Perinatal asphyxia:

predicting and improving outcome

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PERINATAL ASPHYXIA: PREDICTING AND IMPROVING OUTCOME

PERINATALE ASFYXIE: HET VOORSPELLEN EN OPTIMALISEREN VAN DE NEUROLOGISCHE ONTWIKKELING

(met een samenvatting in het Nederlands)

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Als je een schip wilt bouwen,
beveel anderen dan niet om hout te verzamelen,
het werk te verdelen en orders te geven.
In plaats daarvan, leer ze verlangen
naar de enorme, eindeloze zee.

Antoine de Saint-Exupé

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CHAPTER 1

General Introduction

GENERAL INTRODUCTION

Adverse events around birth can have major life-long consequences. The most important cause of morbidity and mortality in term born neonates is perinatal asphyxia (1,2). Perinatal asphyxia (PA) is defined as an oxygen deprivation around birth and can be caused by several perinatal events, such as placental abruption or umbilical cord prolapse (3). PA can lead to brain injury; hypoxic-ischemic encephalopathy (HIE) refers to early clinical symptoms of impaired neurological functions. PA can also result in hypoxic injury of other organ systems causing cardiac ischemia, hepatic failure or acute tubule necrosis (4).

The incidence of HIE varies between 1 to 2 per 1000 term born infants in high income countries and 4 to 26 per 1000 term born infants in middle and low income countries (2). According to the World Health Organization this results in an estimated 1.15 million neonates with newly developed HIE per year and a mortality of 287,000 infants with HIE per year worldwide (2). So, HIE is a major health care problem affecting many neonates and their parents each year.

Pathophysiology of HIE

Brain injury after acute PA is caused by neuronal and glial cell damage that develops in two stages in infants with HIE: acute injury and reperfusion injury (5,6).

The acute period of (fetal) hypoxia around birth may lead to immediate neuronal and glial cell injury by primary energy failure. This primary energy failure increases glutamate release activating the N-methyl D-aspartate (NMDA) receptors which leads to failure of the Na-K-ATPase pump. This results in calcium influx into neuronal cells with subsequent cell death. Next to this immediate cell death, (fetal) hypoxia also leads to lower pH levels in the cell and to the production of pro-radicals such as non-protein-bound iron. Furthermore the hypoxia-induced degradation of adenosine triphosphate (ATP) leads to high levels of adenosine and subsequently to formation of hypoxanthine (5,7,8). See Figure 1.

Upon birth reoxygenation and reperfusion of the brain, in combination with accumulation of the pro-radicals and hypoxanthine, results in a second peak of brain injury, the so-called reperfusion injury (5). The reaction between pro-radicals and now sufficiently available oxygen causes activation of different molecular pathways leading to free radical formation. The accumulated hypoxanthine in combination

with oxygen is converted by xanthine-oxidase into superoxide and uric acid. Superoxide itself is toxic for the brain, but is also responsible for the activation of other destructive pathways that lead to brain injury: superoxide interacts with nitric oxide (NO) leading to the formation of the toxic peroxynitrite and to conversion of non-protein bound iron into hydroxyl free radicals, which is the most toxic free radical in nature (9–12). This early production of free radicals (already starting within the first minutes up to the first hours of life) eventually leads to inflammation, apoptotic activity and inhibition of trophic factors. This impairs repair and neo-neurogenesis (5,13,14). (Figure 1)

The pattern of brain injury might differ depending on the timing and length of the hypoxic event. The majority of infants with HIE suffer from an acute sentinel event leading to injury of the most highly demanding metabolic areas in the brain, such as the deep grey matter including the basal ganglia and thalami (15). Injury of the deep grey matter is more likely to result in motor problems on the long-term (16). Infants with HIE who suffered from subacute-to-chronic asphyxia more often develop watershed injury of the white matter (15) because the borderline areas of the major supplying cerebral arteries (anterior-, middle- and posterior arteries) are especially vulnerable for changes in blood flow. The outcome of infants with only watershed type injury is often better than of infants with deep grey matter type injury. Watershed type injury is more likely to result in cognitive and behavioral problems than in motor problems (15,17). A mixture of both patterns is also possible. In the most severe cases of perinatal asphyxia this can result in near-total brain injury (15).

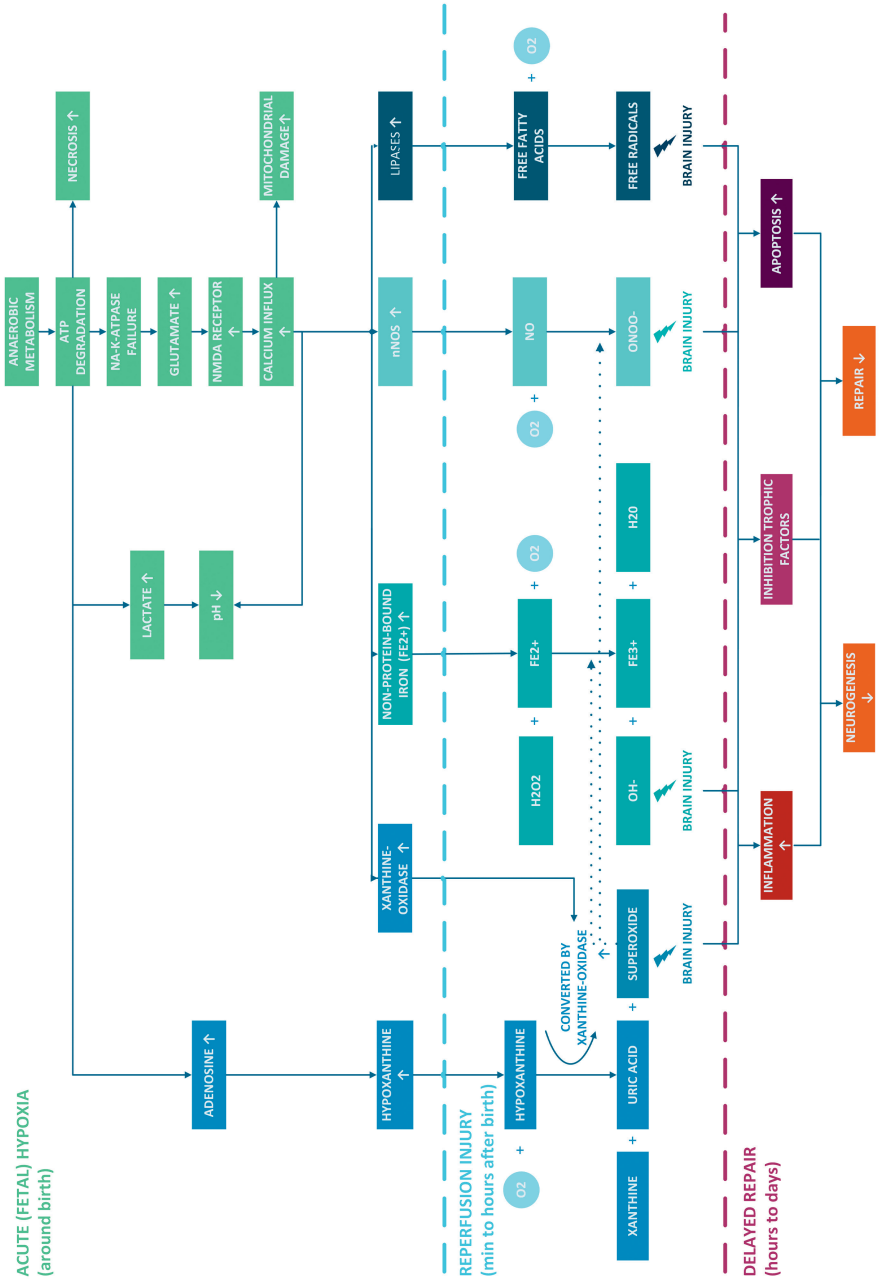

Figure 1: pathophysiological pathways during/after hypoxia-ischemia leading to brain injury.

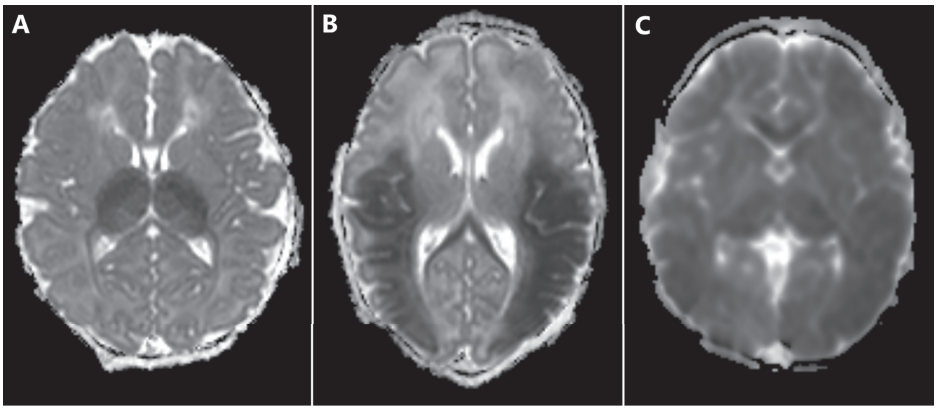

Figure 2: Patterns of brain injury in infants with HIE. A. shows an example of an apparent diffusion coefficient (ADC) map with severe diffusion restriction of the thalami (deep grey matter type injury), B. shows an example of an ADC map with severe restriction of the white matter (watershed type injury), C. shows an example of near total injury. Diffusion restriction results in a low signal intensity on the ADC map.

Neuroimaging

The different patterns of brain injury can be visualized using neuroimaging techniques. Neuroimaging in infants with HIE provides information about the extent and pattern of brain injury and as such is helpful to predict neurodevelopmental outcomes. Predicting neurodevelopmental outcome as early as possible is essential for clinical decision making and counselling of parents. Early biomarkers for neurodevelopmental outcome can also be used as outcome measures in randomized controlled trials (RCT). Shankaran *et al.* showed, in one of the large therapeutic hypothermia trials, that neonatal MRI can be used as a biomarker for six to seven year outcome (18). Neuroimaging is not the only biomarker available for the prediction of neurodevelopmental outcome. In clinical practice, a combination of neuroimaging, neurophysiology and clinical condition (i.e. neurological examination and multi-organ failure) is used to predict neurodevelopmental outcome. The combination of multiple techniques have shown to increase sensitivity and specificity to predict neurodevelopmental outcome. To illustrate, Leijser *et al.* showed that an abnormal amplitude-integrated electroencephalography (aEEG) background pattern (sensitivity 0.93, specificity 0.75) or severely abnormal Magnetic Resonance Imaging (MRI) (sensitivity 0.74, specificity 1.00) had both high predictive values for adverse outcome at two years of age in infants with HIE, but the combination of aEEG and MRI even further increased sensitivity and specificity (19). Although, this thesis

focuses mainly on neuroimaging, one should keep in mind that decisions about clinical care are always based on the triad of neuroimaging, neurophysiology and clinical measures.

The gold standard for neuroimaging in neonates with HIE is MRI. There are several MRI techniques that are known to be associated with neurodevelopmental outcome.

First of all, brain injury on conventional, T1- and T2-weighted imaging can be scored with visual scoring methods. Both the scoring systems of Barkovich *et al.* and Weeke *et al.* are associated with neurodevelopmental outcome and are easy to use in clinical practice (20,21).

Diffusion Weighted Imaging (DWI) is a more quantitative MRI modality and can provide additional information. DWI measures the diffusion of water molecules between the cells in the brain. In case of HIE, cytotoxic edema may lead to swelling of the neurons and thereby to diffusion restriction of the water molecules (high signal on DWI) (22–24). This diffusion restriction can be quantified on the apparent diffusion coefficient (ADC) map, in which the T2 shine through signal is excluded. Alderliesten *et al.* showed that ADC values below $1031*10^{-6}mm^2/s$ in the basal ganglia/thalami area have a sensitivity of 78% and specificity of 95% for an adverse outcome at two years of age (22). It is important to note that the reliability of ADC values is highly dependent on timing: seven to ten days after the hypoxic event there is a pseudo-normalization of the ADC values (23). However, if the MRI is performed too early after birth, the cytotoxic edema might not be fully developed and yet be visible. Therefore, the optimal timing for DWI is between day three and seven after birth.

Magnetic Resonance Spectroscopy (MRS) is another frequently used MRI sequence in infants with HIE. MRS enables us to measure the concentrations of different types of metabolites in the brain (25). Higher lactate peaks are known to be associated with brain injury, but also lower N-acetylaspartate (NAA) levels, which is a neuronal marker for healthy neurons, are associated with neuronal loss. Alderliesten *et al.* showed that the area under the curve of the lactate/NAA ratio for predicting two year outcome was 0.89 (25).

Arterial Spin Labelling (ASL) is an MRI technique showing the perfusion of the brain. In infants with HIE, hyperperfusion of the brain is associated with an

adverse neurodevelopmental outcome at two years of age (26). This technique is less commonly used in clinical practice for infants with HIE because of technical challenges, though it is predictive for outcome (26).

Besides conventional T1- and T2-weighted images, DWI including ADC values and MRS, other sequences are regularly used on clinical indication, such as susceptibility weighted imaging (SWI) to detect bleedings and Diffusion Tensor Imaging (DTI) that provides additional information about white matter integrity. Magnetic Resonance Venography (MRV) can be performed to exclude sinovenous thrombosis and Magnetic Resonance Angiography (MRA) to visualize the arteries (24).

There is a lot of literature about neuroimaging in infants with HIE, but the focus is mainly on supratentorial brain injury. Yet, based on histology it is known that the cerebellum is very vulnerable to hypoxic injury (27). Moreover, we know from preterm born infants that cerebellar injury increases the risk of cognitive and behavioral problems as autism spectrum disorders significantly (28). *Chapter two and three describe cerebellar injury in HIE. Chapter two provides insight in the histopathological pattern of cerebellar injury in HIE and chapter three in the correlation between cerebellar injury based on DWI and histopathology.*

Although MRI is the gold standard in neuroimaging, in some hospitals or countries MRI is not available, or if infants are clinically too unstable for transport, MRI might not be feasible (29). For those infants, cerebral ultrasound (CUS) might offer an alternative since it is an immediately available, bedside and cheaper neuroimaging modality. Additionally, CUS can be performed daily in infants with HIE which enables monitoring of evolving abnormalities. Until now, there is no standardized and validated method to assess brain injury with CUS in infants with HIE yet. *Therefore in chapter four, the development and validation of a new CUS scoring system is described.*

Therapeutic hypothermia

The only current therapy for infants with HIE to reduce brain injury is therapeutic hypothermia (1,30). Neonates are cooled to 33.5°C for 72 hours. This treatment should be started within six hours after birth, the window of therapeutic opportunity, for an optimal effect. The exact working mechanism has not yet been elucidated completely. Nevertheless, there is evidence that therapeutic hypothermia

reduces apoptosis and necrosis, leads to a lower metabolism in the brain and reduces the production and uptake of glutamate, NO and free radicals (31,32).

Multiple clinical trials have demonstrated that therapeutic hypothermia is neuroprotective in HIE by further reducing the incidence of death and neurodevelopmental disabilities at 18-24 months of age, e.g. Azzopardi *et al.* showed that cooling improved survival without neurodevelopmental problems from ~30% to ~45% (1,33–37). Jacobs *et al.* conducted a systematic Cochrane review including 11 RCT's investigating the neuroprotective effect of therapeutic hypothermia in 1505 infants with HIE with a gestational age of 35 weeks and more (31). Therapeutic hypothermia reduced the composite outcome of mortality and severe neurodevelopmental problems at 18 months of age with a relative risk of 0.75 and a number needed to treat of 7 (31). Mortality alone was also decreased in the hypothermia group with a number needed to treat of 11 and a relative risk of 0.75 (31). There was also less CP in the infants with HIE in the hypothermia group that survived with a relative risk of 0.66 and number needed to treat of 8 (31).

There are possible risks of therapeutic hypothermia, such as subcutaneous fat necrosis, bradycardia, hypotension, thrombocytopenia and possibly pulmonary hypertension (31,38,39). However, the benefits on neurocognitive outcome outweigh these risks (30,31).

Long-term neurodevelopmental prognosis

While 18 to 24 month outcome improved since the introduction of therapeutic hypothermia, less is known about the neurodevelopment of infants with HIE at school-age. Even though infants seem to be developing well at two years of age, children might still evolve problems at school-age (40). Therefore, long-term follow-up is essential in children with HIE.

Our research group reported earlier that before therapeutic hypothermia became standard of care, children with HIE without CP that survived also showed significantly more memory and behavioral problems at ten years of age than healthy controls (41,42). Even children with mild HIE had more memory problems at school-age compared to controls (41). So far, it was unclear what type of brain injury led to these memory problems. Gadian *et al.* showed that the hippocampus is an important brain structure for memory and cognition and is also very vulnerable to hypoxic-ischemia

(43). *Therefore, in chapter five we investigate the association between HIE, hippocampal volume at ten years of age and cognitive outcome in the above described cohort.*

We do not have this ten year outcome information of children with HIE treated with hypothermia. Follow-up until six to seven years of age of the TOBY trial, one of the large RCTs investigating the effect therapeutic hypothermia in HIE, showed that significantly more children in the hypothermia group (52%) compared to the control group (39%) survived with an IQ above 85 points (1). Nevertheless, health related quality of life was comparable between the groups (44). Childhood outcome of another trial revealed that 27% of the surviving children treated with hypothermia had an IQ below 70 and 33% in the control group (45). The frequency of CP declined from 29% in controls to 17% in children treated with hypothermia in this study (45).

Although neurodevelopmental outcome in childhood has improved with therapeutic hypothermia, as stated above, there is still a significant number of children with HIE that experience long-term neurodevelopmental problems. Information about which regions of the brain are still vulnerable for hypoxic-ischemic injury despite therapeutic hypothermia would be helpful, for clinicians to better predict outcome and for researchers to develop new neuroprotective strategies. However, childhood MRI studies exploring the association between brain injury and childhood outcomes are lacking. *Therefore, we conducted a prospective observational cohort study assessing cognition, memory, motor functioning and MRI at ten years of age in children with HIE following perinatal asphyxia that were treated with therapeutic hypothermia and those who were born before therapeutic hypothermia became standard of care. The results of this study are presented in chapter six.*

Add-on neuroprotective therapies

Since HIE still is a burden to patients and their family despite the current treatment with hypothermia, additional neuroprotective add-on therapies are essential to further reduce brain injury in neonates with HIE and to optimize their future perspectives.

HIE also comes with a financial burden to society. Based on the six to seven year outcome data of the TOBY trial, the healthcare costs for infants in the United Kingdom who were cooled and those who were not were compared. Healthcare costs were £2549 per child per six months for controls and £1543 per child per six

months for children in the hypothermia group (46). The health care costs in two cooled infants with severe disabilities raised until £20,000 to £40,000 per patient per 6 months (46). Healthcare costs are an additional argument that add-on neuroprotective therapies are necessary. In low and middle income countries therapeutic hypothermia is less feasible because of fewer resources (cooling with ice packs, fans or gel packs) and the neuroprotective effect is not entirely clear because of differences in clinical characteristics (47). This is currently further investigated in the HELIX trial (48). Here, pharmacologic neuroprotection might be a good alternative (49).

With pharmacological interventions in the destructive molecular pathways leading to brain injury (see also Figure 1) additional neuroprotection may be established. In preclinical research several pharmacological interventions have been tested, targeting oxidative, inflammatory/apoptotic and anti-trophic pathways. Several promising drugs and compounds have been or are being investigated in phase II and phase III studies such as Xenon ventilation (an NMDA-antagonist), 2-iminibiotin (a selective nNOS inhibitor), melatonin (seems to have anti-oxidative and anti-inflammatory effects) and erythropoietin (anti-oxidative, anti-inflammation and trophic activities) (5,50–53).

A potential important anti-oxidative neuroprotective agent is allopurinol. Allopurinol is a xanthine-oxidase inhibitor (54). By inhibiting xanthine-oxidase, the conversion of hypoxanthine and oxygen into xanthine, uric acid and the free radical superoxide is reduced upon reperfusion and reoxygenation after (fetal) hypoxia (54). It is hypothesized that allopurinol decreases reperfusion injury by reducing superoxide formation, but also by scavenging free radicals and chelating non-protein-bound iron (5,55,56). *Chapter seven provides an overview of the existing literature about allopurinol as a neuroprotective agent in infants with HIE.* Literature of earlier preclinical and clinical studies in infants with HIE suggests that allopurinol is neuroprotective on the long-term in infants with moderate HIE (57). In these earlier postnatal studies, allopurinol was administered within four hours after birth (58,59). Although there was an effect on the long-term in moderately affected infants, administration of allopurinol within 4 hours after birth is probably too late since the superoxide production reaches its peak around 30 minutes after birth. Therefore, an antenatal study followed, in which allopurinol was administered to the mother in case of imminent fetal hypoxia (60). This study showed a beneficial effect in girls on chemical

biomarkers (S100B) for brain injury, but not in boys whose mothers were treated with allopurinol (60). However, it appeared difficult to antenatally select hypoxic fetuses based on cardiotocography (61) resulting in overtreatment. Therefore, a double-blinded, placebo-controlled RCT "Effect of Allopurinol in addition to hypothermia for hypoxic-ischemic Brain Injury on Neurocognitive Outcome" (ALBINO) trial is now conducted in Europe. In the ALBINO trial, we investigate the neuroprotective effect of early allopurinol on the resuscitation table in neonates with perinatal asphyxia and early signs of encephalopathy (62,63). *The study protocol is further described in chapter eight of this thesis.*

The pharmacokinetics of allopurinol are well known for infants with HIE that are not treated with therapeutic hypothermia, but this knowledge is not yet available for infants with HIE that are treated with therapeutic hypothermia (64). Therefore, as part of the ALBINO trial a pharmacokinetic sub-study was performed. *The results are presented in chapter nine.*

Innovative neuroimaging methods

Add-on neuroprotective therapies will be essential to improve outcome for infants with HIE. However, to evaluate the neuroprotective effect of add-on therapies and to predict long-term outcome, optimization of neuroimaging quality is also important.

MRI with a higher field strength can improve neuroimaging quality. Nowadays MRI with a field strength up to 3.0 Tesla (T) is routinely used in infants. The increase of a field strength of 1.5T to 3.0T has improved MRI quality in infants and enabled us to see more details and pathology (65). In adults, 7.0T has shown to further increase diagnostic value and is now frequently used. According to a growing number of studies, 7.0T MRI enables better visualization of micro-bleedings and the microcirculation at SWI, MRA and MRV, detection of additional metabolites at MRS and better quality of T2-weighted imaging that allows to see more details of the anatomy i.e. the different layers of the cortex can be distinguished (66). In infants 7.0T MRI might also be beneficial to optimize MRI quality, but so far this has never been performed.

A higher field strength in infants might be accompanied with some safety concerns as discomfort and a rise in temperature of the brain, but also with technical

challenges (67). Even though 7.0T MRI is safe in adults (68), we should first investigate the safety and feasibility of 7.0T MRI in infants in a pilot study.

Before starting such a study, we should be able to reliably measure brain temperature during MRI to evaluate the safety. MRS can be used to measure brain temperature in neonates (69,70), but the feasibility of this method without calibration and using different scan protocols is not yet elucidated. *In chapter ten, the feasibility of measuring brain temperature with MRS in infants with HIE is assessed. In chapter eleven, the safety preparations and feasibility of 7.0T MRI in infants are discussed.*

OUTLINE OF THIS THESIS

In part I, different imaging modalities in infants with HIE and the long-term outcome of children with HIE following perinatal asphyxia are investigated. Part II describes the current literature on neuroprotection with allopurinol and the study protocol and pharmacokinetic results of the ALBINO trial. Part III of this thesis investigates and discusses the feasibility of innovative neuroimaging techniques.

Part I: neuroimaging and follow-up

Chapter two describes a specific pattern of cerebellar injury in infants with HIE.
Chapter three shows the correlation of cerebellar injury in infants with HIE based on DWI versus histopathology.
In **chapter four,** the development and validation of a new CUS scoring method is described.
Chapter five discusses the association between HIE, hippocampal volumes at ten years of age and cognitive outcome in a cohort of children born in the "precooling" era.
Chapter six shows the results of a prospective observational cohort study in which we assessed neuropsychological, motor and MRI exams at ten years of age in children that were cooled and children that were born before the cooling era.

Part II: add-on neuroprotection

Chapter seven provides an overview of all literature on allopurinol as neuroprotective agent in neonates with HIE.
Chapter eight describes the study protocol of a large ongoing randomized controlled trial (ALBINO), investigating the neuroprotective effect of allopurinol administered during resuscitation in neonates with HIE.
Chapter nine shows the first results of the ALBINO trial: the pharmacokinetics of allopurinol during therapeutic hypothermia.

Part III: innovative neuroimaging techniques

Chapter ten describes a new method to reliably and non-invasively measure brain temperature in infants with HIE using magnetic resonance spectroscopy.
Chapter eleven discusses the safety preparations and feasibility of 7.0T MRI in infants and shows the first 7.0T images.
Finally, **chapter twelve** summarizes the findings of this thesis and discusses the implications and future directions for research in this field.
Chapter thirteen summarizes the results of this thesis in Dutch.

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Part I

NEUROIMAGING AND FOLLOW-UP

CHAPTER **2**

Uneven distribution of purkinje cell injury in the cerebellar vermis of term neonates with hypoxic-ischemic encephalopathy

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ABSTRACT

Background: In term neonates with hypoxic-ischemic encephalopathy (HIE), cerebellar injury is becoming more and more acknowledged. Animal studies demonstrated that Purkinje cells (PCs) are especially vulnerable for hypoxic-ischemic injury. In neonates, however, the extent and pattern of PC injury has not been investigated. The aim of this study was to determine the distribution of PC injury in the cerebellar vermis of term born neonates with HIE.

Methods: Term born neonates with HIE that underwent postmortem autopsy of the cerebellar vermis were included. Hematoxylin & Eosin (H&E) stained sections of the vermis were used to determine total PC count and morphology (normal, abnormal or non-classified) at the bases and crown of the folia and of the lobules in both the anterior and posterior lobes. Differences in PC count and PC morphology between the anterior and posterior lobe and between the bases and crown were calculated using the paired samples t-test or Wilcoxon-signed rank test.

Results: The total number of PCs were significantly higher at the crown compared to the bases ($p<0.001$) irrespective of the precise location. Besides, PCs at the bases more often had an abnormal morphology. No significant difference between the total number of PCs in the anterior and posterior lobe was observed.

Conclusion: The abnormal PC count and morphology in term neonates with HIE resembles supratentorial ulegyria.

INTRODUCTION

Hypoxic-ischemic encephalopathy (HIE) following perinatal asphyxia is the leading cause of mortality in term neonates with an incidence of 1.5 per 1000 term births (1,2). Therapeutic hypothermia is the current standard of care to reduce brain injury and additional treatment options are still limited (3). Besides high mortality rates, long-term neurodevelopmental disabilities such as cognitive, behavioral and attention impairments and motor dysfunction are still common in neonates with moderate or severe HIE (4–7).

In the clinical setting, HIE is often characterized as cerebral injury (8). However, also cerebellar injury in neonates is being increasingly recognized as a contributor to neurodevelopmental outcome (9,10). For instance, cerebellar injury has been linked to motor deficits, as well as cognitive functioning, behavior, learning and emotional deficits (11,12). However, research about the association between behavioral problems and cerebellar injury in HIE remains sparse (9). In infants born extremely preterm (i.e. below 28 weeks of gestation) a clear association was found between early cerebellar injury and long-term behavioral problems (10,13).

During the third trimester of pregnancy the cerebellum shows a rapid increase in growth, making the cerebellum especially vulnerable to insults that disturb normal development within this time period (e.g. hypoxia-ischemia) (14,15). Surprisingly, acute cerebellar injury is rarely detected on conventional clinical neuroimaging (e.g. T1-, T2-weighted and diffusion weighted magnetic resonance imaging (MRI) scans) (16,17). In contrast, on histopathological level, there is evidence that the cerebellum is injured after severe HIE in infants (18). Of the cerebellar neurons, the Purkinje cells (PCs) are known to be the most susceptible to hypoxic events, which is most likely due to their high metabolic activity and associated oxygen demand (19,20). Postmortem studies in human neonates with HIE on PCs are lacking, but in neonatal mouse models, pronounced reductions in PC number and morphological alterations of PCs in response to hypoxic-ischemia were indeed found (i.e. swelling, autolytic necrosis, cell shrinkage and dark cell degeneration) (24,25). Other animal studies on perinatal hypoxic-ischemia have also demonstrated increased microglia activation, myelination deficits and overall necrosis and apoptosis in the cerebellum (23–25).

Whereas the cerebellar anatomy has been described as homogenous, its susceptibility to cellular damage has been shown to vary between cerebellar lobules. In murine

models, it has been shown that PCs in lobules IX and X were less vulnerable for hypoxic-ischemia (26). Additionally, the lobes of the adult human cerebellum have a functional topographic organization, so the pattern of PC injury might be relevant for functional outcome (25,26). Literature about the topographic organization of the vermis in neonates with HIE is scarce as well as literature about differences in vulnerability of the vermis to hypoxia. Therefore, the aim of this study was to determine the distribution of PC injury in the vermis of term born neonates with HIE.

METHODS

Patient population

In this retrospective cohort study, (near)-term neonates (36 until 42 weeks of gestational age) with HIE were included, who were treated with therapeutic hypothermia (born between 2008 and 2015) or would have qualified for therapeutic hypothermia according to the criteria of Azzopardi *et al.* (born between 2000 and 2008) and who underwent autopsy after neonatal death with hematoxylin and eosin (H&E) staining of the vermis (3). Exclusion criteria were perinatal asphyxia based on major congenital abnormalities, metabolic disorders, chromosomal abnormalities or poor quality of the sections of the vermis. No normotypic age-matched controls were available in this study.

Parental consent was obtained for postmortem histopathological examination. The Medical Ethical Committee (MEC) of the UMC Utrecht confirmed that the 'Medical Research Involving Human Subjects Act' did not apply to this study and therefore an official approval of the MEC was not required (MEC # 18-167). The UMC Utrecht biobank approved the use of the rest tissue (biobank # 18-284).

Histopathological examination

Brains were removed during autopsy and before the slices could be cut they were dehydrated with ethanol 70%. Afterwards they were fixed in a 4% buffered formalin solution for four to six weeks (18). The vermis was cut midsagittally in 5-6 µm thick sections, mounted on coated slides and stained for H&E. From each patient one section was implemented.

The H&E stained sections were used to examine the presence and the morphology of PCs using the light microscope Zeiss AXIO lab A1 and ZEN 1.2 lite software (Zeiss, CA, U.S.A). First, the sections of the vermis were photographed at various locations with

63x magnification (Figure 1). For each patient the pattern of PC injury was examined at two levels: (1) within the lobules (locations examined: the bases of the sulci and crown of lobules II, III, VIII, IX), and (2) within two folia (locations examined: the bases of the sulci and crown of a proximal folia and a distal folia of lobules II, III, V, VI, VIII, IX). The crown is defined as the most convex part of a folia or lobe and the base as the most concave part (Figure 1). The base of a sulcus is the mean value of both sides of the base. The anterior values are the average values of lobules II, III and V and the posterior values are the average of lobules VI, VIII and IX. However, data were only included throughout examination when all values within one lobule were available. Whenever lobular data were available, but data from the folia were missing (or vice versa), only lobular data were included since this was separately investigated.

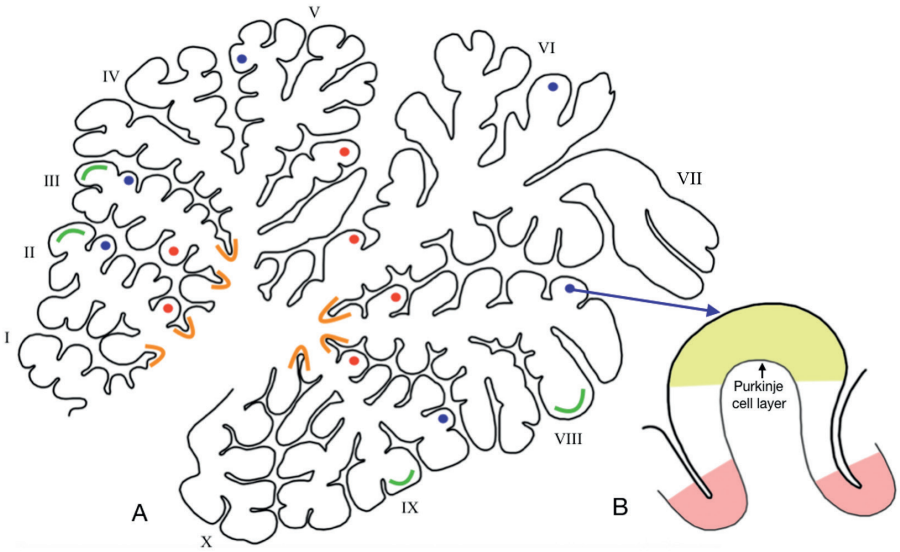

Figure 1: Locations photographed for analyses in the vermis. A. The red dots represent the proximal located folia in the anterior and posterior lobes. The blue dots represent the distal located folia in the anterior and posterior lobes. The orange (concave) curvatures represent the bases of the sulci of the anterior and posterior lobules, the green (convex) curvatures represent the crowns of the anterior and posterior lobules. B. Magnified folia. The yellow bow represents the crown, the pink curvatures represent the bases of the sulci of the folia.

PC classification system

PCs were counted and categorized at the above mentioned locations in the vermis using a newly developed PC classification system based on Hausmann *et al.* (19) by

an experienced neuropathologist (M.L.). PCs were only counted when located in the Purkinje cell layer and when a clear nucleus was visible. PCs were categorized as: normal, abnormal or non-classified (Table 1 and Figure 2). The numbers of normal, abnormal and non-classified PCs were counted for each location using ImageJ software version 1.47 (Wayne Rasband, NIH, USA). Afterwards the numbers were corrected for the measured distance in cells per 100 µm. Also the percentage of abnormal PCs compared to normal PCs was calculated for every location.

Table 1: Purkinje cell classification system.

Category	Definition
Normal	1. An intact cell membrane 2. A basophilic/light stained cytoplasm 3. A clearly identifiable, basophilic/light stained nucleus with intact nuclear membrane 4. An identifiable nucleolus in the nucleus *(In addition to 1 and 2, criteria 3 or 4 should be met to score a PC as normal)*
Abnormal	1. No intact/ruptured cell membrane 2. A hypereosinophilic stained cytoplasm 3. An identifiable nucleolus but no clear nucleus or rest of nucleolus *Presence of activated Bergmann glia and shrinkage of total cell volume was taken into account as an additional confirmation of abnormality, but was not required*
Non-classified	1. The nucleolus and nucleus were not visible or could not be categorized 2. The soma of the PC was clearly visible 3. The cell is located in the PC layer

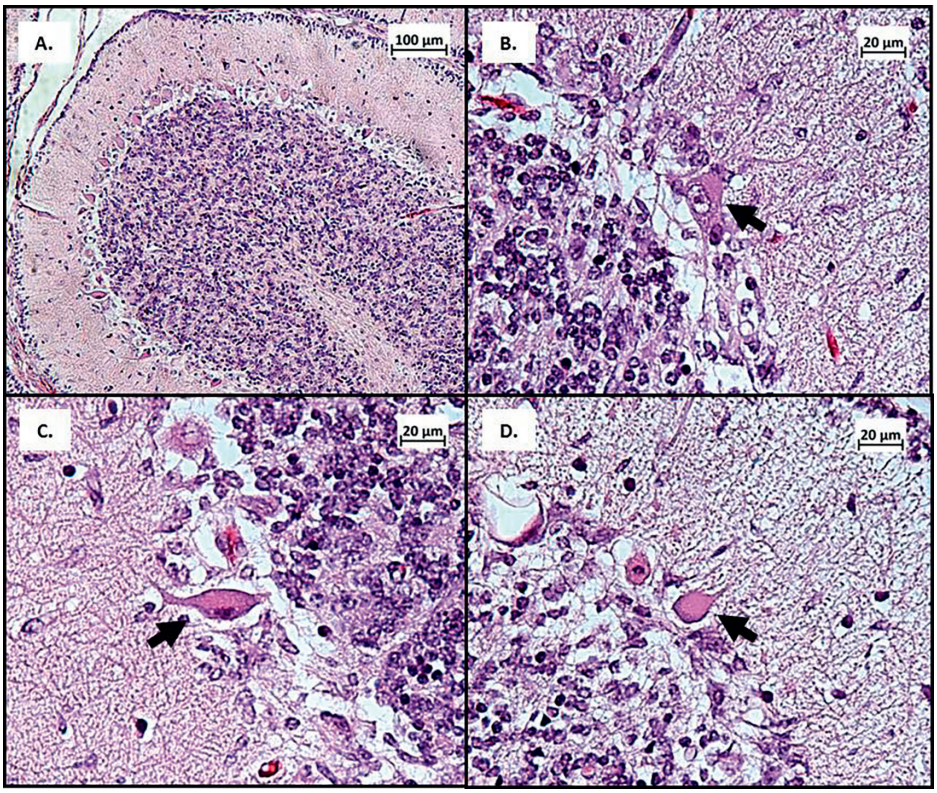

Figure 2: Examples of PC classification system. A. Crown of a proximal located folia of lobe 2. B. Normal PC. C. Abnormal PC. D. Non-classified PC because no nucleus or nucleolus is visible.

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 25 (IBM New York 2013). Differences in PCs in the anterior and posterior lobe were calculated using the paired samples t-test for normally distributed data and a Wilcoxon-signed rank test for data that were not normally distributed. Differences in the number and percentage of PCs at the crown compared to the bases of the sulci within the lobules and within the proximal and distal folia were assessed using a paired samples t-test for normally distributed data and a Wilcoxon-signed rank test for data that were not normally distributed. P-values < 0.05 were considered significant. All data were corrected for multiple comparison analyses by multiplying the p-value with the number of comparisons per statistical test.

RESULTS

Between 2000 and 2015, 22 patients with HIE underwent autopsy and had good quality sections of the vermis stained for H&E available for examination. Clinical data are presented in Table 2. All patients died from irreversible brain injury after redirection of care. Three infants were treated with hypothermia for less than 72 hours, because therapeutic hypothermia was stopped based on clinical decision making. Of the included patients, nine had missing values for some of the lobules. This is also specified in Table 2.

Table 2: Patient characteristics.

Patient characteristics	
Gender (male), n (%)	15 (68.2%)
Gestational age (weeks), median (IQR)	40.0 (1.45)
Mode of delivery, n (%)	
Emergency caesarean section or fetal indication	11 (50.0%)
Normal vaginal delivery	9 (40.9%)
Vacuum extraction	2 (9.1%)
Apgar score at 1 min, median (IQR)	1 (0 – 2.5)
Apgar score at 5 min, median (IQR)	3 (0 – 4.5)
Umbilical cord pH, median (IQR)	6.9 (0.1)
Birth weight (grams), median (IQR)	3352 (429)
Hypothermia, n (%)	
No	5 (22.7%)
Yes, incomplete (>48h)	3 (13.6%)
Yes, complete (72h)	14 (63.6%)
Postnatal age at death (days), median (IQR)	5 (3 – 5.25)
Time of autopsy after death (days), median (IQR)	1 (1-2)
Missing values, n (%)	
Anterior lobes	
Lobe II	9 (40.9%)
Lobe III	9 (40.9%)
Lobe V	4 (18.2%)
Posterior lobes	
Lobe VI	2 (9.1%)
Lobe VIII	2 (9.1%)
Lobe IX	3 (13.6%)

No significant differences were detected between the total number of PCs, i.e. normal, abnormal and non-classified PCs combined, in the anterior and posterior lobes (Table 3). For all analyzed locations, the total number of PCs was significantly higher at the crowns compared to the bases of the sulci (Figure 3). The percentage abnormal PCs was significantly higher at the bases of the sulci than in the crowns in the posterior lobules (p=0.011), as within the central folia (p=0.001) and peripheral folia (p=0.002) of the posterior lobules. In the anterior locations, the increase in abnormal PCs was only significant within the central folia of the anterior lobules (p=0.008).

Table 3: Total PC count per 100 µm in anterior versus posterior lobes.

Location	Total PC count anterior vs. posterior	p-value
Anterior vs. posterior lobules		
All crowns	0.83 (0.72 – 1.01) vs. 1.29 (1.07 – 1.51) #	0.08
All bases of sulci	0.44 (0.27) vs. 0.48 (0.26) *	0.33
Central folia of anterior vs. posterior lobules		
All crowns	1.08 (0.24) vs. 0.86 (0.31) *	0.14
All bases of sulci	0.46 (0.18) vs. 0.43 (0.22) *	≈1.00
Peripheral folia of anterior vs. posterior lobules		
All crowns	0.99 (0.27) vs. 1.11 (0.22) *	0.68
All bases of sulci	0.56 (0.17) vs. 0.56 (0.20) *	≈1.00

The difference in total PC count per 100 µm between lobules II, III, V (anterior) and lobules VI, VIII, IX (posterior) of the vermis are presented. The paired samples t-test for normally distributed data and a Wilcoxon-signed rank test for non-Gaussian data was used. The crowns and bases of the sulci of the proximal and distal folia anterior/posterior are taken together. #Median (IQR), *Mean (SD).

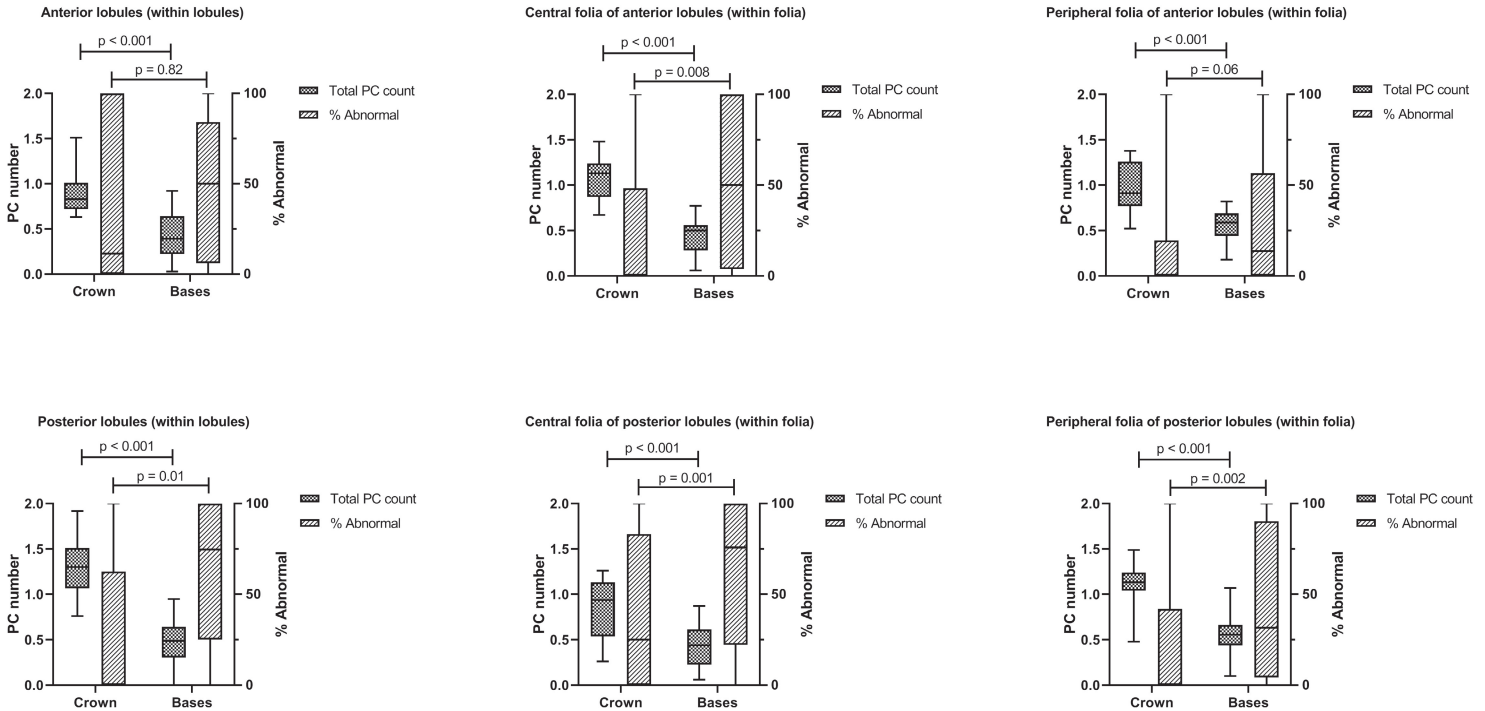

Figure 3: Differences in PC number and abnormalities within lobules and within the folia at different locations and depths. Total PC numbers and percentage of abnormal PC in different areas within lobules II, III, V (anterior) and lobules VI, VIII, IX (posterior) of the vermis are presented. Total numbers are presented as mean (SD), percentages of abnormal PC are presented as median (IQR).

DISCUSSION

In this study we investigated PC injury among different locations in the vermis in (near-) term neonates with HIE. We found that both in the folia and the lobules the total PC count was significantly higher at the crowns compared to the bases of the sulci. For several locations the percentage of abnormal PCs was significantly higher at the bases of the sulci than at the crowns. The total number of PCs did not differ between the anterior and posterior lobes of the vermis. To our knowledge this is the first study that describes differences in total PC count within the lobules and folia in the vermis of term born neonates with HIE.

The lower PC count at the bases of the cerebellar lobules and folia compared to the crowns might well be explained by a combination of normal anatomy and increased vulnerability (29,30). Regional differences in PC density within the cerebellar folia have been shown previously in healthy human adults and 75-day-old rats (29,30). Both studies found more PCs at the crowns compared to the bases. These studies showed that the apparent differences in PC density were caused by the folding of the cortex; this can also (partly) be an explanation for the difference in total PC number in our population (29,30).

In general, our data showed more abnormal PC soma morphology at the bases compared to the crowns. Hence it seems that PCs at the bases of the folia and lobules are more vulnerable to hypoxia. Likewise, Akima *et al.* showed that PCs at the bases of the sulci were more prone to severe ischemic injury in humans between 0 and 89 years of age (31–33). They hypothesized that the vascular architecture of the cerebellar cortex provided by the meningeal arteries could be an explanation for selective PC vulnerability to hypoxia (31,32). Although this hypothesis remains to be tested, they did describe that the bases of the deeper secondary and tertiary sulci are perfused by very small branches from the larger arteries (33). Because the arterial ramifications at the bases of the sulci are smaller than the arteries at the crown, this might partially explain the higher prevalence of infarctions in the bases of the sulci after severe generalized ischemia (33).

The current findings seem remarkably similar to a particular pattern of supratentorial injury called ulegyria, first described by Bresler in 1899 (34). Bresler identified narrowing of the supratentorial cortical gyri because of scar formation in the brain of a mentally impaired patient, which he called ulegyria (34). Ulegyria

is defined by atrophy of the deeper sulcal portions of the cerebral cortex, thereby sparing the crown, and is most pronounced in the watershed regions of the three major cerebral vessels (35–38). More recently, ulegyria has also been described in three infants with intrauterine asphyxia scanned at seven to eight days after birth by Volpe and Pasternak (38). Ulegyria is a hallmark of perinatal asphyxia in term born neonates with HIE, yet it is not exclusive for infancy (36,37). The acute phase of ulegyria is characterized by extensive neuronal loss and signs of hypoxic-ischemic injury at postmortem histopathological stainings, such as neuronal vacuolization, chromatolysis and an influx of macrophages in the bases of the sulci (39). Thereafter, ulegyria is characterized by atrophic changes and gliosis of the subcortical white matter, eventually leading to the so called 'mushroom gyri' with a preserved crown on top of a thin stalk of fibrous glial scar tissue (35,37,39). Ulegyria might be caused by distinctions in cortical perfusion, the crown of the gyri is better perfused than tissue at the bottom of the sulci, thereby causing the typical 'mushroom shaped gyri' after hypoxic events (40). Even though, to our knowledge, ulegyria has not yet been described in the cerebellum, the pattern of extensive PC loss at the bases of the sulci in the vermis (vermian 'ulefolia') seems relatively comparable to ulegyria.

In the present study, no significant differences were found in total PC count between the anterior and posterior lobes of the vermis. Nevertheless, previous studies have shown that the extent of PC death is not uniform over the cerebellum. PC susceptibility to ischemia is partly dependent on whether PCs express zebrin II or not. Zebrin negative PCs were more vulnerable for hypoxic-ischemia in rodent models (26). Furthermore, there is variability in vulnerability of PCs between lobules; PCs in lobules IX and X seem more resistant to death (26). Additional research is needed to investigate differences in the vulnerability of different lobules in the vermis of neonates with HIE.

There were some limitations to this study. The first limitation was the small research population (n=22). This diminished the power of the study and therefore we were not able to investigate the relationship between timing of death and PC injury. The second limitation was that terminal events after withdrawal of care, such as hypoxic events, could have led to additional injury to H&E stained sections of the vermis (18). Due to the variability between death and tissue collection the phenotype scoring system might have been influenced by postmortem timing. Furthermore, it was not feasible to determine the normal distribution of PCs in term born infants

due to the absence of a control group and only little variability in the severity of hypoxic-ischemia in this cohort. Lastly, several neonates were treated with therapeutic hypothermia before withdrawal of care to prevent further brain injury. In this study, hypothermia treated neonates were not separately investigated from non-hypothermia treated neonates, since the latter was just a minor group (n=5). The severity of cerebellar injury could potentially differ between infants who were treated with hypothermia and those who were not.

Additional research is essential to better understand the causes and consequences of ulegyria in the vermis. It must be determined whether ulegyria injury is restricted to the vermis or if this phenomenon is also present in the cerebellar hemispheres. Also, the influence of the pattern of PC injury on neurocognitive outcome should be investigated. Supratentorial ulegyria seems to be associated with epilepsy (41), but the effect of ulefolia on outcome is unknown. In addition, it would be interesting to further quantify pathophysiology of immune responsive cells, oligodendrocytes and all neuronal cell types and establish a potential link to cerebellar ulegyria. Another potential avenue of clinical research would be to investigate subcortical white matter injury in the cerebellum, since subcortical white matter atrophy is an essential hallmark of supratentorial ulegyria.

CONCLUSIONS

In conclusion, we demonstrated that PCs of term neonates with HIE are injured. HIE infants have fewer PCs at the bases of the cerebellar folia and lobules compared to the crown. In addition, PCs at the bases seem to be more vulnerable to hypoxia. This pattern of PC injury is comparable to supratentorial ulegyria.

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DISCLOSURES

The authors have no conflict of interest to declare.

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CHAPTER 3

Cerebellar injury in term neonates with hypoxic-ischemic encephalopathy is underestimated with antemortem diffusion weighted MRI in comparison to postmortem histopathology

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Submitted

ABSTRACT

Background: Postmortem examinations frequently show cerebellar injury in infants with severe hypoxic-ischemic encephalopathy (HIE), while it is less well visible on magnetic resonance imaging (MRI). The primary aim was to investigate the correlation between cerebellar apparent diffusion coefficient (ADC) values and histopathology in infants with HIE. The secondary aim was to compare ADC values in the cerebellum of infants with HIE and infants without brain injury.

Methods: ADC values in the cerebellar vermis, hemispheres and dentate nucleus (DN) of (near-)term infants with HIE (n=33) within the first week after birth were compared with neonates with congenital non-cardiac anomalies, normal postoperative MRIs and normal outcome (n=22). Microglia/macrophage activation was assessed using CD68 and/or HLA-DR staining and Purkinje cell (PC) injury using H&E stained slices. The correlation between ADC values and the histopathological measures was analyzed.

Results: ADC values in the cerebellar hemispheres were comparable. ADC values in the vermis ($p=0.021$) and DN ($p<0.001$) were significantly lower in infants with HIE compared to controls. ADC values in the vermis were correlated with the number and percentage of normal PCs, otherwise ADC values and histology were not correlated.

Conclusion: Infants with HIE had lower ADC values in the vermis and DN than controls. ADC values were only correlated with normal PCs in the vermis.

INTRODUCTION

Despite therapeutic hypothermia, hypoxic-ischemic encephalopathy (HIE) following perinatal asphyxia is still an important cause of mortality and morbidity in term-born neonates (1). HIE can lead to different long-term neurodevelopmental deficits, such as motor impairment, cognitive and behavioral problems and epilepsy (1–3). Future neurodevelopmental outcome can be predicted based on early injury patterns seen on neuroimaging (4,5). Magnetic Resonance Imaging (MRI) is a very accurate and reliable neuroimaging technique to visualize supratentorial patterns of hypoxic-ischemic brain injury (5,6). Especially, diffusion weighted imaging (DWI) is a frequently used MRI sequence in HIE, that has shown to be accurate in detecting brain injury in infants with HIE (7). DWI can visualize the diffusion restriction of water molecules caused by acute cytotoxic edema (8). Furthermore, diffusion restriction can be quantified by lower Apparent Diffusion Coefficient (ADC) measurements (6). Research until now has shown that supratentorial DWI is very well correlated with histopathology (9). Furthermore ADC values of the basal ganglia and thalami are predictive for neurodevelopmental outcome in HIE (10,11).

During the past decades, the main focus of neuroimaging in HIE has been on the supratentorial brain structures. However, recent literature has emphasized the vulnerability of the developing cerebellum for hypoxic-ischemic events (12,13). The cellular and network organization in the cerebellum change rapidly in the third trimester and first months after birth (14–16). The cerebellar cortex receives afferent inputs from the cerebrum, spinal cord, and vestibular nuclei via mossy and climbing fibers (17,18). The Purkinje Cells (PC) are the only output neurons of the cerebellar cortex (19) and are connected to the cerebellar nuclei (the largest is the dentate nucleus (DN)), which predominantly via thalamus are connected back to the cerebral cortex (18).

Historically, the cerebellum is especially known for its role in motor control because cerebellar damage leads to impairments in motor control and posture. However, it has become apparent that the cerebellum also plays an important role in various cognitive functions (20,21). Multiple studies have shown that there is an association between peripartum cerebellar injury and autism spectrum disorders in preterm born infants (22). In a meta-analysis including preterm born infants, hemorrhages in the vermis led to cognitive problems in 80% of the infants (23). Nevertheless, studies about cerebellar injury on MRI in term neonates with HIE are scarce. Clinical MR-sequences as DWI and T1-weighted imaging found little cerebellar injury in neonates

with HIE (10,24,25). More advanced MRI studies have identified cerebellar injury in HIE using diffusion tensor imaging (DTI) in the first month after birth. Also during follow-up, between 5-19 months, atrophy of the vermis was seen in children with perinatal HIE on conventional imaging (21,24).

Only few postmortem histopathological studies have been performed, which all showed extensive cerebellar injury in infants with severe HIE (9,12,25). Especially PCs appear to be vulnerable to hypoxia, which leads to altered morphology or necrosis of these cells following perinatal hypoxia (13,26,27). Also the cerebellar nuclei, e.g. DN, are vulnerable and this is probably important for cerebellar functioning, since the connections between the cerebellar hemispheres and cerebral cortex converge through these nuclei (28)

There seems to be a discrepancy in cerebellar injury in infants with HIE based on MRI and histology. It is important to elucidate whether DWI can reliably diagnose cerebellar injury in infants with HIE, because cerebellar injury might be associated with behavioral and cognitive problems.

Therefore, the primary aim of this study was to investigate the correlation between the cerebellar ADC values in the vermis, DN and cerebellar hemispheres on DWI and the extent of histological cerebellar injury (cell death and inflammation) in infants with HIE. The secondary aim was to compare ADC values in the cerebellum of infants with HIE to neonates without brain injury.

METHODS

Study population

In this retrospective study, we included (near-)term born neonates diagnosed with HIE, who died in the neonatal period and of whom histological material was available and underwent an antemortem DWI of the brain. All infants had to fulfill the therapeutic hypothermia inclusion criteria, although half of them were born before therapeutic hypothermia became standard of care in 2008 (1). Exclusion criteria were a gestational age <36 weeks, suspected genetic abnormalities, absence/poor quality of histological material of the cerebellum or absence/poor quality of the DWI scan.

For the analysis of the DWI scans, we also included a group of neonates with congenital non-cardiac anomalies, without underlying syndromes, with a

normal postoperative MRI (range 3-13; median 7.5 days after birth) and normal neurodevelopmental follow-up at 18-24 months of age as a "control group".

Parental consent for postmortem histopathological examination of the brain was obtained for all patients. This study was approved by the medical ethical committee (#18-167) as a 'non Medical Research Act' study and the Biobank of the University Medical Center Utrecht approved to use the rest biomaterial for this research (biobank #18-284).

Magnetic resonance imaging

MRI was conducted in the first week after birth in the HIE group and within the first two weeks after birth in the control group using 1.5 or 3.0 Tesla (T) MRI scanners (Philips Medical Systems, Best, The Netherlands). Standard scan protocols included at least axial DWI and T1-weighted and T2-weighted images. ADC maps were created as described in Alderliesten *et al.* 2011 (10). The slice thickness was 4mm for both 1.5T and 3.0T MRI.

ADC values were measured in the cerebellar hemispheres, the left and right DN and the vermis using Horos Imaging software (The Horos Project, available from: https://www.horosproject.org). A region of interest (ROI) template was developed and copied to all scans (Figure 1), it was manually adapted per patient if necessary because of the anatomical variances between patients. The anatomy was verified using T1-weighted and T2-weighted MR images and inclusion of cerebrospinal fluid in the ROI was avoided at all times. ADC values were automatically measured within the ROI. Two independent raters measured ADC values in all patients to analyze interrater variability.

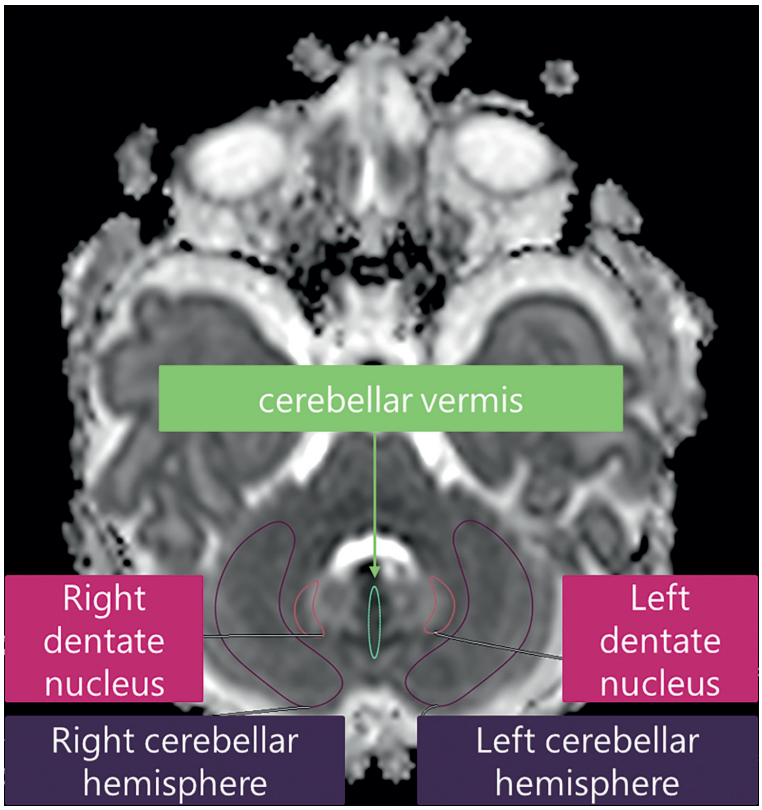

Figure 1: The template for ADC measurements at the level of the pons and vermis that was copied to all scans and slightly adapted per patient.

Postmortem analyses

The histopathological examination of the brains of the infants with HIE was performed in the UMC Utrecht using the following protocol: sections of various brain regions, including cerebellum and vermis, were obtained after removing and fixing the brains in 4% buffered formalin for three to four weeks. After fixation, a transverse section of the vermis and central section of the cerebellar hemisphere with DN were sampled and embedded in paraffin. Histological sections of 5-6 µm thick were cut and mounted on coated slides. Slides were stained with Hematoxylin and Eosin (H&E) or with monoclonal antibodies against CD68 (Novo Castra, Newcastle, UK) and HLA-DR (Dako, Glostrup, Denmark). The H&E staining was used to study the morphology and number of PC's. The CD68 and HLA-DR stains were used to visualize microglia and macrophages activation (13). The tissue was

photographed and analyzed using a standard light microscope (Zeiss AXIO Lab.A1, Oberkochen, Germany) and the ZEN 2.3 Lite software (Zeiss).

H&E staining – scoring Purkinje cells

Pictures were taken at 6 different locations in the vermis and cerebellar hemispheres: 1) at the apex and 2) the base of the anterior lobe, 3) at the apex and 4) the base of the posterior lobe and 5) at the apex and 6) the base of the flocculonodular lobe. All pictures were taken and analyzed at a magnification of 63 times.

PCs were scored as normal or abnormal (Figure 2). A PC was scored normal when the nucleus was clearly visible, light stained with a clear nucleolus and the cellular cytoplasm was slightly eosinophilic and uniformly stained. A PC was scored abnormal when the nucleus was intensively dark stained and shrunken and when there was dark stained, hyper-eosinophilic cytoplasm. PCs were not included in the analyses when we were unable to identify a nucleus, because it was unclear whether this was due to hypoxic-ischemic injury or due to the way the slices were cut.

In every picture the number of normal and abnormal PCs were counted, as well as the total number of PCs. The mean number and percentage for all the analyzed locations within the vermis and hemispheres were used for further analysis. PCs were counted manually using ImageJ (ImageJ program 1.47; National Institutes of Health, USA). In addition, the length of the PC layer was measured with ImageJ to correct for the length of the PC layer and number of PCs per 1000 µm.

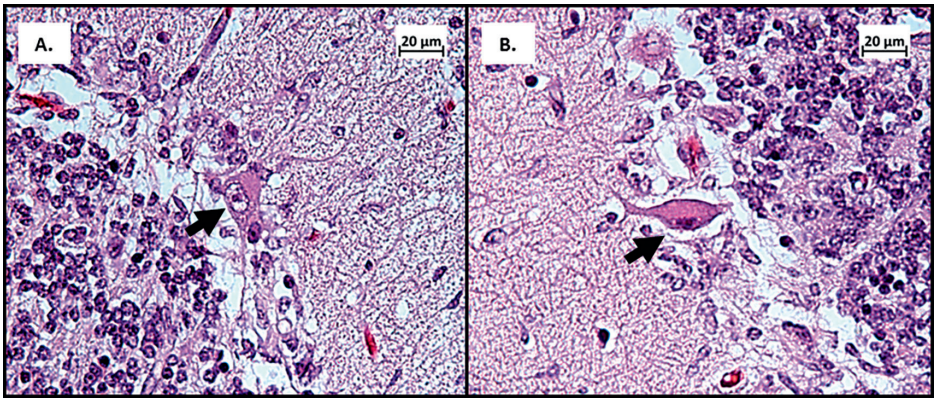

Figure 2: Examples of normal PCs (2A) and abnormal PCs (2B) in lobe 2 of the vermis of a patient with severe HIE who died five days after birth.

H&E staining – scoring DN

The DN pictures were taken at three different locations spread throughout the structure, also with a magnification of 63 times.

Neurons in the H&E stained slices of the DN were also scored normal or abnormal using the same criteria as for PC scoring (see above). Only cells with a transcellular diameter of at least 14 µm were taken into account, because cell characteristics were not reliably assessable in smaller cells. The DN cell counts (normal, abnormal and total) were corrected for the measured surface.

H&E staining – cytotoxic edema

The H&E stained slices of the cerebellar hemispheres, vermis and DN were visually scored for cytotoxic edema. Cytotoxic edema was scored as absent (no, 0 points), minimal or focal cytotoxic edema (mild, 1 point), diffuse cytotoxic edema (moderate, 2 points) or very extensive cytotoxic edema (severe, 3 points) by an experienced neonatal pathologist.

HLA-DR and CD68 staining

The slices stained with monoclonal antibodies for HLA-DR and CD68 were scored according to a previously published method (9). The slices of the cerebellar hemispheres, vermis and DN were scored based on the magnification that was necessary to visualize the stained microglia/macrophages: 0 points were given if there was no staining visible (no staining), 1 point when staining was visible with a magnification of 200x (mild staining), 2 points in case a magnification of 100x was sufficient to visualize staining (moderate staining) and 3 points if the staining was visible with a magnification of 25x (severe staining).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 25 (IBM Armonk, New York, USA). The correlation between the ADC measurements of the two raters was calculated using the Spearman's Rho test. Mean ADC values between infants with HIE and controls, were compared using the independent t-test for parametric data and Mann-Whitney-U test for non-parametric data. The histopathological measurements (number of normal PCs per 1000µm and percentage of normal PCs of the total PCs) were compared between the different locations in the hemispheres and vermis using Repeated Measures ANOVA (between

lobes). The correlation between ADC values and percentage and number of normal PCs were analyzed with Pearson's r coefficient for parametric and Spearman's Rho for non-parametric data. The differences in mean ADC values and count and percentage of normal PCs between the different HLA-DR, CD68 and cytotoxic edema categories were calculated with One-Way Anova or Kruskal-Wallis-H test. P-values below 0.05 were considered statistically significant.

RESULTS

Study population

Thirty-six term neonates with HIE who died and underwent cranial autopsy in the Wilhelmina Children's Hospital between 2000 and 2016 were included in this study. Of those infants, thirty-three infants were included in the final analysis, two infants were excluded because their antemortem MRI was of poor quality and one because the postmortem brain tissue was not available from the pathology department. Twenty-two controls were included. Patient characteristics are described and compared in Table 1.

Table 1: Patient characteristics.

Patient characteristics	Hypothermia (n=20)	No hypo-thermia (n=13)	p-value hypothermia vs. no hypothermia	Controls (n=22)	p-value HIE vs. controls
Gender, n males (%)	12 (60.0%)	9 (69.2%)	0.59	13 (59.1%)	0.73
Gestational age in weeks, mean (SD)	39.8 (1.6)	40.1 (1.3)	0.64	39.6 (1.5)	0.49
Apgar 1 min, median (IQR)	1 (0 - 3)	2 (1 – 4)	0.91	9 (8 – 9)	p<0.001
Apgar 5 min, median (IQR)	3 (0 – 4)	5 (2 – 6)	0.06	10 (9 – 10)	p<0.001
Birth weight in grams, mean (SD)	3406 (523)	3363 (455)	0.81	3272 (686)	0.49
pH, mean (SD)	6.86 (0.19)	7.05 (0.19)	0.01	n/a	n/a
Sarnat, n (%)			0.52		n/a
Moderate	7 (35.0%)	6 (46.2%)		n/a	
Severe	13 (65.0%)	7 (53.9%)		n/a	

Table 1: Continued

Patient characteristics	Hypothermia (n=20)	No hypothermia (n=13)	p-value hypothermia vs. no hypothermia	Controls (n=22)	p-value HIE vs. controls
Predominant type of supratentorial brain injury, n (%)			0.99		n/a
Watershed	3 (15.0%)	2 (15.4%)		n/a	
Basal ganglia thalami	12 (60.0%)	8 (61.5%)		n/a	
Near total injury	5 (25.0%)	3 (23.1%)		n/a	
Field strength MRI, n (%)			<0.001		0.002
1.5 Tesla	0 (0.0%)	11 (84.6%)		0 (0.0%)	
3.0 Tesla	20 (100.0%)	2 (15.4%)		22 (100.0%)	
Postnatal age at MRI in days, median (IQR)	4.0 (3.0 - 4.8)	2.0 (1.0 - 4.5)	0.02	7.5 (5.0 – 9.0)	p<0.001
Postnatal age at death in days, median (IQR)	4.5 (3.0 – 5.0)	4.0 (2.0 – 6.0)	0.95	n/a	n/a
Days between MRI and death, median (IQR)	0.0 (0.0 – 1.0)	1.0 (0.5 – 1.0)	0.05	n/a	n/a

Interrater variability ADC measurements

The correlation of ADC values between the two different observers was good for the cerebellar hemispheres (r=0.87, p<0.001), the vermis (r=0.80, p<0.001) and the DN (r=0.93, p<0.001).

ADC values

Mean ADC values in the DN (HIE: 1004 ± 152x10^{-6} mm^2/s; controls: 1140 ± 82x10^{-6} mm^2/s; p<0.001) and cerebellar vermis (HIE: 785 ± 113x10^{-6} mm^2/s; controls: 859 ± 72x10^{-6} mm^2/s; p=0.009) were significantly lower in neonates with HIE compared to controls, but there was no significant difference in ADC values in the cerebellar hemispheres (HIE: 1072 ± 121x10^{-6} mm^2/s; controls: 1115 ± 89x10^{-6} mm^2/s; p=0.162) (Figure 3A,B). ADC values in the infants with HIE were not correlated with postnatal age at MRI.

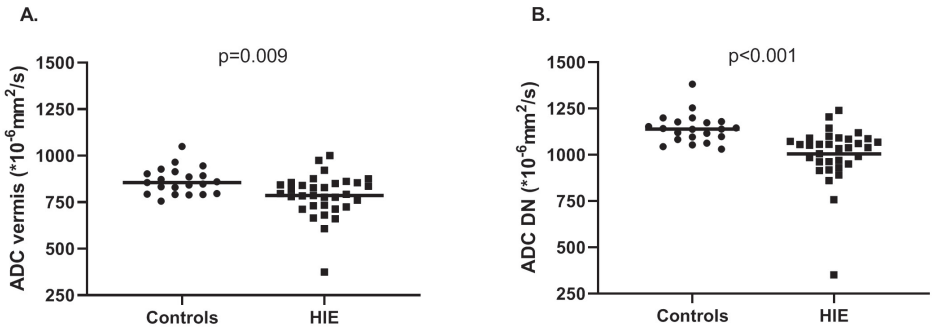

Figure 3: Mean ADC values in infants with HIE (n=33) and controls (n=22) in the vermis (A) and DN (B).

Histopathology

The count of normal PCs per 1000 µm and the percentage of normal PCs were similar in the anterior, posterior and flocculonodular lobe of the vermis (p=0.21) and in the anterior, posterior and flocculonodular lobe of the cerebellar hemispheres (p=0.75). There was no correlation between the timing of death and number of normal PCs per 1000µm (r=0.08, p=0.67) and percentage of normal PCs (r=0.07, p=0.68).

The median histological cytotoxic edema score was 1 (IQR 0-2) in the cerebellar hemispheres, 1.5 (IQR 1-2) in the DN and 2 (IQR 1-2) in the vermis. There were no differences in number and percentage of normal PCs between infants with no, mild, moderate and severe cytotoxic edema in the vermis and cerebellar hemispheres. In the DN, the number of normal neurons (p=0.001) and percentage of normal neurons (p=0.01) were lower in case of more severe cytotoxic edema. The postnatal age of death was not significantly different between infants with no, mild, moderate and severe cytotoxic edema based on the H&E stained slices.

The median CD68 score in the cerebellar hemispheres was 2 (IQR 2-3) and the median HLA-DR score 1 (IQR 0-3). In the vermis, the median CD68 score was 2 (IQR 2-3) and HLA-DR score 2 (IQR 0-3). In the DN the median CD68 score was 2 (IQR 0-3) and HLA-DR score 0 (IQR 0-3). The count of normal PCs per 1000 µm and percentage of normal PCs in the cerebellar hemispheres were comparable between the HLA-DR and CD68 categories in the cerebellar hemispheres. The same applied to the PCs in the vermis and the neurons in the DN. Infants with HIE that had more activation of microglia and macrophages did not have more PC injury compared to infants with HIE with less activation.

The postnatal age at death was not significantly different between the different HLA-DR and CD68 categories, except for the CD68 staining in the DN (p=0.024) in which the patients with score 0 died at a median of 5 days after birth and the infants with score 3 at a median of 3 days after birth.

ADC values versus histopathology

The ADC values in the vermis were significantly correlated with the amount of normal PC per 1000 µm and the percentage of normal PCs in the vermis. In the cerebellar hemispheres and DN there was no correlation between the ADC and the neuron/PC measures (Figure 4A,C,E).

ADC values in the cerebellar hemispheres, vermis and DN did not differ between no, mild, moderate and severe CD68 stained slices of these structures (Figure 4B,D,F). The same applied to HLA-DR staining. The patients with low ADC values in the hemispheres did have severe CD68 staining of the hemisphere and the patient with low ADC values in DN did have severe CD68 and HLA-DR staining of the DN (Figure 4D,E).

The median ADC values in the vermis were not significantly different between infants with no, mild, moderate and severe histopathological cytotoxic edema of the vermis (p=0.08). The mean ADC values in the cerebellar hemispheres were also not significantly different between infants with no, mild, moderate and severe histopathological cytotoxic edema of the hemispheres (p=0.36). The ADC values in the DN were comparable between the cytotoxic edema categories in the DN (p=0.70). See Figure 5.

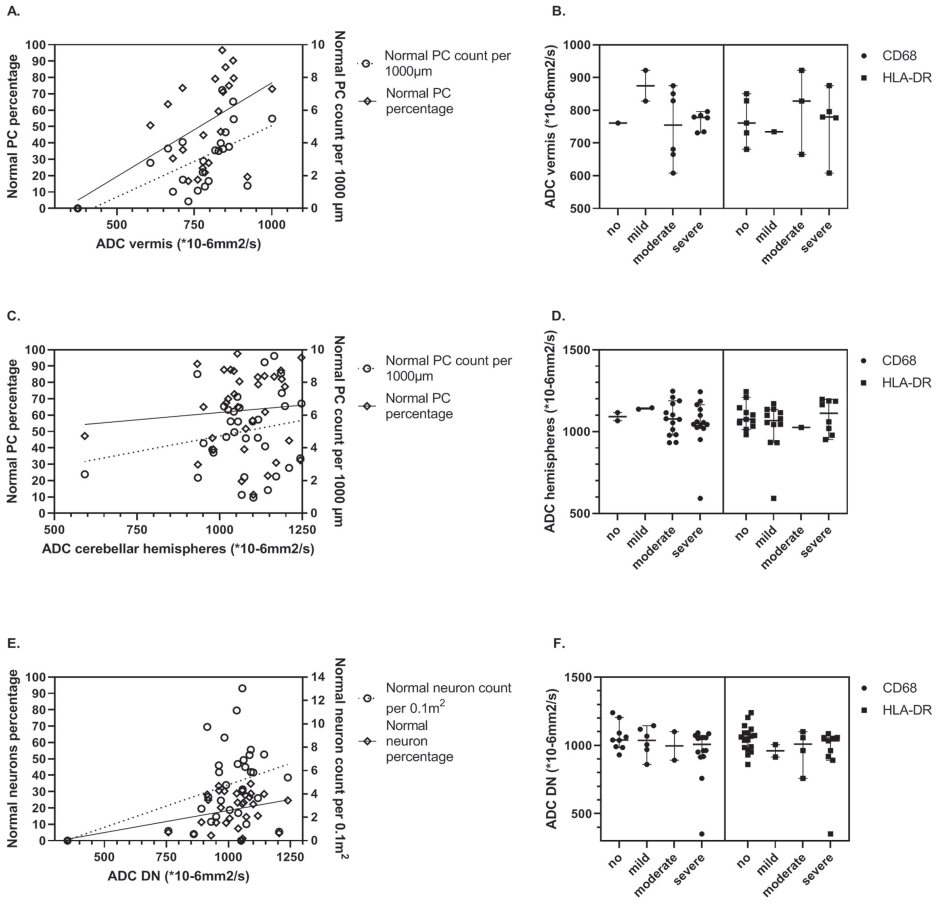

Figure 4: Comparisons between ADC values and histopathology in infants with HIE. The percentage of normal PCs/neurons is presented at the left y-axis and the number of normal PCs/neurons at the right y-axis. A. ADC values in the vermis were significantly correlated with the count of normal PCs per 1000µm (r=0.59, p<0.01) and the percentage of normal PCs (r=0.50, p=0.01) in the vermis. B. ADC values in the vermis were not different among the CD68 categories (p=0.39) or the HLA-DR categories (p=0.89). C. ADC values in the cerebellar hemispheres were not correlated with the count of normal PCs per 1000µm (r=0.14, p=0.46) nor with the percentage of normal PCs (r=0.0, p≈1.00) in the hemispheres. D. The ADC values in the cerebellar hemispheres were not different among the CD68 categories (p=0.59) and the HLA-DR categories (p=0.64) in the hemispheres. E. ADC values in the DN were not correlated with the count of normal neurons per 1000µm in the DN (r=0.29, p=0.11) nor with the percentage of normal neurons (r=0.25, p=0.17). F. The ADC values in the DN were comparable between the different CD68 categories (p=0.46) and the HLA-DR categories (p=0.26) in the DN.

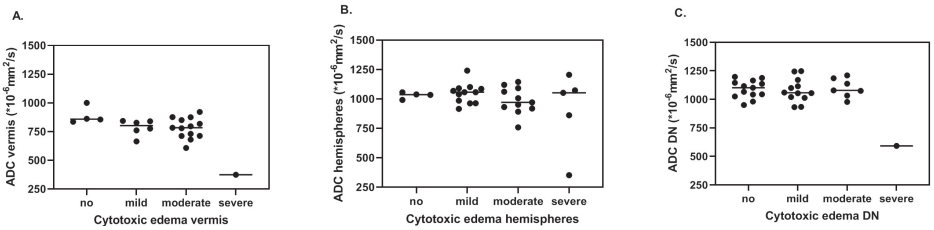

Figure 5: Median ADC values in the vermis (A), cerebellar hemispheres (B) and DN (C) in infants with no, mild, moderate and severe cytotoxic edema based on H&E stained slices.

DISCUSSION

This study showed that infants with HIE had lower ADC values in the vermis and DN compared to controls, but not in the cerebellar hemispheres. ADC values in the vermis were correlated with the number of normal PCs in the vermis, furthermore there were no correlations between ADC values and histology measures.

The lower ADC values in the vermis and DN imply that it is possible to detect hypoxic injury in these structures with ADC maps. There were two patients with clear abnormalities on both the DWI and ADC map, so when cytotoxic edema is very severe, the ADC map will detect this. However, in the hemispheres there were no differences between the HIE group and controls; although in case of severe HIE severe cytotoxic edema in the hemispheres is to be expected (9). We hypothesize that only parts of the hemispheres show diffusion restriction and that by measuring the whole hemisphere, this effect is leveled out.

There was an excellent interrater reliability for ADC measurements. This shows that ADC measurements can be reproduced and that the measurements are reliable.

As expected, PC injury was found in the neonates with HIE during autopsy. PCs fire action potentials at high frequencies of ~100 Hz, and can even achieve frequencies up to 500 Hz when triggered by climbing fibers (29). This high firing rate combined with a large membrane surface sums up to an extraordinary high metabolic demand of PCs and high vulnerability for hypoxic-ischemic injury (30). In addition, the early and high susceptibility of cerebellar PCs for hypoxic-ischemia, as evidenced by the present study, can be explained by their decreased ability to minimize excitotoxic glutamate release in the acute hypoxic-ischemic state (26,31).

Infants with HIE showed staining with monoclonal antibodies for CD68 and HLA-DR in the cerebellar hemispheres, DN and vermis implying microglia and macrophage activation. However, PC injury did not differ between the categories for HLA-DR staining and CD68 staining. This implies that the inflammation is not (solely) caused by PC injury. The PCs are the main output cells of the cerebellum and therefore very important. However, granular cell injury is also described after hypoxic-ischemia and this process might lead to additional inflammation (12,13). CD68 stained macrophages and microglia were in an earlier study especially found in the granular layer and white matter and less frequently in the PC layer, which supports this theory (32). Another explanation might be that CD68 and HLA-DR staining are time dependent (9,32). We did not find an effect of postnatal age at death in our study, except for CD68 staining in the DN but this effect was the opposite of what we expected based on earlier studies (32). The effect of timing between the ischemic insult and death on these CD68 and HLA-DR results cannot be ruled out because of the small sample size. The same applied for histopathological cytotoxic edema. Additional staining methods, such as calbindin, are valuable to further investigate cerebellar injury in the future (12).

ADC values and histopathological injury of the cerebellum were not correlated, except for ADC values in the vermis and number and percentage of normal PCs. This means that even if the DWI and ADC values are in the reference range, this does not rule out cerebellar injury. An underestimation of cerebellar injury with MRI in neonates with HIE is in agreement with earlier literature (9,25,33,34). Kwan *et al.* studied 172 infants with HIE that were treated with therapeutic hypothermia and found cerebellar injury in only 4% of this group using conventional imaging (25). However, histopathological cerebellar injury was found in 72% of the 14 infants that underwent autopsy. This study does support that conventional imaging underestimates cerebellar injury even more than DWI. Alderliesten *et al.* reported cerebellar abnormalities in 61% of the patients with HIE based on DWI and in 83% of the patients based on histopathological analysis (9). In the same study there was a very good correlation between DWI and histopathology for the supratentorial brain structures (9). The reason for this underestimation in the cerebellum is not entirely clear. We found that ADC values did not significantly differ between infants with no, mild, moderate and severe cytotoxic edema based on histopathology. The two patients with severe histopathological cytotoxic edema in the vermis and DN did have very low ADC values. So, cytotoxic edema is only detectable on DWI if the edema is severe and diffuse. Furthermore, histopathological cytotoxic

edema was not associated with PC injury, so even without cytotoxic edema there still might be cerebellar injury

Our study suggests that ADC values of the vermis are most reliable, it does show differences between the HIE and control group and it is associated with the normal PC count and percentage. Additionally, other MR sequences might be more useful to diagnose cerebellar injury in neonates with HIE. For example, Lemmon *et al.* showed that fractional anisotropy based on diffusion tensor imaging (DTI) was associated with cerebellar injury and outcome (24).

There are some limitations to this study. First, this was a retrospective study, meaning that not all microscopic slices were cut at the same position in the cerebellar hemispheres or vermis and only one slice per structure was available. Also, different protocols for DWI were used. Both might have caused some variability in the results. In addition, the control group of infants with a surgical intervention is not a healthy control group. However, by verifying that their MRI showed no supratentorial or infratentorial abnormalities and their neurocognitive follow-up was normal at 24 months of age, it is justifiable to use this group as a control group. Furthermore, for the histopathological group, it would be useful to have a control group; however, there is no autopsy tissue of healthy term born neonates available. Finally, our study population was rather small, especially the number of slices of the vermis was small. A larger study population would have provided more statistical power and would have enabled us to investigate the effect of the postnatal age of death on the results. Nevertheless, to the best of our knowledge, this is one of the largest studies to date comparing MRI to histopathology in neonates with HIE. It is a unique dataset with neonatal human postmortem tissue and we were able to use quantitative MRI measurements, which was proven to be reproducible by different raters.

Future research should focus on elaborate staining of the cerebellum to study the injury in infants with HIE in more depth. Additionally, it would be interesting to explore the pattern of PC injury in the cerebellum in more detail. Finally, more research is needed to study the association between ADC values of the cerebellum and neurocognitive outcome. Prospective research is necessary to answer this question.

CONCLUSIONS

In conclusion, ADC values were lower in the vermis and DN of infants with HIE compared to controls. However, ADC values were not different in the cerebellar hemispheres, even though there was histopathological injury. ADC values in the vermis were correlated with the number of injured PCs. Furthermore, there were no correlations between ADC values and histopathological measures, implying that normal ADC values do not guarantee a lack of injury to the cerebellum.

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DISCLOSURES

Floris Groenendaal is expert witness in cases of perinatal asphyxia. The other authors have no conflict of interest to declare.

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CHAPTER **4**

The development and validation of a cerebral ultrasound scoring system for infants with hypoxic-ischemic encephalopathy

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ABSTRACT

Background: Hypoxic-ischemic encephalopathy (HIE) is an important cause of morbidity and mortality in neonates. When the gold standard MRI is not feasible, cerebral ultrasound (CUS) might offer an alternative. In this study, the association between a novel CUS scoring system and neurodevelopmental outcome in neonates with HIE was assessed.

Methods: (Near-)term infants with HIE and therapeutic hypothermia, with a CUS on day one and day three to seven after birth and available outcome data were retrospectively included in cohort I. CUS findings on day one and day three to seven were related to adverse outcome in univariate and the CUS of day three to seven also in multivariable logistic regression analyses. The resistance index, the sum of deep grey matter and of white matter involvement were included in multivariable logistic regression analyses. A comparable cohort from another hospital was used for validation (cohort II).

Results: Eighty-three infants were included in cohort I and 35 in cohort II. The final CUS scoring system contained the sum of white matter (OR=2.6, 95%CI 1.5-4.7) and deep grey matter involvement (OR=2.7, 95%CI 1.7-4.4). The CUS scoring system performed well in cohort I (AUC=0.90) and II (AUC=0.89).

Conclusion: This validated CUS scoring system is associated with neurodevelopmental outcome in neonates with HIE.

INTRODUCTION

Hypoxic-ischemic encephalopathy (HIE) following presumed perinatal asphyxia is an important cause of morbidity and mortality in neonates and can result in long-term neurological sequelae (1,2). Perinatal asphyxia can be caused by acute or subacute perinatal hypoxia-ischemia that both correspond with different patterns of brain injury (3,4). Acute perinatal asphyxia often results in injury of the deep grey nuclei, such as the basal ganglia and thalamus, or even in near-total brain injury (5). Injury to the deep grey nuclei can lead to dyskinetic cerebral palsy, impaired cognitive outcome and epilepsy (5). Subacute ('chronic') perinatal asphyxia is most often associated with watershed injury with involvement of the cortex and subcortical white matter (5). This usually does not result in motor impairment, but cognitive impairment and language problems occur more frequently and disabilities become apparent in childhood (6-8). In daily practice, the neurological prognosis is predicted based on the triad of neuroimaging, (amplitude-integrated) electro-encephalography ((a)EEG) and clinical features (9). Currently, the gold standard in neuroimaging is magnetic resonance imaging (MRI) (5, 10, 11). MRI predicts neurological outcome in HIE based on conventional imaging i.e. with an MR scoring system (12,13) and quantitatively with apparent diffusion coefficients, arterial spin labelling or magnetic resonance spectroscopy (14-16).

MRI is the gold standard, but an alternative neuroimaging technique is necessary because there are circumstances when the infant is not stable enough to be transported to the MRI unit or MRI is not available, for example in developing countries (17,18). In these situations, cerebral ultrasound (CUS) might offer a bedside and cheaper alternative (18). Currently, CUS is not routinely used to predict outcome in HIE; CUS is not as sensitive as MRI in diagnosing brain injury and may take several days to become apparent (11,19). However, based on the available literature CUS might not only be complementary to MRI but in some cases the only available neuroimaging method in HIE (19-21).

A validated composite CUS scoring system is needed to assess brain injury and to predict outcome with CUS in HIE. To the best of our knowledge, such an ultrasound scoring system is not yet available. The CUS scoring systems that have previously been developed have not been validated (17,22-28). The aim of this study is to assess the association between a novel CUS scoring system and neurodevelopmental outcome in neonates with HIE at the age of two years.

METHODS

Study population

We included (near-) term infants (36 until 42 weeks of gestational age) with HIE who were treated with hypothermia in the University Medical Center Utrecht between January 2008 and July 2014, who had at least one CUS on day one and a second CUS between day three and seven after birth. An additional inclusion criterion was the availability of outcome data, either death or a neurodevelopmental follow-up examination at the age of two years. We excluded infants with metabolic or genetic abnormalities. We used this cohort to create the scoring system (cohort I).

A different cohort with similar clinical characteristics, born in the Erasmus Medical Center in Rotterdam, with at least one CUS between day three and seven and available outcome data was used to validate the scoring system (cohort II). The same inclusion and exclusion criteria applied to this cohort. In both cohorts the CUS scans were part of standard clinical care. The scans were conducted by trained neonatologists, fellows in neonatology and physician assistants with different levels of experience in CUS. In cohort I CUS were performed using an ultrasound machine from Toshiba (Medical System Corporation, Tokyo, Japan) and in cohort II from Esaote (Genova, Italy). Convex 5-10 MHz and linear 15-18 MHz probes were used in both cohorts.

The Ethical Committee of the University Medical Center Utrecht approved this retrospective study and waived the requirement to obtain written informed consent for this study analyzing pseudonymized data.

Development of the CUS scoring system

We searched the literature for possible CUS items to include in the scoring system. The relevance of all these items was discussed by a group of experts including neonatologists and a pediatric neuroradiologist, all with many years of experience in CUS (JD, LdV, FG, PG, ML). Items were categorized into normal-mild, moderate and severe or into absent and present. The same group of experts reached consensus on the definitions of the different categories. For examples of the items see Figure 1 and for definitions see Table 1. Additional antenatally acquired pathology was also scored i.e. porencephaly and atrophy.

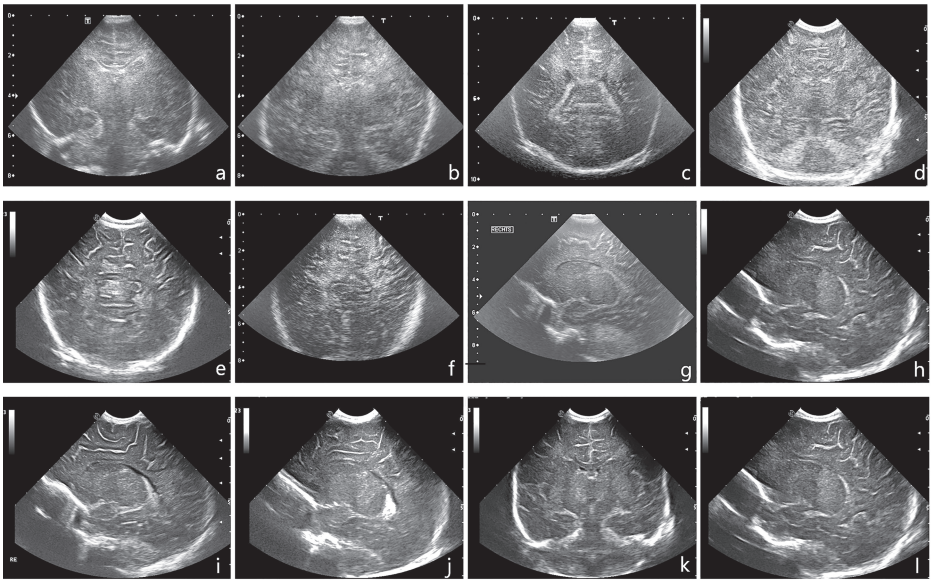

Figure 1: Example images. a. moderate cerebral edema (1 point), b. severe cerebral edema (2 points), c. moderate periventricular white matter (1 point), d. severe periventricular white matter (2 points), e. moderate subcortical white matter (1 point), f. severe subcortical white matter (2 points), g. moderate thalamus (1 point), h. severe thalamus (2 points), i. moderate putamen (1 point), j. severe putamen (2 points), k. "four column sign" which means that both left and right thalamus and putamen are visible at the coronal view as four columns (1 point), l. visibility of the PLIC (1 point). For the scoring sheet and definitions see Table 1.

Table 1: Scoring system. White matter involvement is the sum of edema, periventricular and subcortical white matter damage (0-6 points). Grey matter involvement includes hyperechogenicity of the thalami, putamen, visibility of the PLIC, and four column sign (0-6 points).

	Normal-mildly abnormal (0)	***Moderately abnormal (1)***	***Severely abnormal (2)***	***Total points***
Impaired white/ grey matter differentiation and/or slit like ventricles	Normal differentiation between grey and white matter and open ventricles.	Reduced differentiation between grey and white matter and/or slit like ventricles.	No differentiation between grey and white matter and slit like ventricles.	
Hyperechogenicity periventricular white matter	Normal echogenicity or minor hyperechogenicity.	Moderate or focal hyperechogenicity, not as white as choroid plexus.	Severe and diffuse hyperechogenicity, as white as choroid plexus.	

Table 1: Continued

	Normal-mildly abnormal (0)	*Moderately abnormal (1)*	*Severely abnormal (2)*	*Total points*
Hyperechogenicity subcortical white matter	Normal echogenicity or minor hyperechogenicity.	Focal hyperechogenicity of the subcortical white matter. Moderate differentiation of white and (subcortical) grey matter.	Clear "tramlines" sign; hyperechogenicity of subcortical white matter almost similar to sulci with hyposignal intensity of cortex in between.	
Hyperechogenicity thalamus	Normal echogenicity or minor hyperechogenicity.	Moderate or focal hyperechogenicity thalamus.	The hyperechogenicity is severe and diffuse.	
Hyperechogenicity putamen	Normal echogenicity or minor hyperechogenicity.	Moderate or focal hyperechogenicity putamen	The hyperechogenicity is severe and diffuse.	
	Absent (0)	*Present (1)*		*Total points*
Four column sign	Normal echogenicity to minor hyperechogenicity	On the coronal CUS plane there is a four column sign caused by moderate or severe bilateral hyperechogenicity of the thalamus and putamen.		
Visibility PLIC	The PLIC is not visible as a hypo-echogenic line between the putamen and thalamus.	The PLIC is clearly visible as a hypo-echogenic line between the hyperechogenic putamen and thalamus.		

After the development of the scoring system, a neonatologist, with more than ten years of experience in neonatal neurology and CUS, scored all CUS in cohort I (observer 1). First, the association between all separate items and adverse outcome was determined. Because of multicollinearity all white matter items were summed into a white matter score, the deep grey matter items into a grey matter score and the resistance index remained a separate item. Next, the association between the different composite scores in the scoring system and adverse outcome was calculated.

Furthermore, the additional value of day one CUS to diagnose antenatally acquired pathology of the brain was determined.

Neurodevelopmental outcome

We retrospectively collected clinical parameters of all infants. Neurodevelopmental follow-up was performed with the Bayley Scales of Infant and Toddler Development, third edition (BSITD-III) at the age of two years (29) by a neonatologist and an educational therapist or child psychologist. Adverse outcome was defined as death, cerebral palsy or a cognitive/motor composite score <85 according to the BSITD-III (United States of America norms) at two years of age.

Validation: inter-observer variability

A neonatologist (JD) and a pediatric neuroradiologist (ML), both with an expertise in CUS, scored the day one and day three to seven CUS of cohort I independently of each other (observer one and observer three). Observer one and three did not work in the UMC Utrecht in the period that the CUS were conducted, so they were completely blinded to outcome. Another neonatologist (DV) without a special focus on CUS scored the CUS of 20 randomly selected patients to determine the inter-observer agreement in daily clinical practice (observer two).

Validation of the scoring system in cohort II

The CUS of cohort II were scored by two neonatologists with more than 25 years of experience in reading cerebral ultrasound scans (FG and LdV). They scored the CUS together and reached consensus about the CUS score of all infants. The observers did not work in the Erasmus Medical Center at the moment the CUS were performed, so they were completely blinded to outcome.

Validation: correlation with MRI and histology

Secondary outcomes were the correlation with MRI and histological findings. A correlation between the CUS scoring system and MRI was assessed using the MRI scoring system of Weeke *et al.* (13) in cohort I. In cohort II the diffusion weighted sequences were often of suboptimal quality; therefore, the secondary outcomes were only analyzed for cohort I.

Statistical analyses

SPSS Version 21 was used for statistical analysis (IBM corp., Armonk NY, United States). Differences in baseline characteristics between the two cohorts were calculated using the independent t-test or Mann-Whitney-U test for continuous variables and the χ^2 test for categorical variables. Univariate logistic regression was performed with the CUS items as independent variables and outcome as dependent variable. Non-significant items were excluded from further analysis. The sum of white matter involvement, of deep grey matter involvement and a Doppler ultrasound resistance index (RI) of a cerebral artery ≤0.55 were calculated and included in backward multivariable logistic regression analysis. Variables with a p-value <0.05 were entered in the model and those with a p-value ≥0.1 deleted. The inter-observer variability and the correlation with MRI were calculated using Spearman rank correlation test. Predictive values and ROC curves were determined for cohorts I and II per cut-off value. A p-value <0.05 was considered statistically significant.

RESULTS

Study population

Between January 2008 and July 2014, 145 infants with HIE were treated with hypothermia in the University Medical Center Utrecht. In total, 83 infants were included in cohort I. Infants were excluded because of genetic or congenital abnormalities (n=9), preterm birth <36 weeks (n=12), because one or both of the CUS were not present (n=27), only a few images of an examination were saved (n=2), the quality was insufficient because of suboptimal settings (n=6) or because of missing outcome data (n=6).

In the Erasmus Medical Center, 69 infants with HIE were treated with hypothermia in this period and 35 newborns were included in cohort II. Infants were excluded because of genetic or congenital abnormalities (n=9), preterm birth <36 weeks (n=2), diagnosis of arterial ischemic stroke (n=2), because the CUS between day three and seven was not present (n=15) or because no follow-up data were available (n=6).

The incidence of death due to redirection of care between infants who were included and infants who were excluded from the study did not differ significantly.

Baseline characteristics were comparable between the two cohorts except for Apgar scores (Table 2). The mean Apgar scores were lower in Cohort II. Four infants had a postnatal collapse within two hours after birth, which explains their high Apgar scores. The reason for incomplete hypothermia in all infants was early rewarming because of redirection of care. The reason of death in both cohorts was redirection of care based on a poor neurological prognosis.

Table 2: Baseline characteristics.

Patient characteristic	Cohort I (n=83)	Cohort II (n=35)	p-value
Male, n (%)	50 (60.2)	19 (54.3)	0.55
Gestational age, median weeks^{+days} (range)	40^{+1} (36^{+0}–42^{+4})	39^{+6} (36^{+3}–41^{+6})	0.18
Birth weight, median in gram (range)	3500 (2260-5000)	3425 (1780-4440)	0.36
Mode of delivery			0.07
Emergency caesarian section, n (%)	46 (55.4)	20 (57.1)	
Vaginal delivery, n (%)	25 (30.1)	6 (17.1)	
Vacuum extraction, n (%)	12 (14.5)	9 (25.7)	
Apgar score at 5 minutes, median (range)	3 (0-10)	3 (0-9)	<0.001
First pH, median (range)	6.96 (6.53-7.34)	6.94 (6.60-7.28)	0.67
Thompson score, median (range)	10 (4-19)	11 (5-20)	0.83
Sarnat classification			0.87
Mild, n (%)	5 (6.0)	5 (14.3)	
Moderate, n (%)	58 (69.9)	19 (54.3)	
Severe, n (%)	20 (24.1)	10 (28.6)	
Incomplete hypothermia<72 hours, n (%)	6 (7.2)	0 (0)	0.10
Post menstrual age at CUS day 3-7, median days (range)	4 (3-7)	4 (3-7)	0.49
MRI available, n (%)	77 (92.8)	N/A	N/A
Postmenstrual age at MRI, median days (range)	6 (3-16)	N/A	N/A
Outcome			0.26
Normal, n (%)	54 (65.1)	17 (48.6)	
Adverse outcome < 85, on BSITD-III, n (%)	3 (3.6)	7 (20.0)	
Death, n (%)	26 (31.3)	11 (31.4)	

Development of the CUS scoring system

The following potential CUS items were found in the literature: slit-like ventricles, impaired differentiation white/grey matter, "four column" sign, hyperechogenicity of the thalamus, putamen, subcortical white matter, periventricular white matter, hippocampus, brainstem and vermis, visibility of the posterior limb internal capsule (PLIC) and RI of a cerebral artery ≤0.55 (17, 22-28, 30). We excluded hyperechogenicity of the hippocampus, vermis and brainstem because these structures were rarely depicted on the available CUS images. The RI was scored based on all available ultrasounds, if the RI was ≤0.55 at one of these CUS, this item was scored as abnormal.

The CUS scoring system and neurodevelopmental outcome on day one

Antenatally acquired pathology was found in 14 infants (17%). Germinal layer cysts (n=7), lenticulostriate vasculopathy (n=4), frontal horn cysts (n=2) and porencephaly (n=1) were identified.

On day one, only severe hyperechogenicity of the periventricular white matter on CUS was significantly associated with adverse outcome (OR=5.0; 95%CI 1.4-18.4) in univariate logistic regression. The other items were not significantly associated with adverse outcome. Although it did not reach significance, all infants with hyperechogenicity of the thalamus (moderate n=5; severe n=1), of the putamen (moderate n=2) or a four column sign (n=1) on day one had an adverse outcome.

The CUS scoring system and neurodevelopmental outcome between day three to seven

Between day three and seven after birth, most of the CUS items significantly predicted adverse outcome in the univariate logistic regression analysis (Table 3).

Table 3: Univariate association of the items on CUS (day three to seven) and adverse outcome.

Item	Category	Patients per category, n	OR (95% CI)
Cerebral edema	Normal-mild	70	-
	Moderate	12	14.4 (2.9-72.3)
	Severe	1	
Thalamus	Normal-mild	45	-
	Moderate	32	26.7 (6.7-106.2)
	Severe	5	
Putamen	Normal-mild	66	-
	Moderate	15	7.9 (2.2-28.2)
	Severe	1	
Four column sign	Normal	67	-
	Abnormal	14	10.0 (2.5-39.9)
PLIC	Normal	59	-
	Abnormal	23	11.1 (3.6-34.2)
Periventricular white matter	Normal-mild	32	-
	Moderate	43	9.2 (2.4-34.9)
	Severe	8	16.1 (2.5-103.6)
Subcortical white matter	Normal-mild	42	-
	Moderate	35	4.0 (1.5-11.1)
	Severe	6	8.5 (1.3-54.8)
Resistance index	Normal	60	-
	Abnormal	8	16.3 (1.9-142.6)

In the multivariable analysis the RI (0-1 point), the sum of the deep grey matter (0-6 points) and of the white matter involvement (0-6 points) were included. The deep grey matter involvement was the sum of thalamus, putamen, PLIC and four column sign subscores. White matter involvement included edema, subcortical white matter and periventricular white matter subscores.

The grey and white matter subscores of cohort I on both days are shown in Figure 2 per outcome group.

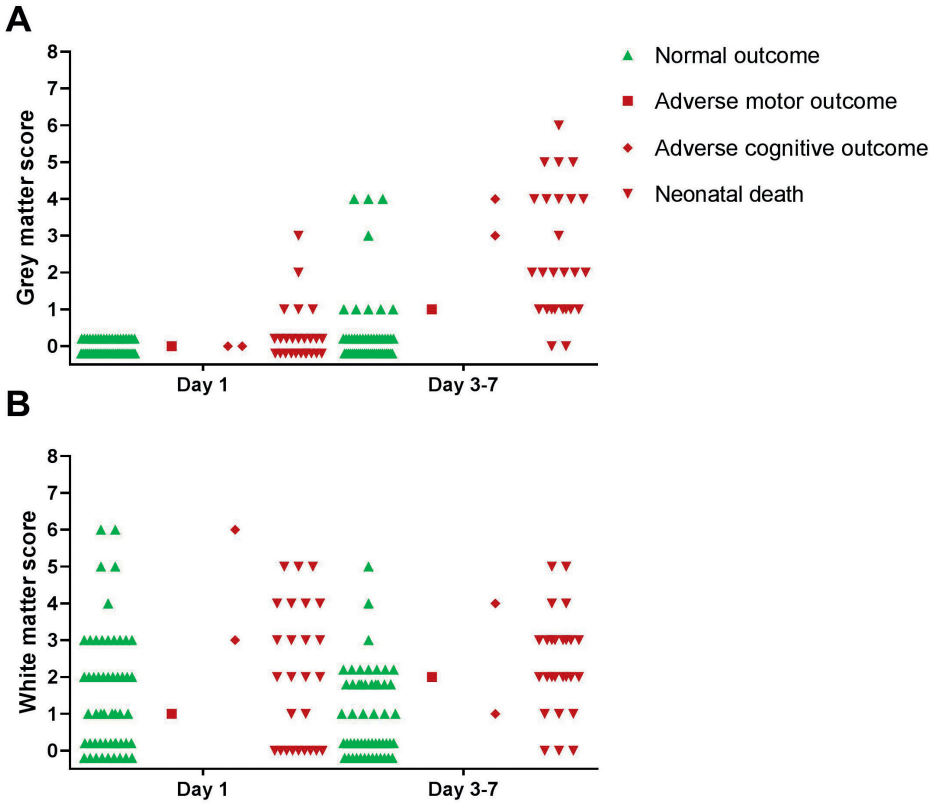

Figure 2: Cerebral ultrasound, outcome and HIE: subscores. Grey matter subscores (A) and white matter subscores (B) of cohort I on day one and day three to seven, categorized for outcome.

The cumulative score of the white matter (OR=2.6, 95%CI 1.5-4.7) and of the deep grey matter involvement (OR=2.7, 95%CI 1.7-4.4) between day three and seven after birth were included in the final scoring system. The RI was not significant in multivariable logistic regression. The probability of an adverse outcome at the age of two years could be calculated using the following formula: $1/(1+e^{-(-3.385+0.960*\text{white matter}+0.995*\text{deep grey matter})})$.

The CUS scoring system performed well in cohort I (area under the curve (AUC)=0.90; 95%CI 0.83-0.98). Table 4 shows the performance per cut-off value.

Table 4: Performance of the model in cohort I and cohort II.

Cut-off value*	≥3	≥4	≥5	≥6	≥7
Cohort I					
Sensitivity	93% (76–99)	79% (60–91)	45% (27–64)	28% (13–47)	17% (7–36)
Specificity	86% (74–94)	88% (76–95)	92% (81–96)	98% (88–99)	100% (91–100)
PPV	79% (62–91)	79% (60–91)	76% (50–92)	89% (59–99)	100% (46–100)
NPV	96% (84–99)	88% (76–95)	75% (62–85)	71% (59–81)	68% (57–78)
Cohort II					
Sensitivity	75% (47-92)	69% (41-88)	63% (36-84)	56% (31-79)	44% (21-69)
Specificity	94% (68-100)	94% (68-100)	100% (76-100)	100% (76-100)	100% (76-100)
PPV	92% (62-100)	92% (60-100)	100% (66-100)	100% (63-100)	100% (56-100)
NPV	79% (54-93)	75% (51-90)	73% (50-88)	70% (47-86)	64% (43-81)

* A cut-off value of ≥3 means that an ultrasound score of 3 or more is defined as abnormal.

Validation: inter-observer variability

Table 5 shows the agreement between the observers in cohort I. There is a moderate inter-observer agreement between all three observers.

Table 5: Agreement between the observers in cohort I.

	Observer 1 vs. 2	Observer 1 vs. 3	Observer 2 vs. 3
Spearman's rho	0.74 (p=0.001)	0.64 (p<0.001)	0.72 (p=0.001)

Validation of the scoring system in cohort II

The predictive values of the scoring system in cohort II, are shown in Table 5. The AUC was 0.89 (95%CI 0.77-1.00).

To exclude the effect of the hospital on outcome, logistic regression was performed with the total CUS score, the hospital and their interaction term included in the analysis. The CUS score was significantly associated with adverse outcome (OR=2.5; 95%CI 1.8-3.4), the hospital and their interaction term were not.

Validation: correlation with MRI and histology

There was a moderate correlation between the CUS and MRI scoring system in cohort I (Spearman's rho=0.67; p<0.001; Figure 3). In the six most severely affected infants, an MRI was not feasible because the infants were clinically too unstable.

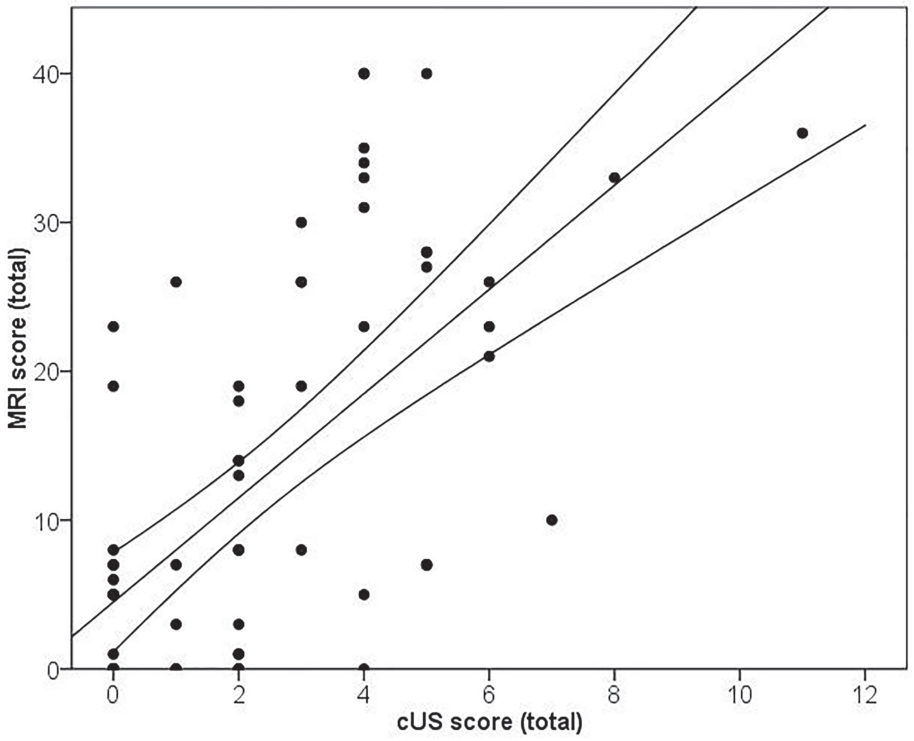

Figure 3: The correlation between the MRI and CUS scoring system in cohort I.

Of the 26 infants in cohort I that died during the neonatal period, 17 infants (65%) underwent postmortem examination. In all these 17 infants, CUS abnormalities were confirmed with histology. The histological damage was more extensive than diagnosed with CUS.

DISCUSSION

We developed a scoring system to structurally score CUS abnormalities in (near-) term infants with HIE. The scoring system was associated with neurodevelopmental outcome and includes composite scores of white matter and deep grey matter involvement, both of which contain multiple separate items. This scoring system was

developed based on the CUS between day three and seven after birth; the CUS on day one after birth was not predictive of adverse outcome. The CUS scoring system was validated in another cohort and the performance was relatively good.

To the best of our knowledge, this is the first CUS scoring system using a validated composite score to predict an adverse outcome. Currently, three CUS scoring systems for infants with HIE are available. A CUS scoring system for asphyxiated infants in Uganda has recently been reported (17). This scoring system was used to identify early HIE-related brain damage but did not provide predictive values (17). Two other scoring systems were developed to score patterns of brain injury in HIE. Leijser *et al.* scored combinations of white and grey matter involvement and compared CUS and MRI (26). The other CUS scoring system by Swarte *et al.* defined six different patterns, for example, the combination of deep grey matter involvement and extensive cortical involvement (28). These scoring systems, did not allow different items to be scored within the categories separately. For example, when describing deep grey matter involvement, this may imply that the left and right thalamus are affected but the basal ganglia are not, while it is of importance to distinguish between just thalamic involvement and thalamic and basal ganglia involvement. Furthermore, they combined white and grey matter involvement, even though different types of brain injury might lead to different outcomes (5). For these reasons, we developed a CUS scoring system based on composite scores, which might be easier to use in clinical practice. The composite scores for deep grey matter and white matter involvement had to be summed because of multicollinearity. However, it remains possible to score the different items separately and to make a distinction between white matter and deep grey matter involvement. Additionally, this is the first CUS scoring system in HIE that is validated in another cohort.

As expected, all scored items on day three to seven in the univariate analysis were significant predictors of adverse outcome. These items have all been described as asphyxia-related brain injury (19, 23, 25, 26, 30-32). Asphyxia-related brain injury is more common in HIE, but as many as 34.2% of controls also showed periventricular hyperechogenicity and 9.2% slit-like ventricles shortly after birth (22). Further, Eken *et al.* correlated hyperechogenicity on CUS with histological findings: hyperechogenicity of the thalamus occurred within 72 hours after birth on CUS (sensitivity 100%, specificity 83.3% of CUS compared to histology), hyperechogenicity of the periventricular white matter within 24 hours (sensitivity 100%, specificity 83.3%) and

hyperechogenicity of the cortex within 72 hours (sensitivity 76.9%, specificity 100%). Additional lesions, not identified by CUS, were found in the brain stem, hippocampus and cerebellum with histology (21). These three items were not included in our CUS scoring system. The "four column sign" and visibility of the PLIC were included as separate items because in some infants the PLIC was visible, but there was no clear "four column sign".

The CUS conducted on day one after birth was not predictive of outcome in this study, which is in agreement with previous studies (17, 22). As mentioned above, it takes 24 to 72 hours before brain injury becomes visible as hyperechogenicity on CUS, unless the onset of the injurious process is of antenatal onset (21). Consequently, CUS within 6 hours after birth had in a previous study a low sensitivity of 42.1% and specificity of 60% (23). Nevertheless, CUS on day one is recommended to identify antenatally acquired pathology (5, 10, 11, 32). We indeed found antenatally acquired pathology in 14 of the 83 infants in cohort I. Most of the antenatally acquired lesions, i.e. germinal layer cysts, did not influence outcome and can also be found in controls (17). However, in one infant a porencephalic cyst was found that led to a mildly asymmetrical motor outcome.

It was of interest to see that the RI was not associated with adverse outcome in multivariable logistic regression analyses. The RI was highly predictive in previous studies with non-cooled infants, but appears to be less predictive in cooled infants, as reported previously (20, 21, 31, 33). Especially the positive predictive value has decreased, so the observed outcome is better than the expected outcome based on an abnormal RI. It has been hypothesized that hypothermia has a direct effect on the cerebral vessels or that hypothermia leads to a better neurodevelopmental outcome but does not lead to a normalization of the RI (33).

The correlations between MRI and CUS in a study of Leijser *et al.* were stronger than in our cohort (0.83 versus 0.67) (26). This might be explained by the fact that they used exactly the same scoring systems for MRI and CUS and in our cohort the scoring systems differed i.e. the cerebellum was included in the MRI scoring system but not in the CUS scoring system. In cohort I there was a moderate correlation between MRI and CUS, but predictive values for MRI in the study of Weeke *et al.* were higher than for CUS in our study (13). The most severely affected infants were too ill to undergo

an MRI. We speculate that these would have shown severe MRI abnormalities, thereby improving the overall association between CUS and MRI.

The performance of the model was good in both cohorts for both the cut-off value of ≥3 which can potentially be used if the number of false negatives should be as low as possible (i.e. for decisions about additional future neuroprotective strategies) and for higher cut-off values that can be preferable if the number of false positives should be as low as possible (i.e. for considering redirection of care in combination with neurophysiology and clinical features if MRI is not possible).

The interrater variability was moderate between all observers implying that the interrater agreement among different hospitals and observers would also be moderate. In a prospective cohort, in which observers can be trained and the quality of CUS can be guaranteed, interrater variability can be further improved.

Our study has several limitations. The first limitation is the retrospective design; no images were routinely taken based on a standard scan protocol. This resulted in poor quality of the images due to non-optimal settings in some cases, the absence of certain anatomical structures on the available images, the absence of CUS on certain days and the absence of follow-up data. As a consequence, a relatively large number of infants had to be excluded. Secondly, neonatal death due to redirection of care was defined as an adverse outcome; the decision was based on a combination of clinical findings, neurophysiology and neuroimaging findings. Even so, we cannot exclude that this leads to some bias because it is not certain that these infants would have experienced problems later in life. However, in cohort I 17 of the 26 infants that died had a postmortem examination and in all infants CUS findings were confirmed. Histopathology showed more extensive damage than CUS, which has also been described for MRI (34).

Thirdly, because of the relatively small sample size and the low incidence of adverse motor or cognitive outcome, our study was not powered enough to perform sub-analyses for the different outcome parameters. Finally, the distinction between the categories ‘normal to mild’ and ‘moderate’ hyperechogenicity remains subjective. Severe hyperechogenicity is easier to distinguish from the other categories. However, this is a reflection of clinical practice and with this scoring system we finally have a method that supports the clinician in their daily routine. Furthermore, this scoring

system provides a tool for prospective clinical trials. Probably, the predictive value of the CUS scoring system will further improve when researchers and clinicians will focus even more on the quality using standard scan protocols in prospective studies.

CONCLUSIONS

In summary, this novel CUS scoring system provides a tool to structurally assess brain injury and predict outcome in HIE if MRI is not feasible or available. It is an easy tool to use in clinical practice and is the first validated CUS scoring system in HIE. In the future, this CUS scoring system should be tested prospectively in infants with HIE.

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DISCLOSURES

Dr. Groenendaal is the founder of 2-iminobiotin and medicolegal expert in cases of perinatal asphyxia. FG also received grant support from Neurophyxia (www.neurophyxia.com) and is an expert witness in legal cases. LdV received royalties for two books, one on cranial ultrasound and the other on aEEG. CR received a lecture fee from Chiesi Pharmacuticals. The other authors have no conflict of interest to declare.

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CHAPTER **5**

The long-term effect of perinatal asphyxia on hippocampal volumes

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ABSTRACT

Background: Hypoxic-ischemic encephalopathy (HIE) in term born infants can lead to memory problems. The hippocampus is important for long-term episodic memory. The primary aim was to investigate the effect of HIE on hippocampal volumes in 9- to 10-year-old children. The secondary aim was to investigate the association between hippocampal volumes and previously found impaired memory and cognitive functions in the current cohort.

Methods: In total 26 children with mild HIE, 26 with moderate HIE and 37 controls were included. The intelligence quotient (IQ) and memory were tested. A 3D-volumetric MRI was obtained. Brain segmentation was performed for hippocampal volumes and intracranial volume. The differences in hippocampal volumes, memory and IQ between the groups were determined. Multivariable linear regression analyses were performed, including hippocampal volume as percentage of intracranial volume as dependent variable.

Results: Smaller hippocampal volumes were found in moderate HIE ($p<0.001$), with a trend toward smaller volumes in mild HIE, compared to controls. In multivariable linear regression analysis, hippocampal volume as a percentage of intracranial volume was significantly associated with long-term visuospatial memory.

Conclusion: Children with moderate HIE had smaller hippocampal volumes than controls, with a trend toward smaller volumes following mild HIE. Reduced hippocampal volumes were associated with poorer long-term visuospatial memory.

INTRODUCTION

The prevalence of hypoxic-ischemic encephalopathy (HIE) after perinatal asphyxia is 1.5 per 1000 live-born term neonates (1). Despite the introduction of hypothermia, the current standard of care, still around 45% of the infants with HIE die or have neurological deficits, such as cerebral palsy (CP), epilepsy or cognitive impairment (2). This risk is especially increased in infants with moderate and severe HIE according to the Sarnat criteria (3).

In the past, it was considered unlikely that cognitive deficits in the absence of CP could be due to HIE, but nowadays, it is widely accepted that infants with HIE can develop isolated cognitive deficits (4). The first landmark paper looking at long-term cognitive outcome following HIE, found that survivors of moderate HIE who did not develop CP had similar receptive vocabulary and perceptual motor skill outcomes as controls, but showed marked delays in reading, spelling and arithmetic (5). Several studies confirmed these findings and it became more accepted that survivors of HIE are at increased risk of cognitive impairment, even in the absence of motor deficits (4,6–9). In addition, several studies have now shown that even children with mild HIE experience more memory problems than controls, as well as behavioral and attention problems (10–13).

We have shown in a previous publication using the same cohort, that HIE especially affects long-term episodic memory, verbal working memory and learning which are all associated with the degree of HIE (10). Several studies, in different populations, have shown that (episodic) memory impairment might be related to smaller hippocampal volumes (14,15). The hippocampus is a specific brain structure that is specifically vulnerable to hypoxia. In addition, some small sample-sized studies suggested smaller hippocampal volumes in HIE compared to controls (6,8,16). However, these groups were heterogeneous and the relation between the hippocampus and memory functioning following HIE has not been fully elucidated.

The primary aim of this study is to evaluate the effect of neonatal HIE on hippocampal volumes in 9- to 10-year-old children. The secondary aim is to investigate whether these hippocampal volumes are associated with the previously found impaired memory and cognitive functions in HIE in the current cohort (10).

METHODS

Study population

This study is a sub-study of a larger follow-up cohort, other results of this cohort have been previously published by van Handel *et al.* and van Kooij *et al.* (10,11,17,18). All participants were born at term (37 to 42 weeks of gestation) between 1993 and 1997 in the Wilhelmina Children's Hospital with HIE following presumed perinatal asphyxia. Perinatal asphyxia was diagnosed when at least three of the following criteria were met: signs of fetal distress (late decelerations on fetal monitoring or meconium-staining amniotic fluid), Apgar score below seven at 5 minutes postpartum, arterial umbilical pH <7.10, delayed onset of spontaneous respiration or multi-organ failure (10,11). Exclusion criteria were small for gestational age, neurological malformations, congenital or chronic diseases influencing outcome, maternal substance use during pregnancy and focal damage with total loss of hippocampal volume.

Children with HIE were divided in two groups. Mild HIE (HIE1) was defined as recovery within 24 hours and a normal electroencephalography (EEG), corresponding with Sarnat stage 1 (3). Moderate HIE (HIE2) was defined as no recovery within 24 hours and an abnormal EEG, the presence of neonatal seizures, corresponding with Sarnat stage 2 (3). Infants with severe HIE were not included in this study. All infants were born and admitted to our level three neonatal intensive care unit before therapeutic hypothermia became standard of care.

Children with the same sex and age, attending regular schools without any special help, were invited as controls. Parents provided details on the perinatal history to exclude children with complications around delivery or referral to a hospital in the first month after birth.

Informed parental consent and child assent were obtained for all participants according to the Declaration of Helsinki. The Ethical Committee of the University Medical Center Utrecht approved the study.

MRI and volumetric measurements

Magnetic resonance imaging (MRI) was obtained between the age of 9 and 10 years, using a 1.5 Tesla Philips system. Brain segmentation was performed using coronal 3D-T1-weighted images (TR: 30ms; TE: 4.6ms, slice thickness: 1.5mm). The left and

right hippocampal volume and the total intracranial volume (ICV) were segmented using FreeSurfer, https://surfer.nmr.mgh.harvard.edu/ (19) (Figure 1).

The total intracranial volume is the volume of the brain including the ventricles and extracerebral space. The total hippocampal volume is the sum of the right and left hippocampal volume. The total hippocampal volume was divided by ICV to correct for any differences in ICV, referred to as "percentage hippocampus/ICV".

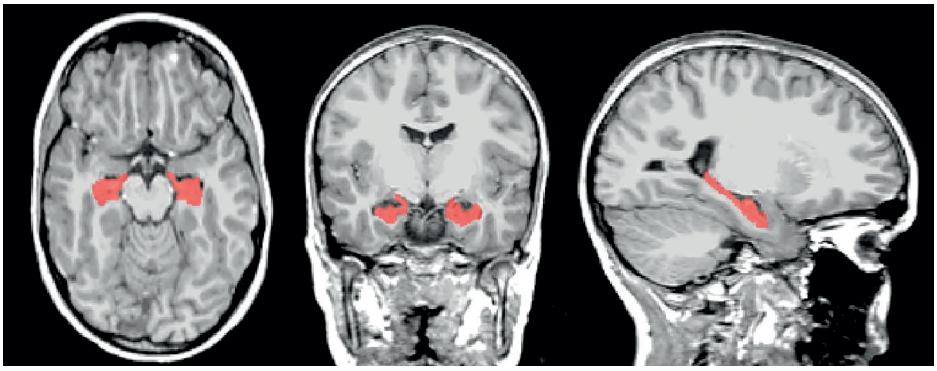

Figure 1: Regions of interest for automated brain segmentation of the hippocampus. Left image: transversal view, middle: coronal view and right: sagittal view.

Memory and Intelligence Quotient tests

For a comprehensive description of the tests, we refer to a previously published study of this cohort (10). In summary, the following neuropsychological tests were performed:

1. Short-term memory:
   a) Verbal short-term memory: Digit Span forward task of the Wechsler Intelligence Scale for Children, Dutch version (WISC-III-NL) (20).
   b) Visuospatial short-term memory: Spatial Memory test of the Kaufman-Assessment Battery for Children (KABC) (21).
2. Verbal working memory: Digit Span backward task of the WISC-III-NL (20).
3. Long-term episodic memory:
   a) Verbal long-term memory: Rey Auditory Verbal Learning Test (RAVLT) (22).
   b) Visuospatial long-term memory: Rey Visual Design Learning Test (RVDLT) and Rey Complex Figure Test (RCFT) (23).
   c) Verbal associative learning: Learning Names of the Revisie Amsterdamse

Kinderintelligentie Test (RAKIT; Amsterdam Child Intelligence Test Revised) (24).
4. Intelligence Quotient (IQ): the Intelligence Scale for Children, WISC-III-NL (25).

Statistical analyses

To detect the differences in baseline characteristics One-Way ANOVA and $\chi 2$ tests were performed. The ANOVA test with Bonferroni post-hoc test, or the Kruskal-Wallis-H test with post-hoc test when appropriate, was used to compare hippocampal volumes, memory and IQ between the groups. We tested asymmetry in hippocampal volumes with a paired samples t-test. Multivariable linear regression was performed to determine the association between HIE, age, sex and hippocampal volumes. Further, we performed univariate and multivariable linear regression analyses to estimate the association between hippocampal volumes and memory and IQ. The percentage hippocampus/ICV, group, age, sex, socioeconomic status based on educational level of the mother (SES) and interaction terms were initially included in the multivariable model. Interaction terms were included to determine whether the effect of hippocampal volume on memory and IQ differed between boys/girls, controls/HIE1/HIE2, SES categories and/or different ages. P-values >0.1 were used to remove and p-values <0.05 to enter the predictors stepwise and bidirectionally in the model. For multivariable linear regression we combined SES categories to limit the number of predictors (Table 1). Statistical analysis was performed using SPSS version 21, and R version 3.1.2. P-values <0.05 were considered statistically significant. All p-values were corrected for multiple comparisons using the Bonferroni correction.

RESULTS

Study population

In total, 164 full-term infants with HIE were admitted to the Neonatal Intensive Care Unit of the Wilhelmina Children's hospital in Utrecht between 1993 and 1997. In the neonatal period 46 children died, including all children with severe HIE. Of the 118 survivors, 81 were examined at 9 to 10 years of age. Of the children not examined, six were too severely affected to participate, seven could not be traced (HIE1: n=5, HIE2: n=2) and the parents of 24 children (HIE1: n=13, HIE2: n=11) refused to participate, mainly because MRI was part of the protocol. Fifty-three controls were included.

An MRI was obtained and was of sufficient quality for volumetric analysis in 26 of the 34 HIE1 infants, 27 of the 47 HIE2 infants and 37 of the 53 controls. In the other

children, MRIs could not be obtained due to fear during or before the MRI (n=11), were of insufficient quality due to movement artefacts (n=31) or not available for segmentation (n=2). One additional child with HIE2 was excluded from volumetric analysis because of a perinatal arterial ischemic stroke, leading to total destruction of the hippocampus. There were no significant differences in baseline characteristics or the degree of HIE between the children whose MRI was used for volumetric analysis and the children of whom no MRI was obtained or whose MRI was of insufficient quality.

Patient characteristics are shown in Table 1. The children with CP were able to perform all tests that were part of the protocol. On conventional imaging, none of the children had pathological lesions of the hippocampus, such as mesial temporal sclerosis.

Table 1: Patient characteristics of the HIE1, HIE2 and control group.

Characteristics	Controls (n=37)	HIE1 (n=26)	HIE2 (n=26)	p-value
Gender, n (%)				0.92
Male	19 (51.4)	13 (50)	12 (46.2)	
Female	18 (48.6)	13 (50)	14 (53.8)	
CP, n (%)	0 (0)	2 (7.7)	2 (7.7)	0.23
Age at follow-up, mean years ±SDS	10.07 ±0.43	9.80 ±0.47	9.77 ±0.55	0.03*
SES 5 categories, median [range]**	4 [2-5]	4 [1-5]	4 [1-5]	0.14
Incidence of epilepsy at school age, n (%)	0 (0.0)	0 (0.0)	1 (4.0)	0.30

* There was a significant trend towards a lower age in the HIE groups (p=0.03), but there were no significant differences in the post-hoc tests between the groups.
** Socioeconomic status (=educational level of mother): 1=no education or primary school, 2=lower technical or vocational training, 3=lower secondary education, 4=higher secondary education, 5=higher education e.g. university. Category 1, 2 and 3 were combined for multivariable linear regression analyses.

Hippocampal volumes in HIE

The total hippocampal volumes in HIE2 were significantly smaller than in controls, with a trend toward smaller volumes in HIE1 (controls: 8.7±0.8ml; HIE1: 8.2±0.9ml; HIE2: 7.6±1.3ml, $p<0.001$, Figure 2A). The hippocampal volume in HIE2 was 12.6% smaller than in the controls. No significant difference in volumes was observed between the HIE1 and HIE2 group.

The total ICV was similar in the three groups (controls: 1498ml±179ml; HIE1: 1456±144ml; HIE2: 1432±205ml, p=1.000, Figure 2B).

When correcting for ICV, differences in the percentage hippocampus/ICV were observed between the three groups, with smaller volumes in HIE (controls 0.58%±0.05 HIE1: 0.56%±0.06; HIE2: 0.53%±0.07; p=0.012, Figure 2C). Again, HIE2 and controls differed significantly in the post-hoc tests. After correcting for ICV, the difference between HIE2 and controls was 8.6%.

Girls had a significantly larger percentage hippocampus/ICV compared to boys in the total group (boys: 0.55%±0.06; girls: 0.58%±0.06; p=0.012).

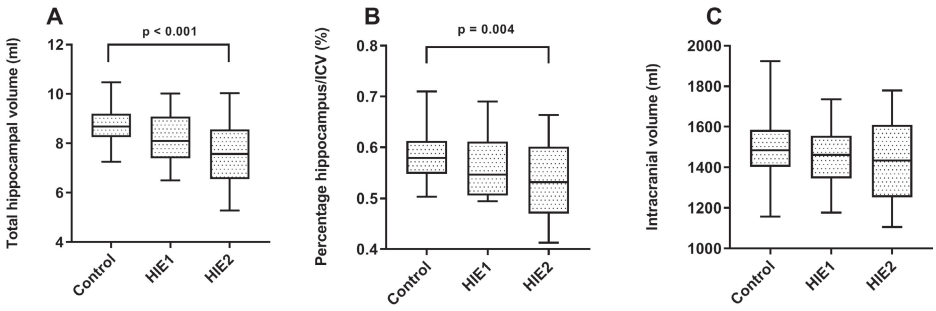

Figure 2: Overview of hippocampal volumes in controls, HIE1 and HIE2. The total hippocampal volumes (A) were significantly smaller in the HIE2 group compared to controls, but the volumes of the HIE1 group were not significantly different. The total hippocampal volume as a percentage of ICV (B) was smaller in the HIE2 group compared to controls, but again the HIE1 group did not differ significantly. The intracranial volumes (C) were similar in the three groups.

Multivariable linear regression with the percentage hippocampus/ICV as a dependent variable and HIE1/HIE2/control, age and sex as independent variables showed that HIE2 and sex were significantly associated with hippocampal volume. HIE2 was associated with a reduction of the percentage hippocampus/ICV of 0.054% (95%CI -0.083% - -0.025%), HIE1 with a reduction of 0.021% (95%CI -0.050%-0.008%) and being a girl with an increase of 0.035% (95%CI 0.011%-0.069%).

Memory and IQ in HIE

Short-term memory, verbal working memory and total verbal long-term memory were comparable between children following HIE and controls and no difference was found between boys and girls. Long-term episodic memory (verbal long-term memory, visuospatial long-term memory and verbal associative learning) was significantly impaired in children with HIE2 compared to the controls. The IQ of the controls was significantly higher than that of children with HIE. The IQ in children with HIE2 was not found to be significantly lower than 100 in a one-sample t-test (p=0.068). IQ was not significantly different in boys and girls (boys: 104±19; girls: 100±18; p=0.292)

The association between hippocampal volumes and cognition

In univariate linear regression both hippocampal volume and ICV were positively associated with IQ (Figure 3), but the percentage hippocampus/ICV was not. All long-term episodic memory tests (verbal long-term memory, visuospatial long-term memory, verbal associative learning) and visuospatial short-term memory were significantly associated with percentage hippocampus/ICV (data not shown).

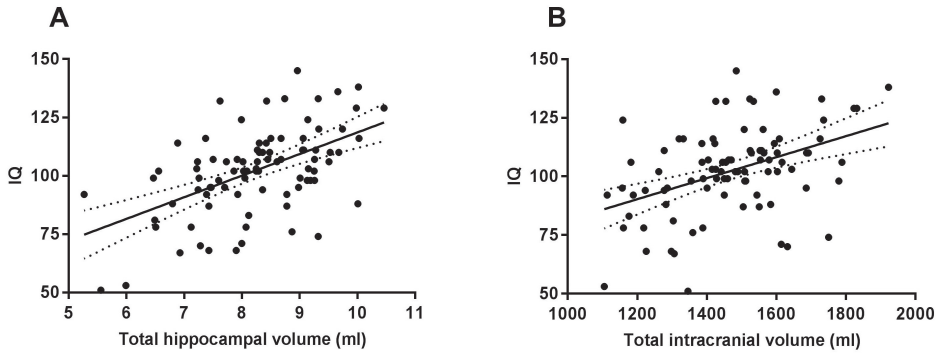

Figure 3: The total hippocampal volume (A) and the total intracranial volume (B) were both significantly associated with IQ. The Pearson's correlation coefficient between IQ and hippocampal volume was 0.52 (p<0.001) and between IQ and intracranial volume 0.43 (p<0.001).

In multivariable linear regression, the degree of HIE was significantly associated with all long-term episodic memory and IQ models. The percentage hippocampus/ICV was a significant predictor for visuospatial long-term memory (Table 2).

Table 2: Predictors included in the final linear regression model per memory or IQ test.

Test	Group[1]	Percentage hippocampus/ICV	Age	SES[2]	Gender	Age* percentage hippocampus/ICV	Group* SES
Verbal short-term memory				x			
Visuospatial short-term memory				x	x		
Verbal working memory							
Verbal long-term memory (immediate)	x						
Verbal long-term memory (total)	x						
Verbal long-term memory (delayed)	x						
Visuospatial long-term memory (immediate)	x	x	x			x	
Visuospatial long-term memory (total)	x	x	x			x	
Visuospatial long-term memory (delayed; RVDLT RECALL)	x	x					
Visuospatial long-term memory (delayed; RFCT RECALL)	x						
Verbal associative learning (immediate)	x			x			x
Verbal associative learning (total)	x						
IQ	x			x			

[1] Group: HIE1, HIE2, controls. [2] Socioeconomic status (educational level of mother): 1=no education or primary school, 2=lower technical or vocational training, 3=lower secondary education, 4=higher secondary education, 5=higher education e.g. university. Category 1, 2 and 3 were combined for multivariable linear regression.

Table 3 shows the models for the memory tests for which the hippocampal volume was a significant predictor. The socioeconomic status and HIE were significantly associated with IQ (IQ = 104.23 + -10.58*HIE1 + -18.76*HIE2 + 4.69*SES2 + 15.17*SES3). For IQ analysis, the percentage hippocampus/ICV was corrected for the age of testing, but was not a significant predictor.

Table 3: The influence of percentage hippocampus/ICV on memory in multivariable linear regression analysis.

Possible items in model	Model visuospatial long-term memory (immediate)[1]	Model visuospatial long-term memory (total)[1]	Model visuospatial long-term memory (delayed)[1]
Intercept	115.04 (p = 0.028)	471.23 (p = 0.021)	3.84 (p = 0.234)
HIE1	-1.54 (p = 0.024)	-5.87 (p = 0.028)	-1.16 (p = 0.129)
HIE2	-2.10 (p = 0.004)	-8.52 (p = 0.003)	-2.27 (p = 0.006)
Age	-11.31 (p = 0.031)	-45.46 (p = 0.028)	n/a
Percentage hippocampus/ICV	-177.17 (p = 0.055))	-808.30 (p = 0.026)	9.95 (p = 0.082)
Age*percentage hippocampus/ICV	19.06 (p = 0.041)	84.58 (p = 0.021)	n/a

[1] ß-coefficients and p-values are shown for the variables included in the final models.

DISCUSSION

In the current study, we reported hippocampal volumes and the association with cognitive outcome at the age of 9 to 10 years in a cohort of children who suffered HIE following presumed perinatal asphyxia. We first showed that hippocampal volumes in children with a history of moderate HIE were significantly smaller compared to controls, with a trend toward smaller volumes following mild HIE. As previously reported, infants with moderate HIE were found to have poorer long-term episodic memory and lower IQ scores than controls (10). Finally, multivariable linear regression showed that the hippocampal volume as a percentage of ICV was positively associated with visuospatial long-term memory, suggesting that poor visuospatial long-term memory following HIE can be mediated by hippocampal damage.

To the best of our knowledge, this is the largest study investigating the effect of neonatal HIE on hippocampal volumes, but smaller studies have been published that are in agreement with our finding that children with HIE have reduced bilateral

hippocampal volumes (6,8,26). For example, Gadian and colleagues examined a smaller group of children and found that severe acute hypoxia was associated with reduced hippocampal volumes and memory problems. This was reported in six school-aged children with memory problems that had experienced hypoxia at, or shortly after, birth and showed bilateral reduction of hippocampal volumes of approximately 40% (6,8). Using MR spectroscopy, Mañeru *et al.* found lower N-acetyl aspartate concentrations and N-acetyl aspartate/choline ratios in the anterior hippocampus of adolescents following HIE, indicating biochemical damage (7). Furthermore, they also confirmed that infants with moderate HIE have bilateral hippocampal atrophy (26). The trend of decreased hippocampal volumes in mild HIE has not been published previously. This finding might explain that several studies found decreased cognitive and memory functions after even mild HIE compared to controls (12). Besides perinatal asphyxia, other conditions leading to neonatal hypoxia, namely acute respiratory distress (ARD) (27), prematurity (28) and congenital heart disease (29), have been associated with decreased hippocampal volumes later in life. Furthermore, smaller hippocampal volumes were found in 12-year-old extracorporeal membrane oxygenation (ECMO) survivors compared to controls (30).

The underlying mechanism explaining the vulnerability of the hippocampus to hypoxia is unknown. It has been hypothesized that the high expression and potentiation of N-methyl-D-aspartate (NMDA) receptors in the hippocampus lead to this higher vulnerability (31–34). During hypoxia there is an excessive release of glutamate, leading to NMDA activation (31). NMDA activation causes an influx of calcium into the cell, resulting in neuronal death by the induction of apoptosis and necrosis (31). Furthermore, NMDA activation induces astrogliosis and microglial reactions, leading to impaired recovery of neurons (32).

The children reported in this cohort were part of a larger study population whose neuropsychological performance has been published previously (10). In the current subgroup of children who underwent MRI, a significantly impaired IQ and long-term episodic memory in the moderate HIE group was found compared to controls. This is in line with the previously reported data, although we now had smaller subgroups (10). Also, a trend of impaired memory functioning in mild HIE was found for some long-term episodic memory subtests. With a mean of 112, IQ scores in controls were significantly higher than in children with HIE and significantly higher than

the reference standard of 100, which is likely to be explained by the fact that their mothers had a higher educational level than the average population. This might partially explain the difference in IQ between children with HIE and healthy controls.

It is well known that infants with HIE have an increased risk of developing cognitive deficits, even in the absence of motor deficits (4,5). In a follow-up study of Robertson *et al.* 40% of the children with moderate HIE without neurological deficits had an impaired IQ and delayed learning skills at 8 years of age in comparison to controls (5). Similarly, Steinman *et al.* showed that 9% of the children following HIE without motor problems had a verbal IQ <70 at 4 years of age (35). Besides IQ, memory also has been shown to be affected in children with HIE (9,10). Mañeru *et al.* found that children with moderate HIE have impaired verbal and visual memory compared to mild HIE and controls (9). It was thought for many years that only infants with moderate and severe HIE are at risk for cognitive impairment (4–6,8,9,26). However, a possible trend of impaired memory function and IQ in mild HIE has been suggested recently (10,12,17). In a large Swedish cohort, even infants with Apgar scores <7 without encephalopathy had an increased risk of an impaired IQ at 18 years of age (36).

The observed predominant deficits in episodic memory in our cohort are in line with the literature (6,8,10,26). Semantic memory is relatively spared in HIE. It is hypothesized that semantic memory depends on the hippocampus and the temporal cortices, but that a spared temporal cortex is sufficient to compensate for hippocampal damage and to develop normal semantic memory functions (37). On the contrary, episodic memory seems to depend fully on the development of the hippocampus and is therefore more affected by hippocampal damage (37,38).

To study the association between HIE, hippocampal volumes and cognition, we first showed that HIE is an important predictor of hippocampal volumes using multivariable linear regression analysis. Next, we studied how the hippocampal volume is associated with IQ and memory using multivariable linear regression. Based on previous reports, we expected reduced hippocampal volumes to be associated with long-term episodic memory impairment (6,38). However, after correcting for age, sex and SES, the percentage hippocampus/ICV was only strongly associated with the episodic subtests for visuospatial long-term memory. In a larger cohort the effect of hippocampal volume on other subtests for long-term episodic memory would maybe also have been significant. The effect of hippocampal volume

on IQ and memory analyzed with multivariable linear regression has not been investigated in HIE before, but has been tested in other neonatal populations. In congenital heart diseases, smaller hippocampal volumes were strongly associated with impaired IQ, verbal and working memory (29) and in ECMO survivors with verbal long-term memory which is a subfield of long-term episodic memory (32). It is of interest that there seems to be an association between hippocampal volumes and memory in different neonatal groups experiencing hypoxia, but that the affected subfields differ.

Some limitations need to be addressed. Although this is the largest study investigating the effect of HIE on hippocampal volumes, the sample size was too small to perform multivariable linear regression in HIE1 and HIE2 separately. In addition, no severely affected children were included, because they either died in the neonatal period or were too severely impaired to be part of the study. The association between hippocampal damage, HIE and memory might be even stronger including this subgroup. Further, segmentation with Freesurfer has some limitations that might influence the quality of the segmentation: Freesurfer uses T1-weighted images that have less contrast between grey and white matter than T2-weighted images and the automated segmentation method is based on the resolution of 3.0 Tesla images instead of the 1.5 Tesla images that were used (39). Segmentation of images with less contrast or a lower resolution might influence the quality of the segmentation. Based on the quality of the scans it was not feasible to measure the volumes of specific subregions of the hippocampus and to relate these to the different memory fields. The segmentation of subregions might have given additional information, since the posterior subregion of the hippocampus might be more important for episodic memory than the anterior subregion (40). Furthermore, previous studies have shown that hippocampal volumes segmented by Freesurfer are larger than manual segmentations (41). However, automatic segmentation with Freesurfer is better reproducible and if the volume of the hippocampus is overestimated, this applies to all three groups so that it remains feasible to compare the groups.

This study shows that children with mild and moderate HIE can experience long-term episodic memory problems, which is associated with hippocampal damage. If hippocampal volumes can be measured in infancy, we may be able to use these measurements to predict memory problems, and intervene at a younger

age. More research is essential to determine at what age hippocampal atrophy first becomes visible. To answer this question, a follow-up study with sequential MRI scans of the brain of neonates with HIE is necessary, using a higher field strength of 3.0 Tesla instead of 1.5 Tesla. Additionally, an even larger follow-up study is necessary to investigate the memory problems in mild HIE and the exact association between hippocampal volumes and all fields of episodic memory. Further, research on the relation between episodic memory and the subregions of the hippocampus would provide us with an even more specific understanding of the long-term effect of HIE on hippocampal volumes and memory and the functional neuroanatomy of the hippocampus.

CONCLUSIONS

In conclusion, children with HIE have decreased hippocampal volumes, as well as impaired long-term episodic memory at the age of 9 to 10 years. Furthermore, hippocampal volumes are associated with long-term visuospatial memory impairment.

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DISCLOSURES

The authors have no conflict of interests to disclose

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CHAPTER 6

The papez circuit and school-age outcome following neonatal hypoxic-ischemic encephalopathy

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ABSTRACT

Background: The Papez circuit is important for memory function, which is often affected following neonatal hypoxic ischemic encephalopathy (HIE). The primary aim of this study was to investigate the association between injury to the Papez circuit, brain volumes, structural connectivity and neurodevelopmental outcome in 10-year-old children with a history of HIE. The secondary aim was to assess school-age outcome and brain development in children with a history of HIE, treated with or without hypothermia.

Methods: Ten-year-old children with HIE were included, who were treated with therapeutic hypothermia (HT group, 22 children) or would have qualified but were born before therapeutic hypothermia became standard of care (non-HT group, 28 children). Children completed neuropsychological and motor assessments and MRI. Grey and white matter volumes were automatically segmented. Mammillary bodies (MB) were scored as normal or atrophic. Tract-based spatial statistics was used to investigate the association between fractional anisotropy (FA) and MB, hippocampal volumes and neuropsychological assessments**.**

Results: Hippocampal volumes and MB atrophy were associated with total and performance IQ, processing speed and episodic memory in both HT and non-HT groups. Normal MB and a larger hippocampus were positively associated with FA values. Performance IQ and visual-spatial long-term memory (delayed recall) were significantly associated with higher FA values. Atrophy of the MB at school-age could be predicted using neonatal MRI.

Conclusion: Children with HIE suffered from neurocognitive and memory problems at school-age, irrespective of hypothermia treatment. Injury to the Papez circuit (hippocampus, fornix and MB) was associated with neurocognition and memory.

INTRODUCTION

Infants with hypoxic-ischemic encephalopathy (HIE) due to presumed perinatal asphyxia are at risk for death, motor problems, cognitive and memory deficits, behavioral problems and epilepsy (1,2). Infants with moderate to severe HIE are treated with whole body hypothermia, which has shown to improve 18-24 month survival without neurological disabilities (1,3,4). However, knowledge about the effect of therapeutic hypothermia on neurological outcome in childhood is limited. Therapeutic hypothermia improves survival and reduces cerebral palsy (CP) and epilepsy at school-age (5,6), but a considerable number of the children treated with hypothermia still has neurocognitive problems at school-age (5,7).

To understand and potentially improve neurodevelopmental outcome in these children, it is important to elucidate which brain structures contribute to these long-term problems. With regards to cognitive function, injury to the hippocampus is associated with a lower intelligence quotient (IQ) and memory scores in children with HIE (8,9). The hippocampus is part of the Papez circuit, which further includes the mammillary bodies (MB), fornix and anterior thalamus. It was recently demonstrated that the MB are often affected following HIE (10). Similar to the hippocampus, MB are known to be important for memory function (11).

Therefore, our primary aim was to investigate the association between injury to the Papez circuit, brain volumes, white matter integrity at 10 years of age and neurodevelopmental outcome in children with a history of HIE with and without therapeutic hypothermia. The secondary aim was to assess neurodevelopmental outcome in 10-year-old children with HIE treated with and without therapeutic hypothermia.

METHODS

Study population

For this observational study, children were eligible if they: 1) were born after a gestational age ≥36 weeks, 2) had been admitted to the neonatal intensive care unit of the UMC Utrecht or Isala Clinics between 2006 and 2009 because of acute perinatal asphyxia, 3) (would have) qualified for therapeutic hypothermia and 4) had reached the age of 10 years. Exclusion criteria were congenital brain abnormalities and/or other (chromosomal/metabolic) anomalies, acquired brain injury due to trauma or infection, severe cerebral palsy making magnetic resonance imaging (MRI)

and neurodevelopmental testing impossible and contraindications for MRI, such as braces, a pacemaker or claustrophobia.

Children born before therapeutic hypothermia became standard of care in 2008, who met the therapeutic hypothermia criteria, were included in the non-hypothermia group (non-HT group). Children that were treated with hypothermia were included in the hypothermia group (HT group).

Written parental informed consent and child assent was obtained for all study participants. The medical ethical committee of the UMC Utrecht approved the study (NL44807.041.14)

Neonatal clinical and MRI data

Baseline characteristics and standard follow-up data were retrospectively collected from the electronic patient files, as well as the neonatal MRI scans if these were available. MRI scans were conducted on a 1.5Tesla (T) in all infants in the non-HT group and partly in the HT group or 3.0T MR scan from 2009 onwards in the HT group (Philips Healthcare, Best, the Netherlands). The scan protocols included diffusion Weighted Imaging (DWI) (slice thickness=4mm, b-values of 0 and 1000s/mm^2 with acquisitive matrix of 128x77 at 1.5T and of 0 and 800s/mm^2 with acquisitive matrix of 112x89 at 3.0T), Inversion recovery or T1-weighted imaging (1.5T Inversion Recovery: slice thickness=2mm, TE=4147ms, TI=600ms, TR=30ms, acquisitive matrix of 256x153; 3.0T T1: slice thickness=2mm, TE=9.4ms, TR=4.6ms, acquisitive matrix of 192x152) and T2-weighted imaging (1.5T: slice thickness=2mm, TE=7565ms, TR=150ms, acquisitive matrix 256x184; 3.0T: slice thickness=2mm, TE=6293ms, TR=120ms, acquisitive matrix 336x234).

When available, the neonatal MRI scans were retrospectively scored according to Weeke *et al.* by two raters who were blinded to childhood outcome (12). This MRI score includes multiple items such as diffusion restriction of the thalamus and hippocampus. Injury to the MB on the neonatal MRI was assessed separately. MB were classified as normal, equivocal or abnormal based on neonatal T1- and T2-weighted sequences and DWI. The definition of normal was a similar signal intensity of the MB as the surrounding tissue and no swelling. The MB were scored “equivocal” if there appeared to be a higher signal intensity of the mammillary bodies on the T2-weighted image or a low signal on T1-weighted imaging but without swelling, so

it was uncertain if this could be a partial volume effect. Abnormal was defined as an increased signal intensity of the mammillary bodies on the T2-weighted image, a low signal on T1-weighted imaging or diffusion restriction on DWI, in combination with swelling of the MB (10).

Neurocognitive tests at 10 years of age

All children completed a full neuropsychological assessment performed by a pediatric neuropsychologist. Intelligence was tested with the Wechsler Intelligence Scale for Children, Third edition (WISC-III-NL) (13). Total IQ (TIQ), Verbal IQ (VIQ) and Performance IQ (PIQ) were derived from the WISC-III-NL. The Processing Speed Index of the WISC-III-NL was used to evaluate the speed of information processing (13). The verbal long-term memory was tested with the 15-words test (14), which consists of five learning trials with immediate recall of words (resulting in a total score) and a delayed recall after 25 minutes (resulting in a delayed score). Verbal Working Memory was measured with the Digit Span task of the WISC-III-NL (13). Visual-spatial Working Memory was tested with the spatial span task of the Wechsler nonverbal scale of ability (15). Visual-spatial long-term memory was assessed with the Rey Complex Figure Test (16). To test attention, four subtests of the Test of Everyday Attention of Children were administered (17).

The child's behavior was evaluated by parents and the teacher using questionnaires. Parents completed the Child Behavior Checklist (CBCL for children aged 6-18 years) and the Behavior Rating Inventory of Executive Function (BRIEF), which is a questionnaire regarding well-organized, purposeful, goal-directed, and problem-solving behavior (18). Teachers completed the BRIEF and the Teacher's Report Form for children aged 6-18 years that is comparable with the CBCL (19,20).

Motor assessment at 10 years of age

Motor performance was assessed with the Bruininks-Oseretsky Test of Motor Proficiency second edition (BOT-2). The BOT-2 scores four different domains: fine motor manual control, manual coordination, body coordination and strength and agility (21). Both a total composite score as composite scores of the four domains were used. Based on standard scores children are scored as well above average, above average, average, below average and well below average.

MRI at 10 years of age

MRI scans were conducted at a 3.0T MRI scanner without sedation (Philips Healthcare, Best, the Netherlands). The scan protocol contained among others 3D-T1-weighted imaging (TE=4.6ms; TR=10ms; slice-thickness=0.8mm, no gap), fluid attenuated inversion recovery (FLAIR) (TE=120ms; TR=10000ms; slice-thickness=4mm, no gap) and DWI (TE=96; TR=3317; using 13 non-diffusion weighted images and 111 diffusion weighted images with b-values of 500 s/mm^2 (15), 1000 s/mm^2 (32) and 2000 s/mm^2 (64)). In addition, a single non-diffusion weighted image was acquired in the opposite phase-encoding direction.

MRI – scoring

The 3D-T1-weighted and FLAIR images were scored according to the scoring method of van Kooij *et al.:* 1) no injury, 2) solitary white matter lesions and/or (focal) thinning of the corpus callosum, 3) watershed injury, 4) deep grey matter injury or 5) focal infarction (22).

Mammillary bodies at 10 years of age were scored based on the sagittal plane of the 3D-T1-weighted MRI. The mammillary bodies were categorized as normal if there was no atrophy or if they appeared smaller but were still detectable. Mammillary bodies were scored as atrophic if they were not visible (Figure 1). Two authors reached agreement on all cases. A third reader scored 18 random patients to determine the interrater variability.

MRI – volumetric measurements

Volumetric segmentation of 3D-T1-weighted MR scans was performed using Freesurfer version 6.0.0 (23). Segmentation of the subcortical deep grey matter structures and parcellation of the white matter and cortex was based on signal intensity differences. Images with large movement artefacts were excluded. This resulted in segmentation of 20 subcortical volumes and parcellation of 40 white matter and 40 cortical volumes. MRI scans with topological defects, skull strip errors or white matter errors were manually corrected prior to segmentation. For more elaborative details of this method, we refer to earlier publications (23). Total volumes refer to the uncorrected volume in ml and relative volumes to the volume of the structure as a percentage of total brain volume (TBV) excluding cerebral spinal fluid.

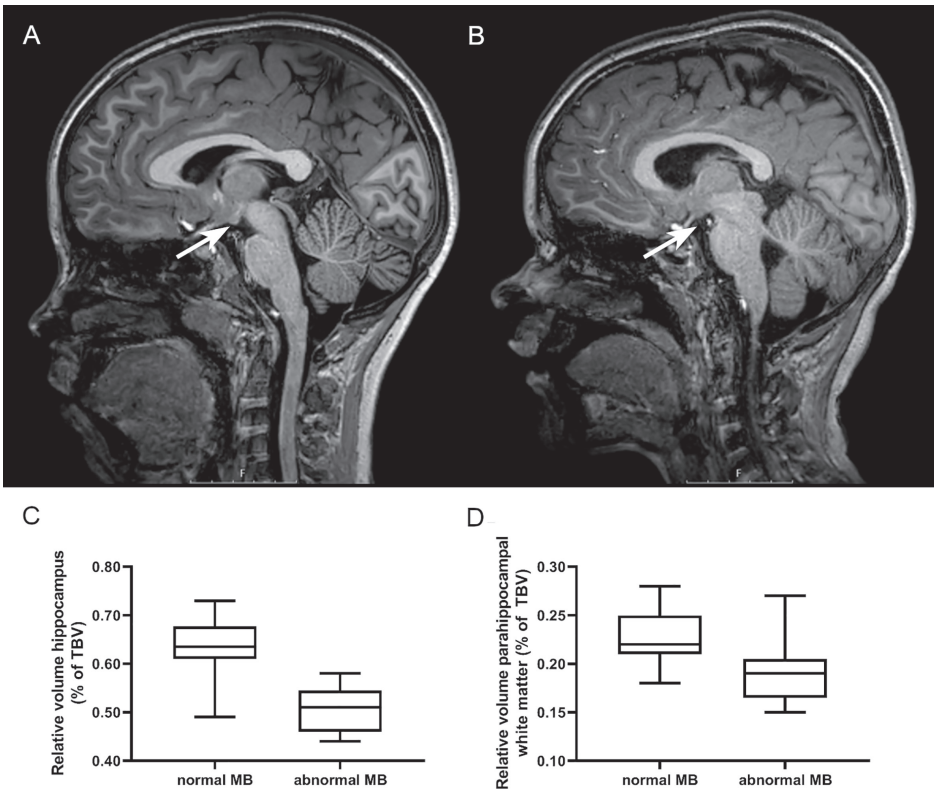

Figure 1: (Para)hippocampal volumes and MB. Example of normal MB (A) and atrophy of the MB (B) at 10 years of age. Differences in relative hippocampal volume (C) and parahippocampal volume (D) between children with normal MB and atrophy of the MB at 10 years of age.

MRI – TBSS

Image processing and analysis of the DWI were performed using tools from the FMRIB's Software Library (FSL v6.0.3) (24), DTI-ToolKit (DTI-TK) (v.2.3.1) and tract-based spatial statistics (TBSS v1.2) (25). FSL was used to correct for susceptibility induced distortions, using the non-diffusion weighted images with opposite-phase directions. Next, the data were corrected for eddy-induced distortions and head movements followed by a brain extraction to remove non-brain tissue. Finally, the tensor was fitted, after which all data were normalized to a template. DTI-TK, which uses a tensor based registration, was used to create a population specific template and to normalize all data. A mean Fractional Anisotropy (FA) map was derived from this template to create a mean FA skeleton. Using TBSS, an FA threshold of ≥0.15 was used to limit the inclusion of non-white matter voxels and voxels with high-inter subject variability. The FA values of each subject were projected on this skeleton for further analysis. A generalized linear model was used to assess the relationship between FA and the clinical variables, hippocampal volume and mammillary body atrophy, corrected for sex and age at scan. Analyses were performed using Randomise and were subject to family-wise-error correction for multiple comparisons following threshold-free cluster enhancement and p-values <0.05 were considered significant.

Statistical analysis

Statistical analysis was performed using SPSS version 25 (IBM corp., Armonk NY, United States). Baseline characteristics were compared between the non-HT versus HT group and normal versus abnormal MB using the independent t-test or Mann-Whitney-U test for continuous variables. For categorical variables the Chi^2-test was used. Outcome measures and volumes were also compared between the groups using the independent t-test or Mann-Whitney-U test depending on the Gaussian distribution.

Univariate analysis was performed with the outcome measure as dependent variable and the brain volume or MB atrophy score as independent variable. Non-significant associations were excluded for the following analysis. Multivariable linear regression was performed including the outcome measure as dependent variable and the brain volume/MB atrophy, therapeutic hypothermia and their interaction term as independent variables. Variables with a p-value <0.05 were retained in the model and those with a p-value ≥0.1 deleted.

Correlations between the different brain volumes were calculated with Pearson's correlation or Spearman's correlation test, depending on the normality.

Lastly, multivariable logistic regression was performed with a binary outcome measure (abnormal <-1SD) as dependent variable and total hippocampal volume or MB atrophy, total white matter, total grey matter, total lateral ventricle and total cerebellar volume as independent variables regarding multicollinearity. This analysis was performed to take other brain structures into account when assessing the effect of either hippocampal volume or MB atrophy on outcome. Again, variables with a p-value <0.05 were retained in the model and those with a p-value ≥0.1 deleted.

In general, p-values <0.05 were considered statistically significant. However, p-values were corrected for multiple comparison by dividing 0.05 by the number of brain volumes/tests.

RESULTS

Neonatal and childhood characteristics

Fifty patients were included in the study. For the non-HT group 38 patients were approached and 28 were included (72%). For the HT group 32 patients were approached and 22 were included (69%). See Figure 2 for the flowchart. In the non-HT group 6 of 38 approached patients (16%) had CP and in the HT group 1 of the 32 approached patients (3%), most of them were too severely affected to take part in the study.

The median birthweight was significantly lower in the non-HT group compared to the HT group. The median Apgar at 1 minute after birth was significantly lower in the HT group and the lactate significantly higher, which suggests that the HT group had more severe perinatal asphyxia.

At school-age, though not significantly different, in the non-HT group 7.1% of the children had epilepsy with anticonvulsive medication and none in the HT group. In the non-HT group 60.7% had no hearing or visual problems compared to 77.3% in the HT group.

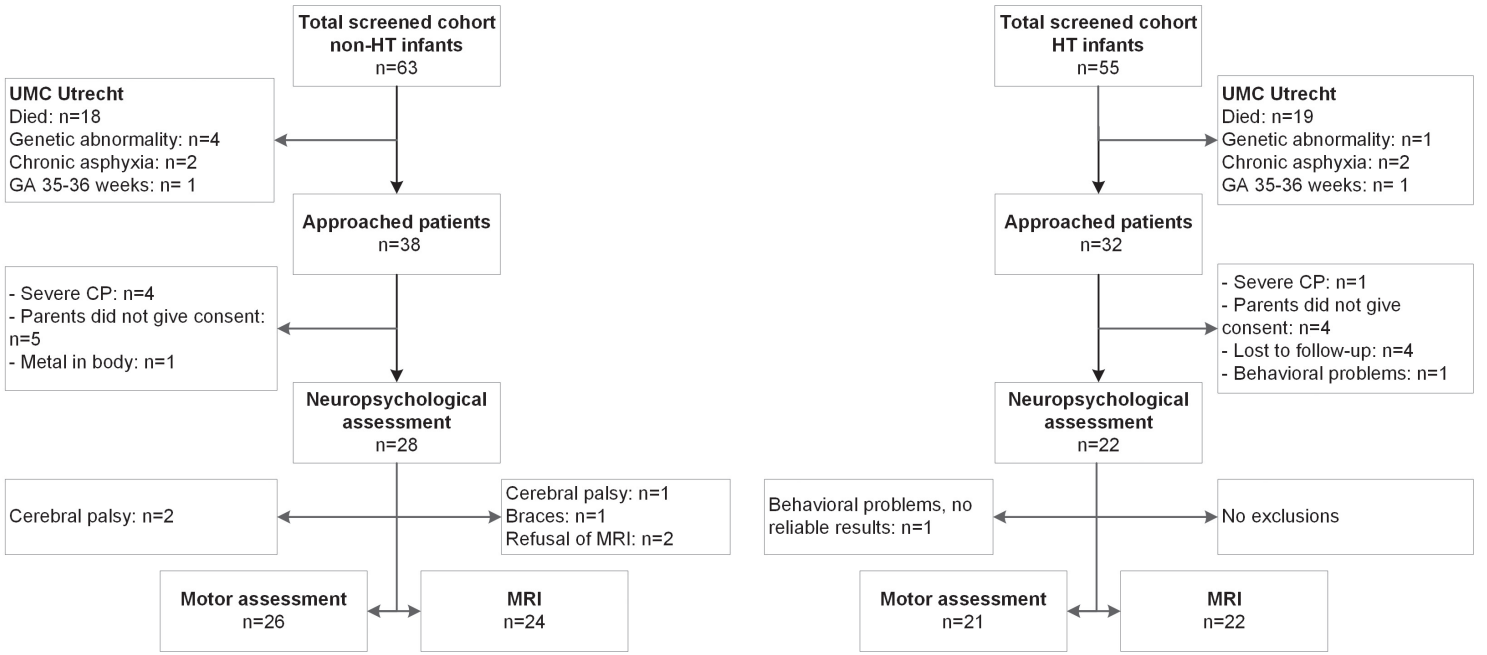

Figure 2: Flow chart. Flow chart of inclusion and exclusion for the children in the non-HT group (left) and for the children in the HT group (right). For Isala Clinics only data were available from the approached children.

Table 1 shows the neonatal and childhood characteristics.

Table 1: Neonatal and childhood characteristics.

Characteristic	non-HT (n=28)	HT (n=22)	p-value
Male, n (%)	17 (60.7)	15 (68.2)	0.59
Gestational age in weeks, median (IQR)	40.86 (39.50-41.61)	40.21 (39.68-41.50)	0.53
Birth weight in gram, median (IQR)	3485 (3103 - 3700)	3710 (3280 – 4410)	0.009 *
Apgar 1 min, median (IQR)	2 (2 – 4)	1 (1 – 2)	0.006*
Apgar 5 min, median (IQR)	4 (3 – 6)	3.5 (3 – 4.75)	0.18
Apgar 10 min, median (IQR)	6 (4.5 – 7.5)	5 (4 – 6)	0.18
Highest lactate within first 12 hours after birth, median (IQR)	7.7 (3.65 – 11.13)	12.4 (5.73 – 19.13)	<0.05*
Lowest pH within first 12 hours after birth, median (IQR)	7.14 (6.99 – 7.34)	6.99 (6.78 – 7.23)	0.06
Seizures, n (%)			0.78
No	5 (17.9)	5 (22.7)	
Yes, subclinical	5 (17.9)	5 (22.7)	
Yes, clinical	18 (64.3)	12 (54.5)	
Worst aEEG background pattern, n (%)			0.05
Normal (continuous normal voltage (+ sleep wake cycling), discontinuous low voltage)	9 (75)	6 (38)	
Abnormal (burst suppression, continuous low voltage, flat trace)	3 (25)	10 (63)	
Neonatal MRI, n (%)			0.01*
No	7 (25)	0 (0)	
Yes	21 (75)	22 (100)	
Total neonatal MRI score (total cohort), median (IQR)	8 (4.5 – 17.5)	4 (0 – 11)	0.03*
Total neonatal MRI score (Utrecht), median (IQR)	8.5 (6.5-19.0)	2.0 (0.0-12.5)	0.01*
Education mother, median (IQR)**	6 (5-6)	6 (5-6)	0.82
Education father, median (IQR)**	5 (5-6)	5 (5-6)	0.69
Age at follow-up study in months, median (IQR)	126 (125 – 127)	125 (123.8 – 127)	0.12

Table 1: Continued

Characteristic	non-HT (n=28)	HT (n=22)	p-value
Weight in kg, median (IQR)	34.8 (31.0 – 40.8)	38.4 (34.3 – 44.9)	0.14
Length in cm, median (IQR)	145.0 (141.3 – 148.0)	146.0 (142.1 – 149.5)	0.16
Head circumference in cm, median (IQR)	53.5 (51.5 – 54.2)	54.1 (53.0 – 54.5)	0.07
Grade repetition or skipping, n (%)			0.42
No	21 (75)	18 (81.8)	
Grade repetition	7 (25)	3 (13.6)	
Grade skipping	0 (0)	1 (4.5)	
Epilepsy, n (%)			0.41
Never	23 (82.1)	19 (86.4)	
In the past	3 (10.7)	2 (9.1)	
At present, without medication	0 (0)	1 (4.5)	
At present, with medication	2 (7.1)	0 (0)	
Audiovisual problems, n (%)			0.46
No	17 (60.7)	17 (77.3)	
Visual problems	9 (32.1)	4 (18.2)	
Hearing problems	2 (7.1)	1 (4.5)	

* p-value < 0.05

** SES according to Verhage classification

The total neonatal MRI score of Weeke *et al.* was significantly higher in the non-HT group compared to the HT group, but significantly fewer neonatal MR scans were performed in this group. MRI was only performed in Isala Clinics in the pre-hypothermia era if brain injury was suspected. In the UMC Utrecht all patients underwent an MRI, analysis of this subgroup showed also higher neonatal MRI scores in the non-HT group.

The MRI score of van Kooij *et al.* at 10 years of age was comparable between the HT (median 2 (range 1-5)) and non-HT group (median 2, (range 1-4), p=0.20). At 10 years of age, atrophy of the MB was present in 17% in the non-HT group and 50% in the HT group**.**

Neurodevelopmental outcome

Table 2 shows the motor and neuropsychological outcomes of the children in the non-HT and HT group. There were no differences in behavioral problems between the non-HT and HT group reported in CBCL and BRIEF questionnaires by parents or teachers.

Table 2: Neurodevelopmental outcome.

Neurodevelopmental outcome	HIE without hypothermia (n=28)	HIE with hypothermia (n=21)	p-value*
Intelligence			
TIQ, mean (SD)	94.3 (15.4)	94.1 (17.1)	0.96
VIQ, mean (SD)	97.7 (12.8)	98.8 (15.5)	0.78
PIQ, mean (SD)	94.0 (15.7)	90.3 (18.4)	0.46
Processing Speed, mean (SD)	102.3 (15.9)	91.1 (14.2)	0.02
Memory			
Verbal long-term memory – immediate recall, mean decile score (SD)	4.7 (3.0)	2.2 (2.3)	0.001**
Verbal long-term memory – delayed recall, mean decile score corrected for age (SD)	3.5 (2.7)	2.2 (2.2)	0.03
Verbal Working Memory, mean norm score (SD)	9.3 (4.1)	9.3 (2.8)	0.97
Visual-spatial Working Memory, mean t-score (SD)	47.7 (9.5)	48.5 (14.1)	0.84
Visual-spatial long-term memory – direct recall, mean t-score (SD)	39.2 (13.8)	32.7 (15.2)	0.12
Visual-spatial long-term memory – delayed recall, mean t-score (SD)	37.0 (11.9)	31.1 (13.9)	0.11
Attention			
Sustained attention, mean (SD)	8.2 (3.1)	8.7 (3.4)	0.55
Selective attention, mean (SD)	9.8 (2.4)	9.4 (2.6)	0.59
Motor outcome			
Total composite score, mean (SD)	48.6 (11.1)	43.0 (9.0)	0.06
Fine Manual Control standard score, mean (SD)	45.5 (9.9)	41.2 (9.3)	0.09
Manual Coordination standard score, mean (SD)	48.9 (8.5)	45.0 (11.1)	0.19
Body Coordination standard score, mean (SD)	43.0 (8.5)	40.3 (9.2)	0.32
Strength and agility standard score, mean (SD)	57.6 (9.9)	52.6 (8.2)	0.07

* For the outcomes of the neuropsychological assessments, after correction for multiple comparison, p-values < 0.004 were statistically significant. For the motor outcome, p-values < 0.01 were statistically significant.

** statistically significant

In the total group, 36.7% of the children scored below average. In the non-HT group, two infants had CP and could not be tested on the BOT-2; they were considered to have an abnormal score. Two of the children in the HT group, had impaired function of one arm because of obstetric brachial plexus injury.

In the total cohort, TIQ was below -2SD in 6.3% of the children and below -1SD in 29.2% of the children. VIQ was below -2SD in 2.1% and below -1SD in 21.3% of the children. PIQ was below -2SD in 8.7% and below -1SD in 37.0% of the children. The processing speed was below -2SD in 2.1% and below -1SD in 22.9% of the children.

MRI – MB atrophy

In the whole study group, 44 patients had a scan at 10 years of age that was considered to be of sufficient quality to score the MB and 39 patients had a neonatal MRI scan as well as an MRI scan at 10 years of age. Of the 44 patients, 38% had atrophy of the MB at 10 years of age.

The majority (91%) of the infants with normal neonatal MB also had normal MB at 10 years, while 76% of the infants with abnormal neonatal MB had atrophy at 10 years of age. The majority (90%) of the infants with equivocal MB on their neonatal scan, had normal MB at 10 years (Table 3).

Table 3: mammillary bodies on the neonatal MRI compared to the MRI at 10 years of age.

Neonatal MB score	Normal MB at 10y	Atrophy MB at 10y
Normal, n (%)	10 (91)	1 (9)
Equivocal, n (%)	9 (90)	1 (10)
Abnormal, n (%)	4 (24)	13 (76)

The third reader on MB scored the same as the others in 83% of the cases. In the three cases where the score was different, the MB was still present but smaller than usual.

Birth weight, Apgar scores and pH did not differ between children with normal MB and atrophy of the MB at 10 years of age. Children with abnormal MB did have higher lactate levels compared to children with normal MB (13.6 vs 8.9 mmol/L, p=0.04). A trend towards increased neonatal MRI scores according to Weeke *et al.* was seen in the children with MB atrophy, but this did not reach significance.

Brain volumes versus neurodevelopmental outcome

Forty-one children (82%) had a 3D-T1-weighted sequence at 10 years of age of sufficient quality for segmentation. In the univariate linear regressions with the brain structure as independent variable and the neuropsychological test score as dependent variable, MB and relative hippocampal volume were significantly associated with multiple neuropsychological tests after correcting for multiple comparisons. The parahippocampal white matter and caudal anterior cingulate cortex were, after correcting for multiple comparisons significantly, associated with processing speed. All structures that showed an association (uncorrected p-value <0.05) with one of the outcome measures are reported in supplemental Table 1.

For all variables that were significant in the univariate linear regression, multivariable linear regression was performed to test the association between the therapeutic hypothermia group and outcome measures. Therapeutic hypothermia was not associated with dependent variable for almost all models, except for verbal long-term memory (immediate recall). A significant regression equation was found for relative hippocampal volume and therapeutic hypothermia ($F(2.37) = 10.2$, $p<0.001$, $R^2=0.36$. A 1% decrease in the relative hippocampal volume resulted in 14.1 points lower verbal long-term memory, whereas therapeutic hypothermia decreased verbal long-term memory with 1.7 points. For the models including the MB score, a significant model was also found for verbal long-term memory (immediate recall): ($F(2,41) =11.2$, $p<0.001$, $R^2=0.35$). Verbal long-term memory was 1.4 points lower in the presence of atrophic MB, and 1.4 points lower following therapeutic hypothermia.

Mammillary bodies and neurodevelopmental outcome

Table 4 shows the differences in outcome measures between infants with normal and abnormal MB.

Mammillary bodies and brain volumes

All volumes assessed by Freesurfer were compared between infants with and without MB atrophy. After correction for multiple comparisons, children with MB atrophy had smaller relative hippocampal volumes ($p<0.001$) and smaller relative parahippocampal white matter ($p=0.001$) (Figure 1). The other relative subcortical, white matter and cortical relative volumes did not differ between the groups.

Table 4: Neurodevelopmental outcome and MB at follow-up

Test	Normal MB at 10y (n=30)	Atrophy MB at 10y (n=14)	p-value
Intelligence			
TIQ, mean (SD)	99.9 (13.5)	80.6 (11.3)	<0.001*
VIQ, mean (SD)	101.8 (13.3)	87.6 (8.9)	0.002*
PIQ, mean (SD)	98.0 (14.2)	76.6 (10.5)	<0.001*
Processing Speed, mean (SD)	100.9 (16.7)	86.7 (10.5)	0.007
Memory			
Verbal long-term memory – immediate recall, mean decile score (SD)	4.4 (2.8)	1.1 (0.3)	<0.001*
Verbal long-term memory – delayed recall, mean decile score corrected for age (SD)	3.6 (2.7)	1.0 (0.0)	<0.001*
Verbal Working Memory, mean norm score (SD)	9.3 (3.3)	9.1 (4.1)	0.90
Visual-spatial Working Memory, mean t-score (SD)	48.2 (12.0)	47.0 (12.2)	0.76
Visual-spatial long-term memory direct recall, mean t-score (SD)	40.8 (14.3)	22.7 (4.9)	<0.001*
Visual-spatial long-term memory delayed recall, mean t-score (SD)	39.9 (11.5)	20.5 (2.4)	<0.001*
Attention			
Sustained attention, mean (SD)	8.3 (3.1)	8.2 (3.7)	0.95
Selective attention, mean (SD)	10.0 (2.1)	8.5 (3.0)	0.11
Motor outcome			
Total composite score, mean (SD)	47.9 (10.0)	38.9 (7.1)	0.004*
Fine Manual Control standard score, mean (SD)	46.5 (9.1)	35.6 (5.9)	<0.001*
Manual Coordination standard score, mean (SD)	49.2 (9.0)	41.2 (10.0)	0.01
Body Coordination standard score, mean (SD)	42.6 (8.1)	36.9 (7.1)	0.03
Strength and agility standard score, mean (SD)	56.6 (9.5)	51.3 (8.7)	0.08

* For the outcomes of the neuropsychological assessments, after correction for multiple comparison, p-values < 0.004 were statistically significant. For the motor outcome, p-values < 0.01 were statistically significant.

** statistically significant

Multivariable logistic regression: Papez circuit and neurodevelopmental outcome
Based on the results above, the association between volumes of the Papez circuit (MB, fornix, hippocampus) and intelligence and memory appeared to be most relevant. These brain volumes were strongly correlated with each other (Supplemental Figure 1). Because of multicollinearity it was therefore impossible to include the different brain volumes in one model.

To correct for other brain volumes, we therefore made two different models. First, multivariable logistic regression was performed with the neuropsychological test results as dependent variable and MB atrophy, total white matter, total grey matter, total volume lateral ventricles and total cerebellar volume as independent variables. Secondly, we performed the same analyses but with total hippocampal volume instead of MB atrophy (Table 5).

Table 5: Multivariable logistic regression models for different neuropsychological tests with MB and with total hippocampal volume.

Outcome measure	Model with MB	Odds ratio (95%CI)	Model with hippocampus	Odds ratio (95%CI)
Cognition				
TIQ	MB atrophy	35.00 (5.53-221.39)	Hippocampus	0.19 (0.07-0.55)
VIQ	Grey matter MB atrophy	0.98 (0.97-1.00) 7.26 (1.1-48.33)	White matter	0.97 (0.95-1.00)
PIQ	MB atrophy	32.50 (5.12-206.16)	Hippocampus	0.19 (0.06-0.53)
Processing Speed	MB atrophy	3.75 (0.90-15.66)	Hippocampus	0.48 (0.23-0.99)
Memory				
Verbal long-term memory – immediate recall	MB atrophy	50.00 (5.40-463.20)	Hippocampus Lateral ventricles	0.24 (0.09-0.65) 1.2 (0.99-1.40)
Verbal long-term memory – delayed recall	MB atrophy	n/a [a]	Hippocampus Grey matter	0.16 (0.05-0.47) 1.02 (1.00-1.03)
Verbal Working Memory	Grey matter Lateral ventricles	0.99 (0.98-1.00) 1.11 (0.99-1.26)	Grey matter Lateral ventricles	0.99 (0.98-1.00) 1.11 (0.99-1.26)
Visual-spatial Working Memory	n.s.	n.s.	n.s.	n.s.

Table 5: Continued

Outcome measure	Model with MB	Odds ratio (95%CI)	Model with hippocampus	Odds ratio (95%CI)
Visual-spatial long-term memory direct recall	MB atrophy	n/a [a]	Hippocampus	0.33 (0.14-0.77)
Visual-spatial long-term memory delayed recall	MB atrophy	n/a [a]	Hippocampus	0.21 (0.07-0.63)

[a] No Odds Ratio's could be calculated, because none of the infants with atrophy of the MB had a normal outcome.

MRI – TBSS

Given the observed association between the hippocampal volume and MB atrophy and neuropsychological outcome, these parameters were used for TBSS. TBSS analyses showed that normal MB and larger hippocampal volumes, corrected for age and sex, had a wide spread positive effect on FA-values throughout the brain. For the neurodevelopmental measures, only PIQ and visual-spatial long-term memory delayed recall were significantly associated with FA values in the TBSS analyses. Age and sex were not associated with FA in any of the analyses. See Figure 3.

DISCUSSION

This study shows that children at 10 years of age with a history of HIE due to presumed perinatal asphyxia have long-term neurodevelopmental problems. This also applied to children who were treated with therapeutic hypothermia. Parts of the Papez circuit, i.e. hippocampal volumes and atrophy of the MB, were strongly associated with especially cognitive and memory problems. Furthermore, hippocampal volumes were significantly smaller in children with MB atrophy compared to normal MB. Even after correction for grey matter, white matter, cerebellar and lateral ventricle volumes, hippocampal volume and MB atrophy remained significantly associated with IQ measures, verbal long-term memory and visual-spatial long-term memory. Finally, children with normal MB or larger hippocampal volumes had significantly higher FA values throughout the white matter.

We were able to show that the volumes of different brain structures were associated with neurocognitive and memory outcomes at 10 years of age. However, after correction for multiple comparisons, only hippocampal volume and MB atrophy were strongly

associated with neurocognitive outcome and episodic memory. In non-cooled infants, the association between hippocampal volumes and episodic memory functioning has been described previously (8,9). The present study confirmed those findings in cooled infants. The MB are also known to be important for episodic memory (26). Recently, Molavi *et al.* described that 13.2% of the infants with HIE had an abnormal signal intensity on their T2-weighted MRI (10). The strong association between subsequent atrophy of the MB and long-term problems has not been described previously in children with HIE. The association between smaller MB volumes and cognitive and memory impairments has recently been shown in adolescents with a Fontan procedure for a single ventricular heart disease (27). The MB are easy to score without additional software or post-processing and atrophy is significantly associated with neurodevelopmental outcome, hippocampal volumes and FA values, making it an easy indicator for neurodevelopmental outcome. Atrophy of the MB is already visible at three months after birth (10). So, the MB are important to routinely assess in infants with HIE and should be added to the available scoring systems.

Besides the hippocampus and MB, the anterior thalamus and fornix are also part of the Papez circuit (11). The thalamus was not associated with outcome in this study, but we were not able to segment the anterior thalamus separately. Also, the fornix could not be reliably segmented with Freesurfer. However, we did find a significant association between normal MB and higher FA values in the fornix and between larger hippocampal volumes and higher FA values in the fornix.

Other brain structures should also be taken into account when assessing brain injury in children with HIE during follow-up, since perinatal asphyxia leads to global hypoxic-ischemia of the brain (28). For example, in the TBSS analyses FA values in the anterior corpus callosum were associated with PIQ and visual-spatial long-term memory, and the volume of the corpus callosum was also associated with processing speed and verbal long-term memory (although not significant after correction for multiple comparisons). Furthermore, the amygdala and parahippocampal white matter were associated with hippocampal volumes and might be related to neurodevelopmental outcome in a larger cohort.

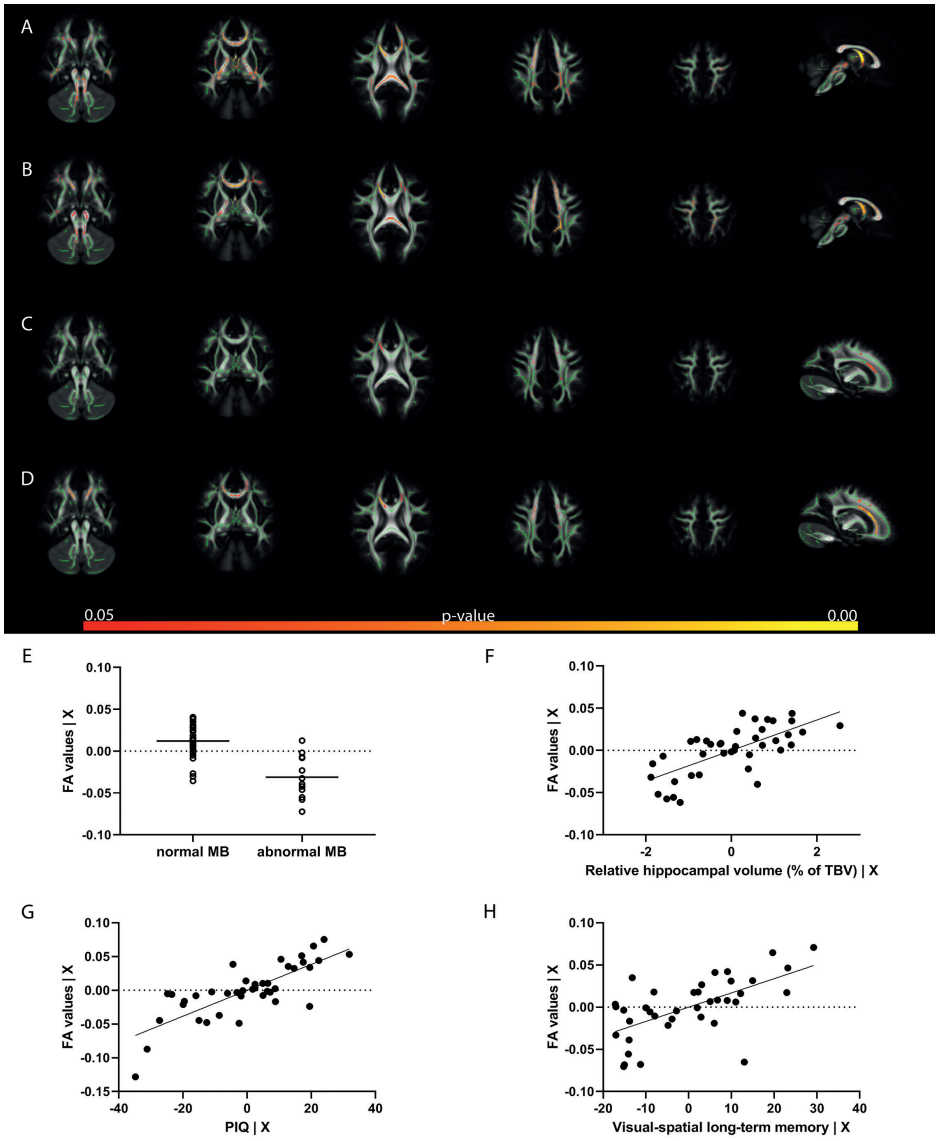

Figure 3: TBSS results. Results of TBSS analyses with five axial views and one midsagittal view for the MB (A) and hippocampus (B) and a parasagittal view for the neurocognitive and memory measures (C, D). All analyses were corrected for age and sex. This Figure shows the associations between FA values and MB (A,E), relative hippocampal volume (B,F), PIQ (C,G) and visual-spatial long-term memory (delayed recall) (D,H). In the plots the unstandardized residuals for the FA values corrected for age and sex are visualized on the y axis, and the MB (E) or the unstandardized residuals for relative hippocampal volume (F), PIQ (G) or visual-spatial long-term memory (H) corrected for age and sex on the x-axis.

Interestingly, FA values conducted with TBSS were only positively associated with PIQ and visual-spatial long-term memory. In the neonatal period, lower FA values in e.g. the posterior limb or the internal capsule and corpus callosum are known to be predictive for long-term outcome (29). Literature about FA values at school-age in children with a history of HIE following perinatal asphyxia is scarce. Laporta-Hoyos *et al.* found that FA values were related to IQ and executive functioning in adults with dyskinetic CP, but not in the controls (30). Attention and information processing were not associated with FA values in both groups (30). The authors concluded that FA values are only associated with cognition in the presence of severe brain injury (30). This might explain the absence of an association between FA and IQ and memory in our population, since brain injury at neonatal age and at 10 years of age was only mild to moderate.

Our study also showed that, although therapeutic hypothermia has proven to be neuroprotective, neurodevelopmental problems at school-age are still prevalent. In the short-term, therapeutic hypothermia reduces death, epilepsy and CP in infants with HIE (6,31). In our cohort, CP was also less often diagnosed in the screened population that was cooled compared to the non-cooled cohort. However, the actual incidence of CP and death cannot be compared due to the study design. The comparisons between the HT and non-HT group should therefore be interpreted with caution, because this is not a randomized controlled trial. We noted that the lactate levels were higher and Apgar scores were lower in the HT group compared to the non-HT group which suggests that the HT group was more severely asphyxiated. Also, more brain injury at the age of 10 years was found in the HT group. This might explain the finding that therapeutic hypothermia was associated with a lower verbal long-term memory (immediate recall).

Nevertheless, we were able to show that memory and cognitive problems can develop at school-age even in children who were treated with therapeutic hypothermia. Shankaran *et al.* described that the incidence of death and CP were lower in 6- to 7-year-old children with HIE that were cooled compared to controls, but cognitive, attention and visuospatial function did not significantly improve (32). The TOBY trial also revealed a better survival with an IQ above 85, but similar intelligence, memory, learning, sensorimotor and visuospatial processing between cooled and non-cooled children at 6-7 years of age (5). Furthermore, quality of life at 6-7 years of age was comparable in the cooled and non-cooled group (33).

Memory and behavioral problems are often not recognized until school-age, emphasizing the need for long-term follow-up. Hayes *et al.* confirmed that children with HIE show more problems at the age of 42 months and above in domains of attention, memory and behavior than was expected based on the 2 year assessment with the Bayley-III (34). Furthermore, it has been reported that one third of the cooled infants without CP still experienced motor problems at the age of 6 to 8 years that were not recognized during the regular 18 month examination (35). Clinicians should realize, that even with a normal 18-24 month follow-up, deficits can still become apparent at school-age.

This study has several limitations. First of all, as mentioned before, the study design is not randomized and does not allow us to draw conclusions about the effect of therapeutic hypothermia at school-age. Also, no data of healthy controls were available for comparison. Secondly, a bias in screening and inclusion criteria might have resulted in slightly different groups. Lactate and Apgar scores suggest that the HT group was more severely affected than the non-HT group. Thompson scores were not conducted in the non-HT group, but the patient files were studied to ascertain that infants would have fulfilled the hypothermia criteria. Furthermore, numbers about neonatal death are difficult to compare, since in the hypothermia era all infants born in level II hospitals that fulfilled hypothermia criteria were transferred to the level III hospitals and before 2008 one cannot exclude that infants who were in a very poor neurological condition were not transferred to the level II hospital and may have died in the level II hospitals. Lastly, the brain volumes were highly correlated. This made it impossible to include different brain volumes in a model and determine which brain volume is most associated with different outcome measures. This may be solved by principal component analysis in future larger studies.

This study has multiple implications for clinical care and follow-up of infants with HIE. First of all, therapeutic hypothermia does decrease neonatal death, CP, and epilepsy, but does not sufficiently protect the brain to prevent cognitive and memory problems at school-age. Different add-on therapies are currently being investigated in larger trials (36), which hopefully help to further improve long-term neurodevelopmental outcome. Furthermore, early atrophy of the MB is strongly predictive for long-term outcome and easy to assess. This might be an early indicator for long-term cognitive outcome, also in infants who are treated with therapeutic hypothermia. Finally, this study underlines the importance of long-

term follow-up into childhood, and maybe even adulthood, to assess definitive outcomes of infants with HIE.

CONCLUSIONS

In summary, children with HIE that were cooled still suffer from neurocognitive and memory problems at 10 years of age. Structures from the Papez circuit were strongly associated with neurocognitive and memory development. Neonatal abnormalities of the MB in the acute phase of HIE can be a biomarker to predict atrophy of the MB, which appears to be associated with neurodevelopmental difficulties at school-age.

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DISCLOSURES

Floris Groenendaal is an expert witness in cases of perinatal asphyxia. He is also co-inventor of 2-iminobiotin for neonatal neuroprotection. The other authors have no conflict of interest to declare.

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SUPPLEMENTAL MATERIAL

Supplemental Table 1: univariate associations between all volumes and outcome measures with p-value <0.05.

Outcome measure	Relative volume (%) or MB atrophy	B-value	R^2 value	p-value
TIQ	Hippocampus	65.8	0.112	0.032
	MB atrophy	-9.6	0.337	<0.001**
	Putamen	-85.7	0.161	0.009
	Caudal anterior cingulate white matter	-122.8	0.147	0.015
	Rostal anterior cingulate white matter	-188.3	0.191	0.005
	Superior frontal white matter	29.8	0.129	0.023
	Insula white matter	-48.6	0.146	0.015
VIQ	MB atrophy	-7.1	0.211	0.002**
	Putamen	-60.5	0.106	0.046
	Superior frontal white matter	26.0	0.133	0.026
PIQ	Hippocampus	88.8	0.190	0.006
	MB atrophy	-10.7	0.356	<0.001**
	Putamen	-76.7	0.119	0.031
	Caudal anterior cingulate white matter	-116.1	0.124	0.030
	Insula white matter	-49.2	0.136	0.023
	Rostal anterior cingulate cortex	-100.6	0.115	0.040
	Frontal cortex	-124.3	0.109	0.046
Processing speed	Hippocampus	97.2	0.234	0.002**
	MB atrophy	-7.1	0.162	0.007
	Amygdala	221.1	0.136	0.019
	Thalamus	54.8	0.100	0.046
	Corpus callosum	160.6	0.108	0.038
	Caudal anterior cingulate white matter	-115.5	0.114	0.036
	Parahippocampal white matter	261.3	0.292	<0.001**
	Caudal anterior cingulate cortex	-153.8	0.279	0.001**
	Parahippocampal cortex	157.2	0.160	0.013
	Pericalcarina cortex	67.0	0.113	0.039
	Rostal anterior cingulate cortex	-96.7	0.136	0.023

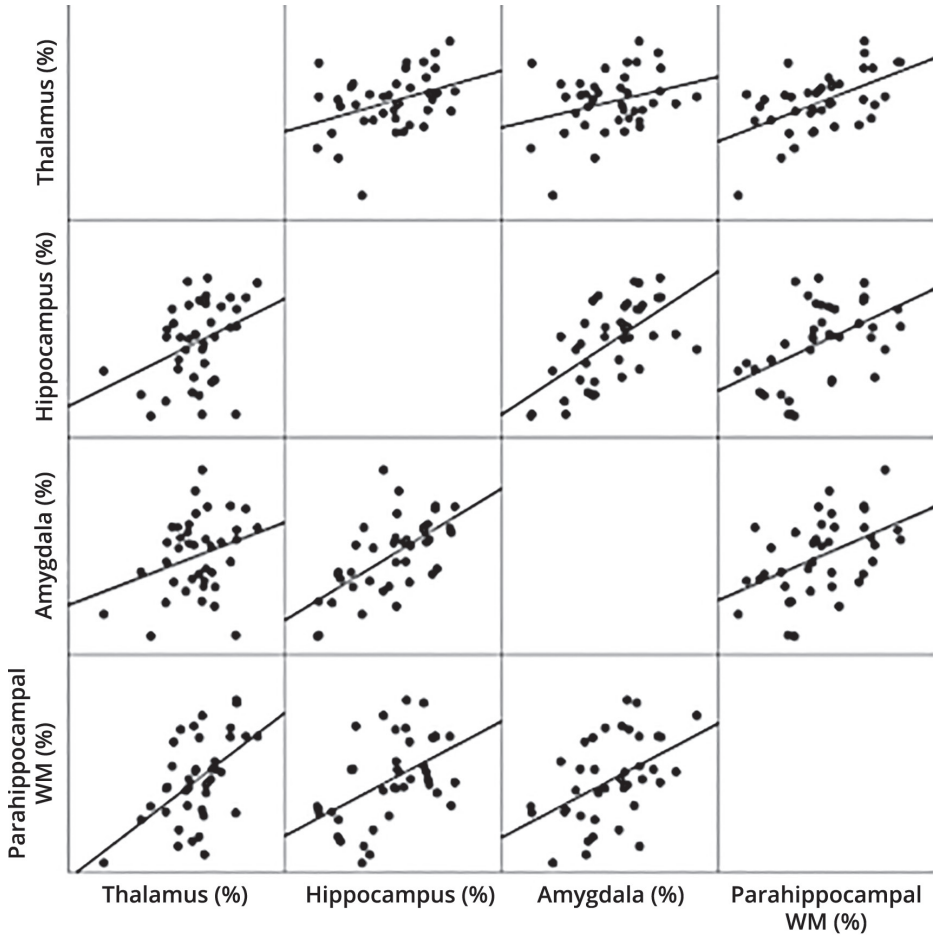
Supplemental Table 1: Continued

Outcome measure	Relative volume (%) or MB atrophy	B-value	R^2 value	p-value
Verbal long-term memory, immediate recall	Hippocampus	19.0	0.284	<0.001**
	MB atrophy	-1.7	0.298	<0.001**
	Corpus callosum	35.7	0.169	0.008
	Isthmus cingulate white matter	22.3	0.141	0.018
	Parahippocampal white matter	41.5	0.226	0.002
	Superior temporal white matter	12.9	0.158	0.012
	Supra marginal white matter	8.9	0.143	0.018
	Caudal anterior cingulate cortex	-22.1	0.176	0.009
	Lateral occipital cortex	-5.9	0.185	0.007
	Posterior cingulate cortex	-14.5	0.128	0.028
Verbal long-term memory, delayed recall	Hippocampus	11.8	0.135	0.020
	MB atrophy	-1.3	0.225	0.001**
	Corpus callosum	27.0	0.120	0.029
	Brain stem	6.8	0.125	0.025
	Isthmus cingulate white matter	22.5	0.178	0.008
	Parahippocampal white matter	38.0	0.234	0.002
	Lateral occipital cortex	-4.6	0.143	0.019
	Medial orbitofrontal cortex	-9.8	0.109	0.043
	Frontal cortex	-24.1	0.149	0.017
Visual-spatial working memory	Precuneus cortex	18.6	0.111	0.047
Verbal working memory	Precuneus white matter	7.9	0.118	0.033
	Superior parietal white matter	5.6	0.150	0.015
	Supra marginal white matter	7.6	0.101	0.048
	Frontal white matter	-104.2	0.109	0.040

Supplemental Table 1: Continued

Outcome measure	Relative volume (%) or MB atrophy	B-value	R^2 value	p-value
Visual-spatial long-term memory, direct recall	Hippocampus	67.1	0.133	0.022
	MB atrophy	-9.1	0.315	<0.001**
	Inferior temporal white matter	56.1	0.127	0.028
	Middle temporal white matter	57.0	0.128	0.027
	Superior frontal white matter	26.4	0.126	0.029
	Lateral orbitofrontal cortex	-42.3	0.161	0.014
	Pars orbitalis cortex	-90.8	0.132	0.027
	Precentral cortex	-24.6	0.110	0.045
	Frontal cortex	-159.7	0.200	0.006
Visual-spatial long-term, delayed recall	Hippocampus	74.4	0.219	0.003
	MB atrophy	-9.7	0.446	<0.001**
	Entorhinal white matter	193.3	0.119	0.036
	Parahippocampal white matter	136.0	0.124	0.033
	Precentral white matter	-28.0	0.112	0.042
	Superior frontal white matter	25.9	0.129	0.029
	Isthmus cingulate cortex	82.7	0.110	0.048
	Lateral orbitofrontal cortex	-32.8	0.126	0.034
	Pars orbitalis cortex	-72.5	0.109	0.049
	Precentral cortex	-30.3	0.215	0.004
	Frontal cortex	-114.5	0.133	0.029

* After correction for multiple comparison, p-values < 0.0025 are statistically significant for the segmentations and p-values of <0.0015 for the parcellations of cortex and white matter.



Supplemental Figure 1: correlations between the different brain volumes

Part II

NEUROPROTECTION

CHAPTER 7

Allopurinol: old drug, new indication in neonates?

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ABSTRACT

Background: Hypoxic-ischemic encephalopathy (HIE) is an important cause of neonatal mortality and neurological morbidity, even despite hypothermia treatment. Neuronal damage in these infants is partly caused by the production of superoxide via the xanthine-oxidase pathway and concomitant free radical formation. Allopurinol is a xanthine-oxidase inhibitor and can potentially reduce the formation of these superoxides that lead to brain damage in HIE.

Methods: The aim of this review is to provide an overview of the animal and clinical data about the neuroprotective effect of allopurinol in HIE and the relevant mechanisms leading to brain injury in HIE.

Results: A possible neuroprotective effect of allopurinol has been suggested based on several preclinical studies in rats, piglets and sheep. Allopurinol seemed to inhibit the formation of superoxide and to scavenge free radicals directly, but the effect on brain damage was inconclusive in these preclinical trials. The neuroprotective effect was also investigated in neonates with HIE. In three small studies, in which, allopurinol was administered postnatally and a pilot and one multi-center study, in which, allopurinol was administered antenatally, a possible beneficial effect was found. After combining the data of 2 postnatal allopurinol studies, long-term outcome was only improved in infants with moderate HIE, therefore, large-scale studies are needed. Additionally, safety, pharmacokinetics and the neuroprotective effect of allopurinol in other neonatal populations are discussed in this review.

Conclusion: The available literature is not conclusive whether allopurinol is a neuroprotective add-on therapy in infants with HIE. More research is needed to establish the neuroprotective effect of allopurinol especially in combination with hypothermia.

Allopurinol: from gout therapy to neuroprotective agent

Allopurinol is a xanthine-oxidase inhibitor that inhibits the production of uric acid. The enzyme xanthine-oxidase converts hypoxanthine into uric acid (1). Allopurinol is almost completely metabolized into oxypurinol by aldehyde oxidase in the liver and is eliminated by the kidneys (1). Oxypurinol is the active metabolite of allopurinol and predominantly inhibits xanthine-oxidase because allopurinol is almost completely metabolized into oxypurinol and oxypurinol has a longer half-life than allopurinol (2).

Historically, allopurinol is a well-known therapy for gout in adults by reducing the concentrations of uric acid and thereby the formation of uric acid crystals (1). Furthermore, allopurinol is widely used as therapy for tumor lysis syndrome and kidney stones. Allopurinol also inhibits the production of free radicals. Therefore, new indications have been investigated for this 'old drug'. Currently, research is focusing on possible cardioprotective and neuroprotective effects of allopurinol by inhibiting the formation of the free radical superoxide production in neonates and adults. Free radicals lead to cell damage by causing oxidative stress i.e. the peroxidation of proteins, lipids and DNA, which in turn causes mitochondrial damage and induce apoptotic pathways (3,4). These free radicals are formed in the presence of oxygen: hypoxanthine and oxygen are converted into uric acid and superoxide by xanthine-oxidase (5). Allopurinol and oxypurinol can both inhibit xanthine-oxidase and thereby the production of superoxide. An additional working mechanism of allopurinol and oxypurinol is free radical scavenging; in neonates as well non-protein-bound iron (NPBI) and the hydroxyl radical seemed to be directly scavenged by allopurinol and oxypurinol (6,7).

From the 1970's onwards, allopurinol was investigated in animal studies because of its possible cardioprotective effect by inhibiting the formation of oxygen radicals (8-13). Following animal studies, it was shown that allopurinol pre-treatment in adult patients undergoing coronary bypass surgery led to a better recovery (14). Also, the hospital mortality, cardiac performance and postoperative recovery, defined as less inotropic and mechanical support, improved after cardiac bypass surgery in allopurinol treated patients (15). On the contrary, other studies did not find an improvement in cardiac function after surgery in these patients (10). Allopurinol was also investigated as a therapy for chronic heart failure and angina pectoris. The hypothesis was that inhibition of the formation of uric acid and the free radical superoxide might prevent endothelial damage and myocardial oxidative stress (16).

However, until this moment, the use of allopurinol as a cardioprotective agent in adults with cardiovascular diseases remains controversial and larger prospective studies are required to determine the cardioprotective effect of allopurinol (17).

The earlier mentioned cardiac studies, which showed that the production of free radicals was leading to hypoxic-ischemic damage of the heart, were the basis of the hypothesis that allopurinol might also be beneficial for the prevention of hypoxic-ischemic damage of the brain. Perinatal asphyxia in the newborn leads to the production of hypoxanthine and the activation of xanthine-oxidase leading to subsequent brain damage (5). Considering that allopurinol can inhibit the xanthine-oxidase pathway, an allopurinol-induced reduction of superoxide might be neuroprotective in hypoxic-ischemic encephalopathy (HIE).

Hypoxic-ischemic encephalopathy

One to eight per 1000 live born neonates experience HIE caused by perinatal asphyxia (18). Perinatal asphyxia is one of the most important causes of death and long-term neurological damage in term born neonates. The current standard of care in perinatal asphyxia is moderate hypothermia for 72 hours, starting within 6 hours after birth. In the TOBY trial, cooled infants were followed up until the age of 6 to 7 years and their outcome was compared to non-cooled infants (19,20). Despite hypothermia, approximately 45% of these neonates had an adverse outcome at the age of 2 years, defined as severely impaired neurological outcome or death, compared to 53% in the non-cooled group (RR 0.86, 95%CI 0.68-1.07) (19). At the age of 6 to 7 years, 55% of the cooled infants that survived experienced neurologic abnormalities compared to 72% in the non-cooled group (20). Neurologic abnormalities were defined as an IQ score below 85, abnormalities in neurologic examination, hearing or vision. The survival rates did not differ significantly (20). In conclusion, hypothermia is an effective neuroprotective strategy for infants with HIE, however still a sizable amount of these infants dies or has an adverse neurological outcome. Therefore, additional neuroprotective therapies are essential to reduce neurological damage in these infants.

Pathophysiology of brain damage after perinatal asphyxia

Brain damage after perinatal asphyxia is caused by hypoxia leading to neuronal cell damage. In neonates with perinatal asphyxia there are two moments of neuronal cell damage: a first peak caused by primary energy failure during the hypoxic event

at birth and a second peak caused by the reoxygenation and reperfusion after birth, called reperfusion injury (21,22).

Figure 1 shows the pathophysiologic processes of brain damage after perinatal asphyxia.

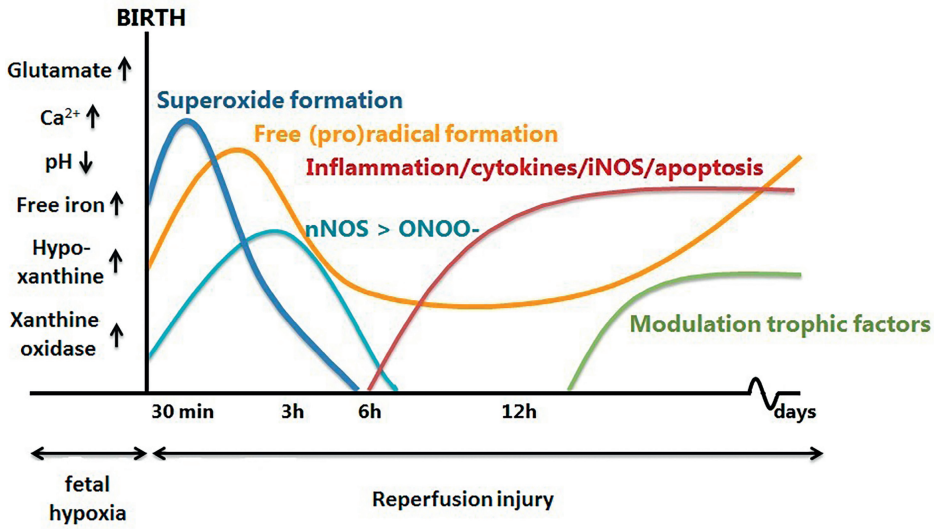

Figure 1: Activated pathways leading to brain damage in HIE (22). (adapted from van Bel and Groenendaal, 2016)

The acute moment of hypoxia during birth (which is caused by deficient oxygen supply e.g. due to placental abruption or other sentinel events) results in primary energy failure and, consequently, the degradation of adenosine triphosphate (ATP) and eventually necrotic cell death (21,22). This leads to the release of excitatory neurotransmitters as glutamate, which results in the over-activation of the N-methyl D-aspartate (NMDA) receptors and failure of the Na-K-ATPase pump (22-24). Both generate a calcium influx into the cells, leading to the activation of enzymes, such as proteases, that initiate predominantly apoptotic cell death (21,25). The acute moment of fetal hypoxia also initiates the production of pro-radicals, such as NPBI, that later on can form free radicals after reoxygenation upon birth (22,26-28). ATP degradation also increases the level of adenosine, which is converted into hypoxanthine via inosine and cumulates during fetal hypoxia (5,29). These elevated hypoxanthine

levels can also lead to production of superoxide after birth because xanthine-oxidase levels also rise during hypoxia (29).

After birth, several pathways are activated because of reoxygenation leading to an excessive production of free radicals and superoxide in HIE. The increased levels of hypoxanthine and xanthine-oxidase in combination with the extra supply of oxygen during reoxygenation lead to the activation of the xanthine-oxidase pathway (22,30-32). Xanthine-oxidase converts hypoxanthine and oxygen into uric acid and superoxide (5), see Figure 2. This inappropriate superoxide production reaches its peak within 30 minutes after birth and plays a central role in the activation of destructive molecular pathways (30-32). The superoxide-derived hydrogen peroxide interacts with pro-radicals such as NBPI resulting in the formation of the very toxic hydroxyl free radical (22). Also nitric oxide (NO), derived from an increased production of endothelial and neuronal nitric oxide synthase, reacts with superoxide to form the toxic compound peroxynitrite ($ONOO^-$) (22,27,33,34). These free radicals and toxic compounds will cause additional neuronal cell damage, but they also activate an inflammatory response leading to the formation of (pro)inflammatory cytokines from about 6-12 hours after birth onwards (22,35). The subsequent apoptotic activity and eventually down regulation of trophic factors also contribute to the neuronal cell injury and start about 12 to 24 hours after birth and can last for days and even weeks (22,36,37). Given this potential pivotal role of superoxide, acute reduction of superoxide formation on top of moderate hypothermia by allopurinol might lead to a reduction of brain damage after perinatal asphyxia.

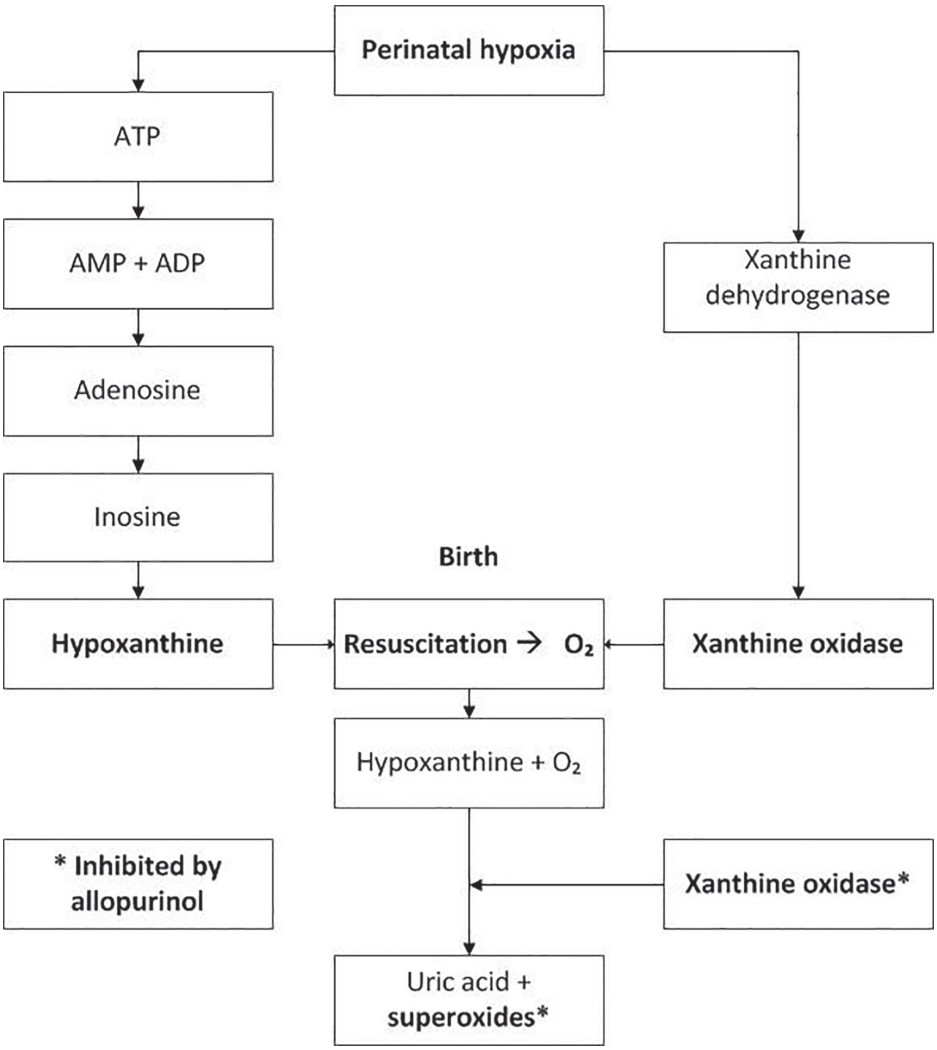

Figure 2: Before birth, during perinatal hypoxia, several pathways are activated, including the production of hypoxanthine and xanthine-oxidase. Because of hypoxia, ATP is converted to adenosine monophosphate (AMP) and adenosine diphosphate (ADP), AMP leads to the production of adenosine and consequently to inosine, which increases the concentration of hypoxanthine in the blood. Also, xanthine-oxidase concentrations rise because of hypoxia by the production of xanthine dehydrogenase. After birth, the supply of extra oxygen during reperfusion leads to the production of superoxide. Xanthine-oxidase causes a reaction in which hypoxanthine and oxygen are converted to uric acid and superoxide. Allopurinol inhibits xanthine-oxidase and thereby reduces the formation of superoxide. Further, allopurinol is thought to have a direct hydroxyl radical scavenger effect and is a non-protein-bound iron chelator.

Preclinical studies – neonatal administration for HIE

The first animal studies investigating the neuroprotective effect of allopurinol in HIE were performed by Palmer and colleagues in 7-day-old rat pups (38-40). In the first studies allopurinol was administrated 30 minutes before inducing hypoxia and this resulted in a reduction of cerebral edema and a lower incidence of infarction (39). Furthermore, ATP increased and the Pi/PCr ratio decreased at 31P-NMR-spectroscopy in rats, which represents a preserved cerebral metabolism (38). In a subsequent study by this group using 7-day-old rat pups, allopurinol was given 15 minutes after inducing hypoxia. This led to reduced atrophy and cerebral edema, as well as less overall brain injury based on a histology scoring system (40). In the studies in which allopurinol was administered before inducing hypoxia core temperature was not measured, so it cannot be excluded that these rats were hypothermic and that this partly has influenced the neuroprotective effect (38,39). In the study of Palmer *et al.* in 1993, the core body temperature was measured before and after allopurinol administration. In the placebo group the temperature varied 0.16±0.18 degrees of Celsius before and after placebo administration and in the allopurinol 0.09±0.32 degrees of Celsius, so these are only minor variations in temperature (40). Hypothermia is therefore an unlikely explanation for the beneficial effect of allopurinol in these rat pups.

The effect of allopurinol in asphyxiated piglets was investigated with phosphorous magnetic resonance spectroscopy, Near Infrared Spectroscopy (NIRS), electroencephalography (EEG) and histology by Peeters-Scholte *et al.* (41,42). After one hour of hypoxia, allopurinol was administered directly during start of reperfusion and 12 hours after reperfusion. On magnetic resonance spectroscopy, a preservation of the cerebral energy status was found and based on T2-weighted magnetic resonance imaging (MRI) less edema in the cortex, striatum and thalami were found in allopurinol treated animals. However, there was no improvement on NIRS, aEEG or histology (41,42). The authors' explanation was that the moderately asphyxiated piglets might have experienced a positive effect on outcome, but that treatment with allopurinol had no effect when the brain is too severely damaged. Although the cerebral energy status might have been preserved, severe brain damage might already have occurred and did not improve after allopurinol. However, no sub-analysis was performed to confirm this hypothesis (41, 42). Rectal temperature remained stable in both groups during the study. Only short-term outcomes until 24 hours after birth were measured (41,42).

Several animal studies have been performed to investigate the neuroprotective mechanisms of allopurinol in HIE. Marro *et al.* showed that uric acid levels were reduced in allopurinol treated piglets with HIE (43). This implicated that the xanthine-oxidase pathway was indeed inhibited after allopurinol administration in piglets, since uric acid is the final product in this cascade. The same group also showed that the Na-K-ATPase pump, that often fails in HIE, was more active in newborn animals suffering HIE following allopurinol administration compared to controls (43,44). As discussed earlier, failure of the Na-K-ATPase pump leads to a calcium influx into the cells which results in cell damage and (pro)radical formation. A decreased failure of the Na-K-ATPase pump might result in less neuronal cell damage. More recently, Marro *et al.* showed that adenosine and inosine levels were higher in allopurinol treated piglets than in controls (45). This suggested that allopurinol also reduced the conversion of adenosine and inosine into hypoxanthine. Lower hypoxanthine levels might result in less superoxide formation. A high adenosine concentration is also thought to be neuroprotective itself, because adenosine can inhibit the production of excitatory neurotransmitters such as glutamate and increases cerebral blood flow during hypoxia (45). Additionally, high levels of allopurinol and oxypurinol also seem to be direct NPBI chelators and hydroxyl radical scavengers (6,7). NPBI levels in cortical tissues were decreased in allopurinol treated lambs with HIE compared to placebo, but plasma NPBI levels did not differ (7). This suggested that allopurinol and oxypurinol were able to cross the blood brain barrier. However, allopurinol in the dose of 20mg/kg did only partly chelate NPBI levels in lambs (7). Both allopurinol and oxypurinol have a hydroxyl radical scavenging effect. The scavenging effect of oxypurinol was stronger than of allopurinol (6).

Whether the possible neuroprotective effect of allopurinol is mainly caused by xanthine-oxidase inhibition or free radical scavenging has not yet been elucidated.

All above mentioned preclinical studies only measured short-term outcome. The measurement of long-term outcomes such as long-term neurobehavioral studies are essential to establish a possible effect of allopurinol in HIE. Further, the effect of postnatal allopurinol has not been investigated as an addition to hypothermia treatment in preclinical research.

Postnatal studies in neonates in HIE

Because of the promising results of allopurinol therapy in animal studies, it was decided to start an open label study in the newborn infant with perinatal asphyxia. The first study in neonates was an unblinded randomized controlled trial (RCT) in which two dosages of 20mg/kg allopurinol or placebo were given after birth to 22 neonates, the first dose up to 4 hours after birth and a second dose 12 hours later (Table 1) (46). The short-term effect of allopurinol was assessed with chemical biomarkers (lipid peroxidation and anti-oxidative parameters), the pattern of the cerebral blood flow was measured with NIRS and electrical brain function with amplitude-integrated EEG (aEEG). NPBI levels were significantly lower two days postpartum and uric acid levels were decreased from 16 hours postpartum onwards in the allopurinol group compared to the controls. However, lipid peroxidation and anti-oxidative parameters were the same in the allopurinol group and in the controls. Moreover, allopurinol induced a relative preservation of cerebral blood flow and electrical brain activity was higher in the allopurinol group suggesting less brain damage. In the allopurinol group 2 of the 11 infants died, whereas in the control group 5 out of 11 died. In conclusion, this study suggested a beneficial effect of allopurinol, without toxic side effects. However, the sample size of this study was small and the study was unblinded (46).

A subsequent multicenter, double-blinded RCT was performed by Benders *et al.* in 2006. Allopurinol was administered to 17 infants in the same dosages at the same time points as in the previous study and a placebo was administered to 15 infants. However, only infants with perinatal asphyxia, multi-organ failure and an abnormal aEEG were included. These strict inclusion criteria led to the inclusion of only severely affected neonates. Therefore, mortality was rather high in this study: 76% in the allopurinol group and 67% in the placebo group, which was not statistically different. Short-term outcomes, namely MRI and cerebral ultrasound abnormalities, seizures and S100ß concentrations, were similar in both groups (47). This suggested that allopurinol might be more effective in moderately affected neonates than in severely affected neonates.

All participants of the two above mentioned studies were seen for neurocognitive follow-up at the age of 4 to 8 years (mean of 5 years and 5 months). Children were tested with a neurological examination and the Wechsler Preschool and Primary Scale of Intelligence or the Wechsler Intelligence Scale for Children. There was

no significant improvement of adverse long-term neurodevelopmental outcome (allopurinol 8% vs. controls 11%, p=1.000) or mortality (allopurinol 54% vs. controls 62%, p=0.376) in the overall analysis. However, when only the moderately asphyxiated infants were analyzed, a significant improvement of long-term outcome was found in the allopurinol treated infants compared to the controls. In moderately affected infants, 65% of controls had a severe adverse outcome, defined as death or severe neurodevelopmental disabilities, compared to 25% of the allopurinol treated infants (p=0.047) (48). This follow-up study suggested a positive effect of allopurinol in moderately asphyxiated infants on neurological outcome, but not in severely asphyxiated infants. This is in line with a previous study on neonatal head cooling after acute perinatal asphyxia in term infants (49) and the previously discussed animal studies (41,42). However, the numbers of this follow-up study were very small, therefore the results should be interpreted with caution (48).

The most recent RCT investigating the benefit of postnatal allopurinol in neonates with HIE was performed by Gunes *et al.* in Turkey. Thirty neonates were treated for three days with two dosages of 20 mg/kg allopurinol per day, with the first dose being administered within two hours after birth. The control group of thirty neonates received a placebo. This study was not blinded in order to enable the investigators to monitor side effects. Free radical production, measured by NO concentrations in the serum, was decreased after the administration of allopurinol compared to controls. NO concentrations in the cerebrospinal fluid were comparable between the groups. Adverse outcome at 1 year of age, was reduced in the allopurinol group compared to placebo (39.3% vs. 53.6%, p<0.05). Adverse outcome was defined as cerebral palsy, Bayley score < -2SD, blindness and/or deafness. Mortality did not differ between the groups. No adverse side effects of allopurinol were seen (50). Although this study is larger than the other two, the sample size is still not large enough to draw conclusions about the neuroprotective effect of allopurinol. Furthermore, a longer neurological follow-up is essential to determine the actual effect of allopurinol on the long-term in HIE. Additionally, pharmacokinetic analysis would have been valuable since this study used other dosing protocols than the previous studies.

It can be concluded from these clinical studies that allopurinol might be neuroprotective in moderately affected infants, but is not effective when the brain damage is too severe. Considering that the onset of free radical production is immediately after the onset of reperfusion, administration of allopurinol within four

hours after birth might be too late to reduce the peak of these toxic metabolites. Importantly, in none of the studies adverse side effects were seen.

As expected, the conclusion of the Cochrane review including the studies of van Bel *et al.,* Benders *et al.* and Gunes *et al.* stated, that there is not enough evidence to draw conclusions and that larger trials are needed (51). In combination with the idea that allopurinol should be given as early as possible, the hypothesis arose that antenatal administration of allopurinol in case of suspected fetal hypoxia might be more effective.

Table 1: Overview or clinical studies with allopurinol in HIE

Study	Population	Moment of administration and dosages	Results
Van Bel *et al*, 1998	Allopurinol (n=11) vs. controls (n=11)	20mg/kg i.v. within 4 hours after birth, 2nd dose of 20mg/kg i.v. 12 hours later (median allopurinol administration 170 min; range 68-210)	Positive effect on free radical formation, electrical brain activity and a relative preservation of cerebral blood volume. No adverse events.
Benders *et al*, 2006	Allopurinol (n=17) vs. placebo (n=15)	20mg/kg i.v. within 4 hours after birth, 2nd dose of 20mg/kg i.v. 12 hours later (median and range not shown)	No effect on short term outcome in severely affected infants. No adverse events.
Gunes *et al*, 2007	Allopurinol (n=30) vs. placebo (n=30)	20mg/kg i.v. within 2 hours after birth, second dose 12 hours later, then every 12 hours a dose for 3 days (median and range not shown)	Better neurological outcome at 1 year of age. Serum NO decreased after allopurinol. No adverse events.
Kaandorp *et al*, 2012	Allopurinol (n=28) vs. placebo (n=26)	Follow-up study of Benders *et al.* and van Bel *et al.* at four to eight years of age	Improved neurological outcome in moderately affected infants. No adverse events.
Torrance *et al*, 2009	Allopurinol (n=27) vs. placebo (n=27)	500mg allopurinol i.v. antenatally (median allopurinol administration 56min before delivery; range 18-190)	Reduction of S100ß levels in therapeutic allopurinol group. No adverse events.

Table 1: Continued

Study	Population	Moment of administration and dosages	Results
Kaandorp *et al*, 2015	Allopurinol (n=113) vs. placebo (n=111)	500mg allopurinol i.v. antenatally (median allopurinol administration 14min before delivery; IQR=7.3-26.9)	In girls S100ß levels and oxidative stress markers are reduced in the allopurinol group compared to controls. No adverse events.

Preclinical studies – antenatal administration for HIE

Pharmacokinetics of allopurinol in mother and fetus were investigated in case of antenatal allopurinol administration in several experimental studies in fetal sheep. Allopurinol crossed the placenta rapidly in a number of animal studies (52-56). After 20mg/kg allopurinol was administered intravenously the maternal allopurinol and oxypurinol concentrations reached their maximum within 20 minutes after birth in sheep. The oxypurinol concentrations in all fetuses were in the therapeutic range (53).

The neuroprotective effect of antenatal allopurinol (20mg/kg weight of the mother) was tested in five sheep and compared to six controls. Allopurinol was administered to the mother during the induction of hypoxia. Brain damage was studied based on histology 48 hours after birth. In this experiment, there was less hippocampal damage in the allopurinol group compared to the placebo group. The dentate gyrus and thalami also appeared to be less damaged based on histological confirmed neuronal necrosis, although this was not statistically significant (57). Moreover, the cardioprotective effect of 20mg/kg allopurinol compared to placebo was also investigated in these 11 sheep after inducing hypoxia with a cord clamp model (53). The allopurinol treated sheep had less cardiac oxidative stress based on fetal blood pressure, heart rate, T/QRS ratio of the fetal electrocardiography and troponin levels. Furthermore, umbilical blood flow was preserved in the allopurinol treated animals, while this was not the case in the control group. These findings suggested a cardioprotective effect of allopurinol in sheep (53). Importantly, core body temperature was not measured, so the influence of hypothermia is unclear in this study. Another study group found a reduced oxygen radical production in 3 fetal sheep after the administration of 400mg allopurinol to the dam (55). Superoxide production was determined with chemiluminescence. The concentrations of superoxide rose until the administration of allopurinol, afterwards it declined until

normal values within 90 minutes. Based on these results, antenatal allopurinol might reduce superoxide production in sheep with HIE (55).

Again, no follow-up has been performed to assess long-term outcome in these animals.

Human antenatal studies in HIE

To investigate the neuroprotective effect of antenatal allopurinol therapy in neonates with suspected fetal hypoxia, a double blinded randomized pilot study was performed including 54 infants based on an abnormal cardiotocography or abnormal fetal scalp sampling, indicating that fetal hypoxia was imminent. The time between allopurinol administration and delivery of the baby varied between 18 and 190 minutes, which is relatively short. Allopurinol rapidly crossed the placenta in these pregnant women, but only in 15 out of 27 allopurinol treated infants therapeutic allopurinol or oxypurinol levels were reached. Apparently, the time between administration of allopurinol and actual delivery was too short for allopurinol to cross the placenta and to reach therapeutic plasma levels in some fetuses. Therefore, the allopurinol treated group was split into two subgroups: infants with therapeutic allopurinol or oxypurinol levels (n=15) and infants with sub-therapeutic allopurinol or oxypurinol levels (n=12). The sum of the allopurinol and oxypurinol concentrations in the umbilical cord were negatively correlated with S100ß concentrations, a biomarker for brain tissue damage ($r=0.59$, $p<0.01$). Furthermore, the S100ß levels were significantly reduced in the therapeutic allopurinol group compared to the other groups. Limitations of this study were the small sample size and the relatively high rate of sub-therapeutic allopurinol or oxypurinol levels (58).

Therefore, a Dutch multicenter, double-blinded, randomized controlled trial was performed in 222 pregnant women with suspected fetal hypoxia during labor, half of which received 500mg allopurinol intravenously and half a placebo. Primary outcomes were the concentrations of S100ß in umbilical cord blood and the concentrations of oxidative stress markers, e.g. neuroketal and 8-isoprostane. In the total group, there was no significant difference in S100ß or oxidative stress marker levels between allopurinol treated infants and controls. In a post-hoc analysis, S100ß and neuroketal levels were significantly reduced in girls in the allopurinol group compared to the controls, but the oxidative stress markers were similar for boys (59). This gender difference is possibly explained by different pathways for programmed cell death and is also seen in other neuroprotective strategies and other neurological

childhood diseases (60, 61). Though this study was not designed to study gender differences, gender differences should be taken into account in future research (59). No adverse events occurred in the allopurinol group (59). A limitation of this study was that most infants had no or only mild asphyxia, which resulted in underpowered results (59). None of the infants were diagnosed with HIE after inclusion. The reason for the relatively high amount of mildly asphyxiated infants is that antenatal monitoring has a poor predictive value for actual perinatal asphyxia with a lot of false positive cases (59). Taking this into account, a very large RCT would be needed to estimate the actual effect of antenatal allopurinol administration.

Although antenatal administration of allopurinol in girls with HIE might be promising, it is not optimal either because of the difficulties to predict which infants will be born with perinatal asphyxia in the daily practice: actual asphyxiated babies might be missed and there will be a lot of overtreatment of fetuses without relevant hypoxia. Therefore early, neonatal allopurinol administration might be a more suitable design for future studies.

Other indications

Allopurinol has also been tested in other neonatal populations in which free radical formation leads to cell damage.

The study of Derks *et al.* that was discussed earlier, already suggested a beneficial cardioprotective effect of antenatal allopurinol in animals. Infants undergoing cardiac surgery experience periods of hypoxia, which is known to lead to brain damage and especially to white matter injury (62,63). Allopurinol might also have neuroprotective effects next to the possible cardioprotective effects in neonates with congenital heart diseases. Serum uric acid levels after allopurinol administration were decreased in infants with a hypoplastic left heart syndrome (HLHS) compared to baseline. This suggests that the xanthine-oxidase pathway is activated in HLHS and that allopurinol can inhibit this pathway (64). In a RCT including infants with a congenital heart disease, allopurinol or placebo was administered before, during and after heart surgery with deep hypothermic circulating arrest (65). Infants with HLHS (n=131) as well as infants with other congenital heart diseases (n=187) were treated in this trial. In the infants with HLHS death, seizures, coma and/or cardiac events occurred in 38% of the infants in the allopurinol group and in 60% of infants in the placebo group (p=0.01) (65). This suggested a neuroprotective and cardioprotective

effect of allopurinol in infants with HLHS. However, when the outcome parameters were analyzed separately, there were no significant differences. Allopurinol was not effective in other congenital heart diseases (65). The authors hypothesized that HLHS infants had a worse cerebral oxygenation status before surgery than infants with other congenital heart diseases, but this has not been confirmed (65).

Marro *et al.* investigated the effect of allopurinol in neonates undergoing extracorporeal membrane oxygenation (ECMO) in a RCT. Allopurinol was given to 11 infants in a dose of 10mg/kg before surgery, 20mg/kg was added to the ECMO circulation and afterwards 5mg/kg was given every 8 hours for 72 hours. Fourteen infants received placebo. This study confirmed that uric acid levels are decreased after allopurinol administration and hypoxanthine levels are increased, suggesting that the xanthine-oxidase pathway was inhibited and consequently the formation of free radicals was possibly reduced. The neuroprotective effect of allopurinol could not be verified, because free radicals and neurological outcome were not determined (66).

In premature born babies, the anti-oxidant system is not fully mature yet, thereby increasing the risk of free radical formation during stress. Therefore, idiopathic respiratory distress syndrome (IRDS) was believed to be caused by free radical formation (67). A study in 1984 suggested a positive effect on mortality in preterm babies with IRDS (67). Later, allopurinol was also tested in premature born babies with a gestational age of 27 to 31 weeks (oral allopurinol 20mg/kg: n=16, placebo: n=17). In this insufficiently powered study, allopurinol had no effect on the incidence of periventricular leukomalacia, periventricular hemorrhage, porencephaly, necrotic enterocolitis, retinopathy of prematurity or bronchopulmonary dysplasia (68).

Pharmacokinetics of allopurinol in neonates

A combined pharmacokinetic study was performed for the cohorts of van Bel *et al.* and Benders *et al.* Almost all neonates reached the target levels (2-13.6µg/ml) after two dosages of 20mg/kg, most of them even reached supra-therapeutic levels. Oxypurinol concentrations could be measured within 1 hour after the first dose of allopurinol. Oxypurinol levels were high for at least 14 hours after a first dose. The half-life of allopurinol was estimated around 7 hours (69). In infants with HLHS the half-life of allopurinol was shorter, around 2.5 hours (64). This difference might be explained by the younger age of the infants with HIE (69). Despite the supra-

therapeutic allopurinol and oxypurinol concentrations in neonates with HIE, no adverse events were seen (46,47).

In the allopurinol treated infants undergoing ECMO, peak serum levels of allopurinol and oxypurinol were 28.4±3.5μg/ml and 15±4μg/ml respectively (70). The allopurinol and oxypurinol levels in ECMO were even higher than in the HIE studies. This is probably caused by the higher dose and frequency of allopurinol administration compared to the previously mentioned cohorts (69,70)

Pharmacokinetics were also studied in 24 mothers delivering full-term neonates and 44 mothers delivering preterm neonates. Allopurinol (500mg) was orally administered to all mothers during early labor. All mothers reached therapeutic allopurinol levels and the levels of allopurinol in the cord blood and in the newborn at 24 hours after birth were therapeutic. Therapeutic levels in cord blood were reached as soon as 23 minutes after allopurinol administration, suggesting a quick placental transfer of oral allopurinol (52).

In the antenatal pharmacokinetic study of Kaandorp *et al.*, 95% of the infants had an allopurinol concentration above the target concentrations in the cord blood (allopurinol ≥ 2 μg/ml and oxypurinol ≥ 4 μg/ml). The target concentrations were reached within 5 minutes after the end of maternal allopurinol infusion (71). In the ALLO-trial, 72% of the pregnant women reached the target blood levels of allopurinol (59).

Safety

In adults, a potentially fatal side effect of allopurinol is the allopurinol hypersensitivity reaction (AHR). This AHR is thought to be caused by potentially immunogenic complexes of oxypurinol and certain human leukocyte antigen (HLA) proteins, particularly HLA-B*58:01 (which is more common in Asians than in Caucasians, similar to the frequency distribution of AHR). AHR predominantly affects the skin and may present as a generalized rash, but can also present as severe exfoliative skin reaction, i.e. as Stevens-Johnson syndrome or toxic epidermal necrolysis. AHR might also present with eosinophilia, leukocytosis, fever, acute hepatocellular injury and/or progressive kidney failure (1).

Most AHR have been diagnosed after daily administration of allopurinol for a duration of more than 2 to 3 weeks, but single cases have been reported after a single day of

allopurinol in adults (72). High doses of allopurinol, pre-existing renal failure and co-medication with ampicillin or amoxicillin seem to increase the risk for AHR.

The rate of occurrence for AHR is frequently cited to be approximately 0.1% in adults treated with allopurinol and this number origins from a report in which 3 out of 1835 hospitalized adults treated with allopurinol had probable AHR (73). More appropriately and more recently, a population-based study reported an annual incidence rate of 4.68 per 1000 new allopurinol users in Taiwan (74). Taking into account that the odds-ratio for AHR in Chinese compared to Caucasian is 70 (75), the incidence is probably less than 1 in 10,000 new Caucasian users. In all antenatal (n=138) and postnatal (n=58) treated infants with HIE, as in other term (n=178) and preterm (n=348) infants, no serious adverse reactions to allopurinol have been reported (46,47,50,58,59,65-68,76).

Consequently, a recent Cochrane review on postnatal allopurinol for HIE concluded that there are no major concerns about safety based on the available data (51).

Nevertheless, because of the high pH of an allopurinol preparation suitable for intravenous administration, perivascular or intra-arterial infusion of allopurinol must be strictly avoided. 4.5% of the allopurinol treated mothers had irritation of the perivascular tissue (71).

Conclusions and recommendations

In conclusion, the neuroprotective effect of allopurinol has been tested by postnatal administration in neonates with HIE and by antenatal administration in mothers with imminent fetal hypoxia. In newborn animals as well as in neonates, allopurinol seems to inhibit the formation of superoxide and scavenge free radicals that both can lead to brain damage. Based on available literature about antenatal and postnatal administration of allopurinol in animals and human neonates, allopurinol might reduce brain damage caused by perinatal asphyxia in term born neonates, but only in those with moderate HIE. Moreover, allopurinol in neonates appears to be safe: even when supra-therapeutic concentrations are reached no adverse events have been seen, but rare side effects may not yet have become apparent.

In summary, the literature is inconclusive whether allopurinol is a suitable neuroprotective therapy in infants with HIE. Antenatal administration may result

in over-treatment, since it is difficult to select the mothers with imminent fetal hypoxia. In contrast, postnatal allopurinol within 4 hours after birth might be too late because the oxygen radicals are formed shortly after birth. Consequently, very early postnatal allopurinol administration, i.e. immediately after resuscitation, might be the most effective mode of application. However, the efficacy of early allopurinol administration has not been investigated yet, neither has the combination of allopurinol and hypothermia been investigated previously in clinical or preclinical studies.

Currently, a European double blinded, placebo-controlled, randomized controlled trial is designed in which 20mg/kg allopurinol intravenously will be administered immediately after or during resuscitation in asphyxiated babies. Neonates undergoing moderate hypothermia will receive a second dose of 10 mg/kg, 12 hours after birth. The primary endpoint will be death or impaired neurodevelopmental outcome at 2 years of age. Secondary endpoints are surrogate markers of brain injury on MRI, cerebral ultrasound, aEEG, multichannel EEG, plasma biomarkers and oxidative stress markers. This trial is essential to determine whether early allopurinol administration is a possible add-on neuroprotective therapy after perinatal asphyxia.

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DISCLOSURES

None of the authors have a conflict of interest to declare.

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CHAPTER 8

Effect of allopurinol in addition to hypothermia treatment in neonates with hypoxic-ischemic brain injury on neurocognitive outcome (ALBINO): study protocol of a blinded randomized placebo-controlled parallel group multicenter trial for superiority (phase III)

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ABSTRACT

Background: Perinatal asphyxia and resulting hypoxic-ischemic encephalopathy is a major cause of death and long-term disability in term born neonates. Up to 20,000 infants each year are affected by HIE in Europe and even more in regions with lower level of perinatal care. The only established therapy to improve outcome in these infants is therapeutic hypothermia. Allopurinol is a xanthine-oxidase inhibitor that reduces the production of oxygen radicals as superoxide, which contributes to secondary energy failure and apoptosis in neurons and glial cells after reperfusion of hypoxic brain tissue and may further improve outcome if administered in addition to therapeutic hypothermia.

Methods: This study on the effects of Allopurinol in addition to hypothermia treatment for hypoxic-ischemic Brain Injury on Neurocognitive Outcome (ALBINO), is a European double-blinded randomized placebo-controlled parallel group multicenter trial (Phase III) to evaluate the effect of postnatal allopurinol administered in addition to standard of care (including therapeutic hypothermia if indicated) on the incidence of death and severe neurodevelopmental impairment at 24 months of age in newborns with a perinatal hypoxic-ischemic insult and signs of potentially evolving encephalopathy. Allopurinol or placebo will be given in addition to therapeutic hypothermia (where indicated) to infants with a gestational age ≥36 weeks and a birth weight ≥2500 gram, with severe perinatal asphyxia and potentially evolving encephalopathy. The primary endpoint of this study will be death or severe neurodevelopmental impairment versus survival without severe neurodevelopmental impairment at the age of two years. Effects on brain injury by magnetic resonance imaging and cerebral ultrasound, electric brain activity, concentrations of peroxidation products and S100B, will also be studied along with effects on heart function and pharmacokinetics of allopurinol after intravenous infusion.

Conclusion: This trial will provide data to assess the efficacy and safety of early postnatal allopurinol in term infants with evolving hypoxic-ischemic encephalopathy. If proven efficacious and safe, allopurinol could become part of a neuroprotective pharmacological treatment strategy in addition to therapeutic hypothermia in children with perinatal asphyxia.

Trial registration number: NCT03162653, www.ClinicalTrials.gov, May 22, 2017

INTRODUCTION

Neonatal hypoxic-ischemic encephalopathy (HIE) as a result of perinatal asphyxia is a major cause of death and long-term disability in term neonates. About 1-4 per 1000 live births and consequently about 5000-20,000 infants per year are affected in Europe (1). In regions with lower level perinatal care it is even more common. HIE affects about one million infants worldwide each year.

Up to now, the only established therapy to improve outcome in infants with HIE is therapeutic hypothermia (2,3). However, despite therapeutic hypothermia and modern supportive neonatal intensive care, 45-50% of the infants with moderate or severe HIE (i.e., 2500-10,000 infants per year in Europe) still die or suffer from long-term neurodevelopmental impairment (NDI) such as cerebral palsy (CP), cognitive or behavioral problems (2,4). Therefore, additional therapies, including pharmacotherapy, are investigated to further improve the neurodevelopmental outcome of infants with HIE.

One of the potential beneficial pharmacological interventions is allopurinol. Allopurinol is a xanthine-oxidase inhibitor, which reduces the production of oxygen radicals, most importantly of superoxide (5). Superoxide radicals damage mitochondria resulting in secondary energy failure and apoptosis affecting neurons and glial cells after reperfusion of hypoxic brain tissue, this is called reperfusion injury (6,7). This reperfusion injury leads to additional brain injury occurring in the hours after birth and may affect much larger areas of brain tissue than the area primarily affected during the sentinel event (7). Superoxide production, which is reduced by allopurinol, reaches its peak within 30 minutes after birth and therefore early administration is important to reduce reperfusion injury (8). Furthermore, allopurinol, especially in higher concentrations, possibly chelates non-protein-bound iron and scavenges the hydroxyl free radicals (9,10). Allopurinol also prevents adenosine degradation, which is an anti-excitatory neuromodulator (11). Thereby, allopurinol might reduce reperfusion injury and improve outcome in neonates with HIE.

Several preclinical and three small clinical studies in neonates with HIE suggested a possible neuroprotective effect of allopurinol (recently reviewed in Annink *et al.* (8)). In the first two studies of van Bel *et al.* and Benders *et al*. allopurinol was administered within four hours after birth. Allopurinol improved neurodevelopmental outcome in infants with moderate HIE, but not in severe HIE

(12-14). Gunes *et al.* administered allopurinol for three days and found improved outcome at one year of age (15). All three studies were conducted before therapeutic hypothermia became standard of care, so the effect of allopurinol in addition to therapeutic hypothermia has not been investigated yet.

Based on the hypothesis that administration within four hours after birth was too late to achieve a full neuroprotective effect, allopurinol was administered antenatally in case of suspected hypoxia in the antenatal allopurinol trial for reduction of birth asphyxia induced brain damage (ALLO-trial) (16). In girls, biomarkers as S100B were reduced in the allopurinol group compared to the placebo group. However, there was substantial overtreatment on the one hand and on the other moderately and severely asphyxiated infants were often missed (16).

Consequently, in this study on the effects of Allopurinol in addition to hypothermia treatment for hypoxic-ischemic Brain Injury on Neurocognitive Outcome (ALBINO), allopurinol will be administered intravenously within 30 (max. 45) minutes after birth to optimize the timing and inhibition of superoxide formation in asphyxiated infants with evolving HIE.

Importantly, in all antenatal and neonatal studies in HIE, no severe side-effects were seen (12,13,15-19). Also, in other neonatal populations, such as preterm infants and infants with congenital cardiac abnormalities, no severe side effects have been reported following (intravenous or oral) administration of allopurinol (20-24). In the ALLO-trial, 4.5% of the mothers who received allopurinol had an irritation of the perivascular tissue, caused by the high pH of the allopurinol solution, but this was reversible in all cases (16). In adults, a rare hypersensitivity reaction to allopurinol has been described after daily administration for a median of two to three weeks (25,26). An allopurinol sensitivity reaction in neonates has never been reported and is expected to be extremely unlikely.

METHODS

Trial objectives

The main objective of the ALBINO trial is to evaluate the effect of early postnatal allopurinol administered in addition to standard of care (including therapeutic hypothermia if indicated) on the incidence of death and severe NDI at 24 months of age in newborns with HIE.

Secondly, safety of early postnatal intravenous allopurinol will be evaluated, as well as the pharmacokinetic profile of intravenous allopurinol and the short-term effect of early allopurinol on brain injury assessed by magnetic resonance imaging (MRI) of the brain, cerebral ultrasound, heart function assessed by echocardiography, electro-encephalography (EEG), and biochemical biomarkers.

Trial design

The ALBINO trial is a European double-blinded randomized placebo-controlled parallel group multicenter trial for superiority of allopurinol versus placebo (mannitol) in addition to therapeutic hypothermia where indicated (Phase III). More than 60 hospitals in 10 countries will participate in this study, and ALBINO may expand to additional sites in further countries, after appropriate approvals have been obtained from ethics committees and authorities.

Population

Term and near-term infants (≥36 weeks) with severe perinatal asphyxia and potentially evolving encephalopathy can be included in the ALBINO trial.

Inclusion criteria

Infants must meet at least one of the following five criteria of severe perinatal asphyxia: 1) umbilical or postnatal blood gas within 30 min after birth with a pH <7.0 or 2) with a base deficit ≥16 mmol/l; 3) need for ongoing cardiac massage at/beyond 5 minutes postnatally; 4) need for adrenalin administration during resuscitation and/or 5) Apgar score ≤5 at 10 minutes after birth.

Further, the infant must meet two out of the following four criteria for potentially evolving encephalopathy to participate in the study: 1) altered state of consciousness (reduced or absent response to stimulation or hyperexcitability); 2) severe muscular hypotonia or hypertonia; 3) absent or insufficient spontaneous respiration (i.e. gasping only) with need for respiratory support at 10 minutes postnatally and/or 4) abnormal primitive reflexes (absent suck/gag/ corneal/Moro reflex) or abnormal movements (i.e. potential clinical correlates of seizure activity).

Exclusion criteria

Infants will be excluded if the gestational age is below 36 weeks, birth weight is below 2500 gram, in the presence of severe congenital malformations or syndromes

requiring neonatal surgery or affecting long-term outcome. Furthermore, infants will be excluded if their postnatal age is > 30 minutes at the end of the screening phase, the neonate is considered “moribund” or “non-viable”, there is a decision of ‘comfort care only’ before study drug administration or if parents decline study participation.

Randomization and allocation concealment
Randomization will be performed with randomization software (Randlist Version 1.2) in blocks of four and stratified per center.

Randomization will be performed by allocation of the next consecutive study medication box (including first and second vial of study medication and two vials with sterile water for reconstitution) to an infant.

Study Intervention
Infants included in the ALBINO trial will receive either allopurinol or placebo (mannitol). Study medication will be administered by intravenous infusion in one or two doses (see Figure 1). The first dose (20 mg/kg body mass reconstituted in 2 ml/kg sterile water for infusion) will be given as soon as possible after birth. The start of infusion of study medication should preferably be within 30 minutes after birth, but no later than 45 min after birth.

The second dose (10mg/kg body mass reconstituted in 1 ml/kg sterile water for infusion) will be administered 12 hours after the first dose. This second dose will only be administered to infants treated with therapeutic hypothermia. Infants who recover quickly and do not qualify for and hence do not undergo hypothermia will not receive a second dose.

Placebo (mannitol) will be given in the same dose, volume and intervals as allopurinol.

Concomitant interventions and medication:
Any concomitant medication that is medically necessary for the patients will be allowed in the study, except open-label allopurinol in any dosage and any application mode.

Where indicated according to respective national standards or treatment protocols, hypothermia treatment (whole body cooling to 33.5°C for 72 hours) should be started as soon as possible according to local protocols (3,27).

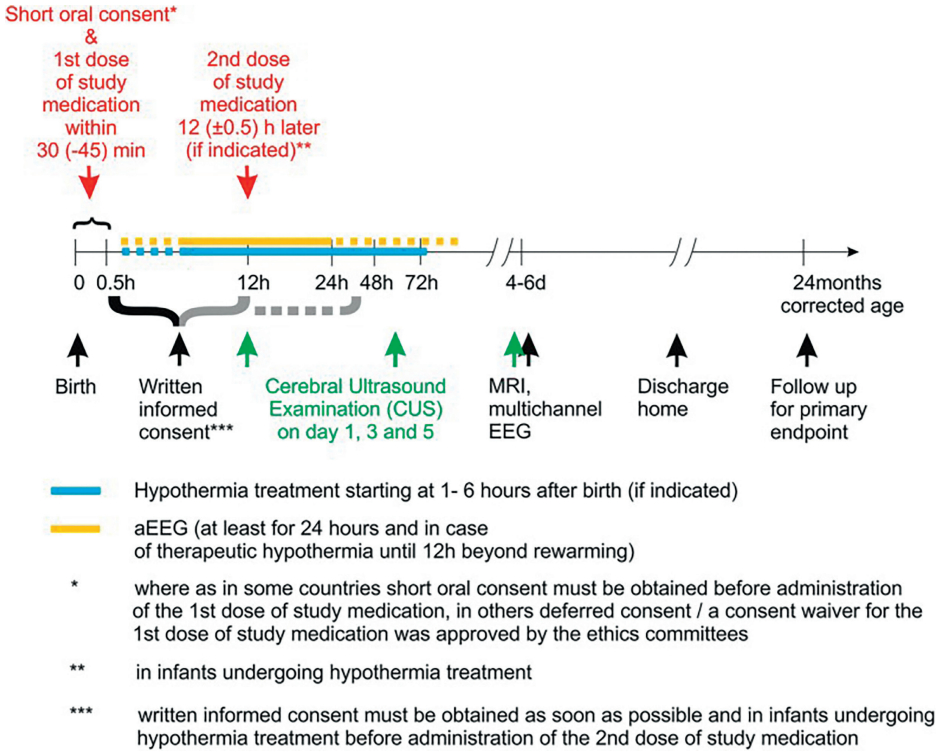

Figure 1: Study interventions in ALBINO.

Primary outcome

The primary endpoint will be death or severe NDI versus survival without severe NDI at the age of two years. Severe NDI is hereby defined as any of the following: cognitive or language delay defined as a cognitive-composite-score or a language-composite-score on the Bayley Scales of Infant and Toddler Development (3rd edition) < 85 and/or cerebral palsy (CP) according to the Surveillance of Cerebral Palsy in Europe (SCPE) criteria.

Secondary and further outcomes

The primary endpoint will be reconstituted as dichotomized composite secondary endpoint (survival without NDI versus death or language-composite-score < 85 or cognitive-composite-score <85 or CP present). Furthermore, the incidences of death and CP and the composite scores derived from the Bayley test (continuous and dichotomized) as well as the Gross Motor Function Classification Score will be analyzed as secondary outcome variables.

Further important secondary outcome parameters are brain injury assessed by MRI of the brain, cerebral ultrasound, amplitude-integrated EEG, full scale multichannel EEG, heart function assessed by echocardiography, concentrations of peroxidation products and S100B which are markers for brain injury in the blood. Furthermore, pharmacokinetics of allopurinol will be investigated in 48 to 52 patients. Finally, the opinions of parents experiencing two different consent procedures will be evaluated.

Parental perspectives

Following study participation, parents will be approached again and asked for their opinion on and satisfaction with the consent procedure to inform future investigators in the field of HIE therapy.

Ethical Considerations

Because allopurinol has to be administered as early as possible after birth to reduce formation of oxygen radicals during reperfusion and because the emergency situation of perinatal asphyxia is very stressful for the parents, the usual procedure of provision of comprehensive oral and written information, time for consideration and full written informed parental consent before study entry is not feasible in the setting of ALBINO. This problem and the various alternative approaches (antenatal consent, short information and oral consent and later full information and written confirmation, waiver of consent for first dose and deferred information and consent), have been discussed with external experts on perinatal HIE as well as medical ethicists and a balance between the need to inform the parents and the feasibility of the study was sought in collaboration with the relevant ethics committees in each participating country.

Community Engagement

Information material, such as posters and flyers, that provides short information for parents, will be available in prenatal clinics and delivery areas and will direct interested parents to the study home page (www.albino-study.eu). The homepage will grant access to nationally approved full parent information material. A press release will inform the community around study sites about the ALBINO trial.

Parents, who do not want to participate in the ALBINO trial, will have the option to deny participation even before delivery verbally or on a ‘declaration of intent’-form

printed on the flyers informing about the study. This can be completed and kept in the maternal health passport to inform the staff in the delivery room.

Form of Consent

According to the relevant ethics committee's decisions, either a deferred consent or an initial short oral consent approach will be used for obtaining parental consent.

The deferred consent procedure has previously been used in emergency research in adults and is in compliance with §30 of the Declaration of Helsinki (28). In the case that a child fulfills the inclusion criteria and meets no exclusion criterion, physicians will administer the first dose of study medication in the delivery room without prior consent (i.e., a 'consent waiver' was granted for the first dose of study medication). Parents will receive detailed information later and will be asked for written informed consent for continued participation in the study (as soon as possible, at the latest before the second dose of study medication if indicated). The deferred consent procedure has been approved in Austria, Belgium, Estonia, Finland and Norway.

In Germany, the Netherlands, Italy, Switzerland and Spain, the ethical committees did not agree on the deferred consent procedure, so in these countries the short oral consent procedure will apply: short oral information (duration <5min) on the indication and the potential benefits and risks of the study medication must be provided to at least one parent and oral (or written) consent of this parent must be obtained before the first dose of study medication can be administered. Again, both parents will receive more detailed information and will be asked for full written consent as soon as possible and at the latest before the second dose of study medication will be administered (if indicated).

Statistical analysis

Sample size, power and study duration

The primary assessment for efficacy will compare the proportions of infants surviving without severe NDI versus those of infants who died or survived with severe NDI at the age of two years.

Based on the above referenced (preliminary) clinical studies from the pre-therapeutic hypothermia era and clinical studies on hypothermic treatment, it is estimated that the combined incidence of death and severe NDI in the control group

will be 37% and 27% in the allopurinol group. Therefore, we calculated with a two-sided X^2-test (alpha=0.05, power 80%) a sample size of 682 infants (341 per treatment group) in which the primary outcome should be ascertained. Assuming a drop-out rate of 10% for loss to follow-up, a total of 760 infants need to be enrolled. And assuming that 10% of the parents will refuse participation after the initial dose of the study drug, 846 infants have to be randomized (see Figure 2).

We estimate a recruitment of about 35 patients per month in approximately 70 study centers (the recruitment of additional study sites is ongoing) and therefore recruitment will last 24 months.

Data analysis

All statistical analyses will be described in detail in a statistical analysis plan completed before closure of the database.

Monitoring safety

An independent Data Monitoring Committee (DMC) will monitor the participants' well-being and the overall risk/benefit-ratio of the study. National monitors will monitor the accuracy and completeness of the data and the safety issues such as the presence of serious adverse events.

Regulatory aspects

Trial sponsor

Sponsor of the ALBINO trial is the University Hospital Tuebingen, Geissweg 3, 72076 Tuebingen, Germany. Contact is available under albino@med.uni-tuebingen.de.

Orphan Drug Designation

The Committee for Orphan Medicinal Products (COMP) has given a positive advice to ACE Pharmaceuticals for the orphan drug designation for allopurinol sodium for treatment of perinatal asphyxia (EU/3/15/1493) and an Orphan Drug Designation has been granted by the European Medicines Agency. The public summary is available at: https://www.ema.europa.eu/documents/ orphan-designation/eu/3/15/1493-public-summary-opinion-orphan-designation-allopurinol-sodium-treatment-perinatal-asphyxia_en.pdf

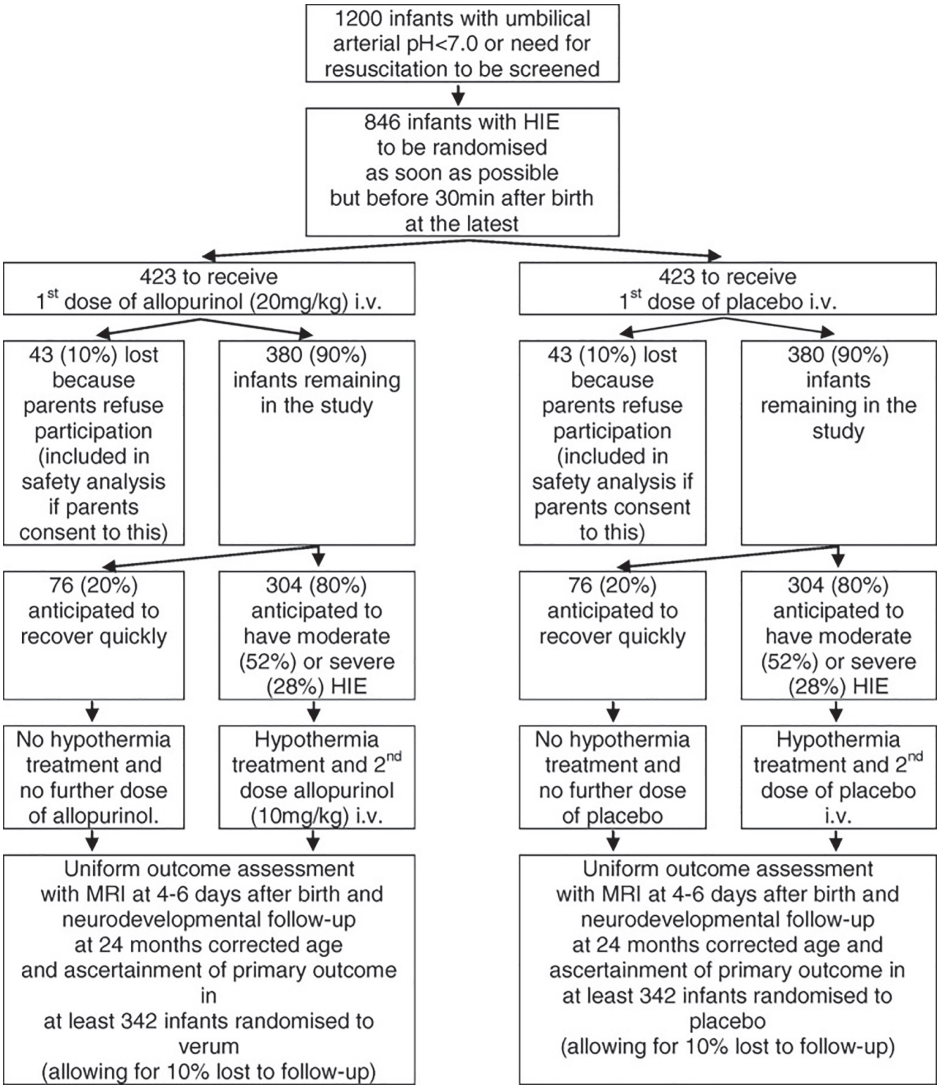

Figure 2: Anticipated trial flow

Scientific Advice from the European Medicines Agency

In November 2015, ACE Pharmaceuticals has requested Scientific Advice and Protocol Assistance from the European Medicines Agency, including questions specifically related to the study protocol and the intended procedure of deferred consent. Written scientific advice was received in May 2016 and after careful consideration by

the Steering Committee, the relevant issues were subsequently incorporated into the study protocol.

Medical ethics committees
At the time of publication, the relevant ethics committees in ten European countries approved the study with either the short oral consent procedure or the deferred consent procedure. Applications for approvals are currently underway in two additional countries.

National Regulatory/Competent Authorities
At the time of publication, eleven European National Regulatory/Competent Authorities approved the study. Application for approval is currently underway in one additional country.

DISCUSSION

ALBINO is a randomized controlled trial investigating the safety and efficacy of allopurinol in (near-) term infants with HIE.

A decision was made for a large phase III trial for efficacy and safety because preliminary clinical data from postnatal and prenatal allopurinol trials already suggested a reduction in brain injury by allopurinol without apparent adverse effects. Another small proof-of-principle or dose seeking study would have added little with respect to safety and clinically relevant outcomes. Survival without NDI was selected as the primary endpoint of this study, because this outcome parameter is most meaningful to the children and their families.

The calculated starting dose was based on previous studies: the doses used in the first studies with allopurinol in neonates undergoing extracorporeal membrane oxygenation and in neonates diagnosed with hypoplastic left heart syndrome (10 and 30 mg/kg birth weight respectively) gave 100% xanthine-oxidase inhibition (21,23). Higher concentrations may be needed for the iron chelating and reactive oxygen scavenging effect of allopurinol. Even with higher doses (up to 40 mg/kg birth weight per day for 3 days) no adverse effects were seen, with special attention to skin rashes and leukopenia (15).

A significant beneficial effect of allopurinol in moderately asphyxiated neonates has been found on long-term (4-5 years) neurodevelopmental outcome by Kaandorp *et al.* (2012), which was a meta-analyses of the study from van Bel *et al.* (1998) and Benders *et al.* (2006) (12,13). These latter trials administered 2 times 20mg/kg birth weight allopurinol with 12 hours interval. The doses of the ALBINO trial are based on these three studies. However, pharmacokinetics of allopurinol during hypothermia have not yet been determined before in neonates with HIE. The second dose in the ALBINO trial (only during hypothermia) is adjusted for the hypothermia treatment which may possibly slow-down allopurinol and oxypurinol metabolism and elimination. In the latter case this would lead to higher circulating concentrations of allopurinol respectively oxypurinol.

In previous studies the plasma concentrations of allopurinol were often supra-therapeutic without any side effect (19,29). These supra-therapeutic levels seem to be important for the direct scavenging of hydroxyl and free iron by allopurinol. However, to ensure that in addition to therapeutic hypothermia plasma concentrations are not lower than in the earlier clinical trials indicating efficacy, blood sampling for pharmacokinetic analyses will be performed in 48 to 52 infants (in selected centers) recruited during the first year of the study and may lead to adaptation of doses.

Mannitol is used as placebo, since its freeze-dried white powder and the reconstituted solution, have the same visual aspects and volume as the freeze-dried sodium salt of allopurinol and its reconstitution solution (10 ml of a colorless, clear solution in a 20 ml vial). The dosage of mannitol is 50 times lower than the dose of mannitol used for neuroprotection (30), and a normal daily dose of intravenous paracetamol will include more mannitol as supporting agent than the dose administered in ALBINO (i.e. 100 ml solution for injection contains 1000 mg acetaminophen and 3670-3850 mg mannitol (31,32). For each single dose of 12.5 mg/kg paracetamol i.v. (33), 45.9-48.1 mg/kg of Mannitol are concomitantly administered).

Inclusion and exclusion criteria were selected to recruit a patient population similar to the TOBY trial of whole body cooling (3), but took into account that the assessment for eligibility has to be done much earlier, i.e. within 30 min after birth.

The ALBINO study group extensively discussed the various ethical implications of need for additional treatment for HIE, need to administer allopurinol very early for best efficacy, need for parental consent to ensure patient autonomy and burden to the parents in the emergency situation of perinatal HIE.

The European Foundation for the Care of Newborn Infants (EFCNI), which is composed of parents, healthcare experts, scientists and politicians, has been asked for advice. The EFCNI endorsed the conduct of the ALBINO trial in a letter of support in September 2016. Because perinatal HIE occurs rarely and unpredictably and because of the need for very early administration of allopurinol, the EFCNI agreed with the approach of deferred consent.

Furthermore, independent ethics experts provided advice. Whereas all experts agreed that regular informed consent by the parents, which includes appropriate time for reflection and further questions is not feasible before administration of the first dose of study medication in the context of ALBINO due to the unpredictable emergency situation. Opinions within the group as well as among external experts ranged from 'deferred consent is unacceptable' to 'deferred consent is justified and the better option', so that the decision was left to the national leading ethics committees in each country.

Currently, we are conducting a survey among parents-to-be and parents of infants with a history of HIE to better understand how parents might feel about deferred versus short oral consent. An additional survey will follow parents of infants enrolled in the ALBINO trial to capture their satisfaction with the various approaches and to inform future trials in similar situations.

CONCLUSIONS

In conclusion, infants with HIE still suffer from death and long-term NDI despite improved standards of care including therapeutic hypothermia. The neurodevelopmental outcome of infants with HIE should be further improved with additional neuroprotective interventions. The aim of the ALBINO trial is to investigate the neuroprotective effect of very early allopurinol within 45 minutes after birth aiming to reduce the formation of the toxic superoxide and subsequent secondary energy failure and apoptosis.

TRIAL STATUS

Protocol version 5: 19. December 2017. Recruitment has started in April 2018 and is expected to be finalized in April 2020. The last patient out (after follow-up) will then be expected in April 2022.

ACKNOWLEDGMENTS

The ALBINO consortium is indebted to Silke Mader and Nicole Thiele from the European Foundation for the Care of Newborn Infants (EFCNI) who granted a letter of support for the ALBINO trial after careful evaluation of the various arguments.

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DISCLOSURES

Y. Jacobs and R. van der Vlught-Meijer are employees of ACE Pharmaceuticals, the company that holds the Dutch marketing authorization registration for Acepurin® (allopurinol 1g/100ml) for intravenous application for treatment of gout. C. van Veldhuizen and B. Laméris are the former owners of ACE Pharmaceuticals. All four contributed to the development of the study protocol. All other contributors declare that they do not have competing interest.

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CHAPTER 9

Pharmacokinetic evaluation of the allopurinol dosing regimen in term infants with perinatal asphyxia in the ALBINO trial

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In preparation

ABSTRACT

Background: Despite therapeutic hypothermia, hypoxic-ischemic encephalopathy (HIE) following perinatal asphyxia remains a major cause of morbidity and mortality. Allopurinol, a xanthine-oxidase inhibitor reducing superoxide formation, might offer add-on neuroprotection. The neuroprotective effect of allopurinol is currently investigated in the randomized placebo-controlled double-blinded ALBINO trial (NCT03162653). The aim of this sub-study was the pharmacokinetic evaluation of the allopurinol dosing regimen in the ALBINO trial.

Methods: In the ALBINO trial, infants received a first dose of allopurinol or placebo within 45 minutes after birth (20mg/kg) and in case of hypothermia a second dose (10mg/kg) 12 hours later. A population pharmacokinetic model was developed for allopurinol and oxypurinol using nonlinear mixed effects modeling (NONMEM, version 7.3). A one-compartment model for allopurinol and oxypurinol was used to describe total body clearance (CL) and volume of distribution (Vd) in neonates normalized to a weight of 3.5kg. The model was used to evaluate whether >66% of patients reached the pre-specified target area under the curve (AUC) between 0-12 hours (43.5mg/L*h for allopurinol and 26.5mg/L*h for oxypurinol).

Results: For this preliminary analysis, fifteen patients who received allopurinol were evaluated. The allopurinol target AUC was reached in all patients. The oxypurinol target AUC was reached in 66.6% of the cooled infants and 100% of the non-cooled infants. The CL of allopurinol was 0.339L/h and of oxypurinol 0.377L/h for an infant of 3.5kg. The Vd of allopurinol was 3.55L and of oxypurinol 5.51L for an infant of 3.5kg.

Conclusion: The dosing regimen used in the ALBINO trial was adequate as the pre-specified target exposure was met.

INTRODUCTION

Hypoxic-ischemic encephalopathy (HIE) following perinatal asphyxia has an incidence of 1-4 per 1000 neonates, and remains one of the main causes of morbidity and mortality in term neonates worldwide (1).

Therapeutic moderate hypothermia (HT) is the only currently proven effective therapy to reduce reperfusion injury in infants with HIE with a number needed to treat for an additional beneficial outcome of 7 (2,3). However, despite therapeutic hypothermia, still 45-50% of the infants with moderate to severe HIE die or develop long-term neurological problems such as motor or cognitive impairments, learning and memory difficulties, epilepsy or behavioral problems (4). Therefore, the search for additional (pharmacological) neuroprotective therapies is essential to further improve their outcome.

Brain injury in asphyxiated infants who develop HIE evolves in two stages: first, the acute (fetal) hypoxia leads to primary energy failure and immediate cell injury, secondly reperfusion and reoxygenation can cause additional brain injury. During the acute (fetal) hypoxia, several compounds and enzymes are formed such as hypoxanthine, pro-radicals and nitric oxide synthase that are subsequently metabolized during reperfusion because of renewed oxygenation (5,6). This leads to the formation of superoxide, free radicals and other compounds that are toxic for the immature brain (5,6). In particular the availability of superoxide plays a central role in the induction of several destructive molecular pathways, resulting in additional brain injury (5,6).

A potentially neuroprotective drug is allopurinol. Allopurinol is a xanthine-oxidase inhibitor and can thereby reduce the production of superoxide substantially (7) (Figure 1). Additionally, allopurinol, especially in high doses, is a direct scavenger for the most toxic free radical 'hydroxyl' and chelates non-protein-bound iron (8,9). Given the proposed positive effects of allopurinol and its metabolite oxypurinol on superoxide and free radical reduction and consequently its preventing action on reperfusion-induced inflammation, the neuroprotective effect of allopurinol in HIE has been investigated and confirmed in several experimental studies (10–17). Based on these mechanisms, postnatal and antenatal clinical studies have been performed (18–23). Postnatal allopurinol administration showed a modest neuroprotective effect in infants with moderate HIE at follow-up (22,24,25). However, the "therapeutic

window" up to 4 hours after birth in the postnatal studies was probably too broad, since the peak of the superoxide formation is within 30-60 minutes after birth (25). Antenatal treatment with allopurinol was thought to prevent superoxide production already during the first minutes after birth, but it turned out to be difficult to select the infants prone for birth asphyxia-induced HIE based on fetal monitoring (21).

We currently investigate the neuroprotective effect of allopurinol administered within 45 minutes after birth in moderately to severely asphyxiated neonates in a large phase III randomized controlled European trial, entitled "Effect of Allopurinol in addition to hypothermia for hypoxic ischemic Brain Injury on Neurocognitive Outcome" (ALBINO, EudraCT-No 2016-000222-19) (26).

In the ALBINO trial, the area under the concentration curve (AUC) of allopurinol and oxypurinol following early allopurinol administration during therapeutic hypothermia was also investigated as pharmacokinetic outcome variable to ensure that target exposure is attained (26).

Pharmacokinetics of allopurinol in (near-)term neonates with HIE has already been studied in earlier trials mentioned above and showed that two intravenous doses of 20mg/kg (within 4 hours after birth and 12 hours later) resulted in supratherapeutic doses without toxicity (18,19,27). However, all these patients were included before therapeutic hypothermia became standard of care and both asphyxia and therapeutic hypothermia affect pharmacokinetics (28).

Therefore, the primary aim of this pharmacokinetic sub-study of the ALBINO trial was to determine whether the pre-defined target AUC of allopurinol (43.5 mg/L*h) and oxypurinol (26.5 mg/L*h) are met (27).

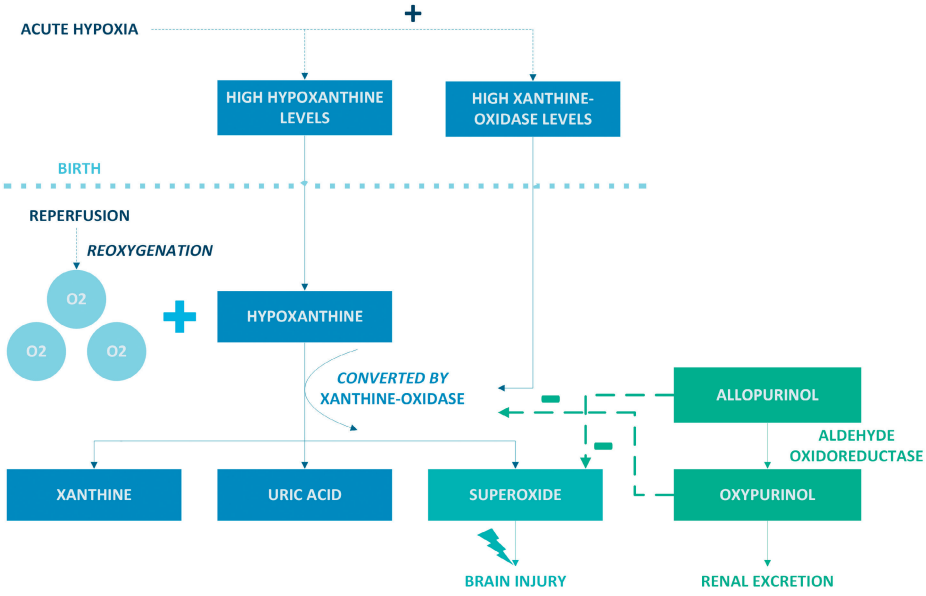

Figure 1: During acute (fetal) hypoxia, hypoxanthine and xanthine-oxidase levels rise because of primary energy failure and ATP degradation. After birth, during reperfusion, hypoxanthine in combination with oxygen is converted by xanthine-oxidase into xanthine, uric acid and the toxic superoxide. Allopurinol and its active metabolite (oxypurinol) inhibit the conversion of hypoxanthine and oxygen into superoxide. Allopurinol and oxypurinol are eliminated via the kidneys.

METHODS

Setting and study design

(Near-)term patients who fulfilled criteria for perinatal asphyxia (see below) and early signs of evolving encephalopathy and were included in the double blind randomized ALBINO trial received an initial dose of study medication (20 mg/kg allopurinol or placebo, mannitol) intravenously during resuscitation within 45 minutes after birth. The second dose of 10 mg/kg allopurinol or placebo was administered 12 hours after the initial dose in infants who needed therapeutic hypothermia. Patients were eligible for the ALBINO trial if they fulfilled one or more of the criteria for perinatal asphyxia: 1) pH < 7.00 or base deficit ≥ 16 mmol/l, 2) need for ongoing cardiac massage for ≥ 5 min postpartum, 3) need for adrenalin administration during resuscitation, 4) Apgar score ≤5 after 10 min postpartum; in combination with two or more early signs of evolving encephalopathy: 1) altered state of consciousness, 2) hypotonia or hypertonia, 3) absent/insufficient spontaneous respiration requiring

respiratory support for ≥ 10 min postpartum, 4) abnormal primitive reflexes/ abnormal movements i.e. seizures. The most important exclusion criteria were gestational age <36 weeks, estimated birth weight <2500 gram or severe congenital abnormalities. Infants who met the inclusion criteria for the ALBINO, but did not meet the therapeutic hypothermia criteria because of a quick and spontaneous recovery, received the initial dose of allopurinol or placebo but did not receive the second dose. Further details of the study protocol are previously published (26). The trial is registered at www.clinicaltrials.gov (NCT03162653).

Therapeutic hypothermia was conducted according to the national treatment protocols (29,30). Therapeutic hypothermia was started within 6 hours after birth to a core temperature of 33.5˚C for 72 hours. Thereafter, patients were slowly rewarmed to normothermia and after rewarming, body temperature was stabilized at 36.5˚C for 24 hours (29,30).

Short oral consent was obtained from at least one parent before administering the study medication. After the first dose, but before the (potential) second dose, full written consent was obtained from both parents. In case parents did not sign informed consent, blood samples collected shortly after birth were destroyed.

The medical ethics committee of the University Medical Center Utrecht and the Central Committee on Human Research approved the study for the Netherlands (NL57237.041.16) and the German Federal Authority (EudraCT 2016-000222-19) as well as the leading Ethics Committee Tuebingen approved the study for Germany.

Target exposure

The pre-defined minimum target AUC of 43.5mg/L*h for allopurinol and 26.5mg/L*h for oxypurinol between 0-12 hours were expected to be reached in more than two thirds (>66%) of the analyzed patients. This minimum target AUC was calculated based on the lowest individual values observed in previous clinical studies of postnatal allopurinol in neonates (18,27).

Timing of this preliminary assessment of pharmacokinetics

According to the ALBINO study protocol the pharmacokinetic study should include 48-52 patients corresponding to about 20-25 infants receiving allopurinol. This sample size was intended to be reached within 12 months after the first included

patient; but as fewer than anticipated study sites contributed to the pharmacokinetic blood sampling, sampling was delayed. In October 2019, 18 months after the first included patient, the ALBINO consortium decided to analyze all available blood samples at that time in a preliminary assessment of target exposure to inform ongoing conduct of the study as a safety measure.

Blood sampling for pharmacokinetics

Blood sampling was combined with clinically indicated blood samples. To limit the number of blood samples per patient there were two sampling schedules: A and B for both patients treated with hypothermia and patients who recovered quickly and were not cooled (Table 1). Investigators had to document the exact date and time of actual blood sampling for each scheduled time interval.

Table 1: Sample intervals

Group	Sampling schedule
No hypothermia	
A	15-60 min, 1.5-4h, 8-12h, 18-24h, 60-72h
B	15-60 min, 1.5-4h, 8-12h, 36-48h, 96-168h
Hypothermia	
A	15-60 min, 1.5-4h, trough level t=12h, 13-14h, 18-24h, 60-72h
B	15-60 min, 1.5-4h, trough level t=12h, 13-14h, 36-48h, 96-168h

For the pharmacokinetic (PK) analyses 0.5 ml blood was collected in MiniCollect® lithium-heparin tubes (Greiner Bio One, Alphen aan den Rijn, the Netherlands). The collected sample was placed in melting ice immediately after collection and centrifuged with a speed of 1500-2000*g for 10 minutes at 4-8°C within 30 minutes after collection. Afterwards the plasma was separated with a pipette and stored in a polypropylene crew cap in a -80°C fridge.

Bioanalysis

Bioanalysis was performed by Ardena Biochemical Laboratory (ABL, Ghent, Belgium). Allopurinol, oxypurinol, xanthine, hypoxanthine and uric acid in human plasma samples were extracted following protein precipitation using ethanol. After precipitation and centrifugation, the supernatant was injected into the LC-MS/MS chromatographic system for the determination of allopurinol, oxypurinol, xanthine

and hypoxanthine. A dilution of the supernatant with mobile phase was performed for the determination of uric acid before injection into the chromatographic system. The chromatographic separation was performed on an Astec® CHIROBIOTIC®V HPLC column using isocratic elution. An API 4000 tandem mass spectrometer equipped with a turbo ion spray probe operating in the negative multiple reaction monitoring mode was used for quantification. The assay was validated according the FDA and EMA guidelines. The lower limit of quantification (LLOQ) was 0.1 mg/L for allopurinol and 0.0934 mg/L for oxypurinol.

Pharmacokinetic modelling

A population PK model was developed for allopurinol and oxypurinol using nonlinear mixed effect modeling (NONMEM 7.3, Icon Development Solutions). Pirana (version 2.9.9) was used as graphical user interface for NONMEM (31). R (version 3.4.3) was used for data handling and visualization.

The estimated pharmacokinetic parameters were: volume of distribution (Vd) and total body clearance (CL) for allopurinol and oxypurinol. The fraction of allopurinol converted to oxypurinol in neonates was unknown. Therefore, oxypurinol parameter estimates were relative to formation fraction (fm), i.e. Vd/fm and CL/fm. Birth weight (BW), normalized to BW of 3.5kg, was used as a description of body size and was related to PK parameters using allometric relationships (32). The exponent defining the relationship of BW and CL was fixed to 0.75 and the exponent defining the relationship of BW and Vd was fixed to 1. Measurements below the LLOQ were included. Values were set to LLOQ/2, which was 0.05 mg/L for allopurinol and 0.0467 mg/L for oxypurinol (33).

Interindividual variability (IIV) was incorporated using a proportional model and was tested for CL and Vd for both allopurinol and oxypurinol. A combination of a proportional and additive error model was used to describe residual unexplained variability. Additive errors were fixed to 0.05 for allopurinol and 0.0467 for oxypurinol to account for the measurements below LLOQ (33). Separate error models were used for allopurinol and oxypurinol. Parameter precision was accessed with sampling importance resampling (SIR) (34). Model adequacy was evaluated through both statistical procedures, goodness-of-fit (GOF) plots and physiological plausibility. Statistical significance was evaluated by the change in objective function value (OFV), which is equal to minus two the log-likelihood. A decrease in OFV of

≥10.8 points, corresponding to a p-value of <0.001, was considered statistically significant (χ^2-distribution with 1 degree of freedom (df)).

Individual PK parameter estimates were obtained as MAP Bayesian estimates using the POSTHOC option of NONMEM. From these estimates the total exposure expressed as AUC value were calculated.

The analytical and modelling facilities will be unblinded to treatment allocation (after signing an appropriate confidentiality agreement), while maintaining blinding of treatment allocation in all clinical centers. The analytical and modelling facilities are not otherwise involved in the ALBINO.

RESULTS

In total, 15 patients received allopurinol, 9 of them were treated with hypothermia. The mean number of samples per patient was 5. Plasma concentrations of allopurinol varied between 0.706 mg/L and 24.4 mg/L, and 10 (14.7%) samples were below LLOQ. Oxypurinol concentrations ranged from 0.146 mg/L to 8.84 mg/L, and 4 (5.9%) samples were below LLOQ. See Table 2 for baseline characteristics.

Table 2: Baseline characteristics

Characteristic	Therapeutic hypothermia (n=9)	No therapeutic hypothermia (n=6)
Gestational age (weeks), median (IQR)	39 (2.0)	40 (1.5)
Birth weight (gram), median (IQR)	3150 (690)	3760 (470)
Male, n (%)	6 (66.7)	1 (16.7)
Lactate level at 12 hours after birth, median (IQR)	16.4 (13.4)	2.8 (3.4)
Thompson score at 2-6 h after birth (before start of therapeutic hypothermia if applicable), median (IQR)	9 (10.00)	5 (0.75)

IQR = interquartile range

Figure 2 shows the time concentration profiles of allopurinol, oxypurinol, hypoxanthine, xanthine and uric acid. Figure 3 shows the allopurinol (A) and oxypurinol (B) concentrations over time within the first 24 hours after birth.

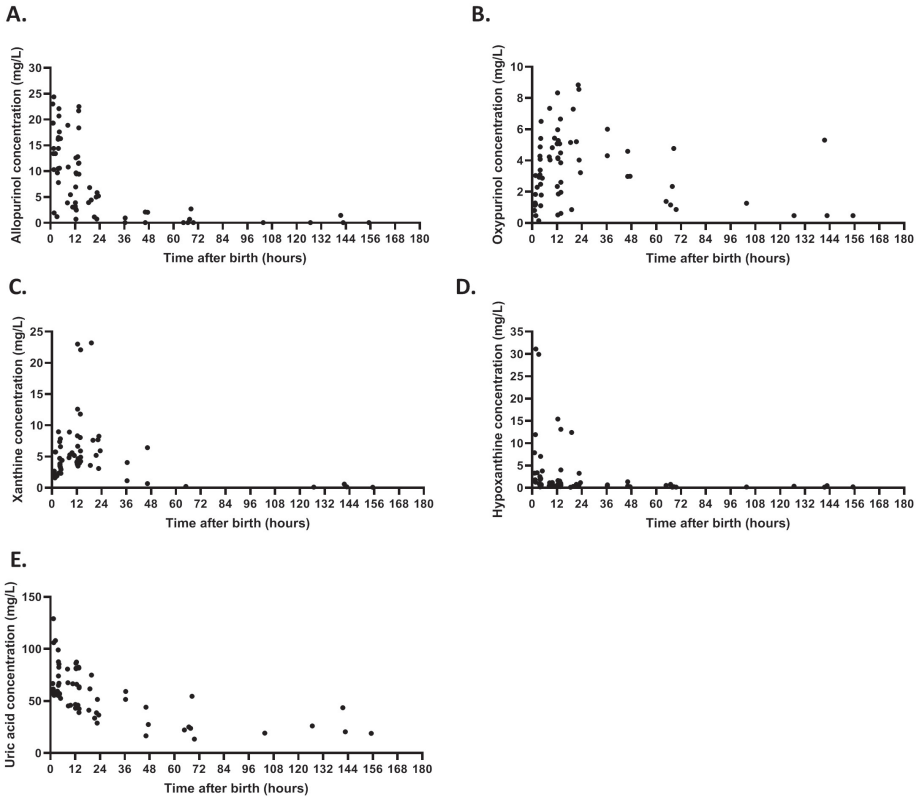

Figure 2: Allopurinol (A), oxypurinol (B), xanthine (C), hypoxanthine (D) and uric acid (E) concentrations over time.

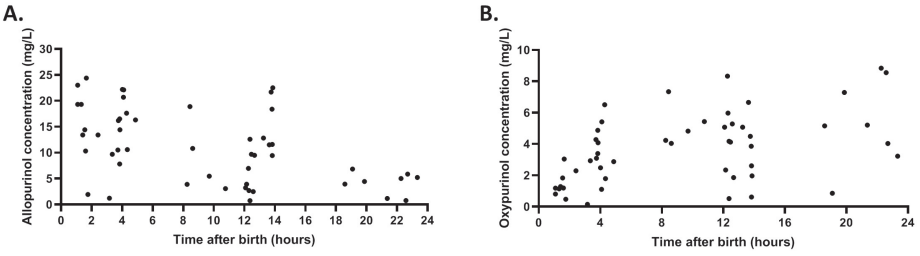

Figure 3: allopurinol (A) and oxypurinol (B) concentrations over time within the first 24 hours after birth.

A one-compartment model for allopurinol and a subsequent one-compartment model for its metabolite oxypurinol were developed to describe the PK of allopurinol and oxypurinol. To reduce the complexity of model, interindividual variabilities of Vd were omitted. Population pharmacokinetic parameters estimated were normalized to a BW of 3.5 kg and are described in Table 3.

Table 3: Population PK parameters (mean) for allopurinol and oxypurinol in infants treated with and without therapeutic hypothermia.

Parameter	Allopurinol		Oxypurinol *	
Structural model	Estimate	SIR 95%CI	Estimate	SIR 95%CI
Clearance (L/h)	0.34	0.27-0.41	0.31	0.21-0.45
Vd (L)	3.79	3.40-4.26	5.54	4.79-6.42
Interindividual variability				
Clearance (%)	34.6		76.2	
Residual variability				
Proportional (%)	15		24	
Additive	0.05		0.0467	

*Parameter estimates for oxypurinol were relative to formation fraction (fm)

Estimated allopurinol AUC values varied between 78 and 264 mg/L*h, and estimated oxypurinol AUC values ranged from 10.6 to 71.6 mg/L*h. The target AUC for allopurinol of 43.5mg/L*h was reached in all infants in the hypothermia groups and no-hypothermia group. The target AUC for oxypurinol of 26.5mg/L*h was reached in 66.7% of the infants treated with hypothermia and in 100% of the infants in the no-hypothermia group. In the total group 12 out of 15 patient (80%) reached the target AUC for oxypurinol.

Table 4: Proportions of infants who reached the target AUC for allopurinol and oxypurinol in infants treated with hypothermia and those who were not.

Target AUC	Total group (n=15)	Hypothermia (n=9)	No-hypothermia (n=6)
Allopurinol (43.5 mg/L*h)	100%	100%	100%
Oxypurinol (26.5 mg/L*h)	80%	66.7%	100%

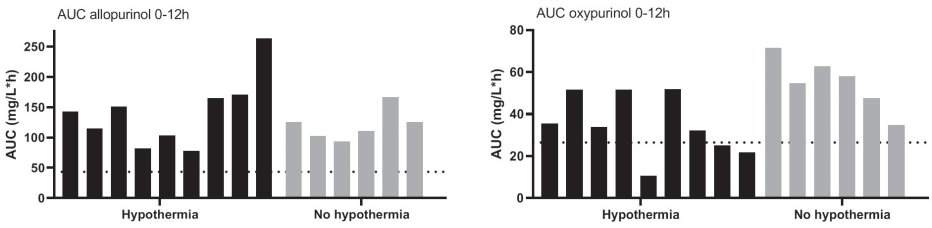

Figure 4: Estimated AUC values of allopurinol and oxypurinol per patient. The target AUC is shown by the dotted line.

One patient had a very low AUC of oxypurinol with oxypurinol concentrations that were about 10% of corresponding concentrations in the other patients. The metabolic ratio (allopurinol to oxypurinol) seemed to be lower both after the first and second dose (data not shown). Subsequently the hypoxanthine, xanthine and uric acid levels of this patient were higher compared to the other patients, suggesting less xanthine-oxidase inhibition.

Figure 5 and 6 show the GOF plots for both allopurinol and oxypurinol.

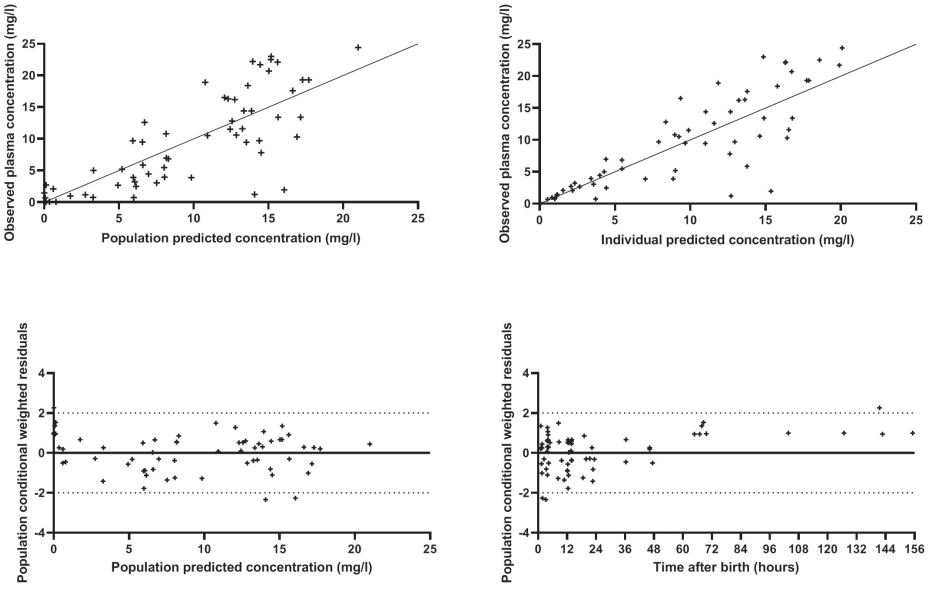

Figure 5: Goodness of fit plots for allopurinol

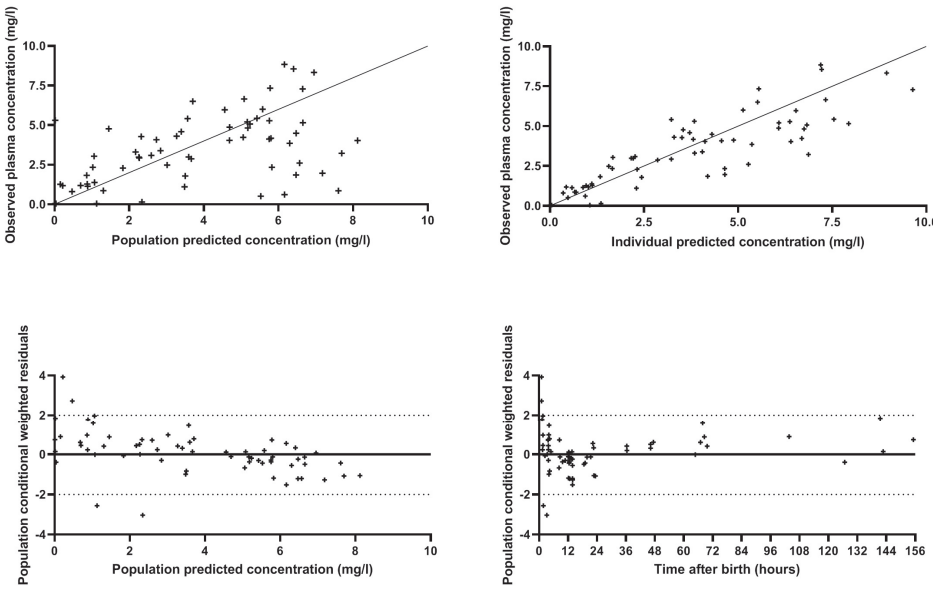

Figure 6: Goodness of fit plots for oxypurinol

DISCUSSION

This study showed that the target concentrations for allopurinol and oxypurinol in the ALBINO trial were met with the current dosing regimen for infants with HIE treated with and without therapeutic hypothermia. Therefore, changes in the dosing regimen in the ALBINO trial were deemed not necessary.

This is the first study assessing the PK of allopurinol in infants with HIE treated with therapeutic hypothermia, as well as the first model including both allopurinol and oxypurinol PK in neonates (26). Nine out of 15 patients had allopurinol concentrations above the upper limit for therapeutic concentrations in adults for gout. High concentrations of allopurinol are important for its ability to directly scavenge free radicals and chelate non-protein-bound iron (8,9). Van Kesteren *et al.* previously described the PK of allopurinol in non-cooled infants with severe HIE (27). Data were used from two postnatal studies in which two doses of 20 mg/kg (first dose within 4 hours after birth and second dose 12 hours later) were administered (18,19,27). Like in the ALBINO PK study, a one compartment model best described their allopurinol data (27). The clearance of allopurinol in the cohort of van Kesteren *et al.* was 0.078 L/h/kg and in our study the clearance was 0.34 L/h (=0.097L/h/kg) for allopurinol.

Van Kesteren *et al.* found an interindividual variability of 60% for allopurinol clearance (27). In the current study, physiological processes were incorporated by adding body weight as a descriptor for body size. Despite this, there is still an interindividual variability of 35% in allopurinol clearance.

In addition to allopurinol PK, we also estimated oxypurinol PK parameters. Oxypurinol clearance was estimated to be 0.31 L/h for a 3.5 kg infant. Since oxypurinol shows an equal ability in inhibiting xanthine-oxidase and has a relatively long half-life, the PK of oxypurinol is responsible for much of the pharmacological activity of allopurinol. An interindividual variability of 76% for clearance of oxypurinol was found. This large interindividual variability of oxypurinol is probably caused by the outlier.

Furthermore, the differences can be explained because of the different study populations. In the study of van Kesteren *et al.* the study population had more severe perinatal asphyxia and HIE and in the ALBINO trial also infants with mild HIE were included because the decision to include infants had to be made much earlier after birth when the degree of HIE cannot be assessed with certainty. The risk of renal failure was therefore higher in the previous studies which might explain the lower clearance. Additionally, in the ALBINO trial therapeutic hypothermia might have had an effect on the PK of allopurinol.

Multi-organ failure is common in infants with moderate to severe perinatal asphyxia and may have an impact on PK in infants with HIE (28,35). Multi-organ failure, such as hepatic failure due to hepatocyte necrosis and renal failure due to acute tubular necrosis, can reduce the clearance of drugs (35).

As mentioned before, therapeutic hypothermia might also influence the pharmacokinetics of allopurinol. Van den Broek *et al.* reviewed the literature on the effect of therapeutic hypothermia on the PK of different drugs and found that absorption can be prolonged, the volume of distribution can be altered, *i.e.* by a lower pH or altered protein binding capacity, and clearance can be reduced (28). Therapeutic hypothermia can result in decreased cardiac output and increased vascular resistance leading to impaired liver and kidney perfusion (36). Also, the activity of liver enzymes and transporters that are important for hepatic clearance can be altered by hypothermia, as well as active tubular secretion (36,37). Favie *et al.*

described that morphine clearance in infants with HIE was about 21% lower during therapeutic hypothermia, which was both dependent on hepatic and renal clearance (38). The clearance of its metabolite was 15% lower in the hypothermia treated infants, which mainly depended on the renal clearance. So both the liver and kidneys seemed to have reduced clearing capacities in the hypothermia group, which has been confirmed by others (28,36,38).

The AUC for oxypurinol depends on the metabolization of allopurinol into oxypurinol and renal clearance which can both be influenced by therapeutic hypothermia and severe asphyxia. Allopurinol is almost completely metabolized by the cytosolic enzyme aldehyde oxidase into oxypurinol, which is present throughout the body, and in high concentrations in the liver. A lower formation of oxypurinol will result in a lower AUC. Oxypurinol is eliminated by the kidneys, so impaired renal clearance due to therapeutic hypothermia or renal failure can lead to a higher AUC for oxypurinol, and in a lesser extent also for allopurinol. Because of the sample size, we were not able to estimate the effect of therapeutic hypothermia and organ failure in our cohort.

In the current study, the uric acid concentrations decreased over time. The normal range for uric acid in neonates is approximately between 10 and 40 mg/L. Some allopurinol treated infants still had higher uric acid concentration. The study of van Bel *et al.* showed that uric acid levels were elevated and decreased over time in both the allopurinol and control group, but in the allopurinol group the concentrations of uric acid were significantly lower at all time points (18). Though allopurinol and oxypurinol inhibit the conversion of hypoxanthine into uric acid, it does not improve excretion of uric acid via the kidneys, which might explain the relatively high concentrations.

There was one specific outlier in our cohort, with a significant lower AUC for oxypurinol, and - to a lesser extent - for allopurinol. The metabolic ratio (allopurinol/oxypurinol ratio) was lower after the first and second dose, however, we should be cautious since oxypurinol has a long elimination half-life. We suggest that this outlier is most likely explained by a dosing error such as leakage during perfusion or a discrepancy between the prescribed and administered first dose, with a correct second dose. Although an additional poor metabolizer status for aldehyde oxidase cannot be excluded. Beedham *et al.* described that polymorphisms in the gene hAOX1, can lead to either poor and fast metabolizer variants (39). Unfortunately,

we were not able to investigate whether the outlier in the ALBINO trial had a polymorphism in hAOX1.

This pilot study had some limitations. The sample size for this pharmacokinetic study was rather small to evaluate the effect of therapeutic hypothermia and disease severity as covariates in the model. Small sample sizes with limited data points are a well-known problem in pediatrics (40). Therefore, the population approach is preferable because it allows to analyze limited data with unequal distribution of samples over time and between individuals. The nonlinear mixed effect model allows to investigate the population pharmacokinetics but also takes into account that the samples come from different individuals (41). Another limitation is that the ALBINO trial is still ongoing, so only limited clinical characteristics were available to prevent unblinding.

We recommend to combine the pharmacokinetic results of the ALBINO trial with the historical data of van Kesteren *et al.* (27) to increase the sample size and investigate the effect of covariates on the pharmacokinetics of allopurinol and oxypurinol. This allows us to draw conclusions about the effect of therapeutic hypothermia and disease severity, and also of maturation and organ recovery, on the pharmacokinetics of allopurinol in neonates with HIE.

CONCLUSION

Dosing regimens in the ALBINO trial are adequate as predefined in the study protocol, also during hypothermia. No dosing adaptations are necessary.

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The bioanalysis of allopurinol and oxypurinol was performed at ABL on the request of the industry partner of the ALBINO consortium to meet Good Laboratory Practice standards according to EMA and FDA requirements. ABL received compensation for their work from the grant from the European Union.

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DISCLOSURE

None of the authors had a conflict of interest to disclose.

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Part III

INNOVATIVE NEUROIMAGING TECHNIQUES

CHAPTER **10**

Brain temperature of infants with neonatal encephalopathy following perinatal asphyxia calculated using magnetic resonance spectroscopy

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ABSTRACT

Background: Little is known about brain temperature of neonates during MRI. Brain temperature can be estimated non-invasively with proton Magnetic Resonance Spectroscopy (^{1}H-MRS), but the most accurate ^{1}H-MRS method has not yet been determined. The primary aim was to estimate brain temperature using ^{1}H-MRS in infants with neonatal encephalopathy following perinatal asphyxia (NE). The secondary aim was to compare brain temperature during MRI with rectal temperatures before and after MRI.

Methods: In this retrospective study, brain temperature in 36 (near-)term infants with NE was estimated using short (36ms) and long (288ms) echo time (TE) ^{1}H-MRS. Brain temperature was calculated using two different formulas: formula of Wu *et al.* and a formula based on phantom calibration. The methods were compared. Rectal temperatures were collected <3hours before and after MRI.

Results: Brain temperatures calculated with the formula of Wu *et al.* and the calibrated formula were similar as well as brain temperatures derived from short and long TE ^{1}H-MRS. Rectal temperature did not differ before and after MRI.

Conclusion: Brain temperature can be measured using ^{1}H-MRS in daily clinical practice using the formula of Wu *et al*. with both short and long TE ^{1}H-MRS. Brain temperature remained within physiological range during MRI.

INTRODUCTION

Over the past decades, magnetic resonance imaging (MRI) has become one of the most important neuroimaging techniques to assess brain injury in high-risk neonates (1,2). MRI has shown to be more sensitive to diagnose brain injury in neonates compared to computed tomography or cerebral ultrasound (2,3). Furthermore, no radiation is used making it safe to use in neonates (2).

Although MRI is safe in neonates, there are some potential risks that should be considered, such as an increase in the temperature of the body and brain (4,5). To conduct an MRI, pulses of radiofrequency (RF) energy are applied to create images (1). This RF energy is partly absorbed by the tissue of the patient, which can potentially lead to an increase in temperature (1). The amount of RF energy in Watt absorbed by 1 kg of tissue of the patient is called the specific absorption rate (SAR). So, a higher SAR increases the risk of a rise in body temperature. Therefore, the SAR level is monitored by the MR scanner and scanning is limited when SAR levels are about to exceed the maximum allowed SAR limits as specified by the FDA guidelines (6).

However, little is known about the exact effect of the SAR on the brain temperature of neonates during MRI. The rectal temperature of term and preterm neonates seems to be similar before and after MRI (4,7). Although body and brain temperatures are correlated, it has not yet been fully elucidated what the exact effect is of MRI on the brain temperature. In addition, the temperature management during MRI is less optimal due to the low temperature in the MR room (18°C) which might also decrease body temperature if no temperature-controlled MR incubator can be used (5).

Recent studies have shown that it is possible to non-invasively measure brain temperature with proton Magnetic Resonance Spectroscopy (^{1}H-MRS) (8–10). The chemical shift of water is temperature dependent, whereas the chemical shift of some metabolites in the brain tissue such as N-acetyl aspartate (NAA) is not. This chemical shift difference (ΔH_2O-NAA) can be used to determine the temperature with an accuracy of 0.5 °C in 1.5T and 3.0T systems (8,11).

The primary aim of this study was to assess the feasibility of measuring brain temperature non-invasively using ^{1}H-MRS in infants with neonatal encephalopathy (NE) following perinatal asphyxia. The clinical feasibility was investigated by

determining whether brain temperatures calculated with a previously developed formula were similar to brain temperatures calculated with a formula developed using phantom calibration. Furthermore, brain temperatures measured using short (36ms) and long (288ms) echo time (TE) ^{1}H-MRS were compared. The secondary aim was to compare the MRS-derived brain temperature in infants with NE with rectal temperature before and after MRI.

METHODS

Design and subjects

In this single-center retrospective study, (near-)term infants with NE following perinatal asphyxia (referred to as NE) who were admitted to the level III Neonatal Intensive Care Unit (NICU) of the University Medical Center Utrecht (UMCU) between December 2011 and June 2017 were eligible for inclusion. Neonates born with a gestational age of ³ 36 weeks, diagnosed with NE following perinatal asphyxia and treated with therapeutic whole-body hypothermia according to international guidelines (12) were included. Furthermore, it was essential that ^{1}H-MRS of sufficient quality and rectal temperature data (not more than 3 hours) before and after the MRI were available for analyses. Infants with metabolic or genetic abnormalities were excluded. All infants were included in the PharmaCool study (www.trialregister.nl NL2421) (13) or 2-STEP study (www.trialregister.nl NL5089) (14) and informed consent of parents for the use of their infants data was available. These studies were approved by the Ethical Committee of the UMCU.

Clinical parameters

All baseline characteristics and rectal temperature data were retrospectively obtained from electronic medical records. The rectal temperatures closest, and not more than 3 hours, before and after MRI were collected. These temperatures were all measured on the NICU using a rectal thermometer or using a rectal temperature probe for continuous temperature monitoring.

Validation of the formula

The local brain temperature was calculated by the formula of the study of Wu *et al.* (8): $T=(-102.89*\Delta H_2O\text{-}NAA)+308.64$; with T as local brain temperature in degrees Celsius and ΔH_2O-NAA being the difference between the spectral positions of water and NAA in parts per million (ppm). This formula is developed in infants with NE using short TE 3.0T MRI.

With phantom measurement in our 3.0T MR scanner, we also calibrated a new formula specific for our scanner, referred to as the calibrated formula, to validate the formula of Wu *et al* (8). A small, in-house developed, spherical phantom with a diameter of approximately 3 cm containing a water solution with creatine, GABA, glutamate, glutamine and NAA with a pH of 7.4 was used. Temperature was measured continuously by securing a Neoptix fiber optic sensor on top of the phantom (Neoptix, Qualitrol Company LLC of Fairport, NY, USA). The phantom was heated in a water bath until the temperature was 45 °C. Thereafter, the phantom was wrapped in towels to slow down the cooling process. The phantom was placed in the isocenter of the MRI and during cooling (from 43 to 30 °C), 20 ^{1}H-MRS scans were conducted using long and short TE alternating. By analyses of the ΔH_2O-NAA and actual measured temperature, a new formula was developed. This formula was compared to the formula of Wu *et al.* to assess whether the two formulas differed (8).

MRI acquisition and brain temperature calculation

MRI examinations were conducted using a 1.5T or 3.0T Achieva scanner (Philips Medical Systems, Best, the Netherlands). As standard of care, all infants with NE who were treated with hypothermia underwent cerebral MRI within the first week after birth with a duration of 30 to 45 minutes. All neonates were scanned in a vacuum mattress to prevent movement artefacts (Med Vac Infant Immobilizer Bag, Radstadt, Austria). During the MRI, the heart rate and oxygen saturation was measured with a pulse oximeter (Nonin, Minneapolis, MN) and respiration rate was observed using the standard Philips equipment (Philips Medical Systems, Best, Netherlands).

Scan protocols included, among others, single voxel ^{1}H-MRS (PRESS, repetition time=2000ms, TE=36ms (short TE) and/or 288ms (long TE), phase cycles=16, 64 measurements, voxel sizes varied between 10x10x10mm and 20x10x10mm, water suppression method = “excitation” (2-water selective pulses followed by spoilers). The region of interest (ROI) for single voxel ^{1}H-MRS was the left deep grey matter, according to Alderliesten *et al.* (15).

The quality of the spectra was visually inspected (visual estimation signal to noise ratio (SNR) of NAA peak should be larger than 3 and measurement of line width should be lower than 10Hz, Figure 1) and spectra with poor quality *i.e.* low SNR or clear artefacts from water suppression were excluded. If the quality was sufficient, jMRUI software version 5.2 was used to analyze the spectrum (16). The peak of NAA

was expected at ~2.02 ppm. As selective excitation was used for water suppression, the residual water peak is still in phase and can be used to determine its frequency position. The positions of the NAA and H_2O peak in the spectrum were determined automatically by using the 'HLSVDPro'-option of the jMRUI software, which determines the spectral position of the fifteen highest peaks automatically (17).

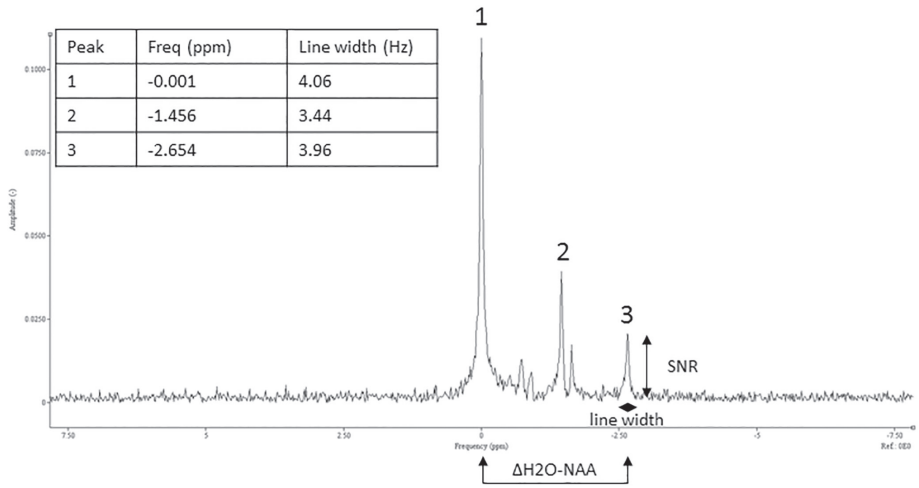

Figure 1: Example of long TE ^{1}H-MRS showing the ΔH_2O-NAA and determination of the quality of the spectrum including visual estimation signal to noise ratio of NAA peak (signal NAA is more than 3 times the noise) and determination of the band width (lower than 10Hz). Peak 1 is the H_2O peak, peak 2 is the choline peak and peak 3 is the NAA peak.

Statistical tests

Statistical analysis was performed using IBM SPSS Statistics for Windows version 25.0 (IBM Corp., Illinois, USA). Linear univariate regression analysis was performed, with the actual temperature of the water bath as independent variable and ΔH_2O-NAA as dependent variable, to develop the new, calibrated formula to calculate brain temperature for validation. Paired sample t-tests for normally distributed and Wilcoxon Signed rank tests for non-Gaussian distributions were performed to compare the brain temperatures calculated with the two formulas and with short versus long TEs. The Pearson correlation for normally distributed parameters and Spearman correlation for non-Gaussian distributed parameters were used to test the association between brain temperatures and rectal temperatures. The Kruskal-Wallis-H test was performed to calculate differences amongst temperature

measurements before, during and after MRI, followed by post-hoc comparison with Wilcoxon-signed rank test when differences were statistically significant. Lastly, multivariable linear regression analysis was performed to explore the association between therapeutic hypothermia and head circumference as independent variables and the difference between rectal temperature before MRI and the brain temperature during MRI as dependent variable, because these might be factors that influence the risk of cooling down during MRI. p-values <0.05 were considered statistically significant. All p-values have been corrected for multiple comparisons by multiplying the p-value with the number of performed tests.

RESULTS

Subjects

Between December 2011 and June 2017, 49 patients with NE, who were treated with therapeutic hypothermia, had ^{1}H-MRS and temperature data available. Of those patients, 13 patients were excluded because of insufficient quality of the ^{1}H-MRS data. So, 36 patients were included in the study. Table 1 shows the baseline characteristics of the included infants. Usually patients were scanned during or after the rewarming process following therapeutic hypothermia (body temperature >35.5˚C). Seven infants had an MRI during therapeutic hypothermia or early rewarming for clinical reasons. In one infant scanned during rewarming, therapeutic hypothermia was stopped before the MRI was conducted and therefore the temperature is higher at the end of the MRI.

Table 1: Baseline characteristics for the two temperature groups during MRI.

Characteristic	Normothermic (n=29)	Hypothermia (n=7)
Male sex, n (%)	15 (51.7)	5 (71.4)
Gestational age in weeks, median (IQR)	40.0 (38.6 – 40.9)	39.7 (37.6 – 40.0)
Birth weight in grams, median (IQR)	3500 (2976 – 3889)	3170 (3100-3575)
Head circumference in cm, median (IQR)	35.0 (32.9 – 36.0)	35.0 (33.0 – 35.3)
Apgar score at 5 minutes, median (IQR)	4.0 (2.8 – 5.0)	4.5 (1.0 – 8.8)
Apgar score at 10 minutes, median (IQR)	5.0 (4.0 – 7.0)	5.0 (4.0 – 5.0)
Postnatal age at MRI in hours, median (IQR)	131.7 (115.0 –152.3)	85.8 (65.7 – 94.0)
Peripheral Temperature before MRI in ˚C, median (IQR)	36.8 (36.6 – 37.1)	33.6 (33.4 – 34.3)

Table 1: Continued

Characteristic	Normothermic (n=29)	Hypothermia (n=7)
Timing temperature before start MRI in hours, median (IQR)	-1.0 (-1.0 – -1.0)	0.0 (-1.0 – 0.0)
Temperature measured with continuous rectal temperature monitor, n (%)	25 (86.2)	7 (100.0)
Peripheral Temperature after MRI in ˚C, median (IQR)	36.5 (36.2 – 36.8)	33.4 (32.8 – 35.0)
Timing temperature after start of MRI in hours, median (IQR)	2.0 (2.0 – 2.0)	3.0 (2.0 – 3.0)
Temperature measured with continuous rectal temperature monitor, n (%)	21 (72.4)	7 (100.0)
Short TE ^{1}H-MRS available, n (%)	20 (68.9)	5 (71.4)
Long TE ^{1}H-MRS available, n (%)	25 (86.2)	6 (85.7)

Calibration

First of all, the phantom was used to calculate a calibrated formula for our scanner. Figure 2 shows the actual temperatures of the phantom plotted against the ΔH_2O-NAA. The following formula is calculated based on these data: T=263-(ΔH_2O-NAA*85.76), in which T is the temperature and ΔH_2O-NAA is the chemical shift between NAA and H_2O in ppm. The r^2 of this curve was 0.96.

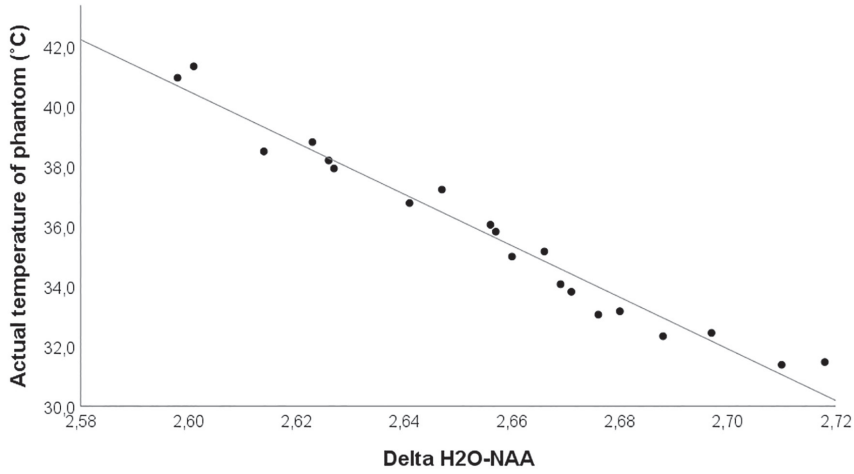

Figure 2: Phantom calibration. The temperatures of the phantom are plotted against the chemical shift difference (ΔH_2O-NAA) to calculate the calibrated formula.

Calculation of brain temperature using MRS in neonates: comparing the formulas

The formula of Wu *et al.* was compared to the calibrated formula to assess whether these were different in neonates, which would imply that calibration is necessary.

The median brain temperature was 35.1˚C (IQR 33.6 – 36.1) according to the formula of Wu *et al.* and 35.0˚C (IQR 33.7 – 35.9) according to the calibrated formula using short TE ^{1}H-MRS (p=0.68). Using long TE ^{1}H-MRS, the median brain temperature assessed according to Wu *et al.* was 35.5˚C (IQR 34.4 – 36.2) and according to the calibrated formula 35.3˚C (IQR 34.4 – 35.9). This difference was just significant (p=0.048).

The difference between the brain temperatures calculated with the two formulas varied for short TE ^{1}H-MRS between 0.01˚C and 0.67˚C (mean=0.06˚C) and for long TE ^{1}H-MRS this varied between 0.01˚C and 0.85˚C (mean=0.15˚C). See Figure 3.

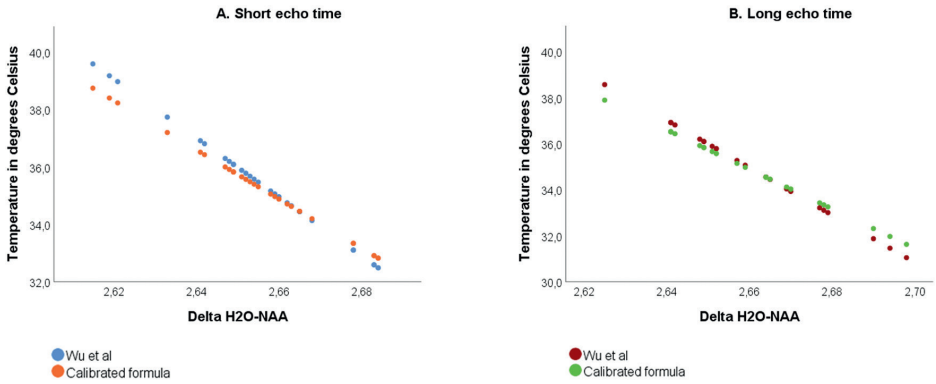

Figure 3: Comparison of the formulas for short and long TE. For each ΔH_2O-NAA the temperature calculated with the formula of Wu *et al.* and the calibrated formula are shown for as well short TE (A) and long TE (B). The intersection point of both curves was (2.664; 34.507).

Calculation of brain temperature using MRS in neonates: short versus long TE ^{1}H-MRS

Twenty patients had both short TE and long TE ^{1}H-MRS available and brain temperatures derived from the different TEs were compared within patients. In normothermic and hypothermic patients, differences in brain temperatures derived from short and long TE ^{1}H-MRS according to Wu *et al.* were not statistically different. The differences in brain temperatures measured with short and long TE ^{1}H-MRS according to the calibrated formula were also comparable for normothermic

and hypothermic patients. However, there were individuals in whom there was a difference of >1 ˚C between short and long TE, see Figure 4.

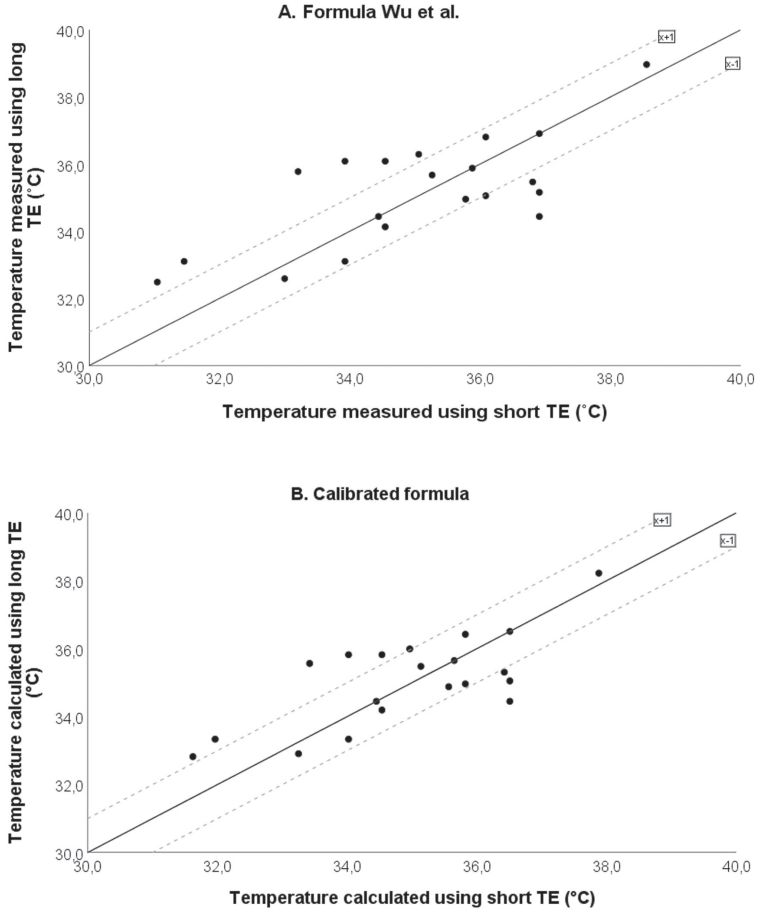

Figure 4: The correlation of brain temperatures calculated based on short versus long TE ^{1}H-MRS using formula of Wu *et al.* (A) and the calibrated formula (B).

Comparison of rectal temperature and brain temperature

Table 2 shows the correlation of the brain temperature, for each method, with rectal temperatures at the NICU. Brain temperatures correlated significantly with temperature before and after MRI when tested in the entire cohort, including both normothermic and hypothermic infants. The rectal temperature before and after MRI were also significantly correlated (r=0.663, p<0.001).

Table 2: Spearman's Rho correlations between brain temperature and peripheral temperature before and after MRI are shown in this Table. The correlations have been tested for both formulas and TE.

Formula	Echo time (TE)	Correlation with temperature before MRI	Correlation with temperature after MRI
Wu *et al.*	Short TE (36ms)	0.648 (p=0.001)	0.617 (p=0.001)
	Long TE (288ms)	0.471 (p=0.009)	0.666 (p<0.001)
Calibration	Short TE (36ms)	0.650 (p=0.001)	0.612 (p=0.001)
	Long TE (288ms)	0.474 (p=0.008)	0.670 (p<0.001)

Afterward, differences in temperatures before, during and after MRI were compared using the Kruskal-Wallis-H test and post-hoc tests for normothermic and hypothermic patients for the different methods (see Table 3).

Table 3: The differences between temperature before, during and after MRI are shown for all methods.

Formula	Hypothermic neonates		Normothermic neonates	
	Before versus during MRI [1]	***During versus after MRI*** [2]	***Before versus during MR*** [1]	***During versus after MRI*** [2]
Wu *et al.*				
Short TE	-1.4°C	+1.3°C	-1.2°C *	+0.9°C *
Long TE	-0.8°C	+0.7°C	-0.8°C	+0.5°C
Calibration				
Short TE	-1.1°C	+1.0°C	-1.4°C *	+1.1°C *
Long TE	-0.6°C	+0.5°C	-1.1°C *	+0.7°C

[1] the temperature difference between the temperature before MRI and during MRS

[2] the temperature difference between the temperature after MRI and during MRS

* p<0.05, corrected for multiple comparisons.

In a multivariable linear regression model the association between therapeutic hypothermia, and head circumference as independent variables and the differences between the temperature before MRI and brain temperature as dependent variable was tested. Head circumference was significantly associated with difference in temperature in which brain temperature was measured using short TE and the formula of Wu *et al.* (head circumference: ß=-0.29, p=0.024) and in the model using short TE and the calibrated formula (head circumference: ß=-0.25, p=0.019). In the models using the brain temperature measured with long TE, none of the variables were associated with temperature differences.

DISCUSSION

This study showed that it is feasible to estimate brain temperature using ^{1}H-MRS in infants with NE with the formula of Wu *et al.* and with as well short as long TE ^{1}H-MRS in magnets with field strengths of 1.5T or 3.0T. Secondly, brain temperature remained within physiological range and was not higher, but even lower in some infants during MRI compared to rectal temperature at the NICU.

Different methods to determine brain temperature were compared to assess the feasibility in clinical practice. A previously developed formula of Wu *et al.* (8) was compared to a new formula developed based on temperature calibration for the 3.0T MR scanner used in our institute for neonatal MRI. A significant difference between brain temperatures calculated with the two formulas would have implied that calibration is essential. Nonetheless, brain temperatures measured with these two different formulas did not differ for short TE ^{1}H-MRS (mean difference 0.06˚C) and the difference for long TE ^{1}H-MRS was statistically borderline significant. However, the mean difference between the formulas for long TE ^{1}H-MRS was 0.15˚C, which is clinically insignificant. Verius *et al.* investigated the need for calibration in 30 healthy volunteers and compared this to previously calibrated formulas. The authors concluded, in contradiction to our results, that calibration is essential (18). This contradiction might be explained by the fact that the formula of Wu *et al.* (8) was based on a similar cohort and scan protocol as ours, and Verius *et al.* (18) compared their formula to studies with different methods, such as different age groups and scanners, than theirs.

A few studies have investigated the use of ^{1}H-MRS to measure brain temperature in neonates, all using different formulas and TEs (7–9). Bainbridge *et al.* measured brain temperatures in neonates with NE on a 1.5T scanner using a TE of 288ms. The authors compared brain temperatures, calculated using two previously developed formulas from calibrations in animals, with rectal temperatures measured shortly after MRI (11,19). They concluded that both formulas correlated well with the rectal temperature, but were not perfect. The explanation of the authors is the difference in field strength: both formulas were developed at ultra-high field MR scanners in animals and the infants were scanned at 1.5T (9). Owji *et al.* measured brain temperatures in infants with NE with and without brain injury using a TE of 288ms on a 3.0T MRI scanner (10). They used a previously reported formula calibrated in rabbits (10,20). So, both studies used formulas calibrated in animals, which might not be most representative for neonatal studies. In this study, we therefore used the formula of Wu *et al.* because they calibrated the temperature in

a phantom study using a TE of 35ms on a 3.0T MRI scanner and tested this formula in neonates with NE, which is more similar to clinical practice. So, hospitals using 3.0T MRI can use their standard ^{1}H-MRS scan and no phantom study is needed before starting to measure brain temperature non-invasively. This will improve the feasibility of ^{1}H-MRS brain temperature measurements in clinical practice.

Furthermore, in previous studies either short (8) or long (9,10) TE ^{1}H-MRS was used, but these two methods were never compared. This study found no statistical differences, but for some individuals there was a difference >1˚C, which is clinically significant. This difference cannot be technically explained. Further research using rectal temperature measurement during MRI in combination with short and long TE ^{1}H-MRS is essential to conclude which TE is more reliable.

The possibility to measure brain temperature is important in clinical practice for the monitoring of safety, as an additional prognostic tool and for evaluation of the effect of therapeutic hypothermia.

This study showed that brain temperature during MRI was not higher than rectal temperature measured within 3 hours before and after MRI in neonates with NE. This suggests that there is no heating of the brain during MRI, which is in accordance with the literature that MRI is safe in different neonatal populations (1,4,5). However, in this study brain temperature was compared to rectal temperature before and after MRI at the NICU. So, we cannot conclude that brain temperature itself did not increase during MRI because of the absence of a baseline measurement of brain temperature.

Furthermore, brain temperature in normothermic infants with NE was even significantly lower during MRI compared to rectal temperature at the NICU, varying between minus 0.6 and 1.4˚C. These findings are in agreement with a study in preterm patients, scanned at 30 weeks of gestation within an MRI incubator, in which 17.3% of the preterm infants became hypothermic with a mean decline in temperature of 0.5˚C during MRI (5). The authors explained the lower temperatures by the cold air that was used for ventilation during MRI instead of the preheated air that is used on the NICU (21). This can also partly explain the decrease in temperature in especially the patients that still received therapeutic hypothermia, because all these infants were ventilated. The decrease in temperature was not statistically significant in infants with therapeutic hypothermia,

but this might be due to the small sample size. In addition, infants are placed in a relatively cool MRI scanner environment (temperature 18°C), which might decrease body temperature. This is supported by the fact that a larger head circumference was associated with a smaller difference between brain temperature and rectal temperature before MRI using short TE. As core temperatures may fluctuate when an infant is exposed to the cooler temperature of the MRI environment, monitoring body temperature during MRI might be recommended to prevent cooling down. An option to prevent body cooling might be the use of a temperature-controlled MRI incubator. A rise in brain temperature in neonates during MRI has never been found, but a decline has also not been described before in NE (4). More research is needed and the results should be interpreted with caution, because the decrease in temperature was not found for all TEs and rectal and brain temperatures were compared.

The lack of information about (brain) temperature during MRI in neonates emphasizes the need for an easy and non-invasive method to measure temperature. This becomes even more important with the use of ultra-high field imaging. Ultra-high field imaging improves the quality of MRI (22), which might also be beneficial in neonates. However, higher field strengths might increase the SAR and thereby the risk of a rise in brain temperature (22). Then, monitoring brain temperature becomes even more important. Additionally, brain temperature measurements can possibly help to assess the severity of brain injury. In adults with stroke, the temperature in injured brain tissue was higher compared to non-injured tissue (23,24). Also in children with epilepsy, the brain temperature in the focal epileptogenic lesions was higher than in controls (25). Therefore, Wu *et al.* investigated brain temperature in infants with NE during and after therapeutic hypothermia. Both during and after therapeutic hypothermia brain temperatures were significantly higher in infants with severe NE compared to moderate NE (8). Subsequently, Owji *et al.* confirmed that infants with NE with brain injury have significantly higher brain temperatures compared to healthy controls. This increase in brain temperature of the injured brain might be explained by the combination of the inflammatory response to injury, chemical reaction in ischemic cells such as the production of oxygen radicals and excitatory amino acids and/or reduced cerebral blood flow leading to less release of heat (24). Furthermore, non-invasive brain temperature measurement during therapeutic hypothermia might provide more information about the actual effect on the brain temperature.

This study has several limitations. The first limitation is the retrospective design in which no standard protocol was followed for rectal temperature measurements. Consequently, infants had to be excluded, because there was no rectal temperature available within 3 hours before or after the MRI, leading to a smaller sample size. With a larger sample size, it would have been more feasible to investigate the effect of brain injury on brain temperature and the effect of more risk factors such as ventilation, sedation and gestational age. Furthermore, it would have been preferable to compare rectal temperature immediately before and after the MRI using a standardized method. Nevertheless, these temperatures do represent the temperature trend of an infant. Another limitation is that the temperature probe could not be placed in the phantom for validation, but only at the surface of the phantom. However, the phantom was small, so it is most likely that the temperature inside the phantom is the same as at the surface. Furthermore, the slope of the calibration curve should remain the same, which is the most relevant.

CONCLUSIONS

In conclusion, this study showed that brain temperature can be measured with ^{1}H-MRS in daily clinical practice without calibration using the previously published formula of Wu *et al*. with both short and long TE ^{1}H-MRS. Furthermore, brain temperature remained in the physiological range during MRI in infants with neonatal encephalopathy following perinatal asphyxia and was in some infants even lower than rectal temperature before and after MRI.

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DISCLOSURES

Floris Groenendaal is expert witness in cases of perinatal asphyxia. The other authors have no conflict of interest to declare.

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CHAPTER 11

Introduction of ultra-high field magnetic resonance imaging in infants: preparations and feasibility

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In press

ABSTRACT

Background: Cerebral MRI in infants is usually performed with a field strength up to 3.0 Tesla (T). In adults, a growing number of studies have shown added diagnostic value of 7.0T MRI. 7.0T MRI might also be of additional value in infants with e.g. unexplained seizures. The aim of this study was to investigate the feasibility of 7.0T MRI in infants. We provide information about the safety preparations and show the first MR images of infants at 7.0T.

Methods: Specific Absorption Rate (SAR) levels during 7.0T were simulated in Sim4life using an infant and adult model. A newly developed acoustic hood was used to guarantee hearing protection. Acoustic noise damping of this hood was measured and compared to the 3.0T Nordell hood and no hood. In the prospective pilot study, clinically stable infants, between term (equivalent) age and the (corrected) age of three months, underwent a 7.0T MRI immediately after their standard 3.0T MRI. 7.0T scan protocols were developed and optimized whilst scanning this cohort.

Results: Global and peak SAR levels in the infant model in centered position and 50mm feet direction did not exceed the levels in the adult model. Hearing protection was guaranteed with the new hood. Twelve infants were scanned. No MRI-related adverse events occurred. It was feasible to obtain good quality imaging at 7.0T for MRA, MRV, SWI, single-shot T2-weighted imaging and MRS. T1-weighted imaging was of less quality at 7.0T.

Conclusion: 7.0T MRI is feasible in infants and good quality scans could be obtained.

INTRODUCTION

Infants who are admitted to the neonatal intensive care unit are at risk for delayed or impaired neurodevelopmental outcome due to brain injury, cerebral malformations, genetic or metabolic disorders (1,2).

MRI is the gold standard to assess brain development, malformations and injury in infants (3). The first neonatal 3.0 Tesla (T) field strength MRI scans were reported in 2004 (4), and 3.0T scanners are now routinely used by many centers. 3.0T MRI has several advantages compared to 1.5T MRI. The quality of the MR images improved because of the increased SNR leading to higher spatial resolution, improved susceptibility contrast and increased chemical shift dispersion leading to improved quality of MRS (5,6). The increased SNR in neonatal 3.0T MRI also led to shorter acquisition times (6).

In adults, the introduction of ultra-high field MRI provided new opportunities, further improving the spatial resolution at 7.0T compared to 3.0T when the same acquisition times were used (7). This provided additional anatomical information (8). At 7.0T the sensitivity to susceptibility is strongly increased enabling the diagnosis of microbleeds and visualization of microvasculature. Due to the increased chemical shift dispersion at 7.0T, additional metabolite peaks could be detected with MRS (5,8–11). Nowadays, accessibility of 7.0T MR scanners in adult research is increasing rapidly and 7.0T MRI is more often used for clinical purposes. The initial safety concerns of using 7.0T MRI in adults were addressed in the past decades. The largest safety concern was an increase in body temperature because of the higher local and global specific absorption rate (SAR), for the same B_1^+ at 7.0T. However, an increase in body temperature has not been reported in ultra-high field MRI in adults or children (5,12). Besides the increased SAR, a higher static magnetic field increases the risk of attracting ferromagnetic object, which can be prevented with screening for ferrometal before MRI (13). It also can potentially influence biological systems, as cardiac and neurophysiological responses, but harmful effects have never been described in follow-up studies in infants (13). Sensory symptoms such as vertigo, headaches and an iron taste due to the varying gradient field (5) were reported by patients undergoing a 7.0T MR scan, however, only 5% rated these symptoms as very unpleasant (14). Acoustic noise protection should be guaranteed during the MR scan, similar to 3.0T MRI.

While studies at 7.0T have now shown to be safe in adults (14,15), literature about safety in children is scarce. In a study of Harris *et al.* 42 children between five and ten years of age underwent a 7.0T MRI which was well tolerated and safe in all children (16). The Food and Drug Administration (FDA) approved 7.0T MRI in infants of one month and older (17). The limit of the main static field in neonates (infants younger than one month) is 4.0T MRI. SAR limits are the same for adults and neonates (17).

We initiated a feasibility pilot study in infants. This study shows the first MR images of infants at 7.0T and provides information about the safety preparations.

METHODS

Preparation - SAR simulation

Using a radiofrequency (RF) simulation, we investigated whether MRI protocols could be translated from the adult to the infant brain without exceeding the SAR limits. Finite difference time domain simulations were performed using Sim4Life (Zurich Med Tech, Zurich, Switzerland) to evaluate transmit efficiency and RF safety limits of the setup, assuming full decoupling to the receiver coils. The geometry and electrical circuits of the Nova head coil (Nova Medical, Inc, Burlington, MA, USA) for 7.0T were implemented in Sim4Life. The simulations were performed on a virtual infant model (Charlie, two months of age, 4.3kg) of the ITIS virtual family (18) in different positions in the coil (Figure 1), using the head coil in quadrature mode. The same simulations were performed on adult heads (male Duke, female Ella) of the virtual family (18) as a reference. Local SAR levels (10g-average) for 1 Watt input power and global SAR levels (average SAR over the whole head) were calculated. Also, the average SAR per B_1^2 was calculated as the average SAR over the whole head divided by the average B_1^2 in a central slice in the brain. Peak local SAR was defined as the highest SAR in the whole infant.

SAR simulations were validated by comparing simulations and measurements of B_1 maps of a spherical phantom and power measurements for data scaling (data not shown).

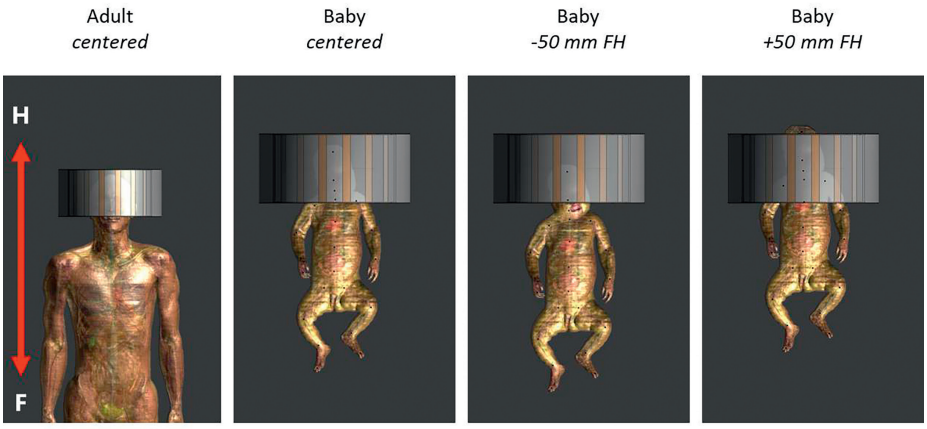

Figure 1: Different positions of the infant model in the RF coil. On the left the adult head and torso is shown which was used as a reference.

Preparation - Acoustic Noise protection

In the 3.0T MRI, acoustic noise protection is guaranteed by Alpine Muffy Baby (Alpine Hearing Protection, Soesterberg, the Netherlands), Natus MiniMuffs (Natus Medical Incorporated, San Carlos, CA, USA) and a hood for acoustic noise protection (19), respectively leading to 6.4-31.6 dB, 7dB and 4-13.6dB reduction. A prototype of the hood (190cm long) for noise protection that fits in the 7.0T MR scanner was developed using a layer of 5cm foam (EASYfoam TC2, Easy Noise Control).

A test setup with a dummy MR bore (old 7.0T MR bore) was made in a sound isolated booth to test the attenuation of acoustic noise with the different hoods and no hood. We conducted all sound level measurements using a Brüel & Kjær Sound Level Meter (type 2250, Brüel & Kjær, Nærum, Denmark). A microphone (Brüel & Kjær, type 4189) was placed in the isocenter of the dummy bore to record the sound volumes in dB(A). Four speakers (Yamaha MSP5A, Hamamatsu, Shizuoka, Japan) were positioned around this test setup: one on each end of the dummy bore and one on both sides (Figure 2). The speakers were separately calibrated on an acoustic noise level of 55dB(A). The acoustic noise was measured with the 3.0T hood, the 7.0T hood and without hood with the acoustic noise coming from the speakers at both ends, both sides and from all four speakers.

We measured the attenuation of the Alpine Muffy Baby and Natus MiniMuffs using the Brüel & Kjaer Type 2250 G4 SLM and Brüel & Kjaer Artificial Ear Type 4153. A stimulus (Decos AudioNigma, Noordwijk, the Netherlands) of 80dB(A) was sent to an artificial ear.

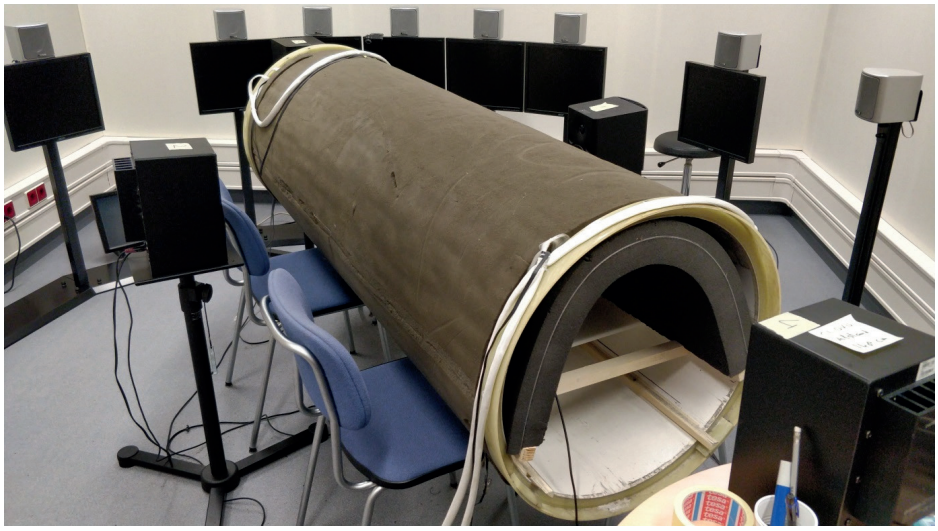

Figure 2: Set up for measuring acoustic noise at the MR table in the presence of the hood. This test setup consisted of a dummy bore with similar dimensions as the 7.0T MR system with a 10mm plastic plate (POM) to create an MR table. The speakers producing the sound positioned at 28cm distance around the dummy bore to mimic the sound produced by the MR scanner.

Study population

Clinically stable infants, between term (equivalent) age and the (corrected) age of three months were included in this pilot study. Infants with respiratory support or an intravenous catheter were excluded. They underwent a 7.0T MRI immediately after their routine 3.0T MRI scan (both Philips Medical Systems, Best, The Netherlands). All infants were sedated with chloral hydrate prior to the 3.0T MRI in combination with the feed-bundle technique, as parts of routine clinical care. An additional dose of chloral hydrate prior to the 7.0T MRI was not allowed by the medical ethical committee. For neonatal scans of the brain at 7.0T, the 2-channel transmit-32-channel receive head coil (Nova Medical, Inc, Burlington, MA, USA) was used. The selection of sequences was based on clinical indication. Details of the scan protocols can be found in Supplemental Table 1. Hearing was protected as described above. Safety parameters were monitored before, during, between and after the MR scans. We monitored heart rate, peripheral

oxygen saturation, temperature (core temperature before and after and abdominal skin temperature during the scans) and comfort scales (20).

This study was approved by the medical ethical committee of the UMC Utrecht (NL66198.041.18) and written informed consent was obtained from parents of all participants.

RESULTS

Preparations – SAR simulation

The global SAR and peak local SAR of the virtual infant model in centered position and 50mm from isocenter in feet direction (-50mm FH) did not exceed the SAR of the adult models. However, when infant Charlie was positioned 50mm from isocenter in head direction (+50mm FH), global SAR levels and peak local SAR levels exceeded those of the adult models (by +13% and +12% compared to Duke, respectively) (Table 1).

Table 1: Global and peak SAR levels.

	Duke centered	Ella centered	Charlie centered	Charlie -50mm FH	Charlie +50mm FH
Global SAR levels					
Average SAR for 1 Watt input power (W kg^{-1})	0.066	0.069	0.062	0.050	0.075
Average SAR per B_1^2 (W kg^{-1} μT^{-2})	0.462	0.465	0.289	0.454	0.474
Average B_1^+ in central slice for 1 Watt input power (μT)*	0.379	0.385	0.466	0.333	0.398
Peak local SAR levels					
Peak local SAR (10g average) for 1Watt input power (W kg^{-1})	0.435	0.398	0.321	0.213	0.487
Peak local SAR (10g average) per B_1^2 (W kg^{-1}μT^{-2})	3.04	2.63	1.48	1.92	3.08

*The power optimization procedure of the MR scanner software calibrates the needed input power to achieve a certain B_1 in the subject. This calibration is based on the average B_1^+ in a central slice of the subject (brain in this case).

The 10g-averaged local SAR in the head of the infant model was lower than in the adult head for all positions. When the infant model was positioned +50mm FH, the local SAR was highest in the neck/shoulder transitions (Figure 3).

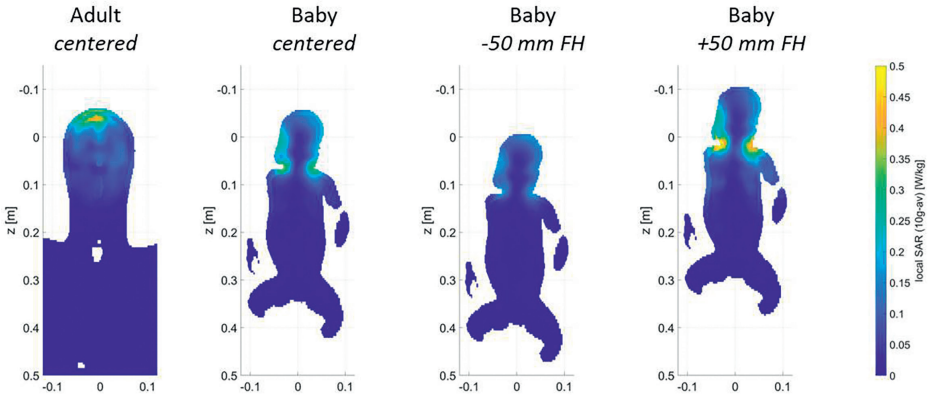

Figure 3: Local SAR levels in adult head (left) and Charlie in the different coil positions. Shifts of an infant in x and y directions are unlikely because of limited space and therefore the results are not included in the Figure. The SAR values when infant Charlie was positioned 50mm in x or y direction were comparable with the +50mm FH position.

The SAR per B_1^2 was lower in the infant model than in the adult models, meaning that less power is needed to reach the same B_1; except for the +50mm FH position.

Preparation – Acoustic Noise protection

The background noise in the sound booth was 28dB(A). The 7.0T hood attenuated the acoustic noise by 8.5dB and the 3.0T hood 7dB (Table 2).

Table 2: Acoustic noise levels in dB(A) with use of no hood, the 3.0T hood and the newly developed 7.0T hood.

	Both ends of the bore	Sides of the bore	Sides and ends of the bore
No hood for acoustic noise protection	60.0dB(A)	58.5dB(A)	62.5dB(A)
3.0T Nordell hood for acoustic noise protection	54.0dB(A)	51.0dB(A)	55.5dB(A)
Prototype 7.0t hood for acoustic noise protection	54.0dB(A)	43.0dB(A)	54.0dB(A)

Without hearing protection the artificial ear measured 80dB(A). The Alpine Muffy Baby reduced the acoustic noise to a level of 56dB(A) and the use of only the Natus Minimuffs resulted in 73.4dB(A). The combination of both, not totally closing of the

artificial ear, led to an acoustic noise level of 58dB(A). If they were both well placed on the ear by using the elastic head band, this decreased to 47.8dB(A).

Feasibility of MRI

Twelve infants have been included with a median gestational age of 28.2 weeks (range 25.0-41.7), median birthweight of 1127 gram (range 585-4570), median postnatal age at MRI of 95 days (range 31-114) and a median weight at MRI of 3322 gram (range 2715-6335). The clinical indications for the MRI scans were: MRI at term equivalent age because of preterm birth before 28 weeks of gestation (n=7); MRI at term (equivalent) age because of white matter injury (n=2); follow-up MRI at three months of (corrected) age because of a thalamic hemorrhage (n=1) or hemorrhage in the temporal lobe (n=1); follow-up MRI at six weeks because of an arterial ischemic stroke (n=1). Temperature, heart rate, peripheral oxygen saturation and comfort scales were stable before, during and after MRI. No serious adverse events related to the MRI occurred.

MRV at 7.0T provided good visibility of the different veins and sinuses i.e. the superficial cerebral veins could be followed in detail (Figure 4A+G).

Also SWI at 7.0T was feasible and showed details of the deep venous circulation, *i.e.* the deep medullary veins (Figure 4B+H).

Single-shot T2-weighted imaging (T2WI) at 7.0T demonstrated good grey and white matter contrast. In one patient with a perinatal arterial stroke, perivascular spaces were seen at 7.0T that were not visible at 3.0T (Figures 4C+I). Otherwise, no clinically relevant additional findings were reported by the radiologist (ML) at 7.0T.

The grey and white matter differentiation at T1-weighted imaging (T1WI) was suboptimal at 7.0T (Figures 4E+K)

MRA showed more peripheral arteries at 7.0T and little noise was visible at 7.0T making it more easy to see the thickness and curves of the arteries (Figures 4D+J).

MRS was of improved quality. For example, the patient shown in Figure 4F+L had an SNR of 19 at 7.0T compared to 6 at 3.0T. It was possible to correctly fit more metabolites with a Cramer–Rao lower-bound <20% at 7.0T, such as N-acetylaspartylglutamate, taurine and glycine.

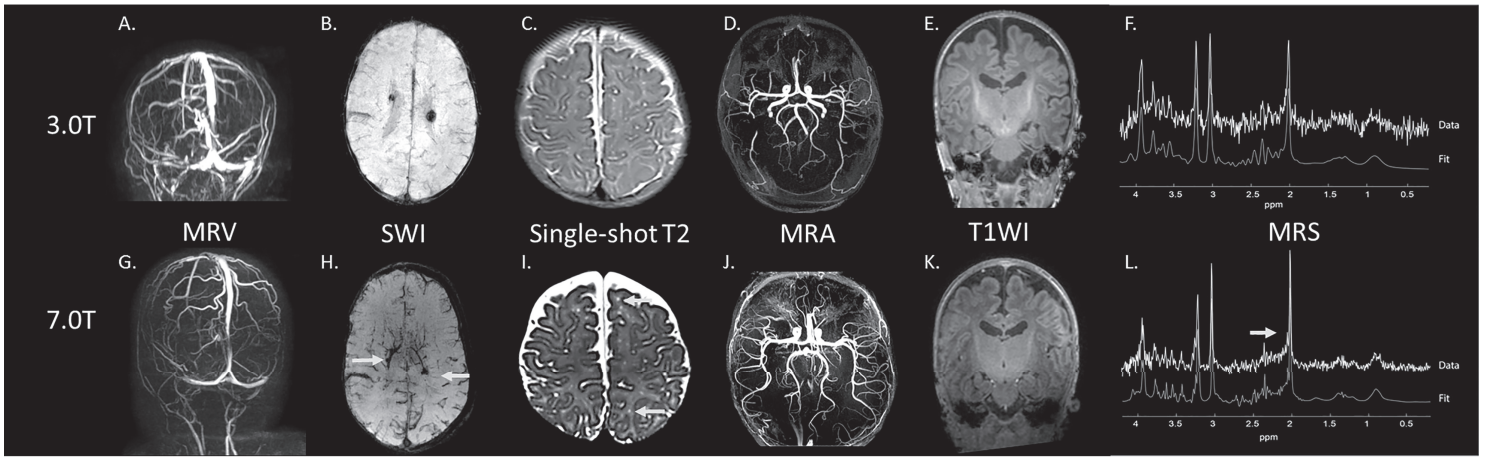

Figure 4: Examples of images of different patients at 3.0T (top row) versus 7.0T MRI (bottom row). Figures 4A and 4G show an MRV at 3.0T respectively 7.0T of a preterm infant at term equivalent age. Figure 4B and 4H show an SWI at respectively 3.0 and 7.0T of a preterm born infant at term equivalent age. Figures 4C and 4I show the six week follow-up single shot T2WI of a term born infant with a perinatal stroke, the arrows point out perivascular spaces perivascular spaces that were better visible at 7.0T compared to 3.0T. Figures 4D and 4J present a three month follow-up MRA at 3.0T and 7.0T of a term born infant with an occipital stroke. Figures 4E and 4K show a T1WI (MP-RAGE) of a preterm born infant at term equivalent age. Figures 4F and 4L show an MRS spectrum at 3.0T and 7.0T of a preterm infant at term equivalent age, both with a comparable ROI in the left basal ganglia/thalami region

DISCUSSION

We demonstrated that scanning infants in a 7.0T scanner is feasible and results in good quality images. Whilst optimization of the sequences is ongoing, we already demonstrated that some sequences showed more details compared to 3.0T MRI.

Prior to scanning, simulated SAR levels at 7.0T were lower in the virtual infant than in adult models (18). When the infants head was farther in the coil than isocenter, or 5cm in x or y direction, SAR levels did exceed the adult situation. Thus, center position of the infant in the coil is essential. Therefore, the position of the infants head was constrained in the coil, making it mechanically impossible to put the infants head farther in the coil than center position. Differences in SAR due to inter-subject variability cannot be completely ruled out. However, previous simulations at 3.0T showed that different sized infant models and different positions did not result in major differences in simulated SAR levels (21).

The SAR simulations had two important limitations. The first limitation is that the Sim4Life model of Charlie uses the dielectric values of adults which might slightly differ from infant dielectric properties. The dielectric values of human infants are unknown and require further research. In the study of Malik *et al.* conversion of adult dielectric values were based on rat data, however these are not validated (21). Secondly, only one virtual model of a two-month-old infant was available, which cannot be completely translated to a term (equivalent age) infant. Head circumference and body composition differ between term (equivalent) age and two months of age. Malik *et al.* showed that term neonates with smaller head size or lower body weight had lower SAR depositions (21), suggesting that the two-month-old infant model does not underestimate SAR values. Regarding body composition, Malik *et al.* simulated the effect of fat percentage on SAR depositions in neonates in two extreme scenarios: one model with only skin and one model with a thick layer of pure fat. The model with only skin had 10% higher peak local SAR depositions (21). The fat percentage of neonates is lower compared to two-month-old infants (22,23). In the worst case scenario, a neonate might have a higher peak local SAR up to 10% compared to a two-month-old infant based on fat composition. This will still not exceed the safety limits of the FDA, since the 7.0T MR scanner of Philips has implemented an additional safety factor larger than 2. Furthermore, the global SAR levels of Charlie in the centered position were 6% lower compared to the adult model

and the peak local SAR 26% lower, leading to an additional safety margin. Adult limits are therefore still safe to use.

Another concern might be that the thermoregulation in neonates is immature compared to adults, so the effect of SAR values on body temperature might differ between neonates and adults (21). Neonates have less isolating subcutaneous fat and a larger surface to body weight ratio, making them more prone to develop hypothermia (21,24–26). The risk of high local peak SAR values in neonates is lower compared to adults since less power is needed in neonates to reach the same B_1^+. Furthermore, the risk of high local peak SAR is reduced by the above described safety margins (Table 1).

No MRI related adverse events occurred in the infants scanned at 7.0T in this pilot study and comfort scales were stable, which are both indicators that infants did tolerate the higher main static field.

The possible improvements in quality of SWI and single-shot T2WI are caused by a shorter T2-relaxation time, improved spatial resolution and increased susceptibility (5). This might enable physicians to assess the extent of injury on a microstructural level, e.g. diagnosing microbleeds, polymicrogyria and thereby improve prediction of neurodevelopmental outcome (27–29).

As expected, the quality of T1WI at 7.0T was worse compared to 3.0T in infants. The T1-relaxation time increases at higher field strengths (13). Furthermore, the brains of neonates have a relatively high water content, which results in less contrast between white and grey matter. To compensate for this longer T1-relaxation time, the repetition time can be increased but this leads to a longer scanning time which is also not preferable in neonates (6,13). On the other hand, this increased T1-relaxation time enables higher quality of angiography, which in the future can help, for example, to evaluate small perforator strokes (6,10).

For MRS the increased chemical shift dispersion at 7.0T results in less overlap between the different metabolite peaks, also the SNR is increased (> 2 fold). Of note is that the maximal required B_1 for MRS cannot be achieved when the infant is in -50mm position in the coil. This can happen if the shoulders do not fit in the head coil when the infant is wrapped in the vacuum matrass. In such cases, the MRS at

7.0T has comparable SNR as at 3.0T, but with the advantage of less overlap between metabolite peaks. Nevertheless, 7.0T MRS enabled more accurate detection of N-acetylaspartylglutamate, taurine and glycine; and possibly other metabolites such as glutamate, GABA and myo-inositol, as has been described in adults (5,6). This could be helpful for the diagnosis of metabolic diseases and neuronal injury, but can also provide information about the biochemical development of the neonatal brain.

In the future, 7.0T might be particularly helpful to answer specific questions about diagnosis or outcome in for example infants with small (perforator) strokes, the diagnosis of metabolic diseases or patients with unexplained neurological symptoms *i.e.* seizures. The clinical implications and additional value of 7.0T in infants, should be investigated in larger 7.0T MRI studies. We did not scan preterm neonates, so safety and feasibility of 7.0T MRI in preterm infants should also be investigated in the future.

CONCLUSIONS

In conclusion, this pilot study shows for the first time that 7.0T ultra-high field MRI is feasible in infants. Good quality images could be obtained, with some sequences providing additional details compared to 3.0T. Positioning of the infant in the isocenter of the coil is important for SAR safety.

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COMPETING INTERESTS

Floris Groenendaal, one of the authors of the manuscript, declares that he is an expert witness in cases of perinatal asphyxia. Fredy Visser works in the ultra-high field MRI team of the department of Radiology in the UMC Utrecht, but is also an employee of Philips Healthcare, Best, The Netherlands. The other authors have no conflict of interest to declare.

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SUPPLEMENTAL MATERIAL

Supplemental Table 1: scan protocols

	3.0T MRI	7.0T MRI
T1-weighted imaging	3D FFE	MP-RAGE
TE	4.6ms	2.1ms
TR	9.4ms	8.0ms
Flip angle	8 degrees	8 degrees
Slice thickness	2.0mm	0.9mm
T2 -weighted imaging	Single shot TSE	Single shot TSE
TE	90ms	292ms
TR	15000ms	2700ms
Flip angle	90 degrees	90 degrees
Slice thickness	2.0mm	1.5mm
SWI	FFEepi	3D-FEepi
TE	30ms	18.7ms
TR	53ms	28ms
Flip angle	15 degrees	14 degrees
Slice thickness	2.0mm	8.0mm
Epi factor	3	3
Reconstruction voxel	0.31x0.31x1.00mm	0.55x0.55x0.27mm
MRA	MRA-3D	3D-Inflow
TE	4.0ms	2.8ms
TR	21.0ms	10.6ms
Flip angle	20 degrees	20 degrees
Reconstruction voxel	0.19x0.18x0.50mm	0.30x0.30x0.20mm
MRV	3D-PCA	3D-PCA
TE	19ms	6.4ms
TR	8ms	10ms
Flip angle	10 degrees	10 degrees
Reconstruction voxel	0.45x0.45x1.00mm	0.75x0.75x0.45mm
Velocity (venc)	7cm/s	7cm/s
Single voxel MRS	PRESS	sLaser
TE	35ms	36ms
TR	2000ms	5000ms
Phase cycles	16	16
Number of averages	64	64
Spectral bandwidth	2000Hz	4000Hz
Number of samples	1024	2048

CHAPTER **12**

General discussion

Conclusions & clinical implications

Future directions

GENERAL DISCUSSION

Perinatal asphyxia resulting in hypoxic-ischemic encephalopathy (HIE) is a worldwide problem affecting the future perspectives of many infants and families (1,2). In 2010, an estimated 1.15 million infants developed HIE after perinatal asphyxia: of these infants 287,000 died, 233,000 developed severe neurodevelopmental deficits and 181,000 experienced mild deficits (2).

Brain injury already starts during (fetal) hypoxia because of primary energy failure but a substantial second wave of injury occurs after birth when perfusion and oxygenation recover. The reperfusion injury is caused by the formation of free radicals (*e.g.* formation of superoxide), neuro-inflammation and secondary energy failure (3,4). Brain injury is commonly assessed using magnetic resonance imaging (MRI), which is an important and indispensable technique to accurately predict neurodevelopmental outcome (5). The only current evidence-based neuroprotective therapy is therapeutic hypothermia. Therapeutic hypothermia has shown to reduce death, severe cognitive problems and cerebral palsy in infants with HIE (1,6–10). Despite therapeutic hypothermia, about 45% of the infants still experience long-term neurodevelopmental problems (1). Therefore, add-on neuroprotective therapies are essential.

The aim of this thesis is to provide new insights in neuroimaging, neuroprotection and long-term neurodevelopmental outcome in infants with HIE. In **chapter one,** the pathophysiology, neuroprotective strategies, neuroimaging and follow-up of infants with HIE are elaborately discussed. Part I of this thesis provides additional information on neuroimaging in HIE and long-term follow-up. In Part II the most important literature on pharmacological neuroprotection with allopurinol in HIE is reviewed and the set-up of the ALBINO trial and the pharmacokinetics of allopurinol are discussed. In part III advanced MRI techniques in neonates are shown and discussed.

Part I: neuroimaging and follow-up

Since many years there is a focus in neonatal research on the prediction of neurodevelopmental outcome in neonates at risk for brain injury. In neonates with HIE many biomarkers have been investigated (11). Discovery of (a combination of) biomarkers that accurately predict(s) neurodevelopmental outcome is valuable for clinical decision making (*e.g.* redirection of care), to select patients at risk for adverse outcome for (future) interventions and early rehabilitation and it will be essential for the counseling of parents about the development of their child. At this moment there is no biomarker that perfectly predicts outcome, which is important to keep in mind while counseling parents.

Neuroimaging is one of the three pillars to predict outcome in neonates with HIE, besides the clinical condition of the patient (e.g. abnormal neurological examination, multi-organ failure) and neurophysiology (12). Many studies have confirmed the important role of multiple MRI sequences in predicting outcome (12–16). Despite the availability of many research on neuroimaging in HIE, there are still gaps in our knowledge. Some of these gaps are addressed in Part 1.

The focus in neonatal neuroimaging following HIE is traditionally on supratentorial brain injury. Based on postmortem histological studies it is known, however, that the cerebellum is also very vulnerable to hypoxic-ischemia (17–19). From extensive studies in preterm infants, we know that cerebellar injury is associated with not only motor problems, but also cognitive and behavioral problems (20). More research is needed on cerebellar injury in HIE and the consequence of cerebellar injury on the long-term neurodevelopmental outcome.

Pathological examination can provide more information on the distribution and extent of cerebellar injury, which might be important for the effect of cerebellar injury on neurodevelopment. Purkinje cells are the main output neurons of the cerebellum and are very vulnerable to hypoxic-ischemia (21). In **chapter two** we describe the distribution of Purkinje cell injury in the cerebellar vermis. Term neonates with HIE were included if they had autopsy including hematoxylin and eosin (H&E) stained sections of the vermis. We found that the number of Purkinje cells was less in the bases of the sulci compared to the crowns of the sulci for both the lobules and folia. There were also more injured Purkinje cells in the bases of the sulci compared to the crowns. The pattern of Purkinje cell injury in the vermis is very

similar to ulegyria in the cerebrum (22). The reason for this distribution is unknown. One hypothesis is that because the arterial ramifications of the more peripheral arteries at the bases of the sulci have a smaller diameter, the Purkinje cells there are more susceptible to hypoxic-ischemia (23–25). Recently, Wright *et al.* described 23 children in whom MRI abnormalities were seen in the bases of the sulci of the cerebellar hemispheres after hypoxic-ischemic events (26). This injury was especially located at the borderzones of cerebellar artery territories and associated with supratentorial watershed injury, therefore they called it cerebellar watershed injury (26). This study underlines our pathological finding that Purkinje cells at the bases of the sulci in the vermis are more susceptible to hypoxia compared to the crowns in neonates with HIE and the hypothesis that this is probably caused by impaired perfusion of the bases of the sulci. The clinical significance should be investigated in the near future as well as the existence of a similar pattern of Purkinje cell injury in the cerebellar hemispheres. It would be interesting to investigate whether this pattern is visible at school-age on MRI and how it effects long-term outcome.

Chapter two confirms that cerebellar injury is detectable with postmortem histopathology; the next question was whether cerebellar injury is visible on MRI. Others published that using conventional imaging, cerebellar injury is very difficult to visualize, but more quantitative methods might reveal more details (27,28). In **chapter three**, we compare cerebellar injury confirmed with histopathology to apparent diffusion coefficient (ADC) values on diffusion-weighted MRI (DWI) in infants with HIE. Infants with congenital non-cardiac anomalies requiring neonatal surgery, with normal postoperative MRI's and normal two year outcome served as controls. ADC values in the vermis and dentate nucleus were significantly lower in infants with HIE compared to the patients without brain injury. ADC values in the cerebellar hemispheres did not differ. When we compared ADC values with histopathology in the infants with HIE, ADC values in the cerebellar vermis in infants with HIE were significantly correlated with Purkinje cell injury. ADC values did not differ in patients with mild and severe inflammation or histopathological cytotoxic edema. So it is important to realize that, even when the DWI seems to be normal and the ADC values are within the normal range, there still might be cerebellar injury. The ADC values in the vermis are most representative for cerebellar injury.

Though MRI might underestimate cerebellar injury, it is an accurate biomarker for brain injury in infants with HIE and the gold standard for neuroimaging. For

situations where MRI is not available or feasible, cerebral ultrasound (CUS) might be an alternative. The benefits of CUS are the lower costs, portability and the ability to monitor evolution of brain injury daily (29). In **chapter four** we describe the development and validation of a cerebral ultrasound scoring system for infants with HIE**.** We retrospectively included (near-)term neonates with HIE that received therapeutic hypothermia in two different hospitals. The final scoring system contains a deep grey matter score (composite score of hyperechogenicity of thalamus and of putamen, hypoechogenicity of the posterior limb of the internal capsule and the four column sign (defined as hyperechogenicity of the basal ganglia and thalami in the coronal view)) and a deep white matter score (including subcortical white matter involvement, periventricular white matter involvement and existence of edema). This score on day three to seven was well associated with adverse neurodevelopmental outcome (AUC=0.90), also in the validation cohort (AUC=0.89). The CUS on day one was not predictive for adverse outcome, but useful to detect antenatal pathology.

Chapter five and six focus on neuroimaging and school-age outcome. It is important to assess neurodevelopment of infants with HIE into childhood, since some deficits can elucidate later in life and children can "grow into their deficits" (30–34). MRI at school-age enables us to investigate the consequences of the perinatal event and helps us to better understand which structures or connections are important for specific problems. This information might allow us to identify neonates at risk for problems at school-age, if the biomarkers at school-age can be related to neonatal injury.

The aim of **chapter five** is to describe the effect of HIE on hippocampal volume in school-age children and to investigate the association between hippocampal volume and cognition and memory. Therefore nine- to ten-year-old children with mild or moderate HIE were included and compared to healthy controls, all born before therapeutic hypothermia became standard of care. Children with moderate HIE had significantly smaller hippocampal volumes compared to controls, also children with mild HIE seemed to have smaller volumes although this did not reach statistical significance. Furthermore the children with HIE had episodic memory problems. The smaller hippocampal volume (as percentage of total intracranial volume) was associated with poor long-term visuospatial memory.

Afterwards, we started a new cohort study to investigate the ten year outcome in a group of children with HIE that were treated with hypothermia and a group that

was not treated with therapeutic hypothermia. In **chapter six** we assess which brain structures are most related to school-age outcome in this group. We included ten-year-old patients with a history of perinatal HIE who were born before therapeutic hypothermia became standard of care (non-HT group) and children who were treated with therapeutic hypothermia (HT-group). All children underwent a neuropsychological examination, motor tests and an MRI including 3D-T1-weighted imaging and diffusion tensor imaging (DTI). We found cognitive and episodic memory problems in both groups. In the group as a whole, smaller hippocampal volumes and atrophy of the mammillary bodies (MB) were associated with poorer cognition and episodic memory. Atrophic MB and smaller hippocampal volumes were also associated with lower FA values throughout the brain, especially in the fornix and corpus callosum. All these results implicate that the circuit of Papez (including the MB, hippocampus, anterior thalamus and fornix) is important for cognitive and memory problems in infants with HIE. Though therapeutic hypothermia reduces death, cerebral palsy and epilepsy, the circuit of Papez does not seem to be fully spared.

When combining the findings in **chapter five and six,** it can be concluded that hippocampal and MB atrophy at ten years of age in children with a history of perinatal HIE are associated with neurocognitive and memory problems, irrespective of therapeutic hypothermia. Atrophy of these structures might be an early biomarker for memory and cognitive problems. It is known that MB atrophy is already visible at three months after birth (35). Currently, we are investigating whether hippocampal atrophy is also detectable at three months after birth, in collaboration with the Sick Kids Hospital in Toronto, Canada. In case atrophy of the MB and hippocampus are both visible at three months of age and are associated with long-term neurodevelopmental outcome, it might be useful to scan all infants with HIE three months after birth to identify the infants at risks for memory problems and start with early rehabilitation. Early memory training might improve memory, because of the plasticity of the hippocampus. For example, taxi drivers in London have larger hippocampal volumes than controls, because their (visuospatial) memory is very well trained (36). The effect of memory training on hippocampal and MB volumes and memory and cognitive functioning in infants with HIE will need further investigation.

Chapter three and six have implications for the current MRI scoring systems (37,38). Based on **chapter six,** it can be advised to add the MB to these scoring systems**.** This also allows to investigate the association of neonatal MB injury and

neurodevelopmental outcome in a large cohort. To be able to score the MB, DWI and T1- and T2-weighted MRI should have a maximum slice thickness of 2mm. When using thicker slices, the MB are often missed. Additionally, the scoring system of Weeke *et al.* also includes an abnormal signal of the cerebellum on diffusion weighted MRI. Based on **chapter two,** one should realize when using this scoring system that even when the cerebellum appears normal on diffusion weighted MRI, there still might be (histopathological) cerebellar injury. It would be interesting to score the vermis separately from the cerebellar hemispheres, since ADC values in the vermis seem most reliable. Other MRI sequences, as magnetic resonance spectroscopy (MRS) and DTI might be more accurate in diagnosing cerebellar injury (27).

The hippocampus, cerebellum and MB were not included in the CUS scoring system in **chapter four**, because the quality of CUS was suboptimal to score these structures and the cerebellar view was often not present. The quality and possibilities of CUS increases rapidly by the use of new techniques, potentially allowing inclusion of these structures in the CUS scoring system in the future as well.

Part II: neuroprotection

Therapeutic hypothermia reduces the risk of adverse outcome in infants with HIE significantly, though still about 45% of the infants with moderate to severe HIE die in the neonatal period or have an adverse neurodevelopmental outcome (1). Add-on neuroprotective agents should be investigated to decrease the risk of an adverse outcome. Part II of this thesis focuses on a promising potential neuroprotective agent, allopurinol.

Allopurinol is a xanthine-oxidase inhibitor and can thereby reduce reperfusion injury (39). Inhibition of xanthine-oxidase leads to less superoxide formation, one of the major toxic compounds leading to brain injury in HIE (39,40). Additionally, allopurinol is a direct scavenger of free radicals and can chelate non-protein-bound iron (41,42). Reperfusion injury starts immediately after birth and superoxide formation already reaches its peak within 30 minutes after birth, so early intervention with allopurinol is probably essential (4). In **chapter seven** this is discussed in more detail, as well as the pathophysiology of HIE and an overview of the preclinical and clinical studies on the neuroprotective effect of allopurinol in neonates with HIE. The available literature on antenatal and postnatal (within four hours after birth) administration is not conclusive (40,43–49). Allopurinol administration within four hours is probably too late (considering the early peak of superoxide formation) and the disadvantage of antenatal allopurinol is that the selection of fetuses with imminent hypoxia based on cardiotocography appeared to be difficult (43,45,48). Nevertheless, infants with moderate HIE treated with postnatal allopurinol had an improved outcome at four to six years of age compared to controls (47). Importantly, allopurinol is regarded to be a safe drug, no serious adverse events have been reported in neonates (40). A larger randomized controlled trial with very early allopurinol administration during resuscitation is needed to determine the neuroprotective effect of allopurinol. Furthermore, there is no information about the effect of allopurinol as add-on strategy in combination with therapeutic hypothermia.

Therefore, the ALBINO trial was designed, registered as NCT03162653 on ClinicalTrials.gov, a large European randomized controlled trial, in which we investigate the neuroprotective effect of allopurinol administration during resuscitation in infants with HIE as add-on therapy next to therapeutic hypothermia. Near-term and term infants with perinatal asphyxia and early signs of encephalopathy receive, after short oral consent, a first dose of allopurinol or placebo within 45 minutes after birth. A second

dose is given to patients treated with therapeutic hypothermia. The protocol of the ALBINO trial is outlined in **chapter eight.** This trial is still ongoing in over 60 hospitals in 13 different countries in Europe. So, we have to wait for the results to conclude whether allopurinol is an effective add-on neuroprotective agent in HIE or not.

Following previous trials on postnatal allopurinol and antenatal allopurinol administration to mothers during imminent hypoxia, its pharmacokinetics are well known (50–52). However, in these studies neonates with HIE were not treated with hypothermia. Pharmacokinetics might be different due to therapeutic hypothermia (53), *e.g.* morphine clearance is lower during hypothermia compared to normothermia (54). Also multi-organ failure caused by the hypoxic-ischemia can lead to differences in pharmacokinetics (55,56). In **chapter nine** we report the pharmacokinetics of allopurinol in the first 15 patients that received allopurinol in the ALBINO trial. The pre-specified target area under the curve concentration (AUC) for allopurinol was met in 100% of the patients undergoing therapeutic hypothermia and 100% of the non-cooled infants. For allopurinol's active metabolite oxypurinol, which is also important for the neuroprotective effect, 66.7% of the patients undergoing therapeutic hypothermia met the target AUC and 100% in the non-cooled group. Based on these results the dosing regimen in the ALBINO trial is adequate for both cooled and non-cooled infants with HIE. The effect of therapeutic hypothermia and of the severity of perinatal asphyxia (multi-organ failure) could not be investigated in this small cohort and will be investigated in the near future by pooling the data of earlier postnatal studies (50) and the current cohort.

Combining the chapters of **Part II**, we can conclude that allopurinol is a promising add-on neuroprotective agent and timing of administration is essential for its effect. There are several additional advantages of allopurinol as neuroprotective agent. First, a study in sheep revealed that hippocampal injury of fetal sheep was reduced by antenatal allopurinol administration to the mother (57). Additional protection of the hippocampus would be valuable considering the results of **chapter five and six.** Another benefit of allopurinol as a possible add-on agent, is that it is a cheap medicine and easy to store and administer, which makes it a feasible treatment strategy in low income countries. Thereby it fulfills the criteria of the world health organization to improve neonatal survival worldwide (58). The results of the ALBINO trial need to be awaited to draw definite conclusion on the neuroprotective effect of allopurinol in term infants with HIE.

Part III: innovative neuroimaging techniques

There has been a rapid innovation in the field of MRI techniques in the past years: more sequences have been developed and applications have been found. It is important to consider the safety of new MRI methods. One of the risks of MRI is an increase of temperature because of the specific absorption rate (SAR), which is the amount of energy absorbed by the body (59). The effect of SAR on brain temperature is rarely studied, but would help to gain more knowledge on safety of innovative MRI techniques. Therefore an easy method to measure brain temperature is needed.

In **chapter ten** we studied the feasibility of measuring brain temperature using ^{1}H-MRS in infants with HIE following perinatal asphyxia. First, we validated a previously reported formula to calculate brain temperature using ^{1}H-MRS (60) and a formula we made based on phantom calibration. Afterwards, we included (near-) term infants with HIE and determined brain temperature of these infants using short and long echo time ^{1}H-MRS. Based on these measurements we were able to conclude that brain temperature did not differ significantly by using the earlier reported formula or the formula based on calibration and neither between short and long echo time ^{1}H-MRS. All brain temperatures remained within the normal range. This is important since it implies that the formula of Wu *et al.* can be used in multiple centers using short or long echo time ^{1}H-MRS without calibration (60). An easy and validated method to measure brain temperature enables researchers and clinicians to collect more safety data during MRI.

Methods to measure safety in neonatal neuroimaging are even more important while investigating safety and feasibility of ultra-high field MRI. In adults ultra-high field MRI has increased diagnostic value and shows more details of the arterial and venous vasculature and anatomy (61). This might also be beneficial in neonates at risk of brain injury, such as neonates with unexplained seizures or neonates with suspected microgyria or metabolic disorders. 7.0 Tesla (T) MRI has never been performed in neonates, so feasibility and safety should be investigated in a pilot study before the additional diagnostic value and clinical impact of 7.0T MRI can be determined in a larger study.

In **chapter eleven** we investigated the feasibility of ultra-high field MRI in neonates between term (equivalent age) and three months post-term and provided the first MR images of neonates at 7.0T MRI. SAR simulations showed that SAR limits in a

virtual model of a two-month-old infant did not exceed the limits of an adult model as long as the infant was positioned in isocenter. Furthermore, it was feasible to obtain good quality images in the first twelve infants scanned at 7.0T. A safety study aiming to include 20 neonates is still ongoing. Awaiting the safety results of this study is essential before other studies can start. The methods of **chapter ten** to measure brain temperature will also be used to compare brain temperature between 3.0T and 7.0T MRI.

In the near future 7.0T MRI might be used for specific indications such as diagnosis of metabolic diseases, detection of small white matter lesions, visualization of smaller arteries and veins. 7.0T MRI might also be useful to detect cerebellar injury, for example ulegyria in the vermis and hemispheres. The clinical significance of this added diagnostic value should be investigated in the future.

CONCLUSIONS & CLINICAL IMPLICATIONS

- Diffusion weighted imaging might underestimate cerebellar injury in neonates with hypoxic-ischemic encephalopathy (HIE), especially in the cerebellar hemispheres. If the apparent diffusion coefficient (ADC) values are measured in the cerebellum, the ADC values in the vermis are most representative for injury.

- The newly developed cerebral ultrasound scoring system to assess brain injury in neonates with HIE was well associated with neurodevelopmental outcome at two years of age. It is an easy tool if MRI is not available or feasible and provides the opportunity for serial bedside monitoring of brain injury with cerebral ultrasound.

- HIE can lead to smaller hippocampal volumes and atrophy of the mammillary bodies (MB) which are associated with memory problems and cognitive deficits at school-age, irrespective of therapeutic hypothermia.

- Atrophy of the circuit of Papez, *e.g.* MB and hippocampal atrophy, could be an early biomarker to predict school-age memory and cognitive problems.

- Allopurinol may be a beneficial add-on neuroprotective therapy, especially when it is administered early after birth. The effect of early allopurinol in HIE is currently investigated in the ALBINO trial.

- The dosing regimen in the ALBINO trial is adequate with 20mg/kg intravenously started within 45 minutes after birth and in case of therapeutic hypothermia with a second dose of 10mg/kg intravenously.

- Brain temperature can be measured using short and long echo time proton magnetic resonance spectroscopy without the need for calibration in infants with HIE. This offers an important tool for MRI safety studies in neonates.

- 7.0T MRI is feasible in neonates and good quality images can be obtained. This might be an additional neuroimaging method for specific indications, such as stroke and metabolic disorders.

FUTURE DIRECTIONS

The future of neuroprotective trials in HIE

Different trial design

Optimal neuroprotection and reduction of brain injury after perinatal asphyxia will ultimately be a combination of therapeutic hypothermia and inhibition of the different destructive pathways with intervening drugs each at their specific optimal points of time and with optimal dosing. Current studies investigate these various pharmaceutical interventions separately as add-on to therapeutic hypothermia. Setting up and conducting such randomized controlled trials is not very effective, because it takes years and requires many resources.

Alternatively, a combination of (pharmacological) add-on therapies might be more effective, since this integrated approach targets the destructive pathways all together leading to optimal protection of the threatened brain (4).

A Master Protocol allows to investigate different therapies in parallel within one study protocol, this design is also commonly used in pediatric oncology (62–64). Drugs that are proven to be effective, as therapeutic hypothermia, will be standard of care and patients can be randomized for the other drugs that are investigated. This approach has several advantages (63,64). First of all, neuroprotective drugs can be investigated more efficient and in parallel, which will save time. Also, the infrastructure, resources and costs can be shared by all research groups that participate in the trial. The sample size will be met sooner with (international) collaboration. Finally, if there is only one research protocol for all infants with HIE in the Netherlands, clinicians will be more likely to remember to ask patients for informed consent, even in the emergency setting.

The Master Protocol also allows to randomize patients within the protocol based on specific (genetic or molecular) biomarkers for individualized target therapies (63). At present, there are no such biomarkers for HIE. In the future genetic or molecular biomarkers might become available that stratify infants with HIE in different groups. For example, some patients with HIE might benefit more from reducing superoxide formation and others from improving regeneration or preventing inflammation.

Before we can start a clinical trial based on a Master Protocol design, the safety of combinations of different pharmaceutical agents and their interactions should be investigated in preclinical experimental research.

Harmonization of consent procedures in the neonatal emergency setting
The informed consent procedure in delivery room research is difficult, but early interventions are sometimes essential, as in the ALBINO trial. Possibilities are to ask all expecting parents for informed consent, use the deferred consent procedure or ask short oral consent. All options have pros and cons considering the impact on healthy pregnant women, patient and parent autonomy, the emotional ability of parents to consent during resuscitation of their newborn and the feasibility of the study in a time critical and stressful situation.

During the ALBINO study, the different ethical committees in Europe and even within the Netherlands had different opinions about the preference for short oral consent or deferred consent. Consensus about the best approach will save time for ethical committees, will harmonize the procedures in international studies and will make clinicians more confident with this approach by becoming more experienced.

Patient participation
Patient participation should become more important for future grant applications and trial design (65). The opinion of parents and patients about the importance of a trial and the burden to parents and patients should always be considered. We have to discuss the primary outcome of trials with parents and patients. What parents and patients consider as clinically relevant might differ from the opinion of researchers and clinicians (65). The aim of research should be to improve the quality of life of patients and their opinion about how their quality of life can be improved is therefore essential. Further, parents of patients formerly admitted to the neonatal intensive care unit should be involved in the design of the informed consent procedure, especially in emergency settings, and the text of the patient information letter. Finally, after each study all participating patients and parents should be able to receive the results, so they are informed about what their participation led to and it will also motivate them to participate in future studies. This can be done by a newsletter, a lay-man summary or by a patient symposium.

Long-term follow-up

Atrophy of the Papez circuit, including the hippocampus, mammillary bodies and fornix, was in our studies associated with school-age cognitive and memory problems in both children treated with and without therapeutic hypothermia. It would be interesting to assess atrophy of the Papez Circuit in children with HIE included in one of the randomized controlled trials for therapeutic hypothermia and compare the atrophy between hypothermia treated infants and controls. Preclinical studies can also help to investigate the effect of therapeutic hypothermia and other add-on therapies on the circuit of Papez.

In the Netherlands, infants with HIE are currently followed in the outpatient clinics until 5.5 years of age. As shown in this thesis and by others, some deficits, such as memory problems or behavioral problems, only become evident at school-age (1,30–32,66,67). Longer follow-up in the outpatient clinic can be useful to screen for these deficits, but this can also be done by other institutions such as school, the rehabilitation center, the local child health care center or the family doctor. Knowledge about these deficits can create awareness that enables earlier detection of problems and earlier therapy or rehabilitation. More research is needed to establish the best therapies to improve memory and executive functioning in these children.

In clinical trials, the primary outcome is often death, neurodevelopmental outcome at two years of age or epilepsy. These are important outcomes, but might not reveal the overall neuroprotective effect of interventions. To assess the actual effect of new interventions, follow-up of patients into school-age (or even up to adulthood) is recommended including a full neuropsychological assessment including memory, executive functioning and behavior. Also the impact on the family might be worthwhile to investigate.

Furthermore, there are no follow-up studies of infants with HIE into adulthood, investigating the presence of psychiatric disorders, career opportunities and social functioning. Infants that were born extremely preterm and term infants with a very low birth weight are known to have an increased risk for developing psychiatric disorders (68). In the future, when the first patients with HIE that were treated with therapeutic hypothermia have reached adulthood, it is important to look further into their adulthood outcome.

Predicting outcome

There are currently many biomarkers for brain injury investigated in infants with HIE, *e.g.* MRI, (amplitude integrated) electric encephalography, monitoring cerebral oxygenation, cerebral ultrasound and evoked potentials (11,69). Research also focuses on clinical parameters as Apgar score, lactate and Thompson score and their relation with the severity of brain injury (69), as well as plasma biomarkers such as S100β, Tau, BNPD and metabolomics (70,71).

For neonates with HIE, most of these data are collected as part of clinical care. Combining these biomarkers can increase the accuracy of outcome prediction in HIE and thereby optimizing the counseling of parents and clinical decision making (72). Machine learning can be a helpful method to develop the most accurate prediction model for infants with HIE.

Ultra-high field neuroimaging needs optimization to further increase the additional diagnostic value. Afterwards, 7.0T MRI might serve as an additional biomarker to predict long-term outcome. It might help us to improve visualization of the anatomy, which might allows us to diagnose ulegyria in the cerebellum. It might also increase our knowledge about brain development in infants with brain injury. DWI might provide additional information about the development of microstructural connectivity, MRS about metabolic maturation and chemical exchange saturation transfer can give information about myelination (73).

An accurate prediction of outcome might become even more important in the future, if the focus will be more on value-based health care.

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CHAPTER **13**

Nederlandse samenvatting

(summary in dutch)

NEDERLANDSE SAMENVATTING (SUMMARY IN DUTCH)

Perinatale asfyxie, zuurstofgebrek rondom de geboorte, kan leiden tot hersenschade. Het klinische beeld wat hiermee gepaard gaat wordt hypoxisch-ischemische encefalopathie (HIE) genoemd. HIE is een wereldwijd probleem en heeft invloed op de toekomstperspectieven van vele pasgeborenen en hun families (1,2). Naar schatting ontwikkelden in 2010 wereldwijd 1,15 miljoen pasgeborenen HIE na perinatale asfyxie: 287,000 pasgeborenen overleden, 233,000 hadden op de lange termijn ernstige neurologische ontwikkelingsproblemen en 181,000 hadden milde ontwikkelingsproblemen (2).

De initiële hersenschade ontstaat tijdens het acute moment van zuurstofgebrek van de foetus tijdens of vlak voor de geboorte. Enkele uren na de geboorte ontstaat er echter een tweede piek in de processen die leiden tot hersenschade, wanneer de bloedsomloop en zuurstoftoevoer worden hersteld door de reanimatie. De nieuwe toevoer van zuurstof in het lichaam van de pasgeborene leidt tot de vorming van vrije radicalen, bijvoorbeeld superoxide, die schadelijk zijn voor de hersenen (3,4). De productie van deze vrije radicalen leidt ook tot een ontstekingsreactie en tot een verminderd herstel van de hersencellen (4). Deze tweede piek van hersenschade, ook wel reperfusieschade genoemd, leidt ook tot een substantieel deel van de hersenschade.

Bij pasgeborenen met HIE is het belangrijk om de uitgebreidheid van eventuele hersenschade in kaart te brengen, omdat dit belangrijk is voor het voorspellen van cognitieve en motorische problemen op de lange termijn. Hiervoor wordt gebruik gemaakt van verschillende technieken. Voor het voorspellen van de neurologische uitkomst bij pasgeborenen is de volgende trias essentieel: beeldvorming van de hersenen, het bepalen van elektrische hersenactiviteit en het klinisch (neurologisch) beeld (5). De gouden standaard voor beeldvorming van de hersenen bij pasgeborenen is MRI. Het is bekend dat we met MRI de motorische en cognitieve problemen op tweejarige leeftijd goed kunnen voorspellen voor pasgeborenen met HIE (6).

Op dit moment is de enige effectieve behandeling om hersenschade bij HIE te verminderen koeling (1). Pasgeborenen met matige tot ernstige HIE worden gedurende 72 uur gekoeld tot 33.5 graden Celsius (7). Deze behandeling moet binnen 6 uur na de geboorte worden gestart. Koeling vermindert de kans op overlijden en ernstige cognitieve en motorische problemen bij pasgeborenen met HIE (1,8–12).

Ondanks koeling, ervaart echter nog steeds 45% van de pasgeborenen met matige tot ernstige HIE op de lange termijn problemen of overlijdt (1). Daarom is aanvullende therapie om de hersenen te beschermen, zogenaamde neuroprotectie, essentieel!

Het doel van dit proefschrift is om nieuwe inzichten te verkrijgen in beeldvorming, neuroprotectie en de lange termijn ontwikkeling van pasgeborenen met HIE.

Deel één van dit proefschrift richt zich op beeldvorming van de hersenen van pasgeborenen met HIE tijdens zowel de acute fase als tijdens de verdere ontwikkeling op schoolleeftijd. Deel twee van dit proefschrift gaat over allopurinol als aanvullende behandeling naast koeling om de hersenen van pasgeborenen met HIE te beschermen. Deel 3 richt zich op de toepassing van innovatieve MRI technieken bij pasgeborenen.

Deel 1: beeldvorming van de hersenen en lange termijn ontwikkeling
De afgelopen decennia is er binnen de neonatale neurologie veel aandacht voor het voorspellen van de neurologische uitkomst bij pasgeborenen met een risico op hersenschade. Dit is belangrijk voor beslissingen rondom palliatieve zorg voor een patiënt, voor het selecteren van patiënten voor (nieuwe) behandelingen en om ouders correcte informatie te geven over de toekomst van hun kind. Op dit moment is er geen enkele techniek die de uitkomst van pasgeborenen met HIE geheel correct voorspelt. Verder onderzoek naar beeldvorming kan bijdragen aan het beter voorspellen van de ontwikkeling van pasgeborenen met HIE.

De focus binnen de neonatale beeldvorming bij pasgeborenen met HIE is vooral op schade van de grote hersenen. We weten echter van autopsie studies, dat het cerebellum (kleine hersenen) ook gevoelig is voor schade na perinatale asfyxie (13–15). Het is belangrijk om hier meer onderzoek naar te doen, omdat cerebellaire schade kan leiden tot motorische, cognitieve en gedragsproblemen (16).

In **hoofdstuk twee** onderzoeken we of Purkinje cellen, de belangrijkste cellen in de cerebellaire cortex die connecties maken met de cortex van de grote hersenen, in bepaalde delen van de vermis (het middelste deel van het cerebellum) van overleden pasgeborenen met HIE gevoeliger zijn voor schade dan in andere delen. Purkinje cellen zijn erg gevoelig voor een zuurstofgebrek (17). Het blijkt dat er in totaal minder gezonde en meer abnormale Purkinje cellen in de top van de lobben

van de vermis aanwezig waren dan in de bodem. Dit is niet eerder beschreven bij overleden pasgeborenen met HIE. Het is nog niet precies duidelijk wat voor consequenties dit heeft voor de ontwikkeling van pasgeborenen met HIE en waardoor dit precies veroorzaakt wordt. Hier moet in de toekomst nog verder onderzoek naar worden gedaan.

Hoofdstuk twee bevestigt histologische cerebellaire schade bij HIE. Onze volgende vraag was of we deze cerebellaire schade ook kunnen aantonen met MRI. Er zijn meerdere onderzoeken gepubliceerd die laten zien dat MRI cerebellaire schade onderschat ten opzichte van histologie (18,19).

In **hoofdstuk drie** vergelijken we de histologische schade met de schijnbare diffusiecoëfficiënt (ADC)-waarden op diffusie-gewogen MRI (DWI) bij overleden pasgeborenen met HIE. De ADC-waarden op DWI zijn een maat voor oedeem van hersencellen en daarmee een indicator voor hersenschade. We vergeleken ook de ADC-waarden tussen overleden pasgeborenen met HIE en controles, dit waren pasgeborenen die een operatie hadden ondergaan in de eerste levensweek met een normale MRI en een normale neurologische ontwikkeling. ADC-waarden in de vermis en nucleus dentatus (één van de cerebellaire kernen) waren lager in de HIE groep dan in de controle groep. De ADC-waarden in de cerebellaire hemisferen waren vergelijkbaar. ADC-waarden in de cerebellaire vermis van pasgeborenen met HIE waren significant gecorreleerd met schade aan de Purkinje cellen in de vermis. Verder was er geen correlatie tussen MRI en histologie. Het is voor clinici dus belangrijk om te realiseren dat pasgeborenen met HIE cerebellaire schade kunnen hebben, ondanks dat de DWI normaal lijkt. ADC-waarden in de vermis lijken het meest betrouwbaar.

MRI onderschat wellicht cerebellaire schade, maar het blijft een accurate voorspeller voor hersenschade en de gouden standaard voor beeldvorming. Er zijn echter situaties dat MRI niet beschikbaar is of MRI niet haalbaar is vanwege de klinische toestand van de patiënt. Echo van de hersenen kan dan een alternatief bieden (20). In **hoofdstuk vier** beschrijven we de ontwikkeling en validatie van een echoscore voor pasgeborenen met HIE. De ontwikkelde echoscore bestaat uit een diepe grijze stof score (composiet score van hyperechogeniciteit van thalamus en putamen, hypo-echogeniciteit van het posterieure deel van de capsula interna en het vier kolommen beeld (gedefinieerd als hyperechogeniciteit van beiden thalami en basale ganglia in het

coronale vlak)) en een witte stof score (composiet score van hyperechogeniciteit van de subcorticale witte stof, periventriculaire witte stof en oedeem). De echoscore op dag drie tot zeven na de geboorte was geassocieerd met een negatieve neurologische uitkomst op twee jarige leeftijd. De echo van de hersenen op dag één na de geboorte was nuttig om antenatale pathologie uit te sluiten.

De focus van **hoofdstuk vijf en zes** is op beeldvorming en de neurologische ontwikkeling op de schoolleeftijd van kinderen met een voorgeschiedenis met HIE na perinatale asfyxie.

Hoofdstuk vijf beschrijft het effect van HIE op het hippocampus volume van kinderen op de schoolleeftijd en de associatie tussen dit hippocampus volume en cognitie en geheugen. Zowel kinderen met milde tot matige HIE als gezonde controles werden geïncludeerd op de leeftijd van negen tot tien jaar. Zij waren allen geboren voordat koeling standaard zorg werd. Kinderen met matige HIE hadden significant kleinere hippocampi vergeleken met controles. Ook kinderen met milde HIE hadden kleinere volumes, maar dit was niet significant. Daarnaast hadden kinderen met HIE meer geheugenproblemen dan controles. Het bleek dat een kleiner hippocampus volume significant was geassocieerd met het episodische geheugen (geheugen voor dagelijkse gebeurtenissen gekoppeld aan een bepaalde plaats of tijd).

Over de neuropsychologische uitkomst op schoolleeftijd van kinderen met HIE die gekoeld zijn, is nog weinig bekend. Daarom hebben we een cohort studie gestart waarin we een groep kinderen van tien jaar hebben gezien die geboren zijn voor het koelingstijdperk (maar wel aan de koelingscriteria voldeden) en een groep kinderen die wel zijn gekoeld. In **hoofdstuk zes** beschrijven we welke hersenstructuren geassocieerd zijn met neuropsychologische uitkomsten in deze groep. Alle kinderen kregen een MRI, een neuropsychologisch onderzoek en een motorisch onderzoek. We vonden problemen in cognitie en episodisch geheugen in beide groepen.
In de totale groep, was atrofie van de hippocampus opnieuw geassocieerd met geheugen en cognitie, maar ook atrofie van de corpora mammillaria was significant geassocieerd met deze uitkomstmaten. De hippocampi en corpora mammillaria maken beiden onderdeel uit van het Circuit van Papez, waartoe ook de fornix en anterieure thalamus behoren. Er was een goede associatie tussen abnormale corpora mammillaria op de neonatale MRI en atrofie op de MRI bij 10 jaar. Atrofie van de corpora mammillaria en hippocampi waren ook significant geassocieerd met

een verminderde witte stof integriteit in het brein, vooral in de fornix en het corpus callosum. Deze resultaten duiden erop dat het circuit van Papez belangrijk is voor de cognitieve uitkomst en het geheugen van kinderen met HIE, bij zowel kinderen die wel als bij kinderen die niet gekoeld zijn.

Atrofie van de corpora mammillaria en hippocampi kan een vroege voorspeller zijn voor geheugen en cognitieve problemen op de schoolleeftijd bij kinderen met HIE. We weten dat atrofie van de corpora mammillaria al bij drie maanden na de geboorte zichtbaar is (21). We zijn momenteel ook aan het onderzoeken of we atrofie van de hippocampus bij drie maanden kunnen aantonen in samenwerking met het Sick Kids Hospital in Toronto, Canada. Wanneer we de atrofie inderdaad vroeg kunnen aantonen, dan is het wellicht mogelijk om vroeger te beginnen met therapie, bijvoorbeeld geheugentraining.

Deel II: neuroprotectie

Zoals eerder beschreven heeft koeling de uitkomst van pasgeborenen met HIE verbeterd, maar nog steeds ongeveer 45% van de pasgeborenen met HIE overlijdt of heeft lange termijn problemen (1). Daarom moeten aanvullende neuroprotectieve therapieën naast koeling worden onderzocht. Een veelbelovende therapie is allopurinol. Allopurinol is een xanthine-oxidase remmer en kan daarmee reperfusieschade verminderen (22). Door het remmen van xanthine-oxidase wordt er minder superoxide gevormd, dat erg schadelijk is voor de hersenen (22,23). Daarnaast is allopurinol een directe katalysator van vrije zuurstofradicalen en niet-eiwit-gebonden ijzer, welke ook beiden verantwoordelijk zijn voor reperfusieschade (24,25). De ontwikkeling van reperfusieschade start direct na de geboorte en de superoxide vorming bereikt zijn piek al binnen 30 minuten na de geboorte. Dus om met allopurinol effectief de superoxidevorming te remmen, moet allopurinol zo snel mogelijk na de geboorte gegeven worden (4).

Hoofdstuk zeven geeft een overzicht van de pathofysiologie van allopurinol en de preklinische en klinische onderzoeken naar het neuroprotectieve effect van allopurinol bij HIE. Op basis van de huidige literatuur kunnen we geen conclusies trekken over de effectiviteit van allopurinol bij HIE (23,26–32). In twee postnatale studies werd allopurinol binnen vier uur na de geboorte gegeven, maar dit was waarschijnlijk te laat gezien het feit dat de superoxide productie al zijn piek bereikt kort na de geboorte (26,28). Pasgeborenen in de allopurinol groep met matige HIE

hadden echter wel een betere uitkomst op vier- tot zesjarige leeftijd, vergeleken met de HIE groep zonder interventie (30). Vervolgens werd de ALLO trial gestart waar voor de geboorte allopurinol werd gegeven aan moeders in geval van foetale nood. Het bleek echter lastig te zijn de juiste patiënten te selecteren op basis van foetale monitoring (31). Allopurinol was in alle eerdere studies veilig (23). Gezien de resultaten van de eerdere studies is vroege allopurinol therapie op de reanimatietafel wellicht de meest effectieve therapie. Er zijn geen studies die het effect van allopurinol naast koeling hebben onderzocht.

Daarom is de ALBINO studie opgezet, geregistreerd als NCT03162653 op ClinicalTrials.gov. Dit is een grote Europese gerandomiseerde, placebo-gecontroleerde studie, waarin het neuroprotectieve effect van vroege allopurinol toediening naast koeling bij pasgeborenen met HIE wordt onderzocht. Het studieprotocol wordt beschreven in **hoofdstuk acht**. Het onderzoek loopt op dit moment in 60 verschillende ziekenhuizen in 13 Europese landen. We moeten de resultaten afwachten van de ALBINO studie om een definitieve conclusie te kunnen trekken over het neuroprotectieve effect van allopurinol bij HIE.

De farmacokinetiek van allopurinol tijdens normothermie is uit de eerdere postnatale en antenatale studies bekend (33–35). De farmacokinetiek van allopurinol tijdens koeling is echter niet bekend en koeling kan potentieel de farmacokinetiek beïnvloeden (36,37), maar ook multi-orgaan falen kan invloed hebben op de farmacokinetiek (38,39). In **hoofdstuk negen** rapporteren we de farmacokinetiek van de eerste 15 patiënten die in de ALBINO studie allopurinol hebben gekregen. De van tevoren opgestelde target concentratie voor allopurinol werd in 100% van de patiënten gehaald. De target concentratie van de actieve metaboliet van allopurinol, oxypurinol, werd in 66.7% van de gekoelde patiënten gehaald en in 100% van de niet gekoelde patiënten. Op basis van deze resultaten is de huidige dosering in de ALBINO studie adequaat voor zowel gekoelde als niet gekoelde patiënten. In de nabije toekomst wordt het effect van onder andere koeling en multi-orgaan falen onderzocht door de huidige data uit de ALBINO studie samen te voegen met de data uit de eerdere postnatale studies (33).

Deel III: innovatieve MRI technieken

MRI technieken hebben zich snel ontwikkeld in de afgelopen jaren: nieuwe sequenties zijn ontwikkeld en nieuwe toepassingen worden gevonden. Het is

belangrijk om de veiligheid te monitoren van deze nieuwe technieken. Een van de risico's van MRI is opwarming, doordat radiofrequente energie wordt opgenomen door het lichaam (40). De hoeveelheid energie die door het weefsel wordt opgenomen noemen we de specifieke absorptie ratio (SAR). Er is weinig bekend over het directe effect van de SAR op de temperatuur van de hersenen. Om de temperatuur van de hersenen beter te kunnen monitoren, is het belangrijk dat er een makkelijke methode beschikbaar is om de brein temperatuur niet-invasief te meten.

In **hoofdstuk tien** onderzoeken we de haalbaarheid van het meten van de brein temperatuur met proton spectroscopie op de MRI (^{1}H-MRS) bij pasgeborenen met HIE na perinatale asfyxie. We hebben een eerder gepubliceerde formule (41) vergeleken met een formule die we zelf hebben gekalibreerd met een fantoom. De brein temperatuur is vervolgens berekend op basis van beide formules voor ^{1}H-MRS met een korte en lange echotijd. De beide formules en beide echotijden leidden tot vergelijkbare temperaturen van het brein. Alle temperaturen bevonden zich daarnaast in de fysiologische range. Deze studie liet zien dat kalibratie niet essentieel is en de formule van Wu *et al.* gebruikt kan worden voor beide echotijden (41). Dit maakt het makkelijker voor onderzoekers om brein temperatuur te meten in toekomstige studies en veiligheid te monitoren tijdens MRI.

Het monitoren van veiligheid is nog belangrijker nu 7.0 Tesla (T) MRI steeds meer gebruikt kan worden. Een hogere veldsterkte kan leiden tot een hogere SAR (42). 7.0T MRI is bij volwassenen veilig en heeft een toegevoegde diagnostische waarde laten zien (42). Voor neonaten, bijvoorbeeld met onverklaarde convulsies of een verdenking op een metabole ziekte, zou scannen op een 7.0T MRI mogelijk tot aanvullende informatie kunnen leiden. 7.0T MRI is echter nog nooit gedaan bij pasgeborenen en daarom moet eerst de haalbaarheid en veiligheid onderzocht worden, voordat de additionele waarde voor de beeldvorming in grotere groepen onderzocht kan worden.

In **hoofdstuk elf** onderzoeken we de haalbaarheid van 7.0T MRI bij pasgeborenen tussen de uitgerekende datum en (gecorrigeerde) leeftijd van 3 maanden. We hebben eerst virtuele SAR simulaties uitgevoegd om te testen of het veilig is om de SAR grenzen van de scanner voor volwassenen aan te houden. De SAR waarden van het babymodel waren lager dan van het volwassene model, zolang de baby in isocentrum lag. Het bleek dus veilig om de SAR limieten van de MRI aan te houden.

Daarnaast was het haalbaar om MRI scans van goede kwaliteit te maken van de eerste pasgeborenen gescand op de 7.0T MRI en de kwaliteit wordt nog steeds verder geoptimaliseerd. De veiligheidsstudie is nog niet afgerond en is essentieel voordat grotere studies kunnen worden opgestart.

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CHAPTER **14**

List of abbreviations

List of coauthors

List of publications

Curriculum vitae

Dankwoord (acknowledgments)

LIST OF ABBREVIATIONS

-50mm FH	=	50mm from isocenter in feet direction
+50mm FH	=	50mm from isocenter in head direction
^{1}H-MRS	=	proton magnetic resonance spectroscopy
ADC	=	apparent diffusion coefficient
ADP	=	adenosine diphosphate
aEEG	=	amplitude integrated electroencephalography
AHR	=	allopurinol hypersensitivity reaction
ALBINO	=	effect of allopurinol in addition to hypothermia for hypoxic-ischemic brain injury on neurocognitive outcome
AMP	=	adenosine monophosphate
ARD	=	acute respiratory distress
ATP	=	adenosine triphosphate
AUC	=	area under the curve
BOT-2	=	Bruininks-Oseretsky test of motor proficiency second edition
BRIEF	=	behavior rating inventory of executive function
BW	=	birthweight
CBCL	=	children's behavior checklist
CD68	=	cluster of differentiation 68
Cl	=	clearance
COMP	=	committee for orphan medicinal products
CP	=	cerebral palsy
CTG	=	cardiotocography
CUS	=	cerebral ultrasound
DDOD	=	department of development and origin of disease
df	=	degrees of freedom
DMC	=	data monitoring committee
DN	=	dentate nucleus
DTI	=	diffusion tensor imaging
DTI-TK	=	DTI-ToolKit
DWI	=	diffusion weighted imaging
ECG	=	electrocardiography
ECMO	=	extracorporeal membrane oxygenation
EEG	=	electroencephalography
EFCNI	=	European foundation for the care of newborn infants
FA	=	fractional anisotropy

FDA	=	Food and Drug Administration
FLAIR	=	fluid attenuated inversion recovery
fm	=	fraction formation
GABA	=	gamma-aminobutyric acid
GOF	=	goodness of fit
H&E	=	Hematoxylin and Eosin
HIE	=	hypoxic-ischemic encephalopathy
HIE1	=	mild hypoxic-ischemic encephalopathy
HIE2	=	moderate hypoxic-ischemic encephalopathy
HLA	=	human leukocyte antigen
HLA-DR	=	human leukocyte antigen DR
HLHS	=	hypoplastic left heart syndrome
HT	=	therapeutic hypothermia
ICV	=	intracranial volume
IIV	=	interindividual variability
IQ	=	intelligence quotient
IQR	=	interquartile range
IRDS	=	idiopathic respiratory distress syndrome
i.v.	=	intravenous
KABC	=	Kaufman-assessment battery for children
LLOQ	=	lower limit of quantification
MB	=	mammillary bodies
MEC	=	medical ethical committee
MRA	=	magnetic resonance angiography
MRI	=	magnetic resonance imaging
MRS	=	magnetic resonance spectroscopy
MRV	=	magnetic resonance venography
NAA	=	n-acetylaspartate
NDI	=	neurodevelopmental impairment
NE	=	neonatal encephalopathy
NICU	=	neonatal intensive care unit
NIRS	=	near-infrared spectroscopy
NMDA	=	n-methyl d-aspartate
NO	=	nitric oxide
NOS	=	nitric oxide synthase
NPBI	=	non-protein-bound iron
OFV	=	objective function value

$ONOO^-$	=	peroxynitrite
OR	=	odds ratio
PA	=	perinatal asphyxia
PC	=	Purkinje cell
PIQ	=	performance intelligence quotient
PK	=	pharmacokinetics
ppm	=	parts per million
RAKIT	=	learning names of the revisie Amsterdamse kinderintelligentie test
RAVLT	=	Rey auditory verbal learning test
RCFT	=	Rey complex figure test
RCT	=	randomized controlled trial
RF	=	radiofrequency
RI	=	resistance index
ROI	=	region of interest
RVDLT	=	Rey visual design learning test
SAR	=	specific absorption rate
SCPE	=	surveillance of cerebral palsy in Europe
SD	=	standard deviation
SES	=	socioeconomic status
SIR	=	sampling importance resampling
SNR	=	signal-to-noise ratio
SWI	=	susceptibility weighted imaging
T	=	tesla
T1WI	=	T1-weighted imaging
T2WI	=	T2-weighted imaging
TBSS	=	tract-based spatial statistics
TBV	=	total brain volume
TE	=	echo time
TIQ	=	total Intelligence quotient
TOBY	=	total body hypothermia for neonatal encephalopathy trial
TR	=	repetition time
UMCU	=	University Medical Center Utrecht
Vd	=	volume of distribution
VIQ	=	Verbal intelligence quotient
WISC	=	Wechsler intelligence scale for children

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CURRICULUM VITAE

Kim Annink was born on May 11th, 1992 in Utrecht, the Netherlands. She grew up in Bilthoven with her younger brother. After graduating in 2009, she started medical school at Utrecht University. During her study, she discovered her passion for pediatrics and research. She performed research projects during her medical training in pediatric hematology under supervision of dr. Marrie Bruin and dr. Katja Heitink, in pediatric infectious diseases under supervision of dr. Tom Wolfs and dr. Tjomme van der Bruggen and in pediatric rheumatology under supervision of professor Joost Frenkel and dr. Nienke ter Haar. After obtaining her medical degree, she was given the opportunity to start as a PhD candidate at the Neonatology department in the Wilhelmina Children's Hospital under supervision of professor Manon Benders, professor Frank van Bel, dr. Jeroen Dudink and dr. Niek van der Aa. Under supervision of professor Manon Benders and professor Frank van Bel, she set up and coordinated the Dutch part of a European randomized controlled trial investigating the neuroprotective effect of early allopurinol in infants with hypoxic-ischemic encephalopathy (HIE) and coordinated the neuroimaging workpackage in all European centers. Furthermore, she worked on several projects on neuroimaging and follow-up in infants with HIE.

During her PhD, Kim followed the PhD Curriculum "training upcoming leaders in pediatric science (TULIPS)" and a post-processing neuroimaging course in Boston, United States of America. She was also active as a student supervisor and organized several public events to give children the opportunity to learn more about research and neonatal neurology.

In June 2020, she started as a resident not in training (ANIOS) at the department of Pediatrics in the Antonius Hospital. Kim is married with Joost Anker and lives in Utrecht.

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