Darbepoetin Trial to Improve Red Cell Mass and Neuroprotection in Preterm Infants

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Lead Study Investigator:	Robin Ohls, University of Utah
Subcommittee Members:	Kurt Schibler, Cincinnati Children's Hospital Medical Center Jean Lowe, University of New Mexico Ed Bell, University of Iowa Seetha Shankaran, Wayne State University Abbot Laptook, Brown University David Carlton, Emory University Mariana Baserga, University of Utah John Flibotte, Children's Hospital of Pennsylvania Abhik Das, RTI Andrew Bremer, NICHD Coordinators: Sandra Beauman and Cathy Grisby
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SECTION 1. ABSTRACT

Study Hypothesis: Preterm infants administered weekly Darbe during the neonatal period will have improved neurocognitive outcome at 22-26 months compared to placebo

Study Design Type: Randomized, masked, placebo-controlled trial

Eligibility Criteria: Preterm infants less than or equal to 24 hours of age born 23-28 6/7 weeks gestation with hematocrit ≤ 60% are eligible for enrollment. Infants with known chromosomal anomalies, known brain or cardiac anomalies, hemorrhagic or hemolytic disease, clinical seizures, congenital thrombotic disease, currently on Epo or Darbe, or systolic blood pressures >100 mm Hg while not on pressor support will be excluded.

Study Intervention/Methods: Enrolled infants will receive weekly Darbe or placebo (sham) dosing. Laboratory tests will be performed on all infants while in the hospital. Transfusions will be administered via protocol. Neurodevelopmental assessment will be performed at 22-26 months.

Primary Outcome: Composite cognitive score on Bayley Scale of Infant Development III.

Secondary Outcomes: Hematocrit, red cell mass, donor exposures, number and volume of transfusions, hospital days, differences in morbidities (ROP requiring intervention, thromboses, hypertension, seizures, ICH, NEC requiring surgery, BPD, culture positive sepsis), moderate and severe neurodevelopmental impairment (NDI), death, NDI or death, and cerebral palsy at 22-26 months. The Data Safety and Monitoring Committee of the NRN, with approval of the Division of Blood Diseases and Resources of NHLBI, will oversee the study. The stopping rules will be set before the study begins.

SECTION 2. CONFLICT OF INTEREST DISCLOSURES

Financial Conflicts of Interest of the Institutions and Investigators: None identified

Plan for Managing Identified FCOIs

SECTION 3. STATEMENT OF PROBLEM

3.1. PRIMARY HYPOTHESIS

Preterm infants administered weekly Darbe starting within 36 hours of birth and continuing to 35 weeks gestational age will have improved neurocognitive outcome at 22-26 months (with deaths assigned the lowest possible score of 54), compared to placebo recipients.

3.2. SECONDARY HYPOTHESES

1. Preterm infants administered weekly Darbe during the neonatal period will have increased red cell mass, decreased transfusions, decreased donor exposures, and decreased volume of transfused red cells compared to placebo infants
2. Preterm infants administered weekly Darbe during the neonatal period will have improved survival without neurodevelopmental impairment compared to placebo infants
3. Preterm infants with peak serum Epo concentrations ≥500mU/mL will have better neurocognitive development than those who have peak serum Epo concentrations <500mU/mL
4. Preterm infants administered weekly Darbe will have a decreased incidence of cerebral palsy compared to placebo infants
5. Preterm infants administered weekly Darbe who receive at least 1 transfusion will have improved cognitive outcome compared to placebo infants who receive at least 1 transfusion

3.3. BACKGROUND AND RATIONALE

Advances in neonatal care have led to significant improvements in the survival of the nearly 60,000 very low birth weight (VLBW) infants born each year in the U.S.[1,2] Improving neurodevelopmental outcomes for these preterm infants continues to be a major goal for neonatal care providers. A subset of these infants sustain a grade 3 or 4 intraventricular hemorrhage (IVH) resulting in an increase in the incidence of developmental delay. Moreover, almost one third of preterm infants with normal head ultrasounds also develop cognitive delay.[3,4] Although a variety of neuroprotective treatment strategies have been evaluated, no specific treatment has been identified to reduce or prevent brain injury in these most vulnerable preterm infants.

A potential neuroprotective therapy involves administering erythropoiesis stimulating agents (ESAs) such as erythropoietin (Epo) and Darbepoetin (Darbe, a longer acting ESA). In addition to stimulating erythropoiesis, ESAs have been shown to be protective in the developing brain in animal models,[5-9]

making it possibly beneficial for very premature infants who are at risk for intraventricular hemorrhage, hypoxic-ischemic injury, and developmental delay. The neuroprotective mechanisms of ESAs include increased neurogenesis,[10] decreased neuronal susceptibility to glutamate toxicity,[11,12] decreased neuronal apoptosis,[13-17] decreased inflammation,[18,19] decreased nitric oxide-mediated injury,[20-22] increased antioxidant response,[23-25] decreased axonal degeneration,[26] and increased protective effects on glia.[27-29]

We evaluated hematologic and neurodevelopmental outcomes at 18 – 22 months corrected age in a multicenter trial of preterm infants randomized to receive Epo, Darbe, or placebo for the first 10 postnatal weeks (Clinical Trials #NCT00334737; IND#100138). During the hospital phase ESA treated infants received fewer transfusions and were exposed to fewer donors[29a]. During the follow up phase ESA treated infants had significantly higher composite cognitive outcomes and lower rates of neurodevelopmental impairment.[29b] Hematocrits were higher in the ESA treated group, and cognitive scores inversely correlated with red cell transfusion volumes. Developmental assessment at 3.5-4 years of age continued to show improved cognitive outcomes in ESA recipients, including higher scores of executive function. We previously published data[30] suggesting a relationship between serum Epo concentrations and cognitive outcomes in ELBW infants. Positive correlations between peak and trough ESA concentrations and full scale IQ remained significant at 4 years. In addition, preliminary data from our laboratory show a neurotropic effect of Darbe that may exceed Epo. Based on preliminary pharmacokinetic and erythrokinetic data on the administration of Darbe to preterm infants, we propose a randomized, masked, controlled study to evaluate effects on red cell mass and developmental outcomes of preterm infants receiving standard hematopoietic doses of Darbe.

Erythropoiesis Stimulating Agents (ESAs)

Darbepoetin alfa

A modified version of Epo, known as darbepoetin alfa (Darbe), was developed by Amgen in 1998.[33, 34] Darbe has a longer serum half-life than Epo, allowing for increased dosage intervals to be used. Studies using Darbe once a week to once every three weeks (as compared to 3 doses per week of Epo) in adult oncology and end stage renal failure patients have demonstrated hemoglobin increases equivalent to that of Epo.[35, 36] Side effects are similar to Epo, and the production of anti-Epo antibodies has not been reported.[37] Some hospital formularies are moving from one ESA, Epo, to the newer long-acting ESA, Darbe. Given its potential for similar efficacy with fewer injections, we considered Darbe to be a beneficial alternative to Epo to prevent anemia in preterm infants. Following our *in vitro* evaluation of fetal and neonatal erythroid progenitor responsiveness to Darbe[36, 38], we evaluated erythrokinetics and pharmacokinetics of Darbe administered to preterm infants.[39-42] Compared with adults, neonates had more rapid clearance, suggesting that dosing in neonates would require a higher unit dose/kg and a shorter dosing interval than is generally used for adult patients. Preterm infants showed a dose-response relationship to Darbe that closely mimicked erythropoietic doses of Epo in preterm infants.[41,42]

ESAs as Neuroprotectants

The initial enthusiasm for Epo came from its effect on erythropoiesis in premature infants. Recently, in vitro, animal, and adult clinical studies suggest that Epo plays a potentially important role in angiogenesis and neurogenesis in the central nervous system.[43, 44] It is now known that Epo and Epo receptors are present in non-hematopoietic developing neural tissue in animals as well as humans.[45-47] Similar to the mechanism leading to erythropoiesis, Epo binds to its receptor in non-hematopoietic tissues, activating cellular mechanisms that include cell maturation, division, and importantly, inhibition of apoptosis.[48, 49] Studies evaluating Epo in adult and neonatal animal models report the prevention of hypoxic-ischemic brain injury, a decrease in infarction volume, reduced vasoconstriction, decreased neuronal apoptosis, and decreased neurologic deterioration in animals treated with high doses of Epo.[14,

[48-53] The mechanisms by which Epo is neuroprotective are under active investigation. It has been shown that neuroprotection in part involves decreased expression of pro-inflammatory and anti-apoptotic genes of injured brain.[54] Epo has protective effects both in the central nervous system and the peripheral nervous system.[55, 56] These broad effects suggest that ***Epo may have multiple mechanisms of protection, some that affect neurons directly, and others that are likely secondary to the effects of Epo on other cells or systems.*** To date, Epo has been shown to increase neurogenesis,[10] decrease neuronal susceptibility to glutamate toxicity,[11,12] decrease neuronal apoptosis,[13-17] decrease inflammation,[18, 19] decrease nitric oxide-mediated injury,[20-22] increase antioxidant response,[23-25] prevent axonal degeneration,[26] and increase protective effects on glia.[27-29] Epo may also provide neuroprotection by regulating blood flow to the brain following injury, as is suggested by Epo neuroprotection in the model of subarachnoid hemorrhage.[57, 58] Jantzie et al showed that Epo signaling promotes critical stages of oligodendroglial lineage development and recovery after prenatal injury, and concluded that Epo treatment may be beneficial to preterm patient populations with developmental brain injury hallmarked by white matter injury.[79] In addition, MRIs performed at 36 weeks corrected age in former preterm infants randomized to 3 high doses of Epo (3000 units/kg IV at 3 hours, 12-18 hours, and 36-42 hours of age) or placebo (Swiss high dose Epo study) revealed decreased white matter and grey matter injury in the Epo recipients,[80] and improved fractional anisotropy.[80a] Developmental outcomes of the infants enrolled in that study remain to be determined.

Human clinical trials of Epo for treatment of adult stroke have been performed. Reduced stroke lesion size and evolutions as well as decreased serum markers of glial damage were seen in adults treated with Epo,[59] however, in a larger RCT[60], increased mortality was noted (16.4% in Epo-treated subject compared to 9.0% in control patients). The FDA placed a hold on high dose Epo studies evaluating neuroprotective effects in 2007 to more closely evaluate initial clinical studies. Exploratory subgroup analysis of the RCT revealed that patients not receiving thrombolysis most likely benefited from EPO during clinical recovery. The glial markers S100B and glial fibrillary acid protein (GFAP) and the neuronal marker ubiquitin C-terminal hydrolase (UCH-L1) were measured by enzyme-linked immunosorbent assay in serum on d 1, 2, 3, 4 and 7 post-stroke. All biomarkers increased post-stroke. Overall, EPO-treated patients had significantly lower concentrations (area under the curve) over 7 d of observation, as reflected by the composite score of all three markers (Cronbach $\alpha = 0.811$) and by UCH-L1. S100B and GFAP showed a similar tendency[78]. The FDA hold on ESA studies for neuroprotection was removed in 2008 and clinical studies were allowed to move forward. No RCTs evaluating Epo in preterm or term neonates have shown a difference in mortality.

Our group was the first to report potential neurodevelopmental improvements in Epo-treated ELBW infants[30] (reviewed in the preliminary studies section). Two other studies have also reported improved outcomes in Epo-treated preterm infants. Brown et al evaluated outcomes of 82 infants <1500 g and ≤ 30 weeks of gestation, evaluated at 2 years after neonatal Epo treatment.[61] Higher MDI scores were associated with higher cumulative doses of Epo, among other factors. Neubauer and colleagues compared 89 Epo-treated with 57 control ELBW neonates at 10-13 years of age.[62] Epo-treated neonates had better outcomes, with 55% of the Epo group assessed as normal *compared to* 39% in the control group ($p<0.05$). IQ scores were also higher in Epo treated patients (90.8 compared to 81.3 in control infants, $p< 0.005$), especially in those infants with grade III-IV IVH.

In addition to hematopoiesis, Epo is also a vascular growth factor. Epo may play a role in the developing human eye, as increased vitreal Epo concentrations have been measured that exceed serum concentrations.[63] Retrospective analyses and meta-analyses have raised concerns about an association between retinopathy of prematurity (ROP) and early Epo administration.[64] However, no randomized controlled trials (RCTs) published in peer-reviewed journals have reported an increased incidence of ROP

in the Epo-treated group, and the most recent meta-analysis of early Epo studies (starting Epo in the first week of life) show no statistical increase in stage 3 or greater ROP.[64] Brown and colleagues performed a retrospective analysis of Epo dose and ROP on the same data base of infants that showed improved MDI, reporting an increase in ROP with increased total dose.[65] In contrast, Neubauer and colleagues[62] reported the incidence of ROP ≥stage III was 11.4% in Epo recipients versus 16.1% in the control group (p=NS). In the Swiss high dose Epo study, there were no differences in any level of ROP between the Epo treated and placebo groups,[65a] nor were there differences in intracranial hemorrhage (any grade) or cystic PVL. The incidence of ROP in Epo-treated infants continues to be closely evaluated.

Darbe as a Potential Neuroprotectant

Recent studies of ESA neuroprotection have evaluated Darbepoetin.[66,67] Both Epo and Darbe cross the blood-brain barrier at a similar rate as albumin, suggesting that they cross the blood-brain barrier by way of extracellular pathways.[66] In an adult animal model of ischemia, Darbe conferred behavioral and histological neuroprotection after focal ischemia.[67] In those animal studies, the darbepoetin doses tested were identical to the doses used in our previous RCT (10 μg/kg/dose), increasing the probability that preterm infants randomized to the Darbe arm are indeed receiving neuroprotective doses. In addition, a study on neuroprotection in term infants with hypoxic ischemic encephalopathy treated with Epo showed promise in decreasing neurodevelopmental morbidity to a similar extent as that seen in recent cooling trials.[68] Epo doses in that term infant study (300-500 units/kg every other day) were comparable to doses used clinically in preterm infants to stimulate erythropoiesis.

Recent clinical studies in term infants with HIE treated with high dose Epo (500-2,500 units/kg/dose) showed equal efficacy at 1,000 and 2,500 units/kg/dose (NEAT Study[69]), and phase III trials are proceeding at 1000 units/kg. Similarly, studies of prophylactic high dose Epo for neuroprotection in ELBW infants reported area under the curve (AUC) values at 1,000 units/kg/dose that were similar to neuroprotective AUC values in animal studies.[70, 71]

The neurodevelopmental consequences of extreme prematurity are enormous. Unfortunately, there are no proven neuroprotective treatments available for these children. Developing and evaluating novel neuroprotective strategies is a critical need in neonatal care with tremendous public health implications. Converging lines of evidence from both animal and human studies demonstrate the potential for ESAs as neuroprotective agents. Mechanisms of neuroprotection may include improved red cell mass, increased oxygen availability to tissues, and decreased adverse impact of transfusions, in addition to the biologic mechanisms of inhibition of apoptosis and increased cell differentiation. Although the safety and beneficial effect of ESAs on development is supported by preclinical and clinical studies, a large RCT evaluating long term neurodevelopmental effects of Darbepoetin in premature infants has not been performed. Our randomized, controlled investigation is powered to answer the question: Do ESAs improve clinically relevant developmental functions? Our primary outcome measures are well standardized and have functional significance. This study differs from previous studies in the following ways:

1. Darbe will be compared to weekly placebo, (those in the placebo group will not receive injections; masking will be maintained by administering drug/placebo behind a curtain and placing a Band-Aid)
2. Dosing will begin at least 1 day earlier on average than in any previously published study – this is potentially important in terms of timing for neuroprotection
3. Correlations will be performed to better understand the relationship between Darbe administration, transfusions, red cell mass, serum Darbe (Epo) concentrations, and cognitive development at 2 years of age.

Preliminary Translational Laboratory Studies

1. Erythropoietin and Darbepoetin Increase Fetal Erythropoiesis in Dose Dependent Fashion: Erythroid progenitor cells were isolated from 12-22 week fetal liver and marrow, and from term (37-41 weeks) and preterm (<32 weeks) cord blood.[74] The number of burst forming units-erythroid (BFU-E) colonies derived from fetal marrow progenitor cells increased significantly with both Darbe ($p<0.01$, 10 vs 50, 100, and 500 ng/mL; Figure 1) and rHuEpo ($p<0.01$, 0.05 vs 0.5, 1.0, and 2 U/mL). Progenitors isolated from fetal liver and from term and preterm cord blood were similarly responsive. Preterm cord blood progenitors were more sensitive than term progenitors to Darbe at every concentration ($p<0.01$).

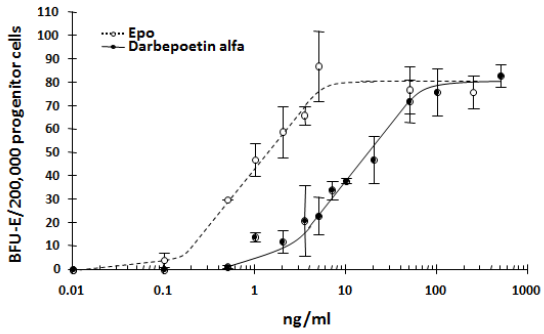


Figure 1. Comparison of Epo versus Darbe in growth of human erythroid progenitors isolated from marrow.

2. Erythropoietin and Darbepoetin Increase Fetal Neurogenesis in Dose Dependent Fashion: We performed *in vitro* studies of Darbe and Epo on human fetal neuronal cell cultures.[73] Cells were incubated with Epo (0-10 units/mL) or Darbe (0-1000 ng/mL) for 7 to 10 days, and cell count and phenotype determined (**Figure 2**, right). Pre-clinical and clinical studies performed by Amgen (the manufacturer of Epo and Darbe) and performed by clinical investigators determined the conversion to achieve equivalent potency is: 1 unit Epo = 100 ng Darbe. Based on this comparison, neuronal cells appeared 10-fold more responsive to Darbe than Epo *in vitro,* suggesting that for neurogenesis, 1 unit Epo would be comparable to 10 ng Darbe (therefore 1,000 units Epo would compare to 10 micrograms Darbe). Both Darbe and Epo increased Epo receptor gene expression (greater with Darbe), and increased expression of the anti-apoptotic gene Bcl. The phenotype of cultured cells did not differ between Darbe and Epo; however nesting gene expression was four-fold greater in Darbe-cultured cells than Epo cultured cells, suggesting increased progenitors.

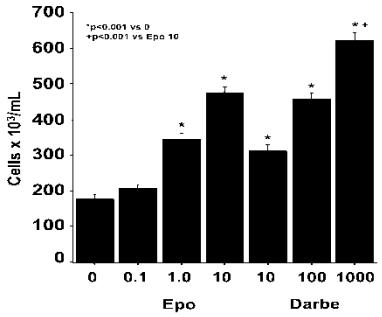


Figure 2. Comparison of Epo versus Darbe in growth of mid-gestation human fetal brain cells.

Preliminary Clinical Studies

1. Short term developmental outcome of ELBW infants treated with early Epo and iron: Working with NRN Centers, we performed a follow-up study of ELBW infants originally enrolled in the NICHD Epo Study[73]. There were no significant differences between treatment groups in weight or length, or in the percentage weighing <10th percentile at the time of discharge or at 18-22 months (corrected age) follow-up (**Table 1**), and no difference was found in the mean head circumference between groups (Table 1, right). However, there was an important difference in the incidence of microcephaly between groups. Over twice as many children developed microcephaly in the placebo group compared to those treated with Epo (32.7% vs 14.3%). To understand this difference, longer term studies that include markers of neuroplasticity will be required.

Table 1. NICHD Follow up Study

Parameter	Epo (n=51)	Placebo (n=51)
weight (kg)	9.8±1.3	10.0±1.5
length (cm)	80.2±3.7	80.8±3.3
HC (cm)	47.0±2.0	46.6±1.7
% Wt < 10%tile*	49.0	50.0
% Ln < 10%tile*	40.8	36.5
% HC < 10%tile*	14.3	32.7

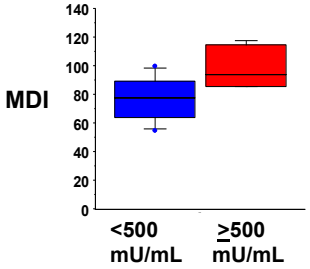
* National Children's Health Study guidelines

2. Developmental outcome correlates with neonatal Epo concentration: we evaluated the relationship between Epo concentrations and neurodevelopmental outcome in the subgroup of infants (those from the University of New Mexico) enrolled in the NICHD Epo study (summarized above).[30] The investigators responsible for neurodevelopmental evaluation were masked to the treatment groups. Serum Epo concentrations were significantly different between groups during the study (**Table 2**). At 18-22 months follow up, Epo recipients (n=7) had BSID II MDI scores of 96±11 and placebo infants (n=8) had MDI scores of 78±7 (p=0.15). There were no differences between groups in anthropometric measurements. Two of six infants in the Epo group and 4 of 6 infants in the placebo group had some form of NDI. Post hoc analysis showed that infants with peak Epo concentrations ≥500 mU/mL (**Figure 3**) had higher MDIs (100±15) than infants with Epo concentrations <500 mU/mL (77±16; p<0.05). This study was the first to demonstrate that elevated serum Epo concentrations to levels studied in animal models of neuroprotection can be achieved using subcutaneous Epo at doses of 400 units/kg 3x/week, which is considered a normal "hematopoietic" dose. Based on this information, we chose Darbe doses that would also achieve Epo concentrations ≥500mU/mL.

Table 2. Epo concentrations and developmental testing

	Epo (n=7)	Placebo (n=8)
Epo conc.	**2,023±652 mU/mL**	**22±4 mU/mL**
MDI scores	**96±11**	**78±7**
PDI scores	**87±13**	**80±7**
Any NDI	**2/6**	**4/6**

Figure 3. MDI scores at 18-22 months follow up. MDI scores were higher in infants with peak Epo concentrations ≥500 mU/mL (right) compared to those with concentrations <500 mU/mL (left; p<0.05).



3. Randomized, masked, dose response study of Darbepoetin in preterm infants: Following single dose pharmacokinetic studies of Darbe administered to VLBW infants,[39, 40] we completed a randomized, masked, dose response study of Darbe in preterm infants ≤1,500 grams birth weight.[74] Sixteen Infants were randomized to one of the following doses: 0, 2.5, 5.0, or 10 μg/kg/dose given once a week SC for 4 doses. We saw a clear dose response relationship in production of reticulocytes (**Figure 4**), with the greatest response occurring in infants receiving 10 μg/kg dose. No adverse effects were reported in any of the infants. The results demonstrated preliminary safety and efficacy, and confirmed the findings of our pharmacokinetic studies, in that higher doses (than are used in adults for erythropoiesis) resulted in a robust biologic response without adverse effects. A Darbe dose of 10 μg/kg SC will generate serum Epo concentrations well above 500 mU/mL, based on single 4 μg/kg SC dose achieving concentrations of 2,000-4,000 mU/mL.[39]

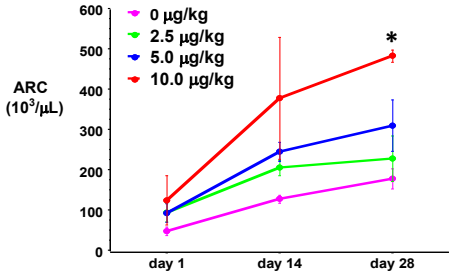


Figure 4. Absolute retic counts (ARC) in preterm infants treated with 0-10 μg/kg doses of Darbe for 4 weeks; *p<0.05 versus 0 and 2.5 μg/kg.

4. Neurocognitive Outcomes at 18-22 Months are Improved in Former Preterm Infants Administered Darbepoetin or Erythropoietin: We evaluated infants at 18-22 months and hypothesized that those

previously randomized to ESAs would have improved neurodevelopmental outcomes compared to placebo.[76, 77] A total of 102 Infants (500-1,250 grams, <48 hours of age) were randomized in masked fashion to Darbe (10 μg/kg, 1x/wk SC), Epo (400 units/kg, 3x/wk SC) or placebo, dosed through 35 weeks postmenstrual age. All infants received supplemental iron, folate, and vitamin E, and were transfused according to a standardized, restrictive transfusion protocol. During the treatment phase, ESA recipients received half the number of transfusions (1.3 versus 2.6) and were exposed to half the donors (0.7 versus 1.4) than infants in the placebo group. The incidence of morbidities, specifically ROP ≥ stage 3, either requiring surgery (2, 1, and 2 infants in Darbe, Epo, and placebo groups, respectively) or regressed (0, 1, and 2 infants in Darbe, Epo, and placebo groups, respectively), was similar in all three groups. Infants were evaluated at 18-22 months corrected age using the Bayley Scales of Infant Development (BSID-III). Object permanence (OP, a measure of executive function and early working memory) was calculated from the BSID-III. Anthropometrics and assessment of cerebral palsy (CP), blindness and deafness were determined. Of the original 102 infants (946±196 grams, 27.7±1.8 weeks gestation) enrolled, 99 were evaluated during the hospital phase, 5 died prior to discharge (1/33 Darbe, 1/33 Epo, 3/33 Placebo) and 14 infants were lost to follow-up (5 Darbe, 3 Epo, 6 placebo). The 80 infants evaluated (29 Epo, 27 Darbe, 24 placebo) were comparable between groups for age at testing, birth weight, gestational age, antenatal steroids, and morbidities associated with prematurity (ICH, BPD, and ROP). Anthropometrics did not differ among groups.

Table 3. Neurodevelopment and neurodevelopmental impairment at 18-22 months

	Darbe	Epo		ESA[a]	Placebo	Unadjusted		Adjusted for Gender, Maternal Education	
	n = 27	*n* = 29	*P**	*n* = 56	*n* = 24	Odds Ratio (95% CI)	*P**	Odds Ratio (95% CI)	*P**
Cognitive score <85	0 (0)	3 (10.3)	0.12	3 (5.4)	6 (25.0)	0.17 (0.04–0.75)	0.02	0.18 (0.04–0.82)	0.03
Cognitive score <80	0 (0)	3 (10.3)	0.12	3 (5.4)	5 (20.8)	0.22 (0.05–0.99)	0.05	0.24 (0.05–1.13)	0.07
Cognitive score <70	0 (0)	1 (3.5)	0.50	1 (1.8)	2 (8.3)	0.20 (0.02–2.32)	0.20	0.24 (0.02–2.93)	0.26
NDI,[b] *N* (%)	3 (11.1)	4 (13.8)	0.88	7 (12.5)	10 (41.7)	0.20 (0.06–0.62)	0.005	0.21 (0.07–0.68)	0.009
CP,[c]	0 (0)	0 (0)	1.00	0 (0)	5 (20.8)	N/A	0.002	N/A	<0.001
Visual deficit	2 (7.4)	0 (0)	0.27	2 (3.6)	1 (4.2)	0.85 (0.07–9.87)	0.91	0.72 (0.06–8.74)	0.80
Hearing deficit	0 (0)	1 (3.5)	0.50	1 (1.8)	1 (4.2)	0.42 (0.03–6.98)	0.54	0.48 (0.03–8.59)	0.62
NDI or death, *N* (%)	4/28 (14.3)	5/30 (16.7)	0.92	9/58 (15.5)	13/27 (48.2)	0.20 (0.07–0.56)	0.002	0.22 (0.07–0.70)	0.01
Moderate NDI,[d] *N* (%)	3 (11.1)	2 (6.9)	0.45	5 (8.9)	9 (37.5)	0.16 (0.05–0.56)	0.004	0.18 (0.05–0.63)	0.008
Moderate NDI or death, *N* (%)	4/28 (14.3)	3/30 (10.0)	0.48	7/58 (12.1)	12/27 (44.4)	0.17 (0.06–0.51)	0.002	0.20 (0.06–0.67)	0.009

* *P* values for comparisons between Darbe and Epo were computed using 2 methods depending on characteristics of the data. For the NDI measures, *P* values were computed using logistic regression with gender and maternal education as covariates. For the remaining measures, *P* values were computed using ANCOVA with only gender as a covariate. Percentages for NDI or death include deaths during initial hospitalization. CI, confidence interval. N/A, not applicable.

[a] Treated groups are combined.

[b] NDI is defined as having either CP, visual deficit, hearing deficit, or a cognitive score <85.

[c] Given the absence of CP cases in the ESA treatment group, odds ratios cannot be accurately estimated. *P* values given for CP were computed using Fisher's exact tests for the unadjusted *P* value and using ANCOVA with gender as the only covariate for the remaining *P* values.

[d] Moderate NDI is defined as having either CP, visual deficit, hearing deficit, or a cognitive score <70.

After adjustment for gender, analysis of covariance resulted in significant differences between groups (**Table3)**: cognitive scores were similar for Darbe (96.2±7.3; mean±SD) and Epo (97.9±14.3) compared to placebo (88.7±13.5; p=0.01 vs ESA recipients; Table 4). OP was higher for the Darbe group compared to the Epo group (p=0.05); both Epo and Darbe groups had higher OP scores than the placebo group (p=0.01). None in the ESA groups had CP, compared with 5 in the placebo group (p=0.002). No differences between groups were noted in blindness or deafness, and there were no infants in the Darbe group with a cognitive score <85. Infants receiving ESAs showed improved cognitive outcomes compared to placebo at 18-22 months. The differences in incidence of CP (22% placebo versus 0% Darbe or Epo) might be explained by potential white matter neuroprotection of ESAs,[80] but this hypothesis remains to be tested.

5. ***Preschool Neurocognitive Outcomes in Preterm Infants Administered ESAs:*** We enrolled children who completed the original RCT into the BRITE Study (Brain Imaging and Developmental Follow up of Infants Treated with Erythropoietin, NCT#01207778), and compared developmental outcomes with children born healthy at term (TC). All participants were assessed using measures of full scale IQ (FSIQ) and general language from the Wechsler Preschool and Primary Scale of Intelligence-III, and an overall measure of executive function, based on tests evaluating inhibitory control and spatial working memory. Rates of neurodevelopmental impairment were compared across groups. Multivariate analysis of variance compared children randomized to ESAs (39), placebo (14), and TC (24). FSIQ and performance IQ (PIQ) were significantly higher in the ESA group compared to the placebo group (**Table 4**). Follow-up analyses revealed that the children receiving ESAs performed better than placebo on executive function tasks. ESA group's performance was below that of TC, but results did not reach significance on executive function. Neurodevelopmental impairment was lower in the ESA than the placebo group (p=0.016).

Table 4. Neurodevelopment in Preschool Children

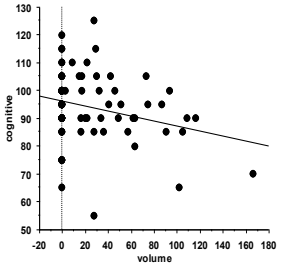
	ESA	Placebo	Term	P_0	P_1
Subjects — no.	**39**	**14**	**24**		
Full-scale IQ	91.49 (18.05)	79.14 (18.53)	102.58 (12.78)	0.034	0.011
Verbal IQ	92.97 (17.00)	79.07 (19.57)	103.92 (10.55)	0.015	0.006
Performance IQ	91.69 (18.30)	82.86 (16.90)	100.83 (15.20)	0.12	0.071
General Language Comprehension	89.82 (16.98)	84.79 (17.11)	101.83 (15.11)	.35	.006
Executive function	99.88 (11.65)	91.52 (13.26)	105.15 (7.79)	0.035	0.083
Working Memory	100.63 (14.58)	91.57 (17.24)	103.92 (12.44)	0.047	0.35
Inhibition	99.13 (15.21)	91.53 (17.61)	106.36 (9.95)	0.11	0.066

Values represent mean standard deviation;
P_0: treated vs placebo; P_1: treated vs term

6. ***Neurocognitive Outcomes in Preterm Infants Administered ESAs in Relation to Transfusion Number and Volume:*** It is unclear if the improved cognitive outcomes seen in the ESA recipients are a result of neuroprotective effects of ESAs, improved oxygenation due to higher hematocrit in ESA recipients, decreased neuronal injury or morbidity due to fewer transfusions, or some combination of the three. We analyzed our transfusion and cognitive outcomes data to identify relationships between transfusions and cognitive outcomes. We hypothesized that cognitive scores would be inversely correlated with the number or volume of red cell transfusions received during the initial hospitalization.

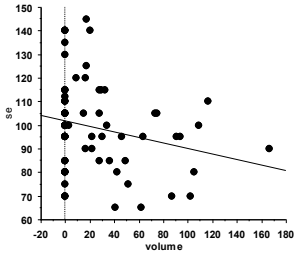
Both composite cognitive scores and social emotional scores were inversely correlated with the volume of red cell transfusions (**Figure 5, A (left) and B (right)**). Moreover, there was a trend towards an inverse correlation between cognitive score and number of transfusions (p=0.08).

Figure 5A. Cognitive score at 2 years vs transfusion volume



R=0.26; p=0.02

Figure 5B. Social Emotional score at 2 years vs transfusion volume



R=0.22; p=0.05

Significant differences were seen in cognitive and language scores between ESA treated and placebo groups when ≥1 transfusions were administered; these differences were not present in infants who did not receive transfusions (**Figure 6, A (left) and B (right) panels**). At 4 years of age, the impact of transfusions was still present but to a lesser extent: differences in full scale IQ between non-transfused ESA and non-transfused placebo trended towards higher scores in the ESA treated group (p=0.07).

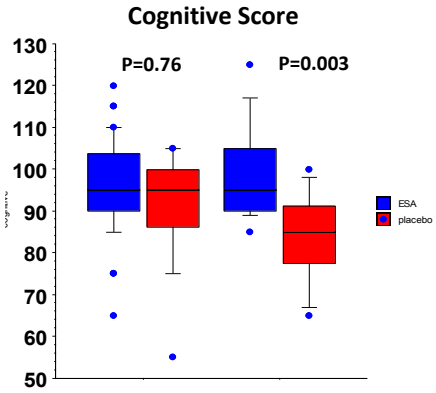


Figure **treated (blue) versus placebo (red), untransfused versus 1 or more transfusions.**

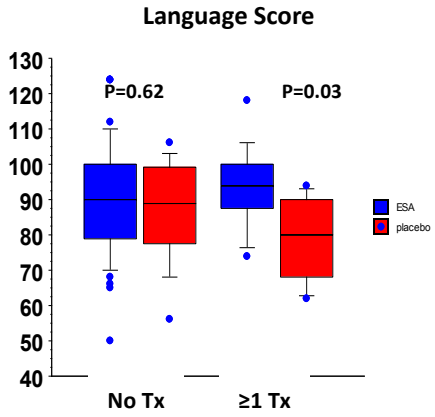
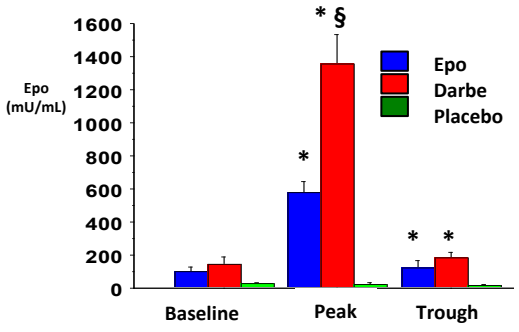


Figure 6B. Language scores in ESA treated (blue) versus placebo (red), untransfused versus 1 or more transfusions.

We speculate that transfusions negatively impact cognitive development, and that ESAs provide neuroprotection from these effects.

7. Epo concentrations correlate with cognitive score at 2 and 4 years: Epo concentrations were measured during the hospital phase. We hypothesized that neurodevelopmental outcomes at 2 and 4 years of former preterm infants previously randomized to ESAs would be positively correlated with peak Epo concentrations. Darbe recipients had the highest peak and trough concentrations (**Figure 8**, **left panel**: ***** p<0.05, ESA vs placebo; **§** p<0.05 Darbe vs Epo). Cognitive scores significantly correlated with **trough** concentrations (**Figure 8**, **right panel**: p<0.05), and there was a trend towards correlation between cognitive scores and **peak** Epo concentrations (p=0.08).



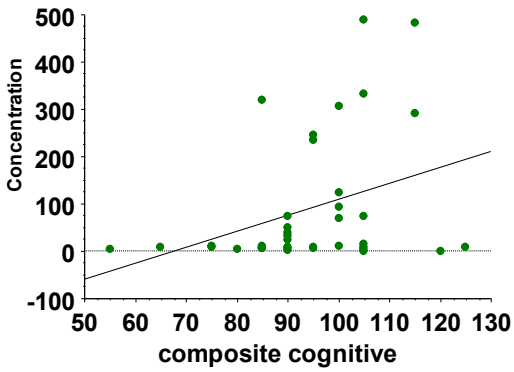
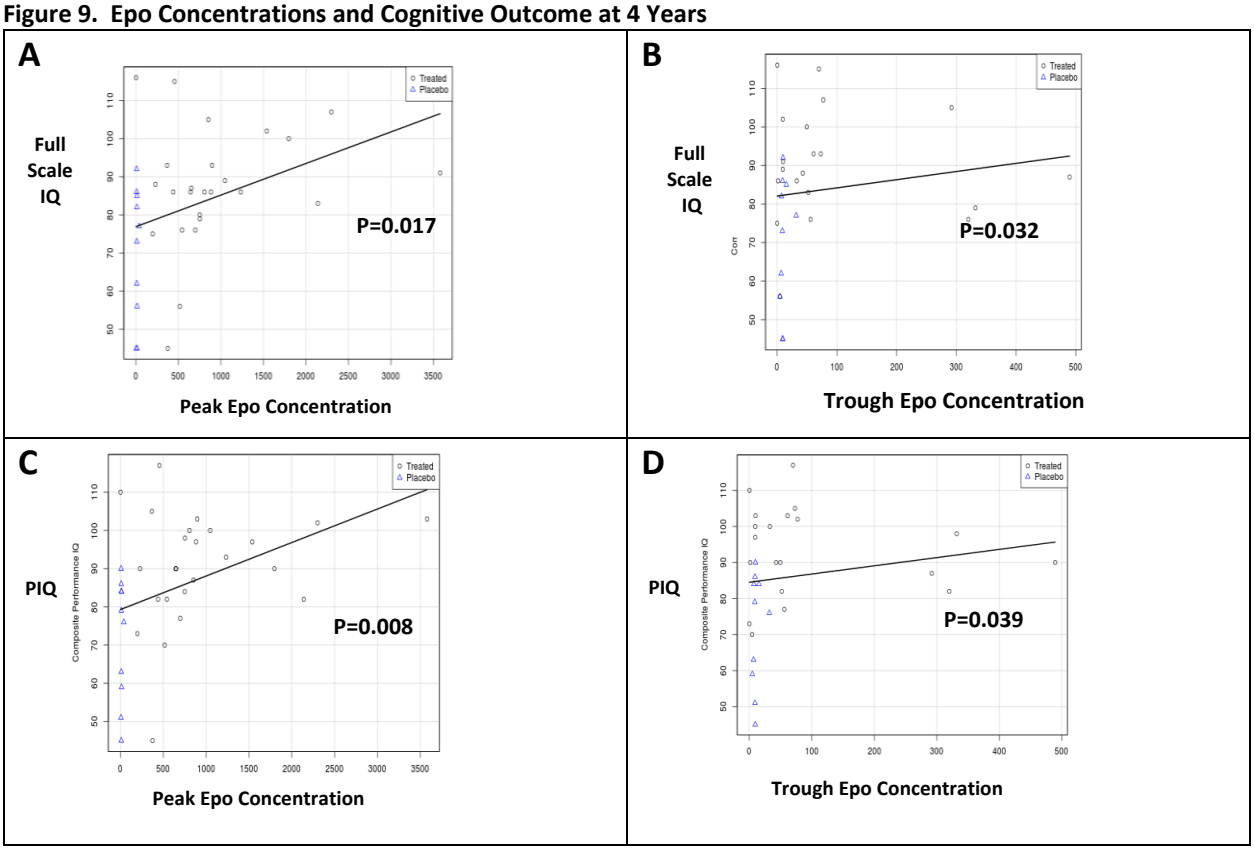


Figure 8. Epo concentrations (left) and relation of Epo trough to cognitive outcome (right) at 2 years

At 4 years of age, full scale IQ (**Figure 9, A and B**) and performance IQ (**Figure 9, C and D**) correlated with peak Epo concentrations (**Figure 9, A and C**), and with trough concentrations (**Figure 9, B and D**).

Figure 9. Epo Concentrations and Cognitive Outcome at 4 Years



Summary and Interpretation of Preliminary Studies

Preliminary studies suggest that ESAs improve short term and preschool neurodevelopmental outcome in premature infants. Our prior work also indicates that Darbe appears safe within established dosing guidelines, and that the doses of Darbe administered should be adequate to achieve serum Epo concentrations ≥500 mU/mL. Before treatment recommendations can be made however, a well designed RCT is needed to evaluate the effect of ESAs on various cognitive domains. Furthermore, understanding the neurologic mechanisms affected by ESAs in former premature infants is necessary to determine how best to modify therapy to achieve optimal neurodevelopmental outcome. The NICHD Neonatal Research Network has the necessary neurodevelopmental testing expertise to investigate potential neurologic effects in former preterm infants.

SECTION 4. METHODS

4.1. STUDY POPULATION

This is a randomized, masked, placebo controlled clinical study in which enrolled infants will receive weekly Darbe or placebo (sham) dosing.

4.1.1. Inclusion Criteria

- 23 0/7-28 6/7 weeks gestation
- ≤24 hours postnatal age

4.1.2. Exclusion Criteria

- Hematocrit > 60%
- Infants with known congenital or chromosomal anomalies, including congenital heart disease and known brain anomalies
- Hemorrhagic or hemolytic disease
- EEG- confirmed seizures
- Congenital thrombotic disease
- Systolic blood pressures >100 mm Hg while not on pressor support
- Receiving Epo or Darbe clinically, or planning to receive Epo or Darbe during hospitalization
- Infants in whom no aggressive therapy is planned
- Family will NOT be available for follow-up at 22-26 months
- Infant unlikely to survive
- Infant enrolled in a conflicting trial

4.2. DETAILED STUDY PROCEDURES

4.2.1. Screening

Pregnant women will be screened for potential delivery between the GA of 23 0/7 – 28 6/7 weeks. In addition, admissions to the NICU (inborn and outborn) with GA 23 0/7 – 28 6/7 weeks will be screened for inclusion/exclusion criteria.

4.2.2. Consent

Parents of infants with GA 23 0/7 – 28 6/7 weeks and who are not known to have any exclusion criteria may be approached for consent before delivery if allowed by the local IRB. This will enable collection of cord blood at delivery. Parents/guardians of infants who meet inclusion criteria and who do not have any exclusion criteria may also be approached for consent after delivery but must be randomized within 24 hours.

Consenting should include informing families that cord blood collected either at delivery or from stored samples, peak and trough samples, and scavenged samples will be sent to the DREAM Lab at UNM to be used for Epo (Darbe measures as Epo) concentrations (and antibody assay if needed). In addition, various lab studies are included as part of the study design. For families that wish to participate in the study but do not want research labs drawn (and for those sites where research labs exceed maximum research blood draw limits [4.0 ml]), only serum ferritin (to adjust iron dosing) and peak Epo concentration will be obtained (1.5 mL total blood volume). Information from clinically-obtained

CBCs/hematology labs will be collected. Serum or plasma remaining after clinical labs are run will be collected and stored (frozen at -80 degrees centigrade) during the study to measure random Epo concentrations, and in the event that antibody assays are run. Deidentified Epo concentration data will be sent to the University of Iowa for pharmacokinetic analyses by Dr. An.

4.2.3. Randomization

After parental consent is obtained, infants will be randomized through the Data Center at RTI in a permutated block design using a web based electronic data capture (EDC) system. Randomized subjects will be stratified by center and within each center by gestation (<26 weeks, 26-28 and 6/7 weeks). Multiple gestation infants will be randomized to the same treatment arm.

4.2.4. Study Intervention and Comparison

Study Drug: Infants will be randomized to one of two groups: Darbepoetin 10 μg/kg/once every week (IV or SC), or Placebo (equal volume normal saline for IV administration, or sham dosing) once a week. Where normal saline for placebo is not available, dextrose 5% in water for IV administration may be used. If the study drug is given IV, it will be given slow IV push. If the study drug is given SC, the study drug will be brought to the bedside in a masked container, and injections will be shielded behind screens and out of earshot from caregivers and parents. An adhesive bandage or gauze will be placed over the true and sham injection sites, or gauze held until no evidence of an injection is visible. For those infants with IV access, IV administration of Darbe or placebo (in equal volume) is allowed. Infants will be treated until 35 completed weeks gestation, discharge, or transfer to another hospital. The first dose of study medicine will be administered as soon as possible, at the latest by 36 hours of age.

Supplementation: Infants will receive parenteral iron dextran or iron sucrose, 3 mg/kg once a week (at the end of the first week) while they are receiving <60 mL/kg/day in enteral feedings. The parenteral iron will be added to TPN solution and run over a 24 hour period to ensure slow delivery, or run over 4 hours separate from TPN. Infants will receive oral iron (5 mg/kg/day elemental iron) when receiving 80-120 mL/kg/day of enteral feedings. Doses of all supplements will be recorded. Infants will receive iron dosing regardless of receiving a PRBC transfusion. Infants will receive study drug regardless of iron dosing (red cell production will continue whether iron stores are adequate or not, but production is more effective if iron stores are adequate). Additionally, Infants will continue to receive iron dosing regardless of whether study drug is withheld (iron is adjusted per algorithm below).

Iron dose monitoring and adjustment: Monitoring for iron overload/insufficiency will occur during the study around days 14 and 42. Additional labs will be sent for ferritin in those cases where ferritins fall below 50 or above 400. Iron supplementation will be adjusted as follows:

Ferritin <50 ng/mL: parenteral iron 4 mg/kg/week; **OR** oral iron 8 mg/kg/day, ***repeat ferritin in 2 weeks***
Ferritin 50-300: parenteral iron 3/mg/kg/week; **OR** oral iron 5 mg/kg/day
Ferritin 301-400 ng/mL: parenteral iron 2 mg/kg/week; **OR** oral iron 4 mg/kg/day
Ferritin >400 ng/mL: hold all iron, ***repeat ferritin in 2 weeks***

Iron doses are similar to the doses and dosing schedule of ELBW infants enrolled in the original NICHD Epo Study, and the recent ESA Study. Ferritin concentrations in the ESA Study were used to adjust iron dosing. A greater number of infants in the placebo group had their iron dose decreased at day 42 due to ferritin >400 ($p=0.045$) and a greater number of infants had their iron dose increased at day 14 ($p=0.024$) and day 42 ($p<0.001$) in the treated groups. There were no differences in incidence of late onset sepsis among groups. A summary of iron dosing is shown in **Table 5.**

Table 5. Ferritin concentrations and iron dosing

	Darbe	Epo	Placebo	P=
Ferritin Day 14	121 [63, 184]	104 [58, 197]	204 [114, 348]	0.003
Ferritin Day 42	50 [27, 77]	61 [33, 99]	127 [67, 216]	0.002
Number of IV iron doses	1.7±1.3	1.6±1.5	1.9±2.3	0.317
oral iron started (days of age)	15.5±8.5	12.9±8.4	14.5±7.7	0.205
Ferritin <50 day 14	7	5	2	0.024
Ferritin <50 day 42	12	13	2	<0.001
Ferritin >400 day 14	1	1	5	0.118
Ferritin > 400 day 42	3	0	5	0.045

Ferritin reported as median [Q_1, Q_3]

Laboratory Studies:

The following labs will be obtained on all enrolled infants: (**1**) CBC with reticulocyte count prior to study drug and at day 14 and 42 (0.5 mL/sample); (**2**) Epo concentration prior to study drug and any time the day of the third dose (trough), and 12±2 hours after the third dose if given SC, or just after the dose if given IV (peak; 0.5 mL/sample); (**3**) ferritin concentration on study day 14 and 42 (0.5 mL/sample). In addition, biomarkers of brain injury and recovery will be run on collected samples (see below). Blood draws prior to first study drug administration can be obtained from the umbilical cord/placenta prior to start of intervention after consent is obtained, or from stored cord blood if this is site practice (1.0 mL). If a retic count can be obtained on the infant's admission CBC, then that CBC can serve as the baseline CBC. Clinically drawn measures of erythropoiesis (e.g., hemoglobin, CBC, retic, total and direct bilirubin) performed over the course of the study will be recorded. For those sites where this exceeds maximum research blood draw limits, only ferritin (to adjust iron dosing) and peak Epo will be drawn. In addition to the timed samples, serum/plasma samples will be collected from clinically drawn labs (scavenged samples) to measure random Epo concentrations, in order to perform population pharmacokinetics. Remaining serum or plasma from the above samples will be collected, frozen and shipped to the University of New Mexico. These samples will be stored until 6 months following the last dose of the last enrolled infant, then destroyed if not used for anti-Darbe antibody analysis. Epo concentration data will be sent to Dr. An at the University of Iowa for pharmacokinetic analyses.

Table 6. Laboratory Evaluation

	Day 1 (before study drug)	Day 14 (dose 3)	Day 42 (dose 7)
CBC and retic count	**X**	**X**	**X**
Epo concentration	**X**	**Peak and trough**	
Ferritin		**X**	**X**

Measuring factors involved in brain injury and recovery:

We will use Meso Scale Discovery (MSD) technology to measure markers of brain injury and recovery using the same serum/plasma samples on which Epo concentrations will be measured. No additional sample volume is required. The following inflammatory markers and growth factors will be considered for measurement: Brain-Derived Neutrotrophic Factor (BDNF), Interferon-gamma (IFN-γ),[81] Interleukin

(IL)-1 β,[82] IL-6, IL-8, IL-10, tumor necrosis factor-α (TNF-α), transforming growth factor (TGF)-β, matrix metalloproteinase (MMP)-2 and MMP-9,[83] macrophage inflammatory protein-1α (MIP-1α), MIP-1β[84], monocyte chemotactic protein-1 (MCP-1)[84] and tissue inhibitor of metalloproteinase (TIMP)-1.[83] Markers of neurotoxicity and brain injury will include: S100B, glial fibrillary acidic protein (GFAP), neuron specific enolase (NSE), Activin A, and Ubiquitin C-terminal hydrolase-L1 (UCH-L1).[85-87]

Not all of the above factors may prove to be informative. We have specifically left off a final list of biomarkers to measure, in order to choose the most informative subset and appropriately manage costs of the MSD assays. The above markers will be measured in a similar population of preterm infants enrolled in the PENUT trial, and we will use those results to inform the final selection of biomarkers to measure.

All donors, transfusions and transfusion volumes will be recorded, and transfusions will be administered according to a transfusion protocol (below). Information regarding delayed cord clamping or cord milking will be collected on each infant enrolled, and centers will be encouraged to perform delayed cord clamping or cord milking.

Prior to discharge, the following data will be collected: length, weight and head circumference; clinical outcomes (days of ventilation, hospital days, incidence of BPD [oxygen at 36 weeks], all stages of ROP and treatments for ROP, incidence of NEC ≥Bells stage 2, any ICH, PDA and treatments of PDA).

The following safety data will be collected through 7 days past the last study dose or to conclusion: thromboses, seizures receiving treatment, hypertension receiving treatment, culture positive sepsis, soft tissue infections at the injection site.

Results of clinically ordered head ultrasound or other head imaging studies, hearing and eye screening will be recorded.

Transfusion Protocol:

Infants in the study will be administered transfusions according to the following protocol. Transfusions may be administered under urgent conditions when the attending clinician feels withholding a RBC transfusion would adversely affect the infant. Transfusions may be considered depending on the infant reaching the following hemoglobin or hematocrit levels, but are not mandatory. The transfusion volume will be 20 mL/kg for all transfusions administered.

Table 7. Transfusion Protocol

<table>
<tr><td>Hct ≤32%
Hgb <11 gms/dL</td><td colspan="3">MAP > 8 cm H_2O (CV) MAP > 14 (HFOV) AND FiO_2 ≥0.40</td></tr>
<tr><td>Hct <27%
Hgb <9 gms/dL</td><td colspan="3">Any mechanical ventilation OR non-invasive ventilation and FiO_2 ≥0.40; undergoing surgery</td></tr>
<tr><td rowspan="2">Hct ≤24%
Hgb ≤8 gms/dL</td><td colspan="3">On respiratory support (≥ 1 LPM and ≥ 0.35 FiO_2)
AND ONE of:</td></tr>
<tr><td>two or more consecutive episodes of tachycardia (heart rate >180) or tachypnea (RR >60)</td><td>Increase in FiO_2 by ≥0.2</td><td>≥2 episodes of apnea and/or bradycardia above baseline</td></tr>
<tr><td>Hct ≤21%
Hgb ≤7 gms/dL</td><td colspan="3">Asymptomatic AND:
Absolute Retic Count < 100,000 cells/µL (<2%) (if clinically available)</td></tr>
</table>

F. Research Design

4.2.5. Blinding/Masking

Primary providers and bedside caregivers will be blinded to randomization group. If the dose will be administered IV and can be masked, the study drug will be administered slow IV push by the bedside nurse. If the dose will be administered subcutaneously, the study drug will be brought to the bedside in a closed container, and injections will be shielded behind screens and out of earshot from caregivers and parents. An adhesive bandage (or 2x2 gauze) will be placed over the true and sham injection sites and either taped or held in place until no evidence of the injection is visible. The parents, medical providers, data collection staff and neurodevelopmental follow up personnel will be masked to the treatment arm.

4.2.6. Control or Monitoring of Co-interventions

Transfusions may be administered outside the protocol under urgent conditions when the attending clinician feels withholding a RBC transfusion would adversely affect the infant. Epo or open label Darbe should not be administered during any enrolled infant's hospitalization.

4.2.7. Primary Outcome

The primary outcome is the Bayley III cognitive score. Deaths will be assigned a score of 54. The trial will analyze all enrolled infants as above, and will randomize infants 23 0/7-28 6/7 weeks GA into two groups (Darbe and placebo) in a 1:1 ratio. The primary hypothesis will evaluate whether infants receiving Darbe have higher cognitive scores at 22-26 months follow up, using a two-tailed test with 5% type 1 error and 90% power.

Study Limitations: This study will have sufficient statistical power to detect a meaningful difference (½ standard deviation or 7.5 points) in BSID III Cognitive scale score at 22-26 months. Loss to follow-up is a risk in any prospective study, however the excellent follow up rates of NRN sites provide the best chance at performing outcome studies.

4.2.8. Secondary Outcomes

Secondary outcomes that will be followed include: hematocrit, red cell mass, number and volume of transfusions, donor exposures, hospital days, and differences in morbidities (thromboses, hypertension, seizures, ICH, NEC requiring surgery, BPD, ROP requiring intervention, culture positive sepsis); NDI, death, NDI or death, and CP at 22-26 months.

4.2.9. Safety Outcomes

Monitored safety events are listed below.

- Death
- Major vessel thromboses
- Receiving treatment for EEG-confirmed seizures
- Receiving treatment for hypertension
- Culture positive sepsis
- Soft tissue infection at the injection site

4.2.10. Compliance Monitoring

Strategies to improve or monitor adherence to the study protocol will include the following:

• Monthly recruitment reports of infants screened and enrolled (accrual figures)

• Monthly reports detailing data received at the Data Center, data consistency, missing data, performance measures and adherence to the study protocol (with appropriate measures taken to preserve the blinding of study personnel and investigators)
• Periodic reports for the DSMC detailing data quality, protocol adherence, participant withdrawals, adverse events, losses to follow-up and study futility/efficacy
• Supplementary blinded reports requested by the study investigators or sub-committee that do not disclose allocation group specific outcomes (primary, secondary or any safety outcomes).

The aforementioned reports will be generated by the NRN Data Coordinating Center at RTI International.

Additionally the following data will be collected to monitor adherence to the protocol: missed dose; missed iron dosing >7 days, doses given more than 2 days before or after a scheduled dose; study drug held per protocol, permanently stopping study drug per protocol; physician request to stop study drug; parental withdrawal from study with or without continued data collection.

4.2.11. Study Specimens

The following labs will be obtained. Research CBC/retic counts and all but ferritins and peak Epo concentrations will be optional for families that wish to participate in the study but do not want research labs drawn or for those sites where research labs exceed maximum research blood draw limits:

(**1**) Ferritin concentration around study day 14 and 42 (0.5 mL/sample)
(**2**) CBC with reticulocyte count prior to study drug and around day 14 and 42 (0.5 mL/sample)
(**3**) Epo concentration and biomarkers prior to initial dose of study drug and before and after the third dose (trough can be obtained any time during the day prior to dosing; please draw in conjunction with clinical labs if possible; peak should be drawn 12±2 hours after SC dosing, or within 30 minutes of IV dosing (0.5 ml/sample)

Blood draws prior to the initial dose of Darbe/placebo can be obtained from the umbilical cord/placenta at birth (1.0 mL). All clinically drawn measures of erythropoiesis (e.g., hemoglobin, CBC, total and direct bilirubin) will be recorded and hematocrit values used to determine red cell mass (Hct x weight x 85 mL/kg). As mentioned above, additional serum/plasma samples will be collected from clinically drawn labs (up to 3 scavenged samples drawn 4-72 hours after a study dose and collected within 7 days of blood draw) to measure random Epo concentrations/biomarkers, in order to perform population pharmacokinetics, and to store in the event anti-Darbe antibody assays are run. Remaining serum or plasma will be collected, frozen and shipped to the University of New Mexico. These samples will be stored until 6 months following the last dose of the last enrolled infant, then destroyed if not used for anti-Darbe antibody analysis or for Epo concentration measurement or biomarkers measurement.

4.2.12. Post-hospital Procedures

Post-hospital procedures will include tracking for 22-26 month neurodevelopmental follow-up, including phone follow up following discharge per site practice. Ancillary studies may be added that include MR Imaging at some sites.

4.2.13. Follow-up at 24 Months

Neurodevelopmental evaluation will be performed by certified follow up examiners at 22-26 months corrected age. Evaluations will consist of the following: BSID III, neurologic evaluation, anthropometric measurements, and assessment of severity of neurodevelopmental impairments (NDI). For the purposes of this study, severe, moderate or mild NDI will be defined as follows (**Table 8**): ***severe NDI*** will be defined by any of the following: a BSID III cognitive score < 70, Gross Motor Functional (GMF) Level of 3-

5, blindness (<20/200 vision) or profound hearing loss (inability to understand commands despite amplification); **m*oderate NDI*** will be defined as a BSID III cognitive score 70-84 and **either** a GMF level of 2 or a hearing deficit requiring amplification to understand commands or unilateral blindness; ***mild NDI*** will be defined by a cognitive score 70-84, **or** a cognitive score ≥ 85 and any of the following: presence of a GMF level 1 or hearing loss not requiring amplification. ***Normal*** *(no NDI)* will be defined by a cognitive score ≥ 85 and absence of any neurosensory deficits.

Table 8. Definition of neurodevelopmental impairment (NDI)

	Normal	Mild	Moderate	Severe
Cognitive	**≥85**	**70-84 or ≥85 and any below**	**70-84**	**<70**
(GMFCS)		**1**	**2**	**3-5**
Vision	**No apparent problem**	**No apparent problem**	**Limited with correction/ unilateral blind**	**Bilateral blind (cannot correct)**
Hearing	**No apparent problem**	**Hearing loss not requiring amplification**	**Moderate, can hear and follow directions with amplification**	**No functional hearing with amplification**

4.2.14. Additional Follow-up Assessments

Estimated mortality for initial hospitalization is 25%. Every effort will be made to maintain contact with the remaining families of enrolled infants so that appropriate follow-up assessments can be made. Estimated follow up rates are 90%.

4.3. POTENTIAL RISKS AND BENEFITS TO SUBJECTS

Side effects are minimal and uncommon in adults receiving Darbe, and are similar to side effects of recombinant Epo, which are hypertension (10%), pain (5%), rash (1%), and rarely, thromboses (≤1%) or seizures (≤1%). Three infants with seizures and two infants with transient hypertension were identified in the Darbe group in the ESA Study (p>0.2 versus placebo group for both side effects). At present there are no other published reports of neonates receiving long term Darbe. There are no long term adverse effects reported of neonates receiving Epo.[62]

Preliminary analyses performed by Amgen reported no anti-Darbe antibodies in any of the infants receiving Darbe in our previous study.[29a] Pure red cell aplasia (PRCA) has been reported in adults who developed antibodies against Epo or Darbe. The incidence of PRCA in adults receiving Darbe is extremely low (14 cases/100,000 patient years[81]), less than half that seen in adults receiving Epo, and there have been fewer than 20 reported cases in the literature. Cases of PRCA after Epo dosing dropped dramatically in 2006 following identification of a specific antigenic single use syringe/stopper. No cases of PRCA have been reported in neonates.

The risks of drawing blood from a vein or giving a subcutaneous shot include discomfort at the site of needle insertion (5%), possible bruising and swelling around the insertion site (5%), skin irritation from the bandaid, and, rarely, an infection (less than 1%). This procedure may be uncomfortable due to local pain at the time of the needle puncture and during the time blood is drawn. There may be other risks to this study which are not yet known. Of the 33 infants we have monitored who have received more than one dose of Darbe, no side effects have been noted.

Potential benefits of Darbe include increased BSID III cognitive scores, improved executive function, decreased transfusions and decreased donor exposures.

SECTION 5. ANALYTICAL PLAN

5.1. STATISTICAL ANALYSIS PLAN

All analyses will be performed on an intent-to-treat basis, with the exception that survivors who are lost to follow up (estimated to be less than 10% in each treatment group) will be excluded from analysis of outcomes evaluated at 22-26 months. The primary outcome is BSID III composite cognitive score. The primary analysis (and all analyses examining outcomes by treatment) will be adjusted for the stratification variables of gestation and center. Since our primary outcome is continuous, we will use general linear mixed models (GLMM) to estimate the adjusted mean difference in BSID III cognitive scaled scores between the two treatment groups; the model will include a random effect for familial clustering. The p value used to assess statistical significance will depend on the alpha spent on interim efficacy analyses (see section 5.5.1.2). If no interim efficacy analyses are conducted, a p value less than 0.05 in favor of the treatment effect will be considered as a statistically significant evidence of treatment benefit. If interim analyses for efficacy are conducted, the p value will be reduced by the alpha spent on those analyses.

Comparisons of secondary outcomes between groups will be considered descriptive, and not formal tests of hypotheses. For continuous secondary outcomes measured serially over time, including hematocrit, CEV, platelet count, and absolute reticulocyte count we will use longitudinal GLMM accounting for the lack of independence between repeated measured on the same participant to obtain estimates of the values over time in each treatment group and adjusted mean differences between the two groups. Because the timing of these measures will vary by infant, days since treatment initiation will be a continuous covariate in the longitudinal models, and quadratic and cubic effects for time may be included depending on the outcome. Interactions between the time effects and treatment group will be included to assess whether the outcome measures have different trajectories of change over time in the two groups. Continuous outcomes measured at one time point, such as length of hospital stay, will be analyzed using similar GLMM models that are not longitudinal.

For categorical outcomes including NDI, death, and other morbidities, and for count outcomes such as number of transfusions and donor exposures, we will use robust Poisson regression implemented in a generalized estimating equations (GEE) model to adjust for familial clustering, and with fixed effects for center and gestational age group, to obtain adjusted relative risk estimates for the treatment effect.

We will examine hematocrit and red cell mass, also known as circulating erythrocyte volume (CEV) and calculated as hematocrit (%) x estimated blood volume (85 mL/kg) x weight (kg). While hematocrit may remain the same or even decrease slightly, preterm infants are constantly growing, and therefore the total number of red cells and CEV increases over time. Each of these outcomes will be measured at multiple points in time, and the repeated measures general linear mixed models to be used in analysis will accommodate variations in the timing of assessments between infants. Analyses examining these outcomes by treatment group will be adjusted for the stratification variables of gestation (<26 weeks, 26 to 28 completed weeks) and center by their inclusion as fixed effects in the statistical models. General linear mixed models (GLMM) will also include a random effect to account for familial clustering due to the randomization of multiple births to the same treatment group.

For outcomes measured serially over time, including hematocrit, CEV, platelet count, absolute reticulocyte count, and transfusion volume, we will use longitudinal GLMM accounting for the lack of independence between repeated measures on the same participant to obtain estimates of the values over time in each treatment group and adjusted mean differences between the two groups. Because the timing of blood draws and transfusions will vary by infant, days since treatment initiation will be a continuous covariate in the longitudinal models, and quadratic and cubic effects for time may be

included depending on the outcome. Interactions between the time effects and treatment group will be included to assess whether the outcome measures have different trajectories of change over time in the two groups.

5.2. SAMPLE SIZE AND POWER ESTIMATES

The table below presents a range of sample size estimates for each arm of the two-arm Darbe study for different underlying assumptions about the study. Key assumptions for this study that are incorporated into each of the sample sizes calculated in the table are: (1) multiples will be randomized to the same arm; (2) 75% of the infants will survive on both arms with survival equal on the two arms and infants who do not survive will have an imputed BSID score of 54; (3) an additional 10% of infants will be lost prior to the follow-up and will be excluded from the analysis under the assumption that the data are missing at random; (4) Composite cognitive scores for survivors on the two arms will have mean values in the range of 85 and 95 with the true standard deviation will be in the range of 10 to 15 among survivors. Under these assumptions, the standard deviation across the mixture of survivors and non-survivors with scores imputed at 54 was found to be in the range of 17 to 20 if the underlying standard deviation among survivors was 10 and between 19 and 22 if the underlying standard deviation among survivors was 15, so three values over the range of 17 to 22 were considered. The adjustment for multiples was estimated to be in the range of 12% to 15%. Note that the final sample sizes incorporate both the multiple randomization effect and the loss to follow-up percentages. Sample sizes were computed for both 80% power and 90% power.

Effect Size		Standard Deviation	Multiple Effect	Sample Size Per Arm	
Survivor	Aggregate			80% Power	90% Power
7.5	5.625	17	12%	179	238
7.5	5.625	17	15%	241	322
7.5	5.625	19.5	12%	234	313
7.5	5.625	19.5	15%	241	322
7.5	5.625	22	12%	299	397
7.5	5.625	22	15%	307	408
10	7.5	17	12%	101	134
10	7.5	17	15%	104	138
10	7.5	19.5	12%	134	178
10	7.5	19.5	15%	137	183
10	7.5	22	12%	169	225
10	7.5	22	15%	174	231

In our previous multicenter study comparing cognitive outcomes at 18 to 22 months, we used information presented in our preliminary data section that showed a difference of 15±15 MDI points among survivors between the two groups. The differences in our current Darbe study are 8±12 points on the BSID composite cognitive score among survivors despite the relatively small sample size. We anticipate that the proposed trial will find a difference at least that large between survivors randomized to receive Darbe compared to those randomized to placebo, but the study is conservatively powered to detect a difference of 7.5 points. When non-survivors with assigned scores of 54 are included, the

overall expected difference between the treatment groups becomes 5.625 points. Using a conservative estimate of differences in BSID III cognitive score of 5.625±19.5 points between Darbe recipients and controls, with 90% power and an α of 0.05, estimating a survival rate of 75% and an additional loss at follow up of 10%, and assuming multiples would be assigned to the same treatment arm, and assigning a score of 54 to non-survivors, a total of 322 (rounded to 325) infants would need to be enrolled in each arm of the study, for a total of 650 infants.

5.3. AVAILABLE POPULATION

The number of eligible NRN admissions is approximately 525 infants per year.

5.4. PROJECTED RECRUITMENT TIME

Based on a consent rate of 50%, enrollment for this study should be completed within 24-30 months of IRB approval at all sites, and all follow up completed by 48-60 months. Compatibility with other IND-associated studies will need to be determined, and could impact enrollment and length of the study.

5.5. STUDY MONITORING PLAN

5.5.1. Reporting Adverse Events

The trial will use NRN standard definitions for adverse events (AEs) and serious adverse events (SAEs), and collect the data on such events using specialized forms for this purpose. All SAEs that are unexpected and/or at least possibly related to the study intervention will be reported to NICHD and the NRN Data Center at RTI within 24 hours, along with a completed Medwatch form. All such events that are marked as possibly related to the intervention will be forwarded to the Chair of the independent NRN DSMC for any further action. The independently appointed DSMC makes recommendations to the Director of NICHD and is entrusted with the following charges:

(i) Review the research protocol, review model informed consent documents, and plans for data and safety monitoring, including all proposed revisions;

(ii) Review methodology used to help maintain the confidentiality of the study data and the results of monitoring by reviewing procedures put in place by investigators to ensure confidentiality;

(iii) Monitor study design, procedures and events that will maximize the safety of the study participants and minimize the risks;

(iv) Evaluate the progress of the study, including periodic assessments of data quality and timeliness, participant recruitment, accrual and retention, participant risk versus benefit, performance of the study site(s), and other factors that may affect study outcome;

(v) Consider factors external to the study when relevant information becomes available, such as scientific or therapeutic developments that may have an impact on the safety of the participants or the ethics of the studies;

(vi) Review serious adverse event documentation and safety reports and make recommendations regarding protection of the safety of the study participants.

5.5.2 Data Monitoring Plan and Stopping Rules

Data Management: Data will be collected using the stated forms, then entered into a centralized web based NRN Data Management System developed and maintained by the NRN Data Center at RTI. The data entry software will perform range checks and consistency checks and errors will be corrected on the spot. The Data Center will perform further consistency checks as well as more sophisticated machine checks designed to detect consistencies across forms. Edit errors detected at the Data Center will be

sent electronically back to the study sites for reconciliation. The final analysis database will be in the SAS format.

Performance Monitoring: Standard reports will be generated from the study database by the Data Center. These include:

- Monthly recruitment reports that provide the number of infants screened and enrolled
- Monthly reports detailing data received at the Data Center, missing data, adherence to study protocols and a variety of performance measures
- Periodic reports for the DSMC that detail data quality and protocol adherence, as well as adverse events, efficacy data and withdrawals or losses to follow-up
- Ad Hoc reports requested by the study investigators that do not disclose treatment group specific outcomes information (primary, secondary or any safety outcomes).

Stopping Rules: Information from clinical labs (CBC and reticulocyte count) are collected to monitor effects of study drugs and procedures. Criteria are in place for holding and/or stopping the study drug.

Criteria for holding the study drug include:

1. Starting with the third dose, the study drug will be held if the known hematocrit on the day of dosing is ≥50% (excluding baseline labs, and not due to a blood transfusion) and will continue to be held until the hematocrit is <50%.
2. If hypertension occurs during the study period in which a clinical decision is made to initiate antihypertensive treatment, the study drug will be held until hypertension resolves with treatment.
3. All local or systemic skin reactions to the drug will be recorded, including soft tissue infections directly related to the injection site. If any reaction to the study medicine occurs which causes a systemic reaction (hypotension, systemic skin reaction, respiratory compromise) the study drug will be held.

Criteria for permanently stopping the study drug:

1. If EEG-confirmed seizures not controlled by anticonvulsant medication occur during the study period, the study drug will be stopped.
2. If hypertension recurs following antihypertensive treatment and resumption of the study drug, the study drug will be stopped.
3. If any major vascular thrombosis is documented, the study drug will be stopped.

5.5.2.1 Safety

Since the primary outcome is available only at 22-26 months follow up, safety will be the primary focus of interim monitoring for this trial. The interim safety analysis will compare the incidence of serious adverse events (SAEs) across the placebo and Darbe groups. The DSMC will conduct pre-specified formal safety looks at the interim data after the first 20 patients enrolled have reached 35 completed weeks gestation, discharge, or transfer to another hospital, and subsequently, after 25%, 50% and 75% of the enrolled patients have reached the same milestone. Pocock stopping bounds will be used as stopping rules for safety, based on the 4 planned interim safety looks at the data. Thus, at each interim safety look, a p value less than 0.0158 from comparing the incidence of SAEs across groups, may be used by the DSMC as evidence of significant harm from the study intervention. SAEs are defined as any adverse event (defined in section 4.2.9) that results in any of the following.

a. Death of infant.
b. Prolonged hospitalization of infant.
c. Persistent or significant disability/incapacity of the infant.

d. Required medical or surgical intervention to prevent any of a through c above.
e. Is considered life-threatening if no medical intervention is provided.

The above analysis will be conducted using robust Poisson regression implemented in a generalized estimating equations (GEE) model to adjust for familial clustering, and adjusting for gestation age group and center to obtain the p value for comparison with the cut-off of 0.0158. Given that there are 15 centers in the NRN, adjustment for center may not be computationally feasible for the first few interim analyses, in which case GLMM with center as a random effect may be attempted if adjustment for center is deemed crucial for inference.

5.5.2.2 Efficacy

Since the primary outcome is only available at 22-26 months follow up, and the projected enrollment period for this study is less than 3 years, interim efficacy monitoring for this trial may become moot if the trial finishes enrollment before substantial primary outcome data is accrued. However, in the event that recruitment is slow, there may be some interim efficacy data available for the DSMC to review. Interim efficacy looks will be performed once 25% of the enrolled infants, regardless of survival, would reach 2 years corrected age, and every 25% thereafter (provided enrollment/intervention is not already complete). To control the overall Type I error rate, a Lan-DeMets alpha spending function with an O'Brien Fleming-type stopping bound will be used. The above analysis will be conducted using GLMM for the primary outcome, adjusting for gestation age group and center, with a random effect for familial clustering, to obtain the p value for comparison with the appropriate stopping boundary.

5.5.2.3 Futility

Just like interim efficacy, interim futility analyses will be limited in usefulness because the primary outcome is obtained at 22-26 months follow up. Thus, it is expected that vast majority of the subjects may be enrolled before the primary outcome can be determined in the first enrolled survivors. Interim futility looks will thus be performed on the primary outcome once 25% of the enrolled infants, regardless of survival, reach 2 years corrected age and every 25% thereafter. Specifically, the conditional power to detect a statistically significant treatment benefit will be calculated at each of these looks based on the accrued data and assuming the hypothesized treatment benefit for the unobserved data. The DSMC can recommend stopping further enrollment for futility if, at any of these interim looks, the conditional power for treatment benefit is less than 0.2.

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