doi:10.1093/brain/awu363 BRAIN 2015: 138; 388–397 | 388

BRAIN
A JOURNAL OF NEUROLOGY

Tract-based spatial statistics to assess the neuroprotective effect of early erythropoietin on white matter development in preterm infants

Ruth L. O'Gorman,[1] Hans U. Bucher,[2] Ulrike Held,[3] Brigitte M. Koller,[2] Petra S. Hüppi,[3,4] Cornelia F. Hagmann[2] and the Swiss EPO Neuroprotection Trial Group

Despite improved survival, many preterm infants undergo subsequent neurodevelopmental impairment. To date, no neuroprotective therapies have been implemented into clinical practice. Erythropoietin, a haematopoietic cytokine used for treatment of anaemia of prematurity, has been shown to have neuroprotective and neuroregenerative effects on the brain in many experimental studies. The aim of the study was to assess the effect of recombinant human erythropoietin on the microstructural development of the cerebral white matter using tract-based spatial statistics performed at term equivalent age. A randomized, double-blind placebo-controlled, prospective multicentre study applying recombinant human erythropoietin in the first 42 h after preterm birth entitled 'Does erythropoietin improve outcome in preterm infant' was conducted in Switzerland (NCT00413946). Preterm infants were given recombinant human erythropoietin (3000 IU) or an equivalent volume of placebo (NaCl 0.9%) intravenously before 3 h of age after birth, at 12–18 h and at 36–42 h after birth. High resolution diffusion tensor imaging was obtained at 3 T in 58 preterm infants with mean (standard deviation) gestational age at birth 29.75 (1.44) weeks, and at scanning at 41.1 (2.09) weeks. Imaging was performed at a single centre. Voxel-wise statistical analysis of the fractional anisotropy data was carried out using tract-based spatial statistics to test for differences in fractional anisotropy between infants treated with recombinant human erythropoietin and placebo using a general linear model, covarying for the gestational age at birth and the corrected gestational age at the time of the scan. Preterm infants treated with recombinant human erythropoietin demonstrated increased fractional anisotropy in the genu and splenium of the corpus callosum, the anterior and posterior limbs of the internal capsule, and the corticospinal tract bilaterally. Mean fractional anisotropy was significantly higher in preterm infants treated with recombinant human erythropoietin than in those treated with placebo ($P < 0.001$). We conclude that early recombinant human erythropoietin administration improves white matter development in preterm infants assessed by diffusion tensor imaging and tract-based spatial statistics.

1 MR Research Centre, Children's University Hospital of Zurich, Switzerland
2 Department of Neonatology, University Hospital of Zurich, Switzerland
3 Horten Centre, University Hospital of Zurich, Switzerland
4 Division of Development and Growth, Department of Paediatrics, University of Geneva, Switzerland

Correspondence to: Dr. med. Cornelia Hagmann Ph.D,
Department for Neonatology,
University Hospital of Zurich Frauenklinikstrasse,
10 8091 Zürich, Switzerland
E-mail: Cornelia.hagmann@usz.ch

Keywords: neuroprotection; brain development; white matter lesion; oligodendrocyte; tractography

Abbreviations: DTI = diffusion tensor imaging; rhEpo = recombinant human erythropoietin; TBSS = tract-based spatial statistics

Received July 10, 2014. Revised August 30, 2014. Accepted October 23, 2014. Advance Access publication December 22, 2014

Introduction

Despite improved survival of preterm infants, the majority still go on to develop some degree of neurodevelopmental impairment later in life (Saigal and Doyle, 2008; Latal, 2009). These neurodevelopmental impairments are associated with periventricular white matter injury (Woodward *et al.*, 2006), which is the most common brain injury observed in preterm infants. Periventricular white matter disease is also frequently accompanied by neuronal/axonal disease, as part of the newly described encephalopathy of prematurity (Volpe, 2009).

In vivo assessment of this neuronal/axonal disease has become possible with the development of new diffusion tensor imaging (DTI) protocols in preterm infants (Huppi and Inder, 2001; Huppi and Dubois, 2006). DTI is a well-studied MRI technique that allows *in vivo* assessment of biological tissues at a microstructural level. In preterm infants, DTI studies have revealed lower fractional anisotropy values relative to those in healthy term control infants (Huppi *et al.*, 1998, 2001; Miller *et al.*, 2002; Mukherjee *et al.*, 2002; Counsell *et al.*, 2003, 2006; Partridge *et al.*, 2004; Berman *et al.*, 2005; Anjari *et al.*, 2007; Dudink *et al.*, 2007; Cheong *et al.*, 2009; Skiold *et al.*, 2010), indicative of altered white matter development.

Tract-based spatial statistics (TBSS) is an automated, observer-independent approach for assessing fractional anisotropy in the major white matter tracts on a voxel-wise basis across groups of subjects (Smith *et al.*, 2006). It has been applied to investigate cerebral microstructure in preterm infants at term equivalent age (Anjari *et al.*, 2007), revealing group differences based on either clinical details such as chronic lung disease (Ball *et al.*, 2010; Alexandrou *et al.*, 2014) or birth weight (Lepomaki *et al.*, 2013) or additional imaging findings such as local white matter abnormalities (Bassi *et al.*, 2011). A recent TBSS study has shown that cognitive and motor outcome correlate with fractional anisotropy in the corpus callosum (van Kooij *et al.*, 2012), suggesting that TBSS of DTI data has the potential to be used as a surrogate biomarker for subsequent neurodevelopmental outcome. In term infants with perinatal asphyxial encephalopathy, TBSS analysis has been used to detect the treatment effects of therapeutic hypothermia in a small group of patients ($n = 10$ per group) (Porter *et al.*, 2010), indicating that TBSS may also be used to evaluate neuroprotective interventions (Ball *et al.*, 2013).

The high incidence of neurodevelopmental impairment in this population is reflected in the ongoing search for neuroprotective interventions that can prevent injury or enhance repair of the immature brain, with the goal of improving long-term motor and cognitive outcome (Gonzalez and Ferriero, 2009). However, to date no such neuroprotective intervention has been implemented into routine clinical care for preterm infants.

Erythropoietin, a haematopoetic cytokine that was originally identified for its role in erythropoiesis, is widely used for the treatment of anaemia in premature infants (Ohlsson and Aher, 2006; Ghezzi *et al.*, 2010). Erythropoietin has been shown to have neuroprotective and neuroregenerative effects on the brain (Dame *et al.*, 2001; Juul, 2004; van der Kooij *et al.*, 2008), and erythropoietin receptors are present on neuron progenitors, neurons, astrocytes, microglia, endothelial cells and erythrocyte progenitors (Juul and Felderhoff-Mueser, 2007). Erythropoietin has anti-inflammatory, anti-excitotoxic, antioxidant and anti-apoptotic effects on neurons and oligodendrocytes and promotes neurogenesis and angiogenesis, which are essential for injury repair and normal neurodevelopment (Shingo *et al.*, 2001; Juul, 2012).

The safety of recombinant human erythropoietin (rhEpo) in preterm infants has been established by two clinical trials with high dose administration (Fauchere *et al.*, 2008; Juul *et al.*, 2008*a, b*). Early high-dose rhEpo is well tolerated, causing no increased mortality or side effects (Fauchere *et al.*, 2008, 2012). More recently, erythropoietin administration has also be shown to be well tolerated by term infants with hypoxic ischaemic or post-asphyctic encephalopathy (Wu *et al.*, 2012).

One clinical trial of rhEpo for treatment of anaemia of prematurity reporting on the effect of early rhEpo on neurodevelopmental outcome has been published. The neurodevelopmental outcome of 20 preterm infants treated with early rhEpo was assessed at 18–24 months of age. No adverse or beneficial effect of rhEpo on outcome could be shown; however, the erythropoietin treatment group included more infants with intraventricular haemorrhage than the control group (Newton *et al.*, 1999; Ohls *et al.*, 2004).

A large retrospective trial indicated clear benefits of rhEpo for preterm infants, demonstrating that infants treated with rhEpo scored significantly better in the overall assessment at school age as well as in the psychological evaluation (Neubauer *et al.*, 2010). These potential neuroprotective effects are thought to arise from a putative reduction in brain injury, but to date the effects of rhEPO on cerebral microstructure in preterm infants have not been established.

The aim of the present study was to assess the effect of early administration of rhEpo on white matter development in preterm infants using TBSS.

Materials and methods

Ethical approval was granted by the local ethical committee (KEK StV-36/04), and the study was approved by the Swiss drug surveillance unit (Swissmedic, 2005DR3179). The trial is registered at ClinicalTrials.gov (number NCT00413946). Full details of the trial protocol can be provided on request. The members of The Swiss EPO Neuroprotection Trial Group are listed in Appendix 1.

Patient selection

Preterm infants in this study represent a subgroup of infants enrolled in the randomized, double-blind placebo-controlled, prospective multicentre study entitled 'Does erythropoetin improve outcome in preterm infants' (NCT00413946). All infants born between 26 and 31 gestational weeks, from whose parents informed consent could be obtained in the first 3 h after birth, were included unless they met exclusion criteria (presence of a genetically defined syndrome, severe congenital malformation adversely affecting life expectancy, and severe congenital malformation adversely affecting neurodevelopment or prior palliative care). A total of 495 preterm infants were recruited into the study between 2005 and 2013, of whom 58 infants were excluded for an exclusion criterion and/or death before term equivalent age (Fig. 1). The primary outcome measure of this study is neurodevelopmental outcome assessed at 2 and 5 years of age. A secondary outcome measure is brain injury and development assessed by conventional MRI at term equivalent age, measured in a subgroup of 165 infants (Leuchter *et al.*, 2014). For this study, only infants with DTI performed in Zurich ($n = 140$) were included. Clinical co-morbidities such as sepsis (blood culture proven), necrotizing enterocolitis, patent ductus arteriosus, and chronic lung disease (defined as oxygen requirement at corrected 36 weeks of gestation) were noted.

Randomization, neuroprotective intervention and blinding

The treatment protocol was based on the previously published safety trial (Fauchere *et al.*, 2008). Study medication was randomly assigned to each patient number in a 1:1 allocation using a computer-based random-number generator. Erythropoetin or an equivalent volume of normal saline (NaCl 0.9%) placebo was administered intravenously before 3 h of age after birth, at 12–18 h and at 36–42 h after birth. A single dose consists of 25 µg (3000 IU) human erythropoietin per kilogram body weight dissolved in 1 ml sterile water. Hospital staff, parents, the outcome assessors and data analysts were kept blinded to the allocation.

MRI studies

In Zurich, cerebral MRI was performed with a 3.0 T GE HD.xt MRI scanner (GE Medical Systems), using an 8-channel receive-only head coil. All infants were scanned under natural sleep using a vacuum mattress. Ear plugs (attenuation: 24 dB; Earsoft; Aearo) and Minimuffs (attenuation: 7 dB; Natus) were applied for noise protection. Oxygen saturation was monitored during scanning, and a neonatologist and/or neonatal nurse were present during the MRI investigation.

The structural MRI protocol included T_1-weighted images acquired with a 3D fast spoiled gradient echo sequence (resolution $0.7 \times 0.7 \times 1.4\,mm^3$, repetition time = 5.7 ms, echo time = 2.6 ms, inversion time = 750 ms, flip angle = 12°), and T_2-weighted images acquired with a fast recovery fast spin echo sequence (resolution $0.7 \times 0.7 \times 1.5\,mm^3$, repetition time = 6600 ms, echo time = 126 ms). The structural magnetic resonance images were assessed for brain injury based on a clinical scoring system which was adapted from Woodward and colleagues (Woodward *et al.*, 2006; Leuchter *et al.*, 2014). The assessor was not aware of the group assignment of the infants.

DTI was performed in a single centre, using a pulsed gradient spin echo echo planar imaging sequence with echo time = 77 ms, repetition time = 9 s, field of view = 18 cm, matrix = 128 × 128, slice thickness = 3 mm. The diffusion encoding scheme included 21 non-collinear gradient encoding directions with b = 1000 and four interleaved b = 0 images.

DTI data demonstrating motion artefacts ($n = 78$) or from infants with cystic lesions ($n = 4$) apparent on structural MRI were excluded from further analysis, resulting in a final group size of 58 infants, of whom 24 were treated with rhEpo and 34 were treated with placebo.

Data analysis

Fractional anisotropy maps were calculated with dtifit and corrected for eddy current distortions with eddy_correct, part of the FSL software library (http://www.fmrib.ox.ac.uk/fsl/) (Smith *et al.*, 2004). The skull was removed from the b0 images using the Brain Extraction Tool (BET) (Smith, 2002) and the corresponding brain mask was applied to the fractional anisotropy maps. The fractional anisotropy maps from all infants were then aligned to the most representative fractional anisotropy image from the cohort, using an automated method implemented in the TBSS pipeline, which applies a non-linear registration of each fractional anisotropy map to all other fractional anisotropy images and then selects the fractional anisotropy image with the lowest mean warp displacement score. An age-appropriate diffusion MRI template was then generated from the average of the normalized fractional anisotropy maps, aligned to the target image corresponding to the most representative subject. The non-linear registrations were performed using FNIRT (Andersson *et al.*, 2007*a*, *b*), which uses a b-spline representation of the registration warp field (Rueckert *et al.*, 1999).

Voxel-wise statistical analysis of the fractional anisotropy data was carried out using TBSS (Smith *et al.*, 2006). After alignment of the fractional anisotropy maps to the neonatal template, a mean image was created and thinned to create a mean fractional anisotropy skeleton, which represents the centres of all tracts common to the group. This skeleton was thresholded at a fractional anisotropy level of >0.15, and voxel-wise cross-subject statistics were used with Randomise (v2.1) to test for differences in fractional anisotropy between infants treated with rhEpo and placebo using a general linear model, including the gestational age at birth and the corrected gestational age at the time of the scan as covariates. A statistical threshold of $P < 0.05$ was applied after family-wise error (FWE) correction for multiple comparisons following threshold-free cluster enhancement (Smith and Nichols, 2009).

Student's *t*-test or the Mann-Whitney test as appropriate for continuous parameters, and chi-square test or Fisher exact test as appropriate for categorical parameters were used to assess differences between clinical and demographic parameters in the erythropoietin and the placebo group. The Pearson coefficient was used to quantify the linear correlation between continuous variables. A multiple linear regression model was fitted to fractional anisotropy, with erythropoietin/placebo as the determinant of interest and adjusted for gender and corrected gestational age. All analyses were performed with the statistical software R for windows (R Core Team, 2013).

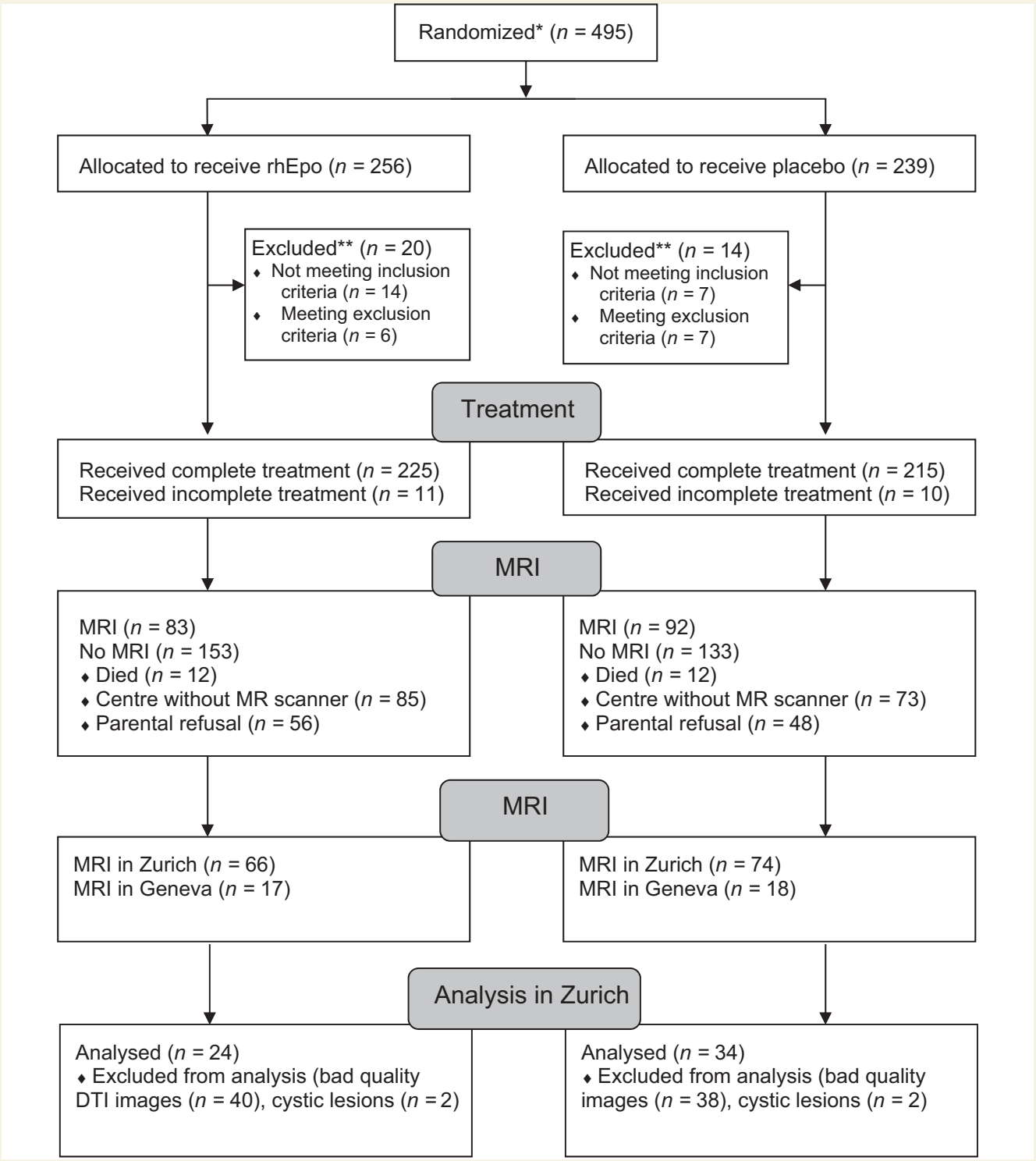

Figure 1 Flow chart of included infants. *Information about screening prior to randomization is not available; **Following randomization, which occurred before 3 h of life, exclusion criteria or the non-compliance with inclusion criteria (due to errors in the reporting of gestational age) were discovered in some infants, and thus they were excluded after randomization. Inclusion criteria: gestational age at birth between 26 and 31 6/7 gestational weeks. Exclusion criteria: presence of a genetically defined syndrome, severe congenital malformation adversely affecting life expectancy, or known to affect neurodevelopment.

Table 1 Comparison of infants with and without TBSS analysis

Variable	Modality	No MRI $n = 437$	TBSS $n = 58$	*P*-value
Gender (female)	*n* (%)	192 (44)	22 (38)	0.235
Gestational age (weeks)	Mean ± SD	28.94 ± 1.8	29.7 ± 1.46	<0.001
Birth weight (g)	Mean ± SD	1176 ± 353	1252 ± 325	0.100
Z-score of birth weight	Mean ± SD	−0.06 ± 0.84	−0.22 ± 0.85	0.185
Broncho-pulmonary dysplasia	*n* (%)	53 (11%)	7 (19%)	0.590
Necrotizing enterocolitis	*n* (%)	10 (3%)	6 (3%)	1
Sepsis	*n* (%)	36 (13%)	24 (14%)	0.837
Meningitis	*n* (%)	2 (1%)	1 (1%)	1
Retinopathy of prematurity	*n* (%)	27 (11%)	13 (8%)	0.383

Results

Patients

The TBSS cohort ($n = 58$) did not differ from the remaining infants in the main study ($n = 437$) with regard to birth weight, chronic lung disease, sepsis, meningitis, necrotizing enterocolitis, or retinopathy of prematurity (Table 1). Infants included in the TBSS study had older mean (SD) gestational age at birth [$n = 58$, 29.29 (1.48) weeks] than the infants in the main study [$n = 437$, 28.74 (1.8) weeks]. Although this difference was only 3 days it reached statistical significance.

Table 2 summarizes the demographic and clinical details of the two groups. No significant differences were found in corrected gestational age at scanning or in any other neonatal or clinical details between the two treatment groups after randomization.

Magnetic resonance

On the basis of the recently published scoring system (Leuchter *et al.*, 2014), 33 (56.8%) infants had normal white matter and 42.2% had abnormal white matter. Three infants had punctate white matter lesions in the cerebellar hemispheres.

Tract-based spatial statisitics

These findings are provided corrected for gestational age at birth and corrected gestational age at scanning. Fractional anisotropy correlated strongly with corrected age at scanning [Pearson correlation coefficient r = 0.74 ($P < 0.001$), Fig. 2]. Preterm infants treated with rhEpo demonstrated increased fractional anisotropy in the genu and splenium of the corpus callosum, the external capsule, the corona radiata and centrum semiovale, the anterior and posterior limbs of the internal capsule, and the corticospinal tract bilaterally ($P < 0.01$, corrected; Fig. 3). Fractional anisotropy was significantly higher in preterm infants treated with rhEpo than in those treated with placebo (Fig. 4). There were no voxels where fractional anisotropy was significantly higher in preterm infants treated with placebo. The multiple linear regression model resulted in an adjusted effect of erythropoietin on fractional anisotropy of 0.02 ($P < 0.001$, Table 3). The amount of variability in fractional anisotropy explained by the model was 70%. No significant differences were found in radial or axial diffusivity between the two groups.

Gender effect

Gender effects were analysed using a multiple linear regression model with fractional anisotropy as dependent variable, gender and intervention (rhEpo or placebo) as independent factors. No significant differences were found in mean fractional anisotropy between male and female infants ($P = 0.46$). Furthermore no interaction between intervention and gender could be shown ($P = 0.11$).

Clinical co-morbidities

Chronic lung disease ($n = 9$) correlated with mean fractional anisotropy; however, after adjusting for gestational age at birth this correlation became insignificant. Sepsis ($n = 8$) and necrotizing enterocolitis ($n = 1$) showed no significant correlation with mean fractional anisotropy.

Discussion

This is the first clinical imaging study showing that early erythropoietin administration can improve white matter development in preterm infants. These results are consistent with experimental imaging data from an immature animal model showing higher fractional anisotropy values in the corpus callosum in erythropoietin-treated animals compared to those treated with placebo (Chatagner *et al.*, 2010). The results are also consistent with the conventional MRI findings showing a reduced risk of white matter and grey matter injury, white matter signal abnormalities, and periventricular white matter volume loss (Leuchter *et al.*, 2014).

To date, many experimental studies investigating erythropoietin as a neuroprotective strategy in adult and neonatal models of brain injury have been published, showing significant promising neuroprotective effects (van der Kooij *et al.*, 2008). One possible mechanism by which erythropoietin may protect against brain injury is by protecting the vulnerable pre-oligodendrocytes. Accumulating evidence from

Table 2 Demographic and clinical parameters of the infants

Characteristic	Reported results	rhEpo group ($n = 24$)	Placebo group ($n = 34$)	*P*-value
Gestational age (weeks)	Mean ± SD	30.17 ± 1.44	29.5 ± 1.44	0.095[b]
Birth weight (g)	Mean ± SD	1337 ± 332	1192 ± 10	0.098[b]
Z-score of birth weight (SD)	Mean ± SD	−0.27 ± 0.71	−0.19 ± 0.95	0·72[b]
Gestational age at MRI (weeks)	Mean ± SD	40.93 (2.09)	41.33 (2.12)	0.49[b]
Gender (= female)	*n* (%)	8 (33)	14 (41)	0.61[a]
Retinopathy of prematurity	*n* (%)	3 (13)	4 (12)	0.77[a]
Sepsis[d]	*n* (%)	3 (13)	5 (15)	0.88[a]
NEC	*n* (%)	0 (0)	1 (3)	0.58[a]
Mechanical ventilation	*n* (%)	8 (33)	13 (38)	0.46[a]
Duration of mechanical ventilation (days)	Median (range)	0 (0–6)	0 (2–12)	0.73[c]
Bronchopulmonary disease[e]	*n* (%)	2 (8)	5 (14)	0.38[a]
Postnatal steroids	*n* (%)	2 (8)	0 (0%)	0.17[a]

Differences of clinical characteristics between groups were evaluated with the chi-square test or the Fisher exact test as appropriate for the categorical variables, and with Student's *t*-test or Mann-Whitney test as appropriate for the continuous variables. SD = standard deviation; IQR = interquartile range (25–75th percentiles); NEC = necrotizing enterocolitis.
[a]Chi-square test or Fisher exact test as appropriate.
[b]Student test.
[c]Mann-Whitney test.
[d] Sepsis proven by positive blood cultures.
[e]Oxygen requirement at corrected age of 36 weeks.

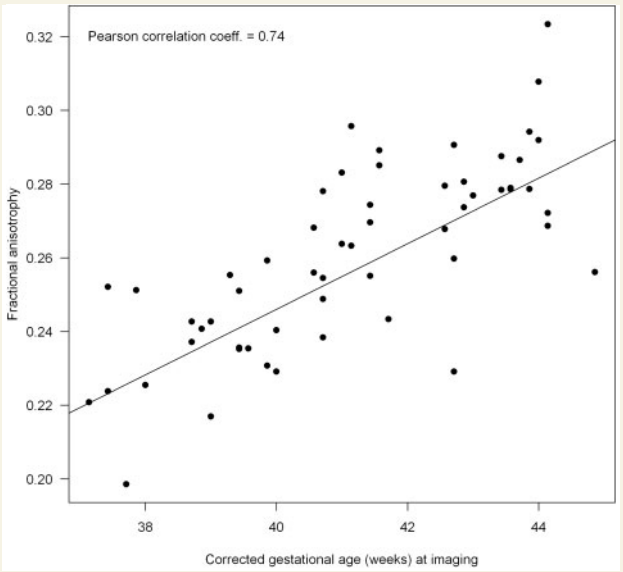

Figure 2 Scatterplot showing the strong positive correlation between fractional anisotropy and corrected gestational age at imaging.

both human (Haynes *et al.*, 2003; Back *et al.*, 2005; Robinson *et al.*, 2006) and experimental models (Back *et al.*, 2002, 2005; Volpe, 2008) suggests that pre-oligodendrocytes are the main cellular target for the periventricular white matter injury common to preterm infants. Given the putative link between periventricular white matter injury and subsequent neurodevelopmental impairment, the pre-oligodendrocytes represent a promising target for neuroprotective therapies.

In a rat periventricular leukomalacia model, erythropoietin has been shown to protect late oligodendrocyte progenitors from hypoxic-ischaemic (HI) injury (Mizuno *et al.*, 2008). Erythropoietin also protects oligodendrocytes from interferon-gamma and lipopolysaccharide toxicity (Genc *et al.*, 2006), improves white matter development *in vivo*, and promotes oligodendrocyte maturation and differentiation in culture (Sugawa *et al.*, 2002). Treatment with erythropoietin also reduced both the incidence and severity of brain injury in a neonatal mouse model of moderate to severe oxidative brain injury (Juul *et al.*, 2008*a, b*). Administration of erythropoietin upregulates erythropoietin receptors and reduces *N*-methyl D-aspartate receptor-mediated excitotoxic injury (Keller *et al.*, 2006) and promotes oligodendrogenesis, leading to attenuated white matter injury with increased neurogenesis after hypoxic-ischaemic injury (Iwai *et al.*, 2010)

Recently, in the neonatal stroke model it was demonstrated that multiple erythropoietin treatments resulted in a shift in subventricular neural stem cells, favouring the production of neurons and oligodendrocytes in injured tissue. Less astrogliosis was observed at both early and late time points, suggesting increased progenitor proliferation, decreased precursor cell death or a change in cell fate choice (Gonzalez *et al.*, 2013).

To assess the effect of rhEpo on white matter development we investigated the fractional anisotropy from diffusion tensor MRI using TBSS. Fractional anisotropy is a scalar value independent of the local fibre orientation, which serves as a marker for white matter tract integrity, thereby providing a relatively objective and straightforward measure for comparison across subject groups (Smith *et al.*, 2006). In this study, fractional anisotropy was calculated from an estimate of the diffusion tensor at each voxel, and compared between erythropoietin and placebo groups using an automated TBSS analysis, as TBSS provides an objective and reproducible voxel-wise analysis of the global white matter integrity. Using this approach, we were able to show that preterm infants treated with

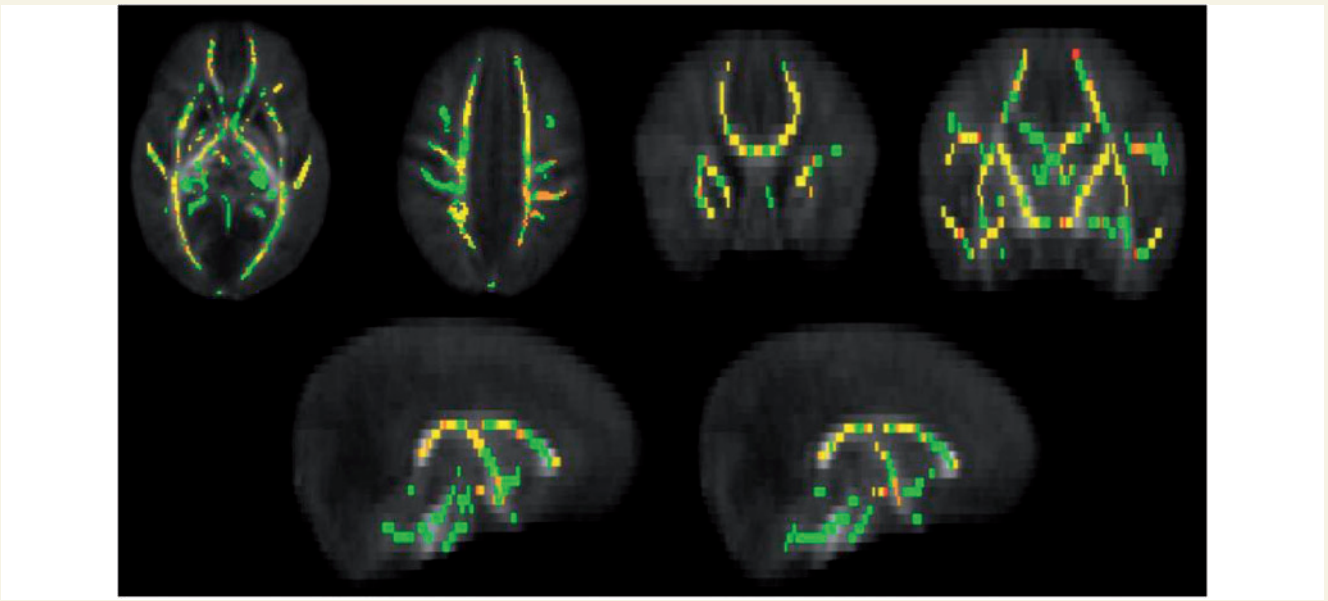

Figure 3 Mean fractional anisotropy skeleton (green) overlaid on the mean fractional anisotropy map in the axial and coronal planes. Regions of the fractional anisotropy skeleton in green represent voxels where there was no difference in fractional anisotropy between infants treated with erythropoietin and placebo. Voxels demonstrating significantly higher fractional anisotropy in the erythropoietin-treated group are overlaid in red-yellow.

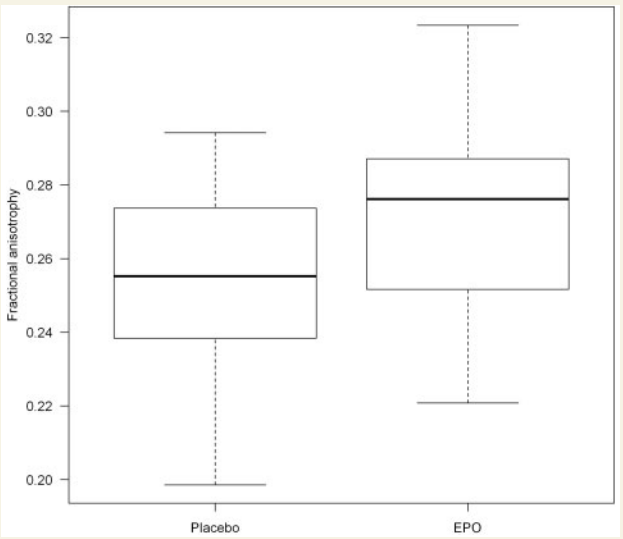

Figure 4 Effect of erythropoietin treatment. Boxplot showing the differences in fractional anisotropy (median and interquartile range) between preterm infants treated with erythropoietin (EPO) and treated with placebo.

Table 3 Results of the linear regression model for fractional anisotrophy

	Coefficient	Standard error	*P*-value
Intercept	−0.132	0.037	0.001
Epo (versus placebo)	**0.019**	**0.004**	**<0.001**
Gender Male	−0.001	0.004	0.793
Corrected gestational age	0.009	0.001	<0.001

erythropoietin have higher fractional anisotropy in most major white matter tracts compared to those preterm infants treated with placebo. As fractional anisotropy is largely dependent on axonal thickness, including premyelination oligodendrocyte wrapping and myelination or axonal attenuation (Sakuma *et al.*, 1991), our results suggest that infants treated with rhEpo have more coherently bundled, more mature fibres along the axis of greatest diffusion, likely reflecting the protective effects of rhEpo on reducing white matter injury and on the promotion of proliferation, maturation and differentiation of pre-oligodendrocytes. These results confirm the conventional MRI findings, with rhEpo treated infants having less white and grey matter injury, fewer white matter signal abnormalities and periventricular white matter loss [reported elsewhere by Leuchter *et al.* (2014) on the entire multicentre cohort].

Cumulative erythropoietin

Cumulative erythropoietin doses have been shown to provide superior neuroprotection when compared to single dose administration following brain injury in a rat model (Kellert *et al.*, 2007; Gonzalez *et al.*, 2009). This improved neuroprotection is likely to arise from multiple effects of erythropoietin during the evolving injury response. Specifically, erythropoietin decreases the early inflammatory response, decreases both early and late neuronal apoptosis

(Juul *et al.*, 2008*a, b*), and stimulates late repair processes such as neurogenesis, angiogenesis and migration of regenerating neurons (Tsai *et al.*, 2006). As the infants in this study were given rhEpo within 2 days of birth and MRI was performed after an interval of several weeks, our data suggest that three early rhEpo doses also improve neuroprotection for preterm neonates, providing a similar long-term improvement to that seen in the neonatal stroke model (Gonzalez *et al.*, 2013). The neuroprotective effect of erythropoietin may be further enhanced by giving rhEpo for a longer period than just within the first 42 h.

Gender effect

Male preterm infants are known to be at higher risk for abnormal neurological outcome than female preterm infants. A recent study has shown that male infants have higher rates for disability and cognitive delay even after adjustment for gestational age at birth and birth weight (Peacock *et al.*, 2012). This observation is in agreement with data from published studies showing constitutional differences between genders, which are not explained by perinatal, neonatal or postnatal factors (Brothwood *et al.*, 1986; Stevenson *et al.*, 2000; Hintz *et al.*, 2006). Therefore, we performed a linear regression to evaluate the interaction between erythropoietin and gender with respect to the mean fractional anisotropy. There was no significant gender effect on mean fractional anisotropy in the measured white matter tracts ($P = 0.46$), and no significant interaction between gender and intervention (rhEpo versus placebo) ($P = 0.23$), indicating that in our sample, gender does not significantly influence the effects of erythropoietin treatment on mean fractional anisotropy. However, the lack of a statistically significant gender effect may also be due to the small numbers in each subgroup (8 and 14 female infants treated with rhEpo and placebo, respectively, 16 and 20 male infants treated with rhEpo and placebo, respectively).

A recent study showed that gestational age in itself was not a strong predictor for adverse brain development as measured by DTI, and that co-morbidities seem to play a larger role in brain development than the degree of prematurity (Bonifacio *et al.*, 2010). In our cohort there was no correlation between mean fractional anisotropy and clinical co-morbidities such as sepsis, necrotizing enterocolitis and chronic lung disease, but this might be due to the small number of infants in our cohort suffering from chronic lung disease (15.5% versus 34.6%; Bonifacio *et al.*, 2010), necrotizing enterocolitis or sepsis (Table 1).

Limitations

One limitation of this study is that the TBSS analysis could only be performed in a subgroup of infants. As the infants were scanned under natural sleep, some DTI studies were incomplete or corrupted by motion artefacts, which made the data unusable. However, we believe that this subgroup is representative of the whole study group, as clinical co-morbidities did not differ between the TBSS and the main cohort. Another limitation is that the TBSS cohort was significantly older than the infants for whom no imaging was performed. However, as the results did not alter after applying a family-wise error (FWE) correction for multiple comparisons following threshold-free cluster enhancement with gestational age at birth as covariate, it seems unlikely that the results are driven by differences in gestational age. A strength of the study is that the magnetic resonance scans of the TBSS cohort were acquired in a single centre; hence, additional variance from between-scanner effects was eliminated.

At present, the neurodevelopmental outcome of this cohort is insufficient to be able to determine the precise relationship between mean fractional anisotropy and motor, cognitive and language outcome. However, as many studies show good correlation between fractional anisotropy and neurodevelopmental outcome (Arzoumanian *et al.*, 2003; van Kooij *et al.*, 2012) in preterm infants, we speculate that preterm infants treated with rhEpo will show improved outcome.

The study used a very short dosing regimen (up to 42 h post-birth) in accordance with the experimental data available at the time of the study design, and therefore did not take into consideration recent experimental data on extended chronic erythropoietin treatment (van de Looij *et al.*, 2014).

Conclusion

Early rhEpo administration improves white matter development in preterm infants assessed by DTI and TBSS. This promising result is consistent with similar results in an immature animal model of neuroprotection through erythropoietin treatment. Neurodevelopmental follow-up at 2 and 5 years are ongoing to further define the neuroprotective effect of erythropoietin.

Funding

None of the authors had any conflicts of interests including any financial interests, activities, relationships, and affiliations. The study was supported by a grant received from the Swiss National Foundation (SNF 3200B0-108176). C.H. was supported by a personal grant ‘For Women in Science’ by the Swiss Academics for Arts and Science, Unesco and L’Oréal. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. All authors had full access to all the data in the study and responsibility for the decision to submit for publication.
ClinicalTrials.gov Identifier: NCT00413946.

References

Alexandrou G, Martensson G, Skiold B, Blennow M, Aden U, Vollmer B. White matter microstructure is influenced by extremely

preterm birth and neonatal respiratory factors. Acta Paediatr 2014; 103: 48–56.
Andersson JL, Jenkinson M, Smith M. Non-linear registration, aka Spatial normalisation FMRIB technical report TR07JA2, 2007a. www.fmrib.ox.ac.uk/analysis/techrep.
Andersson JL, Jenkinson M, Smith M. Non-linear optimisation. FMRIB technical report TR07JA1, 2007b. www.fmrib.ox.ac.uk/analysis/techrep.
Anjari M, Srinivasan L, Allsop JM, Hajnal JV, Rutherford MA, Edwards AD, et al. Diffusion tensor imaging with tract-based spatial statistics reveals local white matter abnormalities in preterm infants. Neuroimage 2007; 35: 1021–7.
Arzoumanian Y, Mirmiran M, Barnes PD, Woolley K, Ariagno RL, Moseley ME, et al. Diffusion tensor brain imaging findings at term-equivalent age may predict neurologic abnormalities in low birth weight preterm infants. AJNR Am J Neuroradiol 2003; 24: 1646–53.
Back SA, Han BH, Luo NL, Chricton CA, Xanthoudakis S, Tam J, et al. Selective vulnerability of late oligodendrocyte progenitors to hypoxia-ischemia. J Neurosci 2002; 22: 455–63.
Back SA, Luo NL, Mallinson RA, O'Malley JP, Wallen LD, Frei B, et al. Selective vulnerability of preterm white matter to oxidative damage defined by F2-isoprostanes. Ann Neurol 2005; 58: 108–20.
Ball G, Boardman JP, Arichi T, Merchant N, Rueckert D, Edwards AD, et al. Testing the sensitivity of Tract-Based Spatial Statistics to simulated treatment effects in preterm neonates. PLoS One 2013; 8: e67706.
Ball G, Counsell SJ, Anjari M, Merchant N, Arichi T, Doria V, et al. An optimised tract-based spatial statistics protocol for neonates: applications to prematurity and chronic lung disease. Neuroimage 2010; 53: 94–102.
Bassi L, Chew A, Merchant N, Ball G, Ramenghi L, Boardman J, et al. Diffusion tensor imaging in preterm infants with punctate white matter lesions. Pediatr Res 2011; 69: 561–6.
Berman JI, Mukherjee P, Partridge SC, Miller SP, Ferriero DM, Barkovich AJ, et al. Quantitative diffusion tensor MRI fiber tractography of sensorimotor white matter development in premature infants. Neuroimage 2005; 27: 862–71.
Bonifacio SL, Glass HC, Chau V, Berman JI, Xu D, Brant R, et al. Extreme premature birth is not associated with impaired development of brain microstructure. J Pediatr 2010; 157: 726–32 e1.
Brothwood M, Wolke D, Gamsu H, Benson J, Cooper D. Prognosis of the very low birthweight baby in relation to gender. Arch Dis Child 1986; 61: 559–64.
Chatagner A, Van der Looij Y, Huppi P, Gruetter R, Sizonenko S. Neuroprotective effects of chronic erythropoietin treatment on neonatal rat following hypoxia-ischemia. Pediatric Academic Societies, Society for Pediatric Research 2011: Baltimore; 2010.
Cheong JL, Thompson DK, Wang HX, Hunt RW, Anderson PJ, Inder TE, et al. Abnormal white matter signal on MR imaging is related to abnormal tissue microstructure. AJNR Am J Neuroradiol 2009; 30: 623–8.
Counsell SJ, Allsop JM, Harrison MC, Larkman DJ, Kennea NL, Kapellou O, et al. Diffusion-weighted imaging of the brain in preterm infants with focal and diffuse white matter abnormality. Pediatrics 2003; 112 (1 Pt 1): 1–7.
Counsell SJ, Shen Y, Boardman JP, Larkman DJ, Kapellou O, Ward P, et al. Axial and radial diffusivity in preterm infants who have diffuse white matter changes on magnetic resonance imaging at term-equivalent age. Pediatrics 2006; 117: 376–86.
Dame C, Juul SE, Christensen RD. The biology of erythropoietin in the central nervous system and its neurotrophic and neuroprotective potential. Biol Neonate 2001; 79: 228–35.
Dudink J, Lequin M, van Pul C, Buijs J, Conneman N, van Goudoever J, et al. Fractional anisotropy in white matter tracts of very-low-birth-weight infants. Pediatr Radiol 2007; 37: 1216–23.
Fauchere JC, Dame C, Vonthein R, Koller B, Arri S, Wolf M, et al. An approach to using recombinant erythropoietin for neuroprotection in very preterm infants. Pediatrics 2008; 122: 375–82.
Fauchere JC, Koller B, Tschopp A, Bucher HU, Group SENT. Safety of high-dose erythopoietin for neuroprotection in preterm infants archives of disease in childhood. fetal and neonatal edition 2012; 97 (Suppl 2): A16.
Genc K, Genc S, Baskin H, Semin I. Erythropoietin decreases cytotoxicity and nitric oxide formation induced by inflammatory stimuli in rat oligodendrocytes. Physiol Res 2006; 55: 33–8.
Ghezzi P, Bernaudin M, Bianchi R, Blomgren K, Brines M, Campana W, et al. Erythropoietin: not just about erythropoiesis. Lancet 2010; 375: 2142.
Gonzalez FF, Abel R, Almli CR, Mu D, Wendland M, Ferriero DM. Erythropoietin sustains cognitive function and brain volume after neonatal stroke. Dev Neurosci 2009; 31: 403–11.
Gonzalez FF, Ferriero DM. Neuroprotection in the newborn infant. Clin Perinatol 2009; 36: 859–80, vii.
Gonzalez FF, Larpthaveesarp A, McQuillen P, Derugin N, Wendland M, Spadafora R, et al. Erythropoietin increases neurogenesis and oligodendrogliosis of subventricular zone precursor cells after neonatal stroke. Stroke 2013; 44: 753–8.
Haynes RL, Folkerth RD, Keefe RJ, Sung I, Swzeda LI, Rosenberg PA, et al. Nitrosative and oxidative injury to premyelinating oligodendrocytes in periventricular leukomalacia. J Neuropathol Exp Neurol 2003; 62: 441–50.
Hintz SR, Kendrick DE, Vohr BR, Kenneth Poole W, Higgins RD. Gender differences in neurodevelopmental outcomes among extremely preterm, extremely-low-birthweight infants. Acta Paediatr 2006; 95: 1239–48.
Huppi PS, Dubois J. Diffusion tensor imaging of brain development. Semin Fetal Neonatal Med 2006; 11: 489–97.
Huppi PS, Inder TE. Magnetic resonance techniques in the evaluation of the perinatal brain: recent advances and future directions. Semin Neonatol 2001; 6: 195–210.
Huppi PS, Maier SE, Peled S, Zientara GP, Barnes PD, Jolesz FA, et al. Microstructural development of human newborn cerebral white matter assessed *in vivo* by diffusion tensor magnetic resonance imaging. Pediatr Res 1998; 44: 584–90.
Huppi PS, Murphy B, Maier SE, Zientara GP, Inder TE, Barnes PD, et al. Microstructural brain development after perinatal cerebral white matter injury assessed by diffusion tensor magnetic resonance imaging. Pediatrics 2001; 107: 455–60.
Iwai M, Stetler RA, Xing J, Hu X, Gao Y, Zhang W, et al. Enhanced oligodendrogenesis and recovery of neurological function by erythropoietin after neonatal hypoxic/ischemic brain injury. Stroke 2010; 41: 1032–7.
Juul S. Recombinant erythropoietin as a neuroprotective treatment: *in vitro* and *in vivo* models. Clin Perinatol 2004; 31: 129–42.
Juul S. Neuroprotective role of erythropoietin in neonates. J Matern Fetal Neonatal Med 2012; 25 (Suppl 4): 105–7.
Juul S, Felderhoff-Mueser U. Epo and other hematopoietic factors. Semin Fetal Neonatal Med 2007; 12: 250–8.
Juul SE, McPherson RJ, Bammler TK, Wilkerson J, Beyer RP, Farin FM. Recombinant erythropoietin is neuroprotective in a novel mouse oxidative injury model. Dev Neurosci 2008a; 30: 231–42.
Juul SE, McPherson RJ, Bauer LA, Ledbetter KJ, Gleason CA, Mayock DE. A phase I/II trial of high-dose erythropoietin in extremely low birth weight infants: pharmacokinetics and safety. Pediatrics 2008b; 122: 383–91.
Keller M, Yang J, Griesmaier E, Gorna A, Sarkozy G, Urbanek M, et al. Erythropoietin is neuroprotective against NMDA-receptor-mediated excitotoxic brain injury in newborn mice. Neurobiol Dis 2006; 24: 357–66.
Kellert BA, McPherson RJ, Juul SE. A comparison of high-dose recombinant erythropoietin treatment regimens in brain-injured neonatal rats. Pediatr Res 2007; 61: 451–5.
Latal B. Prediction of neurodevelopmental outcome after preterm birth. Pediatr Neurol 2009; 40: 413–9.

Lepomaki V, Matomaki J, Lapinleimu H, Lehtonen L, Haataja L, Komu M, et al. Effect of antenatal growth on brain white matter maturation in preterm infants at term using tract-based spatial statistics. Pediatr Radiol 2013; 43: 80–5.

Leuchter RH, Gui L, Poncet A, Hagmann C, Lodygensky GA, Martin E, et al. Association between early administration of high-dose erythropoietin in preterm infants and brain MRI abnormality at term-equivalent age. JAMA 2014; 312: 817–24.

Miller SP, Vigneron DB, Henry RG, Bohland MA, Ceppi-Cozzio C, Hoffman C, et al. Serial quantitative diffusion tensor MRI of the premature brain: development in newborns with and without injury. J Magn Reson Imaging 2002; 16: 621–32.

Mizuno K, Hida H, Masuda T, Nishino H, Togari H. Pretreatment with low doses of erythropoietin ameliorates brain damage in periventricular leukomalacia by targeting late oligodendrocyte progenitors: a rat model. Neonatology 2008; 94: 255–66.

Mukherjee P, Miller JH, Shimony JS, Philip JV, Nehra D, Snyder AZ, et al. Diffusion-tensor MR imaging of gray and white matter development during normal human brain maturation. AJNR Am J Neuroradiol 2002; 23: 1445–56.

Neubauer AP, Voss W, Wachtendorf M, Jungmann T. Erythropoietin improves neurodevelopmental outcome of extremely preterm infants. Ann Neurol 2010; 67: 657–66.

Newton NR, Leonard CH, Piecuch RE, Phibbs RH. Neurodevelopmental outcome of prematurely born children treated with recombinant human erythropoietin in infancy. J Perinatol 1999; 19 (6 Pt 1): 403–6.

Ohls RK, Ehrenkranz RA, Das A, Dusick AM, Yolton K, Romano E, et al. Neurodevelopmental outcome and growth at 18 to 22 months' corrected age in extremely low birth weight infants treated with early erythropoietin and iron. Pediatrics 2004; 114: 1287–91.

Ohlsson A, Aher SM. Early erythropoietin for preventing red blood cell transfusion in preterm and/or low birth weight infants. Cochrane Database Syst Rev 2006; CD004863.

Partridge SC, Mukherjee P, Henry RG, Miller SP, Berman JI, Jin H, et al. Diffusion tensor imaging: serial quantitation of white matter tract maturity in premature newborns. Neuroimage 2004; 22: 1302–14.

Peacock JL, Marston L, Marlow N, Calvert SA, Greenough A. Neonatal and infant outcome in boys and girls born very prematurely. Pediatr Res 2012; 71: 305–10.

Porter EJ, Counsell SJ, Edwards AD, Allsop J, Azzopardi D. Tract-based spatial statistics of magnetic resonance images to assess disease and treatment effects in perinatal asphyxial encephalopathy. Pediatr Res 2010; 68: 205–9.

Robinson S, Li Q, Dechant A, Cohen ML. Neonatal loss of gamma-aminobutyric acid pathway expression after human perinatal brain injury. J Neurosurg 2006; 104 (6 Suppl): 396–408.

Rueckert D, Sonoda LI, Hayes C, Hill DL, Leach MO, Hawkes DJ. Nonrigid registration using free-form deformations: application to breast MR images. IEEE Trans Med Imaging 1999; 18: 712–21.

Saigal S, Doyle LW. An overview of mortality and sequelae of preterm birth from infancy to adulthood. Lancet 2008; 371: 261–9.

Sakuma H, Nomura Y, Takeda K, Tagami T, Nakagawa T, Tamagawa Y, et al. Adult and neonatal human brain: diffusional anisotropy and myelination with diffusion-weighted MR imaging. Radiology 1991; 180: 229–33.

Shingo T, Sorokan ST, Shimazaki T, Weiss S. Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells. J Neurosci 2001; 21: 9733–43.

Skiold B, Horsch S, Hallberg B, Engstrom M, Nagy Z, Mosskin M, et al. White matter changes in extremely preterm infants, a population-based diffusion tensor imaging study. Acta Paediatr 2010; 99: 842–9.

Smith SM. Fast robust automated brain extraction. Hum Brain Mapp 2002; 17: 143–55.

Smith SM, Jenkinson M, Johansen-Berg H, Rueckert D, Nichols TE, Mackay CE, et al. Tract-based spatial statistics: voxelwise analysis of multi-subject diffusion data. Neuroimage 2006; 31: 1487–505.

Smith SM, Jenkinson M, Woolrich MW, Beckmann CF, Behrens TE, Johansen-Berg H, et al. Advances in functional and structural MR image analysis and implementation as FSL. Neuroimage 2004; 23 (Suppl 1): S208–19.

Smith SM, Nichols TE. Threshold-free cluster enhancement: addressing problems of smoothing, threshold dependence and localisation in cluster inference. Neuroimage 2009; 44: 83–98.

Stevenson DK, Verter J, Fanaroff AA, Oh W, Ehrenkranz RA, Shankaran S, et al. Sex differences in outcomes of very low birthweight infants: the newborn male disadvantage. Arch Dis Child Fetal Neonatal Ed 2000; 83: F182–5.

Sugawa M, Sakurai Y, Ishikawa-Ieda Y, Suzuki H, Asou H. Effects of erythropoietin on glial cell development; oligodendrocyte maturation and astrocyte proliferation. Neurosci Res 2002; 44: 391–403.

R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2013. http://www.R-project.org/.

Tsai PT, Ohab JJ, Kertesz N, Groszer M, Matter C, Gao J, et al. A critical role of erythropoietin receptor in neurogenesis and post-stroke recovery. J Neurosci 2006; 26: 1269–74.

van de Looij Y, Chatagner A, Quairiaux C, Gruetter R, Huppi PS, Sizonenko SV. Multi-modal assessment of long-term erythropoietin treatment after neonatal hypoxic-ischemic injury in rat brain. PLoS One 2014; 9: e95643.

van der Kooij MA, Groenendaal F, Kavelaars A, Heijnen CJ, van Bel F. Neuroprotective properties and mechanisms of erythropoietin in in vitro and in vivo experimental models for hypoxia/ischemia. Brain Res Rev 2008; 59: 22–33.

van Kooij BJ, de Vries LS, Ball G, van Haastert IC, Benders MJ, Groenendaal F, et al. Neonatal tract-based spatial statistics findings and outcome in preterm infants. AJNR Am J Neuroradiol 2012; 33: 188–94.

Volpe J. Neurology of the newborn. 5th edn. Philadelphia, PA: Saunders Elsevier; 2008.

Volpe JJ. Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. Lancet Neurol 2009; 8: 110–24.

Woodward LJ, Anderson PJ, Austin NC, Howard K, Inder TE. Neonatal MRI to predict neurodevelopmental outcomes in preterm infants. N Engl J Med 2006; 355: 685–94.

Wu YW, Bauer LA, Ballard RA, Ferriero DM, Glidden DV, Mayock DE, et al. Erythropoietin for neuroprotection in neonatal encephalopathy: safety and pharmacokinetics. Pediatrics 2012; 130: 683–91.

Appendix 1

The Swiss EPO Neuroprotection Trial Group

The following local investigators and hospitals participated in this study (study sites are listed in alphabetical order): Aarau: Kinderklinik Kantonsspital Aarau (Georg Zeilinger, MD; Sylviane Pasquier, MD); Basel: Universitätskinderklinik UKBB (Christoph Bührer, MD; René Glanzmann, MD; Sven Schulzke, MD); Chur: Abteilung für Neonatologie, Kantons- und Regionalspital (Brigitte Scharrer, MD; Walter Bär, MD); Genève: Unité de Néonatologie, Clinique de Pédiatrie HCUG (Riccardo Pfister, MD); Zürich: Klinik für Neonatologie (Jean-Claude Fauchère, MD); Zürich: Zentrum für MR-Forschung (Ernst Martin, MD; Ianina Scheer, MD; Hadwig Speckbacher, MTRA).