RESEARCH PROTOCOL

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Adult mesenchymal stromal cells to regenerate the neonatal brain: the PASSIoN trial (Perinatal Arterial Stroke treated with Stromal cells IntraNasally)

PROTOCOL TITLE 'Adult mesenchymal stromal cells to regenerate the neonatal brain: the PASSIoN trial (Perinatal Arterial Stroke treated with Stromal cells IntraNasally)'

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GCP STATEMENT

This trial will be conducted in compliance with this protocol, Good Clinical Practices and applicable regulatory requirements.

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	**ABR form, General Assessment and Registration form, is the application form that is required for submission to the accredited Ethics Committee (In Dutch, ABR = Algemene Beoordeling en Registratie)**
ADC	**Apparent diffusion coefficient**
AE	**Adverse Event**
AR	**Adverse Reaction**
BSITD-III	**Bayley Scales of Infant and Toddler development version 3**
BPD	**Bronchopulmonary dysplasia**
CA	**Competent Authority**
CCMO	**Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek**
CTF	**Cell Therapy Facility**
CV	**Curriculum Vitae**
DSMB	**Data Safety Monitoring Board**
DTI	**Diffusion Tensor Imaging**
DWI	**Diffusion Weighted Imaging**
EU	**European Union**
EudraCT	**European drug regulatory affairs Clinical Trials**
FA	**Fractional Anisotrophy**
GCP	**Good Clinical Practice**
GMFCS	**Gross Motor Function Classification System**
GVHD	**Graft-versus-host disease**
HAI	**Hand Assessment in Infancy**
HI	**Hypoxic-ischemia**
HINE	**Hammersmith Infant Neurological Examination**
IB	**Investigator's Brochure**
IC	**Informed Consent**
IMP	**Investigational Medicinal Product**
IMPD	**Investigational Medicinal Product Dossier**
MCA	**Middle cerebral artery**
METC	**Medical research ethics committee (MREC); in Dutch: medisch ethische toetsing commissie (METC)**
MSC	**Mesenchymal stromal cell**
NNRN	**Dutch Neonatal Research Network; in Dutch: Nederlands Neonataal**

	Research Netwerk
(S)AE	**(Serious) Adverse Event**
SPC	**Summary of Product Characteristics (in Dutch: officiële productinfomatie IB1-tekst)**
Sponsor	**The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.**
SUSAR	**Suspected Unexpected Serious Adverse Reaction**
PAIS	**Perinatal arterial ischemic stroke**
UMCU	**University Medical Centre Utrecht**
Wbp	**Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgevens)**
WKZ	**Wilhelmina Children's Hospital**
WMO	**Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen**

SUMMARY

Rationale: Perinatal arterial ischemic stroke (PAIS) is an important perinatal cause of long-lasting neurodevelopmental problems. Recent studies report an incidence of PAIS of 1 per 2300 full-term infants born alive. Adverse consequences of PAIS include hemiplegia, cognitive dysfunction, epilepsy and speech problems. In 50-75% of infants, neonatal stroke leads to abnormal neuromotor and -developmental outcome or epilepsy. The estimated annual mortality rate of neonatal stroke is 3.49/100,000 annually. Current treatment options for PAIS mainly focus on controlling convulsions and associated infections. There is no treatment available that leads to reduction of neonatal brain damage in this severely affected group of infants. This leads to life-long consequences of PAIS and forms a large burden for patients and society. The overall aim of this project is to meet this need by developing a cell based treatment strategy.

Animal models of neonatal brain injury provide evidence for the feasibility and efficacy of intranasal mesenchymal stromal cell (MSC) application in the treatment of PAIS. Additionally, results from human trials with MSCs in the treatment of adult stroke or other pathologic conditions provide evidence that MSC treatment is safe. This project aims at making the first step towards clinical application of MSCs to treat PAIS. Successful completion of this project will provide the first evidence of the safety and feasibility of MSCs to treat brain damage in newborn infants.

Objective: This study will assess safety and feasibility of bone marrow-derived allogeneic MSCs, administered by the nasal route, in neonates who suffered from PAIS.

Study design: A phase I/II, open-label, single-arm, single-center intervention study in the NICU at the Wilhelmina children's Hospital / University Medical Centre in Utrecht of (near-)term newborns ≥36 weeks of gestation within the first week of onset of presenting clinical symptoms.

Study population: All (near-)term newborns ≥36 weeks of gestation with or without clinical symptoms of PAIS but with a magnetic resonance imaging (MRI) confirmed PAIS (in the MCA region) will be eligible for this study. Following written parental consent, 10 patients will be included in our study.

Intervention: One dose of 50x10^6 MSCs via the nasal route as soon as possible after confirmation of the MCA-stroke, but within the first week of onset of presenting clinical symptoms. Within 30 minutes after cleaning the nose with saline, using standard procedures operative at the NICU, the MSC will be delivered.

Main study parameters/endpoints: Our primary objective is to determine if MSC treatment in neonates with PAIS is safe in the acute setting, including vital signs at time of treatment, blood sampling before and after MSC treatment and occurrence of any adverse events.

Secondly we will investigate subacute and long-term safety of MSC treatment at 3 months, in terms of SAEs (such as infections) and cerebral tumorigenicity on MRI. Follow-up assessment at 3 months is part of regular care for neonates with PAIS.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: 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 the nasal treatment to apply the MSCs, treatment is not different compared to the standard acting treatment protocol for newborns with PAIS. Nasal application of MSCs has been considered as non-invasive. With respect to possible risks of the present MSC treatment, the most important potential risk factors such as inflammatory actions of MSC therapy with allogeneic human MSCs and an increased risk for malignancies, have been intensively investigated in our own preclinical studies and appear to be non-present at long-term follow up. Additionally, human trials on MSC treatment for adult stroke or other pathologic conditions do not provide evidence for these or other serious adverse events or risks. No single indication has been found in experimental research in the developing animal models that above mentioned complications occur in a higher incidence as compared to non-MSC treated animals, whereas possibility for a substantial better short- and long-term outcome seems very realistic on the basis of previous experimental research data. This study will assess safety bone marrow-derived allogeneic MSCs, administered by the nasal route, in human neonates with PAIS.

INTRODUCTION AND RATIONALE

Introduction

Perinatal arterial ischemic stroke (PAIS) or cerebral infarction is an important perinatal cause of long-lasting neurodevelopmental problems. Recent studies report an incidence of PAIS of 1 per 2300 full-term live births.(Kirton & DeVeber 2009) PAIS has been diagnosed mostly in the first week of life.

PAIS occurs most often in de middle cerebral artery (MCA) with a predilection for the left MCA.(Fernández-López et al. 2014; Benders et al. 2014; Kirton et al. 2011; van der Aa et al. 2014) The most important risk factors are associated with pathological changes that manifest during pregnancy and delivery. Important maternal risk factors include gestational diabetes, pregnancy-induced hypertension and pre-eclampsia, while perinatal factors associated with neonatal stroke are ventouse delivery, emergency Caesarean section, perinatal infections and hypoglycaemia.(Fernández-López et al. 2014; Kirton et al. 2011; Harteman et al. 2012; Martinez-Biarge et al. 2016) Placental pathology has also been linked to PAIS.(Elbers et al. 2011) Coagulation disorders are associated with neonatal stroke, such as genetic polymorphisms of prothrombotic factors (e.g. Factor V Leiden, prothrombin, lipoprotein (a), methylenetetra-hydrofolate reductase or acquired prothrombotic states (e.g. activated protein C). These factors are increasing the risk for PAIS when combined with other risk factors such as perinatal asphyxia, congenital heart diseases or neonatal infections.(Fernández-López et al. 2014)

The disease manifests itself most often with one-sided convulsions in the first week after birth, often accompanied with (asymmetric) hypotonia, lethargy and apnea.(Kirton et al. 2011; van der Aa et al. 2014) When stroke is suspected clinically and on cranial ultrasound, this is usually confirmed by magnetic resonance imaging (MRI). Neuromotor outcome becomes more evident over time and depends largely on the involved cerebral structures. Especially involvement of the internal capsule and the basal ganglia is associated with impaired motor outcome.(figure 1)

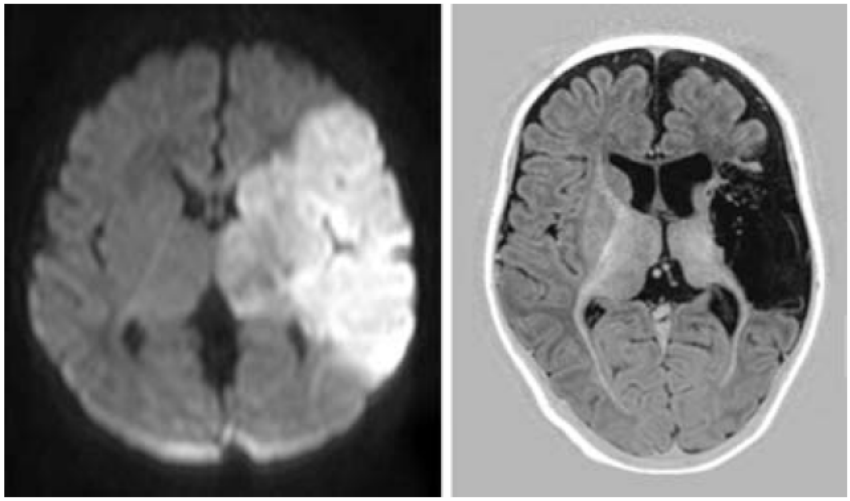


Figure 1: Representative MRI of a full-term infant with a main branch MCA infarct on the left. The first scan was performed during the first week. The diffusion weighted images (left) shows restricted diffusion of the cortex, subcortical white matter and basal ganglia. A second MRI at 3 months of age (right) shows a large cavity with lack of myelination of the posterior limb of the internal capsule.

Adverse consequences of PAIS include unilateral spastic cerebral palsy (USCP) cognitive dysfunction, epilepsy and speech problems. In 50-75% of infants, neonatal stroke leads to abnormal neuromotor and -developmental outcome or epilepsy.(Fernández-López et al. 2014) Recurrent seizures after PAIS are also often reported, with larger areas of stroke being more likely to be associated with postneonatal epilepsy and lower cognitive outcome. (Wusthoff et al. 2011; van Buuren et al. 2013) The estimated annual mortality rate of neonatal stroke is 3.49/100,000.(Lynch & Nelson 2001)

In adult stroke, treatment focuses on thrombolytic therapy and is, after exclusion of hemorrhagic stroke, standard part of care given within a timeframe of 6-8 hours after the event. Newborns usually present with less specific symptoms, which results in a later diagnosis of PAIS compared to adult stroke. Since neonatal stroke is usually diagnosed later, early treatment options, such as hypothermia used in perinatal asphyxia, with a therapeutic window of six hours after birth, cannot be used in neonatal stroke. Current treatment options for PAIS mainly focus on supportive care, such as controlling hypoglycaemia, treatment of convulsions and associated infections. There is no specific treatment available that leads to reduction of neonatal brain damage in this severely affected group of infants. This leads to life-long consequences of PAIS and forms a large burden for patients and society. The overall aim of this project is to meet this need by assessing the safety and feasibility of a cell based treatment strategy for neonates with PAIS.

Rationale

Mesenchymal stromal cells (MSCs) can be isolated from embryonic and fetal tissues, such as placental tissue, fetal blood, umbilical cord blood and stroma (Wharton's Jelly). Furthermore, they can also be isolated from adult tissues like bone marrow. MSCs can differentiate into mesodermal tissue cells (e.g. bone, cartilage, fat), but in vitro experiments have demonstrated that MSCs are, during and after hypoxia-ischemia, also capable to stimulate the formation of neurons, astrocytes, oligodendrocytes and microglia.(Kohyama et al. 2001; Dezawa et al. 2004)

From neonatal rat models of brain injury, we have learned that hypoxic- ischemic (HI) brain injury induces changes in neurovascular environment that promote neurogenesis.(Kadam et al. 2008; Ong et al. 2005; Yang & Levison 2007; Donega, van Velthoven, Nijboer, Kavelaars, et al. 2013) However, after HI brain injury, there is no induction and maintenance of long-term neurogenesis. Therefore it is crucial to assist the brain in regeneration after injury and

also to reinforce development of the maturating brain. In animal HI-model studies, it has been demonstrated that transplantation of murine adult bone marrow-derived MSCs markedly improves neuroregeneration. Treatment with MSCs stimulated formation of new neurons and oligodendrocytes and reduced infarct size by >45%. Although intracranial MSC treatment stimulated formation of new brain cells and differentiation of these cells into new neurons and oligodendrocytes, these newly formed cells were not of transplant origin. These findings indicate that the neuroregenerative effect of MSCs after ischemic brain damage mainly relies on stimulation of endogenous repair mechanisms.(van Velthoven et al. 2010c) This is confirmed by data from our group that show that MSC treatment has profound effects on the expression of growth and differentiation factors in the brain. For example, MSC treatment at 10 days after HI induces upregulation of 35 genes encoding factors that are mainly involved in cell growth and proliferation. MSC treatment at 3 and 10 days after induction of HI brain damage induces expression of 28 additional factors and these are predominantly involved in cell differentiation and nervous system development.(van Velthoven et al. 2010c; van Velthoven et al. 2011) These results show that the first dose of MSC stimulates cerebral cell proliferation, whereas the second injection enhances differentiation and maturation of newly formed cells and integration in a functional network.(van Velthoven et al. 2010c)

Based on these findings, we hypothesized that MSC of donor origin will only transiently survive in the brain and that the neuroregenerative effects of MSC are mediated predominantly by inducing changes in the growth factor milieu of the brain.
The corticospinal tract is the primary transmission route for voluntary movement of the forepaws. We showed that MSC treatment could also partially restore the damaged corticospinal motor tract which led to improved sensorimotor outcome.(van Velthoven et al. 2010c; van Velthoven et al. 2010a)

MSCs have many advantages over other types of stem cells or progenitors. First of all, they can easily be obtained from infants and adults.(Le Blanc & Mougiakakos 2012) Additionally, as described above, MSC treatment have a long therapeutic window, as they can be administered at least until 10 days after brain injury to improve behavioral outcome and lesion volume in neonatal HI mice. This is of great value, since the only available therapies for HI injury have to be given within a few hours. Besides, MSCs are hardly detectable in the brain after 3 days (Donega, van Velthoven, Nijboer, van Bel, et al. 2013), so the risk of Host-versus-Graft is very low. It is also very unlikely that MSCs will cause other adverse effects if they are no longer present in the brain after a short period of time. Our most recent study showed no malignancies in the brains or in other organs of mice treated with MSCs when

assessed at 14 months after start of MSC treatment at 10 days after neonatal HI.(Donega et al. 2015) Furthermore, MSCs have low immunogenicity because they do not express major histocompatibility complex (MHC) class-II antigens.(Rocha et al. 2000; Uccelli et al. 2008; Fleiss et al. 2014) This makes them excellent therapeutic candidates for allogeneic treatment.

Another advantage of MSCs, is that they have proven to be effective in brain injury when administered intranasally. This is beneficial since it has been reported that systemically administered human bone marrow-derived MSCs migrate into peripheral organs but were not able to induce neuroprotective effects in the brain after cerebral ischemia.(Steiner et al. 2012) After systemic administration MSCs will also migrate to peripheral organs, leading to loss of MSCs in these organs (trapping).(Li et al. 2015; Liu 2011; Jiang et al. 2011) This may result in reduced migration of MSCs into the brain and will consequently diminish their neuroregenerative effects on the brain.(Li et al. 2015; Liu 2011; Jiang et al. 2011; Steiner et al. 2012) In newborns, trapping of MSCs in other organs will be more important than in adults. MSCs are attracted by areas of injury in the organ systems,(Wang et al. 2014) PAIS is related to fetal distress and subsequent hypoxia-ischemia.(Harteman et al. 2012; Martinez-Biarge et al. 2016) Other risk factors for PAIS are maternal fever, hypoglycemia and early-onset sepsis/meningitis. (Harteman et al. 2012) These risk factors, as well as hypoxia-ischemia, may lead to diffuse damage to other organ systems. When MSCs are administered systemically, they may be attracted by systemic damage, leading to even more trapping of the MSCs in other injured organs. This makes it difficult to predict if and how many MSCs will migrate to the brain when given systemically. Studies from our group and others have shown that MSCs when given intranasally migrate within two hours after administration to the ischemic brain region.(Donega, Nijboer, van Tilborg, et al. 2014) Intranasal administration of MSCs reduces lesion volume loss and improves sensorimotor outcome.(van Velthoven et al. 2010b; Wei et al. 2015). Efficacy of intranasal versus intracranial MSC treatment on lesion size and motor behavior was similar in the neonatal HI mouse model.(van Velthoven et al. 2012) Studies of our group in the newborn baboon have shown that intranasally administered MSCs have the potential to migrate to the injured brain region of primates (unpublished observations), in accordance with earlier shown studies in mice and rats. Overall, the intranasal administration route provides an effective alternative allowing MSC treatment for brain injury without invasive methods and minimal burden for the patient.

Even though all evidence on efficacy and feasibility of MSCs in the treatment of neonatal brain injury is based on animal models, there is evidence that human MSCs have the same potential. Human MSCs were found to improve sensorimotor function and decrease gray and

white matter volume loss in our mouse model of HI brain injury.(Donega, Nijboer, Braccioli, et al. 2014) These findings are comparable to those of murine MSCs and strongly support the therapeutic potential of hMSCs for neonatal brain injury. Additionally, this study confirms the efficacy of (human) MSCs when administered intranasally.

There are no clinical trials published on MSC treatment for neonatal stroke. However, there are some studies available describing the treatment of adult stroke with MSCs. These studies may support the evidence for the use of MSCs in neonatal stroke, but it is important to realize that results from adult trials can not be directly translated to neonatal care for several reasons. First of all, the newborn brain has much better plasticity capacity than the brain of older children and adults. This causes the newborn to recover more easily after (severe) brain injury than an adult.(Bower 1990; Schneider 1970) Secondly, effectiveness of MSC treatment reduces with increasing age, because endogenous neurogenesis capacity also reduces with age. This causes neonates to have more potential to regenerate their damaged neurons and/or networks compared to adults.(Titomanlio et al. 2011; Bondolfi et al. 2004; Kuhn et al. 1996; Leuner et al. 2007) Lastly, neonatal stroke (PAIS) is a different disease, with different pathology and symptomatology, than adult stroke.(Comi & Johnston 2009)

However, the results from adult trials can be used to support safety and feasibility of MSC treatment in neonatal stroke. Clinical trials with MSC treatment for adult patients with ischemic stroke have shown some evidence for the efficacy of MSCs in the treatment of ischemic stroke. These studies described patients who were treated with MSC at 7-133 days post-stroke.(Bang et al. 2005; Honmou et al. 2011) Although the evidence is sparse, there are some trials, mainly uncontrolled open-label studies, that have demonstrated clinical neurological improvement in patients with acute and chronic stroke after treatment with MSCs.(Bang et al. 2005; Lee et al. 2010) Most importantly, these trials have not demonstrated any serious adverse events, such as neurological deterioration, systemic infections nor tumor development, related to MSC transplantation. This is also concluded from a meta-analysis on over 1200 patients treated with MSCs for various pathological indications. Only transient fever within the first days seemed to be related to MSC transfusion.(Lalu et al. 2012)
There is only one clinical trial describing the use of MSCs in treatment of neonates.(Chang et al. 2014) Chang et al. assessed in a phase I dose-escalation trial the safety and feasibility of intratracheal administration of allogeneic umbilical cord-derived MSCs in preterm infants with high risk for bronchopulmonary dysplasia (BPD). Importantly, there were no serious adverse events or toxicity related to a higher dose of 2 x 10^7 cells/kg.(Chang et al. 2014) These

findings were more recently confirmed by another group treating 3 premature infants with approximately 2 x 10^6/kg bone marrow-derived MSCs intravenously.(Henckel et al. 2015) The authors concluded that intravenous MSC treatment was feasible, well tolerated and no effects on pulmonary and cerebral blood flow could be detected.(Henckel et al. 2015) An ongoing study in Korea is treating preterm infants with intraventricular hemorrhage with allogeneic umbilical cord MSCs, and preliminairy results are promising and show no adverse effects.(Presented at Pediatric Academic Societies Meeting, Baltimore, Monday May 2, 2016 - 8:00 am, E-PAS2016:3090)
Overall, MSC treatment under several pathologic conditions, including stroke, in adults and children appeared to be safe.

In summary, based on the available evidence there seems to be a solid basis for clinical translation. Animal models of neonatal brain injury provide evidence for the feasibility, efficacy and safety of intranasal MSC application in the treatment of PAIS. Additionally, results from human trials with MSCs in the treatment of adult stroke or other pathologic conditions, including BPD in neonates, provide evidence that MSC treatment is safe. This project aims at making the first step towards clinical application of MSCs to treat PAIS. Successful completion of this project will provide the first evidence of safety of MSCs to treat brain damage in newborn infants.

1. OBJECTIVES

Our primary objective is to determine if MSC treatment, in neonates with PAIS, is safe in the acute setting, including vital signs at time of treatment, blood sampling before and after MSC treatment and occurrence of any adverse events or toxicity. Secondly we will investigate safety of MSC treatment at 3 months, in terms of SAEs and tumorigenicity on MRI.

Primary Objective

- *To determine if MSC treatment in (near-)term infants with PAIS is safe in the acute setting.*

  Safety is defined primarily as the absence of treatment-related serious adverse events (SAEs) according to the Consolidated Standards of Reporting Trials (chapter 7.2), secondly as the absence of dose-limiting toxicity, defined as death within 24 hours after MSC transplantation or anaphylactic shock related to the MSC administration. At least, all patients will be regularly and intensively assessed, including vital signs, before and 24 hours after treatment, until discharge from our hospital. Also, blood sampling will be performed before and 24 hours after treatment with MSCs to determine infection and inflammation parameters. Acute safety will be determined until discharge.

Secondary Objectives

- *To determine if MSC treatment in (near-)term infants with PAIS is safe in the subacute setting.*
    - Subacute safety is defined primarily as the absence of treatment-related serious adverse events (SAEs) according to the Consolidated Standards of Reporting Trials (chapter 7.2) until the age of 3 months. Patients will then be asked to report on other SAEs, including infections.
- *To determine if MSC treatment in (near-)term infants with PAIS is safe at 3 months in terms of cerebral tumorigenicity.*
    - To assess safety of MSC treatment on the brain, infants will be scanned using MRI prior to MSC treatment and at 3 months of age. Long-term adverse effects on the brain in terms of tumorigenicity will be determined using this MRI, which is part of regular stroke follow-up program.

2. STUDY DESIGN

2.1 Description

We will perform a single-center, open-label intervention study in the NICU of the Wilhelmina Children's Hospital (WKZ) / University Medical Center in Utrecht (UMCU), the Netherlands to investigate the feasibility and safety of neonatal treatment with allogeneic MSCs in (near-) term neonates with PAIS. Since this is a phase 1/2 safety/feasibility pilot clinical trial, we will assess occurrence of adverse events.

2.2 Duration

The duration of the study for each subject from inclusion until study end will be a maximum of 3 months, comprising a 1 week intervention period and follow-up until 3 months. Subject recruitment will be (approximately) 12-24 months, at the longest, depending on when the intended number of subjects is included. This study is expected to last for approximately 2.5 years, but will only end after all 10 subjects have been included, treated and have been seen for follow-up (MRI and neurodevelopmental testing) at 3 months of age.

2.3 Setting

This study will be executed in the NICU of the WKZ/UMCU, the Netherlands, where (near-) term infants suspected of PAIS following parental consent for the study are admitted. Infants of the other participating hospitals suspected of having PAIS will be transferred to the UMCU for further diagnostic procedures (MRI) and subsequent treatment.

2.4 Study Procedures

Recruitment and inclusion

The NICU of the WKZ is known to have most expertise in neonatal stroke. Therefore, infants suspected of having PAIS will be transferred from hospitals in our region of care and also from other hospitals outside our region, to the UMCU and admitted to our NICU, allowing infants to be part of this study. Patients are eligible for our study if they meet our in- and exclusion criteria, PAIS may have been suspected with cranial ultrasound and will be confirmed within 7 days after onset of symptoms by MRI (including 3D-T1, T2 weighted, diffusion weighted imaging [DWI] and DTI sequences) and this PAIS is characterized by a predominantly unilateral ischemic lesion within the territory of the MCA, with involvement of the corticospinal tracts, cortex, white matter and basal ganglia. Following written parental consent, patients will then be included in our study. Parental consent will be obtained as soon as possible after admission at our NICU. Patients with PAIS that are diagnosed more than one week after birth are not eligible for enrolment.

Intervention period

After inclusion in our study, patients will receive MSCs as soon as possible after confirmation of the MCA-stroke, but within the first week of onset of presenting clinical symptoms. Steps to be undertaken before MSC administration include:

- A bacterial culture taken from both nostrils before administration of MSCs.
- Blood samples will be collected before administration of MSCs. Blood samples are taken routinely in the hospital for clinical purposes, but some extra material (0.5-1 mL) will be collected to determine infection markers CRP, procalcitonin and complete blood count. These samples will be combined with routine blood sampling and taken from routinely placed (arterial) catheters.
- 30 minutes prior to delivery of the MSCs, the nose will be cleaned with saline using standard procedures operative at the NICU.

After these procedures have been performed, infants will receive one dose of 50 x 10^6 MSCs intranasally. We will use a sterile plastic (e.g. 1,0 mL) syringe containing the MSCs to slowly drip the MSCs alternatively in both nostrils.

24 hours after MSC administration, blood samples will be collected again. Blood samples are taken routinely in the hospital for clinical purposes, but some extra material (0.5-1 mL) will be collected to determine infection markers CRP, procalcitonin and complete blood count. These samples will be combined with routine blood sampling and taken from routinely placed (arterial) catheters.

During their stay on the NICU, vital functions of the infant will be monitored, as part of standard clinical care. Cranial ultrasound and neurologic examination will be performed as part of standard clinical care for neonates with PAIS. These procedures will at least all be performed before and 24 hours after MSC treatment, until discharge from the NICU.

All subjects will remain on the NICU of the study center during treatment and for at least 4 days after intervention to monitor acute safety. However, if a subject is transferred to a level II or level I neonatal unit at another hospital during the intervention period, the protocol will be continued under supervision of the principal investigator (PI) and delegated staff.

Safety monitoring

All potential risks are described in chapter 11.1e. These can be summarized in five categories: administration reactions; bacteraemia; systemic infections; post-administration viral infections and ectopic tissue formation. For all infants, these risks will be closely monitored and planned actions are described in chapter 11.2. Additionally, all infants will be monitored more closely by (extra) blood sampling, meaning blood sampling before and 24

hours after MSC administration. Also, we will only continue this study after the first three infants had their second MRI (at three months of age) to assess the size of the infarction and other brain abnormalities on MRI (at three months of age), see paragraph 6.7.

AEs and SAEs occurring during the study will be recorded in the manner described under Section 7.2. All SAEs will be followed up until resolution or until the event is considered stable. SAEs resulting in a life threatening condition or death will be reported to the regulatory authorities and the EC in line with the local regulatory requirements. In addition the investigator will report any SAEs resulting in death and/or a life-threatening situation and any unexpected related SAEs to the regulatory authorities and the Ethics Committee (EC) in line with the local regulatory requirements. An independent data safety and monitoring board (DSMB) has, in addition to an internal safety review committee, been established to carry out further safety surveillance (see Paragraphs 7.2.3 and 7.4).

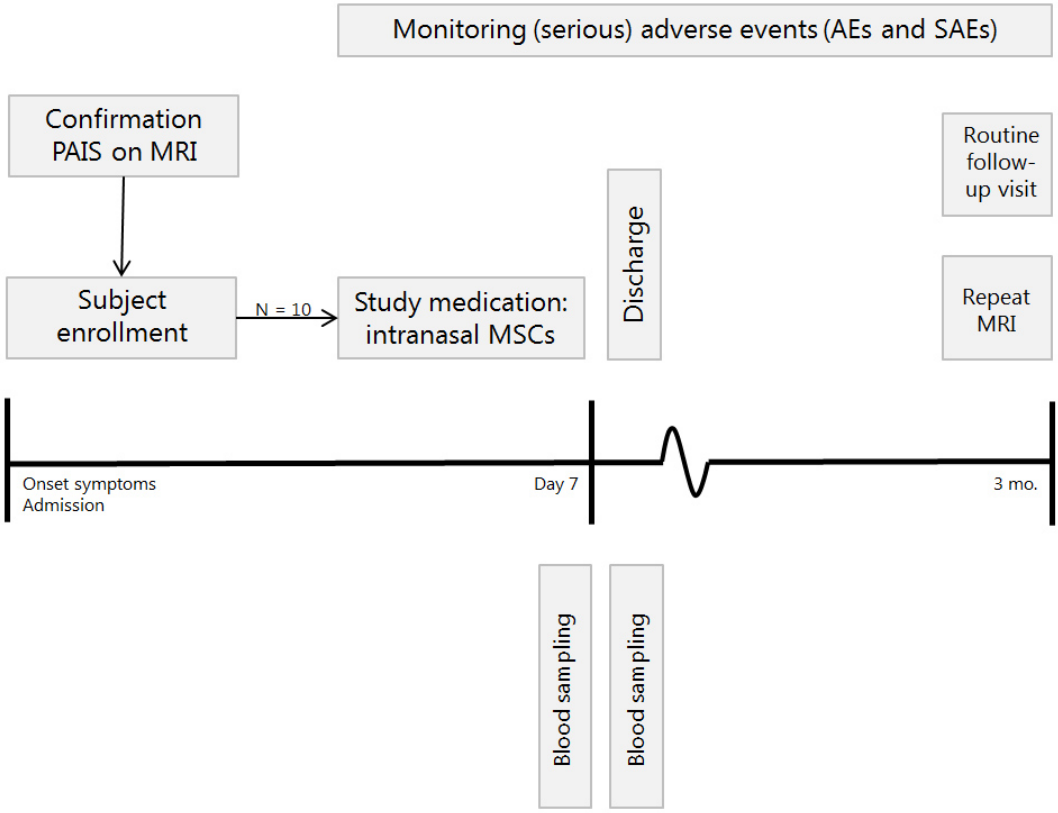
MRI measurements

For secondary parameters, subjects will be scanned on a 3 Tesla MR scanner as soon as possible after clinical suspicion of PAIS and again 3 months after the MSC treatment. Further details on MRI measurements can be found in chapter 6. All infants will return to the WKZ, UMCU for a MRI scan at 3 months of age.

Follow-up

After the intervention period, subjects will be followed until 3 months corrected age. At 3 months neurological assessment will be performed as part of the regular stroke follow-up program. This will also include filming spontaneous and endogenous movements (General Movements). Additionally, fidgety movements will be assessed. Neuromotor development will also be assessed using the HINE. After parental consent, results from these examinations will be used for this study. Subjects in this study will also be followed for at least 2 years of age at regular time points. This follow-up is part of regular care for neonates with PAIS, but these results are not part of this study protocol and are beyond the scope of this study.

2.5 Schematic diagram of study design



3. STUDY POPULATION

3.1 Study Population (base)

Newborn infants born at (near-)term, meaning ≥36+0 weeks of gestation, will be included in the study. Subjects will be recruited from all Dutch NICUs collaborating in the Dutch Neonatal Research Network (NNRN).

3.2 Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria:

- (Near-)Term infants, ≥36+0 weeks of gestation, admitted to one of the Dutch NICUs, diagnosed with PAIS, confirmed by MRI within 7 days after presentation with clinical symptoms.
- PAIS as characterized by a predominantly unilateral ischemic lesion within the territory of the MCA, with involvement of the corticospinal tracts, cortex, white matter and basal ganglia.
- Written informed consent from custodial parent(s).

3.3 Exclusion criteria

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

- Any proven or suspected congenital anomaly, chromosomal disorder, metabolic disorder.
- Presence of an infection of the central nervous system.
- No realistic prospect of survival, (e.g. severe brain injury), at the discretion of the attending physician.

3.4 Sample size calculation

We aim to include 10 infants diagnosed with PAIS on MRI, who will be treated with MSCs via the nasal route.

Since this is a proof-of-principle study, the sample size is not only calculated based on expected power, but on the feasibility to be successful in recruitment of individuals needed for this study in order to complete the protocol in 2 years. It is expected that about 180,000 infants will be born alive per year.(StatLine 2015) Since the incidence of PAIS is 1/2300 there will be about 80 PAIS patients in the Netherlands yearly. A study from our group showed that about 26% of all PAIS patients involved the main MCA branch.(Harteman et al. 2012) Based

on this expectation, we think it will be feasible to recruit 10 PAIS patients in 12-24 months. We furthermore expect only a minimal drop out of initially included patients because PAIS is not life-threatening and follow-up is tightly regulated in all NICUs, including the WKZ.

Data analysis

Data will be analyzed on an intention to treat basis. SAEs are planned to be compared. Statistical analysis is described in detail in chapter 8.

4. TREATMENT OF SUBJECTS

4.1 Investigational product/treatment

The treatment protocol requires that infants will receive MSCs as soon as possible after confirmation of the PAIS, but no later than the end of the first week of onset of presenting clinical symptoms. Steps to be undertaken before MSC administration include:

- A bacterial culture taken from both nostrils before administration of MSCs.
- Blood samples will be collected before administration of MSCs. Blood samples are taken routinely in the hospital for clinical purposes, but some extra material (0.5-1 mL) will be collected to determine infection markers CRP, procalcitonin and complete blood count. These samples will be combined with routine blood sampling and taken from routinely placed (arterial) catheters
- 30 minutes prior to delivery of the cells, the nose will be cleaned with saline using standard procedures operative at the NICU.

After these procedures have been performed, infants will receive one dose of 50 x 10^6 MSCs intranasally. We will use a sterile plastic (e.g. 1,0 mL) syringe containing the MSCs to slowly drip the MSCs alternatively in both nostrils. Blood samples will be collected again at 24 hours after administration of MSCs.

The syringe with MSC will be packaged at the CT-F of the UMC Utrecht and will be delivered to the NICU by the clinical research nurse.

4.2 Use of co-intervention (if applicable)

Not applicable.

4.3 Escape medication (if applicable)

Not applicable.

5. INVESTIGATIONAL PRODUCT

5.1 Name and description of investigational product(s)

Allogeneic bone marrow-derived adult MSCs obtained from the Cell Therapy Facility (CTF) of the UMCU. The CTF will obtain MSC, marrow: a cell product containing mesenchymal stromal cells derived from bone marrow. Mesenchymal stromal cells consist largely of mesenchymal stem cells. However, the International Society for Cellular Therapy (ISCT) states that 'multipotent mesenchymal stromal cells' (MSC) is the currently recommended definition for the plastic-adherent cells isolated from bone marrow and other tissues that have often been labelled as mesenchymal stem cells.(Dominici et al. 2006) We therefore decided to use the same acronym (MSC) as the active substance/cell type necessary for this study is mesenchymal stem cells.

5.2 Summary of findings from non-clinical studies

An overview of findings from non-clinical studies on MSC treatment for PAIS is given in chapter 7 of the Investigator's Brochure (IB).

5.3 Summary of findings from clinical studies

An overview of findings from clinical studies on MSC treatment for PAIS is given in chapter 8 of the IB.

5.4 Summary of known and potential risks and benefits

An overview of findings on potential risks and benefits of MSC treatment for PAIS is given in chapters 7 and 8 of the IB.

In summary, we have shown in our own mouse models of neonatal ischemic infarction that intracerebral application of murine adult bone marrow-derived MSCs markedly improves outcome. Treatment with MSC reduced infarct size by >45%, stimulated formation of new neurons and oligodendrocytes, partially restored cortico-spinal motor tract activity, and improved sensorimotor outcome.(van Velthoven et al. 2010c; van Velthoven et al. 2010a) Additionally, our findings indicate that MSCs can be successfully and rapidly administered to the brain via an efficient non-invasive route, the nasal route.(Donega, van Velthoven, Nijboer, van Bel, et al. 2013) The MSCs subsequently accumulate predominantly in the infarcted area. Notably, MSCs delivered via the nasal route reduce infarct size and improve motor function to the same extent as MSCs administered intracranially.(van Velthoven et al. 2010b)

In general, risks or adverse events related to MSC treatment can be categorized in three groups: acute, subacute and longterm adverse events.

1. Acute adverse events: Administration Reaction/Toxicity
   - Systolic blood pressure >33% decrease or increase from the baseline during or directly following the intranasal administration of the investigational product
   - Diastolic blood pressure >25% decrease or increase from baseline during or directly following the intranasal administration of the investigational product
   - Heart rate >33% decrease or increase from baseline during or directly following the infusion of the investigational product
   - Development of dyspnoea, vomiting, flushing, diarrhea, during or within 1 hour from the intranasal administration of the investigational product
   - Fever ≥ 2 grade increase from baseline during or within 2 hours from the intranasal administration of the investigational product
   - Development of exanthema, or urticaria developing during or within 2 hours from the intranasal administration of the investigational product

Allogeneic hUCB-derived MSC treatment in preterm infants with BPD appeared to be safe and without any acute adverse events.(Chang et al. 2014; Henckel et al. 2015) A large meta-analysis on clinical trials for numerous diseases did not show any evidence for acute severe toxicity due to MSC transplantation.(Lalu et al. 2012) This analysis used both adult and pediatric trials to report on a total of 1012 patients. Including eight randomized control trials, there was no association detected between MSC transplantation and acute infusional toxicity and organ system complications. There was a significant increase in fever with MSCs compared to the control group, but the fever was reported to be transient in all trials.(Lalu et al. 2012)

2. Subacute adverse events:
   - Bacteremia due to bacterial contamination of product. Fever ≥ 2 grade increase from baseline during or within 2 hours from the intranasal administration of the investigational product AND product contamination proven by positive product cultures. In case of contamination patients will be treated with antibiotics in case this is required. As described in the IMPD, there will be extensive quality control in the CTF, e.g. testing for contamination of the product. This risk is therefore considered to be minimal.
   - Systemic infection (e.g. meningitis) due to use of MSCs. We have no evidence from preclinical trials, nor from trials in adult stroke or neonatal BPD, that

MSC treatment increases the risk of infections. However, MSCs might be able to change inflammatory status. In order to monitor more closely, we will take extra blood samples from all subjects at baseline and 24 hours following MSC administration to determine infection markers such as CRP, procalcitonine and complete blood count.

- Post-administration virus infection. This risk is minimal and adherent to all blood (derived) product administration. If a patient develops a new viral infection that is suspected to be due to transfusion of blood products or intranasal administration of the investigational product, a lookback procedure should be initiated by the treating physician according to local regulations for all blood products received. If a MSC donor is proven to be the source of virus infection, this shall be considered a serious adverse event. All donors are being screened for infectious disease markers as indicated by the Wet Veiligheid en Kwaliteit Lichaamsmaterialen (WVKL), the transposition into Dutch Law of the EU Directive 2006/17. Please find specification of control of drug stubstance in chapter 2.1.S.4.1 of the IMPD.

Allogeneic hUCB-derived MSC treatment in preterm infants with BPD appeared to be safe and without any acute or subacute adverse events.(Chang et al. 2014; Henckel et al. 2015) Also from literature on MSC treatment for adult stroke it was shown that MSC treatment has no known risks.(Bang et al. 2005; Honmou et al. 2011) A large meta-analysis on clinical trials for numerous diseases did not show any evidence for severe subacute adverse effects due to MSC transplantation.(Lalu et al. 2012) This analysis used both adult and pediatric trials to report on a total of 1012 patients with ischemic stroke, Crohn's disease, cardiomyopathy, myocardial infarction, Graft-versus-host disease (GvHD) and healthy volunteers. Including eight randomized control trials, there was no association detected between MSC transplantation and organ system complications or infections.

3. Long-term adverse events: Ectopic tissue formation and tumorigenicity

Although concerns that MSCs might transform into tumorigenic cells still exist, there is a general agreement that bone-marrow-derived MSCs can be safely cultured in vitro with no risk of malignant transformation.(Bernardo et al. 2007; Uccelli et al. 2008) So far, there have been no reports in humans of the formation of tumors by in vitro-cultured cells, thus making MSCs amenable for use in transplantation.(see IB and (Lalu et al. 2012)) This is also shown in table 1 of the IB: none of these studies have reported tumorigenesis related to MSC transplantation.

It is also advantageous that MSCs disappear within a short time frame, i.e. do not integrate in the brain, thereby limiting the ability to lead to tumor formation.

From animal models of neonatal stroke we can conclude that no adverse effects have been described for MSC treatment. Our research group has also assessed long-term safety of intranasal MSC treatment.(Donega et al. 2015) At 14 months following HI no significant lesions or neoplasia were observed in the nasal tubinates, brains or other peripheral organs of mice treated intranasally with MSCs. Additionally, it has been shown that MSCs improved sensorimotor and cognitive function for at least 9 and 14 months, respectively. These results provide strong evidence of long-term safety (and efficacy) of MSC treatment following neonatal HI in mice. (Donega et al. 2015) Also a large meta-analysis on MSC treatment for various pathologies does not support tumorigenicity of MSC.(Lalu et al. 2012) Table 1 from the IB summarizes several other studies that performed administration of autologous and allogenic bone marrow derived MSCs in the treatment of several diseases. From these studies with a follow-up time of 2 months to 4 years, it was shown that (adult) patients do not have an increased risk of long-term effects, including tumorigenicity, after allogeneic MSC treatment.

Risk-benefit balance

We can conclude that MSC treatment in newborns with PAIS might be beneficial and no subacute and long-term risks are to be expected based on available evidence. Acute administration reactions, such as subacute temperature increases, are potentially to be expected, but these are expected to be transient, self-limiting and unharmful, based on the available evidence. However, this is the first study in which MSCs will be administered intranasally in humans, so potential risks might be unknown and/or unexpected. Therefore we have planned to perform a pilot (phase I/II) trial to assess safety and feasibility of MSC treatment in newborns with PAIS. Using close monitoring and (preventative) planned actions, as described in chapter 11.1e and 11.2, and tightly regulated stopping rules (see chapter 6.7) we expect to minimize or prevent risks.

5.5 Description and justification of route of administration and dosage

Administration route

MSCs will be administered intranasally to prevent loss of MSC in peripheral organs and to make sure MSCs will arrive rapidly and efficiently at the injured brain region. From the nasal mucosa, MSCs cross the cribiform plate and migrate throughout the brain using the rostral migratory stream.(Danielyan et al. 2009; Danielyan et al. 2011) In an animal model of neonatal brain injury, it was demonstrated that MSCs were detected in the olfactory bulb, in the subventricular zone of the lateral ventricles and more caudally in the ischemic damaged hippocampus within 2 hours after intranasal administration.(Donega, van

Velthoven, Nijboer, van Bel, et al. 2013). We also showed improved sensorimotor outcome and decrease of gray and white matter loss after intranasal treatment with MSCs compared to vehicle treatment. It was therefore concluded that the nasal route is efficient for MSC transplantation after neonatal brain injury.(van Velthoven et al. 2010b) This finding is confirmed by a study of Wei et al, who treated rats intranasally with bone marrow-derived MSCs. At 24 days following stroke, MSCs were found to significantly reduce infarct size, promote angiogenesis, neurogenesis and neurovascular repair. Rats treated with MSCs had improved sensorimotor and social behavioral outcome compared to their placebo-treated controls.(Wei et al. 2015)
Studies of our group in the newborn baboon have shown that intranasally administered MSCs have the potential to migrate to the injured brain regions of primates(unpublished observations), in accordance with earlier shown studies in mice and rats is known that the olfactory anatomy of primates such as the baboon is more identical to human anatomy than rodent olfactory anatomy. We therefore expect the MSCs to migrate in a similar way in the newborn baby when administered intranasally.

It was hypothesized that survival of intranasally applied MSCs is better compared to intracranially applied, because MSCs are allowed to adapt to the brain environment while they migrate to the injured area. Intracranially applied MSCs are administered directly into the lesion site and therefore have no possibility to slowly adapt to the toxic ischemic milieu in the brain.(van Velthoven et al. 2010b) We expect that the risk of anti-donor response is lower when the MSCs are administered intranasally compared to intravenously, because locally administered allogeneic MSCs appear to be non- to weakly immunogenic. (Danielyan et al. 2009; van Velthoven et al. 2012) . It is also hypothesized that the efficacy of intranasally applied MSCs is better compared to systemically applied MSCs because MSCs will not be attracted by other (damaged) peripheral organs, but are only allowed to migrate to the injured brain area. We have found chemotactic factors involved in regulating MSC migration to the lesion after intranasal administration, that mediate effective migration.(Donega, Nijboer, Braccioli, et al. 2014)
In summary, the intranasal administration route provides an effective alternative allowing MSC treatment for brain injury without invasive methods and minimal burden for the patient.

Administration dose
(Near)-term infants with PAIS that are included in this study will receive, 50 x 10^6 MSCs, intranasally. Based on mean body mass, 50 x 10^6 cells/patient is the human dose

equivalent to the dose that was effective in a mouse model of stroke (0.5 – 1.0 x 10^6 cells/mouse).(van Velthoven et al. 2010b; van Velthoven et al. 2013; Donega, van Velthoven, Nijboer, van Bel, et al. 2013; van Velthoven et al. 2014; Donega, Nijboer, van Tilborg, et al. 2014) In another mouse study, intranasally applied human MSCs were most effective when the dosage was 2 x 10^6 MSCs per mouse.(Donega, Nijboer, Braccioli, et al. 2014) In human studies with adult stroke, the dose that was administered varied between 5 x 10^7 MSC/patient (Bhasin et al. 2011) to 12 x 10^8 MSCs/patient (Bang et al. 2005; Honmou et al. 2011; Hess et al. 2014) However, these cells were administered intravenously or intra-arterially, probably leading to loss of cells in peripheral organs.
Studies of our group in the newborn baboon (of about 1.1 kilogram) have shown that one intranasally administered dose of 30 x 10^6 MSCs has the potential to migrate to the injured brain region of primates, in accordance with earlier shown studies in mice and rats.(unpublished observations)
Based on earlier evidence from our clinic, we expect to include infants with a birth weight ranging from 2.5 to 4.5 kilogram (Benders et al. 2014), and a dose of 50 x 10^6 seems appropriate for this weight.

Additional products
Not applicable

5.5.1 Description and justification of source and nature of MSCs

Source of MSCs
For this study, MSCs will be harvested from adult donor bone marrow. This is because MSCs can easily be obtained from bone marrow, expansion is possible and there is a high percentage (>90%) of MSC in stromal cells of the bone marrow. As described in chapter 2.1.S.1. of the IMPD, the MSCs will be harvested from healthy volunteers. The same MSCs are used in another trial in the UMC Utrecht to treat GvHD (see IMPD). The CTF of the UMC Utrecht has gained a lot of experience with MSCs from bone marrow and results from their earlier studies did not show any signs of toxicity of the cells (see chapter 1 IMPD). The MSCs from potential donors that will be used in the trial, were also tested in animal experiments (see IB). Also in other trials, MSCs are widely used. There are more than 500 ongoing studies using MSCs to treat various diseases in adults and children (http://clinicaltrials.gov).

Nature of cells
MSCs have many advantages over other types of stem cells or progenitors. First of all, they can easily be obtained from infants and adults.(Le Blanc & Mougiakakos 2012) Additionally, as described above, MSC treatment have a long therapeutic window, as they can be

administered at least until 10 days after brain injury to improve functional and behavioral outcome and lesion volume in neonatal HI mice. This is of great value, since the only available therapies for HI injury have to be given within a few hours. Besides, MSCs are hardly detectable in the brain after 3 days (Donega, van Velthoven, Nijboer, van Bel, et al. 2013), so the risk of Host-versus-Graft is very low. It is also very unlikely that MSCs will cause other adverse effects if they are no longer present in the brain after a short period of time. Our most recent study shows that no malignancies are found in the brains or in other organs of mice treated with MSCs when assessed at 14 months after start of MSC treatment at 10 days after neonatal HI.(Donega et al. 2015) Furthermore, MSCs have low immunogenicity because they do not express major histocompatibility complex (MHC) class-II antigens.(Rocha et al. 2000; Uccelli et al. 2008) This makes them excellent therapeutic candidates for allogeneic treatment.

5.6 Dosages, dosage modifications and method of administration

(Near-)Term infants with PAIS that are included in this study will receive 50 x 10^6 MSCs in 600µL saline with 20% Human Serum Albumin (HSA) as described in section 5.5. Infants will be put in anti-Trendelenburg (of approximately 10°) position. Thirty minutes prior to delivery of the cells, the nose will be cleaned with saline using standard procedures operative at the NICU. After 30 minutes 0.6 mL MSCs will be slowly dripped into each nostril. This will be done using a sterile plastic (e.g. 1.0 mL) syringe containing the MSCs to slowly drip the MSCs directly in both nostrils.

5.7 Preparation and labelling of Investigational Medicinal Product

All physical, chemical and pharmaceutical properties and formulation of the MSCs are described in chapter 3 of the IB. Description of formulation of all reagents, solvents and other materials can also be found in the IB. Safety procedures during cell manufacture, biosafety qualification testing of cells, storage and handling are detailed in the Investigational Medical Product Dossier (IMPD).

Study product will be labelled in accordance with applicable laws and regulations as proposed in annex 13 of the guideline Good Manufacturing Practice (*2003/94/EG)*.(website *http://ec.europa.eu/health/files/eudralex/vol-4/2009_06_annex13.pdf)* All labels will contain information required for regulatory as well as identification purposes.

5.8 Drug accountability

Not applicable.

6. METHODS

6.1 Study parameters/endpoints

6.1.1 Main study parameter/endpoint

Safety is defined primarily as the absence of treatment-related serious adverse events (SAEs) according to the Consolidated Standards of Reporting Trials (chapter 7.2 and bron), secondly as the absence of dose-limiting toxicity, defined as death within 24 hours after MSC transplantation or anaphylactic shock related to the MSC administration, and thirdly as the absence of adverse events at 3 months of age in terms of cerebral damage and neurodevelopmental outcome (see secondary outcome).

For acute safety, all patients will be regularly and intensively assessed, including blood sampling and vital signs, before and 24 hours after treatment, until discharge from our hospital. Also, blood sampling will be performed before and 24 hours after treatment with MSCs to determine inflammation parameters . Acute safety will be determined until discharge.

6.1.2 Secondary study parameters/endpoints

To determine if MSC treatment in (near-)term infants with PAIS is safe in the subacute setting, patients will be monitored until the age of 3 months. Subacute safety is defined primarily as the absence of treatment-related serious adverse events (SAEs) according to the Consolidated Standards of Reporting Trials (chapter 7.2) until the age of 3 months. Patients will then be asked to report on other SAEs, including infections, during the first three months of age. Whenever their child experiences fever, or other signs of infection/inflammation, there will be tested for bactermia, systemic infections and/or post-administration virus infections.

To determine if MSC treatment in (near-)term infants with PAIS is safe at 3 months in terms of cerebral tumorigenicity, infants will be scanned using MRI prior to MSC treatment and at 3 months of age. Long-term adverse effects on the brain in terms of tumorigenicity will be determined using this MRI, which is part of regular stroke follow-up program. Scans will be assessed by a pediatric radiologist to see if there are any adverse events on the brain (including tumor formation).

All MRI investigations will be performed on a 3.0 Tesla MR system. Infants will be sedated with a combination of chlorpromazine (0.5 mg/kg), pethidine (2 mg/kg), and promethazine (0.5 mg/kg) intramuscularly, which is part of our standard MRI procedure. During MR

examination the neonates will be placed in a vacuum fixation pillow (Med Vac Infant Immobilizer Bag, Radstadt, Austria) to prevent movement. Monitoring will be performed using pulse oximetry (Nonin, Minneapolis, MN) and respiration rate will be observed using the standard Philips equipment (Philips Medical Systems, Best, Netherlands). For hearing protection we will use: Minimuffs® (Natus Medical Incorporated, San Carlos, CA, USA), Earmuffs (EM's 4 Kids, Brisbane, Australia) and a passive acoustic noise dampener, the acoustic hood that properly fitted in the bore of the MRI scanner (Intera, Philips Medical, Best, Holland).

6.1.3 Other study parameters

None.

6.2 Randomisation, blinding and treatment allocation

Not applicable.

6.3 Study procedures

A description of all procedures, techniques and tests that are used to assess the defined study endpoints is given in chapters 6.1.1 and 6.1.2. A schedule including the timeline of all assessments is given in the flowchart of chapter 2.5. In summary, all procedures that subjects must undergo are:

- Intensive monitoring of vital signs at baseline until at least 24 hours after MSC treatment.
- Blood samples will be collected before and 24 hours after administration of MSCs. Blood samples are taken routinely in the hospital for clinical purposes, but some extra material (0.5-1 mL) will be collected to determine infection markers CRP, procalcitonin and complete blood count. These samples will be combined with routine blood sampling and taken from routinely placed (arterial) catheters.
- MRI investigation including 3D-T1, T2 weighted, DWI, DTI and MR-spectoscropy sequences within 7 days after clinical presentation suggestive of PAIS
- Repeated MRI investigation including 3D-T1, T2 weighted, DWI, DTI and MR-spectroscopy sequences at 3 months of age.

These procedures are all part of clinical care for neonates with PAIS.

6.4 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator or physician can decide to withdraw a subject from the study for urgent medical reasons. Withdrawal from this study has no consequences for medical treatment of the patient.

6.4.1 Specific criteria for withdrawal

Not applicable.

6.5 Replacement of individual subjects after withdrawal

Not applicable.

6.6 Follow-up of subjects withdrawn from treatment

Not applicable.

6.7 Premature termination of the study

The study may be terminated prematurely for safety or futility reasons if there are AEs or SAEs as defined by chapter 7.

We will use the following stopping rules:

- If the region of infarction, as defined on T2 weighted MR imaging and/or DWI, increases by >20% after treatment with MSCs in 3 different patients.
- If there appear to be any adverse effects that might be related to admission of MSCs in 3 different patients. These adverse effects include:
  - Systemic infections / meningitis
  - Hemorrhagic transformation of the infarction
  - Gastrointestinal hemorrhage
  - Thromboembolism
  - Formation of malignancies
- Unexpected and unaccountable deterioration of clinical situation, for example persistent seizures, after treatment with MSCs in 3 different patients. Usually, stroke-related seizures will disappear within three days following their onset and treated with anti-epileptic drugs.
- Mortality after treatment with MSCs in one patient will stop the study temporarily until an investigation has been performed.

Continuation of the study will only take place after the first three infants completed their second MRI scan at 3 months of age. This will safeguard that all stopping criteria will be monitored closely and the study may be terminated prematurely in time when stopping criteria are met.

7. SAFETY REPORTING

7.1 Section 10 WMO event

In accordance to section 10, subsection 4, of the WMO, the sponsor will suspend the study if there is sufficient ground that continuation of the study will jeopardise subject health or safety. The sponsor will notify the accredited METC without undue delay of a temporary halt including the reason for such an action. The study will be suspended pending a further positive decision by the accredited METC. The investigator will take care that all subjects are kept informed.

7.2 AEs, SAEs and SUSARs

7.2.1 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to intranasal MSC treatment. All adverse events reported spontaneously by the subject or observed by the investigator or his staff will be recorded.

7.2.2 Serious adverse events (SAEs)

A serious adverse event is any untoward medical occurrence or effect that at any dose:

- results in death;
- is life threatening (at the time of the event);
- requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect;
- Any other important medical event that may not result in death, be life threatening, or require hospitalization, may be considered a serious adverse experience when, based upon appropriate medical judgement, the event may jeopardize the subject or may require an intervention to prevent one of the outcomes listed above.

The sponsor will report the SAEs through the web portal *ToetsingOnline* to the accredited METC that approved the protocol, within 15 days after the sponsor has first knowledge of the serious adverse events.

SAEs that result in death or are life threatening should be reported expedited. The expedited reporting will occur not later than 7 days after the responsible investigator

has first knowledge of the adverse event. This is for a preliminary report with another 8 days for completion of the report.

7.2.3 Suspected unexpected serious adverse reactions (SUSARs)

Adverse reactions are all untoward and unintended responses to an investigational product related to any dose administered.

Unexpected adverse reactions are SUSARs if the following three conditions are met:

1. the event must be serious (see chapter 7.2.2);
2. there must be a certain degree of probability that the event is a harmful and an undesirable reaction to the medicinal product under investigation, regardless of the administered dose;
3. the adverse reaction must be unexpected, that is to say, the nature and severity of the adverse reaction are not in agreement with the product information as recorded in:
   - Summary of Product Characteristics (SPC) for an authorised medicinal product;
   - IB for an unauthorised medicinal product.

The sponsor will report expedited the following SUSARs through the web portal *ToetsingOnline* to the METC:

- SUSARs that have arisen in the clinical trial that was assessed by the METC;
- SUSARs that have arisen in other clinical trials of the same sponsor and with the same medicinal product, and that could have consequences for the safety of the subjects involved in the clinical trial that was assessed by the METC.

The remaining SUSARs are recorded in an overview list (line-listing) that will be submitted once every half year to the METC. This line-listing provides an overview of all SUSARs from the study medicine, accompanied by a brief report highlighting the main points of concern.
The expedited reporting of SUSARs through the web portal ToetsingOnline is sufficient as notification to the competent authority.

The expedited reporting will occur not later than 15 days after the sponsor has first knowledge of the adverse reactions. For fatal or life threatening cases the term will be maximal 7 days for a preliminary report with another 8 days for completion of the report.

7.3 Annual safety report

In addition to the expedited reporting of SUSARs, the sponsor will submit, once a year throughout the clinical trial, a safety report to the accredited METC and competent authority.

This safety report consists of:

- a list of all suspected (unexpected or expected) serious adverse reactions, along with an aggregated summary table of all reported serious adverse reactions, ordered by organ system, per study;
- a report concerning the safety of the subjects, consisting of a complete safety analysis and an evaluation of the balance between the efficacy and the harmfulness of the medicine under investigation.

7.4 Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist. SAEs need to be reported until discharge after the second MRI around the age of 3 months. As described in chapter 7 of the IB, MSCs do not survive for a very long period. When MSCs disappear within a short time frame and they are no longer present in the subject, they are not able/unlikely to lead to (serious) adverse events such as death, tumor formation or disturbances of the immune system. We have therefore decided that after a period of 3 months, AE or SAEs are very unlikely to be linked to the use of MSCs and conclusions regarding safety of MSCs can be drawn.

7.5 Data Safety Monitoring Board (DSMB)

A DSMB will be established to perform ongoing safety surveillance and to perform interim analyses on the safety data. This committee will be an independent committee. The DSMB is composed of 4 members from the UMCU: three are members of the internal DSMB of the UMCU and one member has clinical expertise as paediatric neurologist. Each member has no conflict of interest with the study. The task and responsibility of the DSMB are described in the DSMB charter. CVs of each DSMB member are added to the DSMB charter.

The DSMB may decide to terminate the trial prematurely using the stopping criteria as stated in chapter 6.7 of this protocol.

The advice(s) of the DSMB will only be sent to the sponsor of the study. Should the sponsor decide not to fully implement the advice of the DSMB, the sponsor will send the advice to the reviewing METC, including a note to substantiate why (part of) the advice of the DSMB will not be followed.

8. STATISTICAL ANALYSIS

In this study, 10 newborns will be included who will be treated with MSCs via the nasal route. This is a phase I/II, open-label, single-arm, single-center intervention study to assess the safety and feasibility of intranasal BM-MSCs in neonatal stroke patients.

8.1 Study parameter(s)

Safety is defined primarily as the absence of treatment-related serious adverse events (SAEs) according to the Consolidated Standards of Reporting Trials (chapter 7.2 and bron), secondly as the absence of dose-limiting toxicity, defined as death within 24 hours after MSC transplantation or anaphylactic shock related to the MSC administration, and thirdly as the absence of adverse events at 3 months of age in terms of cerebral damage and neurodevelopmental outcome (see secondary outcome).

For acute safety, all vital signs and blood sample parameters (continuous variables) at baseline and at 24 hours following MSC transplantation will be compared using Wilcoxon signed-rank tests. The occurrence of SAE's, including mortality, toxicity, tumor growth, etc (see chapter 11.1) will be expressed in percentages and analyzed qualitatively.

8.2 Other study parameters

Not applicable.

8.3 Interim analysis (if applicable)

No interim analysis will be performed. Stopping rules are described in chapter 6.7. A DSMB will be established to advice on stopping, as is described in chapter 7.5 and in the DSMB Charter.

9. ETHICAL CONSIDERATIONS

9.1 Regulation statement

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) and other guidelines, regulations and Acts.

9.2 Recruitment and consent

Parents of neonates born in a Dutch hospital and/or admitted to our NICU who are potentially eligible for inclusion into the study will be informed as soon as possible after suspicion of PAIS about the study protocol by a neonatologist involved in the study. These parents include those of infants with or without symptoms of PAIS (***i.e***.one sided convulsions) and a PAIS confirmed with MRI. 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 allowing the parents to read, understand and, if necessary, having received additional information if requested, consent for participation will be asked in case PAIS (in the MCA region) is confirmed within 7 days after the onset of symptoms by MRI (including 3D-T1, T2 weighted, DWI and DTI sequences). Following written parental consent, patients will then be included in our study. Patients with PAIS that are diagnosed more than one week after birth are not eligible for enrolment.

9.3 Objection by minors or incapacitated subjects (if applicable)

Resistance by minors will be conducted by the code of conduct accepted by the “Bestuur van de Nederlandse Vereniging voor Kindergeneeskunde” (NVK) at May 21 2001, and it will be specified in the informed consent letter.

9.4 Benefits and risks assessment, group relatedness

In this study we will include (near-)term newborns with proven PAIS. Current treatment options for PAIS mainly focus on controlling convulsions, hypoglycaemia and associated infections. There is no treatment available that leads to reduction of neonatal brain damage in this severely affected group of infants. This leads to life-long consequences of PAIS and forms a large burden for patients and society. The overall aim of this project is to meet this by making the first step towards clinical application of MSCs to treat PAIS. Successful completion of this project will provide the first evidence of the safety and feasibility of intranasally administered bone-marrow derived MSCs in newborn 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 the nasal treatment to apply the MSCs, treatment is not different compared to the standard acting treatment protocol for newborns with PAIS. Nasal application of MSCs has been considered as non-invasive. With the respect to possible risks of the present MSC treatment, the most important potential risk factors such as inflammatory actions of MSC therapy with allogeneic human MSCs and an increased risk for malignancies, have been intensively investigated in our own preclinical studies and appear to be non-present at long-term follow up. Additionally, human trials on MSC treatment for adult stroke or preterm infants with BPD and other pathologic conditions do not provide evidence for these or other serious adverse events or risks. However, this is the first study administering MSCs intranasally in humans, so potential risks might be unknown and/or unexpected. Using close monitoring and (preventative) planned actions, as described in chapter 11.1e and 11.2, and tightly regulated stopping rules (see chapter 6.7) we expect to minimize or prevent these risks.No single indication has been found in experimental research in the developing animal models that above mentioned complications occur in a higher incidence as compared to non-MSC treated animals, whereas possibility for a substantial better short- and long-term outcome seems very realistic on the basis of previous experimental research data. Therefore we have planned to peform a pilot (phase I/II) trial to assess safety and feasibility of MSC treatment in newborns with PAIS.

9.5 Compensation for injury

The sponsor/investigator has a liability insurance which is in accordance with article 7 of the WMO.

The sponsor (also) has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO and the Measure regarding Compulsory Insurance for Clinical Research in Humans of 24th November 2014). This insurance provides cover for damage to research subjects through injury or death caused by the study.

The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

9.6 Incentives (if applicable)

Not applicable.

10. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

10.1 Handling and storage of data and documents

Data will be handled confidentially (using coding). Where it is necessary to trace data to an individual subject, a subject identification-code list will be used to link the data to the subject. The code will not be based on the patient initials and birth date. The key to the code will be safeguarded by the research team (Principal Investigator), because data and human material will be kept for a longer period of time. The handling of personal data will be complied with the Dutch Personal Data Protection.

10.2 Monitoring and Quality Assurance

A detailed description of monitoring of the study is given in the Monitoringplan and the Monitor proposal (part K6).

10.3 Amendments

A 'substantial amendment' is defined as an amendment to the terms of the METC application, or to the protocol or any other supporting documentation, that is likely to affect to a significant degree:

- the safety or physical or mental integrity of the subjects of the trial;
- the scientific value of the trial;
- the conduct or management of the trial; or
- the quality or safety of any intervention used in the trial.

All substantial amendments will be notified to the METC and to the competent authority.

Non-substantial amendments will not be notified to the accredited METC and the competent authority, but will be recorded and filed by the sponsor.

10.4 Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

10.5 End of study report

The sponsor will notify the accredited METC and the competent authority of the end of the study within a period of 90 days. The end of the study is defined as the last patient's last visit.

In case the study is ended prematurely, the sponsor will notify the accredited METC and the competent authority within 15 days, including the reasons for the premature termination.

Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC and the Competent Authority.

10.6 Public disclosure and publication policy

There are no specific agreements concerning public disclosure or publication policy.

11. STRUCTURED RISK ANALYSIS

11.1 Potential issues of concern

a. Level of knowledge about mechanism of action

From neonatal rat models of brain injury, we have learned that HI brain injury induces changes in neurovascular environment that promote neurogenesis.(Kadam et al. 2008; Ong et al. 2005; Yang & Levison 2007) However, after HI brain injury, there is no induction and maintenance of long-term neurogenesis. Therefore, it is crucial to assist the brain in regeneration after injury and also to reinforce development of the maturating brain. In animal stroke-model studies, it has been demonstrated that transplantation of murine adult bone marrow-derived MSCs markedly improves neuroregeneration. Treatment with MSC stimulated formation of new neurons and oligodendrocytes and reduced infarct size by >45%. Although intracranial MSC treatment stimulated formation of new brain cells and differentiation of these cells into new neurons and oligodendrocytes, these newly formed cells were not of transplant origin. These findings indicate that the neuroregenerative effect of MSCs after ischemic brain damage mainly relies on stimulation of endogenous repair mechanisms.(van Velthoven et al. 2010c) This is confirmed by other data from our group that show that MSC treatment has profound effects on the expression of growth and differentiation factors in the brain. For example, MSC treatment at 10 days after HI induces upregulation of 35 genes encoding growth and differentiation factors that are mainly involved in cell growth and proliferation. MSC treatment at 3 and 10 days induces expression of 28 additional growth and differentiation factors and these are predominantly involved in cell differentiation and nervous system development.(van Velthoven et al. 2010c; van Velthoven et al. 2011) These results show that the first dose of MSC stimulates cerebral cell proliferation, whereas the second injection enhances differentiation and maturation of newly formed cells and integration in a functional network.(van Velthoven et al. 2010c) Importantly, we have shown recently that at 3 days after MSC administration, the number of MSCs in the brain sharply decreases indicating low survival of MSCs for longer time periods (Donega, 2014 Exp Neurol).

Based on these findings, we hypothesize that MSC of donor origin will only transiently survive in the brain and that the neuroregenerative effects of MSC are mediated predominantly by orchestrating changes in the growth and differentiation factor milieu in the brain. The corticospinal tract is the primary transmission route for voluntary movement of the forepaws. We showed that MSC treatment could also partially restore the damaged

corticospinal motor tract which led to improved sensorimotor outcome.(van Velthoven et al. 2010c; van Velthoven et al. 2010a)

An overview of findings from non-clinical studies on MSC treatment for neonatal stroke is given in chapter 7 of the IB. These studies confirm the role of MSCs in stimulating endogenous neuroregenerative mechanisms and thereby reducing cerebral damage, restoring corticospinal motor tracts and improving sensorimotor outcome.

b. Previous exposure of human beings with the test product(s) and/or products with a similar biological mechanism

At present, there are no results of clinical trials available on the treatment of neonatal stroke with MSCs. The only available trial on MSC administration in neonates, showed that allogeneic hUCB-derived MSC treatment in premature neonates with BPD was safe and without any adverse events.(Chang et al. 2014; Henckel et al. 2015) However, many studies have reported on MSC treatment for stroke in adult patients. An overview of findings from these trials is given in chapter 8 of the IB. In summary, these studies show potential of MSCs in the treatment of adult stroke as they improve functional outcome. Even though neonatal and adult stroke do not have a common underlying cause, they both result in brain damage caused by common harmful processes. These include mechanisms of excitotoxicity and neuroinflammation, involving microglial activation, proinflammatory cytokine production and toll-like receptor (TLR) activation.(Fleiss et al. 2014) Comparable to our study, most of these trials administered MSCs days to weeks after the cerebral damage occurred, while brain tissue loss is usually complete after 10 days.(van Velthoven et al. 2010a; Bonestroo et al. 2015) Therefore the main goal of all MSC treatment studies was not to have neuro*protective* effects, but to induce endogenous neuro*regeneration* and repair. We therefore presume that treatment with MSCs in neonatal stroke will have the same biological mechanism of action as in adult stroke and will also lead to improved neuroimaging and functional outcome.

c. Can the primary or secondary mechanism be induced in animals and/or in ex-vivo human cell material?

An overview of findings from non-clinical studies on MSC treatment for neonatal stroke is given in chapter 7 of the IB. These studies show that MSC treatment in animal models of neonatal stroke reduces cerebral damage, restoring corticospinal motor tracts and improving sensorimotor outcome.

d. Selectivity of the mechanism to target tissue in animals and/or human beings

Human MSCs communicate with other cells in the human body and appear to migrate to areas of injury in response to signals of cellular damage and apoptosis.(Kang et al. 2012; Vogel et al. 2010) MSCs migrate selectively into damaged brain lesions, and specific molecular signals, such as stromal cell- derived factor-1 (SDF-1/CXCR4), intracellular signaling and adhesion molecules (selectins and integrins) and proteases have been reported to be involved in the interaction of MSCs to reach, recognize, and function in cerebral ischemic tissue.(Komatsu et al. 2010; Chavakis et al. 2008; Shen et al. 2007; Shyu et al. 2006) It is therefore hypothesized that areas of brain injury caused by PAIS will attract MSCs to migrate to the ischemic brain injury site. MSCs will be administered intranasally to prevent loss of MSC in peripheral organs (Steiner et al. 2012) and to make sure MSCs will arrive rapidly and efficiently at the injured brain region.

e. Analysis of potential effect

Adverse events

From animal models of neonatal stroke we can conclude that no adverse effects have been described for MSC treatment. Our research group has also assessed long-term safety of intranasal MSC treatment.(Donega et al. 2015) At 14 months following HI no significant lesions or neoplasia were observed in the nasal tubinates, brain or other peripheral organs of mice treated intranasally with MSCs and compared with control or vehicle-treated HI mice. These results provide strong evidence of the long-term safety of MSC treatment following neonatal HI in mice.(Donega et al. 2015)

As MSC treatment has never been studied in human neonatal stroke, we can only provide evidence for the safety of MSC treatment in other pathological conditions. There is only one clinical trial describing the use of MSCs in treatment of neonates.(Chang et al. 2014) Chang et al assessed in a phase I dose-escalation trial the safety and feasibility of intratracheal administration of allogeneic umbilical cord-derived MSCs in preterm infants with high risk for BPD. Nine infants were included in this study: 3 patients were given 1 x 10^7 MSCs/kg and 6 patients were given 2 x 10^7 MSCs/kg. After 7 days there was a decrease in inflammatory cytokines (e.g. interleukin-6 and interleukin-8) and BPD severity was lower in patients who received MSCs compared to their historical case-matched controls. Importantly, there were no serious adverse events or toxicity related to a higher dose.(Chang et al. 2014) More recently some preliminary findings from another trial were published, confirming safety and feasibility of MSC treatment in premature BPD

patients.(Henckel et al. 2015) Although these MSCs were administered intravenously, no adverse effects were found.(Henckel et al. 2015)

As described in chapter 8 of the IB, there are many other clinical trials in adults in which MSC are used for the treatment of several diseases. Although the hypothesized mode of action of MSCs in different pathological conditions varies, the most important findings from these clinical trials refer to safety issues. In none of the trials serious adverse events related to MSC treatment were detected during bone marrow harvesting and administration. Some studies reported mild allergic reaction(Duijvestein et al. 2010), probably related to preservation substances in the MSC-product, or self-limiting fever within a few hours after MSC-transplantation.(Shi et al. 2012) In some studies patients had a serious adverse event, most likely not related to MSC administration.(Forbes et al. 2014; Wang et al. 2013; Weiss et al. 2013; Haack-sørensen et al. 2014) Additionally, a large meta-analysis on clinical trials for numerous diseases did not show any evidence for severe adverse effects due to MSC transplantation.(Lalu et al. 2012) This analysis used both adult and pediatric trials to report on a total of 1012 patients with ischemic stroke, Crohn's disease, cardiomyopathy, myocardial infarction, GvHD and healthy volunteers. Including eight randomized control trials, there was no association detected between MSC transplantation and acute infusional toxicity, organ system complications, infection, death or malignancy. There was a significant increase in fever with MSCs compared to the control group, but the fever was reported to be transient in all trials and most often low grade (≤38°C).(Lalu et al. 2012)

No correlation between the dose of MSC and their potential efficacy or side effects could be observed in multiple studies (range 1-8 10^6 cells/kg body weight).(Lalu et al. 2012) It is known that systemic complications such as fever are more common when MSCs were systemically (e.g. intravenously) administered. We found no evidence that fever occurred in our animal studies.(Donega et al. 2015) From our preclinical studies in mice we found that local (intracranial) administration of MSCs at 3 days after brain injury resulted in a decrease of pro-inflammatory cytokines IL-1β, IL-1R and IL-3.(van Velthoven et al. 2011)In vitro we have also found that if MSCs are cultured in a hypoxic-ischemic brain environment, they produce more growth factors and less pro-inflammatory cytokines (IL-1β, IL-6 en TGFb) than MSCs cultured in a normal brain environment.(van Velthoven et al. 2010b) Intranasal administration of MSCs, dampened neuro-inflammatory responses due to hypoxic-ischemic brain injury at 5-18 days after MSC treatment. The number of GFAP and Iba-1 positive cells, as markers for the neuroinflammatory response of glial cells, decreases after administration of MSCs, possibly as a result of anti-inflammatory

functions of MSCs. (Donega, Nijboer, van Tilborg, et al. 2014) In conclusion, it seems that local/nasal MSC treatment has rather an anti-inflammatory effect and will not induce inflammation. We therefore do not expect fever to complicate intranasal administration of MSCs, as a priori chances are low. Additionally, the danger of transient and low grade fever is low.

With respect to possible risks of the present MSC treatment, the most important potential risk factors such as inflammatory actions of MSC therapy with allogeneic human MSCs and an increased risk for malignancies, are intensively investigated in our own preclinical studies and were not found.(Donega et al. 2015) In addition, we found that intranasal administration of human MSCs enhanced secretion of anti-inflammatory cytokines.(Donega, Nijboer, Braccioli, et al. 2014) Additionally, human trials on MSC treatment for adult stroke or other pathologic conditions do not provide evidence for these or other serious adverse events or risks. No single indication has been found in experimental research in the developing animal models that above mentioned complications occur in a higher incidence as compared to non-MSC treated animals, whereas possibility for a substantial better short- and long-term outcome seems very realistic on the basis of previous experimental research data. However, this is the first study administering MSCs intranasally in humans, so potential risks might be unknown and/or unexpected. Therefore, we have planned to perform a pilot (phase I/II) trial to assess safety and feasibility of MSC treatment in newborns with PAIS. Using close monitoring and (preventative) planned actions, as described in chapter 11.1e and 11.2, and tightly regulated stopping rules (see chapter 6.7) we expect to minimize or prevent these risks.

Summary of potential risks

Even though there is no evidence that risks as mentioned below occur in a higher incidence as compared to non-MSC treated animals, these risks will be monitored as part of standard clinical care on the NICU. These risks are based on theoretical mechanisms of action, most likely to occur after systemic administration of MSCs. In general, risks or adverse events related to MSC treatment can be categorized in three groups: acute, subacute and longterm adverse effects.

1. Acute adverse effects: Administration Reaction/Toxicity
    - Systolic blood pressure >33% decrease or increase from the baseline during or directly following the intranasal administration of the investigational product

- Diastolic blood pressure >25% decrease or increase from baseline during or directly following the intranasal administration of the investigational product
- Heart rate >33% decrease or increase from baseline during or directly following the infusion of the investigational product
- Development of dyspnoea, vomiting, flushing, diarrhea, during or within 1 hour from the intranasal administration of the investigational product
- Fever ≥ 2 grade increase from baseline during or within 2 hours from the intranasal administration of the investigational product
- Development of exanthema, or urticaria developing during or within 2 hours from the intranasal administration of the investigational product

2. Subactue adverse effects:
   - Bacteremia due to bacterial contamination of product. Fever ≥ 2 grade increase from baseline during or within 2 hours from the intranasal administration of the investigational product AND product contamination proven by positive product cultures. In case of contamination patients will be treated with antibiotics in case this is required.
   - Systemic infection (e.g. meningitis) due to use of MSCs. We have no evidence from preclinical trials, nor from trials in adult stroke or neonatal BPD, that MSC treatment increases the risk of infections. However, MSCs might be able to change inflammatory status. In order to monitor more closely, we will take extra blood samples from all subjects at baseline and 24 hours following MSC administration to determine infection markers such as CRP, procalcitonine and complete blood count.
   - Post-administration virus infection. This risk is minimal and adherent to all blood (derived) product administration. If a patient develops a new viral infection that is suspected to be due to transfusion of blood products or intranasal administration of the investigational product, a lookback procedure should be initiated by the treating physician according to local regulations for all blood products received. If a MSC donor is proven to be the source of virus infection, this shall be considered a serious adverse event. All donors are being screened for infectious disease markers as indicated by the Wet Veiligheid en Kwaliteit Lichaamsmaterialen (WVKL), the transposition into Dutch Law of the EU Directive 2006/17. Please find specification of control of drug stubstance in chapter 2.1.S.4.1 of the IMPD.

3. Long-term effects: Ectopic tissue formation and tumorigenicity

   Although concerns that MSCs might transform into tumorigenic cells still exist, there is a general agreement that bone-marrow-derived MSCs can be safely

cultured in vitro with no risk of malignant transformation.(Bernardo et al. 2007; Uccelli et al. 2008) So far, there have been no reports in humans of the formation of tumors by in vitro-cultured cells, thus making MSCs amenable for use in transplantation.(see IB and (Lalu et al. 2012)) This is also shown in table 1 of the IB: none of these studies have reported tumorigenesis related to MSC transplantation.

It is also advantageous that MSCs disappear within a short time frame, i.e. do not integrate in the brain, because when they are no longer present, they are not able to lead to tumor formation.

The recent meta-analysis and our own pre-clinical study does not support tumorigenicity of MSC.(Lalu et al. 2012)(Donega et al. 2015) If ectopic tissue formation or a new malignancy is detected in a patient at any time following infusion of the investigational product, the coordinating investigator should be contacted. Information regarding the MSC donor's HLA typing will be provided so that genetic testing for presence of donor-derived MSC can be performed. If MSC donor-derived tissue is present either as malignant tissue or in the malignancy surrounding tissue, this is regarded as a suspect unexpected serious adverse reaction.

f. Pharmacokinetic considerations

Not applicable.

g. Study population

PAIS or cerebral infarction is an important perinatal cause of long-lasting neurodevelopmental problems. The disease manifests itself most often with one-sided convulsions in the first week of life, often accompanied with (asymmetric) hypotonia, lethargy and apnea.(Kirton et al. 2011) When PAIS is suspected clinically this is usually confirmed by MRI. Neuromotor outcome becomes more evident over time and depends largely on the involvement of the corticospinal tracts. Adverse consequences of PAIS include USCP, cognitive dysfunction, epilepsy and speech problems. In 50-75% of infants, PAIS leads to abnormal neuromotor and -developmental outcome or epilepsy.(Fernández-López et al. 2014) Postneonatal epilepsy after PAIS is also often reported, and is associated with larger PAIS.(Wusthoff et al. 2011; van Buuren et al. 2013) The estimated annual mortality rate of neonatal stroke is 3.49/100,000 annually.(Lynch & Nelson 2001) Current treatment options for PAIS mainly focus on controlling convulsions, treatment of hypoglycaemia and associated infections. There is no treatment available that leads to reduction of neonatal brain damage in this severely affected group of infants. This leads to life-long consequences of PAIS and forms a large burden for patients and society. The

overall aim of this project is to meet this need by assessing the safety and feasibility of a cell based treatment strategy for neonates with PAIS.
Patients included in this study will be given MSCs as soon as possible after confirmation of PAIS, but within the first week of onset of presenting symptoms. At time of administration of MSCs, these infants will be admitted to the NICU of our hospital where they can be monitored intensively.

h. Interaction with other products

The potential interactions with other medicinal products, food and other substances have been investigated by the CTF of the UMC Utrecht. No interacting substances were found.

i. Predictability of effect

In this study we will not investigate any therapeutic effects of MSCs. We will only assess safety in terms of (S)AEs.

j. Can effects be managed?

Planned pharmacovigilance actions for potential risks are described below in section 11.2

11.2 Synthesis

Adverse events, observed by the attending physician, which are expected or possibly related to the infusion of the product, are reported to the head of the CTF. Adverse events will be reported in a database, including patient characteristics and product characteristics. Adverse events will be evaluated with the Medical Director of the CTF in their weekly meetings. We will report the observed adverse events to the Dutch Health Inspectorate. Serious adverse events will be reported within 48 hours after the event has happened.

Planned pharmacovigilance actions for potential risks

1. Acute adverse effects: Administration Reaction, during and shortly after administration
Planned actions:

- Monitoring of heart rate and blood pressure. And development of dyspnoea, nausea/vomiting, flushing, diarrhea, during or within 1 hour from the administration of the investigational product

- Monitoring temperature during or within 2 hours from the administration of the investigational product. During admission, temperature will be monitored very strictly, because hyperthermia after hypoxia-ischemia is associated with adverse (neurologic) outcome.(Laptook et al. 2008; Perlman 2006) If the temperature rises above 37.5ºC we will use routinely clinical used precautions to prevent actual hyperthermia, such as lowering environmental temperature or use paracetamol.
- Development of exanthema or urticaria developing during or within 2 hours from the administration of the investigational product.

2. Subactue adverse effects:

- Bacteremia due to bacterial contamination of product.

Planned actions: Monitoring of temperature during the following hours to days after intranasal administration. In case of contamination patients will be treated with antibiotics in case this is required.

- Systemic infection due to use of MSC therapy

Planned actions: Close monitoring of first three patients by extra blood sampling and determination of infection markers. In case of systemic infection patients will be treated with appropriate antibiotics in case this is required, according to the antibiotics protocol used on the NICU.

- Post-administration virus infection.

Planned actions: If a patient develops a new viral infection that is suspected to be due to transfusion of blood products or intranasal administration of the investigational product, a look-back procedure should be initiated by the treating physician according to local regulations for all blood products received. If an MSC donor is proven to be the source of virus infection, this shall be considered a serious adverse event.

3. Long-term adverse effects: Ectopic tissue formation and tumorigenicity

Planned actions: If ectopic tissue formation or a new malignancy is detected in a patient at any time following infusion of the investigational product, information regarding the MSC donor's HLA-typing will be provided so that genetic testing for presence of donor-derived MSC can be performed. If MSC donor-derived tissue is present either as malignant tissue or in the malignancy surrounding tissue, this is regarded as a serious adverse reaction. Treatment will be accordingly to the malignant tissue.

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 the nasal treatment to apply the MSCs, treatment is not different compared to the standard acting treatment protocol for newborns with PAIS.

Nasal application of MSCs has been considered as non-invasive. With the respect to possible risks of the present MSC treatment, the most important potential risk factors such as inflammatory actions of MSC therapy with allogeneic human MSCs and an increased risk for malignancies, are intensively investigated in our own preclinical studies and appear to be non-present at long-term follow up. Additionally, human trials on MSC treatment for adult stroke or other pathologic conditions do not provide evidence for these or other serious adverse events or risks. However, this is the first study administering MSCs intranasally in humans, so potential risks might be unknown and/or unexpected. Therefore, this study will assess safety and feasibility of bone marrow-derived allogeneic MSCs, administered by the nasal route, in human neonates with PAIS. Using close monitoring and (preventative) planned actions, as described in chapter 11.1e and 11.2, and tightly regulated stopping rules (see chapter 6.7) we expect to minimize or prevent these risks.. No single indication has been found in experimental research in the developing animal models that above mentioned complications occur in a higher incidence as compared to non-MSC treated animals, whereas possibility for a substantial better short- and long-term outcome seems very realistic on the basis of previous experimental research data.

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