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Relatório de Estágio
Cord Blood Stem Cells Banking: Purpose, Processing and Cryopreservation

Joana Filipa Figueiredo Pina

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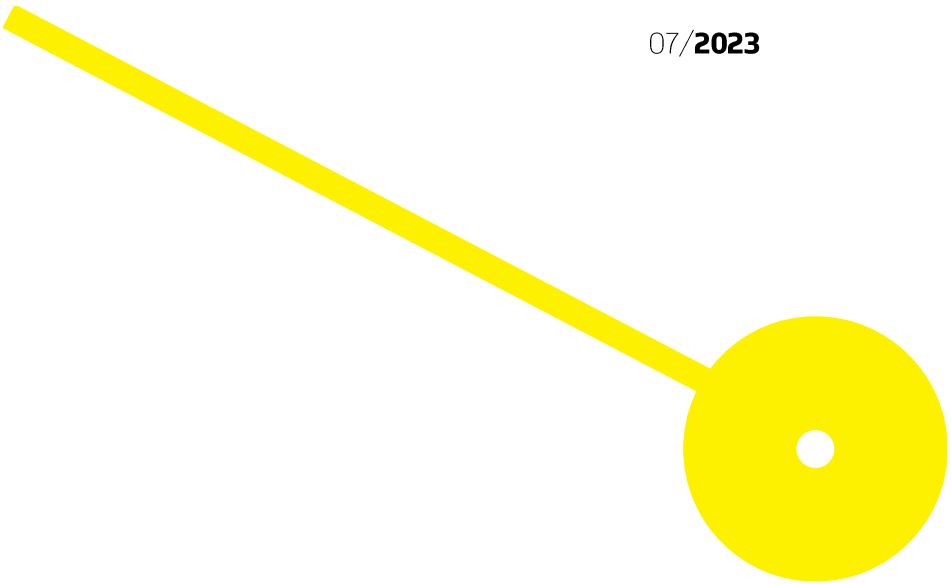

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Cord Blood Stem Cells Banking: Purpose, Processing and Cryopreservation

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Abstract

Stem cells are undifferentiated cells with a high proliferative capacity and the ability to differentiate into any cell type. The umbilical cord is a rich source of stem which is easy, painless, safe and non-invasive for the mother and infant. After birth, instead of being discarded as biological waste, it can be used to obtain multipotent and haematopoietic stem cells. The umbilical cord stem cells are a unique collection, so they should be cryopreserved for their therapeutic potential, as they represent a valuable resource for treating more than 80 diseases, namely solid and non-solid tumors. Stem cells can be stored for several years without significantly altering their properties. For that, there are two types of banks for the cryopreservation of these cells: public and private.

This work consisted of enrolling in the laboratory routine of BebéVida, a Portuguese private bank and FACTNetcord accredited bank that process, cryopreserves and stores umbilical cord blood and tissue. Therefore, it was aimed to learn and perform the related techniques to the processing and cryopreservation of umbilical cord blood, as well as processes inherent to quality assurance. Furthermore, since there are associations between ABO and Rh blood groups and certain diseases, this work also aimed to understand if the fetal blood group were associated with immunological parameters of the cord blood, including platelets, immature red blood cells and immune system cells, nucleated cells, where stem cells are inserted.

In conclusion, the integration into the laboratory routine was successful, with proper contact with processing techniques (AXP) and cord blood cryopreservation. Furthermore, this study also demonstrated that the AXP protocol influences the quality of the unit to be cryopreserved and that the volume collected at the time of collection should be considered. Regarding the blood group study, some blood groups showed significant differences, demonstrating that the presence and absence of A and B antigens on the cell surface may contribute to the difference between the number of immune system cells between different blood types. This highlights the potential impact of ABO blood type on several health conditions, and further research is needed to elucidate the underlying mechanisms.

Keywords: Stem Cells, Umbilical Cord, Cryopreservation, Cryopreservation banks, Quality Assurance.

Resumo

As células estaminais são caraterizadas pela sua elevada capacidade proliferativa e diferenciação. O cordão umbilical é considerado uma fonte rica de células estaminais, cuja colheita é fácil, indolor, segura, e não invasiva para a mãe nem para o bebé. Após o nascimento, em vez de ser descartado como resíduo biológico, pode ser utilizado para obter células estaminais multipotentes e hematopoiéticas, pois dado o seu potencial terapêutico no tratamento de mais de 80 doenças, estas células podem ser criopreservadas, ficando armazenadas por vários anos sem alterar significativamente as suas propriedades. Existem dois tipos de bancos para a criopreservação destas células: os públicos e os privados.

Este trabalho consistiu na integração na rotina laboratorial da BebéVida, um banco português e acreditado pela FACTNetcord que processa, criopreserva e armazena sangue e tecido do cordão umbilical. Assim, pretendeu-se aprender e executar as técnicas relacionadas com o processamento e criopreservação do sangue do cordão umbilical, bem como os processos inerentes à garantia de qualidade. Para além disso, este trabalho visou também compreender de que forma os grupos sanguíneos ABO e Rh podem influenciar diversos parâmetros biológicos, tais como as células do sistema imunitário, as plaquetas, os glóbulos vermelhos imaturos e todas as células nucleadas, onde se inserem as células estaminais.

Em conclusão, a integração na rotina laboratorial foi bem sucedida, com o devido contacto com as técnicas de processamento (AXP) e criopreservação do sangue do cordão umbilical. Além disso, este estudo também demonstrou que o protocolo AXP influencia a qualidade da unidade a ser criopreservada e que o volume coletado no momento do parto deve ser considerado. Relativamente ao estudo do grupo sanguíneo, alguns grupos sanguíneos apresentaram diferenças significativas, demonstrando que a presença e a ausência de antigénios A e B na superfície celular podem contribuir para a diferença entre o número de células do sistema imunitário entre os diferentes grupos sanguíneos. Este facto realça o potencial impacto do tipo sanguíneo ABO em várias condições de saúde, sendo necessário mais investigação para elucidar os mecanismos subjacentes.

Palavras-chave: Células Estaminais, Cordão Umbilical, Criopreservação, Bancos de Criopreservação, Garantia de Qualidade.

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List of Abbreviations

7AAD	7-Aminoactinomycin D
AABB	American Association of Blood Banks
AIHA	Autoimmune Haemolytic Anaemia
BFU-E	Burst Forming Unit – Erythroid
BM	Bone Marrow
BPCCU	Banco Público de Células do Cordão Umbilical
BSC	Biologic Safety Cabinets
CAD	Coronary Artery Disease
CD	Cluster of Differentiation
CFU	Colony Forming Unit
CFU-GEMM	Colony Forming Unit – Granulocyte, Erythroid, Macrophage, Megakaryocyte
CFU-GM	Colony Forming Unit – Granulocyte, Macrophage
CMV	Cytomegalovirus
CPDA	Citrate-Phosphate-Dextrose solution with Adenine
CT3P	Count-Tact 3P Agar
DCC	Delayed Cord Clamping
DGS	*Direção Geral de Saúde*
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
EPS	Expanded Polystyrene Insulation
FACT	Foundation for the Accreditation of Cellular Therapy
FSC	Forward Scatter
GvHD	Graft-versus-Host Disease
HDN	Haemolytic Disease of the Newborn
HES	Hydroxyethyl Starch
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HSC	Hematopoietic Stem Cells

IEB	*Instituto Europeu de Biomedicina*
IMDM	Iscove's Modified Dulbecco's Medium
IPO	*Instituto Português de Oncologia*
IPST	*Instituto Português do Sangue e da Transplantação*
IQ	Installation Qualification
OQ	Operational Qualification
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
MPV	Mean Platelet Volume
MSC	Mesenchymal Stem Cells
NEQAS	National External Quality Assurance Scheme
NRBC	Nucleated Red Blood Cells
PQ	Performance Qualification
RR	Recover Rate
RBC	Red Blood Cells
SC	Stem Cells
SNS	*Serviço Nacional de Saúde*
SSC	Side Scatter
TNC	Total Nucleated Cells
TSA	Tryptic Soy Agan
UC	Umbilical Cord
UCB	Umbilical Cord Blood
UCT	Umbilical Cord Tissue
URS	User Requirement Specification
WBC	White Blood Cells

CHAPTER 1: INTRODUCTION

1. Context and Structure of the Report

This report describes all the work developed during the curricular internship undertaken within the scope of finishing the master's degree in Biochemistry in Health at the *Escola Superior de Saúde*. The internship was conducted in the BebéVida laboratory and began in September 2022.

Initially, there was a contact phase with the facilities and the whole team, understanding the workflow. After, there was a period of theoretical learning, followed by an observational phase. The main objective was to observe the whole process arising from the umbilical cord blood (UCB) and umbilical cord tissue (UCT) samples from the moment they were received until they were cryopreserved and stored. As time progressed, sufficient knowledge was acquired to carry out the different techniques and services with the technical team.

Therefore, this report describes some of the activities performed and is divided into four main chapters. The first chapter introduces the subject of the internship and gives a brief presentation of the laboratory and the objectives of the internship, as well as a bibliographical review of the role of stem cells (SC) and the importance of their cryopreservation in a cryopreservation bank. The second chapter describes some of the laboratory routines performed during the internship, from the collection kits' arrival to the cryopreservation and storage of the samples. The third chapter is about an equipment validation and qualification procedure, which was made necessary by acquiring new equipment in the laboratory. Moreover, chapter four assess the impact of ABO in the immune system parameters regarding the UCB.

2. Institution

BebéVida is a familiar laboratory in Porto with expertise in several areas, especially health and biotechnology, which provides services and is dedicated to processing, cryopreservation and storage of SC from UCB and UCT. In parallel to these services, the laboratory also integrates a foetal seroteca called a maternal-foetal bank, which aims to facilitate the performance of

complementary tests if necessary. In this seroteca, a fragment of the umbilical cord (UC), the infant's plasma and even the maternal blood and plasma are kept.

The company was founded in 2004. However, in 2005, the BebéVida brand's launch took place, with the creation of the first blood collection kit. Before 2011, the laboratory was in Penafiel and called *Instituto Europeu de Biomedicina* (IEB), where the cryopreservation of the first samples was possible.

Over the years, BebéVida has achieved several accomplishments, namely the ISO 9001:2008 certification, awarded by the company *Bureau Veritas* for the laboratory area. In 2011, the laboratory received authorisation from the *Ministério da Saúde* to provide its services and use samples of human origin intended for application in human beings through the *Instituto Português do Sangue e da Transplantação* (IPST) under Law 12/2009. That same year, it received its first distinction with the PME leader status, which continued year after year. In 2019 and 2020, there was a new distinction for the company, with the awarding of the Five Stars prize, distinguished by consumers.

As the area of stem cell cryopreservation has grown, new certifications and services have been established, such as the processing of UCT in 2013. Allied with innovation and the development of science, the laboratory has been improving and became a member of the International NetCord Foundation in 2015, starting the accreditation process of the Foundation for the Accreditation of Cellular Therapy (FACT) NetCord, which was acquired in 2017. In the same year, the laboratory also expanded, leading to the internationalisation process with the reception of samples from Spain.

The company is also involved in various social, psychological, and medical projects, such as BebéVida Sorrisos, which, since 2009, continues to promote and encourage solidarity actions to support entities and causes, such as the Maternity Marathon walk, which supports various associations, such as *Associação Vida Norte* and *Associação Apoio à Vida*, and the launch of the solidarity mascots to raise funds for the *Instituto Português de Oncologia* (IPO) of Porto. Another project was the Solidarity Bank, which allows several low-income families with children suffering from diseases indicated for transplant to benefit from free cryopreservation.

BebéVida's mission encompasses a multifaceted approach to stem cell cryopreservation and research with a strong focus on excellence. In fact, this year, BebéVida is enrolling the process to obtain AABB certification. In addition to SC cryopreservation, BebéVida R&D&I department,

in collaboration with universities and research institutes, try to gain a deeper understanding of SC and its effects on various medical conditions, helping unlock these remarkable cells' full potential.

3. Stem Cells

SC are undifferentiated cells without the functional specialisation characteristic of adult cells. These cells are characterized by self-renewal, with the capacity for continuous proliferation, differentiation into any cell type and plasticity (1), functioning as an internal system to repair damaged tissue and replace dead cells.

SC can divide through symmetric or asymmetric divisions (Figure 1). The symmetric division produces two identical daughter cells that can either be identical to the parent cell or different. The production of two SC identical to the parent stem cell, self-renewal, allows the maintenance of the stem cell population. In comparison, producing two engaged daughter cells will lead to their progressive proliferation and differentiation until terminally differentiated and functional cells are formed. Asymmetric division leads to the formation of a daughter cell identical to the parent cell and a second cell with more restricted potential (2,3).

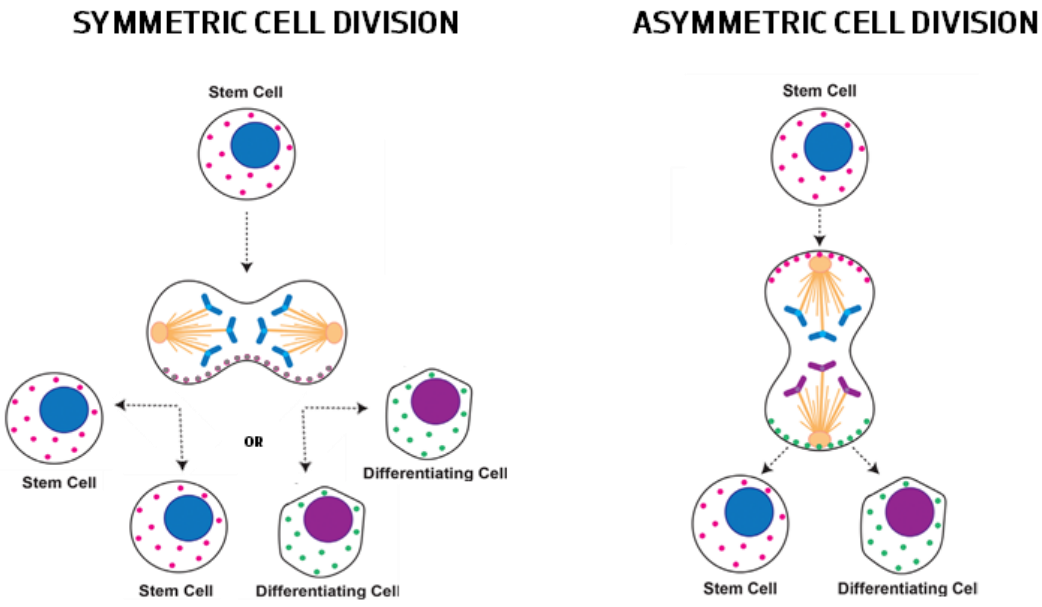

Figure 1: Symmetric versus asymmetric cell division. Adapted from Zion E. et al. (2021) (4).

3.1. Classification of Stem Cells

SC exist from embryo to adulthood, and there are several sources. The main ones are UCB and UCT, bone marrow (BM) and peripheral blood, but they are also present in adipose tissue, nasal mucosa, and dental pulp.

These cells can be classified according to their capacity to produce new cell lineages or potentiality and to their source.

3.1.1. Stem Cells Potency

SC are classified into unipotent, oligopotent, multipotent, pluripotent, or totipotent, in increasing order of competence.

During embryogenesis, the potential of the cells that constitute the embryo decreases (Figure *2*). Totipotent cells proliferate and differentiate into any cell type because of their differentiation potential (5,6). However, after a few days of the first divisions in the embryogenesis, the resulting cells, the blastocyst, become pluripotent, as they cannot originate an embryo independently. These cells have a more restricted differentiation potential than totipotent SC. The blastocyst comprises an outer layer of cells, the trophoblast, which gives rise to extraembryonic tissues such as the placenta and amniotic sac. The trophoblast includes a set of cells called the embryonic bud, composed of pluripotent cells, which originates the epiblast, precursor of the three embryonic germ layers ectoderm, mesoderm, and endoderm, from which all tissues and organs are derived.

Multipotent cells are derived from cells of one of the three germ layers mentioned above. They are called multipotent because they have a capacity for differentiation limited to the cells of the tissue where they are found (5–7).

Cells classified as oligopotent have the proliferative capacity and only differentiate into specific cells, not covering the entire cell lineage. The same does not happen with unipotent SC because these cells exhibit the most limited capacity for differentiation. They also lack undifferentiated proliferation capacity but only proliferate and differentiate into one cell type specific to the tissue they belong (5–7).

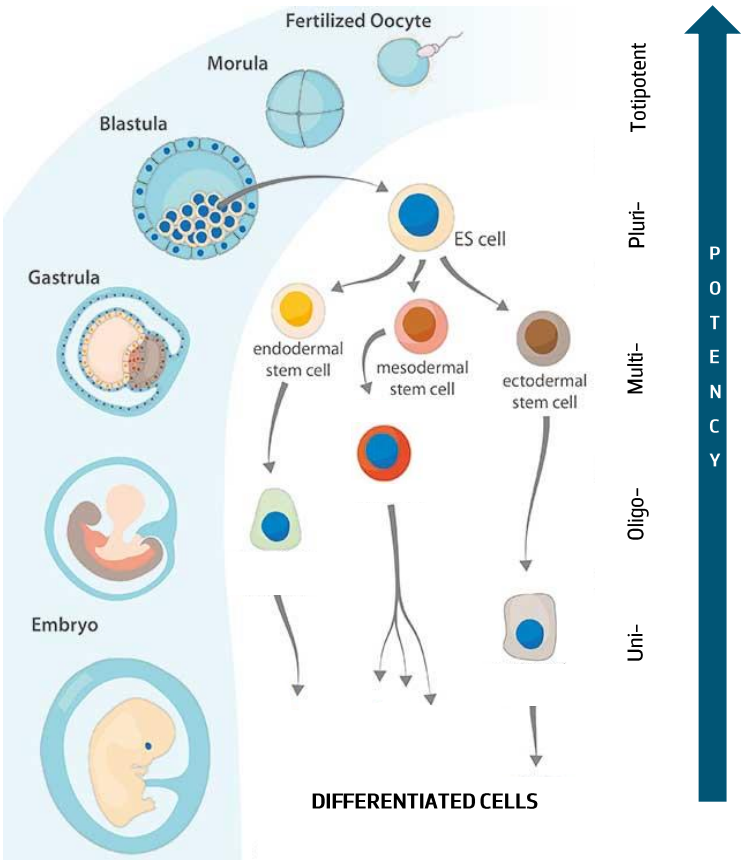

Figure 2: SC potency. Adapted from Enzo Life Sciences Website (2019) (8).

3.1.2. Stem Cells Sources

Another way of characterising SC is according to their source. They can be classified as embryonic, adult, and neonatal SC (6).

Embryonic SC are classified as totipotent or pluripotent due to their high differentiation potential. However, their applications can lead to ethical questions, as they need to be obtained before birth because they can only be found in embryos.

Adult SC are presented in the adult organism in specialised tissues. They have a lower capacity for differentiation, or less potential, giving rise to cells only of the cell lineage of the tissue where they are inserted, such as those of the BM, peripheral blood vessels, and skin. Nevertheless, they are essential in replacing and repairing the damaged tissue in which they are found. Of all the sources identified, the BM, responsible for haematopoiesis, is the oldest source by which SC, such as hematopoietic stem cells (HSC), have been isolated and obtained.

Its therapeutic use in specific diseases has been used in transplantation since the 1950 (9). As for their transplantation, the assumptions for this success are limited since there is a need for a total human leukocyte antigen (HLA) match between the donor–recipient, besides the high probability that after transplantation, the patient will have Graft–versus–Host Disease (GvHD) (10,11). Also, it is an invasive procedure. Another disadvantage of using this source is that the SC per unit volume is lower than other sources.

Neonatal SC are multipotent SC that come from tissues that are, after birth, considered medical waste, such as the placenta and UC. The UC has proven to be a rich source of SC, whose collection is easy, painless, non–invasive, and safe for the mother and infant (12).

3.2. Umbilical Cord

During pregnancy, UC delivers vital nutrients and oxygen to the developing infant and transports away the metabolic waste products produced by the infant (Figure 3).

Typically, the UC measures 50 cm in length and 2 cm in diameter, containing three unbranched vessels: one vein that carries nutrients and oxygen–rich blood and two arteries that deliver waste products and oxygen–depleted blood back to the mother. These blood vessels are adjacent to stromal connective tissue known as Wharton's jelly. Wharton's jelly is a gelatinous connective tissue of an extracellular matrix that includes sulphated proteoglycans and collagen fibres that protects the umbilical vessels from collapsing and provides flexibility to the cord (13).

After birth, instead of being discarded, UC can be used to obtain SC, like HSC and mesenchymal stem cells (MSC). These SC have advantages over other sources, such as immediate availability, no ethical and moral issues, high proliferative and regenerative potential, no strict compatibility requirements, and little exposure to environmental factors since they only exist during gestation (14,15).

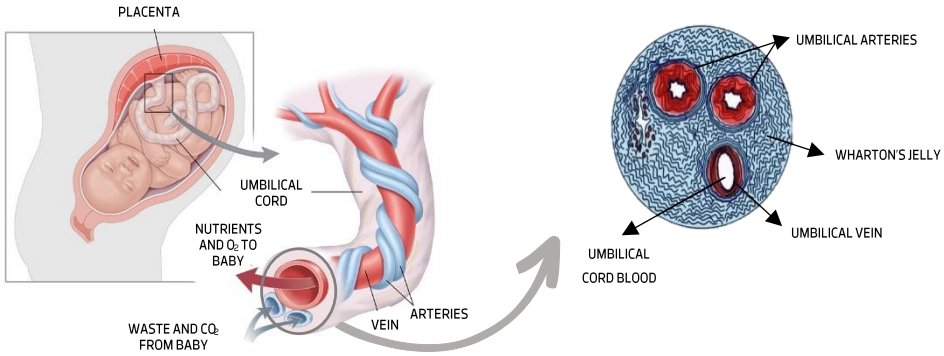

Figure 3: Anatomical composition of the UC. Adapted from BabyCenter Website (2021) (16) and Alatyyat SM. et al. (2020) (15).

3.2.1. Hematopoietic Stem Cells

HSC are cells with unlimited proliferation capacity throughout life. Once classified as multipotent, they can differentiate into all adult and mature blood and immune system cells. Therefore, haematopoietic system is constantly reconstituted, and all the individual's defence functions are ensured.

HSC can be differentiated into different blood cells lines, such as myeloid and lymphoid, as shown in Figure 4. Myeloid cells include monocytes, macrophages, neutrophils, basophils, eosinophils, red blood cells (RBC), and megakaryocytes to platelets. Lymphoid cells include B and T lymphocytes, natural killer cells, and innate lymphoid cells (17,18).

These cells express the cluster of differentiation (CD) 34, a transmembrane phosphoglycoprotein. CD34 protein surface expression is typically lost as cells mature into terminal effectors. For this reason, CD34 is routinely used to identify and isolate human hematopoietic stem/progenitor cells for clinical use (19). Another critical marker used to differentiate cells is CD45, which is in all hematopoietic cells except for mature RBC and platelets (20).

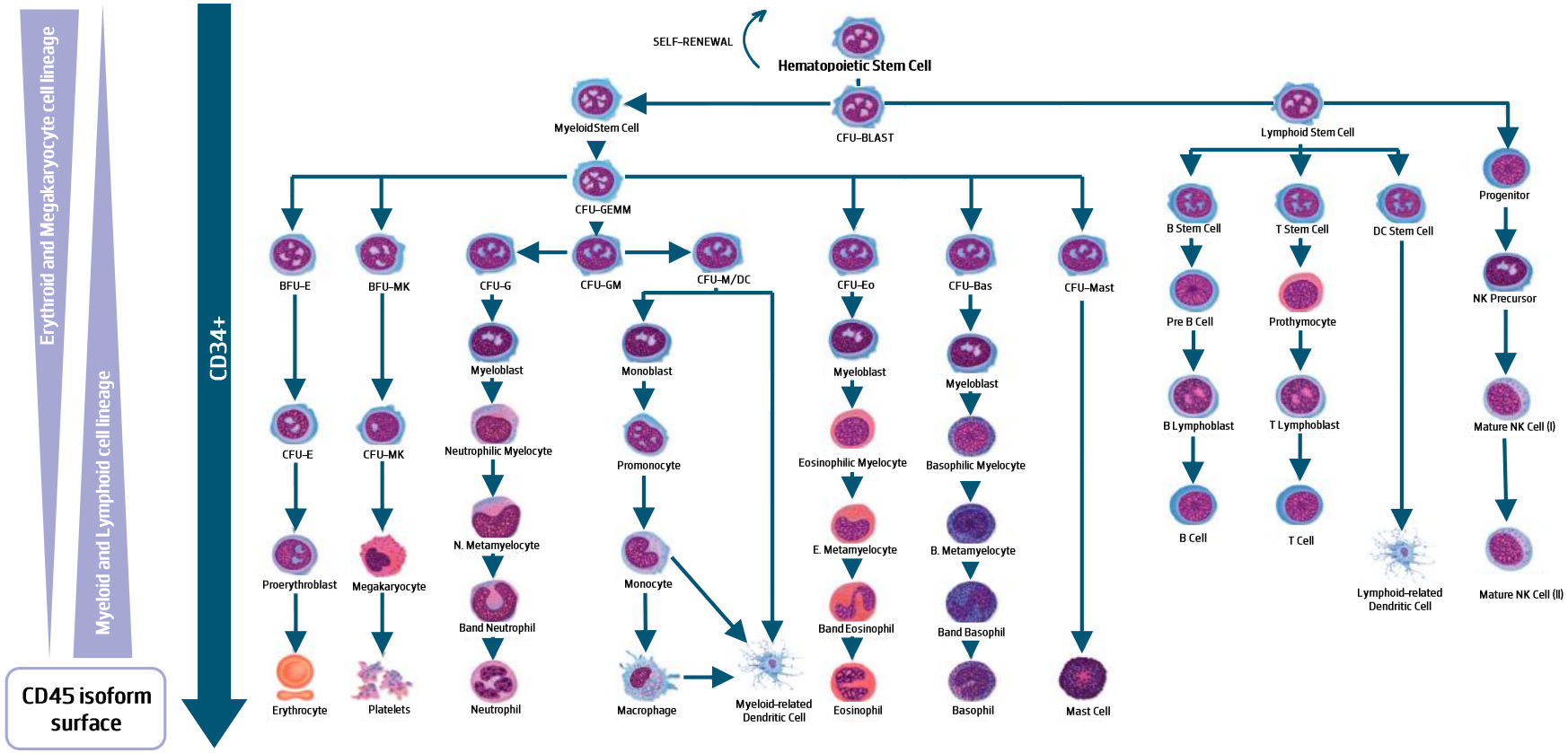

Figure 4: Differentiation process of HSC. Adapted from Genzyme Corporation (2009) (21)

3.3. Therapeutic Applications of Stem Cells

The UCB represents a valuable resource of SC for treating around 80 diseases, such as blood-related diseases, i.e., onco-hematopoietic diseases, such as leukaemia, myeloplasm syndromes, lymphoma, myeloproliferative disorders and BM cancers. However, both UCB and UCT derived stem cells have been demonstrating success in inherited metabolic disorders, inherited disorders of the immune system and other organs, and solid tumours not originating in the blood or immune system (15,22–24).

The transplantation of SC can be done in an autologous transplant, i.e., when the patient without any genetic deficiency receives his cryopreserved cells or in an allogeneic transplant when the patient receives cryopreserved cells from a practically compatible donor, who can be a relative or no relative (25).

UCB first clinical application occurred in Paris in 1988, in a transplant to a 5-year-old child with a rare haematological disease, Fanconi Anaemia. The donor of the UCB sample used was her brother, who had a high HLA compatibility (22,26). The success of the haematopoietic and immunological reconstitution achieved in this patient was considered a milestone in the history of medicine and led to other studies and transplants being carried out for several years worldwide. In Portugal, the first UCB transplantation was carried out at the Lisbon IPO in 1994 in a 3-year-old child diagnosed with chronic myeloid leukaemia. The transplantation of her brother's cryopreserved UCB led to the remission of the disease.

Over the years, UCB-HSC have been demonstrated to have the capacity to regenerate a wide range of other tissue types, and their transplantation into both animals and people has led to appreciable functional gains (27). Numerous clinical trials are underway to effectively treat other diseases such as cerebral palsy, diabetes, stroke, autism, Alzheimer's, and multiple sclerosis, among others. On June 9, 2023, there were 1328 cases in the clinicaltrials.gov database by the terms "Umbilical Cord Blood", where a total of 684 had the status "completed"/"terminated". By the terms "Umbilical Cord Tissue", there were 49 studies, where 17 had the status "completed"/"terminated".

3.4. Cryopreservation Banks

As the UC-SC are only collected at birth, they must be cryopreserved. For this, there are two types of banks, public and private. In Portugal, both private and public entities have to ensure the donor's consent and some other parameters, complying with standards governed by Law No. 12/2009 and being regulated and authorised by the *Direção Geral de Saúde* (DGS).

In both familiar and public banks, the samples are subjected to controls and tests, such as HLA analysis, tests for infectious diseases, minimum cell count, and maternal medical history. Thus, they become characterised units, with a guarantee of quality and uniformity, available to transplant when needed (28).

The cryopreservation banks have contributed intensively to minimising the time spent searching for a donor.

3.4.1. Public Banks

Public UCB banks are non-profit and enable the cryopreservation of UCB from donating a child's UC for unrelated transplant. They become destined for any person, and the sample can be destined for allogeneic use. In these cases, the person or relatives lose the property rights to use the blood donated to the bank, and there is no guarantee that in case of future need, access to their unit of cryopreserved cells will still be available (28,29).

An advantage of public banks is the absence of any payment associated with the whole process and the wide availability of samples to many patients in need of transplantation worldwide. Furthermore, the *Serviço Nacional de Saúde* (SNS) ensures the financing and regulation of the entire process. However, because these banks only store samples for a short time, they sometimes have the disadvantage that no sample is available and compatible with the patient in need.

The first public cryopreservation bank was established in 1991 at the New York Blood Center in the United States of America. Since then, many others have appeared in several countries worldwide. The only current public bank in Portugal is the *Banco Público de Células do Cordão Umbilical* (BPCCU), formerly called Lusocord, which was created by the Portuguese state in

2009. However, years later, it became part of the IPST, which is responsible for all the management and organisation of this bank until now.

3.4.2. Private Banks

Private or familiar banks cryopreserve a newborn's UC–SC that can be used for an autologous or related allogeneic transplants. However, contrary to public banks, in case of therapeutic need, the person is guaranteed the use of their own cells, and the samples are not inserted into public research networks (29).

These banks follow the same processing parameters as public banks for obtaining and cryopreserving the cells. The sample becomes under the parent's care until the infant is of age.

For the establishment, implementation, and regulation of specific quality criteria for the processing and storage of SC from the UCB, there are worldwide organisations with international experts in the field of blood banks, such as the FACT and the American Association of Blood Banks (AABB). Through periodic inspections of laboratory processes, these entities develop accreditations granted to banks that present safety guarantees for the client to request the service. Furthermore, the accreditation increases the quality and criteria guidelines for the quality designed in all the processing phases.

Compared to public banks, private banks are required to comply with the same terms and standards required by the DGS and are also subject to compliance with the criteria governed by the accreditation entities.

BebéVida is one private family blood and cord tissue bank in Porto, Portugal, being the only FACT-accredited family bank and also is enrolling the process to obtain AABB certification.

4. Aims

The main objective of the internship was integrating BebéVida, which consisted to promote a work experience in the laboratory area in a routine context. In addition, it also came to consolidate the knowledge acquired and strengthen autonomous and teamwork workflow.

Therefore, the main goals of this internship were:

1. BebéVida laboratory UCB processing protocols training:
    a. Collection kit reception
    b. UCB automatic processing
    c. UCB cryopreservation
    d. UCB storage
    e. UCB analytical and microbiological screening
2. Equipment qualification and validation procedure
3. Evaluation of UCB parameters:
    a. Effect of AXP UCB processing methodology in Buffy Coat quality
    b. Blood group influence in UCB immune cell profile

CHAPTER 2: LABORATORY PROCESSING ROUTINE

1. Stem Cell Isolation

UCB, once collected, cannot be directly cryopreserved. It must be processed in clean, classified rooms that provide an aseptic environment suitable for safe, contamination-free processing of samples. UCB processing is necessary to concentrate the nucleated progenitor cell fraction without a significant loss of cells and decreased viability, resulting in more viable and functional cells. Therefore, removing the fraction corresponding to plasma and RBC is necessary to obtain a more efficient recovery of this cellular fraction (15). Removing RBC is necessary to improve the remaining cells' quality and functionality. These cells lead to complications during freezing and thawing. They are susceptible to damage and can release haemoglobin, affecting the quality and viability of other cells. Another critical aspect of the concentration of the nucleated progenitor cell fraction is that these processes also allow for testing and quality control, assessing for infectious diseases and other factors that may affect the suitability and safety of the cells for future use (15).

There are different manual and automated methodologies to isolate SC from UCB. Manual processing involves sedimentation agents, such as Hydroxyethyl starch (HES), or the addition of reagents that aid in separating all elements of the blood sample based on a density gradient, such as Lymphopre, Lympholyte, Ficoll-Paque, and Percoll (15,30,31). Density gradient centrifugation is based on the varying densities of the cells within a heterogeneous sample. The sample is stratified on a density gradient medium before being centrifuged, where each cell type will sediment to its isopycnic point. So, when centrifuging, RBC are sedimented in the lowest layer. Slowly, cells with lower densities, platelets, SC, and white blood cells (WBC) overlap, with the plasma retained in the upper part (15,32).

There are also semi-automated methods that are faster, with no need for exogenous additives. An example of a semi-automatic method is the top and bottom systems. The collected UCB is centrifuged after transfer into a triple bag system. High-speed centrifugation is applied to separate the buffy coat layer with simultaneous extraction of RBC at the base and plasma at the top, while the leukocyte layer is kept stable within the original extraction bag throughout the process. The transfer to each bag is done manually (15).

Automatic techniques reduce the number of handling steps for each sample and the time spent separating the different components also have a higher mononuclear cell recover rate (RR). A high cell production success rate is significant for successful transplants and improved patient survival rates. Another advantage of the automatic system is eliminating errors made by the technician. The fact that it is a closed system also reduces the risk of contamination (15). There are several automatic methods, the best known being the AutoXpress Platform (AXP) and the Sepax system (15,33,34). These systems involve a set of processing bags and a central device through which the blood components are automatically separated into different bags during a centrifugation process (34).

2. Services Provided by BebéVida

The decision to cryopreserve UC-SC is always at the parent's discretion. When there is a decision, the process is started, and the choice of service is made. There are four services as presented in Table 1.

Table 1: Services provided by BebéVida and each of its characteristics.

Services			
UCB	UCB + Fragmented UCT	UCB + Isolated UC-Cell Suspension	UCB + Fragmented UCT + Isolated UC-Cell Suspension

These services have different characteristics. Regarding the UCT, there are two distinct processes, the fragmented way and the isolation of the MSC. All these services have free access to the materno-foetal seroteca for complementary tests, where samples of maternal plasma, peripheral blood, plasma from the infant and a fragment of the UC are stored.

3. Documentation

For the guarantee and security between the parties, namely the laboratory and the parents, it is necessary to verify all the documentation. The documentation includes the service provision contract, the clinical questionnaire, the informed consent, the bio-surveillance form, the collection report, and the clinical analyses carried out on the mother in the last trimester of pregnancy.

The contract for the provision of services has a duration of 25 years and has various clauses which set out the rights and obligations of both parties. This contract clarifies all the conditions of the process, i.e. costs, limitations, duties and obligations, information about termination, and indemnities, among others. It is established in the contract that BebéVida respects the legislation and the clinical norms in force concerning the handling of these biological products, as well as having a specialised team and adequate equipment for this purpose. This contract, which has to be signed, requires the filling in the mother's and father's personal information, such as full name, identification number, residence and contacts.

The clinical questionnaire is a mandatory document to be filled out, as it is important to know family history and questions regarding the father and mother. Depending on the information provided, it may lead to non-cryopreservation. The mother and the health professional must sign this document and the informed consent.

Informed consent explains the collection of UCB and UCT, the processing, and its use and purpose. This consent also informs that none of the cryopreserved samples will, under any circumstances, be used for commercialisation. Furthermore, the confidentiality of all clinical information is also ensured, which guarantees the protection of the client's identity and privacy.

Apart from these, other information is essential. Therefore the last analyses of the last trimester of pregnancy are requested. The clinical analyses required are markers for Hepatitis B and C, Cytomegalovirus (CMV), Human Immunodeficiency Virus (HIV) 1 and 2, and Syphilis. The samples will not be stored if the mother is positive for any of these markers.

In advance, these four documents must be sent to the donor selection department for evaluation but are also reviewed on the day the kit arrives. On receipt of the kit comes the bio-surveillance form and the collection report. The bio-surveillance is to be filed if anything occurs

during the transportation of the sample. The collection report has important information for the accurate evaluation of the sample. It informs the infant's and the mother's clinical status at delivery and if there was any complication.

4. Database

A database usually offers structure, accessibility, and speed. Therefore, BebéVida uses a specific database to store all customer data in addition to the paper documentation. Thus, from this database, it is possible to access a given customer's data at any time.

In this database, it is possible to find the following data registration:

- Service chosen, as well as the payment data;
- Associated client number for sample identification;
- Identity of the parents and the infant;
- Date of birth, time of delivery and type of delivery;
- Identification of the hospital entity responsible;
- Date and time of sample reception at the laboratory;
- A temperature range in which the kit (with samples) was exposed;
- Type of product stored;
- Identification of the technicians that performed the different stages of the whole processing, as well as the identification of the biologic safety cabinets (BSC) used;
- Identification of the batch numbers of all materials and reagents used;
- Haemogram's values;
- Analytic results;
- Additional fundamental information for processing, such as volumes collected, the total number of nucleated cells (TNC), and cell RR, among others;
- Validation of samples – cryopreserved/rejected;
- Identification of the location of the samples in the nitrogen tanks;

- Identification of bacteriological controls;

- Values of the different parameters already mentioned in the mother's blood tests in the last trimester, as well as the tests that will be done on the mother and infant's blood afterwards;

- Recording of any occurrences in all the processing stages.

5. Kits

The BebéVida Kit has been improved over the years to simplify the collection of samples, increase the safety of the samples and the volume collected, and become even more environmentally friendly (Figure 5).

It is composed by:

- A set of instructions required for the collection of biological products essential for the medical team;

- A data logger, which is a continuous recording system that monitors the temperature conditions to which the packaging and samples were subjected during transport, from packaging to reception at the laboratory;

- Expanded Polystyrene Insulation (EPS) box for thermal and mechanical protection of biological products during transportation. The box was designed following the UN 3373 standard (Diagnostic Specimens Transport P650);

- A sterile, sealed bag for the collection of the UCB, with a standard double-needle collection system, which simplifies and reduces contamination. For blood preservation, this bag contains a specific amount of anticoagulant, Citrate-Phosphate-Dextrose solution with Adenine (CPDA);

- Three tubes for maternal blood collection, with a specified amount of Ethylenediaminetetraacetic acid (EDTA) and a pre-monitored blood collection system;

- A sterile vial for collection of the UC segment;

- Isopropyl alcohol disinfectant wipe;

- Vial of alcoholic cutaneous solution with chlorhexidine and 70% isopropyl alcohol;

- Physiological serum for cleaning and hydration of the UCT fragment;

- Sterile gauze compresses;

- Thermal gel bags to ensure temperature stability during transport;

- Labels for labelling the biological product with the client's identification number.

All the materials needed to collect UCB, UCT and peripheral blood are sealed and should only be opened by the medical team at the time of delivery. This whole kit has been evaluated and approved in its conformity by Infarmed, according to European legislation for medical devices, and accredited by FACT.

Figure 5: Collecting kit and its composition. Image by BebéVida.

6. Sample Collection, Packaging and Transport

6.1. Collection of the Biological Samples

At the time of delivery, immediately after the UC is cut, the healthcare provider should perform the desired sample collection according to the instructions and use the sterile material provided in the kit. For UCB and UCT collection, the procedure is simple and completely painless.

6.1.1. Umbilical Cord Blood

After clamping the UC close to the infant's abdomen and disinfecting it with the antiseptic solution, the umbilical vein is punctured (Figure 6B), and the most significant possible volume of blood is gravity extracted into the conceived bag (Figure 6A). This extraction lasts a few minutes and, as a rule, does not interfere with other postpartum care.

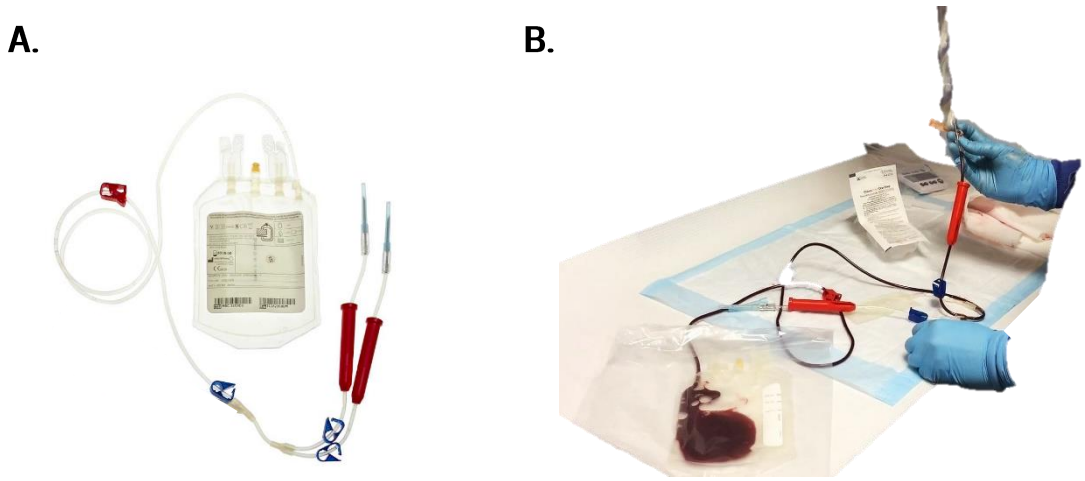

Figure 6: Collection of UCB sample. **A.** Collection bag specifically for the collection of human UCB. **B.** UCB collection ex-utero. Adapted from Macopharma Website (2022) (35).

6.1.2. Umbilical Cord Tissue

If applicable, the UC collection should be a linear section and, if possible, approximately 30 cm long (Figure 7A). The section should be cleaned appropriately and with as little blood as possible. The sample is placed inside the sterile vial provided in the kit and in saline solution (Figure 7B).

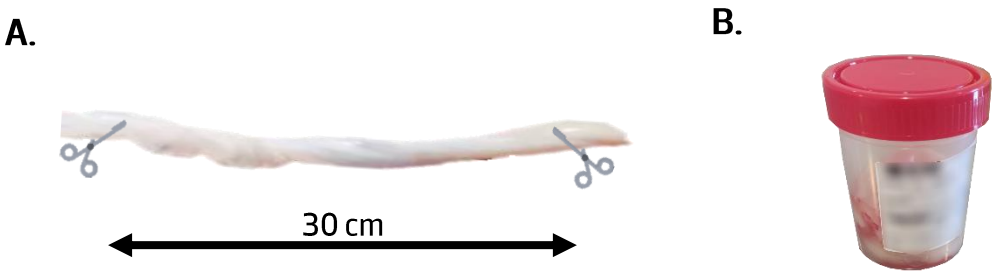

Figure 7: Collection of umbilical cord tissue. A. Size of the fragment required for harvesting. B. Sterile vial for collection of the umbilical cord segment properly identified.

6.1.3. Peripheral Maternal Blood

The collection should be performed on the day of delivery according to the instructions provided. It is done using a pre-assembled extraction system, BD Vacutainer eclipse needle, to make up three BD Vacutainer K2E tubes provided in the kit (Figure 8). This processing should be conducted rigorously to ensure an excellent anticoagulant/blood ratio. Finally, when possible, these tubes should be packaged and sent to the laboratory with the remaining biological samples to obtain the plasma for an external laboratory to perform viral analyses. Additionally, one cryotube of maternal blood and two of its plasma are stored in the materno-foetal seroteca.

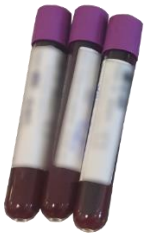

Figure 8: Tubes with peripheral maternal blood.

6.2. Packaging and Transportation of the Biological Samples

Once the samples have been collected, they should be packaged adequately by the health professional, who should place the samples between ice-cold bags. In addition, attention should be paid to labelling since all the kit's internal and external boxes, and each container should have the number and barcode associated with the client.

After packaging and identification, the medical team will deliver the kit to the parents or relatives. They should then schedule the transportation of the biological products to the laboratory as soon as possible because, according to the FACT guidelines, after the collection, the samples (UCB and UCT) need to be processed in 72 hours. Some studies have shown that after collection, the viability of the cells decreases about 50% after 72 hours (36). So, the samples should be cryopreserved within this time.

During transportation, the conservation and integrity of the samples must be ensured.

7. Laboratory Processing

The sample is processed and stored within a few hours as soon as the collection kit is received at the BebéVida laboratory. In this sense, the entire laboratory procedure will be described, from the reception of the collection kits to the moment the samples are stored in the vapour-phase nitrogen tanks (Figure 9).

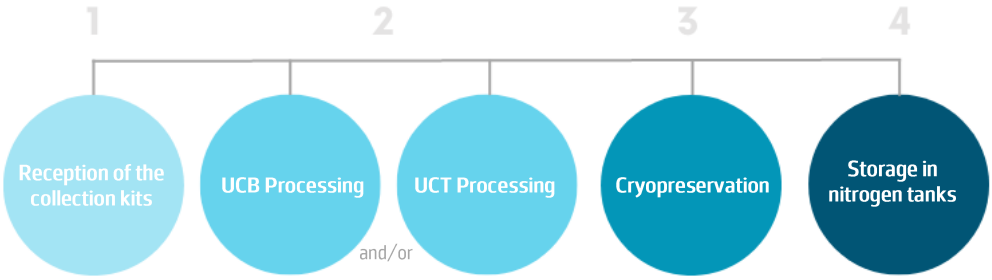

Figure 9: Representative scheme of the laboratory processing steps.

7.1. Reception of the Collection Kits

Once the kits arrive, they are opened in the reception room. It is crucial always to confirm the client number and the contracted service so that samples and information are not exchanged. While the kit is being opened, a specific form is filled in. Afterwards, this data is passed on to the database. The parameters to be considered when filling out the form are:

- Verification of the collection report, confirming the date, time and type of delivery, entity, and type of sample, and whether there was any transfusion during delivery;

- Integrity of the components and containers, such as the blood bag and vials. Special attention to the UCB storage bag, checking for perforations and the existence of blood clots or haemolysis;

- Conformity with the labels provided;

- Travelling conditions (time and temperature recording - data logger).

After all the certifications are made, the bag is sealed, and the needles are removed. The surface of the collection bag is disinfected with 70% alcohol. Subsequently, the sample is dispatched to the type C cleanroom through a pass box, sprayed with isopropyl alcohol.

7.2. Umbilical Cord Blood Processing

The AutoXpress Platform (AXP) is a closed processing system that features a set of sterile, disposable separation bags and a central support device (15). The UCB collected into the collection bag is transferred through a sterile tubing system to one of the three bags that constitute the AXP set, discarding the collection bag. The AXP kit consists of two bags for storing the RBC and plasma, a freezing bag for storing the buffy coat, a clot filter, an inlet filter of the cryoprotectant, and a clot filter as other necessary inlets and valves (Figure 10).

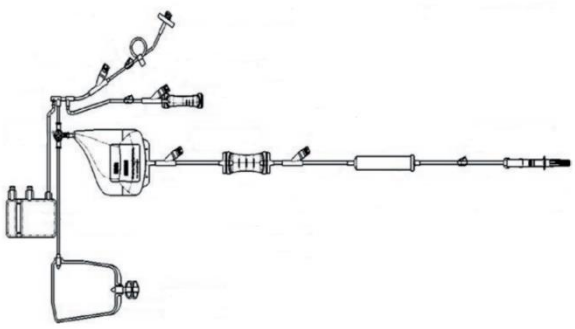

Figure 10: AutoXpress Platform kit. Adapted from Dobrila, L. (2018) (37).

After the transfer, the bags are placed in the different compartments of the AXP device and using a centrifuge, the device is inserted into one of the containers integrated into the device, and a programmed centrifugation process is initiated. Since the centrifuge includes several containers, several samples can be processed simultaneously, thus increasing the efficiency of this process. The centrifugation consists of two steps, a slow centrifugation, and a fast centrifugation, with a total of 30 minutes. The first centrifugation aims to place the three cell fractions in order of density, and the second one separates into different bags by optical density (Figure 11). In the second centrifugation, the first fraction to be directed to the standard bag is the fraction related to the RBC, followed by the fraction corresponding to the buffy coat for the cryogenic bag, with the plasma remaining in the initial transfer bag (33). In this system, the counterbalance is performed by means of plasma to complete the final standard volume of 21 ml in the cryogenic bag. This system is also composed of the software XpressTRAK to support the obtained data. A docking station is necessary for communication between the AXP device and the computer to obtain the data of each sample and the volumes acquired in each bag. At the same time, it charges the device's batteries (15,37).

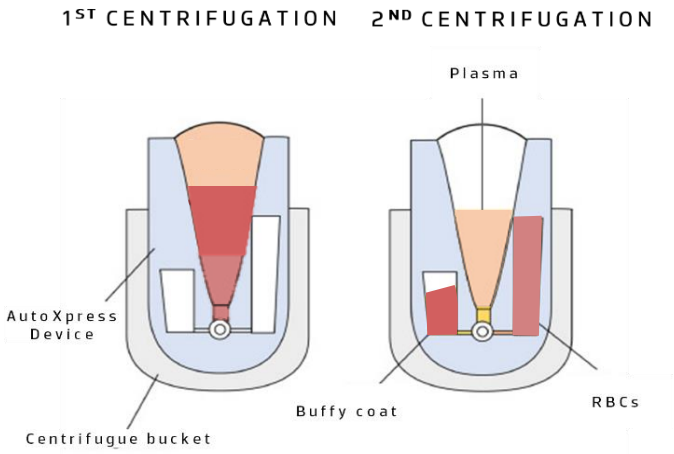

Figure 11: Centrifugation in the AXP method processing. Adapted from Li, A. et al (2021) (33).

Therefore, in order to process UCB using AXP methodology, the blood bag is weighed and UCB volume is calculated (Figure 12A). The initial collection bag is connected to the sterile disposable bag kit AXP within the BSC (Figure 12D). An initial blood count is performed in all samples, and the various haematological parameters are determined using a Mindray BC-6200 haematological analyser. For this, a small representative sample is collected into a previously labelled tube (Figure 12B and C).

Subsequently, the AXP kit is placed vertically outside the chamber, and the blood is filtered and stored in the most oversized bag of the set, as it has a tubing system with a clot filter. It is then sealed, and only the part necessary of the kit is integrated into the AXP device, discarding the initial bag, the air cushion, and the clot filter (Figure 12E).

After placing this kit in the appropriate compartments of the AXP device and packing it with the accessories required for integration in the centrifuge (Figure 12F), these are previously calibrated in the centrifuge cups using weights, if necessary. Then, double centrifugation is performed, 30 minutes in total, always at an average temperature of 10°C (Figure 12G).

Before being placed in the centrifuge, the blood unit ID, client ID, centrifuge ID, lot number and expiry date of the AXP kit processing bags are first entered into the XpressTRAK software. At the end of both cycles, the set of bags is removed from the AXP device, and the equipment is placed in the docking station (Figure 12H). In this way, the data from the processed sample is transferred. From this software, it is then possible to track and record data specific to the separation of each of the samples during and after centrifugation. All the reports are then attached to each customer's file.

The cryopreservation bag is then homogenised in a stemix for a short time. Then, inside the BSC, it is sealed so that the cryopreservation bag (Figure 12I) is separated from the plasma and RBC bags and a representative buffy coat sample is taken into a labelled tube (Figure 12K and L). The tube is then sent to the outer room and analysed by haematological methods. This representative sample, the buffy coat, will also be used for another analysis using flow cytometry.

As for the remaining bags, they are removed and used to perform microbiological screening with aerobic and anaerobic blood culture bottles (Figure 12J). Two cryotubes of plasma are also stored for supplementary analyses, and, if applicable, the remaining plasma is centrifuged and sent to the tissue processing room.

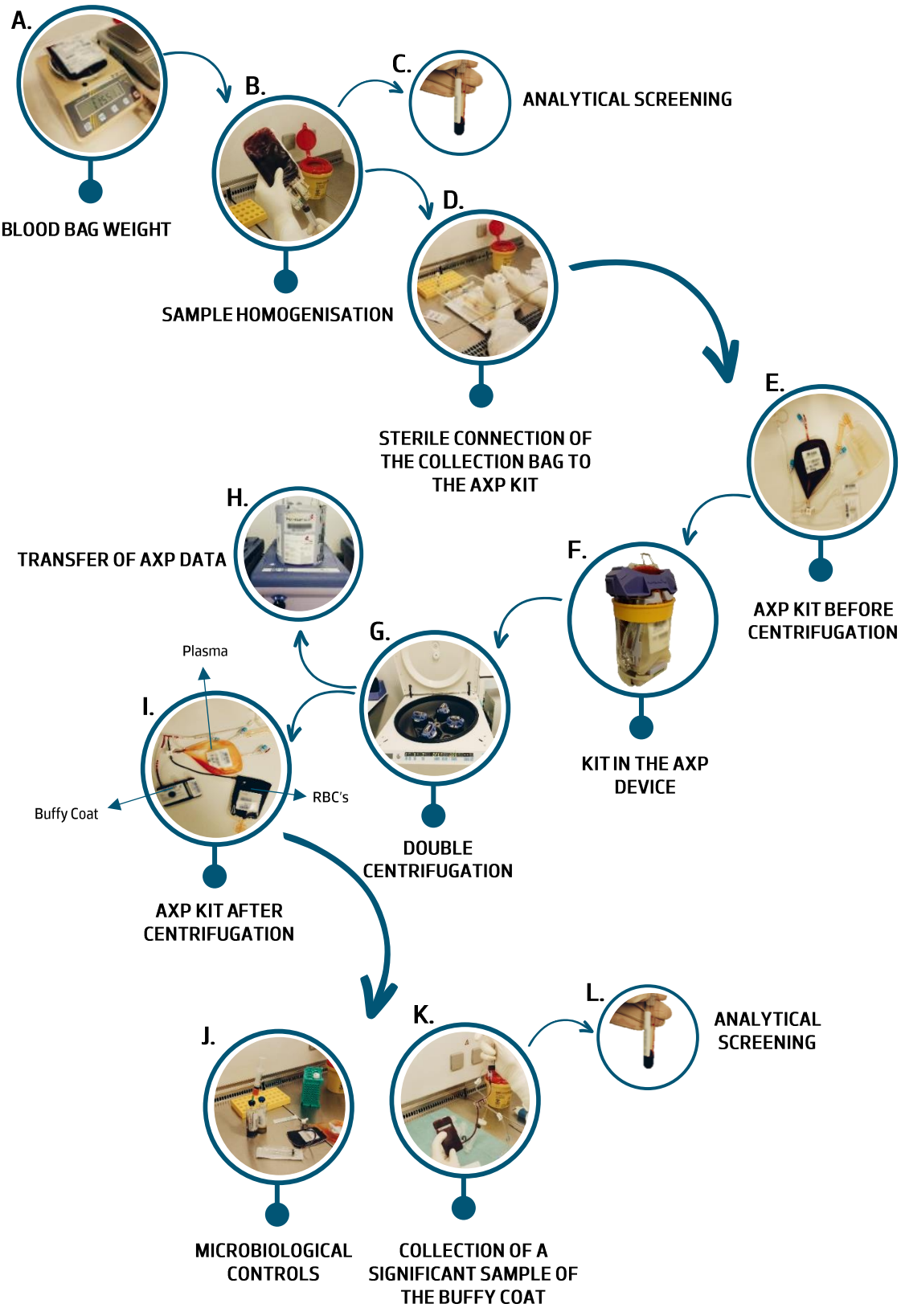

Figure 12: Scheme of the UCB processing.

7.3. Crypreservation of Umbilical Cord Blood

The cryopreservation process is a method that allows cells to be maintained for an extended period of time without significantly altering their properties (38). The stipulated time interval is more or less 25 years (39), but studies are being conducted to see if there are significant adverse effects after 30 to 35 years. Nevertheless, the cell concentration, cryoprotective agents, cooling rate and storage temperature must be considered for successful cryopreservation (40). Regarding cell concentration, before a sample is cryopreserved, it must have a minimum number of cells because after thawing, a percentage of cells die. Therefore, this should already be taken into account when cryopreserving the sample (40).

Another factor is the formation of ice crystals. Cryopreservation aims to avoid the formation of these crystals, osmotic shock, and any damage that might affect the cells (38). One of the detrimental factors for cells comes from the extreme decrease in temperature below the physiological value (36–38°C), which leads to the formation of ice crystals. Therefore, the ability to induce shallow temperatures, necessary for the long-term preservation of cells, must be controlled (40,41).

If the temperature drop is carried out abruptly, ice crystals will be formed in the extracellular and intracellular medium and perforate the plasma membrane causing cell death. On the other hand, if the drop in temperature is carried out slowly, ice crystals are formed in the extracellular spaces and consequently, the solute concentration increase in the extracellular medium. Thus, this modification of the osmotic gradient leads to a high degree of dehydration of the cells, causing their death (38,41). Therefore, to avoid cell death due to the stress of the freezing process, technologies have been developed that allow a controlled cooling of the temperature, and cryoprotective agents have been included to bypass these adversities (Figure 13) (40,41).

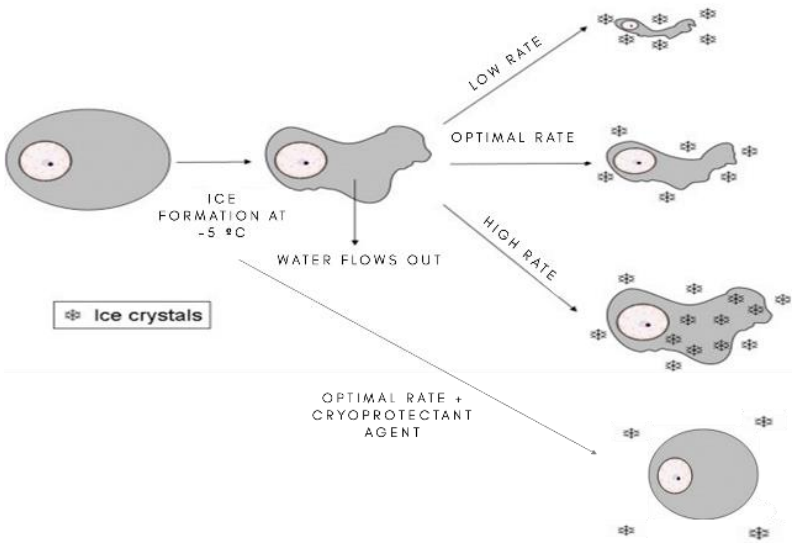

Figure 13: Physical events and cryoinjury of cells during freezing. Adapted from Kosheeka Website (42).

An ideal cryoprotectant should present properties such as high solubility in aqueous medium at low temperatures and high concentrations, low molecular weight, good diffusion capacity in cell membranes, and, ideally, be the least toxic possible. In addition, by interacting with water through hydrogen bonds, the cryoprotectant causes the freezing point to be reduced and fewer water molecules to be accessible to bond. Thus, cryoprotectants limit and delay intra- and extracellular crystal formation (41,43).

Among the conventional cryoprotectants with this intracellular diffusion capacity are glycerol, dimethyl sulfoxide (DMSO), ethylene glycol and propylene glycol (41). DMSO is one of the most used cryoprotectant agents for stem cell cryopreservation. Its use presents some advantages, as it is an agent with a much faster capacity to diffuse into cells than others (44). The DMSO used is at a concentration of 10%. Due to its low molecular weight and physical and chemical properties, it can interact with cell membranes and diffuse rapidly into the intracellular medium. This way, it protects the cells and preserves cell volume by replacing the water inside them (41). However, it is a time and concentration-dependent cytotoxic cryoprotectant since when exposed to the cell suspension at room temperature (37°C) for more than 30 minutes, at a concentration above 1%, cell viability is significantly affected (45).

In the laboratory, DMSO administration is done with the help of syringe injection pumps and equipment to control temperature (stemix) that also keeps the sample in agitation. Thus DMSO is administered gradually at a controlled rate over a 9-minute cycle (Figure 14A and B), and as the sample is subjected to the cryoprotectant, it is cooled and maintained at 4-6°C. Depending

on the number of stemix available and the total number of samples to be processed, there can be more than one cycle of DMSO injection.

As the cryoprotectant addition process is completed, the freezing bags are again placed into the BSC to be sealed. Before sealing the samples, it is crucial to homogenise all the content and remove as much air as possible. The bag is then sealed in such a way that it is bi-compartmentalised, which allows multiple uses. Most SC are stored in the main section, which is intended for clinical use. The smaller section is used in tests, such as histocompatibility tests or for research use. In addition to this sealing, several seals are also performed on a portion of the kit tubing.

In the same way, small fractions are acquired and can also be used for multiple tests and analyses (Figure 14C and D). Next, it is protected inside an overwrap, which is sealed (Figure 14E and F). It is highly resistant to low temperatures and prevents the possibility of cross-contamination from occurring inside the nitrogen tanks. After the sealing, the samples and their labels are transferred by a pass box to the storage room where the sample cooling equipment and the nitrogen tanks are located. There, another technician collects the samples and places them correctly inside aluminium cannisters, with labels surrounding the cannister (Figure 14G). This cannister, also identified, is intended to provide a thermal barrier during freezing or thawing. In addition, it safeguards the physical integrity of the samples between thermal shocks that may occur.

After the sample is appropriately conditioned and identified, it is placed inside an automatic equipment connected to a computer, a controlled rate freezer. Its purpose is to cool the samples by the programmed injection of nitrogen gradually (Figure 14H). Thus, it is possible to automatically optimise the programme we want and the resolution of the freezing curve in progress.

When all the samples are already inside the equipment, the programme is started, and the first freezing phase begins. This stage is based on subjecting the samples to a gradual decrease in temperature, with a controlled cooling rate -1°C per min until -60°C, being the critical phase between -50/-60°C. After this phase, the samples suffer a temperature peak until -156°C, so there is no compromise of the samples during storage.

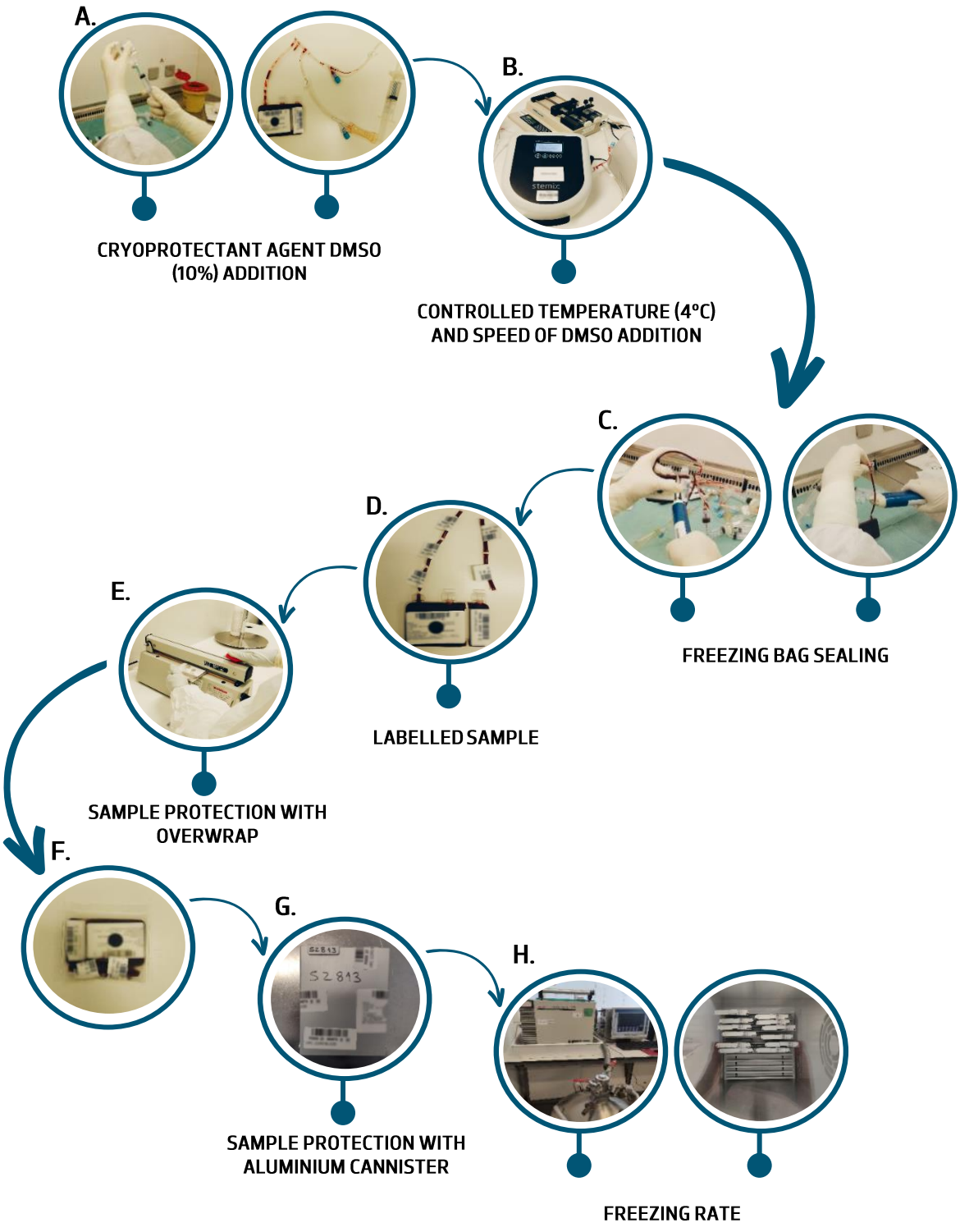

Figure 14: Scheme of the cryopreservation of UCB.

7.4. Sample Storage

The last step of the whole UCB stem cell cryopreservation process is its storage. For this, after the freezing programme is finished, there is the certification that the curve is as intended, without any deviation. Next, the samples are transferred from the equipment to a mobile nitrogen cryostorage that is used to transfer the samples to nitrogen tanks.

Each sample has a predefined position associated with a tank, identified by a letter, a tower, identified by a number and a drawer, represented by a letter (Figure 15). Opening the tanks and placing the samples should be as brief as possible to avoid cell damage caused by exposure to ambient temperature. For that, during storage, the time of exposure of the sample to room temperature is timed.

Then, in the tanks, the samples are subjected to nitrogen in its vapour phase at a temperature of -195°C.

Figure 15: Sample storage.

8. Materno-foetal Seroteca

BebéVida also has a parallel maternal-foetal bank, essentially for maternal blood and plasma samples, infant's plasma, and a fragment of the UC. The storage of these biological products is essential because if it is necessary to perform additional tests, such as haemoglobin diagnosis, genetic tests, viral analyses, and blood group identification, among other analyses, it is easy to thaw these maternal and infant cryotubes.

9. Control Analyses

During processing, the quality and viability of the sample must be guaranteed, and it is essential to perform various control analyses, such as microbiological analyses, haematological evaluation, flow cytometry and a sample stability programme.

9.1. Microbiological Screening

The microbiological analysis is done on the sample and its processing environment (BSC, BSC surface and lab technicians). In case of being positive and after identification, an antibiogram to different antibiotics susceptible to eliminating the microorganism is performed. The results are then transmitted to the laboratory and attached to the client's files. In case of being a resistant micro-organism, the sample is discarded.

9.1.1. Umbilical Cord Blood Sample

To screen for any bacterial or fungal infections in the sample, a complementary diagnostic test, called blood culture, is performed on each client's UCB.

A blood culture is based on the confirmation and identification of the microbial aetiology and helps detect the infection's source. For each sample, two culture bottles are used, BacT/Alert Biomerieux, with aerobic and anaerobic culture medium for better clarity of the results.

The requisitions forms are registered and associated with each client in BebéVida database. At the end of the day, they are sent to an external laboratory that proceeds with the procedure and analysis of the results. First, they will be incubated at 37°C in automated equipment for 5-7 days to detect positive blood cultures. With positive results, the bottle is flagged, and a Gram stain is performed to visualise the morphology, proceeding with identifying the microorganism by molecular diagnostic techniques.

9.1.2. Processing Environment

Every working day, all technicians working on a BSC carries out microbiological controls of air, surface, and contact monitoring. For air and surface monitoring, the sedimentation or contact technique uses Petri dishes with TSA culture medium (Tryptic Soy Agan), a nutritive medium used to grow and isolate aerobic and anaerobic bacteria. Before beginning the laboratory activity, the plates for air control are placed inside the BSC, remaining until the end of all activities, being replaced every four hours to ensure optimal monitoring. The surface control is performed immediately after processing the last sample and is done by contacting the CT3P (Count-Tact 3P Agar) plate with the used surface. To evaluate personal contamination, the glove used to process the last sample is scanned using the TSA contact plate. Subsequently, all plates are incubated at 37ºC for seven days. After this time, the plates are examined, and the growth or absence of bacterial colonies is recorded. With positive results, they are also sent to an external laboratory.

UCB processing is performed in a clean room classified as a class C. The antechambers meet aseptic conditions and strict air quality control according to predefined criteria. Therefore, the rooms have controlled monitoring of the air conditioning, with temperature, humidity and pressure control and the capacity to renew the air constantly. Besides the BebéVida uniform, disposable non-sterile suits, protective shoes, masks and caps are used inside the rooms.

9.2. Haematological Evaluation

As already mentioned, during the UCB processing, a blood count is performed using a Mindray automated haematology analyser interfaced with the software labXper, which sends the data directly to the database. The labXper software allows the analysis of the sample with a high degree of accuracy, selecting and classifying the results according to the settings defined by the user. Furthermore, as this is an automatic method, it has advantages, as it evaluates haematological parameters in greater detail.

A hemogram is a blood test aiming to assess the three main lineages of constituent cells: RBC, leucocytes, and platelets. It is used to look at overall health and find unusual increases or decreases in cell counts.

– The evaluation of RBC includes their count, haemoglobin concentration, occupied volume (haematocrit), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC), as well as the range and distribution of RBC, based on variations in size and haemoglobin concentration.

– The leucocyte evaluation includes the total count and the differential count, that is, the relative and absolute value of the different leucocytes present, such as neutrophils, lymphocytes, monocytes, eosinophils, and basophils.

– The evaluation of platelets includes the count, the volume occupied over the total blood volume (plateletocrit) and also determined the mean platelet volume (MPV) and its distribution.

– It allows the value of reticulocytes, precursor cells of the GV54, to be obtained.

Before the hemogram is performed, the UCB sample should always be homogenised, and then the sample's barcode should be scanned and placed in the equipment. The results obtained in the initial and buffy coat hemogram are essential key parameters regarding sample eligibility, as their values are important during UCB processing. All the data obtained are safeguarded by quality controls carried out at the beginning of each working day.

9.3. Flow Cytometry

Flow cytometry is a process that allows the measurement of multiple parameters in a heterogeneous population acquiring thousands of cells per second, one by one. The principle of this methodology is based on the emission of a beam of light on cells suspended in a flowing liquid medium. Therefore, the performance depends on the scattering characteristics of the light on the cells and the emitted fluorescence, which can derive from monoclonal or polyclonal antibodies conjugated to a fluorochrome directed to surface or intercellular molecules. The light reflectance is thus in accordance with the structural and morphological properties of the cells. At the same time, the fluorescence is based proportionally on the amount of fluorescent probe bound to the cell or cellular component under analysis (46,47).

As a cell passes through the laser, the light will scatter in all directions. The amount of light that scatters frontally, detected by the Forward Scatter (FSC), is proportional to the size of the cell. Laterally scattered light, on the other hand, is picked up through a lens system and collected by

another detector, Side Scatter (SSC), usually placed at 90° to the laser path. This will tell us the granularity and structural complexity within the cell (46).

Thus, through this technique and monoclonal antibodies, it is possible to carry out a count of the SC present in the UCB and their viability. CD34+, CD45+ antibodies and the viability marker 7-Aminoactinomycin D (7AAD) are used for the analysis of each one of the samples.

The CD34+ marker is used since the HSC express only this surface protein. Through cytometry and the fluorescence emitted by the conjugated fluorochrome upon binding to the complementary molecule presented on the cell surface, the number of cells expressing CD34+ is calculated. In order to mark all the progenitor cells, the CD45+ marker is also used, which is a typical marker for these cells. To determine cell viability, the nuclear dye 7AAD is used, since it is a membrane-impermeant fluorescent DNA binding dye that allows live/dead cells discrimination.

Internal quality controls are performed daily to ensure accurate measurements, verify proper performance, and minimise, detect, and correct errors. For this, samples are used to simulate actual samples. The expected results are known and analysed at pre-defined intervals in this case. The comparison between the result obtained and the expected result provides the necessary information to ensure that it produces reliable results. Additionally, a compensation is also performed to correct any kind of spectral overlap between fluorochromes, removing the signal of any given fluorochrome from all detectors except the one used to measuring that dye.

9.4. Umbilical Cord Blood Stability Programme

Another internal control performed in the laboratory is the thawing process of UCB fractions of some donated samples that also went through cryopreservation. These samples are cryopreserved to verify that the complete isolation and cryopreservation protocol was performed correctly and did not affect the number of cells and viability. Thus, these samples will serve as a certification control.

In addition, colony forming units (CFU) testing is performed. This test is intended to confirm that the count of live HSC present in the sample can exert their therapeutic effect and have not been affected by the processing or thawing. Thus, cell culture is performed through a fraction

of the cryopreserved UCB, and it is confirmed that they can form colonies. If no colonies are found, the possible reasons for this are investigated.

9.4.1. Colony Forming Unit

The CFU was performed on three samples in March.

First, the samples were thawed. These were recovered from the nitrogen tanks and thawed in a 37°C water bath. After thawing, they were slowly transferred to a 50ml tube, and the cells were washed with Iscove's Modified Dulbecco's Medium (IMDM) with 2% FBS and centrifuged. Next, the supernatant was removed, and the two previous steps were repeated.

Finally, a sample was taken from the pellet into a crystal tube to perform flow cytometry. From the flow cytometry, the TNC, amount and viability, was determined. With these parameters, it was possible to calculate the sample volume for concentrations 1x 10^5 and 2x10^5.

$$\text{Volume of Cell Stock (mL)} = \frac{1 \times Concentration\ (1\text{x}\ 10^5 \text{ or } 2\text{x}10^5)}{TNC \times \%Viability}$$

The sample volume should be 1 ml. If not, it has to be filled with IMDM. After that, part of the sample was placed in a MethoCult™ tube. MethoCult™ is a semi-solid methylcellulose-based media. It promotes optimal differentiation and growth of hematopoietic progenitor cells in culture.

Four 35 mm Petri dishes were made from each sample, i.e. two of each concentration. Each sample had two 100 mm Petri dishes covered up. Inside was one of each concentration Petri dish and a third 35 mm Petri dish containing sterile water with the lid removed to ensure adequate humidity. The cultures were incubated for 14 days at 37°C, 5% CO_2 and ≥ 95% humidity. After that, the colonies were counted (Table 2) and photographed (Figure 16).

If a UCB sample does not show all three types of colonies, it is not a satisfactory sample.

Table 2: The colonies counting in each sample at different concentrations.

		Samples					
		1		2		3	
		$1x10^5$	$2x10^5$	$1x10^5$	$2x10^5$	$1x10^5$	$2x10^5$
Colonies	CFU-GEMM	2	2	2	4	3	1
	CFU-GM	1	17	11	17	18	17
	BFU-E	6	9	2	7	16	22

As mentioned, HSC can develop and differentiate into a particular lineage, originating all the mature cells in the blood. In a UCB sample, the most often formed colonies when culture after 14 days are CFU-GEMM (Colony Forming Unit – Granulocyte, Erythroid, Macrophage, Megakaryocyte), CFU-GM (Colony Forming Unit – Granulocyte, Macrophage) and BFU-E (Burst Forming Unit – Erythroid). CFU-GEMM produces a colony containing erythroid cells and 20 or more non-erythroid cells, including granulocyte, macrophage, or megakaryocyte cells (48,49). These colonies are often large and more immature. Therefore, have a large capacity to proliferate. This colony often has erythroid cells in the centre and non-erythroid cells surrounding them. However, non-erythroid cells can be concentrated on one side of the erythroid cells (49).

CFU-GEMM is the precursor to other CFUs, which includes the CFU-GM and BFU-E. CFU-GM is a progenitor cell that produces a colony containing more than 40 granulocyte and macrophage cells. These colonies can have a compact and dense centre but can also be spread out. The colonies do not appear red or brown because they do not have erythroid. This precursor originates CFU-G and CFU-M. However, these can't be differentiated in a UCB sample (48,49).

BFU-E contains more than 200 erythroblasts, and each colony arises from a single erythroid progenitor. These red or brown colonies can appear as one distinct compact cluster or with multiple clusters, being difficult to distinguish individual cells in the centre or at the colony's edge (49).

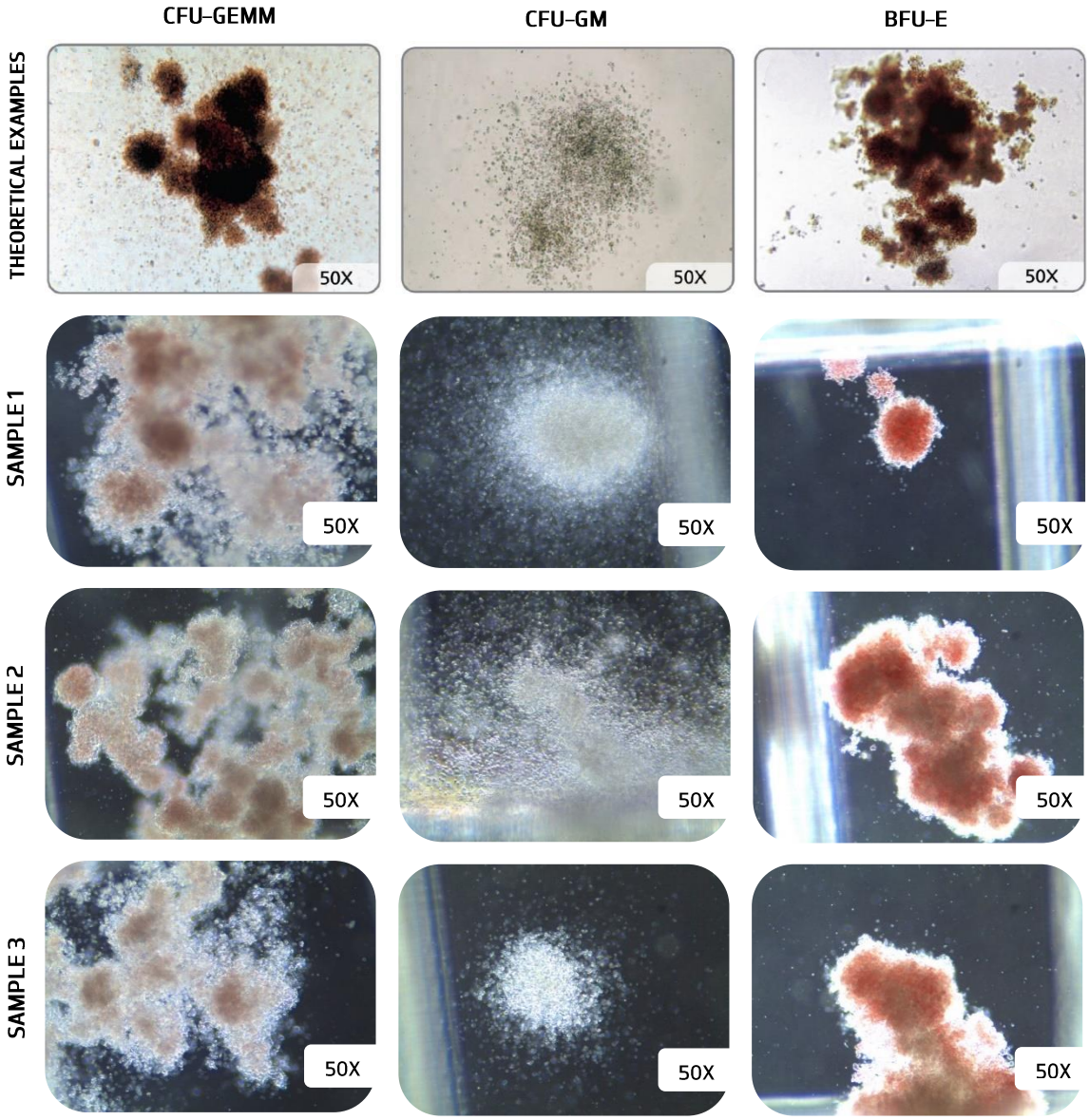

Figure 16: Scheme of theoretical images and images of three samples from the UCB prepared in MethoCult culture medium with colonies of CFU-GEMM, CFU-GM, and BFU-E. Magnification 50X.

10. External Controls

External Quality Control is a programme in which an independent external organisation sends samples for analysis to assess and compare performance between laboratories. They are performed the same way as internal quality control, and the expected results are unknown. The BebéVida laboratory uses the National External Quality Assurance Scheme (NEQAS) for quality control of haemograms and flow cytometry. NEQAS is a quality assurance programme designed to assess and monitor laboratory performance, in this case, in the fields of

haematology and flow cytometry. It provides proficiency testing and external quality assessment schemes for laboratories to evaluate the accuracy and performance of their tests. Participating laboratories receive samples from NEQAS, process them, perform the required tests and report the results to the programme for evaluation. The programme then provides feedback and evaluates the accuracy and consistency of the laboratory's results by comparing them to expected values and the results of other participating laboratories.

Another programme is the Proficiency Testing Programme to standardise hematopoietic CFU assays. This assesses the ability to perform all steps of the CFU assay and upstream events, such as thawing of cells, dilution, inoculation and plating. Participants receive a cell sample, MethoCult™ medium, additional reagents and materials, and detailed instructions for the CFU assay. The results of all participants are equally analysed and compared.

Overall, these programmes play a crucial role in quality assurance and standardisation of laboratory testing in these fields. By participating in these programmes, laboratories can identify areas for improvement, maintain high-quality testing practices and provide reliable services.

CHAPTER 3: EQUIPMENT QUALIFICATION AND VALIDATION PROCEDURE

1. Equipment Qualification Process

A qualification process is defined as a written plan describing all the process of validation, including production equipment and how validation will be conducted, which establishes scientific evidence that a process or a device can consistently deliver a quality product. This protocol should be prepared when an analytical method, process, cleaning plan, or equipment needs to be validated.

When is needed to perform an equipment substitution, it is mandatory to initiate a change control process. This is an essential part of any quality assurance system because it demonstrates to regulatory authorities that validated systems remain under control during and after system changes. In addition, before changes are implemented, the laboratory shall document the expected outcomes and have an established plan to implement, test the change and update any existing validation documentation. If there are no equipment validation reports, then all results have no assurance of consistent outcomes. Therefore, the process of equipment qualification and validation is constituted by:

- Installation Qualification (IQ): documented process that verifies that the equipment has been delivered, installed, and configured correctly, matching the original user requirement specification (URS) design. The documentation of installation includes the details of the supplier and manufacturer, the equipment name, colour, model and serial number, damages if occur, date of installation and calibration, the location of installation and all documents about manuals and certifications, computer-controlled instrumentation, connections and communication with peripheral units, software system installation and accessibility.
- Operational Qualification (OQ): ensure that installed equipment will function perfectly according to its operational specification, with a detailed review of hardware or software startup, operation, maintenance, cleaning, and safety procedures. It also checks that the equipment meets pre-assigned performance criteria and ensures that the testing results are recorded and passed to the laboratory's database.

- Performance Qualification (PQ): ensures that the equipment consistently performs functions according to the mentioned specification, being suitable for daily/routine use and not affecting the quality and validation of results. To this end, a study should be performed under controlled conditions similar to daily sample analysis.

In this case, the BebéVida laboratory needed to change the flow cytometer, from the FACSCalibur cytometer to the FACSCanto II. The FACSCalibur cytometer is equipped with the 488nm excitation Argon laser capable of detecting the physical parameters of size and complexity, but also three distinct fluorescences of the same particle through the 530/30 (FITC), 585/42 (PE), 670LP (PercP) filters and the 633nm Diode laser which detects one fluorescence through the 661/16 filter. Although it is an adequate cytometer and continues to pass all internal and external controls, it was first manufactured in 2000. However, with the exponential technological growth, newer, better, and more efficient forms of new equipment are found. As a result, FACSCalibur was replaced by FACSCanto II. The FACSCanto II operates on a PC-based Windows platform, unlike FACSCalibur, and can analyse eight colours simultaneously, including SSC and FSC. This cytometer has the configuration to capture all measures in the three components, height (H), area (A), and width (W), which simplify the singlet cell separation. This cytometer has three lasers, blue (488nm), red (633nm) and violet (405nm) with a configuration of 4-2-2. The blue laser has four detectors: 530/30 (FITC), 585/42 (PE), 670 (PerCP, PerCP-Cy5.5), and 780/60 (PE-Cy7). The two detectors of the red laser are 660/20 (APC) and 780/60 (APC Cy7), and of the violet laser are two, 450/50 (BD Horizon V4501, Pacific Blue, DAPI) and 510/50 (BD Horizon V500, AmCyan).

To implement the FACSCanto II cytometer, the equipment had to be qualified. Therefore, in the performance qualification, a comparison study was performed between the FACSCalibur and FACSCanto II.

2. Installation Qualification

The new cytometer and the local at which it was to be placed were selected in this process. After meeting all the conditions, the equipment was delivered, installed, and configured correctly. No complications were reported.

3. Operational Qualification

In this qualification, everything necessary was installed according to its operational specifications. A series of analyses, operation, maintenance and safety procedures were conducted on the FACSCanto II cytometer by a technician from the commercial company.

All the technicians were also trained by the specialised trainer, being qualified to handle the new equipment.

4. Performance Qualification

In this qualification phase was performed a comparison study between the FACSCalibur results and FACSCanto II results and the different scenarios of the cytometer validation are agreed upon with the commercial company.

In the study was used a total of 11 samples, including a low and high controls. The parameters analysed were the absolute count of viable CD34+ and CD45+ cells, the percentage of viable CD34+ cells within viable CD45+ cells, and the percentage of viable CD34+ and CD45+ cells.

4.1. Material and Methods

4.1.1. Standard Protocol for Flow Cytometry

Buffy coat from each client is stained with a CD45/CD34 cocktail and 7AAD in a Trucount tube. After the incubation in the dark at room temperature, a lysing solution is added to the tubes promoting the lysis of RBC.

Regarding quality controls, the staining protocol is not quite the same as for the buffy coat. The difference is that the controls are only stained with CD45/34 cocktail.

4.1.2. Acquisition of the Results on FACSCalibur and FACSCanto II Cytometers

After the two cytometers have been turned on and followed the manufacturer's standard operating procedures for each flow cytometer to be ready for use, the daily controls will be

passed on, as well as the BD Calibrite beads in the FACSCalibur and the BD FACS™ 7-color setup beads in the FACSCanto II. After having successfully passed the controls and compensations, in the worklist, is put the identification number of each sample and the Trucount tubes are placed in that same order in the carousel, as well as the High and Low controls.

Once all the samples have been acquired, each client is analysed graphically for each parameter with the appropriate software of each cytometer.

4.1.3. Statistical Analysis

For the statistical treatment of the data obtained, it was used IBM SPSS Statistics v.28 and the programme GraphPad PRISM v.9 to perform the graphics. Shapiro-Wilk test was used to assess data normality. To compare the means of two independent groups, it was used a *t*-Student test. The results are presented as the average ± standard error of the mean (SEM), and the differences were considered statistically significant for *p-value* <0.05.

4.2. Results and Discussion

According to the results demonstrated in Figure 17, there was no statistical significance between the FACSCanto II cytometer and the FACSCalibur cytometer, *p-value* > 0.05. Namely, the *p-value* of the parameter CD34+ viable absolute count was *p-value* = 0.954, the CD45+ viable absolute count was *p-value* = 0.859, the viable CD34+ cells within viable CD45+ cells was *p-value* =0.711, and the viable CD34+ and CD45+ cells were *p-value* =0.101 and *p-value* =0.220, respectively.

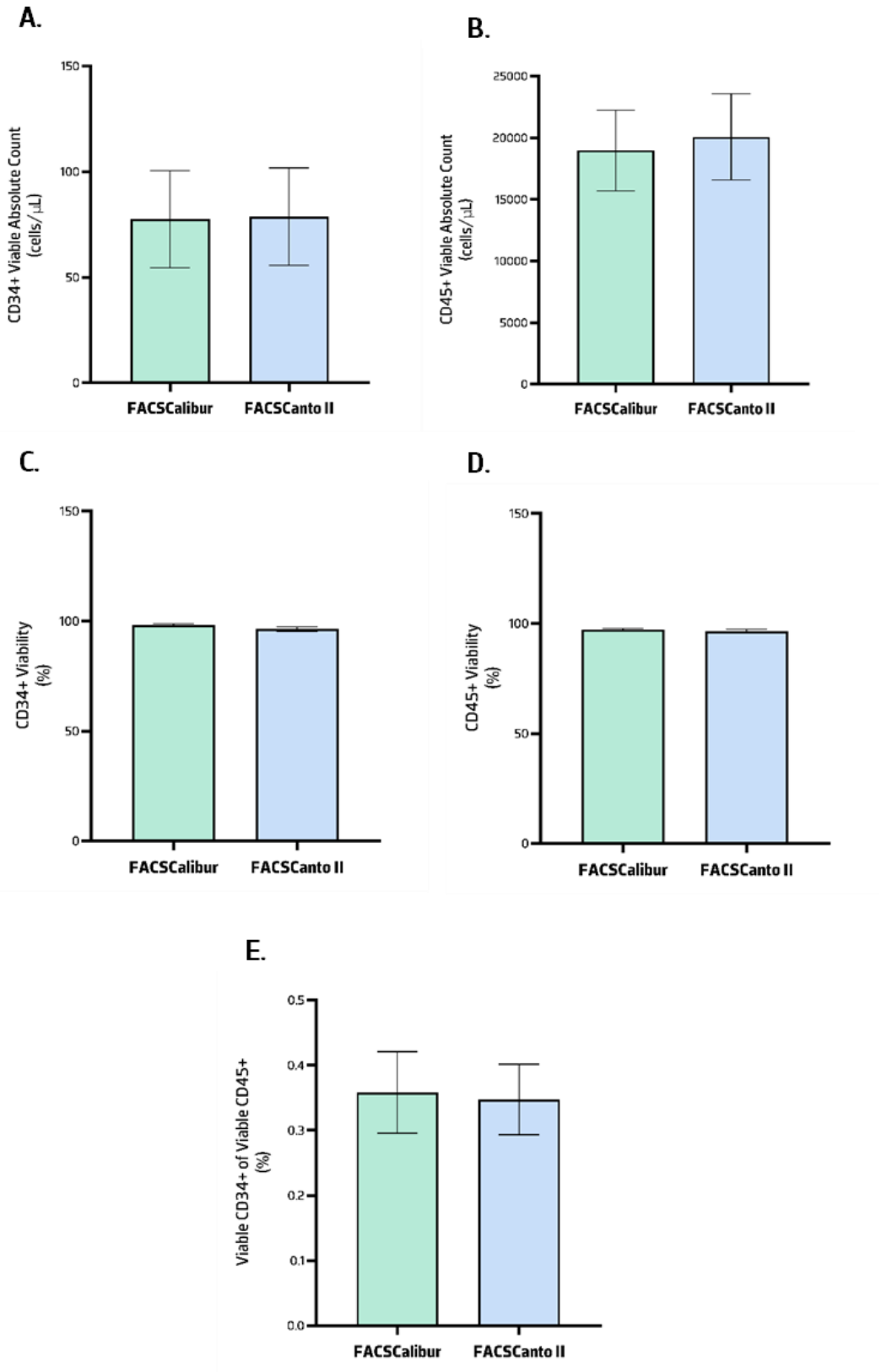

Figure 17: Absolute results of UCB acquisition in FACSCalibur and FACSCanto II.

A – Comparison between FACSCalibur and FACSCanto II CD34+ viable absolute count (n=11). **B** – Comparison between FACSCalibur and FACSCanto II CD45+ viable absolute count (n=11). **C** – Comparison between FACSCalibur and FACSCanto II CD34+ viability (n=11). **D** – Comparison between FACSCalibur and FACSCanto II CD45+ viability (n=11). **E** – Comparison between FACSCalibur and FACSCanto II viable CD34+ of viable CD45+ (n=11). Results are presented as mean ± SEM.

Furthermore, the percentage of variation between the two cytometers was also analysed, which is calculated, for each sample in each parameter, by dividing the FACSCanto II result by the FACSCalibur result. These values are in the Table 3. This variation is important to see if the results differed from one another.

The percentage of variation shows the variation of FACSCanto II regarding the 100% of FACSCalibur. In our study, the % of variation of FACSCanto II in the parameter CD34+ viable absolute count was 2.49 %, the CD45+ viable absolute count was 4.58 %, the viable CD34+ cells within viable CD45+ cells was 0.76%, and the viable CD34+ and CD45+ cells was 1.93% and 0.82%, respectively.

No statistically significant changes were found in the results obtained in the study between the cytometers. This means that the results of the parameters analysed did not differ from one cytometer to another. Also, there was no difference of more than 30% variation concerning the FACSCalibur. Therefore, the equipment consistently performs functions according to the mentioned specification.

Table 3: Absolute values from the comparison study between the FACSCalibur and FACSCanto II cytometer.

Sample ID	Cytometer	CD34+ Viable Abs Cnt (cells/µL)	%Variation CD34+ Viable Abs Cnt	CD45+ Viable Abs Cnt (cells/µL)	%Variation CD45+ Viable Abs Cnt	Viable CD34+ of Viable CD45+ (%)	%Variation Viable CD34+ of Viable CD45+	CD34 Viability (%)	%Variation CD34 Viability	CD45 Viability (%)	%Variation CD45 Viability
1	FACSCalibur	220.61		36641.45		0.60		98.91		97.36	
	FACSCanto II	235.24	106.63%	40001.13	109.17%	0.59	98.33%	99.11	100.20%	96.71	99.33%
2	FACSCalibur	212.58		24955.96		0.85		98.93		98.26	
	FACSCanto II	202.12	95.08%	27154.53	108.81%	0.74	87.06%	99.29	100.36%	96.88	98.60%
3	FACSCalibur	36.76		12588.68		0.29		98.67		93.65	
	FACSCanto II	48.20	131.12%	14006.87	111.27%	0.34	117.24%	92.17	93.41%	90.01	96.11%
4	FACSCalibur	136.69		33571.76		0.41		99.68		98.60	
	FACSCanto II	130.50	95.47%	34478.48	102.70%	0.38	92.68%	99.30	99.62%	98.21	99.60%
5	FACSCalibur	35.29		19297.75		0.18		95.83		98.69	
	FACSCanto II	36.82	104.34%	20361.58	105.51%	0.18	100.00%	100.00	104.35%	98.56	99.87%
6	FACSCalibur	58.74		28861.83		0.20		100.00		97.28	
	FACSCanto II	63.49	108.09%	29485.20	102.16%	0.22	110.00%	95.86	95.86%	96.47	99.16%
7	FACSCalibur	38.90		12787.40		0.27		98.54		98.57	
	FACSCanto II	40.97	105.32%	14824.15	115.93%	0.28	103.70%	97.65	99.10%	98.62	100.05%
8	FACSCalibur	53.70		19139.34		0.28		99.07		98.45	
	FACSCanto II	51.45	95.81%	19290.80	100.79%	0.27	96.43%	98.04	98.96%	97.18	98.71%
9	FACSCalibur	27.73		9647.00		0.29		98.21		96.50	
	FACSCanto II	27.89	100.58%	11080.65	114.86%	0.25	86.21%	93.56	95.27%	95.55	99.01%
10 QC LOW	FACSCalibur	8.19		5604.63		0.15		94.40		96.29	
	FACSCanto II	7.33	89.50%	4755.97	84.86%	0.15	100.00%	90.58	95.95%	92.58	96.15%
11 QC HIGH	FACSCalibur	23.17		5581.70		0.42		98.29		95.80	
	FACSCanto II	22.13	95.51%	5265.17	94.33%	0.42	100.00%	94.02	95.66%	100.00	104.38%
Average			102.49%		104.58%		99.24%		98.07%		99.18%
Standard Error of the Mean (SEM)			3.21%		2.63%		2.62%		0.90%		0.63%
p-value		*0.954*		*0.859*		*0.711*		*0.101*		*0.220*	

5. Conclusion

The equipment qualification and validation process have a crucial role in ensuring the consistent delivery of quality products and maintaining control over validated systems. By following a qualification/validation process, organisations can establish scientific evidence that their devices can consistently produce reliable results. When equipment substitutions are necessary, initiating a change control process becomes mandatory to demonstrate to regulatory authorities that validated systems remain under control. Documentation of expected outcomes, implementation plans, and testing procedures are essential before implementing any changes and updating existing validation documentation. The equipment qualification and validation process verify that the equipment has been correctly delivered, installed, and configured, ensures that the functions are according to its operational specifications, and have to demonstrate consistent daily/routine use performance.

Therefore, in the study conducted between the FACSCalibur and FACSCanto II cytometers for PQ, there were no statistically significant changes between the cytometer, i.e. the results of the parameters analysed do not differ from cytometer to cytometer, it can be concluded that they were not affected. Therefore, the equipment has been shown to consistently perform functions according to the mentioned specification, being suitable for daily use and not affecting the quality and validation of the results. Thus, according to what was reported in the IQ, OP and PQ, the FACSCanto II cytometer was qualified for its intended use and was thus integrated into the laboratory routine.

CHAPTER 4: EVALUATION OF UMBILICAL CORD BLOOD PARAMETERS

This chapter aimed to understand UCB immunological parameters fluctuation regarding UCB characterisation and both manipulation and processing. For that purpose, it was evaluated cryopreserved UCB between October 2022 and March 2023. This gives a total of 910 samples of UCB. Table 4 shows the number of samples according to their ABO and Rh groups.

Table 4: Distribution of the number of samples by ABO and Rh groups.

		ABO Blood group				
		A	B	AB	O	Total
Rh group	Rh +	350	61	36	325	772
	Rh-	60	14	6	58	138
Total		410	75	42	383	**910**

The two aims evaluated in this chapter were:

1- Evaluation of the effect of AXP UCB processing methodology in buffy coat quality. This study evaluated the influence of UCB volume and the influence of the AXP processing method on nucleated cells, WBC, neutrophils, monocytes, lymphocytes, nucleated red blood cells (NRBC) and platelets. The influence of the AXP processing method was further evaluated for TNC and haematocrit. It is to see if the method used to isolate the fraction of SC affects the quality of each sample.

2- Evaluation of blood type influence in UCB immune cell profile. As it has been demonstrated that ABO and Rh blood groups can interfere with various conditions, this study was conducted to understand the influence of blood groups on the UCB immune cell profile. The parameters analysed were the nucleated cells, WBC, neutrophils, monocytes, lymphocytes, NRBC, platelets, and the percentage of the RR.

1. Effect of AXP in Umbilical Cord Blood Processing Methodology in Buffy Coat Quality

1.1. Background

UCB collection, cryopreservation, and storage are essential in regenerative medicine and medical treatments for several reasons (14,15). UCB is a rich source of HSC capable of differentiating into various blood cell types, allowing the treatment of haematological diseases. These cells show greater proliferation potential than those from adult sources, allowing sufficient cells for transplantation and therapeutic applications (5,6). Also, UCB collection is a non-invasive and ethical procedure compared to other sources of SC (12). Therefore, the collection of UCB should be considered as the time of the collection. The ideal time is immediately after the infant's birth when the blood is still circulating in the placenta and umbilical cord. Studies have shown that delaying UCB collection by just a few minutes can significantly reduce the quantity and quality of SC obtained (50).

The decision between delayed cord clamping (DCC) and collecting UCB for SC cryopreservation depends on the parents' preferences and circumstances (51,52). Therefore, late clamping of the UC can lead to benefits such as improved immune function, increased iron stores, reduced risk of anaemia and improved cardiovascular stability. On the other hand, for UCB cryopreservation the clamping cannot be more than 30-60 seconds. Otherwise, it will be collected lower volume of UCB, which can be strongly associated to an insufficient HSC number for therapy use (51,52).

Once collected, the UCB cannot be directly cryopreserved, it has to be processed to concentrate the nucleated progenitor cell fraction without significant cell loss and decreased viability (15). Each processing method has different degrees of efficiency and selectivity in isolating SC. The choice of method can influence the purity and performance of the SC isolated, as inefficient isolation methods can result in substantial cell loss during the process. The AXP system has been shown in some studies to be effective in isolating and concentrating UCB-SC, minimising cell loss and maintaining sample integrity (15,34). The quality and recovery of SC using this system may be influenced by factors such as the volume and quality of the initial cord blood sample (15,37).

Therefore, it was evaluated the influence of UCB volume in total nucleated cells recovery and the influence of AXP processing method in total nucleated cells, WBC, neutrophils, monocytes, lymphocytes, NRBC, and platelets.

1.2. Statistical Analysis

To statistical analysis, it was used the IBM SPSS Statistics v.28 and the programme GraphPad PRISM v.9 to perform the graphics. Kolmogorov-Smirnov test was used to assess the normality of the results.

Spearman correlations were performed to evaluate non-parametric correlations between the UCB volume and the different evaluated parameters. To compare the initial vs final parameters, it was used the Mann-Whitney test. The results distribution shows the data quartiles using a boxplot graphic. Differences were considered statistically significant for *p-value* <0.05.

1.3. Results and Discussion

In order to assess how the collected volume effects the quality of UCB units, it was performed a correlation between the volume and the several cell types including nucleated cells, WBC, NRBC, neutrophils, monocytes, lymphocytes, and platelets (Figure 18).

Since AXP protocol has volume limitations, the minimum UCB volume collected is 40 mL. Therefore, in this study, the samples have a volume between 40-140 mL including the anticoagulant.

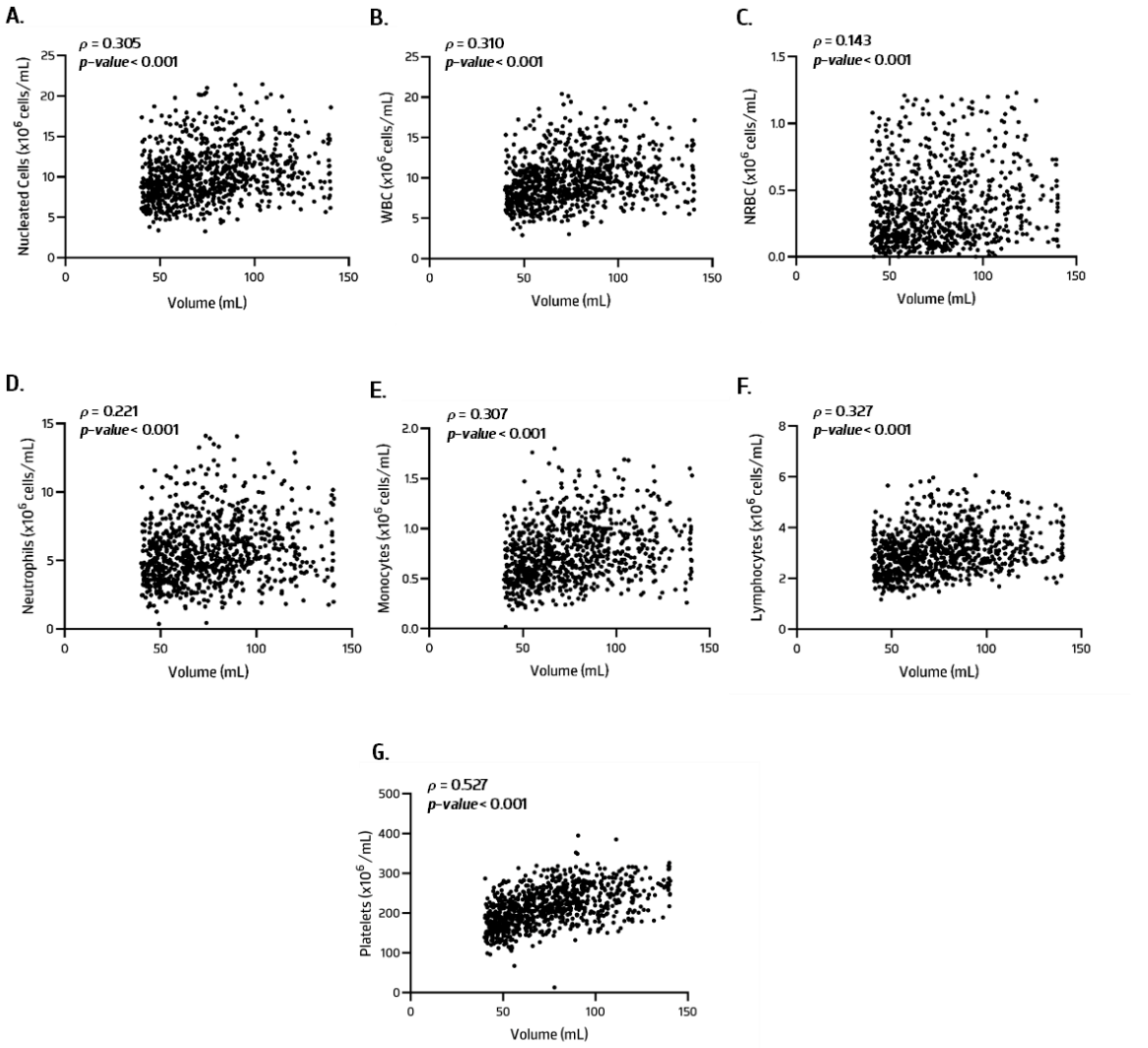

Figure 18: Evaluation of UBC volume influence in UCB immune cell types.

A – Correlation between UCB volume and initial nucleated cells (n=910). **B** – Correlation between UCB volume and initial WBC (n=910). **C** – Correlation between UCB volume and initial NRBC (n=910). **D** – Correlation between UCB volume and initial neutrophils (n=910). **E** – Correlation between UCB volume and initial monocytes (n=910). **F** – Correlation between UCB volume and initial lymphocytes (n=910). **G** – Correlation between UCB volume and initial platelets (n=910). Statistical significance was considered for *p-value* < 0.05.

All correlations between volume and the analysed parameters are positive correlations. As expected, the higher the volume, the more cells the samples will have. In this case, an increase of UCB volume leads to a 30% raise of immune system cells and nucleated cells. Platelets increase by about 50%, and NRBC 10% with the increasing of UCB volume.

In fact, the correlations between UCB volume and the analysed parameters are different. This could be explained by the size of each cell type. For instance, platelets size can explain the 50% increase when increasing the volume of UCB. These are considerably small, between 1 to 3 μm in diameter, compared to immune system cells, which can go between 10 to 20 μm depending

on the cell type. Under normal conditions, the average number of platelets varies between 150 and 450 per mL of blood (53,54). In contrast, immune system cells are only around 9 to 30 per mL of UCB (55). That is, per mL, there are more platelets than immune cells. Hence there is a higher increase in platelets as volume increases. In the case of NRBC, which only increases by 10% with increasing volume, in healthy infants, the number of NRBC in UCB is usually very low or even absent, meaning that the increase in NRBC number with increasing mL is low.

When the parents' choice is to collect UCB for cryopreservation, the blood collection should not interfere with mother and infant security, and late clamping should not compromise the quality and quantity of the sample to be collected. Nowadays, there is still no consensus in the scientific community about the ideal waiting time. In fact, when clamping is performed between 30 and 60 seconds, some studies have shown that it guarantees a safe amount of blood for the newborn and, at the same time, the collection of high-quality UCB units (56). On the contrary, when clamping is performed after 60 seconds, TNC number can be compromised, which negatively impairs the quality of the samples because the blood flow in the cord diminishes, which may lead to the blood starting to coagulate, making its collection difficult (51,52,57). Our results demonstrates that in each mL of UCB volume is lost 30% of WBC (fraction where HSC are founded). Therefore, in each mL collected a significant percentage of WBC is also collected.

The volume of blood collected should always be considered because, in addition to the fact that the more blood collected, the higher the number of cells, there is also a maximum and minimum value, depending on the volume reduction method, for which sample quality is guaranteed. Therefore, more training should be carried out in hospitals on UCB collection to raise awareness that optimised collection increases the therapeutic potential.

To assess whether the quality of each unit of UCB had been influenced by the characteristics inherent to the processing of the reduction of the sample volume by the automatic AXP method, it was compared the initial and the final values for the following parameters: nucleated cells (Figure 19A), NRBC (Figure 19B), Neutrophils (Figure 19C), Monocytes (Figure 19D), Lymphocytes (Figure 19E), Platelets (Figure 19F), TNC (Figure 19G) and the haematocrit (Figure 19H).

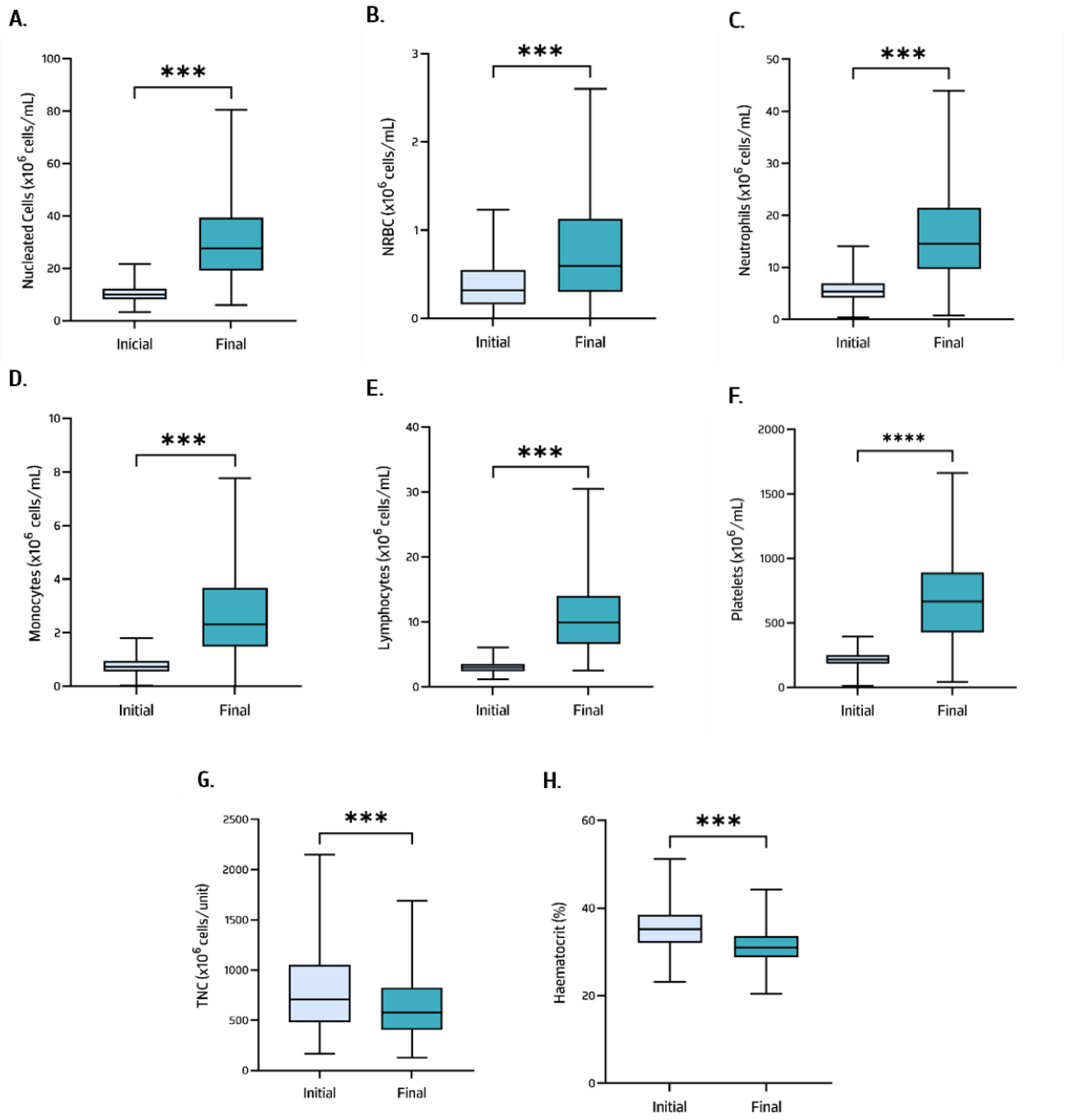

Figure 19: Evaluation of AXP protocol influence in UCB immune cell types.

A – Comparison between initial and final nucleated cells (n=910). **B** – Comparison between initial and final NRBC (n=910). **C** – Comparison between initial and final neutrophils (n=910). **D** – Comparison between initial and final monocytes (n=910). **E** – Comparison between initial and final lymphocytes (n=910). **F** – Comparison between initial and final platelets (n=910). **G** – Comparison between initial and final TNC (n=910). **H** – Comparison between initial and final haematocrit (n=910). Statistically significant is represented by * *p-value*<0.05; ** *p-value*<0.01; *** *p-value*<0.001.

As expected, the results demonstrate that the number of nucleated cells, NRBC, neutrophils, monocytes, lymphocytes, and platelets increase after UCB processing (*p-value* <0.001). The volume reduction protocol aims to reduce the final volume and concentrate the number of nucleated cells, NRBC, neutrophils, monocytes, lymphocytes, and platelets. Therefore, the AXP method effectively performed the cellular recovery of the buffy coat present in the UCB.

Regarding TNC and the haematocrit (Figure 19G and H), there was a decrease in both parameters after UCB processing (*p-value*<0.001).

TNC result was also expected to be diminished after UCB volume reduction, since in this parameter it is evaluated the total nucleated cell number per unit. This TNC decrease can be explained because, in the sample volume reduction, some cells are lost in this process.

For haematocrit, the aim of UCB volume reduction is reduce RBC content in the final sample. Therefore, RBC should be mostly eliminated during the AXP automatic platform method, in which a decrease of RBC can be seen in Figure 19H. This is because the AXP equipment can influence the percentage of the volume occupied by the RBC and the total cell recovery. According to the literature, no method performs 100% cell recovery (58). This is justified by several factors, such as blood volume limits, AXP equipment and centrifuges maintenance and calibration, and technician performance. Lack of maintenance or high sample volume can also lead to the AXP equipment being unable to measure the different densities of the cell fractions, leading to less effective separation and a certain number of RBC being stored in the freezing bag and not being eliminated.

These results are important because they analyse the impact of reduction volume processing in cryopreserved sample quality. Therefore, the choice of UCB reduction volume method is very important because it must guarantee both type and total cell number, viability and the maximum suppression of RBC. Among the automated systems addressed, the Sepax and the AXP platforms achieve a reasonable RR and maintain similar cell viability (15,34). According to Solves P. and colleagues (34), the Sepax method is a method that presents a better performance in comparison with the AXP in terms of cell recovery and RBC separation. However, the main difference is that the Sepax system usually involves the exogenous addition of the HES sedimentation reagent, unlike the AXP platform. The HES is added to the initial sample to increase the volume and subsequently improve the separation and elimination of RBC. Although this method achieves a higher cell recovery, it has also been shown in some studies that it leads to a higher haematocrit in the final sample compared to the AXP method. This higher haematocrit is because this system performs a final compensation for a 21mL volume with the RBC instead of plasma like the AXP. As mentioned, a higher percentage of RBC in the final sample negatively influences the progenitor cells' function and, consequently, the transplant's efficacy (34).

Regarding manual processing, although it is more economical and presents good reproducibility, it requires more time and effort. Despite these disadvantages, it can be a more effective solution for cell recovery since each sample is treated individually and targeted according to its characteristics.

1.4. Conclusion

UCB, a source of HSC with multiple advantages over other existing sources, should be cryopreserved and stored until it is applied in autologous or related transplants. Thus, the collection, processing and storage of UCB must be carried out correctly and never exceed 72 hours to guarantee its integrity and therapeutic efficacy.

Regarding the collection, as has been verified, the volume correlates with the number of the different immune system cells, platelets and NRBC. It has been demonstrated that the higher the volume collected of UCB, WBC increases by about 30%. Therefore, the collection should be careful and timely so that the volume extracted ensures a good number of cells for potential future therapeutics. For this, investing in more training regarding UCB collection in hospitals is important.

After a good collection, the volume reduction methodology, which allows the isolation of HSC and the consequent concentration of progenitor cells without excessive RBC, should have a good RR. In this case, as expected, the AXP method was shown to concentrate, relative to the initial number, nucleated cells, immune system cells, platelets and NRBC. In the case of haematocrit, there is a significant reduction of RBC, demonstrating that the method performs as intended. Regarding the TNC, their total number should not change after volume reduction. However, as is known, no volume reduction method has a RR of 100%, and there are always losses. Therefore, the protocol of the AXP method should be improved for better RR.

2. Blood Group Influence in Umbilical Cord Blood Immune Cell Profile

2.1. Background

The development of the human immune system begins in the early embryonic stages, and it takes time to develop to be capable of efficient immunological response. Given the limited exposure to antigens *in utero* and the inadequate neonatal adaptive immune response, newborns rely significantly on their innate immune response to protect them against infection (59). Innate immunity, also known as genetic or natural immunity, is the immunity born with, as is written into the genes, because a healthy innate immune response depends on the genes that code for the proteins that initiate, execute, and regulate this response, and different people have variations in these genes (60). The main cells of the innate immune system are monocytes, macrophages, neutrophils, eosinophils, basophils, natural killer cells, dendritic cells, and mast cells. At birth, the number of these cells have large reference value ranges compared to adult levels. However, these values decrease after the first few weeks or months of life (61,62).

Foetal growth and development are highly dependent on the mother. During and after the gestation period, multiple molecular and cellular components of maternal origin, including maternal cytokines, antigen exposure and precursor frequency of lymphocytes and antigen-presenting cells, are transmitted across the placenta, enabling passive immunity, which enriches the foetal immune system (63). Therefore, the placenta, the interface between mother and foetus, is crucial for supporting foetal development and immunity in early life, as well as breast milk after birth (64).

ABO blood group antigens are also formed during embryonic development as the blood cells differentiation, and it is the combination of alleles inherited from the parents that determine the antigen expressed on blood cells (65). ABO antigens are specific carbohydrate structures on the surface of RBC and other types of cells and tissues. They constitute the ABO blood group system, which classifies human blood into four main groups: A, B, AB, and O (66).

The ABO antibodies are produced due to exposure to foreign ABO antigens. There are two primary types of ABO antibodies: Anti-A and Anti-B. Antibodies-A are found in the plasma or serum of individuals with blood groups B and O, which lack the A antigen. Antibodies-B are present in the plasma or serum of individuals with blood groups A and O. Individuals with blood

group AB, which possesses both A and B antigens on their RBC, do not naturally produce anti-A or anti-B antibodies since their immune system recognises both A and B antigens. In newborns, the antibodies do not start being produced until 3 to 6 months of age. Therefore, the ones in the serum under four months of age are passively transferred from the mother (67,68).

The gene responsible for the human ABO blood group is located on chromosome 9 and is called ABO glycosyltransferase. The ABO locus has three allelic forms: A, B, and O. It is important to note that the O allele is recessive, but the A or B allele is considered dominant over the O allele (69,70). There are other important genes, such as the *FUT1* gene, which is responsible for the production of the H antigen by encoding the fucosyltransferase enzyme, and the *FUT2* gene, also known as the secretor gene, which encodes the enzyme α(1,2)-fucosyltransferase, which plays a role in the production of the H antigen. Both genes are located on chromosome 19 (66,69,70).

Another blood typing system is the Rh blood group system, alongside the ABO system. In the Rh system, there are two main groups: Rh positive (Rh+) and Rh negative (Rh-), based just on the presence or absence of the Rh D antigen on the surface of RBC. If an individual has the Rh D antigen, they are classified as Rh+ (i.e., A+, B+, AB+, O+), while individuals lacking the Rh D antigen are classified as Rh- (i.e., A-, B-, AB-, O-), being the rarest groups. The Rh D antigen is formed in the RHD gene on chromosome 1 (69,70). However, it is important to note that the Rh system is more complex than just the D antigen. There are over 56 Rh antigens, such as C, c, E, and e, determined by the RHCE gene on chromosome 1 (66).

As mentioned, there are blood groups with a higher population frequency than others. The frequency distribution of ABO and Rh blood groups varies by area and ethnicity worldwide. For example, according to IPST, blood group A is the most common blood group in Portugal and most of Europe, while blood group O is most common in China and the United States, but blood group AB is always the least common (71,72).

Primarily known for its relevance in blood transfusions and organ transplants, the ABO blood group system has been the research subject exploring its associations with various diseases. Numerous studies have indicated potential links between ABO blood groups and cancer, cardiovascular, infectious (bacteria, viruses, and parasites), metabolic, and allergic diseases. In the case of cardiovascular disease, A, B and AB blood groups have been associated with a greater incidence of vascular disorders, including cerebral arterial ischemia, venous

thromboembolism, peripheral vascular disease, angina, and myocardial infarction, compared with group O (73,74). Cardiovascular diseases are strongly associated with metabolic diseases, such as diabetes mellitus, hypercholesterolemia and hypertension, as they are a significant risk factor (75). Nevertheless, the genetic component also has a strong influence, as is the case in the development of coronary artery disease (CAD) (76). As ABO blood groups are transmitted genetically via chromosome 9, so are the ATP-binding cassette 2 (ABCA2) genes involved in cholesterol homeostasis. Thus, genetic risk factors contribute significantly to the hereditary aggregation of CAD, and the inheritance of ABO blood groups may play a role in this context (76,77). In infectious diseases, cell surface glycoconjugates, determined by ABO blood groups, are used by parasites, bacteria and viruses as receptors for binding, affecting interactions between host and pathogen (78). Certain microbial parasites resort to molecular mimicry, using the same blood group antigens as their hosts, which helps them evade immune responses (77). For example, the membranes of gram-negative bacteria, such as *Escherichia coli*, have chemical signatures that resemble blood groups A and B antigens. In *in vitro* experiments, it has been shown that anti-B antibodies can kill *Escherichia coli*, suggesting a potential role for anti-A and anti-B antibodies in destroying gram-negative bacteria in the body (79,80). Concerning allergic diseases, a pattern has emerged indicating that individuals with blood group O are more susceptible to allergic diseases, particularly allergic rhinitis (AR) (81) and asthma (82,83), compared to the other blood group. On the other hand, individuals with non-O blood groups are more susceptible to atopic dermatitis (AD) (84). These results suggest that specific ABO blood groups may serve as risk factors for developing allergic diseases because of immunological mechanisms and glycosylation patterns of blood group antigens.

Previous studies on cancer risk have reported associations between blood group antigens and tumour development, metastasis and prognosis. These antigens participate in cell recognition, signalling and adhesion (85). Their expression varies during cell development, ageing and differentiation, particularly in the context of carcinogenesis and pathological phenomena (77). Normal antigens are lost during malignancy progression, and tumoral antigens are acquired. Therefore, the decrease in antigens is inversely proportional to the metastatic potential of the tumour (68). Sometimes, in individuals not-blood group A, the tumours may possess actual A antigens or "A-like" antigens with properties similar to A antigens. This phenomenon explains the higher incidence of cancer in individuals of blood group A compared to individuals of blood

group O since the A or "A-like" properties of tumour antigens are not recognised as foreign in individuals of blood group A (68,86)

It is important to note that just as with disease, the relationship between blood group and the immune system is complex and is influenced by many genetic, environmental, and individual factors (87). Genetic variability among ethnic groups and populations may contribute to differences in immune system responses and disease risk among blood groups (88).

In fact, blood group can interfere in several conditions and more research is required to understand how the blood group can conditionate immune system response. Therefore, it was aimed to understand the blood group influence in UCB immune cell profile.

2.2. Statistical Analysis

For statistical analysis, it was used the IBM SPSS Statistics v.28 and the programme GraphPad PRISM v.9 to perform the graphics. Kolmogorov–Smirnov test was used to assess data normality. To compare ABO groups, it was used the Kruskal–Wallis test, followed by a post-hoc test. To analyse the influence of RH factor, it was used a Mann–Whitney test. The results distribution shows the data quartiles using a boxplot graphic. Differences were considered statistically significant for $p\text{-}value < 0.05$.

2.3. Results and Discussion

In this study, there was a significant increase of nucleated cells (*p-value* = 0.006 and 0.009, respectively), WBC (*p-value* = 0.006 and 0.008, respectively), NRBC (*p-value* = 0.007 and 0.014, respectively), neutrophils (*p-value* = 0.004 and 0.003, respectively) and monocytes (*p-value* = 0.020 and 0.027, respectively) in blood group AB compared to A and in blood group O compared to AB (Figure 20). However, only statistically significant differences between blood groups A and O (*p-value* = 0.023) were observed regarding platelets. On the other hand, lymphocytes and the percentage of RR, calculated by dividing the number of final TNC with the initial TNC, did not show statistically significant differences between all groups. Contrary to cells from innate immune system, cells of the adaptive immune system (lymphocytes) did not significantly differ between the blood groups (Figure 20).

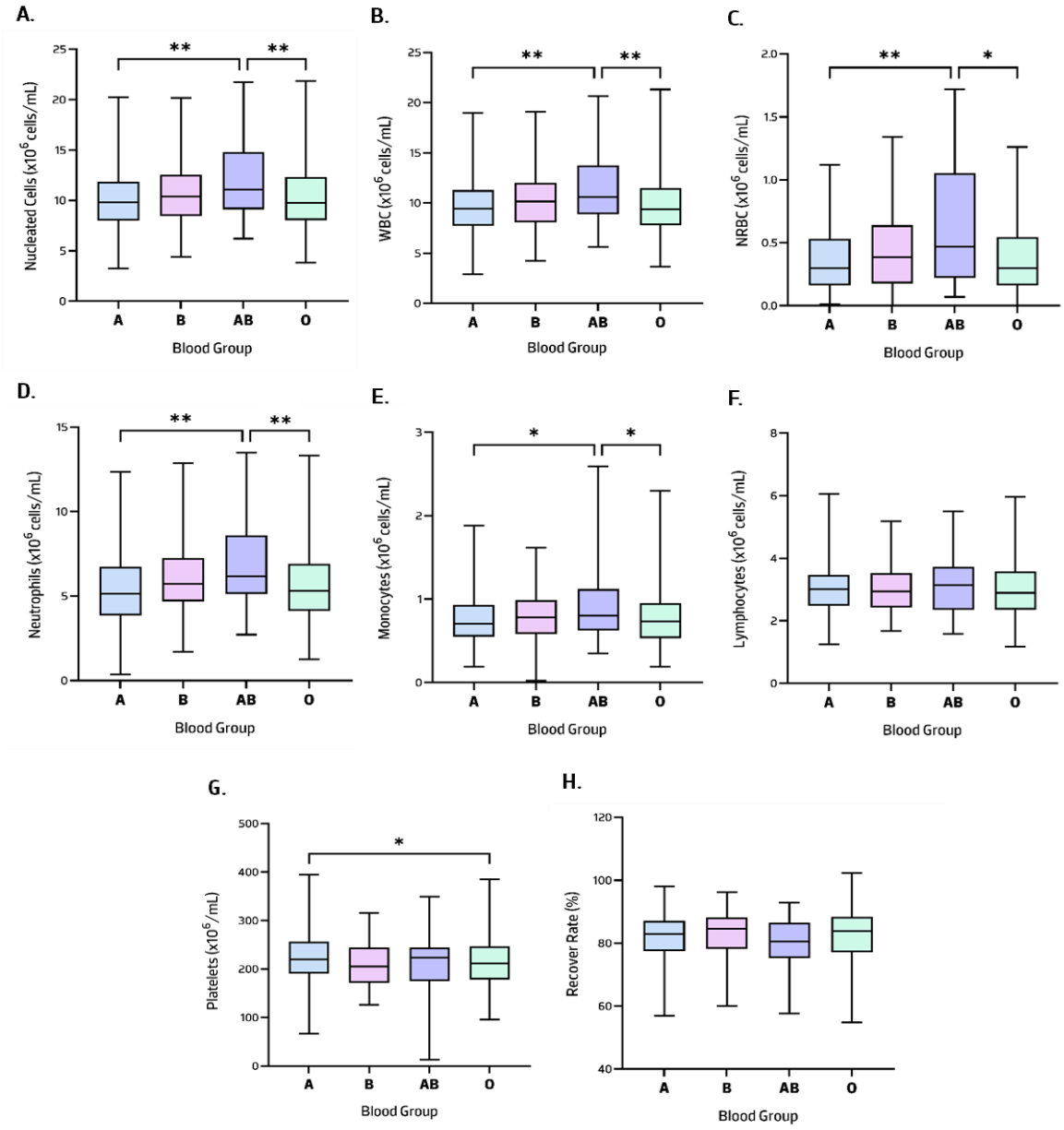

Figure 20: Evaluation of ABO blood groups influence in UCB immune cell types.

A – Comparison between ABO blood groups and nucleated cells (n=910). **B** – Comparison between ABO blood groups and WBC (n=910). **C** – Comparison between ABO blood groups and NRBC (n=910). **D** – Comparison between ABO blood groups and neutrophils (n=910). **E** – Comparison between ABO blood groups and monocytes (n=910). **F** – Comparison ABO blood groups and lymphocytes (n=910). **G** – Comparison between ABO blood groups and platelets (n=910). **H** – Comparison between ABO blood groups and the RR (n=910). Statistically significant is represented by * *p-value*<0.05; ** *p-value*<0.01; *** *p-value*<0.001.

Regarding the presence or absence of Rh factor in each ABO blood group (Figure 21, Figure 22 and Figure 23), group A- presented a significant decrease in the nucleated cells (*p-value* = 0.033), WBC (*p-value* = 0.033), neutrophils (*p-value* = 0.008) and monocytes (*p-value* = 0.034), compared to A+ group. In blood group O, there are a significant decrease between O+ and O- in the NRBC (*p-value* = 0.012). In contrast, there was a significant increase in platelets in the group AB- compared to AB+ (*p-value* = 0.015). Hence, these data point to the fact that RH+, in most cases, is linked to an increase in the number of cells.

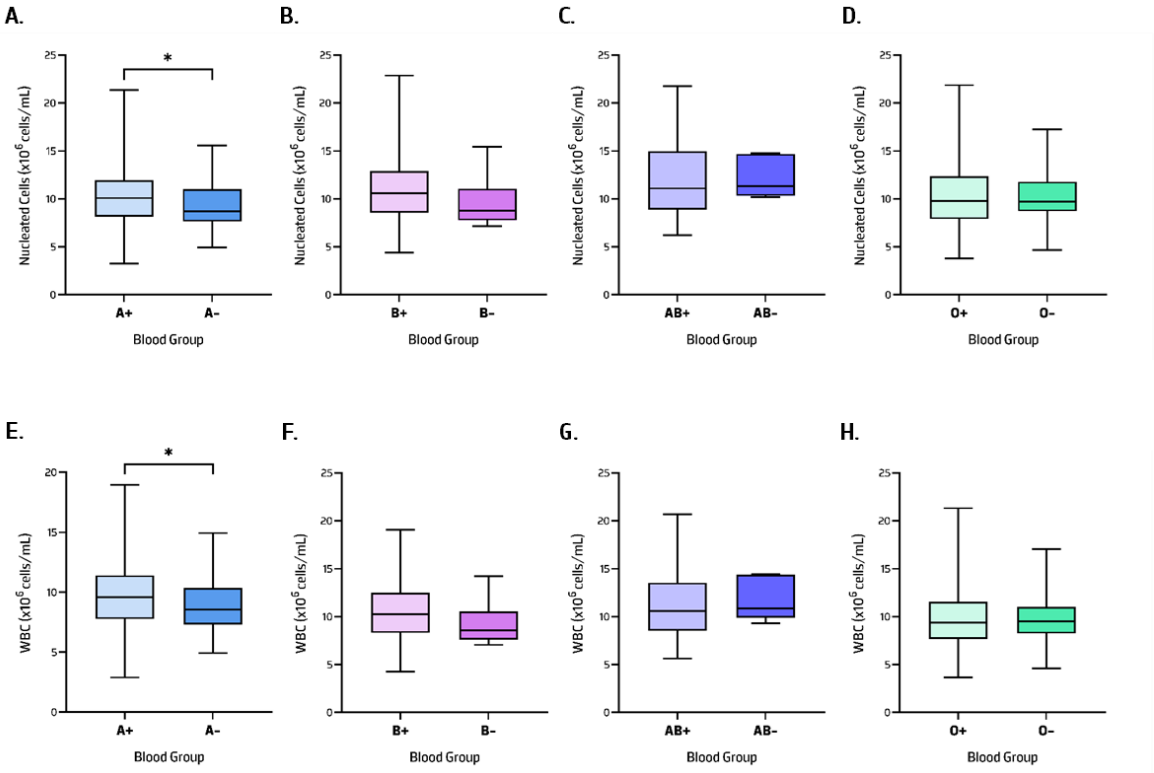

Figure 21: Evaluation of Rh factor influence in nucleated cells and WBC.

A – Comparison between A+ and A- blood group and nucleated cells (n=411). **B** - Comparison between B+ and B- blood group and nucleated cells (n=75). **C** - Comparison between AB+ and AB- blood group and nucleated cells (n=42). **D** - Comparison between O+ and O- blood group and nucleated cells (n=384). **E** – Comparison between A+ and A- blood group and WBC (n=411). **F** - Comparison between B+ and B- blood group and WBC (n=75). **G** - Comparison between AB+ and AB- blood group and WBC (n=42). **H** - Comparison between O+ and O- blood group and WBC (n=384). Statistically significant is represented by * *p-value*<0.05; ** *p-value*<0.01; *** *p-value*<0.001.

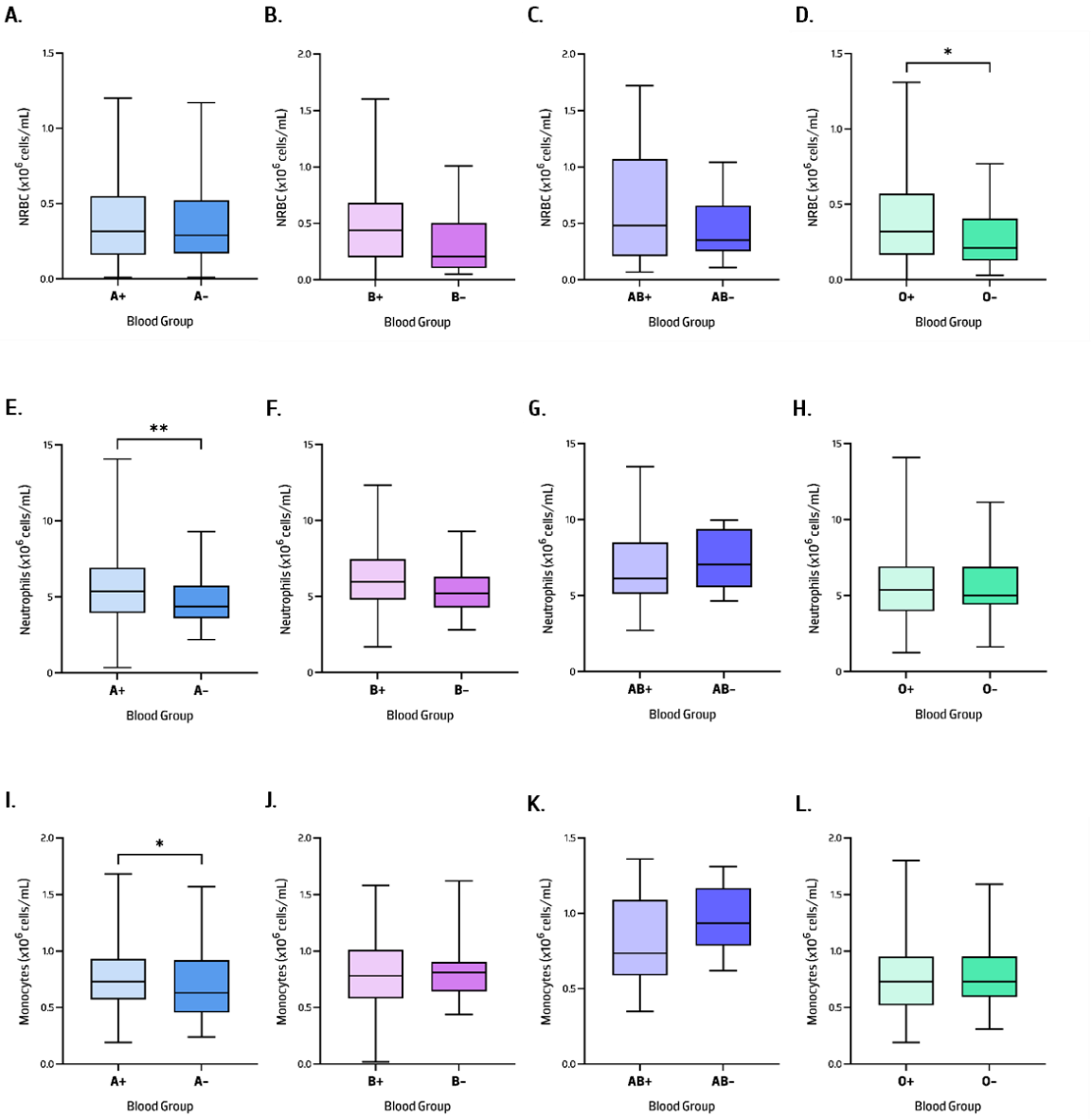

Figure 22: Evaluation of Rh factor influence in NRBC, neutrophils and monocytes.

A – Comparison between A+ and A- blood group and NRBC (n=411). **B** – Comparison between B+ and B- blood group and NRBC (n=75). **C** – Comparison between AB+ and AB- blood group and NRBC (n=42). **D** – Comparison between O+ and O- blood group and NRBC (n=384). **E** – Comparison between A+ and A- blood group and neutrophils (n=411). **F** – Comparison between B+ and B- blood group and neutrophils (n=75). **G** – Comparison between AB+ and AB- blood group and neutrophils (n=42). **H** – Comparison between O+ and O- blood group and neutrophils (n=384). **I** – Comparison between A+ and A- blood group and monocytes (n=411). **J** – Comparison between B+ and B- blood group and monocytes (n=75). **K** – Comparison between AB+ and AB- blood group and monocytes (n=42). **L** – Comparison between O+ and O- blood group and monocytes (n=384). Statistically significant is represented by * *p-value*<0.05; ** *p-value*<0.01; *** *p-value*<0.001.

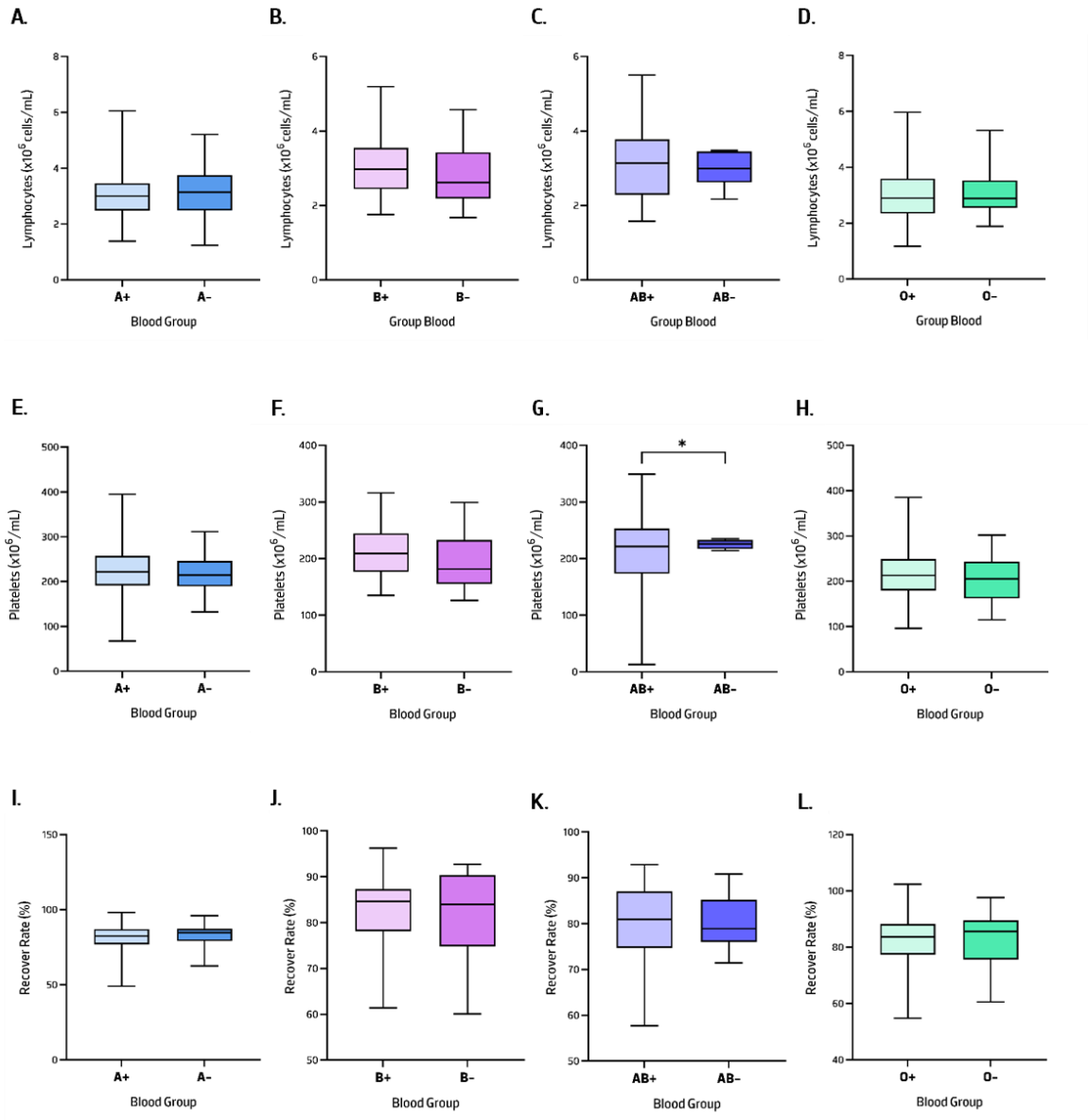

Figure 23: Evaluation of Rh factor influence in lymphocytes, platelets, and RR.

A – Comparison between A+ and A- blood group and lymphocytes (n=411). **B** – Comparison between B+ and B- blood group and lymphocytes (n=75). **C** – Comparison between AB+ and AB- blood group and lymphocytes (n=42). **D** – Comparison between O+ and O- blood group and lymphocytes (n=384). **E** – Comparison between A+ and A- blood group and platelets (n=411). **F** – Comparison between B+ and B- blood group and platelets (n=75). **G** – Comparison between AB+ and AB- blood group and platelets (n=42). **H** – Comparison between O+ and O- blood group and platelets (n=384). **I** – Comparison between A+ and A- blood group and RR (n=411). **J** – Comparison between B+ and B- blood group and RR (n=75). **K** – Comparison between AB+ and AB- blood group and RR (n=42). **L** – Comparison between O+ and O- blood group and RR (n=384). Statistically significant is represented by * *p-value*<0.05; ** *p-value*<0.01; *** *p-value*<0.001.

The differences observed in nucleated cells, WBC, NRBC, neutrophils and monocytes suggest potential associations between the ABO blood group system and immune cell populations. These results raise several points of discussion, such as immune cell composition, the innate immune response, and implications for inflammatory conditions and disease risk and prognosis.

First of all, it is important to note that genetically the alleles of the blood groups are different. The O allele is identical to A, except for deleting one nucleotide base, forming a specific mutation in the *FUT1* gene. This mutation leads to the production of a non-functional or inactive form of the α(1,2)-fucosyltransferase enzyme encoded by the gene (68). Therefore, the enzyme cannot add the fucose molecule to the terminal galactose of the precursor structure, which is required to form the H antigen. Without the H antigen, the A and B antigens cannot be added, leading to the absence of A and B antigens on the surface of RBC (62,68).

In cases of tumour progression, the loss of the A and B antigens contributes to metastasis since it negatively regulates ABO transcription, leading to a loss of transferase A or B activity and an increased accumulation of other antigens that act as ligands for selectins, facilitating the metastatic process (89,90). DNA methylation in the promoter region of the A gene is believed to inhibit transcription of the associated enzyme, resulting in the loss of the A antigen. However, different mechanisms of mRNA reduction have also been found in A tumours, specific to each tumour cell line (91). So, compared to individuals with blood groups O and AB, individuals of blood group A have a higher occurrence of cancer at various sites, including salivary glands, stomach, ovaries, uterus, cervix, and colon/rectum, which appear to involve increased activity of α(1,2)-fucosyltransferase and α(1,4)-fucosyltransferase enzymes (68,86,90).

The ABO blood group polymorphism has also been associated with susceptibility to certain infectious diseases. The presence or absence of A/B antigens and the corresponding absence or presence of anti-A/B antibodies contribute to varying defensive lines against infection (92–94). For instance, individuals of blood group O have an increased risk of cholera, noroviruses and tuberculosis infections (68,77,85). Individuals of blood group A are more susceptible to smallpox and *Pseudomonas aeruginosa* infection (68,77,85,86). Individuals of blood group B have a higher incidence of gonorrhoea, tuberculosis, *Streptococcus pneumoniae*, and salmonella infections (68,77,85,86). All these infections have in common that the interaction

with the antigens present on the surface of the RBC, depending on the blood group, leads to greater susceptibility.

Other infections to which certain blood groups have been found to be more susceptible are infections with SARS-CoV-2, malaria and bacterial meningitis. Individuals with blood groups A, B and Rh+ are more susceptible to SARS-CoV-2 infection, while individuals with blood groups O, AB and Rh- are at lower risk (95–97). The viral spike protein, which facilitates virus entry into host cells, has been found to have similar glycosylation patterns to the A and B antigens. In individuals with blood group O, anti-A and anti-B antibodies can recognise and bind to the viral spike protein's A and B antigen-like structures. This binding may interfere with viral attachment and entry into respiratory epithelial cells, limiting viral replication and reducing the severity of infection (95,96).

Blood group antigens can also influence the expression and function of inflammatory mediators and chemokines. These signalling molecules play a crucial role in the recruitment, activation, and communication of immune cells within the immune system. For example, studies have shown that individuals of blood group O have lower levels of von Willebrand factor (vWF) and factor VIII (FVIII) than other blood groups (77,86,90). vWF and FVIII, which are plasma coagulation glycoproteins, are also involved in inflammation and immune cell recruitment, with vWF playing a crucial role in platelet adhesion and aggregation (98). Studies have shown that individuals of blood group O, who do not possess the A or B antigens, tend to have lower levels of vWF and FVIII in their plasma compared to individuals with blood groups A, B or AB (99). As a result, individuals of blood group O have a lower risk of venous and arterial thromboembolism (100). However, they are at a higher risk of excessive bleeding than group A individuals. On the other hand, individuals with blood group AB have a higher risk of stroke than those with blood group O, which is associated with FVIII levels, because blood group AB is the blood group with higher levels of FVIII (101).

Our results demonstrate that blood group A present higher number of platelet than blood group O, which could be related with higher thrombotic events risk in blood group A. In fact, Vasan and colleagues also demonstrated that individuals with blood group O experiencing lower thrombotic events risk since they present lower platelet levels (100).

Blood group A presented a diminished number of WBC when compared with AB. This could be related to the increased probability of some disease or infection associated with the presence

or absence of antigens. In fact, blood group A is associated with more pathological conditions, and A antigen is also a factor in tumour progression (68,86,90). All the studies described are in adult peripheral blood, but it is interesting to see that already in UCB, it is possible to see a decrease in the number of WBC in group A. This may be why this group is more susceptible to certain diseases due to a low number, compared to the other groups, of WBC cells.

Similarly, to group A, blood group AB also has some predisposition for some conditions. However, group AB predisposition for disease is lower than group A. This may be because group AB has more immune system cells than the other blood groups (Figure 20). A more robust immune system means less predisposition to fewer infections or other conditions. It is still important to emphasise that while it is true that blood group AB carries both A and B antigens, this does not necessarily mean that individuals with blood group AB are susceptible to the common diseases of blood groups A and B (68,85). It may vary depending on the specific disease in question. As for group O, which also, like group A, has a predisposition to some diseases, it can be seen that this group already has many resistances compared to other groups, such as in SARS-CoV-2 infection (95,96) and cardiovascular diseases (73,74,76,77). Contrary to expectations, our study did not reveal significant differences between blood groups A and O. This could be explained by the fact that the samples were from UCB. The combination of genetic factors with environmental and lifestyle factors may contribute to susceptibility to the disease (102,103). As the study was carried out in UCB, there was no interaction with these factors, thus no significant difference between groups A and O. Therefore, it leads us to believe that the differences between these two blood groups may be associated more with environmental and lifestyle factors rather than the blood group itself.

Regarding NRBC, which showed a significant increase between blood groups A and AB (*p-value* = 0.007) and O and AB (*p-value* = 0.014), there is no proven relationship between the blood group and the difference between the number of cells. It is important to note that NRBC are immature RBC with a nucleus, which can be found circulating in the blood of newborns. Sometimes, to help with oxygenation, some immature RBC cells may enter the circulation, increasing the NRBC in circulation. Cases of foetal distress or hypoxia during pregnancy can cause an increase in production as the foetal BM responds to stressful conditions, which can lead to high numbers in UCB (104). Unfortunately, the lack of information in the study about possible events during pregnancy makes it impossible to verify such a situation. Other factors,

such as maternal health conditions, gestational age or specific genetic factors, may contribute to differences in NRBC and RBC counts and the immunological cells.

Regarding the comparison between Rh+ and Rh- groups, there were some parameters with a significant decrease, as was the case of groups A+ and A- in the nucleated cells (*p-value* = 0.033), WBC (*p-value* = 0.033), neutrophils (*p-value* = 0.008), monocytes (*p-value* = 0.034), and the NRBC (*p-value* = 0.012) between O+ and O-. In contrast, the platelets (*p-value* = 0.015) presented a significant increase between the AB+ group and AB-.

The Rh factor can play a role in certain conditions, such as in autoimmune haemolytic anaemia (AIHA) (105,106), where the immune system produces antibodies against the Rh antigen, leading to the destruction of Rh-positive RBC, resulting in anaemia and related symptoms, in the haemolytic disease of the newborn (HDN) (65,107), which can lead to severe complications, and in blood transfusions and organ transplantation, where Rh compatibility is crucial.

Our study suggest that the Rh factor may influence certain aspects of blood composition. However, no studies have been found implicating the Rh factor in immune system cells specifically. While the Rh factor is primarily associated with RBC and its compatibility is significant in certain medical scenarios, its direct impact on immune system cells may not be well-documented or understood.

2.4. Conclusion

In conclusion, these results demonstrated the variations in immune system cells number along the different blood groups. The presence and absence of A and B antigens on the surface of the cells may contribute to this difference, although more research is needed to confirm this hypothesis. Nevertheless, since the blood groups already show different patterns in the immune cells from an early age, i.e., UCB, this preliminary study is important to help understand the onset of certain diseases or predispositions.

Blood group is also often associated with other characteristics or conditions that may influence it indirectly. For example, certain blood groups may be more prevalent in specific populations or geographic regions, and these populations may exhibit different behavioural patterns, lifestyle or genetic characteristics that may contribute to the observed cell type

differences. However, incorporating blood group information into risk assessment and personalised healthcare strategies could improve patient outcomes.

Therefore, it would be interesting to consider other influential factors, such as gestational age, in future studies. It would be particularly interesting to make a temporal analysis of the number of blood cells at different ages in the peripheral blood of the same children in the study because, as it was said, the blood group increases the probability of developing certain diseases or infections and not necessarily the certainty of having them, because extrinsic factors influence and therefore it would be interesting to evaluate these potential risks as well. It is also essential to expand the sample size, ensuring uniform representation of all blood groups, to improve the generalisability of the results. Understanding the underlying mechanisms associated with ABO blood group is also an essential study because it can provide a comprehensive understanding of the relationship between blood groups and immune cell populations.

CHAPTER 5: FUTURE PERSPECTIVES

Regarding laboratory routine, it would be interesting to deepen knowledge concerning UCT processing. Regarding the volume reduction method protocol, the choice of the method is crucial because it has to guarantee a good total number of cells, viability and maximum RBC suppression. Therefore, a constant analysis of UCB processing results must be performed in order to adjust UCB reduction processing protocol. BebéVida laboratory is always concern about their techniques performance and it is always trying to improve their processes.

Regarding the influence of ABO and Rh blood groups on the immune system, this is a topic of ongoing research. Although substantial progress has been made in understanding the effects of blood groups on certain diseases, the future holds exciting perspectives for further research. Preliminary studies on UCB are essential as an early indicator, and early screening for certain diseases with a higher predisposition may be possible. Therefore, it would be interesting to continue to study the same children throughout their development so that predisposition patterns can be seen and certain diseases for which they are most predisposed can be diagnosed earlier.

Advances in genetic and epigenetic research, identifying specific genes, regulatory elements and epigenetic modifications associated with blood groups, are also important to investigate as they may elucidate the biological pathways involved and clarify the mechanisms by which blood groups influence immune responses. Equally, studies of the interactions between immune cells and blood group antigens, the characteristics of immune cells binding to these antigens, and subsequent immune responses will help understand how blood groups modulate immune function. At the same time, the influence of blood groups on disease susceptibility and severity is explored. Ultimately, this knowledge could lead the way for personalised medicine approaches and potentially discover new therapeutic targets.

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