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SYSTEMIC EFFECT OF PROLONGED MODERATE SYSTEMIC HYPOTHERMIA IN NEONATAL HYPOXIC ISCHEMIC ENCEPHALOPATHY

PhD thesis

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Abbreviations

HIE	Hypoxic-ischemic encephalopathy
PCr	Phosphocreatinine
Pi	Inorganic phosphate
OFC	Head circumference
NO	Nitrogen monoxide
NOS	NO synthase
IL1β	Interleukin-1 beta
TNFα	Tumor necrosis factor alpha
CPR	Cardiopulmonary resuscitation
EEG	Electroencephalography
Ic Ca	Intracellular Calcium
MRS	Magnetic resonance spectroscopy
Glu	Glutamate
aEEG	amplitude integrated electroencephalograpy
RR	Risk ration
CI	Confidence interval
NICHD	National Institute of Child Health and Human Development, USA
TOBY	Total body hypothermia
NNT	Number need to treat
MODS	Multiorgan dysfunction syndrome
ASAT	aspartate aminotransferase
LDH	lactate dehydrogenase
HT	Hypothermia
NT	Normothermia
MRI	Magnetic resonance imaging
NSE	Neuron specific enolase
IL	Interleukin
INFγ	Interferon gamma
MCP-1	Monocyte chemotaxic protein 1

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EGF	Endothelial growth factor
VEGF	Vascular endothelial growth factor
AUC	Area under curve
CTG	Cardiotocography
BE	Base excess
CrUSS	Cranial ultrasound scan
ECMO	Extracorporal membrane oxigenisation
CPB	Cardiopulmonary bypass

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1. Induction

1.1. Induction

Neonatal hypoxic-ischemic encephalopathy (HIE) is an important cause of death and neurodevelopmental delay worldwide. Treatment for infants with hypoxic-ischemic encephalopathy was limited to supportive care for a long time, but efforts have been made to develop effective therapies. However, through several experimental therapies for hypoxic ischemic encephalopathy seemed promising none proved consistently successful in clinical studies [1],[2],[3]. Despite the improvement in neonatal care in the last decades, the incident of neurological disabilities related to perinatal brain injury remained unchanged affecting 2-3 in 1000 newborns. However accurate Hungarian national statistical data are not available, the estimated number of infants born with HIE is 150-200 in a year.

Over the past two decades, experimental and clinical evidence has accumulated that a 3-4^{o}C reduction of body temperature maintained for at least 72 hours in newborns with hypoxic-ischemic encephalopathy may reduce cerebral injury and improve neurological outcomes [see later chapters]. Recent clinical trials have demonstrated that prolonged cooling of either the head or the whole body of neonates with HIE is safe and associated with reduced short-term mortality and morbidity at 18 months of age [4].

Neuronal damage and death after asphyxia occurs in different phases and pathways. Initially primary energy failure and oxidative stress are responsible for tissue injury followed by secondary activated long term processes like excitotoxicity, inflammation, and apoptosis. Hypothermia, which decreases the cerebral metabolism and possibly affects on many other ways is the first therapeutic intervention proven being able to improve neurological outcome.

In this work we summarize the pathophysiology of hypoxic ischemic encephalopathy, the evidence behind hypothermia treatment and our observational studies during prolonged systemic hypothermia including markers of multiorgan failure and brain injury, cytokine and cortisol measurements, and changes in morphine metabolism.

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1.2. Pathophysiology

The precise mechanism of neural rescue by moderate hypothermia is uncertain but may be related to the critical relationship between temperature and metabolic rate: for every $1^{o}C$ lowering of the core temperature cerebral metabolism is reduced by approximately 7%, with consequently a lower glucose and oxygen demand [5] [6]. Both necrotic and apoptotic mechanisms are implicated in neuronal injury following neonatal hypoxia-ischemia and reperfusion.

The deprivation of oxygen and glucose caused by the reduction of cerebral blood flow is leading to severe decrease in high energy phosphate deserves including adenosine triphosphate. Due to the energy failure the sodium-potassium-adenosin pump is unable to maintain the polarity of the membrane in neurons and glia cells. The depolarisation results in excessive glutamate release from the axon terminals. The glutamate accumulates within the synaptic cleft, because of increased release and impaired reuptake mechanism. The excessive glutamate (phenomenon called glutamate excitotoxicity) acting on the receptor sites leads to a significant influx of sodium and calcium to the cells. The increased calcium level activates several enzymes including phospholipase, proteases and endonucleases or nitric oxide synthase. The combined effects of cellular energy failure, lactate acidosis, glutamate release, calcium accumulation and oxidative damage disrupt essential components of the cell leading to death.

During reperfusion phase the cerebral energy state usually recover, and the concentration of phosphate metabolites and the intracellular pH normalises. However 6 to 48 hours after the injury the secondary energy failure returns characterised by decrease in the ration of phosphocreatinine/ inorganic phosphate [7, **Figure 1**.].

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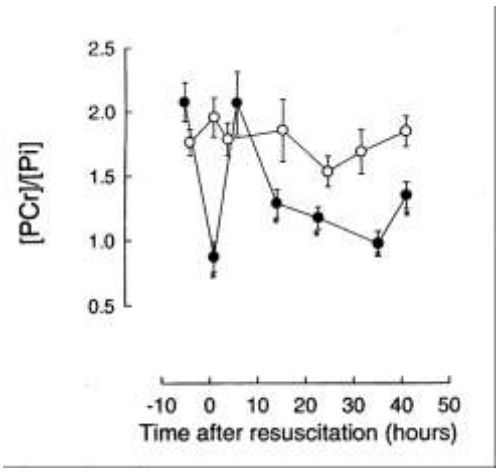

Figure 1. Secondary disturbance of oxidative phosphorylation [7]. Changes in PCr/Pi ratio measured by MR spectroscopy after hypoxic insult and in control group.

In humans, the severity of the secondary energy failure correlates with adverse neurological outcome [8, **Figure 2**.]. Reperfusion causes excessive production of free radicals leading to oxidation of lipids, proteins, DNA, leading to failure in the mitochondria which can be one of the basic mechanisms of secondary energy failure. Two important sources of the oxygen radicals are the by-products of xanthine and prostaglandin synthesis.

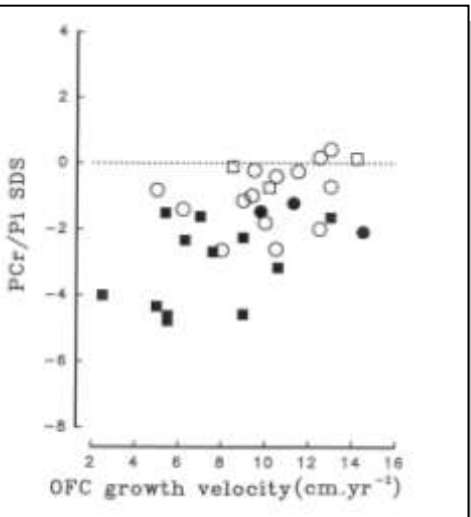

Figure 2. Relation between changes in PCr/Pi and head growth (OFC) in the first year of life [8].

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Oxygen free radicals contribute in tissue injury by membrane fragmentation with oxidation of the polyunsaturated fatty acid components. NO is a weak free radical produced by NO synthase (NOS) which is highly activated after hypoxia and reperfusion. NO combined with superoxide generates peroxynitrite radicals which activates lipid peroxidation and also increases glutamate release. During hypoxia free ferric iron is also released from complex proteins and reacts with peroxides producing hydroxyl radicals also responsible for tissue injury. Mitochondrial injury caused by membrane fragmentation results in energy failure, loss of cell membrane integrity and cytotoxic edema.

Inflammatory mediators play also crucial role in brain injury after hypoxia. Expression of IL1β and TNFα mRNA was observed within 1 to 4 hours after initial insult along with the induction of a and b chemokines followed by neutrophil invasion [9], [10]. Brain microglia and macrophages can be activated rapidly after the initial insult by hypoxia, excess glutamate release or inflammation stimuli. Once activated, microglia/microphages can release various range of toxic mediators responsible for later neuronal damage, and axonal loss characteristic for white matter injury.

The delayed phase of neuronal cell death by apoptosis is lasting for several weeks of the initial insult. Apoptosis is considered to be a major cause of progressive neuronal injury following neonatal hypoxia ischemia however details are still not completely understood.
As mentioned previously, the neuroprotective effect of hypothermia is still not completely understood [11]. A reduction of 3-4^{o}C core temperature clearly decreases the metabolic need of the cells, and maintaining cerebral high energy phosphate levels [12], [7, **Figure 3**.].

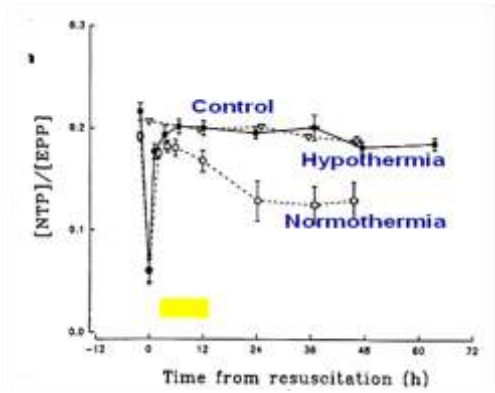

Figure 3. Effect of hypothermia on secondary energy failure [7]. Changes in nucleotide triphosphate and exchangeable phosphate pool ratio on MR spectroscopy after hypoxic insult in HT and NT piglets and control group.

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Hypothermia is also associated with a reduction in free radicals and glutamate levels [7], [13] protecting mitochondrial function and also reduces inflammatory responses.

Animal studies showed that moderate hypothermia decreased apoptosis possibly via inhibiting caspase-3 activation [14] and increasing bcl-2 protein expression [15]. **Figure 4**. is summarizing the underlying processes in pathophysiology and possible target points for treatment.

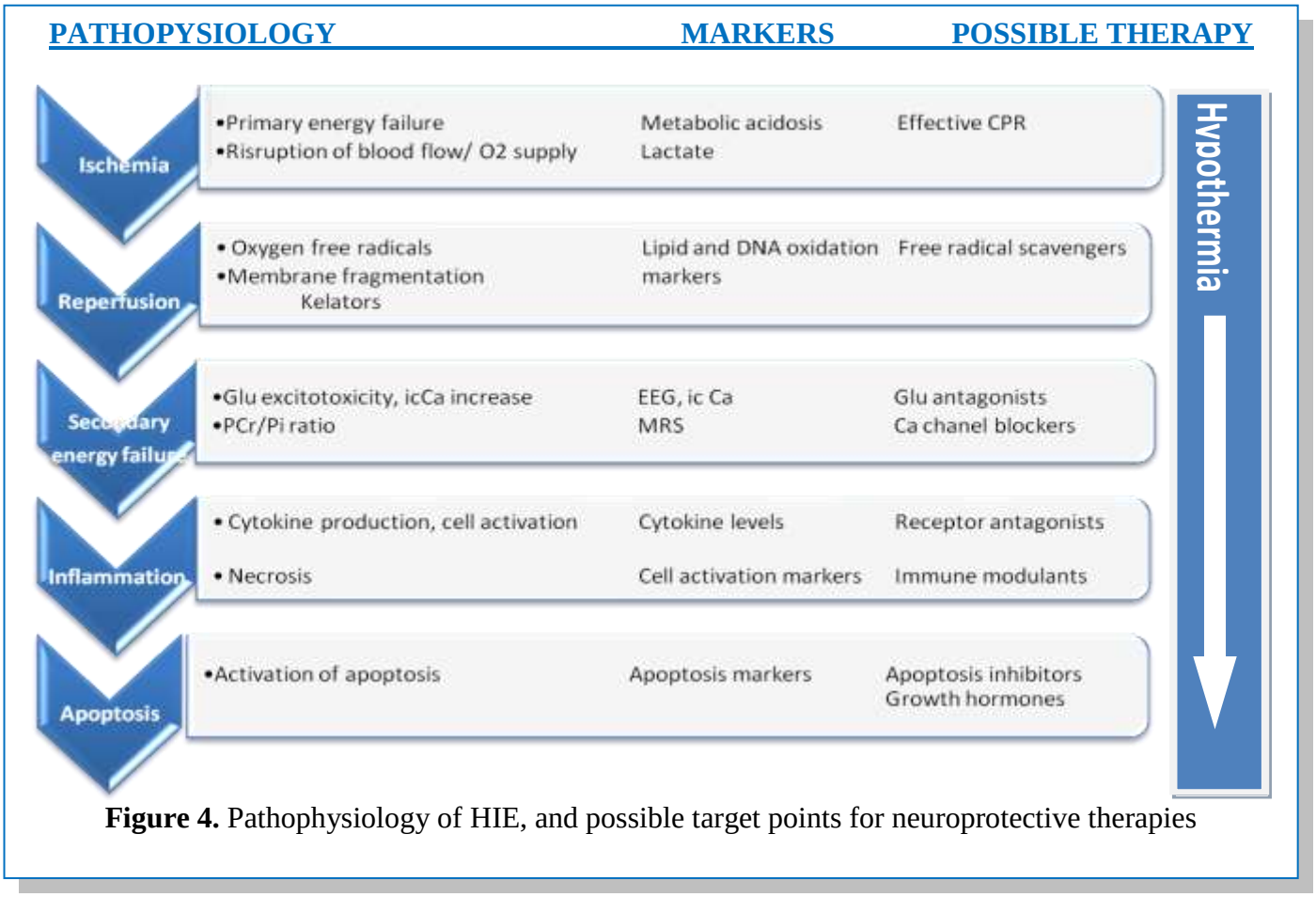


Figure 4. Pathophysiology of HIE, and possible target points for neuroprotective therapies

1.3. Clinical evidence supporting therapeutic hypothermia in newborns

In newborns, therapeutic hypothermia was first described as a method of reanimation by immersion in cold water [16], [17]. Later, experimental studies in adult models of hypoxic ischemic injury suggested that brief periods of post insult hypothermia offered neuroprotection. Moderate systemic or selective cooling of the brain has been shown to reduce brain injury in experimental human adult studies after events like stroke, trauma or cardiac arrest. These results led to a series of studies in piglets, neonatal rats and fetal sheep which showed repeatedly that moderate hypothermia significantly reduced cerebral injury following hypoxic ischemia. Investigators went on to perform a series of clinical studies, first to confirm the safety of prolonged moderate hypothermia in asphyxiated newborns and then to determine therapeutic effect by carrying out randomized controlled trials. However several questions had to be considered before initiating clinical study on neonates with hypoxic-ischemic encephalopathy about the length of the treatment, degree of the hypothermia, starting time of treatment and method of cooling (selective head or systemic). Selective head cooling was thought to be safer without adverse effects, but it is associated with temperature gradient in the brain with possible ineffective cooling of the deeper structures [18]. Major concerns about hypothermia were related to possible adverse effects like reduction of myocardial contractility, increased blood viscosity, coagulopathy, acid-base and electrolyte imbalance and increased risk of infection. To prove the safety of hypothermia pilot studies were performed using different cooling regimes in term neonates [19], [20], [21], [22], [23], [24], [25]. The studies used either selective head cooling (with mild systemic hypothermia) or whole body cooling methods using purposely designed cooling equipment or simple physical cooling with cold bag.

The pilot studies found no evidence of harm from prolonged moderate hypothermia particularly when body temperature was closely controlled; when temperature control was less accurate cardiovascular complications such as hypotension or severe bradycardia occurred [25], [26].

Subsequently several randomized clinical trials with neurological outcomes assessed over at least 18 months followed [27], [22], [28], [29], [30]. Most of these trials were carried out in highly developed countries with a low incidence of hypoxic ischemic

encephalopathy, and so required a large number of participating centers and study enrolment over several years. Each study aimed to determine whether therapeutic hypothermia improved survival but without increasing disability in survivors following neonatal hypoxic ischemic encephalopathy. The studies hypothesized that therapeutic hypothermia would result in a 30% reduction in the combined rate of death and disability in moderately or severely encephalopathic newborns. Uniform infant selection criteria were used: clinical evidence of asphyxia such as prolonged need for resuscitation at birth or severe metabolic acidosis, together with clinically assessed moderate or severe encephalopathy and additionally in most of the studies, abnormal cerebral activity confirmed by amplitude integrated electroencephalography (EEG). Enrollment of infants was complete or exceeded target in most studies but was terminated early in the two most recently reported studies because participating clinicians increasingly lost therapeutic equipoise as neurological outcome data from the completed studies accumulated. A remarkable achievement amongst the trials of therapeutic hypothermia in newborns was the almost complete (>95%) follow-up to 18 months of age of the study participants, which increases confidence in the study findings.

The Cool Cap study [27] applied selective head cooling with mild systemic hypothermia and enrolled 234 infants with signs of encephalopathy and abnormal aEEG. 116 neonates were randomised for head cooling for 72 hours started within 6 hours of life with aim rectal temperature 34-35 C and 118 neonates for conventional care. Primary outcome was a combined end point of death or moderate to several disabilities at 18 months of age. Death or moderate to severe disability occurred in 59 of 108 infants (55%) in the hypothermia group and 73 of 110 infants (66%) in the control group (relative risk 0.61 [95 percent confidence interval 0.34-1.09] p=0.1). However subgroup analysis showed that head cooling had no significant effect on infants with severe encephalopathy based on aEEG (n=46 relative risk 1.8 [95 percent confidence interval 0.49-6.4] p=0.51), but improved outcome of infants with less severe aEEG changes (n=172 relative risk 0.42 [95 percent confidence interval 0.22-0.80] p=0.009).

The NICHD trial by Shankaran et al [30] utilized systemic cooling of term neonates showing clinical evidence and signs of encephalopathy without aEEG monitoring. 208

infants were enrolled and randomised (102 in the hypothermia group and 106 in the control group). Cooling was commenced within 6 hours with blanket connected to a cooling system and was maintained for 72 hours. Aim oesophageal temperature was 33.5 C. Primary outcome was similar, combined death or several disability at 18 to 22 months of age. Infants received hypothermia treatment had significantly less death or severe disability compared to control group (44% vs 62% relative risk 0.72 [95% CI 0.54-0.95] p=0.01). Only this trial showed a significant reduction in the composite primary outcome.

The first two clinical trials showed no significant difference in physiological parameters or adverse events related to hypothermia, but were unable to provide clinically significant evidence for hypothermia treatment to be recommended as standard treatment.

To clarify the role of hypothermia, the TOBY trial [28], [31] was carried out which was the largest randomised multicentre trial, utilizing systemic hypothermia for 72 hours commenced at 6 hours of age enrolling 325 term neonates with clinical signs of hypoxic-ischemic encephalopathy confirmed by aEEG. Our unit was the second largest centre in the trial enrolling 24 infants over 2 years period between 2005 and 2007. Primary outcome was compound end point of death or severe disability at 18 months of age. Of 325 infants, 163 were allocated to cooling and 162 received standard care on normothermia. The rate of survival without neurological abnormality was significantly increased in the cooled group 71/163 (44%), compared to control group 45/162 (28%) (Relative risk 1.57 [95% CI 1.16-2.12] p=0.003). Among survivals cooling resulted in reduced risk of cerebral palsy (RR 0.67 [95% CI 0.47-0.96] p=0.03) and improvement in the Bayley Mental Developmental Index (p=0.03), Bayley Psychomotor Index (p=0.03) and Gross Motor Function Classification Scale (p=0.01). The TOBY trial showed no significant difference in the combined rate of death and severe disability. In the hypothermia group 42 infants died and 32 survived with severe neurodevelopmental disability, whereas 44 normothermic infants died and 42 had severe disability (relative risk 0.86 [95% CI 0.68-1.07], p=0.17). Adverse events were minor and not associated with cooling. Although there was no significant difference in the primary outcome in the TOBY trial, hypothermia resulted in unequivocal improvement of neurological outcomes in survivors.

Recently, as individual trials were insufficient to provide conclusive evidence of efficiency, metaanalysis was carried out with studies identified from the Cochrane Central Register of Controlled Trials, the Oxford Database of Perinatal Trials, PubMed, previous reviews and abstract [4]. The synthesis and metaanalysis of the randomised trials using therapeutic hypothermia is showing striking consistency. The point estimates are remarkably similar and metaanalysis of three trials comprising 767 infants followed to 18 months shows highly significant improvements in neurological outcomes. In the three major trials comparing 767 term infants with asphyxia, cooling reduced the combined rate of death or severe disability (the primary outcome of all the studies) (risk ratio 0.81 [95% CI 0.71-0.93] risk difference -0.11 [95% CI -0.18-0.04] with a number needed to treat of just 9 [95% CI 5-25] p=0.002) [4, **Figure 5.**].

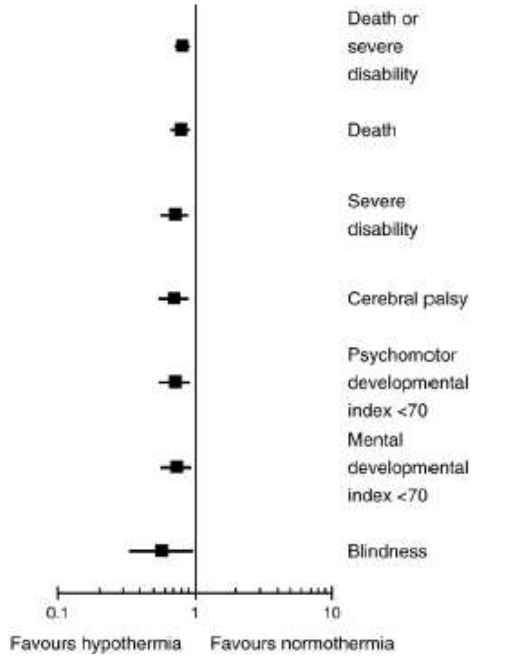
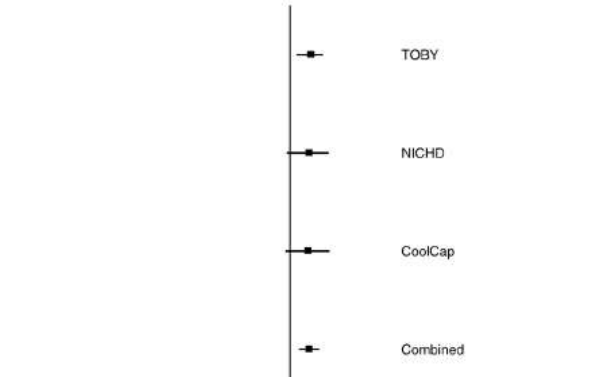


Figure 5. Forrest plot of effect of hypothermia on main neurological outcomes at 18 months [4].

Hypothermia increased normal survival (RR 1.53 [95% CI 1.22-1.93] RD 0.12 [95% CI 0.06-0.18] NNT 8 [95% CI 5-17] p<0.0001) and in survivals reduced the rates of severe disability, cerebral palsy and both mental and psychomotor developmental index <70. In 10 trials comprising 1315 infants mortality was significantly reduced (RR 0.80 [95% CI 0.68-0.94] RD -0.07 [95% CI -0.11, -0.02] with a number needed to treat of 14 [95% CI 8-47] p=0.008) [4, **Figure 6.**]. However, there is some heterogeneity amongst the trials probably because of differences in practice about withholding or continuing intensive care in severely encephalopathic infants.



Study	Hypothermia		Normothermia				
	Total	Events	Total	Events	Relative Risk	95% CI start	95% CI end
CoolCap	116	29	118	20	1.48	0.89	1.93
NICHD	102	32	106	22	1.51	0.94	2.45
TOBY	163	71	162	45	1.57	1.16	2.42
Combined	381	132	386	87	1.53	1.22	2.12

Figure 6. Forrest plot of effect of therapeutic hypothermia on rate of intact survival at 18 months of age. Table gives total cases assessed and rates of events in each trial and on metaanalysis [4].

Reassuringly none of the large randomized trials reported clinically significant complications attributed to hypothermia: Bradycardia to 110-120 beats per minute is normal during therapeutic hypothermia but serious arrhythmia was rarely observed and occurred also in the non cooled groups. Inotropic support was used more commonly in cooled infants but this may have been due to physician bias. Extra cerebral hemorrhage was observed frequently in both cooled and non cooled infants and was mostly mild; however thrombocytopenia was significantly more common with cooling therapy. Cerebral sinus

thrombosis occurred rarely and was not seemingly related to hypothermia. The only possible complication related to the hypothermia was reported in few cases as subcutaneous fat necrosis. Nonetheless none of the trials were powered to detect uncommon complications and although no clear evidence of harm attributed to therapeutic hypothermia has so far emerged from the registries of therapeutic hypothermia established since 2006. However further experience needs to accumulate before clinicians can be reassured about the safety of therapeutic hypothermia especially when it is applied to infants with systemic complications associated with perinatal asphyxia such as pulmonary hypertension or myocardial ischemia.

The final synthesis of the collected data provides creditable evidence that hypothermia treatment is effective and improves neurological outcome in term neonates with hypoxic-ischemic encephalopathy without any known complication.

At this point, the long term follow up of previous clinical trials needs to be completed, but hypothermia has to be confirmed as standard care for neonates with hypoxic-ischemic brain injury in neonatology.

Despite these remarkable results there remain some uncertainties: First no study has reported outcomes yet beyond 18 months of age following therapeutic hypothermia but assessment of cognitive and behavioural functions requires assessment in later childhood. The follow-up rates reported at 18 months may not be achievable later so the effect of therapeutic hypothermia on outcome in later childhood may be less clear cut. Secondly, although the therapeutic effect is remarkable, still over 40% of infants in the trials died or developed disabilities despite therapeutic hypothermia. This partly may be due to selection bias: Since this was a novel intervention, clinicians may have referred the more severely affected infants with prolonged or irreversible asphyxial injury. Thirdly, there is presently very little data on the application of therapeutic hypothermia in resource poor countries, where the incidence of hypoxic ischemic encephalopathy is greatly increased. In a pilot study in Uganda there was an increased mortality in the treatment group, but this may have been due chance allocation in a small study of more infants with severe encephalopathy to the treatment group [[32]]. Field trials are needed before the current data are extrapolated to different environments.

1.4. Organ dysfunction and cell necrosis in asphyxiated neonates

The most significant consequence of the hypoxic insult is irreversible cerebral injury. However, other organs are also severely distressed due to hypoxic-ischemic injury. Cardiovascular instability, pulmonary dysfunction, hepatic impairment, gastrointestinal disorders and acute renal failure may evolve as characteristic components of multiorgan dysfunction (MODS) and failure [33], [34], [35]. MODS contributes significantly to the risk of death in the critically ill neonates including those subjected to asphyxia. Multiorgan failure is mainly due to acute cell necrosis in different organs. Cellular necrosis after hypoxia and reperfusion is the result of cell swelling, disruption of cytoplasmic organelles, loss of membrane integrity and unspecific activation of inflammatory cascade as it is discussed previously.

The diagnosis of MODS is based on clinical signs and laboratory parameters of tissue dysfunction and cell necrosis [33]. As mentioned previously, prolonged moderate systemic hypothermia (HT) is the first therapy proved to be effective in improving outcome of infants with HIE [4], [28]. The neuroprotective effect of HT has been excessively investigated in the last 10 years. However systemic effects have been not completely under focused research.

1.5. Markers for brain injury: S100 and NSE

Assessment of the severity of HIE to provide initial information for indication of hypothermia treatment and preliminary prognosis is usually very important in clinical situations. Hypothermia needs to be initiated as soon as possible within 6 hours of birth, at the moment indication is based on the severity of encephalopathy or abnormality of the amplitude integrated EEG (aEEG). Informing parents and clinical decisions like redirection of care can be particularly challenging. Up to date, brain MRI is considered to be the best tool to assess brain injury; however it is not widely available. Clinical indicators of the severity of asphyxia such as the degree of neurological depression or metabolic acidosis after birth are relatively imprecise predictors of subsequent neurological outcome. Assessment of encephalopathy with aEEG is more and more frequently used, but interpretation can be difficult, especially if sedative or paralytic agents have been used

earlier, so accurate additional sentinel markers are highly awaited. S100B and neuron specific enolase (NSE) may be promising biomarkers of the severity of brain injury and prognosis following asphyxia.

S100B protein is cytosolic calcium binding protein consisting of two monomers, α and β in different combinations. S100B (β β) and S100A (α β) are mainly present in glial cells and specific neuron populations in the central nervous system, but expressed in some extra CNS tissues. Due to its molecular weight (21 kDa), S100B may be only detected in peripheral blood if the integrity of the blood-brain barrier is disrupted. The biological half-life is about 30 minutes. S100B protein can be measured in the blood and urine and is considered a reliable marker of brain damage in adults and newborns. Elevated serum S100B levels are reported after perinatal asphyxia [36], [37], [38], [39], [40], [41], stroke [42], [43], traumatic brain injury [44], cardiopulmonary arrest [45], cardiopulmonary surgery [46].

Neuron specific enolase (NSE) is the neuronal form of intracytoplasmic glycolytic enzyme enolase. NSE is specific for neurons and neuroectodermal cells. The dimeric enzyme consists of 2 subunits with a molecular weight of 78 kDa and biologic half-life about 24 hours. Like S100B, following neuronal injury and impairment of blood-brain barrier integrity NSE is detectable in peripheral blood. Elevated NSE levels are reported after stroke [43], brain injury [44], cardiac surgery [46] cardiopulmonary arrest [47], and perinatal asphyxia [48].

Previous studies mentioned above have shown that serum S100B and NSE may be useful markers for brain injury in infants. Measurements of NSE and S100B proteins are rapid, non-expensive, simple to perform and widely available. These markers may be useful for early assessment of cerebral injury following asphyxia and possibly for monitoring the effect of new therapeutic agents. The influence of moderate hypothermia on these markers in infants is unknown.

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1.6. Role of inflammation

As mentioned previously experimental studies indicate that inflammation is involved in the pathogenesis of hypoxic-ischemic brain injury in the neonate [49], [50]. Both cerebral and peripheral immune responses occur following asphyxia. Microglia and astrocytes become activated and release proinflammatory cytokines and chemokines. Disruption of the blood brain barrier allows infiltration of peripheral monocytes and macrophages into the brain that further enhances the inflammatory response. Immune activation is characterized by increased synthesis of chemokines and cytokines such as interleukin (IL)-1, IL-2, IL-6, tumor necrosis factor (TNF)-α and interferon (IFN)-γ [51]. Levels of inflammatory cytokines are dramatically increased in serum following perinatal asphyxia [52], [53], [54]. The progressing inflammatory response leads to neuronal injury and apoptosis and, therefore, may be a target for neuroprotective therapy in the nearest future.

As demonstrated previously, prolonged moderate hypothermia improves neurological outcome of term infants with HIE. However data about the exact effect of hypothermia on inflammation like cytokine levels in asphyxiated neonates undergoing hypothermia treatment are not reported yet, although one mechanism by which this treatment exerts a neuroprotective effect may be by reducing systemic inflammation.

Simultaneously with inflammatory response, marked endocrine changes may also occur with a possible immune modulator effect. There are increasing data about the significance of low cortisol levels and relative adrenal insufficiency in critically ill preterm and term neonates [55], [56], [57]. These data suggest that hypocortisolaemia can be associated with increased mortality and morbidity. Persistent hypocortisolaemia can also have an adverse effect on systemic inflammatory processes. Data about cortisol levels in asphyxiated neonates are not reported yet, however these infants are often requiring high level of intensive care because of multiorgan failure and are at the risk of relative adrenal insufficiency.

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1.7. Analgesia and drug metabolism during hypothermia

The induction and maintenance of hypothermia may be stressful for the patient, which may counteract the benefits of hypothermia. Thoresen et al demonstrated in piglets that the neuroprotective effect of hypothermia is abolished in the absence of adequate analgesia [58]. Therefore, it is considered highly important to maintain adequate sedation and analgesia during hypothermia. The protocol of the Total Body Hypothermia (TOBY) Study recommends routine treatment with morphine for all infants in the study who required ventilation or showed signs of distress and this is also a common practice in our unit. Assessment of the stress response can be difficult in encephalopathic neonates during hypothermia. Signs of distress include tachycardia, irritability, facial grimacing and shivering. A heart rate consistently above 110-120 beats per minute during hypothermia suggests that analgesia or sedation is required.

Hypothermia influences cellular functions, especially the rate of enzymatic processes. In mammals, cerebral metabolism is reduced by 7% when the body temperature is lowered by 1°C [5], [6]. Data obtained in adults indicate that even short-term hypothermia may have an effect on the metabolism of major analgesics and other drugs [59],[60],[61]. No data are available for neonates concerning the impact of hypothermia on the pharmacokinetics of morphine. However, Thoresen et al reported as unpublished data that the half-life of phenobarbitone in neonates with HIE treated with hypothermia is double that of normothermic infants. The abnormal liver and renal function that often occurs after asphyxia is also likely to alter drug metabolism and excretion.

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2. Aims

Our aim was to perform several observational studies on our study group while participating in the international, randomized TOBY trial. We felt that this trial was one of the last opportunities to do observations comparing a randomized hypothermic and normothermic study group.

As mentioned in the induction, there are several questions not fully investigated in hypothermia. Our plan was to collect more information during prolonged systemic hypothermia about markers of multiorgan failure and brain injury, cytokine responses, and changes in morphine metabolism before hypothermia introduced as standard care. For this purpose additionally to the TOBY protocol we collected serial blood samples at fixed time points in term infants treated with HIE.

Our aim was to find answers for four hypotheses:

1. We hypothesized that as the main pathogenic processes of brain injury and the dysfunction of other organs are partly similar after asphyxia, hypothermia believed to attenuate hypoxic cerebral injury could also protect organs other than brain exposed to hypoxia. There are just few data concerning this issue. Therefore we investigated the effect of hypothermia on some laboratory parameters reflecting cellular necrosis and organ dysfunction characteristic for the failure of internal organs in asphyxiated neonates.

2. Secondly we wanted to evaluate the effect of systemic moderate hypothermia on levels of serum S100B and NSE, their time course, and association with the aEEG and neurodevelopmental outcome as no data was available before.

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3. One of the mechanisms via hypothermia believed to act is the reduction of the inflammation. The aim of our next substudy was to explore the influence of therapeutic hypothermia on serum cytokine and cortisol concentrations.

4. During our pilot study before the TOBY trial we observed severe late hypotension in some infants treated with hypothermia without clear cardiovascular cause. Discussing this we found that we use morphine treatment more often and in higher dose than other study groups, and the late cardiovascular instability can be related to morphine toxicity. Our hypothesis was that morphine pharmacokinetics are altered during prolonged moderate systemic hypothermia in asphyxiated neonates, resulting in excessively high morphine concentrations compared with infants kept at normothermia.

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3. Methods

3.1. Patients and study protocol

Between January 2005 and December 2007, 64 term infants were admitted to the regional level 3 neonatal care unit at the First Department of Pediatrics, Semmelweis University with the diagnosis of hypoxic-ischemic encephalopathy. These infants were screened for evidence of encephalopathy according to a 3-step eligibility system based on clinical and neurologic criteria as used in the TOBY Study [28], [31]. Infants were enrolled within 6 hours of birth if each of the following criteria was fulfilled. (1) infants were ≥36 weeks' gestation with ≥1 of the following: (a) Apgar score of ≤5 at 10 minutes after birth; (b) continued need for resuscitation, including endotracheal or mask ventilation, at 10 minutes after birth; (c) acidosis defined as pH ≤7.0 and/or base deficit ≥16 mmol/L in umbilical cord blood sample or any blood sample within 60 minutes of birth (arterial or venous blood); (2) moderate to-severe encephalopathy consisted of altered state of consciousness (irritability, lethargy, stupor, or coma) and ≥1 of the following: (a) hypotonia, (b) abnormal reflexes including oculomotor or pupillary abnormalities, (c) an absent or weak suck, or (d) clinical seizures; and (3) ≥30 minutes duration of amplitude-integrated electroencephalogram recording showed moderately abnormal or suppressed background amplitude-integrated electroencephalogram activity or seizures. Exclusion criteria from the TOBY were prematurity, congenital malformations, suspected metabolic disorders, absence of parental consent and age of more than six postnatal hours.

From the 64 admitted infants 24 with HIE were enrolled into the multinational, randomized and prospective TOBY Study (Total Body Hypothermia for the Treatment of Perinatal Asphyxial Encephalopathy, ISRCTN 89547571). The study was approved by the national Ethical Committee for Medical Research (591/KO/2004). 40 infants were not enrolled to the study because of the following reasons: no evidence or mild encephalopathy 22/40, unstable infants with severe HIE 9/40, admission after 5 hours of life 2/40, congenital abnormality 1/40 (diaphragmal hernia), parental consent not given 5/40, lack of human resources 1/40.

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Before treatment, each patient's parents provided informed consent to participate in this study. Patient allocation was by central telephone randomization provided by the National Perinatal Epidemiology Unit (Oxford, United Kingdom). Infants allocated to treatment with standard intensive care and hypothermia were cooled to an aim rectal temperature of 33.5°C for 72 hours, called the hypothermia group ((HT) *n* =13).

Hypothermia was maintained by using a cooling mattress (Tecotherm, Munich, Germany) (**Figure 7.)**. Infants allocated to the control group (normothermia (NT); *n* = 11) were treated with standard intensive care on normothermia (37°C). In both groups, rectal temperatures were monitored continuously and recorded each hour for the 72 hours intervention period.

Figure 7. Cooling equipment and setting used in the TOBY trial.

Both groups were treated with the same regimen, except for the hypothermia. All of the infants received morphine-hydrochloride (Biogal-Teva, Budapest, Hungary), as a single loading dose of 50 to 150 μg/kg of body weight before 6 hours of age, followed by continuous infusion at 5 to 30 μg/kg per h. The maintenance dose was adjusted according to physical signs and symptoms of discomfort, such as excessive movement, irritability, or tachycardia assessed by the attending neonatologist. Continuous morphine infusions were stopped after 72 hours or earlier if the infant was extubated and was not distressed.

After randomization, aEEG monitoring was continued and the background activity was assessed in the time points of blood sampling according the following evaluation system and scores were given. Flat = 1, burst suppression = 2, moderately depressed = 3, normal ± sleep-awake cycling = 4. The total score (minimum 5, maximum 20) was calculated in all infants as a marker of recovery on aEEG.

Seizures were controlled with phenobarbital in both groups (starting dose: 20 mg/kg; maintenance dose: 5–10 mg/kg per d). If the infant remained agitated or seizures

persisted, a single dose of midazolam (0.1– 0.2 mg/kg) was administered. The daily cumulative doses of morphine and other drugs were recorded.

Cardiovascular instability was defined as mean arterial blood pressure ≤40 mmHg and was treated with a single or repeated dose of saline (10–20 mL/kg). If hypotension persisted, dobutamine (5–20 μg/kg per min), dopamine (2–10 μg/kg per min), or norepinephrine (0.1– 0.3 μg/kg per min) was administered. When this was insufficient, hydrocortisone (1-2 $mg.kg^{-1}$) was administered, but infants requiring hydrocortisone supplementation were not included for cytokine measurements.

Blood and outer ear swab cultures were obtained at admission from all infants and bacterial infection was excluded. All infants received a regular antibiotic regimen *i.e.* ampicillin and amikacin during the study period.

Venous blood samples were taken at 6, 24, 48 and 72 h after birth for laboratory measurements of full blood count, electrolytes, liver function (ASAT, ALAT), lactate dehydrogenase, creatine kinase, creatinine, uric acid, coagulation and for further investigations (S100, NSE, cytokine and morphine measurements). Samples were collected via venous umbilical catheter. Remaining blood was centrifuged; sera were separated and stored at -80°C until further measurements.

Urine output was monitored in each patient. Enteral feeding was not started during investigation period. Dysfunction of individual organs was characterized according to the following parameters during the first 72 h: catecholamine requirement to maintain blood pressure in normal range (cardiovascular instability); need for oxygen supplementation during the first day (pulmonary dysfunction); elevation of the transaminases (liver involvement); rate of diuresis and serum creatinine levels (renal function) and abdominal distension, gastrointestinal bleeding (gastrointestinal damage). The diagnosis of MODS was established if the investigated parameters suggested the significant impairment of two or more organs additionally to that of the brain.

The severity of encephalopathy during the first 24 hours of age was assessed by Sarnat score and again at 4 days of age by using the TOBY protocol modified encephalopathy score.

Cranial ultrasound scan was performed daily during the investigation period and reported by radiologist consultants. Brain MRI was performed between day 5 and 14 on a Philips 3T scanner obtaining T1 weighted, T2 weighted, and diffusion weighted images. Images were reported by radiologist consultant. Some patients also had MR spectroscopy.

Neurodevelopmental assessment (Bayley Scales of Infant and Toddler Development TM III) was performed between 18-22 months according to TOBY study protocol. Infants were classified into two groups depending on the results of the follow up examination: Survival without severe disability; and severe developmental delay (MDI and PDI <70) or death.

The analysis of the data usually was performed by the statistical software Statistica 8.0 (StatSoft Inc, Tulsa, USA). Anthropometric and clinical parameters were compared with Mann-Whitney and Fischer tests. The level of significance was set at $p<0.05$.

3.2. Laboratory parameters indicating cell necrosis and organ failure

Laboratory measurements of 21 infants were analysed in this substudy by the commercially available tests on a Roche Hitachi 912 system Hitachi 714 automated system. The 3 patients of the 24 enrolled infants who died during the 72 hours investigation period were excluded from this substudy. The analysis of the data was performed by the statistical software Statistica 8.0 (StatSoft Inc, Tulsa, USA). For statistical analysis comparing the markers and area under curve (AUC) we used Kruskal–Wallis test with Dunn's post hoc test and Mann–Whitney U test.

3.3. Markers for brain injury: S100 and NSE

S100B and NSE were measured by enzyme-linked immunosorbent assay (Roche) from frozen sample (100 µl sera) according to the manufactures instructions. The assay's detection limit was 0.02 µg/l for S100B and 1 µg/l for NSE. The coefficient of variation of the control tests was <10% for both measurements. Samples were available in 24 patients (n=13 in HT group and n=11 in NT group). The analysis of the data was performed by the

statistical software Statistica 8.0 (StatSoft Inc, Tulsa, USA). Friedman test with Bonferroni correction was used to assess time-related differences. The analyses were performed both for treatment temperature and outcome data. Spearman correlation was used to assess the relationship between clinical prognostic indicators and S100B and NSE levels.

3.4. Serum cytokine and cortisol measurements

12 cytokines and vasoactive agents (interleukin (IL)-1-α, IL-1-β, IL-2, IL-4, IL-6, IL-8, IL-10, MCP-1, EGF, VEGF, IFN-γ, TNF-α), were measured from frozen sample (100 μl sera) with the Randox Cytokine and Growth factors array (Randox Lifesciences, Crumlin, United Kingdom). The protein chip utilizes a biochip as a reaction platform. This biochip is a 9 mm^2 solid substrate with multiple specific ligands attached at pre-defined sites on the surface and utilizes competitive, sandwich and antibody capture immunoassay formats. The competitive assay uses an enzyme labeled analyte for signal production whereas the sandwich assay is an enzyme labeled antibody. The chemiluminescence signals are simultaneously detected for the full array of tests on each chip. Intra-assay coefficients of variations for each analytes are below 10%. Cortisol levels were measured by a two-step competitive immunoassay with streptavidine microparticles and electro-chemiluminescence detection (Hoffmann-La Roche Ltd, Basel). Intra-assay coefficient of variation was 6.2%.
In this substudy, 6 infants of the 24 enrolled were excluded because of the following reasons: early death: 2, insufficient samples: 1, corticosteroid supplementation because of pressor resistant systemic hypotension: 3 infants.

The analysis of the data was performed by the statistical software Statistica 8.0 (StatSoft Inc, Tulsa, USA). Finally, due to large number of missing data at 48 and 72 hours, these time points were not included into the analysis. Logarithmic transformation was used to normalize cytokine and cortisol data. Cytokine and cortisol levels were analyzed by One Way Repeated Measure ANOVA, where treatment (hypothermia and normothermia) effect was compared over time-points (6, 12 and 24 hours), respectively. Newman-Keuls *post hoc* tests were also performed. Spearman correlation was used to assess relationship between duration (hours elapsed from the time of introduction of hypothermia to the time of the sampling) of hypothermia treatment and cytokine levels.

3.5. Serum morphine measurements

Serum morphine concentrations were determined with an enzyme-linked immunosorbent assay (Opiates Reagent Pack, Abbott Diagnostics, Abbott Park, IL) from the frozen samples. The assay used a stored 6-point calibration curve, and it was linear within the concentration range of 50 and 1000 ng/mL. Control tests were conducted before the analysis of morphine concentrations. The coefficient of variation of the control tests was <10%. SPSS 12, Apache Software Foundation (SPSS Inc, Chicago, IL), was used for statistical analysis.

Morphine measurements were carried out before the end of the TOBY trial from samples available from the first 16 patients (n=10 HT, n=6 NT group). We calculated the area under the curve (AUC) of the serum morphine concentrations and calculated clearance by dividing the total morphine administered by the AUC. Serum morphine concentrations were considered to reach a steady state when the difference between measurements at successive time points was <15%. If morphine concentrations reached a steady state, we also estimated clearance by dividing the infusion rate by the steady-state serum morphine concentration.

A 2-tailed *t* test was used to compare means of normally distributed continuous data, and we used the Mann-Whitney *U* and Kruskal-Wallis tests for nonparametric data. Categorical data were compared with Fisher's exact test. Correlation of the serum morphine concentrations with the morphine infusion rate and treatment with hypothermia was computed by multiple regression analysis.

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4. Results

4.1. Antropometric and clinical parameters in study group

The clinical characteristics of the infants in the HT and NT groups were similar (**Table 1**). The presumed cause of asphyxia was prolonged second stage labour (17/24), shoulder distocia (1/24), placental abruption (3/24), and prolonged fetal bradycardia on CTG (3/24).

Table 1. Anthropometric and initial clinical parameters of 24 asphyxiated newborns treated with systemic hypothermia or on normothermia enrolled to the TOBY trial. There were no significant differences in the parameters except core temperature at the 6th hour of life. Values are median [range], and number of patients. * $p \leq 0.05$

	HYPOTHERMIA N = 13	**NORMOTHERMIA N = 11**	**p**
GA (wk)	38 [36-41]	39[36-41]	ns
Birthweight (g)	3500[2300-4390]	3450[2540-4040]	ns
Vaginal delivery	12/13	7/11	ns
Emergency cesarian section	1/13	4/11	ns
Return of spont. breathing (min)	15[5-60]	32[7-120]	ns
Apgar score 5 min ≤5	8/13	8/11	ns
Apgar score 5 min	5[0-8]	3[0-7]	ns
Apgar score 10 min	6[1-7]	5[0-8]	ns
First measured pH	7.19[6.9-7.34]	7.05[6.8-7.29]	ns
First measured BE (mmol/l)	-12.6[-21- -3.9]	-18.1[-28- -7.0]	ns
First measured lactate (mmol/l)	9.7[0.9-15]	8.3[1.8-15]	ns
Time of randomisation (hrs)	3.3[2.5-5.3]	3.1[2.5-5.5]	ns
Sarnat 1-2 (n/N)	11/13	7/11	ns
Sarnat 3 (n/N)	2/13	4/11	ns
Temperature at 6th hrs (^{0}C)	33.5 [33.2-33.7]*	36.7 [35.7-36.9]*	* 0.0001

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19 of the 24 deliveries were vaginal, including two infants delivered with ventouse extraction, while five infants were by emergency cesarean section (placental abruption in three and fetal bradycardia in two infants). 20 of the 24 infants were boys. 12 of 13 infants survived in the hypothermia group while eight of 11 survived in the normothermia group. The time of admission was (median [range]) 1.8 [0.8-4.4] hours of age in the HT and 1.3 [1.0-4.5] hours in NT groups. The time of randomization was 3.3 (2.5-5.3) hours of age in the HT and 3.1 (2.2-5.5) hours in NT groups. Clinical parameters used to assess the severity of HIE (Apgar scores, pH base excess and lactate within the first hour of life, Sarnat scores) showed no significant difference between the two groups (**Table 1**.).

The rectal temperature of the two groups was significantly different at the 6^{th} hour of life and throughout the observation period (p=0.0001).

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4.2. Effect of hypothermia on multiorgan failure

Laboratory parameters during the first postnatal 72 hours are summarized in **Table 2**. None of the investigated parameters differed significantly at 6 hours of life except uric acid. Shortly, during the investigated period significant differences were obtained in the AUC values (**Figure 8**.) of ASAT median [range] (4314 [1983–22 580] vs. 12 140 [3930–37 440] p = 0.0014), LDH (81 430 [45 780–143 100] vs. 194 300 [80 050–540 700] p = 0.007), creatinine (4520 [3321–6393] vs. 6786 [4317–14 560] p = 0.005) and uric acid (17 720 [9084–24 230] vs. 28 020 [9897–49 670] p = 0.024). ALAT values were significantly different at 72 h of life (19 [11–159] vs. 106.5 [39–214] IU/L p = 0.004). In addition to AUC, postnatal kinetics also differed. LDH values reached their maximum in HT and NT neonates at 24 and 48 h, respectively; peak uric acid levels were measured in HT and NT neonates at 6- and 24 h, respectively.

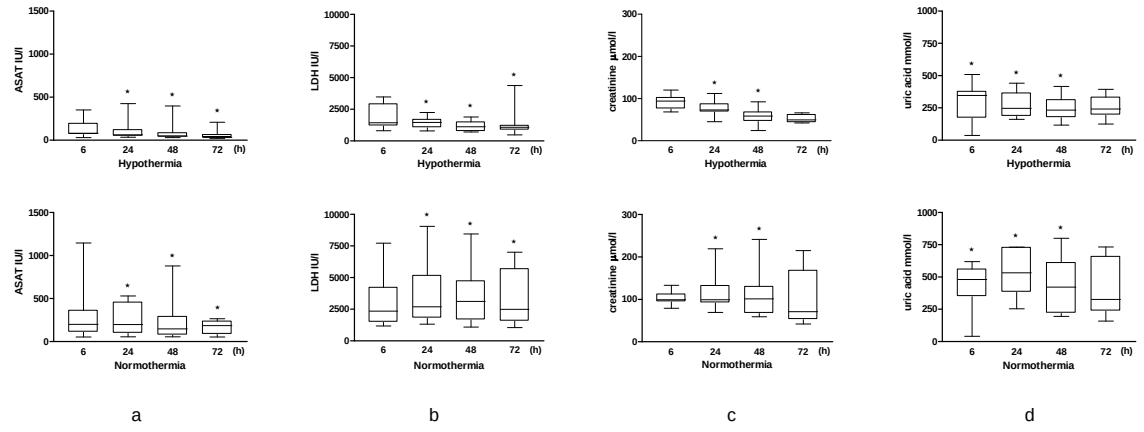

Figure 8. Changes of serum ASAT (a), LDH (b), creatinine (c) and uric acid (d) during the first 72 h of life in asphyxiated neonates treated with hypothermia or on normothermia. Values are given as median [range]. *significant difference between hypothermic and normothermic group, $p < 0.05$.

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Table 2. Median values for investigated laboratory parameters in hypothermic newborns compared to newborns treated on normothermia with standard intensive care in the MODS substudy. Values are median [range]. * p≤ 0.05

Time of blood sampling	HYPOTHERMIA N = 12	NORMOTHERMIA N = 9	p
ASAT (U/l)			
6	80.0 [30-350]	199.1 [53-1144]	ns
24	61.5 [34-424]	198.0 [54-530]	* 0.020
48	47.0 [29-398]	148.5 [54-878]	* 0.012
72	32.0 [20-209]	185.5 [53-265]	* 0.006
AUC	4314 [1983-22580]	12140 [3930-37440]	* 0.014
ALAT (U/l)			
6	21.0 [10-269]	57.0 [16-717]	ns
24	24.0 [9-248]	55.0 [11-270]	ns
48	19.0 [8-187]	68.5 [8-365]	ns
72	19.0 [11-159]	106.5 [39-214]	* 0.004
AUC	1374 [576-12820]	4086 [711-20650]	0.056
LDH (U/l)			
6	1427 [803-3470]	2345 [1159-7714]	ns
24	1454 [787-2242]	2675 [1312-9040]	* 0.014
48	1109 [693-1883]	3111 [1084-8443]	* 0.002
72	1074 [471-4374]	2487 [1048-7000]	* 0.010
AUC	81430 [45780-143100]	194300 [80050-540700]	* 0.007
CK (U/l)			
6	1399 [308-3962]	2320 [595-4989]	ns
24	705 [104-2127]	2394 [336-5505]	ns
48	445 [59-1454]	870 [236-2602]	ns
72	202 [43-1541]	438 [204-2967]	ns
AUC	56000 [4428-122100]	124000 [25050-255200]	0.08

Uric acid (mmol/l)			
6	346 [36-509]	482 [40-619]	* 0.046
24	246 [161-442]	533 [254-731]	* 0.011
48	233 [117-416]	421 [195-800]	* 0.040
72	241 [125-394]	327 [159-733]	ns
AUC	17720 [9084-24230]	28020 [9897-49670]	* 0.024
Creatinine (µmol/l)			
6	94 [68-120]	99 [79-133]	ns
24	73 [45-112]	99 [69-219]	* 0.011
48	58 [24-92]	101 [59-241]	* 0.006
72	49 [42-66]	71 [42-215]	ns
AUC	4520 [3321-6393]	6786 [4317-14560]	* 0.005
Diuresis (ml/kg/h)			
0-23	1.9 [0.5-2.8]	1.7 [0.0-2.3]	ns
24-47	3.2 [1.2-5.6]	2.7 [1.1-4.0]	ns
48-72	3.1[2.3-6.1]	3.7 [0.5-5.3]	ns
AUC	168 [74-238]	142 [74-178]	0.337

The prevalence of organ dysfunction is presented in **Table 3**. It is worth mentioning that acute renal failure and liver impairment affected less HT than NT neonates ($p = 0.03$, and $p = 0.08$, respectively). Four of the 12 HT and 6 of the 9 NT neonates developed MODS ($p = 0.20$).

Table 3. Organ involvements in the hypothermic and normothermic newborns in the MODS substudy. Values are number of patients. *$p \leq 0.05$. Brain: signs of encephalopathy. Cardiovascular: hypotension treated with catecholamine to maintain blood pressure above 40 mmHg for at least 24 hours. Pulmonary: FiO2 > 0.4 for at least 24 hours without primer pulmonary disease. Liver: ASAT> 200 U/l at any time during the first 72 hours of life. Renal: diuresis < 1 ml/kg h-1 for a least 24 hours or creatinine > 100 µmol/l at any time during the first 72 hours of life. Gastrointestinal: abdominal distension, gastrointestinal

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bleeding, clinical and radiographic signs of necrotizing enterocolitis. MODS: involvement of two or more organs additionally to the brain.

Organ	**HT**	**NT**	**p**
Brain	12/12	9/9	ns
Cardiovascular	9/12	5/9	ns
Pulmonary	3/12	1/9	ns
Liver	3/12	6/9	0.08
Renal	3/12	7/9	*0.03
Gastrointestinal	0/12	0/9	ns
MODS	4/12	6/9	ns

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4.3. Effect of hypothermia on S100B and NSE levels, and their relationship with the neurological outcome

Serum S100B levels were greatly increased above the range reported in healthy infants [62], [63] during the 72 hours but were lower in infants treated with hypothermia compared to normothermic infants, although this reached statistical significance only at 48 hours of age (p=0.047) (**Table 4., Figure 9**.). Compared to values measured at 6 hours of age, S100B values decreased over time in both groups (NT: p=0.002, HT: p=0.04).

Table 4. Serum S100B and NSE levels in asphyxiated infants treated with systemic hypothermia or normothermia. Values are given as median [range].

Postnatal age at blood sampling (hour)		**HYPOTHERMIA N =13**	**NORMOTHERMIA N =11**	**P**
S100B µg/l	6	1.03 [0.52 - 52.90]	**4.58 [0.39 – 64.74]**	Ns
	12	0.77 [0.0 - 20.06]	1.04 [0.31 – 31.82]	Ns
	24	0.46 [0.29 – 4.66]	0.78 [0.37 – 20.3]	ns
	48	0.36 [0.23 – 1.20]	0.61 [0.42 – 1.91]	*0.047
	72	0.43 [0.29 – 1.19]	0.70 [0.52 – 0.77]	ns
NSE ng/ml	6	43.86 [23.36 – 132.50]	46.96 [23.92 – 99.20]	ns
	12	43.12 [32.14 – 166.1]	38.58 [26.98 – 141.80]	ns
	24	36.13 [13.94 – 175.1]	38.76 [17.14 – 129.20]	ns
	48	29.44 [21.50 – 110.30]	38.38 [13.82 – 94.42]	ns
	72	23.72 [13.96 – 119.3]	45.16 [22.20 – 62.18]	ns

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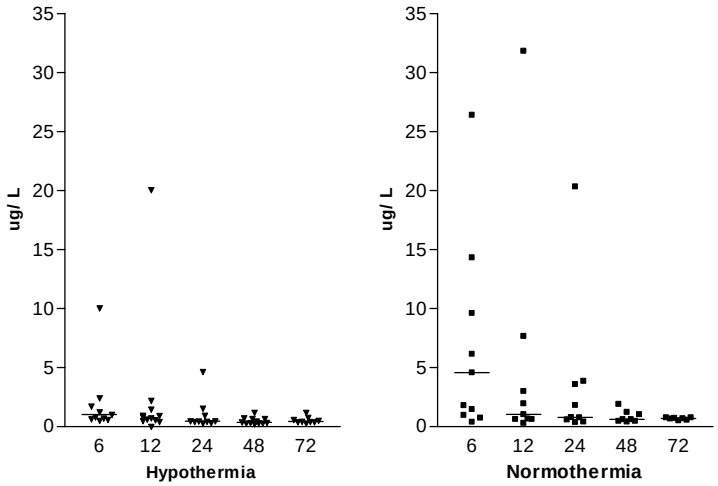

Figure 9. Serum S100B levels in hypothermia and normothermia treatment groups. The median value (indicated by the horizontal lines) and individual values were shown. (At 6 hours of life one outliner value >50 ug/ L in each group is not shown, but these values were included in the analysis).

At all-time points S100B values were very significantly lower in infants with normal outcome compared to levels obtained from infants with severely abnormal neurological outcome or death (6 h $p<0.001$, 12 h $p=0.003$, 24 h $p=0.002$, 48 h $p=0.007$, 72 h $p=0.04$) (**Table 5., Figure 10.**). The association of S100B levels with outcome was greatest at 6 hours of age.

Table 5. Serum S100B and NSE levels in asphyxiated infants with normal neurodevelopmental outcome or severe brain injury/ death. Values are given as median [range].

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Postnatal age at blood sampling (hour)		NO SEVERE DISABILITY N =18	SEVERE DISABILITY OR DEATH N =6	p
S100B µg/l	6	1.01 [0.39 – 14.30]	18.22 [6.16 – 64.74]	*<0.001
	12	0.70 [0.0 – 7.68]	20.06 [3.0 – 31.82]	*0.003
	24	0.48 [0.29 – 3.86]	4.12 [1.82 – 20.32]	*0.002
	48	0.45 [0.23 – 1.24]	1.20 [1.04 – 1.91]	*0.007
	72	0.52 [0.29 – 0.77]	0.77 [0.67 – 1.19]	*0.039
NSE ng/ml	6	38.5 [23.36 – 114.70]	71.14 [32.86 – 132.50]	ns
	12	39.0 [27.34 – 166.1]	42.88 [26.98 – 141.80]	ns
	24	33.82 [13.94 – 175.10]	88.85 [43.78 – 129.20]	*0.036
	48	30.20 [13.82 – 110.30]	68.98 [35.52 – 94.42]	ns
	72	28.56 [13.96 – 119.3]	45.16 [25.24 – 45.58]	ns

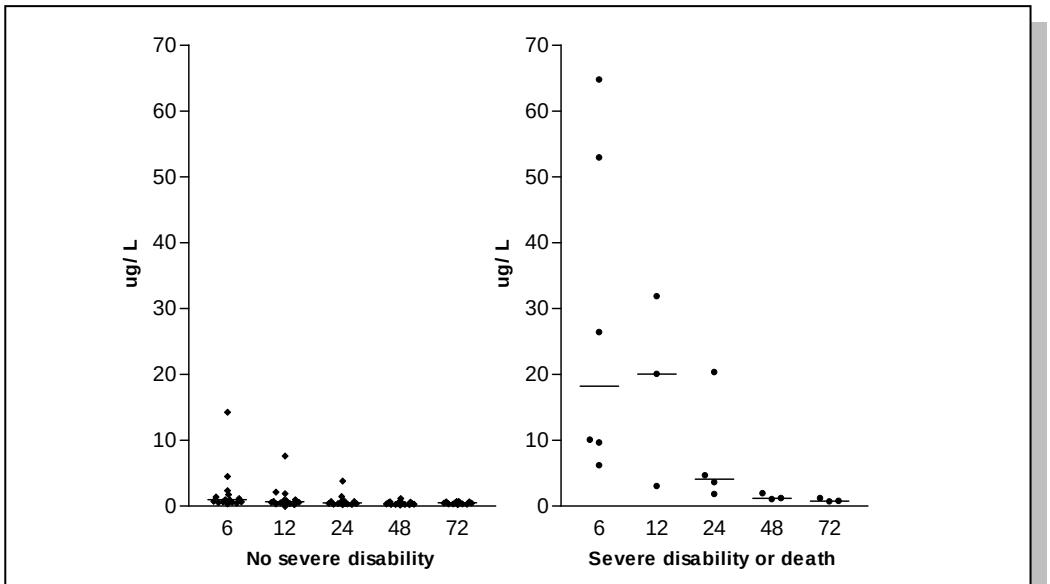

Figure 10. Serum S100B levels in the groups with or without adverse outcome. Median (indicated by the horizontal line) and individual values were shown.

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Serum NSE levels also greatly increased above the range measured in healthy infants in both HT and NT groups. NSE values in infants treated with hypothermia appeared to be lower compared to levels in normothermic infants but the difference was not significant statistically at any time point (**Table 4., Figure 11**.).

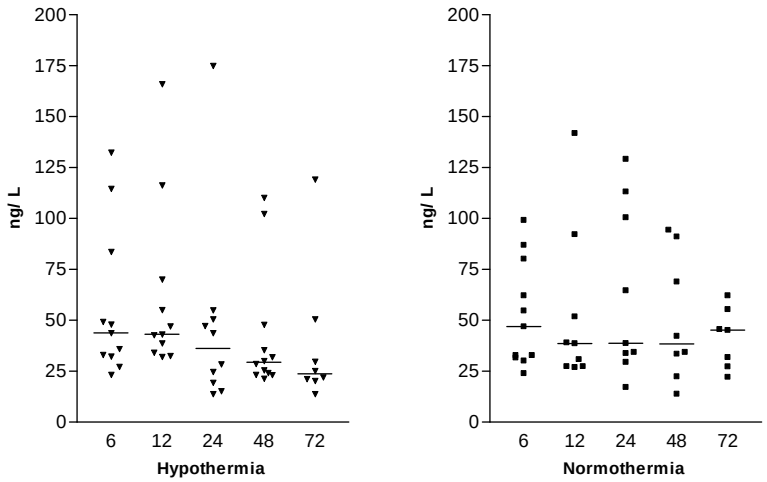

Figure 11. NSE levels in hypothermia and normothermia treatment group. Median (indicated by the horizontal line) and individual values were shown.

NSE values were significantly lower in infants with good outcome at 24 hours of age (p=0.036) but not at other time points (**Table 5., Figure 12**.).

In the combined cooled and non cooled groups, a significant correlation was found between pH within 1st hour of life and S100B values at 6, 24, 72 hours (6 h: R=0.69 p<0.001, 24 h: R=0.61 p=0.005, 72 h: R=0.60 p=0.013), and also with BE within 1st hour of life and S100B values at similar time points (6 h: R=0.69 p<0.001, 12 h: R=0.62 p=0.003, 24 h: R=0.69 p=0.001, 72 h: R=0.67 p=0.004).

Similar significant correlation was found between pH and BE within 1st hour of life and NSE levels at 24 and 72 hours of age (pH 24h: R=0.47 p=0.044, 72h: R=0.69 p=0.004, BE 24h: R=0.60 p=0.006, 72h: R=0.53 p=0.04)

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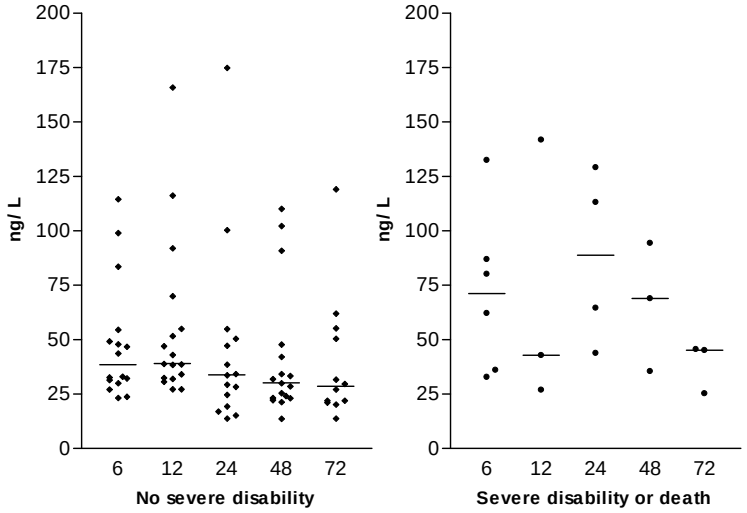

Figure 12. NSE levels in the groups with or without adverse outcome. Median (indicated by the horizontal line) and individual values were shown.

Apgar score at 10 minutes significantly correlated with S100B levels at 6 hours of age (R=0.70, p=0.003). S100B values at 6, 12 and 24 hours, correlated with the Sarnat score (6 h: R=0.66, p=0.002, 12 h: R=0.58, p=0.02, 24 hours: R=0.63, p=0.004).

aEEG scores increased (suggesting significant improvement in background activity) in both groups over time (NT group, p=0.004; HT group, p<0.001). The aEEG returned to normal in nine infants in the HT and four in the NT group during the 72 hour cooling period (p=0.056). aEEG scores were significantly higher in infants with normal outcome at each time point (6h p=0.011, 12h p=0.007, 24h p=0.004, 48h 0.001, 72h 0.046). Total aEEG score was also significantly higher in infants with favorable outcome (16 [8-19] vs. 6.5 [2- 9], p<0.001).

There was no correlation between NSE levels and the Sarnat score nor between S100B or NSE levels and aEEG scores.

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4.4. Effect of hypothermia on serum cytokine concentrations

Clinical characteristics of this substudy group (N=18) were similarly comparable at study entry. Due to technical reasons cytokine measurements were available on 90.0% (38/40) (HT) and 87,5 % (28/32) (NT) of samples. Cytokine values varied widely in each group, causing significant problems in statistical analysis and interpretation. In the case of four cytokines (IL-1α, IL-1β, IL-2, MCP-1), more than 40 percent of the data were below (IL-1α, IL-1β, IL-2) or above (MCP-1) the detection limit at each time points. Therefore no further analysis was performed on these data.

The median values and range of measured cytokine levels in the hypothermia and normothermia groups are presented in **Table 6**.

In this section we would like to present only the results about some main cytokines (IL-4, IL-6, IL-10, INF-γ, TNF-α, VEGF) where significant differences were found during the first 24 hours period.

Table 6. **Cytokine and cortisol levels in hypothermic and normothermic neonates during the first 24 hours.** Values are given as median [range].

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Serum levels	HYPOTHERMIA (n=10)			NORMOTHERMIA (n=8)		
	6 hours	12 hours	24 hours	6 hours	12 hours	24 hours
IL-6 (pg/ml)	31.6 [3.4 – 111.3]	78.5 [16.4 -342.5]	124.2 [42.2 -800.0]	118.9 [25.9 -630.4]	175.9 [17.3-800.0]	145.5 [29.3 -735.6]
IL-10 (pg/ml)	4.3 [1.6 -145.4]	3.0 [1.0 –163.5]	1.7 [1.1 -20.1]	25.6 [1.7 -900.0]	6.6 [0 -72.7]	2.3 [1.26 -26.0]
IL-4 (pg/ml)	3.1 [0 - 9.6]	4.0 [2.4 -6.9]	4.8 [2.4 -6.9]	6.1 [3.7 -16.1]	4.7 [2.6 -7.2]	7.2 [3.1 -11.2]
IL-8 (pg/ml)	447.2 [39- >2900]	198.5 [38- >2900]	149.2 [25.2 -1457]	156.3 [40.9 -850.2]	269.2 [81.2 -2361]	178.5 [28.3 -518.1]
Interferon-γ (pg/ml)	7.2 [0 - 25]	7.5 [0 – 111.0]	9.7 [3.8 – 135.9]	5.8 [0 -11.7]	7.7 [0 -41.7]	8.0 [5.0 -52.9]
TNF-α (pg/ml)	6.4 [3.5 - 94.5]	11.1 [4.9 – 136.6]	8.9 [4.5 – 29.1]	14.7 [6.6 -29.7]	10.4 [6.6 -14.5]	10.1 [4.8 -22.4]
EGF (pg/ml)	163.8 [30.6 -401.4]	125.4 [2.1 -511.9]	91.3 [2.6 -411.6]	97.2 [9.4 -306.4]	52.9 [2.2 -425.1]	92.1 [2.0 -270.1]
VEGF (pg/ml)	233.5 [0.0 -1109.0]	436.7 [24.1 -1322]	243.7 [6.3 -1169]	19.3 [0.0 -417.6]	202.4 [13.1 -492.3]	271.9 [102 -467.7]
Cortisol (nmol/l)	319.7 [157.7-1289]	259.0 [101-756.6]	170.7 [51.2 -781.8]	428.3 [239.6-1432]	204.5 [131 -508.8]	143.2 [51.0 -508.8]

IL-6 levels measured at the 6 hours of age in neonates treated with HT were significantly lower compared to those measured in NT (**Figure 13.**). Serum IL-6 levels at 6 hours after birth, median (interquartile range), were 31.6 pg/ml (3.4-111.3) in hypothermic and 118.9 pg/ml (25.8-630.4) in normothermic neonates. IL-6 levels did not differ between the two groups at other time points.

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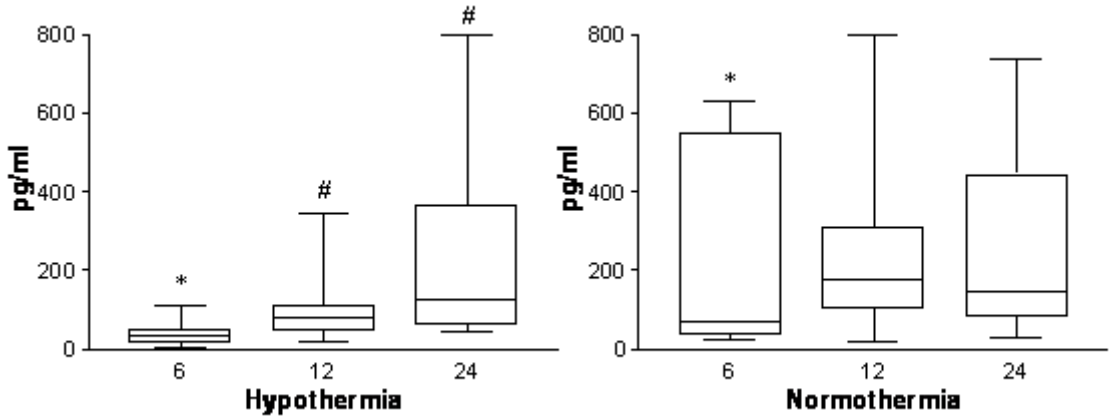

Figure 13. Changes in serum IL-6 in hypothermic and normothermic infants. Median and interquartile range was shown. *, significantly different from normothermia ($p<0.05$), #, significantly different from values measured at 6 hours of age ($p<0.05$).

A significant negative correlation was observed between the duration of hypothermia and IL-6 (R=-0.73, p=0.017) (**Figure 14.**), INF-γ (R=-0.64, p=0.05), and TNF-α (R=-0.67, p=0.04) measured at 6 hours of age and IL-10 at 12 hours of age (R=-0.79, p=0.01).

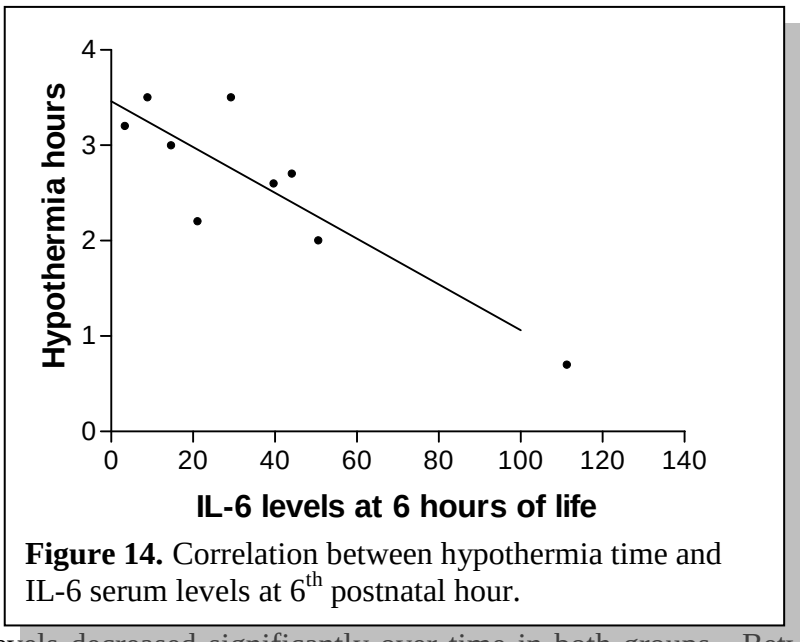

Figure 14. Correlation between hypothermia time and IL-6 serum levels at 6th postnatal hour.

IL-10 levels decreased significantly over time in both groups. Between 6 and 24 hours of age serum IL-10 levels fell from, median (interquartile range), 4.3 pg/ml (1.61-

145.4) pg/ml to 1.7 pg/ml (0-20.1) pg/ml in the HT group, and from, median (interquartile range), 25.6 pg/ml (1.7-900) pg/ml to 2.3 pg/ml (1.26-860) pg/ml in the NT group.

Significantly lower IL-4 levels were detected in the HT group compared to normothermia at each time point without any impact of postnatal age on this cytokine. (p= 0.04).

VEGF levels increased significantly over time in both groups. No treatment or time effect was observed in the levels of IL-8, INF-γ, TNF-α and EGF.

Cortisol levels gradually decreased in both groups during the study period. In normothermic neonates, a significant decrease was present at 12 hours of age, while in hypothermic ones only at 24 hours of age compared to cortisol values at 6 hours of age (**Figure 15.**). At 24 hours of age, almost all neonates 7/8 (87.5%) infants in the normothermic, and 9/10 (90%) of the hypothermia group had cortisol levels lower than 414 nmol/L (=15 μg/dL). However, there was no significant difference in cortisol levels between treatment groups at any time points.

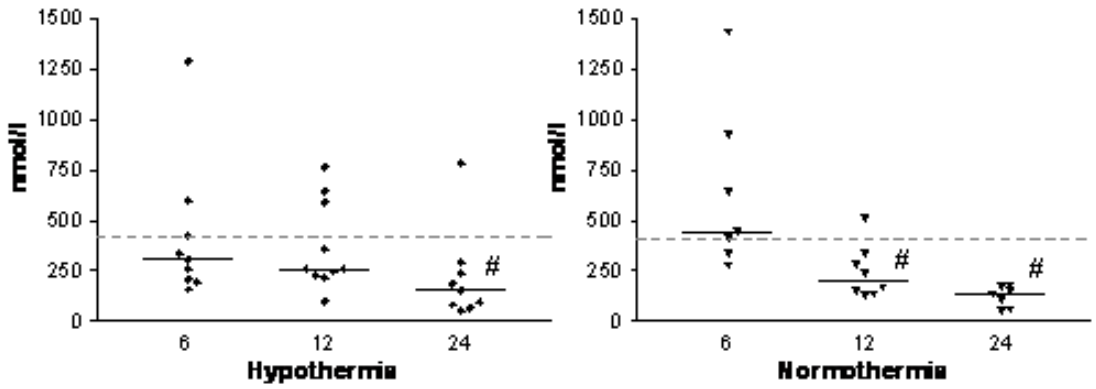

Figure 15. Changes in serum cortizol levels in hypothermic and normothermic infants. # significantly different from values measured at 6 hours of age (p<0.05).

At 6 hours of age, a significant positive correlation was observed between cortisol and IL-10 (R=0.68, p=0.01), and an inverse correlation between cortisol and VEGF (R=-0.55, p = 0.03). At 12 hours of age, an inverse correlation was observed between cortisol and IL-4 (R=-0.52, p = 0.03), and a tendency between cortisol and IL-10 was observed

(R=0.45, p=0.06). At 24 hours of age, positive correlation was observed between cortisol and IL-10 levels (R=0.56, p=0.03).

4.5. Effect of hypothermia on serum morphine concentrations

The clinical characteristics of the infants in the hypothermia (n=10) and normothermia (n=6) groups were similar. The numbers of days ventilated were (mean [SD]) 6 (1.6) in the hypothermia and 4 (2.0) in normothermia group, and the days to oral feeding were 8 (1.3) and 8 (3.5) in the hypothermia and normothermia groups. Nine infants in the hypothermia group and 4 infants in the normothermia group received cardiovascular support with fluid bolus and/or inotropes. The duration of receiving cardiovascular support was 77.4 hours (9.6 hours) in the hypothermia group and 41.8 hours (15.9 hours) in normothermia group (p = 0.09). No infant developed a cardiac arrhythmia or severe hypotension (blood pressure: <30 mm Hg). The median (range) encephalopathy score at age 4 days was 8.5 (3.0–12.0) in the hypothermia group and 5.5 (0.0–15.0) in the normothermia group (p = 0.26). The laboratory parameters including liver function were similar for the 2 groups.

Similar cumulative morphine doses were administered in the hypothermia and normothermia groups (median: 0.58 mg/kg per h; range: 0.31–1.87 mg/kg per h vs median: 0.60 mg/kg per h; range: 0.40–1.08 mg/kg per h; p > 0.1); the median (range) infusion rate was 10 μg/kg per h (4–30 μg/kg per h) in the hypothermia group and 10 μg/kg per h (5–20 μg/kg per h) in the normothermia group (p > 0.1). Infants in the hypothermia group received more morphine at 72 hours than the normothermia group, but the difference was not significant (**Table 7.**). The doses of phenobarbitone and midazolam were also similar during the intervention period. Serum morphine concentrations were not available for 1 infant (hypothermia group) at 12 hours, for 1 infant (hypothermia group) at 48 hours, and for 3 infants (hypothermia group) at 72 hours. In 1 infant (hypothermia group), morphine treatment was only started at 24 hours, and in another infant (normothermia group) treatment was stopped at 48 hours. Therefore, 70 samples were used for the analysis of serum morphine concentrations.

Serum morphine concentrations in the infants treated with hypothermia were higher than in the normothermia group. The morphine concentration at 24 to 72 hours after birth were (median [range]) 292 ng/mL (137–767 ng/mL) in the HT group and 206 ng/mL (88–327 ng/mL) in the NT group (p = 0.014) (**Table 7.**), although there was no difference in the morphine infusion rates (p = 0.56) or cumulative morphine doses between the groups (p = 0.083).

Table 7. Serum morphine concentrations in asphyxiated neonates treated with hypothermia or on normothermia with standard intensive care in the morphine substudy group. All of the infants received a single loading dose of 50 to 150 μg/kg of body weight before 6 hours of age. The morphine infusion rate is the average infusion rate between successive time points. *: The morphine concentrations at 72 hours were normally distributed, and the 2 sided *t* test indicated a significant difference between the hypothermic and normothermic groups (p=0.02).

Postnatal age at blood sampling (hour)	**HYPOTHERMIA N=10**		**NORMOTHERMIA N=6**	
	Morphine infusion rate ($\mu g.kg^{-1}.h^{-1}$)	**Serum morphine concentration ($ng.ml^{-1}$)**	**Morphine infusion rate ($\mu g.kg^{-1}.h^{-1}$)**	**Serum morphine concentration ($ng.ml^{-1}$)**
6	Not applicable	98 [12-185]	Not applicable	65 [44-129]
12	6.85 [0-30]	101 [82-303]	8.33 [1-15]	135 [79-233]
24	7.29 [3-20]	204 [63-562]	8.33 [1-15]	175 [102-300]
48	9.42 [3-20]	228 [172-768]	10 [5.5-20]	216 [160-327]
72	10 [5-30]	373 [149-506]	6.25 [4-10]	222 [89-309]*

The AUC for serum morphine concentrations over the entire observation period was (mean [SD]) 18 608 ng/h per mL (8384 ng/h per mL) in the HT group and 12 135 ng/h per mL (3481 ng/h per mL) in the NT group (p = 0.051). Serum morphine concentrations reached a steady state after 24 hours in the normothermia infants, but they continued to increase in the

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hypothermia group. At the 72nd postnatal hour, serum morphine concentrations for the 7 infants in the HT group and 6 infants in the NT group were (mean [SD]) 373 ng/mL (125 ng/mL) vs 222 ng/mL (73 ng/mL; p = 0.02; **Figure 16.**).

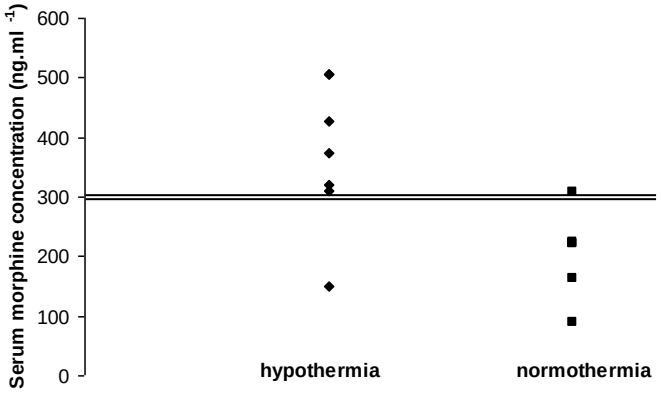

Figure 16. Serum morphine concentrations at 72 hours in asphyxiated neonates treated with hypothermia or on normothermia. Line shows potentially toxic morphine serum levels >300 ng/mL.

Serum morphine concentrations >300 ng/mL occurred in 13 of 42 samples from the hypothermia group and 2 of 28 samples from infants on normothermia (p = 0.025) and in 10 of 25 samples obtained when the morphine infusion rate was ≥10 μg/kg per h compared with 4 of 45 samples at <10 μg/kg per h (p ≤ 0.01). Multiple regression analysis indicated that the morphine infusion rate and treatment with hypothermia (assessed as a continuous variable) strongly influenced serum morphine concentrations with little evidence of collinearity (adjusted r2 = 0.527; infusion rate: r = 0.663; p < 0.0001; hypothermia r = 0.441; p = 0.004; variance inflation factor: 1.015; **Figure 17.**).

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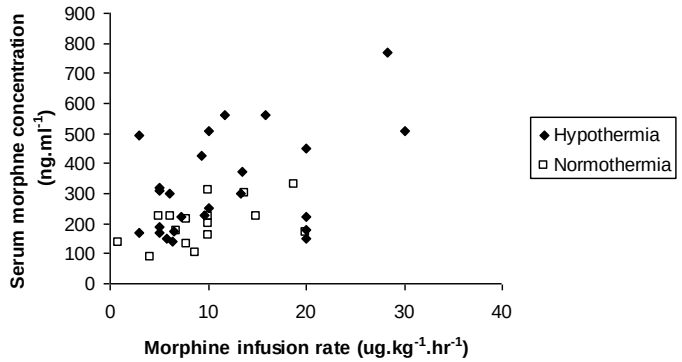

Figure 17. Relation between serum morphine concentrations and infusion rates in asphyxiated neonates treated with hypothermia or on normothermia.

Similar results were obtained when the analysis was repeated with the cumulative dose replacing the infusion rate (cumulative dose $r = 0.646$; $p < 0.0001$; hypothermia $r = 0.264$; $p = 0.017$). Median (range) morphine clearance estimated from the AUC for infants with measurements available at each time point was 0.69 mL/min per kg (0.58–1.21 mL/5–1.33 mL/min per kg) in infants on normothermia ($n = 5$; $p = 0.21$). Steady-state morphine clearance (estimated at 48 hours) was 0.89 mL/min per kg (0.34–1.99 mL/min per kg) in the normothermia group but could not be calculated in the hypothermia group because a steady state was not reached.

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4.6 Follow up and outcome in our study group

Follow up and outcome data are presented in details in **Table 8.**

Cranial ultrasound scan during the 72 hours investigation period showed abnormality (cerebral oedema and/or echogenicity in basal ganglia) in 2/13 neonates in the HT and 4/11 neonates in the NT group. Doppler studies were not done in these cases.

aEEG scores increased (suggesting significant improvement in background activity) in both groups over time (NT group, $p=0.004$; HT group, $p<0.001$). Full recovery on aEEG monitoring to normal background activity was seen in 4/12 in the HT and 1/11 infants in the NT group within 24 hours, and 9/12 versus 4/9 infants during the 72 hours cooling period ($p=0.056$).

aEEG scores were significantly higher in infants with normal outcome at each time point (6h $p=0.011$, 12h $p=0.007$, 24h $p=0.004$, 48h 0.001, 72h 0.046). Total aEEG score was also significantly higher in infants with favorable outcome (16 [8-19] vs. 6.5 [2- 9] $p<0.001$). The median [range] TOBY encephalopathy score at age 4 days was 8.0 [2.0–12.0] in the hypothermia group and 7.0 [0.0–11.0] in the normothermia group.

Brain MRI was performed in 12 of the 13 infants in the HT and eight of the 11 in the NT group. MRI showed hypoxic-ischemic changes (cortical or basal ganglia lesions, or white matter changes) in one infant in the HT and five infants in the NT group ($p=0.018$).

During the first 72 hours of life 1/13 infant (8%) died in the cooled group, and 2/11(18%) in the normothermic group.

Follow up rate at 18-22 months of age was achieved as 100%. At 18-22 months of age 12/13 infants survived in the HT and 8/11 in the NT group. Using Bayley neurodevelopmental assessment in the survivors 1/12 infants in the HT and 1/8 infants in the NT group had severe neurodevelopmental delay. However even in this small population the rate of survivors without neurodevelopmental disability was higher: 10/13 (77%) in the HT and 5/11 (45%) in the NT group ($p=0.08$).

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Table 8. Follow up and outcome data of 24 asphyxiated newborns treated with systemic hypothermia or on normothermia enrolled to the TOBY trial. Values are median [range], and number of patients.

	HYPOTHERMIA N=13	**NORMOTHERMIA N=11**	
CrUSS abnormality within 72 hours (n/N)	2/13 (16 %)	4/11 (36 %)	ns
Abnormal signs on brain MRI (n/N)	1/12 (8 %)	5/8 (62 %)	*0.018
aEEG recovery within 24 hrs (n/N)	4/12 (33 %)	1/11 (9 %)	ns
aEEG recovery within 72 hrs (n/N)	9/12 (75 %)	4/9 (44 %)	ns
Neurology score on day 4	8.0 [2-12]	7.0[0-11]	ns
Need for ventilation (days)	5.5 [4-10]	4.5 [0-9]	ns
Enteral feeding built up (days)	7.5 [6-11]	7.5 [6-14]	ns
Age at follow up (months)	20.5 [18-22]	20.5[18-23]	ns
MDI >85 70-84 < 70	11/12 (92 %) 0/12 (0 %) 1/12 (8 %)	5/8 (62 %) 2/8 (25 %) 1/8 (12 %)	ns
PDI >85 70-84 < 70	10/12 (83 %) 0/12 (0 %) 2/12 (16 %)	5/8 (62 %) 1/8 (12 %) 2/8 (25 %)	ns
Mortality (n/N)	1/13 (8 %)	3/11 (27 %)	ns
Severe disability in survivors (n/N)	1/12 (8 %)	1/8 (12 %)	ns
Severe disability or death (n/N)	2/13 (15 %)	4/11 (36 %)	ns
No severe disability (n/N)	10/13 (77 %)	5/11 (45%)	0.08

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5. Discussion

5.1. Hypothermia decreases the acute cell necrosis caused by hypoxic ischemic insult and may attenuate organ dysfunction in neonatal asphyxia

In this study we tested the hypothesis that hypothermia—that is proven to decrease brain damage following asphyxia—has an additional beneficial effect on the hypoxic damage of other organs than brain. It is well known that in addition to brain injury neonatal asphyxia has a profound effect on the function of major organ systems. Pulmonary, cardiovascular, hepatic, renal and gastrointestinal dysfunction may evolve due to hypoxic-ischemic damage as a result of the temporary lack of oxygen supply. The hypothesized neuroprotective effect of HT is likely due to the reduction of the impaired cerebral cellular energy metabolism. The complex mechanism by which HT works includes the protraction of enzymatic reactions leading to tissue destruction, suppression of free radicals production, protection of lipoprotein membranes against lipid peroxidation, reduction of oxygen demand in low flow regions and reduction of intracellular acidosis. Theoretically, therapeutic HT may be protective against the hypoxic-ischemic injury of all tissues and organs via these mechanisms. At the clinical introduction of hypothermia there were concerns about its possible side effects. HT may increase cardiovascular instability, and thereby the risk of organ hypoperfusion, which, in turn, may aggravate dysfunction. Previous studies, however, documented the safety of therapeutic HT in the treatment of neonatal HIE and dissolved the possibility that HT impairs the function of internal organs. In earlier trials, however, markers of cell necrosis and tissue dysfunction were not assessed systemically during HT; therefore these data were not convenient for kinetic analysis. No study was designed to evaluate the putative beneficial effect of HT on organ damage caused by neonatal asphyxia. In the efficacy trial using whole-body HT Shankaran et al. [30] found that the incidence of adverse events (hypotension treated with vasopressors, occurrence of oliguria, anuria and hepatic dysfunction) was similar in HT and NT neonates. Mortality rate, prevalence of hepatic involvement and renal dysfunction suggested, however, that the enrolled population mainly consisted of neonates who were subjected to a substantially milder asphyxia than those enrolled to other studies. As a result, the prevalence of organ dysfunction and MODS was low in this study. However, data of the Cool Cap study [27]

suggest that in addition to its beneficial effects on brain injury, HT may attenuate the concomitant hypoxic liver damage. In this study selective head cooling combined with mild systemic HT was applied in a population subjected to more severe asphyxia. Hypoxic hepatitis was less prevalent in HT than in NT neonates ($p = 0.02$). The prevalence of MODS, however, was comparable in both groups. In our study we enrolled neonates subjected to moderate or severe asphyxia and treated them with prolonged systemic HT after randomisation and assessed laboratory parameters in a prospective and designed manner. There were no significant differences in the clinical parameters (Apgar score, return of spontaneous breathing, first pH, BE, lactate and Sarnat score) at study entry. The laboratory parameters at 6 h of life (hepatic enzymes, creatinine, lactate dehydrogenase and creatine kinase) also were similar. These facts confirm that the severity of the hypoxic insult was similar in the two groups. High uric acid levels at 6 h of life may reflect energy failure of the cells and anticipate the increase of other monitored parameters of cell necrosis. The observed difference of serum uric acid levels at 6 h of life is probably due to the result of HT being introduced before the first sampling than any theoretical difference of severity between the groups. This hypothesis is further supported by the shifted peak values of LDH and uric acid to an earlier time point in the HT group compared to NT. We demonstrated that the values of serum parameters characteristic for cell necrosis and tissue damage (such as ASAT, ALAT, LDH and uric acid) and renal dysfunction (i.e. creatinine) are lower in asphyxiated neonates on HT than in those on NT.

However, due to low patient number we do not aim to evaluate the clinical significance of these results. Specific trials are required to address this issue. Our opinion is that due to rapid postnatal fluctuations of the laboratory parameters, the appropriate approach to describe cell necrosis caused by hypoxia is to repeat the assessments at predefined postnatal time points. Although the number of patients seems to be relatively low in our study compared to earlier trials, as we collected data in this manner and compared AUC values, we think that this potential fault is outweighed.

In summary we concluded that systemic moderate hypothermia decreases the acute cell necrosis caused by hypoxic ischemic insult and may attenuate organ dysfunction in neonatal asphyxia.

Since our paper, Sarkar et al. [64] compared multiorgan dysfunction in infants receiving selective head cooling with mild systemic hypothermia or moredate systemic hypothermia. They found that there was no significant difference between the two groups in laboratory parameters, coagulopathy, vasopressor treatment, diuresis. They concluded that the two cooling methods have same effect on multiorgan dysfunction, therefore MODS should not be a consideration in selecting the type of therapeutic hypothermia.

5.2. Effect of hypothermia on S100B and NSE levels, and their relationship with the neurological outcome

Similar to previous studies, serum S100B and NSE levels were greatly elevated following perinatal asphyxia, but this is the first study to examine the influence of therapeutic hypothermia on these biochemical markers of neurological injury in neonates. We found that infants treated with moderate hypothermia had lower serum S100B levels compared with normothermic infants and serum NSE did not show clinically significant differences compared with the cooled infants. Serum S100B and NSE levels were significantly higher in the infants who died or had a severely abnormal neurological outcome but the association was stronger for serum S100B levels. These data suggest that serum S100B levels may be a useful biomarker of disease and treatment effect in studies of neuroprotective therapies following perinatal asphyxia.

It is well established in the literature, that S100B and NSE levels are highly elevated in asphyxiated neonates, and correlate with severity. Nagdyman et al. [37] and Qian [38] et al found that asphyxiated term infants had elevated S100B protein levels in their umbilical vein bloods compared to healthy infants, and in the Qian study there was a significant difference in S100B levels between moderate or severe HIE and mild HIE groups. Measuring these protein levels from cord blood may be too early to detect acute hypoxic events, but could help to differentiate acute from antenatal or chronic intrauterine hypoxic injury.

S100B may also be detected in urine following asphyxia. Gazzolo et al. found a significant correlation between urine S100B concentrations from the first urination and the occurrence of neonatal death in normothermic infants. According to their results, a cut off ≥

1μg/l has a sensitivity and specificity of 100% for predicting neonatal death [41]. Collecting urine sample especially in infants with severe HIE can be difficult as they are often anuric or oliguric especially during the first 24 hours after birth. Measurement from serum is easier, and can be carried out at exact time points.

The effect of therapeutic hypothermia on serum S100B and NSE levels has been reported in just two studies in adults. Tiainen et al found that NSE but not S100B levels were lower over the first 48 hours in hypothermic patients after cardiac arrest compared to normothermic ones [65], whilst Derwall et al [66] found in a mixed population of adult patients with out-of-hospital cardiac arrest that mild systemic hypothermia maintained for 24 hours had no influence on S100B serum levels. S100B levels on baseline and 24 and 72 hours later were significantly lower in patients with good neurological outcome compare to those with adverse outcome. Our findings are consistent with these reports.

Hypothermia suppresses metabolism and may suppress protease activity, which might prolong the half life of proteins such as S100B and NSE. Therefore the lower levels of serum S100B levels in the cooled infants compared with the non cooled infants is unlikely to be due to suppression of protease activity by hypothermia and most likely to preserved neuronal and blood brain barrier integrity.

However some studies found higher S100B and NSE levels during ECMO or CPB procedures [46] performed under mild hypothermia, but in these cases it is likely to be related to the procedure and hemodynamic changes, and not to hypothermia treatment on its own. As our neonates did not undergo invasive haemodynamic procedures, we felt that those circumstances are different from our patient group, so have not explored the importance of these results further.

Indeed Kaandorp et al [1] used S100B levels as the primary outcome measure in their ALLO-Trial. They found that the treatment group had lower S100B levels in the cord blood compared to the placebo group and this was considered as the evidence for effectiveness of allopurinol as a neuroprotective agent. However, clinical outcome (neonatal mortality, long-term neurological outcome) was not reported.

Together with the results of other studies, our findings of a strong relationship between serum S100B levels and outcome suggest that serum S100B can be a useful

prognostic marker in infants following perinatal asphyxia. Additionally our observation of lower S100B levels in the cooled group compared with the non cooled group suggest that it may be useful biomarker of treatment effect that may be used in studies of additional neuroprotective therapies. Further larger studies are required to better define these potential roles of S100B following perinatal asphyxia.

5.3. Hypothermia may suppress the early increase of serum IL-6 levels

Our data suggest that therapeutic hypothermia may influence serum cytokine and cortisol levels during the first 72 hours after perinatal asphyxia. Neonatal transition involves a complex systemic inflammatory reaction, but few studies have analyzed the concentration of a range of cytokines at more than one time point during the early perinatal period [67] [68] and data regarding cytokine levels in hypoxic-ischemic encephalopathy are scarce. Previous studies have confirmed that hypoxic-ischemic insult is associated with a marked elevation of IL-6, IL-8 and IL-10 either in plasma [52] [53] [54] or brain [69] [70] [71] that may be correlated with the severity of neurological injury [53]. Xanthou et al measured IL-6, IL-1β and TNF-α levels at 1, 3 and 5 days in asphyxiated and septic newborns, including preterm infants [53]. They found that serum IL-6 levels were higher in both groups compared to healthy controls at 24 hours but were similar at 3 days.

Whilst some *in vitro* studies suggest that mild hypothermia may activate inflammation by increasing IL-1-β, IL-6, IL-12, and TNF-α levels [72], and according to other clinical observations, mild hypothermia increased the production of proinflammatory cytokines in septic patients [73], the majority of experimental studies report a reduction in inflammation with hypothermia [74] [75] [76] [77] [78].

We hypothesized that prolonged moderate hypothermia would influence the inflammatory response following hypoxic-ischemic insult, and this effect may contribute to the beneficial effects of hypothermia on neurological outcome. Akisu et al. reported lower platelet derived growth factor (PDGF) levels in cerebrospinal fluid of asphyxiated neonates treated with hypothermia compared to normothermic neonates [79].

IL-6 plays a key role in stimulating or inhibiting steps in the cytokine cascade; therefore we were particularly interested in the response of this cytokine to treatment with

hypothermia. Our observation that serum IL-6 levels were significantly lower in the hypothermia group at 6 hours of age suggests that hypothermia may immediately prevent or delay the early rise of IL-6 following asphyxia. Moreover, the separate analysis of hypothermia group revealed a significant negative correlation between IL-6 levels at 6 hours of age and the duration of hypothermia, suggesting a "dose dependent" reducing effect of hypothermia on early rise of IL -6 serum levels. These observations are in line with those obtained in animal studies that revealed an increase of neuroprotective effect of hypothermia when it is induced immediately after birth [80] [81]. Our data provide further evidence that one mechanism by which hypothermia exerts neuroprotective effect is the suppression of the hypoxia induced inflammatory response - particularly reducing the early rise of IL-6 levels.

We also found that the anti-inflammatory IL-10 levels fell from 6 to 24 hours after birth but did not observe a statistically significant difference in IL-10 levels between infants treated with hypothermia and normothermia. However, experimental data about IL-10 or on the impact of hypothermia on IL-10 levels are contradictory. Earlier studies showed higher levels of serum IL-10 in asphyxiated neonates compared to normal neonates [51]. Similar increase was observed in serum, and CSF IL-10 levels in adults with stoke [82], but in another study higher serum IL-10 was strongly associated and independently correlated with severe neurological impairment at 48 hours after ischemic stroke [83]. Administration of IL-10 in experimental models of focal brain injury reduced infarct volume, suggesting that IL-10 may have neuroprotective effect [84].

Matsui et al. showed that IL-10 production is reduced by hypothermia but augmented by hyperthermia in cultured neonatal rat microglia during hypothermia [77]. Although studies mainly suggested that IL-10 have a neuroprotective effect after brain injury, in one study the administration of exogenous IL-10 prevented the beneficial effect of hypothermia following traumatic injury in rats [85]. A recent study showed that hypothermia had no influence on selected cytokine levels including IL-10 in piglets, but the IL-10 was identified as prognostic marker for survival with decreasing mRNA levels in survivors [86].

Even less data are available about changes in IL-4 levels after perinatal asphxia. IL-4 similarly to IL-10 is considered as an antiinflammatory molecule, which may provide a negative feedback mechanism to limit the production of proinflammatory cytokines in brain injury. IL-4 levels are significantly higher in neonates compared to adults, irrespective of the presence or absence of asphyxia [51]. A recent study showed no difference after cardiac arrest in piglets between hypothermia and normothermia group [86]. However, in adults with stroke a marked increase in IL-4 levels could be observed compared to healthy controls [87], and IL-4 polymorphism was associated with increased incidence of stroke [88].

There is an important interaction between inflammation and the hypothalamic/pituitary/adrenal system [89] [90]. As cortisol has very important role controlling several steps in the inflammatory cascade, both hyper- and hypocortisolemia may have profound effects on inflammation in neonates with hypoxic-ischemic encephalopathy. Therefore, while studying cytokine kinetics and its interaction with hypothermia, we extended our study to the evaluation of the impact of serum cortisol on cytokine levels as well. Although maternal cortisol rhythm can be detected in the umbilical vein in the fetus [90] earlier studies showed that in healthy neonates marked diurnal cortisol rhythm is not present during the first postnatal weeks [90]. Because of this, random cortisol level in neonates may be adequate to assess the adrenal function during the first week of life. According to our knowledge, no earlier study assessed cortisol levels in asphyxiated neonates treated with hypothermia. Procianoy et al. found elevated cortisol levels in cord blood samples from asphyxiated neonates compared to healthy neonates [91]. This change was assumed to be related to the stress caused by birth asphyxia. However at 12-18 hours of age the cortisol levels were similar in the two groups. Unfortunately cord blood samples were not available for us, and our measurements were performed in later time points. We observed a gradual decrease in serum cortisol levels in both groups during the first 24 postnatal hours although cortisol levels seems to decrease less dramatically in hypothermia group. The sharp decline of cortisol often resulted in serum cortisol levels below 414 nmol/l (= 15μg/dL) at 24 hours of age, which is currently considered as evidence for relative adrenal insufficiency with increased risk for mortality and morbidity [55], [56], [57]. Kamath et al showed that term neonates with congenital diaphragmatic hernia developed

low cortisol levels, and these newborns required more intensive care support for longer time period compared those with normal cortisol levels [57]. Similar observations were reported in term and preterm neonates with critical illness or undergoing surgery [55], [56], [57], [92]. Multiorgan failure and hypotension requiring inotrope support often seen in asphyxiated neonates which can be related partly to relative adrenal insufficiency. Our neonates frequently required volume and inotropic support; however our study was not designed to explore the association of cardiovascular instability and low cortisol levels in asphyxiated neonates.

The lack of major association between cortisol and IL-6 or IL-4 levels suggests that the observed changes in cytokine levels during hypothermia are possibly independent of the adrenal function. It should be noted that based on our observations it cannot be ruled out that in the case of prolonged hypocortisolemia other significant changes and interactions in the inflammatory processes can occur in asphyxiated infants after the first 72 hours.

The main limitation of this study is the small number of neonates, which increases the risk that the observed differences may be by chance. Further studies would be required to confirm these findings, but this now may be difficult since moderate hypothermia is an accepted neuroprotective intervention for neonatal hypoxic-ischemic encephalopathy.

Another factor that might influence the inflammatory response in our study is treatment with morphine. During the first 72 hours we routinely sedated all infants with a morphine infusion. As reported in our other study, morphine can accumulate following asphyxia, particularly in neonates treated with hypothermia and more often after the 24 hours of life. An antiinflammatory effect has been attributed to morphine administration [93]; however, a recent experiment reported opposite results [94]. Our data are insufficient to explore the contribution of morphine administration on cytokine levels.

Our observational study suggests that therapeutic moderate hypothermia rapidly suppresses and modifies the immediate cytokine response to asphyxia. Our main finding, the significant correlation between cytokine levels and duration of hypothermia, suggests that the earlier hypothermia has been introduced, the more pronounced its beneficial immune modulator effect.

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5.4. Elevated morphine concentrations in asphyxiated neonates treated with prolonged moderate systemic hypothermia

We found that neonates with hypoxic ischemic encephalopathy receiving commonly used rates of morphine infusion for 72 hours developed high serum morphine concentrations. Infants treated with moderate systemic hypothermia attained higher and potentially toxic concentrations of morphine compared with normothermic infants, despite receiving similar cumulative morphine doses. Some studies have examined the pharmacokinetics and pharmacodynamics of morphine in neonates but none after HIE [95], [96], [97]. Morphine clearance correlates moderately with gestational age and birth weight, but there is considerable variability between infants, and pharmacodynamics is highly variable, so that dosage is often based on clinical response rather than a fixed regime. In our study we applied a commonly used regimen for morphine administration and altered the dose according to clinical assessment of distress. Although the median morphine infusion rate was 10 μg/kg/h, some infants received ≥30 μg/kg per h on some occasions. It is possible that difficulty in assessing the clinical need for sedation or analgesia in the presence of HIE and treatment with hypothermia resulted in some infants receiving excessive treatment with morphine. Mean morphine concentration required to produce adequate analgesia in healthy term infants is said to be ~125 ng/mL [97]. In our study, the median morphine concentrations in both groups of infants were >200 ng/mL, which is much higher than was observed in nonasphyxiated infants receiving greater morphine infusion rates [95], [98], [99]. Morphine concentrations >300 ng/mL are generally regarded as likely to be toxic and may be associated with adverse cardiorespiratory events, but even lower doses may alter gut motility [95], [97]. We found that that morphine concentration exceeded 300 ng/ mL in several infants who were treated with hypothermia and morphine infusion rates were >10 μg/kg per h. This finding suggests that asphyxiated infants treated with continuous morphine infusion during systemic hypothermia are at the risk of morphine toxicity, if the morphine dose is titrated according to clinical state. We did not observe major complications, such as severe hypotension, that could be ascribed to morphine toxicity [100]. However, the hypothermia group had longer ventilatory requirements, longer need for cardiovascular support, and a worse encephalopathy score at 4 days of age,

although none of these differences were statistically significant. It is conceivable that these observations were related to the higher morphine concentrations in the infants with hypothermia, but we did not explore these findings additional because of the small group sizes. Morphine pharmacokinetics has not been studied previously in infants during hypothermia. In this study we were only able to carry out a limited pharmacokinetic analysis of morphine because of logistic and clinical difficulties: most infants had poor urine output for several hours. The morphine infusion rate was altered often according to the clinical state, and it was inappropriate to obtain more frequent blood samples for pharmacokinetic studies, considering the infants' clinical condition. Because infants in the hypothermia group did not reach steady-state morphine concentrations, we estimated morphine clearance in 2 ways: by using the AUC concentrations in both groups and also from the steady-state morphine concentrations in the normothermic group. The clearance estimated by each method was very similar. The study infants had lower estimated morphine clearance than is reported for non asphyxiated infants [[95]], [[98]]. Moderate or severe asphyxia is often associated with multiorgan dysfunction [[34]], including hepatic impairment, which is likely to influence metabolism and clearance of morphine. As expected, we observed elevation of hepatic enzymes and alterations in other laboratory parameters that reflect acute cellular necrosis and tissue dysfunction, and this probably accounts for low clearance values observed in this study. However, we found that these parameters were less severely abnormal in the hypothermia group, which we have investigated in our MODS substudy. This suggested that hypothermia had an independent effect on the serum morphine concentrations, and this was confirmed on multiple regression analysis. The most likely explanation for the higher morphine concentrations in infants treated with hypothermia compared with those on normothermia is the effect of temperature on drug metabolism. The activity of hepatic drug metabolizing enzymes is strongly impaired at lower temperatures, although no data are available about the kinetic properties of the enzyme (UDP-glucuronosyltransferase) responsible for morphine glucuronidation [[101]]. Although there was no significant difference in estimated morphine clearance between the 2 groups, hypothermia may have reduced hepatic metabolism throughout the 72-hour observation period, whereas hepatic metabolism recovered over the

observation period in the normothermia group. This could explain the continued rise in serum morphine concentrations during the observation period in the hypothermia group, whereas normothermia infants reached steady-state concentrations at 48 to 72 hours. A limitation of our study is that we were unable to measure morphine metabolites. The measurement of the 2 metabolites of morphine, morphine-3-glucoronide (M3G) and morphine-6- glucoronide, requires high-performance liquid chromatography, which was not available to us. Morphine-6-glucoronid, a potent analgesic, was not detected in the plasma of any neonate in 1 study, and only low concentrations were detected in another study, but M3G levels can be detected in neonates [95], [97]. The morphine assay used in this study has some cross-reaction with M3G, which might have inflated our serum morphine measurements but would not explain our observation of increased serum morphine concentrations with cooling. The assay is regularly used by hospital laboratories, and, therefore, our findings are relevant to clinical practice. The therapeutic efficacy of moderate systemic hypothermia was confirmed, and hypothermia now is standard therapy for HIE.

All published studies equivocally report that hypothermia is a safe technique without obvious adverse effects in neonates with HIE. However, our study and unpublished data by Thoresen et al, suggest that hypothermia may result in elevated serum concentrations of morphine and phenobarbitone with undetermined clinical significance. Although no clinical evidence of toxicity was observed in the published large randomized trials of hypothermia, adverse drug-related effects may be difficult to distinguish from those of the underlying severe illness and may be missed if hypothermia becomes standard treatment after HIE, when routine observations may be less rigorous than in the clinical trials.

Clinicians wishing to treat infants with HIE with morphine analgesia or sedation adjusted according to clinical state should be aware of the likelihood of elevated serum morphine concentrations. Units utilizing hypothermia treatment have widely different protocols for analgesia. Our study about morphine levels during hypothermia showed elevated and potentially toxic morphine concentration in asphyxiated neonates at routinely used morphine doses, and clinicians should consider monitoring drug concentrations in some cases. On the other side, minimal analgesia and sedation, therefore less need for

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mechanical ventilation in these neonates can be desirable for clinicians as less invasive method. Animal experiments showed that pain and stress during hypothermia can abolish the neuroprotective effect. Based on these results, cooling without or with not appropriate analgesia should also be avoided because of unknown consequences and possible worse neurological outcome.

Our results highlighted the need to investigate the effect of prolonged hypothermia and HIE on the pharmacokinetics and pharmacodynamics of commonly used drugs that are potentially toxic.

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6. Conclusion

Our studies reported original data about comparing hypothermic and normothermic groups of term asphyxiated neonates in some aspects like markers of multiorgan failure and neuronal injury, inflammatory markers, and morphine metabolism.

1. We concluded first that systemic moderate hypothermia decreases the acute cell necrosis caused by hypoxic ischemic insult and may attenuate organ dysfunction in neonatal asphyxia.

2. We found that infants treated with moderate hypothermia had lower serum S100B levels compared with normothermic infants and serum NSE did not show clinically significant differences compared with the cooled infants. Serum S100B and NSE levels were significantly higher in the infants who died or had a severely abnormal neurological outcome but the association was stronger for serum S100B levels.

3. Our other observation was that serum IL-6 levels were significantly lower in the hypothermia group at 6 hours of age suggesting that hypothermia may decrease immediately the early rise of IL-6 following asphyxia. Moreover a significant negative correlation between IL-6 levels at 6 hours of age and the duration of hypothermia, suggesting a "dose dependent" reducing effect of hypothermia on early rise of IL -6 serum levels.

4. Finally we published first that asphyxiated neonates treated with moderate systemic hypothermia receiving commonly used rates of morphine infusion for 72 hours developed higher and potentially toxic concentrations of morphine compared with normothermic infants, despite receiving similar cumulative morphine doses.

The main limitation of our studies is the very small patient number. However we included all neonates enrolled to the TOBY study during this period with available blood samples. Further studies would be required to confirm some of our findings, like the early changes seen in IL-6 levels, but this now may be difficult for ethical reason since moderate hypothermia is an accepted neuroprotective intervention for neonatal hypoxic-ischemic encephalopathy.

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7. Summary

Our aim was to perform several observational studies on our study group while participating in the international TOBY trial. Our contribution helped to reach the desired patient number in the TOBY trial to answer the question about the efficacy of moderate systemic hypothermia treatment in HIE, and for us provided a reliable background for our publications. Since then, as hypothermia is clarified as standard care in HIE. Our most important observations had some impact on clinical care and future research. We concluded that systemic moderate hypothermia decreases the acute cell necrosis caused by hypoxic ischemic insult and may attenuate organ dysfunction in neonatal asphyxia. This helped to reassure clinicians that severe multiorgan failure is not a contraindication for cooling, but actually may improve organ function. Secondly we found that infants treated with moderate hypothermia had lower serum S100B levels compared with normothermic infants. Serum S100B levels showed strong association with poor neurological outcome. These data suggest that serum S100B levels may be a useful biomarker of disease and treatment effect in studies of neuroprotective therapies following perinatal asphyxia. Finally we published that asphyxiated neonates treated with moderate hypothermia receiving commonly used rates of morphine infusion developed higher and potentially toxic concentrations of morphine compared with normothermic infants, despite receiving similar cumulative morphine doses. These observations resulted changes in the analgesic management of infants treated with hypothermia, being more cautious about morphine dosages. Also has led to more extensive investigations about pharmacokinetics of frequently used drugs during hypothermia in neonates.

At present, several potential neuroprotective agents are waiting for human clinical trials in the near future. As outcome measurements have improved, smaller clinical studies can be carried out to assess the effectiveness of therapies combined with hypothermia. Brain MRI is used as marker for brain injury, which can reduce the length of studies assessing new therapies. New techniques like spectroscopy, diffusion weighted and diffusion tension imaging can provide accurate information about prognosis. Serum biomarkers like S100B protein as we showed can also have future role in monitoring therapy effectiveness.

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Összefoglalás

Munkánk során fő célunk volt a nemzetközi TOBY vizsgálatban való részvétel mellett egyedi megfigyeléseket végezni az adott betegpopulációval kapcsolatban. A TOBY vizsgálatban való részvételünk hozzájárult a kívánt betegszám eléréséhez és a hipotermia hatásosságának bizonyításához, és hiteles hátteret biztosított a nemzetközi publikációinknak. Azóta, a hipotermia stardard terápiává vált a HIE kezelésében. Legfontosabb megfigyeléseink hatással voltak a klinikumra és kutatásra is. Megfigyeltük, hogy a mérsékelt teljestest hipotermia csökkenti a hipoxiás-ischemiás inzultus okozta akut sejtelhalást, és befolyásolja a sokszervi elégtelenség kialakulását. Ez megerősítésül szolgált a klinikusok számára, hogy a sokszervi elégtelenség nem kontraindikációja a hűtéses kezelésnek, sőt inkább a szervfunkciók javulását várhatjuk. Vizsgálataink során a hipotermiával kezelt újszülöttekben alacsonyabb szérum S100B szinteket mértünk, és a szérum S100B szintek szoros korrelációt mutattak a súlyos idegrendszeri károsodással. Ez arra utal, hogy az S100B fehérje alkalmas biomarker lehet az asphyxia okozta idegrendszeri károsodás megítélésében, és terápia hatásosság monitorizálásában. Végül elsőként közöltük, hogy a magasabb és potenciálisan toxikus morfinszintek alakulnak ki mérsékelt teljestest hipotermiával kezelt asphyxiás újszülöttekben rutinszerűen használt morfin infúzió sebességek mellett a normotermiás újszülöttekhez képest, megegyező kumulatív morfin dózisok ellenére. Ezen megfigyelésünk megváltoztatta a hűtött újszülöttek analgéziás kezelését, és fokozott óvatosságot eredményezett az alkalmazott morfin dózisok tekintetében. Emellett kiterjedt farmakokinetikai vizsgálatokhoz vezett az összes, a újszülöttek hipotermiás kezelése során gyakran alkalmazott gyógyszer esetében.

Jelenleg, számos új, potenciális neuroprotektív szer vár humán klinikai kipróbálásra. Mivel az asphyxiával kapcsolatos kimeneteli paraméterek változtak, a jövőben kisebb klinikai vizsgálatok is elegendőek lesznek a hatásosság megítélésére a hipotermiával kombinált terápiák esetében. Az MRI felhasználható az idegrendszeri károsodás markereként, ez pedig szignifikánsan lerövidíti a vizsgálatok időbeli hosszát az új terápiák esetében. Új szekvenciák, mint például a spektroszkópia, diffúziós technikák (DW, DTI, TBSS) megbízható adatokat szolgáltatnak a prognózisról. A szérum markereknek, például az S100B fehérjének - ahogy saját eredményeink is mutatták - a jövőben továbbra is fontos szerepe lehet a terápia hatásosság monitorizálásában.

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