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Use of Therapeutic Hypothermia in routine clinical practice and long-term neurodevelopmental follow-up in infants with neonatal encephalopathy: findings from an Italian Network

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1. Introduction

Neonatal encephalopathy (NE) is a condition resulting in approximately 700,000 deaths/year worldwide in full-term and late preterm neonates. It affects 1–4 per 1000 live births in high-resource settings. Neonatal encephalopathy is an "umbrella" term that does not describe a specific etiology. There are multiple causes of NE including hypoxia-ischemia (leading to hypoxic-ischemic encephalopathy [HIE]), perinatal infections, placental abnormalities, metabolic disorders, coagulopathies, neonatal vascular stroke, but the underlying etiology can remain unidentified in more than half of the cases. However, basing on clinical, electroencephalographic (EEG), and magnetic resonance imaging (MRI) criteria up to 50%–80% of cases of NE can be attributed to HIE (1). To date, the terms HIE and NE are used interchangeably in the literature when describing human and animal newborns (1). We will refer to NE after HIE, and we will further describe in detail the mechanism of hypoxic-ischemic brain injury.

To date, therapeutic hypothermia (TH) is commonly regarded as the optimal therapy to reduce the risk of disability after NE (1). Its efficacy has been proven in 7 randomized controlled studies performed in early 2000; however, only a few network of centers using TH as a standard of care have subsequently described in a large scale the long-term neurodevelopmental outcomes of treated neonates. However, multicenter Italian data regarding neonatal outcome after TH are unavailable. The aim of this study is to report the use of TH and the subsequent neurodevelopmental outcome from an Italian Network.

2. Hypoxic-ischemic brain injury

The hypoxic-ischemic brain injury may occur in different periods:

- Before delivery: in situations where blood supply to the placenta or maternal blood oxygenation are reduced (such as gestosis, maternal diabetes, hypertension anemia, heart disease).
- During labor and delivery: after a dystocic delivery or when placental exchanges fail (after placental abruption, placenta previa, torsion and prolapse of the umbilical cord).
- After delivery: because of respiratory failure, severe congenital heart diseases, septic shock, severe anemia.

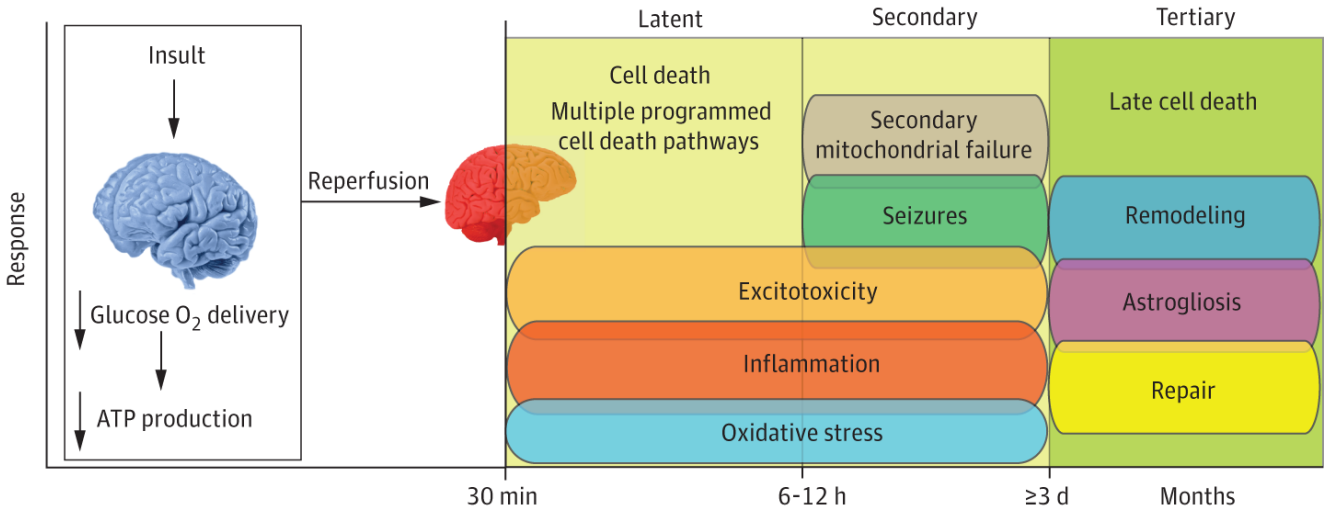
2.1 Pathogenesis of hypoxic-ischemic injury

A reduced cerebral blood flow is the main etiopathogenetic mechanism underlying most neurological disorders resulting from intrapartum hypoxic-ischemic injury. Perinatal events responsible for decreased placental perfusion, impaired oxygen and glucose transport within the umbilical cord (2), lead to asphyxia and fetal acidosis (defined by an umbilical artery pH < 7.0 or a base excess ≥ -12 mmol/L) (3). The fetus responds to hypoxia with a rearrangement of cardiac output in order to preserve blood flow in vital organs; however, if the insult persists, compensatory mechanisms (such as reduction of cerebral vascular resistance) fail, and cardiac output may dramatically decrease, resulting in substantial reduction of the cerebral blood flow. If this reduction is moderate, cerebral arteries can redistribute the flow from the anterior to the posterior circulation to maintain adequate perfusion of the cerebellum, basal ganglia, and brainstem, which are not preserved when an acute hypoxic event causes a sudden decrease in flow (4).

The reduction in cerebral perfusion and oxygen delivery lead to a cascade of biochemical events that ultimately result in cell death. Two main phases of damage can be distinguished: an acute, immediate phase, with an initial energy deficiency, and a secondary phase, with a further energy failure, occurring 6 to 48 hours after the ischemic insult. In the latency period between the two phases, cerebral blood flow and oxygenation are reestablished. Reperfusion allows the transport of therapeutic agents through the cerebral circulatory system, which is why this appears to be the most effective time for the administration of treatments to decrease the extent of brain damage (5).

A third phase can be also identified; it occurs in the months following acute ischemic injury, and it is characterized by late cell death, remodeling of damaged brain areas, and astrogliosis (6).

Figure 1: Phases of HIE damage.



2.2 Diagnosis of hypoxic-ischemic encephalopathy

The pattern of brain damage depends on the severity and the duration of hypoxia and the level of brain maturation achieved by the fetus. The diagnosis of hypoxic-ischemic brain damage is made possible by the combined and integrated use of clinical and instrumental semeiotics (7), and neuroimaging (brain ultrasound, CT, and MRI).

Obstetric factors potentially correlated with intrapartum asphyxia should be identified, by excluding further pathological situations damaging the central nervous system, such as metabolic, genetic, and infectious diseases. Criteria to define intra-partum asphyxia are the followings: 1) umbilical artery pH < 7.0 and base excess ≥ -12 within the first hour of life (to assess the degree of acidosis); 2) hypoxic-ischemic encephalopathy as defined by Sarnat and Sarnat (7), (first clinical classification of HIE), which evaluates different neurological functions, such as consciousness, muscle control, neonatal reflexes, integrity of the neurovegetative system, and presence of seizures.

The classification includes three grade of HIE:

- grade 1 (mild): hyperexcitability, hyperresponsiveness, tremors and clonus (jitteriness) associated with a slight increase in muscle tone, increased tendon reflexes (lively) and spontaneous Moro reflex (exaggerated). Sympathetic nervous system hyperactivity (i.e. tachycardia, mydriasis, and decreased bronchial secretion). Neonatal seizures do not occur, and the EEG is usually normal. These symptoms (consequent to a sympathetic activation), usually last < 24 hours, and have no or mild neurological sequelae (0-5%).

- grade 2 (moderate): lethargy or stupor associated with poor/absent suction, predominantly axial hypotonia, torpid reflexes, incomplete Moro reflex, and frequent seizures (focal or multifocal). Signs of autonomic dysfunction (due to parasympathetic arousal) may be present, such as bradycardia,

reduced heart rate variability, irregularity of breathing, bradypnea/apnea, altered pupillary accommodation, miosis and bronchial hypersecretion. Frequently seizures may occur, with clonic, focal or more often multifocal shocks. The EEG is usually altered by the presence of low-voltage activity with delta and theta rhythms on which point and slow waves are intercalated. The duration of these signs varies from 2 to 14 days. Mild to moderate neurological sequelae may occur in 20-30% of cases.

- grade 3 (severe): stupor or coma is observed, with flaccidity or decerebrate/decorticate posture and marked depression or absence of tendon and archaic reflexes. Suppression of autonomic functions is total (alterations in cardiac and respiratory rhythm with bradycardia and apnea seizures, pupillary hyporeflexia to light stimulus, an expression of profound depression of both components of the autonomic system). Convulsive seizures are frequently diagnosed, often configuring a status epilepticus, which is difficult to control pharmacologically. In the most severe cases, in which cortical damage is imposing, brainstem release phenomena are observed. The EEG is profoundly altered similar to burst suppression or discontinuous type with the presence of paroxysmal discharges, or in some cases totally inactive. The duration of symptoms is variable and often prolonged. This condition is characterized by high early mortality (between 24 and 72 h of life) and, in case of survival, frequently (95-100% of cases) major neurological sequelae (such as infant cerebral palsy (ICP), microcephaly, spastic quadriplegia, and epilepsy) may occur.

Neonatal seizures occur in most patients with moderate-to-severe HIE, usually in the first 24 h of life (8,9).

This classification has a high predictive value (10,11). In fact, neonates with mild HIE usually have a normal outcome, whereas all severe HIE die or develop motor disability and/or cognitive impairment. The prognosis of moderate HIE, on the other hand, is less certain, encompassing a wide range neurodevelopmental outcome.

After moderate HIE, major neurodevelopmental impairment (such as Cerebral Palsy and Mental Retardation) are usually uncommon, but the risk of minor impairments (such as learning and language disorders) is consistent. Limitations of Sarnat's classification include its poor sensitivity in detecting a brain damage established early in pregnancy and premature births (12).

There are additional methods of HIE classification that have been demonstrated to be highly predictive with respect to the neurodevelopmental outcome.

3. Therapeutic Hypothermia

Evidence from animals and subsequently human clinical trials has shown that TH in neonates reduces brain damage and improves neurological outcome related to neonatal hypoxic-ischemic injury (13-15).

The extent of brain damage following an ischemic hypoxic insult is closely dependent on the burden of damage (neuronal necrosis and inflammation) and endogenous protective mechanisms (acute phase response, healing, and neuronal repair).

3.1. Mechanism of action

The neuroprotective effects of hypothermia concern the modulation of mechanisms of irreversible damage through inhibition of the inflammatory cascade, reduction of free radical production, and reduction of metabolic demands resulting in reduced oxygen consumption and carbon dioxide production (16).

Through hypothermia, cerebral energy consumption is reduced by about 5% for every 1°C reduction in body temperature, promoting delay of the onset of anoxic damage. This effect has been demonstrated in many experimental models in rodents, dogs, or pigs undergoing ischemia-reperfusion injury. Part of the neuroprotective effect of TH is related to the blocking of the proinflammatory pathway mainly mediated by TNF-, IL-1, and IL-6 and activating caspases (17). In experimental models, hypothermia prolonged for 72 hours induced the reduction of neuronal necrosis and apoptosis (16). Suppression of cytochrome C and caspase activation in cortex, thalamus, and hippocampus in rats with hypoxic ischemic encephalopathy subjected to hypothermia have been described (18).

In the hypoxic ischemic insult, the severity of encephalopathy depends on the duration and extent of the process. The central role in neuroprotection of TH involves interrupting the damage process by intervening before the secondary damage phase begins (apoptotic neurons have a chance to recover). Although it has not been exactly determined when brain damage becomes irreversible, there are consistent data indicating that the latency phase, before the secondary phase begins, represents the most important window for therapeutic intervention (17). Because the stages of the damage process are not separated from each other, however, the transition to the moment of irreversible cell death is almost imperceptible (16,17).

3.2. The use therapeutic hypothermia

Since the earliest cases, the timing to start TH has been indicated within 6 hours of birth (18).

In the 2010 Conesus, the International Consensus on Cardiopulmonary Resuscitatio (ILCOR) provided the indications for performing therapeutic hypothermia for all in term or near-term infants with development of moderate-to-severe hypoxic-ischemic encephalopathy through specific protocols coordinated by each regional system (19).

All study protocols related to neuroprotection after hypoxic-ischemic encephalopathy have similar inclusion criteria, based on Apgar score, duration of delivery room resuscitation, delivery room blood gas values, neurological examination according to Sarnat, and EEG monitoring. In most cases, exclusion criteria are related to hours of life (cooling should not be performed beyond 6 hours of life), chromosomal alterations, major congenital malformations, or severe intrauterine growth retardation (20).

Two body cooling techniques exist: selective head hypothermia and whole body (or systemic) hypothermia (15). Selective head hypothermia is performed with a helmet, while whole body hypothermia with a thermal mat placed around the infant, both are connected to a temperature regulation system based on the infant's temperature (14). The recommended duration of cooling for both techniques is 72 hours. No advantages have been shown, but increased adverse effects, if systemic hypothermia was used for a longer period (120 hours) or if a lower temperature (32°C) was used (21). All evidences suggested that the response to neuroprotective treatment is time-dependent (therapeutic window) and that efficacy depends on the timing of treatment initiation (within 6 hours of life). Continuous temperature monitoring, even before placement of hypothermic garments, assures the clinician of the safety and quality of treatment.

Specific protocols are also provided for the subsequent rewarming phase: it should be slow and gradual, increasing temperature of 0.5°C/h until it reaches 36.5°C; this process is intended to prevent rewarming-related complications. During the entire cooling period, to ensure the effectiveness of the treatment, the temperature (measured at the esophageal or rectal level) must always remain above 33°C and must be monitored continuously; temperatures below 32°C are less effective in terms of neuroprotection and are associated with severe systemic adverse effects and increased mortality (13,19,22).

A recent study compared infants undergoing systemic hypothermia and infants undergoing selective head hypothermia and showed that brain damage, assessed by brain MRI, was more severe and frequent in patients undergoing the latter type of treatment, suggesting a greater neuroprotective effect of systemic hypothermia (23). Similar findings have been described by Allen et. al, who suggested

that whole body hypothermia appeared to offer the most advantages (24).

3.3 Mortality, neurodevelopmental outcome and TH

Although TH after perinatal asphyxia has reduced the likelihood of an adverse outcome, 45% of infants still die or have neurodevelopmental impairment (25).

To date most data regarding the long-term neurodevelopmental effects of TH in infants with HIE come from randomized-controlled trials or single center studies (26-32). Seven randomized controlled trial and subsequent meta-analyses (13,15,22) demonstrated the effectiveness of TH in reducing mortality and neurological sequelae in infants with HIE. They also found that the neuroprotective effect of cooling was less effective in infants with severe HIE. Severe HIE is likely to be characterized by short latent period duration, high energy demand, and rapid necrosis of gray matter, basal ganglia, thalamus, and white matter neurons. Conversely infants with moderate HIE are those who most benefit of TH (13,22).

Few multicenter studies have been performed in routine clinical practice and even fewer have reported the long-term neurodevelopmental outcome of infants with HIE treated with TH. Randomized clinical trials are considered the gold standard for clinical development, providing the highest level of evidence (33). However, they have limitations in identifying the effectiveness of treatments used in routine clinical practice, because they concern highly selected populations, highly controlled settings and they are optimized to show treatment effects. In contrast, pragmatic trials regarding the real-world evidence may be affected by bias and they may lead to different responses than RCTs by overestimating treatment effects. However, they can provide information on the applicability of the treatment and its effectiveness in routine clinical practice. This enables appropriate generalization to the target patient group for a specific treatment such as therapeutic hypothermia (34).

To date, 5 multicenter studies evaluated the use of therapeutic hypothermia in routine clinical practice (25,35-38) (Table 3). Studies had wide variations according to study design (retrospective or prospective observational studies), number of infants enrolled (from 75 to 1384 infants), period of observation (most studies were not recent and concerned the period between 2006-2015), and percentage of infants with mild HIE who underwent TH (4 to 18%). Case fatalities reported in multicenter studies were lower (2.6 to 22%) as compared with the mortality reported in randomized controlled trial (20 to 37.7%).

Four of these multicenter studies evaluated the neurodevelopmental outcome at 24 months of age and three of them also included a brain MRI study. The outcome for infants with moderate to severe HIE

was available in three studies only; the rates of good outcome was between 63.5% to 71% but the definitions of favorable outcome was quite different. One study (38) is currently ongoing and data are available for brain MRI only.

As already mentioned, although studies differed, they shared common variables: they concerned infants treated a few years after the randomized-controlled trials were performed; they included a proportion of infants with mild HIE. Moreover, rates of favorable outcome were similar between these studies (30 to 40%), with a narrow range as compared to that reported by RCTs (39 to 86%).

Table 3. Multicenter studies evaluating TH in routine clinical practice and long-term outcome.

	Countries	Study design	Infants treated with TH, n	Study period	Case fatalities	Infants with mild HIE, n	Long-term NDO	Brain MRI reported	Rates of infants with moderate HIE and favorable outcome	Rates of infants with severe HIE and favorable outcome
35	UK	P	1384	2006-2011	15-22%	18%	NDO 24 months of age	No	70%*	
36	Swiss	R	103	2011-2013	18%	4%	NDO 18-24 months of age	No	63.5%**	
37	UK	P	75	2014-2015	2.6%	4%	NDO 18-24 months of age	Yes	71%***	
38	France	P	500	2015-2017	-	-	NDO 36 months of age	Yes	ongoing	ongoing
25	Netherlands Sweden	R	173	2007-2014	15.6%	9.2%	24 months of age	Yes	NS****	

CP, cerebral palsy. NDO, neurodevelopmental outcome. NS, not specified. P, prospective observational study. R, retrospective observational study.

**Motor outcome was available for 275 infants (19% of the population) and cognitive outcome was available for 34 patients (2.4% of the population).*

***73% and 32% of infants with Sarnat stage 2 and 3 respectively had a favorable neurodevelopmental outcome. Favorable outcome was defined as Bayley-3 score of 70–84 alone, or a cognitive or language Bayley-3 score ≥85 and a GMFCS level 1 or 2, seizure disorder (without anti-epileptic medication) or hearing deficit with the ability to follow commands without amplification, or as the absence of any of the above.*

****Favorable outcome (defined as the absence of death, CP or cognitive impairment) was available for 69 infants. Cognitive outcome was available for 55 infants.*

***** Favorable outcome was defined as absence of cerebral palsy, Bayley Scales of Infant and Toddler Development, third edition, motor or cognitive composite scores at 2 years of <85*

3.4. Adverse effects

If an adequate ventilation and hemodynamic support, as well as constant temperature monitoring during both cooling and rewarming period are not provided, TH has poor results and it is not recommended because increased mortality (39). Safe procedures require months of multidisciplinary team training and in-depth understanding of the multisystem involvement of perinatal asphyxia together with the potential systemic complications of treatment. Possible adverse effects of TH include hypotension, pulmonary hypertension, increased QT interval, thrombocytopenia, coagulation alterations, skin burns, subcutaneous necrosis, metabolic and electrolyte alterations.

It is important to be able to distinguish events directly related to TH from other clinical problems related to multiorgan dysfunction caused by HIE (40). Finally, it should be mentioned that the pharmacokinetics of some drugs can be changed during cooling. In an observational study, infants with HIE treated with TH who were given normal doses of morphine were found to have significantly higher serum concentrations than the normothermic group. Therefore, attention must be also paid to pharmacological dosages, which may differ from those usually aministered (41). However, recently it has been reported that analgosedation does not negatively affect the neurodevelopmental outcome, but it may contribute to ameliorate neuroprotection during TH (42).

4. HIE and brain MRI

Neonatal brain MRI has been used in infants with neonatal encephalopathy since the late 1980s, and it has been found highly predictive of poor neurodevelopmental outcome beyond early infancy.

Currently brain MRI is routinely recommended in all full-term infants with HIE (25). Following the development of sequences dedicated to the neonatal age, it has been possible to obtain detailed images of brain structures and specific scores for HIE-related changes have been developed. The use of MRI contributes to identify brain lesions in infants with HIE, emphasizing the role of perinatal asphyxia as a cause of brain damage. Brain MRI has been an important biomarker of neurodevelopment impairment for studies related to neuroprotective strategies (43).

The main damage patterns found on MRI in term infants with HIE are (44):

1) Pattern of the basal ganglia and thalamus: this is the pattern that usually follows an acute event and predominantly involves bilaterally the gray matter nuclei (ventrolateral, thalamus, putamen) and the peri-rolandal cortex. The association of hippocampal and trunk involvement is uncommon. This pattern is usually associated with severe disability.

2) Pattern of the last meadows: typically related to prolonged partial asphyxia. The vascular areas of the last meadows (anterior middle cerebral artery and posterior middle cerebral artery) are involved, affecting the white matter and in more severely affected infants also the surrounding cortex. Lesions may be mono/bilateral, posterior/anterior.

3) Pattern with severe involvement of subcortical white matter and cortex, with sparing of periventricular white matter and gray matter: rare. Severe condition that is associated with death or in case of survival to multicystic encephalopathy.

4) Pattern with lesions limited to periventricular white matter.

5) Ischemic stroke, hemorrhagic stroke, venous sinus thrombosis.

Barkovich was among the first to investigate the role of MRI performed early, and by retrospectively reviewing the examination performed in the first 10 days of life in 16 infants with hypoxic-ischemic damage in the perinatal period; he found symmetrical signal abnormalities in all the infants evaluated (45). A central role in the evaluation of MRI images in infants with asphyxia is played by the posterior limb of internal capsule (PLIC). It is an area of high myelination particularly susceptible to hypoxic-ischemic damage given its increased metabolic activity. Myelin is known to be associated with high signal in sequence conventional T1 and reduced signal intensity in sequence conventional T2. The absence of myelin signal at the level of PLIC in a term infant is to be considered as a pathological finding, and in infants with severe basal ganglia and thalami atrophy the normal high signal resulting from myelination of PLIC may not appear. Normally PLIC appears with intense

signal in T1 sequences in at least one third of the length of the posterior arm. The abnormalities are often more easily identified by the end of the first week of life.

Rutherford et al. evaluated the correlation between abnormal signal on brain MRI at the level of the PLIC in infants with perinatal asphyxia and neuromotor outcome at one year of life, finding a pathological outcome in all patients with abnormal signal. Specifically, the patterns included absence of normal signal at the PLIC level, bilateral basal ganglia and thalami (BGT) abnormalities, loss of gray and white matter differentiation at the hemispheric level, and cortical signal alteration (cerebral cortex highlighting). Signal alteration at the level of PLIC predicted pathological outcome with sensitivity of 0.9, specificity 1, VPP 1, and VPN 0.87, being an accurate predictor of neurological outcome in term infants with HIE (46). In contrast, a normal signal was found to be correlated with a normal outcome in all patients. Signal alteration at the level of PLIC is easy to identify: Rutherford reported an high inter-score agreement during image evaluation, and with respect to the number of examinations to which patients should be subjected, it was found that the probability that a single examination could correctly predict outcome was 0.94 (95% CI: 0.88-1.0).

In patients with altered signal intensity at the level of PLIC, signal changes had also been found at the level of BGT, which were more evident after the first week of life, and the severity of motor outcome appeared to be related to the extent of this additional damage (46).

4.1 Scores to predict the neurodevelopmental outcome by brain MRI

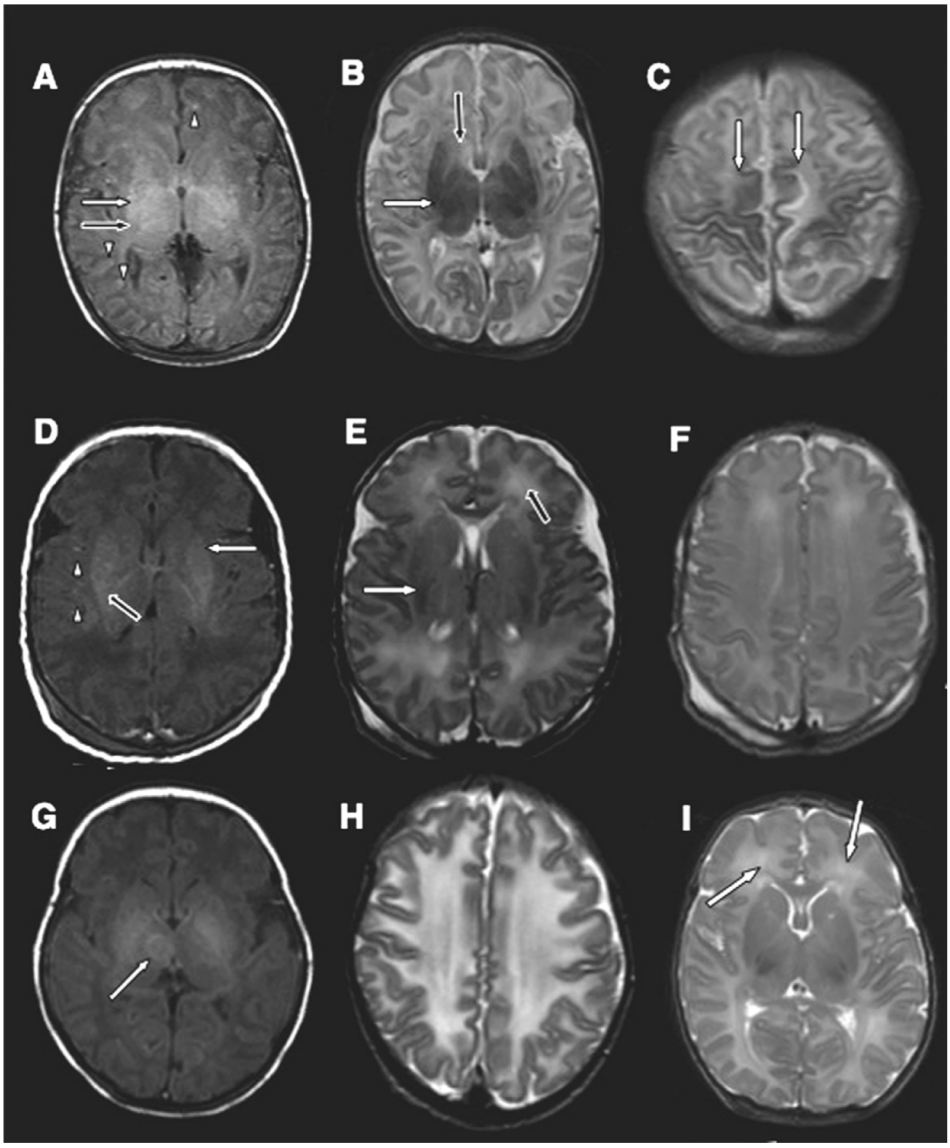
Rutherford et al. in a study (47) on the correlation between prenatal sentinel events and perinatal asphyxia confirmed that in addition to damage to the PLIC, lesions to GBT are the characteristic finding in term infants with hypoxic-ischemic encephalopathy. The evaluation of MRI images (conventional T1 and T2) was based on the analysis of 5 patterns (Figure 2) such as: pattern 1) basal ganglia and thalami lesions associated with severe white matter lesions; pattern 2) basal ganglia and thalami lesions with mild/moderate signal changes at the white matter level; pattern 3) isolated basal ganglia/thalami lesions; pattern 4) moderate/severe alterations at the white matter level; pattern 5) mild alterations at the white matter level or normal examination.

The study showed that events leading to severe acute hypoxia do not result in white matter damage without central gray matter involvement and confirmed the high vulnerability of BGT, trunk, hippocampus and PLIC to acute asphyxia to hypoxic-ischemic events. Loss of normal signal intensity of PLIC was strongly associated with damage to adjacent basal ganglia and was a reliable predictor of pathological motor outcome. Severe white matter damage in the presence of deep gray matter damage suggested a prolonged hypoxic-ischemic mechanism. The identification of damage patterns

1 or 2 had negative prognostic implications: 29% of infants with these patterns had died and 57% had developed cerebral palsy. In contrast, infants with normal patterns had normal outcomes.

Conventional sequences (T1 and T2) are reliable when acquired after the first week of life. Indeed, signal abnormalities in these sequences may not be present in the first week of life. For this reason, scores for the evaluation of images in diffusion sequences have recently been developed. In this case the best time window for acquiring diffusion imaging is between 4 and 7 days of life, since from the second week onward, diffusion abnormalities may no longer be present, with the risk of incurring pseudo-normalization. Scores of diffusion imaging appeared to be able to predict neuromotor outcome (25).

Figure 2. Patterns of brain MRI injury. (47)



A-C, Pattern 1: moderate/severe basal ganglia and thalami damage associated with moderate/severe WM changes and cortical injury. MRI scans of a 7-day-old infant (case 32). A, T1-weighted axial spin echo sequence showing severe bilateral abnormally increased SI in the lentiform nuclei (*white arrow*) and thalami, with loss of normal SI in the posterior limb of the internal capsule (*black arrow*) and cortical highlighting (*arrowheads*). B, T2-weighted axial spin echo sequence showing severely reduced SI in the lentiform nuclei and thalami, abnormal SI in the posterior limb of the internal capsule (*white arrow*), and increased SI in the caudate nuclei (*black arrow*), associated with abnormal SI in frontal WM. C, T2-weighted axial spin echo sequence showing abnormal SI in the cortex around the interhemispheric fissure (*arrows*) and central sulcus, with abnormal subcortical WM on the left. D-F, Pattern 2: basal ganglia and thalami damage associated with mild WM changes with or without cortical injury. MRI scans of a 3-day-old infant (case 30). D, T1-weighted axial spin echo sequence showing excessively high SI in swollen lentiform nuclei (white arrow) and ventrolateral thalamic nuclei, low SI in the posterior limb of the internal capsule (*black arrow*), focal WM changes (*white arrowheads*), and mild cortical highlighting. E, T2-weighted axial spin echo sequence showing abnormal SI throughout the basal ganglia and thalami and the posterior limb of the internal capsule (*white arrow*), with mildly increased SI in the frontal WM (*black arrow*). F, T2-weighted axial spin echo sequence showing focal cortical and subcortical WM SI abnor- malities. G, Pattern 3: focal thalamic lesion with or without cortical injury. T1-weighted axial spin echo sequence in a 6-day-old infant (case 10) showing a limited area of abnormal SI in the right thalamus (*arrow*) and slightly elevated SI in the globus pallidus. H, Pattern 4: predominant WM damage (moderate/severe) with or without cortical injury with or without mild basal ganglia and thalami changes. T2-weighted axial spin echo sequence in an 18-day-old infant (case 17) showing severe abnormally high SI in the WM with low SI in the cortex of the central sulcus. I, Pattern 5: mild WM abnormalities with or without mild cortical changes but with normal basal ganglia and thalami, or normal imaging. T2-weighted axial spin echo sequence in a 5-day-old infant (case 3) showing slightly high SI in the WM.

4.2. Therapeutic hypothermia and brain MRI

As TH has become a standard of care, the most recent studies concerning MRI and infants with HIE involve patients undergoing TH. It has been described how the predictive accuracy of T1/T2 imaging in hypothermic-treated infants does not differ from that investigated in untreated subjects, suggesting that MRI can be used to predict the prognosis even in infants who underwent the treatment. Studies comparing MRI image findings between infants who underwent TH and who did not had a relevant role (48,49). Rutherford et al. evaluating infants enrolled in the TOBY trial described how TH was associated with a reduction in gray and white matter changes and that patients undergoing TH were those who in greater numbers had brain MRI scans in the normal range. Specifically, the study documented a significant reduction of abnormalities at the level of basal ganglia, thalami, and white matter and a reduction of signal abnormalities in cortical gray matter in the group of patients who underwent hypothermia compared with those who did not undergo the treatment. In addition, the accuracy of brain MRI performed in the neonatal period in predicting neurological outcome at 18 months of age was not found to be altered by hypothermia.

Recent studies have focused on the predictive value of analysis of images performed in diffusion scans and they found that findings within the first days of life correlate with long-term motor outcome (50-52).

5. Use of therapeutic hypothermia beyond the limits of recommendations

The use of TH for neuroprotection is increasingly supported also in several adult patient groups, including those with cardiac arrest, stroke, or head injury (53). The latest edition of the Recommendations of the Neonatal Neurology Study Group of the Italian Society of Neonatology for the care of the newborn with hypoxic-ischemic encephalopathy (54), clearly defined the category of patients to be subjected to hypothermic treatment. The indications for treatment consisted in the presence of moderate-severe hypoxic-ischemic encephalopathy and in the presence of specific clinical-laboratory and instrumental conditions (level of resuscitation in the delivery room, severe metabolic acidosis, and severe alterations in electroencephalographic monitoring). The recommendations made explicit that these criteria apply only to infants with gestational age ≥35 weeks, neonatal weight ≥1800 g, and less than 6 hours old. However, they contemplate the possible inclusion of exceptional events in patients outside the above criteria but victims of brain insult, for whom there is no specific therapy to date. These categories include infants less than 35 weeks of gestation age with encephalopathy, infants with perinatal ischemic stroke, and infants with sudden postnatal collapse event.

Perinatal arterial ischemic strokes are the most frequent cause of cerebral palsy in term or late preterm infants (55) and some infants may present with signs of encephalopathy (56). Early diagnosis of ischemic stroke remains a challenge and often, when it is made, the "therapeutic window" has probably been lost (53). In a systematic review and meta-analysis of animal studies covering more than 3000 cases, hypothermia was associated with a 44% reduction in infarcted area (57). A recent preclinical study reported a reduction in the ischemic event in parallel with a reduction in the affected area resulting in improved motor outcome (58), and the first clinical study (59) suggested benefits in long-term cognitive performance. In adulthood the use of hypothermia seems to be associate with increased functional independence (60), but results may be affected by the use of thrombolytic therapy (61).

Regarding prematurity and therapeutic hypothermia, the safety ed efficacy of TH remains uncertain (62). Symptoms and instrumental signs of encephalopathy may overlap with those of prematurity, making the diagnosis difficult. Furthemore, even if encephalopathy may have a different etiology in preterm infants compared to term infants, most clinical studies performed used term infants as a control group (63, 64). To date, the American Academy of Pediatrics states that the extension of hypothermia to preterm infants should be performed only in the research setting (66). The hope is that the results of ongoing randomized trials will shed light on this issue (67).

Finally, for infants affected by sudden unexpected post-natal collapse (68-72) therapeutic hypothermia although in the absence of direct scientific evidence is the only possible treatment to improve the neurological outcome (72-75). Indeed, the brain damage caused by postnatal collapse is similar to the one related to perinatal asphyxia, both from clinical and neuroradiological point of view (76). Although, there have been no randomized clinical studies on the use of hypothermia in patients with sudden unexpected post-natal collapse (either because of the small number of cases or the heterogeneity of underlying causes), several authors suggest that brain cooling should be undertaken in infants with sudden unexpected post-natal collapse who meet clinical and electroencephalographic criteria for hypothermia (72-75,77). Indeed, preliminary results from observational studies in patients with Sudden Unexpected Post Natal Collapse undergoing hypothermia are encouraging (71,72,78,79).

6. Aims of the study

Primary aim. The primary aim of this study was to evaluate characteristics in terms of grade of HIE and EEG abnormalities of infants who underwent TH in a network of Italian NICUs during routine clinical practice.

Secondary aim. As secondary aim we assessed the severe functional disability at 2 years of age in infants treated with TH during routine clinical practice. Severe functional disability was defined as the presence of at least one of the following: cerebral palsy, a BSDI III cognitive composite score < 2 SD or a GMDS-R GQ < 2 SD, bilateral blindness (visual acuity < 6/60 in better eye), bilateral deafness (requiring bilateral hearing aids or unilateral/bilateral cochlear implants) or epilepsy.

7. Materials and Methods

This was a prospective observational study based on the Neuronat network. Neuronat is an Italian network of NICUs aiming to ensure a common neurodevelopmental assessment in infants with HIE treated with TH. The network was set up in 2012 and NICUs included in the network planned seminars and meetings to define and share the study protocol. Currently 8 Neonatal Intensive Care Units (all coming from Emilia Romagna, a northern Italian Region) participate to the network.

In 2015 the study was approved by the ethics committee Area Vasta Emilia Nord (protocol 221/15). Neonatal enrollment was started in 2016 and since 2021 the study has received grants from the Mariani Foundation of Milan. A written informed consent was obtained from the parents of each neonate enrolled in the study. Neonatal data were anonymized.

For data collection an electronic standardized form was ad-hoc developed on REDCap platform. The form included multiple sections regarding: perinatal data, clinical and neurological data during the first hospital admission; neurodevelopmental follow-up data.

7.1. Population

All infants born between January 1st 2016 to December 31st 2019 who received TH and completed the neurodevelopment follow-up at 24 months of age were included. TH was performed based on clinical and EEG criteria according to the Italian Guidelines for using TH (54). According to guidelines infants eligible to TH were those with gestational age ≥35 weeks, birth weight ≥1800 g and the following criteria:

1. Intrapartum asphyxia, confirmed by at least one of the following: 10 min Apgar score ≤ 5, ventilation with an endotracheal tube (or mask) for at least 10 min after birth, severe acidosis (defined as cord pH or any arterial/venous pH ≤ 7.0 or base deficit ≥ 12 mmol/L within 60 min of birth.
2. Moderate to severe neonatal encephalopathy assessed within 6 h of birth, according to Shalak et al (30,80).
3. Moderate to severe EEG abnormalities or seizures confirmed by EEG recording.

We included information regarding sentinel events, defined as an acute event in the perinatal history possibly reducing the placental blood flow, taking place perinatally or causing an immediate delivery. Sentinel events included: placental abrution, uterine rupture, cord prolapsus, amniotic embolism, acute anemia, shoulder dystocia, maternal collapse and sudden unexpected post-natal collapse.

7.2. Neurological examination at admission

Neurological examination in the acute phase included assessment of archaic reflexes, muscle tone (posture, mobilization of body segments), active and passive motility, level of consciousness, and sense-perceptual skills. The prevalence of signs determines the degree of encephalopathy; if neurological signs were equally distributed among the various stages, the degree of HIE was defined by the level of consciousness. Figure 3 provides the definition of the 3 stages of HIE as follows:

-mild HIE: hyperarousal, normal tone, motility and posture, tremor, exaggerated Moro reflex, no autonomic nervous system dysfunction;

-moderate HIE: lethargy, impaired motility, distal flexion/full extension, hypotonia, weak/incomplete primitive reflexes, miosis, bradycardia

-severe HIE: stupor, coma, decerebrate posture, absent motility, flaccidity, absent reflexes, mydriatic/deviated/unresponsive pupils, apnea

Neurological examination to assess the HIE grade was also repeated during hospital stay at 7 days of life.

Figure 3. Neurological examination at admission.

Allegato 1: Esame obiettivo neurologico

(Shalak LF et al, *Paediatrics* 2003; 111:351-357; Shankaran S et al, *N Engl J Med* 2005;353:1574-84)

(da effettuarsi tra 30 e 60 minuti di vita, tra 6 e 24 ore, in 3° e 7° giornata)

1. **Livello di coscienza**
   - Iperallerta *(neonati in piena veglia con difficoltà a dormire,occhi spalancati, sembrano 'fissare' e presentano ridotto ammiccamento)* ☐
   - Letargia *(la risposta agli stimoli è completa ma ritardata, con una soglia aumentata; c'è una riduzione dei movimenti spontanei)* ☐
   - Stupore/coma *(c'è risposta solo a stimoli energici e il tipo di risposta consiste in una retrazione delle estremità o nell'assunzione di una postura decerebrata; assenza di riflessi corneali; spesso c'è necessità di assistenza respiratoria)* ☐
2. **Motilità**
   - Normale/aumentata/tremori ☐
   - Ridotta ☐
   - Assente ☐
3. **Postura**
   - Normale ☐
   - Flessione distale/completa estensione *(atteggiamento delle braccia con flessione ai polsi e estensione ai gomiti, in genere accentuata da stimolazione)* ☐
   - Decerebrata *(atteggiamento rigido con flessione ai polsi, estensione ed intrarotazione delle braccia, estensione delle gambe e flessione forzata plantare dei piedi, opistotono)* ☐
4. **Tono assiale (valutato alla manovra di trazione e/o in sospensione ventrale)**
   - Normale ☐
   - Ipotonia (Fig. 1 a,b) ☐
   - Flaccidità ☐
5. **Riflessi primitivi (riflesso di Moro e/o riflesso di suzione)**
   - Normale/esagerato ☐
   - Deboli /incompleti (Fig. 2 a,b) ☐
   - Assenti ☐
6. **Disfunzione autonomica delle pupille**
   - Assente ☐
   - Miosi ☐
   - Midriasi, deviazione o reattività assente ☐

☐ **EII lieve**: iperallerta, tono, motilità e postura normali, tremori, riflesso di Moro esagerato, non disfunzione del sistema nervoso autonomo

☐ **EII moderata**: letargia, motilità ridotta, flessione distale/completa estensione, ipotonia, riflessi primitivi deboli/incompleti, miosi, bradicardia

☐ **EII severa**: stupore o coma, postura decerebrata, motilità assente, flaccidità, riflessi assenti, pupille midriatiche/deviate/non reattive, apnea

7.3. Electroencephalographic assessment

According to single center local protocols, infants were monitored by amplitude EEG (aEEG) or polygraphyc EEG (pEEG) on admission to NICU and during treatment with TH

Recordings were considered as:

- Normal:
    - aEEG: continuous normal voltage (minimum amplitude greater than 5μV and maximum amplitude greater than 10 to 25μV)
    - pEEG: continuous baseline activity, normal voltage, differentiation of behavioral states
- Mild abnormalities:
    - aEEG: discontinuous normal voltage (minimum amplitude less than 5μ V but demonstrating variability, maximum amplitude greater than 10 μV)
    - pEEG: continuous baseline activity with mild abnormalities (i.e. mild asymmetry, mild voltage depression, or poorly defined sleep-wake rhythm)
- Moderate abnormalities:
    - aEEG: burst suppression (minimum amplitude less than 3-5μV with minimal variability, bursts greater than 25μV of variable density)
    - pEEG: discontinuous activity with IBI <10 sec, sleep-wake cycle not clearly recognizable or clear asymmetry / asynchrony
- Severe abnormalities or seizures:
    - aEEG: Low Voltage (continuous background with both minimum and maximum amplitudes less than 10μ V); isoelectric/flat (primarily isoelectric tracing with amplitudes less than 5μ V); seizures (increase in the minimum amplitude; repetitive seizures appear as a “saw tooth” pattern)
    - pEEG: discontinuous activity with IBI of 10-60 sec, severe depression of baseline activity, absent sleep-wake cycle or inactive track (basic activity <10 microV or severe discontinuity with IBI> 60 sec; repetitive and rhythmic activity lasting> 10 sec with distinct onset, course and conclusion

7.4. Therapeutic Hypothermia

Infants underwent systemic hypothermia performed by CritiCool MTRE (Charter Kontron, Milton Keynes, UK) and they were mantained to a rectal temperature of 33-34°C. Treatment was started as soon as possible within the first 6 hours of life, and continued for a total duration of 72 hours. Thereafter, a period of gradual warm-up (+0.5°C/hour), lasting approximately 6 hours was carried out. During the procedure, analgosedation was usually administered.

The systemic hypothermia device used consists in a special blanket placed that circulates water, which can be cooled or warmed and that achieves uniform cooling of the whole body. The device monitors the neonate's temperature with a rectal probe and maintains the desired target temperature by altering circulating water temperature. Data regarding technical details of TH application were gathered.

During hospital stay and TH treatment we collected data regarding multiple interventions ed evaluations: respiratory assessment and need of respiratory support, cardiovascular assessment and need of cardiovascular support, renal assessment, metabolic and infectious assessment.

7.5. Neurodevelopmental follow-up

Surviving infants were assessed at 24 months of age with neurological examination according to the Amiel-Tison neurological assessment (81) and either with the Griffiths Mental Developmental Scales (GMDS-R, 1996) (82) or the Bayley Scales of Infant and Toddler Development (BSDI III, 2006) (83), depending on the local protocols. Cerebral palsy was defined as a permanent but not unchanging disorder of movement or posture or both and of motor function (the types of CP were spastic [diplegia, hemiplegia, or quadriplegia], dystonic, or athetoid (84)). GMDS-R (0–2 years) provides a General Development Quotient (GQ) of infants' abilities with a mean of 100.5 and an SD of 11.8 and five subscale quotients (Loco- motor, Eye & Hand Coordination, Personal & Social, Hearing & Language, and Cognitive Performance). The BSDI III provides standardized composite scores for each of the domains assessed (cognitive, fine and gross motor, receptive and expressive language, and adaptive), with a mean of 100 and an SD of 15. For both the GMDS-R and BSID-III, the cut-off abnormality was 2 standard deviations (SD) below the normative mean.

All neurodevelopmental follow-up visits were performed by a neonatologist together with a psychologist; a neuropsychiatrist and a physiotherapist with pediatric skills were also present in 79.2 % and 75.2 % of visits respectively.

7.6. Neuroimaging findings

Brain MRI was routinely performed in all infants. All patients included in the study underwent a first brain MRI, under normothermic conditions in the acute phase (4-10 days of life), after TH was discontinued. Conventional (T1-weighted SE, T2-weighted TSE, FLAIR, T2-weighted GE) and diffusion-weighted (DWI) sequences were obtained. The severity of brain damage was assessed by neonatologists together with experienced neuroradiologists basing on MRI reports regarding conventional sequences, and blinded to clinical information. Signal intensity alterations in BGT, PLIC, white matter, and cortex were classified according to the classification of Rutherford et al. (47) into 5 different damage patterns. As per protocol, a second brain MRI was performed at approximately 1 month of life if there were concerns at first investigation. Because conventional sequences obtained in the first days of life may underestimate the brain damage, the second MRI scan (when available) was evaluated.

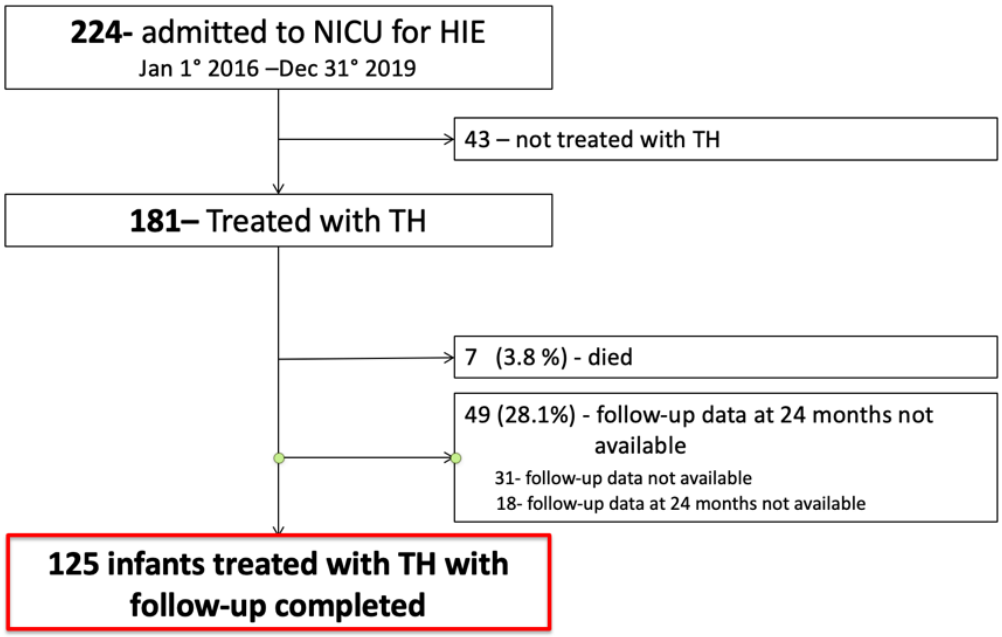
7.7. Statistical Analysis

The characteristics of infants were summarized using descriptive statistics. Continuous variables were expressed as mean ± standard deviation, whereas categorical variables were expressed as frequencies. Differences in the distribution of categorical or continuous variables were compared by using X2 or Mann-Whitney U test (or Kruskal-Wallis tests) respectively. Severe neurodevelopmental outcome and related factors were assessed at uni- and multi-variate logistic regression analysis. We used exploratory strategy for including variables in the multivariable model. To assess multicollinearity, the correlation coefficient and variance inflation factor (VIF) were checked. The odds ratio was used as a measure of association, and it was reported with its 95% confidence interval. A p valued < 0.05 was considered significant. Data were analyzed using STATA software (version 13.0).

8. Results

From January 1st 2016 to December 31st 2019, 224 infants were admitted to NICUs of Emilia Romagna region for HIE; 181 (80.1%) received TH and 7 of them (3.8%) died. Among the remaining 174 surviving infants, 125 (71.8%) completed the neurodevelopmental follow-up at 24 months of age (Figure 4).

Figure 4. Enrollment flow diagram.



From the Natality Report of Regione Emilia Romagna, in the period evaluated 123.000 full-term infants were born from January 1st 2016 to December 31st 2019. The incidence of HIE was 1.8/1000 live births.

Infants who died had a median gestational age of 40 weeks (three of them were born preterm at 33, 34 and 36 weeks of gestational age); 4 out of 7 were outborn. All infants who died were intubated at birth, 6 received chest compression and 5 epinephrine. Six out of 7 had severe HIE (1 infant had moderate HIE at admission); the median age at death was 9 days of life.

In the next sections we will evaluate surviving infants treated with TH (complete follow-up visits, severity of HIE, EEG abnormalities or presence of severe functional disability).

8.1. Demographics and procedure data of the population

Infants treated with TH without a full 24-month neurodevelopmental follow-up were more likely to be delivered by caesarean section and to have cord blood abnormalities.

Most neonatal data (mean gestational age, mean birth weight, metabolic acidosis after birth and need of advanced resuscitation) were consistent according to the enrollment criteria. No infants had positive blood culture. Full standard criteria for TH were met in 79 (48.2%) neonates, while in 85 (51.8%) at least 1 criterion (1, 2 or 3) was missing.

Table 4. Perinatal characteristics and clinical presentation before therapeutic hypothermia was started. Comparison between infants with and without complete follow-up at 24 months of age.

	All infants N=174	Missing	Patients with 24 months follow-up N=125	Missing	Patients without 24 months follow-up (49)	Missing	*p*
Median GA, weeks	40	2	40		40	2	0.883
IQR	38,4;40.8		38,3;41	-	38.8;40.6		
Median Birth weight, grams	3300	6	3300		3410	6	0.614
IQR	2942-3692		2950-3660	-	2840;3810		
Sex, male, N (%)	104 (59.8)	-	74 (59.2)	-	30 (61.2)	-	0.806
Twins, N (%)	4 (2.3)	1	4 (3.2)	-	0	1	0.210
Outborn, N (%)	74 (42.5)	-	49 (39.2)	-	25 (51)	-	0.156
Mother Race		1		1		-	
Caucasian, N (%)	146 (84.4)		109 (87.9)		37 (75.5)		0.119
African, N (%)	17 (9.8)		9 (7.3)		8 (16.3)		
Asiatic, N (%)	10 (5.8)		6 (4.8)		4 (8.2)		
Maternal complications during pregnancy		12		2		10	0.623
Diabetes, N (%)	15 (9.2)		12 (9.7)		3 (7.7)		
High blood pressure, N (%)	13 (8)		12 (9.7)		1 (2.5)		
Others, N (%)	34 (20.9)		21 (17.2)		13 (33.3)		
PROM, N (%)	28 (17.2)	12	20 (16.4)	3	8 (20)	9	0.601
CTG abnormalities, N (%)	96 (60.7)	16	71 (59.7)	6	25 (64.1)	10	0.662
Caesarean Section, N (%)	74 (42.5)	-	45 (36)	-	29 (59.2)	-	**0.005**
Umbilical cord abnormalities, N (%)	17 (10.7)	16	16 (13.8)	9	1 (2.4)	7	**0.041**
Sentinel events		5		1		4	0.639*
Placenta abrution, N (%)	15 (8.8)		11 (8.9)		4 (8.8)		
Uterine rupture, N (%)	6 (3.5)		4 (3.2)		2 (4.4)		
Cord Prolapsus, N (%)	4 (2.4)		2 (1.6)		2 (4.4)		
Amniotic Embolism, N (%)	1 (0.6)		1 (0.8)		0		
Acute anemia, N (%)	4 (2.4)		3 (2.4)		1 (2.2)		
Shoulder dystocia, N (%)	6 (3.5)		3 (2.4)		3 (6.6)		
Maternal collapse, N (%)	2 (1.2)		2 (1.6)		0		
SUPC, N (%)	2 (1.2)		2 (1.6)		0		
Total, N (%)	47 (27)		35 (28)		12 (24.5)		
Apgar 10 minute Median	7	19	7	11	7	8	0.250
IQR	5;8		5;8		5;8		
Resuscitation		-		-		-	
Intubation, N (%)	81 (46.5)		58 (46.4)		23 (46.9)		0.949
Chest compression, N (%)	44 (30.4)		36 (30.4)		8 (16.3)		0.089
Epinephrine, N (%)	22 (25.2)		22 (17.6)		5 (10.2)		0.226
Lowest pH$^{\delta}$ Median	6.98	25	6.99	16	6.98	9	0.947
IQR	6.84;7.15		6.85;7.15		6.81;7.17		
Lowest base excess$^{\delta}$ Median	-15	36	-15	20	-13.7	16	0.414
IQR	-18.4;-10.9		-18.1;-11		-18.7;-8.8		
Body Temp. (°C) at admission		25		15		10	0.385
Median	35		35		35		
IQR	34;35.7		33.7;35.7		34;36		
HIE severity within first 6 h of life, N (%)		-		-		-	0.384
none, N (%)	2 (1.2)		1 (0.8)		1 (2.0)		
mild, N (%)	32 (18.4)		24 (19.2)		8 (16.3)		
moderate, N (%)	118 (67.8)		87 (69.6)		31 (63.3)		
severe, N (%)	22 (12.6)		13 (10.4)		9 (18.4)		
EEG abnormalities at admission, N (%)$^{\pm}$		10		-		10	0.117
0	13 (7.9)		10 (8.0)		3 (7.7)		
1	60 (36.6)		51 (40.8)		9 (23.1)		
2	62 (37.8)		46 (36.8)		16 (41.0)		
3	29 (16.70)		18 (14.40)		11 (28.2)		
Presence of full standard criteria for TH, N (%)	79 (48,2)	10	57 (45.6%)	-	22 (56.4%)	10	0.238

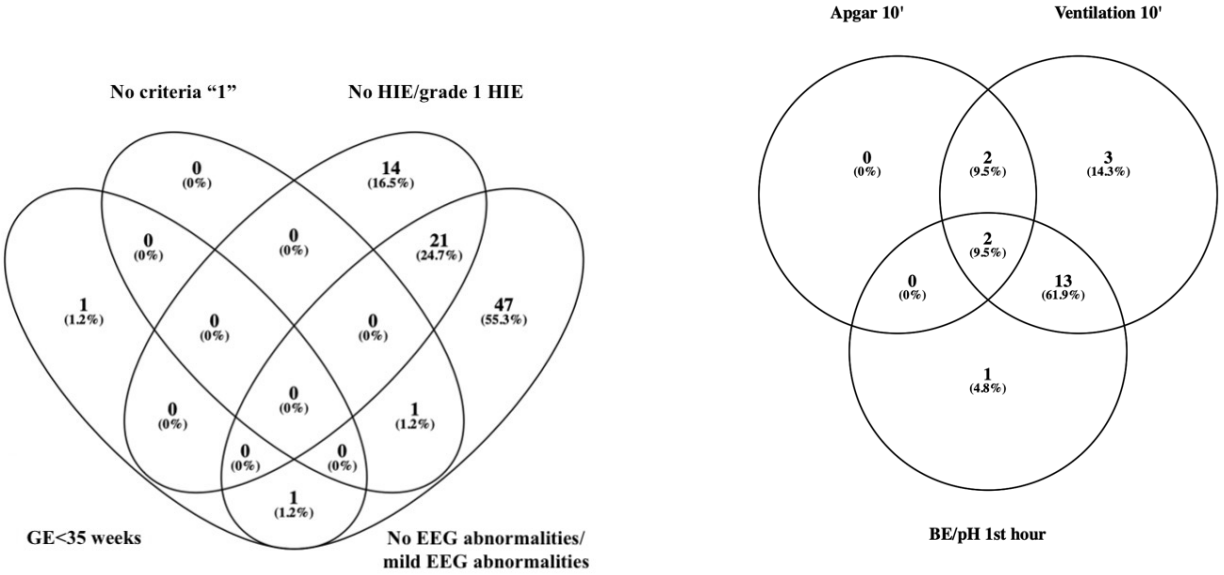
CTG, cardiotochography ; GA, gestational age; IQR, interquartile; PROM, prolonged rupture of membrane; SUPC, sudden unexpected post-natal collapse;

¶ *Two cases were elective caesarean section, in the remaining caesarean sections were urgent or emergent*

[δ]Umbilical cord or arterial, venous or capillary bloods values obtained within the first hour of life
[±]At admission a total of 5 infants had seizures (3 out of 125 infants with completed follow-up, and 2 out of 49 without completed follow-up).
[*]p comparison of the total of sentinel events between the three groups. The comparisons of the single sentinel events between the three groups were non statistically significant.

Figure 5A shows the Venn Diagram reporting the number of infants who did not meet the criteria for each category. Figure 5B shows that 21 infants did not meet both neurological examination and EEG criteria, however they met at least one criterion for asphyxia: 15 out 21 infants (71.4%) had both criteria for need of ventilation and severe metabolic acidosis.

Figure 5. Infants who did not meet criteria for TH.



A. B.

Criteria 1: Intrapartum asphyxia, confirmed by at least one of the followings: 10 min Apgar score ≤ 5, ventilation with an endotracheal tube (or mask) for at least 10 min after birth, severe acidosis (defined as cord pH or any arterial/venous pH ≤ 7.0 or base deficit ≥ 12 mmol/L within 60 min of birth).
A. Infants who did not meet the criteria for TH for each category. B. Criteria 1 was met by 21 infants who did not meet both neurological assessment and EEG criteria.

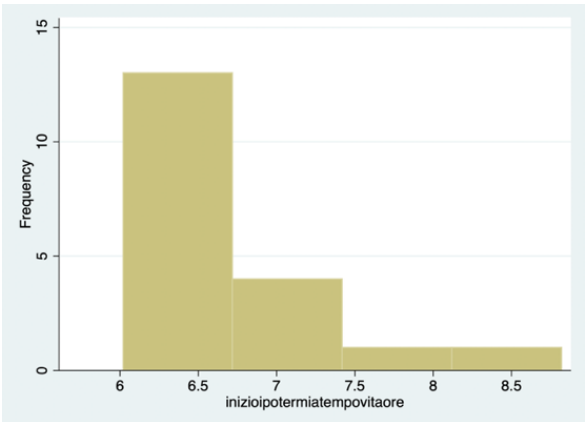
TH was started within 6 hours at a median time of 4.2 hours of life; however, a few infants (30 out of 163, 18.4%) were started (Figure 6) beyond the first 6 hours of life (median time of 6.25 hours, IQR 6.15;6.95). TH was administered significantly earlier in inborn as compared to outborn infants

(3.92 *vs* 5 hours respectively, p=0.011), while body temperature on admission was higher in inborn as compared to outborn infants (35 vs 34.5°C respectively, p=0.011).

Table 5. Management and adverse events of infants treated with TH.

	All infants N=174	Missing	Patients with 24 months follow-up N=125	Missing	Patients without 24 months follow-up (49)	Missing	*p*
Time to initiation TH, (hours)		11		4		7	0.722
Median	4.18		4.25		3.84		
IQR	2.83:5.5		2.87:5.42		2.63;5.62		
TH duration, (hours)		6		4		2	0.070
Median	72		72		72		
IQR	71.3:72		72:72		72:72		
Re-warming duration		24		16		8	0.558
Median	6		6		6		
IQR	6:7,25		6:7,42		6:7		
Respiratory support during TH		28		14		14	**0.005**
None, N (%)	44 (30.1)		41 (36.9)		3 (8.6)		
Non-invasive oxygen support, N (%)	55 (37.7)		39 (35.2)		16 (45.7)		
Invasive ventilation, N (%)	47 (32.2)		31 (27.9)		16 (45.7)		
Analgo-sedation, N (%)	153 (99.4)	20	117 (100)	8	36 (97.3)	12	0.074
Fentanyl, N (%)	152 (98.7)		116 (99.2)		36 (97.3)		
Morphine, N (%)	1 (0.65)		1 (0.85)		0		
Benzodiazepines, N (%)	43 (27.9)		39 (33.3)		4 (10.8)		
Adverse effect during TH		2		-		2	0.332
Bradycardia, N (%)	28 (16.2)		17 (13.6)		11 (23.4)		
Adiponecrosis, N (%)	7 (4.0)		5 (4)		2 (4.2)		
Gastrointestinal bleeding, N (%)	1 (0.58)		1 (0.8)		0		
Pulmonary Hemorrhage, N (%)	3 (1.7)		2 (1.6)		1 (2.12)		
Intraocular bleeding, N (%)	0		0		0		
Thrombocytopenia, N (%)	16 (9.3)		13 (10.4)		3 (6.3)		
Need to increase temperature, N(%)	4 (2.3)		3 (2.4)		1 (2.12)		
Pulmonary hypertension, N (%)	20 (11.6%)		14 (11.20)		6 (12.7)		
Need to interrupt TH, N(%)	1 (0.58)		1 (0.8)		0		

Figure 6. Rates of infants who underwent TH beyond the first 6 hours of life.



Median duration of TH was 72 hours; for 1 infant the treatment was discontinued after 39 hours, because of severe pulmonary hypertension. The median duration of rewarming was 6 hours.

The target body temperature during TH was 33.5°C and 32.2% of infants received mechanical ventilation. Almost all infants (99.4%) received analgo-sedation treatment during TH, mainly through opioids (fentanyl was preferred to morphine) and benzodiazepine were added to opioids only in a few infants (27.9%).

Most common adverse effects related to TH were bradycardia (16.2%), followed by pulmonary hypertension (11.6%), thrombocytopenia (9.3%), adipo-necrosis (4%), pulmonary hemorrhage (1.7%), and gastrointestinal bleeding (0.58%). Few infants (2.3%) needed an increase of controlled temperature during the treatment. Infants without the full 24-month follow-up visits were more likely to have respiratory support, either invasive or non-invasive (p=0.005).

Table 6. Seizures and treatment of seizures between in infants with or without complete follow-up visits at 24 months of age

	All infants N=174	Missing	Patients with 24 months follow-up N=125	Missing	Patients without 24 months follow-up (49)	Missing	*p*
Seizures during TH, N (%)*	59 (34.3)	2	33 (26.4)	0	26 (55.3)	2	**<0.001**
Seizures onset during rewarming	1 (0.58)	3	0	1	1 (2.1)	2	0.103
Onset timing of seizures¶		3		3		-	0.648
Median	12		12		13,5		
IQR	6;24		6;24		6;30		
drugs administered for seizures		3		3		0	-
Phenobarbital, N (%)	40/56 (71.4)		17/33 (51.5)		23/26 (88.5)		
Phenytoin, N (%)	31/56 (55.4)		15/33 (45.5)		16/26 (46.2)		
Benzodiazepines, N (%)	19/56 (33.9)		9/33 (27.0)		10/26 (23)		
Levetiracetam, N (%)	3/56 (5.3)		2/33 (6.0)		1/26 (3.8)		
Lidocaine, N (%)	3/56 (5.3)		3/33 (9.1)		0 (0)		
Vitamins, N (%)	1/56 (1.8)		1/33 (3)		0 (0)		
Others, N (%)	3/56 (5.3)		3/33 (9.1)		0 (0)		

*Clinical and/or encephalographic seizures

¶Two cases were elective caesarean section, in the remaining caesarean sections were urgent or emergent

All infants underwent amplitude integrated encephalography (a-EEG) or conventional encephalography (c-EEG) during TH. The incidence of seizures was 34.3%, the median age at the onset was 12 hours and 1 infant developed seizures during rewarming (73 hours of life). Most commonly administered drugs were phenobarbital (71.4% of cases), phenytoin (55.4% of cases), benzodiazepines, levetiracetam, lidocaine and vitamins (33.9 %, 5.3%, 5.3% and 1.8% respectively). Infants without the full 24-month follow-up visits (either invasive or non-invasive) were more likely to have seizures (55.3%, *vs* 26.4% p<0.001).

The severity of HIE evaluated at 7 days of life is shown in table 7; approximately 20% of infants had moderate or severe HIE; 27.1% of infants had mild HIE and 53% had no signs of HIE at 1 week. The severity of HIE between infants with and without a full neurodevelopmental follow-up (at 24 months of age) did not differ.

Table 7. Comparison of HIE severity between infants treated with TH with or without complete follow-up completed at 24 months of age

	All infants N=174	Missing	Patients with 24 months follow-up N=125	Missing	Patients without 24 months follow-up (49)	Missing	*p*
HIE at 7 days of life		8		5		3	0.063
0	88 (53.0)		71 (59.1)		17 (37)		
mild	45 (27.1)		27 (22.5)		18 (39.1)		
moderate	20 (12.1)		14 (11.7)		6 (13.0)		
severe	13 (7.8)		8 (6.7)		5 (10.9)		

Most infants (134 out of 174, 77%) had pattern 5 of cerebral MRI, while a few had patterns 1 or 2 (20 out of 174, 11.5%). Almost 50% of infants underwent 2 brain MRI scans, and the median timing of the last MRI performed was 30 days of life. Comparing infants with and without neurodevelopmental follow-up completed at 24 months a significant difference was present regarding MRI patterns, number of MRI performed and timing of performance; infants without a full neurodevelopmental follow-up at 24 months had higher incidence of patterns 1 and 2, and were more likely to undergo subsequently further brain MRI study (late).

Table 8. MRI findings in infants treated with TH who had or had not a full 24 months follow-up

	All infants N=174	Missing	Patients with 24 months follow-up N=125	Missing	Patients without 24 months follow-up (49)	Missing	*p*
MRI patterns		-		-		-	
1, N (%)	13 (7.5)		9 (6.4)		5 (10.2)		**0.023**
2, N (%)	7 (4.0)		2 (1.6)		5 (10.2)		
3, N (%)	5 (2.9)		4 (3.2)		1 (2.0)		
4, N (%)	15 (8.6)		8 (6.4)		7 (14.3)		
5, N (%)	134 (77.0)		102 (82.4)		31 (63.3)		
Number of MRI performed		-		-		-	**0.002**
1, N(%)	89 (51.2)		73 (58.4)		16 (32.7)		
2, N(%)	85 (48.8)		52 (41.6)		33 (67.3)		
Days of life at the time last MRI was performed		8		6		2	**0.001**
Median	30		20		38		
IQR	6;41		6;38		10;49		
Range	4-218		4-218		4-181		

8.2. Demographics and procedure according to HIE severity

Demographics and clinical findings at birth of infants with a full neurodevelopmental follow-up at 24 months are shown in table 9 according to HIE severity. Infants with severe HIE were less likely to be male, and more likely to be delivered through cesarean section, to have a low Apgar score at 10 minutes, need for resuscitation, low pH and high base defect, placenta abruption, acute anemia and more severe EEG abnormalities on admission.

Table 9. Demographics and clinical findings at birth according to HIE severity. Only infants with a full neurodevelopmental follow-up at 24 months are included

	Study subjects (N=124) ¶						
	Mild HIE N=24	Missing	Moderate HIE N=87	Missing	Severe HIE N=13	Missing	*P**
GA, weeks Median IQR	40 38,7;41	-	40 38;41	-	40 37.4;40	-	0.381
Birth weight, grams Median IQR	3355 3077-3595	-	3250 2940-3690	-	3150 2715;3350	-	0.346
Male sex, , N (%)	18 (75)	-	51 (58.6)	-	4 (30.8)	-	**0.033**
Twinning, N (%)	0	-	4 (4.6)	-	0	-	0.415
Outborn, N (%)	13 (54.2)	-	28 (32.2)	-	7 (53.8)	-	0.073
Mother Race		-					
Caucasian, N (%)	21 (87.5)		78 (90.7)	1	10 (76.9)	-	0.425
African, N (%)	2 (8.3)		5 (5.8)		2 (15.4)		
Asiatic, N (%)	1 (4.2)		3 (3.5)		1 (7.7)		
Mother diseases during pregnancy							
Diabetes, N (%)	3 (30)	-	7 (26.9)	2	2 (33.3)	-	0.408
High blood pressure, N (%)	3 (30)		8 (30.7)		1 (16.7)		
Others, N (%)	5 (50)		13 (50)		3 (50)		
PROM, N (%)	4 (16.7)	-	14 (16.7)	3	2 (15.4)	-	1.000
Intrapartum fever, N (%)	1 (6.7)	9	7 (12.3)	30	1 (16.7)	7	0.712
CTG abnormalities, N (%)	9 (40.9)	16	51 (61.5)	6	10 (76.9)	10	0.094
Delivery, Cesarean Section, N (%)	6 (25)	-	30 (34.5)	-	9 (69.2)	-	**0.023**
Funiculus abnormalities, N (%)	2 (8.7)	1	12 (15.2)	8	2 (15.4)	-	0.769
Sentinel events		-		-		1	
Placenta abrution, N (%)	2 (8.3)		5 (5.7)		4 (30.7)		**0.012**
Uterine rupture, N (%)	1 (4.1)		3 (3.4)		0		0.773
Funiculus Prolapsus, N (%)	0		2 (2.3)		0		0.649
Amniotic Embolism, N (%)	0		1 (1.1)		0		0.807
Acute anemia, N (%)	0		1 (1.1)		2		**0.047**
Shoulder dystocia, N (%)	2 (8.3)		1 (1.1)		(16.6)		0.124
Maternal collapase, N (%)	0		2 (2.3)		0		0.649
SUPC, N (%)	1 (4.1)		1 (1.1)		0		0.509
Total, N (%)	8 (33.3)		21 (24.1)		0 5 (38.4)		0.430
Apgar 10 minute Median IQR	7 5;8	3	7 6;8	8	5 4;6	-	**0.026**
Resuscitation							
Intubation, N (%)	8 (33.3)	-	40 (46)	-	10 (76.9)	-	**0.039**
Chest compression, N (%)	7 (29.2)	-	20 (23)	-	8 (61.5)	-	**0.020**
Epinephrine, N (%)	3 (12.5)	-	14 (16.1)	-	5 (38.5)	-	0.138
Lowest pH[δ] Median IQR	7.17 6.9;7.24	2	6.98 6.86;7.09	11	6.8 6.75;7.15	2	**0.002**
Lowest base excess[δ] Median IQR	-12.5 -16;-7.6	3	-15.1 -18;-11.5	13	-19.9 -23;-15	3	**0.006**
EEG abnormalities on admission, N (%)							
0	1 (4.2)	-	9 (10.3)	-	0	-	**<0.001**
1	14 (58.3)		35 (40.2)		1 (7.7)		
2	9 (37.5)		34 (39.1)		3 (23.1)		
3	0		9 (10.4)		9 (69.2)		
Presence of full standard criteria for TH, N (%)	0	-	45 (51.7%)	-	12 (92.3%)	-	**<0.001**

CTG, cardiotochography ; GE, gestational age; HIE, hypoxic-ischemic encephalopathy; IQR, interquartile; PROM, prolonged rupture of membrane; SUPC, sudden unexpected post-natal collapse;
[δ]Umbilical cord or arterial, venous or capillary samples were obtained within the first hour of life
[¶]One infant treated with TH but without HIE at admission was not included.
**For continuous variables Kruskal-Wallis test was used to compare groups.*

Table 10 shows the management of TH and adverse events in infants with a full follow-up at 24 months, according to HIE severity. Time to initiation of TH was earlier and rates of respiratory support increased with a higher severity of HIE.

Table 10. Management of therapeutic hypothermia and adverse events. Only infants with a full neurodevelopmental follow-up at 24 months are included

	Study subjects (N=124) (N=124) [¶]						
	Severity of HIE						
	Mild HIE N=24	Missing	Moderate HIE N=87	Missing	Severe HIE N=13	Missing	*p**
Time to initiation of TH (hours)							
Median	5.4	-	4.2	4	3.9	-	**0.009**
IQR	3.9:5.9		2.7:5.3		2.7;4.1		
TH duration, hours							
Median	72	-	72	4	72	-	0.125
IQR	71.7:72		72.2:72		72:72		
Duration of re-warming, hours							
Median	6	22	6	11	6.5	3	0.710
IQR	6:7		6:7,3		6:9		
Respiratory support during TH							
None, N (%)	14 (60.9)	1	26 (33.8)	10	1 (10)	3	**<0.001**
Non-invasive oxygen support, N (%)	7 (30.4)		31 (40.2)		1 (10)		
Invasive ventilation, N (%)	2 (8.7)		20 (26)		8 (80)		
Analgosedation, yes N (%)	23 (100)	1	82 (100)	5	11 (100)	12	-
Adverse effect during TH, N (%)[‡]	5 (20.8)	-	23 (26.4)	-	4 (30.7)	-	0.781

HIE, hypoxic-ischemic encephalopathy; TH, therapeutic hypothermia
[¶]One infant treated with TH but without HIE at admission was not included.
**For continuous variables Kruskal-Wallis test was used to compare groups.*
[‡]Comparisons of single adverse events between the three study groups were not significant.

Seizures during TH were more common in more severe HIE and their onset was earlier (table 11).

Table 11. Comparison of seizures characteristics and management in infants treated with TH. Only infants with a full neurodevelopmental follow-up at 24 months are included

	Study subjects (N=124) [¶]						
	Severity of HIE						
	Mild HIE N=24	Missing	Moderate HIE N=87	Missing	Severe HIE N=13	Missing	*p**
Seizures during TH, N (%)[†]	2 (8.3)	-	23 (26.4)	-	8 (61.5)	-	<0.002
Timing of onset of seizures (hours of life)							
Median	25	-	11	2	10.5	2	0.308
IQR	12;38		7;24		3;18		

[†]Clinical and/or encephalographic seizures

[¶]*One infant treated with TH but without HIE at the admission was not included in this table.*

**For continuous variables Kruskal-Wallis test was used to compare groups.*

MRI findings in infants treated with TH according to the severity of HIE are shown in table 12. A severe cerebral impairment increased with the increasing severity of HIE and vice versa. Infants with severe HIE had an incidence of pattern 1-2 in 45.5% of cases.

Table 12. MRI findings in infants treated with TH. Only infants with a full neurodevelopmental follow-up at 24 months are included

	Infants treated with TH and with neurodevelopmental follow-up completed at 24 months (N=124) ¶						
	HIE grade 1 N=24	*p**	HIE grade 2 N=87	Missing	HIE grade 3 N=13	Missing	*p**
MRI patterns		-		-		-	
1, N (%)	0		4 (4.6)		5 (38.45)		0.002
2, N (%)	0		1 (1.2)		1 (7.7)		
3, N (%)	0		3 (3.4)		1 (7.7)		
4, N (%)	1 (4.2)		6 (6.9)		1 (7.7)		
5, N (%)	23 (95.8)		73 (83.9)		5 (38.45)		
Age (days) at the last MRI							
Median	15	-	21.5	1	20	1	0.891
IQR	7;3.5		5;39		6;33		

¶*One infant treated with TH but without HIE at the admission was not included in this table.*

**For continuous variables Kruskal-Wallis test was used to compare groups.*

8.3. Comparison of the characteristics of infants and procedure based on EEG abnormalities

Table 13. Demographics and EEG abnormalities in infants with a full follow-up (age 24 months)

	Infants treated with TH and with neurodevelopmental follow-up completed at 24 months and different severity of EEG abnormalities (N=125) ¶								*p**
	No N=10	Miss.	Mild N=51	Miss.	Moderate N=46	Miss.	Severe N=18	Miss.	
GE, weeks Median IQR	39.9 39;41.1	-	40 38;40	-	39.8 39;41	-	40 37.8;40.8	-	0.443
Birth weight, grams Median IQR	3295 2970-3650	-	3234 3000-3660	-	3350 2970-3695	-	3200 2366;3500	-	0.389
Sex, male, N (%)	6 (60)	-	33 (64.7)	-	26 (56.2)	-	9 (50)	-	0.703
Twinning, N (%)	0	-	1 (1.9)	-	1 (2.2)	-	2 (11.1)	-	0.281
Outborn, N (%)	2 (20)		21 (41.2)	-	17 (36.9)	-	9 (50)	-	0.456
Mother Race Caucasian, N (%) African, N (%) Asiatic, N (%)	9 (90) 0 1 (10)	-	46 (90.2) 3 (5.9) 2 (3.9)	-	41 (91.2) 2 (4.4) 2 (4.4)	1	13 (72.2) 4 (22.2) 1 (5.6)	-	0.240
Mother diseases during pregnancy, N (%)	2 (20)	-	14 (28)	1	17 (37.8)	1	9 (50)	-	0.261
PROM, N (%)	1 (10)	-	10 (20)	1	9 (20.4)	2	0	-	0.152
Intrapartum fever, N (%)	1 (14.3)	3	3 (9.1)	18	3 (10.3)	18	2 (22.2)	9	0.712
CTG abnormalities, N (%)	7 (77.8)	1	31 (62)	1	20 (47.6)	4	13 (77.2)	-	0.168
Cesarean Section, N (%)	2 (20)	-	17 (33.3)	-	15 (32.6)	-	11 (61.1)	-	0.093
Funiculus abnorm. N (%)	3 (10)	-	5 (10.9)	5	6 (13.9)	3	2 (11.8)	1	0.458
Sentinel events, N (%) Placenta abrution, N (%) Uterine rupture, N (%) Funiculus Prolapsus, N (%) Amniotic Embolism, N (%) Acute anemia, N (%) Shoulder dystocia, N (%) Maternal collapase, N (%) SUPC, N (%) Total, N (%)	 0 0 1 (10) 0 0 0 0 0 3 (30)	-	 3 (5.9) 2 (3.9) 0 0 0 0 2 (3.9) 1 (2) 11 (21.6)	-	 4 (8.7) 1 (2.2) 0 1 (2.2) 1 (2.2) 3 (6.6) 0 1 (2.2) 13 (25.5)	-	 4 (23.5) 1 (5.9) 1 (5.9) 0 2 (4.4) 0 0 0 8 (44.4)	1	 0.155 0.811 0.047 0.635 0.050 0.157 0.406 0.901 0.244
Apgar 10 minute Median IQR	7 5;8	10	7 6;8	4	6 5;7	5	6 5;7	1	**0.007**
Resuscitation Intubation, N (%) Chest compression, N (%) Epinephrine, N (%)	5 (50) 0 0	- - -	18 (35.3) 14 (27.5) 10 (19.6)	- - -	22 (47.8) 12 (26.1) 5 (10.9)	- - -	13 (72.2) 10 (55.6) 7 (38.9)	- - -	0.059 **0.015** **0.025**
Lowest pH[δ] Median IQR	6.9 6.8;7	-	7.02 6.9;7.1	6	7.03 6.9;7.1	4	6.8 6.7;6.9	4	**<0.001**
Lowest base excess[δ] Median IQR	-16 -18.4;-13.4	1	-13.6 -17;-9.9	8	-14.3 -17.7;-11.4	4	-20.8 -24;-17.5	2	**0.007**
HIE severity within first 6 h of life, N (%) mild, N (%) moderate, N (%) severe, N (%)	 1 (10) 9 (90) 0	-	 14 (28) 35 (70) 1 (2)	1	 9 (19.6) 34 (73.9) 3 (6.5)	-	 0 9 (50) 9 (50)	-	**<0.001**
Presence of full standard criteria for TH, N (%)	2 (20%)	-	0	-	37 (80.4%)	-	18 (100%)	-	**<0.001**

Miss., missing cases

**For continuous variables Kruskal-Wallis test was used to compare groups.*

In neonates with a full follow-up (age 24 months), low Apgar at 10 minutes, need of chest compression or epinephrine administration at birth, lowest pH and lowest base excess were more common in neonates with severe EEG abnormalities. They were more likely to require an advanced resuscitation and had the worst presentation in term of clinical and laboratory markers at birth. Grade of HIE severity increased according to EEG abnormalities ($p<0.001$). Full standard criteria for TH were met from 100% and 80.4% of infants with severe and moderate EEG abnormalities respectively; no infants with mild EEG abnormalities met full standard criteria for TH and only 20% of infants with no abnormalities did it.

Table 14. Infants with a full follow-up (age 24 months) and mode of therapeutic hypothermia according to EEG abnormalities.

	Infants treated with TH and with neurodevelopmental follow-up completed at 24 months and different severity of EEG abnormalities (N=125) ¶								*p**
	None N=10	Missing	Mild N=51	Missing.	Moderate N=46	Missing	Severe N=18	Missing .	
Time to initiation of hypothermia (hours)		-		-		-		-	0.586
Median	4.1		4.8		4.2		3.9		
IQR	1.18;6		3.1;5.5		2.6;5.6		2.9;4.9		
TH duration, hours									
Median	72	1	72	-	72	3	72	18	0.688
IQR	72;72.15		72;72		71.8;72		72;72		
Duration of re-warming									
Median	6	0	6	4	6	8	7.5	4	0.104
IQR	5;7		5;7.5		6;7		6;9.5		
Respiratory support during TH									**<0.001**
None, N (%)	6 (66.7)	1	22 (46.8)	4	13 (32.5)	6	0	3	
NIV, N (%)	1 (11.1)		17 (36.2)		18 (22.5)		3 (20)		
Mechanical ventilation, N (%)	2 (22.2)		8 (17)		9 (45)		12 (80)		
Analgosedation, yes N (%)	10 (100)	-	49 (100)	2	42 (100)	4	16 (100)	2	-
Adverse effect during TH, N (%)	4 (40)	-	16 (31.4)	-	6 (13)	-	7 (38.9)	-	0.064

Miss., missing cases. NIV, non invasive ventilation.

**For continuous variables Kruskal-Wallis test was used to compare groups.*

The comparison of perinatal characteristics of infants with complete follow-up at 24 months of age based on the severity of EEG abnormalities found a statistically significant difference in terms of need of respiratory support (the percentage of infants intubated was significantly higher in infants with severe EEG abnormalities), but not in terms of the timing of TH initiation, duration, re-warming, use of analgosedation or adverse effects.

Table 15. Seizures in infants treated with TH. Only infants with a full neurodevelopmental follow-up at 24 months are included

	Study subjects (N=125) ¶								*p**
	No N=10	Miss.	Mild N=51	Miss.	Moderate N=46	Miss.	Severe N=18	Miss.	
Seizures during TH, N (%)†	4 (40)	-	7 (13.7)	-	9 (19.6)	-	13 (72.2)	-	**<0.001**
Onset timing of seizures (hours of life)									
Median	25	1	38	-	7	-	7	3	**0.049**
IQR	12;38		12;70		6;12		4;16		

†Clinical and/or encephalographic seizures

**For continuous variables Kruskal-Wallis test was used to compare groups.*

The comparison of seizures and of their onset timing in infants treated with TH with complete follow-up at 24 months of age based on the grade of HIE found a statistically significant difference. The incidence of seizures increased with the increasing of HIE severity. Timing of onset of seizures decreased according to the burden of EEG abnormality.

Table 16. MRI findings of infants treated with TH. Only infants with a full neurodevelopmental follow-up at 24 months are included

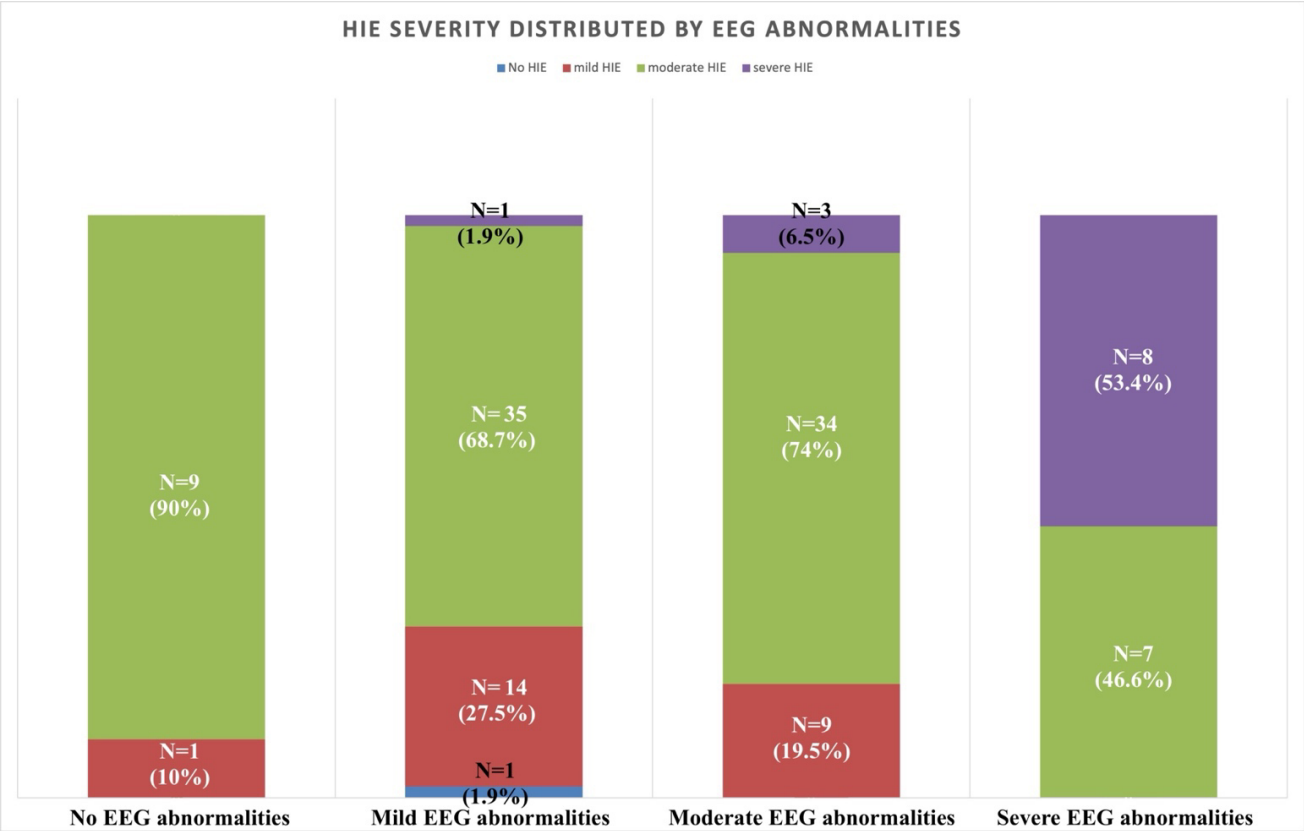
	Study subjects (N=125) ¶								*p**
	No N=10	Miss.	Mild N=51	Miss.	Moderate N=46	Miss.	Severe N=18	Miss.	
MRI patterns									
1, N (%)	1 (10)	-	0	-	2 (4.4)	-	6 (33.3)	-	**<0.001**
2, N (%)	0		0		0		2 (11.1)		
3, N (%)	1 (10)		2 (3.9)		0		1 (5.6)		
4, N (%)	0		4 (7.8)		2 (4.4)		2 (11.1)		
5, N (%)	8 (80)		45 (88.3)		42 (91.2)		7 (38.9)		
Days of life at the time last MRI was performed									
Median	32	1	18.5	3	8.5	2	36	2	0.098
IQR	29;49		6;36.5		5;34		11.5;42.5		

Miss., missing cases

**For continuous variables Kruskal-Wallis test was used to compare groups.*

The comparison of brain MRI findings in infants treated with TH with complete follow-up at 24 months of age based on the severity of EEG abnormalities found a statistically significant difference in terms of MRI patterns: the percentage of pattern with severe cerebral impairment (pattern 1 and 2) increased with the increasing of HIE severity and vice versa.

Figure 7. HIE severity and EEG abnormalities.



A.

B.

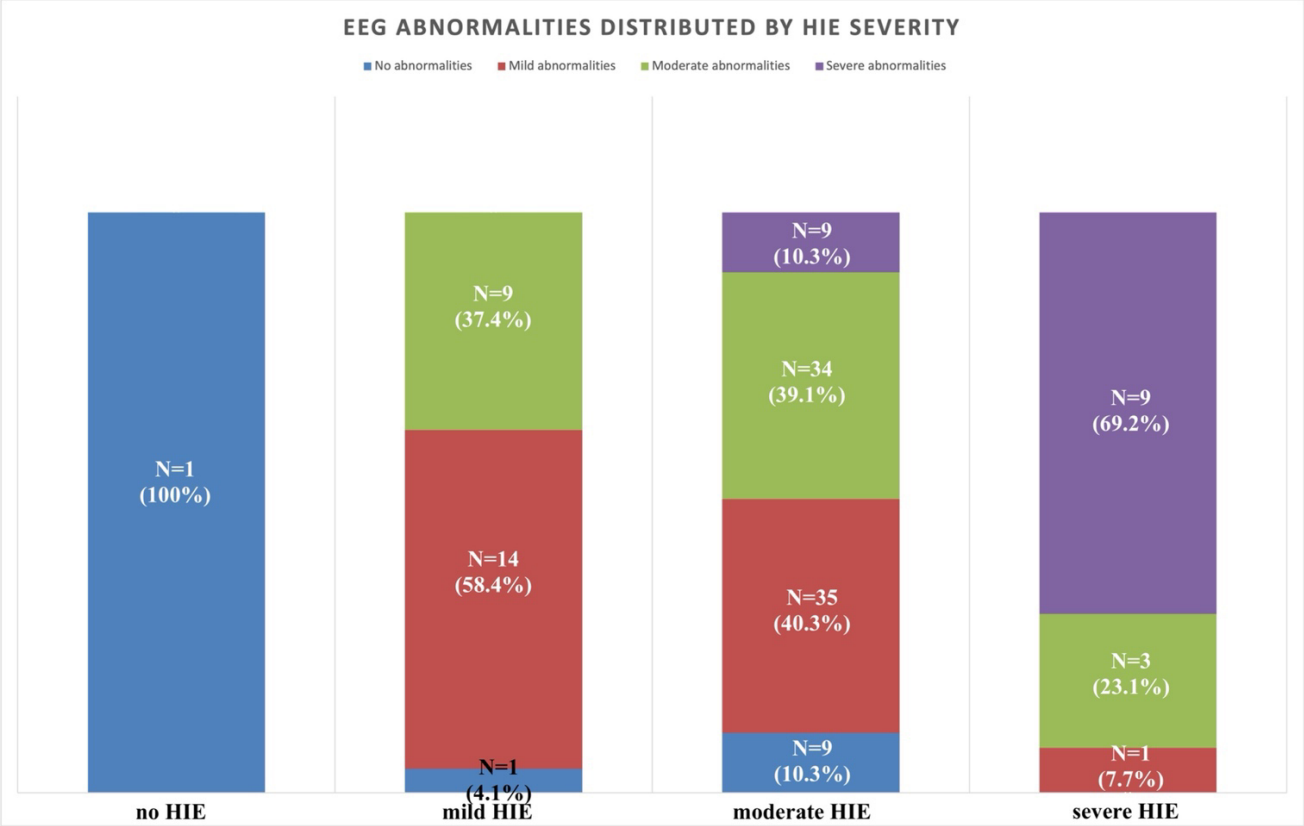


Figure 7 A shows HIE severity according to EEG abnormalities; figure 7 B shows EEG abnormalities according to HIE severity. The severity of HIE and the grade of severity of EEG abnormalities does not always overlap. The grade of severity overlaps in 46% of cases, in the remaining there is not a match on the degree. In particular, moderate HIE was associated to moderate EEG abnormalities in 34 out of 85 (40%) of cases, in the remaining it was associated to absent, mild or severe EEG abnormalities.

8.4. Neurodevelopmental outcome at 24 months of age

A total of 125 infants were evaluated at 24 months of age. Severe functional disability was found in 16 out of them (12.8%). The most frequent impairment was cerebral palsy (CP, 12/125, 9.6%) (Table 16). Among infants with CP, 3 had diplegia, 1 had hemiplegia and 7 had tetraplegia; CP was spastic in 3 cases and dystonic in 5 cases; classification of gross motor function was 2 in 3 cases and 5 in 6 cases.

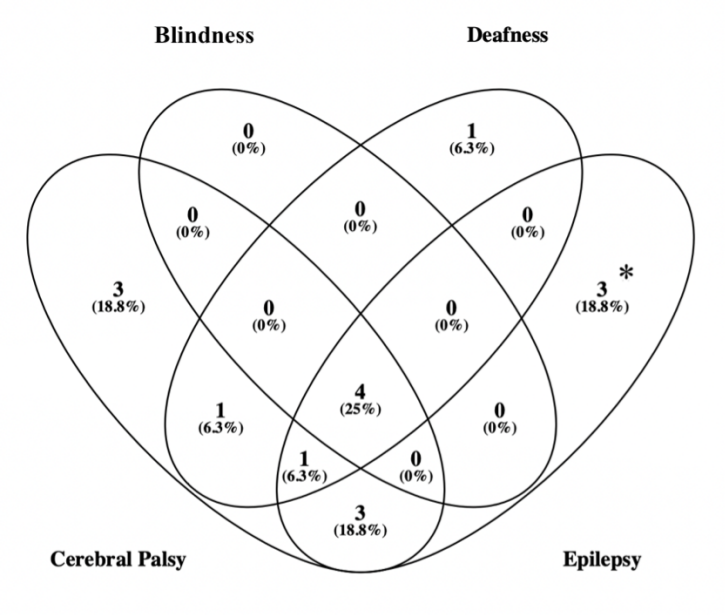
Figure 8 shows the overlap of different neurological impairments: 12 patients out of 16 had CP.

Data regarding cognitive assessment were available for 78 out of 118 infants (69%); infants with tetraparesis and blindness were excluded from cognitive evaluation. Thirty-one infants (39.7%) underwent Griffiths Mental Developmental Scales evaluation, and 47 (60.3%) underwent Bayley Scales of Infant and Toddler Development evaluation. Most infants (72 out 78, 92.3%) had GMDS-R Global quotient or BSDI-III cognitive compositive score above 85, 2 infants had score between 70-85 and 1 infants had score below 70. Mean and SD values are shown in Table 18.

Table 17. Incidence of different impairments.

	Infants treated with TH and with neurodevelopmental follow-up completed at 24 months N=125	Missing
Cerebral palsy, N (%)	12 (9.6)	-
Blindness, N (%)	4 (3.2)	-
Deafness, N (%)	7 (5.6)	-
Epilepsy, N (%)	11 (8.8%)	-
GMDS-R global quotient/BSDI-III cognitive compositive score score<70, N(%) score 70-85, N(%) score >70, N(%)	 1/78 (1.3%) 5/78 (6.4%) 72/78 (92.3%)	 35
Severe functional disability, N (%)	**16 (12.8)**	

Figure 8. Overlapping of neurological impairments in infants with severe functional disability.



**One infant had severe cognitive impairment.*

Table 18. GMDS-R quotients or BSD-III scores

	GMDS-R N = 31	Median, IQR	Missing cases
Patients evaluated with GMDS-R*	Global quotient	102.9 ± 15.4	-
	Locomotor quotient	102.5 ± 18.9	-
	Personal and social quotient	103.0± 14.7	-
	Hearing and language quotient	97.1 ± 21.3	-
	Eye & Hand Coordination quotient	110.2 ± 11.2	-
	Performance quotient	105.9 ± 8.6	1
	BSDI-III N = 47		Missing cases
Patients evaluated with BSDI-III*	Cognitive composite score	100.4 ± 8.7	-
	Motor composite score	95.5 ± 10.7	10
	Language composite score	92.9 ± 11.4	5

Patients with tetraparesis and blindness were excluded.

Table 19. Comparison between infants with and without severe functional disability.

	Infants without severe functional disability N=109	Missing	Infants with severe funciontal disability N=16	Missing	*p*
Outborn, N (%)	42 (38.5)	-	7 (43.8)	-	0.690
Male sex, N (%)	45 (41.3)	-	6 (37.5)	-	0.774
Gestational age					
Mean	40	-	39.5	-	0.375
IQR	38.6;41		37.9;40		
Birth weight					
Mean	3300	-	3200	-	0.349
IQR	2970;3680		2747;3520		
Sentinel events, N (%)		-		1	
Placenta abrution, N (%)	8 (7.3)		3 (20)		0.130
Uterine rupture, N (%)	4 (3.6)		0		1.000
Funiculus Prolapsus, N (%)	1 (0.9)		1 (6.6)		0.241
Amniotic Embolism, N (%)	1 (0.9)		0		1.000
Acute anemia, N (%)	2 (1.8)		1 (6.6)		0.323
Shoulder dystocia, N (%)	3 (2.7)		0		1000
Maternal collapase, N (%)	1 (0.9)		1 (6.6)		0.228
SUPC, N (%)	2 (1.8)		0		1.000
Total, N (%)	29 (26.6)		6 (40)		0.280
Cesarean section, N (%)	36 (33)	-	9 (56.3)	-	0.071
Apgar 10 minute					
Median	7	10	6	1	0.136
IQR	5;8		5;7		
Mother diseases during pregnancy	34 (31.8)	2	8 (50)	0	0.152
PROM, N (%)	19 (17.9)	3	1 (6.3)	-	0.240
CTG abnormalities, N (%)	60 (57.7)	5	11 (73.3)	1	0.248
Resuscitation,		-			
Intubation, N (%)	48 (44)		10 (62.5)		0.167
Chest compression, N (%)	30 (27.5)		6 (37.5)	-	0.411
Epinephrine, N (%)	18 (16.5)		4 (25)		0.405
Lowest pH[δ]					
Median	7	12	6.9	3	**0.023**
IQR	6.87;7.15		6.8;7.1		
Lowest base excess[δ]					
Median	-14.8	15	-18	4	**0.023**
IQR	-18;-11		-24.5;-14.7		
HIE severity within first 6 h of life, N (%)					
no HIE, N (%)	1 (0.9)	-	0	-	**<0.001**
mild, N (%)	24 (22.0)		0		
moderate, N (%)	78 (71.6)		9 (56.3)		
severe, N (%)	6 (5.5)		7 (46.7)		
EEG abnormalities on admission, N (%)[±]					
absent	8 (7.3)	-	2 (12.5)	-	**<0.001**
mild	49 (45.0)		2 (12.5)		
moderate	45 (41.3)		1 (6.3)		
severe	7 (6.4)		11 (68.7)		
Seizures during TH, N (%)	20 (18.4)	-	13 (81.3)	-	**<0.001**
MRI patterns, N (%)					
1	1 (0.9)	-	8 (50)	-	**<0.001**
2	1 (0.9)		1 (6.2)		
3	3 (2.8)		1 (6.2)		
4	5 (4.6)		3 (18.8)		
5	99 (90.8)		3 (18.8)		
Presence of full standard criteria for TH, N (%)	44 (40.4)	10	13 (81.3)	-	**0.002**

CTG, cardiotochography; IQR, interquartile; PROM, prolonged rupture of membrane.

[δ]Umbilical cord or arterial, venous or capillary bloods values obtained within the first hour of life

The comparison of the characteristics between infants with or without severe functional disability at 24 months of age found a significant difference for pH and BE excess (both lower in infants with severe functional disability), HIE severity and EEG abnormalities at admission (they were both more frequently severe in infants with severe functional disability), frequency of seizures during TH (most frequent in infants with severe functional disability), MRI patterns (patterns 1-2 were more frequent in infants with severe functional disability) and presence of full standard criteria for TH treatment (more frequent in infants with severe functional disability).

No infants with mild HIE developed severe functional disability; the rate of severe functional disability in infants with moderate or severe HIE was 16%.

Table 20. Uni- and multi-variate analyses of factors associated with severe functional disability.

	Univariate Analysis			Multivariate Analysis		
	OR	95% CI	*p*	OR	95% CI	*p*
Gestational age at birth	7.642	0.99-58.63	0.050			
Outborn	1.240	0.42-3.58	0.690			
Type of birth*	1.614	0.94-2.75	0.078			
Presence of sentinel event	1.755	0.57-5.35	0.323			
Apgar 10th minute	0.863	0.65-1.13	0.285			
Intubation during resuscitation	2.118	0.71-6.23	0.173			
Timing to start TH	0.802	0.58-1.10	0.172			
Ventilazione prevalente	6.288	2.17-18.16	**0.001**			
BE	0.867	0.77-0.97	**0.018**			
Grade of HIE$^{\chi}$	11.491	3.43-38.42	**<0.001**	5.343	1.05-27.08	**0.043**
EEG anomalies at admission	4.019	1.84-8.77	**<0.001**			
Presence of seizures during TH	19.283	5.01-74.07	**<0.001**	5.474	1.10-27.08	**0.037**
Grade of HIE at 7 days	3.599	1.99-6.50	**<0.001**			
Presence of full standard criteria	6.401	1.73-23.78	**0.006**			
Cerebral MRI patterns	0.270	0.16-0.45	**<0.001**	0.448	0.25-0.78	**0.005**
Sex	0.853	0.28-2.51	0.774			

BE, base excess.

*Vaginal delivery *vs* cesarean section

$^{\chi}$ Grade of HIE during the first 6 hours after birth

Increased base excess, EEG anomalies at admission, presence of seizures during TH, higher grade of HIE at 7 days of life and brain MRI findings were significantly associated with severe functional disability at univariate analysis. At multivariate analysis severe functional disability remained associated with severity of HIE in the first 6 hours of life, presence of seizures during TH and brain

MRI patterns. The sensitivity and the specificity of the final model were 56.3% and 98.17 respectively, positive predictive value and negative predictive value were 81.8% and 93.9% respectively (area under ROC curve: 93%).

Figure 8. ROC curve of multivariate final model.

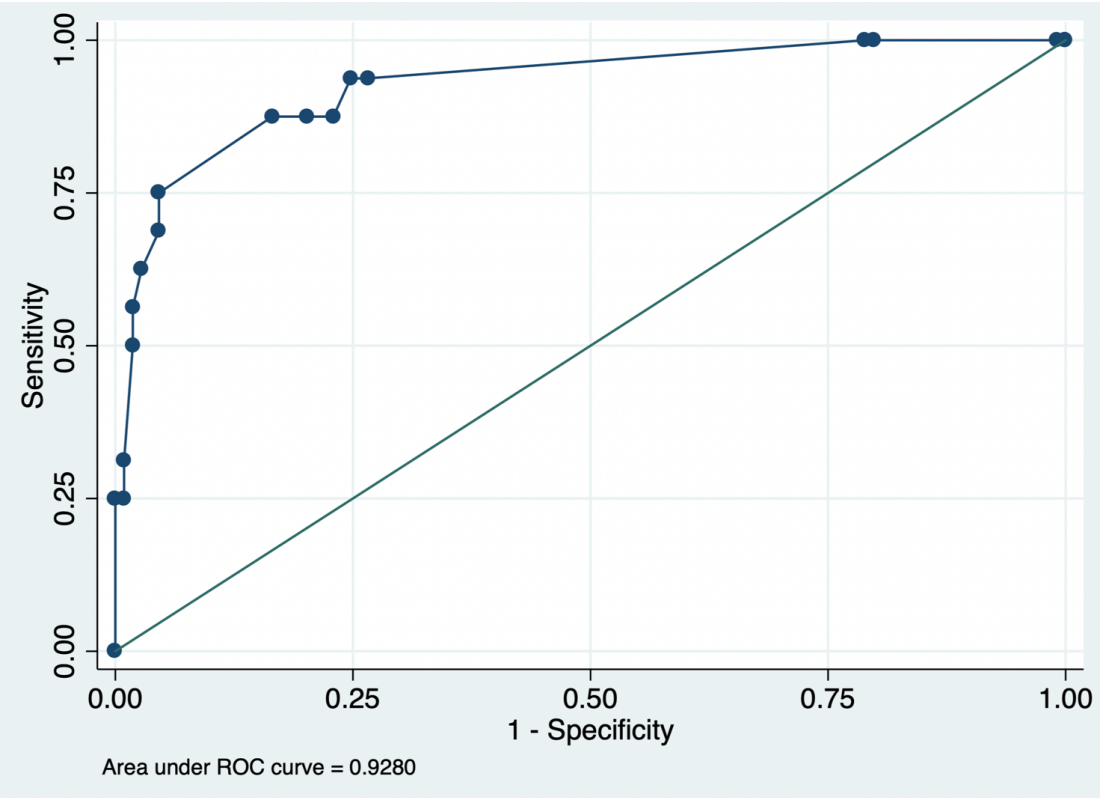
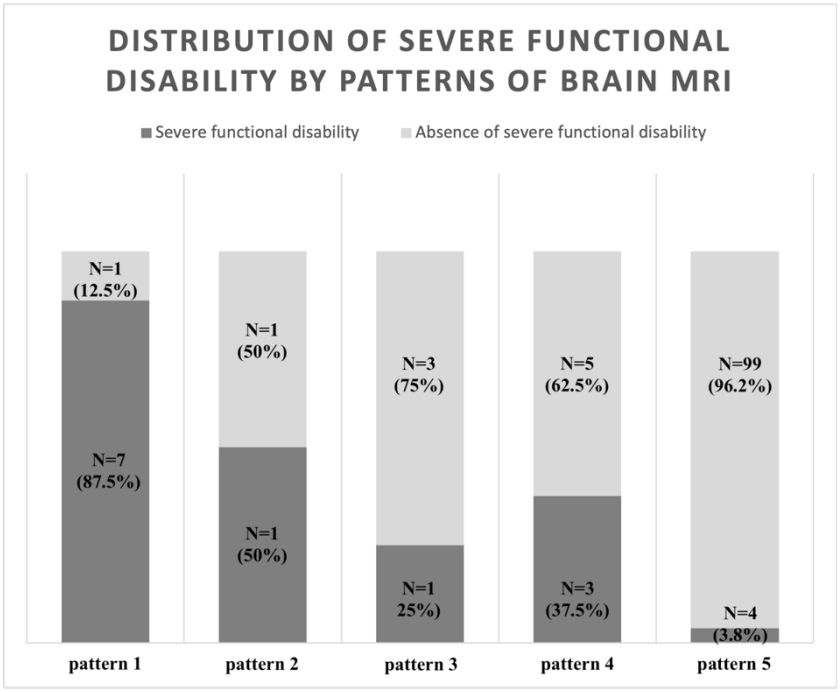


Figure 9 shows cases with severe functional disability according to the pattern of brain MRI.

Figure 9. Cases with severe functional disability according to brain MRI patterns



9. Discussion

Neonatal encephalopathy is a complex syndrome characterized by seizures, an altered level of consciousness, and/or an inability to initiate or maintain spontaneous breathing. TH is the only validated treatment other than supportive intensive care (1). The use of TH has been validated by 7 major randomized controlled trials (26-32) and it has become the standard of care since 2010. The reduction in body temperature to 33-34° C in infants ≥36 weeks gestation with moderate to severe HIE resulted in significant reductions in the risk of death or major neurodevelopmental disability at 18 months of age (RR 0.76 95% CI 0.69- 0.84) and an increase in the rate of survival with normal neurologic function; the number need to treat was 7. The reduction in death or major neurodevelopmental disability was greatest in those with moderate HIE (RR 0.67 95% CI 0.56-0.81) when compared with those with severe HIE (RR 0.83 95% CI 0.74-0.92) (23).

Subsequently, TH has been used worldwide as standard of care for HIE. Nevertheless, a few multicenter studies report observational data on both neonates undergoing TH during routine clinical practice and their long-term neurodevelopmental outcome (37). In particular, no multicenter study has been carried out from Italian NICUs and no information is available on local practices for managing neonates.

The aim of this study was to describe the use of TH during routine clinical practice in Italian NICUs and the long-term neurological outcome. NEURONAT represents an Italian Network which currently includes 8 NICUs from Emilia Romagna, a norther region. In these centers TH is routinely administered in infants with neonatal encephalopathy according to the latest version of the Italian guidelines published in 2012 (54).

9.1 Characteristic of the population evaluated and mortality

In our study the incidence of HIE was 1.8/1000 live births, comparable to that found in the previous literature.

Among 181 infants treated with TH 3.8% died. This rate of mortality was particularly low as compared to the rate of 20 to 38% reported in some RCTs (26-32). More recent studies regarding the use of TH in routine clinical practice found similar results, with a minimum rate of mortality of 2.6% (37,85). These low incidences of mortality could be explained by an increased threshold for enrollment to TH. Consequently, infants with mild HIE (who were not included in the RCTs) are now often treated with TH.

Gestational age over 35 weeks and birth weight over 1800 were criteria for administering TH, and

only two treated infants had gestational age below 35 week (33 and 34 weeks' gestation respectively). Notably, although previous studies reported an incidence of sepsis ranging from 2.5 to 12% in infants treated with TH, (22,86) no infants in our study had culture-proven sepsis. Previous literature, shows that perinatal infections associated with acute hypoxia-ischaemia significantly increase the risk of poor neurodevelopmental outcome as compared with HIE without sepsis in isolation. However current data are limited regarding the outcomes of newborns with early onset sepsis treated with TH. The incidence of sentinel events was 28%, similar to the one previously reported (27-62%) (87-89). We did not find any difference of the incidence of sentinel events basing on EEG abnormalities or severe functional disability. Placenta abruption and acute anemia were more common in infants with most severe HIE, although the low number of cases limits any firm conclusion. Previous reports show an association between sentinel events and brain lesions, particularly in basal ganglia and thalamus (47,87,88), but without worsening of neurodevelopmental outcome at 18 months of age (87). Grass et al. (89) reported lower severity of brain lesions and better neurodevelopmental outcome at 18-36 months of life in infants with sentinel events (as compared to infants without) when treated with TH. These findings were interpreted as neuroprotection of TH in infants with sentinel events (87).

9.2 Therapeutic hypothermia according to guidelines

When TH was administered, target temperature, timing of initiation, duration, and duration of re-warming were in accordance with guidelines (54) for most infants. However, body temperature at admission was 35°C in many infants, sometimes probably because of unavailable portable cooling devices. Furthermore approximately 20% of infants underwent TH after the first six hours of life; this rate is higher with respect to other studies, but similar to that reported by Debillon et al. (90). Interestingly infants with most severe underwent TH earlier than infants with less severe HIE ($p<0.05$); this finding suggest that clinicians recognize patients who have more severe HIE and promptly initiate TH. In contrast, less critically ill patients were evaluated and treated late with TH. However, although late TH may reduce death and disability, early administration (within 6 hours of life) gives the best therapeutic results. (91)

Interestingly, time to TH and body temperature at NICU admission were significantly different between inborn and outborn infants, but their neurological outcome was similar.

Among the 174 surviving infants, about 50% did not meet the full standard criteria according to Italian guidelines: 2 infants were <35 weeks of gestational age at birth, 2 were diagnosed with SUPC, 34 did not have moderate/severe HIE and 73 did not have moderate/severe EEG abnormalities (21

infants did not met both criteria). All but one met at least 1 criteria for asphyxia (10 min Apgar score ≤ 5, ventilation for at least 10 min after birth or severe acidosis). Italian guidelines include special conditions for TH in infants who do not meet full standard criteria i.e. infants with mild HIE and moderate to severe EEG abnormalities, or infants with moderate to severe HIE and absent (or mild) EEG abnormalities. Indeed, 26% of neonates may have a doubtful neurological examination, especially if performed early; thus, guidelines suggest performing an EEG to increase prognostic accuracy and to start TH in case of abnormal EEG recordings (92,93). Neurological examination may vary in the first hours of life, and it is partially related to individual physicians.

In our study, most HIE were moderate (almost 70% of total cases), and they were associated with a wide spectrum of EEG abnormalities (normal 10.3%, mildly abnormal 40.3%, moderately abnormal 39.1% and 10.3% severely abnormal). Similarly, up to 12% of the initial EEG traces may be normal or have artifacts (94), therefore guidelines suggest to rely mainly on neurological examination. Our data confirm that the severity of HIE and EEG abnormalities were not concordant in all cases.

The decision to administer TH is based on many factors evaluated within a narrow range (first 6 hours of life) and includes prenatal history, presence of sentinel events, resuscitation and metabolic status at birth, neurologic and electroencephalographic findings. The 7 original RCTs that demonstrated the efficacy of TH differed in terms of age of infants enrolled, definition of encephalopathy, hypoxia-ischemia, and exclusion criteria; only 3 out 7 studied included EEG evaluation. It is therefore reasonable that clinicians decide whether an infant should undergo TH based on a comprehensive evaluation, within the context of HIE (52).

We found a high rate of infants with mild HIE who underwent TH. In literature the percentage of infants with mild HIE who did not met full criteria for TH is very wide (4 - 75%) (36,37,95,96). Similar to our results, a recent meta-analysis regarding 13 observational studies (97) estimated that 22% of infants undergoing TH had mild HIE. The propensity to treat infants with mild HIE is now reported in many studies (95,98-101). Despite no changes in clinical guidelines (95) the proportions of infants receiving TH after moderate to severe HIE is decreasing over time, while the proportion of those having mild HIE is increasing.

9.3. Neurodevelopmental outcome

Neurodevelopmental outcome data were available for 71.8% of infants, and severe functional disability was found in 12.8% of them. Among infants with severe neurodevelopmental impairment, cerebral palsy was diagnosed in 75% of them. Regarding cognitive outcome, excluding infants with tetraparesis and blindness, only one infant had cognitive score <70, five had a score between 70-85, and the remaining 72 (92.3%) had cognitive score within a normal range. It is known that neonatal encephalopathy is mainly associated with motor disabilities, and our data may be considered reassuring in terms of cognitive outcome at 2 years in infants treated with TH: if cerebral palsy does not occur, the cognitive outcome will be probably within the normal range.

According to what has been previously reported (22) our study confirms that infants with severe HIE (as compared to those with moderate HIE), had the highest risks to develop severe functional disability (53.8% vs 10.3%). Furthermore, in our study 84% of infants had a favorable outcome (defined as absence of severe functional disability). Rates were higher than that reported in previous studies (from 60% to 70%) (35-37). These discrepancies can be explained by slightly different definitions of favorable outcomes. Our data conflict even more from those reported in RTCs, where rates of favorable outcomes range from 15% to 60% (26-32), probably because they do not include infants with mild HIE.

No infants with mild HIE developed severe functional disability; similarly, infants with moderate to severe EEG abnormalities were more likely to develop severe functional disability (18.8% of cases) as compared to of infants with absent or mild EEG abnormalities (6.5% of cases). Furthermore, severe functional disability at 24 months was more likely in infants who met full standard criteria for TH, and only 3 infants (4.6%) without full standard criteria developed severe functional disability.

Whether TH is effective in improving neurodevelopmental outcomes in infants with mild HIE is still debated; a systematic review and meta-analysis did not find any evidence of benefit from TH in terms of outcome (defined as death, moderate or severe disability at or beyond 18 months of age) (102). Authors suggested that TH after mild HIE could improve the outcome, but only large, methodologically robust trials will assess the potential risks or benefits of TH (102,103).

9.4. Prediction of neurodevelopmental outcome

Efforts have been made to predict as soon as possible neuro disabilities in infants with HIE (104-110). Predictors allows to give answers to parents and to select more precisely children who will benefit from an early rehabilitation intervention (111). Prognostic value of single markers remains a problem to be answered: "*the prediction of neurodevelopmental outcome remains one of the major*

challenges for clinician treating newborn with neonatal hypoxic ischemic encephalopathy (HIE)" (105), and the methods used in precooling era changed in the cooling era (104,105). Only neonatal neurologic examination and MRI findings seemed to be the best predictors (105).

We found several factors independently associated at logistic regression with an unfavorable outcome (BE, grade of HIE in the first 6 ours, EEG abnormalities at admission, type of respiratory support during TH, seizures during TH, grade of HIE, pattern of cerebral lesions, grade of HIE at 7 days and presence of full standard criteria for TH).

Our final predictive model (area under ROC curve 97.38%) found a significant statistical difference for severity of HIE in the first 6 hours of life, presence of seizures during TH and brain MRI patterns. The incidence of severe functional disability was clearly associated with a downward trend according to the increasing severity of brain MRI patterns (OR 0.44) and brain MRI confirms to be an important predictor for severe functional disability. Grey matter lesions lead to the highest risks of severe morbidity and death, a finding demonstrated both before and after the introduction of TH (25,47,48,84,88).

Recently, Lugli et al. (110) identified general movements together with EEG findings as the best predictors of neurological impairment at 24 months of age. However, unlike Lugli et al. we did not include general movements assessment and poligraphic EEG, was unavailable in all but one centre; most centres in our study used amplitude EEG both to evaluate infants at admission and during TH. Furthermore, EEG during TH has been demonstrated to be more predictive as compared to EEG recorded soon after birth (94,112). Consequently, 4 infants in the current study developed seizures during TH, but none of them showed EEG abnormalities in the initial recordings.

Interestingly, intubation during resuscitation was not predictive of severe functional disability. Indeed, all infants without EEG abnormalities and approximately 30% of infants with mild EEG abnormalities (22% of these had grade 1 HIE) were intubated during resuscitation, and this was probably the only reason why physicians undertook TH; none of these infants developed severe neurodevelopment disabilities and physician should be aware that intubation itself is not associated with a severe outcome.

9.5 Adverse events

Adverse events did not differ according to the severity of HIE. The most frequent event was bradycardia (up to 28% of infants), while others were less common and had not clinical consequences. TH was discontinued only in 1 neonate because of severe pulmonary hypertension consequent to meconium aspiration syndrome. The newborn had moderate HIE with mild EEG abnormalities on admission and seizures during TH, but did not develop severe functional disability (at age 2 years). Bradycardia was the only event found in infants with mild HIE.

9.6 Strengths and limitations

This study had some limitations. Firstly, the sample size is not large enough, and relatively few infants developed severe neurodevelopmental disabilities. In addition, unfortunately almost 30% of infants did not complete the follow-up visits, and data regarding cognitive outcome were sometimes unavailable. Several reasons may explain the non-compliance to the follow-up visits; in particular some infants were transferred to centers outside the network and some were sent to early rehabilitation intervention during follow-up, thus sometimes missing the following visits. Interestingly, infants without full follow-up visits were more likely to have seizures and severe brain lesions (although the severity of HIE did not differ at 7 days of age). This finding would suggest an underestimation of severe functional disabilities at 24 months. However, it is not uncommon that follow-up visits are missing: a recent study regarding outpatient clinic attendance of patient with CP reported that only 54% of children (from 0 to 4 years of age) attended all follow up visits. In the Neuroprem study (that comes from the same geographical area of Neuronat) the follow-up at 24 months of age in premature infants was incomplete for 43% of neonates (113). Finally, Azzopardi et al. were able to report the motor outcome only for 19% of the infants evaluated (35).

The short duration (24 months) of follow-up is another limitation to have a full information on neurodevelopmental disabilities. This is a relatively short period to assess cognitive outcome of children affected by HIE since minor neurological disfunctions may appear in older age (37,114,115). Regarding the brain MRI evaluation, patterns were scored evaluating MRI reports; specific score related BGT, PLIC, white matter and cortex were missing. The sensitivity and specificity of our model could have been affected, preventing comparisons with other studies. Finally, we did not included investigations regarding the possible etiologies of neonatal encephalopathies different from HIE (116).

The strengths of Neuronat includes its prospective design and a cohort enrolled in very recent years. All but 2 studies (Lytonepal, still ongoing, and Dominguez-Dieppa with a very small cohort of

children), no recent (after 2015) studies report the outcome of infants treated with TH in routine clinical practice. This is particularly important since TH is a relative recent treatment and physicians have progressively gained more confidence with in the last ten years, reducing their fears of adverse effects, and offering TH to an increased number of patients. It is also likely that over time TH began to be administered more efficiently and with potential increased benefits (35,117).

A follow-up until two years of age ensures that no cerebral palsy was undetected, since cerebral palsy is usually diagnosed much earlier. In addition, although the etiology of neonatal encephalopathy is known to be heterogeneous, the population included in this study has consistent characteristics; unlike other studies, such as the TOBY trial, in which some infants with brain malformations or other conditions such as cerebral venous thrombosis underwent therapeutic hypothermia, this was not the case in this study. Finally, even if the results of this study probably are not generalizable to all Italian centres, this is the first study reporting TH and the long-term neurodevelopmental outcome in Italy.

We speculate that that the increasing rates of infants with mild HIE undergoing TH and the growing confidence in using TH explain the good neurodevelopmental outcome found in this study. The network itself may have a relevant role in this process, since it has been shown that networks may improve the quality of care (85).

10. Conclusions

This is the first Italian study reporting the neurodevelopmental outcome of infants treated with TH in a context of routine clinical practice. We found that almost 1 out of 4 infants were not affected by moderate to severe HIE, and none of those with a mild HIE developed severe functional disability.

EEG monitoring plays a crucial and complementary role to the neurological examination in identifying infants who are eligible to therapeutic hypothermia, as well as having an important predictive value for the development of severe functional disability.

Our study confirms that infants at higher risk of severe functional disability were those with severe HIE and EEG abnormalities, despite TH was administered. The severity of HIE, the presence of seizures during TH and brain MRI findings were the most important factors to predict a severe functional disability. Given the observational nature of this study, randomized controlled trials results urge for the evaluation of the real need to treat infants with mild HIE.

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