Universitat Autònoma de Barcelona

Umbilical Cord Blood Platelet Rich Plasma derivatives for therapeutic applications and development of clinical trials for treatment of skin and ocular ulcers

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Universitat Autònoma de Barcelona

PhD THESIS

Title:

Umbilical Cord Blood Platelet Rich Plasma derivatives for therapeutic applications and development of clinical trials for treatment of skin and ocular ulcers

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The future belongs to those who believes in the beauty of their dreams.

Eleanor Roosevelt

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Abbreviations:

BST – Banc de Sang i Teixits

CT – clinical trial

CB – cord blood

CBB – cord blood bank

CBED – cord blood eye drops

CBPG – cord blood platelet gel

GVHD – graft versus host disease

PB – peripheral blood

PBMC – peripheral blood mononuclear cells

PC – platelet concentrates

PG – platelet gel

PL – platelet lysate

PPP – platelet poor plasma

PR – platelet releasate

PRP – platelet rich plasma

Index:

Abstract

Background: There are many advantages to using cord blood (CB) as a source of therapeutic platelet and plasma derivatives for regenerative medicine. These include availability, universal use, young donor source, and virally safe biological material, rich in tissue regenerative factors. CB platelet rich plasma (CB-PRP) derivatives have been investigated as potential therapeutic agents for the treatment of diverse conditions including ocular surface disease and skin ulcers

Material and methods: The aim of this thesis is to validate a bioprocess design for the production of cord blood-derived platelet concentrates (CBPC) in a public CB Bank (BST method). CBPC was defined as a product of 10±5 mL, 1,000±200×10E9/L total platelets, free of erythrocytes and leukocytes. A total of 300 CB units were centrifuged in two steps to enrich for platelets, in compliance with GMP. The samples were tested for the degree of platelet activation present, and the levels of growth factor were analyzed to evaluate their function. CBPC were then activated after thawing with 10% calcium gluconate to generate platelet gels (CBPG) to treat patients with diabetic foot ulcers. Additionally, the molecular characteristics for trophic, angiogenic factors and cytokines of different preparations derived from CB-PRP (platelet poor plasma: CB-PPP; platelet lysate: CB-PL; platelet releasate: CB-PR) were assessed to evaluate their most appropriate clinical application based on functional and immunomodulatory profiles. Finally, a case study evaluating 46 eyes unresponsive to conventional treatments and requiring urgent interventions, who received allogeneic eye drops obtained from CB-PL (CBED) to treat severe ocular surface lesions under compassionate use was performed

Results: A total of 84% of the processed CB units fulfilled the acceptance criteria. Final products contained 1,017±149×10E9 platelets/L in 10±3mL of plasma. Platelet recovery was 50±9%. BST method ensures depletion of white and red blood cells. Platelets showed low levels of activation during processing, but were significantly activated after thawing, further test shows that product are rich in growth factors. For clinical evaluation, a total of 21 CBPG were applied in 3 patients, with no reported adverse events and improvement of ulcers in all of them. Further was investigated the immunomodulatory function of CB-PRP derivatives, for this was used adult PBMCs with CB-PRP preparations that dramatically reduced the activation of NK, NKT and T cells. Of the three preparations CB-PRP that were investigated, CB-PL and CB-PR have higher concentrations of trophic (EGF, bFGF, HGF, TGF-β1) and pro-angiogenic (VEGF, PDGF AB/BB, MMP-2,9, TIMP1-4) factors, CB-PPP has the lowest concentration of all measured analytes. Based on these findings CB-PR seems the most suitable starting material for skin wound patches, while CB-PL and PPP could be used to prepare eye drops for severe corneal pathologies and inflammatory conditions such as ulcers or sever dry eye disease, respectively. Additionally, we evaluated clinical application in compassionate treatment on patients with ocular surface disorders in groups with corneal ulcers (neurotrophic, trauma, burns) which showed full and partial ulcer recovery in 25 (78%) and 6 (19%) eyes respectively. One eye (3%) did not respond to treatment. For groups with chronic conditions (dry eye and oGVHD) improvement was reported in 12 (85%) eyes, and lesions worsened on treatment in both eyes (15%) of one patient. No severe adverse events were directly attributed to CBED

Conclusion: BST method for CBPC production is valid and reproducible, and CB-PRP derivatives are rich in trophic and angiogenic factors; with immunosuppression capacity. Promptly available CBPG and CBED resulted in a well-tolerated allogeneic therapy that showed evidence of safety and efficacy in treated patients. These positive results support final steps of clinical scale-up of both CBPG and CBED as a novel medicinal products of CB banks.

Resumen

Antecedentes: Entre las ventajas en el uso de la sangre del cordón umbilical (CB) como fuente de plaquetas y del plasma para la medicina regenerativa se destacan la disponibilidad, el uso universal, los donantes jóvenes y el material biológico viralmente seguro, rico en factores regenerativos. Los derivados del plasma rico en plaquetas de cordón (CB-PRP) se han investigado como posibles agentes terapéuticos para el tratamiento de diversas afecciones, entre ellas las enfermedades de la superficie ocular y las úlceras de la piel.

Material y métodos: El objetivo de esta tesis es validar un diseño de bioproceso para la producción de concentrados de plaquetas derivados de cordón (CBPC) en un banco de cordón público (método BST). El CBPC se definió como un producto de 10±5mL, 1.000±200×10E9plaquetas/L, libre de eritrocitos y leucocitos. Un total de 300 unidades de cordón fueron centrifugadas en dos pasos para concentrar las plaquetas, en condiciones para cumplimiento de GMP. Las muestras se analizaron para determinar el nivel de activación de las plaquetas presentes, y se analizaron las concentraciones de factores de crecimiento para evaluar su función. Las CBPC se activaron después de la descongelación con un 10% de gluconato de calcio para generar geles de plaquetas (CBPG) para tratar a los pacientes con úlceras de pie diabético. Además, las características moleculares de los factores tróficos, angiogénicos y citoquinas de diferentes preparados derivados del CB-PRP (plasma pobre en plaquetas: CB-PPP; los factores obtenidos a partir de lisado de plaquetas de sangre de cordón: CB-PL y los factores obtenidos a partir de liberación de plaquetas activadas: CB-PR) se evaluaron para determinar su aplicación clínica más apropiada sobre la base de los perfiles funcionales e inmunomoduladores. Por último, se realizó un estudio de casos consecutivo, retrospectivo y multicéntrico en el que se evaluaron 46 ojos que no respondían a los tratamientos convencionales y que requerían intervenciones urgentes, y que recibieron colirio alogénico obtenido del lisado de plaquetas de la sangre del cordón umbilical (CBED) para tratar lesiones graves de la superficie ocular en uso compasivo.

Resultados: Un total del 84% de las unidades de CB procesadas cumplieron los criterios de aceptación. Los productos finales contenían 1.017±149×10E9 plaquetas/L en 10±3mL de plasma. La recuperación de plaquetas fue del 50±9%. El método BST asegura la depleción de los glóbulos blancos y rojos. Las plaquetas mostraron bajos niveles de activación durante el procesamiento, pero se activaron significativamente después de la descongelación, pruebas adicionales muestran que el producto es rico en factores de crecimiento. Para la evaluación clínica, se aplicó un total de 21 CBPG en 3 pacientes, sin que se reportaran eventos adversos y con una mejoría de las úlceras en todos ellos. Además, se investigó la función inmunomoduladora de los derivados del CB-PRP, para lo cual se utilizaron PBMC adultas con preparados de CB-PRP que redujeron drásticamente la activación de las células NK, NKT y T. De los tres preparados CB-PRP que se investigaron, CB-PL y CB-PR tienen concentraciones más altas de factores tróficos (EGF,bFGF,HGF,TGF-β1) y pro-angiogénicos (VEGF, PDGF-AB/BB, MMP-2,9, TIMP1-4), el CB-PPP tiene la concentración más baja de todos los analitos medidos. Sobre la base de estos resultados, el CB-PR parece ser el material de partida más adecuado para los parches de las heridas de la piel, mientras que el CB-PL y el PPP podrían utilizarse para preparación de colirio para patologías graves de la superficie ocular y condiciones inflamatorias como las úlceras corneales o la enfermedad grave del ojo seco, respectivamente. Además, evaluamos la aplicación clínica en el tratamiento compasivo en el grupo de pacientes con úlceras corneales (neurotróficas, quemaduras, traumatismos) que mostraron una recuperación total y parcial de la úlcera en 25 (78%) y 6 (19%) ojos, respectivamente. Un ojo (3%) no respondió al tratamiento. Para los grupos con condiciones crónicas (ojo seco y EICH ocular) se reportó una mejoría en 12 (85%) ojos, y las lesiones empeoraron con el tratamiento en ambos ojos (15%) de un paciente. No se atribuyeron eventos adversos severos directamente al CBED.

Conclusión: El método de BST para la producción de CBPC es válido y reproducible, y los derivados del CB-PRP son ricos en factores tróficos y angiogénicos; con capacidad de inmunosupresión. La disponibilidad inmediata de CBPG y CBED dio lugar a un tratamiento alogénico bien tolerado que mostró pruebas de seguridad y eficacia en los pacientes tratados. Estos resultados positivos respaldan los últimos pasos de la ampliación clínica tanto del CBPG como del CBED como nuevos productos medicinales de los bancos de CB.

I. Introduction:

1. Cord blood for therapy: state-of-the art

1.1. Cord blood for transplantation

Umbilical cord blood (CB) is the blood that remains in the placenta after birth. The umbilical cord, which is formed during prenatal development, can reach approximately 50-70 cm in length and 2 cm in diameter. It has a vein that transports nutrients and oxygen-rich blood from the placenta to the fetus, and two arteries, to return deoxygenated and nutrient-poor blood to the placenta. All this is immersed in Wharton's gel, which consists of connective tissue and mesenchymal cells including stem cells (MSCs) [1].

Therapeutic use started in 1988 in France when the first CB unit was transplanted in a child with Fanconi Anemia [2]. This triggered CB collection for therapeutic purposes, with the objective to treat blood disorders, initially, in pediatric patients, due to low cell dose to transfuse. Later adult transplantation was performed by improving clinical and technical protocols [2]. Importantly, this blood source can be collected without any harm to both mother and new-born. The benefits of this allogeneic blood source for hematopoietic stem cells transplantation (HSCT) is a more compatible engraftment compared to other sources for HSCT [3], with the advantages that CB has more accessibility, allows a lower degree of HLA compatibility, and is associated with a lower risk of graft-versus-host disease, [4] among other benefits.

1.2. Cord blood Banks

The first positive results obtained after transplantation promoted the using of CB source together with bone marrow and mobilized peripheral blood sources. As a consequence, the first pilot program of *Cord Blood Banking* (CBB) was developed in United States and Europe in 90th of past century [5–7]. In 1992, Dr. Rubinstein founded the first CBB in New York Blood Centre, USA. Posterior publications of his study group were based on hematopoietic stem cells concentration before cryopreservation and washing after thawing [8].

In 1995 *EUROCORD* was founded by Professor Gluckman with the objective to create international registers for CB transplantation, to coordinate the research for product improvement and to develop prospective trials to define the best clinical practice [9].

In 1998 *International* ***NetCord*** *Foundation* was founded for standardization of production in CBB. NetCord with Foundation of Accreditation of Cellular Therapy (FACT) jointly

published in 2000 first international standards for CBB, which are renewed approximately every three years that defines the structure of a CBB [10].

Nowadays private and public CBBs exists. The private CB is stored for and paid by donor families for their own use, while the public altruistic donations are volunteer for any patient in need. Most of public CBBs cryopreserve preferentially CB units with very large volumes to guarantee their application to adult patients and therefore discard about three quarters of units collected, since the volume correlates with cell content. It is expected that the discard rate will increase in the future, also because the worldwide inventory of about 780.000 CB units in 139 CBBs already serve to most for the patients required a HSCT [11,12].

After more than 30 years, CB properties have not been sufficiently studied. In this sense, there are many new publications on studying its possible applications [13]. Nowadays, it is known that CB components can be used in non-transfusion application like regenerative medicine or immunotherapy as well as in transfusion.

1.3. Cord blood collection and processing

The CB donations are altruistic and voluntary. The Mother's informed consent signature is necessary to proceed with blood collection. The CB is collected by trained midwifes after childbirth using a "closed system": a venepuncture is performed on the cord, which is connected to a sterile bag, allowing the blood to flow by gravity. Average volume to collect is approximately 75 mL. CB can be collected *in utero*, when the placenta is in the utero or *ex utero*, when the placenta is already delivered [14]. Collected CB, as peripheral blood, consist of leukocytes, erythrocytes, thrombocytes and plasma. Additionally, CB has some stem cells types, especially hematopoietic stem cells used for transplantation.

The transport of the CB units between maternities and CBBs is well organized so that they can be transferred at 4ºC and processed within less than 48 hours. Reception of units is performed by trained technicians, who have to check documents, such as the presence of informed consent signature, transportation sheets and obstetric data, and to verify the weight of the blood bags. If the volume is sufficient a sample extraction is required to check the cell count. Only units that accomplish an acceptance criteria are processed for hematopoietic stem cell cryopreservation, which requires volume reduction by plasma and erythrocytes removal and concentration of nucleated cell fraction. This cell fraction is tested for quality control purposes including cell count and potency assays before final storage in liquid nitrogen below -150ºC in controlled conditions. Samples for virology and

microbiology results, as well as for HLA and blood group phenotype determination, are sent to the corresponding laboratories for safety and identity purposes.

After processing, the units remain in quarantine until serology and microbiology reports are ready. The serology analysis are used to determine infections for HIV1/2; B and C Hepatitis; Treponema Palladium (*Syphilis*); HTLV (I/II); Trypanosoma Cruzi (Chagas) by antibody (IgM/IgG), antigen and NAT determinations. The microbiology tests are used to detect aerobic and anaerobic microorganisms, and fungi.

Distribution for HSCT is organised through international registers. Physicians on behalf of their patients should be directed to their local registers to sort out CB units using primary criteria, as cell content and HLA matching. After request, reference samples are thawed, washed and final quality control is performed by testing again TNC, CD34+ cells counts, viability and CFU assay. The secondary criteria used for CB selection depends on disease status, conditions, age and patient weight (kg). Donor and recipient compatibility both in single and double CB transplantation modalities require matching on HLA-A and -B and HLA-DRB1 high resolution with a minimum identity of 4 out of 6 alleles [14].

1.4. Cord blood beyond transplantation

Placental blood contains valuable therapeutic properties and offers the important advantages of donor to recipient higher HLA compatibility, and lower frequency and severity of GVHD. So, why should novel uses of CB be developed? Despite advantages in HSCT there is higher transplant related mortality, longer patient´s blood parameters (neutrophil and platelet) reconstitution, as well as general disadvantages, such as low cell number and cells immaturity. Additionally the expenses for cryopreservation and expensive bank facilities should be taken in account. Recent reports have shown that CB units using is going down and haploidentical transplantation is rising [15]. The high proportion of valuable biological material routinely and aseptically collected, whose donors were screened for transmissible infection markers, offers the opportunity to develop new applications in transfusion and non-transfusion-regenerative medicine [16]. Some applications are described below.

Nowadays, CBBs like ours receive CB units and less than 20% [16] of which are processed for HSC cryopreservation and introduced in CB registry, and less than 1% per year are transplanted. Preferentially, units with high cell doses are used. Nevertheless, not processed CB and banked cell units can be used in different approaches in research of new

applications [17], such as the use of blood components, advanced cell therapy and BioBank, which is responsible for Biomedical Research (**Figure 1**).

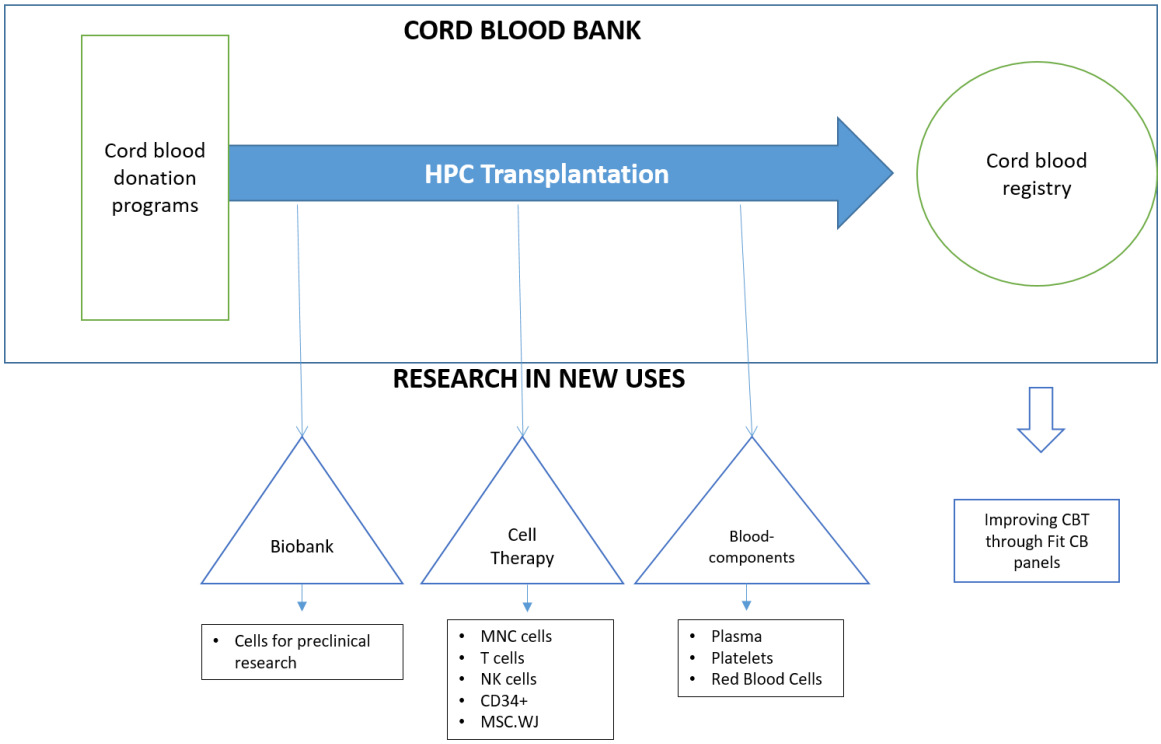


Figure 1. Proposed new cord blood Bank structure

The current perspectives of developing novel reagents and therapeutics from biological materials contained in CB clearly require additional discussion of items such as ownership of CB and its products, maternal and paternal informed consent for the novel uses, for-profit versus not-for-profit processing, and distribution by biotechnology manufacturing industry. Moreover, pathways and procedures should be harmonized at the international level by national competent authorities to regulate the procurement of CB units not suitable for the hematopoietic transplant inventory by industry interested in the development and commercial distribution of novel reagents and therapeutics [17] (**Annex I**).

2. Cord blood derived medicinal products

Once considered as a hospital waste, CB is now a precious source of hematopoietic stem cells to be used in patients affected by malignant and non-malignant diseases. Umbilical CB transplantation (UCBT) in severe blood diseases is regularly performed in paediatric and adult patients [18]. CB has been used also in regenerative medicine, as a treatment for neurologic conditions (autism (Kurtzberg), cerebral palsy (Carrol, Lee), ischemic stroke (Kurtzberg)), in

cardiology (Chronic ischemic cardiomyopathy (Dai)), endocrinology (Crohn's disease (Lee)) [11,17] etc. Other possible applications of CB derived product are illustrated in **Figure 2**.

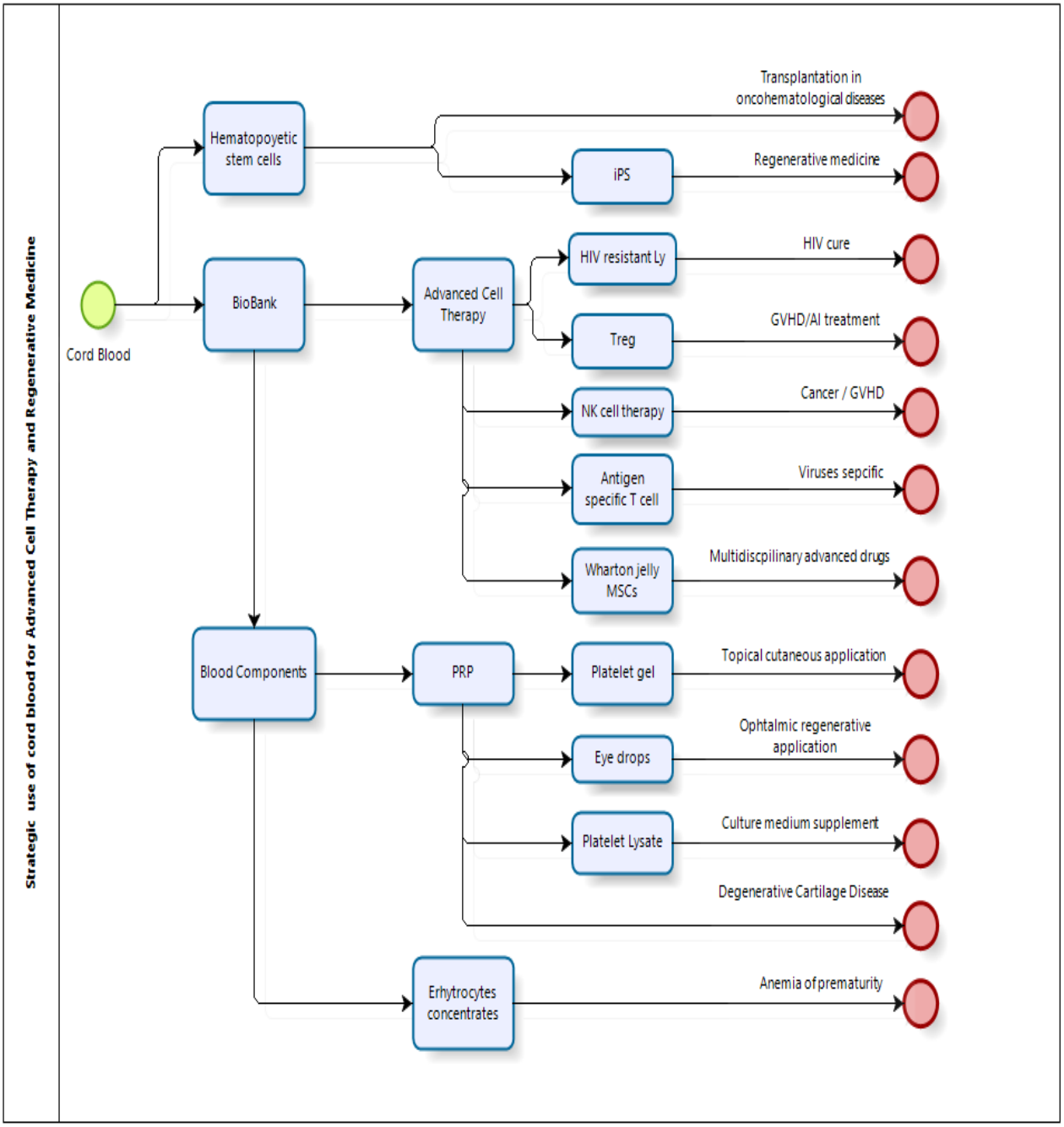


Figure 2. Potential CB applications

2.1. Cord blood as starting material for advanced therapies

CB is a source for development of different human medicinal products. There are a number of proposals, formulated in the scientific literature, about the potential use of CB for medicinal products that cover areas of cellular immunotherapy and regenerative medicine. Examples of advanced cell therapy based on **cryopreserved CB** units as a starting material are:

• *HIV* infection *resistant lymphocytes* after determination of the CCR5 delta 32 mutation (present in 0,6% of the units) that can confer natural immunity to this virus[19].

• *Treg Therapy*: from selected CB units to generate cellular products based on Treg cells for clinical applications. Treg cells have been shown to be useful for the treatment of graft versus host disease (GVHD) and other autoimmune diseases [20].

• *NK cell therapy*: from the selected CB units to generate NK cell products for use in cancer immunotherapy, such as the prevention and treatment of relapses, graft facilitation and reduction of post-transplant complications such as GVHD [21].

• *Antigen specific T-cell bank*: adoptive transfer of T-specific cells has been shown to be viable, safe and effective. There is scientific evidence of a simplified method to manufacture cytotoxic T cells from CB in a single culture in less than 30 days [22]. The method eliminates the need for a live virus or transduction with a viral vector, which makes this approach widely available and applicable to GMP. It can generate cell lines directed to multiple viruses, and is therefore applicable to viral infections that occur after transplantation.

• Banks of *iPS cells from CB progenitors* of frequent homozygous HLA haplotypes: units of CB with homozygous haplotypes could be considered as multi-compatible donors. This may be of interest in regenerative medicine allogenic applications [23]. The incidence of homozygosity for the complete haplotype in a donor panel varies between different countries, from 0,51% to 1,5% of donations in Japan [24]. Such iPSC haplobanks are already available in Japan and Korea [25,26].

• Banks of *mesenchymal stromal cells from Wharton's jelly (WJ-MSC)*: MSCs obtained from Wharton jelly of umbilical cord have a high proliferation valence and they do not produce teratogen or carcinogen after subsequent transplantation [27]. WJ-MSCs have both properties as embryonic and adult stem cells. A recent study demonstrated that spinal cord injury can be improved by WJ-MSC applications [28]. Another publication showed that this therapy improves liver fibrosis in rat model [29]. Therefore, WJ-MSC can be the starting material for the manufacture of advanced drugs therapy [27].

MSCs from CB or amniotic fluid can be used, as well as bone marrow derived MSC, in other applications such as to treat GVHD [30] or can be seeded on biosynthetic scaffolds to treat tracheal defects by generating tracheal cartilage and tendon tissue of diaphragm for bioengineering of trachea [31] and other therapeutic applications [32].

2.2. Multicomponent cord blood bank.

A high proportion of fresh CB units donated and collected in a routine and aseptic way, carefully qualified and selected to detect communicable diseases, and whose data are recorded in fully traceable electronic datasets, offer the opportunity to develop new therapeutic products. We have described above some promising innovative products developed from placental blood components. Given their central position as guarantors of the altruistic procurement of products and their non-profit distribution, public CBB should analyse these opportunities and lead the research and development of their applications.

This principle links very well with the possibility to develop a multicomponent CBB dedicated to CB units collection, fractionation and storage for diverse applications.

Different products with very attractive clinical application can be obtained from fractionations of CB units. The upper component after slow centrifugation is PRP, which can be used for platelet concentration due to beneficial properties in wound healing. This process is described in the next section. The bottom component is the red blood cell concentrate, which is interesting for premature infant transfusion. Premature babies often receive blood transfusions at an early age. In particular, the problem of retinopathy of prematurity (ROP) should be highlighted. A recent article warned that replacing fetal (HbF) and adult (HbA) haemoglobins during transfusion could promote the development of ROP by rapidly increasing the availability of oxygen in the retina. Conversely, maintaining a higher HbF% may be a protection factor against ROP [33]. Also in this context, CB could be safer than adult blood regarding infectious and immunological threats. In Rome, an initial test of red cell bank was performed from units of the CBB that demonstrated safety and efficacy in a clinical cohort [34]. The performance of a sufficiently enhanced clinical trial will likely require collaborative programs between CBB to ensure sufficiently large inventories of CB erythrocyte units to fully match the transfusion requirements of patients. Another program of allogeneic red blood cell transfusion of CB in pediatric patients was developed in the district of Kilisi, Kenya (Hassal, 2015), as a complement to the transfusion of units of red blood cells by local adult donors, not always available, or often not suitable for clinical use. During 12 months, 74 units of CB erythrocytes were transfused in 55 pediatric patients with severe anaemia, without adverse events related to this type of blood component [35].

3. Plasma, platelets and other bioactive components in cord blood

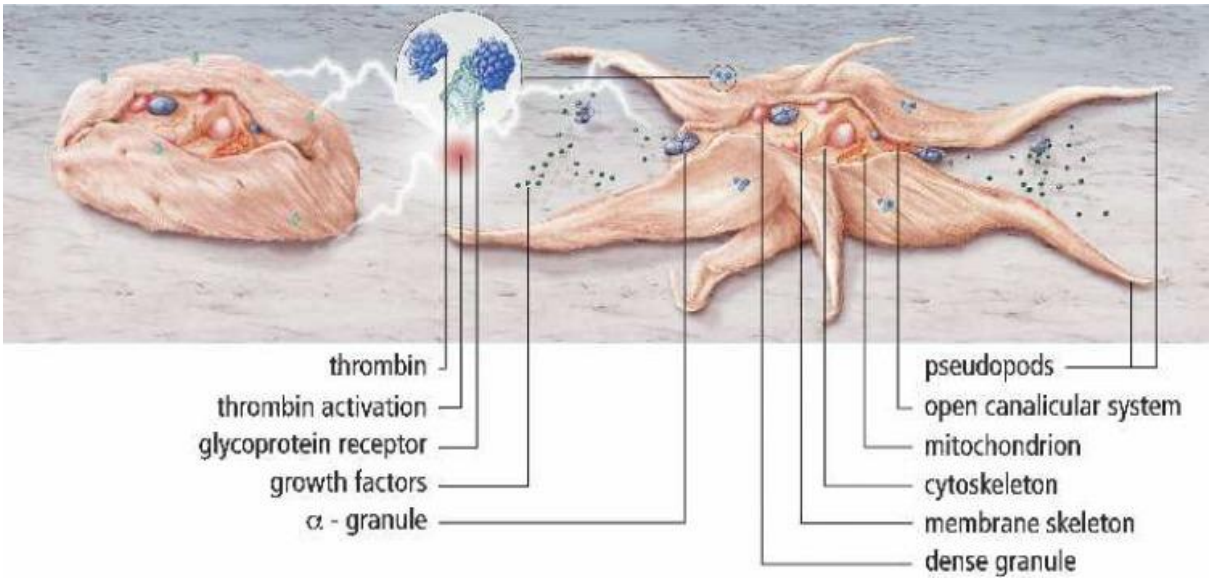
Platelet-rich plasma is a volume of plasma that has a platelet concentration above baseline which was already described by Hematologists in 1970s, with the initial purpose to describe the product to transfusion in thrombocytopenic patients [36]. The first autologous PRP was used in cardiac surgery in 1987. In early 1990s, PRP starts to be used as haemostasis fibrin sealant in open-heart operations [37]. In the 1980s the platelet rich fibrin matrix was used in maxillofacial surgery due to its homeostatic, anti-inflammatory and cell-proliferation effects. Other medicine areas where PRP is being used are in musculoskeletal fields of sport medicine in professionals, paediatric surgery, plastic surgery, genecology, urology, odontology, ophthalmology, and in acute trauma where autologous PRP and gel is spread. Additionally PRP has also been showing positive results of alopecia treatment since 2006 [36]. Different protocols are used to obtain platelet derived factors due to their biological benefits in many areas of medicine. One of the ways to obtain them is from serum, by whole blood collection, coagulation, centrifugation and supernatant – serum – collection [38]. Another method is PRP preparation where the whole blood is collected in anticoagulant solution, such as heparin, citrate buffers and ethylenediaminetetraacetic acid (EDTA) among others. This process is done without coagulation step so the platelets can be collected by gentle centrifugation or concentrated by PRP hard centrifugation [39]. The metabolite profile between preparations from serum and plasma show better reproducibility in plasma samples [40]. The optimized preparation of plasma is performed by collecting whole blood with acid-citrate-dextrose (ACD), and comparing it to EDTA, Heparin, citrate-theophylline-adenosine-dipyridamole (CTAD) or citrate [41].

Normal platelet (Plt) counts in blood range 150-400 x 10^9 platelets/L and average about 200 x 10^9 Plt/L. Today, PRP is defined as a 10(±5)-mL of plasma with a concentration of with 1,000x10^9 Plt/L because of their good scientific results in bone and soft tissue healing [42,43]. Previous studies showed that depending on different centrifuge force and time application, the platelet yield obtained is different. The lower force at 100g during 5 minute had only 17.2±4.2% of recuperation whereas the highest recuperation was on condition 440g during 10 minute with the recuperation of 78.7±5.7%. Controversially, in this condition the number of GFs released (per 10^6 platelets) was minimum indicating that the optimal condition with platelet recovering and growth factors measurement is using less force [39].

The adult and CB platelets markers were studied and no significantly differences were found in many proteins expressed on their surface, indicating that adult peripheral blood platelets can be referenced on analysis of CB platelets [44].

Platelets participate in many non-hemostatic bio-processes, as well as their main role in hemostasis [45], which can include their function in inflammation, immunity and tissue repair [43]. Platelets are small and metabolically active non-nucleated cells, which contain Golgi apparatus, mitochondria, endoplasmic reticulum, many granules and adhesion molecules and numerous surface receptors. Platelets, have mRNA, that is why can synthesize limited amount of molecules [46]. The platelets binds to damaged blood vessels to stop bleeding, as their most important function is to prevent excessive bleeding and take a part in inflammation and immune responses to promote wound healing [47]. Platelets adhesion to extracellular matrix in combination with von Willebrand factors (vWF) connect with platelet glycoproteins (GP) and exposed collagen to form receptor complex. This collagen also binds to platelet GP receptors which provoke platelets activation, following the changing of cell shape and releasing their granules [46] (**Figure 3A**). Platelet activation can be caused by various pathways, there are two principle pathways (**Figure 3B**). The first pathway is interaction between GP VI and collagen induce very strong activation of platelets and releasing of content from their granules [46]. On the other hand, The C-type lectin-2 (CLEC-2) pathway was demonstrated that potentially activates after snake venom application (Batroxombin)[48]. Platelet activation via GP VI and CLEC-2 pathways is through ITAM (immunoreceptor tyrosine-based motif) sequence containing receptors. Many of the platelet functions depends on ITAM signalling. But most soluble agonist, which is released from activated cells, such as thrombin, ADP and others activate platelets through guanine (G) protein coupled receptor (GPCR). The most strong platelet agonist is thrombin and it also participates to convert fibrinogen into fibrin.

A.


thrombin
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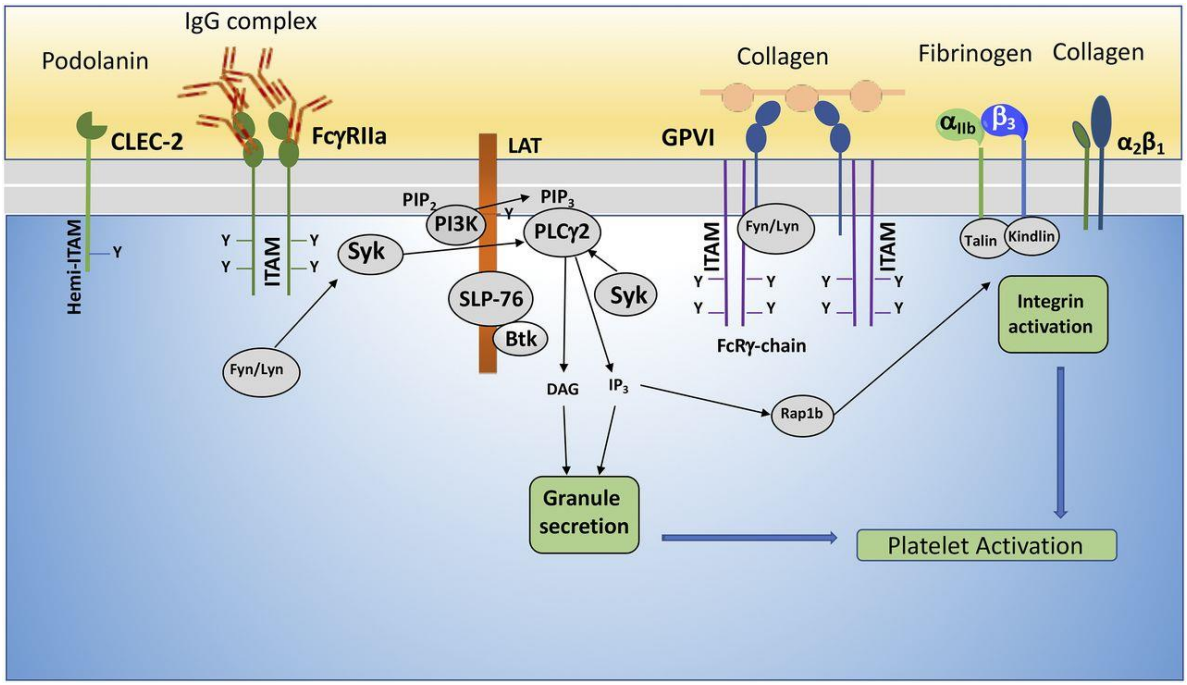
growth factors
α - granule
pseudopods
open canalicular system
mitochondrion
cytoskeleton
membrane skeleton
dense granule

B.


IgG complex
Podolanin
Collagen
Fibrinogen
Collagen
CLEC-2
FcγRIIa
LAT
GPVI
αIIb
β3
α2β1
Hemi-ITAM
ITAM
PIP2
PIP3
PI3K
PLCγ2
Syk
SLP-76
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C.

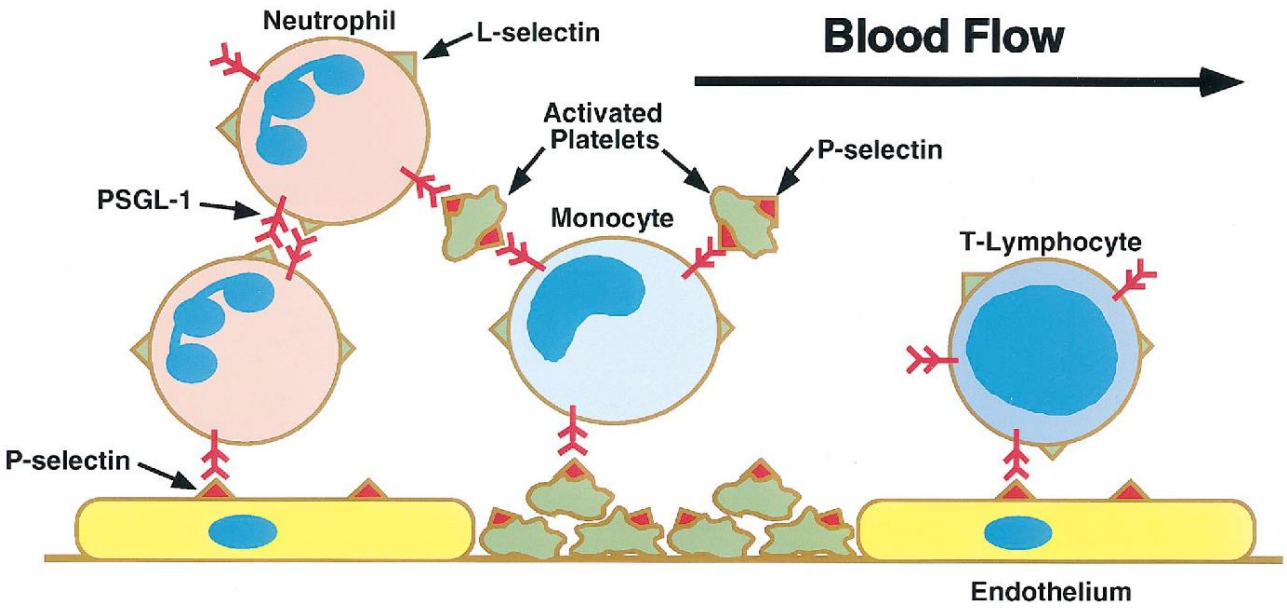

Figure 3: Platelet structure on activation (**A**) [37]; the (**B**) metabolic pathway for alpha granules [49] secretion and (**C**) interaction with immune cells [50].

The activated platelets can interact with other cells which express P-selectin glycoprotein ligand 1 (PSGL-1) such as platelets, neutrophils, monocytes and lymphocytes (**Figure 3C**). This interaction is through mobilization from α-granules to platelet surface – P-selectin (CD62p) molecules. One of the function of platelets, is participating in capture of pathogen by chemokine releasing for neutrophils, with posterior neutrophil extracellular traps formation (NET), which include extrusion of DNA and DNA-associated nuclear proteins [47].

Once activated, it begins releasing platelet granules. There are three types of granules contained in platelets: (a) lysosomes; (b) dense and (c) α- granules[46]. The lysosomes contain acid proteases, glycosidases and cationic acids with their bactericidal function [51]. The dense granules secrets hemostatic molecules, such as serotonin, histamine, ADP, ATP, calcium. But the majority of granules in platelets are α-granules, between 50 and 80 per platelet. The α-granules contains P-selectin, vWF, fibrinogen, coagulation and mitogenic factors, fibrinolytic inhibitors, immunoglobulins, RANTES, growth factors and other molecules [46]. α-granules function is contributing in the hemostasis and in the innate immunity by influencing to leukocytes function [47].

The therapeutic effects of PRP are largely related to growth factors (GF) contained in the platelet granules, especially – transforming growth factor beta (TGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), insulin-like growth

factor (IGF), and nerve growth factor (NGF) which have been shown to contribute to tissue regeneration [52] (**Table 1).**

Table 1. Table GFs in platelet a-granules and their functions [53]

Name	Abbreviation	Function
Platelet derived growth factor	PDGF	Enhances collagen synthesis, proliferation of bone cells, fibroblast chemotaxis and proliferative activity, macrophage activation
Transforming growth factor β	TGF-β	Enhances synthesis of type I collagen, promotes angiogenesis, stimulates chemotaxis of immune cells, inhibits osteoclast formation and bone resorption
Vascular endothelial growth factor	VEGF	Stimulates angiogenesis, migration and mitosis of endothelial cells, increases permeability of the vessels, stimulates chemotaxis of macrophages and neutrophils
Epidermal growth factor	EGF	Stimulates cellular proliferation, differentiation of epithelial cells, promotes cytokine secretion by mesenchymal and epithelial cells
Insulin-like growth factor	IGF	Promotes cell growth, differentiation, recruitment in bone, blood vessel, skin and other tissues, stimulates collagen synthesis together with PDGF
Fibroblast growth factor	FGF	Promotes proliferation of mesenchymal cells, chondrocytes and osteoblasts, stimulates the growth and differentiation of chondrocytes and osteoblasts

The effect of growth factors can vary depending on extracellular environment and how cell types interact. For example, in ocular environment GFs can influence differently than in blood stream.

Additionally, recent studies demonstrate that CB contains higher concentration of hormones and growth factors than PB. Instead PB seems to be richer in proinflammatory factors than CB [54].

Anti-inflammatory properties of CB plasma.

CB plasma contains molecules, which might play an important role in wound healing, such as soluble molecules like the highly polymorphic MHC class I-related chain A and B (MIC A/B) and the unique long 16 binding proteins (ULBP). S. Cox et al demonstrated that soluble molecules of NK cells ligand (sNKG2DL) are present in CB and partly responsible for suppression of NK cells pro-inflammatory function, which may represent a mechanism of fetal-maternal tolerance [55]. NKG2D is the most studied NK cell activating receptor and has diverse ligands, complicating understanding of mechanisms governing NK cell activation. NKG2D interacts with ligands encoded by eight different genetic loci, including MICA/B and ULBP1-6, which are also polymorphic [56–58]. Stress-induced upregulation of NKG2DL expression is sufficient to initiate NK cell activation, degranulation and IFN-g release, therefore inflammatory cascade activation [59]. CB plasma contains detectable levels of soluble NKG2D ligands such as sMIC A / B and sULBP1, which act as immunosuppressive molecules that prevent the reactivity of NK and CD8 + cells [60].

In addition, CB plasmais rich in matrix metaloproteinases (MMP), which have a pro-angiogenic function. MMP are expressed in latent form, and needs activation to participate in initial cascades of wound healing and tissue repair [61]. To regulate function of MMPs, CB plasma also contain inhibitors of MMPs – TIMPs. Recently, was described that systemic factors of CB plasma, as TIMP-2, had capacity to revitalize of aged tissue, such as hippocampus [62].

This anabolic and anti-inflammatory profile and the chance for immediate *off-the-shelf* patient´s access to treatment makes CB an attractive source for treatment.

4. The wound healing

Wound can be defined as damage of anatomical structure and function of tissues, which involves many cell types for wound healing (WH). Wound healing processes occurs differently in normal and pathology states. A normal WH is characterized by an exact replacement while in the pathology WH results in deficiency of healing (i.e. chronic ulcers) or excessive healing (i.e. fibrosis, contractures). The classification of wounds includes acute, chronic or complicated wounds by time of healing. The chronic wounds are increased in elderly population. Those hard to heal wounds develop as a consequence of complex diseases, such as diabetic, vascular or pressure ulcers [63]. The wound healing has some phases, as coagulation and hemostasis; inflammation; proliferation and wound remodelling. Some of the reasons of chronic WH failure are poor nutrition and perfusion, signifying the cells suffering lack of oxygen and nutrients delivered by cardiovascular system. Other reasons such as infections and steroids may have influence for healing. Involving platelets, fibroblast, macrophages and epithelial cells is necessary for wound closure.

5. Cord blood platelet rich plasma derivatives in wound healing

5.1. Mechanism of action

The plasma of CB enriched in platelets may be used in regenerative medicine. Therapeutic properties of platelets in wound healing have been demonstrated in scientific literature [63]. The successful WH depends on proteases, cytokines and GFs interacting [64]. The platelets with their platelets derived factors take an important part in all phases. In the first coagulation and hemostasis phase a release of platelets PDGF, TGF-beta, vasoactive amines, serotonin for vasoconstriction and vasodilatation takes place. In the early inflammation phase, which takes place 24-36 hours after injury, participation of TGF-beta, C3 and C5 components of complement, released from thrombocytes, chemo attract neutrophils, which are phagocyte foreign material and in 48-72 hours the late inflammation phase began. Here some GFs play

a relevant role like PDGF, TGF-beta, leukotriene B4, platelet factor IV, FGF, IL-1 for collagenase regulation, activate keratinocytes, endothelial cells and fibroblast. The next proliferative phase begins after 72 hours and may last about two weeks. In this phase fibroblast migration occur and synthesis of collagen in presence of TGF-beta and PDGF is important. Also in the proliferative phase angiogenesis and granulation tissue formation is observed, playing a central regulatory role presence of molecules, such as FGF, VEGF, PDGF, angiopoetin-1, fibrin, lipid GF, TGF-alpha and beta. The final remodelling phase follows and can last up to 1-2 years. Here which are relevant are GFs like PDGF, TGF-beta and FGF, needed to regulate the process where wound margins becomes closer to each other, owing to fibroblast interaction with extracellular matrix. Finally, platelet derived GFs plays an important role during wound healing, especially FGF and VEGF, which participate in all phases, like production of proteases by endothelial cells; chemotaxis; proliferation; remodelling and differentiation [52,65]. The aim of all this interactions between cells and molecules is finally wound closure (**Figure 4**).

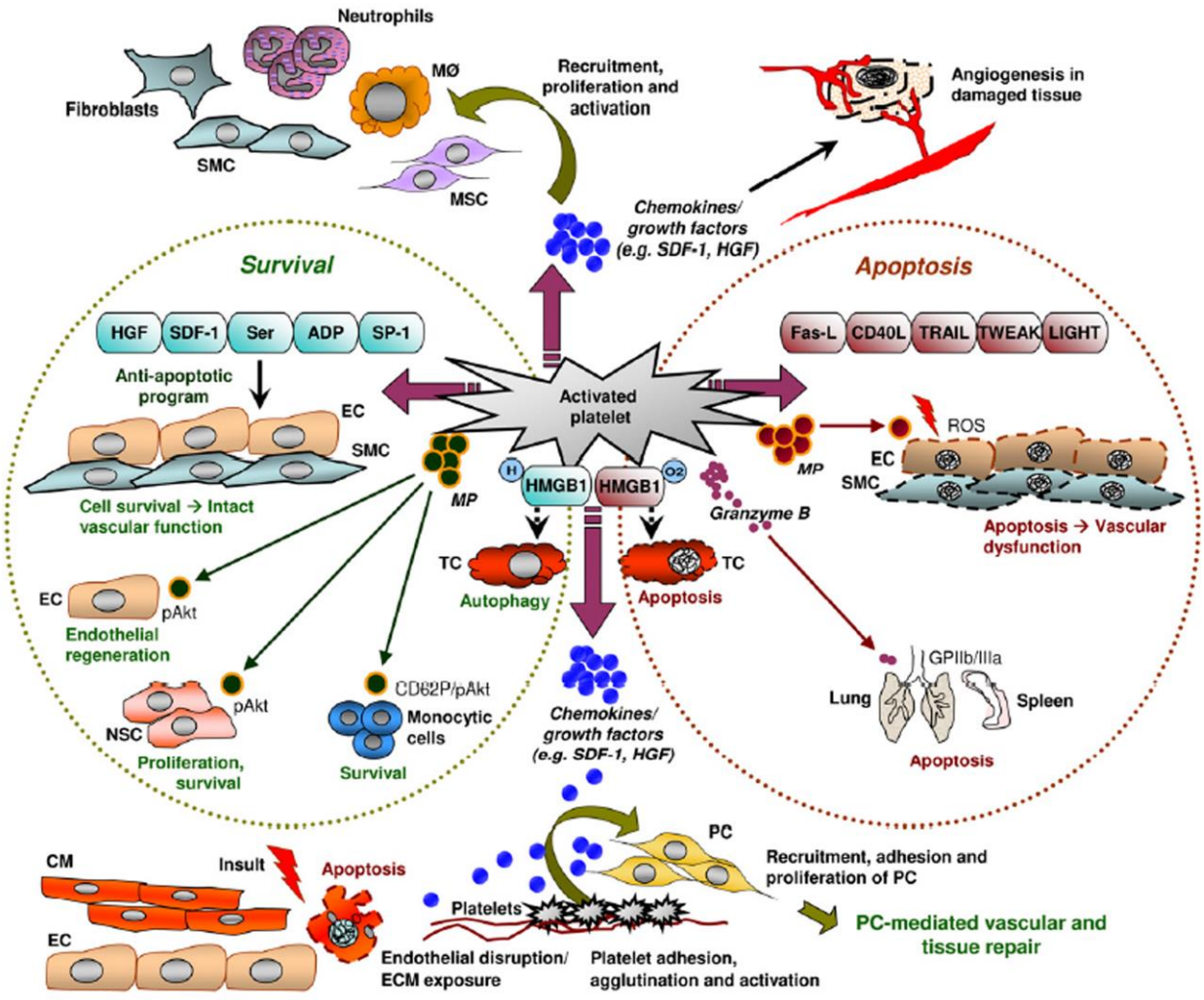

Figure 4. Mechanisms governing platelet mediated tissue repair [66]**:**

5.2. Clinical uses

The PRP may be mixed into a bone graft, layered in as the graft is placed, sprayed on a soft tissue surface, applied on top of a graft, used as eye drops or used as a biologic membrane. However, in case of gel using, clotting of the PRP should be done only at the time of application. Clotting activates platelets, which begin secreting their growth factors immediately [63]. Although peripheral blood (PB) PRP is one of the variants to use for wound healing, the Italian study on proteomic analysis reports that PB PRP is richer in inflammatory factors than CB [67]. CB is an attractive source to collect PRP for clinical use. CB PRP clinical application was also proposed. CB platelets are less studied than adult ones for their role in hemostasis and more recently for their clinical use for wound healing in diabetic patients [68] and in patients with pathologies of ocular surface [69,70].

5.2.1. PRP derived product for skin lesions

Skin is the largest organ of human body, which is characterized by the plasticity and ability to repair its integrity [71], but this healing in some cases requires accelerated results, i.e. big areas involvement; systemic malfunction of some components of wound healing mechanism or chronic ulcers development. PRP can be used when cutaneous integrity is damaged. Studies on scar resolution showed positive results of PRP alone or in complex treatments improvement of scars after burns, post-surgery and acne. This application improves quality of skin, which results in the increase of collagen and elastic fibres. In cosmetic, *in vitro* studies showed that PRP can stimulate human dermal fibroblasts proliferation and increase type I collagen synthesis. PRP injections can induce soft-tissue augmentation, activation of fibroblasts, and new collagen deposition, as well as new blood vessels and adipose tissue formation.

Acute injuries can be developed towards chronic wounds, and are then more difficult to manage, due to its association with skin fibrosis. Chronic wounds is one of the skin lesions more difficult to treat, which mainly develops on heels and often lead to lower extremity amputation. A wound that fails to heal and remains for more than 3 months, is considered a chronic wound [72]. The incidence is rising with aging of population, showing the more affecting after 60 years old. The main reasons of ulceration are arterial, venous disorders, neuropathy and atherosclerotic occlusion (elderly patients), such as diabetes, obesity and smoking among other causes. The ulcers caused by arterial diseases affect the toes and shin. The ulcers based on venous disease develops on malleoli (medial and lateral). In case of neuropathic ulcers, it normally occurs on heel. In all above showed causes, ulcer occurs over pressure points.

The most common chronic ulcers example is diabetic foot ulcer (DFU). On the basis of 2019 prevalence data from the International Diabetes Federation, it is estimated that, annually, foot ulcers develop in 7.3 million people with diabetes worldwide [73] (http://www.diabetesatlas.org). The risk of death at 5 years for a patient with a diabetic foot ulcer is 2.5 times as high as the risk for a patient with diabetes who does not have a foot ulcer. More than half of diabetic ulcers become infected. Approximately 20% of moderate or severe diabetic foot infections lead to some level of amputation. On the basis of outcome data in specialized tertiary care hospitals in Europe, approximately 77% of diabetic foot ulcers heal within 1 year. DFU development depends on many factors (**Figure 5**) [Reviewed in [74].

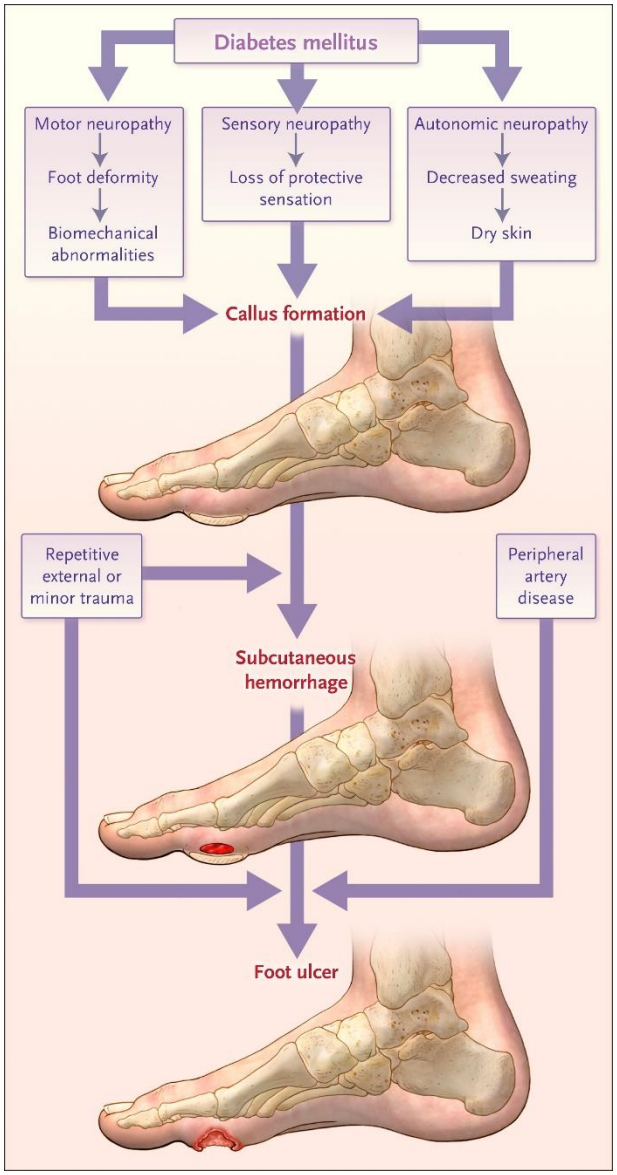

Figure 5. Diabetic foot ulcers development [74]

The DFU depends on its severity classified by Wagner, described in 1970s, comprised in 6 grades. Grade 0 is intact skin; grade 1 includes superficial ulcer development; the 2nd grade is deep ulcer; the 3rd grade is involvement of bone; the grade 4 is forefoot gangrene and the grade 5, where full foot gangrene is present [75].

DFU healing protocol includes cleaning, surgical debridement, off-loading of pressure and vascular restoring (if required). Unfortunately, even after the resolution of a foot ulcer, recurrence is common, which depends on various systemic or local factors. Additionally platelets derived factors can be used to improve this chronic conditions, the early replacement of therapy can be beneficial.

Platelet gel for topical cutaneous applications: the active role played by platelets in tissue regeneration can be delivered by "platelet gel" (PG) application, which can be obtained from autologous or allogeneic adult donors. The biological effects is due to GF trapped in coagula, which release gradually to wound side.

There are many preclinical studies on PRP and PG in dental implants, soft tissue, bone, sinus, muscles and other tissue repair on rabbit, mouse, dog, horse with very confusing results due to physiological differences. Many hospitals from all over the world use autologous gels in surgeries to prevent bleeding, such as cardiac and spinal fusion interventions (reviewed in [37]).

Recalcitrant ulcers of hypmobile patients, which were resistant to standard treatment, treated with PG from pooled allogeneic PC showed a 91±14% size reduction after a 12 week treatment.

Additionally, implementing CBPG therapy has a cost-benefit. This outcome is presented by Greppi et al. (2011), who showed that the use of allogeneic donor platelet gel generated a 90% treatment cost reduction versus conventional treatment and 86% ulcer healing rate in a series of 11 elderly patients affected by pressure ulcers [76].

CB can be also implemented in this area. Small successful preclinical studies in an animal model of pleural injury and clinical studies on the repair of cutaneous ulcers in recessive dystrophic epidermolysis bullosa [77] suggest that the CBPG could represent a valid tool for the treatment of different surgical lesions and skin ulcers.

On chronic ulcer difficult to treat, such as diabetic foot ulcer, CBPG can be used resulting in a 46% ulcer area reduction for Group A and 79% for Group B patients ($p < 0.01$). These observations suggest CBPG application can promote more rapid wound healing than standard care [68].

The bone healing includes GF production by osteoblasts, which as well produces by platelets [78]. Clinical application of PG in reconstructive bone surgery showed positive results in stimulating bone regeneration [79].

Spray application of PRP-derivatives for topical cutaneous applications:

Burns are the damage of skin layers or with involvement of underlying tissues by thermal exposure, which can be classified by degrees [80], which require treatment such as debridement, cooling, cleansing, and proper dressing [81]. Platelet derived growth factors can be sprayed in cutaneous applications. This have advantages, one unit can be applied for more area than gel form. The spray applied on clinical trial with 27 including patients with second degree burns shows significant improvement on PRP spray group compared with placebo. The spray was applied for 4 days. This results indicates that PRP is effective in burns and that spray form is convenient to apply in cutaneous application. The healing rate of PRP group reached nearly 80% and made a breakthrough of 90% in 3 weeks, showing a significant difference compared with the control group ($p<0.05$) [80].

The fibrin sealer was used in cardiac surgery, which was prepared by Vivostat® system. This consists of an automated processor unit, an applicator unit, and a disposable, single-patient–use unit, which includes a preparation set and a Spraypen applicator. For this 120mL of whole blood is required to prepare 4.5mL of sprayed solution. This could provide application within 30 min from blood extraction. Vivostat group had 9% of patients who required a perioperative transfusion while the control group had 25% who required a perioperative transfusion [82], suggesting blood derived components using in this indications is suitable.

5.2.2. Blood derived eye drops for ophthalmic application

The cornea, which takes one-sixth part of the outer wall of the eye, is a transparent, thin, avascular membrane. The basic function of the cornea is to maintain integrity as a protective barrier for ocular content. It is lubricated by tear film, which is produced by ocular glands, and consist of three layers. The upper layer is lipid, the oily component produced by Meibomian gland to prevent tear evaporation and overflow; the middle – aqueous, produced by lacrimal glands through multiple ducts; and the inner – mucin, produced by Goblet cells, which distributed within conjunctival epithelium [83,84]. Most chronic ocular surface pathologies are due to tear film abnormalities, which can occur in case of muco-aqueous deficiency, i.e. Sjögren syndrome, goblet cells destruction, ocular adnexal pathologies, which includes Meibomian glands deficiency and lagophthalmos. The ocular surface integrity and wound healing promotion depends on mechanical and epitheliotrophic characteristics of tear film [85].

In case of dry eye disease (DED), which affects approximately 5-50% of the population in elderly, it occurs due to excessive evaporation by defect of lipid layer or insufficient tear production in aqueous layer, or both [86]. Therefore, integrity of ocular surface is in risk to infection and perforation. The treatment is based on lubrication with artificial tears, hyaluronic acid gels and blood derived products for topical application.

The neurotrophic keratitis is the rare pathology of ocular surface, caused by ocular innervation impairment of trigeminal nerve, which leads to corneal integrity damaging, impairment of healing and following with ulceration, melting and perforation. Those patients have absence of corneal sensation. The causes which lead to neurotrophic keratitis development are intracranial space-occupying lesions, neurological procedures where trigeminal ophthalmic branch can be damaged, herpetic keratitis, chemical burns, physical injuries, corneal dystrophy, chronic use of topical treatments and other systemic conditions, such as diabetes, multiple sclerosis, leprosy and some congenital syndromes which lead to corneal hyposthesia [87]. The main problem of patients with neurotrophic keratitis is that the absence of sensation can provoke worsening such as corneal perforation. There are three stages remarked by Mackie [88]. Stage I includes punctate keratopathy and corneal edema, with the posterior lasting to epithelial hyperplasia, irregularity, neovascularization and stromal scarring. The evolution to Stage II includes persistent epithelial defects with circular shape, which is surrounded with opaque epithelium that can be detached easily with smoothed edges and rolled and stromal edema may also be observed. The Stage III is characterized with corneal ulcer with complications, such as stromal melting, which leads to corneal perforation. (**Table 2**).

Table 2. Mackie Classification for neurotrophic keratitis [87]

Clinical grading of neurotrophic keratitis and management

Stage	Clinical findings	Treatments
I	Corneal epithelial hyperplasia and irregularity Scattered small facets of dried epithelium (Gaule spots) Superficial punctate keratopathy Rose bengal staining of the inferior conjunctiva Increased viscosity of tear mucus Decreased break-up time Superficial neovascularization Stromal scarring Dellen	Discontinuation of all topical medications Use of preservative-free artificial tears Treatment of ocular surface-associated diseases
II	Persistent corneal epithelial defect with smooth and rolled edges Descemet's membrane folds and stromal swelling Anterior chamber inflammatory reaction with hypopyon (rare)	Corneal or scleral therapeutic contact lenses Surgical tarsorrhaphy Palpebral spring, botulinum A toxin injection of the eyelid elevator Amniotic membrane transplantation
III	Corneal ulcer Corneal perforation Corneal stromal melting	Cyanoacrylate glue followed by a soft bandage contact lens Tarsorrhaphy and conjunctival flap Lamellar or penetrating keratoplasty

Persistent corneal epithelial defects can be the cause of several corneal pathologies, such as neurotrophic keratitis, dry eye disease, keratitis due to exposure, ulcers after infection, and limbal cells deficiency. The main reason is the deficiency of some factors, i.e. Vitamin A, epidermal growth factor and neurotrophic factors (Substance P, acetylcholine) [89].

Allogeneic hematopoietic stem cells transplantation can provoke acute or chronic GVHD, which can lead to ocular diseases in about 60% of cases [90], among other complications of multi-organ affectations. Ocular GVHD is one of the most common affectation, which can provoke sever ocular problems. In acute GVHD the inflammation can be related to T-cells response in conjunctiva and lacrimal glands. During the chronic GVHD it seems autoimmune mechanisms involves. The main response is related to excessive IFN-γ expression. Clinically there are signs of excessive fibrosis, collagen deposition, antibody production and suppression of acute inflammation mechanisms. Ocular tissues affected by acute and chronic forms of GVHD include the eyelid and periorbital skin, conjunctiva, cornea, lens, lacrimal system, sclera, uvea, and retina. The main complication of ocular

GVHD is DED (keratoconjunctivitis sicca) [90]. Untreated ocular GVHD can lead to blindness.

There are some urgent sever ocular conditions such as trauma or burns. Ocular chemical burns caused by accidentally introduced corrosive substances to eye and periocular area [91]. It is the emergency which can affect to corneal epithelium and have as an objective restauration of integrity of ocular surface, reduce inflammation, prevention of complications such as scarring and vision loss [92]. Mainly the young population is affected [91].

There are many blood preparations used for topical ocular application, such as serum, autologous and allogeneic PRP eye drops. The use of blood derived therapy was first described in 1975 [93]. Fox et al [94] demonstrated that autologous serum eye drops was effective on patients with DED. Widespread using of autologous serum begins after Tsubbota et al. publishes results on autologous serum treatment of DED and in persistent epithelial defects (PED) [95,96]. There are several studies on dry eye application [97], in PED [98,99], recurrent erosion syndrome [100], chemical injuries [101], superior limbic keratoconjunctivitis [102] and neurotrophic keratitis [87]. Autologous PL used for treatment of refractory ocular GVHD addressed by short-term [103] and long term studies showed efficacies. Moreover no adverse events where observed during a 36 months follow-up procedure [104].

Controversially, the objective clinical measures analysis of the ocular surface treated with autologous serum showed no clear effect, due to data inconsistency [105].

The blood-derived eye drops preparations based on serum/plasma/platelets have therapeutic benefits due to containing many factors and biochemical properties similar to tears (**Table 3**), such as EGF, TGF-β, Vitamin A, fibronectin, pH and other nutrients [85]. Some of them have elevated concentration in blood-derived products (TGF-β and other GFs) (**Table 4**). Autologous serum is the most used preparation worldwide, but there are many contraindication, such as serological positive analysis for HIV, hepatitis C virus etc. Additionally, there are restrictions to perform blood extractions depending on patient gravity (intensive care unit patients), existing of hematological contraindication (anaemia), elderly patients (the vein thinness) or too young patients, among other autologous eye drops preparations inconveniences, such as time for extraction (several visits required), microbiological analysis lasting (14 days) and products variability between each patient. Allogeneic blood-derived components for eye drops preparations was suggested in many publications, which can be processed, tested and stored “ready-to-use”. It is known that conjunctival and corneal surface contains antigens for AB0, which

can lead to inflammation [106]. However many studies did not match the blood groups between donor and patients, and no adverse events reported [85].

The main difference between serum and plasma derived eye drops preparation is in presence/absence of anticoagulant, such as heparin or citrate-phosphate-dextrose solution (CPD), which is commonly used for plasma/platelet preparations for transfusion. Additionally, study on corneal epithelial cells show that diluted platelet releasates products did not present toxicity for human corneal epithelial cells [107].

Table 3. Comparison of tear and serum composition [97]

	Tears	Serum	References
pH	7.4	7.4	Tsubota et al. (1999b), Geerling et al. (2004), López García & del Castillo (2011) and Pan et al. (2013)
Osmolarity	298	296	
EGF (ng/ml)	0.2–0.3 1.9–9.7	0.5	
TGF-β (ng/ml)	2–10	6–33	
Vit A (mg/ml)	0.02	46	
Fibronectin (μg/ml)	21	205	
Lysozyme (mg/ml)	1.4	6	
SIgA (μg/ml)	1190	2	
IGF-I (ng/ml)	157		
SP (ng/ml)	0.157	0.071	
NGF (pg/ml)	468	54	

Table 4. Main growth factors present in blood and their role in ocular surface regeneration [97].

Table 1. Main growth factors present in blood and their roles in ocular surface regeneration.

Proteins	Roles	References
EGF	• Induces corneal epithelial migration and proliferation • Stimulates DNA synthesis of epithelial cells and stromal fibroblasts • Stimulates synthesis of fibronectin by epithelial cells • Chemotactic effect for human epithelial and stromal cells • Anti-apoptotic effect • Induces production of mucin 1 by the Goblet cells	López García & del Castillo (2011)
TGF-β	• Decreases keratocyte migration • Favours chemotaxis of fibroblasts • Induces the production of extracellular matrix by stimulating the production of collagen, fibronectin, and proteoglycans and diminishing its degradation by inhibiting the metalloproteases and other proteolytic enzymes • Promotes the differentiation of myofibroblasts • Anti-inflammatory effect	López García & del Castillo (2011)
Vitamin A	• Prevent processes of squamous metaplasia of the epithelia	Geerling et al. (2004)
PDGF	• Chemotactic effect for monocytes, macrophages and fibroblasts • Synergistic effect with TGF-β to promote myofibroblasts differentiation	
Fibronectin	• Promotes wound healing and phagocytosis • Important role on cell migration during the repair process of corneal epithelium	Phan et al. (1987) and Gordon et al. (1995)
Annexin A5	• Stimulates the secretion of the plasminogen activator-type urokinase facilitating cell migration	
Albumin	• Reduces degradation of cytokines and growth factors	Tsubota et al. (1999b), Shimmura et al. (2003) and Unterlauft et al. (2009)
α2 macroglobulin	• Neutralizes the proteolytic enzymes	Tsubota et al. (1999a) and Poon et al. (2001)
bFGF	• Promotes corneal wound healing increasing cell proliferation and motility	Andresen et al. (1997)
IGF-I	• Acts synergistically with substance P to promote corneal epithelial migration	Yamada et al. (2004)
NGF	• Induces neurite sprouting by neural cells • Restores the function of injured neurons • Induces SP and calcitonin gene-related peptide production in the central and peripheral nervous system enhancing epithelial proliferation • Increases epithelial cell proliferation and differentiation • Promotes fibroblast cell growth	Matsumoto et al. (2004)

EGF = epidermal growth factor, TGF-β = transforming growth factor beta, PDGF = platelet-derived growth factor, bFGF = fibroblast growth factor b, IGF-I = insulin-like growth factor 1, NGF = nerve growth factor.

As there are many studies suggesting application of PLs preparations for ophthalmic use [104,108,109], additionally another interesting study showed albumin eye drops (5%) efficacy in persistent epithelial defects and corneal ulcers [110].

The comparative study of autologous serum and CB serum eye drops on sever DED showed to be more effective CB derived product, presenting capacity to goblet cell density increasing compared to autologous serum application results [111].

The use of CB derivatives as medicinal ophthalmic solutions has been previously reported [108,112–114]. In the clinical setting CB derived eye drops shows to be a safe and effective source of neurotrophic factors to treat severe corneal injuries of different origins. Additionally, previous reports and controlled clinical trials describe CB therapeutic superiority when compared to autologous serums in ocular disorders.

5.3. Other application of cord blood PRP derivatives

There are some attractive applications for CB plasma and platelets for biomedical areas due to their trophic properties, such as applications described below:

- Plasma and cord platelets as a *cell and tissue culture medium*: Platelets contain a large number of biological molecules, including coagulation factors and adhesion molecules, enzymes, chemokines, cytokines and growth factors that collectively constitute the so-called "loading of platelet granules", which play an important role in the physiology and pathological processes related to haemostasis, immunity, angiogenesis and tissue repair. Recently, a greater interest in its use was generated due to the concern caused by the traditional use of fetal bovine serum as a supplement in the media for the cultivation of advanced therapy medicinal products, since it carries the risk of xenoimmunization and transmission of non-human pathogens to the recipients of these therapies [115]. The CB constitutes a unique source for its properties for *ex vivo* cell growth. For its development as a culture medium, it is necessary to harmonize the national and international regulatory channels that allow its acquisition and use in different jurisdictions.
- CB PRP is also an attractive product for injections in *degenerative cartilage diseases*, which is characterized by pain, loss of function and substantial social impact. Cartilage has limited regeneration properties. Many studies focused to demonstrate safety and efficacy of PRP treatment for pathological degeneration of cartilage to accelerate tissue repair with beneficial effects in knee, hip and other joints compared to conventional treatment [116].
- CBPG it seems to be effective in treatment of *Pleural tissue repair in vitro* and *in vivo*, the study group Rosso et al. showed positive results with inflammation decreasing and regeneration of damaged tissue after CBPG application [117].
- *Oral mucositis* is severe complications after radio- and chemotherapies on patients who required hematopoietic stem cells transplantation and characterized by painful ulceration of oral mucosa. After eight day application of CBPG with symptoms improvement in single case report [118].
- Many other application can be applied on the fields where peripheral blood PRP is used, such as thin endometrium [119] and others.

II. **Hypothesis**

Altruistic donated cord blood (CB) units collected within the CBB programme and discarded for hematopoietic stem cells cryopreservation can be used to obtain PRP for regenerative medicine, due to their potential biological value. The CB fractionation is feasible in public cord blood banks (CBB). CB-PRP derived medicinal products can be standardized, into a robust and reproducible method, and transformed material can maintain functional abilities *in vitro and in vivo*. Thus, CB-PRP derived medicines are clinically safe and effective in cutaneous (chronic ulcers) and ophthalmic (ocular surface pathologies) topical application.

III. **Objectives**

Main objective:

The objective of the study is to validate a manufacturing procedure, to biologically characterize CB-PRP derivatives and to evaluate their therapeutic potential in cutaneous and ophthalmic clinical indications.

Secondary objectives:

- To validate a standardized production of CB-PRP based in Good Manufacturing Practices and to establish a Standard Operating Protocol (SOP) for routine production.
- To characterize CB-PRP bioactive components in their different derivatives: CB-PPP, CB-PL, CB-PR.
- To evaluate cord blood platelet gel (CBPG) in chronic skin ulcers
- To evaluate clinical aspects of cord blood eye drops (CBED) in complex ocular surface pathologies

IV. Materials & Methods

The results are presented by compendium of publications and includes next manuscripts:

1. Cord blood derived platelet concentrates as starting material for new therapeutic blood-components prepared in a public cord blood bank: from product development to clinical application.
2. Cord Blood Platelet Rich Plasma Derivatives for Clinical Applications in Non-transfusion Medicine
3. Clinical evaluation of allogeneic eye drops from cord blood platelet lysate

Paper 1. *Cord blood derived platelet concentrates as starting material for new therapeutic blood-components prepared in a public cord blood bank: from product development to clinical application*

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PMID: **32281925**

RESUMEN:

Título: Concentrados de plaquetas derivados de la sangre del cordón umbilical como material de partida para nuevos componentes sanguíneos terapéuticos preparados en un banco público de sangre del cordón umbilical: desde el desarrollo del producto hasta la aplicación clínica

Antecedentes: existen múltiples ventajas al usar sangre de cordón umbilical (CB) como fuente de derivados de plaquetas y plasma para la medicina regenerativa. Estos incluyen disponibilidad, universalidad, fuente de donantes jóvenes y material biológico viralmente seguro, rico en factores regenerativos tisulares.

Materiales y métodos: el objetivo de este estudio es la validación de un diseño de bioprocesos para la producción de concentrados de plaquetas derivadas de sangre del cordón umbilical (CBPC) en un banco de sangre de cordón umbilical público. El CBPC se definió como un producto de 10 ± 5 mL, 1000 ± 200x10^6 / mL de plaquetas totales, libre de eritrocitos y leucocitos. Se centrifugaron un total de 300 unidades de sangre de cordón umbilical, en dos pasos, para enriquecer las plaquetas, en conformidad con las buenas prácticas de manufacturación. Las muestras se analizaron para determinar el grado de activación de las plaquetas y la presencia de las concentraciones de factores de crecimiento para evaluar su función potencial. A continuación, tras descongelación se activaron CBPC con gluconato de calcio al 10% para generar geles de plaquetas (CBPG) para tratar pacientes con úlceras del pie diabético.

Resultados: tras el procesamiento, el 84% de los productos cumplieron con los criterios de aceptación. Los productos finales contenían 1017±149x10^6 plaquetas/mL en 10±3 mL de plasma. La recuperación plaquetaria fue del 50 ± 9%. Los métodos aquí descriptos aseguran la concentración mínima de los leucocitos y los eritrocitos hasta una concentración residual de 0,2±0.1x10^6 / mL y 0,03±0.02x10^9 / mL, respectivamente. Las plaquetas mostraron bajos niveles de activación durante el procesamiento, pero se activaron significativamente después de la descongelación, indicado por un aumento de la expresión de CD62p.

Los factores de crecimiento EGF, VEGF, bFGF, PDGF AB/BB y TGF-β1, se encuentran en concentraciones de 1706 ± 123 pg / mL; 1602 ± 227 pg / mL; 314 ± 26 pg / mL; 30 ± 1,5 ng / mL; 24 ± 2 ng / mL (media ± SEM), respectivamente. Para la evaluación clínica, se aplicaron un total de 21 CBPG en 3 pacientes, sin eventos adversos informados y mejoría de las úlceras en todos ellos.

Conclusiones: en este estudio diseñamos y validamos un método de sistema cerrado altamente reproducible para fabricar CBPC de alta calidad adecuado para aplicaciones clínicas utilizando unidades de sangre de cordón umbilical no aptas para trasplante en un banco de sangre de cordón público.

Original article

Cord blood-derived platelet concentrates as starting material for new therapeutic blood components prepared in a public cord blood bank: from product development to clinical application

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Background - There are many advantages to using cord blood (CB) as a source of therapeutic platelet and plasma derivatives for regenerative medicine. These include availability, universal use, young donor source, and virally safe biological material, rich in tissue regenerative factors.

Materials and methods - We aimed to validate a bioprocess design for the production of cord blood-derived platelet concentrates (CBPC) in a public Cord Blood Bank (CBB). CBPC was defined as a product of 10±5 mL, 1,000±200×10^9/L total platelets, free of erythrocytes and leukocytes. A total of 300 CB units were centrifuged in two steps to enrich for platelets, in compliance with Good Manufacturing Practice. The samples were tested for the degree of platelet activation present, and the levels of growth factor were analysed to evaluate their potential function. CBPC were then activated after thawing with 10% calcium gluconate to generate platelet gels (CBPG) to treat patients with diabetic foot ulcers.

Results - After processing, 84% of the products fulfilled the acceptance criteria. Final products contained 1,017±149×10^6 platelets/mL in 10±3mL of plasma. Platelet recovery was 50±9%. The methods described here ensure depletion of white and red blood cells down to a residual concentration of 0.2±0.1×10^6/mL and 0.03±0.02×10^6/mL, respectively. Platelets showed low levels of activation during processing, but were significantly activated after thawing, as indicated by an increase in CD62p expression. The growth factors EGF, VEGF, bFGF, PDGF AB/BB and TGF-β1 were at concentrations of 1,706±123 pg/mL; 1,602±227 pg/mL; 314±26 pg/mL; 30±1.5 ng/mL; 24±2 ng/mL (mean±standard error of mean), respectively. For clinical evaluation, a total of 21 CBPG were applied in 3 patients, with no reported adverse events and improvement of ulcers in all of them.

Discussion - We designed and validated a highly reproducible, closed system method to manufacture high quality CBPC suitable for clinical applications using CB units not suitable for transplantation in a public CBB.

Keywords: *cord blood plasma, platelet rich plasma, platelet gel, regenerative medicine, cord blood banking.*

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INTRODUCTION

Cord blood (CB) is used as an important source for haematopoietic stem cell transplantation in children and adults with cancer, bone marrow (BM) failure syndromes, haemoglobinopathies and other genetic metabolic disorders[1]. Cord Blood Banks (CBB) are responsible for the collection, processing, storage and distribution of CB-derived haematopoietic progenitor cells (HPC) for transplantation[2]. Due to the high number of cells required for engraftment, only a small fraction of the collected donations meet the strict cellular criteria required for clinical use.

Thus, large amounts of donated units are discarded. However, these could be used for other applications whilst also avoiding ethical concerns by utilising donated materials otherwise destined to be discarded as medical waste. In addition, as a source of new medicinal products, these CB units have the advantage that they are an accredited source; they were collected by trained midwives following validated procedures[3], hold appropriate informed consent, and fulfill the rigorous quality criteria required for human use. Other advantages are inherent to the nature of the starting material: absence of safety risks for the donor, easily accessible source, very low risk of transmissible infectious diseases, and low immunogenicity[2]. One promising approach is to use CB components for novel clinical applications. Evidence is already available for the therapeutic efficacy of CB plasma and platelets associated with their anti-inflammatory and regenerative properties[4,5]. Platelet rich plasma (PRP) has been used as a regenerative product in several applications, with many publications suggesting that platelets may offer beneficial effects on wound healing[6]. Developing a well-defined, off-the-shelf product from an allogeneic source like CB may contribute to the standardisation of such new therapeutic approaches[7]. This would also solve issues related to autologous donations, such as problems in obtaining therapeutic products during long-term treatments, or in situations where the plasma or the platelets may have deteriorated due to an underlying disease.

There are a number of reports on the use of CB components, including anti-inflammatory treatment for osteoarthritis[5], wound healing enhancer in ocular surface lesions[8], part of biological scaffolds to promote tissue regeneration with bedsores[9], diabetic foot ulcers (DFU)[10], or their use in mucocutaneous lesions related to graft-vs-host disease (GvHD)[11].

In order to increase the use of such products for clinical use, we propose to recover and process CB units not suitable for transplantation and to generate an intermediate product, cord blood platelet concentrate (CBPC), as starting material for further development of different medicinal products (eye drops, platelet gel, and other platelet and plasma derivatives). Here, and using the experience of a multicentre standardisation programme carried out by the Italian cord blood network[7], we describe and validate a procedure to concentrate platelets in a small volume of plasma with a defined amount of platelets, free of erythrocytes and cells. The objective was to achieve a process of CBPC production compliant with current Good Manufacturing Practice (GMP). To evaluate their biological activity, we assessed the levels of platelet activation and growth factors (GF) present. Finally, we assayed their healing properties in the context of a clinical pilot study for the treatment of DFU.

MATERIALS AND METHODS

Sample collection: raw material

Cord blood units were collected in authorised maternity hospitals within the Concordia programme. Our Blood and Tissue Bank (BST) is accredited by FACT-Netcord and also holds the Spanish CAT Foundation certification. Before delivery, mothers signed an informed consent for donation that allows the use of these samples for research and validation purposes. Qualified health care professionals collected CB units while placenta was still in utero using validated procedures[3]. All samples were transferred from the BST's authorised Biobank, following local regulations and after approval from the *Hospital de la Vall d'Hebron*'s ethics committee (ref.: 192/2014).

All processed CB units were selected among those excluded by the quality control criteria for haematopoietic stem cell cryopreservation. The most frequent exclusion criteria were total nucleated cell and total $CD34^+$ cell counts below 1.5×10^6 and 4×10^6, respectively. These CB units also had to comply with the following inclusion criteria to be eligible for producing CB PRP: less than 48 hours from collection, >50 mL volume (excluding anticoagulant citrate-phosphate-dextrose, CPD), absence of visible

haemolysis, and platelet concentration >150×10⁶/mL. The target product profile (TPP) is shown in **Table I**.

Cord blood platelet concentrate manufacturing

The objective of CB processing was to obtain platelet concentrates (CBPC) within the ranges defined above. A total of 300 CB units were included for processing following a method based on a previously described two-step centrifugation protocol[7] (**Figure 1**). Modifications were made to this protocol, including using an irradiated pre-fabricated kit, and additional sampling for virology testing from CB product to ensure safety, following local laws for medicinal products (2001/83/CE). First, whole cord blood (WCB) was transferred into a 150 mL bag (Fenwal Inc., Lake Zurich, IL, USA) and centrifuged at 210 *g* for 10 min to isolate a leucocyte poor and platelet rich plasma (PRP). PRP, which is an intermediate product, was transferred to another 150 mL bag using a manual plasma extractor while the pellet containing the majority of nucleated cells and the red blood cell (RBC) fraction was discarded. Then, the PRP was centrifuged at 2,000 *g* for 15 min, the platelet poor plasma (PPP) was transferred to another 150 mL bag and the platelet pellet was re-suspended in an appropriate volume of PPP (as defined below) to obtain a standard final concentration of 800-1,200×10⁶ platelets/mL in the CBPC (**Table I**). The appropriate volume of PPP required for resuspension was determined according to the initial platelet count multiplying the PRP volume by a reduction factor (0.25, 0.33, 0.40 and 0.50 for ranges of 150-199, 200-249, 250-299, and >300×10⁶ platelet/mL) to achieve a range of volume of 10±5 mL, and stored in special bags to facilitate clinical application (PRPS Biomed Device SrL, Modena, Italy). CBPC were then stored into sealed security wraps (PRPS000 Biomed Device SrL, Modena, Italy) at –80 °C for subsequent evaluation. All procedures were performed in GMP-compliant facilities.

Product safety was evaluated by serology for infectious disease markers in maternal and CB samples (for HIV-1/2, HCV, HBs and HBc, CMV, HTLV I-II and *Trypanosoma cruzi* antibodies, *Treponema pallidium*, and nucleic acid testing for HIV, HBV and HCV). For sterility testing, a mixed sample from PPP and residual erythrocyte bag was used to determine the presence of aerobic and anaerobic bacteria, and fungi (BacTalert,

Table I - *Acceptance criteria for cord blood platelet concentrate manufacturing*

Type of sample	Parameter	Acceptance criteria
WCB	Time from collection	<44 hours
	Signed Informed consent	Present
	Volume	75-150 mL (including CPD)
	Visible haemolysis	Absence
	Platelet count	≥150×10⁶/mL
CBPC	Volume	10 (±5) mL
	Platelet count	800-1,200×10⁶/mL
	Leukocytes	≤0.5×10⁶/mL
	Erythrocytes	≤0.1×10⁶/mL
	Virology	Negative
	Haemoculture	Negative
Maternal blood	Virology	Negative

WCB: whole cord blood; CPD: citrate-phosphate-dextrose.

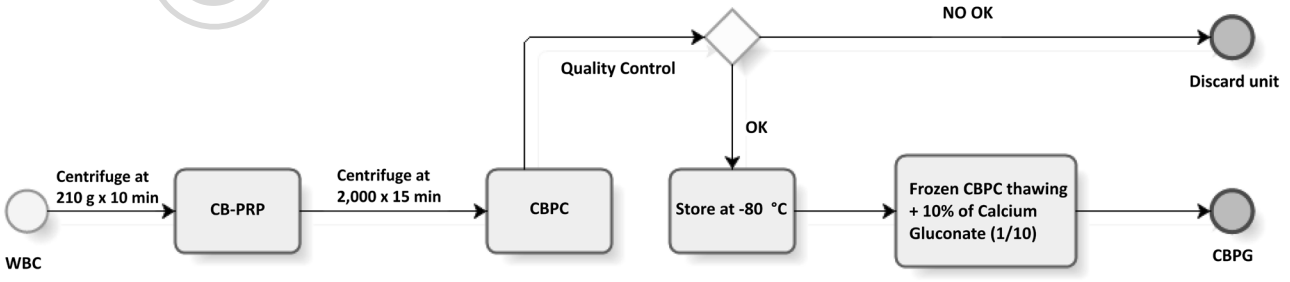

Figure 1 - Manufacturing flow of cord blood platelet gel (CBPG) for clinical application
WCB: whole cord blood; CB-PRP: cord blood-platelet rich plasma; CBPC: cord blood platelet concentrate; min: minutes.

Blood Transfus 2020; 18: 208-16 DOI 10.2450/2020.0305-19

Biomerieux Inc., Durham, UK). The final product was also characterised for cell counts and a blood sample from a residual bag used for RBC immunophenotyping (ABO and Rh blood group). Cell count was performed using a haematology analyser validated for the CBB activity (LH750 model, Beckman Coulter Inc., USA). Acceptance criteria for the final product are detailed in **Table I**.

In vitro evaluation of cord blood platelet concentrate

Validation of the manufacturing process was performed by determining platelet recovery, leukocyte and erythrocyte contamination, level of platelet activation, and GF content.

Platelet activation by flow cytometry

As a part of the validation of CBPC manufacturing, the activation of preserved platelets was demonstrated before and after freezing. To do this, five CB units were assayed at different stages of the manufacturing process, using flow cytometry for assessing the platelet activation phenotype of samples from: whole CB (WCB), PRP, PC before freezing and after thawing to analyse platelets surface and platelet activation levels of CD41aPE+ CD62pAPC+ positive and negative control IgG isotype (Beckton Dickinson, USA) markers antibody[12]. Platelets were used a positive control, which was activated with its own thrombin in the presence of anticoagulant.

Growth factor measurements by Luminex

The next validation step consisted of the determination of platelet-derived GF content in platelet releasates of CB. After thawing at 37 °C in a waterbath, the unit was activated using 10% calcium gluconate (1/10). To generate platelet releasates, clots were consolidated in approximately 10 min. Samples were then kept at room temperature for one hour and subsequently centrifuged at 5,000 *g* for 15 min. Supernatant was collected to measure EGF, VEGF, bFGF, PDGFAB/BB and TGFβ1 in multiplex (R&D Systems, Abingdon, UK) using a Luminex 100IS analyser (Luminex Corp., Austin, TX, USA) following the manufacturer's instructions.

Clinical evaluation of cord blood platelet concentrate

Preparation of platelet gel

For clinical application, 21 units of CBPC meeting the acceptance criteria were used. After the quarantine period of 2 weeks to discard transmissible diseases, units were released for clinical use. Upon request, released CBPC were thawed at 37 °C in a water bath, and then activated using 10% calcium gluconate 1: 3 vol: vol. A platelet gel was formed in approximately 10 min. This investigational product, called Cord Blood Platelet Gel (CBPG), could then be used for application to a DFU. After application to the side of the ulcer, a hydrophobic dressing (Mepilex®Lite, Molnlycke Health Care, Sweden) was used to prevent product absorption.

Clinical trial

A pilot clinical trial was conducted in order to demonstrate safety and efficacy as part of product validation. To this end, we obtained the approval from the Spanish Medicines Agency (AEMPS) and from the Ethics Committee of the Hospital de la Santa Creu I Sant Pau (HSP), (EC ref.: 15/043; EudraCT: 2015-000510-22; clinicaltrials.gov identifier: NCT02389010). The study had an open-label, two-arm, randomised design and was conducted at the HSP Barcelona, Spain, in patients with DFU. The inclusion criteria were an ulcer classified as at least Wagner II stage[13], and adult diabetics (>18 years) with certain clinical and laboratory parameters, without tumours or osteomyelitis. The primary objective of the study was evaluation of safety and the secondary objective was to evaluate efficacy by measuring reduction of the ulcer area. The experimental treatment was applied topically twice a week for one month (8 applications in total). The control arm was the standard treatment consisting of cleaning with Povidone-Iodine (Topionic®, Barcelona, Spain) and "wound discharge", and use of a shoe inner sole was adapted to the ulcer. Both arms received 8 treatment visits after screening and one follow-up visit at month 4. The visit consisted of wound cleaning, photographic documentation with measurements, and recording in a Data Collection Logbook of: stage (according to Wagner), size of ulcer, if there was tunneling, presence of necrotic, granulation tissue, exudate, and evaluation of pain. In addition, laboratory analysis was performed before starting treatment, and at 1-month and 4-month evaluations.

We randomised 11 patients: 6 to CBPG (3 withdrawn) and 5 to control arm (2 withdrawn). The study was terminated before completion of the complete sample size due to slow enrolment.

Analysis of the feasibility of cord blood platelet concentrate production

To evaluate the capacity to maintain a defined size of CBPC stock, a retrospective analysis was performed using data from CB units registered in the Programa Concordia. This analysis was based on the calculation of the number

of units received from 2014 to 2018 that fulfilled the required volume of >75 mL (including anticoagulant) and a time of collection to reception up to 44 hours. The number of patients with DFU that could potentially require CBPG treatment was based on the yearly report of hospital activity from the Functional Unit of the Diabetic Foot of the HSP. The cost of production was calculated on the basis of expenses for kits, consumables, testing, installations, maintenance, and labour provided by the Finance Department of the BST.

Statistical analysis

Results shown are presented as mean and standard deviation (SD) on cell count, unit volume or standard error of mean (SEM) on assays of platelet activation and GF measurement. U-Mann-Whitney test was used to compare processing stages. Due to low recruitment, non-statistical analysis was applied to compare two arms of the clinical trial, and percentage of size reduction was shown.

RESULTS

Validation of cord blood platelet concentrate manufacturing

Of the WCB selected for processing, 300 fulfilled the acceptance criteria for CBPC production. The mean platelet count in WCB was 240±46×10^6/mL and final PC product showed a platelet count of 1,017±149×10^6/mL, resulting in a 50±9% platelet recovery in a final volume of 10±3mL. An almost negligible amount of red and white blood cells were present (see **Table II**). Following the described protocol, more than 98% of initially measured leukocyte and 99% of the erythrocyte content were depleted from the CBPC.

Amongst the 300 WCB units processed, only 29 units (9.6%) did not fulfill CBPC acceptance criteria, thus demonstrating that the manufacturing procedure was robust. The main reason for failure was visible haemolysis (21 products). In a further 8 units it was due to other factors including volume, platelet counts, erythrocyte and/or leukocyte contamination. In addition, 20 units (6.6%) failed to pass quarantine due to a reactive serology or positive results in microbiological cultures. The remaining 251 (83.7%) units of CBPC met the acceptance criteria. All products used for the clinical application displayed the pre-defined target criteria.

In vitro evaluation of cord blood platelet concentrate

Platelet activation

To measure activation, we compared percentage of expression of CD62p at different stages of the manufacturing process. The proportion of CD41a+CD62p+ events was 15%±4 SEM, which rose slightly to 25% ±6 SEM after the first centrifugation step (PRP) and significantly increased to 39%±5 SEM after the second centrifugation step (PC) (p=0.01 with respect to the starting sample). This suggests that loss of GF during CBPC processing was limited. After thawing, 81% of the stored platelets were recovered. These platelets showed high levels of activation (percentage CD62p+ within the CD41a population was 80% ±2 SEM; p=0.0015 with respect to the fresh CBPC) (**Figure 2**), suggesting the stored CBPC retain the ability to activate. This also suggests a good functionality for GF release. Furthermore, CBPC after thawing were able to form gels after addition of calcium, demonstrating capacity to be that of clinical applications.

Growth factor measurements

Determination of GF was performed in platelet releasates obtained from CBPC. The mean platelet count was 1,077×10^6/mL±122 SD, the EGF had a concentration of 1,706 pg/mL±123 SEM; VEGF –1.602 pg/mL±227 SEM; bFGF –314 pg/mL±26 SEM; PDGF AB/BB –30 ng/mL±1.5 SEM; TGFβ1 –24 ng/mL±2 SEM.

Clinical evaluation of cord blood platelet concentrate

Eleven patients were randomised in the clinical study (6 to CBPG and 5 to standard procedure (STD) (*Online*

Table II - *Product validation parameters and results (n=300)*

Parameter	Volume	Platelets ×10^6 mL	Leukocytes ×10^6 mL	Erythrocytes ×10^6 mL
WCB	85 (±14) mL	240±46	11.2±2.8	3,1±0.3
CBPC	10 (±3) mL	1,017±149	0.2±0.1	0.03±0.02
Yield	12±4%	50±9%	2±1%	0.1±0.05%

WCB: Whole cord blood; CBPC: cord blood platelet concentrates; n: number. Values are expressed as mean±standard deviation.

Blood Transfus 2020; 18: 208-16 DOI 10.2450/2020.0305-19

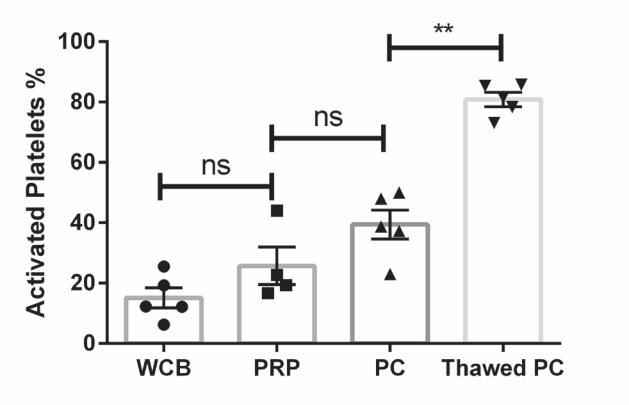

Figure 2 - Graphical representation of platelet activation throughout manufacturing processing
Significant differences in percentage of activated platelets between fresh platelet rich plasma (PC) and thawed PC (mean±standard error of mean). Statistical significance assessed by U-Mann-Whitney test. **p=0.0015; ns: not significant. N=5.
WCB: whole cord blood; PRP: platelet rich plasma.

Supplementary **Table SI**). Five of them withdrew due to clinical protocol violation (3 CBPG, 2 STD). From the remaining 6 patients, 3 of them received CBPG, and 3 STD. Clinical outcome of patients receiving STD resulted in one being infected and withdrawing from the study, whilst another had reduction of the ulcer area (62%) at 4 weeks but this was followed by an increase in area at the 4 month follow-up visit (75% of the initial ulcer area remaining at this time point). Finally, the last patient included in the STD arm had good evolution with ulcer closure at 4 weeks and at the 4-month follow-up visit. In contrast, in the experimental treatment (CBPG) arm, all 3 patients improved. In one (BST-10), the ulcer remains open but with a reduction in area to 44% and to 71% at the 4th and 16th week follow-up visit, respectively. The ulcers of the other two patients had completely healed at different time points: patient BST-08 by the 3-week visit and BST-10 at 4 months. There were no reported adverse events in the CBPG treated patients (*Online Supplementary* **Table SI**) and signs of efficacy were observed. No exudate, necrotic tissue or pain was reported.

As an example, **Figure 3** shows the evolution of the ulcer of patient BST10. This patient suffered from a chronic DFU that failed to heal after different previous treatments, and entered the study in Wagner II stage with 10.4 cm^2. The patient was randomly-assigned to CBPG treatment and showed a 20% ulcer area reduction after the first 8 applications (1 month of treatment, two applications per week). The patient continued receiving CBPG and at the two month follow-up visit the ulcer had continued to improve with a remaining area of 37%. CBPG administration was stopped but the ulcer was completely closed by the 4-month follow-up visit, showing the CBPG medicinal product had maintained its effect. No safety issues were reported.

Feasibility of regular production of cord blood platelet concentrate in a public Cord Blood Bank

According to the historical data of Programa Concordia, a median of 4,973 CB units were collected every year in the last 5 years. Of these, 11% fulfill the strict criteria for haematopoietic stem cell transplantation processing. Interestingly, 62% (up to 3,621 units) of the total CB units received would have been eligible for CBPC production according to the volume and time criteria defined in the Materials and methods section (*Online Supplementary* **Table SII**). This large number suggests the feasibility of a sustained CBPC production within the environment of a public CBB.

The Functional Unit of the Diabetic Foot of the HSP treated 137±10 patients per year during 2015-2017 (Report of HSP activity); 24±7 of them had a DFU (Patient Register Database). Following our estimation of production (3,621 units per year), taking into account 8 units per patient, this would require approximately 240 units per year.

Finally, we analysed the direct costs of CBPC production, including consumables, quality controls (cell count), and safety assays (virology, microbiology and fungal detection in CB samples), facilities and equipment, maintenance and labour costs of technicians and supervisors. This initial cost assessment showed that the direct cost of manufacturing a clinical grade CBPC using this validated procedure is € 156.1 ($172.98) (*Online Supplementary* **Table SIII**), with €1,248 ($1,383) per treatment course. Such costs are highly competitive compared to the cost of treating the ulcers using alternative therapies, where DFU treatment can reach >$ 3,000[14].

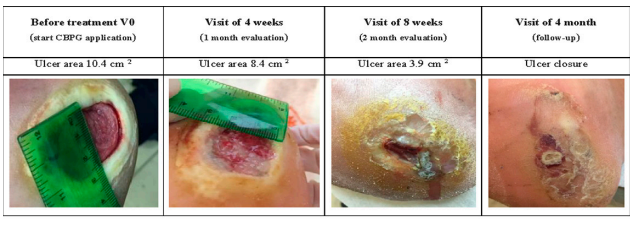

Figure 3 - Patient BST-10 with chronic diabetic foot ulcers (DFU), who received platelet gel (CBPG) treatment

DISCUSSION

Wound healing is a clinical condition which still has no satisfactory therapeutic solution, especially in patients with chronic ulcers[9,15]. We developed this project to answer a clinical need for new products for difficult-to-treat skin wounds. To achieve this, we developed a TPP[16].

In this study, we focused on developing a scalable CBPC production protocol according to this defined TPP from CB units that are otherwise discarded for haematologic application at the CBB[2,17]. We first described a manufacturing process that obtains a product with very low content of leukocytes and erythrocytes, within a defined range of platelet content. This medicinal product fulfills safety requirements, including negative results for infectious disease markers and microbiological contamination before unit release. In this regard, the GMP-compliant processing of CB platelets was hugely facilitated by the experience and established procedures of the CBB that had successfully completed rigorous accreditation schemes. Thereafter, the product was described in the investigational medicinal product dossier for a clinical trial to test the healing potential of CBPG in DFU patients. The gel consists of platelet-derived factors trapped in coagula, which are continuously released at the wound site after application.

Cord blood platelet concentrate is a new tool for tissue engineering and regenerative medicine applications. Here we also describe the feasibility of CBPC production as well as the versatility of its therapeutic applications based on the possibility to conveniently preserve off-the-shelf products in the CBB to further produce different formulations of CB plasma, according to the desired final use. CBPC offers some advantageous benefits for patients because of: i) its safety; ii) the fact that pregnant donor women were previously evaluated for presence of transmissible diseases; iii) the fact that quality control analysis can be easily carried out before product release; iv) the immediate availability; and v) the unique properties of CB plasma[18] due to the presence of angiogenic GF, and immunomodulatory cytokines[19] with recognised beneficial effect to wound healing[20]. Importantly, the possibility of standardising CBPC manufacturing to yield a well-characterised product also provides the chance to reduce product variability; this is in contrast to autologous PRP applications where treatment dose and composition change on an individual basis.

Platelet rich plasma is a well-known biological product typically used in the autologous application settings[21,22] for therapeutic purposes. The medical use of platelet GF has been described for eye drops[23], platelet gel[24,25], and supplements of culture media[26,27], and for advanced therapy medicinal products (ATMPs), amongst others. In this sense, there are several recently described clinical applications of autologous PRP, including chronic wound healing[10,20], skin and soft tissue repair[25], treatment of inflammatory pathologies, and even anti-ageing medicine applications[22]. However, the clinical application of autologous PRP has some disadvantages, such as the variability in the raw materials and processing protocols, lack of characterisation of the final product applied to the patient, and the contraindication for some patient populations to obtain blood for PRP preparation (haematologic malignancies, elderly patients with limited mobility[9]). Improvements in wound healing based on platelet properties, after treatment with peripheral blood platelet gel, have been reported elsewhere[10,20], although the scientific evidence[28] is scarse. Parazzi *et al.* also showed by proteomic analyses that adult plasma is richer in inflammatory factors compared to CB[18]. In this regard, the use of CBPC manufactured with the methodology proposed here would easily overcome the aforementioned disadvantages due to the standardisation of platelet content in a defined volume, the validated manufacturing protocol, and its potential universal use.

In addition, as set out above, CBPC preserves the content of platelets after thawing, indicating the suitability of the proposed manufacturing protocol to ensure a controlled dose of platelets in the final product for the patient. Thus, our processing protocol yielded CBPC units with at least 800×10^6 platelets/mL in all cases, demonstrating the reproducibility of our protocol. More importantly, the capacity of those platelets to express activation markers after thawing suggest that GF release, the putative active ingredient, occurs at the moment of application of the medicinal product and not in an uncontrolled manner during processing. Despite this, it is still not clear whether the observed expression of platelet activation marker is a result of the physiological platelet activation triggered by the temperature conditions of preservation[29] or because of the platelet membranes breaking after thawing[30], or even due to both mechanisms occurring at the same

Blood Transfus 2020; 18: 208-16 DOI 10.2450/2020.0305-19

time. In the future, other preservation strategies, such as freeze-drying methods, could be tested to improve presentation. In addition, our analysis of GF levels after activation of CBPC with calcium gluconate supports the preservation of platelet function and showed comparable levels of factors in CBPC compared to the ranges described as reference values[31], therefore suggesting that current clinical applications using autologous PRP might potentially be replaced with CBPC.

On the other hand, the very low number of leukocytes, and the almost complete absence of erythrocytes in our products, assures a low risk of potential immune and inflammatory reactions after allogenic applications, even without HLA or ABO group compatibility, thus enforcing their universal use. In this sense, a safety profile for the use of CBPG has been observed in our pilot clinical trial on DFU patients as a part of product validation and observed product functionality.

Provided that product safety remains a key concern, all the steps of the manufacturing process were designed to be performed in compliance with GMP.

According to these data, it is suggested that allogeneic products be applied on wound healing. Currently, there are several kits commercially available for the preparation of autologous PRP, with prices ranging from $175 to $1,150 US[32], excluding the costs of virology and microbiology testing, labour, and product characterisation. Therefore, the cost analysis of the CBPC product presented here shows it to be competitive and suitable for use in public health environments with negligible safety risks. The limitation of our study is the lack of an accurate local cost-to-benefit analysis, which is not feasible at this stage considering the small number of patients that we have treated with CBPG so far. However, encouraging data have been reported by Greppi *et al.*, who showed that the use of allogeneic donor platelet gel generated a 90% reduction in treatment cost vs conventional treatment, and 86% ulcer healing in a series of 11 elderly patients affected by pressure ulcers[9].

Our data also assessed the feasibility of a public CBB to regularly produce this therapeutic blood component for clinical use. Furthermore, the full development of this product in the catalogue of a CBB would result in a substantial increase in the efficiency of the CB collection programmes. In our analysis, the current 11% of clinical conversion would increase to 73%, if CBB included the production of CBPC to their routine processing of CB cells for transplantation. In this regard, we propose a new generation of CBBs to be used in other contexts beside transplantation[33].

CONCLUSIONS

In conclusion, here we demonstrate the feasibility of obtaining CBPC by implementing GMP protocol using conventional equipment typically present in a CBB. This methodology allows for the large-scale production that is required for conducting future clinical trials to assess efficacy and potential new applications.

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AUTHORSHIP CONTRIBUTIONS

SQ, DS conceived the initial study; DS, LR designed and performed data analysis of experimental in vitro assays; DS, SQ, EF, LR prepared documentation for both Regulatory Authority and Local Ethics Committee approvals; DS, LR, MC, EV designed and performed CBPC scale-up manufacturing and quality control batch release; DS, RC, ET, JG, JRE designed clinical trial and participated in patient recruitment, treatment and follow-up; DS, LR, JV manuscript writing that was revised and edited. All Authors discussed and revised the final version of the manuscript.

The Authors declare no conflicts of interest.

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Paper 2. *Cord Blood Platelet Rich Plasma Derivatives for Clinical Applications in Non-transfusion Medicine*

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RESUMEN:

Los derivados de plasma rico en plaquetas de la sangre del cordón umbilical (CB-PRP) se han investigado como agentes terapéuticos potenciales para el tratamiento de diversas afecciones, incluidas las enfermedades de la superficie ocular y las úlceras cutáneas. Se han desarrollado procesos para la formulación de varias preparaciones de CB-PRP, que tienen diferentes composiciones y atributos. Aquí se describen las características moleculares de estas preparaciones y se hacen recomendaciones en cuanto a su aplicación clínica más apropiada basada en perfiles funcionales e inmunomoduladoras. Se muestra que la incubación de células mononucleares de sangre periférica (PBMC) adultas, con las tres preparaciones, redujeron drásticamente la producción de INF-γ y la expresión de NKG2D y CD107a en las células NK, NKT y T, disminuyendo así su activación. Se propone que este resultado está asociado a los altos niveles de ligandos de NKG2D solubles presentes en el plasma. De las tres preparaciones que investigamos, los factores obtenidos a partir de lisado de plaquetas de sangre de cordón (CB-PL) y los factores obtenidos a partir de liberación de plaquetas activadas (PR) tienen concentraciones más altas de factores tróficos y pro-angiogénicos mientras que el plasma pobre en plaquetas CB (PPP), tiene la concentración más baja de todos los analitos medidos. En base a estos hallazgos, proponemos que CB-PR es la materia prima más adecuada para parches de heridas en la piel, mientras que CB-PL y PPP pueden usarse para preparar colirio para patologías severas de la superficie ocular y patologías inflamatorias como úlceras corneales u ojo seco severo, respectivamente.

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Cord Blood Platelet Rich Plasma Derivatives for Clinical Applications in Non-transfusion Medicine

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Cord blood platelet rich plasma (CB-PRP) derivatives have been investigated as potential therapeutic agents for the treatment of diverse conditions including ocular surface disease and skin ulcers. We have developed processes for the formulation of several CB-PRP preparations, which have different composition and attributes. Here we describe the molecular characteristics of these preparations and we make recommendations as to their most appropriate clinical application based on functional and immunomodulatory profiles. We show that incubation of adult peripheral blood mononuclear cells (PBMCs) with all three preparations dramatically reduced the production of INFγ and the expression of NKG2D and CD107a in NK, NKT, and T cells thus diminishing their activation, we propose that the likely mechanism is the high levels of soluble NKG2D ligands present in plasma. Of the three preparations we investigated, CB platelet lysate (PL) and platelet releaseate (PR) have higher concentrations of trophic and pro-angiogenic factors, CB platelet poor plasma (PPP) has the lowest concentration of all analytes measured. Based on these finding we propose that CB-PR is the most suitable raw material for skin wound patches, while CB-PL and PPP can be used to prepare eye drops for severe ocular surface pathologies and inflammatory conditions such as corneal ulcers or severe dry eye disease, respectively.

Keywords: cord blood, platelets rich plasma, plasma, platelets, ocular surface disease, skin ulcer, immunomodulation

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INTRODUCTION

Human platelet rich plasma (PRP) has been recognized as a rich source of trophic factors (1, 2), which aid healing in several indications including some ocular surface disorders (3, 4), such as severe dry eye disease and corneal ulcers, skin wounds (5) and orthopedic applications. Initially, autologous PRP was used to treat corneal injuries (6) but more recently allogeneic preparations have been favored (7–9). PRP can be obtained readily from both adult peripheral and cord blood (CB). Comparisons of PRP obtained from these two sources suggest that they differ both in the type and in the amount of factors they contain (10). These and other studies argue that CB-PRP has therapeutic advantages over adult PRP as the latter

contains more pro-inflammatory molecules which can aggravate certain conditions, while CB-PRP contains higher levels of anti-inflammatory molecules [reviewed in (11)].

CB-PRP contains soluble molecules of NK Group 2, member D (NKG2D) ligands (sNKG2DL), which are in part responsible for NK, NKT, and T cells suppression and may represent a mechanism of fetomaternal tolerance (12). Further work elucidated the differential roles of the various types of sNKG2DLs and revealed soluble unique long 16 binding proteins (sULBP1) as the most abundant soluble ligand in CB-PRP that directly downmodulated NK cell cytotoxicity in a dose-dependent manner (13). Additionally, we showed that polymorphisms in Major Histocompatibility Complex Class I-related Chain A (MICA) which encode either valine (Val) or methionine (Met) at residue 129 affected the ability of soluble MICA (sMICA) to suppress NK cell function. Met is known to bind with strong affinity to NKG2D whereas Val has weaker binding (14).

Depending on the processing protocols used to separate the PRP from whole blood, a variety of preparations can be made which have different properties. An initial low speed centrifugation of whole CB units yields CB-PRP which can be further processed. CB-PRP contains a variety of growth factors (GF), cytokines and other immunomodulatory factors that can act on the proliferation, differentiation and function of both immune and other cells such as fibroblasts. CB-PRP also contains albumin, transferrin, fibronectin, fibrinogen, minerals, adiponectin, vitamin A and E, several fatty acids and platelets (15), which contribute as trophic factors in wound healing mechanisms of epithelial cells of the cornea and its stromal tissue (16). These elements have also been implicated in cutaneous scarring formation (17). Thus, the availability through CB banking and the therapeutic benefits of the factors contained in CB-PRP derived products make CB an ideal product source for the treatment of a variety of diseases and inflammatory conditions. These benefits can be enhanced by processing the PRP both by concentration of the platelets and their lysis, which releases platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), epithelial growth factor (EGF), vascular endothelial growth factor (VEGF), and transforming growth factor beta (TGFβ) among others, all of which have been shown to enhance tissue regeneration (18).

The purpose of this study was to compare the profile of GFs, cytokine and immune elements of CB-PRP derived preparations to determine which ones would be best suited for clinical applications. We formulated, from CB-PRP, three different preparations: (a) platelet poor plasma (CB-PPP), (b) platelet concentrate lysate (CB-PL), and (c) platelet concentrate releaseate (CB-PR).

The concentrations of various GFs, cytokines and soluble NKG2D ligands, together with their immunomodulatory effects on Natural Killer (NK) cells and $CD8^+$ T cells were determined in the different CB-PRP preparations. Taken together, the results can help us determine the optimal CB-PRP derived preparations for different clinical applications for ocular surface diseases and skin wound healing.

MATERIALS AND METHODS

Ethical Considerations

PRP derived samples from individual CB units were obtained from BioBank (BST). The informed consent for the use of samples for research was obtained in accordance with the requirements of the Declaration of Helsinki and local laws at the time of collection. This study was approved by the BST local Research Ethics Committees (HCB/2017/0785).

CB Unit Collection

CB units were collected in authorized maternity hospitals. Before delivery, mothers signed an informed consent for donation that allows the use of these samples for research and validation purposes. Qualified health care professionals collected CB units while placenta was still *in utero* using validated procedures (19). The collection bag contains 25 mL of citrate phosphate dextrose (CPD) as anticoagulant.

CB Unit Verification

Full serological and microbiological analysis of the CB units was carried out to exclude infection. Tests were performed for the presence of Hepatitis A, B, and C viruses, human immunodeficiency virus, *Treponema Pallidum* (Syphilis), *Trypanosoma cruzi* (Chagas disease), and cytomegalovirus as well as aerobic and anaerobic bacterial and fungi (BacTAlert, Biomerieux, INC. Durham). Quarantine was applied as a minimum for 2 weeks. Only units, which were non-reactive, were further processed.

Preparation of CB-PRP Derivatives

Briefly, a total of 20 whole CB (WCB) units were used for the experiments. Units were treated individually and from each unit we derived all 3 preparations described. Each unit was centrifuged at low speed (210 g) for 10 min, the supernatant collected constitutes the PRP. The PRP was then centrifuged at high speed (2,000 g) for 15 min to obtain two fractions, a PPP and a platelet pellet, which was resuspended in PPP to obtain a platelet concentrate (CB-PC) in range of 800–1,200 $\times$ 10^9 platelets/L in 10 ($\pm$5) mL and then stored at -80°C. CB-PC fraction was frozen for quarantine. Only microbiology and serology results negative samples of CBPC were used for preparation of platelet lysate (CB-PL) and platelet releaseate (CB-PR). **Figure 1** shows a schematic of the processing of CB to obtain the different preparations tested.

CB-PPP Preparation

The remaining PPP was frozen at -80°C for future analysis; this product is CB-PPP.

CB-PL Preparation

CB-PC samples underwent 3 freeze ($\sim$-80°C)/thaw ($\sim$37°C) cycles (20) to lyse platelets and release GFs followed by a centrifugation step at 5,000 g for 15 min. The collected GF rich supernatant free of intact platelets was subsequently stored at -80°C for future use. This CB-PL, diluted with Plasmalyte (Baxter, USA) (1:1, v:v) is used to prepare CB eye drop (CBED), which was used in clinical trial [ClinicalTrails.gov ID

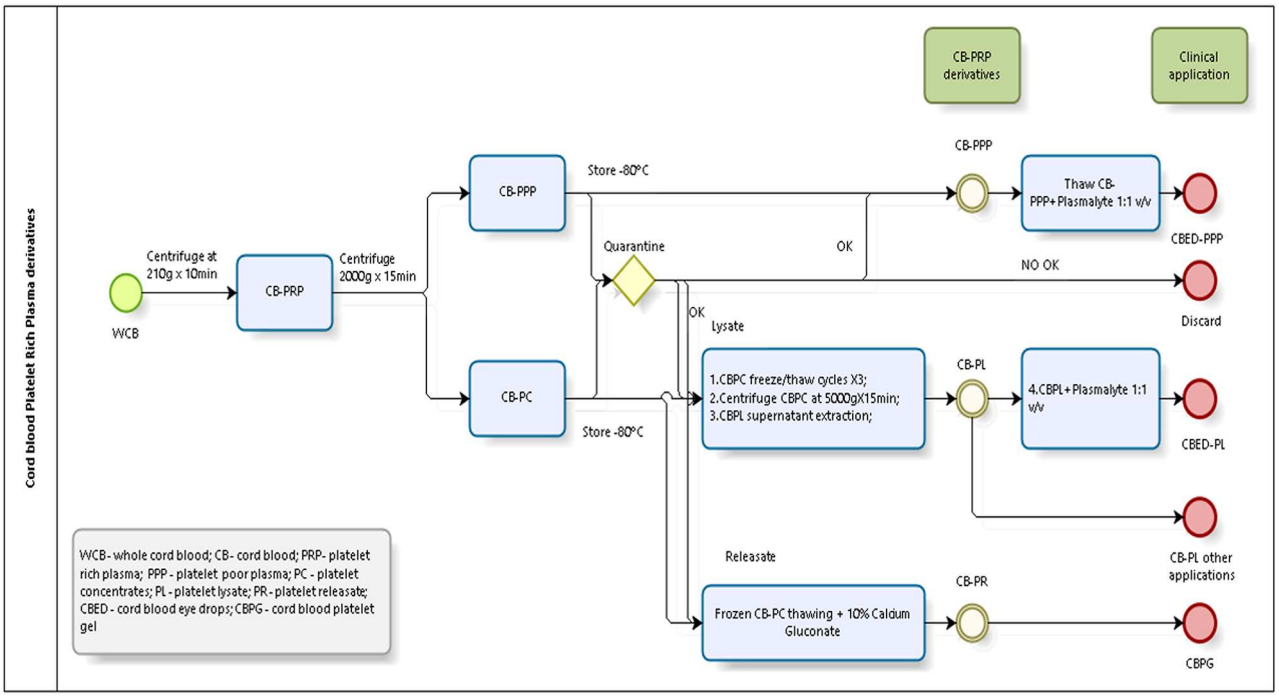

FIGURE 1 | CB-PRP derivatives processing scheme.

NCT03084861]. For this study was used only CB-PL, without dilution as the active ingredient.

CB-PR Preparation

CB-PC underwent one freeze/thaw cycle (which in our experience is insufficient to cause major platelet lysis and allows the storage of this fraction ready for calcium gluconate activation), the resulting fraction termed platelet releasate was treated with 10% calcium gluconate (Braun®, Terrassa, Spain) (1:10, v:v) and incubated for 1 h at room temperature. This jellification step forms the basis for the clinical skin patch, which has been used for clinical trial (ClinicalTrails.gov ID NCT02389010). For the purpose of the analyte measurement in this study, the releasate was also treated with 10% calcium gluconate (as above) but in the presence of heparin at 0.61 IU/mL to prevent clotting and thus the jellification of the plasma and trapping GFs, which would have confounded the measurements of factors in this preparation. After a centrifugation step (5,000 g for 15 min) the supernatant free of intact platelets (checked by automatic counter-Beckman-Coulter) was used for analyte measurements or stored at −80°C until needed.

CB-PRP Derived Products Cytokines and GF Quantification

CB-PRP preparations of CB-PPP, CB-PL, and CB-PR from single units were analyzed using magnetic beads of Luminex kits (R&D Systems, Abingdon, UK) according to manufacturer's recommendations, to determine concentration of following analytes: (plate 1) PDGF AB/BB, EGF, bFGF, VEGF, IL1/6/10, TNFα; (plate 2) TGFβ1; (plate 3) HGF; (plate 4) MMP2/9; (plate 5)TIMP1-4.

PBMC Functional Analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood (WB) from consenting, healthy adult male or female volunteer donors by density-gradient centrifugation using Lympholyte®-H solution (Cedarlane, ON, Canada). PBMCs were then used for co-culture with the various CB-PRP derived preparations for functional analysis. For assessment of cellular function of CD3- CD56bright/dim NK cells, CD3+ CD56+ NKT cell and CD3+ CD56− T cells, healthy adult donor PBMCs were plated in RPMI (Lonza, Slough, UK) containing 10% heat-inactivated fetal calf serum (FCS) supplemented with 1% penicillin and streptomycin (complete media) and containing human IL-2. Test cultures using CB-PRP preparations: CB-PPP, CB-PL or CB-PR were diluted with an equal volume of complete media containing 200 IU IL-2 per well (200 μl volume) in 96-well U-bottom plates. Results were normalized against PBMCs cultured with complete media containing 200 IU IL-2 per well and expressed as percentage of maximum expression (for measurement of NKG2D, CD107a or IFN-γ expression). All cultures were incubated at 37°C with 5% CO_2 for 48 h before Phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation. Duplicate cultures were carried out for each experiment without PMA and ionomycin stimulation to assess levels of NKG2D and baseline CD107a expression. NKG2D expression on the relevant cells was determined using the unstimulated cultures and CD107a background levels in

unstimulated cultures were subtracted from the values obtained for equivalent stimulated cells. Three independent experiments were carried out using four different, healthy PBMC donors, each tested with CB-PPP, CB-PL, CB-PR, or media only and data points represent donor means for each replicate test sample.

Flow Cytometry

Briefly, cells were labeled with fluorochrome-conjugated antibodies in Phosphate-buffered saline (PBS) with Bovine Serum Albumin (BSA) (0.5%) for 10 min at 4°C. Antibodies (BD Biosciences, Oxford, UK) were as follows: CD3 (SK7), CD56 (B159), CD107a (HA4A3), NKG2D (BAT221, Miltenyi Biotec, Bisley, UK). Viability was assessed using Annexin V and 7-AAD. For quantitation of CD107a, cells were re-suspended in complete media containing 100 ng/ml PMA, 1 μg ionomycin and 0.1% 2-mercaptoethanol (stimulated) or complete media with 0.1% 2-mercaptoethanol (non-stimulated) for 2 h at 37°C. Fluorescence minus one (FMO) controls (where samples are stained sequentially with all antibodies except one) were used to set gates. Acquisition was performed using a Fortessa II flow cytometer (BD Biosciences, Oxford, UK) and analysis was carried out using FlowJo version 10.5.0 (Tree Star Inc., OR, USA). The gating strategy used for analysis of lymphocyte subtypes was performed as described in our previously study (12) and is shown in **Supplementary Figure S1**.

Soluble NKG2DL and IFN-γ Detection and Quantification Assays

Soluble MICA/B (DY1300/DY1599) and ULBP1 (DY1380) were quantified in CB-PRP derivatives directly and IFN-γ (DY285) was measured in PBMC stimulated supernatants using Duoset ELISA kits (R&D Systems, Abingdon, UK), according to manufacturer's instructions.

MICA-129 (Valine or Methionine) SNP Genotyping

Genomic DNA for MICA genotyping was obtained from WCB using QIAamp® DNA blood minikit (Qiagen, GmbH, Hilden, Germany). The SNP rs1051792 (G/A) causing substitution of *Val* (G) for *Met* (A) at position 129 of the MICA gene was genotyped using a TaqMan® assay (Applied Biosystems, Foster City, CA, USA). PCR amplification conditions were as recommended by the manufacturer and reactions contained the forward primer 5′-GCTCTTCCTCTCCCAAAACCT-3′ and reverse primer 5′-CGTTCATGGCCAAGGTCTGA-3′ and the two allele-specific dye labeled probes FAM-5′-AATGGACAGTGCCCC-3′ and VIC-5′-AATGGACAATGCCCC-3′. Results were obtained and interpreted using the CF96 real-time PCR system (Biorad, Hercules, CA, USA). Confirmatory typing was performed using DNA extracted from WCB and polymerase chain reaction (PCR) amplification and sequencing of exon 3 of the MICA gene. Results were used to determine the presence of "A," "G" or both encoding *Met* or *Val* at residue 129 (ATG or GTG, respectively). Exons 2–6 of the MICA gene were amplified as previously described (21) using a forward primer located in intron 1 (5′- CACCTGTGATTTCCTCTTCCCCAGAGC-3′) and reverse primer in the 3′ untranslated region (5′-CTAACAATTTGC AGCMTCCAACAAC-3′). Cycle sequencing was performed using standard protocols and exon 3 forward sequencing primer (5′- CCCTGGGCTGAGTTCCTC-3′) and reverse sequencing primer (5′- ATAGCACAGGGAGGGTTT-3′).

Statistical Analysis

Results are shown as mean with standard error of the mean (SEM) and were evaluated using Graphpad Prism 8 (Graphpad Software, CA, USA. Datasets were analyzed using both paired and unpaired non-parametric statistical analysis as the sample size is too small to assume normal distributions. As we had 3 categories of sample, we used Friedmans's test for the paired comparisons and a Kruskal-Wallis for the unpaired analysis. Significance levels are indicated as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****), unless the exact p-value is given. For comparisons between genotypes only we used Mann-Whitney.

RESULTS

Comparison of Cytokine and GF Concentration Among the Different Preparations of CB-PRP Derivatives

The prepared CB-PC units contained $1{,}004 \pm 76 \times 10^9$ platelets (PLT)/L ($n = 10$) and were within the standard range of 800–$1{,}200 \times 10^9$ PLT/L, which was used to prepare CB-PL and CB-PR.

The levels of trophic and wound healing factors (group I), pro-angiogenic molecules (group II), pro-inflammatory (group III.A) and anti-inflammatory (group III.B) GFs and cytokines for all three preparations were analyzed and compared, the results are shown in **Table 1**, **Figure 2**, we have included statistical analysis of results both for paired and unpaired samples on the table, but the graph shows only significance for the paired samples. For comparative purposes the levels in tears of some of these factors are as follows: EGF (200–3,000 pg/mL), TGFβ (2–10 ng/mL), HGF (200–500 pg/mL), VEGF (2–19 pg/mL), PDGF (90–1,700 pg/mL) (3). In CB-PL preparations the concentration of most factors tested was higher than that observed in CB-PPP, however this did not reach significance in HGF, MMP-2, TIMP-1, 2, and 4 or IL6 and was lower for MMP-9. There were no statistically significant differences in concentration in any of the analytes examined between CB-PR and CB-PL, except for HGF, MMP-2, and TIMP-2 and 4, which were higher in CB-PL (**Table 1**, **Figure 2**).

As expected, for processed CB-PPP samples, that had $8 \pm 3 \times 10^9$ PLT/L in 24 ± 4 mL ($n = 10$), all measured analytes had lower concentrations, with the exception for HGF, TIMP-2 and 4 which were no different and MMP-2 and 9, which were higher in CB-PPP than in CB-PR (**Table 1**). Pro-angiogenic factors showed low concentrations, except for MMP-9. Both pro and anti-inflammatory cytokines have significantly higher concentration in CB-PL than CB-PPP, but are similar between CB-PL and CB-PR (**Table 1**, **Figure 2**). In adult serum the concentrations of these factors are as follows: EGF (500–1,000 pg/mL), TGFβ (6–50 ng/mL), HGF (100–1,000 pg/mL), VEGF (1,000–5,000 pg/mL), PDGF (30–100 ng/mL) (3). While levels of EGF, VEGF, HGF are comparable between CB-PL, CB-PR, and

TABLE 1 | Comparative composition of CB-PRP derivatives.

Analyte	Sample	Concentration mean ± SEM	Paired			Unpaired		
			p-Value CB-PPP vs. CB-PL	*p*-Value CB-PPP vs. CB-PR	*p*-Value CB-PR vs. CB-PL	*p*-Value CB-PPP vs. CB-PL	*p*-Value CB-PPP vs. CB-PR	*p*-Value CB-PR vs. CB-PL
Group I (trophic and wound healing factors)								
		pg/mL						
EGF	CB-PPP	93.5 ± 5	0.0004	0.0110	>0.9999	0.0002	0.008	>0.9999
	CB-PL	858 ± 66						
	CB-PR	800.6 ± 54						
bFGF	CB-PPP	179.5 ± 5	0.0004	0.0110	0.9999	0.0001	0.0011	>0.9999
	CB-PL	348 ± 26						
	CB-PR	310 ± 21						
HGF	CB-PPP	1446 ± 204	0.0553	0.29669	0.0002	>0.9999	0.6702	0.1399
	CB-PL	1676 ± 227						
	CB-PR	1127 ± 116						
TGF-β1	CB-PPP	8767 ± 929	0.0002	0.1365	0.1365	0.0003	0.0126	0.8665
	CB-PL	58646 ± 3553						
	CB-PR	49461 ± 3949						
Group II (pro-angiogenic factors)								
		pg/mL						
VEGF	CB-PPP	329 ± 17	<0.0001	0.0760	0.0760	<0.0001	0.0038	0.7279
	CB-PL	1046 ± 367						
	CB-PR	798 ± 90						
PDGF AB/BB	CB-PPP	3388 ± 535.5	<0.0001	0.1017	0.1017	<0.0001	0.0569	0.0447
	CB-PL	37267 ± 1817						
	CB-PR	10276 ± 752						
		ng/mL						
MMP-2	CB-PPP	170 ± 8	>0.9999	0.0012	0.0140	>0.9999	0.0905	0.1607
	CB-PL	153 ± 9						
	CB-PR	140 ± 7.7						
MMP-9	CB-PPP	101 ± 12	0.0286	0.0005	0.7158	>0.9999	0.8959	>0.9999
	CB-PL	88 ± 12						
	CB-PR	83.4 ± 13						
TIMP-1	CB-PPP	157 ± 12	0.0733	0.0005	0.4008	0.0100	0.0003	>0.9999
	CB-PL	332 ± 18						
	CB-PR	372 ± 11						
TIMP-2	CB-PPP	48 ± 2.6	0.2969	0.0553	0.0002	0.8156	0.0720	0.0024
	CB-PL	48.7 ± 2						
	CB-PR	38.8 ± 2.5						
TIMP-3	CB-PPP	1.1 ± 0.01	0.0029	0.0065	>0.9999	0.0019	0.0033	>0.9999
	CB-PL	2.8 ± 0.2						
	CB-PR	3.0 ± 0.3						
TIMP-4	CB-PPP	1.8 ± 0.2	0.2969	0.0553	0.0002	0.8156	0.7047	0.0667
	CB-PL	2.0 ± 0.2						
	CB-PR	1.5 ± 0.2						
Group III.A (pro-inflammatory GFs and cytokines)								
		pg/mL						
IL-6	CB-PPP	77 ± 10	0.0140	>0.9999	0.1017	0.0778	>0.9999	0.4621
	CB-PL	98 ± 6						
	CB-PR	82 ± 5						

(Continued)

TABLE 1 | Continued

Analyte	Sample	Concentration mean ± SEM	Paired			Unpaired		
			p-Value CB-PPP vs. CB-PL	*p*-Value CB-PPP vs. CB-PR	*p*-Value CB-PR vs. CB-PL	*p*-Value CB-PPP vs. CB-PL	*p*-Value CB-PPP vs. CB-PR	*p*-Value CB-PR vs. CB-PL
IL-1α	CB-PPP	40.5 ± 1.0	<0.0001	0.1017	0.1017	<0.0001	0.0081	0.6702
	CB-PL	88 ± 8						
	CB-PR	69 ± 4						
TNF-α	CB-PPP	103 ± 1.6	0.0024	0.0024	>0.9999	0.0008	0.0009	>0.9999
	CB-PL	123.6 ± 4						
	CB-PR	122 ± 2						
Group III.B (anti-inflammatory cytokines)								
		pg/mL						
IL-10	CB-PPP	43.5 ± 0.8	0.0004	0.0110	>0.9999	<0.0001	0.0020	>0.9999
	CB-PL	72 ± 8						
	CB-PR	63.8 ± 3						

Non-parametric comparison test. n = 10/product type.

adult serum, the concentration of PDGF is lower in CB-PR. TGFβ is slightly higher in CB-PC derived products and comparable to adult serum levels in CB-PPP samples.

Effect of CB-PRP Preparations on Donor Cell Viability

After measuring the levels of GFs and other factors, we investigated the effect of the PRP derivatives preparations on immune cells, specifically on NK cells. We and others have established that the immunomodulatory effects of plasma leading to suppression of NK cells are exerted through both the high concentration of TGFβ and NKG2D engagement with sNKG2DLs. Therefore, we examined the effects of the different preparations on the phenotype and activity of NK, NKT, and T cells.

Initially we examined the effect of incubation of PBMCs in the presence of the different preparations to ensure they had no toxic effects and viability was comparable. We incubated the cells in standard cell culture conditions, with 50% solution of each PRP preparation, diluted with complete media containing IL-2. We then determined the percentage of live lymphocytes by gating CD3-CD56 bright/dim NK cells, CD3+ CD56+ NKT cells and CD3+ CD56– T cells cultured with each of the CB-PRP preparations. The results, shown in **Figure 3**. show a general improvement in viability when cells were cultured with CB-PRP-supplemented media compared to media only. For all cell types investigated, viability was significantly improved in the presence of CB-PR compared to media only. While the presence of CB-PL was only significantly better compared to media alone in CD3– CD56 bright NK cells, CB-PPP was significantly better only in CD3-DC56 dim NK cells.

The viability of CD3– CD56 bright NK cells was not significantly different between cells grown in CB-PR or CB-PL when samples were compared either paired or unpaired. Growing them in the presence of CB-PL or CB-PR increased the viability significantly compared to CB-PPP, again regardless of samples being paired or unpaired. For CD3– CD56 dim NK cells the presence of CB-PR improved viability significantly compared to CB-PPP and CB-PL again regardless of sample pairing, and there was no significant difference between CB-PPP and CB-PL in these cells. The viability of CD3+ CD56+ NKT was highest in cells grown in CB-PR but the difference was only slightly significant between CB-PR and CB-PL when samples were analyzed paired. For CD3+CD56– T cells, again CB-PR is significantly better than CB-PL, but no different than CB-PPP whether samples are analyzed paired or unpaired.

Overall, incubation of PBMCs in the presence of all three CB-PRP preparations had a beneficial effect on viability and no detrimental or toxic effect.

Incubation With CB-PPP, CB-PL, and CB-PR Shows Differential Reduction in NKG2D Expression

Generally, incubation of peripheral blood CD3– CD56 dim and bright NK cells, CD3+ CD56+ NKT cells and CD3+ CD56– T cells with CB-PRP derivatives resulted in reduced expression of NKG2D relative to media only (**Figure 4**). Significant differences were observed between the different preparations of CB-PRP with all cell types, with CB-PL reducing NKG2D expression the most. While incubation with CB-PPP only reduced NKG2D expression to 94.7% ± 0.25 of maximal expression in CD3– CD56 dim NK cells, CB-PR reduced it to 78.6% ± 2.05 ($p = 0.0052$) and CB-PL to 77.04% ± 2.57 ($p = 0.0010$). Similar NKG2D expression patterns were seen in CD3– CD56 bright NK cells after incubation with the 3 preparations. Analysis of CD3+ CD56+ NKT cells showed a similar reduction in NKG2D expression after treatment with both CB-PL and CB-PR (49.3% ± 2.3% and 54.7% ± 1.5%, respectively) which was significantly more pronounced than culture with CB-PPP ($p = 0.0004$ and $p = 0.0110$, respectively in paired analysis). A similar profile to CD3+ CD56+ NKT cells was observed for CD3+ CD56– T cells, though the overall reduction in expression was less dramatic.

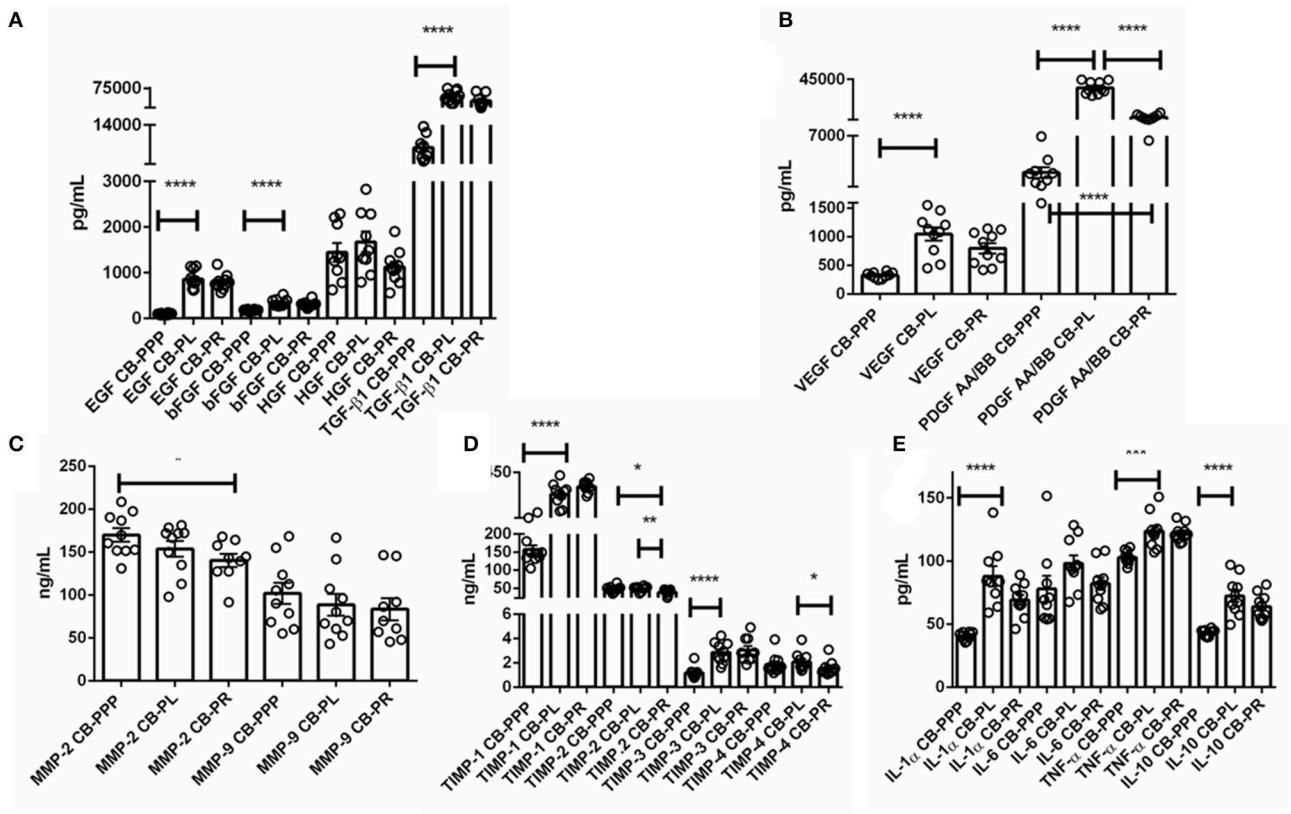

FIGURE 2 | (A) Trophic and wound healing factors in CB-PRP preparations in pg/mL, growth factors analyzed were EGF, bFGF, HGF, and TGF-β1. **(B–D)** Concentration of the angiogeneic group of factors in CB-PRP derived preparations. Results show concentration in pg/mL and ng/mL of each factor. The analytes measured were **(B)** VEGF and PDGF in pg/mL; **(C)** MMP-2 and MMP-9 in ng/mL; **(D)** TIMP1-4 in ng/mL. **(E)** Concentration of inflammatory and anti-inflammatory cytokine group of analytes in CB-PRP preparations. Data points represent concentration mean of IL-1α, IL-6, and TNF-α pro-inflammatory and IL-10 anti-inflammatory cytokines. CB-PRP preparations investigated were, cord blood platelet poor plasma (CB-PPP; $n = 10$), cord blood platelet lysate (CB-PL; $n = 10$) or cord blood platelet releasate (CB-PR; $n = 10$). Each sample was measured in duplicate in two separate experiments. Data points represent concentration mean. Statistical analysis was performed using non-parametric one-way ANOVA (Kruskal-Wallis test with Dunn's *post-hoc* test for unpaired samples and Friedman's for paired samples). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Incubation With CB-PL, CB-PPP, and CB-PR Reduces Expression of CD107a and IFN-γ

Incubation of all cell types examined with CB-PL resulted not only in a significant reduction in the expression of NKG2D, but also in a dramatic, and statistically significant, reduction in the expression of CD107a indicating a reduction in activation potential as shown in **Figure 5**. For example, percentage of maximum expression of CD107a in CD3- CD56bri NK cells incubated with CB-PL was 34.2% ± 3.4 SEM compared to 72.8% ± 4.01 SEM with CB-PPP ($p = 0.002$) and 41.4% ± 3.81 SEM with CB-PR ($p = 0.539$). Similar differences were seen in CD3– CD56 dim NK cells and CD3+ CD56+ NKT cells. Compared with NK and NKT cells, a smaller reduction in CD107a expression was seen in CD3+ CD56– T cells and there was no significant difference between CB-PR and CB-PL. However, CD107a expression, in the presence of both CB-PL (52.9% ± 4.14) and CB-PR (54.48 ± 3.8), was significantly reduced compared to incubation with CB-PPP (83.3% ± 2.2) ($p = 0.0024$). We measured the total IFN-γ produced after stimulation of cultures following the incubation period and results are shown in **Figure 6**. All CB-PRP preparations resulted in reduced IFN-γ production compared to complete media only, though there were no significant differences between CB-PR and CB-PL, treatment with both these preparations resulted in significantly less IFN-γ production compared to CB-PPP.

CB-PRP Preparations CB-PL, CB-PPP, and CB-PR Contain sNKG2DLs

We previously reported that CB plasma contains soluble NKG2DLs such a sMICA, sMICB, and sULBP1 (12, 22). Using ELISA, we measured the concentration of these ligands in the different preparations of CB-PRP. For this analysis we used two sets of preparations for CB-PL and CB-PR with a total of 20 and 19 samples, respectively. One set of CB-PPP was too dilute for the limit of detection of the assay, therefore only 10 samples were utilized. We found that sMICA ligands were present in most samples of CB-PPP, CB-PL, and CB-PR and all contained sMICB

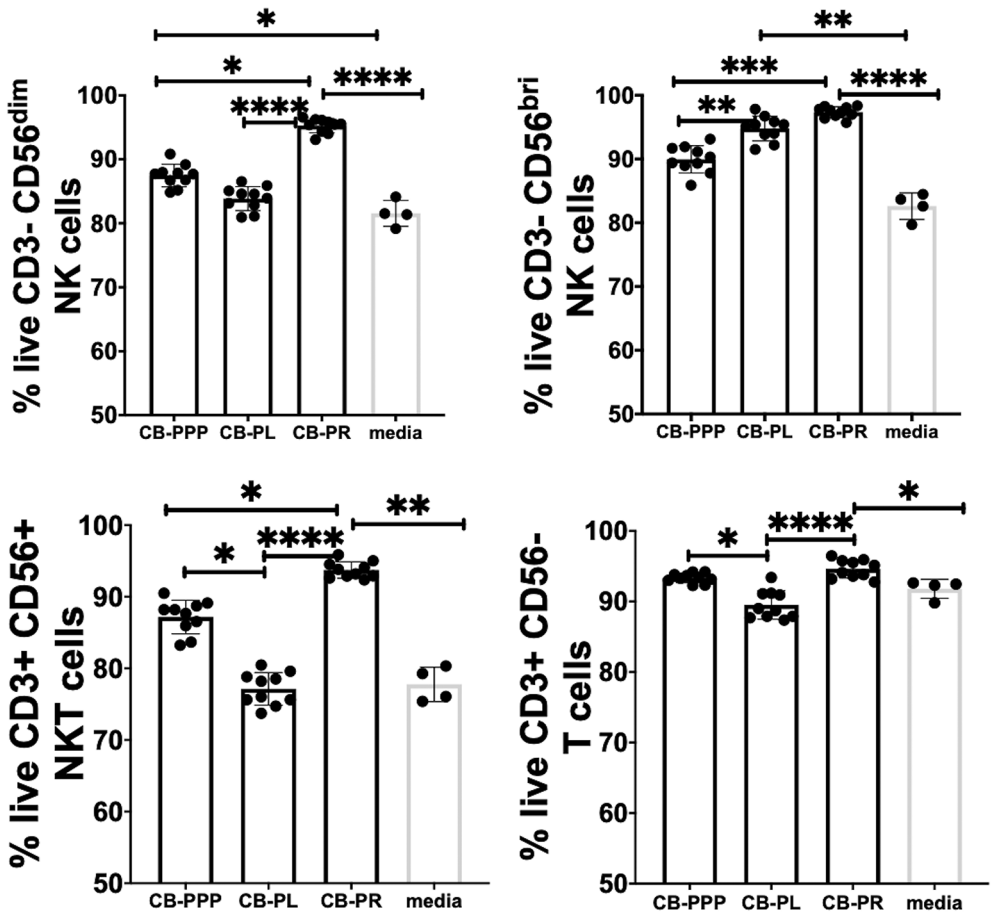

FIGURE 3 | Viability of CD3- CD56 dim, CD3- CD56 bright NK cells, CD3+ CD56+ NKT cells, and CD3+ CD56- T cells from adult donor PBMCs ($n = 3$) after incubation with cord blood plasma preparations or complete media only. Results show percentage of live cells (Annexin V negative, 7-AAD negative) for each cell type. Cord blood plasma preparations investigated were cord blood platelet poor plasma (CB-PPP; $n = 10$), cord blood platelet lysate (CB-PL; $n = 10$), or cord blood platelet releasate (CB-PR; $n = 10$). PBMCs were incubated with each preparation diluted 50:50 with media and supplemented with IL-2, or with complete media and IL-2 only for 48 h prior to antibody staining and flow cytometry analysis. Each experiment was repeated with four different PBMC donors and data points represent donor means. Statistical analysis was performed using non-parametric one-way ANOVA (Kruskal-Wallis test with Dunn's *post-hoc* test for unpaired samples and Friedman's for paired samples). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

and sULBP1 (**Figure 7A**). There was no significant difference in levels of sMICA or sMICB among the different CB-PRP preparations but significantly more sULBP1 could be detected in CB-PL (3.85 ng/ml ± 0.20) compared to CB-PPP (2.62 ng/ml ± 0.35) ($p = 0.0058$).

MICA Polymorphisms and Their Effects on IFNγ Production

The MICA polymorphism encoding either valine (Val; V) or methionine (Met; M) at residue 129 of the MICA protein is thought to influence functional differences in NK cell activation (14). We categorized CB-PRP preparations (CB-PL, CB-PPP, and CB-PR) as either Val homozygous (V/V) or homozygous and heterozygous samples containing Met (M/M and V/M) and examined the amount of IFN-γ produced by NK cells after PMA and ionomycin stimulation compared to media alone (**Figure 7B**). As expected per previous results the production of IFNγ is reduced by the presence of the CB-PRP derived preparations (∼60%), but the reduction is not uniform across preparations (incubation with CB-PR leads to ∼50% less INFγ for example) but it is not significantly different within preparations for the different genotypes. In agreement with our previous study, we did detect a difference in reduction of IFN-γ according to genotype with CB-PPP. Samples typed as V homozygous showed less of a reduction in production of IFN-γ (mean 52% ± 2.4 SEM) compared to M homozygous and V/M heterozygous samples (mean 42% ± 1.5 SEM) and was statistically significant ($p = 0.0074$). Overall, 50% of samples tested were V/V and 50% M/M or M/V genotype.

DISCUSSION

In this study we have assessed and compared the properties of 3 different CB-PRP preparations, CB-PPP, CB-PL and CB-PR.

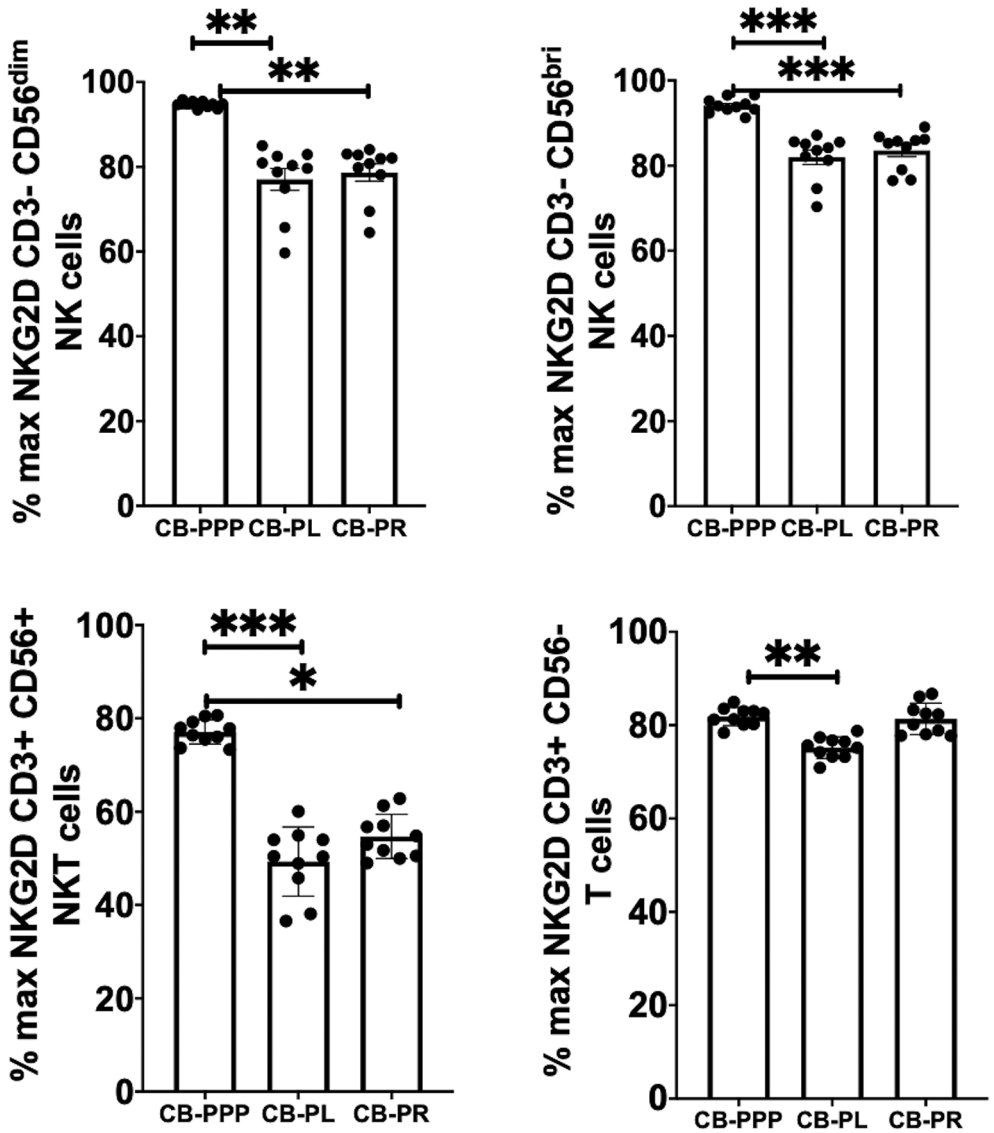

FIGURE 4 | Expression of NKG2D on NK, NKT and T cells from healthy donors is reduced by incubation with, CB-PPP, CB-PL, and CB-PR. Data represents analysis of CD3– CD56 dim, CD3– CD56 bright NK cells, CD3+ CD56+ NKT cells and CD3+ CD56– T cells in adult donor PBMCs ($n = 4$) after incubation with CB-PRP preparations or complete media only. Results show percentage of maximum expression relative to complete media for each cell type from unstimulated cultures. CB-PRP preparations investigated were cord blood platelet poor plasma (CB-PPP; $n = 10$), cord blood platelet lysate (CB-PL; $n = 10$), or cord blood platelet releasate (CB-PR; $n = 10$). PBMCs were incubated with each preparation diluted 50:50 with media and supplemented with IL-2, or with complete media and IL-2 only for 48 h prior to antibody staining and flow cytometry analysis. Each experiment was repeated with four different PBMC donors and data points represent donor means. Statistical analysis was performed using non-parametric one-way ANOVA (Kruskal-Wallis test with Dunn's *post-hoc* test for unpaired samples and Friedman's for paired samples). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

We measured the concentrations of various factors which are thought to contribute to the activity of CB-PRP. We found that as expected the CB-PPP preparation had lower concentrations of most analytes, while there was less differences between CB-PL and CB-PR. In general, the concentrations of the key analytes were higher in CB-PRP preparations than in tears and comparable to those in adult serum. This is similar to other reported studies (23, 24), though we measured additional, previously not reported analytes.

Further we looked at the immunomodulatory properties of the 3 preparations, by investigating their effects on the activation of NK, NKT, and T cells. We found that all 3 preparations reduced the expression of activation markers NKG2D and CD107a in all cell types investigated to different degrees and further they also decreased the expression of INFγ in NK cells. As we used the same unit to make all three preparations, and the paired and unpaired analysis give almost identical results, it is most likely the effects are due to the PRP preparation and only some of the effect

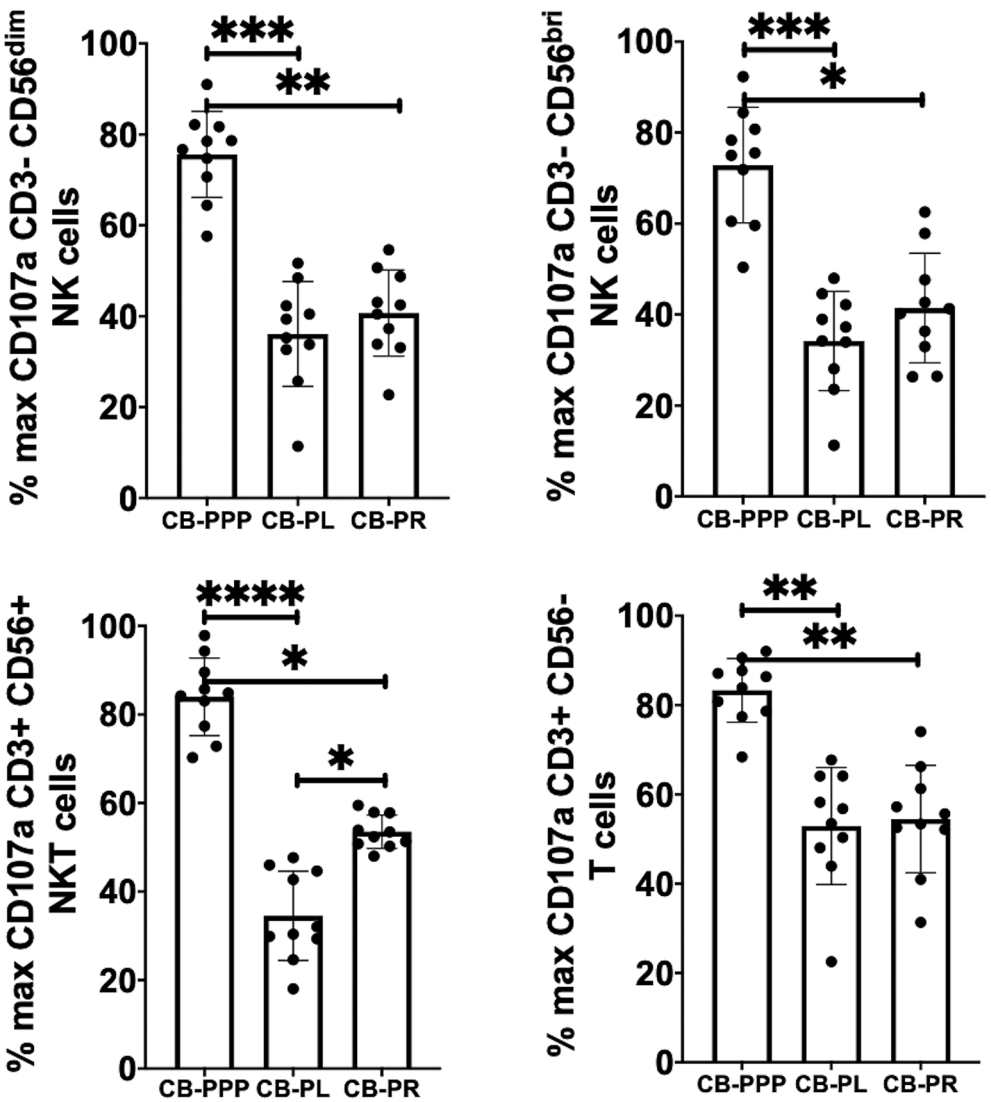

FIGURE 5 | Expression of CD107a after PMA and ionomycin stimulation on NK, NKT, and T cells from healthy donors is reduced by incubation with, CB-PPP, CB-PL, and CB-PR. Data represents analysis of CD3– CD56 dim, CD3– CD56 bright NK cells, CD3+ CD56+ NKT cells and CD3+ CD56– T cells in adult donor PBMCs ($n = 4$) after incubation with CB-PRP derived samples or complete media only. Results show percentage of maximum expression relative to complete media for each cell type following 2 h stimulation with PMA and ionomycin. CB-PRP preparations investigated were cord blood platelet poor plasma (CB-PPP; $n = 10$), cord blood platelet lysate (CB-PL; $n = 10$), or cord blood platelet releasate (CB-PR; $n = 10$). PBMCs were incubated with each preparation diluted 50:50 with media and supplemented with IL-2, or with complete media and IL-2 only for 48 h prior to antibody staining and flow cytometry analysis. Each experiment was repeated with four different PBMC donors and data points represent donor means. Statistical analysis was performed using non-parametric one-way ANOVA (Kruskal-Wallis test with Dunn's *post-hoc* test for unpaired samples and Friedman's for paired samples). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

is due to individual variations between individuals. As we have reported previously, we believe this reduction is caused by the presence of soluble NKG2D ligands (MICA, MICB and ULBP1) in the CB-PRP preparations, but it would be prudent to have a larger sample size to confirm this. Studies in tumor biology do corroborate this idea, as soluble NKG2D ligands produced by tumor cells have been reported to downregulate NK cell responses (25, 26).

To understand better the properties of these PRP preparations and the processing methodology, it would be important to measure the concentrations of the GFs at different stages during the processing and see how the different steps affect the final product.

The mechanism of action of CB-PRP in terms of immunomodulation has been investigated by us and others (12, 22). The anti-inflammatory molecule transforming growth factor beta (TGFβ), is an obvious candidate to exert such a function as it is known to be present in fairly high levels in PRP (27–29). Some studies have looked at the effect of TGFβ on the induction of T-regulatory (Tregs) cells as a plausible

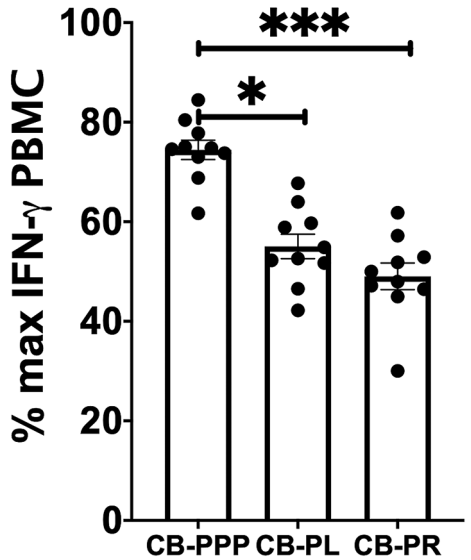

FIGURE 6 | IFN-γ production by PBMCs from healthy donors is reduced by incubation with CB-PPP, CB-PL and CB-PR samples. Results show percentage of maximum IFN-γ expression in culture supernatants relative to complete media following 2 h stimulation with PMA and ionomycin. CB-PRP preparations investigated were cord blood platelet poor plasma (CB-PPP; $n = 10$), cord blood platelet lysate (CB-PL; $n = 10$), or cord blood platelet releasate (CB-PR; $n = 10$). PBMCs were incubated with each preparation diluted 50:50 with media and supplemented with IL-2, or with complete media and IL-2 only for 48 h prior to antibody staining and flow cytometry analysis. Each experiment was repeated with four different PBMC donors and data points represent donor means. Statistical analysis was performed using non-parametric one-way ANOVA (Kruskal-Wallis test with Dunn's *post-hoc* test for unpaired samples and Friedman's for paired samples). No significant differences between the different CB-PRP preparations were found. $^{*}p < 0.05, ^{***}p < 0.001$.

mechanism to explain the overall anti-inflammatory properties of PRP preparations (7). In contrast, in our previous studies we were able to demonstrate that CB-PRP preparations contain soluble molecules that act as ligands for NK cell receptors and result in reduction of NK cell activation potential (22). These soluble molecules, which have an activating role when expressed on the cell surface, interact with NKG2D and directly down-modulate cytotoxicity of cells bearing this receptor in a dose-dependent manner, independent of a similar effect exerted by TGFβ (12). Further work delineating the differential roles of the various types of NKG2D ligands (NKG2DLs) revealed that soluble ULBP1 (sULBP1) was the most abundant ligand in CB-PRP. Furthermore, we also found that a second NKG2DL termed MICA, also supressed NK function, but this ability was affected by a genetic polymorphism in the *MICA* gene itself (12). A dimorphism in MICA gives rise to two possible variants of the protein with either a methionine (Met) or a valine (Val) at residue 129. This difference has been found to have functional consequences, with the affinity for the receptor being higher on the Met containing protein (14). Indeed, investigations found that PRP preparations from donors who had at least one Met allele were able to down regulate the expression of NKG2D, CD107a, and INFγ in NK cells significantly more than PRP preparations form Val homozygote donors, revealing a functional difference affecting the anti-inflammatory potential of sMICA variants (12).

The use of PRP as a treatment for eye (9) and skin lesions (30) has been established for some time. Though initial studies were carried out mainly on autologous PRP preparations, as a means of avoiding transmission of pathogens, the use of allogeneic PRP has been favored in recent years (9, 31). The use of allogeneic PRP has several advantages, and it offers a means of standardizing the preparation and quality control to make dosing regimens more accurate. Amongst the sources of PRP, CB has been studied closely in the last decade or so, as it has all of the advantages of other allogeneic sources, with the extra advantages of being essentially a waste by product which requires very little extra effort to prepare; having special properties and being less likely to have unexpected transmissible viral pathogens, as mothers are checked during pregnancy.

Currently we are using CB-PRP preparations on two separate clinical trials for two indications: an eye drop treatment for Neurotrophic Keratopathy (NCT03084861) and a gel patch for diabetic foot ulcers (NCT02389010). However, there is a need to establish the likely differences in preparations and how the biochemical content and functional attributes of each preparation may affect their use. We propose that for skin ulcers, the most appropriate preparation would be platelet releasate (PR) in gel form (CB-PG), where the GFs content is released gradually (32). This contains the highest concentrations of both angiogenic as well as trophic and wound healing factors and the lowest concentration of inflammatory molecules. For skin regeneration several phases are required, first there is a vasoconstriction and platelet aggregation phase to stop bleeding, followed by an inflammatory phase which recruits several cells (mainly neutrophils) into the site followed by a proliferative phase where there is new tissue formation (33). The second phase requires pro-inflammatory molecules such as TNFα and INFγ, as well as factors that promote collagen degradation, followed by a re-epithelialisation phase where fibroblasts are recruited to the site and with the help of growth factors induce the multiplication of this fibroblast, which in turn deposit new collagen. In chronic wounds there is a clear disruption to this process therefore providing these factors exogenously should have a positive effect. Consequently, preparations with high levels of TNFα, for example, which increase inflammation, as well as metalloproteinases and their inhibitors, such as the MMPs and TIMPs, which degrade collagen, should aid in the induction of the wound healing cascade (34). High levels of bFGF and EGF consolidate the effect by promoting fibroblast proliferation. The necessary neo vascularisation is also promoted by VEGF, though the levels of this factor are modest in the CB-PR preparation. Importantly the CB-PR preparation reduces the expression of NGK2D activating receptor as well as CD107a in NK cells only modestly, thus the necessary immune cell activation and IFNγ production is not blocked by this preparation. Previous work using platelet lysate preparations in *in vitro* models of wound

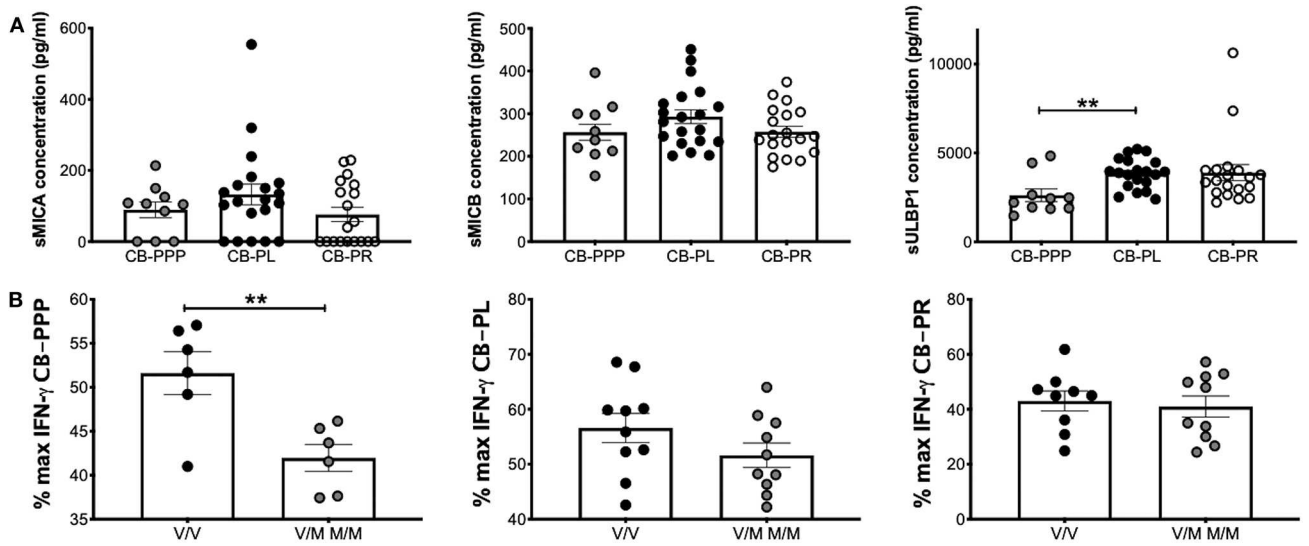

FIGURE 7 | CB-PRP preparations (CB-PPP, CB-PL, and CB-PR) contain immunosuppressive soluble NKG2D ligands. **(A)** ELISA analysis of soluble NKG2D ligand content (sMICA, sMICB, and sULBP1) in CB-PPP ($n = 10$), CB-PL ($n = 20$), and CB-PR ($n = 19$) revealed similar quantities of sMICA and sMICB. Significantly higher concentration of sULBP1 was found in CB-PL compared with CB-PPP. **(B)** MICA genotyping was performed with DNA obtained from whole CB used to prepare CB-PL ($n = 20$), CB-PR ($n = 19$), and CB-PPP ($n = 12$). MICA-129 valine homozygotes (V/V) were compared with heterozygote and homozygote MICA-129 methionine genotypes (V/M or M/M) for each preparation. Statistical analysis was performed using non-parametric one-way ANOVA (Kruskal-Wallis test with Dunn's *post-hoc* test) or Mann Whitney test. $^{**}p < 0.01$.

healing demonstrated that the combination of factors present in platelet lysates was able to promote wound healing in a dose dependent manner (35). CB-PC derivatives (lysate and releasate) are rich in GFs, and are attractive sources for many clinical applications in degenerative conditions, such as joint and cartilage pathologies (36, 37), where injection of this product can be beneficial.

For the treatment of ocular conditions, preparations need to be appropriate to the indication being treated. On the one hand, ocular ulcers and other conditions that have damaged the ocular surface where regeneration is required need special preparations (38). These should contain sufficient quantities of EGF, bFGF, and a certain quantity of inflammatory cytokines to activate the regenerative process. However, they must not promote the excessive proliferation of fibroblasts leading to scarring, as it would impair the function of the cornea. The CB-PL preparation has moderately high levels of EGF, bFGF, HGF, PDGF, and VEGF and fairly high levels of TGFβ (compared to CB-PPP and CB-PR), mostly in line with those found in natural tears, except for VEGF which is much higher in PL. The CB-PL preparation has a dramatic effect on NK, NKT and CD3 T-cells. It reduces the expression of both NKG2D and CD107a substantially indicating a reduction on the activation potential of these cells, thus likely reducing overall inflammation and disease progression but without compromising viability.

For ocular diseases where the corneal surface is not yet compromised, such as dry-eye disease, which is usually caused by a deficiency of the lacrimal functional unit and is accompanied by ocular surface inflammation and irritation due to lack of lubrication (39), the CB-PPP preparation may offer an optimal topic treatment, though here we analyzed the raw material, the topical eye drops used for clinical application are diluted in Plasmalyte, which may dilute the fibrinogen and the pro-thrombin present in the CB-PRP avoiding their possible negative effects. So far, studies have not shown any safety issues caused by the presence of fibrinogen which is present in autologous PRP preparations too (40, 41), however, further studies are necessary to measure this more accurately. This preparation contains low levels of pro-angiogenic and wound healing factors, it also reduces the activation of NKT cells by about 50%, which may arrest the progression of disease caused by the recruitment of Th17 cells. Previous studies in animal models of severe dry eye disease, showed that activated NK and NKT cells up-regulated IL-6 and IL-23 and created an environment where dendritic cells could skew T cell activation to a more pathogenic Th17 phenotype (42). In the absence of NK and NKT cells, disease progression was less severe (43), as no pathogenic Th17 cells were activated (42). Additionally, in the presence of CB-PRP derivatives preparations there is a substantial reduction (∼50%) in the production of INFγ by immune cells, which would otherwise contribute to the inflammatory process. The CB-PPP preparation contains the lowest levels of the pro-inflammatory cytokines IL-6, IL-1α, and TNFα, and substantial amounts of soluble NKG2D ligands (ULBP1, MICA, and MICB), making it ideal to treat conditions where inflammation has a major role in disease progression.

In conclusion, CB has unique properties and can be used as a source of plasma and platelet to formulate preparations for

regenerative medicine, that can be used for the topical treatment of several eye conditions that affect the ocular surface, such as severe dry eye disease, corneal ulcers and burns. Furthermore, these preparations could also serve as an optimal adjuvant to help in skin wound healing and for articular regenerative processes. Through differential processing of CB-PRP production, we have formulated three blood derived preparations which have different and interesting biochemical and immunomodulatory properties which can be investigated in translational applications. Further work in evaluating the safety and efficacy of these preparations must now be undertaken.

Additionally, future studies should include: (a) *in vivo* safety and efficacy studies in models of corneal and skin wounds. (b) the assessment of vitamin A concentration on final preparations, as it is known to be light sensitive (44), but an essential ingredient in eye drop preparations and (c) the effects of the preparations on the immune response in general.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

Plasma samples from individual CB units were obtained from BioBank (BST). The informed consent for the use of samples for research was obtained in accordance with the requirements of the Declaration of Helsinki and local laws at the time of collection. This study was approved by the BST local Research Ethics Committees (HCB/2017/0785).

AUTHOR CONTRIBUTIONS

DS and SC designed, carried out experiments, and wrote the manuscript. DH analyzed results, designed, and wrote manuscript. RC-M design study, analyzed results, and wrote manuscript. SQ, LR, and AM designed study and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00942/full#supplementary-material

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The reviewer JB declared a past co-authorship with one of the authors SQ to the handling editor.

Paper 3. *Clinical evaluation of allogeneic eye drops from cord blood platelet lysate*

Dinara Samarkanova, Sara Martin, Laia Bisbe, Luciano Rodriguez, Carmen Azqueta, Ruth Coll, Ricardo Casaroli-Marano, Alejandro Madrigal, Paolo Rebulla, Sergio Querol, on behalf for Barcelona CBED Study Group

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RESUMEN:

Antecedentes - Los tratamientos actuales para varias lesiones corneales muestran una eficacia limitada. Aquí se reporta la evaluación clínica de la eficacia de una nueva preparación de colirio preparada en un banco público de sangre de cordón umbilical.

Material y Métodos - Se realiza un estudio de casos multicéntrico, retrospectivo y consecutivo evaluando 33 pacientes (46 ojos) que no respondían a los tratamientos convencionales y que requerían intervenciones urgentes, que recibieron colirios alogénicas preparados a partir del lisado de plaquetas de sangre del cordón umbilical (CBED) para tratar lesiones de superficie ocular severas bajo uso compasivo. El CBED se preparó con unidades de sangre de cordón umbilical donadas para el trasplante de células madre hematopoyéticas que no cumplían con la dosis mínima de células madre requerida para este uso. Los pacientes fueron agrupados por condiciones agudas (úlceras neurotróficas: grupo I; otras úlceras corneales: grupo II; quemaduras corneales: grupo III), y condiciones crónicas (enfermedad de injerto ocular contra huésped: grupo IV; síndrome de ojo seco grave: grupo V). Se instruyó al personal sanitario y a los pacientes para que administraran 1-2 gotas de CBED en cada ojo afectado cada dos horas, hasta 4-6 veces al día. El tratamiento inicial de 19 días podía repetirse según la evaluación clínica.

Resultados - Los pacientes recibieron una mediana de 19 viales diarias de CBED, intercuartil 19-57, rango 19-442. Los pacientes del grupo I-II-III mostraron una recuperación total y parcial de la úlcera en 25 (78%) y 6 (19%) ojos respectivamente. Un ojo (3%) no respondió al tratamiento. Para los grupos IV-V se reportó una mejoría en 12 (85%) ojos y las lesiones empeoraron con el tratamiento en ambos ojos (15%) de un paciente. No se atribuyeron eventos adversos severos directamente al CBED.

Discusión - La disponibilidad inmediata de CBED resultó en un tratamiento alogénico bien tolerado que mostró evidencia de eficacia en esta cohorte de pacientes. Estos resultados positivos apoyan estudios adicionales sobre CBED de lisado plaquetario como un producto novedoso de los bancos de CB. Se está llevando a cabo un ensayo clínico prospectivo en queratitis neurotrófica (NCT03084861) para confirmar estos datos preliminares.

Clinical evaluation of allogeneic eye drops from cord blood platelet lysate

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Key words: cord blood, eye drops; corneal ulcers; ocular surface diseases; neurotrophic keratitis; dry eye; ocular chemical burns.

Short tittle: Eye drops from allogeneic cord blood platelet lysate

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Abstract:

Background - Current treatments for several corneal lesions show limited efficacy. Here we report the clinical evaluation of the efficacy of a novel eye drop preparation produced in a public cord blood (CB) bank.

Material and Methods - We performed a multicentre, retrospective, consecutive case study evaluating 33 patients (46 eyes) unresponsive to conventional treatments and requiring urgent interventions, who received allogeneic eye drops obtained from cord blood platelet lysate (CBED) to treat severe ocular surface lesions under compassionate use. CBED were prepared from CB units donated for hemopoietic stem cell transplantation that did not comply with minimum stem cell dose required for this use. Patients were grouped by acute conditions (neurotrophic ulcers: group I; other corneal ulcers: group II; corneal burns: group III), and chronic conditions (ocular graft-versus-host disease: group IV; severe dry eye syndrome: group V). Health personnel and patients were instructed to administer 1 to 2 drops of CBED to the affected eye 4 to 6 times per day for 19 days. A further cycle of 19-day treatment could be repeated according to initial clinical response'.

Results - Patients received a median of 19 CBED vials, interquartile 19-57, range 19-442, to complete the therapy. Group I-II-III patients showed full and partial ulcer recovery in 25 (78%) and 6 (19%) eyes respectively. One eye (3%) did not respond to treatment. For groups IV-V improvement was reported in 12 (85%) eyes and lesions worsened on treatment in both eyes (15%) of one patient. No severe adverse events were directly attributed to CBED.

Discussion - Promptly available CBED resulted in a well-tolerated allogeneic treatment that showed evidence of efficacy in this cohort of patients. These positive results support further studies on CBED from platelet lysate as a novel product of CB banks. A prospective clinical trial in neurotrophic keratitis (NCT03084861) is ongoing to confirm these preliminary data.

Introduction.

Serum eye drops, most frequently obtained from patient's autologous blood, have been used for the treatment of severe ocular surface lesions [1]. This use is based on the evidence that blood serum and natural tears show similar values of pH and osmolarity, and share a number of constituents including growth factors and vitamin A [2]. The latter help to restore an environment that promotes re-epithelialization and supports ocular surface health. These factors are likely to be responsible for the therapeutic benefits observed in comparison to commercial conventional ocular lubricants [3].

However, the availability of autologous serum is usually delayed by as much as two weeks, due to completion of microbiological controls. Moreover, autologous serum eye-drop preparation may not be feasible in patients that are positive for serological markers of infectious diseases, in the very young or the elderly and in patients requiring intensive care treatment, for whom autologous blood collection may be difficult or impossible. In these circumstances, an allogeneic source could be considered.

There are studies suggesting the use of healthy blood donors as source to obtain allogeneic eye drops, as reviewed by Giannaccare et al [1]. An attractive alternative to this readily available source is the use of umbilical cord blood (CB), that presents unique biological characteristics including key growth factors and anti-inflammatory molecules [4,5]. In this regard, previous studies have described the therapeutic effects of CB serum derived eye drops [6,7].

We investigated the novel approach of using CB platelet lysate rather than serum as source material for eye drops. This source is particularly convenient for public CB banks, where large numbers of anticoagulated CB units can be repurposed for this use from those unsuitable for haemopoietic stem cell transplantation [8–10]. They show routinely controlled quality and safety profiles [11], ideal to generate allogeneic, off-the-shelf, CB eye drops (CBED).

This article reports a preliminary clinical evaluation of CBED prepared in our CB bank that were used to treat a consecutive case series of patients with severe ocular surface lesions unresponsive to conventional treatments.

Material and Methods

Study design and patient selection

This is a consecutive case series study analysing clinical outcomes of patients treated with CBED for severe refractory surface ocular lesions. Some of these patients were treated after failing to comply with strict inclusion criteria of an ongoing randomized clinical trial to evaluate safety and efficacy of CBED for neurotrophic keratitis (NCT03084861), and others were included using a compassionate scheme. Patients' physicians proposed use of CBED if patient: a) showed severe pathology of the ocular surface; b) was unresponsive to conventional treatment (artificial tears, lubricant gels, therapeutic contact lenses) or to other blood derivatives like autologous or, if possible, allogeneic serum; c) required urgent interventions, that precluded production of autologous serum eye drops; d) showed full understanding of the therapy conditions and agreed to adhere to treatment protocol and comply with the scheduled control visits.

The series included 33 consecutive patients (46 eyes) treated during November 2015 to April 2019 in 5 ophthalmologic centres that constituted the Barcelona CBED study group (see appendix I). Following informed consent, a request was submitted to obtain compassionate use authorization from the Spanish Drug Agency (AEMPS) for each case.

After approval, a batch of frozen CBED was released from the CB bank and sent to the patient's ward for immediate start of the treatment.

Preparation of CBED

In Spain, human plasma for non-substitutive (transfusional) use is regulated as a 'special medicine' and its manipulation requires the compliance with specific norms issued by AEMPS. In this regard, the Banc de Sang i Teixits (BST) holds Investigational Drug Approval for the therapeutic use of CBED (PEI 16-116). In addition, BST obtained a non-exclusive license from Episkey s.r.l (Lovero, Italy) to manufacture, use and distribute CB derived plasma and platelet components.

CBED manufacturing process requires a stock of stored CB platelet concentrates (CBPC) as starting material. Full manufacturing validation of CBPC has been published elsewhere [10]. The CBPC used for manufacturing CBED contained 1,000 x 10^9/L platelets in 5-30 mL and were negative for infectious disease markers including HIV-1/2, HCV, HBc, CMV and HTLV I-II antibodies, HBsAg, nucleic acid testing for HIV,

HBV and HCV (triple NAT), antibodies anti Trypanosoma Cruzi (Chagas disease), anti-Treponema Pallidum (Syphilis); and negative cultures for aerobic and anaerobic bacteria and fungi. The CBED vials were produced under GMP conditions, in class C clean rooms of the BST facilities.

The CBPC underwent 3 frozen/thawing cycles (frozen without cryoprotectant at -80 °C and thawed in a water bath at 37 °C) [12] to obtain a platelet lysate (CBPL) rich of anti-inflammatory and tissue regenerative factors. Finally, the CBPL was centrifuged at 5000g for 15 min (Allegra® X-15R centrifuge, equipped with a SX4750A ARIES swinging bucket rotor, Beckman Coulter Inc, Indianapolis, IL, USA) to sediment the platelet stroma and the CBED were obtained by vol/vol dilution of the supernatant with Plasmalyte (Baxter SL, Valencia, Spain). The volume of CBED obtained from a single CB unit (defined as "one batch") was dispensed into a commercial kit of 20 vials (COL20, BioMed Device s.r.l., Modena, Italy) which were frozen without antimicrobials or other preservatives. The volume of CBED after Plasmalyte dilution defined the number of vials that could be generated in each batch. Each individual vial contained between 350-500 µL. Assuming that one drop is equal to 30µL (COL-20 guide), each vial contained 11-16 drops. A treatment of 4-6 applications per day in both eyes required 8-12 drops/day. This means that one vial contains the necessary quantity for one day therapy. Each CBPC derived-batch could include up to 40 vials. Finally, the vials were packed in boxes containing each 19 vials and stored frozen until release. One vial per batch was long term stored for regulatory purposes. Specification for CBED batch release were: platelets $\leq 15x10^9$/L, leukocytes $\leq 0.5x10^9$/L, erythrocytes $\leq 0.01x10^{12}$/L and proven sterility.

A total of 119 CBPC were used for preparing the required CBED. Of them, 20 (13%) did not comply with acceptance criteria (10% due to microbiology positive results and 3% due to platelet count exceeding the limit) and were discarded. From the CBED processes that met acceptance criteria, 161 individual packages of 19 vials/each were finally shipped during the study period. Cost of CBED manufacturing amounted to 202.5 euro per batch of 19 vials.

Clinical protocol

After treatment approval by AEMPS, CBED was shipped from the CB bank to patient's ward and stored in hospital freezers. Alternatively, if the patient's condition indicated that CBED could be self-administered at home, instruction was given to patient or

companion on CBED frozen storage in a domestic freezer. To start treatment, one vial was thawed at room temperature. During the day of use, the thawed vial was capped and stored in a domestic refrigerator in a sterile plastic bottle to prevent any contamination and maintain growth factor stability[13]. Health personnel or patients were instructed to administer 1-2 drops into each affected eye 4-6 times along day, with a minimum of at least two hours between applications. Each morning a new vial was thawed for use. The initial treatment duration period was 19 days, which could be repeated if some degree of improvement was observed during clinical evaluation. Improvement was defined as positive variation of clinical symptoms, ulcer size or keratopathy reduction pre and post administration. Used vials were returned to the CB bank for verification.

A common protocol for clinical follow-up was established with all ophthalmology clinics (**Figure 1**). First, an ophthalmologic examination was carried out within 2-3 days after starting application to check tolerance of treatment and to detect adverse events. Follow-up visits were carried out weekly during the first treatment course and once monthly if the treatment was repeated.

Clinical data collection and statistical analysis

Treated patients were grouped by condition: neurotrophic ulcers (group I), other corneal ulcers (group II), corneal burns (group III), ocular graft-versus-host disease (GVHD) (group IV), and severe dry eye syndrome (DES) (group V).

For the retrospective data collection, a purpose-designed form with instructions to homogenize data interpretation was sent to each participant clinician to recover the following information: a) visual acuity (Snellen chart) where possible; b) qualitative corneal esthesiometry (norm, hypo or anesthesia), according to the investigator's criteria; c) evaluation of corneal ulcer/desepithelization area (in mm^2) and keratopathy (in affected quadrants) by positive staining with fluorescein using slit lamp biomicroscopy; d) clinical variables (corneal inflammation, conjunctivalization, corneal neovascularization, pain) assessed according to semi-qualitative or semi-quantitative scale; e) presence of complications: thinning, perforation, melting, calcifications, infections, and vascularization; f) presence of adverse events associated with the treatment, and g) subjective self-reports. In addition, clinical data were collected using information obtained during programmed visits weekly during first treatment course, and then monthly during the extension of the therapy if applicable). If available, ocular

surface images were collected.

The clinical outcomes were evaluated during and after treatment. For groups I, II and III, outcomes were arbitrarily defined in terms of ulcer closure as recovery (100% ulcer closure), improvement (some degree of reduction), or failure (if no change or worsening was appreciated). For patients with chronic conditions in groups IV and V, the outcomes were only defined as improvement or failure according to clinical evaluation changes observed pre and post CBED therapy.

Descriptive statistics are reported using mean and standard deviation or median and range.

Results

The consecutive case series included 33 patients (46 eyes), 20 males and 13 females, aged 1 month to 92 years (mean 61 ± 23). A total of 1,897 CBED vials obtained from 99 CB units were used during the study period. Patients received a median of 19 CBED vials, interquartile 19-57 (range 19-96 for Groups I-II-III and 38-442 for Groups IV-V). Clinical outcomes by etiology group are reported in **Table 1**.

In groups I-II-III, full and partial ocular surface ulcer recovery were observed in 25 (78%) and 6 (19%) of 32 eyes respectively. One eye (3%) did not respond to treatment. In groups IV and V, improvement and failure were reported in 12 (85%) and 2 (15%) eyes respectively. Overall (groups I-V), no response to CBED treatment was reported in 3/46 eyes (6.5%).

Individual data and outcomes from patients enrolled in groups I-II-III and IV-V are reported in **Tables 2** and **3** respectively. Pictures from selected patient's eyes before and after CBED treatment are shown in **Figures 2** and **3**. Additional observations from selected cases are reported below by etiology group.

In group I, CCU8 showed corneal stromal infiltrates 21 days after starting CBED treatment, but achieved complete healing after topical antibiotic treatment. CCU20 presented with a peripheral corneal ulcer that was persistent in spite of intensive therapy with topical tobramycin, ciprofloxacin, autologous serum eye drops, poly-carboxymethylglucose sulfate (Cacicol®) and tarsorrhaphy. Application of CBED showed efficacy within 5 days with full ulcer closure. The epithelial defect did not reappear at follow-up.

In group II, CCU4, presenting exposure ulcers in both eyes, demonstrated substantial structural improvement and reduction of inflammation; the left eye had severe corneal infiltration with melting in the lower temporal quadrant of cornea that healed after 19 days CBED treatment (**Figure 2 c-f**). CCU27 presented a corneal trauma with wide surface de-epithelization, which was unresponsive to conventional treatment (**Figure 2 a-b**). After 2 weeks of intensive CBED topical administration, a complete recovery of ocular surface integrity and corneal transparency was noted, with no recurrence at follow-up.

In group III, CCU23 showed bilateral damage (**Figure 2 g-j**) and received a combination of amniotic membrane transplantation every 5 days and an hourly application of CBED. The right eye had 360º limbic ischemia, notable eyelid edema,

associated with intense conjunctival edema, and loss of corneal transparency. Improvement of both ocular surfaces was observed with this combined therapeutic approach. CCU25, that failed CBED treatment, showed desepithelization of total cornea, limbus and part of conjunctiva. CCU33 presented ocular burn of grade IV-V (Dua scale) with bilateral epithelial defect which did not respond to amniotic membrane transplantation. After CBED application, 100% recovery was achieved in both eyes (**Figure 2 k-n**).

In group IV, CCU2 presented with bilateral severe limbic deficiency with corneal neovascularization and conjunctivalisation affecting both eyes. After CBED treatment disease progressed and ended in a corneal perforation with fungal infection. The adverse event of CCU2 was severe and was resolved after antifungal treatment and amniotic membrane transplantation. This event was considered not related to CBED and due to disease progression.

In group V, CCU14 presented with severe DES due to hypovitaminosis A that was unresponsive to conventional therapy. This patient was also unable to use autologous serum due to HCV infection. Improvement was evident after 19 days CBED treatment but recurred after CBED withdrawal. CBED was resumed and controlled recurrence during follow-up (**Figure 3**).

Patients self-reports included positive outcome (general improvement) in all cases, except CCU2 and CCU25

Discussion

This study showed encouraging outcomes from the compassionate use of novel allogeneic CBED prepared from platelet lysate for the treatment of a consecutive series of 33 patients (46 eyes) with severe ocular surface lesions refractory to conventional therapies. Full ulcer healing was observed in 78% of the treated eyes from 26 patients with neurotrophic keratitis, corneal ulcers of different etiology and corneal burns. Clinical improvement was noted in 85% of the treated eyes from 7 patients with GVHD and severe DES.

The CBED compassionate use was approved by the Spanish national drug agency AEMPS based on the patients' urgent need for effective treatment, which prevented the use of autologous serum in 19 patients who could not wait until the completion of blood collection and manipulation procedures and of sterility testing required before serum release[14]. Moreover, autologous serum could not be used in 9 additional cases due to practical reasons (positive infectious markers, elderly age, severe comorbidities) or concern that autologous serum from GVHD patients could contain noxious pro-inflammatory factors. This concern was also supported by the failure of previous treatment with autologous serum in 2 and 3 additional patients with severe DES secondary to Sjögren syndrome, as reported in other studies[15], and neurotrophic keratitis respectively.

A number of studies compared allogeneic eye drops obtained from different serum and plasma sources and evaluated their immune modulation properties[14,16]. Interestingly, previous studies showed that adult peripheral blood is richer in inflammatory factors than CB plasma [5] and that CB plasma contains unique molecules not present in peripheral blood, such as NKG2D ligands (MIC, ULBP1), that play an important role in immune suppression [17]. This feature supports the preferential use of CB in conditions associated with abnormal inflammation.

Our clinical observations corroborate previous investigations [16] showing that the administration of 20% CBED prepared from CB serum to 14 patients with persistent corneal defects was effective in 86% of the cases, with no significant complications. Significant improvements after 1 month of treatment with 20% CBED from CB serum of 30 patients with severe corneal epithelial damage were also reported [18]. CBED at 20% serum dilution were also used in 33 eyes presenting chemical burns, demonstrating the safety and lack of toxicity of this treatment. Complete epithelialization was achieved

in 12 of 18 cases in shorter times compared to artificial tears and autologous serum [19]. Additional positive results with CB serum were reported from patients with severe DES associated with neurotrophic keratitis [19] and GVHD [20]. A reduction in the frequency of recurrences from 2.24 ± 1.09 to 0.5 ± 0.79 was also reported in patients treated with artificial tears and CB serum respectively, during a follow-up of 14.7 ± 2.5 months [21]. Studies comparing autologous and CB serum in 92 eyes demonstrated higher therapeutic effectiveness in CB serum eye drop recipients [22]. Thus, there is scientific evidence to support the use of topical CB serum in severe ocular surface diseases. The clinical outcomes from our trial are aligned with those described in the literature [2,16,18,23].

Operationally, the selection of platelet lysate as source material for the preparation of CBED offers the advantage of using a large proportion of anticoagulated CB donations containing too few stem cells to make their long term banking convenient for public CB banks[10].

There are several limitations in our study, including the small number of patients tested in each of the conditions, a variable degree of patients' clinical symptoms at presentation, and the lack of concurrent controls treated with conventional therapy only. In spite of these limitations, it is encouraging to note that positive outcomes were obtained in a large proportion of this cohort of patients who had failed all applicable therapeutic options.

While the currently available data suggest that CB serum and platelet lysate could be provisionally considered complementary sources for the production of CBED, additional studies should be performed to test different dosages and administration schedules in different conditions. Moreover, blinded randomized clinical trials are necessary to conclusively determine the respective clinical efficacy of biological ED prepared from different blood sources. These comparative studies should also include highly expensive recombinant molecules that recently became available in the market[24].

Conclusions

This study provides preliminary evidence on the safety and efficacy of CBED prepared from platelet lysate as a new therapeutic blood component manufactured in a public cord blood bank. These preliminary data support the development of further studies to

obtain regulatory approval for routine CBED clinical use. A prospective randomized clinical trial is currently ongoing in patients suffering from neurotrophic keratitis (NCT03084861).

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Authorship Contributions:

SQ, DS, LR, RCB – designed study; SM, LB, JP and Study group (Appendix I) – patients recruitment and follow up; SM, LB, JP, RC, PR, DS – analysed data; AM, PR, RCB – results discussion; SQ, PR, LR, SM, RC, DS – wrote manuscript. All authors discussed and revised manuscript.

[SQ – Sergio Querol; LR – Luciano Rodríguez; RCB – Ruth Coll; RC – Ricardo Casaroli; SM – Sara Martin; LB – Laia Bisbe; JP – Javier Puig; AM – Alejandro Madrigal; PR – Paolo Rebulla; DS – Dinara Samarkanova].

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Conflict of interest

Paolo Rebulla is a co-inventor of a patent on platelet fractions from cord blood and holds shares of Episkey, a start-up company aimed at developing novel reagents and therapeutics from human blood

Appendix I: The Barcelona Cord Blood Eye Drop (CBED) Study Group

This is a multicentre retrospective case series study and the following people are considered as co-authors representing the Barcelona CBED study group:

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Alexandra Arango	–	*Hospital Universitari German Trias y Pujol, Badalona, Spain*
Nevena Romanic	–	*Hospital Universitari German Trias y Pujol, Badalona, Spain*
Anna Mones	–	*Hospital Universitari German Trias y Pujol, Badalona, Spain*
Silvia Bover	–	*Hospital Santa Caterina, Salt, Girona, Spain*
Teresa Torrent	–	*Hospital Dr Josep Trueta, Girona, Spain*
Veronica Mas	–	*Hospital Dr Josep Trueta, Girona, Spain*
Miriam Barbany	–	*Hospital Mutua de Terrassa, Terrassa, Spain*
Irene Sassot	–	*Hospital Mutua de Terrassa, Terrassa, Spain*
Daniela Ortiz	–	*Hospital Universitari Joan XXIII, Tarragona, Spain*

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FIGURE LEGENDS

Figure 1. Algorithm of decision for CBED treatment

Figure 2. Clinical outcomes. CCU4, 75 years old patient with corneal ulcer in both eyes after eye exposure pre (**a** and **c**) and post (**b** and **d**) CBED application. CCU27, 1-month old patient with corneal ulcer by trauma pre (**e**) and post application (**f**). CCU23, 3 years old patient presenting an acute bilateral corneal burn pre (**g** and **i**) and post (**h** and **j**) CBED application on right eye (RE) and left eye (LE). CCU33, 44 years old presenting acute bilateral corneal burn pre (**k** and **m**) and post (**l** and **n**) CBED treatment on right (RE) and left (LE) eyes.

Figure 3. Clinical outcomes. CCU14, a patient with severe DES, hypovitaminosis A and HCV infection, three weeks after treatment discontinuation (A) and two days post resuming CBED application (B).

Table 1. Clinical outcomes after CBED treatment by patient etiology group.

Group	Etiology	Patients n (%)	Eyes n (%)	Recovery n (%)	Improvement n (%)	Failure n (%)
I	Neurotrophic ulcers	18	19	14 (73.7)	5 (26.3)	0 (0)
II	Corneal ulcers (others)	4	6	5 (83)	1 (17)	0 (0)
III	Corneal burns	4	7	6 (80)	0 (0)	1 (20)
Total for groups I-II-III		**26**	**32 (100)**	**25 (78)**	**6 (19)**	**1 (3)**
IV	Ocular GVHD	3	6	na	4 (67)	2 (33)
V	Severe DES	4	8	na	8 (100)	0 (0)
Total for groups IV-V		**7**	**14 (100)**	**na**	**12 (85)**	**2 (15)**

GVHD: graft versus host disease; DES: dry eye syndrome; na: not applicable

Table 2. Patients' general data and clinical follow-up: Groups I-II-III.

Group	CCU, Gender, Age	Eye	Pre CBED ineffective treatments added to conventional therapy* (CT); comments on lesions before CBED use; infectious pathologies	Applied vials	Clinical follow-up (pre / post CBED)					Outcome	Notes
					CI	CN	CO	Pain	Ulcer % reduction		
I	1, M, 80	L	ASED, allogeneic serum ED	40	+++/+	+/++	++/+++	+++/-	100	R	-
I	3, F, 59	L R	ASED	60	-/-	-/-	-/-	-/-	100	R R	-
I	5, F, 91	L	No pre-CBED treatment added to CT	19	-/-	-/-	++/+++	-/-	100	R	1
I	6, M, 69	L	No pre-CBED treatment added to CT	19	-/-	-/-	+++/++	-/-	100	R	-
I	7, M, 34	L	No pre-CBED treatment added to CT	19	-/-	-/-	+++/-	-/-	100	R	-
I	8, M, 51	R	No pre-CBED treatment added to CT	19	-/-	-/-	-/+++	-/-	100	R	2
I	9, M, 27	L	AM graft. Corneal recurrent erosions	19	+/++	++/+++	+/++	+/+	65	I	3
I	13, M, 32	R	No pre-CBED treatment added to CT	38	+/-	-/-	++/+	+++/+	93	I	4
I	16, M, 66	L	Temporary tarsorrhaphy	38	-/-	-/-	++/+	-/-	100	R	
I	18, F, 91	L	No pre-CBED treatment added to CT. Persistent epithelial defect secondary to corneal surgery	19	-/-	-/-	++/+	+/+	100	R	-
I	19, M, 56	L	AM graft. Bilateral herpetic keratitis with corneal abscess and hypopyon after keratoplasty; HIV+, HCV+	38	++/++	-/-	++/++	-/-	81	I	-
I	20, F, 63	L	ASED. Temporary lateral tarsorrhaphy	19	-/-	-/-	+++/+	-/-	100	R	-
I	22, M, 77	R	AM graft. Temporal lateral tarsorrhaphy; Lues+	19	-/-	-/-	++/+	-/-	83	I	-

I	24, F, 85	R	AM graft	19	-/-	-/-	-/-	+++/-	100	R	-
I	28, M, 41	R	No pre-CBED treatment added to CT; HIV+, Lues+	19	-/-	-/-	-/-	-/-	100	R	5
I	29, M, 86	L	No pre-CBED treatment added to CT; HIV+	19	-/-	-/+	-/-	-/-	67	I	-
I	30, F, 85	R	No pre-CBED treatment added to CT	19	-/-	-/+	-/-	+++/-	100	R	-
I	31, M, 55	L	No pre-CBED treatment added to CT	19	++/-	+/+	++/+	++/-	100	R	-
II	4, M, 75	L R	Temporary lateral tarsorrhaphy (LE); intensive care patient	19	+++/+	L -/++ R -/-	L ++/++ R -/-	nav	100	R R	6
II	10, M, 51	L R	GvHD. Topic cyclosporine and topic tacrolimus; corneal erosion with calcification complicated with corneal ulcer	76	+/-	-/-	+/+	+/+	L= 96 R=100	I R	-
II	27, F, 1mo	L	Topic ciprofloxacin and acyclovir	38	-/-	-/-	-/-	nav	100	R	-
II	32, F, 76	L	No pre-CBED treatment added to CT	19	-/-	-/-	+++/++	-/-	100	R	-
III	23, M, 3	L R	AM graft	96	+++/-	-/-	L +/- R +++/++	nav	100	R R	-
III	25, M, 62	R	AM graft	57	++/++	-/-	+/+	-/-	na	F	7
III	26, M, 50	L R	AM graft	19	++/-	-/-	L -/- R +/-	+/-	100	R R	-
III	33, M, 44	L R	AM graft	95	+/-	-/-	++/+	nav	L=100 R=100	R R	-

*CT: conventional treatment (artificial tears, lubricant gels, therapeutic contact lenses)

Abbreviations: ASED: autologous serum eye drops; CCU: unique patient number; Gender: M: male, F: female: ED: eye drops; Eye: L: left, R: right; Outcome: R: recovery; I: improvement; F: failure; AM: amniotic membrane; CI: conjunctival inflammation; - absent; + mild; ++ moderate; +++ severe; CN: corneal neovascularization; - absent; + 1 quadrant; ++ 2 quadrants; +++ 3 quadrants; CO: corneal opacity; - absent; + mild; ++ moderate; +++ severe; Pain: - absent; + mild; ++ moderate; +++ severe; na: not applicable; nav: not available.

Notes of Table 2:

1. Bacterial keratitis reported at 3 weeks, resolved with antibiotic treatment.
2. Infection reported at 3 weeks; mild persistent epithelial defect.
3. Clear improvement during days 1-10, then no changes.
4. Patient with Dawn syndrome. CBED treatment requested as a bridge to ASED availability.
5. Endured treatment in patient with glaucoma with maintenance of ulcer closure.
6. Suspension of treatment at 10 days due to infection and restarted after antibiotic application. Pain evaluation not available (intensive care unit patient).
7. Ulcer size not available (intensive care unit patient).

Table 3. Patients' general data and clinical follow-up: Groups IV-V.

Group	CCU, Gender, Age	Eye	Pre CBED ineffective treatments added to conventional therapy* (CT); infectious pathologies	Applied vials	Clinical follow-up (pre / post CBED)			Outcome	Notes
					Pain	Photophobia	Visual acuity		
IV	2, F, 46	L R	Allogeneic serum ED; topic cyclosporine and topic tracrolimus	20	+/+++	+++/++	HM/HM HM/HM	F F	1
IV	11, M, 36	L R	No pre-CBED treatment added to CT	249	+++/+	+++/+	0.5/0.5 0.5/0.5	I I	2
IV	15, M, 63	L R	No pre-CBED treatment added to CT	57	++/+	++/+	L: 0.3/0.6 R: 0.5/0.8	I I	3
V	12, F, 92	L R	No pre-CBED treatment added to CT	442	++/+	++/+	0.3/0.45 0.3/0.45	I I	4
V	14, F, 65	L R	No pre-CBED treatment added to CT; HCV+	154	++/+	+++/+	L: 0.7/0.8 R: 0.7/0.8	I I	-
V	17, F, 58	L R	ASED and autologous platelet concentrate ED	38	++/+	++/+	L: 0.9/1.0 R: 0.8/0.7	I I	-
V	21, F, 70	L R	ASED; topic cyclosporine	57	+++/++	nav	L: 0.6/0.9 R: 0.4/0.6	I I	-

*CT: conventional treatment (artificial tears, lubricant gels, therapeutic contact lenses)

Abbreviations: ASED: autologous serum eye drops; CCU: unique patient number; Gender: M: male, F: female: ED: eye drops; Eye: L: left, R: right; Pain and Photophobia: + mild, ++ moderate, +++ severe; Outcome: I: improvement, F: failure; ; nav: not available; HM: visual acuity determined by hand movement.

Notes of Table 3:

1. A severe case with calcification of ocular surface who failed cornea transplantation. Fungal infection during treatment with corneal perforation at 3 weeks. Finally operated to put keratoprotesis (Boston) as last option.

2. Currently on treatment.

3. No improvement with 1st batch. After treatment stop, new course with 38 vials with improvement.

4. Currently on treatment. Photophobia decreasing.

Figure 1.

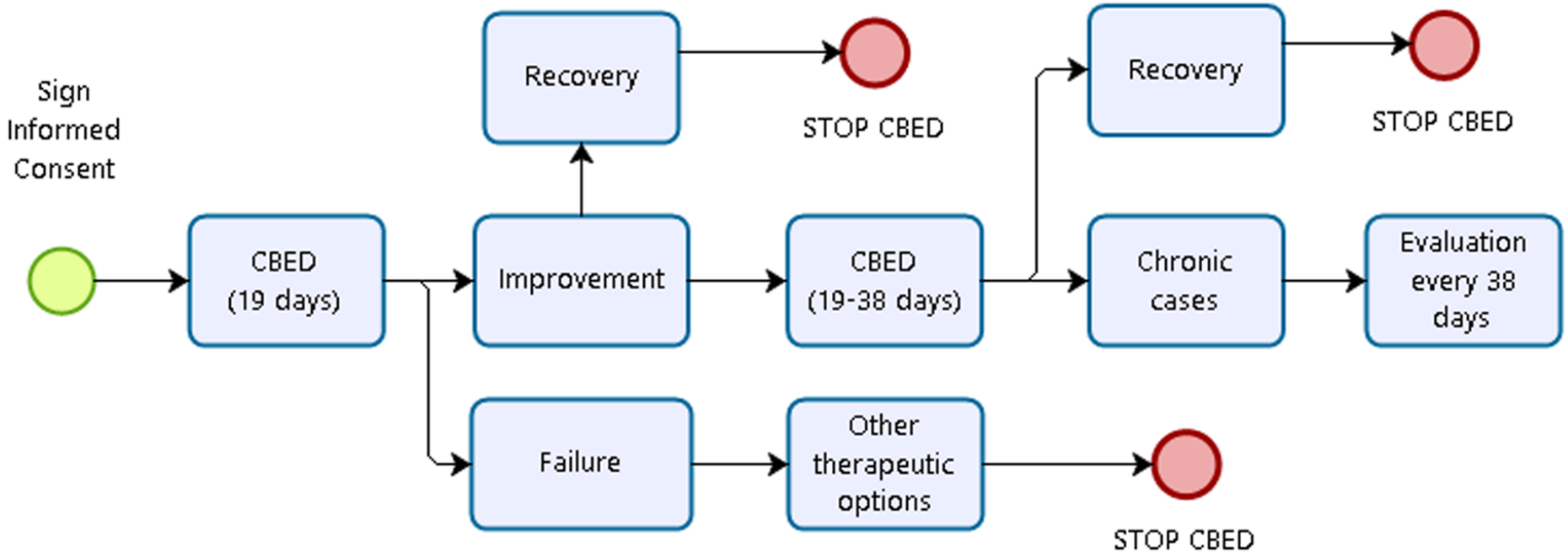
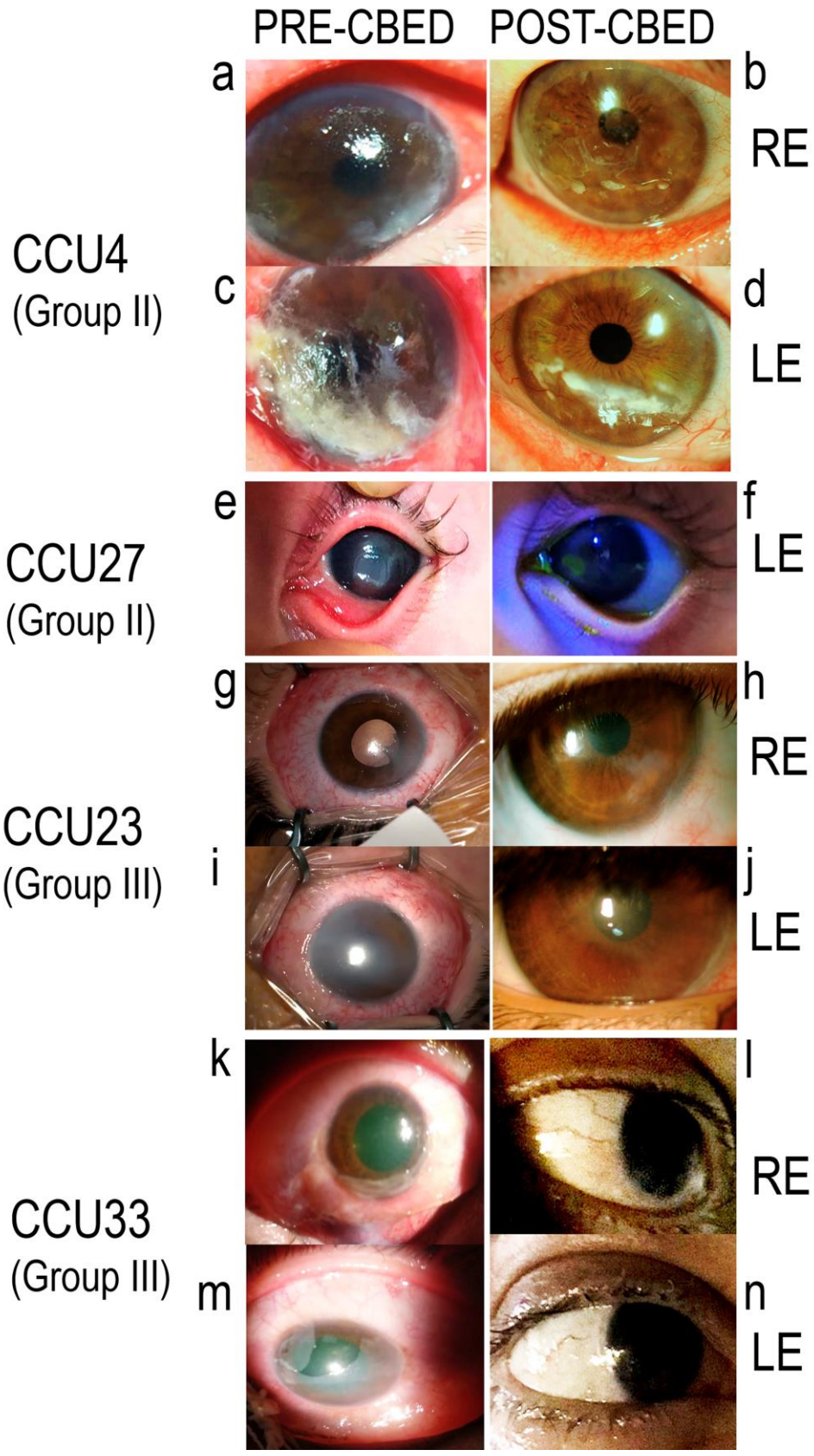

Sign Informed Consent
CBED (19 days)
Improvement
Recovery
STOP CBED
CBED (19-38 days)
Recovery
STOP CBED
Chronic cases
Evaluation every 38 days
Failure
Other therapeutic options
STOP CBED

Figure 2.


PRE-CBED
POST-CBED
a
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CCU4
(Group II)
c
d
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LE
CCU27
(Group II)
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h
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CCU23
(Group III)
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CCU33
(Group III)
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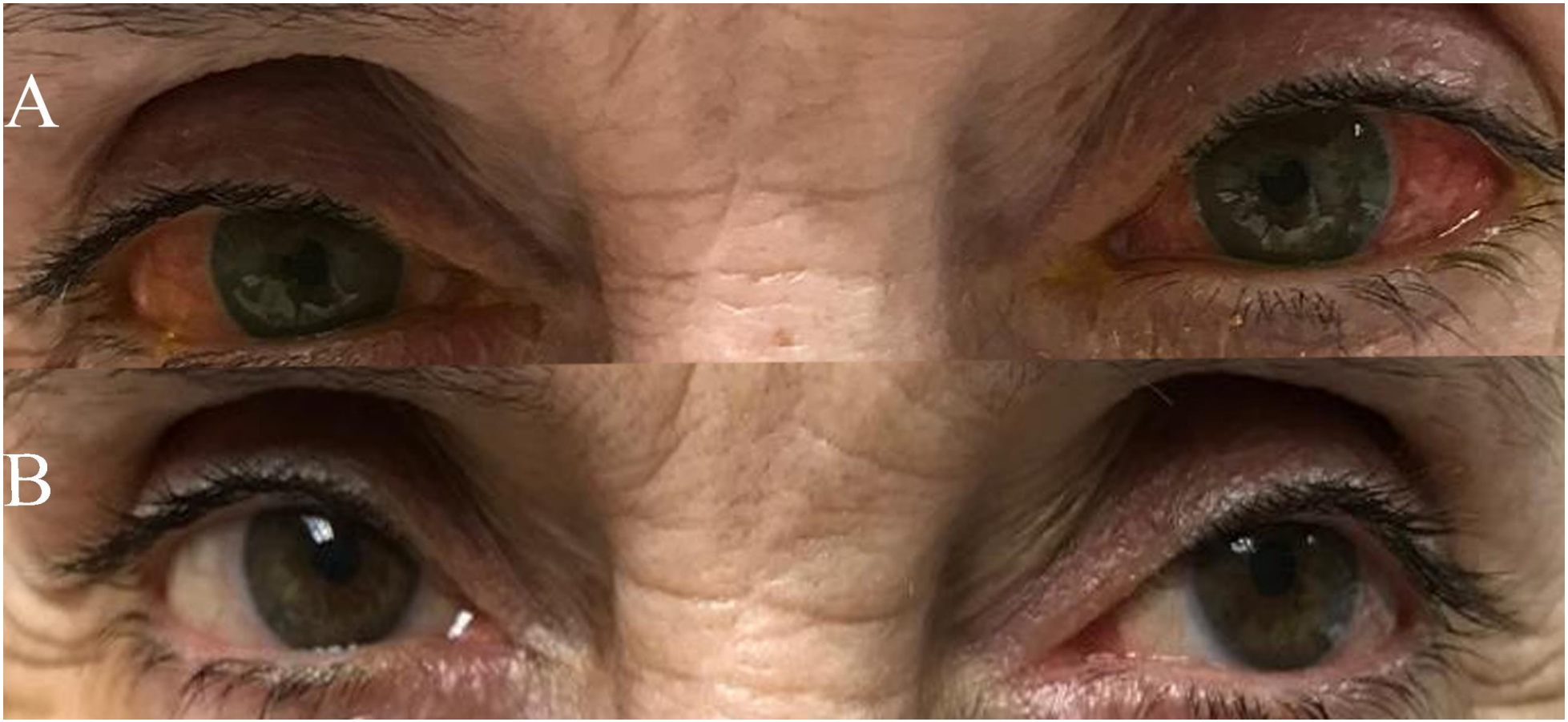
LE

Figure 3.



V. Results summary

In this Thesis cord blood platelet rich plasma (CB-PRP) derived products standardisation, characterisation and clinical application have been investigated. CB-PRP has been used to obtain frozen cord blood platelet concentrates (CBPC) and plasma. This CBPC is used as raw material to prepare medicine such as eye drops or gel parches. We assessed and compared the properties of 3 different CB-PRP derived preparations, platelet poor plasma (CB-PPP), platelet lysate (CB-PL) and platelet releasate (CB-PR). We measured the concentrations of various factors which are thought to contribute to wound healing and angiogenesis.

We developed a target product profile (TPP) for production in BST within a standardized range of platelets with concentrations of 800-1200x10^9/L in 10±5mL in CBPC, with almost absence of leucocytes and erythrocytes ($\leq$0.5x10^6leu/L and $\leq$0.1x10^{12}RBC/L, respectively). The units were selected among those discarded for cryopreservation of hematopoietic stem cells for transplantation. CBPC was used either for manufacturing of CB-PL, which is active ingredient of CBED, or for CB-PR, in case of CBPG application. Additionally CB-PPP was assayed as another possible medicine. The CBPC units directly used after thawing. For this platelets were activated to form gel (CBPG) and apply to diabetic foot ulcers (DFU). In the case of CBED, the CBPC units underwent three freeze-thaw cycles to obtain PLs rich in growth factors, which were diluted with Plasmalyte (50% v/v) and had TPP: 0-15x10^9 platelets/L, 0.35-1.0mL/vial, with negligible amount of leucocytes and erythrocytes. This frozen CBED vials were distributed for patient's self-administration, or for application by health personnel in case of intensive care unit cases.

Finally these active ingredients (CB-PL and CB-PR) were applied on patients with skin and ocular surface disorders, by applying CBPG and CBED respectively, to evaluate safety and efficacy signs.

CBPC preserves the content of platelets after thawing, pointing to the suitability of the proposed manufacturing protocol in order to assure a controlled dose of platelets in the final product applied to the patient. Thus, our processing protocol yielded CBPC units with at least $800x10^9$platelets/L in all cases, demonstrating the reproducibility of our protocol. More importantly, the capacity of those platelets to express activation markers after thawing suggest that GF release, the putative active ingredient, occurs at the moment of application of the medicinal product and not in a uncontrolled manner during processing. Notwithstanding, it is currently unclear whether the observed expression of platelets activation marker is a result of the physiological platelets activation triggered by the temperature conditions of preservation [43] or because of the platelet membranes breaking after thawing, [120] or even due to both mechanisms occurring at the same time. In the future, other preservation strategies such as freeze-drying methods may be tested to improve presentation. Additionally, our analysis of GF levels after activation of CBPC with calcium gluconate supports the preservation of platelets' function and showed comparable levels of factors in CBPC compared to the ranges described in references [121], therefore suggesting that current clinical applications using autologous PRP might be potentially substituted with CBPC.

On the other hand, the very low number of leukocytes and almost absence of erythrocytes in our products assures a low risk of potential immune and inflammatory reactions after allogenic applications, even without HLA or AB0 group compatibility, thus enforcing their universal use (Described in *Paper 1* [122]).

We found that as expected the CB-PPP preparation had lower concentrations of most analytes, while there were less differences between CB-PL and CB-PR. In general, the concentrations of the key analytes were higher in CB-PRP preparations than in tears and comparable to those in adult serum. This is similar to other reported studies [123,124], though we measured additional, previously not reported, analytes.

The CBED contain high concentrations of active molecules and GFs which are important for corneal healing. Moreover, CBED promoted cell migration and growth with no cytotoxic effects on a human epithelial corneal cell line. Furthermore, they showed a suppressive immune response on NK and CD8+ T cells. These interesting anti-inflammatory and regenerative properties are required in the treatment of epithelial corneal lesions.

Further we looked at the immunomodulatory properties of the 3 CB-PRP derived preparations, by investigating their effects on the activation of NK, NKT and T cells. We found that all 3 preparations reduced the expression of activation markers NKG2D and CD107a in all cell types investigated to different degrees and further they also decreased the expression of INFγ in NK cells. As we used the same unit to make all three preparations, and the paired and

unpaired analysis gave almost identical results, it is most likely the effects are due to the PRP preparation and only some of the effect is due to individual variations between individuals. We suggested that this reduction is caused by the presence of soluble NKG2D ligands (MICA, MICB and ULBP1) in the CB-PRP preparations, but it would be prudent to have a larger sample size to confirm this (Described in *Paper 2* [125]).

The next step was the evaluation of clinical safety and efficacy of both CB platelet gel (CBPG) and CBED. The CBPG was applied on clinical trial on patients with diabetic foot ulcers. Our data assessed the feasibility of a public CBB to regularly produce this therapeutic blood component for clinical use. Furthermore, the full development of this product in the catalogue of a CBB would result in a substantial increase in the efficiency of the CB collection programmes. In our analysis, the current 11% of clinical conversion would increase to 73% if CBB included the production of CBPC for their routine processing of CB cells for transplantation. In this regard, we propose a new generation of CBBs to be used in other contexts beside transplantation [17].

Another product which had clinical application is eye drops formulation – CBED. This study showed encouraging outcomes from the compassionate use of novel allogeneic CBED prepared from platelet lysate for the treatment of a consecutive series of 33 patients (46 eyes) with severe ocular surface lesions refractory to conventional therapies. Full ulcer healing was observed in 78% of the treated eyes from 26 patients with neurotrophic keratitis, corneal ulcers of different aetiology and corneal burns. Clinical improvement was noted in 85% of the treated eyes from 7 patients with GVHD and severe DES (Described in *Paper 3* [126]).

Finally, responding to hypothesis of present work, the CB fractionation is feasible in public cord blood banks (CBB). CB-PRP derived medicinal products can be standardized, transformed material can maintain functional abilities *in vitro* and *in vivo*. Thus, CB-PRP derived medicines are clinically safe and effective in cutaneous (diabetic foot ulcers) and ophthalmic (ocular surface pathologies: ulcers, severe dry eye, ocular GVHD) topical application.

VI. Discussion

The Barcelona CBB (from the Programa Concordia BST) is one of the largest in Europe and the third more important in the world in number of transplants. BST collects CB units from about 66 maternities. BST receives every year more than 2000 units and our estimation is that almost 90% of them can be used yearly for PRP manufacturing.

PRP is a well-known biological product typically used in the autologous application settings [127,128] for therapeutic purposes. The medical use of platelet GF has been described for eye drops [114], platelet gel [37,129] and supplements of culture media [115,130] and for advanced therapy medicinal products (ATMPs), amongst others. In this sense, there are several recently described clinical applications of autologous PRP, including chronic wound healing [52,131], skin and soft tissue repair [37], treatment of inflammatory pathologies and even anti-aging medicine applications [128]. However, the clinical application of autologous PRP has some disadvantages, such as the variability in raw material and processing protocols, lack of characterization of the final product applied to the patient, and the contraindication for some patient populations to obtain blood for PRP preparation (hematological malignancies, aged hypomobile patients [76]). Improvements in wound healing based on platelet properties, after treatment with peripheral blood platelet gel, have been reported elsewhere [52,131], although the scientific evidence [108] is scant. Also, Parazzi et al showed by proteomic analyses that adult plasma is richer in inflammatory factors compared to CB [54].

The mechanism of action of CB-PRP in terms of immunomodulation has been investigated [59,60]. The anti-inflammatory molecule transforming growth factor beta (TGFβ), is an obvious candidate to exert such a function as it is known to be present in fairly high levels in PRP [132–134]. Some studies have looked at the effect of TGFβ on the induction of T-regulatory (Tregs) cells as a plausible mechanism to explain the overall anti-inflammatory properties of PRP preparations [135]. In contrast, Cox et al were able to demonstrate that CB-PRP preparations contain soluble molecules that act as ligands for NK cell receptors and result in reduction of NK cell activation potential [60]. These soluble molecules, which have an activating role when expressed on the cell surface, interact with NKG2D and directly down-modulate cytotoxicity of cells bearing this receptor in a dose-dependent manner, independent of a similar effect exerted by TGFβ [59]. Further work delineating the differential roles of the various types of NKG2D ligands (NKG2DLs) revealed that soluble ULBP1 (sULBP1) was the most abundant ligand in CB plasma. Furthermore, was also found that a second NKG2DL termed MICA, also supressed NK function, but this ability was affected by a genetic polymorphism in the MICA gene itself [59]. A dimorphism in MICA gives rise to two possible variants of the protein with either a methionine (Met) or a valine (Val) at residue 129. This

difference has been found to have functional consequences, with the affinity for the receptor being higher on the Met containing protein [136]. Indeed, investigations found that PRP preparations from donors who had at least one Met allele were able to down regulate the expression of NKG2D, CD107a and INFγ in NK cells significantly more than plasma samples form Val homozygote donors, revealing a functional difference affecting the anti-inflammatory potential of sMICA variants [59].

The use of PRP as a treatment for eye [108] and skin lesions [137] has been established for some time. There are many preclinical studies on PRP and PG existing in dental implants, soft tissue, bone, sinus, muscles and other tissue repair on rabbits, mice, dogs and horses with very confusing results due to physiological differences. Hospitals worldwide use autologous gels in surgeries to prevent bleeding, such as cardias and spinal fusion interventions (reviewed in [37]). The PG from pooled allogeneic PCs has been used on recalcitrant ulcers of hypomobile patients showing a 91±14% ulcer size reduction after 12 weeks of treatment. Additionally, the implementation of CBPG therapy seems to have a positive cost-benefit result. This is in agreement with Greppi et al. (2011), who showed that the use of allogeneic donor platelet gel generated a 90% treatment cost reduction versus conventional treatment, and 86% of ulcer healing in a series of 11 elderly patients affected by pressure ulcers[76]. Small successful preclinical studies in an animal model of pleural injury and clinical studies on the repair of cutaneous ulcers in recessive dystrophic epidermolysis bullosa [77] suggest that the CBPG could represent a valid tool for the treatment of different surgical lesions and skin ulcers. On difficult to treat chronic ulcer, such as diabetic foot ulcer, CB derived platelet gel can be used resulting in an ulcer area reduction of 46% for Group A and 79% for Group B patients ($p < 0.01$). These observations suggest CBPG application can promote more rapid wound healing than standard care [68].

A number of studies compared allogeneic eye drops obtained from different serum and plasma sources and evaluated their immune modulation properties [114,138]. The main differences in composition between the plasma product and the allogeneic serum described in the literature provided are the presence of coagulation and anticoagulant factors in the product. We consider that the presence of coagulation and / or anticoagulant factors is not a source of toxicity, nor will it condition the efficacy or safety of the product because the anticoagulant containing the product is derived from citrate with a phosphate buffer. This reagent is widely used in blood banks and is a constituent part of all blood derivatives administered to patients. Its toxicity profile is widely studied [139] and does not present an associated risk. Coagulation factors are widely used in blood transfusion in an allogeneic context (for example,

cryoprecipitate administered to patients with hemophilia) that present an extremely low profile of isoimmunization even when administered intravenously [139].

Our clinical observations corroborate previous investigations [114] showing that the administration of 20% CBED prepared from CB serum to 14 patients with persistent corneal defects was effective in 86% of the cases, with no significant complications. Significant improvements after 1 month of treatment with 20% CBED from CB serum of 30 patients with severe corneal epithelial damage were also reported [140]. CBED at 20% serum dilution were also used in 33 eyes presenting chemical burns, demonstrating the safety and lack of toxicity of this treatment. Complete epithelialization was achieved in 12 of 18 cases in shorter times compared to artificial tears and autologous serum [70]. Additional positive results with CB serum were reported from patients with severe DES associated with neurotrophic keratitis [70] and GVHD [141]. A reduction in the frequency of recurrences from 2.24 ± 1.09 to 0.5 ± 0.79 was also reported in patients treated with artificial tears and CB serum respectively, during a follow-up of 14.7 ± 2.5 months [142]. Studies comparing autologous and CB serum in 92 eyes demonstrated higher therapeutic effectiveness in CB serum eye drop recipients [69]. Thus, there is scientific evidence to support the use of topical CB serum in severe ocular surface diseases. The clinical outcomes from our trial are aligned with those described in the literature [97,114,140,143].

Normal tears contain GF, MMPs and TIMPs which maintain corneal homeostasis [144,145]. Tear composition can be mimicked by other substances of human origin like PRP derivatives. These have the advantage of containing some similar GF, cytokines and structural proteins that are immunomodulatory and can be enhancers of wound and corneal healing [146–148]. The benefit of this cytokine profile is widely described in the literature; GFs like PDGF, TGF-β, bFGF and VEGF have been shown to enhance regeneration in other tissues [52]. A topical supplement of EGF, usually found in tears, has the ability to induce and improve the process of corneal epithelial healing both *in vitro* and *in vivo* [149].

Together these findings suggest that topical application of those GFs would be beneficial as a treatment for corneal and cutaneous lesions.

CBPC is a new tool for tissue engineering and regenerative medicine applications. Herein we also describe the feasibility of CBPC production as well as the versatility of its therapeutic applications based on the possibility to conveniently preserve "*off-the-shelf*" products in the CBB to further produce different formulations of CB-PRP, according to the desired final use. CBPC offers some advantageous benefits for patients because: A) its safety, B) the fact that pregnant donor women were previously evaluated for presence of transmissible diseases, C)

quality control analysis can be easily made before product release, D) immediate availability and E) the unique properties of CB plasma [54] due to the presence of trophic and angiogeneic GF, and immunomodulatory cytokines[55] with recognized beneficial effect to wound healing [52]. More importantly, the possibility of standardizing CBPC manufacturing to yield a well characterized product also provides the chance to reduce product variability. This is in contrast to autologous PRP applications where treatment dose and composition change on an individual basis.

Currently we are using CB-PRP preparations on two separate clinical trials for two indications: an eye drop treatment for Neurotrophic Keratopathy (NCT03084861) and a gel patch for diabetic foot ulcers (NCT02389010). However, there is a need to establish the likely differences in preparations and how the biochemical content and functional attributes of each preparation may affect their use. We propose that for skin ulcers, the most appropriate preparation would be platelet releasate (PR) in gel form (CB-PG), where the GFs content is released gradually [150]. This contains the highest concentrations of both angiogenic as well as trophic and wound healing factors and the lowest concentration of inflammatory molecules. For skin regeneration several phases are required; first there is a vasoconstriction and platelet aggregation phase to stop bleeding, followed by an inflammatory phase which recruits several cells (mainly neutrophils) into the site followed by a proliferative phase where there is new tissue formation [151]. The second phase requires pro-inflammatory molecules such as TNFα and INFγ, as well as factors that promote collagen degradation, followed by a re-epithelialisation phase where fibroblasts are recruited to the site and with the help of growth factors induce the multiplication of this fibroblast, which in turn deposit new collagen. In chronic wounds there is a clear disruption to this process, therefore providing these factors exogenously should have a positive effect. Consequently, preparations with high levels of TNFα, for example, which increase inflammation, as well as metalloproteinases and their inhibitors, such as the MMPs and TIMPs, which degrade collagen, should aid in the induction of the wound healing cascade [152]. High levels of bFGF and EGF consolidate the effect by promoting fibroblast proliferation. The necessary neo vascularisation is also promoted by VEGF, though the levels of this factor are modest in the CB-PR preparation. Importantly the CB-PR preparation reduces the expression of NGK2D activating receptor as well as CD107a in NK cells only modestly, thus the necessary immune cell activation and IFNγ production is not blocked by this preparation. Previous work using PL preparations in *in vitro* models of wound healing demonstrated that the combination of factors present in PL was able to promote wound healing in a dose dependent manner [65]. CBPC derivatives (lysate and releasate) are rich in GFs, and are attractive sources for many clinical applications in degenerative

conditions, such as joint and cartilage pathologies [116,153], where injection of this product can be beneficial.

For the treatment of ocular conditions, preparations need to be appropriate to the indication being treated. On the one hand, ocular ulcers and other conditions that have damaged the ocular surface where regeneration is required need special preparations [154]. These should contain sufficient quantities of EGF, bFGF and a certain quantity of inflammatory cytokines to activate the regenerative process. However, they must not promote the excessive proliferation of fibroblasts leading to scarring, as it would impair the function of the cornea. The CB-PL preparation has moderately high levels of EGF, bFGF, HGF, PDGF and VEGF and fairly high levels of TGFβ (compared to CB-PPP and CB-PR), mostly in line with those found in natural tears, except for VEGF which is much higher in PL. The CB-PL preparation has a dramatic effect on NK, NKT and CD3 T-cells. It reduces the expression of both NKG2D and CD107a substantially indicating a reduction on the activation potential of these cells, thus likely reducing overall inflammation and disease progression but without compromising viability.

For ocular diseases where the corneal surface is not yet compromised, such as dry-eye disease, which is usually caused by a deficiency of the lacrimal functional unit and is accompanied by ocular surface inflammation and irritation due to lack of lubrication [155], and ocular graft versus host disease, where autoimmune components take a place, the CB-PPP preparation may offer an optimal topic treatment, though here we analysed the raw material, the topical eye drops used for clinical application are diluted in Plasmalyte, which may dilute the fibrinogen and the pro-thrombin present in the CB-PRP avoiding their possible negative effects. So far, studies have not shown any safety issues caused by the presence of fibrinogen which is present in autologous PRP preparations too [156,157], however, further studies are necessary to measure this more accurately. This preparation contains low levels of pro-angiogenic and wound healing factors, it also reduces the activation of NKT cells by about 50%, which may arrest the progression of disease caused by the recruitment of Th17 cells. Previous studies in animal models of sever dry eye disease, showed that activated NK and NKT cells up-regulated IL-6 and IL-23 and created an environment where dendritic cells could skew T cell activation to a more pathogenic Th17 phenotype [158]. In the absence of NK and NKT cells, disease progression was less severe [159], as no pathogenic Th17 cells were activated [158]. Additionally, in the presence of CB-PRP derivatives preparations there is a substantial reduction (~50%) in the production of INFγ by immune cells, which would otherwise contribute to the inflammatory process. The CB-PPP preparation contains the lowest levels of the pro-inflammatory cytokines IL-6, IL-1α and TNFα, and substantial

amounts of soluble NKG2D ligands (ULBP1, MICA and MICB), making it ideal to treat conditions where inflammation has a major role in disease progression.

To better understand the properties of these PRP preparations and the processing methodology, it would be important to measure the concentrations of the GFs at different stages during the processing and see how the different steps affect the final product.
The limitation of our study on CBPG in DFU treatment is the lack of an accurate local cost-to-benefit analysis, which is not feasible at this stage considering the small number of patients that we have so far treated with CBPG.
There are several limitations in our study with CBED, including the small number of patients tested in each of the conditions, a variable degree of patients' clinical symptoms at presentation, and the lack of concurrent controls treated with conventional therapy only. In spite of these limitations, it is encouraging to note that positive outcomes were obtained in a large proportion of this cohort of patients who had failed all applicable therapeutic options.

In summary, CBED and CBPG are standardized products, which have unique properties and can be used as a source of plasma and platelet to formulate preparations for regenerative medicine that can be used for the topical treatment of several eye conditions that affect the ocular surface, such as severe dry eye disease, corneal ulcers and burns. Furthermore, these preparations could also serve as an optimal adjuvant to help in skin wound healing and for articular regenerative processes. Through differential processing of CB-PRP production, we have formulated three blood derived preparations which have different and interesting biochemical and immunomodulatory properties which can be investigated in translational applications. Further work in evaluating the safety and efficacy of these preparations must now be undertaken.

Future studies should include: a) *in vivo* safety and efficacy studies in models of corneal and skin wounds; b) the assessment of vitamin A concentration on final preparations, as it is known to be light sensitive [160], but an essential ingredient in eye drop preparations; c) the effects of the preparations on the immune response in general; d) further investigations of anti-inflammatory properties, such as soluble HLA-G concentration measurement; e) application of products CBPG, CBED and CB-PPP in more powerful controlled, randomized clinical trials to show its efficacy; e) new formulations for each cutaneous (lyophilisation; spray) and ocular applications (lyophilisation).

VII. Conclusions

1) A purpose-designed method was validated (the BST CB-PRP method) for large-scale production of CB-PRP derivatives. This method is feasible, reproducible and implemented under GMP conditions, which allows for clinical scale manufacturing required to perform clinical trials to assess safety and efficacy in defined clinical applications. Using data generated AEMPS approved two IMPD files with PEI numbers assignment.
2) The study demonstrated unique biological properties of CB plasma and platelets defined by the presence of GFs and cytokines suitable for use in clinical setting. Through differential processing of CB-PRP production, we have formulated three blood derived preparations (platelet lysate, platelet releasate and plasma). Main properties are a well balance between trophic, angiogenic and anti-inflammatory molecules that support their application in regenerative medicine like topical treatment of eye conditions that affect the ocular surface, such as severe dry eye disease, corneal ulcers and burns. Furthermore, these preparations could also serve as an optimal adjuvant to help in skin wound healing and for articular regenerative processes.
3) The consecutive case series study on ocular surface lesions provides preliminary evidence on the safety and efficacy of CBED prepared from platelet lysate as a new therapeutic blood component manufactured in a public cord blood bank. Data showed an improvement on more than 80% of patients treated. These preliminary data support further development to obtain regulatory authorization of use for CBED in special clinical situation where an urgent therapy is required.

VIII. **Future investigations**

1. To gain more evidence on the efficacy to make these products available for clinical use:
    a. Design controlled clinical trial for evaluation of safety and efficacy of CBED to early treatment of severe presentations of ocular surface lesions to avoid invasive therapies and obtain AEMPS authorization for hospital use.
    b. To perform preliminary evaluation of CBPG in chronic skin ulcers or in complicate surgical procedures like post-amputation in peripheral vascular diseases and others. CBPG in prospective, controlled clinical trial with an objective to obtain the clinical applications.
2. Improvement of manufacturing process and quality control:
    a. Developing a more practical technology, such as lyophilization of platelet lysate, spray or cream formulations for facilitating their topical use.
    b. Deepen in knowledge of *in vitro* and *in vivo* characterization of CB-PRP derived products, such as soluble HLA-G measurement; and designing clinical scale up for new indications (i.e. burns, extremity amputations, other surgical conditions etc.).
    c. And later, to perform more advanced clinical trial to evaluate safety and efficacy of platelet derived growth factors in formulation which could be topically applied in big areas on skin surface lesions, i.e. for burns or other surgical indications.
3. Developing new products over the described methodology:
    a. Cord blood platelet poor plasma (CB-PPP) is an attractive source as anti-inflammatory topical medicinal product for ocular surface alterations, where GFs are not required, such as ocular graft versus host and dry eye diseases. It will be necessary to focus research in this chronic inflammation and study the potential role of diverse PRP-derivatives.
    b. Additionally, take in advantage the fractionation of cord blood and clinical necessities, will be developed red blood cells concentrates (CB-RBC) for transfusion in preterm infants suffering anemia of prematurity.
    c. And finally, as a complementary research, development of eye drops, gel and spray formulations from blood bank surplus obtained from adult platelet concentrates not used for transfusion. This need to be accompanied by the comparison of biological and clinical characteristics and properties between both sources. This will require assessing their safety and efficacy on well-designed clinical trials.

IX. References

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X. Annex

Annex I. Paper 4. *Rapid review: next generation of cord blood banks; transplantation and beyond*.

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Rapid review: next generation of cord blood banks; transplantation and beyond

Sergio Querol and Dinara Samarkanova

KEY IDEAS

- Cord blood (CB) is a medicinal product of human origin with unique cellular properties such as the presence of multipotent stem cells, naive immune cells, and fetal blood components.
- CB transplantation provides high rate of donor chimerism, and a good balance of graft-versus-host (GVH) and graft-versus-leukemia (GVL) effects.
- Use of CB for transplantation has decreased in recent years as haplo-identical stem cell transplants have achieved similar short-term clinical outcomes. For most patients, however, the optimal stem cell source remains unclear.
- CB inventories can be used as a starting material to develop new cellular medicines, and units with low cellular content can be converted to produce blood components like platelet-rich plasma and red blood cell (RBC) units for special indications.

CORD BLOOD HAS UNIQUE PROPERTIES

After birth, placental tissues contain residual amounts of fetal blood that can be recovered through venipuncture of the cord vein. This fetal blood has unique cellular characteristics. First, it contains a high number of hematopoietic progenitor cells as well as progenitors from other tissues. These cells are rare, but most donated CB units contain enough multipotent hematopoietic stem cells to convey long-term engraftment. Additionally, fetal blood contains a unique lymphocyte profile. Most of the lymphocytes are naïve—recent thymic emigrants with a balanced number of conventional and regulatory T cells. This lymphocyte population is unusual as it is highly diverse and exceptionally capable of expansion[1]. These unique features make fetal lymphocytes attractive as a source for allogeneic cellular therapy. Finally, blood components derived from CB donations are again unique: the plasma contains placenta-derived peptides that are inhibitory ligands of the NKG2d receptor (soluble MIC-A, MIC-B, ULG1), which is necessary for T and NK cell expansion. This confers anti-inflammatory properties to CB.[2] And last but not least, CB-derived RBCs contain fetal hemoglobin, the natural oxygen-carrying protein present in the fetus and newborn. This open the possibility of using these cells to meet the transfusion requirements of prematurity.[3]

CORD BLOOD TRANSPLANTATION

Worldwide, more than 40,000 CB transplants have been reported. CB allows transplantation without strict HLA matching as is required using other hematopoietic cell sources, with a relative low incidence of acute and particularly chronic Graft-versus-host disease (GVHD). A remarkable graft-versus-leukemia (GVL) effect is conferred due to the excellent fetal-like T cell activity and neonatal innate immunity. Accordingly, CB may be the optimal graft source for the treatment of pediatric patients with lysosomal storage diseases,[4] and promotes more long-term disease-free survival with less chronic GVHD incidence in patients suffering high-risk leukemias including those with positive minimal residual disease.[5] In spite of this, the use of CB for transplantation has decreased in recent years, as haplo-identical stem cell sources have achieved similar short-term clinical outcomes.[6] Still, there is no consensus on the best stem cell source for most patients; the debate continues.[7] Only long-term outcome analyses with quality-of-life measures will be able to determine the value and cost of CB transplantation compared with other therapies. In addition, improved graft availability, informed graft selection, and emerging techniques to enhance engraftment are likely to improve outcomes and decrease the costs associated with CBT. [8]

CB has additional benefits: it is the only stem cell source with no risk to the donors; it provides universal access to therapy for almost all ethnic groups; and, as "an off-the-shelf" product, it can provide immediate access to

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therapy. CB banks must therefore maintain high-quality inventories of units containing high numbers of progenitor cells, with a broad genetic diversity to cover the entire potential patient population.

OTHER USES OF THE CORD BLOOD INVENTORIES

From the units comprising a CB registry, only a small number are used, typically fewer than 1% per year.[9] Units containing a high number of cells are used preferentially. In our inventory at the Barcelona Cord Blood Bank, the 34% of CB units containing at least 1.5 x 10^9 total nucleated cells were used in 76% of all CB transplants performed (around 2,000). As a result, many CB units remain "dormant" in our inventory. This opens the possibility of converting them into the starting material for advanced therapies. To do that, it is necessary to have an appropriate signed consent at the time of donation allowing for this conversion. Discussion with local medicine agencies is required. There are many attempts to produce medicinal products derived from cryopreserved CB, including: allogeneic NK cells; T regs for tolerance treatments; antigen-specific T cell lines; CB-derived universal CAR-T cells; off-the-shelf, expanded, CB-derived CD34+ cells; and MNCs to treat condition like autism, cerebral palsy, or stroke.[10] In our institution, we have started two approaches for developing cell banks: first, development of Good Manufacturing Practice (GMP)-grade induced pluripotent stem cells (IPSCs) from confirmed homozygous donations. This project aims to create seven cell lines that could cover one fourth of the Spanish population. Availability of these clinical-grade cell lines will allow researchers to differentiate tissue-specific stem cell progenitors. A second interesting project is the mesenchymal stem cell bank.[11] Wharton's Jelly-derived mesenchymal stromal cells have the potential to be used in several different situations: GVHD treatment, heart regeneration with direct perfusion or through generation of tissue and cell composites, neural regeneration in spinal cord section, Crohn's fistula, and many others.

RECYCLING CORD BLOOD UNITS AS SOURCE OF NEW BLOOD COMPONENTS

CB banks generate many units that are not used to renew the cord blood inventory for transplantation. These units have a very high intrinsic quality providing they were altruistically donated, aseptically collected after a careful donor screening for transmissible diseases, and the data are registered in fully traceable electronic data sets. Such CB units offer the opportunity for novel product development. The availability of thousands of such units prompted different groups to determine the feasibility of obtaining CB-derived blood components by fractionation.

- Platelet gel was successfully assessed in an animal model of pleural injury and later in clinical studies on the repair of skin ulcers in recessive dystrophic epidermolysis bullosa,[12] suggesting it could represent a valid tool for the treatment of different surgical lesions and skin ulcers.
- CB-derived platelet lysate and serum have been used as eye drops. Initially, serum was used in different conditions like severe dry-eyes, ophthalmic chronic GVHD, and Sjögren's syndrome with reported significant improvements both in corneal healing and pain during 1 month of therapy.[13]

Finally, another attractive component is CB-derived red blood cells (RBCs). Investigators at a public CB bank in Rome, Italy, developed studies to define the feasibility and safety of a program of allogeneic transfusion of CB-RBCs in premature newborns.[3] This program was prompted by evidence suggesting that transfusing RBCs from adult donors to premature babies may have a causative role in the development of retinopathy of prematurity[14] as well as necrotizing enterocolitis and bronchopulmonary dysplasia. These risks have been associated with increased oxygen delivery by adult versus fetal hemoglobin.

ETHICAL, LEGAL, AND SOCIAL ISSUES

Developing novel reagents and therapeutics from CB will require additional discussion of points such as ownership of CB and its derivatives, maternal and paternal informed consent for novel uses, for-profit versus not-for-profit processing, and distribution by the biotechnology industry. Moreover, pathways and procedures should be harmonized at the international level to regulate the procurement of CB units not suitable for hemopoietic transplantation by companies interested in developing and distributing novel reagents and therapeutics.

CONCLUSION

CB is a human biological material with unique properties that make it attractive for use in transplantation and in some emerging regenerative medicine applications. Until now, CB banks were primarily focused on building large inventories of well-characterized units based on their content of hematopoietic stem and progenitor cells as an alternative source to adult donors for allogeneic bone marrow transplantation. Today, new uses of CB-derived components and GMP products are foreseen.

CONFLICTS OF INTEREST

The authors have disclosed no conflicts of interest.

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Annex II. Ethical considerations

CB is an attractive source to elaborate medicinal products, such as PRP derived drugs for regenerative medicine. This Doctoral Thesis was based on Project for CB-PRP derived products manufacturing validation, characterization and to evaluate clinical safety and efficacy on cutaneous and ocular surface pathologies. For *in vitro* research, we submit to Ethic committee of Hospital Universitari Vall d´Hebron with authorization number ref.: 192/2014; Hospital Clinic HCB/2017/0785 and sample transfer approval for research.

BST had an approval of clinical trials with CBPG to treat diabetic foot ulcers by Hospital Santa Creu y Sant Pau Ethic Committee 15/043 and AEMPS (Nº EudraCT: 2015-000510-22; PEI:15-034); CB eye drops had approval to treat neurotrophic keratitis by Hospital Vall d´Hebron Research Institute ethic committee with Nº268 and AEMPS (Nº EudraCT: 2016-001841-23; PEI:16-116).

The Italian CB program through its national blood centre in Rome has developed the NUPLA project to study the possibility to investigate CB-PRP products. The BST has signed a collaboration agreement to jointly develop it and invites participation to the Spanish CB program.

Annex III. Requirements for CB-PRP projects development

1. Public cord blood banks for large scale production of PRP derivatives

BST is a non-profit public company of the Ministry of Health, which was created in 1995. The BST has established a world-renowned cord blood bank (Barcelona CBB). Our CBB receives, processes, stores and distributes the stem cells obtained from CB, which are used in transplants to cure patients with diseases such as leukemia, lymphoma and other bone marrow pathologies. The BST makes sure that the CB obtained is properly typed and preserved so that it can be transfused in the best possible conditions. BST also coordinates the Concordia program, a CB donation program that collect and transport CB donations from over 66 maternity wards in Catalonia and other regions of Spain as well as the Principality of Andorra.

The Concordia-BST CBB is the third largest in Europe and one of the most important in the world in number of transplants. Up to 1867 HSC transplants were performed until end of 2019.

A high proportion of fresh CB units are donated and collected in a routine and aseptic way, and are carefully qualified and selected to detect communicable diseases, and whose data are recorded in fully traceable electronic datasets, offer the opportunity to develop new products. Currently, only 10% of collected units are stored for transplantation. Availability of many of these products open the way for developing other applications like those presented in this Thesis.

It is, therefore, expected that 70-80% of CB units donated for allogeneic HSCT may be unsuitable for this use and ultimately discarded. These units can be used for the preparation of CB-PRP to treat different pathologies characterized by impaired tissue regenerative processes and/or inflammatory profiles [150].

2. *Concordia* Program

Concordia is an inter-territorial cooperation programme for CB donations based on the bonds of mutual trust between the Department of Health of the Catalan Government and the health departments of other regions in Spain: the Balearic Islands, Aragón, Navarre, Extremadura, Cantabria and the Principality of Andorra. The programme has the following benefits: allows for rapid, efficient start-up of donation programmes; responds to the growing demand for this type of blood; maintains a high level of quality; facilitates transplants and promotes cooperative clinical and biological research on CB; allows participants to share technical protocols and co-design dissemination and professional training programmes, and to jointly develop programmes for exchanging knowledge and experiences.

Within the programme, the BST is responsible for cryopreservation, quality control, storage and distribution. Fulcrum key point of this initiative, it aims to optimise research efforts, performance efficiency and financial profit by unifying all donations at one central location.

National and international quality certifications guarantee that the CB units are screened to verify compliance with the quality standards of the CAT (Committee on Accreditation in Transfusion jointly supported by ONT) in Spain and FACT-Netcord at the international level.

A total of 66 maternity wards in 6 different Spanish regions and the Principality of Andorra are registered with the Concordia Programme.

The programme holds a stock of over 20,000 premium-quality CB units that are made available to any patient who needs them via national and international registers and almost 2,000 units from the Barcelona CBB were used in transplants in more than thirty countries.

3. The Research BioBank at *Banc de Sang i Teixits*

The Biobank at BST is in operation with full authorization from 2010 with an objective to support Biomedical Research (**Figure 1**).

Research Biobank is part of the research structure of BST, the only authorized transfusion centre throughout Catalonia. BST is in charge of the activities related to the extraction and processing of human blood and its components, whatever their destination, and their treatment, storage and distribution. The tissue establishment that is dedicated to the preparation, preservation and storage of cells and tissues for use, among other things, it is responsible for properly documented clinical research procedures. It also acts as a centre for the collection and processing of umbilical CB and in other areas such as immunobiology, molecular diagnostics, cell therapy regenerative medicine and breast milk bank.

Therefore, an appreciable number of samples voluntarily donated for research purposes are transferred to the Research Biobank in order to provide to the scientific community the biological material needed in optimum conditions ensuring the competitiveness and excellence of the research, as well as guarantee the rights of donors. For performing these research, the Scientific Committee and the ethics committee of the biobank have approved the projects that allowed the availability of hundreds of samples not used for transplantation.

4. Spanish regulations for allogeneic PRP and their derivatives

Last decades there was an increase in the use of platelet-derived growth factors for clinical applications. Spanish drug and sanitary products agency (AEMPS) established the framework for the usage of the PRP in Spain, and regulated the obligations that must be respected by its manufacturers and the minimum information that the patients should receive. As allogeneic PRP derivatives are considered as a medicine and requires an approval by the AEMPS, this needs special manufacturing conditions based in good manufacturing practices (GMP).

The directive 2001/83/EC, establishing a community code on medicines for human use and Law 29/2006, on guarantees and rational use of medicines and health products define medicine for human use as *"any substance or combination of substances that is presented as having properties for the treatment or prevention of diseases in humans or that can be used in humans or administered to humans in order to restore, correct or modify physiological functions exerting a pharmacological, immunological or metabolic action, or establishing a medical diagnosis"* [161].

Specific investigational dossier needs to be completed with information, which is mandatory for its approval, like preclinical validation, production details, quality control and clinical indication. BST requested to AEMPS the license of different PRP derivatives and AEMPS required according their recommendations [161]:

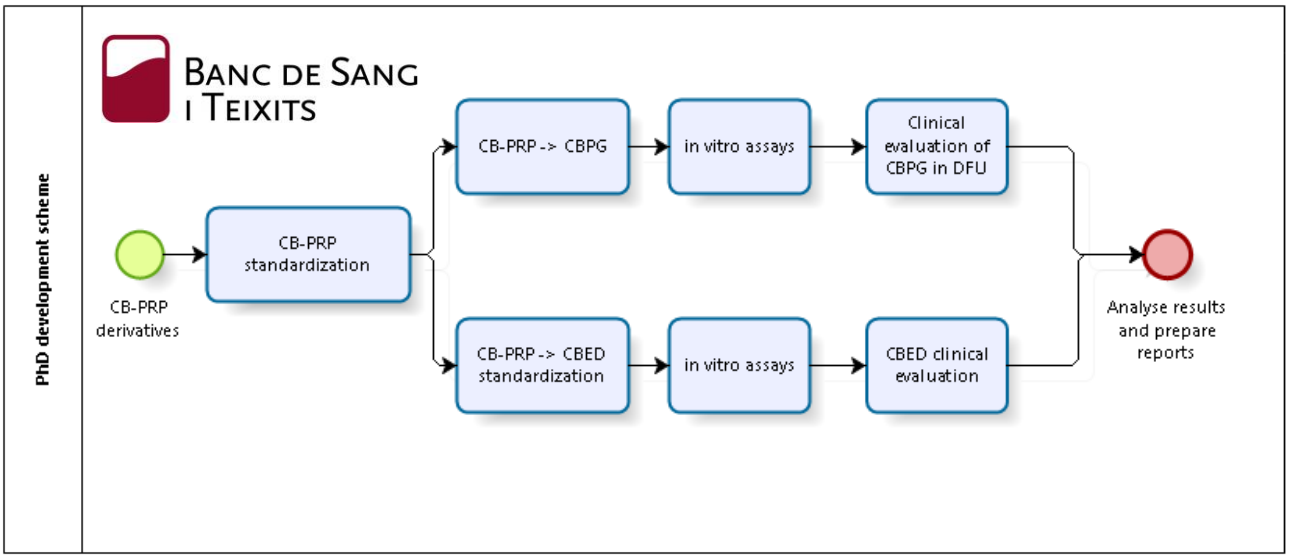
- If closed system manufacturing kits are used, should be presented Technical sheet.
- If manufacturing kits are used in open step system, and it performs in blood banks, "CAT" accreditation is required.

The BST obtained authorizations for clinical trials performing on PEIs 15-034 and 16-116 authorizations contingent on maintaining CAT accreditation of the blood bank.

5. CB-PRP projects development

The BST started this project in 2014 following an agreement with Italian Study Group [42], and the Episkey company who holds a patent for the use of PRP derivatives for clinical use presented in 2010 [162]. Following this, and within present Thesis, *in vitro* validations and clinical trials were performed under sponsorship of BST. **Figure 6** shows CB-PRP projects development to perform this PhD thesis.

Figure 6. The PhD Projects development plan


PhD development scheme
BANC DE SANG I TEIXITS
CB-PRP derivatives
CB-PRP standardization
CB-PRP -> CBPG
in vitro assays
Clinical evaluation of CBPG in DFU
CB-PRP -> CBED standardization
in vitro assays
CBED clinical evaluation
Analyse results and prepare reports

Success is not final, failure is not fatal: it is the courage to continue that counts

Winston S. Churchill