Advances in perinatal stroke

From early prediction to future therapies

UMC Utrecht Brain Center

Nienke Wagenaar

ADVANCES IN PERINATAL STROKE

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Colophon

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Advances in perinatal stroke

From early prediction to future therapies

Vooruitzichten voor het perinataal herseninfarct: van vroege voorspelling naar toekomstige therapieën

(met een samenvatting in het Nederlands)

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Als ik iets zou mogen zeggen, het is geen rechte lijn
De grens maar iets verleggen, gaat maar zelden zonder pijn
Want de allermooiste bloemen groeien vlak langs het ravijn
En om die te kunnen plukken, moet je durven bang te zijn
Veldhuis & Kemper

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If you look closely at the tail of the frog, you see that he has none.
Lao-Tse

CHAPTER I

GENERAL INTRODUCTION

INTRODUCTION

Perinatal stroke comprises a spectrum of brain injury caused by a cerebrovascular event around birth. Its origin can be both hemorrhagic or ischemic, although it is most commonly referred to as perinatal cerebral injury of ischemic origin. This was confirmed by an international workshop on this subject describing ischemic perinatal stroke as "a group of heterogeneous conditions in which there is focal disruption of cerebral blood flow secondary to arterial or cerebral venous thrombosis or embolization, between 20 weeks of fetal life through to the 28th postnatal day, confirmed by neuroimaging or neuropathological studies."[1] Within this timeframe, classification of perinatal stroke is possible based on timing of onset: fetal ischemic stroke is diagnosed before birth; neonatal ischemic stroke is diagnosed after birth but before the 28th postnatal day; and presumed perinatal ischemic stroke (PPIS) is diagnosed after 28 postnatal days, but the event is presumed to have occurred between the 20th week of fetal life and the 28th postnatal day.[1] The use of the definition proposed by the workshop also results in differentiation of two types of perinatal stroke: perinatal arterial ischemic stroke (PAIS) as induced by arterial blood flow disruption; and cerebral sinovenous thrombosis (CSVT) caused by venous blood flow disruption.[2]

Hemorrhagic brain injury in the (preterm) neonate, most frequently caused by periventricular hemorrhagic infarction (PVHI), is not considered perinatal stroke under the workshop definition, although this is part of an ongoing debate. PVHI results from hemorrhage in the vulnerable medullary veins surrounding the ventricles.[3] Hemorrhage in the germinal matrix may cause a restriction of periventricular blood drainage, thereby inducing hemorrhagic-ischemic brain injury in the white matter and eventually resulting in white matter lesions.[4]

This thesis mainly focuses on perinatal stroke of ischemic and arterial origin and therefore encompasses the diagnosis of PAIS. However, some other chapters include patients with other forms of unilateral or asymmetric brain lesions, in whom both PAIS and PVHI are the most common diagnoses and have equally severe impact on neurodevelopmental outcome. Therefore, when referring to perinatal stroke in this thesis, data or results from studies on prediction of outcome from part I can most often be applied to patients with both PAIS and PVHI.

Epidemiology/incidence

The incidence of PAIS is reported around 1:2300 – 1:5000 live births, and this wide spread in incidence results from differences in definition and diagnosis of PAIS.[2] The exact pathophysiology of arterial blood flow disruption leading to PAIS is unknown, but it has been described that the occlusion is often temporarily. Many risk factors

have been determined that are related to complicated deliveries or intrapartum events.[1,5] PAIS is more often found in males than females (3:2) and has a left-sided predominance. Most studies report PAIS as a condition of the term neonate, but preterm infants have also been described to be affected by PAIS.[6]

Presentation

Most infants affected by PAIS present with seizures in the first days after birth, usually hemi convulsions. Other, often aspecific symptoms include apneas, bradycardia, hypotonia and feeding difficulties. Some infants remain asymptomatic and their lesion may be detected by (routine) ultrasonography during the first days after birth. Seizures or other symptoms are sometimes subtle, leading to a delayed or missed diagnosis. Those infants who develop neurological deficits that can be attributed to presumed perinatal stroke present later in life, depending on the severity of the sequelae. These neurological deficits include early hand preference, hypertonia, seizures or cognitive delay.

Diagnosis

Infants that present with seizures during the first days after birth, are highly suspected of brain injury. Seizures after PAIS mainly distinguish themselves from other underlying pathology, such as diffuse brain injury caused by HIE or metabolic disorders, by a delayed onset (>12 hours) and lateralization.[7] Next to the history of the patients in presentation of the symptoms, several neuro-imaging and neuromonitoring modalities have been recommended to differentiate between different types of brain injury.[8] However, other diagnostics including blood sampling should be performed to rule out other non-neurologic conditions that may underlie epileptic activity, such as infection or meningitis, electrolyte disturbances, hypoglycemia, metabolic disorders, congenital abnormalities or syndromes.

Cranial ultrasound

Cranial ultrasound in a fast and non-invasive tool that can be used at the bedside in infants suspected of cerebral injury. Asymmetry in echogenicity in the deep white matter is often the first sign of PAIS, although the onset of visualization of these abnormalities may take a few days. Therefore, cranial ultrasound has a relatively low detection rate in the first three days after birth/injury (around 70%), while it improves between day 4 and 10.[9] Furthermore, small strokes in the deep gray matter or cortex, may be missed by cranial ultrasound, although this is dependent on the experience of the sonographer. Doppler ultrasound may improve detection of PAIS and evaluate severity of the lesion as it detects asymmetric changes in cerebral blood flow after PAIS.[10,11]

MRI

Magnetic resonance imaging is considered the gold standard to diagnose PAIS.[12] The most frequently used sequences to detect PAIS include T1- and T2-weighted imaging (T2WI) and diffusion weighted imaging (DWI).(Figure 1) On T2WI, the first signs of PAIS on MRI are increased signal intensity in the affected area (usually white matter, deep gray matter and/or cortex), as well as reduced contrast between the cortical gray matter and the white matter. On the other hand, first signs of PAIS on T1WI are decreased signal intensity in the cortex and white matter. These abnormalities usually appear within 24-48 after onset of injury/symptoms, and their patterns may change over time. Dudink et al. presented an overview of the evolution of PAIS patterns on MRI, which was shown to be remarkably consistent among patients.[13] DWI is highly sensitive to acute ischemic injury and may therefore be able to detect PAIS before abnormalities appear on conventional T1WI or T2WI. High signal intensity on DWI and low signal intensity on the derived apparent diffusion coefficient (ADC) map often appear within 24 hours and remain present until 6-10 days after injury. After that stage, ADC values return to normal again, known as pseudo-normalization, resulting in limited use of DWI to diagnose PAIS after one week after onset of symptoms.[14] Diffusion tensor imaging (DTI) is used to visualize and quantify the integrity of white matter tracts. As PAIS may lead to destruction or degeneration of these tracts, such as the corticospinal tract, DTI can be used to demonstrate asymmetries in the white matter organization and integrity between hemispheres.

Advanced imaging modalities can contribute to the diagnosis of PAIS.(Figure 2) For example, MR angiography (MRA) provides a reconstruction of the cerebral arteries and visualizes the location of arterial occlusions or other anatomical variations.[15,16] MR spectroscopy is able to detect increased lactate levels and decreased N-acetyl-aspartate to choline ratios in the ischemic areas following PAIS, even up to a few weeks after injury. A few studies have used perfusion weighted imaging, including arterial spin labeling (ASL), to demonstrate changes after PAIS, but it's use in diagnosis or prognosis still needs to be studied further.[17–19]

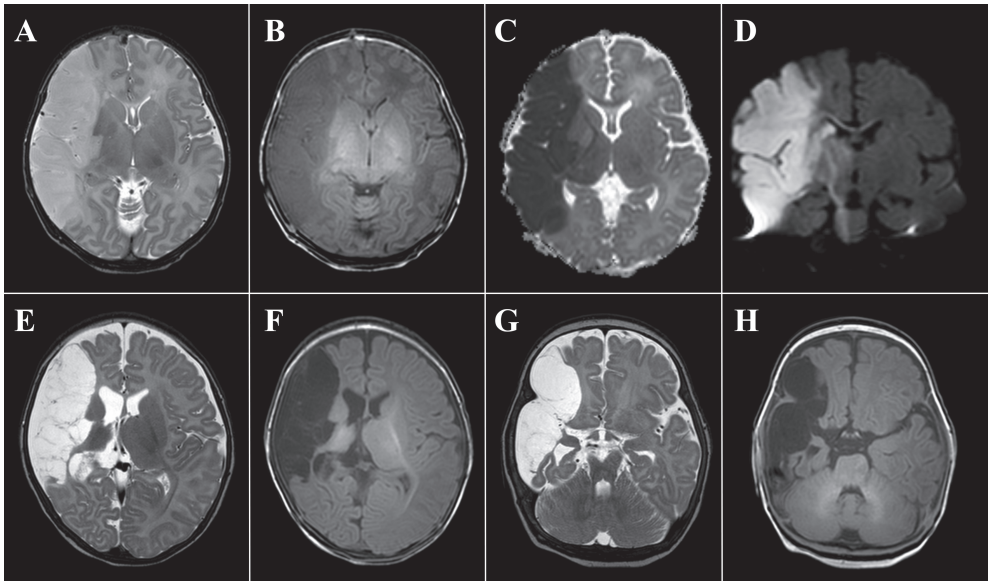

FIGURE 1 | MRI findings in an infant with PAIS. An infant presented with hemi convulsions in the left arm and leg at 26 hours after birth. The MRI, including T2-weighted imaging (A), T1-weighted imaging (B) and diffusion-weighted imaging (ADC map) (C), was performed at 72 hours after initial presentation and showed a main branch middle cerebral artery stroke in the right hemisphere. DWI shows clear restricted diffusion in the descending corticospinal tract, referred to as "pre-Wallerian" degeneration (D). Three months later, a follow-up MRI was performed that demonstrated cystic evolution of the middle cerebral artery territory (E+F), including atrophy of the basal ganglia and thalamus. At three months of age, multicystic encephalomalacia can be seen in the former stroke area (F). Degeneration of the corticospinal tract (Wallerian degeneration) led to atrophy of the right cerebral peduncle (G) and base of the pons (H). This child was at high risk of an adverse outcome, and was eventually diagnosed with cerebral palsy and cognitive deficits at the age of two.

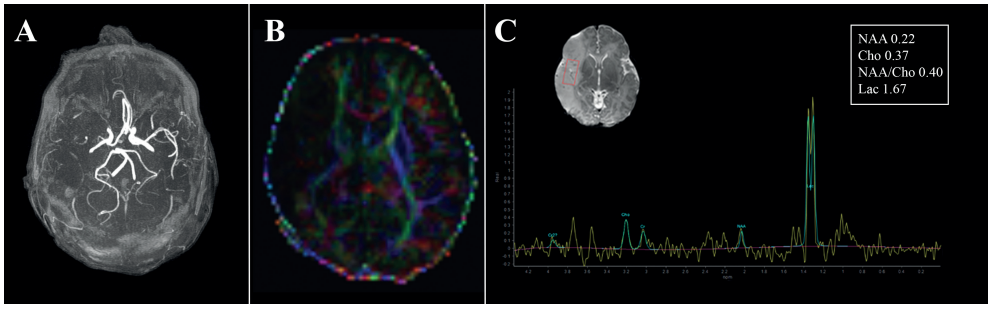

FIGURE 2 | Advanced MRI modalities used to diagnose PAIS. Examples of additional advanced MRI modalities that can be used following perinatal arterial ischemic stroke, shown in an infant with a right middle cerebral artery stroke. Magnetic resonance angiography (MRA) demonstrated absent cerebral blood flow in the right middle cerebral artery (A). Diffusion tensor imaging (DTI) is able to create color-coded fractional anisotropy maps, that demonstrated decreased anisotropy in the optic radiation and an absent posterior limb of the internal capsule in the right hemisphere (B). MR spectroscopy detected increased lactate levels and decreased N-acetyl-aspartate to choline ratios in the ischemic area of the right hemisphere.

Neuromonitoring: NIRS and aEEG

Near-infrared spectroscopy (NIRS) is used to measure cerebral oxygenation and perfusion at the bedside. It monitors regional oxygen saturation and can be applied uni- or bilaterally.(Figure 3) NIRS is proven to be sensitive to cerebral oxygenation changes in newborns with HIE and in adults with strokes, but its use for term infants with PAIS remains understudied.[22,23] Electroencephalogram (EEG) and amplitude-integrated EEG (aEEG) can be used to detect seizures after first clinical symptoms, but also provides information on the location (and possible origin) of the seizures.[20] As described above, seizures after PAIS mainly distinguish themselves from other underlying pathology, such as HIE, by delayed onset (>12 hours) and lateralization.[7] Two-channel aEEG (figure 4) is recommended to detect asymmetries in background pattern, which is more likely to occur after focal injury such as PAIS, than after global injury resulting from hypoxic-ischemic encephalopathy (HIE), meningitis or inborn errors of metabolism.[21]

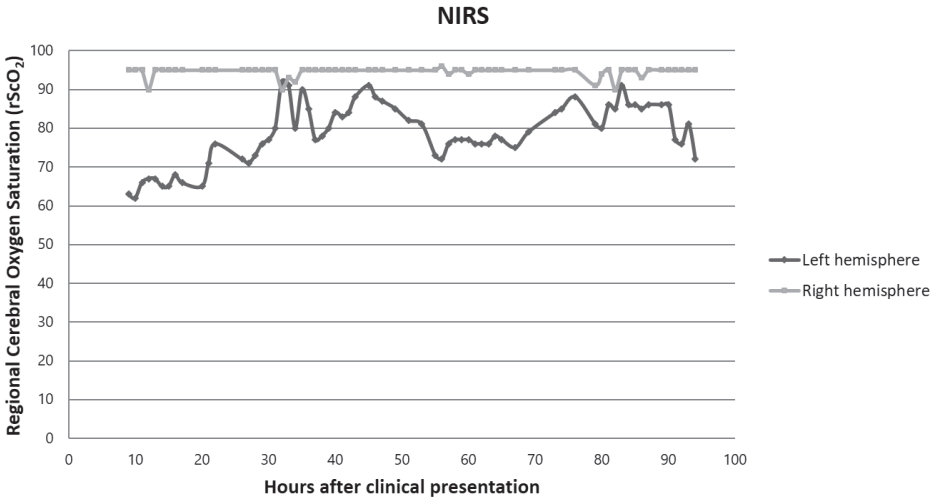

FIGURE 3 | Near-infrared spectroscopy in a neonate with right-sided PAIS. An infant presented with left-sided hemi convulsions at 26 hours after birth caused by PAIS in the right middle cerebral artery territory. Nine hours after initial presentation, the infant was admitted to the neonatal intensive care united and monitored with near-infrared spectroscopy (NIRS). Although the regional cerebral oxygen saturation ($rScO_2$) fluctuated between 60% and 90% in the left hemisphere, it remained >90% in the right hemisphere, indicating hyperperfusion or a loss of oxygen extraction in the right hemisphere caused by PAIS.

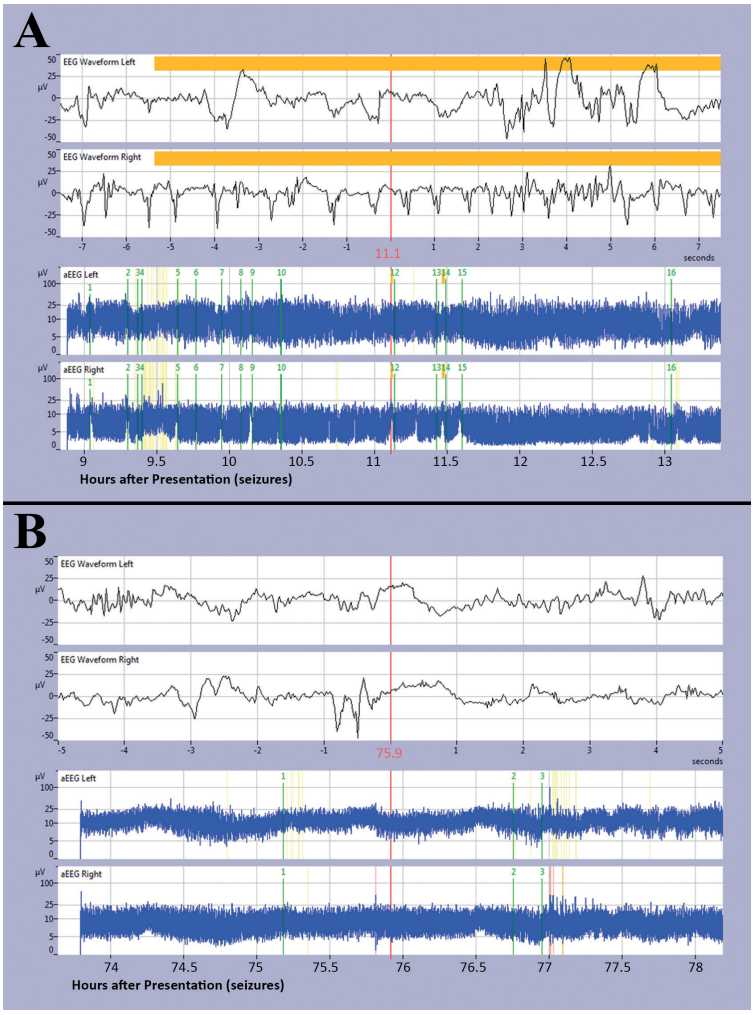

FIGURE 4 | Amplitude-integrated electroencephalogram in a neonate with right-sided PAIS. An infant presented with left-sided hemi convulsions at 26 hours after birth. Nine hours after initial presentation, the child was admitted to the neonatal intensive care united and monitored with amplitude-integrated electroencephalogram (aEEG). Differences in background pattern between left and right can be observed in the early stage (A) and later stage (B). Overall, the background pattern is worse in the right hemisphere, which was found to be caused by a stroke in the middle cerebral artery territory. Subclinical and clinical seizures were observed in the initial stage for which phenobarbital (A, green lines 8 and 11) and midazolam (A, green line 15) were given, with clear rhythmic epileptic activity at, for example, 11.1 hours after clinical presentation.

Prognosis

PAIS can have important consequences for neurological development. Most studies focus on the development of motor abnormalities after PAIS: unilateral spastic cerebral palsy (USCP) or hemiplegia has been reported to occur in around 30% of cases.[24,25] Infants with PVHI are equally at high risk for developing cerebral palsy as studies report a rate of 28-47% of USCP after PVHI.[26–28] Development of USCP depends greatly of the size and location of the lesion, but also on involvement of specific structures, such as the motor tracts or basal ganglia.[29] Children with USCP after PAIS are at risk of developing an additional disabilities on other domains.[30]

Infants with PAIS are also at risk of learning difficulties due to cognitive or language deficits. Impaired language or cognition has been described in 30-50% of cases, and is dependent on size of the lesion, but also on the presence of post-neonatal epilepsy.[24,31–34] It has also been shown that intellectual abilities in PAIS patients decrease over time, which may be related to the onset of post-neonatal epilepsy, but also to increasing intellectual demands at school-age.[35,36]

Although recurrence of PAIS is uncommon, recurrence of seizures is frequently described. Post-neonatal epilepsy is found in about 27% of PAIS patients, with a mean onset at four years of age and has a mean duration of around 10 years.[37,38] Most epileptic activity responds well to anti-epileptic drugs, although drug-resistant epilepsy is described in about 10% of cases.[24,39,40] This often follows after West syndrome, a triad of infantile spasms, hypsarrhythmia on EEG, and intellectual disability, and requires aggressive drug treatment or cranial epilepsy surgery. Risk factors for development of epilepsy remain a topic of debate, but may include size of the lesion, and neonatal seizure burden.[37,38,41]

Visual impairment is also associated with PAIS, and most commonly includes visual field defects, which is found in up to 42% of cases.[42] Development of such defects are associated with larger lesions and involvement of specific brain structures such as the optic radiation, or the occipital (visual) cortex.[42] Other visual or visuospatial skills after PAIS have not been well studied. Little is known on the risk of other developmental disabilities after PAIS, such as adverse behavior, attention or executive functions. Higher rates of deficits in attention and execute function were described after PAIS, and negative risk factors for these included larger infarct size and comorbid epilepsy.[43]

Chapter one of this thesis provides an overview of neurodevelopmental outcome of PAIS in six different outcome domains.

In contrast to PAIS, mortality rates of PVHI range between 30% and 60%, most often associated with extent of the PVHI. However, other risk factors for increased mortality include lower gestational age and associated complications such as circulatory failure

or infections.[2,44–46] As described above, studies reported high rates of USCP after PVHI ranging between approximately 30% and 50%,[2,27,28] and this risk was mainly associated with several lesion characteristics on cranial ultrasound.[2] Infants with PVHI are at risk of other neurodevelopmental disabilities, comparable to consequences of PAIS. These additional sequelae include cognitive dysfunction, language disorders, epilepsy and visual abnormalities.[45,47]

Although quality of life is expected to be generally better after perinatal than adult stroke, reduced quality of life is mainly reported in infants with disabilities. Overall, the degree of disability, but also cognitive dysfunction, mainly determine parental and family reported outcomes.[48,49] Parents often experience a sense of guilt or blame regarding the origin of their child's disability, but also traumatic experiences or depression may result from the inability to assign specific causes for the perinatal events.[39] Access to information, discussion and education are important interventions during follow-up after perinatal stroke, that may reduce the burden of this disease on patients, their families and society during their lifetime.

Prediction of outcome

Many studies on PAIS focus on early risk evaluation to predict who are most likely to develop adverse neurodevelopmental outcome. This is important in order to adequately counsel families and caregivers, but also to select those who might benefit from early intervention programs or therapies. The chapters from part I of this thesis focus on early prediction of outcome after perinatal stroke. As adverse motor outcome is the most well-known complication following perinatal stroke, most outcome prediction studies have used USCP as their primary end point, and studied the use of ultrasound, MRI and clinical motor evaluation as their predictors of interest. A recent review from Novak et al. described that infants with cerebral palsy should be identified as soon as possible, preferably before five months of age, and recommended a combination of neuro-imaging (preferably MRI) and early motor assessment as the gold standard to diagnose cerebral palsy.[50] However, as most clinicians do not officially diagnose cerebral palsy before the age of two years, this mainly implies the need for reliable parameters to select those at high risk of developing CP. Furthermore, there is also a growing interest for the use of MRI or other monitoring tools to predict other neurodevelopmental disabilities after unilateral or asymmetric perinatal brain lesions such as PAIS and PVHI.

Conventional MRI

MRI has proven especially useful in the prediction of abnormal motor development in later life, which is further demonstrated in chapter one of this thesis. Overall, extent, size and location of the ischemic lesion are associated with adverse motor

outcome.[51,52] Several studies have demonstrated that involvement of the corticospinal tract (CST) and basal ganglia on MRI increased the risk for the development of USCP.[25,53,54] Changes in the signal intensity of the corticospinal tracts are often the result of secondary network injury after perinatal stroke. This phenomenon is referred to as pre-Wallerian degeneration, as it precedes anterograde degeneration of the descending axons in the white matter, as a result of injury to their cell bodies within ischemic stroke area, first described as Wallerian degeneration.[55,56] (Figure 1) These early changes are best visualized using DWI: several studies have demonstrated that involvement of the descending CST at the level of the posterior limb of the internal capsule or cerebral peduncle (Figure 1D) is highly associated with poor motor outcome or USCP.[57–60] Quantification of ADC values in the CST was also highly associated with future motor development: decreased ADC values in the cerebral peduncle were found to be associated with the development of USCP.[61,62] Involvement of basal ganglia has also been related to adverse motor outcome, but the exact pattern on MRI is less well described. Most studies evaluating involvement of the basal ganglia have used conventional imaging with T1WI or T2WI, and found that a lesion at or including the region of the basal ganglia was associated with an increased risk of USCP.[51,52,54]

The use of MRI for prediction of other neurodevelopmental outcomes is less well studied. Overall, size of the ischemic lesion on MRI increases the risk for cognitive deficit, epilepsy and behavioral problems, but there is no consensus on an exact definition or cut-off for size of the lesion.[34,40,42,43] Evidence on involvement of specific brain structures or regions in relation to cognition, epilepsy or behavior is sparse: involvement of the basal ganglia was associated to cognitive deficits, while cortical and multifocal infarction was related to epilepsy.[36,63] Chapter one demonstrates the association of specific brain region on MRI with several domains of adverse outcome.

Advanced MRI

DTI is able to quantify integrity of white matter tracts and several studies have described the association between fractional anisotropy (FA) within these tracts and motor development. DTI analyses after PAIS and PVHI have been performed using tractography or tract-based spatial statistics (TBSS) showing that decreased levels of FA in the ipsilesional CST or increased asymmetry between FA values of the CSTs (Figure 2B) were related to development of USCP.[62,64–67] These FA changes usually take time to develop, and neonatal DTI may therefore underestimate the effects of perinatal stroke on future development.[66] In contrast, DTI measurements at three months of age are related to motor, cognitive and visual development.[42,66] To study the role of DTI in comparison with other MRI sequences, we used several quantitative MRI parameters including DTI tractography at three months of age for

the prediction of USCP after unilateral perinatal brain injury, including both PAIS and PVHI, in chapter four.

Other advances in perfusion MRI techniques that have been used in perinatal stroke patients are MRA and Arterial Spin Labeling (ASL). A study from our group found that ASL is able to detect hypo- and hyperperfusion after PAIS. Another study found that abnormal findings on MRA increased the risk for cerebral palsy[15]. However, the association of perfusion techniques like ASL or MRA with the development of adverse outcome needs to be elucidated further. Chapter six describes the use of NIRS to detect oxygenation changes, most likely related to perfusion changes, after PAIS, and may be a first step to continue exploring the field of perfusion MRI scanning.

Neuromonitoring

Early neuromonitoring with either NIRS, aEEG, or their combination, have proven important early predictors for long-term neurodevelopmental outcome in neonates with other subtypes of cerebral injury, such as HIE.[68,69] However their predictive ability for outcome after PAIS needs to be elucidated further, which is studied in chapter six of this thesis. This chapter describes the course of brain activity and oxygenation from aEEG and NIRS in the first five days after clinical symptoms of PAIS, and relates these to neurodevelopmental outcome.

Clinical assessment

Several groups have studied the use of early motor behavior as a predictor for future motor disabilities. General Movements have been described at three months of age and abnormal patterns are strongly related to development of USCP.[70,71] Early binary hand function assessments are also often used to diagnose USCP, but many of these tests can only be performed after 18 months of age.[72] Other studies focus on unilateral hand function as a measure for future development of USCP specifically, and new tools are currently being developed to be used in risk evaluation after perinatal brain injury.[73,74] In chapter three, four and five of this thesis the use of early hand assessment for the prediction of USCP is described in infants with unilateral perinatal brain injury, including PAIS and PVHI. As assessment of early motor function requires the need for a trained observer, recent focus of studies has shifted to the use of automated measurements to assess motion.[75] This includes the use of video-based assessment, wearable sensors and machine learning. The ability of these tools to predict adverse motor outcome early after perinatal stroke, remains however unstudied. Chapter five involves a pilot study with accelerometry using wearable sensors in infants with unilateral brain injury at three months of age, in order to detect early movement asymmetries.

Therapy

Treatment strategies after PAIS focus on the management of (sub)clinical seizures, concurrent infections or hypoglycemia. Until now, no curative therapies are available to reduce cerebral injury after PAIS. Total body hypothermia used as a neuroprotective therapy after HIE is not applied after PAIS, as it is known to exert neuroprotective properties only when administered within 6 hours after the hypoxic-ischemic event.[76,77] PAIS patients usually present around 24-48 hours after birth, and although the exact timing of onset of PAIS is unknown, clinical presentation after onset lies most likely beyond the timeframe of effective hypothermia. In adults with ischemic stroke, early treatment with thrombolytic therapy is effective to improve neurological outcome when administered within 4.5h after onset of ischemia.[78] However, again, delayed clinical presentation limits the role for thrombolytic therapy after PAIS.

New potential therapeutic options therefore focus on restoration of brain injury after the ischemic event. Focus of treatment has shifted from neuroprotection to neuro-regeneration, in order to reduce lesion volume and improve neurodevelopmental outcome after PAIS. Several strategies are currently being studied that include the use of growth factors and stem cells. This thesis outlines the potential use of these neuro-regenerative agents in the context of PAIS or other neonatal brain injury. Chapter seven provides an overview on existing literature on the use of stem cells for neonatal cerebral injury, and the steps necessary to bring this therapy to the clinic. Chapter eight focuses on the therapeutic potential of growth factors and stem cells for PAIS patients specifically. Most studies demonstrated efficacy of stem cell therapy for neonatal brain injury in animal rodent models of (hypoxic)-ischemic brain injury. In order to translate these results into clinical practice, models with larger animal species are needed to demonstrate that stem cells are likely to exert similar beneficial properties in humans. In preparation of studies in human neonates, chapter nine of this thesis provides evidence of an effective migration route of mesenchymal stem cells to the injured brain in an animal model of non-human primates.

Erythropoiesis-stimulating agents, such as erythropoietin and darbepoetin, are one of the most promising neuro-regenerative growth factors that are currently studied by many groups and in several neonatal populations.[79] Erythropoietin (EPO) is a cytokine, which is produced in the developing brain where it functions as an important growth factor, stimulating neurogenesis and angiogenesis. Furthermore, EPO is upregulated after hypoxia, during a process mediated by hypoxia inducible factor-1 (HIF-1), and it has described to exert several neuroprotective and neuro-regenerative abilities by increasing angiogenesis and promoting neurogenesis.[80] Our group has previously performed a pilot safety and feasibility trial in newborns with PAIS and demonstrated that intravenous administration of EPO is safe.[81] Although it was not the aim of that

study, some trend was observed towards feasibility of EPO to improve lesion volume.[81] Future efficacy trials need to demonstrate the efficacy of erythropoiesis-stimulating agents to decrease cerebral injury and improve neurodevelopmental outcome after PAIS. The set-up and clinical protocol of a multicenter randomized controlled trial for PAIS patients treated with darbepoetin or placebo to improve neurological deficits is outlined in chapter 10.

The functional consequences of perinatal stroke not only depend on the size, location and extent of the lesion, but also on the response of the brain after such injury. It has been reported that the immature or developing brain is more capable to reorganize after brain injury than the adult brain. Especially after unilateral brain injury, the unaffected hemisphere is able to (partially) take over certain function to compensate for the affected hemisphere. Several therapies aim at stimulating plasticity of the developing brain after unilateral brain lesions, focusing mostly on reorganization of the motor tracts. These therapies aim to increase extensive activity, which has been shown to stimulate plasticity.[82,83] Examples include physical or occupational therapy, bimanual therapy or constraint-induced movement therapy, that have all proven effective in improving motor performance in infants with USCP.[84,85] Transcranial magnetic stimulation, in combination with MRI, provides information about the reorganization patterns of the corticospinal tract(s) after perinatal stroke. These patterns are predictive of potential functional outcomes, but may also select infants eligible for activity based training.[86] Moreover, interventions that aim to stimulate reorganization should be initiated early in life, when plasticity of the developing brain is highest. This underlines the need for early prediction tools to select infants at risk, who are most likely to benefit from these interventions.

OUTLINE OF THIS THESIS

Perinatal arterial ischemic stroke (PAIS) is an important cause of neurodevelopmental disabilities in several domains, including motor, cognitive and behavior, as outlined in this introduction. Early risk evaluation of these consequences is important in order to adequately inform parents and caregivers, and to select those who might benefit from early intervention strategies. Part I of this thesis describes the use of several early parameters in order to improve prediction of outcome, and select those at risk, with a combination of neuro-imaging, neuro-monitoring and clinical and functional motor assessment.

Part II of this thesis focuses on the improvement of neurodevelopmental outcome by the development of new intervention strategies, including growth factors and stem cells. An overview of several new potential therapies is outlined, as well as the last translational steps to bring these therapies into clinical practice. The final chapter concludes with the study protocol of an ongoing international multicenter randomized controlled trial that studies the effect of a new potential therapy that may improve the outcomes of infants with PAIS.

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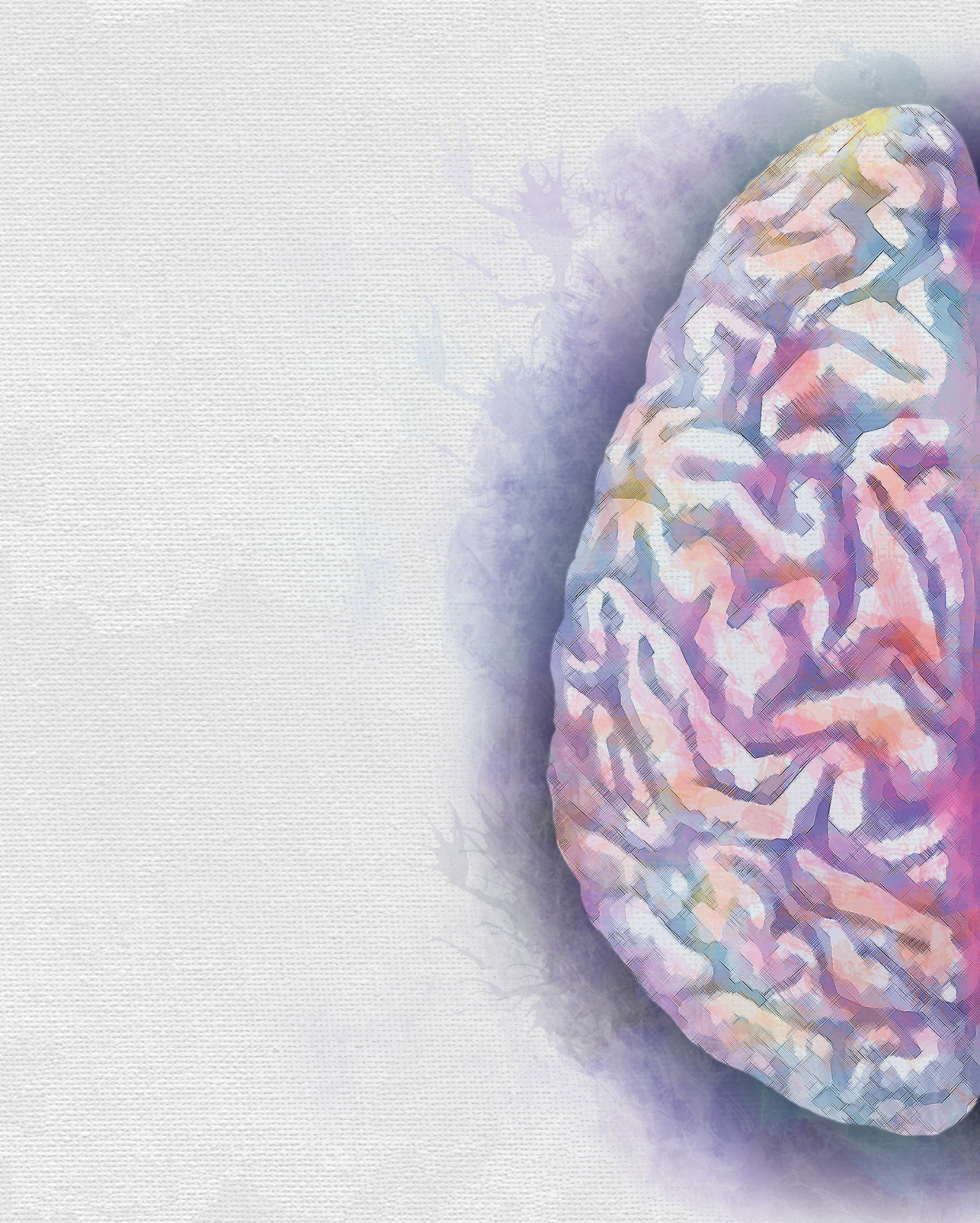

My interest is in the future because I am going to spend the rest of my life there.
Charles Kettering

PART I

EARLY PREDICTION OF OUTCOME AFTER PERINATAL STROKE

CHAPTER 2

NEURODEVELOPMENT AFTER PERINATAL ARTERIAL ISCHEMIC STROKE

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ABSTRACT

Objectives: Perinatal arterial ischemic stroke (PAIS) leads to cerebral palsy in about 30% of affected children and has other neurological sequelae. Most outcome studies focus on middle cerebral artery (MCA) stroke without differentiating between site and extent of affected tissue. The aim of this study was to report outcomes after different PAIS subtypes.

Methods: Between 1990-2015, 188 full-term infants, from two centers (London [n=79] and Utrecht [n=109]) had PAIS on their neonatal MRI. Scans were re-evaluated to classify stroke territory and determine specific tissue involvement. At 18-93 (median 41.7) months, adverse neurodevelopmental outcomes were recorded as one or more of cerebral palsy, cognitive deficit, language delay, epilepsy, behavioral problems or visual field defect.

Results: The MCA territory was most often involved (90%), posterior or anterior cerebral artery (PCA/ACA) territory strokes occurring in 9% and 1% respectively. Three infants died and 24 had scans unavailable for re-evaluation or were lost to follow-up. Of 161 infants seen, 54% had an adverse outcome. Outcomes were the same between centers. Main branch MCA stroke resulted in 100% adverse outcome, while anterior, middle, posterior and cortical MCA strokes, perforator stroke and PCA/ACA stroke had adverse outcomes in only 29-57%. The most important outcome predictors were involvement of the corticospinal tracts and basal ganglia.

Conclusions: Although neurodevelopmental outcome was adverse in at least one domain with main branch MCA stroke, in other PAIS subtypes outcome was favorable in 43-71% of children. Site and tissue involvement is most important in determining outcome in PAIS.

INTRODUCTION

Perinatal arterial ischemic stroke (PAIS) is an important cause of long-lasting neurodevelopmental problems.[1,2] With increased use of neuro-imaging techniques, especially magnetic resonance imaging (MRI), the incidence from hospital-based studies is now considered to be about 1 in 2300-5000 live born full-term neonates with a low mortality rate.[3,4]

Adverse consequences of PAIS include cerebral palsy (CP), usually of a hemiparetic type, cognitive dysfunction, epilepsy, language, visual and behavioral problems are reported to occur in 50-75% of infants.[1] Several groups have described MRI parameters that help in predicting adverse outcome after PAIS.[5,6] More specifically, the development of CP mainly depends on involvement of the corticospinal tracts (CST) at the level of the PLIC or cerebral peduncles.[2,7–11] Visual field defects occur most often when PAIS clearly involves the optic radiation.[12,13]

PAIS most often occurs in the territory of the middle cerebral artery (MCA) and most studies on outcome focus on main branch MCA stroke.[1,14] As occlusion of the proximal segment (M1) of the MCA will lead to infarction of the entire MCA region, including the basal ganglia and CST, development of unilateral CP can be reliably predicted. However, often only more distal MCA segments, or the anterior (ACA) or posterior cerebral artery (PCA) are involved resulting in relatively characteristic lesion patterns, that can be recognized on MRI.[15] Most studies on outcome in PAIS do not differentiate between these lesion patterns involving various sites and extent of affected tissue. We hypothesized that outcome of PAIS primarily depends on the brain area that is affected by the stroke. Therefore, the aim of this study was to report on neurodevelopmental outcomes of different subtypes of PAIS in full-term infants, taking into account its lesion site and involvement of well-defined important brain structures.

PATIENTS AND METHODS

The neonates in this study comprised two cohorts of full-term newborn infants that were admitted to the neonatal intensive care unit or referred for neurological assessment to Queen Charlotte´s/Hammersmith Hospitals in London, United Kingdom (n=79) or the Wilhelmina Children's Hospital of the University Medical Center in Utrecht (UMCU), the Netherlands (n=117) between October 1990 and January 2015. All infants had acute symptoms in the first week after birth, most often (hemi)convulsions, but in a few infants their symptoms were less neurologically specific. All had PAIS confirmed on their neonatal MRI. Eight infants were excluded

due to congenital syndromes with known adverse outcome (n=4) or other significant brain lesions (n=4), resulting in a total cohort of n=187 infants. Infants who died in the neonatal period (n=3), whose neonatal MRI scan could not be re-evaluated (n=4) or who were lost-to-follow-up (n=20) were excluded from further analyses. This resulted in a total study cohort of 161 full-term neonates.

Informed verbal parental consent was obtained to perform an MRI for clinical purposes. The institutional review board of the UMCU approved the use of MRI data for anonymous data analysis and waived the requirement to obtain written informed consent. In London, neonatal MRI scans were performed after written informed consent and permission to use these scans and clinical data for research.

Magnetic Resonance Imaging

In both centers, MRI was performed on a 1.0T, 1.5T or 3 Tesla whole-body system (Philips Medical Systems, Best, the Netherlands, or Picker System, Cleveland, OH, USA), using a scanning protocol including at least T1-weighted, T2-weighted and diffusion weighted imaging (DWI). In general, infants were sedated to minimize movement artefacts. As we included infants over a long time period, the MRI protocol was not always the same; imaging details have been reported previously.[9,16,17]

Evaluation of MRI data

Neonatologists experienced in neonatal brain MRI (FC and LdV) re-evaluated each (of both centers) MRI scan. The lesions were assessed in three planes if possible. Based on the shape, extent and localization of the area of signal intensity changes, all infants were classified to one of the stroke subtypes shown in figure 1 based on their most predominant stroke pattern.

Classification was mostly based on vascular territory of specific named arteries that resulted in characteristic infarctions as described by Govaert et al.[15] However, we felt that consistency of involvement of particular anatomical structures was more important and the involvement of specific anatomical hallmarks was also used for classification. A hemispheric lesion in the MCA territory located posterior to the central sulcus was attributed to the posterior branch of the MCA while involvement anterior to the central sulcus was attributed to the anterior branch. When the full central sulcus was involved but not regions more anterior or posterior this was attributed to the middle branch MCA. If the anterior or posterior areas and middle branch MCA were involved, the most predominant branch was chosen, and central sulcus involvement noted separately. Involvement of the central sulcus region was best assessed in the parasagittal plane. Where more than one MCA branch artery was involved this was also noted separately as multiple lesions. Small punctate lesions were not considered part of the spectrum of 'multiple lesions'.

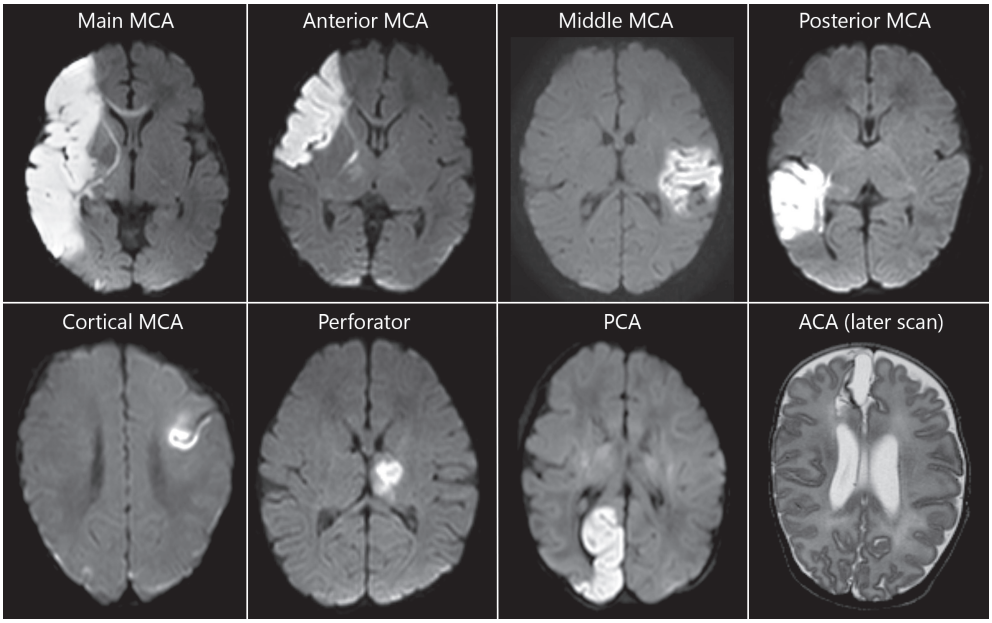

FIGURE 1 | Classification of stroke territory subtypes:
Stroke subtypes were classified based on infarction territories as:

- Main MCA: complete MCA infarction
- Anterior MCA branch: partial MCA infarction anterior to the central sulcus
- Middle MCA branch: partial MCA infarction involving the central sulcus
- Posterior MCA branch: partial MCA infarction posterior to the central sulcus
- Cortical MCA branch: superficial MCA infarction involving only the cortex, without involvement of the striatum
- Perforator branch: perforator stroke involving only the deep gray matter (thalamus and/or basal ganglia)
- Posterior cerebral artery (PCA) / Anterior cerebral artery (ACA): non-MCA infarction

In these examples, corticospinal tract involvement is seen in the main MCA stroke, and the anterior and posterior MCA branch strokes. Secondary network injury to the thalamus is seen with the main branch and the anterior MCA stroke.

MR images were also evaluated for involvement of specific regions we considered likely to be of major importance in predicting outcome, i.e. the CST, the central sulcus region, thalami and basal ganglia, as described previously[2,8], mainly by visual inspection of the DWI from MRI scans done in the first week MRI and from T1- and T2-weighted images when the MRI was acquired later.[7] Involvement of the CSTs has been described previously.[11] Only when the middle part of PLIC and cerebral peduncle were affected, carrying the main motor tracts, was this classified as involvement (figure 2) for the purposes of this study.

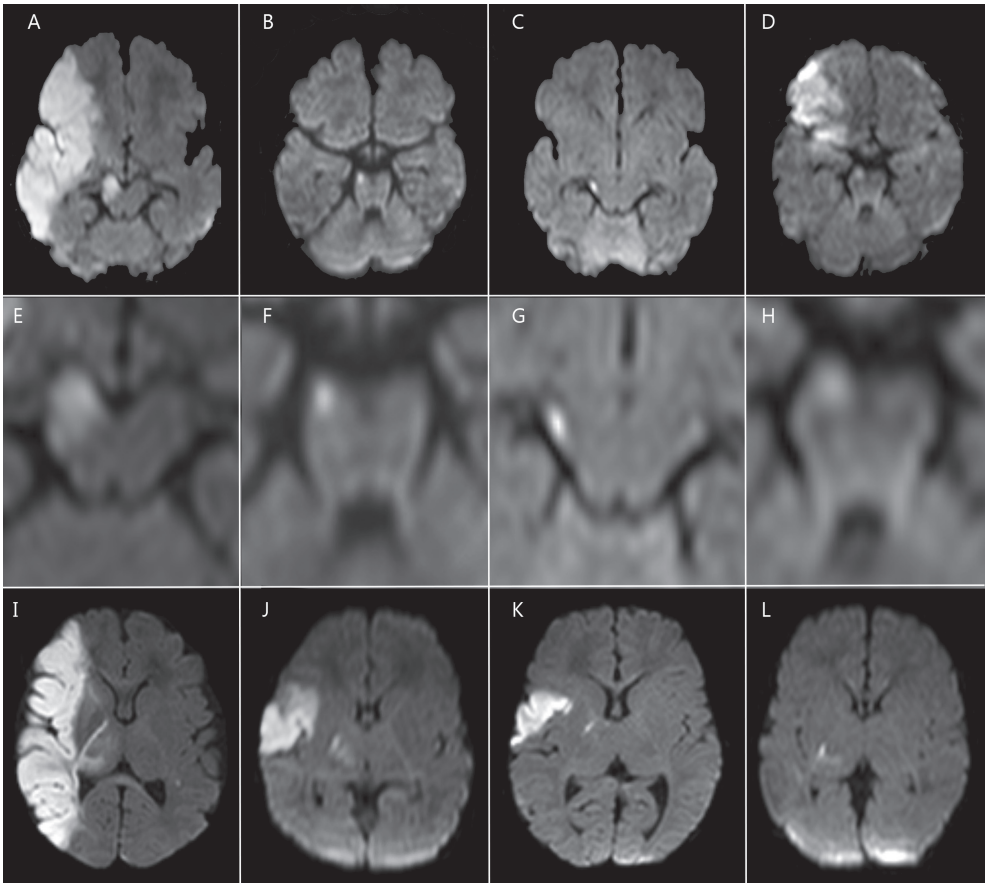

FIGURE 2 | Classification of cerebral peduncle involvement. Involvement of the cerebral peduncle was only scored when the full (A+E) or middle third of the cerebral peduncle was involved (B+F), as described by Kirton et al.[11]. Lateral (C+G) or median (D+H) peduncle abnormalities were not defined as 'cerebral peduncle involvement' in our analyses. Involvement of the PLIC was only scored when the full (I) or middle (J) part of the PLIC was involved and not when there was only anterior (K) or posterior (L) PLIC involvement.

Often with hemispheric strokes, signal changes were also seen in the thalami, particularly the pulvinar, that are likely secondary to "network" injury rather than part of the primary stroke.[18] Examples of primary thalamic stroke (perforator stroke) and secondary network injury to the thalamus are shown in figure 1 (anterior MCA). Bilateral lesions were described as bilateral stroke when stroke lesions were equally severe or as smaller contralateral lesions when one region of stroke predominated.

Neurodevelopmental outcome

Neurodevelopmental outcome was determined during routine follow-up appointments. We only used data from after 12 months until 7 years.

Cognitive development was determined using the Developmental Quotient (DQ) of the Griffiths Mental Development Scale (GMDS), calculated using all subscale scores except locomotion, the Bayley Scales of Infant and Toddler Development third edition (BSITD-III) or the Wechsler Preschool and Primary Scale of Intelligence (WPPSI).[19–21] For all cognitive tests, Z-scores were calculated to allow comparison of the data for statistical analyses. Cognitive delay was defined as a Z-score below -1, corresponding to -1 SD. Language delay was defined as a language score on the GMDS of <-1 SD, >15 points on the Dutch language screening instrument [22] or a diagnosis of speech- and/or language disorders.

CP was diagnosed using criteria from the European CP Network[23] and severity determined using the Gross Motor Function Classification System (GMFCS).[24] Behavioral problems were asked about at each clinic visit and when appropriate children were referred to a clinical psychologist; additionally in the UMCU cohort parents completed the Child Behavior Checklist.[25]. Only behavioral assessments after two years of age were taken into account. Post-neonatal epilepsy was classified as (recurrence of) seizures, diagnosed on EEG for which regular medication was given. Visual field defects included hemianopia and quadrantanopia, diagnosed by a specialized visual development unit in London or pediatric ophthalmologist specialized in assessing visual field defects in infancy in Utrecht. An adverse outcome was defined as the presence of one or more of the following: CP, cognitive deficit, language delay, epilepsy, behavioral problems or visual field defect at latest follow-up.

Statistical Analyses

Descriptive statistics are summarized as percentages of the available study cohort or as median and IQR where appropriate. Stroke subtypes and other imaging features were compared with outcome parameters by using X^2 tests, independent t tests, or Mann-Whitney U tests (for non-parametric variables). Binary logistic regression analysis was performed to determine independent MRI predictors for adverse outcome, which were expressed as odds ratios (ORs) with 95% confidence intervals (CI). These regression analyses were performed for the total cohort and also separately for those without main MCA branch infarction.

Statistical analyses were performed with SPSS, version 21 (IBM Inc, Armonk, New York). P values <.05 were considered to be statistically significant.

RESULTS

The total study cohort consisted of 161 term-neonates born at a median of 40.3 weeks' gestation and a median birth weight of 3440 grams. (Table 1) There were no differences in infant clinical parameters between the two centers, so results are reported for the total cohort. In Utrecht, 30 infants received erythropoietin as part of an intervention study or off-label use.[26] These infants did not differ in stroke patterns and also had equal rates of adverse outcome (unpublished data) and were therefore not reported separately.

TABLE 1 | General characteristics of the study population.

Characteristics	Total (n=161)
Gestational age	40.3 [39.0-41.1]
Birth weight	3440 [3040-3700]
Birth weight Z-score < -1 SD	41 (26)
Head circumference at birth	34.8 [33.5-36.0]
Male	103 (64)
Apgar score at 1 minute	7 [5-9]
Apgar score at 5 minutes	9 [7-10]
Seizures	142 (88)
Postnatal day at first seizures	1 [0-2]
Hypoglycemia*	28 (17)
Postnatal day at MRI	5 [3.5-7]
MRI >7 days after first symptoms or birth	22 (14)
Side of stroke lesion:	
Right	51 (32)
Left	103 (64)
Bilateral	7 (4)
Lesion subtype	
Main middle cerebral artery (MCA)	31 (19)
Anterior MCA branch	17 (11)
Middle MCA branch	21 (13)
Posterior MCA branch	28 (17)
Cortical MCA branch	21 (13)
Perforator branch	27 (17)
Posterior CA / Anterior CA	16 (10)

Data reported as median [Interquartile Range] or number (percentage), where applicable.
*Hypoglycemia was defined as a blood glucose <2 mmol/L.

MRI findings

MRI was performed at a median of 5 (IQR 3.5-7) days after birth. The MCA was most commonly involved, and most often affected were the main branch, posterior, and perforator branches (19%, 17%, 17% respectively) and less often the anterior, middle, or one of the cortical branches (11%, 13%, 13% respectively). PCA stroke was found in 14 cases (9%) and ACA stroke in 2 cases (1%). Clinical characteristics are given in Table 1 and details of involvement of specific regions in Table 2. Involvement of the CST could not be determined in 5 infants (3%), who had an MRI in the second week after birth without DWI abnormalities and T1 or T2-weighted imaging of insufficient quality to assess the CST. Infants that were lost-to-follow up did not differ in terms of stroke pattern classification, basal ganglia/thalamus or corticospinal tract involvement.

TABLE 2| MRI features per stroke territory subtype.

	Total (n=161)	Main MCA branch (n=31)	Anterior MCA branch (n=17)	Middle MCA branch (n=21)	Posterior MCA branch (n=28)	Cortical MCA branch (n=21)	Perforator branch (n=27)	PCA / ACA (n=16)
CST involvement :								
PLIC alone	28 (18)	0 (0)	8 (47)	5 (24)	9 (32)	0 (0)	6 (26)	0 (0)
PLIC and peduncle	46 (30)	28 (100)	3 (18)	6 (29)	4 (14)	0 (0)	2 (9)	0 (0)
Basal ganglia and/or thalamic involvement:								
BG alone	25 (16)	1 (3)	5 (29)	1 (5)	1 (4)	0 (0)	17 (63)	0 (0)
Thalamus alone	17 (11)	0 (0)	1 (6)	3 (14)	2 (7)	0 (0)	8 (30)	3 (21)
BG and thalamus	41 (26)	30 (97)	2 (12)	1 (5)	6 (21)	0 (0)	2 (7)	0 (0)
Central sulcus involvement	68 (43)	31 (100)	7 (41)	21 (100)	9 (35)	0 (0)	0 (0)	0 (0)
Bilateral lesions:								
Smaller lesions	26 (18)	10 (39)	1 (6)	1 (5)	5 (19)	4 (19)	2 (8)	3 (21)
Bilateral stroke	7 (5)	2 (8)	1 (6)	1 (5)	0 (0)	0 (0)	2 (8)	1 (7)
Multiple lesions	58 (39)	12 (46)	5 (31)	6 (30)	12 (46)	7 (33)	7 (28)	9 (64)

Neonatal MR images were re-evaluated for involvement of the corticospinal tracts (CST) at the peduncle and the posterior limb of the internal capsule (PLIC) (n=156), basal ganglia (BG) (n=159) and central sulcus region (n=157). In 148 infants we could assess the presence of multiple and/or bilateral lesions.

Clinical Characteristics per stroke subtype

Infants with cortical MCA infarction were born at significantly later gestational age (mean 40.7±1.3 vs. 40.0±1.4 weeks, $p<0.03$) compared to other subtypes.

Hypoglycemia was more often found in infants with PCA infarction compared to the other subtypes (58% vs. 16%, $p<0.0001$), and more often in main branch MCA

strokes compared to other subtypes (36% vs. 16%, p<0.02), while the incidence of hypoglycemia was not significantly different between PCA and main branch MCA stroke (p>0.1).

Seizures at presentation were less common in perforator stroke compared to the other subtypes (62% vs 94%, p<0.0001). Also, perforator stroke was less often left sided compared to the other subtypes (48% vs. 71%, p<0.03). Other characteristics from table 1 were not significantly different between stroke subtypes.

TABLE 3 | Cognitive developmental score after the age of 12 months per time-point.

	Age at testing (months)	GMDS: DQ (without Locomotor subscore)	BSITD: Cognitive Composite Score	WPPSI: Total IQ score	Z-score	Z-score < -1 SD
12-18 months (n=83)	15.2 [13.0 - 17.6]	98.2 [92.2 - 110.0]			-0.15 [-0.65 - 0.83]	15 (18)
Around 2 years (n=123)*	24.0 [21.1 - 25.0]	98.5 [91.1 - 104.7]	105.0 [95.0 - 113.8]		-0.03 [-0.67 - 0.67]**	17 (13)
Around 3-4 years (n=71)	41.1 [36.0 - 42.4]	98.9 [88.6 - 107.5]			-0.09 [-0.95 - 0.64]	14 (19)
Around 5-7 years (n=64)	67.0 [65.0 - 70.0]			102.0 [88.0 - 111.0]	0.10 [-0.80 - 0.73]	11 (17)
Latest follow-up (n=160)	41.7 [24.6 - 66.0]				-0.04*** [-0.95 - 0.68]	37 (23)

Data presented as median [IQR] or number (percentage), where applicable. DQ, Developmental Quotient; GMDS, Griffiths Mental Development Scale; BSITD, Bayley Scales of Infant and Toddler Development; WPPSI, Wechsler Preschool and Primary Scale of Intelligence. * Around 2 years, 106 tested with the GMDS and 28 tested with the BSITD-3. ** When tested with both GMDS and BSITD-III, a Z-score was calculated for latest test.

*** Z-score could not be calculated in three infants due to severe delay (<-2 SD).

Neurodevelopmental outcome

All infants were seen between 12 months and 7 years with a median age of 41.7 months when last seen (Table 3). There were no differences in outcomes between centers. At their latest follow-up 49 infants (30%) had developed CP; GMFCS levels were determined for 40 infants: 90% level I, 8% level II, 3% level IV (one child with bilateral main branch MCA). There were no infants in this study who were first diagnosed with CP beyond two years of age. Cognitive test-results were available for 157 infants and three infants could not be tested due to severe delay (<-2 SD). This resulted in 37 infants (23%) with a cognitive Z-score <-1 SD and 13 infants (8%) with a cognitive Z-score <-2 SD (Table 3). At their latest follow-up, 87 of 161 infants (54%)

had an adverse outcome: 38 infants (24%) had one or a combination of adverse outcomes without having CP. More details on adverse outcome domains per stroke subtype are given in Table 4. Further analyses were performed using outcome results from each patient's last follow-up.

Overall, 50 of 87 (57%) infants with adverse outcome developed sequelae in multiple domains. Of the 49 infants with CP, 35 (71%) had another adverse outcome, most commonly a cognitive deficit (n=22, 45%). Visual field defects did not occur in isolation, and most often in combination with CP (13/17). Although adverse outcomes commonly co-occurred, only four children were affected in all six developmental domains.

Infants with language delay had increased risk of cognitive delay (OR 11.8; 95% CI 4.7 – 29.2), but excluding those with main MCA branch stroke, the odds for cognitive delay were 6.5 times increased with language delay (95%CI 2.1 – 20.1). Post-neonatal epilepsy also increased the risk for cognitive delay (OR 9.1; 95% CI 3.1 – 26.6), but this was only significant in the main MCA branch stroke group.

TABLE 4 | Adverse outcome domains per stroke territory subtypes.

PAIS type and outcomes (number with data)	Total (n=161)	Main MCA branch (n=31)	Anterior MCA branch (n=17)	Middle MCA branch (n=21)	Posterior MCA branch (n=28)	Cortical MCA branch (n=21)	Perforator branch (n=27)	PCA / ACA (n=16)
Cerebral palsy (n=161)	49 (30)	31 (100)	2 (12)	4 (19)	6 (21)	0 (0)	4 (15)	2 (13)
Cognitive deficit (n=160)	37 (23)	17 (57)	1 (6)	3 (14)	8 (29)	3 (14)	2 (7)	3 (19)
Language delay (n=145)	34 (23)	15 (58)	4 (25)	2 (10)	5 (20)	3 (17)	3 (11)	2 (17)
Post-neonatal epilepsy (n=151)	18 (12)	12 (41)	1 (6)	0 (0)	3 (12)	0 (0)	0 (0)	2 (13)
Behavioral problems (n=126)	31 (25)	10 (37)	4 (31)	1 (6)	6 (25)	2 (13)	3 (17)	5 (42)
Visual field defect (n=96)	17 (18)	12 (48)	0 (0)	0 (0)	2 (14)	0 (0)	0 (0)	3 (27)
Combination of adverse outcomes (n=161)	50 (31)	26 (84)	3 (18)	2 (10)	8 (29)	2 (10)	2 (7)	7 (44)
Within normal range (n=161)	74 (46)	0 (0)	9 (53)	13 (62)	12 (43)	15 (71)	18 (67)	7 (44)

Data presented as number (percentage). Number of infants tested per outcome domain are presented in the first column.

MRI parameters associated with neurodevelopmental outcome

Analyzing all infarcts together, univariate analyses showed associations between several MRI parameters and neurodevelopmental outcome domains (Table 5). Involvement of the cerebral peduncles and combined involvement of the basal ganglia and thalami were both related to almost all adverse outcome domains with ORs ranging between 3.8 and 115.6 (Table 5).

Multivariable modelling

For the total cohort, several MRI parameters were significantly and independently associated with different outcome domains, as described in Table 6. Since there was involvement of the CST, basal ganglia and central sulcus in all main MCA branch infarcts, multivariable analyses were repeated separating all infants with main MCA branch infarction from the others (Table 6).

TABLE 5 | Univariate associations between the MRI parameters and neurodevelopmental outcome domains.

	Involvement on MRI:							
	PLIC	**Cerebral peduncle**	**Basal Ganglia**	**Thalamus**	**BGT**	**Central Sulcus**	**Bilateral lesions**	**Multiple lesions**
Cerebral palsy	6.7 (1.2-38.7)	115.6 (35.2–379.4)	5.5 (1.6-19.4)	NS	102.2 (27.8-376.2)	16.7 (6.7–41.6)	2.3 (1.0–5.2)	NS
Cognitive deficit	NS	6.1 (2.7–13.5)	NS	NS	5.9 (2.5–14.1)	4.2 (1.9–9.4)	NS	NS
Language delay	NS	4.7 (2.0–10.8)	NS	NS	7.2 (2.7–18.8)	4.1 (1.7–9.4)	NS	NS
Post-neonatal epilepsy	NS	11.4 (3.5–37.4)	NS	NS	11.8 (3.1–44.9)	4.1 (1.4 –12.2)	4.4 (1.5–12.9)	2.9 (1.0-8.6)
Behavioral problems	NS	NS	NS	NS	3.8 (1.5–9.5)	NS	NS	NS
Visual field defect	NS	8.4 (2.4–28.6)	NS	NS	7.8 (2.0–30.7)	3.5 (1.0-11.8)	NS	NS
Adverse outcome in any domain	NS	17.7 (5.9–52.9)	NS	NS	68.6 (8.9–526.5)	4.9 (2.4–9.7)	NS	NS

Data presented as Odds Ratio with (95% confidence interval). BGT, basal ganglia and thalami; PLIC, posterior limb of the internal capsule; NS, non-significant.

TABLE 6 | Logistic regression models for neurodevelopmental outcome domains with best fit.

Outcome domain	MRI parameters	Total cohort (n=161)		Subgroup (n=130) excluding main branch MCA stroke	
		OR	*95% CI*	*OR*	*95% CI*
CP	Cerebral peduncle BGT	63.0 21.0	10.7–369.4 4.1–106.6	34.4 6.9	5.6–208.9 1.1–45.1
Cognitive deficit	BGT	5.9	2.5-14.1	4.4	1.2-16.9
Language delay	BGT	7.1	2.7-18.8	NS	NS
Post-neonatal epilepsy	Cerebral peduncle Bilateral lesions	13.9 3.6	2.9–67.6 1.1–11.7	9.7 15.9	1.0–101.1 2.3–110.8
Behavioral problems	BGT	3.8	1.5–9.5	8.9	1.9–41.2
Visual field defect	BGT	7.8	2.0–30.7	NS	NS
Adverse outcome in any domain	Cerebral peduncle BGT	4.0 27.9	1.1–14.7 3.2–244.0	NS 17.1	NS 2.1–141.1

Data presented as odds ratio (OR) with 95% Confidence Interval (CI). BGT, basal ganglia and thalamus; NS, non-significant.

In infants with main MCA branch infarction, no specific MRI parameters were associated with different adverse outcome domains. In the other subgroup (n=130), CP was still associated with involvement of the cerebral peduncle (OR 34.4; 95%CI 5.6 – 208.9) and combined basal ganglia and thalamus (BGT) involvement (OR 6.9; 95%CI 1.1 – 45.1). Adverse cognitive outcome was associated with combined BGT involvement (OR 4.4; 95%CI 1.2 – 16.9). Language delay and visual field defects were no longer associated with MRI features but post-neonatal epilepsy remained associated with involvement of the cerebral peduncle (OR 9.7; 95%CI 1.0 – 101.1) and bilateral lesions (OR 15.9; 95%CI 2.3 – 110.8). Behavioral problems were associated with combined BGT involvement (OR 8.9; 95%CI 1.9 – 41.2). Combined involvement of the BGT increased the risk of adverse outcome in at least one domain (OR 17.1; 95%CI 2.1 – 141.1) (Table 6 and Figure 3).

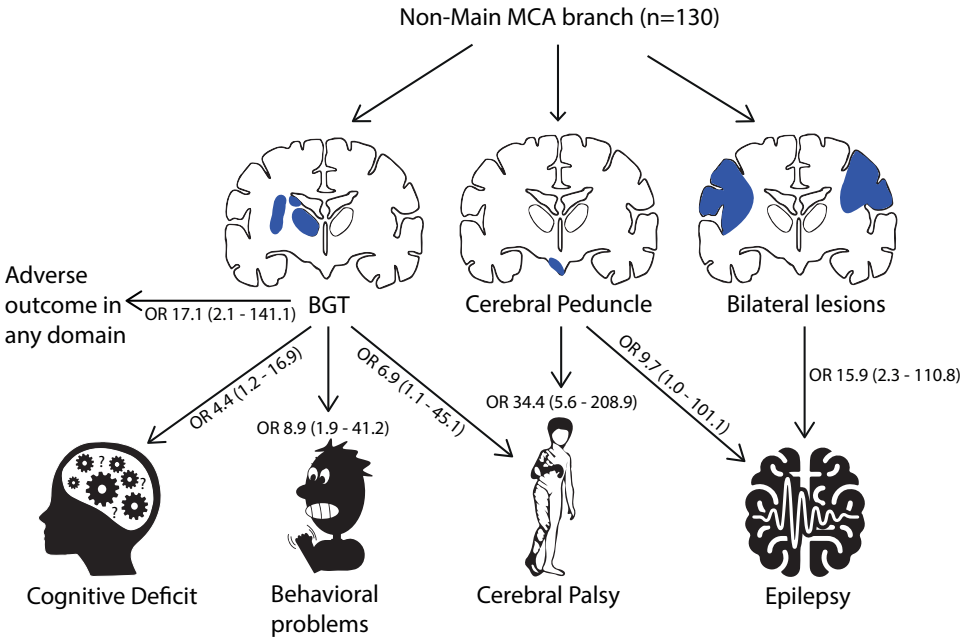

FIGURE 3 | Graphic representation of the logistic regression models for neurodevelopmental outcome domains. Specific brain regions as seen on early MRI increase the odds for cerebral palsy, epilepsy and behavioral problems in subgroup of 130 infants excluding those with main branch MCA stroke. Presented as odds ratio (OR) with 95% Confidence Interval (CI). BGT, basal ganglia and thalamus.

DISCUSSION

In this study we have demonstrated that an adverse outcome in full-term infants with PAIS depends on stroke territory: the site, extent, and location of the lesion. To the best of our knowledge, this is the first study providing a precise overview of a spectrum of different outcome domains per stroke pattern in a large cohort of full-term infants from two centers. Additionally, our study highlights the use of early neonatal MRI, and especially DWI, to predict neurodevelopmental outcome, as outcome is not only dependent on stroke territory, but also on the involvement of specific brain regions, such as the CST.

It is of great importance to clinicians and parents to evaluate promptly the risk of an adverse outcome in patients with PAIS, because early intervention strategies, which may attenuate unfavorable development, need to be appropriately focused.[27] It is also important when possible to reassure parents that outcomes are likely to be good. Several studies on neurodevelopmental following PAIS are available, but these report a wide range of abnormal neurodevelopment, mainly because there is no

distinction between specific stroke sub-types.[28,29] This makes risk-estimation difficult for the individual child. Our study has distinguished several specific PAIS subtypes and described incidence rates per outcome domain for them, enabling more personalized prediction of long-term development. In the literature the incidence rate of CP after PAIS is around 30%, comparable to our study.[8,17,28,29] However, infants with main MCA branch infarction will all develop CP, while this percentage ranges between 0-21% in other stroke subtypes. Our data provides a firm basis for informing parents of infants with main MCA branch infarction differently about future prospects than parents of infants with other stroke subtypes.

Involvement of the CST on neonatal MRI was seen in the majority of patients with more extensive stroke subtypes, as reported in other studies.[8,30] The CST signal changes are best seen on early DWI and have been described as 'pre-Wallerian degeneration'. When they are seen in the middle third of the cerebral peduncle they are always associated with the development of hemiplegic CP [7,9] As 'pre-Wallerian degeneration' is the result of anterograde degeneration of the descending axons of injured cell bodies within the infarcted areas, it was found more often in infants with larger infarctions (affecting the complete motor cortex). Infants with other stroke subtypes, not resulting in involvement of the CST in our cohort did not develop CP. We performed multivariable modelling separately for those without main MCA branch infarction, to determine individual MRI risk factors for adverse outcome in milder subtypes. For these subtypes, involvement of the cerebral peduncle was still a risk factor for CP and epilepsy, illustrating the importance of 'pre-Wallerian' degeneration in the prediction of adverse outcome in those with less widespread stroke.

Involvement of the BGT increased the risk for CP, in agreement with the literature.[8,31,32] In our cohort, basal ganglia and thalamic involvement most commonly occurred with larger infarcts (main and partial MCA branches) and was most often part of the primary stroke. But BGT involvement could also be a manifestation of secondary injury to connectivity pathways, e.g. corticothalamic or corticostriatal networks, particularly in the thalami and best seen on DWI.[18,31,33] The increased risk for CP most likely stemmed from the larger stroke than just the basal ganglia or thalami involvement. This is supported by several studies showing that primary stroke lesions restricted to the BGT (i.e. perforator stroke) are usually not associated with adverse motor outcome.[17,34] In our cohort only 15% of infants with perforator stroke developed unilateral spastic CP, all related to additional involvement of the PLIC.

A limited number of studies have reported on long-term cognitive outcome in infants with PAIS [28,35–37]. We found that cognitive delay occurred in 23% of all PAIS patients, but in 57% of those with a main branch MCA stroke, when last seen at a median age

of 41 months. Other studies have reported even higher rates of cognitive impairment after PAIS at school-age.[37,38] As most infants were still young when last seen, we did not see a trend over time. Multivariable analysis revealed that cognitive delay was related to BGT involvement. However, this seemed to reflect larger strokes, as BGT involvement was most often seen in main and partial branch MCA strokes. Other studies have shown that larger infarct volume was associated with adverse cognitive development in PAIS.[39–42] We also found that posterior MCA branch and PCA strokes had higher rates of cognitive delay compared to other non-main MCA subtypes, indicating that not only volume but also location of affected tissue plays an important role in cognitive development. A recent study by Stephan-Otto et al. reported that stroke in regions posterior to the central sulcus, close to the arcuate fasciculus, may account for language deficits after PAIS.[43] In our cohort, rates of language delay were not higher in posterior compared to anterior MCA branch strokes, but many children were too young for detailed speech- or language assessment. It was of interest that infants with language delay had 6.5-10 times increased risk of cognitive delay, demonstrating that language and cognition are closely related. However, cognitive delay might also precede language delay or share a common origin, and exact causative mechanisms need to be studied further. Development of post-neonatal epilepsy increased the risk for cognitive delay as described previously, but this was limited to infants with main MCA stroke.[44]

This study has several limitations inherent to its retrospective design. Infants were only eligible if they were admitted to the neonatal intensive care unit or referred for neurological assessment, excluding infants with (smaller) infarcts that may not have caused neonatal symptoms; also preterm infants were not included.[45] However, our strict inclusion criteria resulted in a homogeneous group of term infants with PAIS. This study focused on DWI from MRI, while early DWI may not always be possible in all institutions. However, we were often able to see signal intensity changes in CST on T2 weighted sequence as well, especially when the MRI was done in the second half of the first week. The use of Apparent Diffusion Coefficient (ADC) maps from DWI to assess acute ischemic injury is recommended, to avoid T2 shine through and other artefacts. As the ADC map was not always available for our cohort, we used DWI for all infants. When ADC maps were available, they were used to verify DWI signal abnormalities, as is recommended in clinical practice. Infants that performed well were sometimes discharged from follow-up and cognitive, language and behavioral problems may have been under-diagnosed.[37,38] Long-term outcome studies with a prospective design are needed to determine whether early predictions in PAIS patients remain stable over time. Even with this large study some sub-groups were small and we cannot exclude that some associations between brain regions and outcomes might have been significant.

CONCLUSION

In a large cohort of term-born infants from two centers, we have demonstrated that neurodevelopmental outcomes vary between PAIS subtypes. Although neurodevelopmental outcome was invariably adverse in at least one domain with main branch MCA stroke, in other PAIS subtypes outcome was normal in 43-71% of children. This study provides clinicians with important information for more precise risk-evaluation of neurodevelopment in PAIS patients based on the tissue involved assessed from early MRI allowing better counseling of parents in the neonatal period, personalized planning of therapeutic interventions and long-term support for behavioral and cognitive difficulties.

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CHAPTER 3

MR IMAGING FOR ACCURATE PREDICTION OF OUTCOME AFTER PERINATAL ARTERIAL ISCHEMIC STROKE: SOONER NOT NECESSARILY BETTER

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ABSTRACT

Two full-term infants with perinatal arterial ischemic stroke (PAIS), with cerebral sinovenous thrombosis of the superior sagittal sinus in one of them, are reported. Diffusion weighted imaging (DWI)-MRI, performed within 24 hours following onset of seizures and repeated 48 hours later, clearly showed restricted diffusion within the middle cerebral artery territory on both MRIs, but clear patterns of signal intensity changes in the descending corticospinal tracts on the second MRI only. Since involvement of these structures is essential for prediction of motor outcome, we may need to reconsider optimal timing of MR imaging for prediction of neurodevelopmental outcome after PAIS.

INTRODUCTION

Perinatal stroke consists of perinatal arterial ischemic stroke (PAIS) and cerebral sinovenous thrombosis (CSVT), and both are associated with unfavourable neurodevelopmental outcome. Adverse sequelae of PAIS include unilateral spastic cerebral palsy (USCP) cognitive dysfunction, epilepsy and speech problems. In 40-75% of infants, PAIS or CSVT lead to abnormal neuromotor and -developmental outcome.[1–3] Early MRI is used for diagnostic purposes, and for dating PAIS, but is also increasingly emphasized as an important prognostic value to predict the neurodevelopmental outcome of the infant.[4,5]

PAIS, and especially middle cerebral artery (MCA) infarction, often involves the descending corticospinal tracts (CST).[4–6] MRI and especially diffusion weighted imaging (DWI) offers the advantage of evaluating the posterior limb of the internal capsule (PLIC) and the cerebral peduncles, i.e. the presence of "pre-Wallerian" degeneration. Involvement of these structures is strongly correlated with adverse motor outcome.[4–9] CSVT is often associated with neurological comorbidity, such as hypoxic-ischemic encephalopathy, venous infarction and thalamic haemorrhage.[2,10] The presence of such neurological comorbidity at diagnosis predicts poor outcome in patients with CSVT.[2]

The aim of this short communication is to describe two newborns with PAIS who both had two early MRIs showing delayed onset of "pre-Wallerian" degeneration on sequential MRIs performed within 72 hours after onset of symptoms.

CASE DESCRIPTIONS

Case I

This boy was born at 40^{+4} weeks of gestation after an uncomplicated pregnancy by secondary caesarean section due to slow dilatation of the cervix and meconium stained amniotic fluid. His birth weight was 3570 gram (p15), length 53 cm (p50), head circumference 36 cm (p50). He had good Apgar scores of at 9, 10 and 10 at 1, 5 and 10 minutes, respectively. He was admitted to the neonatal unit in a level II hospital for observation, where he developed fluctuations in temperature, frequent apneas and lethargy, suspected for seizures. Amplitude integrated EEG (aEEG) showed epileptic activity originating from the left hemisphere. Cranial ultrasound and an early CT-scan showed an area of decreased attenuation in the distribution of the left MCA. He received phenobarbital to control seizure activity, and antibiotics and anti-viral medication until meningitis was ruled out by negative cerebrospinal fluid cultures, and he was transferred to our level III neonatal intensive care unit.

The first MRI was performed on the third day of life, around 20 hours after the onset of apneas (figure 1). In the complete left MCA territory, DWI and T2 weighed imaging showed increased signal intensity in the cortex and white matter. The basal ganglia including globus pallidus and putamen were also mildly affected. The left PLIC showed only very mild highlighting on DWI and T2 imaging, while some asymmetry was found laterally in the cerebral left peduncle (figure 1D-E). A small cortical lesion was also found in the upper right hemisphere, not affecting the corticospinal tracts. Reduced flow of the left MCA was seen on MR Angiography (MRA). The infant was included in an imaging study and the MRI was repeated on day 5, around 72 hours after the onset of clinical seizures and 48 hours after the first scan, to additionally perform arterial spin labeling.[11] DWI and T2WI showed the same area of increased signal intensity in the main left MCA territory. DWI hyperintensity in the PLIC had extended, showing a clear asymmetrical pattern. Additionally, on DWI, the left cerebral peduncle now also clearly showed restricted diffusion, including the middle part.

The aEEG did not show any seizure activity, but the background pattern showed a continuous normal voltage with sleep-wake cycling on the right, while there was discontinuous normal voltage pattern on the left.

After the second MRI, the child was included in a pilot trial and treated with erythropoietin (EPO) 1000 U/kg per day for three days to improve neuroregeneration.[12] The infant improved clinically, could be fully breastfed and was discharged home one week after onset of seizures. A thrombophilia screen was performed, showing normal clotting time and homocysteine, no MTHFR, factor V Leiden or prothrombin mutation.

Repeat MRI at the age of three months showed cysts in the left hemisphere, especially in the region of the anterior branch of the MCA, and Wallerian degeneration of the left descending CST (Figure 1). On neurological assessment there was a hand preference for his left hand, suggestive of development of USCP, which was subsequently confirmed at two years of age, with Manual Ability Classification System score of 2, Gross Motor Function Classification System score of 1 and Hand Assessment for Infants (HAI) of 83% (whereas 0% equals no asymmetry).[13]

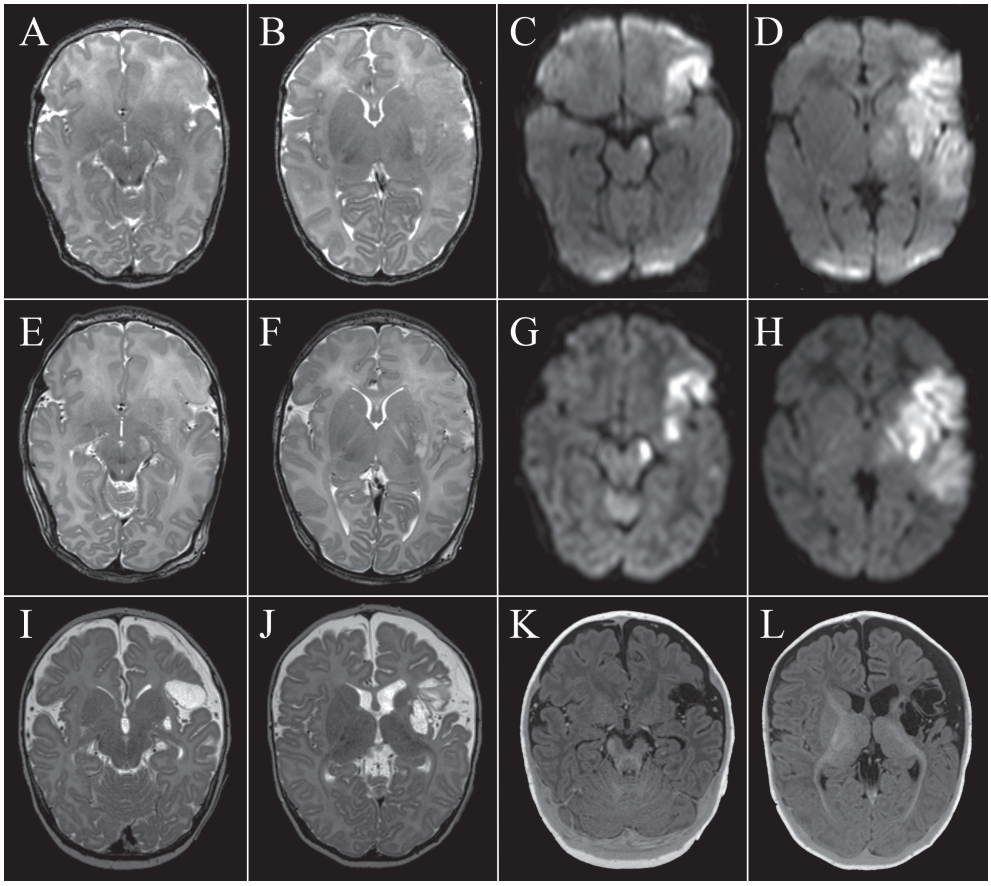

FIGURE 1 | MRI of Case 1. MRI of a newborn (case 1) with a left sided MCA PAIS who had three MRIs: the first within 24 hours (upper row), the second at 72 hours after onset of symptoms (middle row) and the last at three months of age (bottom row). At 72 hours there was an increase in signal intensity in the right cerebral peduncle (A versus E) and PLIC (B versus F) on axial T2 weighted imaging compared to the first scan. On early axial DW imaging mild lateral signal changes can be observed in the cerebral peduncle (C), while a profound hyperintense signal was seen at 72 hours after onset of seizures (G). Signal changes in the PLIC on DW imaging were very clear after 72 hours (H), but not on the first MRI (D). Repeat MRI (bottom row) at the age of three months showed cysts in the left hemisphere, especially in the region of the anterior branch of the MCA (I-J). On Inversion Recovery T1 weighted imaging (axial view) Wallerian degeneration of the left descending CST, involving the cerebral peduncle (K) and PLIC (L) is noted.

Case 2

This male infant was born at 42^{+1} weeks' gestation after a secondary caesarean section because of slow dilatation of the cervix, meconium stained amniotic fluid, and fetal heart rate abnormalities following induction of labor. His birth weight was 3190 grams (<p5), length 51 cm (p3-p50), head circumference 35 cm (p3-p50). Apgar scores were 4, 7 and 9 at 1, 5 and 10 minutes, respectively. He recovered quickly after stimulation, insufflation breaths and CPAP (with max 40% O_2) and no longer needed respiratory support after 5 minutes. He was admitted to the neonatal unit in a level II hospital, where he developed frequent apneas and desaturations 26 hours after birth, suggestive of seizures. He was transferred to our neonatal intensive care unit and was intubated because of respiratory insufficiency. Cranial ultrasound (9 hours after onset of apneas) showed a large wedge-shaped area of increased echogenicity in the distribution of the right MCA, and reduced flow in the superior sagittal sinus. aEEG showed epileptic activity originating from the right hemisphere.

The first MRI was performed at 50 hours of age, 24 hours after the onset of apneas (Figure 2). DWI and T2 weighed imaging showed increased signal intensity in the cortex and white matter of the complete MCA territory in the right hemisphere. The basal ganglia including globus pallidus, putamen and part of the thalamus were also mildly affected. Very subtle restricted diffusion of the PLIC and right peduncle were noted (Figure 2D-E). On MRA there was interrupted flow of the right MCA. MR Venography (MRV) showed no flow in the anterior part of the superior sagittal sinus, suggestive of CSVT.

The infant was started on low molecular weight heparin (Dalteparin, 200 U/kg subcutaneously) to prevent propagation of the thrombus. Clinical seizures were controlled following administration of phenobarbital and midazolam and subsequently lidocaine on day 2 that controlled ongoing subclinical seizures on aEEG. There was a persistent asymmetry in background activity due to decreased activity on the right side. On day 2, activity on the right side was still mildly suppressed, but sleep-wake cycling had returned.

To assess the effect of therapy on CSVT, the MRI was repeated around 72 hours after the onset of clinical seizures and 48 hours after the first scan. DWI and T2WI showed the same area of increased signal intensity in the main MCA territory, including parts of the thalamus. In contrast, on DWI, the PLIC, anterior limb of the internal capsule and right cerebral peduncle now also clearly showed restricted diffusion. MRV showed recurrence of flow in the superior sagittal sinus.

After the second MRI, the child was treated with EPO.[12] The infant improved clinically, was extubated, started to bottle feed, and was discharged home on day 10 after birth. A thrombophilia screen was performed, showing a transient increase in homocysteine

(13.7 μmol/L, normal upper level 10 μmol/L), but no MTHFR, factor V Leiden or prothrombin mutation.

At the age of three months, the infant returned for a repeat MRI, which showed a large area of cavitation in the right MCA region and Wallerian degeneration (Figure 1). On neurological assessment, he showed a clear preference for using his right hand, with intermittent fisting of the left hand, suggestive of a development of unilateral spastic cerebral palsy (USCP). The HAI at three months of age was 58%.

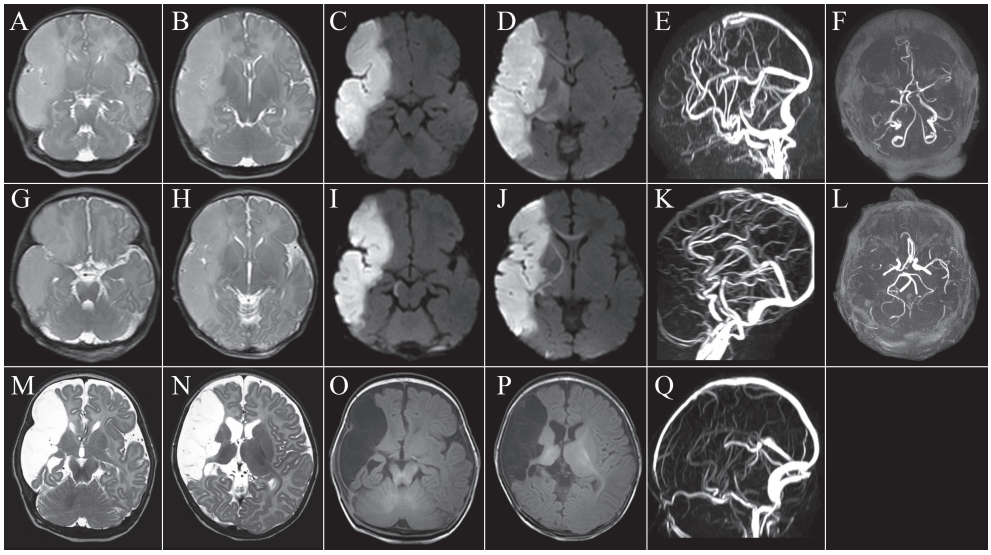

FIGURE 2 | MRI of Case 2. MRI of a newborn (case 2) with a rare combination of PAIS and CSVT who had three MRIs: the first at 24 hours (upper row) and the second at 72 hours after onset of symptoms (middle row) and the third at three months of age (bottom row). Compared to the first scan there was an increase in signal intensity in the right cerebral peduncle (A versus G) and PLIC (B versus H) on axial T2 weighted imaging at the second scan. On early axial DW imaging very little signal intensity changes were observed in the cerebral peduncle (C), while a hyperintense signal was seen at 72 hours after onset of seizures (I). Signal changes in the PLIC on DW imaging were clear after 72 hours (J), but not on the first MRI (D). MRV (sagittal view) showed initially no flow in the anterior part of the superior sagittal sinus (E), and recurrence of flow in the sinus on the second MRV after treatment with low molecular weight heparin (K). On MR angiography (axial view) there was persistent hypoperfusion of the right MCA until 72 hours after onset of seizures (F and L). At the age of three months a large cyst had developed in the right hemisphere (M-N). Additionally, there was Wallerian degeneration seen in the descending right CST involving both the cerebral peduncle (O) and the PLIC (P). On MRV flow in the superior sagittal sinus had restored completely (Q).

DISCUSSION

In this case report on two PAIS patients with main branch MCA stroke, DWI showed different patterns of CST involvement at 24 and 72 hours after the onset of clinical seizures. Also, the unusual combination of PAIS and CSVT is reported in the second infant, with recurrence of flow across the superior sagittal sinus on the second scan.

CT was performed in the level II hospital, but this neuro-imaging technique is not recommended as radiation is involved and CT will provide less information than MR imaging which is considered the gold standard.[14] Within the first week after PAIS, especially T2WI and DWI are most useful for detection and prediction of motor outcome. T2WI will show high signal intensity in the affected cortex and white matter, which can first be observed from 24 to 48 h onwards.[7] Changes on DWI can precede those on conventional T2WI and are often seen more clearly during the first 24–72 h as high signal intensity on DWI or low signal intensity on the derived apparent diffusion coefficient (ADC) map.[14] The lowest ADC can be observed around day 3, followed by a slow increase and pseudo-normalization of ADC values.[7,15] Clinicians are inclined to perform an MRI as quickly as possible in order to make a diagnosis in an infant presenting with neonatal seizures.[16] However, MR imaging also plays an important role in the prediction of motor outcome, especially in the development of USCP, and the cases presented have shown that "pre-Wallerian" degeneration may take time to develop and may not yet be present 1-2 days after onset of PAIS.

Wallerian degeneration was first described by Augustus Waller in 1850 and refers to anterograde degeneration of axons and their myelin sheaths, secondary to proximal axonal or cell body injury.[17] Wallerian degeneration of the descending CST and cerebral peduncle is first seen several weeks after cortical injury, and is associated with development of USCP. This was shown by Bouza et al. in a group of 20 infants and later by Kirton et al. [4,18] They described that the degree of Wallerian degeneration in the CSTs shown with MRI, well beyond the neonatal period, was correlated with the presence and severity of USCP later in life. Therefore, a follow-up MRI after several weeks is recommended for optimal prognosis of motor outcome. DW imaging is sensitive to early changes of cytotoxic edema and therefore able to depict acute injury. Several studies have now shown that DWI can show subsequent axonal ischemia to the descending CST and the term "pre-Wallerian" degeneration was coined.[4,5,19,20] The presence of these DW changes in the CST, in particular the PLIC and the middle part of the cerebral peduncle, were found to be predictive of developing USCP.[4]

Our second infant presented with an unusual combination of main branch MCA infarction and CSVT. We performed a second MRI shortly after the first scan to evaluate the recurrence of flow in the sinus. This gave us the opportunity to obtain two early MRI scans, within the first week after birth. The first scan was performed at 24 hours after onset of seizures. Although DW imaging clearly showed acute injury in the MCA region, we could hardly find DW changes in the descending CST. This absence of early "pre-Wallerian" degeneration was also shown by Kang et al. in 2 adult patients and suggested by Husson et al.[6,21] On a repeat MRI at 72 hours after seizure onset, we found clear signal changes in the PLIC and cerebral peduncle. The increase of DWI abnormalities was also found in the first case. This delay in "pre-Wallerian" degeneration is in agreement with experimental data of Tuor et al. who showed in neonatal rats a delay of 24 hours for the decline in apparent diffusion coefficient (ADC) values and concomitant cellular correlates of degenerating neurons in the cerebral peduncle compared to the ADC values in the affected cortex. They proposed associated axonal ischemia as an explanation for this delay, induced by ischemic cell bodies of the axons in the descending motor pathways that lie within the primary motor cortex in the ischemic region. Subsequently, diffusivity reductions in the cerebral peduncle increase in magnitude over the first few days, but might not be visible on DWI or not yet involve the middle part of the peduncle the first day after hypoxia-ischemia.[20,21] However, these findings are important for prognosis of an adverse outcome.[4,5] This supports the different DWI findings in our two newborn infants with PAIS. It is of interest and rather unexpected that an increase in signal intensity was already present in the mesencephalon on the T2 weighted sequence on the first MRI in the first patient.

In summary, we have shown that early DWI-MRI, performed within 24 following onset of seizures and repeated 48 hours later, clearly showed restricted diffusion within the MCA territory, but showed different patterns of signal intensity changes at the level of the PLIC and cerebral peduncles. When infants present with clinical seizures, an MRI scan is usually performed as soon as possible in order to make a diagnosis.[16] However, MR imaging in suspected PAIS may even be more important to predict neurodevelopmental outcome and its timing should depend on when it is most sensitive to see "pre-Wallerian" degeneration. As our data suggest that for accurate prediction of outcome 'sooner is not necessarily better', as early MRI may underestimate the full extent of the injury and one might underestimate or even miss "pre-Wallerian" degeneration, in the PLIC and cerebral peduncle. In case of suspected PAIS, timing of the MRI therefore needs to be well considered to optimize prediction of neurodevelopmental outcome.

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CHAPTER 4

EARLY PREDICITON OF UNILATERAL CEREBRAL PALSY IN INFANTS WITH ASYMMETRIC PERINATAL BRAIN INJURY – MODEL DEVELOPMENT AND INTERNAL VALIDATION

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ABSTRACT

Objective: Early diagnosis of unilateral cerebral palsy is important after asymmetric perinatal brain injury (APBI). Our objective is to estimate the risk of unilateral cerebral palsy (UCP) in infants with APBI during the first months of life using neuroimaging and clinical assessment.

Methods: Prognostic multivariable prediction modelling study including 52 infants (27 males), median gestational age 39.3 weeks with APBI from Sweden (n = 33) and the Netherlands (n = 19). Inclusion criteria: (1) neonatal MRI within one month after term equivalent age (TEA), (2) Hand Assessment for Infants (HAI) between 3.5-4.5 months of (corrected) age. UCP was diagnosed ≥ 24 months of age. Firth regression with cross-validation was used to construct and internally validate the model to estimate the risk for UCP based on the predictors corticospinal tract (CST) and basal ganglia/thalamus (BGT) involvement, contralesional HAI Each hand sum score (EaHS), gestational age and sex.

Results: UCP was diagnosed in 18 infants (35%). Infants who developed UCP more often had involvement of the CST and BGT on neonatal MRI and had lower contralesional HAI EaHS compared to those who did not develop UCP. The final model showed excellent accuracy for UCP prediction between 3.5-4.5 months (area under the curve, AUC = 0.980; 95% CI 0.95-1.00).

Conclusions: Combining neonatal MRI, the HAI, gestational age and sex accurately identify the prognostic risk of UCP at 3.5-4.5 months in infants with APBI.

INTRODUCTION

Unilateral perinatal brain injury is commonly diagnosed in the neonatal intensive care unit. Most frequent forms of unilateral brain injury include perinatal arterial ischemic stroke (PAIS) in term born infants and periventricular haemorrhagic infarction (PVHI) in preterm infants. However, also other conditions such as white matter injury or parenchymal haemorrhages may lead to asymmetric brain injury in newborns. Infants with such asymmetric perinatal brain injury (APBI) are at high risk to develop unilateral cerebral palsy (UCP).[1–3]

UCP is usually diagnosed at the age of two to four years or even later in mildly affected children, while asymmetric hand use is typically reported much earlier by clinicians and parents.[4–6] Early diagnosis of UCP is important to adequately counsel families and to provide access to early interventions.[1,7,8] Increased knowledge about high plasticity in the young brain suggests that activity-based training should occur at an early age in order to be effective, in parallel with the development of the corticospinal tract.[9,10] New treatment strategies for neonatal brain injury, such as neuroprotective or neuroregenerative repair, have also become available and new motor assessment tools are needed to accurately and objectively study their effect.[11,12]

Magnetic resonance brain imaging (MRI) during the neonatal period is increasingly used as a predictive tool to identify infants at high risk for UCP [13–15]. Involvement of specific regions, such as the corticospinal tracts or the basal ganglia/thalamus, have been associated with adverse motor development and UCP [14,16,17]. To improve the prediction of UCP at an early age, a combination of MRI with standardized clinical assessments is recommended.[5] A promising clinical tool for prognosis of UCP is the newly developed Hand Assessment for Infants (HAI). The HAI is the first standardized assessment quantifying hand function in terms of asymmetry and measuring both hands use in infants at high risk for UCP from 3-12 month of age.[18] This makes the HAI especially suitable for infants with unilateral or asymmetric perinatal brain injury, in contrast to more commonly used motor assessment tools, which do not measure asymmetric hand use.[19]

The aim of this study is to develop and internally validate a multivariable prediction model to estimate the prognostic risk of UCP in infants with APBI following the recommendation of combining neuroimaging with a clinical assessment for the prediction of UCP as early as possible.[5,20,21] It is hypothesised that neonatal brain imaging in combination with an early assessment of hand asymmetry using the HAI and additional infant characteristics can predict UCP during the first months of life in infants with APBI. If so, this method may have the potential to predict the risk of UCP and facilitate individualized treatment that focuses on the infant's specific needs and prognosis.

MATERIALS AND METHODS

Participants

This prognostic multivariable prediction modelling study included a convenience sample of 52 infants with evidence of APBI from the Karolinska University Hospital and Södersjukhuset in Stockholm (n = 33), Sweden and the Wilhelmina Children's Hospital of the University Medical Center in Utrecht (UMCU), the Netherlands (n = 19), within April 2008 and May 2016. APBI was diagnosed after MR investigation in the neonatal unit during the first month after term equivalent age (TEA). In Stockholm, all infants were recruited from the national stroke follow-up program based on neurological signs and MRI evidence of APBI, and referred to the occupational therapy department for HAI assessments. In contrast, in Utrecht, only infants with high risk of UCP based on MRI findings were included and followed by HAI assessments. Inclusion criteria were: (1) an MRI within one month of TEA and (2) early assessment of hand function between 3.5-4.5 months of (corrected) age using HAI. Exclusion criteria were major congenital malformation or surgery before the first symptom was apparent. No children have been included in any specific training program prior to the investigation, five infants received erythropoietin as part of a safety and feasibility trial (Table 1).[12] UCP was diagnosed based on a clinical assessment by an experienced child neurologist or rehabilitation specialist at ≥ 24 months in compliance with international European guidelines.[22]

Ethical approval

Ethical approval was granted from the Regional Ethics Committee Stockholm (2008/148-31), and was applied for, but not required by the Medical Ethical Committee Utrecht (WAG/th/14/038370) because HAI assessment and MR imaging are considered standard medical care for infants with APBI who were considered at high risk of UCP.

Magnetic resonance imaging of the brain (MRI) and evaluation of MRI data

The MRI assessment was performed as part of the clinical examination of APBI. In the UMCU, MRI was performed on either a 3 Tesla whole-body system (Philips Medical Systems, Best, the Netherlands), using a coronal or axial scanning protocol that consisted of at least a T1-weighed imaging (T1WI), T2WI and DWI. Infants at the UMCU were sedated for the MRI to avoid movement artefacts. MR imaging details for the Utrecht group have previously been described [23]. In the Karolinska University Hospital and Södersjukhuset, MRI was performed using a 1.5 Tesla MRI system with protocols including T1WI and T2WI. Infants were not sedated, instead positioned in bean bags after being fed breastmilk or formula.

All images were re-evaluated by two experts in the field of neonatal neurology (LdV, NW) through visual inspection of specific regions that are known to be predictive of adverse motor outcome: the corticospinal tract (CST), basal ganglia and thalamus (BGT)[14,24]. The assessors were unaware of the clinical diagnosis and functional outcome. Visual inspection of the DWI was done when the MRI was performed during the first week after symptom onset or of the T1- and T2-weighted images when the MRI was acquired later. Involvement of the CSTs was determined at the level of the posterior limb of the internal capsule (PLIC) and the cerebral peduncle as described previously [17,25]. Involvement of the BGT was noted when there was involvement of the basal ganglia and/or the thalamus.

Hand Assessment for Infants (HAI)

The HAI is a newly developed standardized observation-based assessment for infants 3-12 months of age at risk of developing UCP [18]. It assesses the degree and quality of goal-directed manual actions performed with each hand separately as well as both hands together.

In a semi-structured, video-recorded 10-15 min play session 12 unimanual and 5 bimanual items are tested and scored on a 3-point rating scale [18]. The sum score is Rasch-transformed into an interval level logit-based Both hands measure, BoHM (0-100 HAI-units) with higher scores indicating better performance. For unimanual items, each hand is scored separately resulting in the Each hand sum score, EaHS (0-24 points). Based on the EaHS an asymmetry index, AI, (0-100 percentage difference) is calculated.[18] The HAI showed excellent validity and reliability of scores for the evaluation of bilateral hand use in infants from 3-12 months of age at risk of UCP, and showed very good predictive validity for UCP in infants at risk [18].

HAI data was collected at 3.5-4.5 months of corrected age. The time of the initial assessment varied depending on different clinical routines. HAI assessments were video-recorded and subsequently scored by experienced assessors from Utrecht and Stockholm, who were unaware of the clinical diagnosis.

Statistical analysis

Descriptive summary measures were reported either as mean with standard deviation (SD) or median with interquartile ranges [IQR] depending on their distribution.

To investigate the predictive validity of the qualitative evaluation of involvement of the CST and BGT on neonatal MRI, predictive values, accuracy, and likelihood ratios were calculated from contingency tables.

Firth penalized regression was applied to construct a multivariable prediction model to estimate the prognostic risk of UCP at ≥ 24 months and at the same time consider the quasi-complete separation in the predictor CST involvement, i.e. all infants that did not show CST involvement on the MRI, did not develop UCP. As Firth penalized regression does not allow for variable selection, the model was based on all available and relevant clinical predictors (CST, BGT, HAI, gestational age, sex). A single model constructed from the sample data may be overly optimistic in predicting UCP. To limit overfitting, we applied tenfold cross-validation, i.e. dividing the sample data in ten subsets, where nine serve to construct the model and the tenth is used to evaluate its accuracy; this procedure is repeated for each subset and results in reduced model coefficients. Receiver operating characteristics (ROC) curve analysis was performed and the area under the curve (AUC) calculated to evaluate the model accuracy. Statistical analysis was performed in Stata IC 15.

RESULTS

Participants

Baseline characteristics of all 52 infants are summarized in Table 1. PAIS and PVHI were the most common forms of ABPI in our cohort with 26 and 11 infants (50% and 21%) affected respectively. Other diagnoses (n = 15) included parenchymal haemorrhage (n = 5), white matter injury (n = 3), watershed injury (n = 2), subdural haemorrhage (n = 2), antenatal PVHI leading to porencephalic cyst (n = 1) and thalamic haemorrhage (n = 1). At the age of 24 months 18 infants (35 %) had developed UCP. More preterm infants (61%, n = 11) than term infants (39%, n = 7) developed UCP ($p<0.01$), and more males than females (66% vs. 33%) developed UCP, but this difference did not reach statistical significance ($p>0.05$). The HAI was collected across the whole age range of 3.5-4.5 months with the majority of assessments at the lower age (Table 1). Median [IQR] scores of the HAI (contralesional EaHS, AI and BoHM) at 3.5-4.5 months of age were lower in infants who developed UCP compared to those who did not develop UCP (all $p<0.002$) (Table 2).

TABLE 1 | Descriptive data of participants.

	Total (n = 52)	Stockholm (n = 33)	Utrecht (n = 19)
Male	27 (52)	17 (52)	10 (53)
Gestational age at birth (in weeks)*	39.3 [33.5, 40.5]	40.3 [38.1, 40.5]	32.3 [26.1, 37.5]
Preterm (< 37 weeks of gestation)*	19 (37)	6 (18)	13 (68)
Diagnosis			
Perinatal arterial ischemic stroke (PAIS)	26 (50)	19 (58)	7 (37)
Periventricular haemorrhagic infarction (PVHI)*	11 (21)	2 (6)	9 (47)
Other	15 (29)	12 (36)	3 (16)
Laterality of lesion			
Left	25 (48)	13 (40)	12 (63)
Right	24 (46)	17 (51)	7 (37)
Asymmetric bilateral	3 (6)	3 (9)	0 (0)
Erythropoietin	5 (10)	0 (0)	5 (26)#
UCP diagnosis*	18 (35)	6 (18)	12 (63)
Corrected age at HAI assessment			
15-16 weeks	18 (35)	12 (37)	6 (32)
16-17 weeks	9 (17)	5 (15)	4 (21)
17-18 weeks	9 (17)	5 (15)	4 (21)
18-19 weeks	5 (10)	2 (6)	3 (16)
19-20 weeks	11 (21)	9 (27)	2 (10)
Postnatal age at MRI scan*	5.0 [3.0, 10.0]	5.0 [3.0, 6.8]	10.0 [5.0, 34.0]

Data presented as median [Interquartile Range] or number (percentage), where applicable. UCP – unilateral cerebral palsy, GA - gestational age *differences between Stockholm and Utrecht group ($p<0.05$); #Two infants who received EPO did develop UCP.

TABLE 2 | Descriptive data from neonatal MRI and HAI.

	Total (n = 52)	No UCP (n = 34)	UCP (n = 18)
No CST involvement*	24 (46)	24 (71)	0 (0)
CST involvement:	28 (54)	10 (29)	18 (100)
PLIC alone	12 (23)	4 (12)	8 (44)
PLIC and cerebral peduncles	16 (31)	6 (18)	10 (56)
No BGT involvement*	27 (52)	23 (68)	4 (22)
BGT involvement:	25 (48)	11 (32)	14 (78)
HAI contralesional EaHS*	15 [9 - 18]	17 [15 - 19]	7 [5 - 10]
HAI ipsilesional EaHS	17 [15 - 19]	18 [15 - 19]	17 [13 - 19]
HAI asymmetry index*	12 [5 - 47]	6 [0 - 15]	59 [46 - 71]
HAI BoHM*	51 [40 - 59]	57 [51 - 61]	38 [35 - 48]

Data presented as median [Interquartile Range] or number (percentage), where applicable.
* significant difference between infants with and without UCP, $p<0.01$.
UCP – unilateral cerebral palsy, GA - gestational age, CST - corticospinal tract, BGT - basal ganglia/thalamus, PLIC - posterior limb of internal capsule, HAI – Hand Assessment for Infants, EaHS – Each hand sum score, BoHM – Both hands measure

Predictive validity of CST and BGT involvement on neonatal MRI

All infants who developed UCP showed CST involvement, while infants who did not develop UCP predominantly showed no CST involvement (71%, n = 24) (Table 2). CST involvement on neonatal MRI had excellent sensitivity (100%), but only moderate specificity (71%) and likewise an excellent NPV (100%), but moderate PPV (64%) to predict the presence of UCP at ≥ 24 months of (corrected) age with an accuracy of 81% (Table 3). MRI performance increases the likelihood to identify UCP in infants that later developed UCP to a minor extent (LR+ 3.4). In infants who developed UCP, 78% (n = 14) showed BGT involvement, while in infants who did not develop UCP, 32% (n = 11) showed BGT involvement. BGT involvement showed somewhat lower values with 78% sensitivity and 68% specificity, 56% PPV and 85% NPV with 71% accuracy (Table 3).

TABLE 3 | Predictive value of MRI parameters for UCP.

	Sen (%)	Spec (%)	PPV (%)	NPV (%)	Acc (%)	LR +	LR -
CST involvement	100	71	64	100	81	3.4	0.0
BGT involvement	78	68	56	85	71	2.4	0.3

MRI - magnetic resonance imaging, UCP - unilateral cerebral palsy, CST - corticospinal tract, BGT - basal ganglia and/or thalamus, Sen - sensitivity, Spec – specificity, PPV – positive predictive value, NPV – negative predictive value, Acc – accuracy, LR +/- – positive / negative likelihood ratio.

Prognostic risk of developing UCP

The final model included all available predictors, including gestational age (in weeks), male sex, CST and BGT involvement observed from neonatal MRI, and the contralesional HAI EaHS between 3.5-4.5 months. ROC analysis for this model yielded an AUC of 0.980 (95% CI 0.953-1.00, Figure 1). The equation of the final model to estimate the prognostic risk of developing UCP between 3.5-4.5 months of (corrected) age is: *invlogit = 2.19 + 3.49*CST + 1.85* BGT – 0.51*contralesional HAI EaHS – 0.04 gestational age + 1.81*sex*.

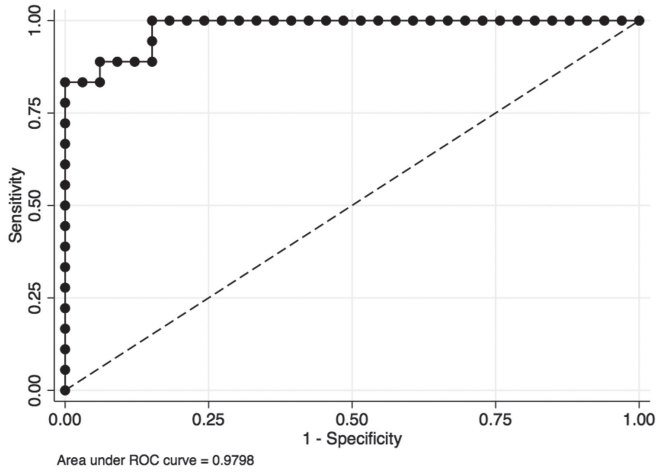

FIGURE 1 | Receiver operating characteristics (ROC) curve displaying sensitivity and 1-specificity of the final prediction model for UCP at 3.5-4.5 months. A nomogram based on the final model presented in the equation above serves to estimate the prognostic risk or probability of an individual infant (Figure 2). For further explanation of the nomogram, see also Appendix 1. The sensitivity and specificity at various thresholds of the prognostic risk of UCP at ≥24 months is displayed in Figure 3.

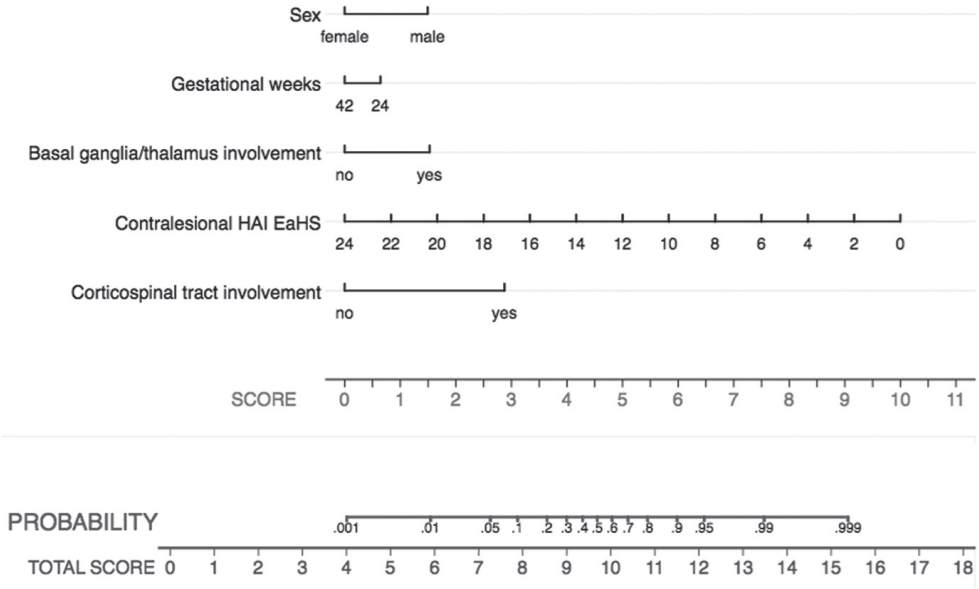

FIGURE 2 | Nomogram relating potential predictors to the prognostic risk score of UCP. Nomogram relating potential predictors (sex, gestational weeks at birth, basal ganglia/thalamus involvement on neonatal MRI, contralesional HAI Each hand sum score (EaHS) between 3.5-4.5 months (corrected) age, corticospinal tract involvement) to the prognostic risk score of UCP. For each predictor, read the points assigned on the 0–11 'Score' scale (green) and then sum these points. Find the sum score on the 0-18 'Total score' (blue) scale and then read the corresponding 'Probability' (prognostic risk, purple) of UCP above it. Application of the nomogram is explained in a practice example displayed in the appendix. UCP = unilateral cerebral palsy.

An alternative to the final model with all available data at one month of term equivalent age (thereby excluding HAI) yielded an AUC of 0.842 (95% CI 0.733-0.950). In contrast, another variant of the model excluding the MRI evaluation of CST and BGT still including HAI EaHS between 3.5-4.5 months, gestational age and sex resulted in a similar performance as the complete final model, but with wider confidence intervals (AUC of 0.930, 95% CI 0.859-1.00).

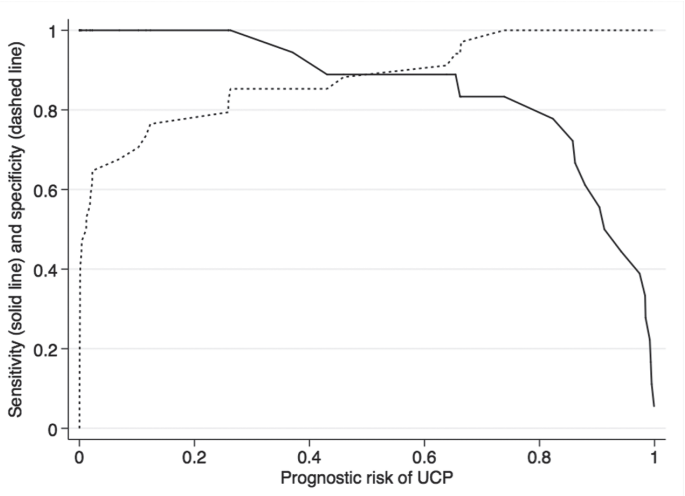

FIGURE 3 | Graph displaying the sensitivity and specificity of the prognostic risk (probability) of UCP.

DISCUSSION

A multivariable prediction model, that was developed and internally cross-validated, was able to calculate with high accuracy the risk of UCP already from 3.5 months of age based on CST and BGT involvement observed from neonatal MRI, a contralesional HAI EaHS between 3.5-4.5 months, and the infant's gestational age and sex. However, external validation is required before implementation of this model and its accompanying nomogram in clinical practice.

MRI is recognized as the most reliable neuroimaging method to predict CP and several distinct MRI parameters have been associated with CP in later life. For example, brain injury with involvement of the CSTs at the level of the PLIC and/or cerebral peduncles is associated with CP.[6,14,26] In our study, no UCP was seen in the absence of CST involvement (100% NPV). However, involvement of the CST resulted in 10 infants who did not develop UCP, and therefore a low specificity (71%) and PPV, leading to considerable overdiagnosis of UCP. These findings are similar to

other research reporting that absence of CST involvement resulted in typical motor development in 94% of infants, while CST involvement had lower sensitivity (67%) and associated PPV.[17] BGT involvement is another MRI parameter that has been associated with adverse motor outcome in neonatal brain injury[16,17,26], but was much less predictive in our cohort. This may be due to a heterogeneous group of infants, including a large number of preterm infants with PVHI in our cohort, for whom the predictive value of BGT involvement has not yet been established. Additionally, in our cohort CST and BGT involvement was perhaps low due to timing of the MRI. The MRI was not always performed in the acute phase after injury, when predictive ability of MRI (including DWI) is highest, but sometimes >7 days in term infants or around TEA in preterm infants. By dichotomizing the evaluation of brain structures (CST, BGT) additional information on the location and size of the lesion, which are also assumed to be predictive for the extent of UCP, could not be taken into account.

Although MRI is a good predictor, a prediction model for UCP based on neonatal MRI combined only with gestational age and sex, can indeed within one month of TEA predict UCP with good accuracy, but with less confidence. This seems helpful for very early preliminary risk estimation, as the MRI is performed much earlier than the assessment of hand function. Prediction performance increases further at 3.5-4.5 months in the same model using HAI instead of MRI. However, when information about CST and BGT involvement from neonatal MRI and asymmetric hand function measured by HAI are combined in a final model the prediction performance becomes excellent. Such a combination of imaging and standard assessment has also been recommended in recent guidelines[5]. It needs to be noted though that this prediction model is valid only for the predictors measured at specific time-points, i.e. MRI within one month of TEA and the contralesional HAI EaHS between 3.5-4.5 months of (corrected) age. An earlier model including HAI prior to 3.5 month of age shows insufficient predictive ability due to large variations of voluntary upper limb actions at this early age.

Asymmetric hand function has been described as one of the earliest clinical manifestations of UCP.[27] The HAI enables us to measure this asymmetric hand function in infants as early as three months of age.[18] Indeed, our results show that of all three HAI scales (contralesional EaHS, BoHM and the AI) were significantly different between infants who developed UCP and those who did not. The contralesional EaHS was the most predictive HAI scale for infants with risk for UCP when investigating the statistical model. Due to large variations of voluntary upper limb actions before 3.5 month of age, this study only included HAI scores from 3.5 months onwards. HAI values typically increase by age and asymmetric hand function may also become more obvious, thereby changing the predictive ability of the model at other ages. This will be elaborated upon in future studies of our own group (personal communication).

Recently, we have described three distinct developmental trajectories of hand function in infants with UCP over the first year of life (3-12 month). Although, the future severity level of UCP could not yet be detected at 3-4 months of age due to large variation and overlap between curves, at six months of age the trajectories were clearly delineated.[28] Additionally, normative values of different HAI scores are established and can be used to further compare the development of infants at risk of UCP and typical developing infants.[29] In future, HAI may not only serve as a predictive tool, but also as an outcome measure of early intervention. It has already been used in a study demonstrating improvement of manual ability after constraint induced movement therapy in infants younger than 12 months of age.[7] Overall, accumulating results of these studies will increase our knowledge about the development of upper limb function in children with UCP.

A limitation of this study is that the cohort consisted of infants with different diagnoses of APBI, and consequences of these types of injury on motor behaviour differ. Around 30-50 % of children who suffer from PAIS develop UCP.[16,17,30] Unilateral PVHI in preterm infants leads to UCP in 50-70% of children.[31–33] Other diagnoses, such as white matter injury, are less likely to lead to UCP, depending on site and extent of the injury. To account for these differences, we focused on MRI parameters that can be applied to various forms of brain injury such as involvement of the CST and BGT. Differences in recruitment might have caused selection bias, though combining of infants from two sites had the advantage to increase variation and to identify various prediction factors in order to build a more clinically relevant model. To address potential selection bias, the model was tested separately in both groups in a post hoc-analysis and showed very similar performance (AUC for Utrecht 0.97, 95% CI 0.920-1.00; AUC for Stockholm 0.96, 95% CI 0.901-1.00).

Erythropoietin is another factor that might be assumed to influence the outcome, but in this study treatment with erythropoietin did not add to the prediction model. It would also be of interest to investigate General Movements (GMs) assessment or Hammersmith Infant Neurological Examination (HINE) as they are currently the most predictive tools of CP.[5] Unfortunately, this data was inconsistently collected in our clinical cohort and could not be included in this study. It has to be noted that this is a convenience, hospital-based cohort including high risk infants. Infants without a neonatal event would most likely have been referred to other health care services at later age and are not the target group for this model. An important next step in this research is to externally validate the model in a similar population before implementation in clinical practice.

Clinical implication

In general, the diagnosis of UCP is based on clinical signs from neurological examination and medical history in accordance with national guidelines. This first explorative study strengthens the interpretation of the signs, showing that neonatal MRI gives good information about UCP development already at one month TEA, but prediction performance increases considerably with complementary information about hand asymmetry between 3.5-4.5 month of age measured by HAI.

A clinically relevant threshold for sensitivity and specificity of the prognostic risk of UCP depends on the context and the actions that follow. For early treatment, one wants to choose a lower threshold at higher sensitivity to not miss any infant that could benefit from early intervention that does not harm the infant. If on the other hand one would like to inform the parents, one would like to choose a higher threshold at higher specificity in order to minimize the number of false-positives and thus not unnecessarily worry parents.

As a next step, this model needs to be externally validated and possibly refined in a larger sample with similar participants in order to implement the nomogram into clinical practice, to enable clinicians to early inform families about their infant's risk to develop UCP, and refer those with a high probability to early intervention programs. The case in Appendix 1 may help to illustrate the further use of the nomogram in clinical practice.

CONCLUSIONS

A combination of a qualitative evaluation of the CST and BGT from neonatal MRI, a contralesional HAI Each hand sum score, gestational age and sex of the infant can already between 3.5-4.5 months of (corrected) age predict the prognostic risk of UCP in infants with APBI.

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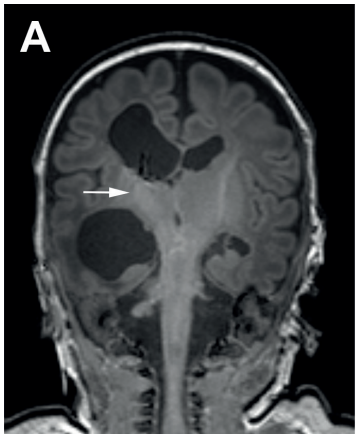
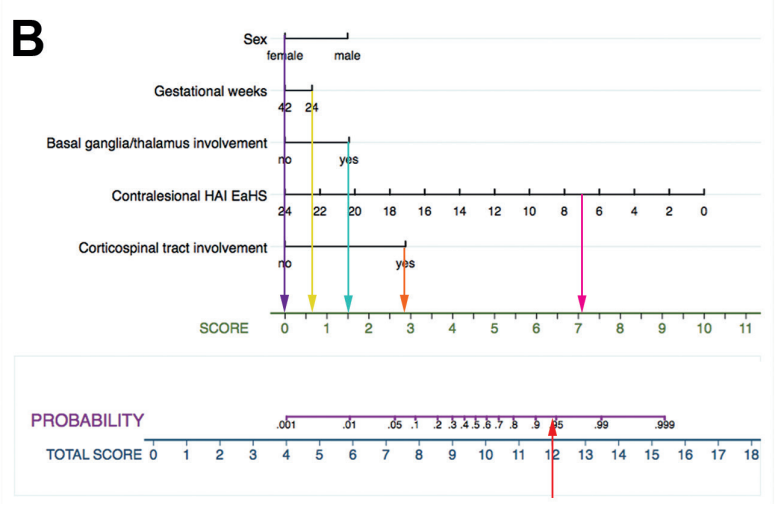
APPENDIX

Clinical Case explaining the use of the Nomogram

A baby **girl** was born at **25+3** weeks of gestation. She was born after an emergency caesarean section due to decelerations on cardiotocography. Her birth weight was 900 grams and her Apgar scores were 4/7/9 after 1/5/10 minutes. She had her MRI around term equivalent age that showed clear **asymmetry of the corticospinal tracts (CST)** at the level of the PLIC (A). The **basal ganglia and thalamus (BGT) were also affected**, as they showed clear asymmetry. She was discharged and returned for follow-up at 17 weeks of corrected age, when the HAI was performed. The **HAI Each hand sum score (EaHS)** of the left (**contralesional) hand was 7**.





Read from the nomogram (B) by drawing a vertical line (arrow) from each predictor scale to the 0-11 'Score' scale and read:

- For sex, 'Score' scale for being female (purple) is **0**
- For gestational age 'Score' scale for 25 gestational weeks (yellow) is **0.6**
- For basal ganglia involvement (yes/no) 'Score' scale for yes (turquoise) is **1.5**
- For HAI contralesional Each hand sum score (EaHS) 'Score' scale (pink) for the 7 EaHS is **7.1**
- For corticospinal tract involvement (yes/no) 'Score' scale for yes (orange) is **2.8**.
- Sum these scores**: 0** + **0.6 + 1.5 + 7.1 + 2.8 = 12.**
- Find the sum score of 12 points on the 0-18 'Total score' (bottom line).
- Read the assigned prognostic risk of developing UCP from the 'Probability' scale by drawing an orthogonal line (red arrow) from 12 'Total score' scale to the 'Probability' scale: **0.94**.

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CHAPTER 5

EARLY PREDICITON OF UNILATERAL CEREBRAL PALSY IN INFANTS AT RISK: MRI VERSUS THE HAND ASSESSMENT FOR INFANTS

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ABSTRACT

Objective: Neonates with unilateral perinatal brain injury (UPBI) are at risk for developing unilateral spastic cerebral palsy (USCP). This study compares several predictors for USCP later in life.

Methods: 21 preterm and 24 term born infants with UPBI were included, with an MRI scan including diffusion tensor imaging (DTI) performed at term equivalent age or around three months after birth, respectively. T2-weighted images and DTI-based tractography were used to measure the surface area, diameter and fractional anisotropy (FA) of both corticospinal tracts (CST). The Hand Assessment for Infants (HAI) was performed before 5, between 5-8 and between 8-12 months of (corrected) age. Asymmetry indices were derived from all techniques and related to USCP at ≥2 years of age.

Results: MRI measures and HAI scores were significantly lower for the affected compared to the unaffected side. Before five months of age, FA asymmetry on DTI yielded the highest area under the curve compared to conventional MRI and HAI.

Conclusion: Prediction of USCP after UPBI is reliable using asymmetry of the CST on MRI, as well as clinical hand assessment. Before five months of age, DTI-tractography provides strongest predictive information, while HAI specifically aids to prognosis of USCP at later age points.

INTRODUCTION

Unilateral perinatal brain injury (UPBI) is commonly diagnosed in newborn infants, but the type of injury varies with gestational age (GA). In term born neonates, perinatal arterial ischemic stroke (PAIS) is the most frequent form of UPBI, which is the result of focal disruption in arterial cerebral blood flow.[1,2] In preterm neonates, periventricular hemorrhagic infarction (PVHI) is the most common unilateral brain lesion, which results from impaired venous drainage of the vulnerable medullary veins in the germinal matrix.[3,4]

UPBI may lead to adverse neurodevelopmental outcome, including unilateral cerebral palsy (USCP). Several studies showed that 30- 50% of infants with PAIS and around 28-47% of preterm infants with PVHI developed USCP, which is mainly dependent on extent of the lesion and (secondary) tissue involvement.[5–8] Although no interventions specifically aim to treat UPBI and prevent the development of USCP, new therapies that target neuroprotection or –regeneration are on its way.[9,10] Other early-intervention programmes include constraint-induced movement therapy (CIMT), which is specifically developed for patients at risk for developing USCP.[11] These intervention strategies should be initiated as early as possible, when plasticity of the developing brain is highest. To start therapy as early as possible, early identification of those at risk for developing USCP is required.

MR imaging is a reliable technique for early diagnosis and prediction of motor outcome after UPBI, both in the acute phase as on follow-up.[4,6,12] On follow-up scans, degeneration of axons in the corticospinal tracts (CST), known as Wallerian degeneration, is associated with development of USCP.[5,6,13,14] However, most studies have used a qualitative evaluation of CST involvement on MRI, while objective quantitative measures of Wallerian degeneration are preferable to increase clinical use. Kirton et al. has demonstrated a quantitative measure of Wallerian degeneration at the level of the cerebral peduncle that corresponds to adverse motor outcome.[15] Diffusion tensor imaging (DTI) can be used to reconstruct white matter fiber tracts, such as the CST, in vivo and allow for quantative measurement of diffusion parameters in these tracts.[16] DTI tractography offers more detailed information on brain connectivity compared to conventional MRI, is able to provide a quatitative measure of white matter integrity and can therefore aid in early prognosis of USCP in infants with UPBI.[16–19]

In the literature, a combination of neuroimaging and standardized clinical assessments is recommended for most reliable prediction of USCP at an early age.[20] A relatively new instrument that may contribute to diagnosing motor impairment in young children is the hand assessment for infants (HAI).[21] The HAI scores the function of both hands separately in play-related tasks and provides a scale of asymmetry

between hands. The HAI is a non-invasive clinical test that can be performed at multiple timepoints from three to twelve months of age.[21,22] This makes HAI suitable to quantify the development of asymmetric hand function early in life, although its predictive ability compared to early neuroimaging is unknown.

In this study, we aim to investigate the predictive value of several quantitative asymmetry indices based on conventional MRI, DTI and HAI in infants with UPBI to predict USCP later in life. As many studies have described the predictive value of one measure to predict USCP, this study specifically aims at comparing several quantitative measures in a large group of infants specifically at risk for unilateral CP, additionally studying whether these prediction measures differ between term and preterm infants with UPBI.

METHODS

Patients

For this study we selected all infants with UPBI on their neonatal MRI, who were born between August 2011 and May 2016 and underwent HAI assessment in the Wilhelmina Children's Hospital in Utrecht, the Netherlands, at some point during their first year of life. Parents signed informed consent for the use of their child's data for research purposes. A waiver of authorization to conduct this study was approved by the Medical Ethical Committee Utrecht because HAI assessment and MR imaging (including DTI) are considered standard medical care for infants with UPBI who were considered at high risk of UCP.(WAG/th/14/038370)

Clinical and obstetric characteristics were obtained from the infants' charts (Table 1). Prematurity was defined as infants born before 37 weeks of gestation. Infants had their early MRI when they were admitted to the neonatal intensive care unit at the Wilhelmina Children's Hospital (Utrecht, The Netherlands) and as soon as they were stable enough for transportation to the MRI scanner. A follow-up MRI was obtained several weeks later, which corresponded to term equivalent age (TEA) in preterm infants and three months postnatal age in term infants. Based on previous reseach in term and preterm neonates, the follow-up scan was chosen as optimal timepoint for MRI measurements and DTI tractography.[17,23,24] MRI measurements used in this study therefore refer to the follow-up MRI scan.

Imaging methods

MRI was performed on either a 1.5T or 3T Philips Achieva MR system (Philips Medical Systems, Best, Netherlands) equipped with a 8-channel SENSE head coil and included both conventional MR imaging and DTI sequences. The conventional axial or coronal

MR protocol consisted of at least a T1-weighted imaging (T1WI), T2WI and diffusion weighted imaging (DWI), of which the scanning protocols details have previously been reported.[25,26]

Single-shot echo planar DTI was acquired in either 32 or 45 noncollinear directions with one of the following scanning protocols; 1.5T, 32 directions: repetition time (TR): 6817ms, echo time (TE) = 87 ms, voxel size = 1.98 × 1.98 × 2 mm^3, b value 800 s/mm^2, SENSE factor of 2.5. On 3T, 32 directions: TR: 5685 ms, TE = 70 ms, voxel size = 1.41 × 1.41 × 2 mm^3, b-value 800 s/mm^2, SENSE factor of 2. 3T, 45 directions: TR: 6.500 ms, TE = 80 ms, voxel size = 2 × 2 × 2 mm^3, b value 800 s/mm^2, SENSE factor of 1.4.

Intensive care was continued throughout the examination with the attendance of a neonatologist or physician assistant, and the heart rate and transcutaneous oxygen saturation were monitored by pulse oximetry in all infants (Nonin, Minneapolis, MN) as well as respiration rate (Philips ACS-NT, Best, The Netherlands) A vacuum pillow (Med-Tec, Orange City, IA) was used to prevent head movement. Minimuffs (Natus Medical, San Carlos, CA) were used for hearing protection. Preterm infants at TEA were sedated using oral chloralhydrate 50 to 60 mg/kg, according to clinical protocol. At three months after birth, the term infants were sedated throughout the examination with an i.m. injection of 0.1 ml/kg of a combination of pethidine (2 mg/kg body weight), chlorpromazine (0.5 mg/kg body weight) and promethazine (0.5 mg/kg).

Conventional MRI analysis

Using T2-weighted images, brain stem sections were analyzed using a previously described technique [15,27]. When T2WI were unavailable, T1WI or inverse recovery T1WI images were used. Asymmetry of the cerebral peduncle diameter and surface area was used as a proxy of CST asymmetry. A first line was drawn paralel to the fronto-medial part of each peduncle, towards the midbrain (figure 1A, line AB). A second line (figure 1A, line BC) was drawn perpendicular to this line, crossing the peduncle. The area frontolateral to this line was considered to be the 2D-surface area of the cerebral peduncle (figure 1B) Finally, a third line was drawn, parallel to line BC at the broadest part of the peduncle (line D), reflecting the width of the peduncle (figure 1A). Interrater reliability was determined between two researchers by repeating volume and diameter measurements of both cerebral peduncles in 15 infants.

DTI analysis

DTI data were processed with ExploreDTI.[28] The diffusion-weighted images were realigned to the b0 image to correct for subject motion and eddy current–induced geometric distortions. In this process, the diffusion tensor was fitted for each voxel after adjusting the diffusion gradients for the b-matrix rotation.[29] Furthermore, the DTI data were registered to a single-subject neonatal DTI template (freely available

http://cmrm.med.jhmi.edu), using a rigid registration method, to correct for any angulation-asymmetries. A quality check was performed using the outlier profile. Diffusion weighted images with >0.5% outliers were discarded; and if the number of discarded diffusion weighted images exceeded 10% of the total number of images (32 or 45), the whole DTI scan was excluded from further analyses. Whole brain tractography was performed, using a 1 mm step size.[30] Propagation of the fibers was stopped if a voxel with a FA value <0.1 was entered or if the angle of a fiber between two consecutive steps exceeded 40 degrees.

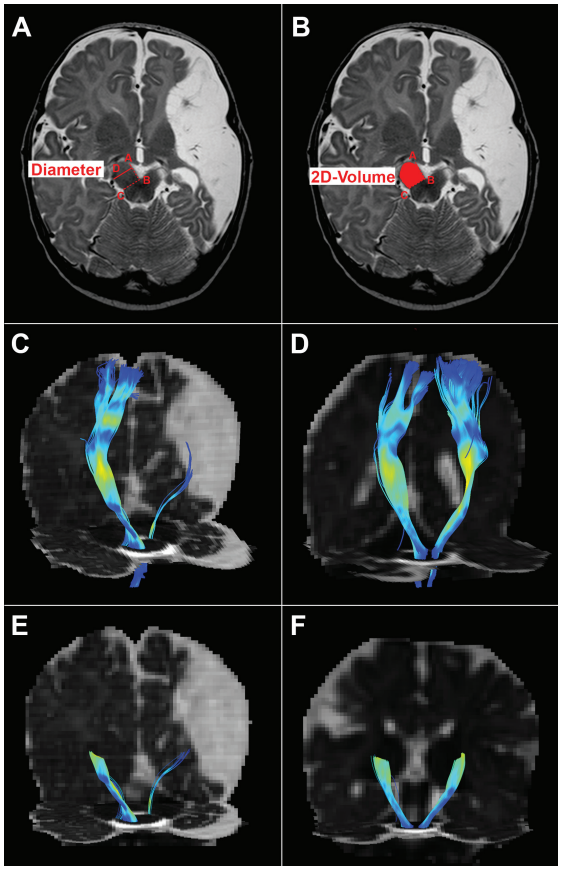

FIGURE 1 | Example of MRI measurements. Cerebral peduncle measurements were performed on axial T2-weighted images (1A-B). First, a line was drawn paralel to the medio frontal part of each peduncle, towards the midbrain (figure 1A, line AB). A second line (figure 1A, line BC) was drawn perpendicular to this line, crossing the peduncle. The area frontolateral to this line was considered to be the 2D-surface area of the cerebral peduncle (figure 1B) Finally, a third line was drawn, parallel to the second at the broadest part of the penduncle, reflecting the width of the peduncle (line D, figure 1A). Tractography in CSTs of an infant who developed USCP (1C) and an infant with normal outcome (1D) using diffusion tensor imaging (DTI). After tractography of the full CST, segments between the PLIC and cerebral peduncle were selected for FA measurements. Clear FA asymmetry is shown in the infant who developed USCP (1E) compared to a typical developing infant (1F).

Following the whole brain tractography, the CST was identified using two regions of interest (ROI) as described before.[17] The first ROIs was drawn around the frontal part of the middle cerebral peduncles at the level of crossing pontine fibres where the anterior and posterior parts of the peduncles were completely seperated on the colourmap. The second ROI was drawn around the posterior limb of the internal capsule (PLIC) two to three slices below the corpus callosum. In case tissue loss hindered specific drawing of any of the ROIs based on visible landmarks, a ROI was drawn based on the location and size of the corresponding ROI in the unaffected hemisphere. Fibers passing the two ROIs were identified as the CST. Any fibers continuing into the other hemisphere, the cerebellum and the medial lemniscus or single aberrant fibers that were considered not to be part of the CST were removed. The segment between the two ROIs was used for further analysis. The FA was calculated for each CST segment separately and used to calculate an asymmetry index (AI). If no FA could be calculated from the affected CST, this resulted in an AI of 100%. Interrater reliability was determined between two researchers by repeating tractography, segment selection and subsequent FA measurements in 20 CSTs of 10 infants.

Assessment of early motor development

Motor development was assessed using the HAI <5 months, between 5-8 and between 8-12 months of corrected age. The HAI assessment was performed by a pediatric occupational therapist with expertise in HAI evaluation and who was unaware of the MRI results. The HAI consists of a 15-minute play-related session that stimulates unilateral and bilateral upper limb movement.[22] The assessment was video recorded and scored afterwards. The HAI scores 12 items for each individual hand (Contralesional/Ipsilesional each Hand Sum Score, score 0-24 for each hand), and 5 bimanual items creating a total score for both hands combined (Both hand measure, score 0-100) and an AI. For the study, the AI was re-calculated using the each Hand Sum scores for the unaffected and affected hand.

Development of USCP

After discharge from the NICU, children were seen at regular time intervals in the neonatal follow-up clinic. USCP development was assessed at the age of two years or above by a pediatric neurologist (LdV) who was unaware of the DTI and HAI results using criteria from a report from 2007.[31] Severity of USCP was classified using the Gross Motor Function Classification System (GMFCS).

Statistics

For all conventional MRI, DTI and HAI measures, an asymmetry index (AI) was calculated as: asymmetry index = ((measure unaffected [MU] - measure affected [MA])/(MU+ MA))*100%, where affected refers to the affected hemisphere and contralesional hand. IBM SPSS Statistics® v25 (IBM Corp., Armonk, NY,) was used for statistical analysis. Patient characteristics were summarized as counts with percentages for categorical variables, means ± standard deviation (SD) for parametric data, and as median ± interquartile ranges (IQR) for nonparametric data. To test for differences between groups, chi-square test, one sample T-tests, paired sample T-test, independent T-tests, one-way ANOVA or the nonparametric variant were used. Receiver operater characteristic (ROC) curves were created using Prism GraphPad Software (version 7.04 for Windows, GraphPad Sofware, Inc.) to determine sensitivity and specificity at various AI thresholds. Each threshold value is midway between two values. Optimal cutoff values were determined by calculating the maximal Youden's Index using Youden's J = Sensitivity + Specifity – 1. When cutoffs resulted in similar Youden's Index, the cutoff yielding in highest sensitivity was chosen since these predictors are part of a screening test. AUC from ROC curves were compared using a calculation as described by Hanley et al.[32]

RESULTS

After admission to the department of Neonatoly in the Wilhelmina Children's Hospital in Utrecht, 48 infants with UPBI were considered at risk of developing USCP based on MRI parameters such as involvement of the corticospinal tract, and therefore referred for HAI assessment by the attending physician. Of these, 45 children had HAI data available and were included in this study.(Table 1) Of those, 24 were born at term (mean GA 39.5±1.5 weeks) and 21 were born preterm (mean GA 28.7±3.7 weeks). Infants were diagnosed with PVHI (n=18), PAIS (n=18) or other diagnoses including thalamic hemorrhage (n=4), antenatal PVHI with porencephalic cyst (n=3), parenchymal hemorrhage based on COL4A1 mutation (n=1) and herpes encephalitis with diffuse unilateral lesions (n=1). PHVI was mainly observed in preterm infants (n=16), while PAIS was more common in term born infants (n=15).

After a follow-up of at least two years, 27 children (60.0%) were diagnosed with USCP. USCP was diagnosed in 12 term (50%) and 15 (71.4%) preterm born infants. GMFCS could be determined in 23 USCP patients (85%) and was most often grade I (67%).

TABLE 1 | Patients characteristics.

	Total (n=45)	Term (n=24)	Preterm (n=21)
Male	27 (60.0%)	14 (58.3%)	13 (61.9%)
Gestational age (weeks)*	34.5 (±6.1)	39.5 (±1.5)	28.7 (±3.7)
Birth weight*	2308 (±1153)	3191 (±555)	1299 (±753)
Diagnosis:*			
PAIS	18 (40.0%)	15 (62.5%)	3 (14.3%)
PVHI	18 (40.0%)	2 (8.3%)	16 (76.2%)
Other	9 (20.0%)	7 (29.2%)	2 (9.5%)
(Sub)clinical seizures*	22 (48.9%)	18 (75.0%)	4 (19.0%)
Follow-up MRI available	40 (88.8%)	20 (83.3%)	20 (95.2%)
Age at follow-up MRI			
Postmenstrual age (weeks)	47.9 (±5.8)	53.0 (±1.6)	42.9 (±3.7)
Postnatal age (days)	97.1 (±19.3)	93.4 (±14.2)	100.8 (±23.0)
Good quality follow-up DTI	37 (82.2%)	19 (79.2%)	18 (85.7%)
Corrected age at HAI (weeks):			
1: <5 months (n=44)	14.9 (±2.2)	14.5 (±2.5)	15.3 (±1.8)
2: 5-8 months (n=32)	28.1 (±2.9)	27.0 (±2.3)	29.5 (±3.1)
3: >8 months (n=32)	42.1 (±5.1)	42.4 (±6.0)	41.9 (±4.1)
USCP	27 (60.0%)	12 (50.0%)	15 (71.4%)
GMFCS Grade I	18 (66.7%)	8 (66.7%)	10 (66.7%)
GMFCS Grade II	4 (14.8%)	2 (16.6%)	2 (13.3%)
GMFCS Grade III	1 (3.7%)	0 (0%)	1 (6.7%)

Data are given as a number (percentage) or mean (±standard deviation). PAIS, perinatal arterial ischemic stroke; PVHI, periventricular hemorrhagic infarction; MRI, magnetic resonance imaging; DTI, diffusion tensor imaging; HAI, hand assessment for infants; USCP, unilateral spastic cerebral palsy; GMFCS, gross motor function classification system. *significant difference between term and preterm infants, $p<0.05$.

MR imaging

An MRI scan was acquired in 22 preterm infants (100%) and 20 term born infants (83.3%) at a mean age of 98.5 (±22) and 93.4 (±14.2) days, respectively. This corresponded to a corrected age of 42.9 (±3.7) weeks for preterm and 53.0 (±1.6) weeks for term infants ($p<0.0001$). A DTI scan of sufficient quality was available in 38 infants (82.6%), as DTI images were not obtained (n=2) or didn't reach quality requirements (n=2).

Conventional MRI asymmetry indices

Median diameter and volume of the affected (ipsilesional) cerebral peduncle were significantly lower compared to the non-affected cerebral peduncle (Table 2). Also,

within subgroups of term and preterm infants, most differences between affected and non-affected cerebral peduncle persisted.(Table 2) Median AI of peduncle diameter was higher in infants who developed USCP while peduncle volume measurements did not differ between infants with and without USCP.(Table 3). There were no differences in cerebral peduncle AI between term and preterm infants (p>0.05). Cerebral peduncle volumes did not significantly differ between researchers in 15 subjects (mean difference -2.1±11.6, p>0.1), and their AI was also similar (mean difference -0.8±5.9, p>0.1). Cerebral peduncle diameter measurements differed between researchers in 15 subjects (mean difference -0.5±0.7 mm, p<0.01), but AI between researchers was similar (mean difference 0.7±3.5 mm, p>0.1).

TABLE 2 | Measures of MRI, DTI and HAI in the affected and non-affected side.

	Affected			Non-affected		
	Total	**Term**	**Preterm**	**Total**	**Term**	**Preterm**
Peduncle surface in mm² (n=40)	76.1 (62.6 – 98.4)*	87.2 (69.3 – 116.3)$a	68.0 (57.0 – 78.9)a	89.6 (66.4 – 109.8)*	106.4 (94.4 – 117.2)$a	69.6 (63.2 – 84.1)a
Peduncle diameter in mm (n=40)	9.6 (8.6 – 10.5)*	9.9 (9.0 – 10.7)$	9.3 (8.5 – 10.2)#	10.3 (9.5 – 11.3)*	10.9 (10.2 – 11.9)$a	9.8 (9.0 – 10.5)#a
FA CST (n = 37)	0.31 (0.26 – 0.40)*	0.38 (0.28 – 0.42)$	0.30 (0.22 – 0.33)#	0.41 (0.35 – 0.44)*	0.44 (0.42 – 0.45)$a	0.36 (0.33 – 0.39)#a
HAI <5 months (n=44)	9.0 (6.0 – 12.8)*	10.0 (5.0 – 12.8)$	9.0 (7.0 – 15.0)#	14.0 (11.0 – 17.0)*	12.0 (11.0 – 15.8)$a	16.5 (12.5 – 18.5)#a
HAI 5-8 months (n=32)	13.5 (6.5 – 17.8)*	14.5 (5.0 – 20.3)$	12.0 (8.8 – 15.0)#	22.0 (21.3 – 23.0)*	22.0 (21.8 – 23.0)$	22.0 (20.8 – 23.0)#
HAI >8 months (n=32)	16.0 (5.5 – 21.8)*	15.0 (3.0 – 23.0)$	16.0 (12.0 – 20.0)#	24.0 (23.0 – 24.0)*	24.0 (22.3 – 24.0)$	23.5 (23.0 – 24.0)#

Measurements are presented as median (interquartile range). * Difference between affected/non-affected within total group; $difference between affected/non-affected within term infants; #difference between affected/non-affected within preterm infants; adifference between term and preterm infants. FA, fractional anisotropy; CST, corticospinal tract; HAI, hand assessment for infants.

DTI asymmetry indices

FA values of the CST were also significantly lower ipsi- versus contralesional in the total cohort, but also when analyzing term and preterm infants separately.(Table 2) FA measurements differed between term and preterm infants in the non-affected CST only. Median AI of FA values were higher in infants who developed USCP compared to those with normal motor development (Table 3). There were no differences in FA AI between term and preterm infants (p>0.05). FA values did not significantly differ between researchers in 20 measurements (mean difference 0.02±0.06, p>0.1),

and AI was also similar between researchers in these 10 subjects (mean difference -8.0±27.8, p>0.01).

HAI asymmetry indices

HAI was performed at a corrected age of 14.9 (±2.2), 28.1 (±2.9) and/or 42.1 (±5.1) weeks.(Table 1) HAI scores at all timepoints were significantly lower for the affected (contralesional) hand compared to the non-affected (ipsilesional) hand in the total cohort, but also when analyzing term and preterm infants separately.(Table 2) The ipsilesional each Hand Sum Score <5 months of age differed between term and preterm infants, while other HAI scores did not significantly differ. AI of HAI scores at all timepoints were higher in infants who developed USCP compared to those who did not.(Table 3) There were no differences in HAI asymmetry indices between term and preterm infants (p>0.05 at all timepoints).

TABLE 3 | Asymmetry indices.

Asymmetry Index (%)	Total (n=45)	No USCP (n=18)	USCP (n=27)
Peduncle surface (n=40)	3.0 (-2.9 – 14.4)	1.1 (-3.9 – 7.1)	6.7 (1.1 – 15.0)
Peduncle diameter (n=40) $	3.0 (0.9 – 5.6)	1.4 (-0.1 – 3.4)	3.9 (2.2 – 11.3)
FA CST (n = 37)*	8.2 (1.5 – 22.5)	-0.2 (-0.8 – 3.5)	16.6 (8.0 – 34.4)
HAI <5 months (n=44)*	9.7 (0.0 – 42.6)	0.0 (0.0 – 5.9)	38.8 (7.6 – 56.7)
HAI 5-8 months (n=32)*	23.3 (5.4 – 54.1)	2.4 (0.0 – 5.4)	34.3 (21.9 – 65.8)
HAI >8 months (n=32)*	18.0 (2.1 – 53.7)	0.0 (0.0 – 2.1)	31.4 (18.0 – 77.4)

Median (IQR) of asymmetry indices (AI) per predictor for total cohort, and subgroups of infants who developed USCP and those who did not. Differences between subgroups are marked with * p<0.0001 or $p<0.05. USCP, unilateral spastic cerebral palsy; FA, fractional anisotropy; CST, corticospinal tract; HAI, hand assessment for infants.

ROC analyses USCP

ROC analyses were performed for all asymmetry indices in relation to outcome in the total cohort, and for term and preterm infants separately. Optimal cut-off values per AI and their corresponding prediction measures, including sensitivity, specificity, positive and negative predictive values (PPV and NPV) are presented in table 4 and Figure 2.

Comparing asymmetry indices

To compare the different predictors for USCP, area under the curve (AUC) of ROC curves were analyzed. Overall, the AUC of the HAI >8 months of age was highest being 1 and cerebral peduncle measurements yielded lowest AUCs (Table 3). However, as predicting USCP as early as possible is clinically most relevant, we

focused on comparing prediction measures at the earliest time period (<5 months of age). At this age, the FA AI on DTI yielded the highest AUC when compared to both conventional MRI indices ($p<0.05$) and HAI in the first 5 months ($p>0.05$). When analyzing the preterm infant subgroup, only the FA AI was found to be a significant predictor, where the peduncle surface and diameter and the early HAI were not significant predictors. For term infants, peduncle diameter measurements yielded the highest AUC before 5 months of age, although conventional MRI, DTI and HAI asymmetry measurements yielded comparable AUC ($p>0.05$) (Table 4).

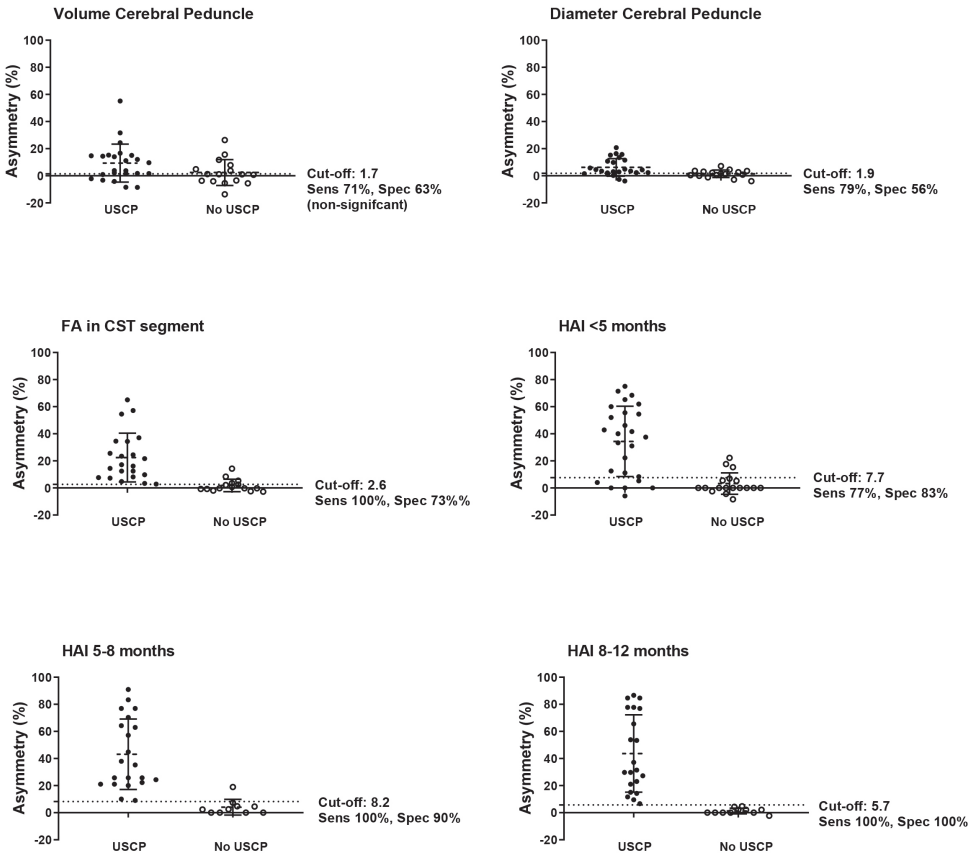

FIGURE 2 | Graphic representation of asymmetry indices for total cohort. Per asymmetry index, optimal cut-offs to predict unilateral spastic cerebral palsy were calculated using Youden's index and sensitivity (sens) and specificity (spec) were calculated. FA, fractional anisotropy; CST, corticospinal tract; HAI, hand assessment for infants; USCP, unilateral spastic cerebral palsy.

TABLE 4 | Predictive performance of asymmetry indices in the prediction of unilateral spastic cerebral palsy.

Asymmetry Index	Total cohort (n=45)	Term (n=24)	Preterm (n=21)
Peduncle surface (n=40)	AUC 0.67* Cut-off 1.7% Sens 71% Spec 63% PPV 74% NPV 59%	AUC 0.79 Cut-off 10.9% Sens 70% Spec 90% PPV 88% NPV 75%	AUC 0.61* Cut-off 1.5% Sens 64% Spec 67% PPV 82% NPV 44%
Peduncle diameter (n=40)	AUC 0.74 Cut-off 1.9% Sens 79% Spec 56% PPV 73% NPV 64%	AUC 0.97 Cut-off 4.7% Sens 90% Spec 100% PPV 100% NPV 91%	AUC 0.51* Cut-off 1.9% Sens 64% Spec 50% PPV 75% NPV 38%
FA CST (n = 37)	AUC 0.94 Cut-off 2.6% Sens 100% Spec 73% PPV 85% NPV 100%	AUC 0.92 Cut-off 2.6% Sens 100% Spec 70% PPV 75% NPV 100%	AUC 0.99 Cut-off 6.3% Sens 92% Spec 100% PPV 100% NPV 83%
HAI <5 months (n=44)	AUC 0.85 Cut-off 7.7% Sens 77% Spec 83% PPV 87% NPV 71%	AUC 0.93 Cut-off 2.0% Sens 92% Spec 83% PPV 85% NPV 91%	AUC 0.74* Cut-off 19.9% Sens 64% Spec 100% PPV 100% NPV 55%
HAI 5-8 months (n=31)	AUC 0.99 Cut-off 8.2% Sens 100% Spec 90% PPV 95% NPV 100%	AUC 0.97 Cut-off 6.9% Sens 100% Spec 86% PPV 92% NPV 100%	AUC 1.00 Cut-off 14.2% Sens 100% Spec 100% PPV 100% NPV 100%
HAI >8 months (n=32)	AUC 1.00 Cut-off 5.7% Sens 100% Spec 100% PPV 100% NPV 100%	AUC 1.00 Cut-off 8.0% Sens 100% Spec 100% PPV 100% NPV 100%	AUC 1.00 Cut-off 5.7% Sens 100% Spec 100% PPV 100% NPV 100%

Per index an area under the curve (AUC) is presented, where * presents non-significant AUC. Optimal cut-offs were calculated using Youden's index and using this cut-off, sensitiviy (sens), specificity (spec), positive predictive value (PPV) and negative predictive value (NPV) were calculated. FA, fractional anisotropy; CST, corticospinal tract; HAI, hand assessment for infants.

DISCUSSION

This study cohort consisted of infants with UPBI who were at high risk of developing motor disabilities in later life. Early risk evaluation is important to adequately counsel families and caregivers, and select those infants that might benefit from early intervention strategies to attenuate adverse outcome. These interventions focus on rehabilitation strategies to stimulate activity-dependent plasticity of the developing brain, that have proven effective when initiated within the first 3-8 months of life.[11] This study aimed to describe the predictive ability of several quantitative asymmetry measures in order to provide simple cut-offs for the selection of high-risk infants.

UPBI leads to USCP by either primary injury of motor areas, or secondary injury of axons of the CST, often referred to as Wallerian degeneration.[13,14] MRI has proven especially useful in evaluating CST involvement, which is highly associated with the development of USCP.[12] However, as visual qualification of CST involvement requires knowledge and experience, quantifying Wallerian degeneration seems preferable.[15] Using a method described by Kirton et al., we indeed found that asymmetry of the descending CST a the level of the cerebral peduncle on conventional MR imaging corresponded to poor motor outcome.[15,27] However, in our study only diameter, and not volume, of the cerebral peduncle reached significant predictive ability for USCP. This may be explained by the fact that our cohort consisted of preterm infants, in whom Wallerian degeneration is less well described.[33] Additionally, we used the complete frontal parts of the cerebral peduncle as a proxy of CST asymmetry, but this structure also consists of other tracts (e.g. frontopontine fibres, corticonucleur fibres, etc) that are not necessarily affected by UPBI.

As findings from us and others suggest, visual inspection of CST asymmetry on conventional MRI is unable to correctly predict USCP in all infants.[24] Kirton et al. therefore suggested the use of 'function-specific' imaging with DTI tractography to improve prediction, since it is able to specifically assess motor tracts.[15] As DTI quantifies detailed white matter connectivity, it may also be more sensitive to subtle changes that increase the risk of adverse motor outcome, but are not visible on conventional imaging. We confirmed that before five months of age, DTI yielded the highest predictive ability with excellent prediction (AUC 0.94) with 100% NPV, which was comparable to our previous studies.[17,18] These are optimal conditions for a screening instrument because it means that after selecting high-risk infants with DTI, no infants with USCP will be missed for early intervention.

As the follow-up scan is often performed when clinical parameters are also available, we compared both measures for this study. The combination of neuro-imaging and clinical assessment is also recommended as the golden standard for CP prediction.[20]

Before five months of age, clinical motor assessment is recommended with use of the General Movements (GM) and after five months of age with the Hammersmith Infants Neurological Examination (HINE).[20,34] However, these tests are not designed for the specific diagnosis of hemiplegia, which is the result of UPBI. Diagnosing hemiplegia focuses on evaluation of movement asymmetry, which is often the first clinical sign of USCP in young infants. Cioni et al. found reduced segmental movements during fidgety movement period (9-16 weeks post term) to be more predictive for development of hemiplegia than global abnormalities in GM.[34–36] HAI also aims to assess these segmental distal movements of the upper limbs by scoring the use and quality of both hands separately, however during goal-directed movements, and providing a clinical measure of asymmetry. We found a specificity to predict USCP of HAI asymmetry <5 months of age of 77%, lower than the specificity of HINE and GMs at this age (ranging between 95%-98%).[20,34] This can potentially be explained because goal-directed intentional movements are still emerging before five months age, and can therefore be non-asymmetrical due to their bilateral immaturity. As recently published normative reference values showed that the majority of infants acquire all skills of HAI only after six months of age, predictive value of HAI may increase after this age.[37] Indeed, our study shows that after eight months of age, HAI is able to correctly detect all infants who will develop USCP, comparable to other studies. This demonstrates that HAI may have a potential role to diagnose USCP before the first year of age, although other studies are needed to validate this. A recent study from Hay et al. also described that addition of an asymmetry score to the HINE also helps to distinguish infants with hemiplegia from controls after 5 months of age.[38] The comparison of HAI and these HINE asymmetry scores also needs to be studied further.

This study described differences in predictive ability for MRI asymmetry indices between term and preterm infants. This could be explained by timing of the scan: although postnatal age at the time of MRI did not differ, the postmenstrual age at time of the MRI was approximately 10 weeks shorter for preterm compared to term infants. Consequently, preterm infants had less-developed brains with less myelin, resulting in lower FA values and smaller peduncle measures compared to term infants. Due to these differences, it may be more difficult to detect asymmetry in preterm infants by conventional imaging, while DTI is more sensitive to minor asymmetry changes in white matter connectivity.

We also found differences between term and preterm infants in the predictive ability of HAI. Hand function asymmetry was highly predictive before five months of corrected age in term infants, while it was not in preterm infants. This could potentially be a result of differences in lesion type, which is closely linked to prematurity. PVHI was the most prevalent type of UPBI in preterm infants, while it was PAIS for term

infants. It has been described that periventricular lesions, such as PVHI, usually result in milder USCP than lesions in the cortex, subcortical area and basal ganglia, as seen in PAIS.[39] Additionally, hand function after early brain injury is influenced by reorganization of the CST, which is affected by both lesion type and age of onset of the lesion.[40] Future studies are necessary to investigate the distinctive effects of UPBI type and premature birth on asymmetric hand function.

There are some limitations to this study. The HAI is a relatively new instrument that requires a trained assessor to perform the assessment (10 minutes testing + 30 minutes assessment) during clinical follow-up. Therefore, only infants at high-risk of developing USCP based on MRI findings were followed-up by HAI in our hospital, and eligible for this study. The results of this study should therefore be validated in a population at lower risk before extrapolation to the general population. Secondly, extensive MRI post-processing techniques such as DTI tractography assumes strict scanning protocols without movement artifacts, leading to exclusion of several cases. Furthermore, this study focused on the use of follow-up MRI instead of early MRI within days after birth, limiting its role for selecting candidates for early neuroprotective therapies. Previous studies have shown that asymmetry in FA of the CST is already predictive of motor outcome after UPBI when DTI is obtained within 4 weeks after birth.[17,24,27] However, as DTI is prone to movement-artifacts, it is more difficult to perform in preterm infants before term-equivalent age.[41,42] Additionally, DTI is especially sensitive to injury, and tractography is often unreliable in the presence of acute edema/injury. Furthermore, secondary injury to the descending CST takes time to develop.[43] This resulted in the use of follow-up MRI including DTI for this study, while conventional MRI tools may potentially be more useful when obtained earlier in life

CONCLUSION

Prediction of USCP in children with UPBI can be done by asymmetry of the CST on conventional MRI and DTI, as well as clinical hand assessment. This study revealved that before five months of age, DTI-tractography provides strongest predictive information in both preterm and term born infants, while HAI specifically aids to prognosis of USCP at later time points. Therefore, HAI might be of additional value in the infants of whom follow-up MRI and/or DTI is not performed or its result is uncertain. Combining several quantitative asymmetry indices could potentially select all infants with UPBI who are at high risk for developing USCP that could benefit from intervention strategies.

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CHAPTER 6

ACCELEROMETRY TO QUANTIFY ASYMMETRIC UPPER LIMB MOVEMENT AT THREE MONTHS OF AGE IN INFANTS WITH UNILATERAL BRAIN INJURY; A FEASIBILITY STUDY

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Submitted

ABSTRACT

Objective: Asymmetric upper limb movement is often the first clinical sign of abnormal motor development after unilateral perinatal brain injury. This study aimed at comparing quality of hand function to quantitative measurements of upper limb motion in infants with unilateral perinatal brain injury.

Methods: 16 infants (mean gestational age 34.7 ± 4.7 weeks) with an MRI-diagnosis of unilateral perinatal brain injury were simultaneously assessed with the Hand Assessment for Infants (HAI) and bimanual accelerometry at 3.5 (± 0.4) months of corrected age. Asymmetry indices between both upper limbs were calculated from HAI and accelerometry using: [(ipsilesional score – contralesional score) / (contralesional + ipsilesional score)] * 100%. Hand preference was scored as none (44%), mild (25%) or clear (31%) by an experienced pediatric neurologist.

Results: the HAI score and number of accelerations were both lower in the contralesional limb (13 and 3638 units) compared to the ipsilesional limb (15 and 6994 units). Asymmetry indices were higher in the group with clear hand preference compared to no/mild hand preference. Both HAI and accelerometry detected clear hand preference when asymmetry was above 20% with negative predictive value of 100% and positive predictive value of 100% for HAI versus 71% for accelerometry.

Conclusions: Accelerometry is feasible to detect upper limb asymmetry in infants with unilateral brain injury at the age of three months. More studies are needed to validate the relation between quantity and quality of upper limb asymmetries, and their predictive value for future development of motor disabilities.

INTRODUCTION

Perinatal brain injury is still a common problem in the neonatal intensive care unit and puts newborns at risk of developmental disabilities in later life.[1,2] Neonatal care focuses on monitoring infants at risk, early in life in order to assess brain injury, predict adverse outcome and select those who might benefit from intervention strategies.[3–5] These methods include neuro-imaging with cranial ultrasound or MRI, continuous neuro-monitoring including aEEG and NIRS, as well as clinical parameters of motor behavior.[6–10] Non-invasive and objective bedside methods are preferable because they allow continuous risk evaluation at an early stage, independent of the clinical condition of the patient.

There is emerging evidence that motor patterns early in life are predictive of abnormal neurodevelopment such as motor disabilities.[11,12] General Movements (GMs) is a monitoring tool to qualitatively assess motor behavior in a clinical setting as early as one to three months, which is found to be predictive of future neurodevelopment.[13] This method was mainly developed for research settings, and its clinical use is limited due to high inter-observer variability.[14,15] More recently, studies have been conducted that analyze motion of neonates using wearable sensors.[16] Main advantages of these devices include quantitative assessment of motion without the need for trained observers, and their potential to monitor continuously and in non-hospital settings. Most studies so far focus on the assessment of spontaneous movements or sleep-wake patterns in, mostly, preterm born infants.[17,18] However, the feasibility of movement monitoring sensors in neonates at risk of motor disabilities remains unknown.

This study focuses on infants at risk of developing motor disabilities, in particular unilateral spastic cerebral palsy (USCP), due to unilateral perinatal brain injury. In these infants, movement asymmetry, especially in the upper limbs, is often the first clinical sign of abnormal motor development at an early age.[12,19] As GMs do not allow assessment of asymmetric movements in infants, new instruments have been developed that specifically aim to evaluate unilateral and bilateral upper limb function in the first year of life.[20,21] The Hand Assessment for Infants (HAI) is such an instrument that scores the quality and function of both hands independently and provides a scale of asymmetry between hands.[22] This study aimed at comparing quality of hand function to quantitative measurements of upper limb motion by using simultaneous HAI assessment and accelerometry in infants with unilateral perinatal brain injury. This is the first study to describe feasibility of accelerometry in measuring upper limb asymmetry as early as three months of age.

METHODS

The participants of this study were infants with unilateral perinatal brain injury as confirmed on MRI around term-equivalent age and who were seen at the outpatient clinic at around 3 months corrected age for routine follow-up with HAI. All patients had previously been admitted to the neonatal intensive care unit (NICU) of the Wilhelmina Children's Hospital, University Medical Center in Utrecht. Parents signed informed consent for participation in an accelerometer study and approved the use of their child's HAI data for research purposes. A waiver of authorization to conduct this study was approved by the Medical Ethical Committee Utrecht because HAI assessment is considered standard medical care for infants with unilateral brain injury who are considered at high risk of USCP.(WAG/th/14/038370) During routine follow-up, early hand preference or asymmetric upper limb development was determined by a pediatric neurologist (LSdV) who is specialized in neonatal development with >20 years of experience, who was unaware of HAI scores and accelerometry data. It was diagnosed following asymmetry in distal movements in upper and lower extremities, tone and hand fisting on the side contralateral to the brain lesion. Hand preference was used as a first clinical sign of unilateral motor abnormalities and scored as: none, mild or clear.

HAI Assessment

Motor development of the upper limbs was qualitatively assessed using the HAI. The HAI assessment was performed by a pediatric occupational therapist with expertise in HAI evaluation and who was unaware of the MRI results. HAI consisted of a 10-minute play-related video-recorded session that stimulated unilateral and bilateral upper limb movement.[23] Using the video-recording, the HAI was scored afterwards on17 items with 12 items for each individual hand (Contralesional/Ipsilesional each Hand Sum Score [EaHS], score 0-24 for each hand), a total score for both hands combined (Both Hand Sum Score [BoHS], score 0-48) as well as a scaled total score (Both Hands Measure [BoHM], score 0-100) and an asymmetry index (AI).[23] Recently published normative reference values for the contralesional/ipsilesional EaHS and the BoHS were used to determine the number of infants below -1 standard deviation (SD).[24]

Accelerometry

Two triaxial accelerometers (Actical® Version 2.12, Respironics, Bend, OR, USA) were used to measure upper limb acceleration in 3-dimensional space. The accelerometers were placed around the infant's wrist using a neonatal soft foam band (Precision®, PDC Healthcare, Valencia, CA, USA). The accelerometer has a size of 29x37x11mm and weighs 16 grams. The acceleration signal of all three directions was sampled at a rate

of 32 Hz (1/0.03 second). Recorded data were stored in the system memory of the accelerometer until they were transferred to a computer for subsequent processing using analysis software (Actical Software, Version 2.10, Respironics). Acceleration counts were available for each hand per epoch of 15 seconds.

Procedure

Infants were placed in a baby seat and their wrists were uncovered from any clothing. A video camera was placed on a tripod, straight in front of the infant.(Figure 1) After attaching two accelerometers to the left and right wrist, the marker button was pressed to make sure video recordings could be matched to accelerations in time. HAI assessment was performed by presenting objects mostly in midline, or stimulating both hands equally for grasping, reaching, etc. The observer aimed for at least 10 minutes (600 seconds) of continuous simultaneous video recording and accelerometry. However, if participants were crying or expressed another form of discomfort, observations were paused or stopped prematurely. At the end of the assessment, the marker button was pressed again to mark the end of accelerometry.

Data analysis

Depending on their distribution, descriptive measures were reported either as mean with standard deviation (SD) or median with interquartile ranges [IQR]. For the study, an AI between ipsilesional and contralesional (unaffected and affected, respectively) hand/arm was calculated for HAI and accelerometry: [(ipsilesional score – contralesional score) / (contralesional + ipsilesional score)] * 100%. For HAI, the AI was re-calculated using the each EaHS values and for accelerometry, the AI was calculated using total count of accelerations. An AI <0 is the result of a preference for the ipsilesional (unaffected) hand, whereas an AI >0 is the result of a preference for the contralesional (affected) hand. To test for differences between groups, chi-square test, one sample T-tests, paired sample T-test, independent T-tests, one-way ANOVA or the nonparametric variant were used. Associations between continuous variables were assessed using linear regression. Receiver operator curves were used to calculate cut-offs for asymmetry indices and accelerometry scores in prediction of clinical hand preference: the optimal cut-off was determined by calculating the maximal Youden's Index using Youden's J = Sensitivity + Specificity – 1. When cutoffs resulted in similar Youden's Index, the cutoff yielding in highest sensitivity was chosen since these predictors are part of a screening test. For EaHS and BoHS on HAI, the cut-off was set at -1SD, in accordance with recently published reference values.[24] Statistical analyses were performed with IBM SPSS Statistics® v25 (IBM Corp., Armonk, NY,); a p-value <0.05 was considered significant.

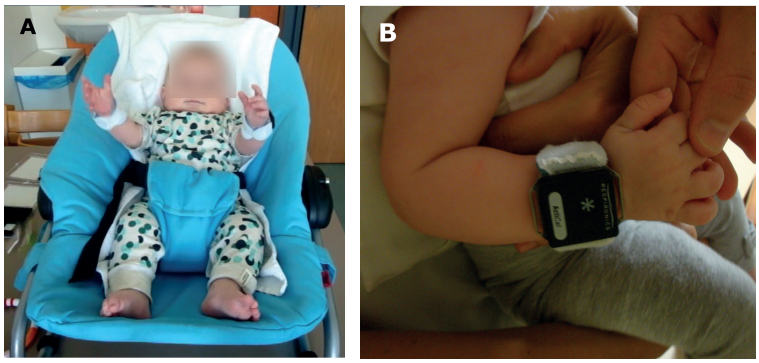

FIGURE 1 | Study set-up of accelerometry during HAI assessment. Infants were placed in a baby seat, their wrists uncovered from any clothing and positioned in midline towards the camera (A) Two accelerometers were attached to the left and right wrist (B).

RESULTS

Sixteen infants born at a mean gestational age of 34.7 ± 4.7 weeks were included in this study during follow-up at a mean corrected age of 3.5 ± 0.4 months. Patient characteristics are presented in table 1. There were seven infants without hand preference (44%), four with mild preference (25%) and five with clear hand preference who were likely to develop USCP (31%). The characteristics are reported in table 1 and were equally distributed among the subgroups.

TABLE 1 | Baseline characteristics.

	Total group (n=16)
Male	11 (69%)
Gestational age (weeks)	34.7 ± 4.7
Born <36 weeks of gestation	7 (44%)
Birth weight (grams)	2271 ± 975
Diagnosis:	
PAIS	8 (50%)
PVHI	5 (31%)
Focal of punctate white matter injury	3 (19%)
Affected hemisphere / side lesion:	
Left	8 (50%)
Right	8 (50%)
Corrected age at FU (months)	3.5 ± 0.4

Data presented as number (percentage) or mean ±SD where applicable. PAIS = perinatal arterial ischemic stroke; PVHI = periventricular hemorrhagic infarction; FU = follow-up.

HAI

Overall, the EaHS was higher in the ipsilesional compared to the contralesional hand (median 15 [IQR 13 – 17] versus median 13 [IQR 7 – 17], but the difference did not reach statistical significance ($p>0.05$).(Table 2) BoHS and BoHM values are presented in table 2. Gestational age was not correlated with HAI scores, because HAI scores are calculated taking corrected age at time of assessment into account. As expected, all HAI scores increased with increasing age at time of the assessment, but this correlation was not significant. As HAI is a qualitative measure, duration of the assessment was also not associated with HAI scores. Contralesional EaHS was lower in infants with clear hand preferences (median 4, IQR 3-8) compared to those with no/mild hand preference (median 14, IQR 13-18, $p<0.001$), while the ipsilesional and bimanual scores did not significantly differ between hand preference subgroups. (Table 2) Seven infants had a contralesional EaHS <-1 SD of norm value: five of these infants (100%) had a clear hand preference, while two others (18%) did not ($p<0.01$). Three infants hand an ipsilesional EaHS below -1SD, all without clear hand preference. BoHS was <-1SD in eight infants: five (100%) vs. three (33%) were found to have clear vs. no/mild hand preference ($p<0.02$). Predictive values for HAI scores for prediction of clinical hand preference are shown in table 3.

Accelerometry

Accelerometry lasted a median of 645 (IQR 604 – 686) seconds (10.8 minutes). Overall, the total amount of accelerations was higher in the ipsilesional compared to the contralesional arm (median 6994 [IQR 3987 – 10895] versus median 3638 [IQR 2349 – 6566], $p<0.05$.(Table 2) There was no correlation between the duration of the assessment and the amount of accelerations in the ipsilesional arm ($p>0.05$), but increased duration of the assessment was associated with accelerations in the contralesional arm (coefficient 11.1, 95%CI 7.0-15.3). Therefore, the number of accelerations per second was calculated per arm: this was still higher in the ipsilesional compared to the contralesional arm ($p<0.05$).(Table 2) Gestational age and corrected age at assessment were not associated with the amount of accelerations per arm. The amount of accelerations (per second) of the contralesional arm was lower in the groups with clear hand preferences (median 3.9 IQR 3.0 – 4.7) compared to those with no/mild hand preference (median 8.4, IQR 5.0 – 9.8, $p<0.01$), while the amount of accelerations of the contralesional arm was similar for subgroups.(Table 2) Examples of accelerometer data are shown in figure 2. ROC analysis found an optimal cut-off of 5.5 accelerations/second to predict clinical hand preference with a sensitivity of 100% and specificity of 73% at the contralesional arm.(Table 3)

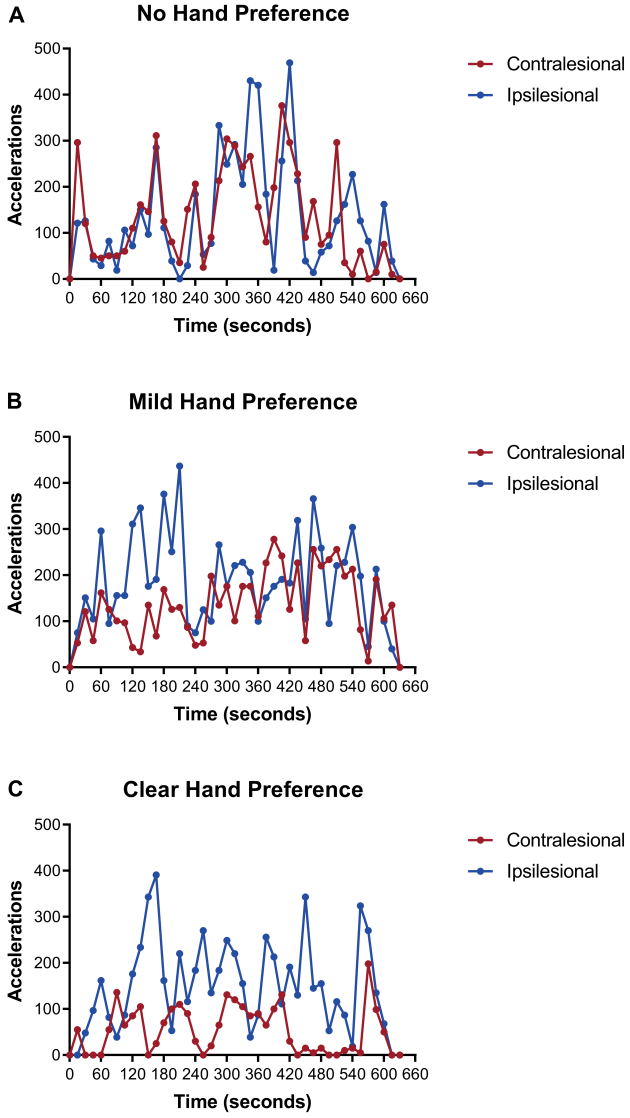

FIGURE 2 | Example of accelerometry of both contralesional (red) and ipsilesional (blue) arm in infants with no (2A), mild (2B) and clear (2C) hand preference. In these cases, symmetry index was -0.5%, 13.5% and 48.5% respectively.

Association of asymmetry and hand preference

Median AI of HAI and accelerometry are presented in table 2. Infants with clear hand preference all showed more than 30% asymmetry on HAI and all infants without or with mild hand preference were within -20% to 20% asymmetry. Overall, infants with

clear hand preference had a median AI of 56% (IQR 36% - 73%) on HAI, while this was 0% (IQR -6% - 0%) for those with no/mild hand preference (p<0.001).(Figure 3A) ROC analyses found a cut-off of 20% on HAI to predict clinical hand preference with 100% sensitivity and 100% specificity (Table 3).

TABLE 2 | Median scores from HAI and accelerometry within subgroups.

	Total group (n = 16)	No/mild hand preference (n=11)	Clear hand preference (n=5)
Duration of assessment (sec)	645 (604 – 686)	645 (630 - 735)	600 (480 – 683)
EaHS contralesional$^{\theta}$	13 (6 – 17)	14 (13 – 18)	4 (3 – 8)
EaHS contralesional <-1SD*	7 (44%)	2 (18%)	5 (100%)
EaHS ipsilesional	15 (13 – 17)	14 (12 – 17)	15 (15 – 17)
EaHS ipsilesional <-1SD	3 (19%)	3 (27%)	0 (0%)
BoHS (n=14)	25 (18 – 31)	29 (21 – 33)	19 (18 – 23)
BoHS <-1SD (n=14)$^{\#}$	8 (57%)	3 (33%)	5 (100%)
BoHM	45 (34 – 53)	49 (44 – 54)	35 (33 – 41)
AI HAI using EaHS (%)$^{\theta}$	0.0 (-2.8 – 38.9)	0.0 (-5.6 – 0.0)	55.6 (36.1 – 72.8)
Accelerations contralesional*	3638 (2349 – 6566)	5437 (3252 – 7491)	2131 (1513 – 3155)
Accelerations ipsilesional	6994 (3987 – 10895)	7940 (3005 – 12701)	6146 (4767 – 9366)
Accelerations/second contralesional*	5.6 (4.0 – 9.5)	8.4 (5.0 – 9.8)	3.9 (3.0 – 4.7)
Accelerations/second ipsilesional	11.1 (5.9 – 13.6)	9.7 (4.7 – 13.6)	11.7 (8.9 – 14.7)
AI Accelerometry (%)*	13.8 (0.4 – 48.8)	8.9 (-17.2 – 14.2)	48.9 (39.0 – 59.2)

Data presented as median (IQR) or number (percentage), where applicable. AI = asymmetry index calculated by [(ipsilesional score – contralesional score) / (contralesional + ipsilesional score)] * 100%. EaHS = each hand sum score; BoHS = both hands sum score; BoHM = both hands measure; HAI = hand assessment for infants; SD = standard deviation. $^{\#}$ p<0.02, * p<0.01, $^{\theta}$ p<0.001.

Infants with clear hand preference showed more than 29% asymmetry on the accelerometry. One infant with no and one infant with mild hand preference showed an asymmetry on accelerometry of 64% and 40% respectively. All other infants without or with mild hand preference (n=9) had an accelerometry AI between -20% - 20%. Overall, infants with clear hand preference had a median AI of 49% (IQR 39% - 59%) on accelerometry, while this was 8% (IQR -17% - 14%) for those with no/mild hand preference (p<0.01).(Figure 3A) ROC analyses found a cut-off of 20% asymmetry on accelerometry to predict clinical hand preference with 100% sensitivity and 82% specificity (Table 3). Asymmetry on accelerometry was significantly associated with asymmetry on HAI (B=0.6, 95%CI 0.1 – 1.1).(Figure 3B)

TABLE 3 | Predictive value of HAI and accelerometry for hand preference.

	Cutoff	Sens	Spec	PPV	NPV
EaHS contralesional	13	100%	82%	71%	100%
EaHS ipsilesional*	13	0%	73%	0%	62%
BoHS	27	100%	73%	63%	100%
AI each hand scores (%)	20%	100%	100%	100%	100%
Accelerations/second contralesional	5.5	100%	73%	63%	100%
Accelerations/second ipsilesional*	5.5	0%	73%	0%	62%
AI Accelerometry (%)	20%	100%	82%	71%	100%

AI = asymmetry index calculated by [(ipsilesional score – contralesional score) / (contralesional + ipsilesional score)] * 100%. EaHS = each hand sum score; BoHS = both hands sum score; Sens = sensitivity; spec = specificity, PPV = positive predictive value; NPV = negative predictive value. * non-significant association.

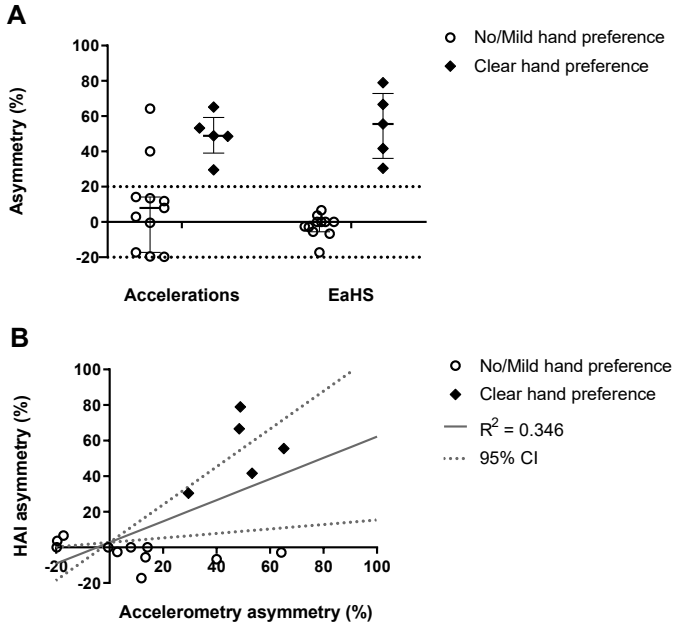

FIGURE 3 | Association between hand preference and asymmetry. Median (IQR) asymmetry index of accelerations (left) and Each Hand Sum Score (EaHS) on HAI (right) between ipsi- and contralesional hand differed between subgroups of hand preference (A). In A the dotted line represents an optimal cut-off of 20% asymmetry. There was an association between asymmetry on accelerometry and HAI with Nagelkerke R^2 = 0.35 (B). HAI = hand assessment for infants.

DISCUSSION

This study demonstrates feasibility of accelerometry in measuring upper limb movement asymmetry during hand assessment as early as three months of age in a group of infants with unilateral perinatal brain injury. Overall, the number of accelerations per second in the contralesional arm is significantly lower than in the ipsilesional arm, comparable to a qualitative score of hand functioning. Furthermore, we aimed at comparing asymmetry of upper limb movement and quality of hand functioning in infants with no/mild and clear clinical hand preference at the age of three months. We found that both measures are able to detect clear hand preference when asymmetry is above 20%.

In infants with unilateral brain lesions, asymmetry between left and right limbs is usually the first sign of asymmetrical motor development.[19,25] These asymmetries become more apparent when the nervous system matures and voluntary motor activity emerges. As the development of voluntary motor behavior corresponds to a decreasing spontaneous pattern of GMs[26], assessing GMs seems less suitable to detect movement asymmetry. The HAI is a qualitative score that assesses upper limb functioning separately and provides a score of each hand that can be used to calculate asymmetry.[22] A study describing normative reference values for HAI showed that in a large group of typically developing infants, the difference between EaHS on HAI was maximal 1-5 points.[24] This is comparable to our study: those with no/mild hand preference had a difference of 0-5 points, while this was 7-15 points for the infants with clear hand preference. We also found that all infants with clinical hand preference displayed >20% asymmetry on HAI. The positive predictive (PPV) value of the AI was higher (100%) than the PPV of the EaHS or BoHS values on HAI (71%) for prediction of clinical hand preference. Reference values for asymmetry on HAI have not been described yet[24], but based from our observations a cut-off of 20% is useful to detect asymmetric motor behavior at the age of three months. More studies on HAI asymmetry at different time points and in other populations including typically developing infants are needed to confirm this.

Classification of hand preference and HAI scoring, but also observation of GMs, is based on observations of movement quality, which proposes a risk of observation bias and high inter-observer variability.[15] Therefore, this pilot was designed to study feasibility of accelerometry to detect movement asymmetry at three months of age in infants at risk of developing motor disabilities. Our main aim was to validate the ability of wearable sensors to detect and quantify upper limb movement at a young age, and we have shown variability in accelerations during a video-recorded session, as described before.[27,28] Trujilo-Priego et al. described no differences in the amount of accelerations in the left and right arm during the first year of life in

typically developing children, in agreement with other data.[29] Our results confirm that there were no differences between left and right in infants without clinical hand preference, but additionally show that the number of accelerations is overall lower in the contralesional arm, in infants with unilateral brain lesions. This suggests that accelerometry is able to detect asymmetries that are consistent with unilateral brain lesions in our study population as early as 3 months of age.

Several other groups found that accelerometry in the first months of life is able to detect abnormal motor development. Heinze et al. described that accelerometry between 1-6 months of age had 88-92% detection rate of at-risk infants with brain abnormalities who were later diagnosed with cerebral palsy at two years of age.[30] Gravem et al. studied ten preterm born infants at 30-43 weeks of gestation with five accelerometers for one hour and related motion algorithms from accelerometry with GMs.[31] In their study, accelerometry was found to have high accuracy and specificity to detect a cramped-synchronized pattern on GMs, which is highly related to abnormal motor development.[31,32] All these studies focused on spontaneous motor activity during the first months after birth, in contrast to our setting of active play stimulating voluntary movement. As described before, asymmetric development usually appears at a time when spontaneous movements disappear, making our study set-up more applicable to infants at risk of unilateral motor disabilities.

We observed in our study that asymmetry in motion quantity from accelerometry corresponded to asymmetry in motion quality from HAI and clinical assessment. However, two cases were found to have more than 20% asymmetry on accelerometry while they were not asymmetric clinically or on the HAI. One of these was a child with PWML who developed a strong head preference with plagiocephaly and showed mild hand preference. The other was a child with PAIS without clear hand preference, without a clear explanation for increased movements in one of his arms and without evidence of a USCP at 18months of age. From this study we may conclude that accelerometry at this early age is a potentially excellent screening instrument for hand preference with high sensitivity and low specificity. A potentially excellent screening instrument means that no infants will be missed for further follow-up or possible early intervention strategies.[4] However, the ability of movement quantity asymmetry to predict motor disabilities or even USCP later in life remains unknown. Infants from our cohort will be followed over time to see their motor development and future studies are necessary to compare predictive abilities of the motor assessment tools used in this study.

Our study has several limitations. The first is that we have so far only collected accelerometry data at one timepoint in a relatively small number of infants. Others described dynamics and variability of spontaneous movements in term infants in

the first months of life.[27,28] They described non-linear, unstable and unpredictable movements of both upper and lower limbs, but also found that the dimensions and dynamics change over time. As our study is ongoing, we are planning to collect more simultaneous HAI and accelerometry data over time at different stages of development. HAI is a qualitative score after active stimulation, and the observer often stimulates the subject to show their best stages of upper limb development, proposing a risk of manipulation of the accelerometry data by overstimulation of the ipsilesional arm. Although accelerometry was able to detect all children with clinical hand preference, Asymmetry Indices should be interpreted with care. Perhaps future studies should also include a time-window of spontaneous movements without active stimulation. Another limitation is the lack of future motor development, as described above. A gold standard of motor outcome, such as the development of unilateral spastic cerebral palsy, is needed to study the predictive ability of both HAI and accelerometry in our study population. However, this pilot study was performed to show feasibility to detect movement asymmetry. Furthermore, our group did not consist of healthy controls because all were diagnosed with some form of unilateral brain injury and therefore considered at risk of motor disabilities. This might have biased our data, although infants without hand preference had low asymmetry on HAI and accelerometry. However, to extrapolate our results to the general population, infants without MRI abnormalities may need to be included in a future study as well.

Easily applicable and lightweight accelerometers may be useful as clinical tools to assess asymmetric and potentially abnormal patterns of motor activity in infants at risk of motor disabilities. Their application may add a new and objective method that is less time-consuming, automated and without the need of experienced observers. Early prediction of abnormal motor development with wearable motion sensors could identify those that might benefit from early preventative strategies or treatments, and possibly monitor the effects of these interventions. However, the use of accelerometry might also be useful to detect other neurological conditions such as tonic-clonic or rhythmic movements due to epileptic activity or developmental disorders including autism, both at intensive care or out-of-hospital settings.

CONCLUSION

In summary, accelerometry is able to detect movement upper limb asymmetry in infants with unilateral brain injury at the age of three months. More studies are needed to validate the relation between quantity and quality of upper limb asymmetries, and future development of motor disabilities including cerebral palsy.

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CHAPTER 7

BRAIN ACTIVITY AND CEREBRAL OXYGENATION AFTER PERINATAL ARTERIAL ISCHEMIC STROKE ARE ASSOCIATED WITH NEURODEVELOPMENT

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ABSTRACT

Objectives: In infants with perinatal arterial ischemic stroke (PAIS) early prognosis of neurodevelopmental outcome is important to adequately inform parents and caretakers. Early continuous neuro-monitoring after PAIS may improve early prognosis. Our aim was to study early cerebral electrical activity and oxygenation measured by amplitude-integrated electroencephalography (aEEG) and near-infrared spectroscopy (NIRS) in term neonates with PAIS and relate these to the development of cerebral palsy and cognitive deficit.

Methods*:* aEEG Patterns and regional cerebral oxygen saturation ($rScO_2$) levels of both hemispheres were studied for 120 hours from the first clinical symptoms of PAIS (i.e. seizures) onwards. Multivariable analyses were used to investigate the association between aEEG, NIRS, clinical variables and neurodevelopmental outcome.

Results: In 52 patients with PAIS (gestational age 40.4 ±1.4 weeks, birth weight 3282 ±479 grams), median time to a continuous background pattern was longer in the ipsilesional compared with the contralesional hemisphere (13.5 vs. 10.0h, $p<0.05$). $rScO_2$ decreased over time in both hemispheres but less in the ipsilesional one, resulting in a $rScO_2$-asymmetry ratio of 4.5% (IQR -4.3% – 15.9%, $p<0.05$) between hemispheres from day 3 after symptoms onwards. Both time to normal background pattern and asymmetry in $rScO_2$ were negatively affected by gestational age, size of the PAIS, use of anti-epileptic drugs and mechanical ventilation. After correction for size of the PAIS on MRI, a slower recovery of background pattern on ipsilesional aEEG and increased $rScO_2$-asymmetry between hemispheres was related with an increased risk for cognitive deficit (< -1 SD) at a median of 24.0 (IQR 18.4-24.4) months of age.

Conclusions: Recovery of background pattern on aEEG and cerebral oxygenation are both affected by PAIS, and related to neurocognitive development. Both measurements may provide valuable early prognostic information. Additionally, monitoring cerebral activity and oxygenation may be useful in identifying infants eligible for early neuroprotective interventions and to detect early effects of these interventions.

INTRODUCTION

Perinatal arterial ischemic stroke (PAIS) is defined as an acute symptomatic insult in an arterial territory confirmed by neuroimaging that occurs in 1:2300 newborns.[1,2] Most frequently, neonates with PAIS present with seizures within the first days after birth.[3–5] PAIS can lead to severe morbidity, such as cerebral palsy (CP), epilepsy, cognitive, behavioral and language impairments.[1,6]

Early prognosis is important to adequately inform parents and caretakers, as well as to initiate new early intervention strategies that aim for neuroprotection or neuroregeneration.[7] Early estimation of the prognosis is currently based on neuroimaging with MRI using diffusion weighted imaging (DWI). However, neuroimaging with MRI is not always possible in neonates who are unstable. Besides, use of early MRI mainly focuses on involvement of the corticospinal tracts, such as pre-Wallerian degeneration, to predict motor development, while neuroimaging predictors for cognitive outcome are far less established.[6,8,9] Furthermore, neuroimaging only provides useful predictive information at the time of scanning, while early installed continuous neuro-monitoring after PAIS may improve early prognosis of neurodevelopmental outcome.

This study will focus on amplitude integrated electroencephalography (aEEG) and near-infrared spectroscopy (NIRS), both noninvasive, continuous techniques to monitor cerebral activity and oxygenation in unstable neonates before neuroimaging can be performed in conditions such as hypoxic-ischemic encephalopathy.[10–12] aEEG-patterns and NIRS have shown to be related to neurological outcome in several neonatal disorders.[10,12–17] Nevertheless, aEEG and NIRS monitoring are still not standard practice in PAIS in most centers across the world.[10,17] Therefore we will investigate aEEG-patterns and NIRS values during the first five days after clinical symptoms of PAIS and relate these to neurodevelopmental outcome. We hypothesize that early neuro-monitoring after PAIS may improve early prediction of neurodevelopmental outcome.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Patients

This is an observational retrospective cohort study that included term neonates (>36 weeks of gestational age), who were admitted to the tertiary neonatal intensive

care unit (NICU) of the Wilhelmina Children's Hospital (Utrecht, Netherlands) with suspected PAIS based on clinical seizures between January 2009 and March 2018. The diagnosis of PAIS was confirmed in all infants by neuroimaging with MRI including DWI. For this study, infants with aEEG and/or NIRS recordings available were selected. Anti-epileptic drugs (AEDs) were administered per clinical protocol when neonates showed signs of clinical and aEEG confirmed seizures. The number of AEDs was noted. In the Netherlands, phenobarbital is first-line AED after neonatal seizures, while second line AED was either midazolam or lidocaine.

Information on relevant clinical parameters was collected from the patient chart. Social economic status was approximated based on ZIP code, provided by the 'Social and Cultural Planning Agency' in the Netherlands.[18] Furthermore, using neonatal MRI, PAIS was classified based on size and site of the lesion. A lesion was labelled large when it affected the whole territory of the middle cerebral artery (MCA); medium when it affected the MCA territory partially or the area of the anterior cerebral artery (ACA) or posterior cerebral artery (PCA); or small when the lesion was defined as a cortical or perforator stroke based on our previous work.[6] Lesions were located anteriorly or posteriorly to the central sulcus, or were classified as central when they involved the central sulcus, the deep gray matter or the complete MCA territory. The presence of bilateral lesions was also recorded.

Between July 2009 and April 2016, 27 infants were part of a safety and feasibility study and treated with 3x 1000IU recombinant human erythropoietin.[7] All parents signed consent for this study and these infants were monitored with aEEG and NIRS as part of the study protocol. After initiation of this trial, neuromonitoring with both NIRS and aEEG became part of standard clinical care for all infants with (suspected) PAIS. The research question as addressed in this study was not part of the initial trial, but performing this study was waived by the ethical committee of our hospital. Two infants were part of a randomized placebo-controlled trial that was recently initiated in our center studying the effect of darbepoetin after PAIS. (DINOSAUR, ClinicalTrials.gov NCT03171818)

Main analyses

Neuromonitoring included simultaneous, bilateral continuous assessment of regional cerebral oxygen saturation ($rScO_2$) and electrical activity, which became standard clinical practice for infants with suspected PAIS in our neonatal intensive care unit. Neuromonitoring is applied by the attending nursing staff as soon as possible after admission. To allow comparison of the patients, data were calibrated from the time of the first clinical symptoms (usually seizures). Recordings were used up till five days (120 hours) after onset of symptoms.

For data processing, SignalBase® v.7.8.1 (University Medical Center Utrecht, Netherlands) was used. The first 120 hours after onset of PAIS were divided into 20 epochs of 6 hours each. From each epoch, one hour of continuous data was randomly chosen as representative for this epoch and data were checked for the absence of clinical and subclinical (suspected) seizures and artifacts. These periods were used for analyzing patterns of the aEEG and mean $rScO_2$ in both hemispheres.

Monitoring brain activity using aEEG:

The aEEG (BrainZ Monitor, BRM3, Natus CA, Seattle, WA) signal was recorded from two frontal and two parietal electrodes, (F3-P3 and F4-P4), according to the international EEG 10-20 classification.[10,19] The aEEG recordings in both hemispheres (ipsi- and contralesional of the stroke) were scored on background pattern and sleep wake cycling by an experienced neonatal aEEG reviewer (LV). BGP was divided into: flat trace, burst suppression-, burst suppression+ , discontinuous normal voltage and continuous normal voltage, as described by Hellstrom-Westas et al.[16] In further analyses, continuous normal voltage is referred to as a normal background pattern. Sleep-wake cycling was described as being absent, imminent or present, as reported by Osredkar et al.[20] In further analyses, present sleep-wake cycling is referred to as normal.

Monitoring cerebral oxygenation and perfusion using NIRS:

Cerebral oxygenation was monitored by NIRS (INVOS 5100C, Medtronic, Minneapolis, MN) with a bilateral sensors placed over the frontoparietal cortex (small adult SomaSensor SAFB-SM, Medtronic, Minneapolis, MN).[21] Simultaneously noninvasive BP, HR and SaO_2 were measured. Median values for each variable were calculated per epoch.

The mean percentage above or below the reference range for $rScO_2$ of 55-85%[22] was calculated for each epoch. To correct for interindividual baseline differences, the asymmetry index (%)) between $rScO_2$-level at the ipsilesional and contralesional hemisphere was calculated (($rScO_2$ ipsilesional– $rScO_2$ contralesional) / $rScO_2$ contralesional) * 100%. An asymmetry index in $rScO_2$ ($rScO_2$ –asymmetry) of 0% indicates no difference between hemispheres. Positive values indicate higher $rScO_2$ levels in the ipsilesional hemisphere, while negative values indicate higher $rScO_2$ levels in the contralesional hemisphere.

Neurodevelopmental outcome

Neurodevelopmental outcome was determined during routine follow-up between 15 and 25 months of age. Cognitive development was determined by using the developmental quotient of the Griffiths Mental Development Scale (GMDS),

calculated by using all subscale scores except locomotion, or the Bayley Scales of Infant and Toddler Development, Third Edition (BSITD-III), as previously described[6]. Cerebral palsy (CP) was classified as unilateral spastic cerebral palsy by a pediatric physiotherapist and neonatal neurologist who were unaware of neuromonitoring data. Unfavorable outcome was defined as cognitive deficit (<-1SD on GMDS or BSITD-III) or development of CP.

Statistical analysis

IBM SPSS Statistics® v25 (IBM Corp., Armonk, NY,) and R 3.0.0 for Windows with the *nlme* package (The R Foundation of Statistical Computing, www.r-project.org) were used for statistical analysis. Nonparametric variables were log-transformed in order to achieve normal distribution. Patient characteristics were summarized as counts and percentages for categorical variables, means ± standard deviation (SD) for parametric data, and as median ± interquartile ranges (IQR) for nonparametric data. To test for differences between groups, chi-square test, one sample T-tests, paired sample T-test, independent T-tests, one-way ANOVA or the nonparametric variant were used. Linear and binary regression analyses were performed to test the association between variables. To assess the effect of time on $rScO_2$-asymmetry a mixed model analysis was performed, which allows to control for the number of observations per patient. Linear, quadratic, and cubic functions were explored for obtaining the best fitting data. Binary logistic regression models for cognitive deficit were compared using Omnibus Tests of Model Coefficients where a baseline model with size of lesion was compared to models including neuro-monitoring variables. Receiver operating characteristic (ROC) curves were created using Prism GraphPad Software (version 7.04 for Windows, GraphPad Software, Inc.) to determine sensitivity and specificity at various cut-offs per outcome parameter. Estimated p-values <0.05 were considered statistically significant.

RESULTS

A total number of 62 PAIS patients were initially eligible. However, 10 infants had to be excluded, because of concomitant syndromes (n=3), other severe brain abnormalities (n=4), cardiac abnormalities requiring surgery (n=2) or death in the neonatal period due to severe hypoxic-ischemic encephalopathy (n=1). Hence, we included 52 patients. aEEG data were available in 49 patients, NIRS data in 39 patients, and 36 infants had simultaneous aEEG and NIRS available. All patient characteristics are displayed in Table 1.

TABLE 1 | Baseline characteristics.

Male (%)	28 (54)
Gestational age (weeks)	40.4 (39.3 – 41.0)
Birthweight (gram)	3268 (2981 - 3567)
Perinatal asphyxia* (%)	7 (14)
Hypothermia** (%)	2 (4)
Apgar score at 5 minutes	9 (7-10)
Hypoglycemia <2.0	9 (21)
First clinical symptoms of seizures (h after birth)	24.0 (10.5-48.0)
aEEG:	
Duration before start monitoring after seizures (h)	11.4 (4.9-42.1)
Total duration monitoring (h)	92.7 (66.7-115.1)
NIRS:	
Duration before start monitoring after seizures (h)	17.7 (5.8–51.4)
Total duration monitoring (h)	66.8 (50.8–92.6)
Number AEDs:	
0-1	32 (62)
>1	20 (38)
Mechanical ventilation	12 (23)
Left-sided PAIS	31 (60)
Size of PAIS:	
Small	18 (35)
Medium	25 (48)
Large	9 (17)
Artery involved:	
MCA	39 (75)
PCA	4 (8)
Perforator stroke	8 (15)
PICA	1 (2)
Erythropoietin:	
Safety and feasibility study	27
DINOSAUR	2
None	23

Gestational age and birthweight are represented as mean (SD). Other data as n(%) or median (IQR) where applicable. *Perinatal asphyxia was defined as an Apgar-score ≤5 at 5 minutes or metabolic acidosis (cord pH < 7.0 and/or base deficit (BE) ≤-16 mmol/L). **Hypothermia was applied when criteria for cooling were met including signs of encephalopathy within 6 hours after birth.

aEEG

Figure 1 demonstrates aEEG patterns over time in both hemispheres. At 24 hours after clinical symptoms of PAIS, 57% of the recordings showed a continuous normal voltage in the ipsilesional hemisphere, while this was 65% in the contralesional hemisphere. At the end of day 5 after symptoms, 71% of the aEEG recordings showed full recovery for both background pattern and sleep-wake cycling in the

ipsilesional and 78% in the contralesional hemisphere.(figure 1) Median time to recovery to normal background pattern was longer for the ipsilesional than for the contralesional hemisphere (13.5 [interquartile range (IQR) 0.0 – 42.2] versus 10.0 [IQR 0.0 – 38.4] hours, p<0.03) and also time to a mature sleep-wake cycling was longer in the ipsilesional compared to the contralesional hemisphere (58.8 [IQR 32.4 – 120.0] versus 54.5 [IQR 32.4 – 93.2] hours, p<0.05). In all infants, time to recovery of the aEEG pattern was always longer in the ipsilesional hemisphere.

The effect of clinical parameters that are described in Table 1 on the aEEG pattern was assessed. The aEEG patterns per lesion size subtype are presented in supplemental figure I and II (Available Online). Median time to a normal background pattern of the ipsilesional hemisphere was significantly longer in patients with large lesions compared to those with small lesions (59.3 [IQR 16.8 – 88.3] versus 0.0 [0.0 – 31.7] hours, p<0.03). Lower gestational age, more than one AED and mechanical ventilation all resulted in a longer time to normal background pattern of both hemispheres (all p<0.03). Time to normal sleep-wake cycling was not related to clinical parameters. After multivariable analyses, gestational age (coefficient -12.4, 95%CI -18.3 – -6.5), >1 AED (coefficient 19.8, 95%CI 0.3 – 39.3) and mechanical ventilation (coefficient 22.0, 95%CI 0.2 – 43.8) were significantly and independently associated with time to normal background pattern.

NIRS

Mean $rScO_2$ values over time are presented in figure 2. Overall, the mean level of $rScO_2$ was higher on the ipsilesional side (72.8±10.8%) compared to the contralesional one (69.7±10.3%) (p<0.001). This resulted in a median $rScO_2$-asymmetry of 4.2% (IQR -5.0% – 15.4%). From day 3 after clinical symptoms onward, there was a significant difference in $rScO_2$ levels between ipsi- and contralesional hemisphere.(Figure 2) The variables gestational age, time in days, size of the lesion, birth asphyxia, use of AED and mechanical ventilation were all associated with an increased $rScO_2$-asymmetry (respectively p<0.001, p<0.003, p<0.001, p<0.02, p<0.01 and p<0.001). The side and location of the lesion, presence of bilateral lesions or erythropoietin administration, did not affect $rScO_2$-asymmetry. After mixed model analyses, the best model to explain variance in $rScO_2$-asymmetry included large (versus small) size of the lesion (coefficient 16.9, 95%CI 9.8 – 23.9), >1 AED (coefficient 5.6, 95%CI 0.2 – 11.0), and time in days (coefficient 2.9, 95%CI 1.4 – 4.3). $rScO_2$-asymmetry data were normalized using log-transformation, but this did not affect the model and are therefore not presented here.

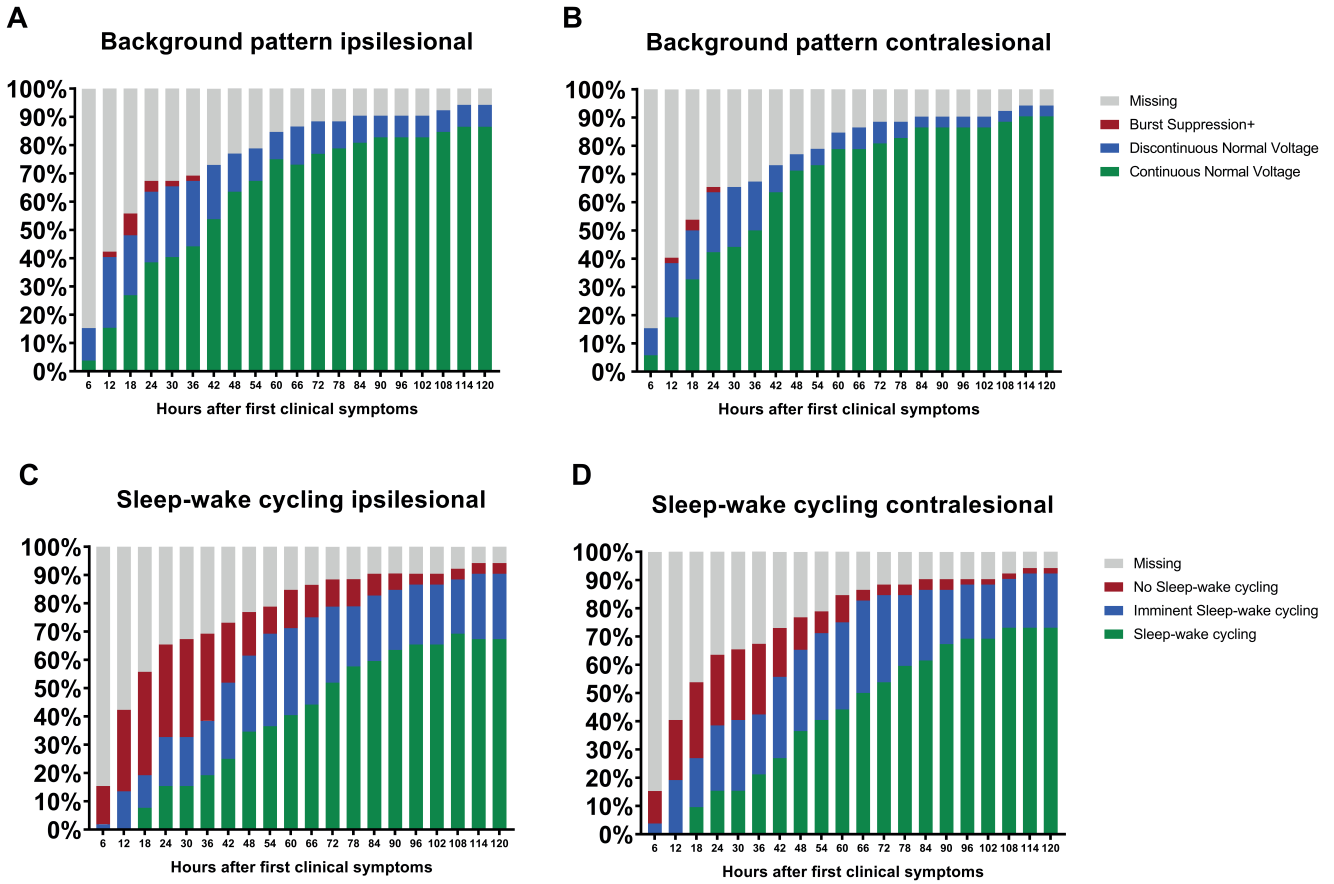

FIGURE 1 | aEEG patterns over time in the ipsilesional and contralesional hemispheres. aEEG patterns are classified based on background pattern (1A-B) and sleep-wake cycling (1C-D) in periods of 6 hours starting from first clinical symptoms.

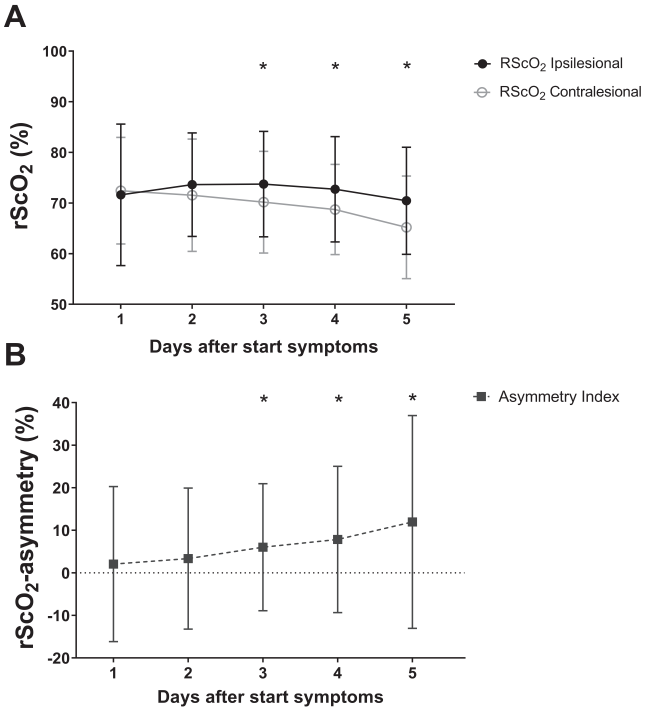

FIGURE 2 | NIRS parameters in ipsi- and contralesional hemispheres. $rScO_2$ (2A) and $rScO_2$-asymmetry (2B) on NIRS over first 5 days after clinical symptoms of PAIS. * = Significant difference between hemispheres. $RScO_2$ = regional oxygen saturation. $rScO_2$-asymmetry is the asymmetry index (%)) between $rScO_2$-level at the ipsilesional and contralesional hemisphere.

Reference values of $rScO_2$

The $rScO_2$ of the ipsilesional hemisphere was on average 12.8% of the time above the upper reference value of 85%, against 6.3% on the contralesional side ($p<0.001$). On the other hand, the contralesional side was on average 8.5% of the time below the lower reference value of 55%, against 5.4% of the ipsilesional side ($p<0.03$).

Neurodevelopmental outcome

Neurodevelopmental outcome data was available for 44 infants (85%); two infants were lost-to-follow-up, and six others were not yet 15 months of age. Of these, 25 infants (57%) were tested using the BSITD-III and 19 infants (43%) were tested with the GMDS. At a median age of 24.0 (IQR 18.5-24.4) months 12 infants had adverse outcomes: 9 infants (17%) developed CP, and 8 infants (15%) had an impaired cognitive outcome.

Large (versus small) lesions were associated with increased risk of an adverse outcome, including CP (OR 90.0, 95% CI 4.8 – 1683.9) and cognitive deficit (OR 20.0, 95% CI 1.6 – 248.0). Other clinical variables, including erythropoietin or social-economic status, were not significantly related to neurodevelopmental outcome.

aEEG in relation to outcome

In patients with adverse outcome (both CP and cognitive deficit), the time to CNV was longer in the ipsilesional hemisphere compared with patients with a normal outcome.(Table 2) Time to CNV in the contralesional hemisphere was also longer in patients who developed cognitive deficits compared to those with normal cognition. (Table 2) Time to normal background pattern of the ipsilesional hemisphere was used for ROC analyses to predict CP and cognitive deficit using optimal cut-offs.(Figure 3)

NIRS in relation to outcome

The $rScO_2$ in the ipsilesional hemisphere was significantly higher in patients with an unfavorable outcome compared with those with a favorable outcome, while $rScO_2$ values at the contralesional hemisphere did not relate to outcome.(Table 2) $rScO_2$-asymmetry was higher in patients who had an adverse outcome compared to those with normal development. Mixed model analyses revealed that time (day) after PAIS did not add to the relation of NIRS parameters and outcome: ipsilesional $rScO_2$ and $rScO_2$-asymmetry were associated with CP and cognitive deficit on all 5 days after PAIS symptoms.(Table 2; Figure 4) Only on day 2, ipsilesional $rScO_2$ did not differ between infants with a good and adverse cognitive outcome.(Figure 4)

As ipsi- and contralesional $rScO_2$ values became different from day 3 onward after PAIS symptoms, $rScO_2$-asymmetry at day 3 was used to calculate optimal cut-offs for the prediction of CP and cognitive deficit.(Figure 3)

TABLE 2 | Association of neuromonitoring parameters to neurodevelopmental outcome.

Outcome	Unfavorable outcome		CP		Cognitive Deficit	
	No	Yes	No	Yes	No	Yes
Time to CNV Ipsi[#] (h)	**17.6 (20.6)**	**51.7 (46.4)**	**19.6 (27.0)**	**55.6 (41.1)**	**19.4 (23.8)**	**60.8 (48.1)**
Time to CNV Contra[#] (h)	16.1 (19.6)	40.8 (43.1)	18.3 (26.4)	41.0 (36.8)	**16.6 (22.1)**	**51.0 (43.1)**
Time to SWC Ipsi[#] (h)	61.8 (36.8)	79.4 (50.4)	**59.8 (39.6)**	**92.6 (38.9)**	64.6 (38.5)	76.4 (53.5)
Time to SWC Contra[#] (h)	59.6 (35.1)	72.6 (49.5)	57.8 (38.1)	83.5 (40.5)	62.4 (37.1)	67.2 (51.5)
$rScO_2$ ipsilesional[#] (%)	**70.2 (9.6)**	**78.5 (11.9)**	**69.9 (9.4)**	**80.4 (11.6)**	**71.3 (9.6)**	**79.4 (13.7)**
$rScO_2$ contra-lesional (%)	69.1 (10.8)	70.3 (10.7)	69.4 (10.7)	69.8 (11.0)	69.9 (10.5)	68.3 (11.7)
$rScO_2$-asymmetry[#] (%)	**2.8 (13.8)**	**15.8 (22.7)**	**2.5 (13.5)**	**17.8 (23.1)**	**3.2 (13.8)**	**22.0 (24.4)**
$rScO_2$ ipsilesional day 3 (%)	**72.0 (9.9)**	**79.0 (11.1)**	**71.5 (9.9)**	**81.5 (9.8)**	**72.9 (9.7)**	**80.1 (12.6)**
$rScO_2$ contra-lesional day 3 (%)	68.7 (10.9)	72.7 (10.0)	69.1 (10.7)	72.2 (10.5)	69.9 (10.7)	70.9 (10.9)
$rScO_2$-asymmetry day 3 (%)	**4.2 (12.7)**	**13.3 (17.0)**	**3.7 (12.4)**	**15.5 (16.8)**	**4.3 (12.5)**	**18.9 (17.5)**

Data are presented as mean (SD). Time to a SWC = Time to mature sleep-wake cycling on aEEG. Time to CNV = Time to a continuous normal voltage background pattern on aEEG. Bold = Significant difference between subdivisions of that outcome domain ($p<0.05$). Non-normally distributed data ([#]) were tested by non-parametric tests.

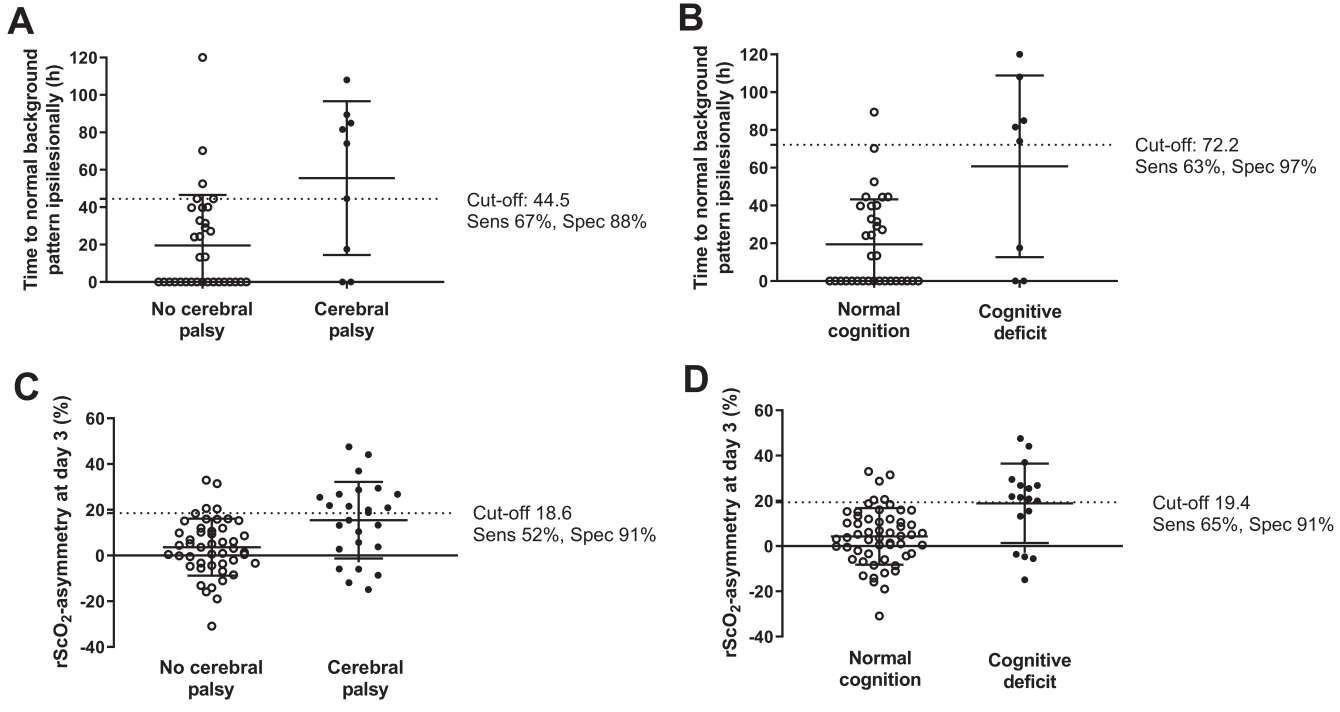

FIGURE 3 | Cut-off values for aEEG and NIRS parameters to predict adverse neurodevelopmental outcome. Time to a normal background pattern (continuous normal voltage) on aEEG and $rScO_2$-asymmetry on day 3 predicted cerebral palsy (CP) (3A-C) and cognitive deficit (3B-D).

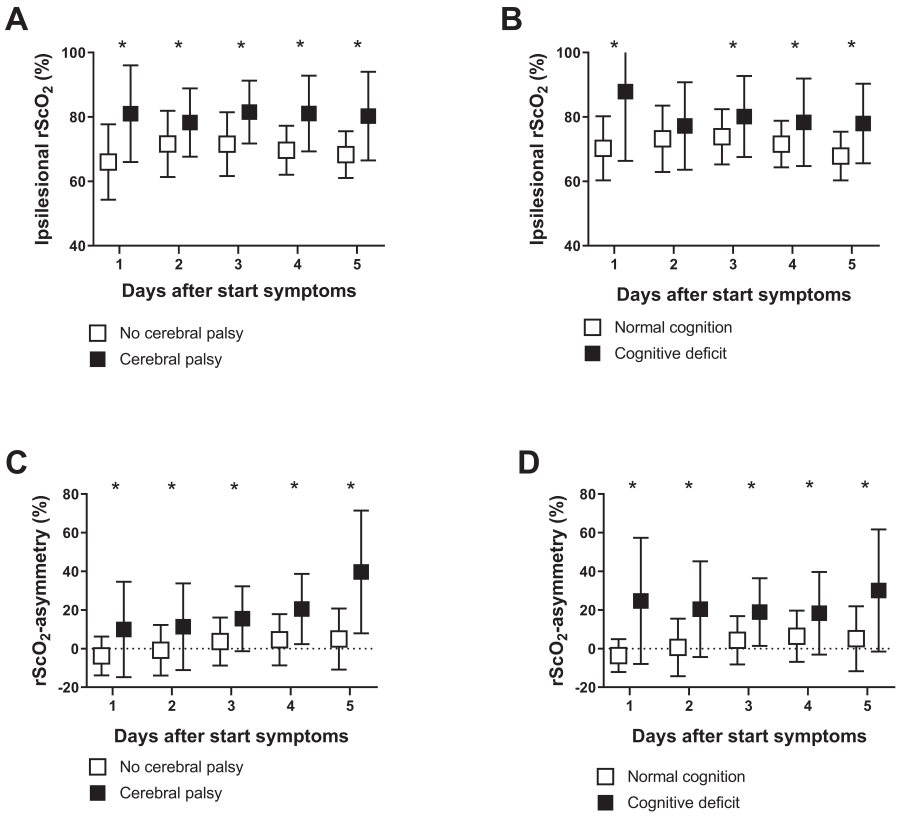

FIGURE 4 | NIRS parameters in the first five days after clinical symptoms of PAIS in relation to neurodevelopmental outcome. Ipsilesional $rScO_2$ is increased in infants with CP (4A) and cognitive deficit (4B). Asymmetry ratio of $rScO_2$ increases in infants with CP (4C) and cognitive deficit (4D).

Multivariable analyses

Multivariable analyses revealed that after correction for size of the lesion, both time to normal background pattern on aEEG and rScO2-asymmetry on NIRS were still associated with cognitive outcome. Increased time to normal background pattern of the ipsilesional hemisphere (per 24 hours OR 1.9, 95% CI 1.0 – 3.6, p=0.05; Nagelkerke R^2 = 0.39) and $rScO_2$–asymmetry at day 3 (per 10% OR 1.8, 95% CI 1.1 – 3.0; Nagelkerke R^2 = 0.58) were still associated with cognitive deficit after correction for size of the lesion. Both models were significantly better associated with cognition than size of lesion alone (large vs small lesion OR 20.0, 95%CI 1.6 – 248.0; Nagelkerke R^2 = 0.25) (p=0.04 and p=0.02 respectively). A model combining neuromonitoring data revealed that both time to normal background pattern (per 24 hours OR 9.6, 95% CI 1.9 – 49.6) and $rScO_2$–asymmetry at day 3 (per 10% OR 7.9, 95% CI 1.3 – 45.9) were independently related to cognitive deficit when correcting for size of lesion (Nagelkerke R^2 = 0.89). This model was significantly better than a model with size of lesion alone (p<0.0001).

DISCUSSION

In this retrospective cohort study, aEEG and NIRS measurements were studied in term neonates that presented with clinical seizures due to PAIS. To the best of our knowledge, this is the first study that describes the course of aEEG and NIRS parameters in both hemispheres during the first five days after clinical symptoms due to PAIS. Differences in cerebral activity and oxygenation were found between the ipsilesional and contralesional hemisphere, that were also affected by size of PAIS, gestational age, use of AEDs and mechanical ventilation. Moreover, aEEG and NIRS values were found to be associated with neurocognitive development at 15-24 months of age, even after correction for size of the lesion.

The $rScO_2$ decreased during the first five days after clinical symptoms of PAIS in both hemispheres, but less in the ipsilesional than in the contralesional hemisphere. This was also reflected by the $rScO_2$-asymmetry, resulting in a significant $rScO_2$-asymmetry from day 3 onward. A relatively low $rScO_2$ of the ipsilesional hemisphere can be explained by a state of hypoperfusion in the first few hours after PAIS.[23] A subsequent relative rise of $rScO_2$ of the ipsilesional hemisphere can be due to hyperperfusion (due to 'luxury perfusion')[23,24] This state of luxury perfusion is likely to affect both hemispheres, in line with abnormal brain activity as measured by aEEG bilaterally. Alternatively, high $rScO_2$ can also be due to reduced oxygen consumption by the damaged brain tissue of the ipsilesional hemisphere. De Vis et al. demonstrated higher $rScO_2$ values accompanied by hypoperfusion, as measured by NIRS and arterial spin labelling on day 5-6, in the ipsilesional hemisphere in 3 out of 4 cases with PAIS.[25] We hypothesize that the persisting relatively high $rScO_2$ ipsilesional is explained by initial luxury perfusion, gradually progressing to a normal/low perfusion state accompanied by decreased O_2 consumption. The contralesional $rScO_2$ (initially as high as ipsilesional) reflecting the same initial luxury perfusion state of the brain, with subsequent normalization of perfusion accompanied by rather stable O_2 consumption during day 0-5. Impaired autoregulation of the brain after injury could be a plausible explanation for this phenomenon and would be an interesting aspect to explore in future studies.

In healthy term infants, normal background pattern and sleep-wake cycling is present in 99% and 96% of infants directly or within a few hours after birth.[26] Overall in our cohort, PAIS led to a disruption of background pattern and sleep-wake cycling in both hemispheres, with more than 40% of the infants not having a normal background pattern within 1 day after the onset of the first clinical symptoms of PAIS. Additionally, only 59% showed mature sleep-wake cycling 72 hours after first symptoms. Several studies reported an impaired sleep-wake cycling and a suppressed background pattern in infants with PAIS or clinical seizures.[27,28] In line with our results, cerebral

activity was more severely affected in the ipsilesional hemisphere, especially after large PAIS.[28]

Cerebral activity and oxygenation were not only negatively influenced by PAIS and size of the lesion, but also by administration of AEDs and mechanical ventilation. Seizures and the use of AED medication, in particular midazolam, are known to be associated with apneas and the need for mechanical ventilation.[29] The effect of AED on BGP is known to be more pronounced in infants with more severe brain injury. This explains the effect of AEDs and mechanical ventilation on AR- $rScO_2$-asymmetry, as the damaged ipsilesional hemisphere responded more to sedatives than the contralesional hemisphere.[30] In this study, the use of AEDs and use of sedation during mechanical ventilation are clinical factors associated with neuromonitoring that could be managed during NICU admission. Although both will have an initial negative affect on background activity, more rapid control of convulsions will ultimately improve recovery of background activity. However, our results warrant critical evaluation of the use of AEDs and sedation after PAIS, as these might negatively influence neurodevelopmental outcome.

Neuroimaging using MRI is the gold standard to diagnose PAIS and various studies have demonstrated its predictive ability for neurodevelopmental outcome.[6] Our study confirms that size of the lesion on neonatal MRI is indeed strongly related to cognitive and motor outcome at 15-25 months of age. In univariate analyses, unfavorable outcome was associated with increased time to normal background pattern and increased $rScO_2$ in the ipsilesional hemisphere, in line with several other studies in other neonatal populations.[12–17] Our group previously reported that combining aEEG and NIRS parameters in infants with hypoxic-ischemic encephalopathy in a multivariable model was associated with neurological outcome[12,17], which is comparable to our multivariable model. After correction for size of the lesion, time to normal background pattern at the ipsilesional hemisphere and $rScO_2$-asymmetry were still independently associated with impaired cognition. MRI predictors for cognitive deficit after PAIS usually include size or location of the lesion, but more specific regions of interest on MRI have not been well established.[6,9] Our study is the first to demonstrate that NIRS and aEEG may add to prediction of cognitive outcome. It also provides targets for potential new therapies to improve cognition, for example neuroprotective agents at times of increased $rScO_2$-asymmetry to protect the brain from O_2-radicals produced after luxury perfusion.

The study cohort consisted of patients that presented with clinical symptoms of PAIS, mostly hemi-convulsions or apneas, thereby excluding those without any evident clinical symptoms, and mostly likely those with smaller infarcts.[31] This reduced the number of eligible infants and thereby the statistical power of our study. However, the

advantage is that our registration data are homogeneous as all patients presented with symptoms of PAIS, allowing comparison of neuromonitoring data over time. Since the exact moment of PAIS is unknown, onset of seizures was used as the start of our study period, but some infants may exhibit seizures after PAIS later than others. Although this may have influenced our results, timing of seizure onset after perinatal stroke needs to be elucidated in future studies.

Neuromonitoring data were collected as part of standard clinical care and recordings were usually started several hours after onset of clinical symptoms, because most infants were outborn. This resulted in many missing data between symptoms and start of the recording during the first day after symptoms. Patients with smaller strokes often showed a normal background pattern on aEEG at the start of the recording, so the exact effect of PAIS on the background pattern during the first hours is unknown in these infants. Consequently, the average difference in time to normal background pattern reported here is still conservative, however do reflect standard clinical practice after PAIS.

For this study, outcome parameters such as development of USCP and cognitive deficit were collected between 15-25 months of age. Follow-up studies are continued till school-age to see whether neurological outcome remains stable over time, or other disabilities present later in life (e.g. attention disorders), and how neuromonitoring tools relate to these.

CONCLUSIONS

PAIS affects cerebral activity and oxygenation in both hemispheres. As neuromonitoring parameters are related to neurodevelopmental outcome, these results illustrate that aEEG and NIRS monitoring may provide useful information for early prognosis of PAIS, especially of cognition. Before drawing firm conclusions, more prospective research is necessary with a larger study population and with more complete registrations during several days. However, continuous neuromonitoring is a promising way to optimize care. Due to the prognostic value, aEEG and NIRS could potentially be used to monitor the effect of early interventions.

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I can't change the wind,
but I can adjust my sails to
always reach my destination.
Jimmy Dean

PART II

FUTURE THERAPIES FOR PERINATAL STROKE

CHAPTER 8

PROMOTING NEUROREGENERATION AFTER PERINATAL ARTERIAL ISCHEMIC STROKE: NEUROTROPHIC FACTORS AND MESENCHYMAL STEM CELLS

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ABSTRACT

Newborns suffering from perinatal arterial ischemic stroke (PAIS) are at risk of neurodevelopmental problems. Current treatment options for PAIS are limited and mainly focus on supportive care, as presentation of PAIS is beyond the time window of current treatment strategies. Therefore, recent focus has shifted to interventions that stimulate regeneration of damaged brain tissue. From animal models it is known that the brain increases its neurogenic capability after ischemic injury, by promoting neural cell proliferation and differentiation. However, neurogenesis is not maintained at the long-term, which consequently impedes full repair leading to adverse consequences later in life. Boosting neuroregeneration of the newborn brain using treatment with neurotrophic factors and/or mesenchymal stem cells may be promising novel therapeutic strategies to improve neurological prospects and quality-of-life of infants with PAIS. This review focuses on effectiveness of neurotrophic growth factors, including EPO, BDNF, VEGF, GDNF, and mesenchymal stem cell therapy in both experimental neonatal stroke studies and first clinical trials for neonatal ischemic brain injury.

INTRODUCTION

Perinatal arterial ischemic stroke (PAIS) is an important cause of perinatal morbidity which occurs in 1 in 2500 - 4000 live births, forming a large burden for patients and society worldwide.[1,2] PAIS manifests itself most often with one-sided seizures in the first week after birth, often accompanied with (asymmetric) hypotonia, lethargy and apnea.[1,3,4] Using neuro-imaging, it is commonly identified as a focal wedge-shaped cerebral lesion that leads to loss of all cell types in that part of the brain. PAIS is most often detected in the middle cerebral artery (MCA) with a predilection for the left MCA.[3–5] The exact underlying pathology is unknown, but the most important risk factors are intrapartum or peripartum complications, such as prolonged rupture of membranes, thick meconium and abnormal cardiotocography.[6] In 50-75% of infants, neonatal stroke leads to abnormal motor and neurodevelopmental outcome including cerebral palsy, cognitive dysfunction, behavioral disorders and epilepsy.[5] Current treatment options for PAIS mainly focus on supportive care, such as controlling hypoglycemia and treatment of (subclinical) seizures. As these treatments offer limited protection, additional therapeutic strategies for PAIS are urgently needed. Early neuroprotective treatments mainly aim at preventing production of free radicals and apoptotic cell death. For example, therapeutic hypothermia, started early after birth, has shown benefit in newborns with hypoxic-ischemic encephalopathy.[7] However, hypothermia is only beneficial when applied within six hours after a well-documented hypoxic-ischemic insult followed by moderate to severe encephalopathy.[8] As cerebral abnormalities evoked by PAIS often do not present themselves within this short therapeutic timeframe, these neuroprotective treatment strategies are not applicable to PAIS. Due to the relative late diagnosis, therapeutic focus has rather shifted to interventions that stimulate repair of the damaged newborn brain.[9] In most recent years, preclinical research has aimed to develop additional therapies that boost endogenous regenerative pathways, as these will be critical for improving outcome in the severely affected PAIS patients.

Neuroregeneration

Neural stem cells (NSC) residing in the subventricular zone (SVZ) and subgranular zone (SGZ) of the hippocampus are self-renewing and are capable of differentiating into neurons, astrocytes, and oligodendrocytes. These processes of neurogenesis and gliogenesis continue throughout life but decrease with age. After (hypoxic-) ischemic brain injury, the brain enhances its neurogenic capability by promoting proliferation of young precursors in the SVZ and SGZ, but also in the striatum, cortex, hippocampus.[10,11] Growth factors, which regulate several cellular processes including apoptosis, inflammation, angiogenesis, cell differentiation and proliferation, are increasingly expressed following exposure to hypoxia-ischemia and thereby aid in

boosting neurogenesis.[12] However, long-term neurogenesis is not maintained after hypoxic-ischemic (HI) brain injury, which consequently impedes full repair. It is therefore crucial to assist the brain during regeneration of injured areas. Stimulation of neurogenesis by exogenous administration of neurotrophic factors has been studied in the context of neonatal brain injury, especially in preclinical research. Furthermore, boosting endogenous neuroregeneration by administration of stem cells has also gained considerable attention in the treatment of neonatal brain injury. Therefore, this review will provide an up-to-date evaluation of preclinical and clinical evidence for potential future neuroregenerative therapies for neonatal stroke comprising neurotrophic factors and stem cells.

Animal models

When investigating neonatal brain injury, the use of highly reproducible and clinically relevant animal models is crucial. The most commonly used rodent model for neonatal brain injury is the Rice-Vannucci model, which combines permanent unilateral carotid artery ligation with exposure to systemic hypoxia in newborn pups.[13,14] This model however contains a systemic hypoxic component that better reflects HI encephalopathy caused by perinatal asphyxia in human neonates rather than PAIS. Therefore, specific models of neonatal stroke have been designed in newborn rodents that use permanent or transient occlusion of the MCA (MCAO) leading to (more) focal ischemia. Studies have shown contrasting variability between HI and stroke models and also the pathophysiological underlying mechanisms differ between HI encephalopathy and stroke.[15] As this review aims at covering specifically PAIS, the experimental data discussed in this paper will focus on regenerative therapies in animal models of neonatal *stroke* only.

Promoting neuroregeneration: animal models of neonatal stroke

Erythropoietin (EPO)

EPO is originally known for its role in erythropoiesis and it has long been used to treat anemia in premature infants.[16,17] However, non-hematopoietic effects of EPO have also been shown. EPO is produced in the developing brain by multiple cell types including neurons, astrocytes, oligodendrocytes and microglia and it promotes growth of the central nervous system.[18,19] EPO is upregulated after cerebral injury, a process regulated by transcription factor HIF-1, a factor stabilized by hypoxic conditions.[20,21] *In vitro,* EPO has been proven to exert neuroprotection against neuronal injury as it can reduce free radical formation, inflammation, and apoptosis in neuronal cultures.[22] In vitro and in vivo studies have demonstrated that EPO not only prevents ischemia-induced cell death (i.e. acts neuroprotective) but also stimulates neuronal differentiation of neural progenitor cells.[23–25] Experimental rodent studies using

neonatal stroke models have shown that treatment with EPO substantially reduces infarct volume[26,27] as well as improves motor and cognitive function.[28,29] A review from our group summarized neuroregenerative effects of EPO in neonatal experimental *in vivo* studies, including MCAO models.[30] In general, EPO administration after MCAO in postnatal day(P)7-10 rat pups was found to improve neurogenesis, as measured by increased brain volume up to 70% (table 1).[30] More recently is was shown that delayed EPO treatment, up to one week after the onset of neonatal stroke, improved histological as well as functional outcome which underlines the involvement of EPO as a trophic factor stimulating neurogenesis.[31] Gonzalez et al. also demonstrated that EPO treatment after neonatal stroke in rats stimulated neural progenitor cells proliferation in the subventricular zone and migration of these progenitors to the site of the injury, again emphasizing the neuroregenerative effects of EPO.[32]

Brain-derived neurotrophic factor (BDNF)

BDNF is a neurotrophic factor known to promote neural cell proliferation and survival in the developing human brain.[33,34] BDNF protein expression is especially high in the hippocampus, but BDNF can affect survival and proliferation of several neural cells, including cerebellar and cortical neurons.[33,35] The neurogenic effects of BDNF are mediated via activation of two different receptor pathways: the p75 neurotrophin receptor and the tyrosine kinase receptor B (TrkB), that activates MAPK pathways. BDNF levels rapidly increase in response to brain injury in neonatal rats[36,37], leading to reduced neuronal apoptosis and increased neuronal survival.[37] *In vitro*, BDNF improves survival of hypoxic-hypoglycemic hippocampal neurons by reduction of apoptosis through TrkB.[38] Other *in vitro* studies demonstrated that BDNF enhanced neurite outgrowth of neonatal cortical neurons in the presence of astrocytes.[39]

To the best of our knowledge, BDNF has never been studied in neonatal rodent models of PAIS. However, in neonatal rat models of focal cerebellar injury, BDNF treatment stimulated axonal regrowth leading to re-innervation of the cerebellum (table 1).[40] In addition a neonatal rat model for motorneuron axotomy demonstrated the beneficial effect of BDNF on motor neuron survival up to 30 days after injury.[41] A neonatal mouse model mimicking periventricular leukomalacia in preterm newborns, showed that intraparenchymal injections of BDNF (at P5) reduced cortical gray and white matter lesions with 36% and 60% respectively. Protective effects of BDNF treatment in this excitotoxic mouse model were associated with TrkB receptor/MAPK pathway activation and reduced apoptosis.[42] However, timing of BDNF administration seemed crucial, as BDNF treatment at postnatal day P0 exacerbated neuronal death and at P10 did not have any effects on periventricular leukomalacia in neonatal mice.[42] Therefore more preclinical evidence for beneficial effects of exogenous BDNF administration is needed to determine the exact treatment regimen and possible risks for neonatal stroke.

TABLE 1 | Neurotrophic factor therapy in animal models of neonatal stroke.

Paper	Growth Factor	Animal model	Type of ischemic injury	Dosing and timing of treatment
Dixon & Sherrard, Exp. Neurology 2006	BDNF	Neonatal rat: P15 or P30	Unilateral transection of olivocerebellar pathways	1 dose of 1µL of rh-BDNF (4 µmol/L) at 24h after injury
Husson et al., Cerebral Cortex, 2005	BDNF	Neonatal mouse: P5	Focal excitotoxic lesions: Periventricular leukomalacia	1 dose of 0.5, 5 or 50ng rh-BDNF simultaneously with excitotoxic drugs
Bemelmans et al., J of Neurosci Res, 2006	BDNF	Neonatal mouse: P5	Focal excitotoxic lesions: Periventricular leukomalacia	1 dose of 10 ng or 25 ng of LV-BDNF (with BDNF level reaching 0.086-0.118 pg/mg protein) 3 days before inducing lesions (P2)
Sola et al., Pediatr Res, 2005	EPO	Neonatal rat: P7	Permanent MCAO	1 dose of 1000 U/kg rh-EPO at 15 min. after injury and 2 doses of 100, 1000, or 5000 U/kg once a day for 2 days
Chang et al., Pediatr Res, 2005	EPO	Neonatal rat: P10	Transient MCAO: 45 minutes	1 dose of 5 U/g rh-EPO immediately upon reperfusion
Gonzalez et al., Dev Neurosci, 2007	EPO	Neonatal rat: P10	Transient MCAO: 45 minutes	1 dose of 5 U/g rh-EPO immediately upon reperfusion
Gonzalez et al., Dev Neurosci, 2009	EPO	Neonatal rat: P10	Transient MCAO: 45 minutes	1 dose of 5 U/g rh-EPO immediately upon reperfusion or 1 dose of 1 U/g immediately upon reperfusion followed by 1 U/g at 24h and 7 days after injury.
Larpthaveesarp et al., Neurobiol Dis, 2016	EPO	Neonatal rat: P10	Transient MCAO: 180 minutes	3 doses of rh-EPO 1000 U/kg started one week after injury
Wen et al., Neuroscience, 2006	EPO	Neonatal rat: P7	Permanent MCAO	3 doses of rh-EPO 1000 U/kg started 15 min. after injury and repeated once a day for 2 days
Dzietko et al., Trans Stroke Res, 2013	VEGF	Neonatal rat: P10	Transient MCAO: 90 minutes	1 dose of 1.5µg/kg rh-VEGF at day 8 after injury (P18)

	Dosing and timing of treatment	Administration route	Effect
	1 dose of 1μL of rh-BDNF (4 μmol/L) at 24h after injury	Intracerebellar injection	BDNF induced axonal growth by transcommissural olivocerebellar reinnervation at 7 days after injury.
	1 dose of 0.5, 5 or 50ng rh-BDNF simultaneously with excitotoxic drugs	Intracerebral (intraparenchymal)	BDNF reduced 36 and 60% of cortical and white matter lesions, involving TrkB receptors, MAPK pathway and reduced apoptosis at 120h after injury. At P0 BDNF exacerbated neuronal death. At P10 BDNF did not have an effect.
	1 dose of 10 ng or 25 ng of LV-BDNF (with BDNF level reaching 0.086-0.118 pg/mg protein) 3 days before inducing lesions (P2)	Intraventricular delivery of BDNF –expressing lentiviral vector	BDNF induced protection against ischemic injury at 5 days after injury. Viral-mediated gene transfer was more efficient for neuroprotection than the intraparenchymal route.
	1 dose of 1000 U/kg rh-EPO at 15 min. after injury and 2 doses of 100, 1000, or 5000 U/kg once a day for 2 days	Intraperitoneal	EPO reduced the number of apoptotic cells and decreased infarct area and volume at 3 days after injury providing significant neuroprotection. 1000 U/kg rh-EPO for 3 days was the most effective dose.
	1 dose of 5 U/g rh-EPO immediately upon reperfusion	Intraperitoneal	EPO preserved hemispheric brain volume and improved functional outcome (by decreasing forelimb asymmetry) at 2 weeks after injury.
	1 dose of 5 U/g rh-EPO immediately upon reperfusion	Intraperitoneal	EPO preserved hemispheric brain volume, increased neurogenesis and decreased gliogenesis at 6 weeks after injury.
	1 dose of 5 U/g rh-EPO immediately upon reperfusion or 1 dose of 1 U/g immediately upon reperfusion followed by 1 U/g at 24h and 7 days after injury.	Intraperitoneal	EPO increased brain volume and improved functional outcome (spatial learning and memory performance) at 2-4 months after MCAO.
	3 doses of rh-EPO 1000 U/kg started one week after injury	Intraperitoneal	Delayed EPO treatment improved histological and functional outcome at 4 weeks after MCAO.
	3 doses of rh-EPO 1000 U/kg started 15 min. after injury and repeated once a day for 2 days	Intraperitoneal	EPO reduced infarct volume and improved sensorimotor function recovery at 6-12 weeks after MCAO. The effect was more beneficial in female compared to male rats.
	1 dose of 1.5μg/kg rh-VEGF at day 8 after injury (P18)	Intracerebroventricular	VEGF enhanced angiogenesis, endothelial proliferation and vessel volume leading to improved brain injury recovery at 1 week after injury.

Paper	Growth Factor	Animal model	Type of ischemic injury	Dosing and timing of treatment
Matheson et al., J of Neurobiol, 1997	GDNF	Neonatal rat: P1	Sciatic nerve axotomy	Pretreatment: 1 dose of 25µL of GDNF (1mg/mL). Post-injury: Gelfoam soaked in 1mg/mL GDNF immediately after injury; and reinjection with 5µL (1mg/mL) on P4, P5 and P6.
Morcuende et al., Neuroscience, 2013	GDNF and BDNF	Neonatal rat: P0 (6-24h old)	Extraocular motorneuron axotomy	BDNF and GDNF both 1 dose of 5µg immediately after injury

BDNF, brain-derived neurotrophic factor; EPO, erythropoietin; GDNF, glial-derived neurotrophic factor; LV-BDNF, lentiviral-mediated gene transfer of BDNF; MAPK, mitogen-activated protein kinase; MCAO, occlusion of the middle cerebral artery; P, postnatal day; TrkB, tyrosine kinase receptor B; VEGF, vascular endothelial growth factor; rh, recombinant human.

Vascular endothelial growth factor (VEGF)

VEGF is a factor produced by neurons and astrocytes in the developing brain. Experimental data indicate that VEGF is involved in several stages of neurodevelopment, including migration, differentiation, synaptogenesis and myelination.[43] Furthermore, VEGF stimulates vascular processes like angiogenesis and vasculogenesis by stimulating endothelial cell proliferation and migration via Flk1 and by improving vascular stabilization via Ang1. [44,45] VEGF also enhances blood-brain barrier maintenance. These vascular processes together contribute to an optimal microenvironment for NSCs to drive neuronal regeneration, thereby demonstrating an important role for VEGF in brain repair.[46,47] Experimental studies demonstrate that VEGF expression is increased in the brain after neonatal stroke in vivo.[48] Interestingly, increased VEGF expression after neonatal cerebral ischemia is associated with NSC proliferation and differentiation.[49] Furthermore, it has been shown that inhibition of the VEGF receptor-2 after neonatal stroke worsened injury, increased cell death and reduced endothelial cell proliferation in 10-day old rats, indicating a role for VEGF signaling in recovery and repair after ischemic brain injury.[50]

Dzietko et al. have shown that Intracerebroventricular (i.c.v.) VEGF treatment enhanced angiogenesis, endothelial proliferation and vessel volume leading to improved recovery of brain injury after MCAO in P10 rats (table 1).[51] Experimental *adult* stroke models have demonstrated that timing of VEGF administration is crucial: early administration leads to brain edema, whereas late application has desired neuroprotective effects.[52] In contrast, in newborn rats with HI early VEGF administration (i.e.at 5 minutes to 3 days after injury) resulted in decreased brain

	Dosing and timing of treatment	Administration route	Effect
	Pretreatment: 1 dose of 25µL of GDNF (1mg/mL). Post-injury: Gelfoam soaked in 1mg/mL GDNF immediately after injury; and reinjection with 5µL (1mg/mL) on P4, P5 and P6.	Pretreatment: subcutaneous. Post-injury: Implantation of GDNF-soaked Gelfoam into nerve stump	GDNF improved neuronal survival in dorsal root ganglions to nearly 100% at 7 days after injury.
	BDNF and GDNF both 1 dose of 5µg immediately after injury	Implantation of BDNF or GDNF-soaked Gelfoam into orbit	BDNF and GDNF both rescued extraocular motorneurons at 30 days after injury. GDNF was more potent for survival than BDNF. Combining GDNF and BDNF was not more effective.

damage, possibly via reduced neuronal apoptotic cell death in the cortex and hippocampus.[53,54] These studies indicate that in the neonatal brain VEGF does not only support neuroregeneration after brain injury via angiogenesis but may also exert neuroprotective properties by reducing apoptosis via the Akt/ERK signaling pathway. Overall, late administration of VEGF seems most favorable in neonatal stroke, as diagnosis is usually late after assumed onset of PAIS. However, the exact timing of VEGF for neonatal stroke remains to be studied.

Glial-derived neurotrophic factor (GDNF)

GDNF is a member of the transforming growth factor β superfamily, produced by glial cells and neurons, and plays an important role in neuronal differentiation during normal development.[55] *In vitro*, GDNF was found to increase the number of surviving neonatal rat corticospinal motor neurons.[56] After central or peripheral nervous system injury, GDNF also promotes survival and recovery of several types of mature neurons, including motor and dopaminergic neurons.[57,58] In neonatal rats with hypoxic-ischemic brain injury, GDNF levels in serum and brain were upregulated by 48 hours and returned to normal by 7 days.[59] and increased GDNF levels were associated with reduced neuronal apoptosis, indicating that GDNF may reduce neonatal brain injury.[59]

Although GDNF has not been studied in the treatment of neonatal stroke in rodents (table 1), GDNF treatment induced nearly 100% neuronal survival of dorsal root ganglions after sciatic nerve axotomy in newborn rats.[60] In neonatal rats, GDNF was

also found to rescue extraocular motorneurons from axotomy-induced cell death at 30 days after injury.[41] To translate GDNF treatment to the clinic for the treatment of PAIS, more preclinical research is needed to overcome several potential hurdles. For example, in contrast to EPO and BDNF, GDNF does not cross the blood-brain barrier which makes exogenous administration of GDNF more difficult.[61] Additionally, GDNF exerts only transient effects, so repeated administration into the cerebral or ventricular space would be required.[62]

MSC therapy

Overall, the levels of growth factors that are naturally available during brain development and which are upregulated after an ischemic insult are unable to accomplish full repair of the injured neonatal brain. As described above, boosting neuroregeneration after neonatal brain injury by exogenous administration of single growth factors has been shown beneficial in many experimental studies. These factors can stimulate repair of the neonatal brain by promoting neurogenesis and stimulating neural cell survival (figure 1). Moreover, numerous studies have been performed to explore the potential of multipotent stem cells as a therapy for neonatal brain injury. Experimental data strongly indicate that stem cells secrete a plethora of trophic factors that can boost neuroregenerative processes in the injured neonatal brain.

Multipotent stem cells are capable of self-renewal and can commit to differentiate into cell types of a discrete lineage. For example, hematopoietic stem cells give rise to several blood cell types, such as erythrocytes, lymphocytes and neutrophils. Other multipotent stem cell types include NSCs and mesenchymal stem cells (MSCs). MSCs can differentiate into cells of the mesoderm, such as bone, cartilage or fatty tissue, but it has been demonstrated that MSCs are also capable of developing into neuronal cells, given specific conditions.[63] MSCs display various characteristics that can be favorable as a regenerative therapy for neonatal brain injury. Firstly, MSCs are found in several birth-related tissues, including the placenta, umbilical cord and Wharton's Jelly.[64] These resources seem ideal for easy and non-invasive isolation of MSCs and allow them to be used in an autologous manner if collected at time of birth. Moreover, MSCs do not express MHC class II, making them excellent candidates for allogenic transplantation, as they do not cause immune responses/ graft-versus-host disease.[65] Given their potent neuroregenerative properties and favorable immunological profile, MSCs seem to be promising for neuroregenerative medicine in neonatal brain injury. [64] In animal models of neonatal brain injury, MSC therapy has been shown to improve motor function and cognitive behavior by stimulating neurogenesis, gliogenesis, and axonal remodeling as will be discussed below. Therefore, MSCs could function as miniature factories of a mixture of growth factors to repair neonatal brain injury in a tailor-made way.

MSCs have been shown to be effective in repairing brain tissue after neonatal stroke (table 2). Kim et al. treated P10 rats i.c.v. with umbilical cord-derived MSCs at 6 hours after permanent MCAO.[66] At day 28, they observed that MSC transplantation had significantly attenuated brain infarct volume measured by MRI and had improved functional motor performance.[66] Other studies have focused on non-invasive routes of MSC administration, such as intranasal MSC treatment, which specifically targets the brain.[67] It has been shown that MSCs migrate rapidly towards the lesioned brain area, i.e. within 2 hours, after intranasal administration.[68,69] Wei et al. intranasally administered bone marrow-derived MSCs to P7 rat pups at 6 hours and 3 days after induction of stroke.[70] At P24, MSC treatment had significantly reduced infarct volume and blood-brain barrier disruption, and increased angiogenesis leading to neurovascular repair and improved cerebral blood flow. MSC treatment also stimulated neurogenesis, leading to better sensorimotor and social functions.[70] In addition, van Velthoven et al. showed that intranasal MSC treatment at 3 days after neonatal stroke also significantly reduced infarct size and white and gray matter loss in newborn rats, leading to improved motor performance at 28 days after the infarct.[71,72]

From a mechanistic point of view it is important to note that the number of MSCs at the lesion site drastically decreases at 12 hours after intranasal administration and the majority of cells does not survive more than 72 hours.[69,73] These findings indicate that the regenerative effects of MSCs in the neonatal brain are not caused by integration of transplanted MSCs themselves, but rather by their paracrine effects. It was demonstrated that MSC administration after neonatal HI injury in mice specifically regulates cerebral expression of genes regulating both proliferation and survival.[73] *In vitro*, MSCs that promote axon growth in developing rats were shown to express BDNF and VEGF.[74] Furthermore, MSCs that were cultured in presence of ischemic vs control brain extracts show a specific upregulation of several growth factors indicating that MSCs can adapt their secretion profile according to the tissue milieu.[75] We hypothesize that the secretion of several neurotrophic factors by MSCs modulates the neurovascular niche to promote endogenous repair of the injured neonatal brain.

TABLE 2| Mesenchymal stem cell therapy in animal models of neonatal stroke.

Paper	Therapy	Animal model	Type of ischemic injury	Dosing and timing of treatment	Administration route	Effect
Kim et al., Pediatr Res, 2012	Human umbilical cord blood-derived MSCs	Neonatal rat: P10	Transient MCAO: 60 minutes	1 dose of 1 x 10^5 MSCs in 10µL of PBS at 6 hours after injury	Intraventricular	MSC transplantation decreased brain infarct volume (by MRI), improved histological abnormalities and improved functional motor outcome.
Wei et al., Cell Transplant, 2015	Rat hypoxic-preconditioned bone marrow-derived MSCs	Neonatal rat: P7	Permanent MCAO	2 doses of 1 x 10^6 MSCs in 100µL of PBS at 6 hours and 3 days after injury	Intranasal	MSCs reduced infarct size, promoted angiogenesis, neurogenesis, neurovascular repair and improved local blood flow. Additionally, MSCs improved sensorimotor function at 17 days after injury.
Van Velthoven et al., Stroke, 2013	Rat MSCs (GIBCO, bone marrow-derived?)	Neonatal rat: P10	Transient MCAO: 90 minutes	1 dose of 1 x 10^6 MSCs in 20µL PBS (as 2 doses of 5µL per nostril) at 3 days after injury	Intranasal	MSCs reduced infarct volume, white and gray matter loss and improved motor deficits until 28 days after injury.
van Velthoven et al., J Neurosci Res 2017	Rat MSCs (GIBCO, bone marrow-derived?)	Neonatal rat: P10	Transient MCAO: 90 minutes	1 dose of 1 x 10^6 MSCs in 20µL PBS (as 2 doses of 5µL per nostril) at 3 days after injury	Intranasal	MSCs attenuate white matter injury (on MRI) and enhance somatosensory function 28 days after injury.

MCAO, occlusion of the middle cerebral artery; MRI, magnetic resonance imaging; MSC, mesenchymal stem cell; P, postnatal day; PBS, phosphate-buffered saline.

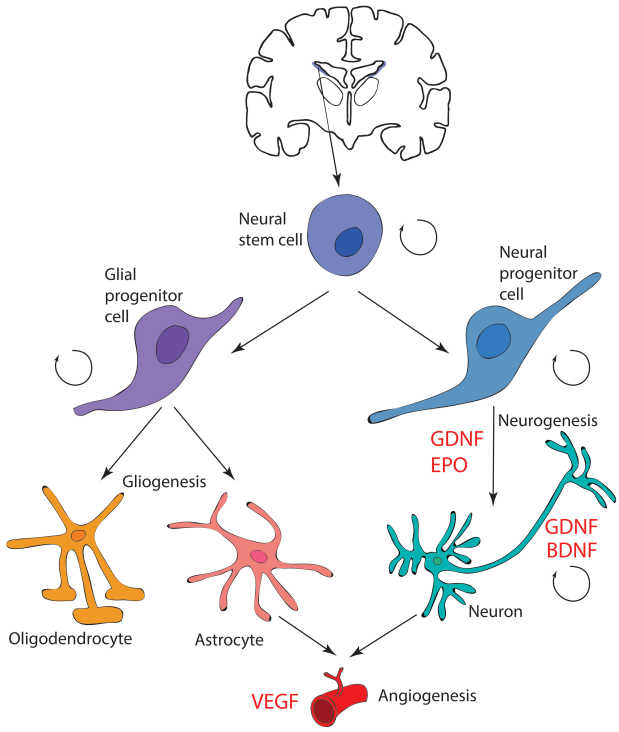

FIGURE 1 | The effects of neurotrophic factors on neurogenesis and gliogenesis. Neural stem cells from the subventricular zone differentiate into two lineages of progenitor cells: glial and neural progenitor cells, resulting in gliogenesis and neurogenesis respectively. EPO stimulates neuronal differentiation of neural progenitor cells. GDNF is also involved in neurogenesis by increasing the number of neurons, but additionally promotes survival and recovery of several types of mature neurons. BDNF is involved in neural cell proliferation and survival. VEGF mainly stimulates vascular processes like angiogenesis and vasculogenesis.

Animal models of adult stroke

In parallel to *neonatal* stroke, quite extensive preclinical and clinical research has been performed to study neuroregenerative therapies for the treatment of *adult* ischemic stroke. It is important to realize that results from adult trials cannot be directly translated to neonatal care for several reasons. First of all, the newborn brain has much greater plastic capacity than the brain of older children and adults. This causes the newborn to recover relatively more easily after brain injury than an adult.[76,77] Secondly, effectiveness of neuroregenerative treatments reduces with growing age, because endogenous neurogenesis capacity declines with age. In other words, the neonatal brain has more potential to regenerate damaged tissue than the adult brain.[78–81] Lastly, PAIS is a different disease than adult stroke, with different pathology and symptomatology.[82] Despite these differences, the results from experimental adult stroke studies provide biological plausibility support for potential effectiveness of interventions in PAIS and will therefore be discussed shortly below.

Neurotrophic factors

Preclinical studies in rodent models of adult stroke have also shown potential effectiveness of administration of the discussed factors EPO, BDNF, VEGF and GDNF in stroke recovery.[83–87] However, neuroregenerative effects of many more (hematopoietic) growth factors, such as G-CSF, EGF, FGF-2 and IGF were described for adult stroke as well. All these factors are upregulated after focal cerebral ischemia and potentially play a neuroregenerative role by stimulating neurogenesis and angiogenesis, and inhibiting neuronal death.[88] A review from 2011 provides an overview on experimental studies of acute ischemic stroke in adult rodents using hematopoietic growth factors and neurotrophins. The review summarizes results of studies which demonstrate that these factors both reduce infarct size, brain edema and apoptosis, as well as stimulate cell proliferation, survival of new mature neurons and neovessel formation, subsequently leading to improved clinical outcome.[88] The authors conclude that these growth factors were shown to be potentially effective in experimental models of adult stroke, but the therapeutic potential of many of these growth factors still needs to be investigated in experimental *neonatal* stroke. Although a few reports are available on the beneficial effects of G-CSF [89–91], bFGF [92] and IGF-I [93,94] in neonatal HI brain injury, evidence is limited to date and therefore beyond the scope of this review.

MSC therapy

A large meta-analysis from Vu et al. identified 46 studies that report on the use of MSC treatment in animal models of adult stroke.[95] Of these, 44 reported that MSCs significantly improved neurological outcome. Effect sizes varied significantly with administration route and species, and had a median of 0.9 for reduction of infarct volume to 1.8 for Neurological Severity Score. The authors concluded that the effect of MSC therapy was very robust and consistent over different studies, species, routes and treatment protocols, and translation of MSC treatment in ischemic stroke in (adult) humans should be further enhanced.[95]

Promoting neuroregeneration in neonatal clinical trials

Neurotrophic factors

EPO has been studied most often clinically in the context of neonatal brain injury. Our group recently performed a clinical study in 20 full-term infants with PAIS, who were treated with three doses of recombinant human erythropoietin (rhEPO) 1000U/kg i.v.[96] Most importantly, no adverse effects of rhEPO were observed. Furthermore, volumetric MRI measurements of the stroke area were smaller in 10 rhEPO-treated infants compared to 10 non-treated PAIS historical controls, but this effect did

not reach significance.[96] A randomized controlled trial (RCT) from our group is currently undertaken to show the effect of darbepoetin on stroke tissue loss and neurodevelopmental outcome in PAIS patients (ClinicalTrials.gov: NCT03171818). Other groups have described the use of rhEPO or darbepoetin in the context of HIE.[97–100] A few RCTs are currently studying full-term infants with perinatal asphyxia who are receiving hypothermia with rhEPO as an add-on therapy and the results are promising.[101,102] Most importantly, it was concluded that repeated rhEPO treatment regimens were well tolerated without any serious adverse events .[97,99–103]

Currently, there is no evidence from human studies on treatment of PAIS with BDNF, VEGF or GDNF. However, altered levels of neurotrophic factors (including EPO, VEGF and BDNF) in serum, CSF and/or cord blood have been described for neonates with perinatal asphyxia, hydrocephalus and intraventricular hemorrhage, which were, in some studies, related to higher severity of brain injury.[104–109] Other types of neonatal brain injury including PAIS, are also very likely to alter levels of neurotrophic factors in serum as a response to brain damage, but this remains to be studied. Studies in adult humans have also found correlations between neurotrophic factors and progenitor cells in serum and severity and outcome of ischemic stroke[110,111], suggesting that neurotrophic factors may serve as predictive parameters in clinical care. This also seems a potential field of interest when setting up future studies in neonatal stroke.

MSC therapy

At present, no results are available of clinical trials on the treatment of neonatal stroke with MSCs. There is only one clinical trial describing the use of MSCs in treatment of neonates.[112] Chang et al. showed in a phase I dose-escalation trial the safety and feasibility of intratracheal administration of allogeneic umbilical cord-derived MSCs in nine preterm infants with high risk for bronchopulmonary dysplasia (BPD) compared to historical case-matched controls. Importantly, no serious adverse events or toxicity related to a higher dose were observed in this study.[112] The same study group is currently performing a Phase I study in preterm neonates with severe intraventricular hemorrhage using i.c.v. administered umbilical cord-derived MSCs (ClinicalTrials.gov: NCT02274428).

In addition, the group of Cotten et al. has treated neonates with HIE with autologous umbilical cord blood cells and first results are promising as no safety concerns were observed.[113] Although the exact cell types in the cord blood were not specified, it is hypothesized that cord blood contains MSCs among other progenitor cells.[114]

A large meta-analysis reported on a total of 1012 adult and pediatric patients with various pathological conditions including ischemic stroke, who were treated with either autologous or allogeneic MSCs and did not show any evidence for severe adverse effects due to MSC transplantation.[115] Including eight RCTs, the only

significant side effect was an increased risk of transient fever after MSC administration compared to the control group.[115] We hypothesize that systemic complications such as fever are probably more common when MSCs are administered systemically (e.g. intravenously) in comparison to local applications.

Clinical trials in adult stroke

In adult humans, several groups studied EPO as a treatment strategy for acute ischemic stroke, however results are conflicting. [116] [117] [118] Treatment with other neurotrophic factors has not been studied for adult stroke yet. A Cochrane review on stem cell transplantation for adult ischemic stroke identified three very small published RCTs, of which two only reported subgroups of patients.[119,120] Currently, more clinical trials studying efficacy and safety of MSCs for ischemic stroke are on their way, and safety reports until now are reassuring. [121] Results from adult stroke studies may provide supportive evidence for the feasibility and safety of interventions for PAIS. However, as described above, we feel that results from adult trials cannot be directly translated to neonatal care and detailed information on regenerative medicine for adult ischemic stroke therefore goes beyond the scope of this review.

Future perspective

Current data indicate that MSCs may improve neurological outcome after neonatal stroke by secretion of several growth and/or neurotrophic factors that boost neuroregenerative processes. However, as only few study groups have focused specifically on neonatal stroke (as opposed to HIE), replication of preclinical study results seems mandatory. These studies should mainly focus on optimizing dosing regimens and finding the optimal time window and route of administration for treatment after neonatal stroke. When these experimental results become available, clinical trials should first address safety issues regarding the use of neurotrophic factors and/or MSCs in neonatal stroke specifically. With respect to rhEPO, these steps have already been undertaken, and our current RCT will show the potential effect of erythropoiesis-stimulating agents on stroke recovery in newborns in the near future (ClinicalTrials.gov: NCT03171818).

Most recently, research has focused on combining MSC therapy with additional neurotrophic factors by administration of MSCs that overexpress a neurotrophic factor. For example, BDNF-overexpressing MSCs were intranasally administered to newborn rats with transient MCAO and were found to potently reduce infarct volume, white and gray matter loss and improve motor deficits compared to vehicle-treated rats.[72] Even though BDNF-overexpressing MSCs were not significantly better than 'normal' MSCs in improving neonatal stroke injury in this study, additional experiments are required to further elucidate the possible additional benefits of MSCs transduced

with neurotrophic factors. Results of studies assessing the effects of modified MSCs in other animal models of e.g. adult stroke or neonatal hypoxia-ischemia may serve as an example for future preclinical testing in neonatal stroke.(Table 3) For instance, in an adult acute ischemic stroke model administration of MSCs transduced with the EPO gene decreased infarct volume and improved neurological function to a significant larger extent when compared to treatment with either vehicle, normal MSCs or a combination of MSCs + rhEPO.[122] Another rodent study showed that treatment with GDNF-modified MSCs for adult stroke was effective in reducing apoptotic cells numbers and improving functional outcome, more potently than normal MSCs.[123] In line, other studies have shown that administration of VEGF-overexpressing NSCs improved functional outcome after hypoxic-ischemic brain injury in neonatal rats significantly more than normal NSCs.[47,124] We hypothesize that in future MSC therapy for neonatal brain injury may be improved by manipulating these cells to produce enhanced levels of growth factors or by combining administration of several types of MSCs with specific neurotrophic factors. For example, it was shown that combining NSC transplantation with exogenous BDNF administration improved the nervous function recovery after hypoxic-ischemic injury in neonatal rats more than NSCs alone.[125] As opposed, Ahn et al. demonstrated that MSC therapy in combination with knockdown of BDNF was ineffective in improving outcome after intraventricular hemorrhage in neonatal rats, indicating that BDNF has a pivotal role in MSC therapy for neonatal brain injury.[126] The interplay between several neurotrophic factors, MSCs or the manipulation of MSCs to stimulate neurotrophic factor production, needs to be further elucidated in preclinical studies to optimize regenerative treatment strategies for neonatal brain injury

TABLE 3 | Modified stem cells in animal models of stroke.

Paper	Stem cells	Animal model	Type of ischemic injury
Ahn et al., Cell Transplant, 2016	MSCs + BDNF-knockdown	Neonatal rat: P4	Intraventricular Hemorrhage
Wang et al., Chinese J Of Pediatr, 2008	BDNF + NSCs	Neonatal rat: P1	Hypoxic-Ischemic injury: carotid ligation + hypoxia.
Velthoven et al., Stroke, 2013	BDNF-overexpressing MSCs	Neonatal rat: P10	Transient MCAO: 90 minutes
Cho et al., Brain Res, 2010	EPO-modified MSCs	Adult rat	Transient MCAO: 120 minutes
Zheng et al., Behav Brain Res, 2012	VEGF-transfected NSCs	Neonatal rat: P7	Hypoxic-Ischemic injury: carotid ligation + hypoxia.
Yao et al., Neur Regen Res, 2016	VEGF-transfected NSCs	Neonatal rat: P7	Hypoxic-Ischemic injury: carotid ligation + hypoxia.
Wang et al., Front Integr Neurosci, 2011	GDNF-modified MSCs	Adult rat	Transient MCAO: 120 minutes

BDNF, brain-derived neurotrophic factor; EPO, erythropoietin; GDNF, glial-derived neurotrophic factor; HI, hypoxia–ischemia; MCAO, occlusion of the middle cerebral artery; MSC, mesenchymal stem cell; NSC, neural stem cell; P, postnatal day; PBS, phosphate-buffered saline; VEGF, vascular endothelial growth factor.* Full-text only available in Chinese.

	Dosing and timing of treatment	Administration route	Effect
	1 x 10^5 MSCs on P6	Intraventricular	After BDNF knockdown, MSCs were no longer effective. BDNF has a pivotal role in MSC-working mechanism.
	Unknown dose* at 7 days after injury	Intracerebral?*	BDNF stimulated survival of NSCs. BDNF stimulated proliferation and differentiation of NSCs. Functional recovery was also more improved with BDNF at 4 weeks after injury.
	1 x 10^6 MSCs in 20µL PBS (as 2 doses of 5µL per nostril) at 3 days after injury	Intranasal	BDNF-overexpressing MSCs reduced infarct volume, white and gray matter loss and improved motor deficits. However BDNF-overexpressing MSCs were not significantly better than normal MSCs at 28 days after injury.
	1 dose of 6 x 10^5 EPO-MSCs in 5µL at two weeks after injury	Intracerebral (intraparenchymal)	MSCs transduced with the EPO gene decreased infarct volume and improved neurological function at 20-35 days, even to a larger extent than normal MSCs.
	1 dose of 1 x 10^5 NSCs in 2µL at 3 days after injury	Intracerebral (intraparenchymal)	VEGF-overexpressing NSCs improved functional outcome more than NSCs alone.
	1 dose of 1 x 10^5 NSCs in 2µL at 3 days after injury	Intracerebral (intraparenchymal)	VEGF-overexpressing NSCs improved histopathological changes of HI and improved learning and memory abilities more than unmodified NSCs.
	1 dose of 500µL of MSC-suspension (5 x 10^6 cells/mL) with or without GDNF-gene expression vector at 72 hours after injury	Intravenous	MSC and GDNF-modified MSCs both improved behavioral data after three days. At 14 days after injury, GDNF-modified MSCs were more effective in reducing apoptotic cells than normal MSCs.

CONCLUSION

In conclusion, due to their neuroregenerative properties, growth factors and stem cells have a relative large therapeutic window, making them excellent candidates for novel treatment strategies to improve neurological prospects and quality-of-life of infants with PAIS.

While experimental neonatal stroke studies and first clinical trials show clear benefits, large promising potential and safety of therapies using e.g. VEGF, (rh)EPO or MSCs, their effectiveness in neonatal PAIS needs to be confirmed. The current hypothesis is that MSCs can improve neurological outcome after neonatal stroke by functioning as miniature factories secreting a wide array of growth and/or neurotrophic factors that boost neuroregenerative processes. Recent studies indicate that modification of MSCs e.g. by overexpression of specific neurotrophic factors might be even more beneficial to treat neonatal brain injury. More research is needed to determine the safety, therapeutic window and dosage of modified MSCs and to compare the potential of overexpression of the specific growth factors. An important issue for optimization of MSC-based repair treatments is to determine the potential of overexpression one specific growth factor versus combinations of growth factors or even combinations of different overexpressing MSCs at different times after the insult for effective tailor-made treatment of neonatal stroke, to eventually combat the devastating consequences of PAIS.

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CHAPTER 9

REPAIR OF NEONATAL BRAIN INJURY: BRINGING STEM CELL-BASED THERAPY INTO CLINICAL PRACTICE

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ABSTRACT

(Hypoxic)-ischemic brain injury is one of most important causes of neonatal mortality and long-term neurological morbidity in full-term infants. At present, only hypothermia in infants with perinatal hypoxic-ischemic encephalopathy has shown benefit as a neuroprotective strategy. Otherwise, current treatment options for neonatal brain injury mainly focus on controlling (associated) symptoms. Regeneration of the injured neonatal brain with stem cell-based therapies is emerging and experimental results are promising. At present, increasing efforts are made to bring stem cell-based therapies to the clinic. Among all progenitor cell types, mesenchymal stromal (stem) cells seem to be most promising for human use given their neuroregenerative properties and favorable safety profile. This review summarizes the actual state, potential hurdles and possibilities of stem cell-based therapy for neonatal brain injury in the clinical setting.

INTRODUCTION

Worldwide, 2.9 per 1000 term infants suffer from neonatal (hypoxic-)ischemic brain injury, including both hypoxic-ischemic encephalopathy (HIE) and perinatal arterial ischemic stroke (PAIS). These disorders are important causes of perinatal mortality and long-lasting neurodevelopmental problems and form a large burden for patients, their families, and society at large.[1,2] Current treatment options for neonatal (hypoxic-)ischemic brain injury mainly focus on supportive care, such as controlling hypoglycemia, treatment of convulsions, and associated infections. Therapeutic hypothermia, started within the first six hours after birth, has shown to be beneficial for neonates with HIE.[3] Pharmacological neuroprotective therapies have been explored in animal models and may be promising in improving outcome in neonates in the future.[4] However, current therapeutic possibilities are limited and no treatment is available that leads to restoration of (hypoxic-)ischemic brain damage in neonates.

Cell Therapy

Increasing experimental evidence shows that regeneration of the developing injured brain with stem cell-based therapies is promising and may serve as an effective treatment strategy. Stem cells have an intrinsic potential for self-renewal and are capable of differentiating into several cellular phenotypes.[5]

Stem cell types

Embryonic stem cells are derived from the inner mass of the blastocyst and they are truly pluripotent: able to self-renew indefinitely and to give rise to cell types from each of the three germ layers (ectoderm, endoderm, and mesoderm).[6] Given their pluripotent capacity, embryonic stem cells seem the most obvious choice for repair of brain injury, but can induce formation of teratomas after transplantation. Their application therefore raises considerable ethical concerns.[7] Multipotent adult stem cell types arise from embryonic stem cells and are subdivided in hematopoietic, neural and mesenchymal stem cells (MSC). A major advantage of multipotent neural stem cells is their possibility to derive all neural lineages, but their accessibility in humans is limited and they also carry a significant risk of tumor formation.[8] As a detailed discussion of the potential benefits and hazards of several types of stem cells is beyond the scope of this review, we refer to Fleiss et al.[5] who have published an overview of the safety aspects and therapeutic values of major stem cell types.

Among all progenitor cell types, MSCs seem to be most promising for near-future use in humans given their potent neuroregenerative properties and favorable immunologic profile.[9] MSCs can differentiate into mesodermal tissue cells (e.g. bone, cartilage, fat), but experiments have demonstrated that MSCs are also capable, given

specific in vitro conditions, of developing characteristics associated with neuronal cells.[9,10] In vivo administration of MSCs is associated with increasing numbers of neurons, astrocytes and oligodendrocytes, and it is hypothesized that MSCs stimulate this formation of new brain cells by paracrine effects (see below) rather than by transformation into various cell types themselves.[11–13] This review will therefore focus on the use of MSCs to treat neonatal brain injury.

Physiological aspects of MSCs

MSCs are relatively easy to isolate, have neuroregenerative properties and exert important immunomodulating and anti-apoptotic effects.[5,11,14,15] MSCs are a heterogeneous cell population and originate from birth-associated tissues including the placenta, umbilical cord blood and umbilical cord stroma (Wharton's Jelly), but also from adult tissues including bone marrow, peripheral blood and fat tissue.[16] MSCs can be characterized by their adhesion to plastic when cultured, specific surface markers and lack of expression of major histocompatibility (class-II antigens).[17] An advantage of cell-therapy with MSCs is that both autologous and allogeneic transplantation is possible. Autologous transplantation is of special interest for neonatal brain injury as MSCs from the neonate's own umbilical cord (blood) can be used for transplantation. MSCs have also shown to be safe for allogeneic transplantation, given their favorable immunologic profile as shown in experimental studies and clinical studies for various pathologies.[18,19] However, safety studies on autologous MSC administration in neonates with neurological disorders have not been performed yet.

For clinical application, MSCs can be harvested from different tissue sources, but MSCs from birth-associated tissues, especially from the placenta and the umbilical cord, may have some advantages.[16] For example, they can be obtained non-invasively and without ethical concerns. Additionally, MSCs from birth-associated tissues have higher proliferative potential and faster self-renewal compared to MSCs from adult tissues.[20] Another advantage of MSCs from birth-associated tissues is that they have more primitive properties, which resemble embryonic rather than adult stem cell characteristics, which could be beneficial as adult MSCs show reduced differentiation capacity with increasing age.[16,20] In summary, in contrast to adult MSCs, MSCs from birth-associated tissues can be obtained in large quantities without limitations, which make them excellent potential candidates for use in neonatal care.

Mechanisms of action of MSCs

From neonatal rodent models of brain injury, we have learned that hypoxic-ischemic brain injury induces changes in the neurovascular environment that promote (transient) neurogenesis, i.e. increased cell proliferation and differentiation.

However, hypoxia-ischemia does not induce and maintain long-term neurogenesis, resulting in a residual cerebral lesion. Therefore, it is crucial to assist the neonatal brain in regenerative processes after injury and to reinforce proper development of the maturating brain. It is hypothesized that aiding endogenous regenerative mechanisms will subsequently lead to improvement of functional outcome.

Rodent studies have shown that lesion size does no longer increase after 4 days after a neonatal hypoxic-ischemic event.[21] Therefore the time window for neuroprotective strategies, i.e. prevention of cell death and inflammation, lies before 4 days after the insult. However, MSC transplantation at day 10 after hypoxia-ischemia is still able to reduce brain damage.[22] This clearly indicates that MSC treatment rather stimulates endogenous brain repair than acting neuroprotective. The regenerative mechanism of MSC transplantation must therefore be based on paracrine effects of MSCs on the endogenous repair system, i.e. boosting a growth-promoting environment for neural stem cells in neurogenic niches, instead of integration or direct differentiation of transplanted MSCs themselves into new neuronal cells. This is supported by several studies that demonstrated that MSCs migrate to the ischemic boundary zone, induce changes in brain environment and support neurogenesis.[11,23] MSC treatment after hypoxia-ischemia markedly induced cell proliferation in hippocampus and cortex, stimulated neuronal cell differentiation and formation of new neuroblasts within the subventricular zone.[11–13,24]

Paracrine effects of MSCs are thought to include the production of a plethora of factors (the secretome) involved in reduction of apoptosis and neuro-inflammation, promotion of neurogenesis, angiogenesis and synaptogenesis, and reduction of scar formation after brain damage.(Figure 1) The secretome of MSCs includes several growth factors such as vascular endothelial growth factor, brain-derived neurotrophic factor, nerve growth factor, basic fibroblast growth factor as well as anti-inflammatory cytokines.[25] A study from our group used PCR-array analysis to demonstrate upregulation of gene expression profiles associated with cell proliferation (e.g. Spp1 and IL17), neurogenesis (e.g. NRCAm and NGF), migration (e.g. CXCR4) and neuronal survival (e.g. glial derived neurotrophic factor) in the damaged area of the brain after MSC treatment. On the contrary, genes involved in inflammation (e.g. IL1B) were downregulated.[23] Other studies have hypothesized that MSCs inhibit apoptosis by transferring mitochondria during hypoxia-ischemia.[26] These findings together support the view that MSCs induce neurogenesis and reduce neuroinflammation.

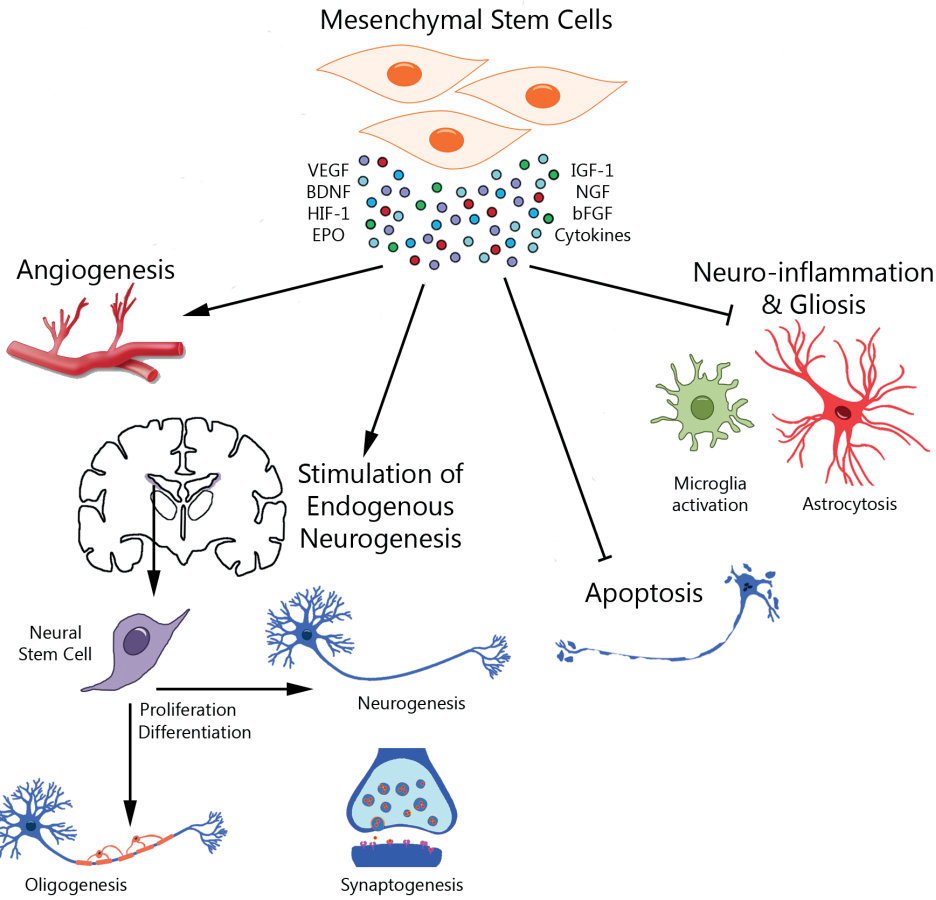

FIGURE 1 | Potential mechanisms for mesenchymal stem cells to induce repair of neonatal brain injury, including induction of angiogenesis, stimulation (⟶) of neurogenesis in the subventricular zone (SVZ) and reduction (⟝) of apoptosis, neuro-inflammation and gliosis. These pathways are mediated by the secretome of mesenchymal stem cells, which consists of many growth factors such as vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), hypoxia-inducible factor 1 (HIF-1), erythropoietin (EPO), insulin-like growth factor 1 (IGF-1) nerve growth factor (NGF), basic fibroblast growth factor (bFGF) and anti-inflammatory cytokines.

Besides beneficial effects of MSCs on cellular level, several studies have shown that MSCs improved functional outcome after brain injury in newborn rodents. MSCs given at day 10 after hypoxic-ischemia were effective in reducing white and grey matter loss at 28 days after hypoxia-ischaemia.[22] The effects on lesion size were associated with improved sensorimotor function of MSC-treated mice after hypoxic-ischemia compared to vehicle-treated littermates.[22] Functional improvements after MSC administration were long-lasting: motor and cognitive performance had

further improved in MSC-treated mice compared to vehicle-treated littermates up to 14 months after hypoxia-ischemia.[19] These experimental studies provide strong evidence for short- and long-term efficacy of MSC treatment after neonatal (hypoxic-) ischemic brain injury.

Taken together, MSCs can exert beneficial effects via multiple paracrine routes that together create an environment that facilitates tissue regeneration, subsequently leading to improved functional outcome.

Delivery of MSCs for therapy

Routes of delivery

MSCs can be delivered into the brain via different routes, using either systemic (e.g. into the bloodstream or peritoneal cavity), or local administration (e.g. intraparenchymal/ intracranial, intracerebroventricular, intrathecal, intranasal). In experimental settings, several research groups have used systemic administration of MSCs for neonatal brain injury, as MSCs have proven to migrate to injured brain regions.[27] Intravenously administered MSCs migrated mainly to injured ischemic brain areas and significantly improved functional outcome in a model of adult stroke.[12] A major disadvantage of systemic application of MSCs is loss of MSCs in other organs, which lowers effective cell numbers in the brain.[28] Especially in neonates suffering from systemic inflammation or birth asphyxia, the peripheral organs also undergo a hypoxic period, leading to multi-organ dysfunction. We hypothesize that this enhances loss of MSCs in these organs, which impedes delivery of MSCs to the brain. Therefore, local administration of MSCs seems more effective. As intracranial injection of MSCs (frequently used in rodents) is not feasible for clinical application, a less invasive local transplantation method is preferred. In clinical trials so far, MSCs or other stem cells (i.e. from autologous cord blood) have been administered mostly intravenously or intrathecally to treat cerebral palsy.(Table 1)

TABLE 1 | Several clinical trials are recently being initiated using stem cell therapy to treat neonates with several neurological disorders.

Study group	Indication	Control group	Age at inclusion
Samsung Medical Centre, Korea NCT02274428	Preterm infants with grade 3-4 intraventricular hemorrhage (n=9).	None	23-34 weeks of gestation
Duke University Medical Centre, Durham, NC, USA NCT00593242	Term neonates with HIE and hypothermia (n=23)	Concurrent cooled infants (n=33)	34-40 weeks of gestation
Duke University Medical Centre, Durham, NC, USA	Infants with severe congenital hydrocephalus	None	6 days – 4.5 years
Duke University Medical Centre, Durham, NC, USA	Young children with acquired neurological disorders (n=184)	None	6 days -9.5 year
Duke University Medical Centre, Durham, NC, USA	Krabbe's disease: asymptomatic neonates (n=11) and symptomatic infants (n=14)	Matched untreated affected children (siblings)	Neonates: 12 – 44 days Infants: 142 – 352 days
New York Medical College, Vallhala, New York, USA NCT02434965	Term neonates with severe HIE within 6h of birth (n=20)	None	≥36 weeks of gestation
Hospital Universitario "Dr. Jose E. Gonzalez", Monterrey, Mexico. NCT01506258	Term neonates with oxygen deprivation	None	37 – 42 weeks of gestation
University of Texas, Houston, USA NCT01700166	Stroke in children	None	6 weeks – 6 years

	Cells used	Status	Efficacy	Adverse effects	Study period
	Pneumostem®: Allogeneic human umbilical cord blood derived mesenchymal stem cells. Single intraventricular injection of low ($5x10^6$) or high ($10x10^6$) dose.	Ongoing	Aim: acute adverse events and death or shunt operations after 1year.	-	October 2014 - December 2016
	IV infusion of autologous Umbilical Cord Blood cells as soon as possible after birth and 24, 48 and 72 hours postnatally. 1-4 doses of 1-$5x10^7$ cells/dose.	Completed, results have been published.[36]	Similar outcomes after 1 year in both groups.	None.	January 2009 - June 2012
	IV infusion of Autologous Umbilical Cord Blood in 2-4 doses of a median of 0.1-13.3 x 10^7 cells/kg.	Completed, results have been published.[37]	All babies experienced developmental delays (as expected).	None.	October 2006 - August 2014
	IV infusion of Autologous Umbilical Cord Blood cells in 1-2 doses of a median of 0.1-13.3 x 10^7 cells/kg.	Completed, results have been published.[38]	None described.	1.5% acute anaphylactic reactions infusion. No adverse events until 12 months	March 2004 - December 2009
	IV infusion of Allogeneic Umbilical Cord Blood cells. Neonates received 22x10^7 cell/kg and infants received 17x10^7 cells/kg.	Completed, results have been published.[39]	In neonates very promising, but in infants minimal improvement.	4/14 infants progressive disease, 2/14 infants GvHD, 2/14 infections.	August 1998 - August 2004
	IV infusion of Autologous Cord Blood and Human Placental Derived Stem Cells (HPDSC). Collected after birth. 2 doses of HPDSC at day 2 and day 7. Autologous UCB were given in 3 doses: day 0, day 3 and day 7. Dosage depended on cell collection per patient.	Ongoing	Aim: safety and tolerability, neurological condition at 2 year.	-	January 2016 - June 2019
	IV infusion of autologous hematopoietic stem cells / autologous cord/placental blood within 48h after birth (unknown dosage).	Completed, no results available.	-	-	January 2012 - April 2013
	IV infusion of autologous human umbilical cord blood-derived stem cell (unknown dosage).	Withdrawn before inclusion started.	-	-	September 2012 - December 2015

Intranasal

More recently, intranasal delivery is emerging as an effective administration method for several therapeutic substances, including insulin, oxytocin, orexin, growth factors and neuro-peptides to treat central nervous system diseases.[29] Intranasal application might provide improvement over systemic routes as MSCs are targeted directly to the brain, preventing loss of MSCs in peripheral organs. Given the rapid distribution of MSCs from the nasal cavity towards ischemic brain lesions within 2 hours,[24] migration of MSCs through the brain tissue using the rostral migratory stream, seems unlikely. Therefore, in agreement with literature, several possible routes are proposed for MSCs to reach the brain after intranasal application: by following the tracts of the olfactory or trigeminal nerve, the meningeal circulation and/or via absorption into cerebrospinal fluid.[24,30]

Intranasal MSC treatment, when compared to vehicle treatment, improved sensorimotor and cognitive function and decreased grey and white matter loss in neonatal mice with hypoxic-ischemic brain injury.[11,31] The beneficial effects of intranasal MSC treatment were similar to intracranial delivery of MSCs in the lesion area.[14] Therefore it was concluded that the nasal route is a rapid and efficient way for MSC delivery after brain injury in neonatal rodents.

As humans have a less developed olfactory bulb in comparison to rodents, our group has also demonstrated that intranasally administered MSCs have the potential to migrate to injured brain regions in a newborn primate model of hypoxic-ischemic brain injury, in accordance with studies in rodents (unpublished observations). Although not investigated to date, it is expected that intranasally delivered MSCs will induce similar beneficial effects in the primate brain as observed in rodent models.

In summary, intranasal administration provides an effective and rapid alternative for MSC transplantation, allowing non-invasive MSC delivery for brain injury with minimal burden for patients.

Therapeutic window

Most available neuroprotective therapies for (hypoxic-)ischemic brain injury have to be started within a few hours (e.g. hypothermia) to be effective. In adult animal models, administration of MSCs at 3 to 24 hours after middle cerebral artery occlusion reduced the number of apoptotic cells in the ischemic penumbra.[12,15] However, the therapeutic window of MSC treatment is much wider as it has been shown that MSCs improved functional outcome and lesion volume in neonatal hypoxic-ischemic mice when administered at least until 10 days after induction of the insult.[22] In addition, it has been shown that administration of MSCs at 17 days after induction of brain damage was not effective anymore as a result of lacking chemotactic signals within

the brain lesion. So, the therapeutic window was determined to be at least 10 days but shorter than 17 days.[22] Although it might be hard to translate this window one to one to humans, as rodents of two to three weeks old correspond to a human child of approximately 0.5 to two years (depending on which parameter is used to relate age between the species), we hypothesize that the time window for MSC treatment after neonatal brain injury may be at least months in humans.[32]

Dosing

The appropriate effective MSC dose depends on several factors, including administration route, timing of injury and treatment. In neonatal rodent models of stroke, the most effective dose of intranasally administered MSCs to improve functional outcome was 0.5 –1.0 x 10^6 cells/pup (5 - 15 grams of bodyweight).[22,31] Other studies indicate that intracranial administration of 1.0 x 10^5 MSCs into the neonatal mouse brain at 10 days after the insult is sufficient to improve functional outcome.[11] Intravenously administered MSCs in adult rat models of stroke varied in dose, but were shown to improve functional outcome when given at3 x 10^6 MSCs/rat.[12,15] In clinical studies for adult stroke, the intravenous dose varied between 0.6 to 1 x 10^8 MSCs/patient.[33,34] Taking into account an average weight of 75kg, these clinical studies have used dosages of 8 to 13 x 10^5 cells/kg, which seem rather low. Extrapolating experimental adult rat data (using a weight of around 300g per rat) to humans, an effective intravenous dose would be around 1 x 10^7 MSCs/kg. Intratracheally administered MSCs for bronchopulmonary dysplasia in preterm infants were found to be safe and feasible when dosage was 1 to 2 x 10^7 cells/kg body weight, which was comparable to animal models.[35] Overall, it is hypothesized that higher doses of systemically administered MSCs are required compared to local MSC administration because of potential loss of MSCs in peripheral organs after systemic administration.

Safety aspects

The most important potential risk factors of MSC treatment are thought to be inflammatory reactions when using allogeneic cells and formation of malignancies. These risks have been intensively elaborated on in preclinical studies and appear to be absent at follow up. Our research group has assessed long-term safety in a mouse model of neonatal (hypoxic-) ischemic brain damage: at 14 months no lesions or neoplasia were observed in the nasal turbinates, brains or other peripheral organs of mice treated intranasally with MSCs.[19] Because animal studies have demonstrated that MSCs are hardly detectable in the brain at 3 days after transplantation,[24] the risk of Graft-versus-Host Disease or tumorigenicity seems low.

Additionally, human trials on MSC treatment in adults do not provide evidence for serious adverse events or risks. A large meta-analysis from 2012 on clinical trials for numerous diseases did not show any evidence for severe adverse effects.[18] This study used both adult and pediatric trials to report on a total of 1012 patients with various conditions, including neurological disorders, who were treated with either autologous or allogeneic MSCs. Including eight randomized control trials, MSC transplantation was not associated with acute infusional toxicity, organ system complications, infection, death or malignancy. A significant fever was observed after systemic MSC treatment compared to the control group, but the fever was reported to be low and transient in all trials.[18]

In summary, no indications have been reported in experimental animal models that complications occur in a higher incidence after MSC transplantation as compared to vehicle-treated animals. Additionally, in clinical trials adverse events, i.e. transient fever, were only observed when systemic MSC administration was used. Despite the benign safety profile of MSC therapy, safety and feasibility assessment of MSC treatment for neonatal brain injury should be confirmed in well-conducted clinical trials.

Clinical application of stem cells in neonates

Several clinical trials are recently being initiated using stem cell therapy to treat neonates with neurological disorders (HIE, intraventricular hemorrhage, stroke, hydrocephalus or acquired neurological disorders) (Table1). There is large variety in terms of study design, type of cells, dosing and timing of administration. Most studies use intravenous administration, but local intraventricular administration is used in an ongoing Korean trial for preterm infants with intraventricular hemorrhage. (ClinicalTrials.gov registration number NCT02274428) Several studies use autologous cord blood or stem cells derived from cord blood, including the group from Duke University treating neonates with HIE, congenital hydrocephalus and acquired neurological disorders including Krabbe's disease. Their results regarding safety seem promising: all infusions were well-tolerated and no adverse events were observed.[36–39] However, efficacy of autologous umbilical cord blood therapy on improvement of outcome and survival was only found significant in asymptomatic neonates with proven Krabbe's disease.[39] Another study using autologous cord blood to treat HIE in neonates is registered , but to the best of the authors' knowledge, results have not been communicated so far.(ClinicalTrials.gov registration number NCT01506258) The Korean study group has reported the intratracheal use of allogeneic MSCs in preterm neonates with high-risk for bronchopulmonary dysplasia.[35] Nine infants were included in this study: 3 patients were given 1 x 10^7 MSCs/kg and 6 were given 2 x 10^7 MSCs/kg. After 7 days pro-inflammatory cytokines were decreased (e.g. interleukin-6 and

interleukin-8) whereas the bronchopulmonary dysplasia severity score had improved in patients receiving MSCs compared to historical case-matched controls. No serious adverse events or toxicity related to a higher dose were observed.[35]

FUTURE PERSPECTIVES

The treatment of neonatal (hypoxic-)ischemic brain injury with cell-based therapies is emerging and experimental and preliminary clinical trial results are promising. MSCs have various characteristics that are favorable to be used as a regenerative therapy in neonatal (hypoxic-)ischemic brain injury: MSCs are present in several tissues, can relatively easily be harvested in large numbers and have low immunogenicity which makes them excellent candidates for allogeneic transplantation. Preclinical research has shown that MSC therapy has potential to repair neonatal brain lesions by boosting the endogenous regenerative capacity of the immature brain, thereby improving functional outcome at the long-term. Other approaches, such as manipulation of MSCs or use of MSC-derived exosomes have recently been investigated aiming at improvement of MSC effectivity or the use of a cell-free approach in experimental settings. Additionally, first results from clinical trials regarding safety aspects from both autologous and allogeneic cell transplantation are reassuring. However, some consideration should be taken for application of MSCs in human neonates, as positive bias in many reports may fuel unrealistic expectations. Several clinical trials are underway to evaluate safety and efficacy of MSC therapy for neonatal brain injury, providing the first steps towards clinical application of MSCs for severely affected neonates, to ultimately improve their quality of life.

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CHAPTER 10

MESENCHYMAL STEM CELLS ARE EFFICIENTLY DELIVERED TO THE INJURED NEWBORN NON-HUMAN PRIMATE BRAIN AFTER INTRANASAL APPLICATION

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In preparation

ABSTRACT

Background: Intranasally applied mesenchymal stem cells (MSCs) reduce brain lesion size and improve functional outcome in rodent models of neonatal hypoxic-ischemic (HI) brain injury. Whether the intranasal route can be used for effective MSC application in human neonates with brain injury is still unknown. As the anatomy of the rodent and human olfactory system differ, delivery efficiency or migration routes of the MSCs may vary between rodents and humans.

Objective: We investigated whether human MSCs are able to migrate to cerebral lesions when applied via the nose in a non-human primate model of neonatal ischemic brain injury. We also tested whether pre-treatment of the nose with hyaluronidase enhanced MSC migration after intranasal application in the rodent and baboon model.

Methods: HI brain damage was induced using a bilateral carotid artery occlusion model with systemic hypoxemia in two newborn baboons (birthweight 940-1040 gram) at postnatal day 5-7. Intubated animals were kept in the intensive animal care unit with cardiovascular/aEEG monitoring under the care of an experienced neonatologist. At 24 hours after the insult, the baboons were treated intranasally with 30 x 10^6 PKH-26 or PKH-67 labeled human umbilical cord-derived MSCs with or without hyaluronidase pretreatment of the nose. Eighteen hours after intranasal MSC application, animals were sacrificed, and their brains removed, dissected and snap-frozen. Coronal sections of 10 µm were cut, counterstained with DAPI and analyzed for presence of the PKH signal, and/or stained for antibodies against human CD29 and HLA-G. In our mouse study, HI brain injury was induced in 9-day-old mice and 1 x 10^6 MSCs were administered intranasally at 10 days post-HI with or without hyaluronidase pretreatment of the nasal cavity. Motor behavior and lesion volume were measured at 28 days post-HI.

Results: In the baboon, PKH signal, and/or CD29- and HLA-G- positive signal, was detected at 18 hours after intranasal administration in the bulbi olfactorii, forebrain and hippocampal area in both animals, indicating a rapid and effective migration of MSCs into the injured newborn baboon brain with and without hyaluronidase pretreatment of the nose. In rodents, pretreatment with hyaluronidase did not enhance the beneficial effects of intranasal MSC therapy on motor behavior and lesions size after HI compared to littermates treated with MSCs without hyaluronidase pretreatment.

Conclusion: This study provides evidence that intranasally administered MSCs migrate rapidly to injured brain regions of newborn baboons, in agreement with previous rodent studies. Rodent and baboon data further indicate that hyaluronidase pre-treatment of the nasal cavity is not required for efficient migration of MSCs towards lesioned brain areas. Our pilot study underlines the therapeutic potential use of non-invasive intranasal MSC administration as a treatment of HI brain injury in neonates.

INTRODUCTION

Neonatal brain injury in term born infants is most commonly caused by hypoxic-ischemic encephalopathy (HIE) or perinatal arterial ischemic stroke (PAIS), and it often leads to neurodevelopmental disabilities including cerebral palsy, cognitive deficits or the development of epilepsy.[1–3] Treatment options for these conditions are very limited, as they only consist of therapeutic hypothermia in infants with HIE and additional symptomatic treatments in PAIS.[4,5] Experimental studies using neonatal rodents provide evidence that treatment with mesenchymal stem cells (MSCs) reduces brain lesion size and improves motor and cognitive outcome after hypoxic-ischemic (HI) brain injury.[6,7] Our group has previously demonstrated that MSC transplantation after neonatal brain injury improved neurogenesis and oligodendrogenesis by secretion of several trophic factors that stimulate endogenous repair.[8]

Different administration routes have been suggested to deliver MSCs to the injured neonatal brain. A local route is preferred, due to the risk of loss of cells in peripheral affected organs after systemic (e.g. intravenous) administration.[9,10] However, many rodent studies have used intracranial administration of MSCs to apply cells directly in lesioned brain areas, which is a less desired route for clinical application in neonatal brain injury due to its invasive nature. A promising route of application of cells or agents into the brain is the intranasal route.[11–13] We and others have shown that intranasally applied MSCs are capable of migrating rapidly and specifically from the nasal cavity towards brain lesions after HI in rodents.[14,15] Moreover, the intranasal route of MSC application has shown to be effective in reducing hypoxic-ischemic brain damage and improving functional outcome in small animals.[14,16] Whether the intranasal route can be effectively used for MSC application in human neonates with brain injury is still unknown. As rodents depend significantly more on their olfactory system than humans, the olfactory anatomy between rodents and humans differs. Therefore, efficient migration of MSCs via the intranasal route, as observed in rodents, cannot be translated directly to humans. In order to validate intranasal MSC administration and allow translation of intranasal MSC therapy to clinical application, the current study investigated whether human MSCs are able to migrate to cerebral lesions when applied via the nose in a non-human primate model of neonatal ischemic brain injury.

In our previous rodent studies, hyaluronidase pretreatment of the nose was applied prior to intranasal MSC administration to promote penetration of the MSCs into the brain.[11,14] Since hyaluronidase is not yet approved for intranasal use in human neonates, in this study we also investigated whether hyaluronidase pretreatment of the nose is required for effective migration of intranasally applied MSCs to the injured areas. To investigate this second question, both primate and rodent models were used in which MSCS were given with and without hyaluronidase pretreatment of the nose.

MATERIALS AND METHODS

Ethics

All experiments were performed according to the international guidelines for animal research. The primate experiments were approved by the Institutional Animal Care and Use Committee (Protocol Number: 14047x) at the University of Texas Health Science Center [San Antonio, Texas, USA]; UTHSCSA) and were conducted in accordance with accepted standards of humane animal use, as described before.[17] All efforts were made to minimize suffering. The rodent study was approved by the Experimental Animal Committee Utrecht (Utrecht University, Utrecht, the Netherlands).

MSCs

24h-cultured human umbilical cord-derived MSCs were provided on the day of intranasal delivery to the animals as a cell suspension in phosphate-buffered saline at 4°C (Chiesi Pharmaceutics S.p.A, Italy). The MSCs used in this study meet the release criteria set by the International Society for Cellular Therapy (i.e. more than 90% of the MSC are CD73, CD90 and CD105 positive).[18] Furthermore, the MSCs were tested to ensure sterility (i.e. negative for bacteria, fungi, mycoplasma and endotoxin).

Directly before intranasal administration, MSCs were labeled with either PKH-26 (red, baboon 1) or PKH-67 (green, baboon 2) using the fluorescent cell linker kit according to manufacturers' instructions (Sigma-Aldrich Chemical Co., Steinheim, Germany). Briefly, under sterile conditions, MSCs were resuspended in the provided diluent and incubated with the dye for 5 minutes at room temperature. The staining reaction was quenched with the addition of fetal calf serum followed by DMEM culture media. Labelled cells were then washed, counted, and finally resuspended at a concentration of 30 x10^6 cells/400µl in sterile PBS.

For rodent studies, bone marrow-derived MSCs from C57Bl/6 mice were purchased from Invitrogen (Life Technologies, United Kingdom). Characterization of cell specific antigens has been described previously by our group: they were negative for myeloid and hematopoietic cell lineage specific antigens and stained positively for Sca-1, CD90, CD29, CD44, and MHC class I.[7] The cells were cultured according to the instructions of the manufacturer. Passage 4 MSCs were administered to the animals.

Primate study

Care of term animals (baboons)

Two baboons were obtained from the Southwest National Primate Center at the Texas Biomedical Research Institute (San Antonio, TX, USA). Studies were performed

at the UTHSCSA using the same animal care procedures as described before.[17] Term baboons were delivered at approximately 185 days gestation via spontaneous vaginal delivery and survived for about 6-7 days. Animals were cared for by their mothers for up to 72 hours before being transferred to UTHSCSA. After transfer, animals received 24-hour nursery care and were monitored by veterinary staff daily. Animals were housed in a temperature-controlled environment and were fed Similac (Abbott, Abbott Park, Illinois) formula orally every 3-4 hours. After experimental procedures, animals were euthanized with pentobarbital followed by exsanguination and immediately necropsied.

Experimental procedures

At postnatal day 5-7 baboons of either sex underwent bilateral carotid artery occlusion and systemic hypoxemia to create HI brain damage as described previously.[19–21] Animals were kept in the intensive animal care unit under constant control of an experienced neonatologist. Under general anesthesia (isoflurane) and sedation with ketamine (2mg, Putney, Portland, USA) and Midazolam (0.02mg, Akorn, Lake Forest, USA) every 2–6 hours, animals were exposed to permanent occlusion of the left carotid artery for 90 minutes (baboon 1) or 30 minutes (baboon 2) before subsequent clamping of right carotid artery for 60 minutes. Systemic hypoxia aimed to reach a saturation of 70-80% for 90 minutes and was started during the transient occlusion (baboon 1) or was initiated prior to occlusion of the carotids and continued for 180 minutes (baboon 2). This change in experimental protocol was necessary as another animal (not reported in this study, but similar to baboon 1) had insufficient evidence of brain damage. Therefore, the time between left and right carotid artery clamping was reduced and the duration of hypoxemia was prolonged in baboon 2. A detailed overview of the experimental protocol per animal is shown in figure 1.

In baboon 1, 24 hours after the induction of brain injury, each nostril was treated with 200 µl of hyaluronidase (500 IU, Sigma-Aldrich, St. Louis, MO) in PBS to increase permeability of the nasal mucosa. Thirty minutes later, 200µl of the PKH-labeled human umbilical cord-derived MSC suspension was carefully administered in droplets to each nostril ($30x10^6$ MSCs in total volume of 400 µl sterile PBS). The MSCs were given slowly in droplet under light anesthesia. In baboon 2, no hyaluronidase pretreatment of the nose was performed, so directly at 24 hours after the induction of brain injury 400µl of the PKH-labeled MSC suspension (see above) were administered (200 µl per nostril, $30x10^6$ MSCs in total; for details see baboon 1).

At 18 hours after intranasal MSC treatment, animals were sacrificed under humane conditions with overdose pentobarbital and the brain was immediately removed. The brain was first cooled in cold saline for 10 minutes before being sliced in 13-14 coronal sections. Each slice was divided in 8 pieces; 4 pieces per hemisphere

and divided in dorsal and ventral plus central and more distal (cortical) parts, see brain map in Figure 2. All brain pieces were placed in OCT in cryo-molds, frozen in isopentane, and stored at -80C.

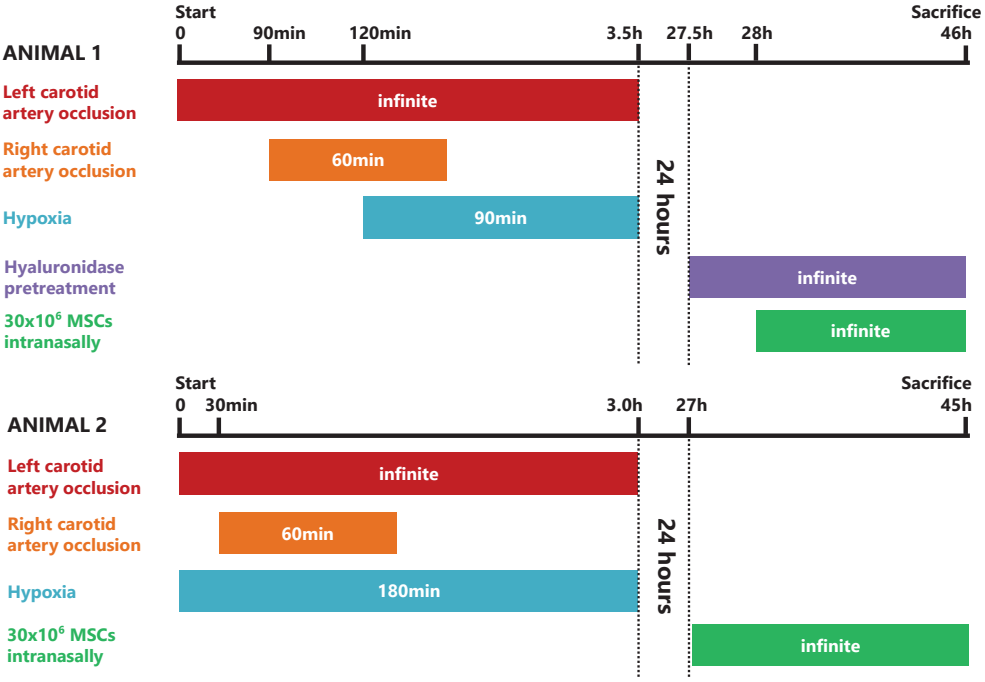

FIGURE 1 | Overview of the experimental procedures per animal. Animals were exposed to permanent occlusion of the left carotid artery, subsequent transient occlusion of the right carotid artery, followed by systemic hypoxemia of 70-80% to create hypoxic-ischemic brain injury. 24 hours after the injury, animals were treated intranasally with $30x10^6$ human umbilical cord derived MSCs with (animal 1) or without (animal 2) pretreatment of the nose with hyaluronidase. Animals were sacrificed 18 hours later.

10

Histology and MSC tracking (baboon)

Coronal cryosections of 10 µm were cut at a cryotome and counterstained with DAPI. Sections were qualitatively analyzed for presence of the PKH signal by using confocal microscopy. Fluorescent images were captured using an EMCCD camera (Leica Microsystems, Benelux) and Softworx software (Applied Precision, Washington).

In addition, to further confirm the presence of human MSCs in the baboon brain tissue, coronal cryosections of 10 µm of baboon 2 were stained with antibodies against CD29 and HLA-G, two markers that are expressed on human umbilical cord-derived MSCs and do not cross react with endogenous baboon stem cells or other cells in the baboon brain.[22–26] CD29 is a human-specific membrane marker which is present on human MSCs but absent on endogenous cells in the baboon brain.

HLA-G is an human-specific gene, which has a pseudogenic variant in baboons and is therefore not expressed in the baboon brain.[22–27] Stainings were performed with a monoclonal mouse antibody against human-CD29 (Novus Biologicals; TS2/16 clone) and a rabbit-polyclonal antibody against human-HLA-G (Abcam; ab135736), followed by secondary anti-mouse or anti-rabbit antibodies labelled with Alexa Fluor® 488 (green) or 594 (red), respectively (Abcam ab150113 or ab150080). Sections were counterstained with DAPI. Fluorescent images were captured using an EMCCD camera (Leica Microsystems, Benelux) and Softworx software (Applied Precision, Washington).

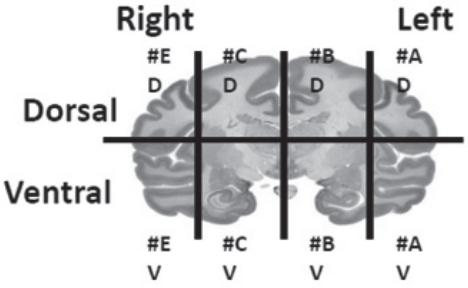

FIGURE 2 | Overview of Baboon brain slices. The baboon brains were sliced in 13-14 coronal slices. Each slice was divided in 8 pieces; 4 pieces per hemisphere and divided in dorsal (D) and ventral (V) plus central (#B and #C) and more distal (#A and #E) parts.

Rodent study

Experimental procedures

At postnatal day 9 C57Bl/6j mice underwent permanent occlusion of the right common carotid artery under isoflurane anesthesia followed by 45 min hypoxia (10% O2) to create unilateral hypoxic-ischemic brain damage, as described previously.[7,14] Sham-control animals underwent anesthesia without occlusion of the carotid artery and without hypoxia (n=12). Animals of both sexes from 10 litters were randomly assigned to one of 3 HI experimental groups (i.e. (1) vehicle-treatment (n=12), (2) MSC treatment with hyaluronidase pretreatment (n=13) or (3) MSC treatment without hyaluronidase pretreatment (n=13). A total of 50 animals was used in this study. At 10 days after HI, animals were treated intranasally with 1 x 10^6 mouse MSCs (in a total volume of 12 ml, 3 ml per nostril applied twice) or with vehicle solution (PBS; total volume of 12ml; 3 ml per nostril, applied twice). The nose of the MSC-treated animals was either pretreated with hyaluronidase (100 U in PBS; 3 ml per nostril, applied twice; Sigma-Aldrich, St. Louis, MO) or not. MSCs were administered 30 minutes after hyaluronidase treatment. Animals were tested at 4 weeks after induction of HI for

sensorimotor behavior after which they were sacrificed by pentobarbital overdose for histological assessment of the brain.

Sensorimotor function

Unilateral sensorimotor function was evaluated in the cylinder rearing test (CRT) at 28 days after HI. Mice were placed in a plexiglass cylinder and weight-bearing left (impaired), right (unimpaired) or both paw(s) contacting the wall during full rears were scored during 5 minutes. The test was videotaped and scored by 2 researchers blinded to treatment. Minimal number of full rears had to be 10 within 5 minutes, animals that did not reach this number were tested again. Preference to use the non-impaired forepaw, a measure of motor impairment, was calculated as ((right-left) / (right+left+both)) x 100%.

Histology

After pentobarbital overdose, mice were transcardially perfused with PBS followed by 4% paraformaldehyde (PFA) in PBS. Brains were post-fixed in PFA for 24 hours. PFA-fixed brains were dehydrated and embedded in paraffin. Paraffin-embedded coronal sections (8 mm) were cut at the level of the hippocampus (-1.85mm from bregma) and were stained with hematoxylin-eosin (Klinipath, Duiven, the Netherlands). Full section photographs were made and both HE-stained hemispheres were outlined using Adobe Photoshop CS5 software to calculate the areas of the contra- and ipsilateral hemispheres. Ipsilateral HE-positive area loss was calculated as follows: [1 - (area ipsilateral HE-positive staining/area contralateral HE-positive staining)] x 100%. For the rodent study, MSCs were not labeled with PKH, because presence of PKH-labeled MSCs within 12 hours at the lesion site was demonstrated in our previous studies.[14]

Statistical analysis

Mouse data are presented as mean ±SEM. Histological and sensorimotor data between experimental groups were compared using one-way ANOVA with Bonferroni post-hoc analysis. $P<0.05$ was considered statistically significant.

RESULTS

Migration of MSCs into the baboon brain after intranasal application

To determine whether MSCs migrate from the nasal cavity to the brain in newborn baboons with ischemic brain injury, we used PKH-26- (baboon 1) and PKH-67-labeled MSCs (baboon 2) and analyzed brain sections at 18 hours after intranasal administration. Figure 3 shows that PKH-positive signal could be detected in the

forebrain, hippocampus and deep mid brain (pons) in both animals at 18 hours after intranasal application of PKH-labeled MSCs. These data indicate that MSCs were able to migrate rapidly (within 18 hours) from the nasal cavity into the brain parenchyma. As baboon 2 did not undergo pretreatment of the nose with hyaluronidase, these data furthermore show that hyaluronidase pretreatment is not required for MSC migration from the nasal cavity.

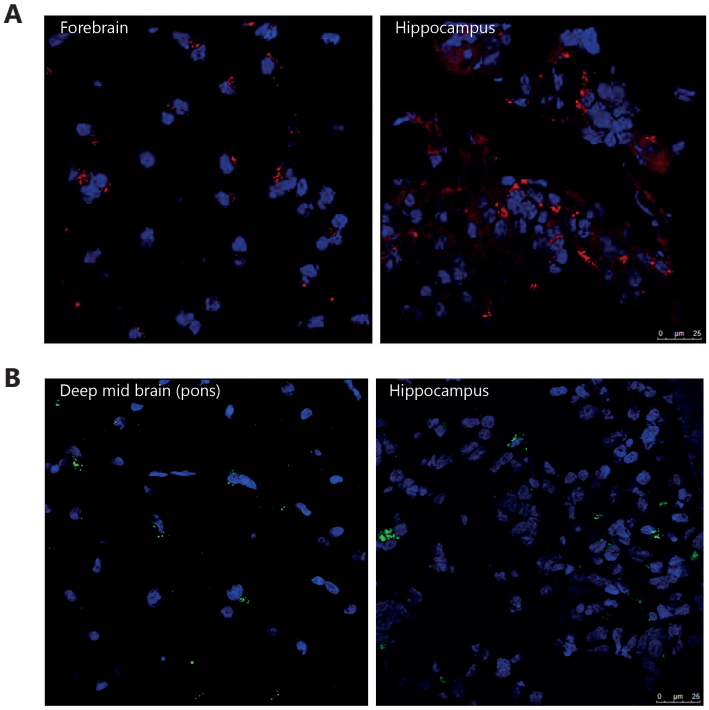

FIGURE 3 | PKH-signal detection in the damaged brain of a newborn baboon. PKH-positive signal, indicative of intranasally administered MSCs, could be detected in the forebrain, hippocampus and deep mid brain (pons) in baboon 1 (A; red signal by PKH-26) and baboon 2 (B; green signal by PKH-67).

To further illustrate the presence of transplanted human stem cells in the baboon brain after intranasal application, we confirmed the PKH data in baboon 2 by showing CD29-positive human cells in the hippocampus, frontal deep brain and parietal cortex (Figure 4A). In addition, also human HLA-G-positive cells were detected in the hippocampus and parietal cortex (Figure 4B). A double staining with CD29 and HLA-G antibodies showed that both markers were expressed by the same cells, which further confirmed that human MSCs were found in the baboon brain parenchyma, as illustrated in Figure 5 in the hippocampal region.

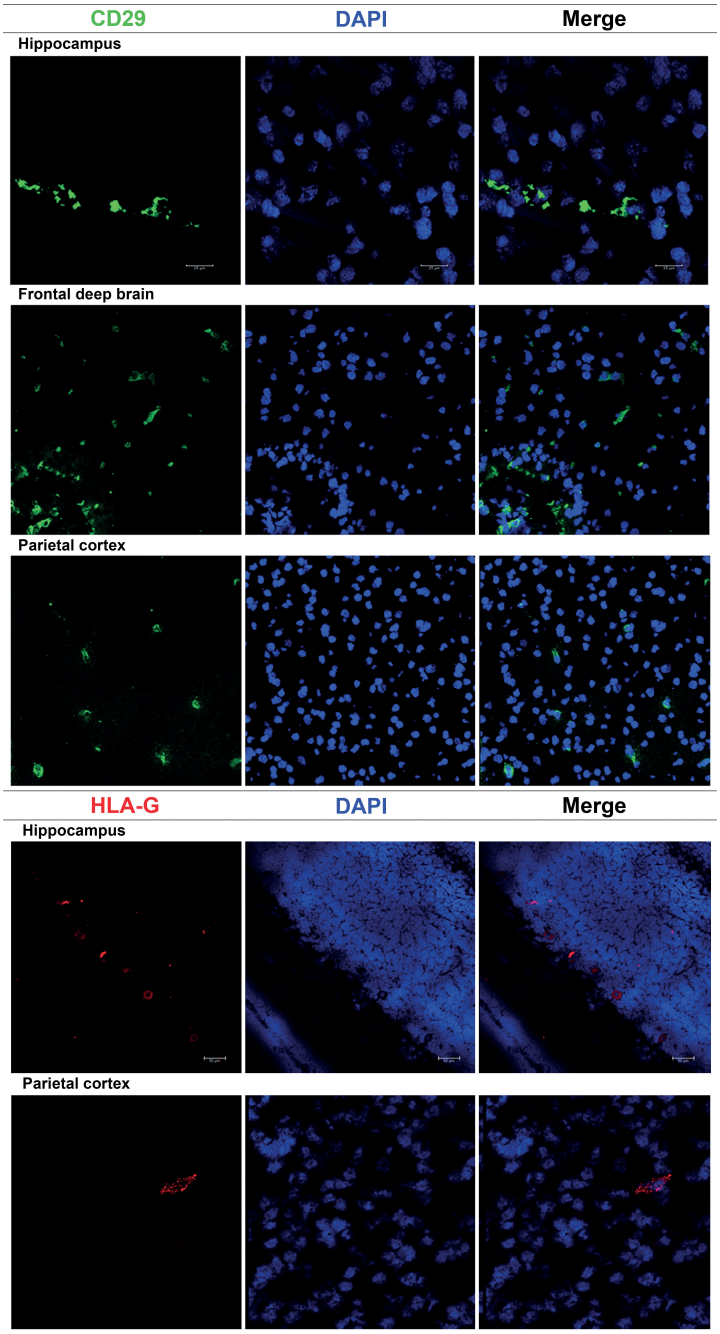

FIGURE 4 | Additional cell-stainings to confirm human cell migration in the baboon brain (part I). PKH data in baboon 2 were confirmed by showing CD29-positive cells in the hippocampus, frontal deep brain and parietal cortex (Figure 4A), as well as HLA-G-positive cells in the hippocampus and parietal cortex (Figure 4B).

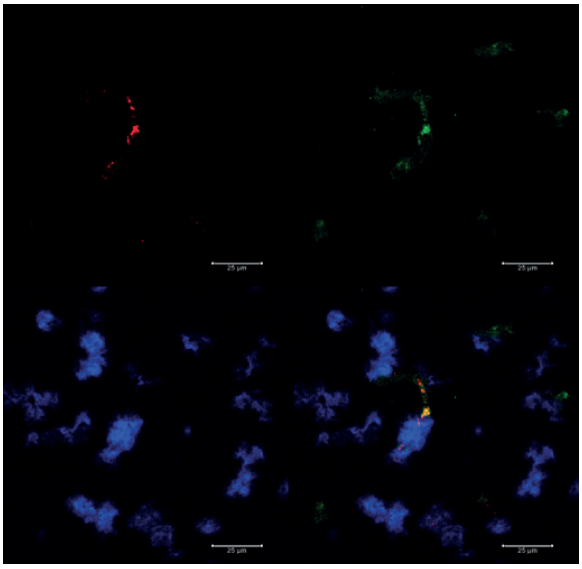

FIGURE 5 | Additional cell-stainings to confirm human cell migration in the baboon brain (part II). Double staining with CD29 (green) and HLA-G (red) antibodies showed that both markers (yellow, overlay) were expressed by the same cells in the hippocampal region.

Omitting hyaluronidase pre-treatment does not affect efficacy of intranasal MSC treatment in neonatal mice with HI brain injury

In neonatal mice, HI resulted in 28% area loss of the ipsilateral hemisphere (Figure 6A). Intranasal treatment with $1x10^6$ mouse MSCs resulted in a significant reduction of ipsilateral area loss to 13% in animals that received hyaluronidase pretreatment, indicating repair of the HI-induced lesion (Figure 6A). Importantly, no significant difference in the beneficial effect of MSC treatment on hemispheric area loss between animals with and without hyaluronidase pre-treatment was observed. Animals without hyaluronidase pretreatment showed 15% ipsilateral area loss (figure 6B).

Sham-operated mice did not show any preference to use either of the forepaws during rearing in the cylinder rearing test (Figure 6B). HI brain damage resulted in 30% preference to use the non-impaired forepaw in neonatal mice treated with vehicle, indicating motor behavior deficits. Intranasal treatment with $1x10^6$ MSCs significantly reduced forepaw preference to around 15%, indicating a potent improvement in motor behavior. Most importantly, there was no significant difference in the beneficial effect of MSC treatment on motor behavior between animals that received hyaluronidase pre-treatment of the nose and those that did not receive hyaluronidase (figure 6B).

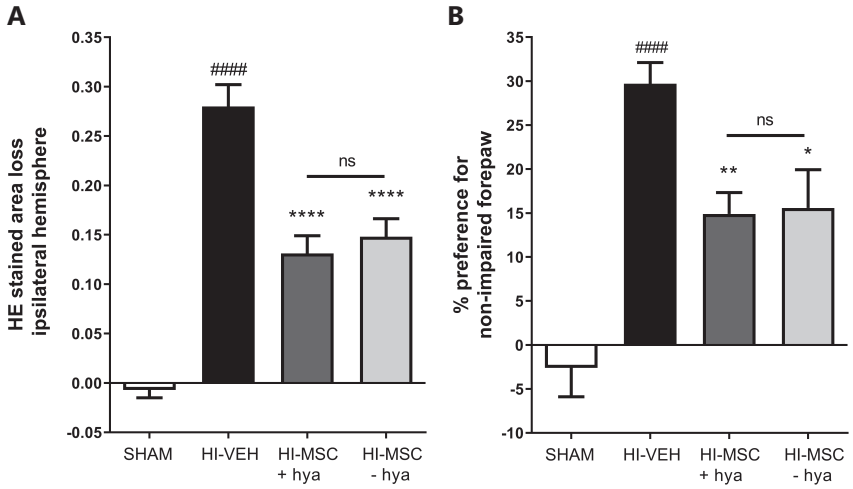

FIGURE 6 | Effect of intranasally administered mesenchymal stem cells (MSCs) on lesion size and sensorimotor function after hypoxic-ischemia (HI) in neonatal mice. Nine-day old mouse pups were subjected to hypoxic-ischemic brain damage and were treated intranasally with MSCs or vehicle at 10 days after HI. MSCs were applied with (+) or without (-) pretreatment of the nose with hyaluronidase. Part A: Quantification of infarct size by analysis of HE+ area loss in the ipsilateral (damaged) hemisphere at 28 days after induction of HI. Part B: Preference for use of the non-impaired paw was determined as a measure of sensorimotor impairment at 28 days after induction of HI. ####<0.05 compared to sham-operated group. *<0.05, **<0.01, ****<0.0001 compared to vehicle treatment. No significant difference was observed in MSC treatment with versus without hyaluronidase.

DISCUSSION

This study demonstrates for the first time that intranasally administered human umbilical cord-derived MSCs migrate within 18 hours into the brain parenchyma of newborn primates with hypoxic-ischemic brain injury, showing that the intranasal delivery route of cells is effective in species resembling the human. Both the baboon and rodent data of this study show that hyaluronidase pretreatment of the nasal cavity prior to MSC application is not required for effective migration of cells from the nasal cavity into the brain tissue. Namely, labeled MSCs were found back in the brain of the baboon pretreated with hyaluronidase as well as in the baboon that had not been pretreated with hyaluronidase. In line with our baboon data, our rodent data demonstrated that therapeutic effectiveness of intranasally administered MSCs to improve motor impairments and reduce lesion size is independent of hyaluronidase pre-treatment of the nose.

Intranasal delivery is emerging as a promising non-invasive administration route for therapies including insulin, hormones, growth factors and neuropeptides to

treat several neurologic conditions.[28] Other studies, including work from our own group, have demonstrated the potential of intranasal administration of stem cells to treat brain injury.[14,15,29–33] Although the exact migratory route of cells from the nasal cavity towards the brain tissue is unknown, it is hypothesized that the cells bypass the blood-brain barrier by following the tracts of the cranial nerves (I or V), into the cerebrospinal fluid or meningeal circulation.[11,28] Most importantly, it has been demonstrated that intranasally administered MSCs are effective in reducing brain tissue loss and improving regeneration in newborn rodent models of hypoxic-ischemic brain injury, preterm white matter injury and ischemic stroke.[14,15,33,34] Furthermore, in a mouse model of neonatal hypoxic-ischemic brain injury, intranasal administration was equally beneficial as intracranial transplantation of MSCs directly into the brain lesion.[35]

To determine whether intranasally applied MSCs migrate from the nasal mucosa to the lesion site in newborn baboons, we used the cell tracking dye PKH-26 or PKH-67, as described previously by our group and others.[14,29,36] Our previous data show that PKH-26 labeled MSCs migrate rapidly and specifically to the damaged brain regions in neonatal mouse pups with hypoxic-ischemic brain injury.[14] An advantage of using PKH membrane labeling is that it does not affect cell proliferation or survival, and it does not leak from the cell membrane to transfer to other cells, in contrast to other commonly used cell tracking dyes.[31,37] In the two newborn baboons subjected to brain injury, the PKH signal was indeed detected in the deep mid brain (pons), hippocampus and forebrain, demonstrating effective and rapid (within 18 hours) migration of MSCs from the nasal cavity. We confirmed the presence of human MSCs in the brain parenchyma of baboon 2 after intranasal application by using human MSC-specific immunostainings. We were not able to use antibodies against generally used specific membrane markers that define MSCs, i.e.CD73, CD90 and CD105[18] due to the cross-reactivity of commercially available antibodies to both transplanted human and resident baboon cerebral cells. We therefore used immunostainings against CD29 and HLA-G to which a human-specific antibody was available, or which was only expressed in human cells, respectively. With these immunostainings we showed the human MSCs to be present in the hippocampus, frontal deep brain and parietal cortex, confirming our data on MSC migration with PKH labeling.

Our group has previously shown that intranasally applied MSCs specifically migrate to areas of brain damage in neonatal mice with HI brain injury, probably due to chemotactic signals produced by the lesion.[14,31] We have also shown that exogenous MSCs do not migrate into the brain parenchyma in sham-operated mice, indicating that (HI) injury and subsequent chemotactic signals produced in the lesion,are required for MSC migration to the brain.[31] Furthermore, MSCs were not detected in the contralateral (i.e. non-injured) hemisphere of the unilaterally damaged HI mice,

although the MSCs were administered intranasally in both nostrils or intraventricularly in both ventricles, indicating that MSCs migrate specifically to the lesion site.[31,38] Our newborn baboon model of bilateral carotid artery occlusion is thought to generate global bilateral hypoxic-ischemic brain injury. In the baboons, MSCs were detected in both hemispheres, although predominantly in the deep midbrain and hippocampal/cortical regions. Human MRI studies have shown that acute neonatal hypoxic-ischemic brain injury usually affects the deep grey nuclei (basal ganglia and thalamus) and the perirolandic cortex, and often also involves the hippocampus.[39] As our primate baboon model is supposed to closely resemble acute neonatal HI brain injury, it is of no surprise that MSCs migrate to those deep mid-brain areas most affected. Ongoing studies into the histopathology of the baboon brains will yield more insight into the exact brain injury patterns in this model.

In this study we also addressed the question whether hyaluronidase pretreatment of the nasal cavity is a prerequisite for effective migration of cells into the brain. The current study shows that hyaluronidase pretreatment did not significantly benefit migration of intranasally applied MSCs in a model of hypoxic-ischemic brain injury in newborn primates and rodents. This is in contrast to a study in adult mice that showed that pretreatment with hyaluronidase 30 minutes before MSC administration significantly increased migration of MSCs from the nasal cavity to the olfactory bulb, while other regions including the thalamus, hippocampus and cerebral cortex showed only slight improvement.[11] Although we did not quantify the number of MSCs in the primate brains, PKH-signal seemed to be equally present in the deep mid brain of the injured primates that were treated with or without hyaluronidase. More importantly, we show here that effectiveness of MSCs on reduction of sensorimotor deficits and lesion size was equal with or without hyaluronidase pretreatment of the nose in newborn rodents subjected to HI. We suggest that if omitting hyaluronidase pretreatment from study procedures would diminish the number of migrating MSCs reaching the injured brain regions, this number must be small and will not affect effectiveness of intranasal MSC administration. Hyaluronidase is being used in clinics to improve drug efficacy because the enzyme modifies the permeability of connective tissue through the hydrolysis of hyaluronic acid, a polysaccharide found in the intercellular ground substance of connective tissue.[40] No known risks of intracutaneous [41], intraocular [42,43] or intranasal [40] hyaluronidase administration in human adults have been found. However, as hyaluronidase increases the permeability of the nasal mucosa, it hypothetically also increases the risk for other (potentially pathogenic) cells to penetrate the brain more easily.[11,44] Additionally, intranasal use of hyaluronidase is not yet registered in human newborns, so a clinical trial without the use of hyaluronidase in newborns with brain damage would be preferable. Our current study indicates that a future human trial using the intranasal route for MSCs administration, does not require hyaluronidase pretreatment.

This study confirms that the nasal route is effective for MSC administration in newborn baboons, but the amount or percentage of cells that reach the brain is unknown, as this was beyond the scope of our research project. As the MSCs were distributed over many sections of the brain, it would be a very time-consuming procedure to determine how many cells were present in the brain. Also, PKH labeling of MSCs membranes is not a suitable method to quantify cells in the tissue as such, as it does not allow to visualize whole cells but presents as a patchy membrane labeling. In future studies, more quantitative techniques like Q-PCR or nanoprobe labeling might be used to define the percentage of MSCs reaching the brain, and possibly other organs. Moreover, the exact number of MSCs that is required to be effective in restoring brain tissue and function is also not established. Efficacy of intranasal MSC administration on repair of brain injury needs to be studied further in clinical trials.

In rodent models described in this and other studies, beneficial effects of intranasal MSCs on both functional and anatomical outcome can be studied relatively easy on the long-term (e.g.at 4-5 weeks after injury).[14,31,45] A limitation of the baboon model, in comparison to rodent studies, is that long-term follow up of the baboons to study efficacy of MSC treatment in a larger number of animals is much more difficult due to ethical and financial reasons. Studying efficacy of intranasal MSC therapy in newborn baboons was therefore beyond the scope of our paper. Future studies could set out to search for earlier markers of MSC efficacy after brain damage in newborn primates, such as neuroimaging techniques and/or biomarker studies to assess the effect of intranasal MSC treatment in this model.

Although it was not the aim of this study, it would be of interest to determine whether MSCs migrate specifically from the nasal cavity towards the lesioned brain areas. Extensive pathological assessment of the baboon brain after bilateral carotid occlusion procedure will be focus of our future studies.

In sum, our pilot study has provided evidence that in a model of newborn baboons, PKH-labeled human MSCs migrate from the nasal cavity to the brain after hypoxic-ischemic injury, in accordance with previous rodent studies, showing that the intranasal route of MSC delivery is operative in an animal closely resembling the human patient. These data warrant the near-future use of non-invasive intranasal MSC administration in treatment of neonatal brain injury in clinical trials.

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CHAPTER II

DARBEPOETIN FOR ISCHEMIC NEONATAL STROKE TO AUGMENT REGENERATION (DINOSAUR); A MULTICENTER RANDOMIZED PLACEBO-CONTROLLED TRIAL

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In preparation

ABSTRACT

Background: Perinatal arterial ischemic stroke (PAIS) often leads to adverse neurodevelopment, such as motor and cognitive delay. Currently, there is no curative treatment available that leads to restoration of brain damage and improved outcome in this severely affected group of infants. This leads to life-long consequences of PAIS and forms a large burden for patients, families and society. The overall aim of the current project is to meet this need by developing a new treatment strategy.

Methods/Design: The DINOSAUR trial is an international multicenter, randomized placebo-controlled intervention study in the Netherlands and Canada that will investigate whether darbepoetin alfa compared to placebo can reduce brain injury in (near-)term newborns ≥36 weeks gestation with MRI-confirmed middle cerebral artery (MCA) PAIS. Two doses of darbepoetin alfa (10μg/kg) or placebo will be administered intravenously: the first dose within the first week after birth, the second one week later. Primary outcome measure is change in lesion size between the time of onset of the insult and 6-8 weeks of age, estimated using advanced volumetric MRI techniques. Secondary outcome measures are reorganization of the corticospinal connectivity using diffusion tensor imaging, and neurological outcome measures of cognitive and motor development at 18 months of age.

Discussion: This trial will determine if intravenous darbepoetin alfa started within the first week after birth is effective in reducing brain damage in (near-) term newborns ≥36 weeks of gestation with MRI-confirmed middle cerebral artery PAIS, as compared to a placebo.

BACKGROUND

Perinatal arterial ischemic stroke (PAIS) is a disease of the newborn, which occurs just before or around birth, leading to significant long-lasting neurodevelopmental morbidity. It is defined as acute symptomatic, focal cerebral arterial infarction around birth and 28 days after birth that is confirmed by neuroimaging.[1,2] PAIS is now diagnosed in approximately 1 in 2300-5000 full-term, live born neonates, due to increased use of neuroimaging techniques in particular MRI.[3] In 50-75% of infants, PAIS, depending on size and location leads to abnormal neuromotor and -developmental outcome, such as unilateral spastic cerebral palsy (USCP), cognitive delay, epilepsy, and language and behavioral deficits.[2,4]

In adult stroke, treatment focuses on thrombolytic therapy and is, after exclusion of hemorrhagic stroke, standard part of care given within a timeframe of 4.5 hours after stroke onset.[5] However, a critical issue, especially in PAIS, is the lack of exact knowledge about time spent between actual occurrence of stroke and the diagnosis. The most common cause of stroke in neonates is a transient occlusive thrombus, that may occur during or prior to the perinatal period.[6,7] Magnetic resonance imaging studies, in particular diffusion weighted imaging, in human neonates have demonstrated that the onset of injury is usually around the time of delivery, with cerebral tissue breakdown continuing through six weeks of age.[8] However, neonates present usually later with clinical symptoms, most often during the first week after birth[3], resulting in a later diagnosis of PAIS compared to adult stroke. Late diagnosis of PAIS also limits the use of early treatment options, such as hypothermia, which is used within a therapeutic window of six hours after birth for patients with perinatal asphyxia. In the acute setting, therapeutic strategies for PAIS consist of treating associated symptoms, such as hypoglycemia, seizures and infections. There is no specific curative treatment available that leads to restoration of neonatal brain damage in this severely affected group of infants. The severe life-long consequences of PAIS form a large burden for patients, families and society. The overall aim of this project is to meet the urgent need to reduce the burden of PAIS by developing a novel and potentially effective treatment strategy.

Erythropoiesis-stimulating agents (ESA), such as erythropoietin (EPO) and darbepoetin, have been proven to exert neuroprotection as they can reduce (neonatal) hypoxia-ischemia-induced free radical formation and detrimental pro-inflammatory and apoptotic activity.[9] EPO also stimulates neuroregeneration via trophic effects, such as angiogenesis, oligogenesis and neurogenesis.[10] In vitro and in vivo studies have demonstrated EPO stimulated neuronal differentiation of neural progenitor cells.[11,12] Experimental studies using models of neonatal stroke have shown a substantial anatomical reduction of infarct volume [13] as well as improvement of cognitive

function in rats after early EPO treatment of 1000U/kg within one hour after injury.[14] Moreover, delayed EPO treatment, at one week after the onset of neonatal stroke, has also shown improvement of anatomical as well as functional outcomes in rats.[15] This study provided evidence that late ESA treatment is an option for neonatal brain injury without clear timing of the ischemic event. It underlines the potency of EPO as a trophic drug stimulating neurogenesis.[16] No studies have provided evidence that ESA are effective in reducing brain damage when administered beyond 7 days after the ischemic event. Therefore, in this RCT, study medication will be administered as soon as possible after diagnosis but within 7 days after birth.

In human adults with stroke, recombinant human EPO (rhEPO) was shown to be both safe and beneficial [17], whereas rhEPO therapy (using high dosages) in extremely preterm infants improved long-term outcome[18–20] and reduced risk of brain injury as well as improved white matter integrity on MRI.[21,22] A few RCTs are currently studying rhEPO as an add-on therapy in full-term infants with hypoxic-ischemic encephalopathy (HIE) after perinatal asphyxia who are receiving hypothermia and their results look promising.[23–25] rhEPO was well-tolerated and no deaths or serious adverse effects were observed.[23,25,26] It was concluded that repeated rhEPO reduced the risk of disability (assessed at 6-24 months of age) for infants with moderate HIE.[24–27] Although administration routes between trials vary between intravenous and subcutaneous and also doses vary within trials, doses between 300 and 2500U/kg were considered safe in neonatal human trials.

In our center we have performed a pilot study in 21 infants with PAIS who received open label treatment with rhEPO showing feasibility and safety.[28] No adverse effects of rhEPO were observed, i.e. no differences in hemoglobin, hematocrit or blood counts between treated and non-treated matched controls.[28] Darbepoetin is another ESA that is promising as a promotor of neuroprotection in both term and preterm infants.[29] It has been shown that EPO and darbepoetin have similar effects, but darbepoetin is more potent, requires lower dosing and is more universally available across countries.[29–31] Baserga et al. published the safety and pharmacokinetic results of the DANCE-trial (Darbepoetin Administered to Neonates Undergoing Cooling for Encephalopathy), a randomized placebo-controlled study with moderate to severe hypoxic-ischemic encephalopathy (HIE) in term infants.[32] They reported no adverse effects and concluded that darbepoetin administration of 2 doses of 10 ug/kg was safe.[32] An overview of all neonatal studies using ESAs to improve neurodevelopmental outcome is shown in Table 1. Overall, all these studies indicate potential beneficial effects of ESAs on neurodevelopmental outcome in infants with (or at risk of) perinatal brain injury, without causing adverse effects. However, the beneficial effect of ESAs for PAIS needs to be confirmed by a large randomized placebo-controlled trial.

METHODS/DESIGN

Aim of the study

The primary objective is to investigate if intravenous treatment with darbepoetin alfa started within the first week after birth is effective in reducing brain damage in our study population of PAIS patients, as compared to placebo treatment. Our study population of PAIS patients was defined as (near-) term newborns ≥36 weeks of gestation with MRI confirmed middle cerebral artery (MCA)- PAIS, with specific in- and exclusion criteria as outlined below. The specific research questions that will be addressed in this study are:

1. Is intravenous darbepoetin alfa given within the first week after birth effective in reducing lesion size at 6-8 weeks of age in PAIS patients, as compared to a placebo?
   Changes in lesion size will be estimated using advanced volumetric magnetic resonance (MRI) techniques at two timepoints: as soon as possible and at 6-8 weeks after clinical presentation. Changes in lesion size will be corrected for brain growth, as described earlier by our group.[28]

2. Does intravenous darbepoetin alfa, given within the first week after birth, affect reorganization of corticospinal connectivity at 6-8 weeks of age in PAIS patients, as compared to a placebo?
   Diffusion Tensor Imaging (DTI) can provide information about the organization of the brain and it is sensitive to microstructural changes, even in the absence of abnormalities on conventional MR images.[33] Maturation of white matter after stroke and its consequences for white matter connectivity and rewiring can also be visualized using DTI.[34,35] DTI will be used to detect whether darbepoetin alpha treatment stimulates white matter connectivity, e.g. by evaluating fractional anisotropy of the corticospinal tracts.[36] Postprocessing software is developed within the Imaging Sciences Institute (ISI) and UMC Utrecht Brain Center.[36–38]

3. Does intravenous darbepoetin alfa given within the first week after birth improve sensorimotor skills and cognitive development up to 18 months of age after PAIS as compared to a placebo?
   Neurodevelopmental outcome, including sensorimotor skills and cognitive development, will be determined using the Bayley Scales of Infant and Toddler development version 3 (BSITD-III) and the Peabody Stroke Outcome Measure (PSOM) as well as a full neurological assessment at 18 months corrected age. Other measures of neurodevelopmental outcome will be collected including the development of cerebral palsy, and a selection of hand function tests, up to18 months of age.

TABLE 1 | Overview of trials studying the effect of ESAs to improve neurodevelopmental outcome in neonates.

Study	Study design	Neonate	Indication	Drug	Timing
Melo J Matern Fetal Neonat Med 2005 (only abstract)	Prospective cohort study	Preterm infants < 34 weeks or <1500g	None specific	EPO 400U/kg	Start?, single dose
Warwood et al. Journal of Perinatology 2005	Prospective cohort study	Preterm infant <32wk and <1500g (n=12)	None (Hb<10.5g/dl)	Darbepoetin 1ug/kg or 4ug/kg	Between day 26-62, single dose
Warwood et al. Journal of Perinatology 2006	Prospective cohort study	Preterm neonates <32 wk (n=7) and Term/near-term neonates (n=3)	None (Hb<10.5g/dl)	Darbepoetin 4ug/kg	Between day 3-28 (median 8.5), single dose
Baserga et al. Pediatric Research 2015	Randomized controlled trial	Newborn with hypothermia for HIE ≥36 weeks: n=10 per arm	HIE and hypothermia	Darbepoetin 2ug/kg or 10ug/kg or placebo	Start<12 hours, second dose after 7d
Wu et al. Pediatrics 2012 AND Rogers et al. Pediatric Neurology 2014	Prospective open-label phase I study	Newborn with hypothermia for HIE (n=24)	HIE and hypothermia	EPO 250U/kg or 500U/kg or 1000U/kg or 2500U/kg	Start < 24 hours, up to 6 doses every 48 hours

Method of administration	Effect on neuro-development	Effect on blood	Safety	Hospital and date
subcutaneous	x	x	x	Teaching Hospital, University of Sao Paulo, date unknown.
subcutaneous	x	x	No adverse events	McKay Dee Hospital in Ogden, UT and LDS Hospital/ Intermountain Medical Center in Murray, UT, USA, November 2004-April 2005
intravenous	Neurological outcome not reported.	No changes in reticulocyte count.	No adverse events, no changes in vital signs or immature/ absolute reticulocyte count, no rashes	McKay Dee Hospital in Ogden, UT and LDS Hospital/ Intermountain Medical Center in Murray, UT, USA, July 2005-November 2005
intravenous	x	No polycythemia, neutropenia	No SAEs, AEs similar across groups (related to HI). No polycythemia, neutropenia nor sepsis.	Between October 2012-December 2013 in one of 8 participating centers: University of Utah Hospital (N = 6); Primary Children's Hospital (N = 4); Intermountain Medical Center (N = 8); Monroe Carell Jr Children's Hospital at Vanderbilt (N = 6); University of New Mexico Children's Hospital (N = 1) and Presbyterian Hospital (N = 3); Seattle Children's Hospital (N = 1); and McKay Dee Hospital-Intermountain Healthcare (N = 1).
intravenous	Outcome described by Rogers et al. Pediatric neurol 2014: no deaths and low rate (4.5%) of moderate-severe neurodevelopmental disability.	x	No more systemic complications after EPO (compared to historic controls). No intracranial hemorrhages or CSVT on MRI after EPO.	In one of the 5 centers: University of California, San Francisco (N = 10); Seattle Children's Regional Hospital and Medical Center (N = 5); Children's National Medical Center (N = 4); Children's Hospital of Oakland (N = 3); and Santa Clara Valley Medical Center (N = 2), date unknown.

Study	Study design	Neonate	Indication	Drug	Timing
Wu et al. Pediatrics 2016 AND Mulkey et al. Journal of Pediatrics 2017	Randomized controlled trial	Term newborn ≥36 weeks with hypothermia for HIE: n=24-26 per arm	Moderate / severe HIE and hypothermia	EPO 1000U/kg	Start < 24 hours, total of 5 doses on day 1,2,3,5,7
Zhu et al. Pediatrics 2009	Randomized controlled trial	Newborn with HIE: n=83-84 per arm	HIE (no hypothermia)	EPO 300U/kg (n=52) or EPO 500U/kg (n=31) (combined results only)	Start <48 hours, every other day for 2 weeks
Elmahdy et al. Pediatrics 2010	Prospective case-control with 3 groups	Term newborn 38-42 weeks (n=15) Control: 1. healthy newborns (n=15) 2. HIE without EPO (n=15)	Mild/ moderate HIE (no hypothermia)	EPO 2500U/kg	Start 4-6h, daily for 5 doses
Benders et al. The Journal of Pediatrics 2014	Prospective open-label phase I study	Term newborn 37-42 weeks (n=21) Subgroup controls: matched untreated patients (n=10)	MRI-confirmed perinatal arterial ischemic stroke	EPO 1000U/kg	Start after MRI (diagnosis), repeated at 24h and 48h
Ohls et al. Pediatrics 2013, 2014 and 2016 AND Gasparovic Pediatr Radiology 2018	Randomized controlled trial	Preterm newborns 500-1250g birth weight and ≤48 hours of age: n=33 per arm	None specific (profylactic neuroprotec-tion)	Darbepoetin 10ug/kg or EPO 400U/kg or Placebo	Start ≤48h of age, through 35'weeks gestation. Darbe 1x/week, EPO 3x/week.

Method of administration	Effect on neuro-development	Effect on blood	Safety	Hospital and date
intravenous	Brain MRI at day 5: less injury in EPO group. At 12 months: better motor scores in EPO-group. At 12 months no longer correlation brain injury on DWI and outcome in EPO group.	Hematocrit the same at day 6 between groups.	Similar death rate among groups. No adverse events related to Epo.	In one of 7 centers: Children's National Health System (n = 9); University of California, San Francisco (n = 9); Seattle Children's Hospital (n = 8); Arkansas Children's Hospital (n = 8); Washington University, St Louis (n = 8); Stanford University (n = 6); and Kaiser Permanente Santa Clara (n = 2), January 2012-November 2012.
Subcutaneous 1x, then intravenous	Death/disability same between doses. EPO improved long-term outcome for moderate HIE.	No negative hematopoietic side effects	Well-tolerated, no negative hematopoietic side effects	2 neonatal centers in Zhengzhou, China, August 2003-January 2007
intravenous	At 2 weeks: improved EEG and decreased blood NO-concentrations for EPO compared to HIE; no difference in MRI. At 6 months: after EPO fewer neurologic and develop-mental abnormalities.	Less red blood cell transfusions in EPO group.	Well-tolerated, no side effects.	Tanta University Hospital Egypt, October 2007-December 2008
intravenous	Subgroup analysis in n=10: no differences in residual infarction volumes on MRI or neurodevelopmental outcome.	No differences in coagula-tion fuction and blood cell counts.	No differences in vital functions.	Wilhelmina Children's Hospital Utrecht and Isala Clinics Zwolle, the Netherlands, September 2009-October 2011
subcutaneous	18-22months: higher cognition in darbe and epo compared to placebo. No CP in ESA recipients. 3.5-4yr: IQ (full-scale and performance) higher in ESA group than in placebo. At 4-6yr: no differences in metabolite level on MR-spectroscopy.	Less transfusions, higher hematocrit and ARC in ESA-groups.	Similar incidence of ROP.	University of New Mexico in Albuquerque, NM; McKay Dee Hospital in Ogden, UT; LDS Hospital/ Intermountain Medical Center in Murray, UT; University of Colorado in Denver, CO. Enrollment: July 2006-May 2010

Study	Study design	Neonate	Indication	Drug	Timing
Lowe et al. Jrn of Pediatr 2017	Randomized controlled trial	Preterm newborns 500-1250g birth weight and ≤48 hours of age: n=35/n=14 (ESA/placebo)	None specific (profylactic neuroprotection)	Darbepoetin 10ug/kg or EPO 400U/kg or Placebo	Start ≤48h of age, through 35'weeks gestation. Darbe 1x/week, EPO 3x/week.
Juul et al. Pediatrics 2008 AND McAdams et al. Journal of Perinatology 2013	Prospective open-label phase I study	Preterm infant <1000g (n=30) Controls n=30	None specific (profylactic neuroprotection)	EPO 500U/kg or EPO 1000U/kg or EPO 2500U/kg	Start < 24 hours, 3 doses every 24 hours
Fauchere et al. Journal of Pediatrics 2015 AND Natalucci et al. JAMA 2016	Randomized controlled trial	Preterm newborns 26-32 weeks: n=214-229 per arm	None specific (profylactic neuroprotection)	EPO 3000U/kg or placebo	Start <3h after birth, repeated at 12-18 and 36-42h after birth
Leuchter et al. JAMA 2014	Randomized controlled trial	Preterm newborns 26-32 weeks: n=214-229 per arm. Subanalyses: n=165 (n=77 treatment, n=88 placebo)	None specific (profylactic neuroprotection)	EPO 3000U/kg or placebo	Start <3h after birth, repeated at 12-18 and 36-42h after birth
Wehrle et al. BMJ open	Randomized controlled trial	Preterm newborns 26-32 weeks: n=214-229 per arm. Eligible: n=365 (n=191 treatment, n=174 placebo). Controls: term (n=185)	None specific (profylactic neuroprotection)	EPO 3000U/kg or placebo	Start <3h after birth, repeated at 12-18 and 36-42h after birth
O'Gorman et al. Brain 2014	Randomized controlled trial	Preterm newborns 26-32 weeks: n=214-229 per arm. MRI-DTI subanalyses: n=58.	None specific (profylactic neuroprotection)	EPO 3000U/kg or placebo	Start <3h after birth, repeated at 12-18 and 36-42h after birth

Method of administration	Effect on neuro-development	Effect on blood	Safety	Hospital and date
subcutaneous	Behavior: better on behav. Symptoms and externalizing scales. More effect with low SEC.	-	See above.	See above
intravenous	Less ICH and WMI, neurodevelopment by McAdams et al J of Perinatol 2014: improved cognitive and motor scores on BSITD-II/ III in linear regression compared to matched controls	No effect on hematocrit or blood transfusions.	No adverse events	Patients were enrolled at the University of Washington Medical Center and Providence Everett Medical Center NICUs, January 2006-March 2007
intravenous	2yr outcome by Natalucci et al: no difference in mental development between groups. Also no differences in motor outcome, hearing/ visual impaiment, anthropometric growth.	At day 7-10 higher hematocrit, reticulocyte and white blood cells and lower platelet	Short-term outcome not different: mortaility, ROP, IVH, sepsis, NEC, BPD.	Five Swiss perinatal centers: 3 university hospitals (Basel,Geneva, and Zurich) and 2 districthospitals(Aarau and Chur) between September 2005-December 2012.
intravenous	Subanalyses in 165 infants: MRI at term-equivalent age: after EPO less abnormal scores in white and gray matter. Epo reduced brain injury on MRI.	x	No difference in deaths between EPO and placebo at TEA.	Five Swiss perinatal centers: 3 university hospitals (Basel,Geneva, and Zurich) and 2 districthospitals(Aarau and Chur) between September 2005-December 2012.
intravenous	Protocol for new analyses at 7-12 yr of age: executive function. Sec: IQ, fine motor, etc.	x	x	Five Swiss perinatal centers: 3 university hospitals (Basel,Geneva, and Zurich) and 2 districthospitals(Aarau and Chur). Currently enrolled for new analyses.
intravenous	DTI at TEA: increased fractional anisotropy in corpus callosum, PLIC and corticospinal tract after Epo compared to placebo. Epo improved white matter development.	x	x	Five Swiss perinatal centers: 3 university hospitals (Basel,Geneva, and Zurich) and 2 districthospitals(Aarau and Chur) between September 2005-December 2012.

Study	Study design	Neonate	Indication	Drug	Timing
Brown Pediatrics 2009	Retrospective cohort study	Preterm newborns ≤30 weeks and <1500gram (n=366)	None specific	EPO 250-400U/kg Total dose in first 6 weeks was variable: median 3750U/kg (IQR 3250-4800U/kg)	Variable: start median 10 days (IQR 8-13.3). 3x/week until enteral intake was established
Andropoulos et al. Journal of Thoracic and Cardiovascular Surgery 2013	Randomized controlled trial	Newborns≥35 weeks, <30 days with congenital cardiac malformation (n=59 in total)	congenital cardiac malformation undergoing cardiopulmonary bypass surgery	EPO 500-1000U/kg or placebo	3 doses: 12-24h preoperatively, immediately after surgery (or post-op day 1) and 24 hours after dose 2 (or post-op day 3)
Bierer et al. Pediatrics 2006	Randomized controlled trial	Preterm newborns ≤1000g birth weight (n=16)	None specific	EPO 400U/kg or Placebo	Start ≤4days of life, through 35'weeks gestation, 3x/week.
Newton et al. Journal of Perinatology 1999 (abstract only)	Randomized controlled trial	Birth weight of ≤1250 g, gestational age at birth of ≤33 weeks	Anemia of prematurity	EPO 100U/kg or placebo	Start unknown, 2-5x/week
Neubauer et al. Annals of Neurology 2010	Retrospective case-control study	Preterm newborns ≤1000g birth weight that survived for FU (n=146 in total)	Stimulation of erythropoiesis	EPO 1750-21500 U/kg in total or no treatment	Exact start? Early start (<day 14, n=77) or late start (>day 14, n=12) over 15-121 days.
He et al. Zhongguo Dang Dai Er Ke Za Zhi 2008 (abstract only)	Prospective nonrandomized controlled trial	Preterm infants: n=22 per arm	None specific (profylactic neuroprotection)	EPO 250IU/kg	Start day 7, 3x/week for 4 weeks
Malla et al. Journal of Perinatology	Randomized controlled trial	Term neonate with moderate-severe HIE (N=50 per arm)	HIE without hypothermia	EPO 500U/kg or placebo	Start 6h of age, 5x every other day

Method of administration	Effect on neuro-development	Effect on blood	Safety	Hospital and date
subcutaneous	At 25 months: mental development on Bayley-2 was positively associated with 6-week rhEPO dose	x	x	NICU at Presbyterian/ St Luke's (Denver, CO) between January 1995-December 1998
intravenous	Pre/post-op MRI not different between groups. At 12-months (n=42): cognition, language and motor scores on Bayly-3 were not different.	x	Similar safety profiles: adverse events not related to EPO (equally distributed among groups).	Texas Children's Hospital, Houston, Texas, USA, September 2006-February 2011
subcutaneous	At 18-22months: Mental&psychomotor development did not sign. differ between groups. Posthoc: epo concentration ≥500mU/ mL correlated with higher mental development.	Higher serum epo con-centrations, fewer blood transfusions after epo-treatment.	At 18-22 months: similar neonatal morbidities (including ROP and IVH) and anthropometric measurements.	University of New Mexico, Albuquerque, New Mexico, USA, August 1997-March 2000.
subcutaneous or intravenous	No differences in neurologic/cognitive outcome, or growth. All infants treated with rhEPO were neurologically normal.	x	x	?
subcutaneous or intravenous	At 10-13 years: EPO group (with IVH) higher developmental and psychological assessment. Children without IVH: no difference with/without Epo.	x	x	Children's Hospital "Auf der Bult", Hannover, Germany, January 1993-December 1998
intravenous	At TEA: neurological assessment higher in EPO-group. At 6 months: fine motor higher in EPO-group. At 12 mnths: gross motor, fine motor and language higher in EPO-group.	x	x	Department of Neonatology, Zhangzhou Municipal Hospital Affiliated to Fujian Medical University, Zhangzhou, Fujian, China. Date unknown.
intravenous	At 19 months: 40%vs70% death or mod-sev disability, less CP and less AED use. Less MRI abnormalities after EPO.	RBC elevated after EPO, normalized by 1 month.	Adverse events similar in groups.	Sheri Kashmir Institute of Medical Sciences, Srinagar, Kashmir, India, December 2012-November 2015.

Study	Study design	Neonate	Indication	Drug	Timing
Hong-yan Lv. Et al. Neural regen research 2017	Randomized controlled trial	Term neonates with hypothermia (n=20-21 per arm)	HIE with hypothermia	EPO 200U/kg	Start at 24h 1x/ day for 10 days
Juul et al. Neonatology 2018 (HEAL trial)	Randomized controlled trial	Term newborn ≥ 36 weeks (n=500)	Moderate / severe HIE and hypothermia	EPO 1000U/kg	Start < 24 hours, total of 5 doses on day 1,2,3,4,7
El Shimi et al. Journ Mat-Fet Neonat Medicine 2014	Randomized controlled trial	Term neonates with HIE (n=30) and controls (n=15)	HIE with/ without hypothermia	EPO 1500U/kg, hypothermia or supportive care	EPO 1 dose at day 1, hypothermia 72h
Avasiloaiei et al. Pediatr Int. 2013	Prospective open-label study	Term neonates with HIE (n=67)	HIE without hypothermia	group 1: supportive; group 2: sup. + pheno-barbital during 4h; group 3: sup. + EPO 1000U/kg	EPO 1x/day for 3 days
Clinical Trials number: NCT01732146	Randomized controlled trial	Newborn with HIE ≥36 weeks : n=120	HIE (with hypothermia)	EPO 1000-1500 U/kg or Placebo	Start <12 hours after delivery, 3 doses every 24 hours

Method of administration	Effect on neuro-development	Effect on blood	Safety	Hospital and date
intravenous	Neonatal behavriour at day 7-28 better after EPO. No differences at 9 months of motor, language, social skills.	Serum tau protein at day 8+12 lower after EPO.	Not reported.	Neonatal Intensive Care Unit, Maternal and Child Health Care Hospital of Handan City, China. August 2014 to August 2015
intravenous	Only protocol for trial available: reduction of death/neurodev impairment at 24 months	Only protocol for trial available: blood samples taken baseline, days 2 + 4	Only protocol for trial available, study not performed/published yet	In one of several centers including: Children's National Health System (n = 9); University of California, San Francisco (n = 9); Seattle Children's Hospital (n = 8); Arkansas Children's Hospital (n = 8); Washington University, St Louis (n = 8); Stanford University (n = 6); and Kaiser Permanente Santa Clara (n = 2), currently enrolling.
subcutaneous	Hypothermia best survival (60%) compared to EPO (30%). Better MRI score and neuro-muscular function at 3 months after hypo-thermia compared to EPO.	Not reported.	Fewer side-effects than hypothermia?	Ain Shams University Hospital, Cairo, Egypt. September 2007 - February 2010.
subcutaneous	At 18 months: more delay in control group; no difference EPO vs phenobarb group.	Serum anti-oxidant higher after EPO.	Lower mortality after EPO vs control.	Cuza-Voda Clinical Hospital of Obstetrics and Gynecology NICU, Iasi, Romania; 1 Jan 2010- 30 Sept 2011
intravenous	Primary outcome = survival without neurologic sequelae within 24 months. Secondary = Mortality, moderate/severe sequelae, aspect of brain lesions on MRI.	x	Secondary outcome: tolerance of treatment at 24 months.	Cochin Hospital Paris. Study is ongoing.

Study design

The proposed project will undertake an international multicenter, randomized placebo-controlled intervention study conducted in the University Medical Center Utrecht (UMCU), a tertiary hospital in Utrecht, the Netherlands; the Alberta Children's Hospital, a tertiary hospital in Calgary, British Columbia, Canada; and the Hospital for Sick Children, a tertiary and quaternary hospital in Toronto, Ontario, Canada. Other centers may potentially be added in the future. The inclusion period will be 2 years, with 18 months follow-up for each infant. The DINOSAUR trial is registered under NCT03171818 at ClinicalTrials.gov.

Study population

Infants suspected of having PAIS will be transferred to the participating centers. In order to be eligible to participate in this study, a subject must meet all of the following criteria:

- Newborns ≥ 36+0 weeks of gestation within the first week of life
- MRI-confirmed diagnosis of acute PAIS, in the MCA region with involvement of the cortical spinal tract (e.g. PLIC and/or peduncles) within one week after birth. Involvement of the corticospinal tract should be determined based on DWI, or conventional T1- or T2WI.
- Written informed consent from custodial parent(s)

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- Evidence of HIE on MRI (independent of hypothermia therapy)[39]
- Any proven or suspected major congenital anomaly, chromosomal disorder, metabolic disorder;
- Presence of a serious infection of the central nervous system;
- No realistic prospect of survival, at the discretion of the attending physician.
- Infant for whom withdrawal of supportive care is being considered.

Interventions

Randomisation and blinding

After written informed consent, eligible neonates will be automatically randomized to two groups: active study medication (darbepoetin alpha), or placebo (saline) in a block design. Block sizes are unknown to the study team. The infants will be stratified by gender and study site, to ensure that the study groups will be approximately equally distributed based on these variables. The key to the randomization list will be kept by

the local pharmacy and will be available only in case of suspected adverse reactions. This will be a double-blinded study, and blinding will be maintained during the full study-period of 18 months. This means that those researchers involved in collecting outcome parameters, such as MRI data and neurodevelopmental outcome, will also be unaware of treatment allocation. For safety monitoring, a data safety and monitoring board (DSMB) is installed to assess adverse events in their relation to study medication administration. The DSMB consists of 4 independent and experienced researchers who are not involved in trial management or data collection. An interim-assessment will take place after the primary outcome parameters have been collected for all infants and the randomization code will be broken by the pharmacy and shared with the members of the study team who are not involved in follow-up data collection. All patients and health care providers will still be blinded.

Treatment regimen

Trial medication will be prepared and distributed by each of the study site's local pharmacy, according to Good Clinical Practice (GCP) and Good Manufacturing Practice (GMP) guidelines. Infants included in this study will receive two intravenous doses of 10 ug/kg/each of darbepoetin alpha (active study medication) or saline (placebo). The initial dose of study medication will be administered as soon as possible after the MRI diagnosis of PAIS, but at least within one week after birth. The second dose will be administered 7 days after the first dose. Active study medication consists of darbepoetin alfa 50µg/0.5mL solution for intravenous injection in a pre-filled syringe (Aranesp, Amgen®). The placebo group will receive two doses of saline intravenously in a volume equal to study medication. Since the study population is expected to weight between two and five kilograms, volumes will range between 0.2-0.5 mL, and administered manually via i.v. catheters.

To check for contra-indications before each study medication administration, blood pressure will be monitored, blood samples will be drawn, and an MR venography or ultrasound with sinovenous Doppler will be performed. These contra-indications are criteria for withholding the study drug and include:

- Neutropenia (ANC < 500/ul)
- Polycythemia (hematocrit of >65%)
- Hypertension (blood pressure 2D greater than the mean for age)
- Cerebral sinovenous thrombosis

Outcome measures and procedures

The timeline of all study procedures is summarized in Figure 1. The primary outcome measure is change in lesion size, based on neuro-imaging, and will be determined at

the start of the intervention and at 6-8 weeks of age using MR imaging. Secondary outcome measures include DTI at the MRI of 6-8 weeks and standardized follow-up until 18 months of age.

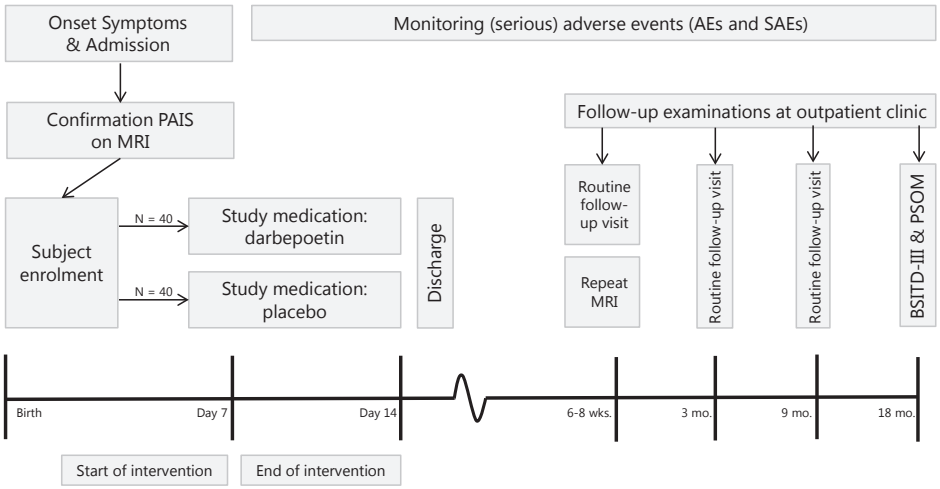

FIGURE 1 | Flowchart of the DINOSAUR study protocol. Timeline of all study procedures of the DINOSAUR trial: first dose of study medication will be administered within one week after birth, and the second dose one week later. MRI = magnetic resonance imaging, PAIS = perinatal arterial ischemic stroke, AE = adverse event, SAE = serious adverse event, BSITD-III = Bayley scales of infant and toddler development version 3, PSOM = pediatric stroke outcome measure.

Neuroimaging

Changes in lesion size will be estimated using advanced volumetric MRI techniques, comparing the MRI obtained after clinical presentation with the second MRI at six to eight weeks of age. These two MRI scans will be performed as part of routine clinical care: The first scan to diagnose PAIS, before the start of the study medication, and the second scan at six to eight weeks after birth (to obtain growth measures and to verify established injury and assess structural connectivity). All MRI investigations will be performed on a 1.5 or 3.0 Tesla MR system, and each scanning protocol consists of at least conventional MRI imaging (3D T1- weighted, or inversion recovery (IR), and T2-weighted imaging, with a maximum slice thickness of 2 mm; and DW-Imaging with a maximum slice thickness of 4 mm including b-values of 0 and 800-1000m^2/s, no gap). Each child will be scanned on the same scanner for both MRI scans. Lesion size percentages and differences between scans will be collected using each child's own scan. These percentages will be calculated using the same methods and formulae as described in our pilot study.[28]

DTI

DTI parameters will be used to quantify development and maturation of important white matter tracts such as the corpus callosum, the cortical spinal tracts and optic radiation. Our group has previously performed tractography and tract-based spatial statistics to show that fractional anisotropy (FA) measurements from DTI in several white matter regions were associated with neurodevelopmental outcome.[36,38] These techniques will be used to assess changes in DTI parameters in both hemispheres between MRI scans and compare them between treatment groups. Based on DTI we will also be able to perform connectivity analysis of the cortical network at 6-8 weeks and compare this between treatment groups.

Neurodevelopment

Follow-up data on neurodevelopmental outcome, including sensorimotor skills and cognitive development, will be collected up until 18 months of age:

- At three months: General Movements, the Pediatric Stroke Outcome Measure (PSOM).
- At nine months: Griffiths scale of Infant and Toddler development, PSOM
- At 18 months of age: the Bayley Scales of Infant and Toddler development version 3 (BSITD-III) and, when available, a full neurological assessment. The development and severity of cerebral palsy (CP) will be determined using the Gross Motor Function Classification System.

In some centers, data from specific hand function tests such as the hand assessment for infants (HAI) and the assisting hand assessment will also be collected between three and 18 months of age.

Statistical methods

Data will be analyzed according to the "Intention-to-Treat" principle, primarily answering the question whether or not darbepoetin treatment for neonatal stroke is better than placebo. Analysis of the primary effect of darbepoetin treatment on volumetric measurements will be conducted using linear regression and/ or an independent T-test or Mann-Whitney U test, where appropriate. Change in volumes of several brain tissue classes will be the dependent variable and an indicator variable for having received darbepoetin treatment as independent variable. For secondary outcome, changes in DTI parameters (including FA values) in several white matter regions between the two scans will be compared between treatment groups using independent T-test or Mann-Whitney U test, where appropriate. The analysis of neurodevelopmental outcome at 18 months will be analyzed using linear regression with neurodevelopmental outcome as the dependent variable and an indicator

variable for having received darbepoetin treatment as independent variable. Since this is an RCT, and possible cofounders are therefore assumed to be equally divided over treatment groups, we do expect to correct for confounders. The development of CP is also compared between treatment groups. An independent T-test or Mann-Whitney U test will be used where appropriate.

Sample size

The primary outcome of this study is change of infarct size between the two MRIs performed before and after treatment. We hypothesize that intervention with darbepoetin results in a reduction in infarction size. As we have described in our pilot rhEPO study, the estimated percentage of stroke tissue at the age of 3 months that had dissolved was not different between groups (median 109% vs 114%, mean 105±28% vs 120±34%).[28]

However, in our RCT we will only focus on those PAIS patients with cortical spinal tract involvement. Our pilot study included seven patients in each group with corticospinal tract involvement (and were still matched to historical controls). Using these numbers, the estimated percentage of stroke tissue at the age of 3 months that had dissolved had a mean of 108±8% vs 116±13%. When we use these numbers to calculate a sample size with a power of 0.8, we will need a sample size of 34 infants per study group, so 68 infants in total. Drop-out or missing outcome rate is expected to be around 15%. Therefore, the intended inclusion number is 40 infants per study arm leading up to 80 PAIS patients in total.

DISCUSSION

PAIS in newborn infants leads to severe consequences in later life, and currently there are no treatment options to reduce brain injury and improve outcome in these patients. This study is an RCT that compares the effects of intravenous darbepoetin treatment versus placebo on reducing brain damage in (near-)term newborns ≥36 weeks of gestation with MRI-confirmed middle cerebral artery PAIS. This is the first study that aims to demonstrate regeneration of brain tissue in this group of patients. In our pilot study, we have demonstrated that ESA-therapy with rhEPO had no adverse effects in neonates with PAIS, but we did not find a beneficial effect of rhEPO on stroke volume compared to matched controls. Although the pilot study was not designed to demonstrate such an effect, percentages of stroke volume loss were used to calculate sample size for this study.

Our cohort consists of (near-)term neonates ≥36 weeks with PAIS. PAIS is less often diagnosed in preterm infants, which could perhaps be explained by the fact that

they less often present with neonatal clinical seizures.[40] Furthermore, the beneficial effect of ESAs on reduction of brain damage after hypoxic-ischemic injury is less well studied in animal models of preterm brain injury. To limit heterogeneity within our cohort, only (near-)term neonates within their first week of life will be included in this trial. Additionally, differences between centers, such as MRI scanners and clinical care, may increase variation of outcome parameters. Therefore, randomization for study medication will be stratified per study site. Randomization will also be stratified per gender for two reasons: PAIS is more common in males [1,41] and rodent studies have shown differences in efficacy of EPO to improve sensorimotor function and reduce brain injury after neonatal stroke.[42]

The base of this dosing is based on several earlier preclinical and clinical studies. First of all, in neonatal rat models of hypoxic-ischemic brain injury, it was shown that three doses of 5,000 U/kg rhEPO given daily subcutaneously or intraperitoneally immediately after the injury results in neuroprotection at 48 hours and 7 days in 7 day-old rats.[43,44] In addition, darbepoetin was shown to be most, and equally as rhEPO, effective when administered at a dose of 10ug/kg intraperitoneally in adult male rats at two hours after middle cerebral artery occlusion (MCAO).[45] Preclinical studies in mice have shown that half-life of darbepoetin was three times longer than rhEPO, which allows darbepoetin to be administered only once every week.[31,46] In human newborn studies pharmacokinetic parameters of 1000U/kg rhEPO or 10ug/kg Darbepoetin IV were comparable to 5000u/kg IP/SC rhEPO in a newborn rodent model.[23,32,47,48] A dose of rhEPO 1000U/kg IV and Darbepoetin 10ug/kg IV produced pharmacokinetic values (AUC and Cmax) that were most comparable with established neuroprotective levels reported in preclinical studies.[23,32,47,48] Additionally, a single dose of rhEPO 5000U/kg IP in adult rats with MCAO, reached a cerebrospinal fluid (CSF) concentration of 2% after 30 minutes, which had previously shown to be neuroprotective.[43,44,49] A dose of 10ug/kg darbepoetin IV given to a human newborn HIE patient (during hypothermia) reached an CSF-EPO-concentration of 2.7% after 17 hours[32], which is comparable or even higher than EPO-CSF concentrations in rodents that were determined necessary for neuroprotection. Given that no harmful drug effects have been noted clinically in other studies of ESAs in both term and preterm born neonates[19,20,23,24,32,50], we are convinced that repeated dosing of rhEPO 1000 U/kg i.v. or 10ug/kg darbepoetin i.v. is a safe and effective dose, suitable for future neuroprotection studies in human neonates, including term born infants with PAIS.

The extra burden of the present study for the included infants is considered to be very limited to non-existent given the fact that besides administering the study medication, treatment is not different from the standard acting treatment protocol for newborns with PAIS. With respect to possible risks of ESA treatment, the most important potential risk factor such as polycythemia has been investigated in

our own pilot study and was not present. No indication has been found in other human studies that complications occur in a higher incidence after darbepoetin treatment as compared to non-treated controls, whereas possibility for a substantial better short- and long-term outcome seems very realistic on the basis of previous research data. The overall aim of this project is to develop a treatment strategy for PAIS patients within the first week after birth to reduce brain injury and improve neurodevelopmental outcome.

DECLARATIONS

Ethical approval and consent to participate

This study will be conducted according to the principles of the Declaration of Helsinki (version 2013: www.wma.net) and in accordance with the Medical Research Involving Human Subjects Act ('WMO' in Dutch) and other guidelines, regulations and Acts. The study protocol has been reviewed and approved by the Ethics Committee of the University Medical Center in Utrecht (the Netherlands), the Regional Ethics Board in Calgary (Canada) and the Ethics Committee of the Hospital for Sick Children in Toronto (Canada).

Parents of neonates (born and) admitted to the NICU of a study center will be informed as soon as possible after suspicion of PAIS about the study protocol by a neonatologist involved in the study. Parents of neonates from other hospitals (in the Netherlands), who are also potentially eligible for inclusion into the study, may also be informed as soon as possible after suspicion of PAIS about the study protocol by the neonatologist in their own center. Transportation to the study site will be part of neonatal care for PAIS patients and parents will be informed more specifically in the study center by a neonatologist involved in the study.

The parents will receive both the patient information letter describing the research proposal as well as an informed consent form. As soon as possible but allowing for an appropriate period of time for parents to read, understand and, if necessary, receive additional information if requested, consent for participation will be asked in case PAIS (in the MCA region) is confirmed by MRI (including 3D-T1, T2 weighted, DWI and DTI sequences). Following written parental consent, patients will then be included in our study. Parents may decide to withdraw their child from the study at any time for any reason without any consequences.

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Think it over, think it under.
Winnie-the-Pooh

CHAPTER 12

GENERAL DISCUSSION, CONCLUSIONS AND DIRECTIONS FOR FUTURE RESEARCH

GENERAL DISCUSSION

Despite major advances in perinatal and neonatal care, perinatal brain injury remains one of the most important complications for newborns admitted to the neonatal intensive care unit (NICU) with many consequences for their future life. Perinatal arterial ischemic stroke (PAIS) is a form of perinatal brain injury that is commonly encountered in term born neonates at the NICU, with an incidence varying between 1:1600 to 1:5000 depending on the use of neuro-imaging for diagnosis.[1] Periventricular hemorrhagic infarction (PVHI) is the most frequent form of perinatal brain injury following preterm birth, with an incidence of approximately 1-3% of preterm born infants.[2] The first chapter of this thesis introduces PAIS and PVHI, as they are commonly grouped in the perinatal stroke spectrum, and generally affect only one hemisphere of the brain.

Even though PAIS and PVHI do not share a common origin, their implications on development can be equally severe and include the risk for motor and cognitive disabilities, as well as post neonatal epilepsy or behavioral problems. For families and caretakers, early counseling on prognosis after perinatal brain injury is important, in order to manage expectations, prepare for possible future disabilities, and most importantly, to start intervention programs as soon as possible. These interventions may reduce severity of adverse (motor) outcomes, prevent complications or improve quality of life with modifications to daily life, functioning or care. Examples include physical or occupational therapy, bimanual therapy or constraint-induced movement therapy, that have all proven effective in improving motor performance in infants with cerebral palsy.[3] This is in line with animal studies demonstrating that extensive activity stimulates plasticity of the developing brain, e.g. by reorganization of the corticospinal tract.[4,5] The process of reorganization occurs at an early age after injury, emphasizing the need for early selection of those infants at risk for motor disabilities to start interventions as soon as possible. Infants at risk for other disabilities, such as cognitive deficits or epilepsy, may also benefit from early prognosis to offer interventions such as custom education, reduction of provocative stimuli or anti-epileptic drugs. The first part of this thesis focused on the use of parameters from neuro-imaging, neuro-monitoring and clinical functioning to predict neurodevelopmental outcome in several domains. These parameters may serve as tools to select those infants at risk for adverse outcome, who are most likely to benefit from (new) early intervention strategies. The second part of this thesis proposed potential new treatment options that are currently being studied in animal models, or are on the edge of translation to clinical care.

PART I: EARLY PREDICTION OF OUTCOME AFTER PERINATAL STROKE

Many studies have described that patients with perinatal stroke are at high risk of developing neurological disabilities, but the incidence and spectrum of these disabilities have a wide range.[1,6,7] In order to make a more personalized statement on expected neurodevelopmental outcome, it is important to demonstrate which factors are most important in determining outcome after PAIS. First of all, PAIS can affect several arterial territories: most commonly the middle cerebral artery (MCA), and less often the posterior (PCA) or anterior cerebral artery (ACA).[1,8] Furthermore, the location of the occlusion of the artery determines the affected infarct area, i.e. an occlusion to the proximal M1 segment of the MCA will lead to a larger infarct area than more distal occlusions.[9] Additionally, location of the infarct area is important as it determines the effect of the brain lesion on functioning. Until now, most studies do not differentiate between PAIS subtype, and therefore do not take into account the extent or location of the lesion, when reporting on neurodevelopmental outcome in future life.

In **chapter two** we aimed to provide clinicians with more precise risk-evaluation of neurodevelopment after PAIS by describing several outcome domains in different PAIS subtypes. This study was performed in a large international cohort of 161 term neonates from two different centers with seven different subtypes of PAIS, based on early MRI scans. The number of infants who developed cerebral palsy, cognitive deficits, language delay, epilepsy, behavioral problems and visual field defects was noted and compared between PAIS subtypes. We found that overall, 54% of infants developed an adverse outcome in one or more of the domains. However, 100% of the infants with a main MCA stroke involving the complete MCA territory, had an adverse outcome, while this was much lower, ranging between 29-57% in the other subtypes. More specifically, we found a rate of cerebral palsy of around 30% in our PAIS population, comparable to other studies.[6,10] However, 100% of infants with a main MCA stroke developed cerebral palsy, while this percentage was only 0-21% in all other subgroups. With this study, we distinguished several specific perinatal stroke subtypes and described incidence rates per outcome domain for them, enabling more personalized and tailor-made prediction of long-term development. Furthermore, we also found that, based on diffusion weighted imaging (DWI), involvement of the corticospinal tracts and basal ganglia were most important outcome predictors for adverse outcome. Other groups also found involvement of the basal ganglia after PAIS on MRI to be associated with cerebral palsy[10,11], but we found that basal ganglia involvement was, next to cerebral palsy, also associated with increased risk for cognitive deficits and behavioral problems. Involvement of the descending corticospinal tract at the level of the cerebral peduncle was associated

with an increased risk for cerebral palsy, as described by others[12], but we also found a relation to development of epilepsy. This study focused mainly on early DWI to distinguish different perinatal stroke subtypes and involvement of specific regions, but an early MRI may not always be possible in all institutions. Other early prediction markers, apart from MRI, may therefore be useful to select infants at risk for adverse outcome after PAIS. **Chapters four to seven** focus on such parameters.

As described in chapter two, MRI, and especially DWI, offers the ability to assess the corticospinal tract for involvement after PAIS. In the nineteenth century, the British scientist Waller described that injury to the neuronal cell bodies or proximal axons, could lead to secondary anterograde degeneration of axons and their myelin sheaths.[13] This phenomenon was later referred to as "Wallerian" degeneration and is usually seen in the descending corticospinal tract and cerebral peduncle starting from several weeks after cortical injury. Several studies have now shown that DWI is also able to detect subsequent axonal ischemia to the descending corticospinal tract before degenerative changes appear, and this is therefore referred to as "pre-Wallerian" degeneration. Both pre-Wallerian and Wallerian degeneration are strongly associated with the development of motor disabilities later in life.[11,12,14–16] **Chapter three** reported on two full-term infants with PAIS, in whom DWI was performed very early, within 24 hours after clinical presentation (i.e. seizures) and repeated 48 hours later. Although both scans showed clear patterns of PAIS within the MCA territory, restricted diffusion in the descending corticospinal tracts was only present on the second scan. These cases showed a delayed onset of "pre-Wallerian" degeneration and thereby provided important information about timing of the MRI after PAIS. In most hospitals, clinicians are inclined to perform an MRI as soon as possible in order to make a diagnosis in a neonate with clinical seizures. However, as MRI parameters are also strongly associated with neurodevelopmental outcome, as demonstrated in chapter two, and therefore play an important role in prognostic counselling, our study suggested that scanning 'sooner is not necessarily better'. As this study only described two patients, further research in larger cohorts needs to establish the optimal timing of MRI scanning, balancing between early diagnosis and prognosis of PAIS.

In **chapter four**, we have continued the use of MRI-based parameters for the prediction of motor outcome after asymmetric perinatal brain injury, in combination with clinical hand assessment. This combination of neuro-imaging and clinical assessment, is often described as the golden standard to detect abnormal motion development, or to diagnose cerebral palsy.[17] We included 52 infants with PAIS, PVHI or another unilateral brain lesion from two different centers in Europe, who all received an early MRI within one month after term-equivalent age and underwent clinical hand evaluation around four months of corrected age. Hand function was

scored using a new standardized instrument for upper limb assessment, known as the Hand Assessment for Infants (HAI).[18] In our multivariable analyses, we found that combining neuroimaging and clinical assessment improved the accuracy for the prediction of unilateral cerebral palsy over the use of one of these parameters alone. We constructed a prediction model using involvement of the corticospinal tracts and basal ganglia from MRI, in combination with gestational age, sex and the contralesional hand score from HAI, that yielded an area under the curve (AUC) in the prediction of unilateral cerebral palsy of 0.98. To improve clinical application of this model, we proposed a nomogram as a tool for the prediction of unilateral cerebral palsy in the future, although this needs to be externally validated in a different cohort first.

The HAI is the first standardized assessment that measures the degree and quality of hand function in both hands separately and together in infants before the first year of age. Using a video-recorded 10-15-minute play session, it provides a separate contra- and ipsilesional each hand score, a both hand measure and a measure of hand asymmetry between 3-12 months of age. This makes the HAI especially suitable for young infants with unilateral brain injury, as movement asymmetry, most often in the upper limbs, is frequently the first clinical sign of abnormal motor development at an early age.[19,20] HAI is therefore more useful than other, more commonly used, motor assessments that do not specifically aim to describe asymmetric upper limb functioning, or only do this beyond the age of one year.[21] **Chapter four** was the first of three chapters (**chapter four, five and six**) that described the use of the HAI for the prediction of unilateral cerebral palsy, and the results seem promising.

In **chapter five** we continued the use of MRI parameters and clinical hand assessment with HAI for the prediction of unilateral cerebral palsy in infants with unilateral brain lesions. However, in this chapter we moved away from qualitative evaluation of the MRI, and assessed the descending corticospinal tract on MRI using a quantitative evaluation by measuring its volume, diameter and integrity. Since visual qualification of corticospinal tract involvement requires knowledge or experience for scoring, these quantitative MRI measurements were considered less variable, more objective and therefore advantageous. We included 21 preterm and 24 term born infants with unilateral brain lesions who had an MRI scan at around three months postnatal age: term equivalent for preterm and three months after birth for term born infants. The MRI included diffusion tensor imaging (DTI), a sequence that enabled us to study white matter integrity from fractional anisotropy (FA) measures in the descending corticospinal tract. HAI was performed at three timepoints: before 5, between 5-8 and between 8-12 months of (corrected) age. When we compared asymmetry indices of all these techniques, we found that at the earliest timepoint, before five months of age, FA asymmetry on DTI yielded the highest value to predict unilateral

cerebral palsy compared to volumetric measurements of the corticospinal tract and HAI scores. HAI specifically aided to the prognosis of unilateral cerebral palsy at later age points, and although this was not the aim of the study, could potentially be used to predict severity of unilateral cerebral palsy in the first year of life.

A new quantitative measure of upper limb asymmetry at an early age was developed in **chapter six** of this thesis: the measurement of upper limb motion by accelerometry. We included 16 infants with unilateral brain lesions on MRI in a pilot study on upper limb asymmetry that compared qualitative function by HAI to quantitative motion detection at three months of age. Asymmetry on both HAI and accelerometry was more profound in infants that exhibited clinical signs of unilateral motor abnormalities. For both measures, a cut-off of >20% asymmetry was proposed to reliably detect motor abnormalities, although future studies with larger number of patients and controls are needed to confirm this. Our population mainly consisted of infants at risk of developing unilateral cerebral palsy, as they were all found to have a unilateral or asymmetric brain lesion on MRI. It would be of interest to see if our cut-off of >20% asymmetry on both HAI and accelerometry also applies in the general population. Overall, we found that the positive predictive value of the HAI to detect early motor abnormalities was higher than that of the accelerometry. However, due to its ability to quantify motion without the use of a trained observed, we are convinced that accelerometry may be of additional value in future clinical use. Beside prediction of cerebral palsy, it may also play a role in continuous monitoring of abnormal motion patterns on the NICU, detect rhythmic motions due to epileptic activity or detect repetitive behavioral patterns in developmental disorders. In line with **chapter five**, we favor the use of quantitative measurements for outcome prediction, that can be applied without the use of an experienced (and possible biased) observer, and are not prone to inter-observer variability. Motion sensors such as accelerometers are safe, easily applicable and their potential application for detecting subtle motion asymmetries at an early age needs to be confirmed further. Apart from their additional value to clinical care, motion sensors could potentially replace some of the motor assessments in the future. For example, when motion devices are applied in the home-setting, they may be able to select those infants at risk for motor disabilities for additional follow-up assessments in the hospital. This may reduce the screening burden on the observational therapists, limit the number of infants who undergo unnecessary assessments and improve cost-effective early detection of unilateral cerebral palsy.

In **chapter seven** we reported on the use of early continuous neuro-monitoring for the prediction of motor and cognitive outcome in the first five days after PAIS. Patterns of cerebral electrical activity on amplitude-integrated encephalography (aEEG) and regional cerebral oxygenation ($rScO_2$) saturation on near-infrared

spectroscopy (NIRS) were found to be affected by PAIS: background pattern recovery was longer and asymmetry in $rScO_2$ between hemispheres increased. We also found that in this cohort of 52 term infants with PAIS, neuro-monitoring parameters were negatively related to cognition, which was not described before. As demonstrated in this study, neuro-monitoring can be applied directly after clinical presentation, and can potentially be used for prediction of cognitive outcome very early in life, even before MRI scanning can be performed. An early screening instrument should preferable have low numbers of false-negatives, resulting in relatively high sensitivity (potentially at a cost of specificity), to limit the number of infants missed for follow-up evaluation to confirm a diagnosis of adverse outcome. This was in contrast to our findings, as we demonstrated neuromonitoring predicted adverse outcome with very high specificity (88-97%) but quite low sensitivity (52-67%). Future prospective studies are needed to confirm our findings before drawing firm conclusions on the use of neuromonitoring as a prediction tool for adverse outcome after PAIS. These studies should potentially apply neuromonitoring as soon as possible after clinical presentation in all stroke subtypes, not only including those with seizures, but also continue beyond a timeframe of five days to be able to detect more differences over time. Additionally, aEEG and NIRS could also be used to monitor the effect of early intervention directly at the bedside. For example, the use of agents that influence oxygenation or brain activity might have a direct effect on neuromonitoring that potentially also affects future outcome. Therefore, neuromonitoring should perhaps be implemented as an endpoint in intervention trials.

When combining the chapters of Part I of this thesis, we can conclude that several early measures can be used for the prediction of outcome in infants with unilateral perinatal stroke as caused by PAIS and PVHI. These measures can be obtained starting at the first days to weeks after presentation. Most measures, either qualitative or quantitative, focused on asymmetry between ipsi- or contralesional hemispheres or upper limbs, as it is often the earliest sign of unilateral brain injury and strongly related to abnormal motor development.[19,20] Qualitative measures included scoring of involvement of the corticospinal tract on MRI, upper limb functioning by HAI and brain activity on aEEG. Quantitative measures included volume, diameter and integrity of the corticospinal tract on (DTI)-MRI, upper limb motion using accelerometry and cerebral oxygenation by NIRS. All measures were found to correlate to motor or cognitive outcome, but their predictive values were not all mutually compared. Only chapter five compared conventional MRI to DTI and HAI, and found that FA asymmetry on DTI yielded the highest predictive value for cerebral palsy compared to volumetric measurements of the corticospinal tract and to HAI scores. However, in chapter six we described that asymmetry on HAI at three months of age was strongly related to clinical motor outcome, with a negative and positive predictive value of

100%. For future research, more studies should focus on comparing or combining prediction measures for adverse outcome after perinatal stroke. For example, we described in chapter five that clinical upper limb assessment with HAI might be of additional value in infants in whom (follow-up) MRI and/or DTI is not performed or its result is uncertain.

Additionally, the measures of brain activity and oxygenation from chapter seven are performed in the first five days after clinical presentation, much earlier than most MRI scans or clinical assessments can be obtained. It might be helpful to develop a prediction protocol that includes several different parameters at different time points, in order to provide each patient with an accurate and specific prognosis on his or her expected outcome on several domains. This protocol may be able to sum up all risk factors providing the clinician with a score that predicts the risk for adverse outcome. The earliest measures, such as aEEG, NIRS and early MRI, may select those at risk for adverse outcome for additional assessments later in life. These additional assessments could include HAI or accelerometry to increase accuracy of the prognosis from 3-12 months of age. On the other hand, those without risk factors on early selection tools may be protected from the use of extra assessments at follow-up. Other prediction tools that may be added to the proposed prediction protocol are transcranial magnetic stimulation, which provides information on the brain's ability to reorganize after perinatal stroke, or advanced imaging tools such as connectivity analysis of brain networks. These tools should be developed further in their individual ability to predict outcomes after perinatal stroke, but also combined with other prediction parameters to assess their additional value.

Several of our studies have shown excellent ability to predict adverse outcome after perinatal stroke. Many imaging and monitoring tools can be used to estimate risks and provide families and caretakers with tailor-made prognosis of future outcome on several domains after PAIS or PVHI. However, others remain skeptical on the use of early prediction of future outcome. Families might be affected by false hopes, unnecessary worries or be overwhelmed by information at a time their child is admitted to the hospital or experience parenthood for the very first time. Prediction of outcome should therefore be considered with care and balanced between doing good and doing harm. Whether infants benefit from early prediction might also depend on availability of interventions that could influence future perspectives for those most at risk of adverse development. This will be discussed in part II of this thesis.

PART II: FUTURE THERAPIES FOR PERINATAL STROKE

Up until now, treatment options after PAIS mainly focused on supportive care, such as treating seizures, hypoglycemia or infections, but these treatments have no long-term protective or curative effect on the brain. Early neuroprotective treatments, such as therapeutic hypothermia, aim at preventing production of free radicals and apoptotic cell death in the brain. In order to be effective, therapeutic hypothermia requires initiation early after a well-documented hypoxic-ischemic insult that leads to moderate to severe encephalopathy.[22,23] Patients with PAIS usually present between 12-48 hours after birth, far beyond the therapeutic timeframe, making these early neuroprotective treatment strategies not applicable to PAIS. Therefore, therapeutic focus has rather shifted to interventions that stimulate repair of damage instead of protecting the newborn brain from early damage.[24]

After hypoxic-ischemic brain injury, the brain automatically enhances its self-renewing capability by promoting proliferation of new precursor cells in endogenous stem cell niches of the brain.[25,26] These processes are mediated by the expression of several neurotrophic factors that regulate cellular processes such as apoptosis, inflammation, angiogenesis, cell differentiation and proliferation.[27] The expression of these growth factors in the brain is increased after hypoxia-ischemia and aids in boosting neurogenesis in the newborn brain, resulting in greater compensatory capacities than the mature adult brain after injury.[27] However, the newborn brain still lacks the capacity of full repair after hypoxic-ischemic injury and assisting this process may improve repair and subsequent outcome. **Chapter eight** provided an overview of preclinical and clinical evidence of new therapeutic agents that aim to stimulate neurogenesis after ischemic brain injury. Animal models of perinatal stroke using a permanent or transient occlusion of the MCA, resembling focal ischemic injury in PAIS, were discussed. Our study showed that in literature administration of neurotrophic growth factors, including erythropoietin (EPO), brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF) and glial-derived neurotrophic factor (GDNF) improved histological and functional outcome in rodent models of PAIS. Additionally in chapter eight, the potential of multipotent MSCs was discussed, as we proposed that these cells secrete a plethora of trophic factors that can boost endogenous repair of the injured neonatal brain. The review described several studies that demonstrated the beneficial effect of MSCs in reducing infarct volume and improving functional outcome in rodent models of perinatal stroke. We also provided an overview of clinical trials with neurotrophic factors and MSCs in neonatal ischemic brain injury that have been performed or are still ongoing. Until now, only EPO has been studied as a therapy in clinical human trials focusing mainly on HIE and only one trial of our own group has demonstrated feasibility of EPO treatment for perinatal stroke.[28,29] Other neurotrophic factors or MSCs may be

used as potential future therapies to treat PAIS. As only a few study groups have demonstrated a potential effect of MSCs or growth factory therapy specifically for perinatal stroke (instead of HIE), more evidence from preclinical and clinical studies is needed before effectiveness in PAIS can be confirmed.

In **chapter nine** we elaborated further on MSCs as potential therapy to stimulate repair of the neonatal damaged brain. In this study we reviewed the current state, potential hurdles and first steps to bring stem cell-based therapy into the clinic for neonatal brain injury. We described that of all potential multipotent cell types, MSCs seem most promising for near-future use in human neonatal clinical trials, due to their favorable safety profile and paracrine effects to stimulate endogenous repair of the damaged brain. Practicalities for clinical use of MSCs were also discussed in this study, including administration route, dosing and time window. Intranasal administration of MSCs seems promising, as it is a non-invasive route that targets MSCs to migrate directly into the brain, preventing loss of MSCs in other peripheral organs, as seen following systemic (e.g. intravenous) administration. The intranasal route was been proven effective to restore brain damage and improve motor outcome in neonatal rodents with hypoxic-ischemic brain injury. Intranasal delivery was shown to be equally effective as local delivery of MSCs by intracranial injection. Future studies need to demonstrate whether this route is also effective in species with less developed olfactory systems, resembling human neonates. This was discussed in chapter ten.

We provided an overview of human trials studying safety and feasibility of cell-therapy in neonates with disorder such as HIE, intraventricular hemorrhage, hydrocephalus or acquired neurological disorders. More recently, a Korean group published the results of a pilot study demonstrating the use of intraventricular MSC administration to treat severe intraventricular hemorrhage (including PVHI) in preterm infants between 24 and 31 weeks of gestation.[30] They demonstrated that administration of up to 1 x 10^7 MSCs intraventricularly was safe, there were no serious adverse effects such as toxicity or mortality. Even though there was no control group, the authors demonstrated feasibility of MSC therapy as some infants showed continuous improvement and were discharged without further interventions. These promising results are not yet available for neonates with PAIS, but our own group about to start the first clinical trial studying the safety and feasibility of intranasal MSC therapy for term neonates with PAIS. This PASSIoN (Perinatal Arterial Stroke Treated with Stromal Cells Intranasally) trial aims to include ten infants with PAIS and treat them with 50 x 10^6 MSC intranasally within one week after presentation (ClinicalTrials.gov identifier NCT03356821). Future studies are also needed to evaluate the optimal administration route, dosing, time window and type of cell therapy to be effective in reducing brain damage after perinatal stroke.

In **chapter ten** we report that the intranasal route is effective for MSC administration in a baboon model of neonatal brain injury. This study was necessary to translate results from rodent studies on intranasal MSC administration to a clinical application, as the olfactory anatomy differs largely between rodents and humans. Two newborn baboons underwent bilateral carotid artery occlusion followed by systemic hypoxia at postnatal day 5-7 to create global hypoxic-ischemic brain injury. Twenty-four hours later, the animals were treated with 30 x 10^6 umbilical-cord derived MSCs intranasally. The MSCs were labeled with a fluorescent marker (PKH) prior to intranasal application, and this signal could be detected in the brain at 18 hours following administration. The results from chapter ten confirmed previous data from our groups that demonstrated that PKH-labeled MSCs migrated specifically to the damaged brain areas in a neonatal rodent model of hypoxia-ischemia.[31] Although, due to ethical reasons, we were not able to study efficacy of the MSCs to restore brain injury and improve function in the primate model, this study did demonstrate that the intranasal route is effective to deliver MSCs to the damaged brain in an animal model resembling the human patient. This step will allow us to soon study safety and feasibility of intranasal MSC treatment for perinatal stroke in a clinical trial.

In **chapter eleven** we outlined the study protocol of the DINOSAUR trial (registered at ClinicalTrials.gov as NCT03171818), an international, multicenter randomized controlled trial that studies the effect of darbepoetin alfa compared to placebo in the reduction of brain injury in (near-)term neonates with PAIS. This study succeeds an earlier pilot study in which 21 infants with PAIS who received open label treatment with recombinant human EPO (rhEPO).[28] The earlier study did not show any adverse effects of rhEPO between treated infants and their historically matched controls.[28] Term infants with PAIS are eligible for the study when their infarct is located in the MCA territory and involves the corticospinal tract, selecting only those most at risk for adverse outcome (chapters 2-5). The ongoing DINOSAUR trial, as we described here, needs to confirm a beneficial effect of erythropoietin-stimulating agents (ESA) for PAIS, and may provide evidence for the first therapeutic agent that aims to stimulate neuroregeneration after PAIS and subsequently improve neurodevelopmental outcome.

Part II of this thesis focuses on the development of new therapeutic agents for PAIS and the last steps necessary to bring these new therapies to the clinic. These potential therapies focus on stimulating repair after neonatal brain injury, as patients with PAIS usually present beyond the time window of early neuroprotective interventions. Chapter eight outlined two major research lines along which future therapies may be developed: on one hand neurotrophic agents and on the other hand cell-based therapy using MSCs. The use of neurotrophic agents seems promising, as many human clinical trials have been performed, or are currently underway, that study the

effect of erythropoietin to improve neurodevelopmental outcome. As summarized in chapter eight, RCTs comparing EPO to placebo after HIE demonstrate that EPO is safe, well-tolerated and may result in less brain damage on MRI and improved neurodevelopmental outcome at one year of age.[32,33] However, the results of the very recently finished trial studying the effect of rhEPO in over 800 preterm neonates are disappointing: rhEPO did not result in neurodevelopmental benefits when compared to a placebo.(Oral communication by Prof. S. Juul at Pediatric Academic Societies Meeting, Baltimore 2019) Perhaps the administration of one neurotrophic factor is not enough to make a difference. As described in chapter eight and nine, MSCs could function as miniature factories that produce a mixture of growth factors. Their secretome includes several growth factors such as VEGF, BDNF, nerve growth factor, basic fibroblast growth factor as well as anti-inflammatory cytokines.[34] When MSCs are able to reach the damaged areas of the brain, they might be able to adapt to their environment and produce exactly those growth factors that are necessary to increase endogenous neurogenesis. In this way, MSCs could be able to exert beneficial effects via multiple paracrine routes that together create an environment that facilitates tissue regeneration, subsequently leading to improved neurodevelopmental outcome in a tailor-made way.

Timing of therapy remains an important issue when treating neonatal brain injury. From preclinical work from our own group, we have demonstrated that MSCs are unable to reach the brain when administered beyond 17 days after hypoxic-ischemic brain injury, and subsequently do not have an effect on lesion reduction or functional outcome.[31] This was explained by the lack of chemotactic signals (such as CXCL10), which probably play an important role in cell migration to the brain.[35] Several groups have studied the effect of cell therapy for the treatment of cerebral palsy in older children and these results are not yet convincing. Although most studies have shown that stem cell administration is safe and well tolerated, the effects on motor outcomes are contradictory.[36] A review from 2016 described a small significant effect of stem cells on short term improvement of gross motor skills after cerebral palsy.[37] However, there were many heterogeneities between type of cells, treatment window, dosing and other procedures in these studies, limiting the body of evidence on treatment of cerebral palsy with stem cells. The lack of an acute brain injury in older infants with cerebral palsy, limiting the amount of potential chemotactic attractants for cells to reach the brain, may be an important reason for disappointing results. It also emphasizes the need for cell therapies that can be administered at an early stage to be able to repair brain injury and reduce the risk for developing cerebral palsy.

The chapters in part II of this thesis mainly outline therapies that eventually aim to repair damaged brain tissue after perinatal stroke. As outlined in chapter eleven of this thesis, this effect could potentially be measured by serial volumetric brain tissue

measurements on MRI. The underlying hypothesis is that restoration of tissue volume loss will eventually lead to improved neurodevelopmental outcome. The association between brain tissue volume and outcome parameters has been investigated in several studies on preterm infants: overall smaller brain volumes are associated with worse cognitive and motor outcomes.[38,39] However, the exact relation between tissue loss or remaining brain tissue and outcomes remains largely unstudied in PAIS patients. Perhaps the amount of brain tissue does not automatically reflect the level of functional outcome. It would be helpful to determine specific brain tissue volumes cut-off that are associated with good outcomes, to know what therapeutic studies should eventually aim for to improve outcome after neonatal brain injury.

Most recent studies reporting on adverse neurodevelopmental outcome after perinatal stroke conclude their work with recommendations for the development of new therapies to improve these adverse outcomes. However, a study from 2014 demonstrated that school-aged children who suffered from PAIS did not demonstrate lower scores of quality of life compared to their peers.[40] Although the authors described this as a 'disability paradox', it reminds us that researchers are not always aware how patients and their families experience disabilities caused by PAIS. Work from others demonstrates that quality of life after perinatal stroke is decreased in infants with disabilities compared to others, and this level depends mainly on degree of the impairment.[41–43] The ultimate question is what new therapeutic agents should eventually aim for. Perhaps, therapies should be focused more on quality of life and participation in society, than on restoration of brain tissue or improving intelligence quotients. It would be helpful if more studies reporting on neurodevelopmental outcome, or on the results of intervention studies, would take these parameters into account as well.

CONCLUSIONS

The following conclusions can be drawn from this thesis:

- Neurodevelopmental outcome is invariably adverse in at least one domain with main branch MCA stroke, while outcome was normal in 43-71% of children with other PAIS subtypes. (chapter 2)
- The most important outcome predictors are involvement of the corticospinal tracts and basal ganglia on MRI. (chapter 2 and 4)
- Optimal timing of MRI imaging in PAIS patients for prognosis may be not as soon as possible, but more likely beyond 48 hours after presentation. (chapter 3)
- A combination of neuroimaging and clinical assessment improves accuracy for the prediction of unilateral cerebral palsy. (chapter 4)
- Before five months of age, FA asymmetry on DTI yielded the highest value to predict unilateral cerebral palsy, compared to conventional MRI and clinical hand function assessment. (chapter 5)
- Accelerometry is feasible to detect upper limb asymmetry in infants with unilateral brain injury at the age of three months. (chapter 6)
- Cerebral electrical activity and oxygenation are both affected by PAIS in the first five days after presentation, and related to neurocognitive development. (chapter 7)
- Mesenchymal stem cells could function as miniature factories of a mixture of growth factors to repair neonatal brain injury in a tailor-made way. (chapter 8)
- Stem cell-based therapy is a promising near-future treatment strategy for neonatal brain injury. Mesenchymal stem cells have most favorable characteristics among other stem cell types for clinical use. (chapter 9)
- Mesenchymal stem cells are efficiently delivered to the injured newborn non-human primate brain after intranasal application, in accordance with previous rodent studies. (chapter 10)
- The DINOSAUR trial will determine if intravenous darbepoetin alfa started within the first week after birth is effective in reducing brain damage in (near-) term newborns ≥36 weeks of gestation with MRI-confirmed middle cerebral artery PAIS, as compared to a placebo. (chapter 11)

FUTURE DIRECTIONS FOR RESEARCH

Prognosis, diagnosis and severity

Many studies that aim to predict future neurodevelopmental outcome after perinatal stroke, use binary measures to determine eventual outcome. Infants are classified as having an adverse outcome or not. However, variation in outcome severity might be even more relevant to patients, their families and caretakers. For example, the Gross Motor Function Classification Score (GMFCS) classifies cerebral palsy into five categories: patients with GMFCS class I are able to walk unaided, while those in GMFCS class V are wheelchair bound and often unable to maintain antigravity head and trunk postures. Most infants with unilateral PAIS or PVHI usually remain within GMFCs classes I-III (chapter 2). Furthermore, severity of learning disabilities may eventually determine whether patients are able to attend mainstream education or not. However, when counselling families on the prognosis of neurodevelopmental outcome, we often do not anticipate on the severity of their disability, and prediction tools are not yet equipped to do so. Future studies should focus not only on optimizing prediction of adverse outcome, but also on grading these disabilities to give a realistic prognosis for future development. Recent work from Sakzewski et al. demonstrated that the HAI is able to distinguish three trajectories of hand development for infants with unilateral cerebral palsy based on their level of functioning.[44] The HAI may therefore be an optimal early prediction tool to not only predict the development of cerebral palsy, but also the degree of impairment. Such tools should also be further developed for other neurodevelopmental disabilities such as cognition or epilepsy.

Term versus preterm born infants

Chapter five of this thesis demonstrated that there were differences between term and preterm born infants in the ability of asymmetry indices on MRI and hand function to predict unilateral cerebral palsy. These could be explained in several ways. First, the set-up of that study was that infants had an MRI scan around three months postnatal age, which resulted in differences of postmenstrual age at time of scanning. Term infants were approximately ten weeks older at the time of the scan than the preterm born infants, subsequently leading to more developed and mature brains with increased myelinization that may result in improved detection of asymmetry on MRI. Furthermore, PVHI, a disease of the preterm infant and restricted to the white matter, usually results in milder forms of cerebral palsy when compared to PAIS in term infants where the lesion is located in the cortex, subcortical area and basal ganglia.[45] This may lead to differences in the predictive ability of hand function for the prediction of cerebral palsy between term and preterm infants. The predictive measures for outcome after unilateral brain lesions as described in part

I of this thesis, should therefore always be considered in their specific (preterm or term) population, and may not simply be extrapolated to all infants with unilateral perinatal brain injury.

Additionally, brain plasticity, such as reorganization of white matter tracts, is affected by age of onset and the type of brain injury, which differed between preterm and term born infants in our study population.[46] PAIS is more often diagnosed in term infants, while PVHI is the most frequent form of unilateral brain injury in preterm infants. Variation in brain plasticity between populations is an important factor to take into account when predicting adverse outcome, but it may also be important to target new therapies that aim for stimulating neuroregeneration. The use of growth factors and stem cells to treat (hypoxic-)ischemic brain injury, as outlined in chapter eight and nine of this thesis, is found to be potentially effective for term infants. Many ongoing studies focus on developing these therapies for term born infants with HIE and perinatal stroke. Preterm infants have been shown to benefit from ESA administration by improved outcome, but the use of other (cell-based) therapies for preterm infants with (unilateral) perinatal brain injury, remains less well studied. As brain plasticity is hypothesized to be larger in the preterm compared to the term brain, neuroregenerative therapies may potentially be even more effective after preterm brain injury. Additionally, variation in pathophysiology of perinatal brain injury may also result in differences between affected brain tissues. For example, PVHI is mainly restricted to the white matter, while PAIS usually affects the cortex, white matter as well as deep grey matter structures. Some therapies may be able to regenerate only specific brain cell populations, such as neurons or oligodendrocytes, resulting in differences between neurogenerative therapy potential in term and preterm populations. Future research should focus on the development of clinically relevant preterm models of brain injury to study the effect of new neuroregenerative therapies on restoration of brain tissue and function.[47]

Optimization of therapy

Until now, most clinical trials focus on studying the effect of a new therapeutic agent to improve outcome compared to a placebo. However, comparing different therapies among each other is also essential to optimize future strategies to improve outcome after perinatal stroke. Additionally, combining several strategies may also be an interesting option to explore. For example, differences between the optimal therapeutic window between therapeutic agents may result in a treatment protocol that includes several therapies at different time points. The earliest strategies may focus on protecting the brain from additional or secondary injury, such as reduction of hyperperfusion leading to formation of reactive oxygen species or target radical formation. After that, neurotrophic factors that stimulate repair may be used to

treat the injured neonatal brain. These can either focus on establishing an optimal environment for endogenous repair such as increasing angiogenesis or reducing scar formation; but also on direct repair by neurogenesis, synaptogenesis or oligogenesis. Final steps to improve outcome after perinatal brain injury should focus on brain plasticity by stimulating function. Directions for future research include integration of these different treatment targets to take advantage of all steps that have been taken to develop a therapeutic strategy to eventually improve outcome after perinatal stroke.

Furthermore, optimization of MSC therapy may also be a key to success. There is an ongoing debate on the optimal source of MSCs for use in treating neonatal brain injury. MSCs can be obtained from several different tissue sources including the bone marrow, umbilical cord or adipose tissue. Some studies suggest that MSCs from birth-associated tissue such as the umbilical cord may be more potent that those from adult tissue in terms of additional primitive properties with higher proliferation and differentiation rates.[48,49] However, a recent study that compared the neuronal induction potential of MSCs from different neonatal and adult sources, did not confirm this.[50] This study demonstrated that MSCs from both bone marrow and the umbilical cord have high potential to generate neuronal cells and could both be used to treat brain diseases. Recent studies suggest that breast milk contains MSCs, among other cell-types, that could provide beneficial effects on neurodevelopment after intranasal administration.[51] As breast milk can be easily obtained for neonates, it might be an interesting source for MSCs to explore in future studies. Identifying the most suitable cell type and cell source for treating perinatal stroke is essential to optimize therapy and eventually improve outcome.

Several studies investigate a combination of MSC therapy with additional neurotrophic factors by administration of MSCs that overexpress a neurotrophic factor (e.g. BDNF-overexpressing MSCs, EPO-transduced MSCs or GDNF-modified MSCs)[52–54] Until now, most of these MSCs transduced with neurotrophic factors have been used to treat adult stroke, but we hypothesized that future MSC therapy for neonatal brain injury may be improved by manipulating these cells to produce enhanced levels of specific growth factors. Additionally, combining administration of several types of MSCs with specific neurotrophic factors may also optimize therapy. Gonzalez et al. recently demonstrated that combined administration of MSCs and EPO improved cognition more than MSCs therapy alone in a rodent model of perinatal stroke.(Oral communication at Pediatric Academic Societies Meeting, Baltimore 2019) Currently, this group is studying whether combined administration of MSCs and EPO is more effective than EPO alone for improving outcome. The interplay between several neurotrophic factors and MSCs to stimulate brain repair and improve outcome, needs to be further elucidated in (pre)clinical studies to optimize regenerative treatment strategies for perinatal stroke.

The advantages of collaboration

Multidisciplinary teams are not only important to take care of newborns presenting with clinical symptoms of perinatal stroke, they are also essential in the follow-up of these infants. Rehabilitation specialists, including physio- and occupational therapists, play an important role to diagnose disabilities and enhance functioning in daily life. Although many patients across the world suffer from consequences of perinatal brain injury, the research field of neonatal neurology remains relatively small. Collaboration with different research groups across the world is essential to exchange knowledge on diagnosis and prognosis, but also to learn from others on the development of new treatment strategies. As we have described in chapter two and four, combining cohorts of infants affected by perinatal stroke improved our ability to predict outcomes. Furthermore, the ongoing DINOSAUR trials as outlined in chapter 11 will hopefully be the first to demonstrate an effect of a new therapy in a large group of PAIS patients. This trial is made possible due to joint effects of several teams across the world that aim for the ultimate common goal to improve the future of infants affected by perinatal stroke. Future research should not only focus on improving international and interdisciplinary collaborations, but also aim to involve patients and families, as they are crucial to direct research questions that they will eventually benefit from.

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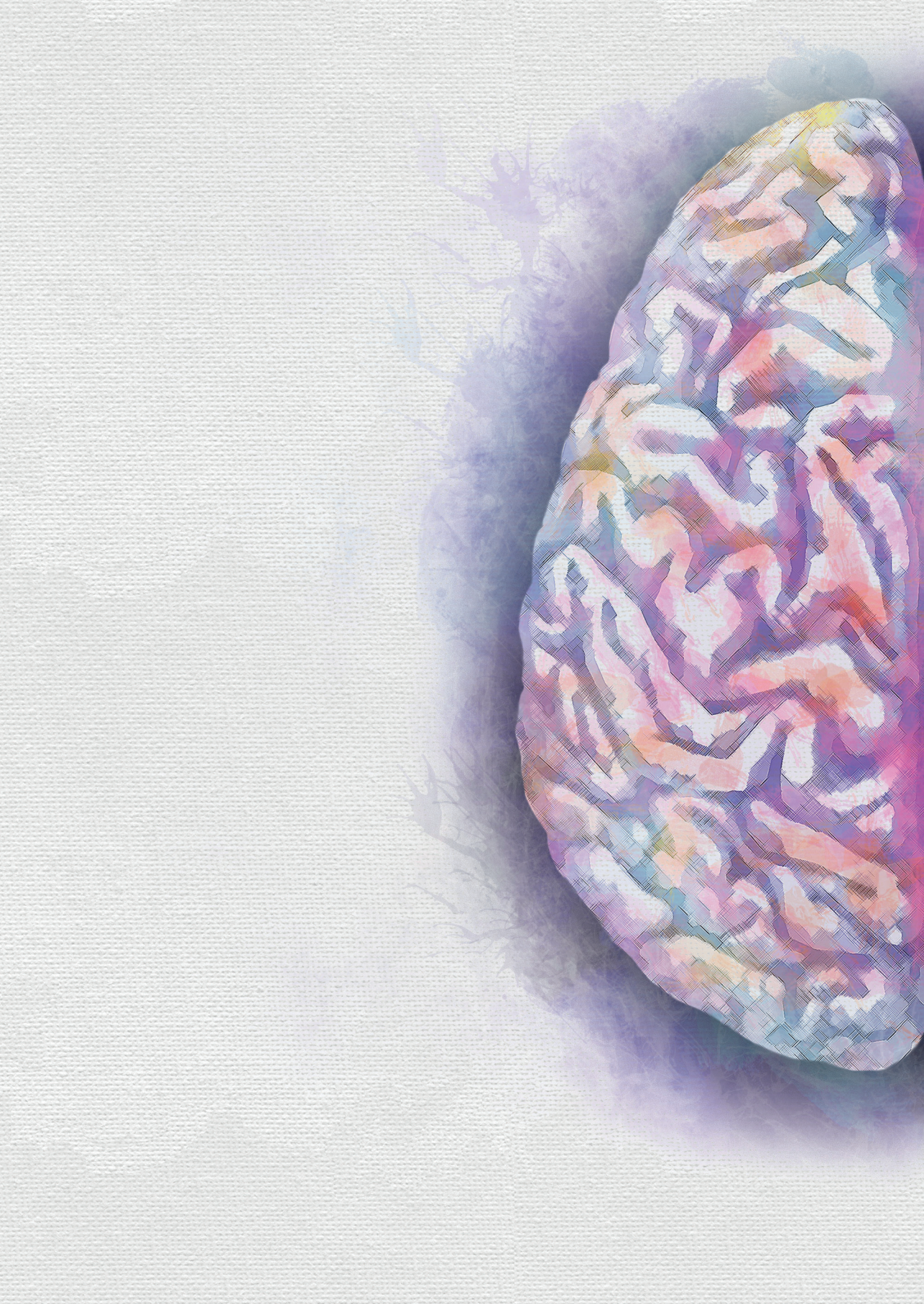

CHAPTER 13

NEDERLANDSE SAMENVATTING
(SUMMARY IN DUTCH)

OVERZICHT VAN DIT PROEFSCHRIFT

Het perinatale arteriële ischemische herseninfarct (PAIS) is een belangrijke oorzaak van neurologische ontwikkelingsstoornissen in verschillende domeinen, waaronder motoriek, cognitie en gedrag. Het vroegtijdig voorspellen van deze gevolgen is belangrijk om ouders en verzorgers adequaat te kunnen informeren en om patiënten te selecteren die baat hebben bij vroege behandelstrategieën. Deel I van dit proefschrift beschrijft verschillende vroege parameters die helpen om kinderen met het hoogste risico op een ongunstige ontwikkeling te selecteren. Hierbij wordt gebruik gemaakt van een combinatie van beeldvorming, hersenbewaking en functioneel onderzoek.

Deel II van dit proefschrift richt zich op het verbeteren van de neurologische ontwikkeling met nieuwe behandelstrategieën, waaronder neurologische groeifactoren (neurotrofines) en stamcellen. Er wordt een overzicht gegeven van verschillende nieuwe potentiële behandelingen, evenals de laatste translationele stappen om deze therapieën naar de kliniek te brengen. Het proefschrift wordt afgesloten met het onderzoeksprotocol van een lopende gerandomiseerde multicenter studie naar een nieuwe potentiële therapie om de uitkomsten van neonaten met PAIS te verbeteren.

NEDERLANDSE SAMENVATTING

Ondanks grote ontwikkelingen in perinatale en neonatale zorg, blijft perinatale hersenschade één van de belangrijkste complicaties voor de pasgeborene op de neonatale intensive care unit (NICU), met vele gevolgen voor de toekomst. Het perinataal arterieel ischemisch herseninfarct (PAIS) is een vorm van perinatale hersenschade die redelijk vaak voorkomt bij op tijd geboren zuigelingen op de NICU met een incidentie tussen de 1:1600 tot 1:5000, afhankelijk van het gebruik van beeldvorming voor het stellen van de diagnose.[1] Het periventriculair hemorragisch infarct (PVHI), ook wel een intraventriculaire hersenbloeding met betrokkenheid van het hersenparenchym genoemd, is de belangrijkste vorm van hersenschade bij prematuur geboren kinderen, met een incidentie van ongeveer 1-3%.[2] Zoals in het eerste hoofdstuk van dit proefschrift wordt geïntroduceerd, worden de ziektebeelden PAIS en PVHI vaak samen beschreven als het perinatale herseninfarct en treffen ze vaak maar één van beide hersenhelften.

Ook al hebben PAIS en PVHI niet dezelfde oorzaak, de gevolgen die ze kunnen hebben op de ontwikkeling kunnen even groot zijn, zoals motorische en cognitieve handicaps, epilepsie of gedragsproblemen. Het is belangrijk om gezinnen en zorgverleners vroegtijdig te informeren over de prognose van perinatale hersenschade zodat zij realistische verwachtingen hebben en zijn voorbereid op eventuele handicaps. Nog belangrijker is een vroegtijdige prognose om zo snel mogelijk met interventieprogramma's te kunnen beginnen. Deze interventies kunnen de ernst van (motorische) beperkingen verminderen, complicaties voorkomen of de kwaliteit van leven verbeteren door aanpassingen door te voeren in het dagelijks leven, het functioneren of de zorg. Interventies die bewezen effectief zijn voor het verbeteren van de motoriek bij pasgeborenen met een perinataal herseninfarct zijn bijvoorbeeld fysio- of ergotherapie, bimanuele therapie of constraint-induced movement therapie.[3] Deze interventies stimuleren met intensieve fysieke activiteit de plasticiteit van het ontwikkelende brein, bijvoorbeeld door reorganisatie van de corticospinale baan, zoals eerder is aangetoond in dierstudies.[4,5] Reorganisatie vindt plaats na het ontstaan van de schade, wat impliceert dat er behoefte is aan vroege selectie van kinderen die risico lopen op motorische handicaps zodat zij zo snel mogelijk met interventies kunnen starten. Kinderen die risico lopen op andere handicaps, zoals leerproblemen of epilepsie, kunnen mogelijk ook profiteren van een vroege prognose zodat interventies kunnen worden aangeboden, zoals aangepast onderwijs, prikkelreductie of anti-epileptica. Het eerste deel van dit proefschrift richt zich op het gebruik van verschillende variabelen die de neurologische ontwikkeling in verschillende domeinen kunnen voorspellen, door gebruik te maken van beeldvorming en monitoring van de hersenen, evenals het klinisch functioneren.

Deze variabelen kunnen helpen om hoog-risico gevallen vroegtijdig op te sporen, omdat zij mogelijk het meest zullen profiteren van (nieuwe) vroege interventie programma's. Het tweede deel van dit proefschrift beschrijft potentiele nieuwe behandelopties die momenteel in dierstudies worden onderzocht en op de rand staan van vertaling naar de kliniek.

DEEL I: VROEGE VOORSPELLING VAN ONTWIKKELING NA HET PERINATAAL HERSENINFARCT

Verschillende onderzoeken hebben uitgewezen dat patiënten met een perinataal herseninfarct een groot risico lopen op het ontwikkelen van neurologische stoornissen, maar de incidentie en het spectrum van deze handicaps kennen beide veel variatie.[1,6,7] Om een individuele uitspraak te kunnen doen over de verwachte neurologische uitkomst, is het belangrijk om aan te tonen welke factoren de ontwikkeling na een perinataal herseninfarct het meest beïnvloeden. Allereerst is het arteriële stroomgebied van het infarct belangrijk: meestal treft de occlusie de arteria cerebri media (ACM), en minder vaak de arteria cerebri posterior (ACP) of anterior (ACA).[1,8] Verder bepaalt de locatie van de occlusie uiteindelijk het getroffen infarctgebied: zo zal een occlusie in het proximale M1-segment van de ACM bijvoorbeeld leiden tot een groter infarctgebied dan meer distale occlusies.[9] Daarnaast bepaalt de locatie van het infarctgebied grotendeels het effect van de hersenlaesie op het functioneren. Tot nu toe maken de meeste studies geen onderscheid tussen verschillende infarct-subtypes en houden ze daarom geen rekening met de omvang of de locatie van het infarct bij het beschrijven van neurologische ontwikkeling op lange termijn.

Het doel van **hoofdstuk twee** was om zorgverleners een nauwkeurigere inschatting te kunnen laten maken van het risico op een ongunste neurologische ontwikkeling na PAIS door verschillende uitkomstdomeinen in verschillende PAIS-subtypen te beschrijven. Deze studie beschreef een groot internationaal cohort van 161 voldragen neonaten uit twee verschillende centra met zeven verschillende PAIS-subtypen, die werden ingedeeld op basis van vroege MRI-scans. We verzamelden gegevens over het aantal kinderen met cerebrale parese, cognitieve of taalachterstand, epilepsie, gedragsproblemen en gezichtsvelddefecten en vergeleken deze tussen de PAIS-subtypes. We ontdekten dat in de hele groep 54% van de kinderen een ongunstige ontwikkeling had in één of meer van de genoemde domeinen. Honderd procent van de kinderen met een infarct in het volledige ACM-stroomgebied hadden een ongunstige ontwikkeling, terwijl dit percentage veel lager was voor de andere subtypen, variërend van 29% tot 57%. We zagen dat cerebrale parese bij ongeveer 30% van onze PAIS-populatie voorkwam, vergelijkbaar met andere studies.[6,10] Toch ontwikkelde 100% van de kinderen met een volledig ACM infarct een cerebrale parese, terwijl dit percentage slechts 0-21% bedroeg in alle andere subgroepen. Met deze studie was het mogelijk om een onderscheid te maken tussen verschillende specifieke subtypen van het perinatale herseninfarct, en de incidentie van de verschillende uitkomstdomeinen per subtype te beschrijven. Hierdoor is een meer persoonlijke en op maat gemaakte voorspelling van de ontwikkeling op lange termijn mogelijk.

Daarnaast vonden we ook dat betrokkenheid van de corticospinale baan en de basale kernen de belangrijkste voorspellers voor een ongunstige ontwikkeling waren. Deze betrokkenheid kon op basis van diffusie-gewogen opnames (DWI) worden bepaald. Andere onderzoeksgroepen hebben al eerder beschreven dat betrokkenheid van de basale kernen op de MRI geassocieerd was met cerebrale parese[10,11], maar wij ontdekten daarnaast dat betrokkenheid van de basale kernen geassocieerd was met een verhoogd risico op cognitieve- en gedragsproblemen. Anderen hebben al eerder een associatie beschreven tussen betrokkenheid van de corticospinale banen op het niveau van de hersenstam en een verhoogd risico op cerebrale parese[12], maar wij vonden daarnaast een verband met de ontwikkeling van epilepsie. In deze studie werden voornamelijk vroege DWI-scans gebruikt om de verschillende subtypen van PAIS en de betrokkenheid van specifieke regio's te onderscheiden, maar een vroege MRI is misschien niet altijd mogelijk in alle instellingen. Afgezien van MRI zouden andere vroege risicofactoren daarom nuttig kunnen zijn om neonaten met PAIS te selecteren die risico lopen op een ongunstige ontwikkeling. De **hoofdstukken vier tot zeven** richten zich op dergelijke parameters.

Zoals beschreven in hoofdstuk twee biedt MRI, en met name DWI, de mogelijkheid om betrokkenheid van de corticospinale baan na PAIS te beoordelen. In de negentiende eeuw beschreef de Britse wetenschapper Waller dat schade aan de cellichamen (soma) of proximale axonen van neuronen kan leiden tot secundaire anterograde degeneratie van distale axonen en hun myelineschedes.[13] Dit fenomeen werd later "Wallerse" degeneratie genoemd. "Wallerse degeneratie" wordt meestal na enkele weken na de schade in de cortex gezien in de corticospinale baan en de cerebrale pedunkel. Andere onderzoeken hebben aangetoond dat DWI ook in staat is secundaire axonale schade te detecteren in de corticospinale baan voordat er daadwerkelijk degeneratieve veranderingen optreden. Dit fenomeen wordt op de DWI daarom "pre-Wallerse" degeneratie genoemd. Zowel pre-Wallerse als Wallerse degeneratie zijn sterk geassocieerd met de ontwikkeling van motorische handicaps op latere leeftijd.[11,12,14–16] In **Hoofdstuk drie** beschreven we twee voldragen pasgeborenen met PAIS, die al binnen 24 uur na klinische presentatie (d.w.z. trekkingen) een DWI scan kregen, en bij wie deze 48 uur later werd herhaald. Op beide scans was een herseninfarct in het ACM-stroomgebied duidelijk zichtbaar, maar het lage diffusie signaal in de corticospinale baan was alleen te zien op de tweede scan. In deze twee patiënten was er dus sprake van vertraging bij het ontstaan van "pre-Wallerse" degeneratie. Deze studie geeft daardoor belangrijke informatie over de timing van de MRI na PAIS. Zorgverleners in de meeste ziekenhuizen zijn geneigd om zo snel mogelijk een MRI te maken bij pasgeborenen met klinische convulsies, zodat er snel een diagnose kan worden gesteld. In hoofdstuk twee hebben we echter laten zien dat MRI-parameters ook sterk geassocieerd zijn met

neurologische ontwikkeling en daarom een belangrijke rol spelen in prognostische counseling. Deze studie suggereerde daarom dat het eerder maken van een MRI-scan 'niet noodzakelijkerwijs beter is'. Aangezien deze studie slechts twee patiënten bevatte, moet verder onderzoek in grotere groepen patiënten de optimale timing van de MRI-scan verder vaststellen, door een afweging te maken tussen vroege diagnose en prognose van PAIS.

We vervolgden het gebruik van op MRI gebaseerde parameters voor de voorspelling van motorische ontwikkeling na asymmetrisch perinataal hersenletsel in **hoofdstuk vier**, ditmaal gecombineerd met klinische handfunctie. Deze combinatie van beeldvorming en klinisch onderzoek, wordt in de literatuur beschreven als de gouden standaard voor het opsporen van een abnormale motorische ontwikkeling, of het diagnosticeren van cerebrale parese.[17] In dit onderzoek includeerden we 52 pasgeborenen met PAIS, PVHI of een andere eenzijdige hersenlaesie uit twee verschillende ziekenhuizen in Europa. Deze patiënten kregen allemaal een vroege MRI-scan binnen een maand na hun uitgerekende datum, en ondergingen een handfunctie-onderzoek rond de gecorrigeerde leeftijd van vier maanden. De handfunctie werd gescoord met behulp van de Hand Assessment for Infants (HAI), een nieuw gestandaardiseerd instrument dat de functie van de bovenste extremiteiten beoordeelt.[18] In onze multivariabele analyses vonden we dat het combineren van beeldvorming en de klinische handfunctie de nauwkeurigheid van de voorspelling van eenzijdige cerebrale parese verbeterde ten opzichte van het gebruik van één van deze parameters. We stelden een voorspellingsmodel samen waarbij we de volgende parameters combineerden: betrokkenheid van de corticospinale baan en basale kernen op de MRI, zwangerschapsduur, geslacht en de contralesionale handfunctie score van HAI. Dit model was in staat om eenzijdige cerebrale parese te voorspellen met een 'area-under-the-curve' (AUC) van 0.98. Om de klinische toepassing van dit model te verbeteren, hebben we een nomogram ontworpen wat als hulpmiddel kan dienen voor de voorspelling van eenzijdige cerebrale parese. Voordat dit model kan worden gebruikt, moet het eerst in een ander cohort worden gevalideerd.

De HAI is de eerste gestandaardiseerde test die vóór het eerste levensjaar de mate en kwaliteit van de handfunctie in beide handen afzonderlijk en samen meet. Tijdens een gefilmde speel-sessie van 10-15 minuten op de leeftijd van 3-12 maanden, wordt een afzonderlijke contra- en ipsilesionale handscore bepaald, evenals een bimanuele handscore en een maat van asymmetrie tussen beide handen. Deze verschillende scores maakt de HAI vooral geschikt voor neonaten met eenzijdig of asymmetrisch hersenletsel, omdat asymmetrie in de bewegingen van de bovenste ledematen vaak het eerste symptoom is van een abnormale motorische ontwikkeling op jonge leeftijd.[19,20] De HAI is daarom nuttiger dan andere motorische test-instrumenten die niet specifiek gericht zijn op het beschrijven van asymmetrie tussen de bovenste

ledematen, of die dit pas doen op oudere leeftijd.[21] In **hoofdstuk vier** wordt de HAI als eerste van drie opvolgende hoofdstukken (hoofdstuk vier, vijf en zes) gebruikt als voorspeller van eenzijdige cerebrale parese, en de resultaten lijken veelbelovend.

Hoofdstuk vijf is een vervolg op de vraag of we eenzijdige cerebrale parese bij neonaten met eenzijdige hersenschade kunnen voorspellen met behulp van MRI-parameters en klinische handfunctie-scores van de HAI. In dit hoofdstuk vertrouwden we echter niet meer op de kwalitatieve evaluatie van de MRI, maar we beoordeelden de betrokkenheid van de corticospinale baan op MRI door het volume, de diameter en de integriteit ervan te meten. Het doen van dit soort kwantitatieve metingen heeft een groot voordeel ten opzichte van kwalitatieve scores, omdat het kennis en ervaring vereist om de betrokkenheid van de corticospinale baan visueel te kunnen beoordelen. Kwantitatieve metingen zijn dus objectief en minder variabel. In deze studie werden 21 premature en 24 voldragen zuigelingen met eenzijdige hersenlaesies geïncludeerd. Zij hadden allemaal een MRI-scan gehad op de postnatale leeftijd van ongeveer drie maanden: dit kwam overeen met de uitgerekende datum voor prematuren en de leeftijd van drie maanden na de geboorte voor voldragen zuigelingen. De MRI bestond onder andere uit een sequentie genaamd 'diffusion tensor imaging (DTI)'. Deze sequentie stelde ons in staat om de integriteit van de witte stof te bestuderen door het meten van fractionele anisotropie (FA) in de corticospinale baan. Daarnaast werd de HAI uitgevoerd op drie momenten: op de (gecorrigeerde) leeftijd vóór 5 maanden, tussen 5-8 maanden en tussen 8-12 maanden. Bij het vergelijken van de asymmetrie-indices van al deze technieken ontdekten we dat vóór de leeftijd van vijf maanden, de middels DTI bepaalde FA-asymmetrie in de corticospinale baan het sterkst voorspellend was voor eenzijdige cerebrale parese. De waarde van FA-asymmetrie voor het voorspellen van eenzijdige cerebrale parese was namelijk hoger in vergelijking met volumetrische metingen van de corticospinale baan en HAI-scores. HAI was beter in staat om op de latere tijdspunten het ontwikkelen van cerebrale parese te voorspellen. Hoewel we dit niet hebben onderzocht in deze studie, zou de HAI mogelijk kunnen worden gebruikt om de ernst van cerebrale parese voor het eerste levensjaar te voorspellen.

In **hoofdstuk zes** ontwikkelden we een nieuwe kwantitatieve maat voor het meten van asymmetrie van de bovenste ledematen op jonge leeftijd: het meten van beweging van de bovenste ledematen door middel van accelerometrie. Een accelerometer meet versnellingen van beweging. We includeerden 16 baby's met unilaterale hersenschade op MRI in deze eerste studie over asymmetrie van de bovenste ledematen. Het doel van de studie was om op de leeftijd van drie maanden kwalitatieve handfunctie gemeten met HAI te vergelijken met kwantitatieve bewegingsdetectie. Uit de studie bleek dat pasgeborenen met klinische tekenen van een handvoorkeur een hogere maat van asymmetrie op zowel HAI als accelerometrie hadden ten opzichte van

kinderen zonder handvoorkeur. De resultaten van de studie suggereerden van een afkapwaarde van> 20% asymmetrie op beide meetinstrumenten voldoende zou moeten zijn om motorische afwijkingen betrouwbaar te kunnen detecteren. Om deze resultaten te bevestigen, is nader onderzoek nodig met meer patiënten en controles. Onze populatie bestond namelijk vooral uit neonaten die een hoog risico hadden op het ontwikkelen van cerebrale parese, omdat ze allemaal een unilaterale of asymmetrische hersenlaesie op de MRI hadden. Het zou interessant zijn om te zien of onze afkapwaarde van >20% asymmetrie op de HAI en accelerometrie ook van toepassing is in de algemene populatie. In de studie vonden we dat de positief voorspellende waarde van de HAI om vroege handvoorkeur te voorspellen hoger was dan die van de accelerometrie. Toch zijn we ervan overtuigd dat accelerometrie van toegevoegde waarde kan zijn in de toekomst, omdat de accelerometer beweging kan kwantificeren zonder dat iemand hiervoor hoeft te worden opgeleid. Naast voorspelling van cerebrale parese kan accelerometrie mogelijk ook een rol spelen bij de signalering van abnormale bewegingspatronen bij pasgeborenen op de NICU, het detecteren van ritmische bewegingen als gevolg van epileptische activiteit of het herkennen van repeterende bewegingspatronen als gevolg van gedragsproblemen zoals pervasieve ontwikkelingsstoornissen. Net als in hoofdstuk vijf concluderen we de voorkeur te geven aan het gebruik van kwantitatieve metingen voor de voorspelling van ontwikkeling. Deze metingen kunnen namelijk worden gedaan zonder een ervaren (en mogelijk vooringenomen) waarnemer die scores geeft, en de metingen worden niet beïnvloed door variabiliteit tussen de waarnemers. Bewegingssensoren zoals accelerometers zijn veilig en gemakkelijk in gebruik. In de toekomst moet hun potentie om subtiele bewegingsafwijkingen op jonge leeftijd te kunnen detecteren verder worden onderzocht. Afgezien van hun toegevoegde waarde in de zorg, zouden bewegingssensoren in de toekomst mogelijk ook bepaalde motorische testen kunnen vervangen. Als bewegingssensoren namelijk in de thuissituatie worden gebruikt, kunnen ze kinderen met een verhoogd risico op motorische handicaps vroegtijdig selecteren voor aanvullende beoordeling in het ziekenhuis. Dit kan de screeninglast op therapeuten verminderen, het aantal kinderen dat onnodige motorische onderzoeken ondergaat beperken en de kosteneffectiviteit van het vroegtijdig opsporen van eenzijdige cerebrale parese verhogen.

In **hoofdstuk zeven** beschreven we of vroege continue hersenbewaking in de eerste vijf dagen na PAIS een voorspelling kan doen over de motorische en cognitieve ontwikkeling. PAIS bleek van invloed te zijn op het achtergrondpatroon van de elektrische hersenactiviteit op amplitude-geïntegreerde encefalografie (aEEG) en ook op de regionale cerebrale zuurstofsaturatie ($rScO_2$) saturatie op near-infrared spectroscopy (NIRS). Hoe groter het infarct, hoe langer het herstel van het achtergrondpatroon en hoe hoger de asymmetrie in rScO2 tussen de hemisferen. We

ontdekten ook de parameters van het aEEG en de NIRS negatief waren gerelateerd aan cognitie in dit cohort van 52 voldragen pasgeborenen met PAIS. Dit was nog niet eerder beschreven. Met deze studie toonden we aan dat hersenbewaking met aEEG en NIRS direct na klinische presentatie van het perinatale herseninfarct kan worden toegepast, en daarmee potentieel kan worden gebruikt voor het voorspellen van cognitieve ontwikkeling. Deze voorspelling kan dus al heel vroeg in het leven worden gedaan, zelfs voordat een MRI-scans wordt gemaakt. Een vroeg screeningsinstrument heeft bij voorkeur een laag aantal vals-negatieven, wat resulteert in een relatief hoge sensitiviteit (mogelijk ten koste van de specificiteit), om het aantal gemiste neonaten die voor vervolgonderzoek in aanmerking komen te beperken. Dit was tegengesteld aan de bevindingen van onze studie: hersenbewaking voorspelde een ongunstige ontwikkeling juist met een heel hoge specificiteit (88-97%) maar een vrij lage sensitiviteit (52-67%). Voordat we definitieve conclusies kunnen trekken over het gebruik van hersenbewaking als voorspellingsinstrument voor een ongunstige ontwikkeling na PAIS, zijn eerst nieuwe prospectieve studies nodig om onze bevindingen te bevestigen. In deze studies zou hersenbewaking zo snel mogelijk na klinische presentatie moeten worden gestart in alle infarct-subtypes, niet alleen bij neonaten met epileptische aanvallen. Daarnaast zou de bewaking niet al na vijf dagen moeten worden gestopt, om meer verschillen in de tijd te kunnen detecteren. Bovendien kunnen aEEG en NIRS ook worden gebruikt om het effect van vroege interventies te meten. Zo kan het gebruik van middelen die de hersenactiviteit of oxygenatie beïnvloeden, een direct effect hebben op het aEEG of de NIRS, wat dan mogelijk weer de toekomstige ontwikkeling kan beïnvloeden. Daarom moeten interventiestudies hersenbewaking misschien implementeren als een eindpunt.

Als we de **hoofdstukken uit deel I** van dit proefschrift combineren, kunnen we concluderen dat verschillende vroege metingen kunnen worden gebruikt voor de voorspelling van neurologische ontwikkeling bij pasgeborenen met een unilateraal perinataal herseninfarct veroorzaakt door PAIS of PVHI. Deze metingen kunnen worden gedaan vanaf de eerste dagen tot weken na de klinische presentatie van het kind. Zowel de kwalitatieve als kwantitatieve metingen waren vooral gericht op detecteren van asymmetrie tussen de ipsi- of contralesionale hemisferen of bovenste ledematen. Dat komt omdat asymmetrie vaak het eerste teken is van unilateraal hersenletsel en sterk gerelateerd is aan abnormale motorische ontwikkeling.[19,20] Kwalitatieve metingen omvatten bijvoorbeeld het beoordelen van betrokkenheid van de corticospinale baan op de MRI, functie van de bovenste ledematen met HAI en hersenactiviteit op het aEEG. Kwantitatieve metingen omvatten bijvoorbeeld het meten van het volume, de diameter en de integriteit van de corticospinale baan op de (DTI) -MRI, beweging van de bovenste ledematen met behulp van accelerometrie en cerebrale zuurstofsaturatie met behulp van NIRS. Al deze metingen bleken samen

te hangen met de motorische of cognitieve ontwikkeling, maar hun voorspellende waarde werd niet altijd onderling vergeleken. Alleen hoofdstuk vijf vergeleek conventionele MRI met DTI en HAI, en ontdekte dat FA-asymmetrie op DTI de hoogste voorspellende waarde voor cerebrale parese had het in vergelijking met volumetrische metingen van de corticospinale baan en HAI-scores. In hoofdstuk zes beschreven we echter dat asymmetrie op de HAI bij de leeftijd van drie maanden sterk gerelateerd was aan klinische motorische presentatie, met een negatief en positief voorspellende waarde van 100%. Toekomstig onderzoek moet zich meer richten op het vergelijken of combineren van verschillende metingen die een ongunstige ontwikkeling na een perinataal herseninfarct kunnen voorspellen. We hebben bijvoorbeeld in hoofdstuk vijf beschreven dat een klinische handfunctie test zoals de HAI van toegevoegde waarde kan zijn bij neonaten waarbij een (DTI-)MRI niet kan worden gemaakt of als het resultaat daarvan onduidelijk is.

Bovendien worden de bewaking van hersenactiviteit en oxygenatie uit hoofdstuk zeven uitgevoerd in de eerste vijf dagen na klinische presentatie, veel eerder dan de meeste MRI-scans of klinische onderzoeken kunnen worden verkregen. Het kan handig zijn om een voorspellingsprotocol te ontwikkelen met verschillende parameters op verschillende tijdstippen, zodat elke patiënt een nauwkeurige en specifieke prognose krijgt over zijn of haar verwachte ontwikkeling op verschillende domeinen. Dit protocol kan bijvoorbeeld alle risicofactoren bij elkaar optellen en de zorgverlener een score geven die het risico op een ongunstige ontwikkeling voorspelt. De vroegste metingen, zoals aEEG, NIRS en vroege MRI, kunnen de kinderen selecteren die het grootste risico lopen op een ongunstige ontwikkeling. Zij komen dan in aanmerking voor aanvullende onderzoeken op een later tijdstip. HAI of accelerometrie zijn voorbeelden van dit soort aanvullende onderzoeken, die vervolgens de nauwkeurigheid van de prognose vanaf de leeftijd van 3-12 maanden kunnen vergroten. Aan de andere kant kunnen patiënten zonder risicofactoren bij de vroege metingen worden beschermd tegen het gebruik van aanvullende onderzoeken. Andere hulpmiddelen die mogelijk bijdragen aan het voorgestelde voorspellingsprotocol, zijn transcraniële magnetische stimulatie (wat informatie biedt over het aanpassingsvermogen van de hersenen na een perinataal herseninfarct), of geavanceerde beeldvormingstechnieken zoals connectiviteitsanalyses van hersennetwerken. Deze hulpmiddelen moeten verder worden ontwikkeld om te zien wat hun individuele vermogen is om ontwikkeling na een perinataal herseninfarct te kunnen voorspellen, maar ook moet er worden onderzoek wat hun toegevoegde waarde is in combinatie met andere voorspellingsparameters.

Veel van onze studies hebben laten zien dat we heel goed in staat zijn om een ongunstige ontwikkeling na een perinataal herseninfarct te voorspellen. We kunnen verschillende beeldvormingstechnieken en andere onderzoeken gebruiken voor

een risicoschatting en om families en verzorgers een op maat gemaakte prognose te geven over de toekomstige ontwikkeling op verschillende domeinen na PAIS of PVHI. Toch blijven sommige zorgverleners sceptisch over het geven van vroege prognoses betreffende ontwikkeling in de toekomst. Gezinnen kunnen te maken krijgen met valse hoop, onnodige zorgen of worden overweldigd door informatie op een moment dat hun kind is opgenomen in het ziekenhuis of zij het ouderschap voor het eerst ervaren. Het geven van een voorspelling over de toekomst moet daarom zorgvuldig worden overwogen en er moet een afweging worden gemaakt tussen goed doen en schade berokkenen. Of pasgeborenen uiteindelijk baat hebben bij het geven van een vroege prognose kan ook afhankelijk zijn van de beschikbaarheid van interventies. Deze interventies zouden de toekomstige vooruitzichten kunnen beïnvloeden voor juist die patiënten die het grootste risico lopen op een ongunstige ontwikkeling. Dit wordt besproken in deel II van dit proefschrift.

DEEL II: TOEKOMSTIGE THERAPIEËN VOOR HET PERINATAAL HERSENINFARCT

Tot nu toe waren de behandelopties na PAIS voornamelijk gericht op ondersteunende zorg, zoals de behandeling van epileptische aanvallen, hypoglykemieën of infecties. Helaas zorgen deze behandelingen op de lange termijn niet voor bescherming of genezing van de hersenen. Vroege behandelingen die wel bescherming van de hersenen bieden (neuroprotectie), zoals therapeutische hypothermie, zijn gericht op het voorkomen van de productie van vrije radicalen en van het afsterven van cellen in de hersenen. Deze neuroprotectieve behandelingen moeten zo snel mogelijk worden geïnitieerd nadat de hypoxisch-ischemische gebeurtenis die leidt tot hersenschade (meestal rondom de geboorte) heeft plaatsgevonden.[22,23] Meestal presenteren patiënten met PAIS zich tussen de 12-48 uur na de geboorte, ver voorbij het therapeutische tijdsbestek van vroege neuroprotectieve behandelstrategieën, waardoor deze niet van toepassing zijn op patiënten met PAIS. De focus van nieuwe behandelopties is daarom verschoven naar interventies die het herstel van hersenschade stimuleren in plaats van de hersenen beschermen.[24]

Het brein zal zich na hypoxisch-ischemische schade proberen te herstellen door gebruik te maken van zijn zelfvernieuwende (regeneratieve) vermogen. Dit doet het door de vermenigvuldiging (proliferatie) van neurale stamcel-voorlopers te bevorderen.[25,26] Deze processen worden gemedieerd door verschillende neurologische groeifactoren (neurotrofines), die op hun beurt weer allerlei andere processen reguleren, zoals apoptose, inflammatie, angiogenese, cel-differentiatie en cel-proliferatie.[27] Als de hersenen worden blootgesteld aan hypoxie of ischemie, wordt de expressie van deze neurotrofines verhoogd.[27] Dit proces helpt bij het stimuleren van neurogenese in de hersenen van pasgeborenen, en zorgt er uiteindelijk voor dat de hersenen van een pasgeborene zich beter kunnen herstellen na schade dan die van een volwassene. Toch is zelfs het pasgeboren brein niet in staat om zich volledig te herstellen na hypoxisch-ischemische schade. Als we dit proces weten te verbeteren, dan kunnen we herstel bevorderen en daarmee mogelijk de gevolgen van hersenschade verminderen. **Hoofdstuk acht** gaf een overzicht van preklinisch en klinisch onderzoek naar nieuwe therapeutische middelen die erop gericht zijn neurogenese na ischemische hersenletsel te stimuleren. De diermodellen van het perinataal herseninfarct die hier werden beschreven, gebruikten meestal een permanente of tijdelijke occlusie van de ACM, zodat het daaruit volgende letsel lijkt op een perinataal herseninfarct in neonaten. Onze studie toonde aan dat er in de literatuur veel bewijs is voor het gebruik van verschillende neurotrofines, zoals erytropoëtine (EPO), brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF) en glial-derived neurotrophic factor (GDNF). Verschillende

studies in knaagdiermodellen hebben aangetoond dat deze neurotrofines zorgen voor histologisch en functioneel herstel na PAIS. Daarnaast werd in hoofdstuk acht de mogelijkheid van behandeling met multipotente mesenchymale stamcellen (MSC) besproken. Deze cellen zouden namelijk een overvloed aan neurotrofines kunnen uitscheiden, wat het endogene herstel van het beschadigde neonatale brein waarschijnlijk stimuleert. Het review in hoofdstuk acht beschreef verschillende onderzoeken die hebben aangetoond dat MSC's een gunstig effect hebben op het verminderen van de infarct-grootte en het verbeteren van de motoriek bij knaagdiermodellen van het perinataal herseninfarct. We gaven ook een overzicht over verschillende klinische studies die zich richten op het gebruik van neurotrofines of MSC's bij neonaten met ischemisch hersenletsel. EPO werd tot nu toe het vaakst onderzocht bij neonaten met hersenschade, waarbij de meeste onderzoeken zich richtten op de toediening ervan na hypoxisch-ischemische hersenschade door zuurstoftekort bij de geboorte (perinatale asfyxie).[28] Slechts één studie, namelijk van onze eigen onderzoeksgroep, heeft het gebruik van EPO-behandeling bij het perinataal herseninfarct onderzocht.[29] Misschien kunnen andere neurotrofines of MSC's ook worden gebruikt als mogelijke toekomstige therapieën voor PAIS. Omdat slechts een paar onderzoeksgroepen hebben geprobeerd om de effectiviteit van neurotrofines of MSC's voor de behandeling van het perinatale herseninfarct aan te tonen (in plaats van perinatale asfyxie), is er meer bewijsmateriaal uit preklinische en klinische studies nodig voordat de effectiviteit van deze nieuwe behandelingen in PAIS kan worden bevestigd.

Hoofdstuk negen vervolgden we met een review over MSC's als mogelijke nieuwe behandeling voor het stimuleren van herstel na neonatale hersenschade. In deze studie gaven wij een overzicht van de huidige kennis, mogelijke hindernissen en de eerste stappen die nodig zijn om stamcel-gebaseerde therapie voor neonatale hersenschade in de klinische praktijk te brengen. We beschreven dat MSC's het meest veelbelovend lijken voor toekomstig gebruik in klinische studies bij neonaten vergeleken met veel andere potentiële multipotente celtypen. Dit komt door hun gunstige veiligheidsprofiel en doordat ze door paracriene werking het endogene herstel van de beschadigde hersenen stimuleren. Praktische zaken voor klinisch gebruik van MSC's werden ook in deze studie besproken, waaronder de toedieningsroute, de dosering en het tijdsinterval waarin de cellen kunnen worden toegediend. Intranasale toediening van MSC's lijkt veelbelovend, omdat het een niet-invasieve route is die ervoor zorgt dat de MSC's direct naar de hersenen kunnen migreren. Hierdoor wordt verlies van MSC's naar andere perifere organen voorkomen, wat vaak voorkomt als de cellen systemisch (bijvoorbeeld intraveneus) worden toegediend. De intranasale route is bovendien effectief gebleken om hersenschade te herstellen en de motorische uitkomst te verbeteren bij neonatale knaagdieren

met hypoxisch-ischemisch hersenletsel. Daarnaast was intranasale toediening net zo effectief als directe toediening van MSC's in de hersenen met een intracraniële injectie. Toekomstige studies zullen moeten aantonen of de intranasale route ook effectief is bij diersoorten met minder ontwikkelde reukorganen, zoals de menselijke neonaat. Hierover ging hoofdstuk tien.

In hoofdstuk negen hebben we ook een overzicht gegeven van humane studies die de veiligheid en de haalbaarheid van celtherapie bestudeerden bij pasgeborenen met allerlei neurologische aandoeningen zoals perinatale asfyxie, intraventriculaire bloedingen en hydrocephalus. Heel recentelijk heeft een Koreaanse groep de resultaten van een eerste klinische studie gepubliceerd waaruit bleek dat het intraventriculair toedienen van MSC's veilig en haalbaar was bij premature neonaten met een ernstige intraventriculaire bloeding (zoals PVHI) die geboren waren bij een zwangerschapsduur tussen de 24 en 31 weken.[30] Ze toonden aan dat de intraventriculaire toediening van maximaal 1 x 10^7 MSC's veilig was en dat er geen ernstige bijwerkingen waren, zoals toxiciteit of mortaliteit. Ondanks dat de studie geen controlegroep had, toonden de auteurs aan dat MSC-therapie de potentie heeft om schade te herstellen, aangezien sommige neonaten continu herstel toonden en zonder verdere interventies naar huis werden ontslagen. Deze veelbelovende resultaten zijn helaas nog niet beschikbaar voor neonaten met PAIS, maar onze onderzoeksgroep staat op het punt om de eerste klinische studie te beginnen met als doel om de veiligheid en haalbaarheid van intranasale MSC-therapie voor voldragen neonaten met PAIS aan te tonen. Deze PASSIoN-studie (Perinatal Arterial Stroke Treated With Stromal Cells Intranasally) beoogt tien kinderen met PAIS te includeren en hen binnen één week na presentatie intranasaal te behandelen met 50 x 10^6 MSC (ClinicalTrials.gov nummer NCT03356821). Toekomstige onderzoeken zijn verder nodig om het optimale celtype, toedieningsroute, dosering en tijdsinterval waarbinnen de cellen kunnen worden toegediend te evalueren, zodat celtherapie optimaal kan worden ingezet om hersenschade na een perinataal herseninfarct te verminderen.

In **hoofdstuk tien** beschreven we dat de intranasale route effectief is voor MSC-toediening in een baviaanmodel van neonatale hersenschade. Deze studie was noodzakelijk om de resultaten van knaagdierstudies over intranasale MSC-toediening te vertalen naar een klinische toepassing, aangezien de anatomie van het reukorgaan grotendeels verschilt tussen knaagdieren en mensen. Op de leeftijd van 5-7 dagen ondergingen twee pasgeboren bavianen bilaterale occlusie van de halsslagader gevolgd door systemische hypoxie, wat zorgde voor hypoxisch-ischemisch hersenletsel. Vierentwintig uur later werden de dieren intranasaal behandeld met 30 x 10^6 MSC's. Deze MSC's waren afkomstig uit de navelstreng, en voorafgaand aan de intranasale toediening gelabeld met een fluorescente marker

(PKH). Het PKH- signaal kon 18 uur na toediening in de hersenen van beide bavianen worden teruggevonden. De resultaten uit hoofdstuk tien bevestigden eerdere gegevens van onze onderzoeksgroep, welke aantoonden dat met PKH-gelabelde MSC's specifiek naar beschadigde hersengebieden van pasgeboren knaagdieren migreerden na hypoxisch-ischemische hersenschade.[31] Ondanks dat we, vanwege ethische bezwaren, niet in staat waren om de werkzaamheid van de MSC's op het herstellen van hersenschade en verbeteren van de motoriek in het primatenmodel te beoordelen, liet deze studie wel zien dat de intranasale route effectief is voor het vervoeren van MSC's naar de beschadigde hersenen van een diersoort die lijkt op de menselijke patiënt. Na deze stap kunnen we hopelijk binnenkort de veiligheid en de haalbaarheid van intranasale MSC-behandeling voor het perinataal herseninfarct in een klinische studie bestuderen.

In **hoofdstuk elf** schetsten we het studieprotocol van de DINOSAUR-studie (geregistreerd op ClinicalTrials.gov als NCT03171818), een gerandomiseerde, internationale, multicenter studie die de werkzaamheid van darbepoëtine alfa vergelijkt met placebo bij het herstellen van hersenschade bij (bijna-)voldragen neonaten met PAIS. DINOSAUR is een vervolg op een eerdere studie die de veiligheid van recombinant humaan EPO (rhEPO) onderzocht bij 21 kinderen met PAIS in een open-label setting.[29] In die studie werden geen nadelige effecten van rhEPO gevonden toen de behandelde neonaten werden vergeleken met hun historisch gematchte controles.[29] Op tijd geboren kinderen met PAIS komen in aanmerking voor de DINOSAUR studie wanneer het infarct zich in het ACM-stroomgebied bevindt en de corticospinale banen betrokken zijn. Door deze criteria worden de kinderen geselecteerd die het hoogste risico lopen op een ongunstige ontwikkeling (zie hoofdstuk 2-5). De hier beschreven DINOSAUR-studie hoopt een gunstig effect van erytropoëse-stimulerende middelen (ESA) aan te tonen, en hiermee bewijs te leveren voor het eerste therapeutische middel dat neuroregeneratie na PAIS stimuleert en daarmee de neurologische ontwikkeling verbetert.

Deel II van dit proefschrift richt zich op de ontwikkeling van nieuwe behandelingen voor PAIS en de laatste stappen die nodig zijn om deze therapieën naar de kliniek te brengen. Deze potentiële therapieën richten zich op het stimuleren van herstel na neonataal hersenletsel, omdat patiënten met PAIS zich meestal pas presenteren na het korte tijdbestek waarin neuroprotectieve interventies mogelijk zijn. Hoofdstuk acht schetste twee belangrijke onderzoekslijnen waarlangs toekomstige therapieën kunnen worden ontwikkeld: enerzijds middels neurologische groeifactoren (neurotrofines) en anderzijds middels MSC's. Het gebruik van neurotrofines lijkt veelbelovend, omdat er al veel klinische studies hebben onderzocht, of momenteel onderzoeken, of EPO de neurologische ontwikkeling verbetert. Zoals we hebben samengevat in hoofdstuk acht, zijn er al verschillende gerandomiseerde studies bij

perinatale asfyxie patiënten uitgevoerd die EPO vergelijken met een placebo. Deze studies tonen aan dat EPO veilig is, goed wordt verdragen, kan leiden tot minder hersenschade op de MRI en zorgt voor een betere neurologische ontwikkeling na één jaar.[32,33] Toch zijn de resultaten van een zeer recent onderzoek naar de werkzaamheid van rhEPO bij meer dan 800 te vroeg geboren neonaten teleurstellend: rhEPO zorgde niet voor neurologische voordelen ten opzichte van een placebo.(Prof. S. Juul, Pediatric Academic Societies Meeting, Baltimore 2019) Misschien is het toedienen van één neurotrofine niet genoeg om het verschil te kunnen maken. Zoals beschreven in hoofdstuk acht en negen, kunnen MSC's functioneren als mini-fabrieken die een mix van neurotrofines uitscheiden, zoals VEGF, BDNF, nerve growth factor (NGF), basic fibroblast growth factor (b-FGF) en anti-inflammatoire cytokines.[34] Wanneer MSC's in staat zijn om de beschadigde delen van de hersenen te bereiken, kunnen ze zich misschien wel aanpassen aan hun omgeving en precies de juiste neurotrofines produceren die nodig zijn om de endogene neurogenese te verbeteren. Op deze manier zijn MSC's in staat om via meerdere paracriene wegen hun werkzaamheid uit te oefenen. Deze wegen samen creëren de ideale omstandigheden voor herstel van hersenweefsel en een betere neurologische ontwikkeling.

Timing van de behandeling is een belangrijk punt van aandacht bij de behandeling van neonataal hersenletsel. Uit preklinisch onderzoek van onze eigen groep hebben we aangetoond dat MSC's niet in staat zijn om de hersenen te bereiken wanneer ze langer dan 17 dagen na hypoxisch-ischemisch hersenletsel worden toegediend.[31] Daardoor hebben ze vervolgens ook geen effect op het verminderen van hersenschade of het verbeteren van de motorische functie in knaagdieren.[31] Dit werd verklaard door het ontbreken van chemotactische signalen (zoals CXCL10), die waarschijnlijk een belangrijke rol spelen bij het migreren van cellen naar de hersenen.[35] Verschillende groepen hebben gekeken naar de werkzaamheid van celtherapie voor de behandeling van cerebrale parese bij oudere kinderen en de resultaten hiervan zijn nog niet overtuigend. Hoewel de meeste onderzoeken hebben aangetoond dat stamceltoediening veilig is en goed wordt verdragen, zijn de resultaten voor de motorische ontwikkeling tegenstrijdig.[36] Een review uit 2016 beschreef dat stamceltherapie voor cerebrale parese een klein significant effect had op het verbeteren van de grove motoriek op korte termijn.[37] Er waren echter veel verschillen tussen de studies in het gebruik van verschillende type cellen, het tijdsinterval, de dosering en procedures, waardoor de hoeveelheid bewijsmateriaal voor de behandeling van cerebrale parese met stamcellen beperkt is. Het ontbreken van een acute laesie bij oudere kinderen met cerebrale parese kan een belangrijke reden zijn voor tegenvallende resultaten. Hierdoor is waarschijnlijk de hoeveelheid chemotactische signalen in de hersenen te beperkt, waardoor cellen niet of in mindere mate tot de hersenen worden aangetrokken en ze het beschadigde hersengebied

slechts in beperkte mate bereiken. Deze resultaten benadrukken ook dat er behoefte is aan celtherapieën die in een vroeg stadium kunnen worden toegediend om hersenletsel te repareren om zo het risico op het ontwikkelen van cerebrale parese te kunnen verminderen.

De hoofdstukken uit deel II van dit proefschrift beschrijven voornamelijk therapieën die als doel hebben om beschadigd hersenweefsel na een perinataal herseninfarct te herstellen. Het effect van de behandeling kan bijvoorbeeld worden vastgesteld door met seriële MRI-scans het volume van het hersenweefsel te meten, zoals beschreven in hoofdstuk elf van dit proefschrift. De onderliggende hypothese van deze methode is dat het verminderen van de hoeveelheid weefselverlies uiteindelijk zal leiden tot betere neurologische ontwikkeling. Het verband tussen het volume van het hersenweefsel en de neurologische ontwikkelingsparameters is onderzocht in verschillende onderzoeken bij te vroeg geboren baby's: over het algemeen hangen kleinere hersenvolumes samen met slechtere motorische en cognitieve scores.[38,39] Er is echter weinig bekend over de exacte relatie tussen weefselverlies of overgebleven hersenweefsel en neurologische uitkomsten bij patiënten met een perinataal herseninfarct. Het is goed mogelijk dat de hoeveelheid hersenweefsel niet per se samenhangt met het niveau van functioneren. Het zou bijvoorbeeld nuttig kunnen zijn om te bepalen welke afkapwaarden van specifieke hersenweefselvolumes geassocieerd zijn met een gunstige ontwikkeling, zodat dit streefwaarden zouden kunnen zijn voor interventiestudies bij neonatale hersenschade.

De meeste onderzoeken die gaan over de ongunstige gevolgen van een perinataal herseninfarct, eindigen tegenwoordig met de aanbeveling dat er nieuwe therapieën moeten worden ontwikkeld om deze ongunstige gevolgen te verbeteren. Een studie uit 2014 toonde echter aan dat PAIS patiënten op de schoolgaande leeftijd geen lagere kwaliteit van leven hadden dan hun leeftijdsgenoten.[40] Hoewel de auteurs van deze studie dit beschreven als een 'handicap-paradox', herinnert deze studie ons eraan dat onderzoekers dat niet altijd rekening houden met de beleving van de PAIS-veroorzaakte handicap zoals die door patiënten en hun families wordt ervaren. Andere studies tonen namelijk aan dat de kwaliteit van leven na een perinataal herseninfarct verminderd is bij kinderen met een handicap vergeleken met kinderen met een normale ontwikkeling, en dat dit ook nog afhangt van de mate van beperking.[41–43] De belangrijkste vraag is waar nieuwe therapeutische behandelingen uiteindelijk naar moeten streven. Misschien moeten therapieën meer gericht zijn op verbetering van kwaliteit van leven of bevordering van participatie in de samenleving dan op herstel van hersenweefsel of verhoging van punten op een intelligentietest. Het is daarom aan te raden dat er meer rekening wordt gehouden met deze parameters in ontwikkelings- en interventiestudies bij patiënten met perinatale hersenschade.

CONCLUSIES

De volgende conclusies kunnen getrokken worden aan de hand van dit proefschrift:

- Neonaten met een herseninfarct in het volledige arteria cerebri media stroomgebied zullen zich altijd ongunstig ontwikkelen in ten minste één neurologisch domein, terwijl voor neonaten met een ander type perinataal herseninfarct de ontwikkeling bij 43-71% van de kinderen gunstig zal zijn. (Hoofdstuk 2)
- De belangrijkste voorspellers voor neurologische ontwikkeling na een perinataal herseninfarct zijn betrokkenheid van de corticospinale baan en basale kernen op de MRI. (Hoofdstuk 2 en 4)
- Om het best een uitspraak te kunnen doen over de prognose na een perinataal herseninfarct ligt het optimale moment voor het maken van de MRI scan waarschijnlijk op tenminste 48 uur na klinische presentatie. (Hoofdstuk 3)
- Een combinatie van beeldvorming en klinische beoordeling van de handfunctie verbetert de nauwkeurigheid om eenzijdige cerebrale parese te voorspellen. (Hoofdstuk 4)
- Vóór de leeftijd van vijf maanden is asymmetrie van de corticospinale banen op DTI de beste voorspeller van eenzijdige cerebrale parese, in vergelijking met conventionele MRI en klinische beoordeling van de handfunctie. (Hoofdstuk 5)
- Het is mogelijk om met accelerometrie op de leeftijd van drie maanden asymmetrie van de bovenste ledenmaten te detecteren bij kinderen met unilateraal hersenletsel. (Hoofdstuk 6)
- In de eerste vijf dagen na presentatie worden hersenactiviteit en -oxygenatie beïnvloed door het perinataal herseninfarct en daarnaast zijn deze parameters gerelateerd aan cognitieve ontwikkeling. (Hoofdstuk 7)
- Mesenchymale stamcellen zijn een soort mini-fabrieken van verschillende neurotrofines die neonatale hersenschade op maat kunnen herstellen. (Hoofdstuk 8)
- Stamceltherapie lijkt in de nabije toekomst een veelbelovende behandeling voor neonatale hersenschade. Mesenchymale stamcellen hebben van alle typen stamcellen de gunstigste kenmerken voor klinische toepassing. (Hoofdstuk 9)
- Mesenchymale stamcellen komen na intranasale toediening aan in de beschadigde hersenen van een aan de mens verwante primaat, in overeenstemming met eerdere knaagdierstudies. (Hoofdstuk 10)
- De DINOSAUR-studie zal uitwijzen of intraveneuze toediening van darbepoëtine alfa binnen de eerste levensweek hersenschade, veroorzaakt door een herseninfarct in de arteria cerebri media, zal verminderen bij (bijna) voldragen pasgeborenen. (Hoofdstuk 11)

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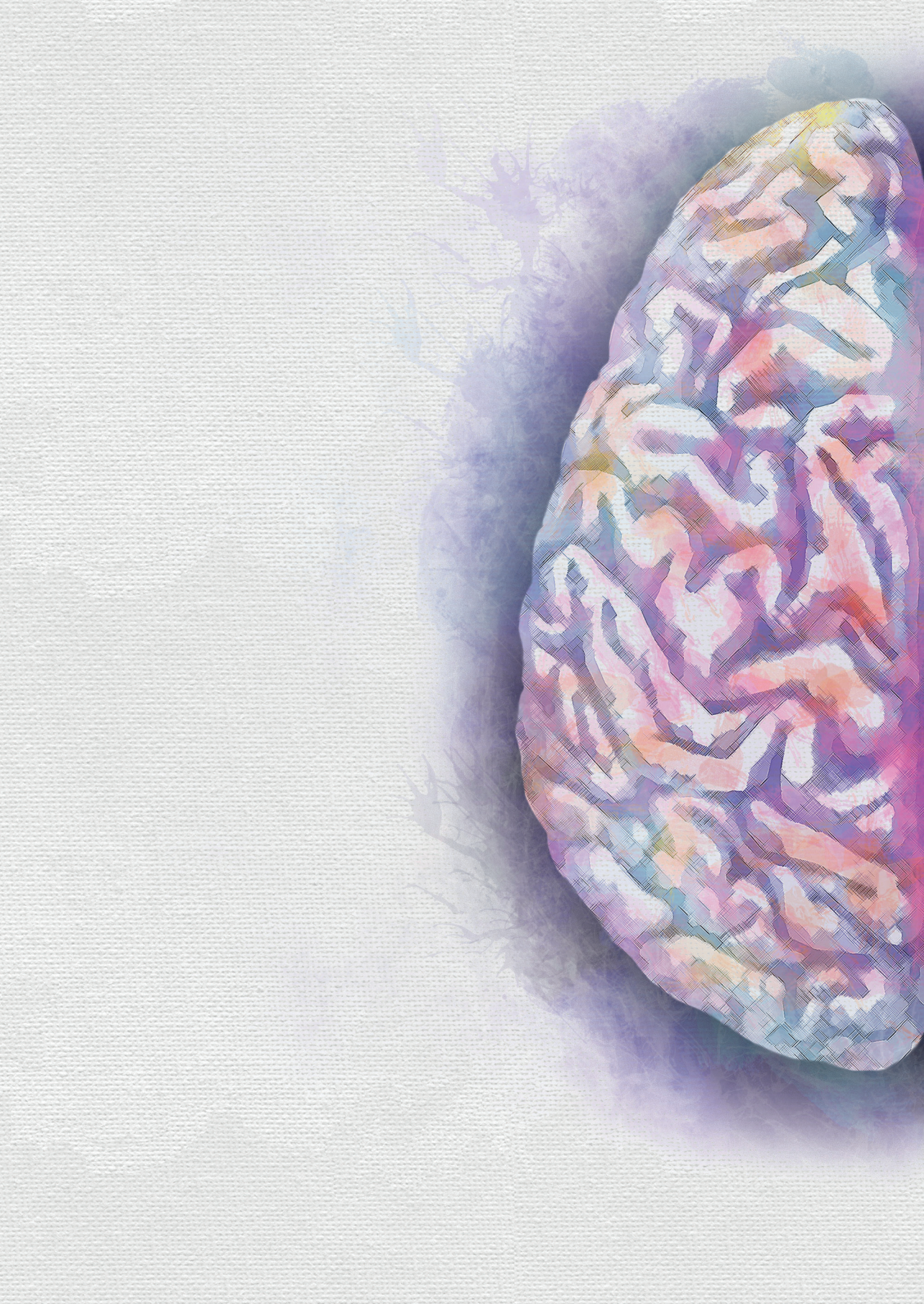

CHAPTER 14

LIST OF ABBREVIATIONS

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CURRICULUM VITAE

DANKWOORD
(ACKNOWLEDGEMENTS)

LIST OF ABBREVIATIONS

ACA: anterior cerebral artery
ADC: apparent diffusion coefficient
AED: anti-epileptic drug
aEEG: amplitude integrated electroencephalography
AI: Asymmetry index
APBI: asymmetric perinatal brain injury
AUC: area under the curve
BDNF: brain-derived neurotrophic factor
BGT: basal ganglia and/or thalamus/thalami
BoHM: Both hands measure
BPD: bronchopulmonary dysplasia
BSITD-III: Bayley Scales of Infant and Toddler Development, Third edition
CP: cerebral palsy
CSF: cerebrospinal fluid
CST: corticospinal tract
CSVT: cerebral sinovenous thrombosis
DINOSAUR: Darbepoetin for Ischemic Neonatal Stroke to Augment Regeneration
DWI: diffusion weighted imaging
DTI: diffusion tensor imaging
EEG: electroencephalography
EaHS – Each hand sum score
EPO: erythropoietin
FA: fractional anisotropy
GA: gestational age
GDNF: Glial-derived neurotrophic factor
GM: general movement
GMDS: Griffiths Mental Development Scales
HAI: Hand Assessment for Infants
HI: hypoxic-ischemia/ischemic
HIE: hypoxic-ischemic encephalopathy
IQR: interquartile range
IVH: intraventricular hemorrhage
MCA: middle cerebral artery
MCAO: middle cerebral artery occlusion
MRA: magnetic resonance angiography
MRI: magnetic resonance imaging
MSC: mesenchymal stem cell
NICU: neonatal intensive care unit

NIRS: near-infrared spectroscopy
NS: non-significant
NSC: Neural stem cells
OR: odds ratio
PAIS: perinatal arterial ischemic stroke
PASSIoN: Perinatal Arterial Stroke treat with Stromal cells IntraNasally
PCA: posterior cerebral artery
PLIC: posterior limb of the internal capsule
PSOM: Pediatric Stroke Outcome Measure
PVHI: periventricular hemorrhagic infarction
RCT: randomized controlled trial
rhEPO: recombinant human erythropoietin
ROC: receiver operator curve
ROI: region of interest
rhEPO: recombinant human erythropoietin
$rScO_2$: regional cerebral oxygen saturation
SD: standard deviation
SGZ: subgranular zone
SVZ: subventricular zone
TEA: term equivalent age
TrkB: tyrosine kinase receptor B
UPBI: unilateral perinatal brain injury
UMCU: University Medical Center Utrecht
UCP: unilateral cerebral palsy
USCP: unilateral spastic cerebral palsy
VEGF: Vascular endothelial growth factor
WPPSI: Wechsler Preschool and Primary Scale of Intelligence

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CURRICULUM VITAE

Nienke Wagenaar was born on July 8 1990 in Venlo, the Netherlands. After graduating from high school, she moved to Utrecht and started studying Medicine at Utrecht University. During several research internships she became interested in the field of neonatal neurology, and she was accepted as a PhD-candidate at the department of Neonatology in the Wilhelmina Children's Hospital in Utrecht in February 2015. Her research focuses on perinatal stroke, a condition that leads to many adverse consequences in later life, and currently has no therapy available. Nienke and her research team, led by profs. Benders and de Vries, are developing new strategies to treat these vulnerable newborns. Her future goal is to create her own research trajectory, in combination with working as a pediatrician. Nienke would like be a motivated clinical researcher who contributes to the field of pediatrics with a sense of interest, inspiration and passion. She completed her PhD-thesis in the summer of 2019 and has since started working as a resident at the department of pediatrics of the St. Antonius Hospital in Nieuwegein. Apart from her career, Nienke enjoys travelling the world, sports, exploring new hobby's, and spending time with friends. She lives happily together with Oscar in Utrecht.

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Bringing something we must learn.
And we are led to those who help us most to grow
If we let them and we help them in return."
— *Stephen Schwartz*

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A mind that is stretched by a new experience
can never go back to its old dimensions.
Oliver Wendell Holmes, Jr.

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