Pediatrician 1 and masked to clinical data, will conduct clinical assessments from admission and daily during hospitalization.

Also, as per protocols, routine blood samples will be collected at specified time points (6, 12, 24, 48,72 and 96 hours) and analyzed. The laboratory technician from each hospital will determine serum NSE levels on the first (1-day NSE), second (2-day NSE) and third (3-day NSE) days of life using immunoassay equipment.

Additionally, aEEG recordings (initiated immediately upon admission and continuing for at least 48 hours of life) and MRI images (at 7 days of life) will be performed as part of routine clinical practice and management, but will not be analyzed by the researchers at this stage.

The analysis of aEEG recordings and MRI images will take place once the recruitment period concludes, approximately 1 year from the start of the recruitment period. aEEG and MRI assessment will be conducted by the corresponding pediatrician from HUJT (referred to as Pediatrician 3) and two radiologists, also from HUJT, respectively.

Stage 4: Follow-up (1 year, October 2025–September 2026)

The follow-up will occur at 18 months of age for each patient, resulting in a total period of approximately 2 to 2 and a half years between patient recruitment and total data collection.

During this phase, patients will undergo evaluation at the center where they were initially treated. The corresponding pediatrician (specifically, Pediatrician 2) will conduct the outcome evaluation through neurodevelopmental assessment, staging infants into categories of normal development, mild to moderate delay, or severe delay through BSID-III scoring system.

Stage 5: Data analysis (3 months, October 2026–December 2026)

The principal investigator will consolidate all collected data, including clinical assessments, aEEG recordings, NSE levels, and MRI findings. Subsequently, statistical analysis related to NSE levels in reference to neurodevelopmental outcomes will be conducted by a specialized statistician from IDGIBI. Following the analysis, the results will be presented to the principal investigator for discussion and conclusions.

All researchers involved in the study will meet to discuss the statistical data. A thorough interpretation and discussion of the findings will contribute to a meaningful conclusion.

Stage 6: Results publication (5 months, January 2027–May 2027)

Finally, the results will be compiled and authored in an article by the principal investigator. The article will undergo further editing and will be submitted for publication within an estimated period of 3 months. Simultaneously, the project results and conclusions will be submitted to scientific journals, the Sociedad Española de Neonatología (SENEO) and the European Society of Paediatric and Neonatal Intensive Care (ESPNIC) for publication. The findings and their derived conclusions will also be presented at specific national and international conferences.

3.7.3. Chronogram

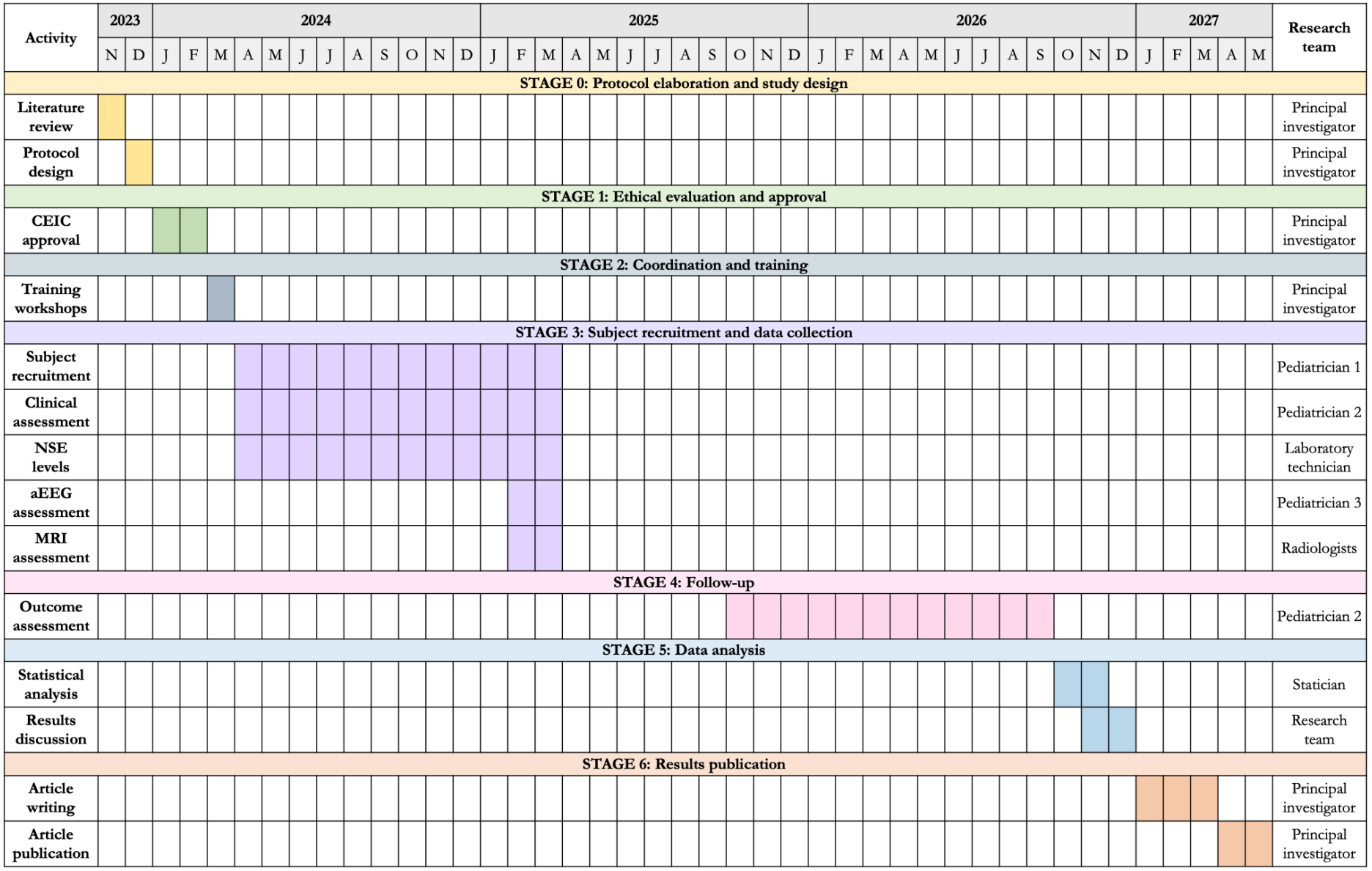


Figure 7. **Chronogram**

3.8. Budget

The study's budget includes personnel, material, and dissemination expenses, as seen in **Table 6**.

3.8.1. Personnel expenses

The main research team consists of pediatricians, radiologists, and laboratory technicians working in the hospitals participating in the study. Additionally, most of the activities outlined in the work plan are integrated into the routine clinical care provided by hospital professionals for the appropriate management and treatment of newborns with HIE. Therefore, none of these activities will imply any additional cost.

Regarding pediatricians, providing information to parents, conducting clinical assessments, monitoring and analyzing brain function through aEEG, collecting serial blood samples, and performing MRIs are actions routinely carried out in clinical practice. Moreover, these actions are recommended in clinical practice guidelines and specified in hospital protocols. Hence, they would not result in any extra charges.

For laboratory technicians, the determination of NSE is not a routine test but is performed upon request by specialists. Therefore, it also would not incur any additional cost.

As for radiologists, their primary task is to analyze and characterize the findings observed in the images, specifically those obtained from MRI. In particular, the evaluation of areas of interest (BGT, PLIC, WM, and cortex) is already part of the routine description of images in newborns with HIE. Consequently, this would not lead to any additional charges.

Furthermore, all participants in the research team are not expected to receive extra payment, as the motivation to be part of the study is considered to be driven by scientific prestige and intellectual gains rather than economic incentives.

Finally, a qualified statistician from IDGIBI will be hired to perform the statistical analysis. Considering a rate of 40€ per hour, working approximately 60 hours per month for two months, the estimated cost for the statistician is around 5,000€.

3.8.2. Material expenses

Within the material costs, printing expenses and the determination of serum NSE are included. On the one hand, printing costs include the information sheet, informed consent form, and data collection sheet. Approximately 100 double-sided copies of each document will be printed, taking into account the recruitment of approximately 90 patients and allowing for potential losses or errors. Therefore, for an approximate cost of 0,10€ per copy, the total cost amounts to 30€.

On the other hand, the determination of NSE in serum requires specific immunoassay kits, which are already available at the hospital, given that this biomarker is determined in specific cases in adults (such as cardiac arrest or stroke), so there would be no need to buy any additional equipment. Since the NSE determination will be performed three times for each patient (1-day, 2-day and 3-day NSE), with approximately 90 patients, this results in a total of 270 NSE determinations. As it is a test already included under request, NSE determination will not result in any extra cost.

3.8.3. Results dissemination costs

In this section, publication fees and congress registration will be taken into account.

On the one hand, a publication fee of 1,000€ should be considered for publishing an article in a journal, such as SENEO or ESPNIC.

On the other hand, it is necessary to anticipate a registration fee for congresses, approximately 450€ for attending national conferences like the SENEO/SEEN congress, and around 600€ for attending international conferences, such as the ESPNIC congress.

Item		Amount	Cost	Subtotal	Total
Personnel expenses					
Research team (pediatricians, radiologists and laboratory technicians)		-	0 €	0 €	4,800 €
Statistician		60hours/month 2 months	40€/hour	4,800 €	
Material expenses					
Printing material (information sheet, informed consent form, data collection sheet)		300	0,10€/copy	30 €	30 €
NSE determination		270	0 €	0 €	
Results dissemination costs					
Publication fees		2	1,000 €	2,000 €	4,100 €
Congresses registration	SENEO/SEEN congress	2	450 €	900 €	
	ESPNIC congress	2	600 €	1,200 €	
					8,930 €

Table 6. **Budget summary regarding personnel, material and results dissemination costs**.

3.9. Limitations and strengths

3.9.1. Limitations

The present study presents several limitations that need to be acknowledged.

Firstly, there is a selection bias due to the small and non-probabilistic recruitment of the sample chosen in this research, given that it is designed as a preliminary study. However, the present study might serve for a future larger multicentric study that would allow for more precise and significant estimations and conclusions, making it more representative of the study population.

Additionally, as this is a multicenter study, there may be differences in the assessment of tests, especially those that are more subjective and dependent on the examiner, such as clinical assessment, aEEG assessment, or neurodevelopmental assessment, as these will be evaluated by different pediatricians in different hospitals. Moreover, there could be errors in data collection and information filling in the data collection sheet. To minimize inter-observer variability, all investigators participating in the study should be adequately informed and trained about the foundations or references on which such evaluations will be based, aiming for maximum uniformity. Regarding this inter-observer variability, the assessment of MRI images can also be somewhat subjective. Therefore, it has been established that the evaluation will be conducted by 2 specialist radiologists who will assess the images simultaneously to avoid subjectivity and inter-observer variability.

In addition, the study may be affected by some of the covariables explained earlier, which, although not the main focus of the study, will be studied and evaluated to draw correct conclusions and possibilities for improvement in future studies.

There are also some potential limitations regarding the variability of the study concerning the determination of NSE, which are not the subject of the study but should be considered. One of them is that the blood concentration of NSE depends on the permeability of the blood-brain barrier (BBB). However, it is true that NSE is released into the blood due to a combination of brain injury and disruption of the BBB caused by hypoxic-ischemic insult [36,41]. Thus, elevated NSE levels are associated with poor neurological prognosis.

Finally, certain technical confounding factors [43], not under investigation, such as sample processing, the effect of blood hemolysis, and concentration reduction due to storage time, should be considered in future studies.

3.9.2. Strengths

Prior to this study, there are no investigations which specifically study serum NSE values as a prognostic predictor for neurodevelopmental outcomes.

There is a recent study [43] that explores the association between NSE values in CSF and the neurodevelopmental prognosis. The present study aims to conduct a similar investigation, but focusing on serum NSE values, avoiding the limitations and potential risks associated with lumbar puncture for the NSE determination in CSF.

Another recent study [23] examines the serum biomarker but assesses the prognostic capacity of serum NSE values, aEEG, and MRI collectively —not specifically focusing on the biomarker alone— to predict neurodevelopmental outcomes in newborns with HIE. Additionally, this study is conducted in China, involving a population that differs from the European population. Hence, this study aims to adjust the results to our specific population.

Therefore, this preliminary study has been designed to investigate the prognostic capacity of serum NSE to predict favorable or unfavorable neurodevelopmental outcomes. Depending on the results, it will enable for a larger-scale research to conduct further investigation on this topic, involving more patients, hospitals, and researchers to draw more significant conclusions.

In addition, as an added value, the implementation of this study does not entail additional risks for patients (unlike lumbar puncture for NSE determination in CSF, for example). It also does not require a significant additional workload for specialized pediatricians in clinical practice since it only involves adding the determination of the biomarker to routine blood tests that are already performed. Lastly, it does not involve acquiring new or specific materials, nor the need for specialized personnel for analysis, as the determination of NSE in serum is already performed in hospital laboratories (in adults, for example) as an additional test in routine biochemistry.

3.10. Health impact

It should be emphasized that the birth and hospitalization of a newborn with perinatal asphyxia resulting in hypoxic-ischemic encephalopathy constitute an unexpected, stressful and traumatic experience for parents.

As previously mentioned, providing parents with a clear prognosis is not only their right but also an essential aspect of medical practice. Estimating prognosis allows for anticipation of the newborn's clinical evolution and potential challenges during childhood, particularly regarding neurodevelopmental outcomes. Additionally, it enables medical professionals to offer guidance to parents about the likely trajectory of the condition.

Prognosis estimation is fundamental for decision-making regarding treatment, facilitates individualized planning for both short and long-term follow-up, and helps establish potential needs or rehabilitation interventions.

In conclusion, prognosis estimation in HIE is an important aspect of medical care with implications for parents, medical professionals, and the well-being of the newborn. It serves as a guiding tool for planning, decision making, and providing care throughout the management of HIE.

As mentioned earlier, predicting the prognosis in patients with HIE is not straightforward and is not based on a single test but rather on a combination of them. Its strength increases as more items are added to its assessment. Specifically, clinical evaluation, aEEG, or MRI alone do not provide a very clear prognosis, but when considered together, they allow for a more accurate prognosis.

Therefore, adding the determination of NSE as a prognostic predictor would further enhance the accuracy of the prognosis, enabling adjustments in the management and treatment in clinical practice. This would also provide parents or legal representatives with more informed insights regarding their children, for both in short and long-term evolution and outcome.

3.11. Feasibility

This study is considered feasible to conduct , as there are no major obstacles:

1) The planned 1-year duration is deemed sufficient to recruit the required number of patients from the 6 participating hospitals..
2) The 6 participating hospitals provide the necessary tools and experience needed to facilitate the conduct of the study.
3) Utilizing SAP and REDCap systems provides structured databases, ensuring that all clinical information and imaging studies are easily uploaded and shared, meeting the data requirements of the study.
4) The study will be executed by a team of specialists, including:
    a) 1 principal investigator guiding the overall study.
    b) 13 pediatricians responsible for clinical, aEEG, and outcome assessment, following predefined guidelines for uniform and homogeneous evaluations.
    c) MRI assessments will be carried out simultaneously by 2 radiologists to minimize subjectivity and inter-observer variability.
    d) NSE determination will involve 6 laboratory technicians using immunoassay equipment.
    e) Statistical analysis will be performed by an experienced statistician from IDGIBI using the SPSS program.
5) The planned duration of 2 to 3 years is designed to prevent the emergence of new studies with similar objectives during the study, ensuring its completion before it becomes obsolete.

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Annexes

Annex 1

1.1. Categorical grading for HIE

Gravity		Mild	Moderate	Severe
Clinical signs	**Alertness**	Normal	Lethargy or moderate stupor	Serious stupor or coma
	Muscular tone	Hypotonia (mostly superior proximal distribution) or hypertonia	Hypotonia (mostly superior proximal distribution)	Serious global hypotonia or flaccid
	Motor response	Normal or slightly diminished	Diminished, normal quality	Absent or stereotyped
	Reactivity	Normal or hiperexcitability: exaggerated myotatic reflexes, tremor, shakes	Diminished myotatic reflexes, altered primitive responses	Mostly absent myotatic reflexes and primitive responses
	Signs of brainstem dysfunction	Absent	Absent	Present or absent
	Clinical seizures	Absent	Present or absent	Present or absent
aEEG items	**Baseline tracing**	Normal (CNV) or moderately altered (DC)	Moderately altered (DC) or seriously altered (BS, CLV, FT)	Seriously altered (BS, CLV, FT)
	Sleep-wake cycles	Present or absent	Absent	Absent
	Electric seizures	Absent	Present or absent	Present or absent

Table 7. **Categorical grading for HIE**. Adapted from García-Alix A., Arnáez, J., Neurología Neonatal de un Vistazo, Ed Cabeza de Chorlito; 2022 [21]

*aEEG registration refers to the worst aEEG registration recorded between 30 minutes and 1 hour before clinical evaluation.

1.2. Numerical grading for HIE

ALERTNESS: apply the stimuli progressively increasing the intensity, leaving sufficient time between each application to fully observe the patient's reaction				
0	**2**	**4**	**6**	**8**
Wakes without difficulty and remains alert for more than 30s	Wakes with mild difficulty in response to non-nociceptive stimuli. Alertness is slightly reduced (7-30 s). Crying gradually subsides without requiring consolation	Clear difficulty in waking up to nociceptive stimuli. Upon awakening, alertness is maintained for a few seconds (<6 s). If crying occurs, it abruptly ceases	Wakes up with significant difficulty in response to nociceptive stimuli and quickly returns to sleep upon discontinuation of the stimulus. Does not cry	Does not awaken in response to nociceptive stimuli
POSITION (MUSCULAR TONE): with the patient in supine position, observe the position of the legs and arms				
0	**2**	**4**	**6**	**8**
Appropriate flexion and adduction of the limbs	Poor flexion and adduction of the upper limbs	Poor flexion and adduction of the upper and lower limbs	Severe hypotonia or tonic posture (not persistent)	Flaccid posture or maintained tonic posture (decerebration or decortication)
SPONTANEOUS MOTOR ACTIVITY: without applying stimuli to the patient, observe their pattern of spontaneous movements concerning whether it involves different parts of the body, if the movements occur in different directions and speeds (complexity and variability), and if there appears to be continuity between the movements (fluidity)				
0	**2**	**4**	**6**	**8**
Fluid, variable, and complex movements	Fluid and variable movements. There are excessive tremors and jerks	Diminished, monotonous with poor variability and complexity	Very decreased activity	No activity or continuous tremor at rest
MOTOR RESPONSE TO STIMULI: observe the motor response when applying stimuli of progressive intensity				
0	**2**	**4**	**6**	**8**
Alternating and vigorous limb movements	Normal motor response, but limited movements	Withdrawal movements that extend beyond the stimulated limb	Withdrawal movements limited to the stimulated limb	Absent or stereotyped activity, reminiscent of decorticate or decerebrate postures
MIOTATIC REFLEXES: observe the ease of response, amplitude, and extension of the reflexogenic zone				
Patellar reflex Position the knee slightly flexed and tap the tendon		**Adductor reflex** Place a finger on top of the tendon and strike above		**Aquiline reflex** Strike the finger placed on the plantar surface of the foot
0	**2**	**4**	**6**	**8**
Normal	Hyperactive	Hypoactive	Absent	Immediate response without latency or habituation
RESPIRATORY PATTERN				
0	**2**	**4**	**6**	**8**
Spontaneous or Kussmaul	-	Periodic breathing	-	Central hyperpnea, apneustic, Biot, ataxic, or apnea
CLINICAL SEIZURES				
0	**2**	**4**	**6**	**8**
Absent	-	-	Single (≤1/h)	Repeated (>1/h) or status
ELECTRICAL SEIZURES IN aEEG: with suspicion of seizures in the integrated by amplitude tracing, it is mandatory to confirm the pattern of waves in the unprocessed EEG line for at least 10 seconds, supporting the diagnosis of a seizure				
0	**2**	**4**	**6**	**8**
Absent	-	-	Single (≤1/h)	Repeated (>1/h) or status
BACKGROUND PATTERN IN aEEG: Observe the pattern of the background tracing and the upper and lower margins of amplitude (voltage) of activity in the EEG				
0	**2**	**4**	**6**	**8**
CNV, sleep-wake cycles	CNV, no sleep-wake cycles	DC	BS	CLV or FT

Table 8. **Numerical grading for HIE**, where a total score of 8 points differentiates mild and moderate HIE, and 30 points differentiate moderate and severe HIE. Adapted from García-Alix A., Arnáez, J., Neurología Neonatal de un Vistazo, Ed Cabeza de Chorlito; 2022 [21]

Annex 2

2.1. Assistance to the newborn with perinatal asphyxia

As a summary, all the steps for monitoring and managing a newborn with perinatal asphyxia or hypoxic-ischemic encephalopathy are summarized in the following figures.

Figure 8 summarizes the sequential steps and stages to be completed in the first 6 hours of life in a newborn with possible HIE. **Figure 9** explains the chain of cerebral neuroprotection (more specific for hypothermia code), and **Figure 10** summarizes all the steps to be followed as time progresses from the moment of birth in a newborn with HIE.

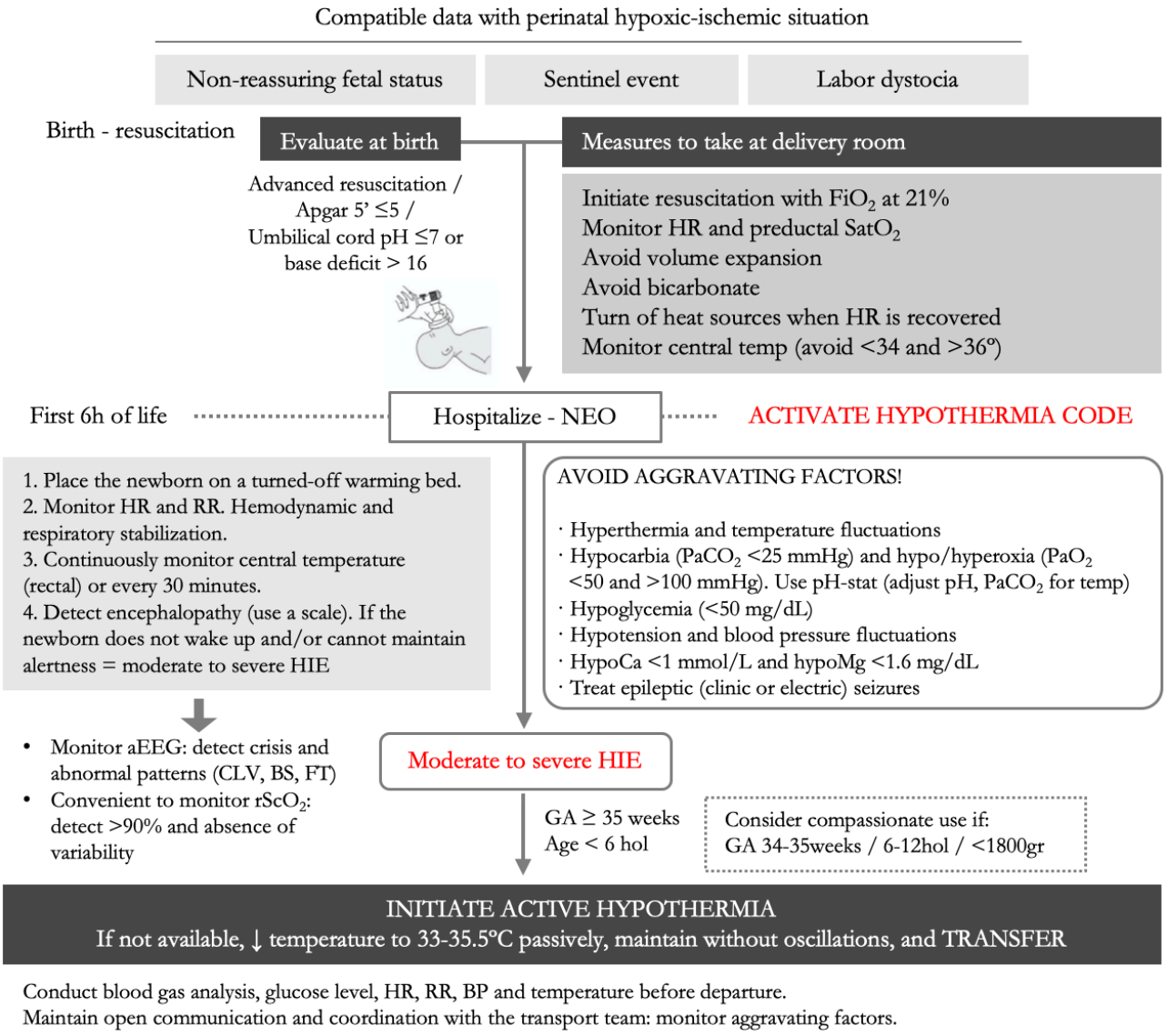

Figure 8. **Protocol of actuation in the first 6 hours of life**. Adapted from García-Alix A., Arnáez, J., Neurología Neonatal de un Vistazo, Ed Cabeza de Chorlito; 2022 [21]

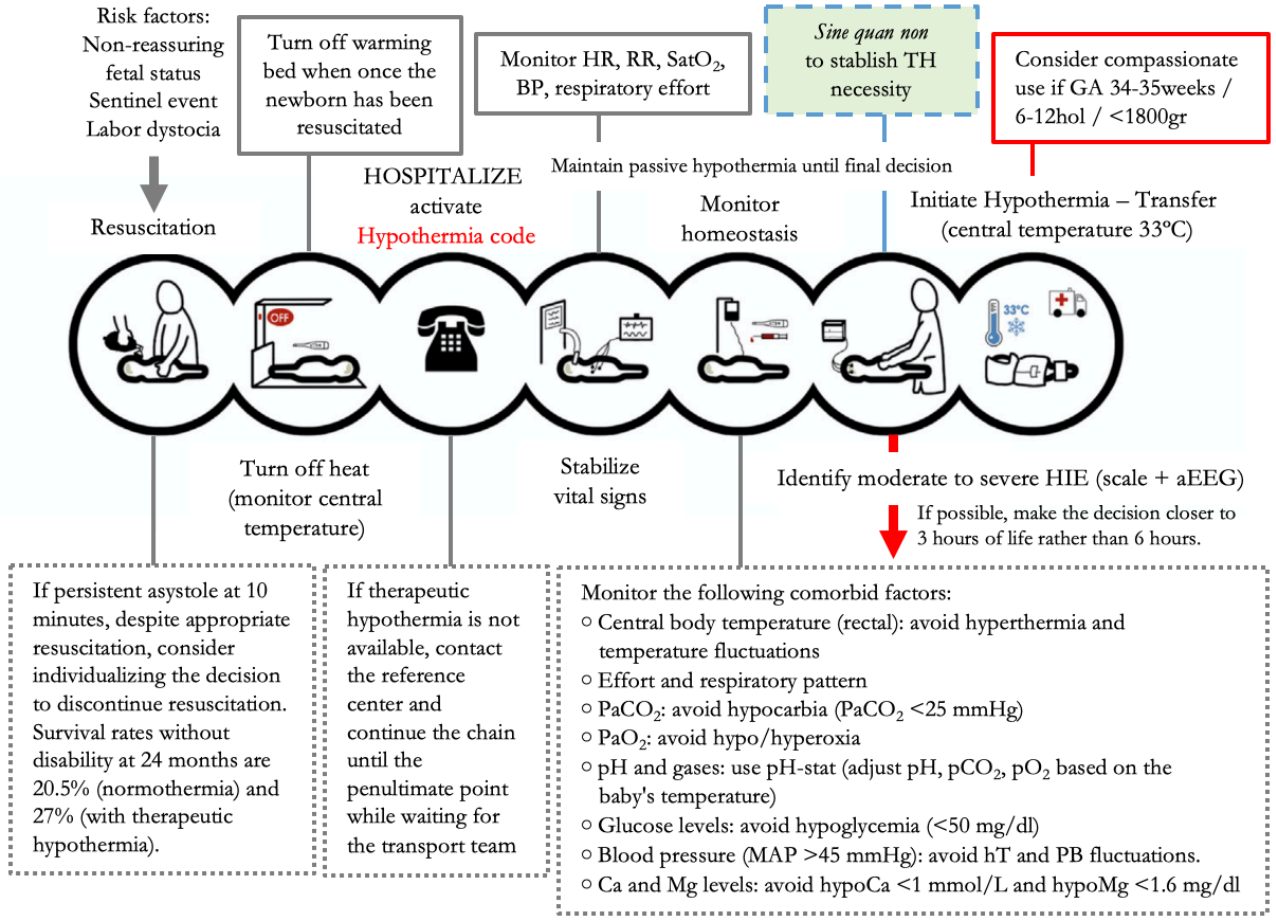

Figure 9. **Cerebral neuroprotection chain**. Modified from García-Alix A., Arnáez, J., Neurología Neonatal de un Vistazo, Ed Cabeza de Chorlito; 2022 [21]

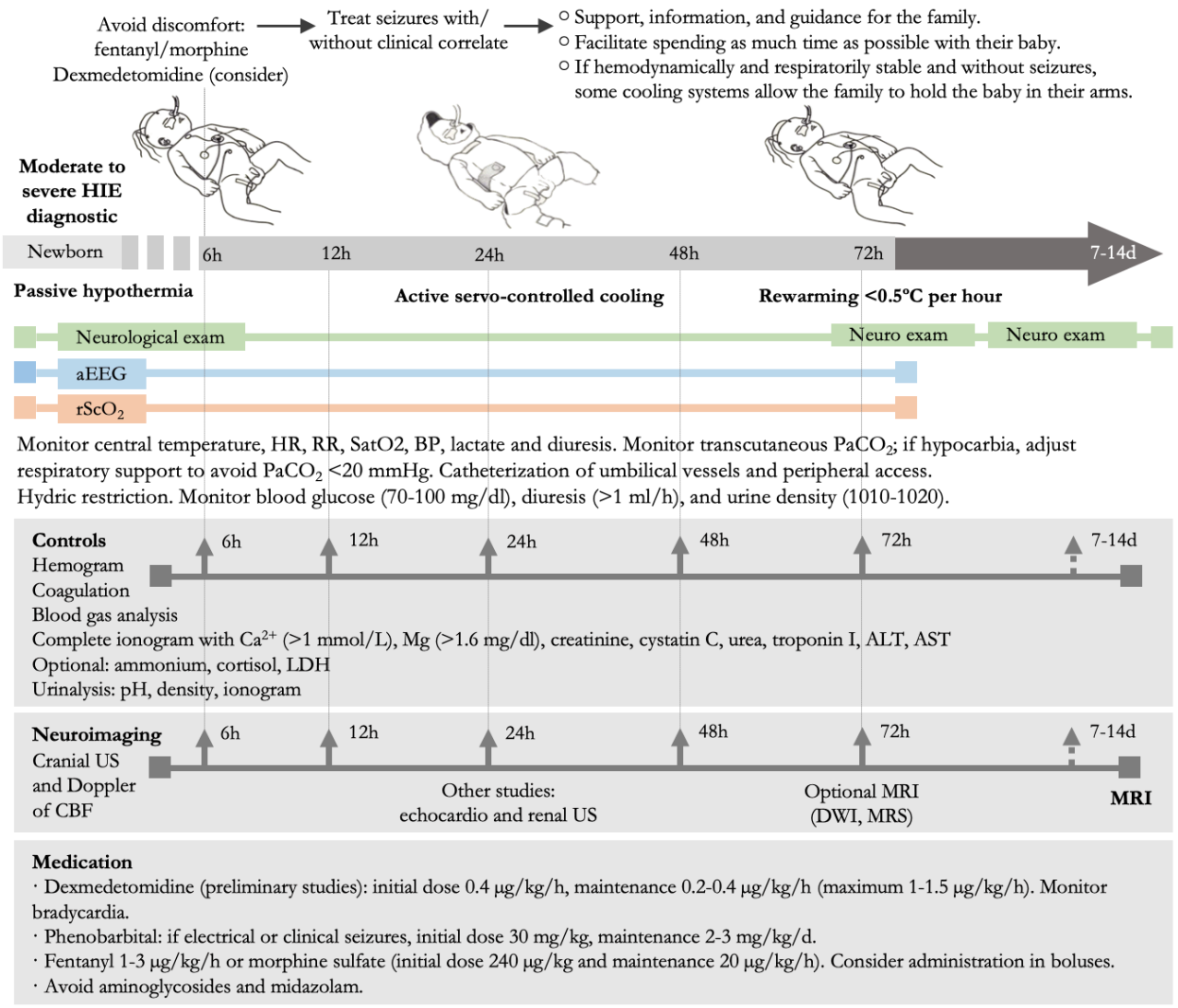

Figure 10. **Management and control of moderate to severe HIE**. Modified from García-Alix A., Arnáez, J., Neurología Neonatal de un Vistazo, Ed Cabeza de Chorlito; 2022 [21]

Annex 3

3.1. Interpretation and classification of aEEG recordings

The classification and interpretation of aEEG tracings have been described in various studies. The proposal by Toet M. et al. [56,57] is the most widely used in textbooks and publications. This classification is based on the visual recognition of patterns and the amplitude of the tracing. In aEEG, we can assess three main factors: the baseline tracing, sleep-wake cycling and seizures.

3.1.1. Baseline tracing

The baseline tracing is the predominant type of electrocortical activity in the aEEG recording.

A) **Continuous normal voltage** (CNV): continuous activity with a minimum amplitude around 5 μV and a maximum amplitude of 25 μV.

This corresponds to the normal trace of a full-term newborn.

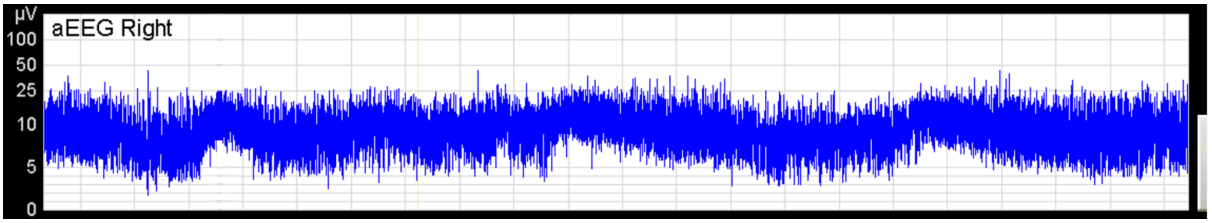

Figure 11. **Continuous normal voltage pattern**. Extracted from Bruns et al., Application of an Amplitude-integrated EEG monitor (Cerebral Function Monitor) to neonates. J Vis Exp. 2017;(127)1–9 [58].

B) **Discontinuous** (DC): discontinuous background with variable minimum amplitude, predominantly below 5 μV and maximum amplitude above 10 μV.

This trace is considered moderately altered because it can appear in a normal preterm infant, in an infant receiving sedation or anticonvulsant treatment. This trace is often considered transient towards normality (if medication is withdrawn or the child continues to mature his brain function) or towards a severely pathological trace (as it can appear in the initial phases of HIE).

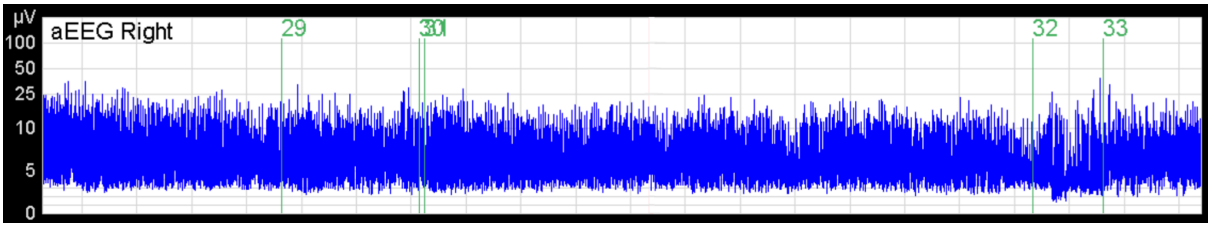

Figure 12. **Discontinuous pattern**. Extracted from Bruns et al., Application of an Amplitude-integrated EEG monitor (Cerebral Function Monitor) to neonates. J Vis Exp. 2017;(127)1–9 [58].

C) **Burst-suppression** (BS): discontinuous background periods of very low voltage (or inactivity) with minimum amplitude lower than 5 μV and bursts of higher amplitude higher than 25 μV. Burst-suppression is considered severe when the burst density is higher than 100 bursts/hour. Mild burst-suppression when the burst density is less than 100 bursts/hour.

The trace is severely pathological when persistent, but it can also occur in the initial phases of hypoxic-ischemic encephalopathy with subsequent normalization of the trace. The longer the child has this trace, the less likely it is to improve.

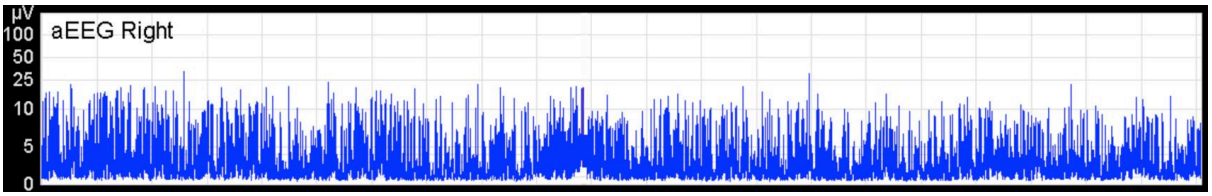

Figure 13. **Burst-suppression pattern**. Extracted from Bruns et al., Application of an Amplitude-integrated EEG monitor (Cerebral Function Monitor) to neonates. J Vis Exp. 2017;(127)1–9 [58].

D) **Continuous low voltage** (CLV): continuous background pattern of very low voltage around or below 5 μV.

This is a very pathological trace.

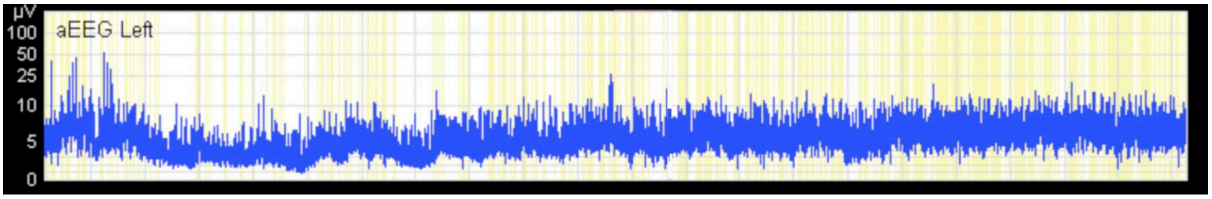

Figure 14. **Continuous low voltage pattern**. Extracted from Bruns et al., Application of an Amplitude-integrated EEG monitor (Cerebral Function Monitor) to neonates. J Vis Exp. 2017;(127)1–9 [58].

E) **Inactive** or **flat trace** (FT): very low voltage, mainly inactive (or isoelectric) tracing with activity below 5 μV.

This is a severely pathological trace.

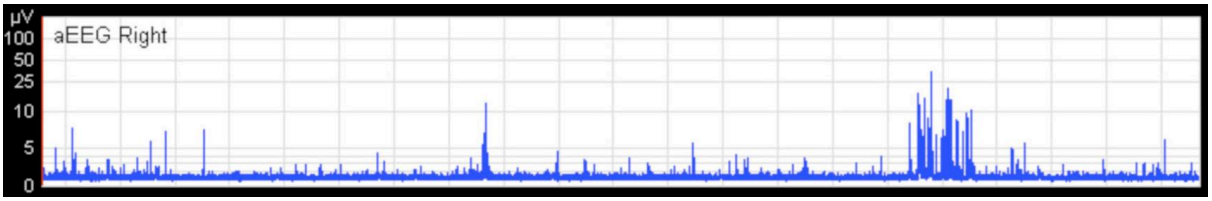

Figure 15. **Flat trace pattern**. Extracted from Bruns et al., Application of an Amplitude-integrated EEG monitor (Cerebral Function Monitor) to neonates. J Vis Exp. 2017;(127)1–9 [58].

3.1.2. Sleep-wake cycling

Sleep-wake-sleep cycling (SWC) in the EEG is characterized by smooth sinusoidal variations, mostly in the minimum amplitude. The broader bandwidth represents discontinuous background activity during quiet sleep, and the narrow bandwidth corresponds to the more continuous activity during wakefulness and active sleep.

A) **Developed** or **present SWC**: clearly identifiable sinusoidal variations between discontinuous and more continuous background activity, with cycle duration ≥20 minutes.

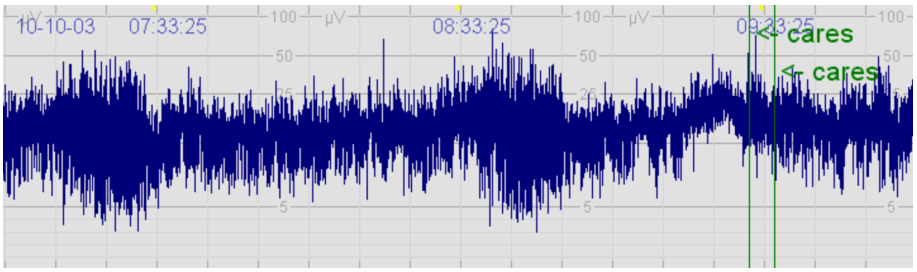

Figure 16. **Developed or present sleep-wake cycling**. Extracted from Ametller Malfaz E. Monitor d'electroencefalograma d'amplitud integrada (EEGa). Girona: Unitat de Cures Intensives Neonatal i Pediàtrica del HUJT; 2015; 1–15 [59].

B) **Immature** or **imminent SWC**: some, but not fully developed, cyclic variation of the lower amplitude, but not developed as compared with normative gestational age representative data.

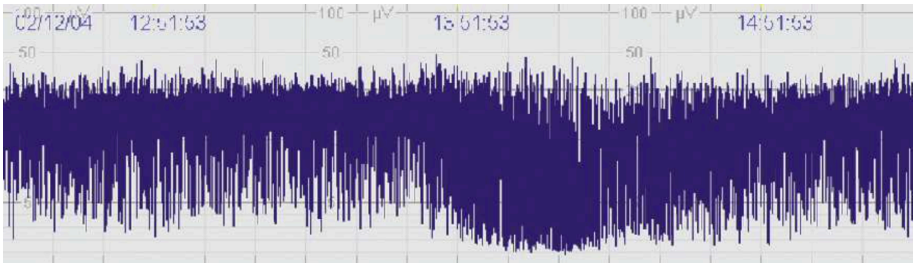

Figure 17. **Immature sleep-wake cycling**. Extracted from Ametller Malfaz E. Monitor d'electroencefalograma d'amplitud integrada (EEGa). Girona: Unitat de Cures Intensives Neonatal i Pediàtrica del HUJT; 2015; 1–15 [59].

C) **No SWC**: no cyclic variation of the aEEG background.

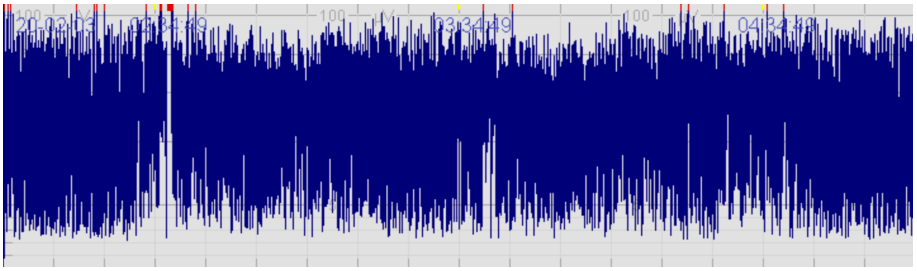

Figure 18. **No sleep-wake cycling**. Extracted from Ametller Malfaz E. Monitor d'electroencefalograma d'amplitud integrada (EEGa). Girona: Unitat de Cures Intensives Neonatal i Pediàtrica del HUJT; 2015; 1–15 [59].

3.1.3. Seizures

Seizures correspond to epileptic seizure activity in the aEEG usually seen as an abrupt rise in the minimum amplitude and a simultaneous rise in the maximum amplitude, often followed by a short period of decreased amplitude. The raw EEG should show simultaneous seizure activity, with a gradual wild-up and then decline in frequency and amplitude of repetitive spikes or sharp-waves or activity with duration of at least 5 to 10 seconds.

A) **Single seizure**: solitary seizure.

B) **Repetitive seizures**: single seizures appearing more frequently than at 30-minute intervals.

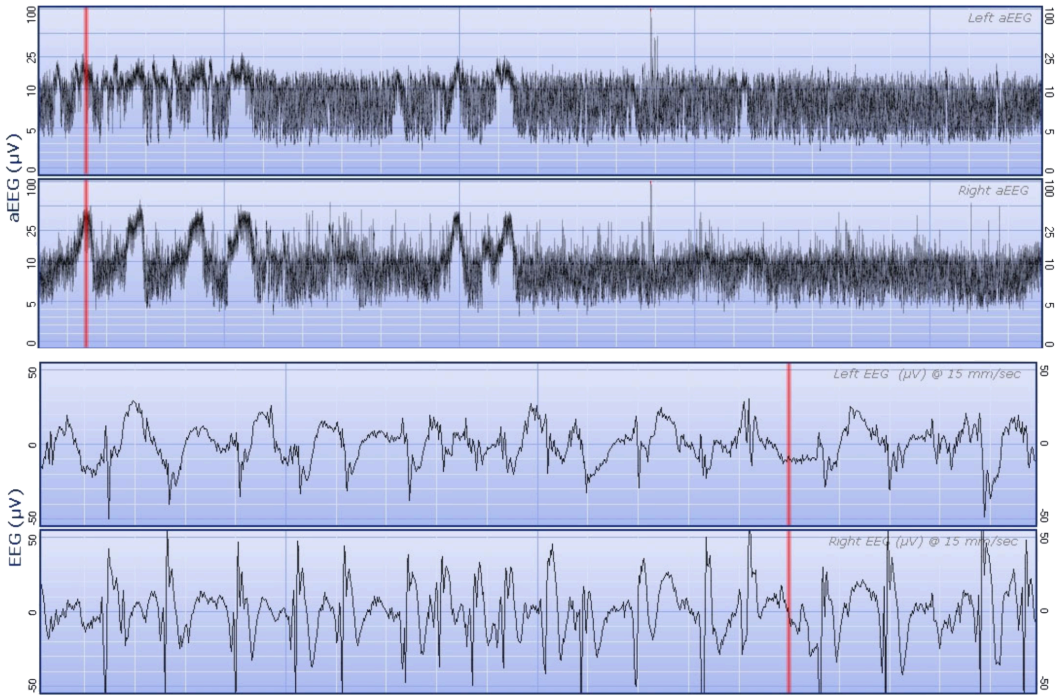

Figure 19. **Repetitive seizures**. Extracted from Griffiths N., aEEG Monitoring in Newborn Infants: Practice Guideline. Nurse Educator. 2023. 1–13 [60].

C) **Status epilepticus**: continuously ongoing seizure activity for more than 30 minutes.

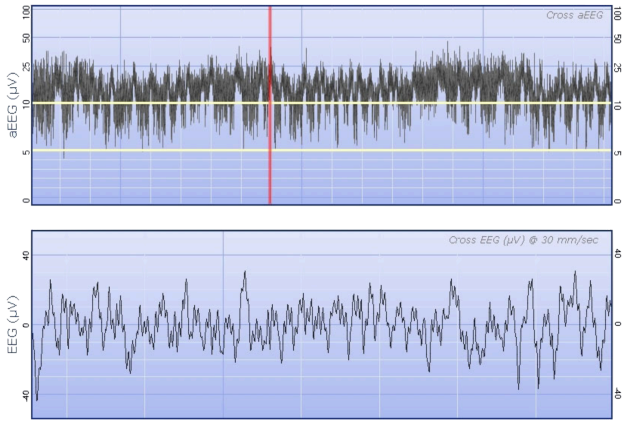

Figure 20. **Status epilepticus** . Extracted from Griffiths N., aEEG Monitoring in Newborn Infants: Practice Guideline. Nurse Educator. 2023. 1–13 [60].

3.2. Artifacts

Artifacts in aEEG recordings can be numerous and varied. Identifying and interpreting them correctly is crucial for an accurate reading, as they can render the record non-evaluative.

1) **Technical issues**

    a) **Electrode contact loss**: loss of contact with electrodes.

2) **Biological artifacts**

    a) **Electromyography** (EMG): the most frequent artifact, secondary to the contraction of scalp, frontal, masseter, or neck muscles.

    b) **Sweating**: significant slow oscillations across all channels.

    c) **Arterial artifact**: electrodes located very close to temporal arteries or arterioles generate a pulsatile and rhythmic waveform. This can be resolved by changing the electrode position.

    d) **Electrocardiographic**: the action potential generated by the heart is transmitted finely to the scalp, resulting in rhythmic spikes that can be confused with seizure activity.

    e) **Ballistocardiographic**: the heart's movement itself transmits fluctuations recorded on the scalp.

3) **Environmental artifacts**

4) **Alternating current**: caused by the 50 Hz electrical current. Always use an alternating current filter to minimize this artifact.

5) **Poor electrode contact**: causes instability in impedances and leads to very sharp waves. Easily corrected by repositioning the electrodes.

Annex 4

4.1. Annink scoring system for cranial US

Item	**Normal-midly abnormal** (0 points)	**Moderately abnormal** (1 point)	**Severely abnormal** (2 pints)
Impaired white/grey matter differentiation and/or slit-like ventricles	Normal differentiation between grey and white matter and open ventricles	Reduced differentiation between grey and white matter and/or slit-like ventricles	No differentiation between grey and white matter and slit-like ventricles
Hyperechogenicity periventricular white matter	Normal echogenicity or minor hyperechogenicity	Moderate or focal hyperechogenicity, not as white as choroid plexus	Severe and diffuse hyperechogenicity, as white as choroid plexus
Hyperechogenicity subcortical white matter	Normal echogenicity or minor hyperechogenicity	Focal hyperechogenicity of the subcortical white matter. Moderate differentiation of white and (subcortical) grey matter	Clear "tramlines" sign; hyperechogenicity of subcortical white matter almost similar to sulci with hyposignal intensity of cortex in between
Hyperechogenicity thalamus	Normal echogenicity or minor hyperechogenicity	Moderate or focal hyperechogenicity thalamus	The hyperechogenicity is severe and diffuse
Hyperechogenicity putamen	Normal echogenicity or minor hyperechogenicity	Moderate or focal hyperechogenicity putamen	The hyperechogenicity is severe and diffuse
Item	**Absent** (0 points)	**Present** (1 point)	
Four column sign	Normal echogenicity to minor hyperechogenicity	On the coronal cUS plane there is a four column sign caused by moderate or severe bilateral hyperechogenicity of the thalamus and putamen	
Visibility PLIC	The PLIC is not visible as a hypo-echogenic line between the putamen and thalamus	The PLIC is clearly visible as a hypo-echogenic line between the hyperechogenic putamen and thalamus	
White matter involvement (0–6points)	Includes the sum of edema, periventricular and subcortical white matter damage		
Grey matter involvement (0–6points)	Includes hyperechogenicity of the thalamus, putamen, visibility of the PLIC, and four-column sign		

Table 9. **Annink scoring system to assess the severity of findings on cranial US between 3 and 7 days of life**. Adapted from Annink KV et al., The development and validation of a cerebral ultrasound scoring system for infants with hypoxic-ischaemic encephalopathy. Pediatr Res. 2020 [32]

Annex 5

5.1. Information sheet

FULL D'INFORMACIÓ PER EL/LA PACIENT

TÍTOL DE L'ESTUDI	Predicció del neurodesenvolupament en recent nascuts a terme amb encefalopatia hipòxico-isquémica a través dels valors d'enolasa neuronal específica en sèrum
INVESTIGADORA PRINCIPAL	Ariadna Reberté Guillamón
CENTRE	☐ Hospital Universitari Josep Trueta (HUJT, Girona) ☐ Hospital Sant Joan de Déu (HSJD, Barcelona) ☐ Hospital Universitari Vall d'Hebron (HUVH, Barcelona) ☐ Hospital Universitari Germans Trias i Pujol (HUGTP, Barcelona) ☐ Hospital Universitari Parc Taulí (HUPT, Barcelona) ☐ Hospital Universitari Joan XXIII (HUJ23, Tarragona)

INTRODUCCIÓ

Ens dirigim a vostè per a informar-li sobre un estudi d'investigació en el qual se'l convida a participar (en aquest cas, al seu fill/a), el qual ha estat aprovat pel Comitè d'Ètica i Investigació Clínica.

La intenció d'aquest document és donar-li tota la informació de manera correcta, clara i precisa per a què pugui decidir si accepta o no participar en l'estudi. Així doncs, li demanem que llegeixi aquest document atentament i que ens consulti qualsevol dubte que li sorgeixi.

PARTICIPACIÓ VOLUNTÀRIA

El/La convidem a participar en aquest estudi ja que el seu fill/a ha estat recentment diagnosticat d'encefalopatia hipòxico isquèmica del recent nascut.

La seva participació en aquest estudi és totalment voluntària i en tot moment pot decidir o no participar, o retirar-se d'aquest un cop estigui dins, sense que això suposi un canvi en l'atenció sanitària del seu fill/a.

OBJECTIUS D'ESTUDI

Aquest estudi pretén resoldre principalment un objectiu: avalauar el valor predictiu de l'enolasa neuronal específica (un biomarcador de dany neuronal) en sang en predir un neurodesenvolupament normal o un retard d'aquest en recents nascuts amb encefalopatia hipòxico-isquèmica, com és el cas del seu fill/a.

DESCRIPCIÓ DE L'ESTUDI

L'estudi inclou un total d'aproximadament 90 pacients, els quals seran suficients per a poder resoldre el nostre objectiu principal. Aquests hauran estat valorats i diagnosticats prèviament d'encefalopatia hipòxico-isquèmica per un/a pediatra especialista.

Aquests pacients s'informen en la Unitat de Cures Intensives Neonatals (UCIN) de l'hospital on han estat atesos i són convidats a participar en l'estudi. Durant la seva hospitalització, seran avaluats en quant a la gravetat de l'encefalopatia hipòxico-isquèmica, la qual serà categoritzada subjectivament pel pediatra especialista en lleu, moderada o severa, segons el protocol de cada hospital.

Per a l'avaluació de l'objectiu principal descrit anteriorment, els pacients seran sotmesos a 3 analítiques de sang, ja realitzades de forma rutinària en pacients amb encefalopatia hipòxico-isquèmica, en el primer, segon i tercer dia de vida del nadó. Els nivells d'enolasa neuronal específica seran determinats en aquestes 3 mostres de sang, a través d'un equip específic d'immunoanàlisi. La determinació del biomarcador no canviarà en cap cas l'actuació pel diagnòstic, seguiment i tractament del nadó hospitalitzat a la UCIN.

Als 18 mesos del naixement del seu fill/a, un/a pediatra especialista li realitzarà en consulta una valoració del neurodesenvolupament a través de l'escala BSID-III, la qual valora el desenvolupament motor, cognitiu i de llenguatge.

ACTIVITATS DE L'ESTUDI

La seva participació en aquest projecte tindrà una durada d'uns 18 mesos, des de l'hospitalització en la UCIN fins la valoració del neurodesenvolupament a consulta amb el/la pediatra especialista. Per tant, els passos a seguir són:

- Llegir atentament i signar el consentiment informat en cas que vulgui participar.
- Procediments derivats de l'hospitalització, donat que el seu fill/a ingressa en la UCIN perquè pateix una malaltia greu, que posa en perill la seva vida i necessita un tractament i/o vigilància especial.
- Determinació del biomarcador en sang, realitzat a través de mostres de sang rutinàries.
- Monitorització de la funció cerebral a través d'un electroencefalograma integrat per amplitud (aEEG), específic per la condició que pateix el seu fill/a.
- Realització d'una ressonància magnètica al cap de 7 dies de naixement, per valorar la possible afectació neurològica deguda a la condició que pateix el seu fill/a.
- Seguiment als 18 mesos del naixement del seu fill/a en consulta amb el/la pediatra especialista per realitzar una valoració del neurodesenvolupament.

RISCOS I BENEFICIS

RISCOS GENERALS

Poden ser necessàries mesures o tècniques que denominem de "suport vital", que no estan lliures de riscos que vostè ha de conèixer. En el cas concret del seu fill/a li explicarem quines d'aquestes actuacions seran utilitzades i per quin motiu, sempre que la urgència ho permeti.

RISCOS ESPECÍFICS

Aquests riscos són variables en freqüència i gravetat, depenent de la tècnica i del propi pacient però els més freqüents són:

- Els derivats de la col·locació de catèters en venes i artèries que poden donar lloc a complicacions com hemorràgies, coàguls o infecció.
- Intubació i ús de respiradors, utilitzats per ajudar a substituir la pròpia respiració, també poden tenir efectes no desitjats com infeccions pulmonars, fugida d'aire per trencament del pulmó, obturacions o lesions de la tràquea.
- Reaccions adverses, fonamentalment a medicaments, per reacció al·lèrgica o efectes secundaris.
- Toracocentesi: punció de l'espai pleural per fins diagnòstics i extracció d'aire o líquid amb fins terapèutics. Pot tenir efectes no desitjats com fugida d'aire per punció del pulmó, fugida d'aire sota la pell, hemorràgia pulmonar, lesió de vasos intercostals o lesió de les vísceres abdominals.
- Pericardiocentesi: tractament del taponament cardíac i anàlisi del líquid extret per fins diagnostics. Pot tenir efectes no desitjats com lesió del miocardi, punció d'una artèria coronària¡a, arítmies, lesió del pulmó o lesió de les vísceres abdominals.

Les persones que cuiden el seu fill/a coneixen aquestes possibilitats i estan atents a la seva possible aparició per combatre-les, cosa que generalment transcorre amb èxit. Tot i que els efectes secundaris poden agreujar la situació del pacient, els possibles beneficis d'aquestes mesures o tècniques superen àmpliament els riscos que comporten, és per aquest motiu que només es solen utilitzar en pacients greus.

CONTACTE EN CAS DE DUBTE

Si durant la seva participació té algun dubte o necessita obtenir més informació, pot posar-se en contacte amb el/la pediatra especialista que tractarà amb vostè/s durant l'hospitalització del seu fill/a o amb la investigadora principal. Se li proporcionarà un paper amb les dades de contacte.

PROTECCIÓ DE DADES PERSONALS

Tant els responsables de l'estudi com el centre s'asseguraran del compliment de tots els principis contemplats en la normativa de protecció de dades nacional i europea. les seves dades seran accessibles només pels membres de l'equip de recerca i s'afegiran a la base de dades de forma anònima.

Gràcies a la seva participació, en un futur es podrà predir millor el neurodesenvolupament en un pacient amb la mateixa condició que el seu fill/a, l'encefalopatia hipòxico-isquèmica, gràcies a la determinació de biomarcadors, com és l'enolasa neuronal específica en aquest estudi. D'aquesta manera, es podrà fer una predicció més acurada del pronòstic, així com millorar l'atenció i el tractament d'aquests pacients, essent més o menys agressius segons requereixi la situació.

Annex 6

6.1. Informed consent form

Dades del/la pacient
Nom i cognoms:
Nº HC:

CONSENTIMENT INFORMAT

TÍTOL DE L'ESTUDI	Predicció del neurodesenvolupament en recent nascuts a terme amb encefalopatia hipòxico-isquémica a través dels valors d'enolasa neuronal específica en sèrum
INVESTIGADORA PRINCIPAL	Ariadna Reberté Guillamón
CENTRE	☐ Hospital Universitari Josep Trueta (HUJT, Girona) ☐ Hospital Sant Joan de Déu (HSJD, Barcelona) ☐ Hospital Universitari Vall d'Hebron (HUVH, Barcelona) ☐ Hospital Universitari Germans Trias i Pujol (HUGTP, Barcelona) ☐ Hospital Universitari Parc Taulí (HUPT, Barcelona) ☐ Hospital Universitari Joan XXIII (HUJ23, Tarragona)

Jo, _____ , pare/mare/tutor de _____ :

- ☐ He llegit i entès el full d'informació que se m'ha entregat sobre l'estudi.
- ☐ He pogut fer les preguntes pertinents sobre l'estudi i s'han respost satisfactòriament.
- ☐ He rebut suficient informació sobre l'estudi.
- ☐ Entenc que la meva participació (o la del meu fill/a) és voluntària i puc reconsiderar aquesta decisió i abandonar en qualsevol moment.
- ☐ Signo aquest document de consentiment informat de manera voluntària per manifesta el meu dret (o del meu fill/a) de participar en aquest estudi de recerca.

Presto la meva conformitat per a participar a l'estudi i confirmo que he llegit el full d'informació i estic conforme amb el seu contingut:

Signatura i DNI del responsable legal:
DNI: _____

Signatura del/la metge que informa i nº de col·legiat:
Nº col·legiat: _____

Data: ___ / ___ / ___

Annex 7

7.1. Data collection sheet

Dades del/la pacient
Nom i cognoms:
Nº HC:

FULL DE RECOL·LECCIÓ DE DADES

TÍTOL DE L'ESTUDI	Predicció del neurodesenvolupament en recent nascuts a terme amb encefalopatia hipòxico-isquémica a través dels valors d'enolasa neuronal específica en sèrum
INVESTIGADORA PRINCIPAL	Ariadna Reberté Guillamón
CENTRE	☐ Hospital Universitari Josep Trueta (HUJT, Girona) ☐ Hospital Sant Joan de Déu (HSJD, Barcelona) ☐ Hospital Universitari Vall d'Hebron (HUVH, Barcelona) ☐ Hospital Universitari Germans Trias i Pujol (HUGTP, Barcelona) ☐ Hospital Universitari Parc Taulí (HUPT, Barcelona) ☐ Hospital Universitari Joan XXIII (HUJ23, Tarragona)

INFORMACIÓ GENERAL

Sexe:

☐ Masculí
☐ Femení

Data de naixement: ___ / ___ / ___

Hora de naixement: ___ : ___ h

Edat gestacional: ____ setmanes

Pes: ____ grams

INFORMACIÓ PERINATAL

Via de part:

☐ Part vaginal
☐ Part per cessària
☐ Part instrumentat
☐ Altres: _____

Distòcia de part:

- ☐ No
- ☐ Sí: _____

Esdeveniment sentinella:

- ☐ No
- ☐ Sí: _____

Estat fetal no tranquilitzador:

- ☐ No
- ☐ Sí: _____

Necessitat de reanimació avançada:

- ☐ No
- ☐ Sí: _____

Test Apgar: 1 minut _____ , 5 minuts _____ , 10 minuts _____

pH cordó umbilical: _____

Dèficit de bases: _____

Annex 8

8.1. Baseline characteristics and outcome data

Characteristics	**Neurodevelopmental outcome**			**P value**
	Normal (n)	**Moderate delay** (n)	**Severe delay** (n)	
Sex				
Male				
Female				
Weight (g)				
Gestational age (weeks)				
5-min Apgar score				
pH				
Sentinel event				
Labor dystocia				
Advanced resuscitation				
Therapeutic hypothermia				
Adverse event				
HIE severity				
Mild (grade I)				
Moderate (grade II)				
Severe (grade III)				
aEEG				
Abnormal pattern				
Absence of SWC				
Seizures				
MRI				
Rutherford score				
Moderate-severe injury				
Global injury				
BSID-III				
Motor				
Cognition				
Language				
NSE values				
1-day NSE				
1-day NSE				
1-day NSE				

Table 10. **Baseline characteristics and outcome data**, where values are expressed as absolute and relative frequencies, median and interquartile range, or mean and standard deviation, defined in reference to normal neurodevelopment, mild to moderate delay and severe delay, according to BSID-III score.