Umbilical or adult donor RBC to transfuse extremely low gestational age neonates. A randomized trial to assess the effect on ROP severity.

Version 5, August 5, 2021

Version number	Revision type (Approval/Revision)	Revised paragraph (page)	Date
1			01.06.2021
2	Revision requested by EC	Synopsis: study design, sample size and statistical analysis Par. 2.1 Study design Par. 3.1Treatment allocation Par. 4.2 Sample size Par. 4.3 Interim analysis Par. 4.6 Outcome analysis Par. 4.8 Analysis set Par. 5.5 Access to data.	24.06.2021
3	Revision	Page 1: the list of participating centers has been removed. Page 2 Synopsis: the list of participating centers has been removed. Par. 2.5.1. Patient recruitment and CB-RBC production. The list of participating centers has been removed. (centers are listed in the "List of participating Centers")	08.07.2021
4	Revision requested by EC	Study Type page 1 and Study design page 3 (phase II/III eliminated)	
5	Revision requested by EC	Par. 2.8 In Vitro Study Par. 4.3. Interim analysis Annex 3	05.08.2021

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ADMINISTRATIVE INFORMATION

Title	**UMBILICAL OR ADULT DONOR RBC TO TRANSFUSE EXTREMELY LOW GESTATIONAL AGE NEONATES. A RANDOMIZED TRIAL TO ASSESS THE EFFECT ON ROP SEVERITY.**
Acronymn	**BORN (umBilical blOod to tRansfuse preterm Neonates) study.**
Trial registration	ClinicalTrials.gov Identifier: NCT05100212
Study type	Investigator initiated, interventional, randomized, controlled, double-blind trial on safety and efficacy of allogeneic cord blood red blood cell transfusions.
Protocol version	Version 5, August 5, 2021
Study Promoter	Fondazione Policlinico A. Gemelli IRCCS
Study Support	Fresenius HemoCare Italia SRL
Principal Investigator	Luciana Teofili
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Approval date 22/07/2021

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STUDY SYNOPSIS

Title	**Umbilical or adult donor RBC to transfuse extremely low gestational age neonates. A randomized trial to assess the effect on ROP severity.**
Acronym	**BORN (**um**B**ilical bl**O**od to t**R**ansfuse preterm **N**eonates**) study**
Background and rationale	Extremely low gestational age neonates (ELGAN, i.e., born before 28 gestation weeks) are among the most heavily transfused pediatric patients. In this clinical setting, repeated RBC transfusions independently predict a poor outcome, with a higher risk for mortality and morbidity. Recent studies from our own and other groups highlighted a close association between low levels of fetal hemoglobin (HbF) and severity of retinopathy of prematurity (ROP) and bronchopulmonary dysplasia (BPD), two disabilities that frequently complicate preterm birth. This association is not surprising, considering that 1) preterm neonates have a highly immature antioxidant reserve and both ROP and BPD rely on the oxidative damage as underlying mechanism; 2) in comparison with HbA, HbF is endowed with higher oxygen affinity, greater redox potential, higher tetrameric stability, and higher ability to generate unbound nitric oxide, all functions potentially protective in presence of an oxidative challenge; 3) in normal prenatal life, developing organ and tissues are exposed exclusively to HbF until last weeks of gestation; 4) in preterm neonates, the switch of the synthesis from HbF to HbA occurs around their due date, i.e., several weeks after the premature birth; 5) when preterm neonates receive transfusions, their tissues are abruptly exposed to high levels of HbA. We have recently run a pilot trial demonstrating as a proof-of-concept that transfusing cord blood red blood cell concentrates (CB-RBC) effectively prevents or restrains the HbF loss consequent to adult donor standard transfusions (A-RBC).
Hypothesis	Transfusing CB-RBCs instead of A-RBC may lower the incidence of severe ROP and BPD in ELGANs needing transfusions.
Study design	Interventional, randomized, controlled, double-blind, with an adaptive design to evaluate safety and efficacy of allogeneic CB-RBC transfusions.
Outcomes	**Primary outcome**: incidence of severe ROP (stage 3 and higher) in CB-RBC and A-RBC arms at 40 weeks of PMA or discharge, which occurs first. **Secondary outcomes**. Incidence of ROP requiring treatment at 40 weeks of PMA or discharge, which occurs first. Incidence of BPD at 40 weeks of PMA or discharge, which occurs first. Incidence of a composite outcome of death, severe ROP, BPD, and necrotizing enterocolitis (NEC) at 40 weeks of PMA

	or discharge, which occurs first. HbF threshold predicting severe ROP and BPD at 32 and 36 weeks of postmenstrual age (PMA). Intervals between two consecutive transfusions. Post-transfusion hematocrit increase. Gene expression profile in endothelial cells exposed to CB-RBC or A-RBC surnatants.
Randomization and treatment allocation	Arm A, comparator: Adult-RBC transfusions Arm B, intervention: CB-RBC transfusions Patients are randomized at a ratio of 1:1 between two arms. Random allocation is stratified per center and gestational age at birth (greater or lower than 26 weeks).
Study population	Extremely low gestational age neonates (ELGAN, defined as neonates born <28 gestation weeks).
Inclusion criteria	– gestational age (GA) at birth <28 weeks – signed informed consent of parents
Exclusion criteria	one or more of the followings: – maternal-fetal immunization, hydrops fetalis – major congenital malformations associated or not with genetic syndromes – previous transfusions – hemorrhage at birth – congenital infections – out-born infants – health care team deeming it inappropriate to approach the infant's family for informed consent. – severe IgA deficiency – any life-threatening comorbidity or any other medical condition which, in the opinion of the investigator, makes the patient unsuitable for inclusion
Sample size	The estimated sample size is 146 patients (73 per arm). An interim analysis is planned after the first 58 patients (29 per arm) are enrolled, to confirm that the sample size is adequate to the planned power study.
Statistical analysis	Continuous variables will be expressed as median with relative interquartile range (IQR) and categorical variables as n (%). The comparison of the proportion of patients free from severe ROP and other comorbidities in the two arms is made through Cochran-Mantel-Haenszel test. The association between HbF and risk for prematurity-associated diseases (ROP, BPD, NEC, IVH) is investigated by logistic regression analysis and expressed as

	an odds ratio with a relative 95% confidence interval (95% CI). The best predicting cut point value of HbF is identified for each condition by the AUROC (Area Under the Receiver Operating Characteristics) analysis. Interval between two consecutive transfusions and hematocrit increase after CB-RBC or A-RBC transfusions in the two arms are compared by the Mann-Whitney U test.
Study duration	18 months

1. INTRODUCTION

1.1. STUDY BACKGROUND. Extremely preterm birth is associated with a high risk for mortality and adverse functional outcome.[1] Along with the decline of mortality, attention has been focused on long-term morbidities and related functional impairments, which lifelong affect the quality of life of "born too soon" neonates.[1] Among diseases complicating the clinical course after premature birth, retinopathy of prematurity (ROP) and bronchopulmonary dysplasia (BPD) oddly influence the neurodevelopmental outcomes of affected patients.[1] ROP is one of the most important causes of childhood blindness.[2] ROP develops in the immature retina for vessel proliferation that follows the vasoconstriction caused by hyperoxia. Prematurity and low birth weight are the most important determinants of ROP, whereas genetic and environmental factors such as oxygen supplementation are likely to play a role in determining the severity of the disease.[3] BPD occurs when preterm newborns require the use of a ventilator or oxygen therapy for support. The high amounts of inhaled oxygen and pressure may overstretch the alveoli, causing inflammation and damage to the alveoli and the blood vessels around them.[4] Respiratory distress at birth, requirement for invasive mechanical ventilation and supplemental oxygen, infections, hemodynamically significant patent ductus arteriosus also are relevant factors to BPD development and outcome.[5] Recently, studies about ROP physiopathology focused attention on low circulating concentrations of insulin-like growth factor-1 (IGF-1), an anabolic hormone with mitogenic, differentiating, anti-apoptotic and metabolic effects.[6] Low serum IGF-1 levels are associated with poor general growth, poor brain growth, as well as ROP, and other neonatal morbidities including BPD.[7,8,9] In extremely preterm infants, ROP severity correlates with high early plasma glucose levels, insulin insensitivity, and low IGF-1 levels.[10] Also, the IGF binding protein-3 (IGFBP-3), acting independently of IGF-1, has been shown able to prevent in vivo oxygen-induced vessel loss and promote vascular regrowth after vascular destruction[11]. Indeed, IGF-1 gained attention as a potential therapeutic target for ROP.[6] Despite this solid biological rationale, the treatment with recombinant human (rh) IGF-1/rhIGFBP-3 failed to achieve a significant reduction of severe ROP. The unexpected decrease of severe BPD, and grades 3-4 intraventricular hemorrhage (IVH) was conversely observed.[12,13] Preterm birth is invariably complicated by anemia.[14] The impaired erythropoietic function in is primarily responsible for the prematurity anemia, as well as for the scarce responsiveness to the several attempted therapeutic approaches.[14] Like in a vicious circle, anemia is further worsened by concurrent clinical complications (infections, hemorrhages, inflammatory reactions), and, in the critical care context, by the frequent blood withdrawal for tests. As a result, the vast majority of neonates born at gestation age <28 weeks receive at least one RBC transfusion, with the median number ranging from 3 to 8 during their hospitalization.[15,16] Recently, two large, randomized trials have shown that transfusion strategies based on higher hemoglobin threshold do not help reduce the likelihood of death or disability of extremely-low-birth-weight neonates,[17] nor improve survival without neurodevelopmental impairment.[18] In general, the association between transfusions and the poorer outcome seems to be unquestionable.[19–28] In general, detrimental effects of transfusions have been ascribed to components released during the storage (such as microparticles, cytokines, reactive oxygen species, free iron) which are able per se to elicit in recipients the activation of the innate immune system and inflammatory response.[29,30] In preterm neonates, the increase of many of these mediators and markers of oxidative stress has been documented.[31–33] A body of growing evidence from retrospective and prospective studies suggest a connection between ROP and BPD severity and low levels of fetal hemoglobin (HbF). First, Stutchfield et al. (prospective study, 42 infants 32 gestational weeks) reported a higher risk for severe ROP in neonates experiencing prolonged exposure to low HbF levels (61,7%) until 36 weeks of postmenstrual age (PMA), whereas the initial HbF was only weakly associated with ROP.[34] Jiramongkolchai et al. (prospective study, 60 infants 33 gestational weeks) showed that neonates with HbF levels below 31.5% at 31-weeks of PMA have a 7.6 increased risk to develop mild and severe ROP and that this risk raises at 12.3 times in neonates with HbF levels below the same thresholds at 34-weeks of PMA.[35] Hellstrom et al (retrospective study, 452 infants 30 gestational weeks) reported that mean HbF values during the first 5 days of life significantly predicted BPD (with an area under the curve for HbF value and BPD development of 87.1%).[36] Notably, this effect was independent from the increased oxygen exposure: an HbF increase of 10% reduced the risk for BPD development with an odds ratio of 0.64 (95% CI 0.49 to 0.8).[36] It deserves to be mentioned that the synthesis of HbF is regulated by IGF-1 and other IGF family members, which preferentially promote the transcription of γ genes.[37] The IGF family factors, more than the hypoxia-erythropoietin axis, regulates erythropoiesis in mammals during fetal life.[38] In non-transfused neonates, HbF largely predominates over adult hemoglobin (HbA) until their due date.[39] Conversely, in those receiving red blood cell (RBC) transfusions, HbF progressively decreases proportionally to its level before transfusion.[40] HbF has attracted renewed attention for its ability to modify the clinical course of sickle cell disease and thalassemia.[41,42] The evolutionary advantage that this protein confers over co-inherited hemoglobinopathies resides in its extraordinary stability.[42] Even though HbF and HbA have a very similar three-dimensional structure, amino acids sequence specific for and chains deeply modifies their chemical and physical properties. The distinctive HbF characteristics go far beyond the higher oxygen affinity of HbF and involve a higher efficiency of HbF in maintaining its tetrameric integrity, preventing the release of toxic free heme groups, as well as the critical redox effect due to its pseudo peroxidase activity.[43–45] This protective mechanism neutralizes peroxides and removes radicals through a peroxide radical-self termination reaction that generates more stable molecules.[45] Notably, HbF properties are pivotal for the progressive adaptation to the post-natal oxygen-rich environment of premature neonates, whose enzymatic antioxidant systems are still highly immature.[46] In addition, HbF exerts a greater ability than HbA in vessel tone regulation, through a more efficient generation of unbound nitric oxide via oxidative denitrosylation.[47] All these properties make it possible that HbF levels as low as 30% efficaciously protect from vasocclusive crises in adults and children affected by sickle cell disease.[41] Despite the growing evidence on the negative effect of decreasing HbF, RBC transfusions are up so far, the mainstay for the treatment of anemia of preterm neonates and remain unavoidable in several patients.

1.2. STUDY RATIONALE. Since years our group has been working on the hypothesis that cord-RBC transfusions, preventing the HbF loss, may positively influence the outcome of transfused neonates.[48] In 2014 our group conducted a pilot non-randomized study assessing the feasibility of using RBC concentrates obtained from allogeneic umbilical blood to transfuse preterm neonates.[49] Those units solidary donated at our cord blood bank, unsuitable for transplant purpose for a low total nucleated cell content, were fractionated and used for transfusing neonates with 30 gestational weeks at birth. The main conclusions were: i) the cord blood-based transfusion approach is overall feasible ii) CB-RBC and A-RBC elicit similar hematocrit (Hct) increments and post-transfusion intervals iii) the availability of ABO-RhD-matched units represents the main critical issue, so that cooperation among cord blood banks is necessary for a valid implementation of this program.[49] We then refined our methods and recently published them.[50] The interest in the topic was growing, and other blood banks in Europe contacted our center to start fractionation of cord blood for transfusion purposes.[51] In 2018, we started a proof-of-concept study aiming at establishing i) if cord-RBC transfusions prevent as expected the HbF drop, and ii) to which extent they could limit the HbF loss in case of multiple transfusions (the CB-TrIP study, NCT 03764813). HbF was monitored three times a week and the primary outcome was the median value of HbF at post-menstrual age of 32. Twenty-five neonates with gestational age at birth 30 weeks were enrolled and nine received transfusions: at each transfusion request, cord-RBC or adult-RBC units were given depending on whether ABO/Rh matched units were available.[40] An editorial signed by an eminent European neonatologist was dedicated to the study and positively commented this transfusion approach.[52] The main conclusions were: i) cord-RBC transfusion elicits significantly higher HbF levels not only in patients receiving exclusively CB-RBC but also in those transfused with CB-RBC and A-RBC units. ii) HbF values < 61.7% were significantly associated with ROP (OR 0.913, 95%CI 0.846-0.986) iii) every A-RBC increased the risk for low HbF of about 10 folds, whereas this risk was lower if transfusions included A- and CB-RBC.[40] Overall, our data demonstrated for the first time that transfusing cord-RBC can prevent or restrain the HbF depletion in preterm neonates receiving transfsuions.[40] These observations constitute the rationale for proposing a randomized trial to assess the ability of CB-RBC to decrease the incidence of severe ROP.

1.3. BENEFIT / RISK ASSESSMENT OF THE TREATMENT

1.3.1. Benefit of CB-RBC. Potential benefit of CB-RBC transfusion is to prevent ROP development and/or progression to a more severe form. Benefits of CB-RBC are conceivably connected with the presence of fetal Hb instead of adult Hb. At the time of writing, this benefit is to be proven yet in large population of ELGANS, but is based on small pilot studies,[34,40,53] and on retrospective data.[36,54] An additional advantage for CB-RBC transfusions is that they are obviously obtained from CMV-negative donors.

1.3.2. Risks of CB-RBC. Whereas a plenty of studies reported that cord blood transfusion is safe and efficacy, it is not routinely used for transfusion purpose and cord RBC concentrates are not included among blood products in the current regulation. Filters and bags used in this study are distributed by Fresenius for standard RBC transfusions, so that their use in this protocol is intended as "off label". The operative instruction included in this protocol (annex 1) is designed to obtain CB-RBC units fulfilling the same quality and safety requirements currently requested by National and European regulations for RBC concentrates, in terms of hemoglobin content, hematocrit, residual leukocytes and hemolysis rate at the end of storage. For this purpose, fractionation parameters of CB units processed during the study will be tightly monitored, in order to optimize the configuration of automated blood separator Compomat G5 (annex 1) and make CB-RBCs perfectly compliant to the above-mentioned standards. Due to the different type of collection setting, all CB-RBC units are screened for bacterial and fungal contamination beside HBV, HCV, HIV and syphilis infections. Since CB-RBC units are released only after the achievement of microbial test negative results, they may have a slightly longer storage than A-RBC. Nevertheless, there are no data favoring a disadvantage for that. Further potential risks connected to CB-RBC transfusion are those relative blood product transfusion in general (including A-RBC), and include allergic/febrile reaction (estimated frequency 1 in 100), transmission of infectious disease (HIV, HBV, HCV, syphilis, etc.; estimated frequency 1 in 1.000.000), transfusion associated circulatory overload (TACO, estimated frequency 1 in 100) and transfusion associated acute lung injury (TRALI, estimated frequency 1 in 100.000). Hemolytic severe reactions could also occur if ABO incompatible RBC (either A-RBC or CB.RBC) are transfused. Any precaution will be undertaken to minimize these risks.

2. STUDY DESCRIPTION

2.1. STUDY DESIGN. This is an interventional, randomized, controlled, double-blind phase II/III study with an adaptive design to evaluate safety and efficacy of allogeneic CB-RBC transfusions. The clinical trial is endowed with an *in vitro* study on biological effects of different types of RBC products.

2.2. STUDY TYPE. The study is a no-profit investigator-initiated trial sponsored by Fondazione Policlinico A. Gemelli IRCCS and supported by Fresenius HemoCare Italia. Fresenius supplies participating centers the equipment necessary for CB-RBC production, including the automated blood component separator Compomat G5 and transfusion devices (filters, bags). Moreover, Fresenius provides the technical assistance for setting automated cord blood fractionation procedures during the study. Finally, Fresenius contributes a grant to cover part of the costs for the insurance policy and for study management.

2.3. STUDY OBJECTIVES. The study objective is to demonstrate *in vivo* a protective role for fetal Hb against ROP and other complications of preterm birth (free radical diseases). Likewise, the *in vitro* study will investigate the effect of CB-RBC and A-RBC surnatants on the activation and oxidative stress response in endothelial cells.

2.4. STUDY OUTCOMES

2.4.1. Primary outcome. Incidence of severe ROP (stage 3 and higher) in CB-RBC and A-RBC arms at discharge or 40 weeks of PMA, which occurs first.

2.4.2. Secondary outcomes: Incidence of ROP requiring treatment (laser therapy or anti-VEGF administration) in CB-RBC and A-RBC arms at discharge or 40 weeks of PMA, which occurs first. Incidence of BPD in CB-RBC and A-RBC arms at discharge or 40 weeks of PMA. Incidence of a composite outcome including any of the following: death, severe ROP, BPD, necrotizing enterocolitis (NEC) at discharge or 40 weeks of PMA, which occurs first. HbF threshold predicting severe ROP and BPD. Interval between two consecutive transfusions in the CB-RBC or A-RBC arms. Hematocrit increase after CB-RBC or A-RBC transfusions. *In vitro* differential gene expression profile in endothelial cells exposed to surnatants recovered from either CB-RBC or A-RBC units.

2.5. STUDY POPULATION. Extremely low gestational age neonates (ELGAN, defined as neonates born <28 gestation weeks) are eligible for the study.

2.5.1. Patient recruitment and CB-RBC production.

2.5.1.1. Patients are recruited at Neonatal intensive Care Units participating to the study (centers are listed in the attached “Lists of participating centers”)

2.5.1.2. CB units are collected and processed at public Italian Cord Blood Banks participating to the study (centers are listed in the attached “Lists of participating centers”)

2.5.2. Patient inclusion and exclusion criteria.

2.5.2.1. Inclusion criteria. The inclusion criteria are gestational age (GA) at birth between 24+0 and 27+6 weeks and signed informed consent of parents.

2.5.2.2. Exclusion criteria. One or more of the followings: maternal-fetal immunization, hydrops fetalis, major congenital malformations associated or not with genetic syndromes, previous transfusions, hemorrhage at birth, congenital infections, out-born infants, health care team deeming it inappropriate to approach the infant's family for informed consent**.**

2.5.3. Concomitant therapies. Patients enrolled in the study receive standard therapy, i.e. treatments recommended in protocols in use at each center. These may include erythropoietin administration, O_2 therapy management, as well criteria for transfusion. The O_2 saturation target in enrolled patients is evaluated according to the PMA and is not superior to 95%. The enrollment of participants in other interventional trials is not allowed.

2.6. INTERVENTION DESCRIPTION. Patients enrolled in this study are randomized 1:1 to receive standard A-RBC transfusions (Arm A, comparator) or CB-RBC transfusions (Arm B, intervention). Patients in arm B receive CB-RBC units until the completion of 31 weeks of PMA (31+6). In case of unavailability of an ABO/Rh matched CB-RBC unit, patients in arm B receive A-RBC. CB-RBCs are produced as described in Annex 1, are screened for HIV, HBV, HCV, and syphilis and tested for microbial cultures before release. Before distribution, A-RBC and CB-RBC are γ-irradiated as per center procedure, and transfused within 24 hours from irradiation. In both A-RBC and CB-RBC arms, transfusion therapy is managed according to protocols defined in each center, including transfusion triggers, blood request, unit match, unit distribution, and infusion time. The final products are delivered to the NICU in dedicated bags containing not identifiable RBC types (either cord or adult).

2.6.1. CB-RBC UNITS PRODUCTION. CB-RBC consists of filtered, leuko-depleted, irradiated RBC concentrates suspended in saline adenine glucose-mannitol (SAG-M) additive solution and obtained through automated fractionation of allogeneic cord blood according to the procedures described in the Annex 1. Blood cell separator Compomat G5, transfusion filters and bags are kindly provided by Fresenius and used “off label” for fractionation, filtration and storage of cord blood. The BioR Flex filter is a CE marked filter for leukocyte reduction of red cell concentrates; CompoFlex 4F RCC are pediatric bags not made with di(2-ethylhexyl) phthalate (DEHP), CE marked and distributed for the storage of units destined to pediatric transfusion. In contrast to adult blood donations, the volume of cord blood units is highly variable and continuous adjustments of the fractionation protocol may be required to achieve standardized cord blood products. For this purpose, it is necessary the cooperation between blood banks and Fresenius technical assistance. During CB-RBC unit production, blood banks continuously monitor parameters relative to fractionation and storage of CB-RBC units. The specialists of Fresenius may consequently tune the configuration parameters of Compomat G5 cell separators in use at each production site. This allows to optimize and standardize the quality of CB-RBC units distributed in the different centers. Processing parameters are recorded in dedicated electronic report forms and include complete cell blood count (CBC) before and after fractionation, residual leukocyte after filtration (assessed according to center procedures), hematocrit at distribution, free hemoglobin and CBC at the end of storage.

2.6.2. A-RBC UNITS PRODUCTION. A-RBC units consist of filtered buffy coat removed RBC concentrates suspended in SAG-M and obtained according to procedures in use at each center. Before distribution, A-RBC units are transferred through sterile connection into CompoFlex 4F RCC pediatric bags as well. In case of either A-RBC or CB-RBC units, a label indicating the Hct value of the unit is affixed on the bag.

2.7. ASSESSMENT OF ROP, BPD IVH and NEC. Diagnosis and staging of ROP, BPD, IVH and NEC is carried out according to criteria listed in Annex 2.

2.8. IN VITRO STUDY OF EFFECT OF SURNATANTS ON ENTOTHELIAL CELLS. Endothelial gene expression profile is investigated as previously reported, using commercially available assays exploring the oxidative damage and endothelium activation.[55] Experiments will be performed at cell laboratory of Cord Blood Bank of Policlinico A. Gemelli IRCCS and bioinformatics analysis of data will be carried out at Bioinformatics department of Fondazione Policlinico A. Gemelli IRCCS, as detailed in the Annex 3 of the present protocol (version 5 of August 5, 2021).

3. STUDY MANAGEMENT. A study flow-chart is shown in Annex 4.

3.1. TREATMENT ALLOCATION AND RANDOMIZATION. Treatment allocation will be randomized between arms A and B with a ratio of 1:1. Randomization sequences will be generated at Fondazione Policlinico Gemelli IRCCS through the REDCap web application, uploading the allocation table through the randomization module (ref: https://www.project-redcap.org/). Randomized stratification will be performed according to center and gestational age (< or ≥ 26 weeks, given the higher incidence of ROP in neonates born before this gestational age). Twins will be assigned to the same arm. The assignment to the intervention will be unmasked to transfusion providers (blood banks). Conversely, the assignment to the intervention will be blinded to care providers at NICU, all medical staff involved in the assessment of clinical outcomes and analysts.

3.2. **DATA RECORDING.** Epidemiological, clinical, and laboratory data of enrolled patients are recorded in customized eCRF (electronic Case Report Form) specifically designed for the study. Data are collected and managed using REDCap electronic data capture tools hosted by the Research Unit 1 at Fondazione Policlinico Universitario A. Gemelli, IRCCS (https://redcap-irccs.policlinicogemelli.it/).[56,57] This is a secure, web-based application designed to support data capture for research studies, providing 1) an intuitive interface for validated data entry; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for importing data from external sources. All patients enrolled in the trial have the following data recorded:

3.2.1. **Baseline visit:** date of birth, gestational age at birth (weeks); birth weight (grams); gender; Apgar index measured at 1 and 5 minutes; Hb, Hct, and HbF at birth (g/dL), CRIB score (gender, gestational age in weeks, birth weight in grams and base excess), antenatal maternal steroid administration for jaline membrane disease prophylaxis, clinical chorioamnionitis, post-natal steroids.

3.2.2. **Weekly assessment**: Hb, Hct, and HbF value (obtained at blood gas analysis and expressed as % of total Hb) twice a week; date of transfusion; Hct values before and after transfusion

3.2.3. **End study visit** (discharge or 40 weeks of PMA, which occurs first): maximal stage of retinopathy of prematurity,[58] necrotizing enterocolitis,[59,60] bronchopulmonary dysplasia,[61] and intraventricular hemorrhage,[62] ROP treatment, erythropoietin treatment, microbiologically documented infections, ventilator support, (invasive, non-invasive), O_2 therapy (days), death.

3.3. **DATA MANAGEMENT**. Each participating site must maintain appropriate medical and research records for this trial and regulatory/institutional requirements for the protection of confidentiality of study subjects. Investigators responsible for the study at each site will assure that data are recorded in complete, accurate, and timely manner in dedicated eCRF. The Principal Investigator is responsible for assessing the accurate and timely data collection.

3.4. **DATA MONITORING.** Each study site agrees to allow monitors from Monitoring Unit of Fondazione Policlinico Gemelli (FPG) IRCCS direct access to the study records and medical records from those patients enrolled in the clinical study. In accordance with the applicable regulations and good clinical practice (GCP), the monitor shall periodically contact the center. The duration, nature and frequency of such visits/contacts shall depend on the rate of recruitment, the quality of the documents in the possession of the center, and its adherence to the protocol. Through these contacts, the monitor must: control and evaluate the progress of the study, examine the collected data, conduct Source Document Verification (SDV), identify every problem and find solutions. The aims of the monitoring activity are to verify that: the rights and well-being of the subject are respected, the study data are accurate, complete and verifiable by original documents and the study is conducted in accordance with the protocol and any approved amendments, GCP and the applicable regulations.

3.5. ADVERSE EVENTS. Due to the nature of the investigated product (cord blood) adverse events are managed according the Italian regulation on Transfusion Surveillance, and recorded in the dedicated section of Sistema Informativo dei Servizi Trasfusionali - SISTRA (DM 21/12/2007).

4. STATISTICAL ASPECTS

4.1. HYPOTHESIS. The experimental hypothesis is that CB-RBC transfusions result in a lower incidence of severe ROP (stage 3 and higher, as evaluated at 40 weeks of PMA or discharge, which occurs first) than A-RBC transfusions.

4.2. SAMPLE SIZE.

4.2.1. Sample size calculation. The sample size has been calculated based on the primary outcome. Our recent observations suggest that high HbF values more than transfusion numbers are critical for developing severe ROP.[40,53] For the sample size calculation, we considered data on severe ROP incidence recorded in the Vermont Oxford Network dataset, at our Neonatal Intensive Care Unit (NICU),[40,49,53] and in previous studies.[12] In the current study, we considered a significant reduction of severe ROP incidence after the treatment (primary endpoint), and therefore the alternative hypothesis: Incidence (Severe ROP | A-RBC) > Incidence (Severe ROP | CB-RBC). In the study design, we accounted for three incidence values, according to different gestational age's strata 38% (24 weeks), 20% (24-25 weeks), and 5% (26-27 weeks). In the treated branch, we expect a reduction of proportion of cases to 10%, 5%, and 1% for the 24, 24-25, and 26-27 weeks stratum, respectively. Considering these three strata and the expected reduction in the treated sample per stratum, we estimated an effect size for the proportion difference of $h = 2\arcsin\left(\sqrt{p_0}\right) - 2\arcsin\left(\sqrt{p_1}\right) = 0.5$, corresponding to a moderate effect size according to Cohen (1988),[63] where p_0 and p_1 are the marginal proportions of severe ROP cases in the untreated and treated subjects, respectively. Without considering multi-centricity, with a significance level of 0.05, and a power of 0.8, a sample size of n = 2×31.3 ≈ 63 subjects would be sufficient to detect a moderate effect (h = 0.5). Considering a mortality rate of 15% during the study period, n is increased to 2 (31.3 + 0.15×31.3) ≈ 72 subjects. Including the random effect due to the 6 centers involved in this study, using a Cochran-Mantel-Haenszel test,[64] with the same proportions, significance level, and power as the previous test, we calculated a total sample size of **146 subjects (73 per arm)**.

4.2.2. Sample size adjustment. Should the results of interim analysis (see next paragraph) highlight that the planned sample size is not adequate to the study power, it will be re-evaluated. The adaptive design of this study was considered justifiable by the steering committee for several reasons: first, the innovative experimental hypothesis makes difficult to get a precise estimate of the extent of clinical effects of cord blood transfusions. In addition, no pilot studies providing preliminary data are available so far. Finally, considering that the study was meant to address an unmet need in a very fragile category of patients, it is highly desirable to avoid running an under-powered trial.

4.3. INTERIM ANALYSIS. An interim analysis has been planned with a twofold objective to confirm the safety of CB-RBC transfusions and re-evaluate the planned sample size. For these purposes, we scheduled a preliminary analysis when the first 58 enrolled patients are evaluable for the primary outcome. This estimation was performed considering the above reported reduction of the ROP incidence in different patient strata, and applying a proportion test [65] with fixed h = 0.5, significance level of 0.1, and power of 0.8. This resulted in a sample size of n = 2×25 = 50 neonates (25 per arm) which was further increased to 58 total patients (29 per arm) taking into account a mortality rate of 0.15.

4.3.1. Interim analysis management. When 58 patients have been discharged or have reached the 40° week of PMA (which occurs first), data will be analyzed by an independent third-party (a scientist from the Bioinformatics team of Fondazione Policlinico A. Gemelli IRCCS who are not directly involved in the study). If no severe adverse events related to the treatment occur (as defined by article 2 of Directive 2001/20/EC), and data suggest a clinical response that warrants further investigation, the study will proceed. Moreover, should data raise a doubt on the dependability of the planned sample size, this will be adjusted to ensure the desired study power.[66] Finally, on the basis of results at interim analysis the study duration will be revaluated.

4.4. STATISTICAL ANALYSIS.

4.5. Descriptive statistics. Continuous variables will be expressed as median with relative interquartile range (IQR) and categorical variables as n (%).

4.6. Outcome analysis. Based on the above-mentioned design, the comparison of the proportion of patients free from severe ROP and other comorbidities is made between A versus B through Cochran-Mantel-Haenszel test. The association between HbF (expressed as the median value of repeated measures collected from birth to PMA of 32 or 36 weeks) and risk for prematurity-associated diseases (ROP, BPD, NEC, IVH) is investigated by logistic regression analysis and expressed as an odds ratio with a relative 95% confidence interval (95% CI). The best predicting cut point value of HbF will be identified for each condition by the AUROC (Area Under the Receiver Operating Characteristics) analysis. Interval between two consecutive transfusions and hematocrit increase after CB-RBC or A-RBC transfusions in the two arms are compared by the Mann-Whitney U test.

4.7. Analysis of *in vitro* study data. Regarding the in vitro study, bioinformatics analysis of RNA expression data is carried out by Ingenuity Pathway Analysis and Differential Expressed Gene analysis.

4.8. ANALYSIS SETS. The analysis will be carried out in three different sets.

a. All enrolled patients, independently if they were or not transfused
b. All enrolled patients receiving transfusions, including major protocol deviations (i.e. patients in the CB-RBC arm receiving one or more A-RBC transfusions due to CB-RBC transfusion unavailability)

c. All enrolled patients receiving transfusions, excluding major protocol deviations (i.e. patients in the CB-RBC arm receiving one or more A-RBC transfusions due to CB-RBC transfusion unavailability)

5. ETHICS AND DISSEMINATION

5.1. RESEARCH ETHICS APPROVAL. The study is conducted with the approval of the Ethics Committee, after verification of compliance with the European Union Clinical Practice Standards and in accordance with ICH Good Clinical Practice (GCP) and the ethical principles expressed in Declaration of Helsinki. The study is carried out adhering to local legal requirements and the applicable national law, whichever represents the greater protection for the individual. Study protocol, patient information and informed consent are submitted to the appropriate Ethical Committees for approval. Ethical Committees are informed about any relevant changes in the study protocol which could interfere with the patient's safety.

5.2. PROTOCOL AMENDMENTS. Is the PI responsibility to communicate any protocol amendments (e.g., changes to eligibility criteria, outcomes, analyses) to investigators, EC/IRBs, trial participants, trial registries, and regulators.

5.3. INFORMED CONSENT FOR PARENTS. Parents of eligible neonates, adequately informed in clear, simple and understandable words of the technical terms used, are invited to provide written informed consent. Parents are provided with a description of the general aims of the research, the methodology, the procedures, the indication of any benefits or possible risks and adverse effects. In addition to the consent to participate to the study, all parents have to sign the consent to receive blood products in use at each center. The physicians treating neonates are responsible for information of the parents and for obtaining of the Informed Consent. In the event that parents revoke the consent to the processing of data for research purposes, data collected are not included in the analysis. Finally, in accordance with the law on the protection of personal data (Legislative Decree 30/6/2003 No. 196, Guidelines for the processing of personal data in the context of clinical trials of medicinal products - 24 July 2008 - OJ No. 190 of August 14, 2008; 2016/679 European Regulation, as well as the Deliberation of the Guarantor (Del.52 of 24/7/08), parents are required to sign specified consent for dealing personal data of neonates. Centers of experimentation, in accordance with the responsibilities established by the rules of good clinical practice (legislative decree 211/2003), process personal data, especially those on health and, only to the extent that they are indispensable in relation to the objective of the study, other data related to the demographic characteristics, exclusively according to the realization of the study.

5.4. CONFIDENTIALITY. All subject related information including records, reports, etc. will be kept strictly confidential. All records are kept in a secure, locked location and only research staff have access to the records. Subjects are identified only by means of a coded number specific to each subject. All computerized databases identify subjects by numeric codes only, and are password protected. Upon request, subject records can be made available to the study audit, monitoring representatives of the study promoter, or representatives of regulatory agencies (CNS, Ministry of Health).

5.5. ACCESS TO DATA. Only people officially registered as study investigators or data manager can receive a user login to access the REDCap web platform and enter/manage data. An exception is made for the Bioinformatics team responsible for the interim analysis, who have direct access to data limitedly for this purpose. Source documentation should support the data collected on the CRF's. Source documents include all recordings of observations or notations of clinical activities and all reports and records necessary for the evaluation and reconstruction of the clinical history.

5.6. INSURANCE. After study approval by local EC, the sponsor stipulates an insurance policy with Lloyd's Insurance Company S.A to cover all risks connected with CB-RBC therapy. This policy covers all neonates enrolled in this trial.

5.7. DISSEMINATION POLICY. Investigators are responsible for communicating trial results to participants, healthcare professionals, and the public through scientific publications. Authorship eligibility is defined according to ICMJE guidelines.

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ANNEX 1 - BORN STUDY

PRODUZIONE DI EMAZIE CONCENTRATE DA SANGUE DI CORDONE OMBELICALE

Questo documento descrive le modalità di produzione e la validazione delle emazie concentrate da sangue di cordone ombelicale (ECC), ottenute dopo filtrazione delle unità di sangue di cordone con il filtro BioR Flex 01 BBS (Fresenius Kabi) e successiva scomposizione automatizzata con Compomat G5 (Fresenius Kabi).

A. Criteri di idoneità delle unità di sangue di cordone ombelicale: tutti i seguenti

1. Idoneità a donazione allogenica oppure
2. Scartate da donazione allogenica ma solo se per patologie autoimmuni o fecondazione assistita eterologa.
3. Raccolta da meno di 44 ore (la produzione di ECC deve avvenire entro 48 ore dalla raccolta);
4. assenza di coaguli/emolisi al controllo visivo;
5. volume SCO ≥67 mL (incluso l'anticoagulante);
6. ematocrito pre-manipolazione ≥33%.

B. Materiali e apparecchiature

1. Forniti dalla Fresenius

- ✓ Scompositore automatizzato Compomat G5
- ✓ Filtro per emazie BioR Flex Fresenius
- ✓ Sacche CompoFlex 4F RCC Storage System
- ✓ SAG-M Solution 200 mL (da aliquotare in transfer bag)

2. A carico della Banca del Cordone

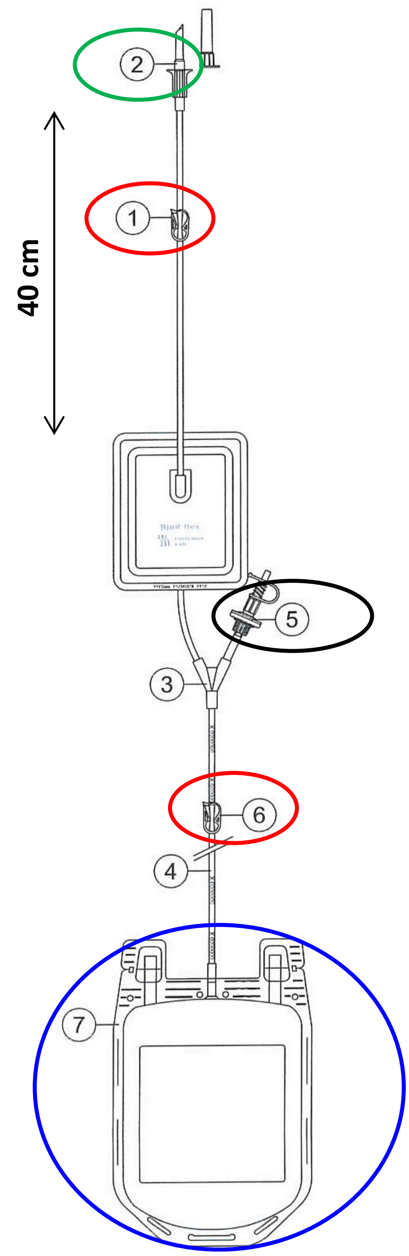
- ✓ Cappa a flusso laminare
- ✓ Centrifuga per sacche
- ✓ Connettore sterile per sacche
- ✓ Saldatore per sacche
- ✓ Transfer bag da 150 mL o volume inferiore
- ✓ Altri materiali: guanti, siringhe, aghi, sterile sampler ("spike"), garze
- ✓ Roller clamp
- ✓ Soluzione fisiologica

C. Istruzione operativa

C1. Valutazione dell'unità e filtrazione

Eseguire un campionamento per emocromo completo. Se l'ematocrito ≥33%, procedere come illustrato di seguito.

Figura A



✓ Attendere che l'unità SCO raggiunga la temperatura ambiente

✓ Prendere il filtro BioR Flex Fresenius (**Fig. A**)

✓ Dopo l'apertura del kit, chiudere le clamp del filtro indicate con **(1)** e **(6)** nella figura A

✓ Prendere un kit CompoFlex 4F RCC Storage System (**Fig. B**)

✓ Rimuovere la sacca di raccolta del kit del filtro BioR Flex **(7)**, sostituendola tramite connessione sterile con una sacca del kit CompoFlex 4F RCC Storage System (4 sacche da 150 mL preconnesse - **Fig. B**) che verrà posizionata mantenendo la stessa distanza dal filtro della sacca rimossa.

✓ Connettere tramite connessione sterile l'unità SCO al filtro al di sotto dello spike **(2)**, **garantendo che il tubo che connette la sacca SCO e il filtro sia lungo almeno 40 cm**

✓ Appendere la sacca ad un'altezza di circa 180 cm

✓ Aprire le clamp **(1)** e **(6)** per permettere la filtrazione per gravità (richiede circa 4-5 minuti)

✓ Quando il filtro si è svuotato aprire la valvola al di sotto del filtro **(5)** per svuotare la linea

✓ Chiudere la clamp **(6)** sopra la sacca di raccolta, allocandola il più vicino possibile alla sacca.

✓ Mescolare gentilmente la sacca di raccolta. Non è necessario rimuovere l'aria presente all'interno

✓ Saldare la sacca di raccolta allo stesso punto di saldatura iniziale mantenendo la clamp (6) vicino alla sacca,

✓ Staccare la sacca di raccolta dal filtro e pesarla.

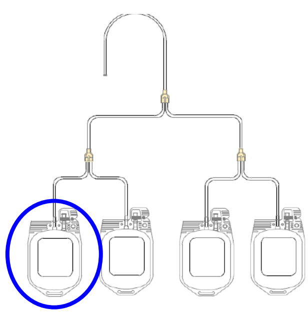


Figura B

C2. Scomposizione automatizzata e risospensione in SAG-M

✓ Prendere una transfer bag e rimuovere lo spike lasciando la massima lunghezza del tubo

✓ Connettere sterilmente la transfer bag alla sacca SCO filtrato che porta con se la clamp

✓ Recuperare tutto il prodotto filtrato nella sacca di raccolta (eventualmente aiutarsi con lo "stripping" del tubo) e posizionare la clamp il più vicino possibile alla sacca dello SCO filtrato

✓ Chiudere la clamp e avvolgerla con della garza

✓ Preparare l'unità per la centrifugazione posizionando la sacca tra due transfer bag riempite con soluzione fisiologica (è possibile utilizzare transfer bag da 150 mL o 250mL per allocare le sacche in maniera adeguata a seconda del cestello utilizzato).

✓ Posizionare l'unità nel cestello e bilanciare la centrifuga

✓ Sottoporre l'unità a centrifugazione con i seguenti parametri di impostazione:

2979 *g*, 10 minuti, accelerazione 7*, senza freno, temperatura 20-24°C.

* NB per centrifughe in cui non è possibile settare il parametro "accelerazione" attivare l'ARC (Automatic Rate Control)

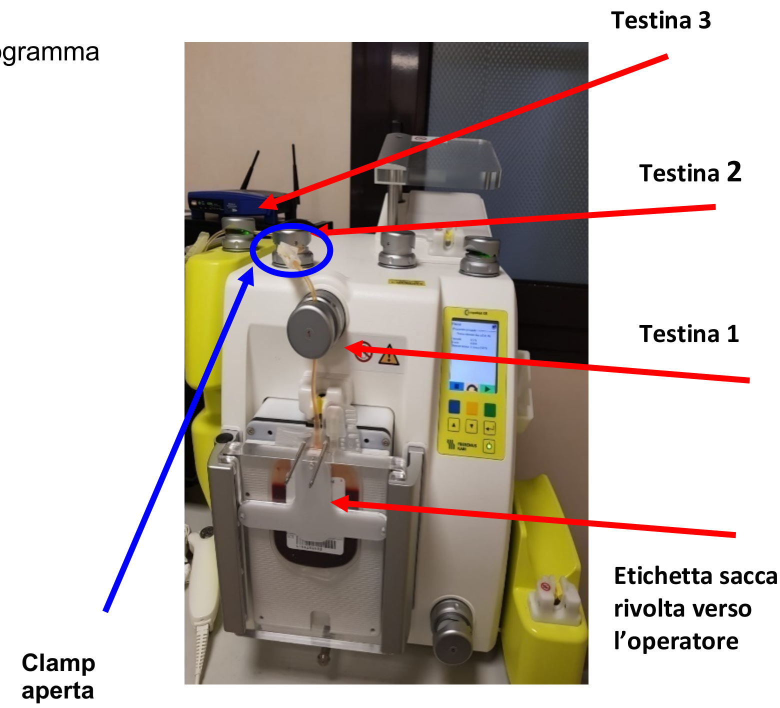
✓ Durante la centrifugazione accendere il Compomat G5 e selezionare il programma di scomposizione **sulla base del volume dello SCO pre-filtrazione**:

– **programma 33** per unità di sangue di cordone con volume compreso **tra 67-79 mL;**

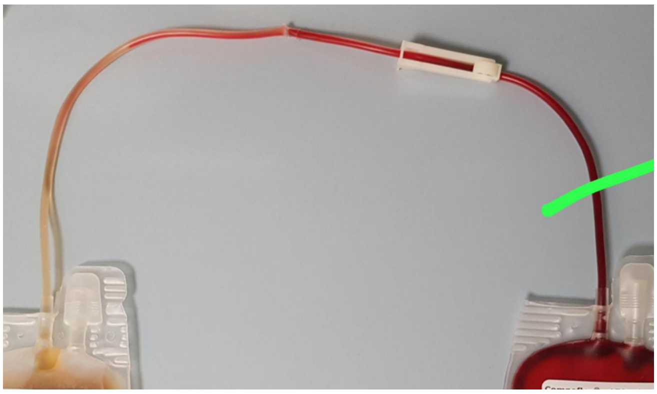
– **programma 26** per unità di sangue di cordone con volume **≥80 mL**;

✓ Posizionare la sacca centrifugata sul CompoMat G5, con le etichette della sacca verso l'operatore, facendo passare la tubistica nella testina 1, 2 e 3 e poggiando la sacca di scarto accanto alla testina 3.

✓ Aprire la clamp e avviare il programma



✓ Al termine della procedura staccare la sacca contenente le emazie concentrate e pesarla (**N.B: tanto minore è il volume di partenza dello SCO tanto meno plasma dovrà essere recuperato: il segno verde identifica un punto corretto di saldatura**)



✓ Pesare la sacca di emazie concentrate (orientativamente, la tara della sacca con tubo come da foto è di 15 g) e calcolare il volume delle emazie concentrate

✓ Connettere sterilmente una sacca con SAG-M (precedentemente aliquotato) e aggiungere nel rapporto: emazie: SAG-M=2:1.

✓ Eseguire il campionamento sul prodotto finale utilizzando il tubicino

– emocromo completo
– emoglobina libera su surnatante
– conta dei globuli bianchi residui.

✓ Eseguire le emocolture **utilizzando lo scarto di plasma** ottenuto dopo scomposizione.

C3. Campionamento a fine conservazione (+14 giorni dalla produzione dell'unità di emazie)

✓ Per le unità che non vengono trasfuse, a fine conservazione (14 giorni dalla produzione) eseguire i seguenti controlli

– emocromo completo
– emoglobina libera su surnatante

D. Criteri di validazione delle unità di emazie concentrate

Al fine di procedere alla validazione delle unità di ECC utilizzabili ad uso clinico, si utilizzano i seguenti criteri di validazione

Test	**Tipologia di campione**	**Valore soglia**
Emocromo pre-manipolazione	SCO pre-filtrazione	Ematocrito ≥33%
Fenotipo AB0 (test diretto)	SCO pre-filtrazione	NA Non è possibile eseguire la prova indiretta dell'AB0, come richiesto dalla normativa vigente
Fenotipo Rh completo	SCO pre-filtrazione	
Antigene Kell (se applicabile, ricerca antigene Cellano)	SCO pre-filtrazione	
Test di Coombs diretto	SCO pre-filtrazione	NEG
Controllo gruppo sacca	ECC	Concorde con tipizzazione SCO
Emocoltura aerobi	ECC-plasma	NEG
Emocoltura anaerobi	ECC-plasma	NEG
Emocoltura miceti	ECC-plasma	NEG
HBsAg	Sangue materno	NEG
Anticorpi anti-HCV	Sangue materno	NEG
Test sierologico per la ricerca combinata di anticorpo anti HIV 1-2 e antigene HIV 1-	Sangue materno	NEG
Anticorpi anti-Treponema Pallidum (TP) con metodo immunometrico	Sangue materno	NEG
HBV-DNA	Sangue materno	NEG
HCV-RNA	Sangue materno	NEG
HIV-RNA	Sangue materno	NEG

E. Requisiti informatici

Variabili in relazione al sistema informativo utilizzato

In caso EmoNet possono essere usati come parametri di configurazione

- ✓ Profilo di frazionamento unità SCO: da eseguire fino a 48 ore prima della raccolta
- ✓ Unità di ECC: scadenza a 14 giorni dalla produzione

In caso di **irradiazione** non si modifica la data di scadenza, poiché eseguita al momento dell'assegnazione al paziente

✓ Profilo esami per validazione: vedi tabella al punto D

F. Dati del frazionamento delle unità SCO da raccogliere durante lo studio nelle e CRF dedicate alle unità

Dati pre-frazionamento		
Identificativo della Banca		
Identificativo unità SCO		
Data e ora di raccolta		
Data e ora inizio processazione		
Volume unità SCO		
Durante il frazionamento	**Tipo di esame**	**Tipologia di campione**
Ematocrito % Emoglobina g/dl MCV fl WBC 10^9/l RBC 10^12/l PLT 10^9/l	Emocromo	SCO pre-filtrazione
Volume emazie post-filtrazione		
Ematocrito % Emoglobina g/dl MCV fl WBC 10^9/l RBC 10^12/l PLT 10^9/l	Emocromo	Emazie concentrate sospese in SAG-M
Globuli bianchi residui WBC/µl	Come da procedura locale	Emazie concentrate sospese in SAG-M
Emoglobina libera	Come da procedura locale	Surnatante delle emazie concentrate sospese in SAG-M
Al termine della conservazione	**Tipo di esame**	**Tipologia di campione**
Ematocrito % Emoglobina g/dl MCV fl WBC 10^9/l RBC 10^12/l PLT 10^9/l	Emocromo	Emazie concentrate sospese in SAG-M
Emoglobina libera	Come da procedura locale	Surnatante delle emazie concentrate sospese in SAG-M

Annex 2

ROP assessment. Diagnosis and staging of ROP will be carried out according to the International Classification, based on an ordinal scale with higher numbers indicating a more severe outcome: 0.1.2.3.3+, 4 and 5.1 Severe ROP indicates stages 3 and higher. ROP is assessed every week from PMA of 31 to 40 weeks.

1. International Committee for the Classification of Retinopathy of Prematurity. The International Classification of Retinopathy of Prematurity revisited. Arch Ophthalmol. 2005 Jul;123(7):991-9.

BPD assessment. BPD is diagnosed by the need for oxygen use during the first 28 days after birth.[1] Definitions of mild, moderate, and severe BPD are based on the National Institute of Child Health and Human Development criteria for preterm infants born before 32 weeks of gestation.[2]

1. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001; 163(7):1723-9.
2. M.C. Walsh, S. Szefler, J. Davis, M. Allen, L. Van Marter, S. Abman, et al. Summary proceedings from the bronchopulmonary dysplasia group. Pediatrics, 2006; 117:S52-S56

IVH assessment. The presence of cerebral hemorrhage is weekly assessed by ultrasound scan and graded between 0 and 4 according with the Papile /Bowerman criteria[1,2]

1. Papile LA, Burstein J, Burstein R, Koffler H. Incidence and evolution of subependymal and intraventricular hemorrhage: a study of infants with birth weights less than 1,500 gm. J Pediatr. 1978 Apr;92(4):529-34. doi: 10.1016/s0022-3476(78)80282-0. PMID: 305471.
2. Bowerman RA, Donn SM, Silver TM, Jaffe MH. Natural history of neonatal periventricular/intraventricular hemorrhage and its complications: sonographic observations. AJR Am J Roentgenol. 1984;143:1041-52.

NEC assessment. NEC is diagnosed and staged according to the modified Bell's criteria.[1,2]

1. Bell MJ, Ternberg JL, Feigin RD, Keating JP, Marshall R, Barton L, Brotherton T. Neonatal necrotizing enterocolitis. Therapeutic decisions based upon clinical staging. Ann Surg. 1978;187(1):1-7
2. Kliegman RM, Walsh MC. Neonatal necrotizing enterocolitis: pathogenesis, classification, and spectrum of illness. Curr Probl Pediatr. 1987;17(4):213-88.

Annex 3

***In vitro* evaluation of effects of CB-RBC and A-RBC surnatants on gene expression profile of endothelial cells.**

This study will be conducted at Fondazione Policlinico Universitario A. Gemelli. Experiments will be performed at cell laboratory of Cord Blood Bank of Policlinico A. Gemelli IRCCS and bioinformatics analysis of data will be carried out at Bioinformatics department of Fondazione Policlinico A. Gemelli IRCCS. The experimental hypothesis is that surnatants collected from CB-RBC and A-RBC may differ for the content of soluble molecules and microparticles released during the storage, resulting in different Damage Associated Moleculars Patterns. The expression of genes involved in pathways of inflammation, vessel tone regulation and oxidative stress response will be investigate in endothelial cells exposed to CB-RBC or A-RBC surnatants. Surnatants collected at different time of storage will be evaluated.

Considering the explorative nature of the study, a formal sample size calculation has not be performed. However, a minimum number of 16 pairs for each RBC type and relative controls will be analyzed. Circulating endothelial progenitors will be grown from cord blood mononuclear cells using the units donated at the Blood Bank of the same Hospital. CB units unsuitable for either transplant of transfusion purposes will be used. Endothelial cells (EC) will be obtained from CB units and confluent layers at passages II will be used. Packed RBC will be obtained by fractionating whole blood units from adult donors or CB units from full term infants as reported in the Annex 1. Both packed RBC types will be stored at +4°C for 7 days. Then, cord and adult packed RBCs will be centrifuged to collect the supernatant. EC will be exposed for 6 hours to 7-day supernatants from cord or adult RBCs units, or to culture medium, as a control. Total RNA will be extracted and reverted using RT^2 First Strand Kit (Qiagen). Gene expression profile will be assessed by commercially available PCR Arrays for genes of endothelial activation (Human Endothelial Cell Biology RT2Profiler PCR Array, PAHS 015Z, Qiagen), and oxidative response (Human Oxidative Stress Plus RT2Profiler PCR Array, PAHS 065Y, Qiagen), as previously reported.[55] Each array includes primers for 84 test genes and five housekeeping genes.

Bioinformatics analysis of RNA expression data will be carried out by the Ingenuity Pathway Analysis software. The mean gene expression value will be considered as input for a heatmap plot by the online tool Heatmapper (Clustering method: Complete Linkage, Distance Measurement Method: Spearman Rank Correlation). In silico analyses with Ingenuity Pathway Analysis (IPA, Qiagen) will be performed to decipher upstream regulators, i.e. likely regulators that are connected to dataset genes through a set of direct and indirect relationships. Results will be expressed as Z-score (i.e. the match of observed and predicted up/ down regulated patterns).[55]

The study will be conducted in agreement with the following ICH Harmonised Tripartite Guidelines for Good Clinical Practice, the EU Directive 2001/20/EC, 2005/28/EC and the Declaration of Helsinki.

The study will be submitted for approval of the Ethics Committee of Fondazione Policlinico A. Gemelli IRCCS as an annex of the clinical study “Umbilical or adult donor RBC to transfuse extremely low gestational age neonates. A randomized trial to assess the effect on ROP severity” version 2 (June 2, 2021) and will start after the approval.

Annex 4. Study flow diagram