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Ex vivo manufacture of human neutrophils: optimizing the cost of production

A thesis presented by

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Dedication

To my loved parents, Pedro Robles and Sandra Ovalle, and sister, Xcaret Robles, for giving me the motivation for pursuing this degree, for the unconditional support, encouragement, and love.

To my uncle Salvador Robles, rest in peace, for teaching me that all challenge may by faced with a smile on the face.

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Abstract

Neutrophils are the most abundant leukocyte in human bloodstream. Nevertheless when neutrophils absolute count (ANC) values are inferior to $1.5x10^6$ cells/mL, an immunocompromising medical condition known as neutropenia occurs. The aim of this research is to evaluate the impact the quantity of *ex vivo* produced neutrophils, from $CD34^+$ cells isolated from umbilical cord blood, have in the cost of goods (CoG) for stem cell factor in the production process. To set a cornerstone for the development of cell therapy to treat neutropenia. To achieve this, $CD34^+$ cells were isolated from umbilical cord blood (UCB). To obtain $CD34^+$ cells, mononuclear cells (MNCs) were isolated by Ficoll density gradient separation. Posteriorly $CD34^+$ cells were isolated either from fresh or thawed MNCs, using 1 or 2 successive MS columns during the CD34 enrichment process. Isolated $CD34^+$ cells were cultured in Stemline II medium supplemented with stem cell factor (SCF), granulocyte colony stimulating factor (G-CSF), and thrombopoietin peptide mimetic (Tpo), 100 ng/mL each one. Finally the impact of using immobilized stem cell factor in *ex vivo* neutrophil production was *in silico* evaluated. Freshly isolated $CD34^+$ cells resulted in both higher purity (94.5%) and 254,280 $CD34^+$ cells than thawed cells. The cultured cells successfully produced mature neutrophils, exhibiting a CD11b+/CD15+ phenotype, with functional ROS production capacity. Additionally freshly processed cells produced higher expansion in the final cell number, 3,264.88 ± 545.57 fold increase. Regarding the *in silico* evaluation, the concentration of immobilized SCF and the number of cells produced resulted in the most critical factors to be optimized to effectively reduce the production costs associated to *ex vivo* produce neutrophils. According to the results of this research purer $CD34^+$ cell fractions and the usage iSCF at low concentrations could considerably reduce the

CoG associated to SCF in the *ex vivo* production of neutrophils. Thus, the panorama to produce a technical and economically feasible cell therapy is clearer.

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Abbreviation List

Term	Abbreviation
Analysis of variance	ANOVA
Absolute neutrophil count	ANC
Acute myelogenous leukemia	AML
Allogenic neutrophil transfusion	ANT
Biosafety level 2	BSL2
Biotinylated SCF	bSCF
Bovine serum albumin	BSA
Cluster differentiation	CD
Common lymphoid progenitor	CLP
Common myeloid progenitor cells	CMPCs
Cost of goods	CoG
Deoxyribose nucleic acid	DNA
Dimethyl sulfoxide	DMSO
Dihydrordamine-1,2,3	DHR 1,2,3
Ethylenediamine tetra acetic acid	EDTA
Ex vivo manufactured neutrophils	EVMNs
Fc Receptor	FcR
Fetal bovine serum	FBS
Food and Drug Administration	FDA
Forward Scatter	FSC
Graft versus host disease	GVHD
Granulocyte colony-stimulating factor	G-CSF
Granulocyte macrophage colony-stimulating factor	GM-CSF
Granulocyte-macrophage progenitor cells	GMPCs
Growth factor	GF
Hanks´ balanced salt solution	HBSS
Hematopoietic stem cells	HSCs
Huma leukocyte antigen	HLA
Immobilized Stem Cell Factor	iSCF
Interleukin-3	IL-3
Interleukin-6	IL-6
Interleukin-8	IL-8
iSCF concentration	[iSCF]
Isolation Buffer	IB
Lipopolysaccharide	LPS
Lysis Buffer	LB
Macrophage antigen 1	MAC-1

Macrophage colony-stimulating factor	M-CSF
Macs Buffer	MB
Matrix metalloproteinases	MMPs
Mean fluorescence intensity	MFI
Metamyelocyte	MM
Mononuclear cells	MNCs
Multinational Association for Supportive Care in Cancer	MASCC
Myelocyte	M
Myeloperoxidase	MPO
Neutrophil	N
Neutrophil elastase	ELANE
Neutrophil extracellular traps	NETs
Neutrophil gelatinase-associated lipocalin	NGAL
Nicotinamide adenine dinucleotide phosphate	NADPH
Peripheral blood	PB
Phorbol myristate acetate	PMA
Phosphate buffered saline	PBS
Phycoerythrin	PE
Promyelocyte	PM
Propidium iodide	PI
Reactive oxygen species	ROS
Relative centrifugal force	RCF
Revolutions per minute	RPM
Side Scatter	SSC
Stem cell factor	SCF
Thrombopoietin	Tpo
Transfusion-related acute lung injury	TRALI
Trimethoprim–sulfamethoxazole	TMP-SMZ
Tumor necrosis factor α	TNF-α
Umbilical cord blood	UCB
Wiskott-Aldrich syndrome protein	WASP

1. Chapter 1: Introduction

1.1 Neutrophils and their biological relevance in human health

The immune system main function is to protect human body against pathogens. To accomplish such function, this complex system is conformed by different cell types, which recognize and neutralize pathogens in order to keep the human body healthy (IQWiG, 2006). One of the most critical cell types that comprise the immune system are neutrophils. These cells are the most abundant white blood cells in humans, comprising up to 60% of leukocytes (McCracken and Allen, 2014). In normal circumstances, healthy adults trend to present a neutrophil blood count in a range between 1.7-6.2x10^9 cells/L (Wakeman et al., 2007). Neutrophils are the first response line in healthy humans against bacterial and fungal pathogens. Additionally, they play a key role in inflammatory and wound healing processes (Sampson, 2000) due to the vast products released by neutrophils; lipids, cytokines, proteases, and reactive oxygen species (ROS).

1.1.1 Neutropoiesis *in vivo*

Neutrophils are produced from hematopoietic stem cells (HSCs) through a process called hematopoiesis. HSCs can self-renew, proliferate and exhibit multipotency. This last characteristic, defined as the capacity of stem cells to differentiate into several cell types according to their tissue of origin (Spinelli et al., 2014), confers HSCs the capacity to selectively produce two different types of lineage: lymphoid cells and myeloid cells. Lymphoid common progenitors will produce cells belonging to the immune system such as: B cells, T cells, natural killer cells, and dendritic cells. While myeloid progenitor cells differentiate into myeloid blood components: erythrocytes, platelets, macrophages, eosinophils, basophils, and neutrophils (Reya, 2003). In **Figure 1,** the HSC different lineages are illustrated, with emphasis on growth factors and lineage markers that regulate each final cellular fate.

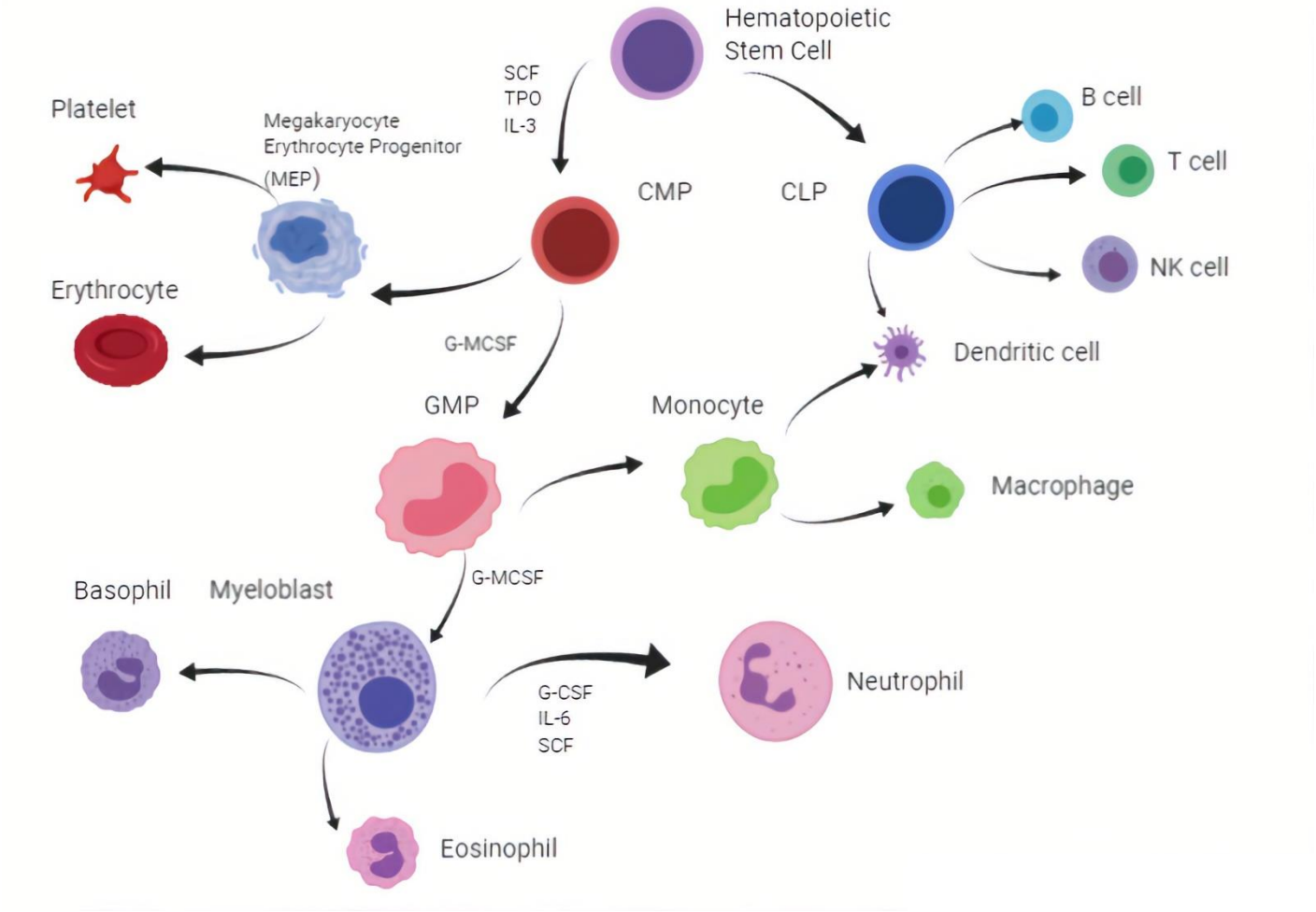


Figure 1. Hematopoietic stem cell lineage. Growth factors involved in neutropoiesis are shown. (CMP) Common myeloid progenitor; (CLP) Common lymphoid progenitor; (GMP) Granulocyte macrophage progenitor. Image created in BioRender.com.

One characteristic of HSCs is positivity (expression) of the trans membranal protein cluster of differentiation (CD) 34, which is almost exclusively expressed by blood derived cells, including HCSs. The expression of CD34 is gradually lost once HCSs starts differentiating into more specialized cell lineages. Other surface protein

markers expressed by HSCs include low expression levels of CD38, CD19, and CD90 (Carvalho et al., 2009; Seita and Weissman, 2010). The main function of HSCs in adult humans is to maintain homeostasis through the renewal of blood components. Therefore, a population of HSCs is stored within the bone marrow, where all initial differentiation takes place (Ogawa, 1993). The process in which HCSs differentiate into mature neutrophils is called neutropoiesis. Neutropoiesis process is shown in **Figure 2.** The first step in this differentiation process is the specialization of HSCs into common myeloid progenitor cells (CMPCs) (**Figure 3**). To achieve this cellular fate HCSs need to be exposed to interleukin-3 (IL-3), granulocyte macrophage colony-stimulating factor (GM-CSF), and macrophage colony-stimulating factor (M-CSF). Following this first step CMPCs need to be exposed to IL-3, stem cell factor (SCF); and thrombopoietin (Tpo) in order to be differentiated into granulocyte-macrophage progenitor cells (GMPCs). GMPCs exposure to GM-CSF will direct cellular fate into myeloblasts. Finally, in order for myeloblasts to differentiate into neutrophils, they need to be exposed to G-CSF, GM-CSF, interleukine-6 (IL-6); and SCF (Kayshansky, 2006; Robb, 2007; Miranda and Johnson, 2007; Laiosa, Stadfeld, and Graf, 2006; Ushach and Zlotnik, 2016). **Table 1** summarizes the biological role of each growth factor within neutropoiesis.

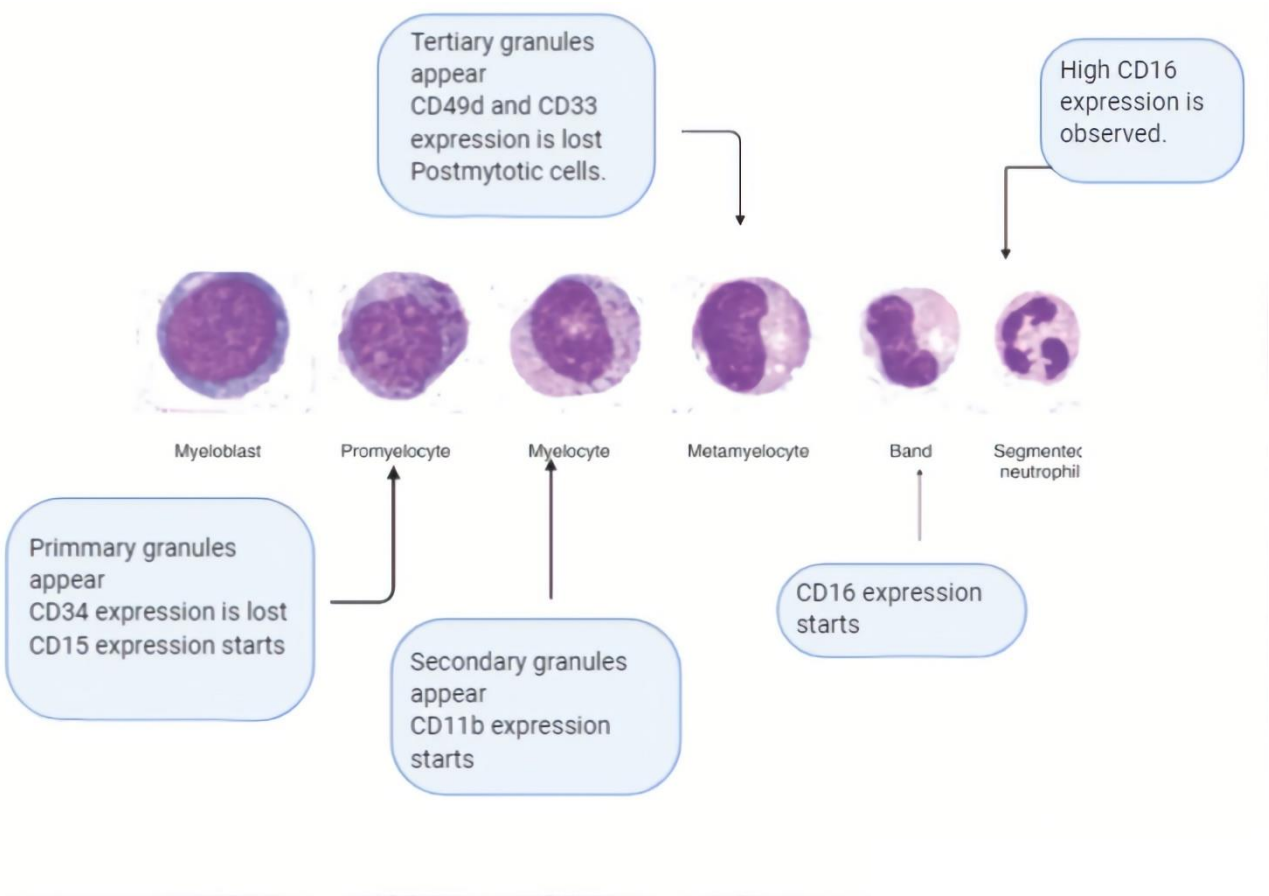


Figure 2. Neutropoiesis process. Neutrophil differentiation process from myeloblasts. Each cell stage is described in a matched box along with the characteristic traits for each cell stage. Image created in BioRender.com.

Table 1. The biological function of key GFs involved during neutropoiesis

Growth Factor	Biological Function	Reference(s)
IL-3	• Required for survival of primitive HSCs • Stimulates hematopoiesis.	Rao and Mufson, 1998. Kenneth et al., 2012
GM-CSF	• Induces myeloblast differentiation. • Promotes HSC proliferation	Gangadhar et al., 2014. Vincent et al., 2008.

	• Promotes myeloid maturation and survival	Mizoguchi and Andoh, 2013.
M-CSF[1]	• Regulates myeloid population. • Induces macrophage colony formation • Induces GMPCs differentiation	Ushach and Zlotnik, 2016.
SCF[2]	• Promotes and regulate hematopoiesis. • Influences migration of melanocytes, germ cells, hematopoietic stem cells.	Broudy, 1997.
Tpo[3]	• Promotes early in development hematopoietic lineages. • Principally regulates megakaryocytes and platelet production.	Kato et al., 1998.
GCSF[4]	• Regulates the production of neutrophilic granulocytes. • Regulates response to inflammation and other stimuli.	Demetri and Griffin, 1991.
IL-6[5]	• Increases myeloid linage production over lymphoid lineage. • Inhibits lymphopoiesis. • Increases the production of proinflammatory cytokines.	Maeda et al., 2009.

[1]Macrophage colony-stimulating factor. [2]Stem cell factor. [3]Thrombopoietin. [4]Granulocyte colony-stimulating factor. [5]Interleukin 6.

During embryogenesis, neutrophils are produced inside the spleen, thymus and liver (Lawrence et al., 2018). However in this dissertation only adult neutropoiesis *ex vivo* will be approached.

1.1.2 Neutrophil morphology

Neutrophils diameter is in the range between 9 and 15 μm, with an average of 8.85 μm (Niemiec et al., 2015; Wickramasinghe and Erber, 2011). Mature neutrophils are post-mitotic cells and do not proliferate. Neutrophil precursor cells turn into post-mitotic cells from the metamyelocyte stage. Another characteristic present among neutrophils is their polymorphous nucleus (**Figure 3-N**). Depending on the development stage, neutrophils present a band or a segmented (multilobe) nucleus. For mature neutrophils present in bloodstream, the nucleus is segmented in 3 to 5 interconnected lobes (Smith, 2010). Neutrophils are also characterized by their high granule content, between 200-300 granules per cell. For this reason neutrophils are commonly named as neutrophilic granulocytes or granular leukocytes. Granules contain antimicrobial proteins, respiratory burst components, and inflammation mediators such as lysozyme and matrix metalloproteinases (MMPS) (Faurschou and Borregaard, 2003). Once neutropoiesis reaches advanced stages, called? Promyelocyte, neutrophil completely abrogates the expression of CD34. In contrast, the expression levels of membrane marker proteins CD11b, CD14, CD15, CD66 and CD16 are increased. These proteins serve to positively purify and identify neutrophils (Lakschevitz et al., 2016). A microscopy image prepared using 20ytospin is presented in **Figure 3.**

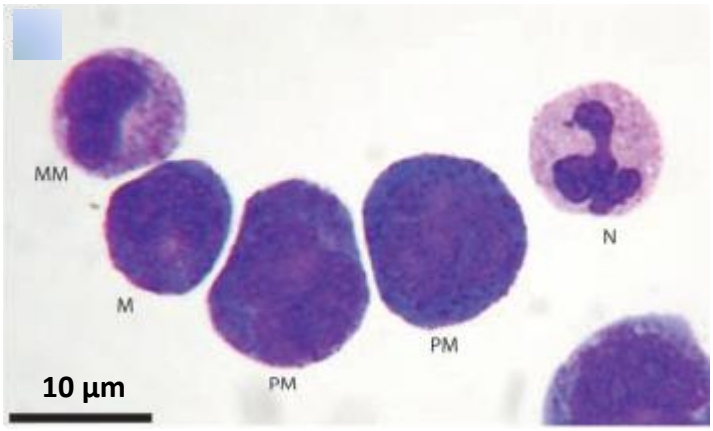


Figure 3. Leishman stained slide, where neutropoiesis process is observed. Different stages of neutropoiesis can be observed. The last stage corresponds to a mature neutrophil, where the segmented nucleus can be easily observed. MM, metamyelocyte; M, myelocyte; PM, promyelocyte; N, neutrophil. Adapted from Timmins et al., 2009.

1.1.3 Neutrophilic granules

One of the principal morphological characteristic of neutrophils is the presence of granules. Granules are specialized organelles composed by a phospholipid by-layer, which inside them proteins and oxidative burst components are contained. Neutrophilic granules may be classified as primary, secondary, or tertiary granules. Granule classification depends on their content and in the moment granules appear during neutropoiesis.

Primary granules are formed at the promyelocyte stage (Faurschou and Borregaard, 2003). The function of primary granules is closely related to intracellular killing of pathogenic microorganisms through phagocytosis. Primary granules contain proteins with antimicrobial activity such as, lysozyme, myeloperoxidase (MPO), neutrophil elastase (ELANE), defensins, and several type of proteases and

hydrolases. The principal function of primary granules is to fuse with the phagosome to aid during phagocytosis (Khanna-Gupta and Berliner, 2018).

Specific granules can be subclassified into secondary granules, formed at myelocyte stage; and tertiary granules, formed at metamyelocyte stage (Faurschou and Borregaard, 2003). Secondary and tertiary granules are more associated to chemotaxis, since both subtypes are secretory granules.

Secondary and tertiary granules, also known as specific or secretory granules, are more associated to chemotaxis. Secondary granules are formed at myelocyte stage and are characterized by containing proteins like lactoferrin, neutrophil gelatinase (MMP8), lysozyme, neutrophil gelatinase-associated lipocalin (NGAL) and iron chelators (Faurschou and Borregaard, 2003; Aktor, 2012). Finally, tertiary granules are formed at the metamyelocyte stage (Faurschou and Borregaard, 2003). Tertiary granules contain neutrophil gelatinase (MMP9) (Khanna-Gupta and Berliner, 2018). More recently a fourth type of granule has been described, and are commonly called secretory vesicles. These vesicles are exocytosed upon chemotactic stimuli, and are characterized by mainly contain plasma proteins and ficolin-1 (Rovig et al., 2009).

1.1.4 Lifespan and storage *in vivo*

One remarkable feature of neutrophils is their short lifespan, some authors have reported neutrophil *in vivo* lifespan ranging between 7 hours to 11 days (Hidalgo et al., 2019). Hence to keep neutrophil homoeostasis, neutrophils are produced in the bone marrow at a rate between $5x10^5$ to $1x10^6$ cells per day (McCracken and Allen, 2014; Liew and Kubes, 2019). Total neutrophil count in humans is divided into two subpopulations, the circulating and the marginated pool. The first one corresponds to the population of neutrophils that resides in human peripheral blood. The marginated pool corresponds to the population of neutrophils that have marginated outside of the blood and into organs. Organs that include marginated neutrophil are the liver, spleen, and the lungs. In homeostasis marginated and circulating pools are in equilibrium, about $7x10^9$ cells/kg of body weight (Hidalgo et al., 2019). An anormal

rise in the total number of neutrophils in the blood stream is known as neutrophilia (Rifkin, 2010). Depending on inflammation and stimuli, neutrophil can shift between both pools, this process is known as shift neutrophilia. Another type of neutrophilia is known as true neutrophilia. In this scenario unmatured neutrophils are released from the bone marrow into the blood torrent. Independently on the type neutrophilia occurs when the neutrophil count is above 10,000 cells/μL. Neutrophilia can be triggered by as a response to inflammation pathogenic process, stress, and medication, or as a regulatory process for neutrophil production (Rifkin, 2010; Naeim et al., 2018). Inflammatory mediators that trigger neutrophilia include interleukine-8 (IL-8), tumor necrosis factor α (TNF α), complement component C5a, granulocyte colony stimulating factor (G-CSCF), and lipopolysaccharide (LPS) associated to pathogenic bacteria (Summers et al., 2010). Neutrophils apoptosis is regulated by Cas8 and Cas9. Apoptosis in neutrophils is key to maintain homeostasis (McCracken and Allen, 2014).

1.1.5 Mechanisms for antimicrobial innate immunity

Neutrophils are recruited into infection or inflammation sites by responding to different types of stimuli such as membrane phospholipids, platelet-derived factors, products released by phagocytes and activated lymphocytes such as lymphokines, interleukine-8 (IL-8), and tumor necrosis factor (Wickramasinghe and Erber, 2011). Neutrophil arsenal against pathogens include phagocytosis, intracellular killing, production of reactive oxygen species (ROS), or producing neutrophil extracellular traps (NETs). Granules confer neutrophils a vast arsenal of proteins and cytotoxic compounds to fight infections and regulate inflammatory processes (Wright et al., 2010).

1.1.5.1 Phagocytosis and intracellular killing

Phagocytosis is defined as the process in which a cell, in this case neutrophils, engulf a particle or a pathogen for its elimination. This process include 4 principal

steps: first the recognition of the pathogenic agent, followed by the extension of the cell membrane to surround such pathogen, afterwards the cell membrane fully engulf the pathogenic agent, forming the phagosome and finally the phagosome fuses with granules, liberating granule content required for the pathogen elimination (Kessel et al., 2014).

For pathogen recognition neutrophils depend mainly in Fc receptors, which recognize immunoglobulins, and β2 integrins, for to recognize complement coated particles (Lee et al., 2003). Following particle recognition neutrophils enter into a brief latent phase prior pathogen engulfment. By phagocytosis neutrophils can engulf particles up to 10 μm of diameter (Herant et al., 2006).

Once the phagosome is formed, granule proteins are liberated inside it for bacterial killing. For instance MPO catalyzes the production of hypochlorous acid. Another example is lactoferrin which is involved in sequestering iron molecules, preventing bacterial growth. Lysozyme binds to the peptidoglycan comprising the bacterial membrane integrity (Kessel, 2014).

1.1.5.2 Extracellular killing mechanisms

Additionally to intracellular killing, neutrophils can also produce ROS and secret granule content for extracellular killing (Hellebrekers, Vrisekoop, and Koenderman, 2018); when stimulated neutrophils activate NAPH NOX2 complex in order to produce ROS. Neutrophils are capable to produce a wide range of ROS for extracellular killing, including superoxide, hydrogen peroxide, hypochlorous acid, chloramines, and organic radicals. Additionally neutrophils can produce neutrophil extracellular traps (NETs) to trap and killing pathogenic microorganisms. NETs are networks mainly composed of chromatin fibers. These fibers are released by neutrophils upon stimulation. By this strategy neutrophils kill pathogens without great release of cytotoxic agents. NETs contain more than 30 proteins with associated antimicrobial activity, such histones, ELANE, serine protease, cathelicidins, among others (Papayannopoulus, 2017). During the process of NET production and

release, cellular death of the producing neutrophil occurs, this specific cell death process is known as NETosis (Kettle and Hampton, 2016; Papayannopoulos, 2017). Although both NETosis and apoptosis are programmed cellular death fate there have several differences. For instance NETosis is not dependent of caspases and the nuclear membrane is disintegrated (Gallucci et al., 2020). Finally, neutrophils release chemokines and cytokines, such as IL-1β and TNFα, in order to recruit other immune cells to combat pathogens (Uciechowski and Rink, 2016).

1.1.6 Neutropenia

Neutropenia is a severe medical condition that occurs when blood absolute neutrophil count (ANC) is lower than 2 standard deviations of the normal mean of the population ANC. In adults, neutropenia occurs when the ANC is under 1.8 $x10^9$ cells/L. Neutropenia can be classified as mild or severe depending of the neutrophil count, between 1-1.5$x10^9$ and less than 5$x10^8$ cells/L, respectively. Neutropenia is a severe health threat since it predisposes patients to opportunistic infections??, generally febrile infections that in healthy individuals would not represent a threat. Hence, organisms normally considered as mutualistic symbionts inhabiting the skin, gut, and nasopharynx tract may turn pathogens under neutropenic conditions. If neutropenia remains untreated, it increases the risk to develop acute myelogenous leukemia (Hunter and Avalos, 2000; Williams, 2016). Depending on the cause of the neutropenia, it can be classified into three groups: congenital, ethnic, and acute or induced. Ethnic neutropenia, also known as benign congenital neutropenia, occurs when low values for ANC are lower to the reference values, without increasing the incidence of infection in patients. Variability of ANC values may vary among different ethnic groups for instance European-American women scored an ANC value 11% higher than African-American women (Grann et al., 2007). Ethnic groups that may be prone to this condition are white American children, African descent adults, west Indian descendants, and middle eastern people (Haddy, Rana, and Castro, 1999).

1.1.6.1 Congenital neutropenia

Also called chronic neutropenia, is it caused by congenital immunodeficiency disorders which disrupt neutrophil production, function, or migration rate. Severe congenital neutropenia has an incidence between 0.0005% and 0.00077% depending on the ethnic group of the sample. Congenital neutropenia is usually diagnosed before the first two years of life (U.S. National Library of Medicine, 2019). Most of the cases associate to congenital neutropenia, 40% to 55%, are related to ELANE mutations. Congenital neutropenia due to ELANE mutations can also be subdivided into two classes: permanent severe and cyclic neutropenia. Permanent severe neutropenia is characterized by the neutropoiesis process arrest at promyelocyte stage. This type of neutropenia is associated with anemia, chronic bacterial and fungal infections, and the high risk of leukemic transformation. In contrast cyclic neutropenia is more associated to stomatologic disorders and periods where the neutrophil count is normal, thus resulting in a less severe type of neutropenia (Donadieu et al., 2011).

Another gene associated to congenital neutropenia is HAX1. This mitochondrial protein with antiapoptotic activity is a key regulator of myeloid cells death. A mutation of this protein in myeloid cells will result in cells with low capacity to dissipate the mitochondrial membrane potential, thus making them more sensitive to apoptosis induced by caspases. This type of neutropenia is commonly associated to neurological conditions such as epilepsy (Klein, 2009). Although less frequent, mutations in one the following genes also lead to congenital neutropenia and other associated syndromes: glucose-6phosphate, Wiskott-Aldrich syndrome protein (WASP), growth factor independence-1, adenylate kinase-2, and G-CSF receptor (Donadieu et al., 2011; Klein, 2009). A mutation in the previously mentioned genes will provoke stage arrest in neutropoiesis.

1.1.6.2 Induced neutropenia

Induced or acute neutropenia develops when an abrupt fall in neutrophils occurs, as consequence said organism will be immunosuppressed. The most common causes

for acute neutropenia are myelotoxic chemotherapeutic drugs, such as cancer chemotherapy and immunosuppressive agents used to treat autoimmune syndromes, being these drug types the most common causes in the United States of America (Williams, 2016).

Chemotherapies are designed to target cell with high proliferation rate, as tumoral cells, nevertheless there exist healthy cells that maybe targeted by chemotherapy. Neutrophils are one of them. With a daily bone marrow generation rate of $1x10^6$ cells/ day, neutrophils are severely affected by chemotherapy. The principal chemotherapy agents associated with the development of neutropenia include paclitaxel, carboplatin, docetaxel, cyclophosphamide, prednisone, doxorubicin, vincristine, quinidine, methimazole, alkylating agents, camptothecins among other regimens specially used for myeloid malignancies (Hashiguchi et al., 2015; Crawford, Dale, and Lyman, 2004; Kuhn, 2002). These drugs induce neutropenia principally by causing DNA damage. Mechanism of action of drugs most commonly associated to neutropenia development are shown in **Table 2.** Recently rituximab has been associated to neutropenia development, although the mechanism of neutropenia induction has not been clearly stablished (Moore, 2016). Patients with chemotherapy induced neutropenia, or febrile neutropenia, are among the principal cause of deaths associated to chemotherapy usage. The principal pathogens that threat patients with this type of neutropenia are gram negative bacteria (Timmins et al., 2009; Crawford, Dale, and Lyman, 2004).

Table 2. Drugs with higher incidence of neutropenia and their respective mechanism of action.

Chemotherapy agent	Target	Cause of neutropenia	References
Paclitaxel	β-tubulin	Mitosis arrest in bone marrow cells	Fernandez et al., 2005; Rowinsky et al., 1993.
Docetaxel	β -tubulin	Mitosis arrest in bone marrow cells	Fernandez et al., 2005; Rowinsky et al., 1993.

Carboplatin	Interferes with DNA repair	Interferes with cytokine encoding genes such as IL-3, IL-6, and TNF	Cheng et al., 2017.
Doxorubicin	Disrupts topoisomerase II DNA repair mechanism	Induces ROS production in hematopoietic stem cells	Faraji et al., 2016; Shi et al., 2011
Alkylating agents	DNA damage by crosslinking and DNA fragmentation	Targets cell with high expression levels of NAPDH	Brock and Hohorst, 1967.

1.1.6.3 Current treatments of neutropenia

Initial process to mitigate the effects of neutropenia is the use of broad spectrum antibiotics combined with aminoglycosides in order to treat and prevent infections. Patient management and specific treatment for neutropenia will depend on the severity and the risk associated for each patient. Multinational Association for Supportive Care in Cancer (MASCC) has developed a risk evaluation system based in score. Patients with a score lower than 21 are considered as low risk patients, in contrast those patients with a score higher than 21 are cataloged as high risk. In most of the cases low risk patients are treated with oral antibiotics, being fluoroquinolones the first treatment options. For high risk patients intravenous type IV antibiotics are administered (Lucas et al., 2018). Prophylaxis treatment based on fluoroquinolones reduced infection related mortality by 62% when compared to patients who received placebo or no treatment for neutropenia. Additionally fluoroquinolone based treatment was compared to trimethoprim–sulfamethoxazole (TMP-SMZ) treatments. Results shown that risk of developing resistance to TMP-SMZ was higher than the risk to develop resistance to fluroquinolones. Most importantly fluroquinolones have become the first antibiotic strategy for neutropenia due to their lack of myelotoxicity. The most common fluroquinolones used to aid neutropenia management are ciprofloxacin, ofloxacin, norfloxacin, and pefloxacin

(Gafter-Gvili et al., 2005). Additionally to antibiotics, antimycotic compounds are administered to neutropenic patients to prevents and treat fungal infections, being fluconazole and itraconazole the most common compounds administered.

Prophylaxis has been proven as a good strategy to reduce infection-associated mortality in neutropenic patients. However, using antibiotics and antimycotic compounds does not increase ANC. Hence neutropenic patients must undergo other treatment options to restore their ANC to normal values. As mentioned before G-CSF regulated and promotes neutrophil production and maturation. Thus, since 1989 it stated to be used as an experimental therapy for cyclic neutropenia. Results of clinical trials from that time indicate that G-CSF increased neutrophil count from 10 to 12 fold in neutropenic patients. Additionally G-CSF administration decreased the neutropenic period from 21 to 14 days (Hammond et al., 1989). Since then the Food and Drug Administration (FDA) approved G-CSF, under the commercial name of Filgrastim, for the treatment of neutropenia and for HSCs mobilization for transplantation (Mehta, Malandra, and Corey, 2015). Currently, G-CSF administration is the most frequent treatment for neutropenia, either acute or chronical. However, some adverse effects had been associated to this therapy, for example prolonged exposure to G-CSF has been associated to risk of developing acute myelogenous leukemia (AML) (Dale et al., 2003). Nevertheless, the main drawback of using G-CSF is that this therapy does not restore neutrophil normal count until after 10-15 days from administration, period in which patients will remain immunocompromised (Pelus, 2010; Mehta et al., 2016).

A solution to close this 14 day gap of immunosuppression is allogenic neutrophil transfusion (ANT). However the use of ANT to treat neutropenia remains uncertain and controversial. In 2012, Strauss analyzed the results of clinical trials for therapeutic ANT, from seven studies analyzed, only 3 showed a superior survival rate in patients who received ANT over those who do not (Strauss, 2012). Nevertheless the correct implementation of this approach is limited by the availability of donor neutrophils (Hubel et al., 2000). Neutrophil low availability is principally

associated to two factors; the low amount of peripheral blood neutrophils that donors are capable to donate and difficulty of separating neutrophils from the rest of the cells. Another factor against the therapeutic use of ANT consists in the short life span neutrophils have once they are extracted from donor blood, maximum 8 to 10 hours depending on storage conditions. Neutrophil processing time in laboratory affects the functionality of donated neutrophils (Estcourt et al., 2016). Risks related to ANT include the immunological reactions hosts may present. Alloimmunization, graft versus host disease (GVHD), and transfusion-related acute lung injury (TRALI); are among the adverse effects related to allogenic transfusions (Marcucci et al., 2004). Donors are exposed to several health threatening factors. To donate neutrophils, a donor must be first summited to cell mobilization, which is a process where the use chemicals or growth factors cause stem cells to migrate from bone marrow to the blood. In the particular case of ANT, cell mobilization must be performed using dexamethasone and G-CSF. Both count with several side effects including increased risk of developing cataracts, severe headaches, and depression (Dale, 2016).

1.1.7 *Ex vivo* manufactured neutrophils cell therapy

During the last decade, cell therapies have merged as an alternative to treat life-threatening conditions where conventional medicine fails. Contrary to drugs and bioactive compounds, cell therapies can respond dynamically and adapt their phenotype *in vivo*. During the last decade almost 200 cell based therapies were submitted to testing in Europe. For specific information related to cell therapies, please refer to **Appendix 1**.

In the case for neutropenia, the pursuit of developing a cell therapy is no new. For instance, Dick, Prince, and Sabroe (2008), developed a protocol for *ex vivo* production of neutrophil from adult bone marrow $CD34^{+}$ cells. This protocol for producing neutrophils lasted for 17 days, in order to achieve differentiation stem cells were cultured in Stemspan medium supplemented with SCF, Tpo, IL-3, and G-CSF

at concentrations of 100 ng/mL, 10 ng/mL, 10ng/mL, and 10 ng/mL respectively. Morphology analysis of the cells at day 17 shown that 76.1% of the cells, were either segmented or band neutrophils. Results from respiratory burst and phagocytosis analysis reported the limited ability of *ex vivo* manufactured neutrophils to kill *E. coli* and *S. pneumoniae* when compared to peripheral blood neutrophils. An alternative source for $CD34^+$ cells is umbilical cord blood (UCB). This approach represents several advantages toward obtaining adult HSCs, either from bone marrow or peripheral blood. The main advantage of UCB is that represents a non-invasive quick way to obtain HSCs. Other benefits from UCB include higher tolerance for human leukocyte antigen (HLA), reduced GVHD, and reduced risk of infection transmission (Csaszar, Cohen, and Zandstra, 2013).

An attempt to produce *ex vivo* manufactured neutrophil starting from UCB $CD34^+$ was performed in 2009 by Timmins et al. In this protocol, freshly isolated $CD34^+$ cells were cultured for 15 days in StemLine II medium supplemented with 100 ng/mL of SCF, Tpo, and G-CSF. With this condition the cell expansion number achieved was 5,860. Phenotype analysis by flow cytometry of the produced cells indicate the expression for neutrophil markers CD11b and CD15. Functional assays of the manufactured neutrophils by ROS production indicate that the produced cells were capable to produce respiratory burst. These results indicate that *ex vivo* manufacture neutrophils starting from UCB $CD34^+$ are functional as PB neutrophils.

In more recent years a 4 stage culture of UCB $CD34^+$ to produce *ex vivo* manufactured neutrophils was developed and optimized (Jie et al., 2017). The principal problem of developing a commercially feasible cell therapy, based on *ex vivo* manufactured neutrophils is the high costs associated to its production. For example, the production cost of $2x10^{10}$ cells equivalent to a single therapeutic dose, following the protocol described by Timmins and collaborators (2009), could range between US$1,607 and US$7,448. The principal contributor to these elevated production costs is the acquisition of the required materials to achieve cell survival and differentiation. These materials include culture medium, supplements, and

growth factors. **Figure 4** adapted from Torres-Acosta et al., 2019, shows a breakdown of the production costs associated to *ex vivo* manufacturing neutrophils, the contribution of materials to the total cost is significantly greater than other factors.

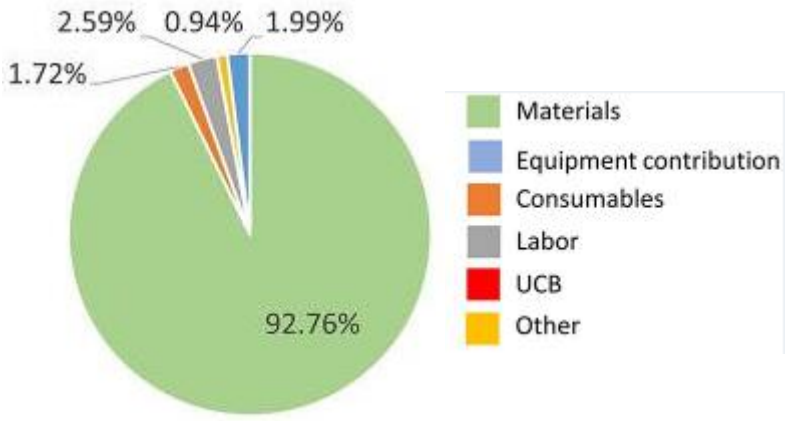


Figure 4. Production cost breakdown of ex vivo manufactured neutrophils. Adapted from Torres-Acosta et al., 2019. In this scenario a 30% discount on the materials acquisition was considered and the cost of acquiring UCB is zero, since is donated.

1.1.8 Stem cell cryopreservation, current controversy

As mentioned in the previous section, it is possible to produce *ex vivo* neutrophils by differentiating $CD34^+$ cells isolated from UCB. According to Mexican government, during 2019, 2 million 169 thousand births occurred. Under normal circumstances UCB will be discarded. This is critical since it opens the possibility to use UCB as raw material for a potential cell therapy. By obtaining UCB, isolate $CD34^+$ cells, and cryopreserved them in order to produce *ex vivo* neutrophils on demand. Nevertheless, there is controversy whether cryopreservation has a negative impact over $CD34^+$ hematopoietic stem cells.

During 2002, Hunt and collaborators performed a research with the aim of evaluating the impact cryopreservation and post-thaw recovery had over hematopoietic $CD34^+$

cells. Results from this research indicate that under certain conditions, high cooling rates and high cryoprotectant concentrations, the recovered $CD34^+$ cells may contain a non-clonogenic population, reducing the capacity of these cells to proliferate (Hunt et al., 2002).

A clinical trial performed during 2016 in Malaysia evaluated the effect that cryopreservation had on the viability of autologous transplanted $CD34^+$ cells. Results from this clinical trial suggest that cryopreservation had a negative impact on both the viability and the number of cells obtained post-cryopreservation (Zulkafli et al., 2016).

In contrast, results from a research performed by Berens and collaborators in 2016 indicate that $CD34^+$ cells are resistant to cryopreservation and post-thaw isolation. According to the results divulgated by these researchers, 93% of the $CD34^+$ cells were successfully recovery after cryopreservation (Berens et al., 2016).

1.2 Immobilization of SCF as a solution

Current neutropenia treatment approaches represent several disadvantages. The need to develop a commercially feasible cell therapy to treat this medical condition rises. Nevertheless, clinical scale *ex vivo* production involves high cost of production. The immobilization of growth factors used during neutrophil differentiation could reduce the usage of growth factors during the culture, hence high costs associated to the manufacture of neutrophils.

Worrallo et al. (2017) reported a growth factor immobilization method. In this research SCF was biotinylated trough NHS-PEG biotin, posteriorly biotinylated SCF was conjugated among streptavidin coated magnetic micro beads. The capacity of immobilized SCF (iSCF) to induce cell proliferation and cell differentiation was evaluated. For this purpose SCF dependent cell lines were cultured and the final number of cells produced was measured. Soluble 200 ng/mL of SCF served as positive control. This presentation of SCF reach a proliferation cell response of $1.5x10^6$ cell/mL. Although iSCF failed to achieve the maximum soluble SCF cell

response, an optimum concentration of 2.9 ng/mL; cell response matched 65% or $1.0x10^6$ cells/mL of the control response. These results suggest that usage of iSCF could considerably reduce the use of SCF in cultures without reducing the final number of cells produced. Additionally, immobilized growth factors used during the experiments remained bioactive after periods of time superior to 120 hours, which opens the possibility to retrieved them by the use of magnets to be reused in further cultures. For more information related to SCF immobilization techniques please refer to **Appendix 1.**

1.3 Thesis outline:

1.3.1 State of the problem and justification.

Neutropenia is a serious threat to public health, expenses related to this condition can rise up to US$19,110, per admission (Timmins et al., 2009). Neutropenic patients are prone to opportunistic pathogens, which normally would not suppose a threat to their health. In Mexico, up to 60% of cancer patients would develop acute neutropenia (Accardi et al., 2008) and 1 in 14 from those neutropenic patients would die from a neutropenic infection (Caggiano et al., 2005). Current strategies designed to treat neutropenia present several areas of opportunity. Hence an alternative is the possibility of developing a cell therapy based on producing *ex vivo* neutrophils to treat neutropenic patients. Although the main disadvantage for this approach are the elevated production costs associated to expand and differentiate hematopoietic $CD34^+$ cells into mature neutrophils.

Thus, in the present thesis is proposed to optimize the process of acquiring $CD34^+$ isolated from UCB HSCs, to stablish a small scale culture using soluble SCF to differentiate $CD34^+$ cells into neutrophils, and to perform an *in silico* analysis of the economic impact of immobilizing SCF to streptavidin coated magnetic beads. SCF was chosen over G-CSF and Tpo due to impact SCF has over the cost breakdown involving the culture medium and the supplements required to differentiate $CD34^+$ stem cells to neutrophils. Additionally state of the art literature indicates

immobilization of SCF could significantly reduce the usage of this GF without affecting in a significant way the final number of cells produced. In **Figure 5** a pie chart is presented showing the cost breakdown of preparing the culture media required for differentiation.

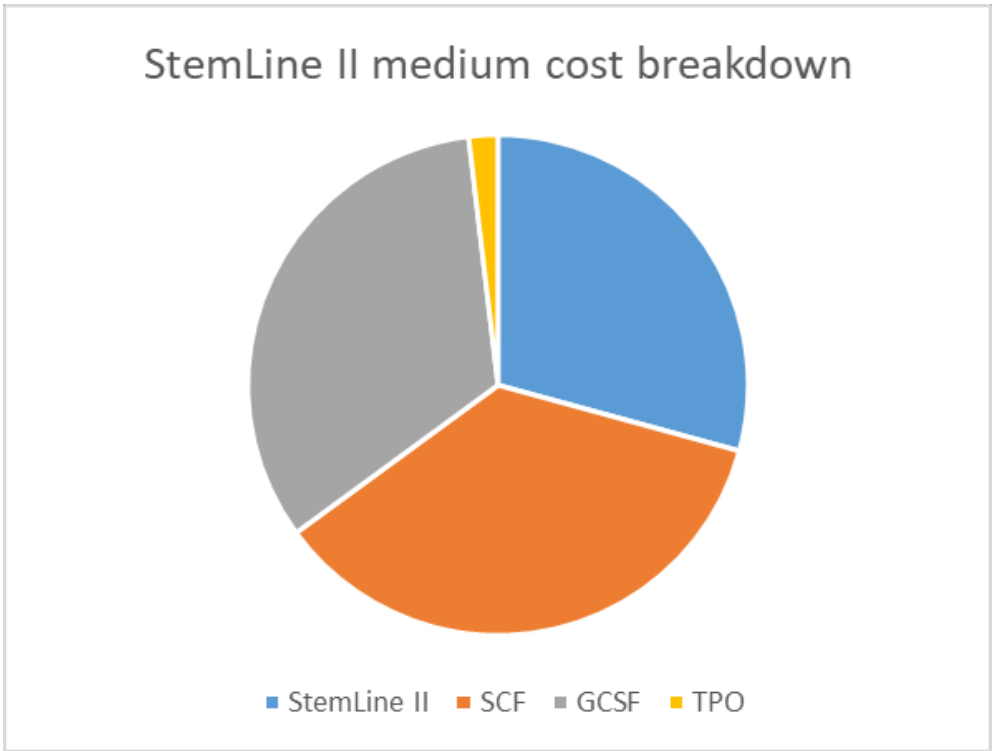


Figure 5. Supplemented Stem Line II medium cost breakdown. The pie chart is a cost breakdown of the supplemented medium used at FEMSA Biotechnology Center to produce *ex vivo* manufactured neutrophils.

1.3.2 Hypothesis

Simulated production costs associated to immobilize SCF for *ex vivo* neutrophil production will be economical and technologically feasible and according to the simulated scenarios, a cost effective alternative compared to using soluble SCF to differentiate $CD34^{+}$ cell into neutrophils.

1.3.3 General objective

To evaluate the impact of various parameters, including final cell yield and Stem Cell Factor processing costs, on an economic analysis looking at optimizing the cost of neutrophil production.

1.3.4 Specific Objectives

1. To stablish a cell bank with $CD34^+$ cells from human cord blood.
2. To identify the impact of cryopreservation on human hematopoietic CD34+ cell yield and purity.
3. To establish *ex vivo* neutrophil differentiation culture protocols from human $CD34^+$ cells, using soluble growth factors.
4. To evaluate *in silico* the impact of using immobilized stem cell factor in *ex vivo* neutrophil production.

2 Chapter 2: Materials and Methods

2.1 Umbilical cordon blood ethic protocol

A specific protocol for the use of umbilical cord blood was submitted and approved by Committees of Research and Ethics in Research from the School of Medicine of Tecnologico de Monterrey. The ethic protocol, official approval, the consent form for the use of umbilical cord blood and the consent for the use of peripheral blood are presented in **Appendix 2, 3, 4, and 5, respectively**.

2.2 Umbilical cord blood collection

UCB was obtained from full-term pregnancy births at the San Jose and Zambrano Hellion Hospitals in Monterrey, upon informed consent. All the samples were submitted to a deidentification process collecting ob/gyn, as described in the approved ethic protocol. Falcon tubes of 50 mL were heparinized using 250 units of heparin (Inhepar). UCB was obtained from umbilical cord after the baby was born. A 50 mL syringe was used by the obstetrician to obtain the blood from the cord. Cord blood samples were kept at room temperature until cell isolation process within 12 h of collection.

2.3 Isolation of $CD34^+$ cells from UCB

The process of isolating human $CD34^+$ hematopoietic stem cells is subdivided into 3 successive sections: isolation of mononuclear cells (MNCs) from UCB, magnetic labelling of $CD34^+$ cells using the MiniMACs separation kit from Miltenyi (Cat. No. 130-042-501), and the positive enrichment of $CD34^+$ cells from the rest of MNCs in the sample using the MiniMACs separation kit from Miltenyi (**Figure 6**) (Mata et al., 2019).

In order to increase both the amount of $CD34^+$ cells and the purity of retrieved sample, this process was optimized, in which two factors were evaluated. The first

one being storage status of MNCs: either thawed or fresh cells. While the second one is the number of MS columns (Miltenyi, Cat. No. 130-042-501) used for $CD34^+$ isolation: a single column or 2 successive columns. **E**xperimental design is shown in **Table 3.** The dependent variables measured were the number of cells isolated and the purity of $CD34^+$ cell population post-enrichment.

Table 3. Experimental design for optimization of $CD34^+$ cells isolation process

Storage Condition	1 MS Column	2 MS Columns
Thawed	T1	T2
Fresh	T3	T4

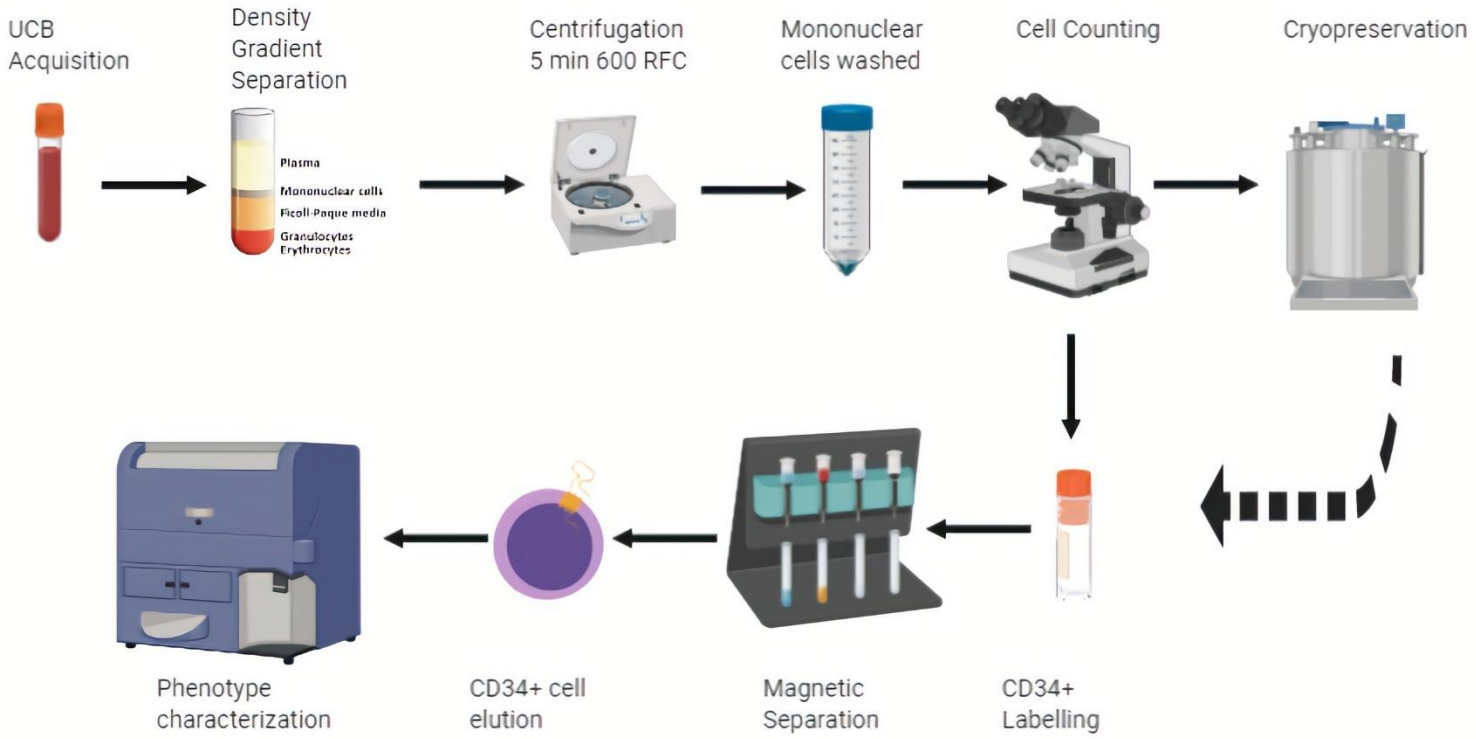


Figure 6. $CD34^+$ isolation process from UCB diagram. Image created in BioRender.com.

2.3.1 Buffers and Stains preparation

The isolation buffer (IB) was prepared using 500 mL of sterile phosphate buffered saline (PBS)(Gibco, Cat. No. 14190-144) and by adding 2mM ethylenediamine tetra acetic acid (EDTA)(Sigma, Cat. No. EDS-100g), 0.372g / 500mL of PBS. This buffer was stored in the fridge at 4°C until use.

Lysis buffer (LB), which is used to lyse remaining red blood cells post MNCs enrichment, was prepared by adding NH_4Cl (Sigma, Cat. No. A9434-500g) at a concentration of 8.02 g/100 mL, $NaHCO_3$ (Sigma, Cat. No. S5761-500g) at a concentration of 0.84 g/100 mL, and EDTA at a concentration of 0.37 g/100 mL into sterile deionized water (Gibco, Cat. No. 10977-023). This buffer was a 10x stock and stored at 4°C. 1x dilution was prepared fresh on the same day as isolation.

MACS buffer (MB), to prepare this buffer 2.5 mL of bovine serum albumin (BSA) (Roche, Cat. No. 10775835001.00) were added to 47.5 mL of IB to make a total of 50mL.

To prepare pure Leishman stain, 5 g of Leishman´s stain powder (Sigma, Cat. No. L6254) were dissolved in 2.5 L of methanol, the solution was stirred with a magnetic stirrer overnight. The solution was kept at room temperature in a sealed glass container to avoid evaporation. Wright´s stain was prepared by adding 1.5 g of Wright´s stain in 500ml of methanol. Wright´s stain was kindly donated by QBP. Rosario Salazar from Universidad Autonoma from Nuevo Leon (UANL) hematology department.

2.3.2 Mononuclear cell isolation from fresh UCB

Blood samples were processed inside a biological safety level II (BSL2) hood. First, 15 mL of room temperature IB were added per 10 mL of original blood. Gradient separation was used to isolate the mononuclear cell fraction from the diluted UCB.

To achieve this, diluted UCB was carefully underlaid using an equal volume of the original blood (for example, 5 mL for 10mL diluted blood), and Ficoll (GE Healthcare, Cat. No. 17-1440-03) with the aid of a serological pipette, two phases were created inside the tube. Once the two layers are formed inside the tube, the pipette was carefully removed from the tube without disturbing the phases. Afterwards, the tube was centrifuged for 30 min at 18°C, 450 RFC, and brake deceleration set at 1.

Following this centrifugation step, 4 layers were formed inside the tube. From top to bottom, the first layer corresponded to plasma, followed by a buffy coat containing the MNC fraction, afterwards, the Ficoll layer, and finally the pellet, which is composed of erythrocytes and granulocytes (see **Figure 7**).

The plasma supernatant was carefully discarded by vacuum and the buffy coat containing the MNCs was retrieved using a 1 mL transfer. The collected buffy coat was placed into a new 50 mL Falcon tube containing 10 mL of cold LB, and topped up to 50 mL with cold LB. Then, the solution was centrifugated for 5 mins at 600 RCF. After centrifugation, the supernatant was discarded and the pellet resuspended in 10 mL of IB. Posteriorly, the tube was topped to 50 mL using IB. This washing step was repeated two more times. After the final wash the supernatant was discarded and the pellet resuspended in 2mL LB to lyse remaining red blood cell contaminants. The remaining LB (8 mL) was added to the tube and left for incubation for a total of 7 min. Once the incubation time passed the falcon tube was topped up to 50 mL with LB, followed by a 5 min centrifugation at 300 RCF. Supernatant was discarded and the pellet resuspended in LB. This washing step was repeated 3 times in total. Before the 3rd centrifugation, volume was measured and 20 µL of cell suspension was aliquoted in order to count the collected mononuclear cells.

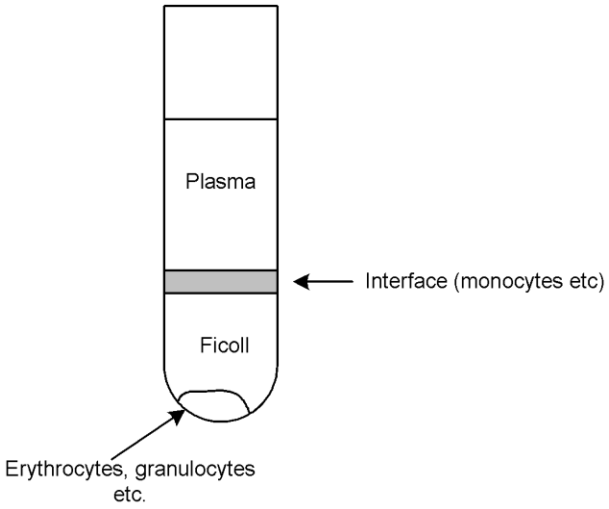


Figure 7. Layers formed after centrifugation using Ficoll gradient separation.

2.3.3 Magnetic labelling of CD34+ cells

After the isolation, the MNCs pellet was resuspended in 300 µL of MB. Afterwards 100 µL of Fc-receptor (FcR) blocking reagent (Miltenyi, Cat. No. 130-042-501), were added to the cell suspension. This suspension was incubated in the dark for 20 min at 4°C. Then, cells were labelled by adding 100 µL of anti-$CD34^+$ antibodies-coated magnetic micro-beads (Miltenyi, Cat. No. 130-046-703). This suspension was incubated in the dark for 30 min at 4°C. After, cell suspension was transferred into a new 15 mL Falcon tube and topped to 10 mL with cold MB. Washing was done by centrifuging for 5 min at 400 RCF. Finally, the supernatant was discarded and the pellet resuspended in 3 mL of MB.

2.3.4 Magnetic enrichment of CD34+ cells

This protocol was realized using MiniMACs separation kit from Miltenyi, which consist in a magnetic separator, a magnetic stand, and depending on the protocol chosen, 1 or 2 MS columns, especially designed columns for selection of cells, used to elute the magnetically labelled cells. MS columns contain a special matrix composed by ferromagnetic spheres that generates a strong magnetic field when

the column is placed in the magnetic separator. MS columns have a maximum cell capacity of $2x10^8$ cells. The first step of this process is to prepare the first MS column. Inside the BSL2 hood, the magnetic separator was attached to the magnetic stand, then, with aseptic technique the MS column was placed in the separator. A beaker is placed underneath the column to collect the unlabeled cell fraction. Once the MS column is ready a pre-separation filter (Miltenyi, Cat. No. 130-041-407) is placed on the top of the column to remove cell clumps or debris which may clog the column. Finally, the column is equilibrated by adding 0.5 mL of MB on top of the column, without touching the filter and let it run through it.

The labelled cell suspension previously prepared is applied into the MS column. After the cell suspension has passed through the column, wash it by applying 0.5 mL of MB into the column, repeating 3 times. After the 3rd wash, the column is removed from the magnetic separator and placed in a new 15 mL Falcon tube, in order to elute the $CD34^+$ cell fraction. For this step, it is critical to get the maximum possible separation between the magnetic stand and the column. To elute the cells, 1 mL of MB were added and half of this volume was allowed run through the column, then, pressure was applied using the plunger at the top of the column to push the second half of the solution trough the column. Finally the eluted fraction was centrifuged for 5 min at 400 RCF, the supernatant was discarded and the pellet was resuspended in 0.5 mL of MB.

For treatments T2 and T4 steps described in this section were performed twice. Pre-separation filter is not required before applying cells into the 2nd column. Finally, a small volume was collected, approximately 20 µL, for cell counting and according to cell concentration the required volume containing approximately 20,000 cells for flow cytometry purity check.

2.4 Cell freezing

For optimal cell freezing inactivated fetal bovine serum (FBS; Invitrogen, Cat. No. 10099141) and the required cryo-vials are pre-cooled during at 4°C 30 min previous

cell freezing. On the same way Mr. Frosty container (Sigma, Cat. No. CLS432008) was pre-cooled 30 min at -20°C. After counting, cells were centrifuged for 5 min at 400 RCF. Pellet was resuspended in 800 µL of PBS and transferred into the pre-cooled cryovial. Afterwards 100 µL of previously cooled FBS were added to the cells. Finally 100 µL of dimethyl sulfoxide (DMSO) (Sigma, Cat. No. D248) were added into the cryo-vial. Suspension inside the cryo-vial were gently mixed by pipetting. Cell containing cryo-vials were putted in Mr. Frosty container and placed at -80°C for 24 h. Finally, the cryovials were transferred to a vapor phase/liquid nitrogen tank.

2.5 Isolation of neutrophils from peripheral blood

Peripheral blood (PB) blood neutrophils were used as positive control, for phenotype validation and ROS production assay. To obtain these cells, blood samples were donated from volunteer upon signing informed consent (**Appendix 5**). PB was obtained from the median cephalic and median cubital veins using sodium citrate anti-coagulated vacutainers with volume of 4 mL (BD Biosciences, Cat. No. 363083). Approximately 12 mL of blood were collected per sampling. PB neutrophil isolation process is based on gradient separation. Inside the hood, the collected PB was into a 50 mL Falcon tube and diluted with LB in a 1:1 ratio. Then, 15 mL of Ficoll were added following the procedure detailed in **2.3.2**. Once layering was complete the tube was centrifuged for 30 mins at 18°C, 450 RCF, and brake deceleration set at 1. The layers containing plasma, buffy coat, and Ficoll were discarded, and the red pellet was resuspended in 2 mL of LB. Afterward, additional 8 mL of LB were added and the solution was incubated in the dark for 10 minutes at room temperature. Once the incubation time passed, the tube was topped to 50 mL with Hanks balanced salt solution (HBSS) (Gibco, Cat. No. 13175-103) and centrifuged for 5 min at 300 RCF. Following the centrifugation, supernatant was discarded and the pellet was resuspended in 2 mL of LB. The tube was topped to 50 mL with HBSS and centrifuged for 5 min at 300 RCF. Finally, the supernatant was discarded and the

pellet dispersed with 1 mL of HBSS. Once the pellet was resuspended, a small sample was taken to perform cell counting.

2.6 Neutrophil differentiation from human CD34$^+$ cells

To start neutrophil differentiation cultures, isolated CD34$^+$ cells are seeded at a concentration of 10^4 cells/mL in 24 wells (Corning, Cat. No. 3473) and 96 wells (Costar, Cat. No. 3370) culture plates, 1 and 200 µL per well respectively. Culture plates were maintained in a humidified incubator at 37°C and 5% CO_2 content. Culture medium used to achieve cell differentiation consisted in StemLine II medium (Sigma, Cat. No. S0192) supplemented with 100 ng/mL each of SCF (Sigma, Cat. No. S7901-10UG), G-CSF (Filatil, Lot. No. 8515190601), and TPO peptide mimetic (JPT, batch no. 090418BC10). Culture medium was also supplemented with 50 µL of 2mM of glutamine (Gibco, Cat. No. 35050-061) and 50 µL of 100X penicillin-streptomycin (Gibco, Cat. No. 15240-062). Fresh culture medium was added after day 5, and every 2nd day from then. Cell counting was performed before adding media and/or passaging cells. First, the volume of media left in each well was measured using a pipette. Then cells were gently resuspended and a 20 µL aliquot was counted on a hemocytometer using trypan blue. For cell concentrations lower than 10^6 cells/mL, cell suspension was diluted 1:2 with fresh medium. For cell concentration that exceeded 10^6 cells/mL, the cultures were diluted to a final concentration of $5x10^5$ cells/mL (Timmins et al, 2009).

2.7 Cytospin slide process optimization and slide preparation

To develop a cytospin preparation protocol, an optimization process was implemented. This process was based in the cytospin centrifuge (Thermo scientific, Cytospin 4) technology, which is based on gravity and centrifugal force. Thus this centrifuge deposits cells charged into a chamber into the bottom of a glass slide, allowing visualization through microscopy. To achieve this, a qualitative experimental design was organized taking into consideration 3 factors: 1-cell

concentration, 2-centrifuge program, and 3-staining method. Tested cell concentrations were: 1,000 cells/µL, 100 cells/µL, and 10 cells/µL. The total volume in the cytospin chamber remained constant at 200 µL/ chamber. Two centrifuge programs were tested: 1,100 RPM for 5 minutes (P1) and 1,000 RPM for 4 min (P2). Staining method factor consisted in 2 different protocols: Leishman´s and Wright´s stains. For the Leishman´s staining protocol, slides were immersed for 2 min into pure Leishman stain. Afterwards slides were transferred into a container with Leishman´s stains diluted 1:6 into PBS pH 6.8, and remain there for 8 min. Finally slides were washed using PBS pH 6.8. For the Wright´s staining protocol, slides was covered with Wright´s stain for 5 min. Afterwards, PBS pH 6.8 was gently applied over the slides, ventilated manually until the surface color changed to a chromatic tone, and left to incubate for 10 min. Finally, slides were washed with PBS pH 6.8. All implemented treatments are shown in **Table 4.**

Table 4. Experimental design for Cytospin preparation optimization process.

Cells/µL	P1 (1,100 RPM for 5 min)		P2 (1,000 RPM for 4 min)	
	Wright	Leishman	Wright	Leishman
1,000	T5	T6	T7	T8
100	T9	T10	T11	T812
10	T3	T14	T15	T16

2.8 Phenotype and morphology validation

2.8.1 $CD34^+$ purity assessment by flow cytometry

After the protocol described in **2.3.4** was completed, a qualitative measure of $CD34^+$ molecule presence on enriched cells was performed using flow cytometry. Enriched cells were stained using an anti-human $CD34^+$ antibody conjugated to phycoerythrin (BD Biosciences, Cat. No. 560941). In order to determine $CD34^+$ cell content on enriched cells, a fraction of the enrichment was set aside and divided into two

different Eppendorf 1.5 mL tubes and kept on ice. Tube #1 remained unstained as negative control. The content of tube #2 was centrifuged for 5 min at 450 RCF. Posteriorly the supernatant was discarded and the pellet resuspended in 50 µL of PBS plus the required volume anti CD34-PE antibody, in order to have a keep the ratio at 10^6 cells per 20 µL of antibody. Once the antibody anti CD34-PE was added, cell was incubated in dark at 4°C for 20 min. Following the incubation time, 1mL of PBS was added to the tube containing the stained cells and centrifuged for 5 min at 450 RCF. The supernatant was discarded and the pellet was resuspended in 500 µL of PBS, proceeded to centrifuge for 5 min at 450 RCF. Finally the supernatant was discarded and the pellet resuspended in 200 µL of PBS.

2.8.2 Neutrophil morphology assessment by microscopy

Cell morphology was evaluated by visualizing microscopy. Cytospin slides used for this purpose were prepared using the configuration that produced slides with best quality from section **2.7**. To evaluate cell morphology 206 cells were observed and characterized according to Anderson´s Hematology Atlas (Christine and Poulsen, 2013).

2.8.3 Neutrophil phenotype assessment by flow cytometry

At day 15, the total number of neutrophils was counted. Cells were resuspended at 500,000 neutrophils/testing tube. Antibodies for CD15 (BD Biosciences, Cat. No. 555401) and CD11b (BD Biosciences, Cat. No. 557321) attach to FITC and PE respectively, were used to confirm the desired phenotype. Both CD15 and CD11b volume reactions were of 10 µL per 500,000 cells. Propidium iodide (PI) fluorochrome (BD Biosciences, Cat. No. 556547) was used to determined live/death populations, election of PI to determine cell viability is due to its capacity to stain the nucleus of death cells, due to permeability of a comprised plasma membrane, that loses integrity in necrotic and late apoptotic cells (Rieger et al., 2011). Once the corresponding volume calculation was performed, the respective cell solution

volume was placed into a micro tube and proceed to centrifugate for 5 min and 450 RCF. Then the supernatant was discarded and the pellet resuspended in 500 µL of PBS, proceed to centrifuge for 5 minutes at 450 RCF. Posteriorly the supernatant was discarded and the pellet resuspended in 80 µL of PBS plus 10 µL of each antibody corresponding to each reaction tube, except PI. **Table 5** shows an example of the reaction tube with each corresponding antibody. Test tubes containing cell solution with antibodies were left for incubation in the dark for 20 min at 4°C. After the incubation, 500 µL of PBS were added. The solution was washed in PBS twice. Following the last centrifugation supernatant was discarded, cell pellet was resuspended in 200 µL of PBS, and PI was added to the corresponding tubes. 10,000 cells were acquired on the flow cytometer (BD Biosciences, FACSCelesta) equipped with three lasers: violet, wavelength of 405 nm; blue, wavelength of 488 nm; and red, wavelength of 640 nm.

Table 5. Reaction tubes with PB neutrophils, positive control and compensation settings, and *ex vivo* manufactured neutrophils.

Sample	Tube number (per essay)	Antibody
Peripheral Blood Neutrophils	1) Negative control	Unstained
	2) Compensation control	Anti 11b-PE
	3) Compensation control	Anti15-FITC
	4) Compensation control	PI
	5) Positive control	PE+FITC+PI
Ex vivo manufactured Neutrophils	6)	Unstained
	7)	PE+FITC+PI

2.8.4 ROS production analysis

In order to test biological activity of the day 15 culture, ROS production was measured. Isolated PB neutrophils were used as positive control of cells that can produce superoxide. For both control and *ex vivo* manufactured neutrophils, three

tubes containing 200,000 cells per tube, were prepared and analyzed in the cytometer. Cell suspension volumes were added to the tubes were calculated to achieve 200,000 per test tube. The first tube contained unstained and inactivated neutrophils. The second tube contained neutrophils with 25 µL of 45 µg/mL dyhydrordamine (DHR 1,2,3; Thermofischer, Cat. No. D632). Finally the last tube contained neutrophils with 25 µL of DHR and 10 µL of 10 µg/mL phorbol myristate acetate (PMA) (Sigma, Cat. No. P8139). Finally, the tubes were incubated in the dark at 37°C for 15 min. PB neutrophils tubes and *ex vivo* manufactured containing tubes were analyzed through flow cytometer using the blue laser and 530/30 and 575/25 collection filters (Vowells et al., 1995).

2.8.5 Flow cytometry data analysis

Flow cytometry gating strategy was designed using FACSDiva cytometry software. In order to perform a proper analysis for each test, the minimum required stored events was set at 10,000 events per tube. Once this minimum number of events have been passed through the cytometer and recorded by FACSDiva, the data was exported into Flow-Jo, a specialized software designed to analyze raw data generated by the cytometer. Specific data analysis varied depending on the aspect analyzed, $CD34^+$ fraction purity, neutrophil phenotype validation, or ROS production evaluation. The general strategy for data processing was the same for the three type of data. The first step to analyze this data on Flow-Jo was to identify the population of interest in dot plot generated by the analysis of cell size, forward scatter (FCS), against cell granularity, side scatter (SSC). Since the obtained sample after the $CD34^+$ enrichment consisted in a mix of different cell types, the entire population was initially analyzed. When neutrophils were analyzed, the population of interest was identified as those cells with high granularity, From the selected population only single events were analyzed, in order to positively select these events a dot plot generated by FSC-A (area) and FSC-H (height) must be generated. To select single events from this population a new gate included the dots arranged in a linear

behavior were selected. From this single event-based population, phenotype and bioactivity analysis was performed. The final step for general gating was to select and analyze only live cells. Alive cells were those negative for PI stain, as PI stains the DNA of dead/Dying cells. Is critical to properly establish negative and positive control values before analyzing sample tubes. Positive and negative selection was done on the control tubes, first by determining the negative values by the creation of gates using unstained controls.

2.9 Absolute and relative growth of the culture.

Post day 5 and every 2nd day from there, fresh medium was added to cultures for cell expansion. During these days, before adding fresh medium to the culture, culture volume was measured and cells were counted. The resulting cell concentration for every day for each well, including day 15th, was analyzed in Microsoft Excel, in order to determine cell growth measured in absolute and relative fold increase. To evaluate the number of cells generated by this culture protocol an algorithm was developed. Each replicate was analyzed independently, posteriorly average relative and absolute growth were calculated for each day. The volume corresponding per each day was determined at the sampling day for each well independently.

2.10 In silico evaluation of cost of goods of using immobilized SCF.

In order to determine the economic impact of using iSCF to produce *ex vivo* neutrophils, a simulation of several scenarios was required. The first step in this simulation was to identify variables that may affect the production cost of such neutrophil production. In this project 6 variables were identified as important to determine the effect of cost of goods (CoG) of using iSCF. The first variable identified was the acquisition cost the biotinylated SCF (bSCF), this variable is identified as an external variable since the cost is determined by the supplier. According to the supplier each mol of SCF may contain 0.5 to 0.75 moles of biotin, the relationship between SCF and biotin was identified as the second variable. Since the biotin:SCF

ratio is determined by the supplier and cannot be modified in the lab is an external variable. Since bSCF would be immobilized to streptavidin coated magnetic microbeads, the cost of acquiring such beads was also identified as a variable, since it cannot be controlled it is considered an external variable. The interaction between biotin and streptavidin is widely known as been one of the strongest noncovalent interactions, this interaction would determine the amount of bSCF immobilized to the magnetic beads. The immobilization yield was identified as another important variable that may affect the CoG, since the immobilization would be performed in the laboratory, its yield is internal but is not completely controlled. The following variable identified was the concentration of the resulting iSCF ([iSCF]) to be supplemented in the culture media in order to induce cell differentiation. iSCF concentration would be determined at the laboratory and could be controlled, therefore is an internal variable. The final variable identified is how the cultured cells will respond to the iSCF. This cell response will be measured in the number of cells produced at the end of the culture. Cell response is an internal variable and can be indirectly controlled by modifying the iSCF concentration. For this *in silico* analysis cell response was defined as a percentage of the maximum number of cells produced by the soluble culture. **Table 6** displays the previously identified 6 variables, their nature and its abbreviation.

Table 6. Variables identified to have an impact in the CoG of iSCF in the *ex vivo* production of neutrophils

Factors involved in SCF CoG		
Variable	**Nature**	**Abbreviation**
iSCF concentration	internal	[iSCF]
Cell response	internal	Cell response
Biotinylated SCF cost	external	$Cost_{bSCF}$
Biotin relationship to SCF	external	Biotin:SCF
Magnetic Bead Cost	external	$Cost_{beads}$
Immobilization yield	internal	Immb. Yield

After variable identification, the cost of immobilizing SCF was estimated. The first step to calculate immobilization costs was to determine the amount of materials required for the immobilization. The two main materials taken into account for the immobilization step were the recombinant biotinylated SCF and the streptavidin coated magnetic beads. According to supplier information SCF to biotin ratio is equal to 0.59. Using this information was possible to determine the maximum SCF that can be bound per mg of beads, 7.08 nmol of SCF/mg of bead, which equals to 153.63 µg of SCF/mg of beads. Taking in consideration the number of beads per mg, 50,000,000/mg of beads, and the bead concentration, 10 mg of beads/mL of suspension; it was possible to determine the required volume to immobilize 1 µg of SCF, this volume equals to 0.91 µL of bead suspension. With these quantities **Table 7** shows the presentation, cost, and immobilization cost for immobilizing 100 ng of SCF.

Table 7. Variables required for the calculation of immobilizing SCF.

Material	Presentation	Cost (USD)	Required amount	Immobilization cost ($/100ng of SCF)
Biotinylated SCF	25 µg	$350.00	1 µg	$1.4
Magnetic Beads	1 mL	$276.14	0.00091 mL	$0.02

Once immobilization cost for iSCF was calculated, cost of goods of such iSCF was estimated in terms of the amount of iSCF used in culture, which may range between 12.5-100 ng/mL. The CoG of iSCF was calculated using **Equation 1.**

Equation 1. Equation used to calculate the CoG of using iSCF. a) The complete form of equation 1 indicating the name of all the variables considered for the model.

b) Simplified form of equation 1, all the constant values considered for the model were substituted.

$$a)\ CoG = \frac{[iSCF] * \left(\frac{Cost_{bSCF}}{\mu g\ of\ bSCF\ acquired} + \frac{Bead\ Excess * \ \mu g\ of\ SCF\ to\ immobilize * Immb.yield * Cost_{beads}}{Biotin{:}SCF * mg\ of\ beads * Bead\ capacity}\right)}{Cell\ response * \ 10^9}$$

$$b)\ CoG = \frac{[iSCF] * \left(\frac{Cost_{bSCF}}{25} + \frac{1.4 * Cost_{beads} * Immb.yield}{Biotin{:}SCF * 1{,}536}\right)}{Cell\ response * 10^9}$$

2.10.1 Sensitivity analysis of the variables

Following the initial economical simulation, a sensitivity analysis was performed. The aim of this analysis is to determine the individual impact each of the six variables have over the CoG. To perform the sensitivity analysis an optimistic and pessimistic scenario was simulated per variable, while the rest of the parameters remain constant. **Table 8** displays each variable with their respective pessimist, normal, and optimist scenario. The optimist and pessimist scenario for the acquisition cost of bSCF and the magnetic beads consisted in an increase or decrease of 20% from the actual sale price. Regarding the biotin:SCF relationship the pessimist scenario was 0.5 and the optimist was 1, which is the range of biotin:SCF the supplier guaranties. The optimist and pessimist scenario from the 3 remaining variables were defined as the respective lower and higher values listed in section **2.10.**

Table 8. Maximum and minimum values defined for the optimist and pessimist scenarios required for the sensitivity analysis.

Variable	Minimum	Base	Maximum
$Cost_{bSCF}$ ($/ng)	280	350	420

$Cost_{beads}$ ($/mg)	220.9116	276.1395	331.37
[iSCF] (ng/mL)	12.5	50	100
Immobilization yield	90%	95%	100%
Biotin:SCF	0.5	0.75	1
Cell response (%)	50%	75%	100%

Once the 3 variables with more influence were identified an *in silico* simulation was performed. The aim of this simulation is to generate a robust amount of data, where random scenarios simulated many possibilities that could substitute experimental data in an initial evaluation. This simulation consisted in the generation of 100 random scenarios, were the 3 variables had a random value within their respective range. Posteriorly the CoG of the resulting scenarios were analyzed in Microsoft Excel and Origin Lab.

2.11 Statistical analysis

To determine whether two means were different or not a hypothesis test based on T test was performed. To determine if an independent variable had an effect over the dependent variable an analysis of variance (ANOVA) was performed. Bothe statistical tests was performed in Microsoft Excel.

3. Chapter 3: Results

3.1. $CD34^+$ cell isolation

The first step to establish a human $CD34^+$ cell bank is to isolate $CD34^+$ cells from UCB. A total of 6 samples of UCB were obtained by attending full term deliveries upon informed consent signed by the parents (see **Appendix 4**). A minimum of 26 mL and maximum of 40 mL of UCB were obtained from deliveries with an average blood volume of 36 mL per sample. Overall from these samples a total of $1.3x10^8$ MNCs were obtained. On average $2.17x10^7 \pm 1.04x10^7$ cells were obtained per cord. Relevant information about each sample and its current status is presented in **Table 9.** Additionally **Figure 8** presents the number of MNCs obtained per cord.

Table 9. Information for each UCB sample.

UCB Sample	Collection Date and Time	UCB Collected Volume	Sex of newborn	MNCs isolated	Collected cells ($x10^5$)/mL of obtained blood	$CD34^+$ cell isolation condition	Time on cryopreservation	$CD34^+$ cells isolation date
UCB1	13/03/19; 8:40 am	30 mL	Male	$53x10^6$ cells	17.6	Thawed	8 months	15/11/19
UCB2	04/05/19; 8:48 am	26 mL	Female	$17 x10^6$ cells	6.53	NA	MNCs currently cryopreserved	NA
UCB3	10/06/19; 6:13 am	40 mL	Male	$17.05 x10^6$ cells	4.26	Thawed	7 months	15/01/20

UCB4	15/07/19; 8:55 am	40 mL	Male	9.97 x10^6 cells	2.49	NA	MNCs currently cryopreserved	NA
UCB5	22/01/20; 9:16 am	40 mL	Female	14.4 x10^6 cells	3.6	Fresh	NA	22/01/20
UCB6	17/03/20; 9:22 am	40 mL	Female	19 x10^6 cells	4.75	Fresh	NA	17/03/20

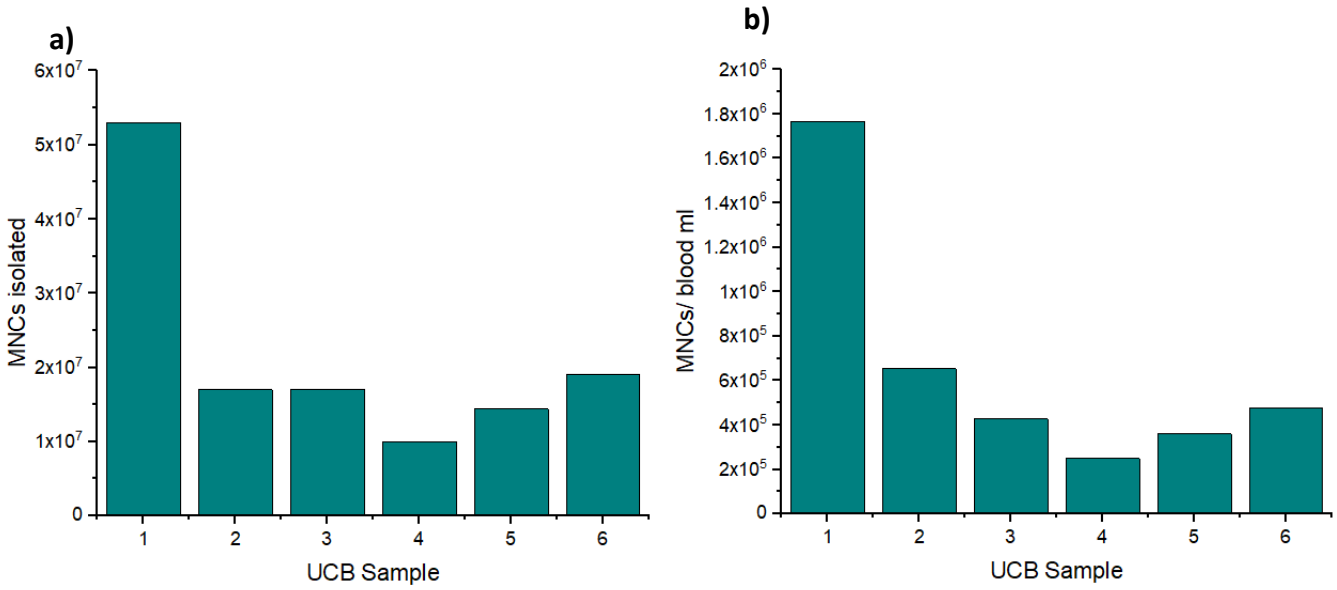


Figure 8. MNCs obtained for each UCB sample. a) Total number of MNCs isolated from each UCB obtained. b) Number of MNCs isolated from each UCB obtained per mL of blood for each sample**.**

3.1.1. Optimization of CD34+ cells enrichment

To maximize the number of $CD34^+$ cells isolated from isolated MNCs, an optimization process was performed. $CD34^+$ were isolated from directly post

mononuclear cell enrichment (fresh MNCs), or from thawed MNCs that were collected in a previous occasion and processed using either a single column, or 2 successive columns. The total number of cells isolated after $CD34^+$ enrichment is presented in **Figure 9.** The average normalized number of cells isolated after $CD34^+$ enrichment was significantly higher for freshly processed MNCs. For freshly processed cells 0.0204 cell were isolated for every initial MNC, in contras for post-thawed cells only 0.002845 cells were isolated for every initial MNC, this results indicate that the isolation yield for freshly processed cells is significantly higher than for post-thawed cells. Therefore freshly processing the cells could reduce the number of cells lost during the isolation process (T_c=7.23>T_{st}=4.30). In contrast, there was no significant difference between the average normalized number of cells isolated using 1 MS column (0.0131 cells/MNCs) and those cells isolated using 2 MS columns (0.0101 cells/MNCs) (T_c=0.239<T_{st}=4.30). These results suggest that processing the cells freshly considerably increase the total number of cells obtained after CD34 enrichment. In order to calculate the total amount of isolated $CD34^+$ cells, they purity of the $CD34^+$ cell fraction was assed by flow cytometry.

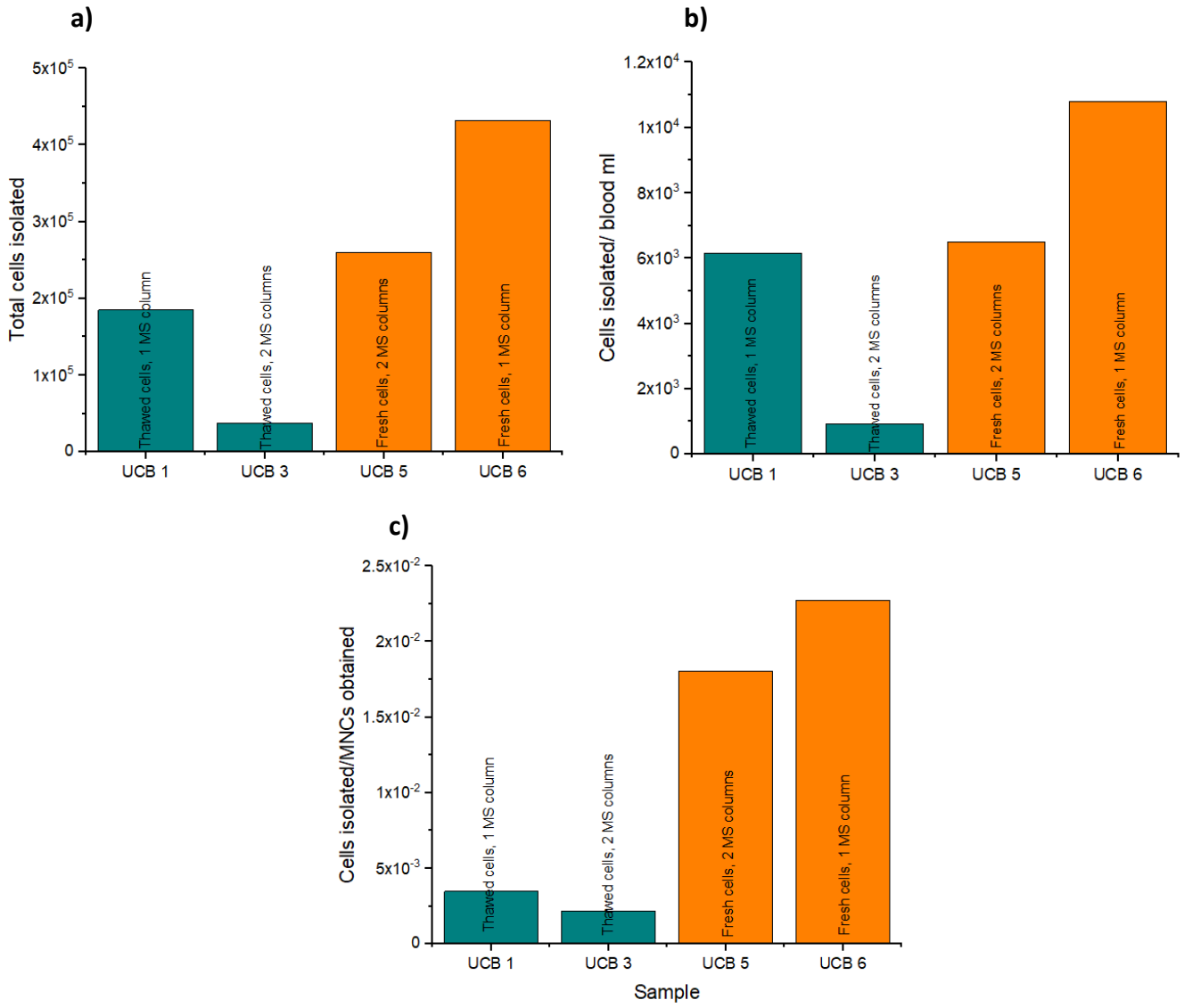


Figure 9. Number of cells isolated after CD34⁺ enrichment. a) Total number of cells isolated from CD34⁺ enriched MNCs. b) Cells isolated after CD34⁺ enrichment per mL of blood for each sample. c) Cells isolated after CD34⁺ enrichment per MNCs previously obtained. Blue graphs represent samples processed after cryopreservation, orange graphs represent samples processed freshly.

3.1.2. CD34⁺ cell fraction purity

The gating strategy for identifying CD34⁺ cells is presented in **Figure 10.** From the total isolated population only, the single events were taken in account for analyzing the CD34⁺ population. The expression of the surface marker CD34 was assessed by measuring the intensity of fluorescence of PE, which is conjugated to an anti-CD34 monoclonal antibody.

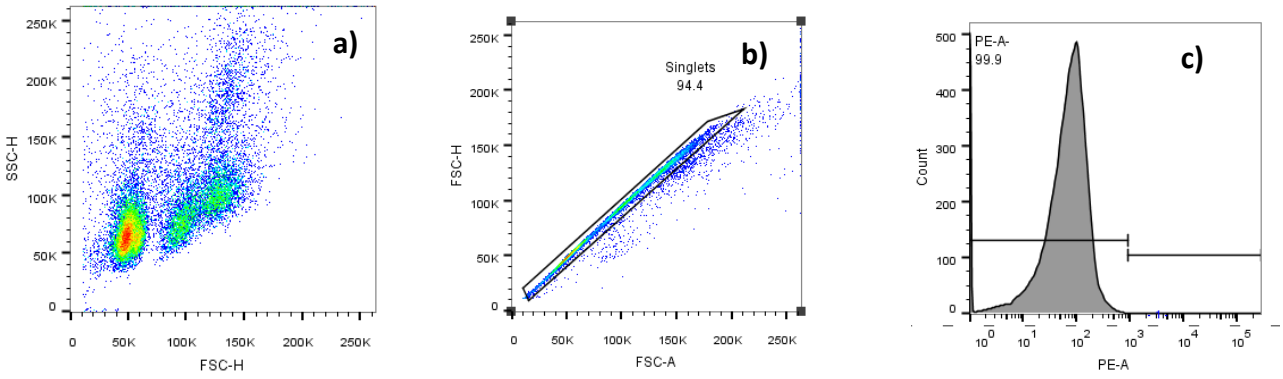


Figure 10. Gating strategy for the identification of the CD34⁺ cell fraction. a) Overall population. b) Single events selected for analysis. c) Histogram showing the median fluorescence intensity of PE.

The purity of the CD34⁺ cell fraction obtained from 3 different treatments is presented in **Figure 11**. The cells isolated freshly with a single MS column resulted significantly purer than those isolated from thawed MNCs. the resulting CD34⁺ cell population obtained from freshly isolated cells with 2 MS columns presented a purity of 94.5%, in contrast cells isolated from thawed cells using 1 and 2 MS columns presented a purity of 19.4% and 20.7%, respectively. These results suggest that isolating CD34⁺ from freshly processed MNCs has a significant positive effect in the resulting purity of both the total number of cells obtained and the purity of the resulting CD34⁺ cell fraction. Looking at yields from thawed cells, the use of 1 or 2 MS columns does not affect the purity of the obtained CD34⁺ fraction.

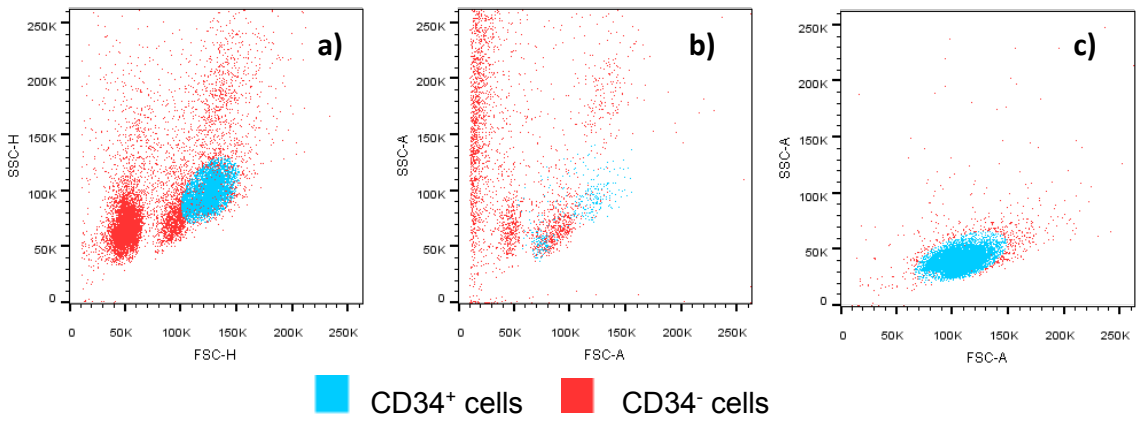


Figure 11. Back-gating on SSC/FSS of $CD34^+$ cells analyzed through flow cytometry. a) Cells isolated from thawed MNCs using 1 MS column. b) Cells isolated from thawed MNCs using 2 MS columns. c) Cells isolated from fresh MNCs using 2 MS columns.

3.1.2.1. Number of cells isolated

Once the purity of the $CD34^+$ cell fraction has been assessed, the total number of $CD34^+$ cells was calculated by multipliying the obtained purity by the total number of cells recuperated after enrichment. The number of $CD34^+$ cells obtained per treatment is presented in **Figure 12**. The total number of $CD34^+$ cells isolated from freshly processed MNCs were 254,280 cells and resulted higher than the number of cells obtained from thawed MNCs using either 1MS column (36,960 cells) or 2 MS columns (9,750 cells). To proper analize the effect each treatment has over the $CD34^+$ cells isolation yield a normalization was performed. Hence, the following data shows the number of $CD34^+$ cells isolated per the number of MNCs before the enrichment was performed. From freshly processed MNCs it was possible to obtain 0.0176 $CD34^+$ cells for every MNC initially processed. In contrast, for post-thawed cells only $6.97x10^{-4}$ $CD34^+$ cells for every MNC initially processed were obtained using 1 column and $5.71x10^{-4}$ $CD34^+$ cells for every MNC initially processed were obtained using 2 columnns. There is a consistent increment in the total number of

CD34⁺ cells and the yield of CD34⁺ cells isolated/MNCs processed in freshly processed cells over cryopreserved cells, this increment was calculated at 6.97-fold and 25.3-fold respectively.

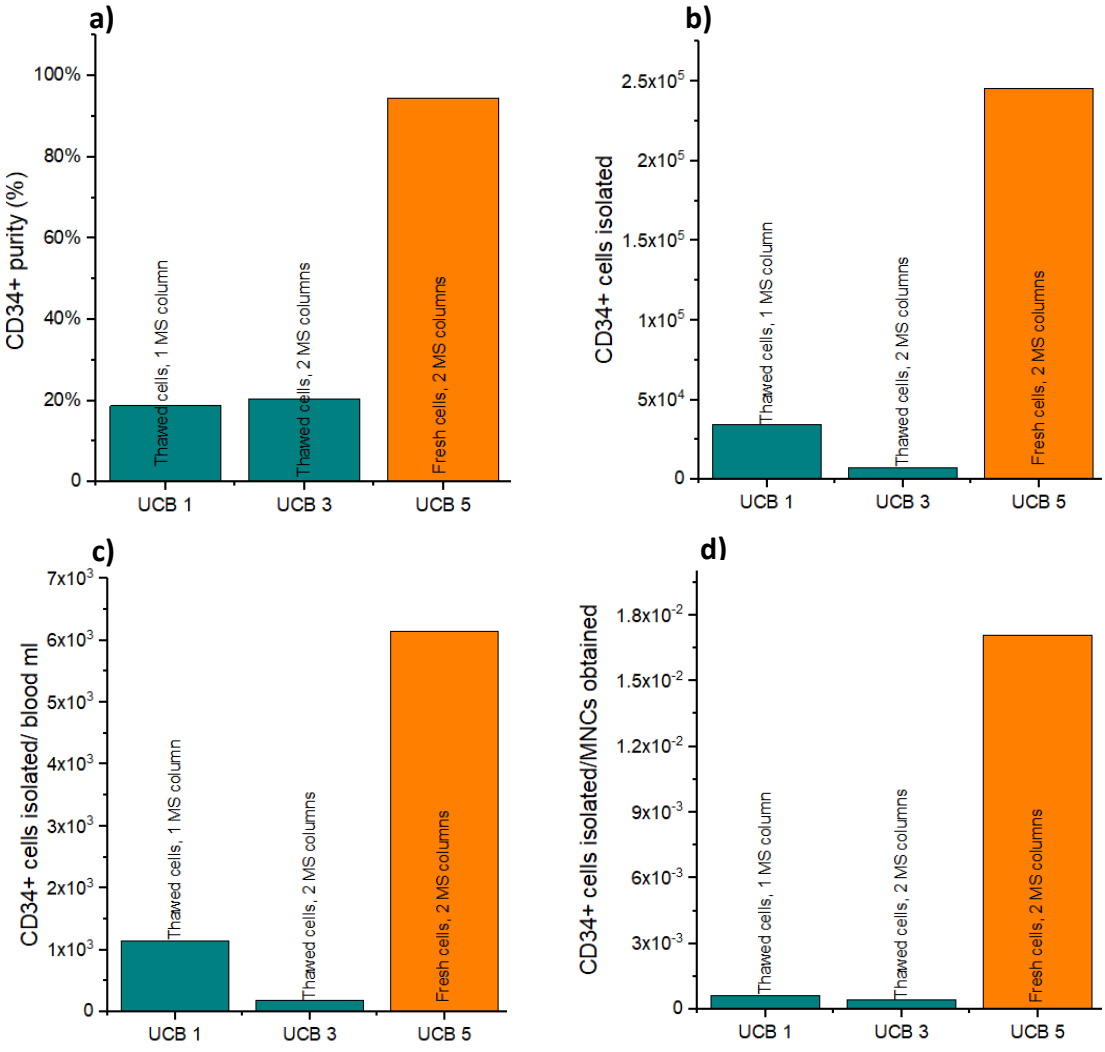


Figure 12. CD34⁺ cells isolated per UCB sample. a) Purity of collected CD34⁺ fraction enriched from MNCs. b) Total number of CD34⁺ cells obtained from each UCB sample. c) CD34⁺ cells isolated after CD34⁺ enrichment per mL of blood for each sample. d) CD34⁺ cells isolated after CD34⁺ enrichment per MNCs previously obtained.

Additionally these results indicate a positive correlation between the number of $CD34^+$ cells isolated and the purity of the fraction obtained. According to these results the purity is principally affected by processing of MNCs. Results show that cryopreserving and posterior post-thaw processing of MNCs for isolating $CD34^+$ cells had a negative effect in both the total number of cells isolated after enrichment and the purity of the $CD34^+$ fraction obtained. In consequence the total number $CD34^+$ cells obtained will also result affected.

3.2. Differentiation process of $CD34^+$ cells to neutrophils

$CD34^+$ enriched cells were put into a 15-day differentiation culture, which allow to evaluate the effect of the isolation treatments and population purity over the number and phenotype of neutrophils produced.

3.2.1. Expansion curves

As mentioned in the previous section, here it was follow a 15-day differentiation protocol, starting with human hematopoietic $CD34^+$ stem cells. **Figure 13** displays both the absolute and the relative growth curves achieved for the cultured cells. Cells were passaged first after 5 days in culture, then every 2 days after final collection at day 15. Regarding relative expansion, the 3 curves behaved in a similar way. All of the curves achieved maximum relative expansion at day 5, average fold increase of 11.10. Following day 5 the relative expansion was considerably lower and trended stabilization, with an average fold increase of 3.54, 3.18, 2.09, 2.07, and 2.31 for days 7, 9, 11, 13, and 15 respectively. This behavior is expected since $CD34^+$ cells were cultured in a culture medium supplemented with growth factors that promote proliferation followed by differentiation into neutrophils. Hence, a decrease over the relative expansion is expected since neutrophils and its latest precursors, metamyelocytes and banded neutrophils, are post-mitotic cells. The maximum relative expansion at day 5 was 16.19 ± 2.06 and was obtained by $CD34^+$ cells isolated from fresh MNCs using 2 MS columns, UCB5, in contrast lower relative

expansion at day 5 was,5.81 ± 2.35 and was obtained from cells isolated from thawed cells using 2 MS columns, UCB3. While those cells isolated from thawed cells using 1 MS column, UCB1, achieved a maximum relative expansion of 11.29 ± 3.01. As mentioned previously the behavior of the relative expansion curves over time suggest $CD34^{+}$ cells stop dividing after day 5 of the culture protocol and start differentiating into post-mitotic cells.

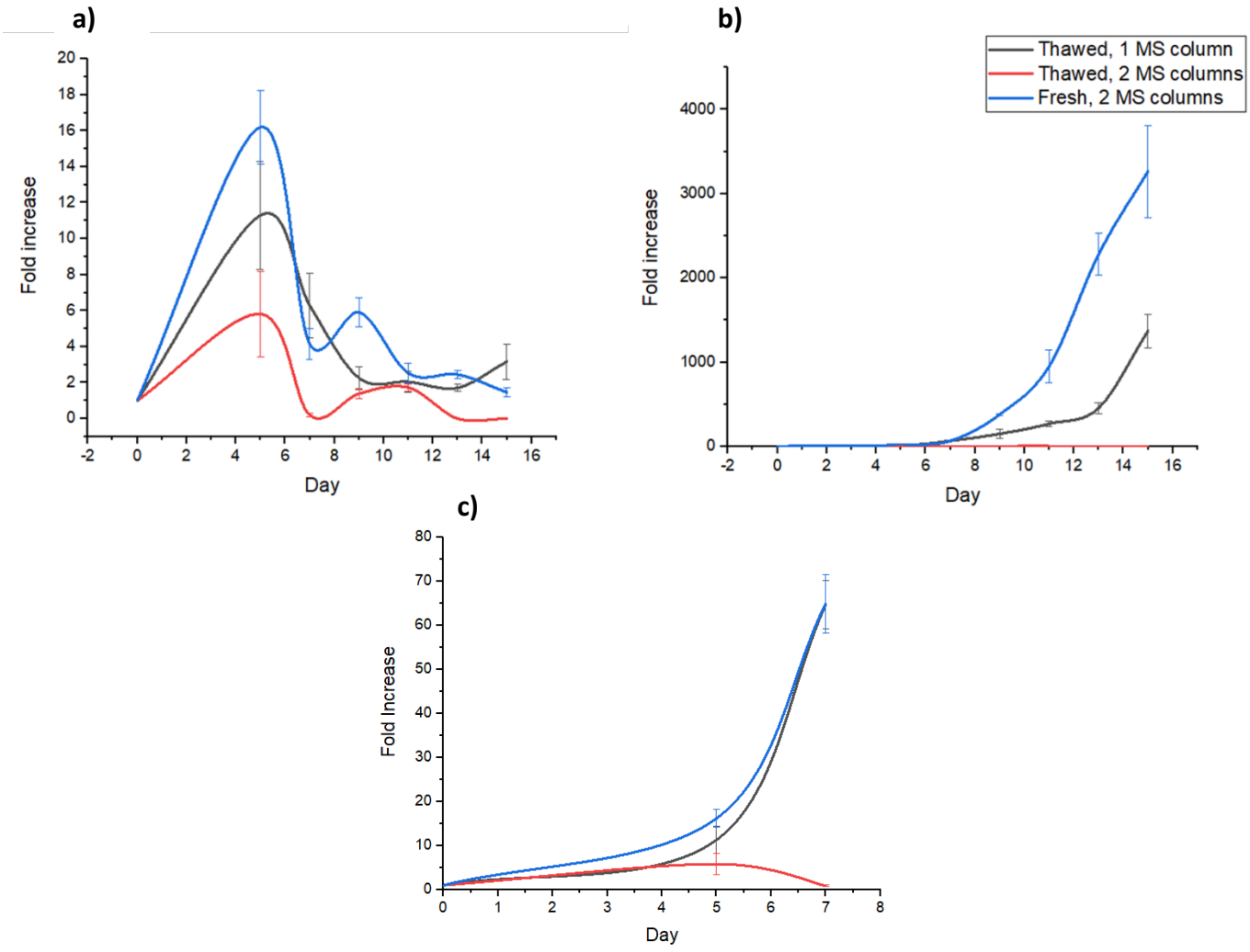


Figure 13. Relative and absolute expansion curves obtained from the differentiation cultures. a) Relative expansion curve. b) Absolute expansion curve. c) Absolute

expansion curve, showing the absolute expansion during the first 7 days of the cultures.

In contrast, absolute expansion curves showed significant differences between the 3 cultures. Maximum absolute expansion was 3,264.88 ± 545.57 fold increase, and was obtained from those isolated from fresh cells using 2 MS columns (UCB5, 94.5% purity). Whereas minimum absolute expansion was 1.91 ±0.2485 fold increase, and was achieved by those cells isolated from thawed cells using 2 MS columns (UCB1, 20.7% purity). In fact this culture was killed on day 13 when no viable cells were observed. Regarding cells isolated from thawed cells using 1 MS column (UCB1, 19.4% purity) absolute expansion achieved was 1,368.78 ± 203.15 fold increase. The difference between the absolute expansion obtained by UCB5 (3,264.88) and UCB1(1,368.78) was 1,896.1. The difference between both samples is higher than the $LSD_{0.05,4}$= 1,228.30. An ANOVA analysis determined that the purity of $CD34^+$ cells culture has a significant impact in the final number of neutrophils produced (F_c=18.36> $F_{0.05,4,5}$=5.19). These results strongly suggest that isolating $CD34^+$ cells from thawed cells, considerably affects the overall number of neutrophils produced from the differentiation process. Additionally the results indicate a strong positive correlation between the purity of the $CD34^+$ cell fraction initially cultured and the final number of neutrophils produced trough the differentiation process.

3.2.2. Neutrophil phenotype assessment

Phenotype characterization of the produced cells is required to ensure that cell differentiate into neutrophils. Neutrophil morphology and phenotype were confirmed through microscopy and flow cytometry, respectively.

3.2.2.1. Neutrophil phenotype assessment by microscopy

Multiple cell differentiation stages of neutropoiesis at day 9 of culture can be appreciated in **Figure 14.** Neutropoiesis evaluation by microscopy at day 9 of the differentiation process indicated that some segmented and band neutrophils were already present. Neutrophils isolated from peripheral blood served as positive control, to compare the morphology. Although the majority of the cell stages appreciated at day 9 correspond to myelocyte and promyelocyte stages, it was possible to appreciate a segmented neutrophil with a segmented nucleus perfectly formed. Results indicate previously isolated $CD34^+$ cells are responding adequately to the *ex vivo* differentiation process. Additionally, this information explains the drop in the relative expansion curve after day 5, since most of the cell stages observed have morphology similar to metamyelocytes, which are postmitotic cells. At this point of cell differentiation most of the cells observed in the microscopic field exhibit granule presence within their cytoplasm.

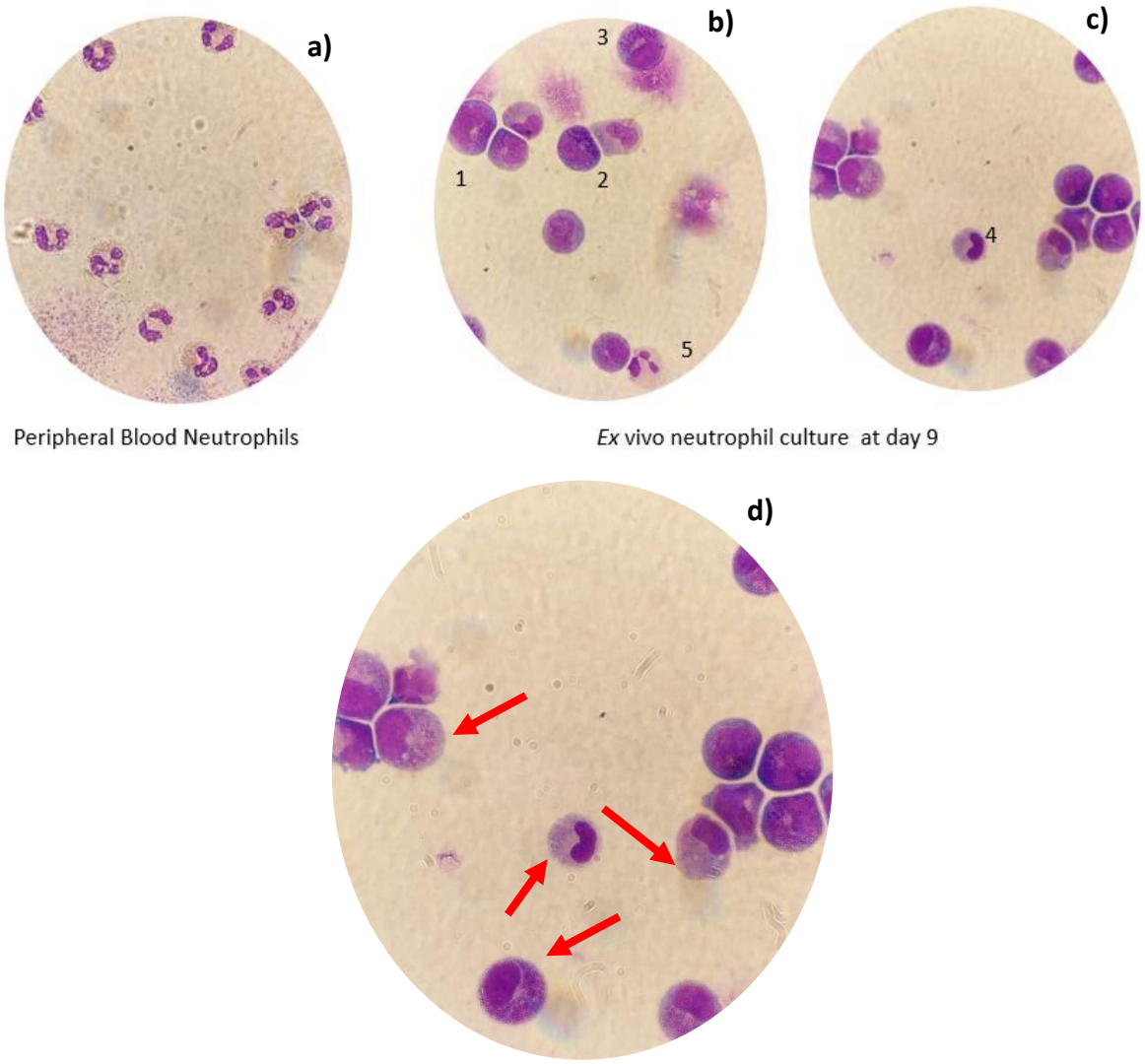


Figure 14. Cytospin slide of peripheral blood and *ex vivo* manufactured neutrophils observed under a microscope at 100x magnification. a) Peripheral blood segmented neutrophils. b) *Ex vivo* manufactured neutrophils at day 9 of differentiation; 1) Promyelocyte, 2) Myelocyte, 3) Metamyelocyte, 5) Segmented neutrophil. c) *Ex vivo* manufactured neutrophils at day 9 of differentiation, 4) band neutrophil. d) *Ex vivo* manufactured neutrophils at day 9, arrows indicate the presence of granules.

At the end of the culture (day 15), a final Cytospin slide preparation was performed to evaluate the morphology of *ex vivo* produced cells. **Figure 15** displays the slide,

in the same way, peripheral blood neutrophils slides were prepared to serve as positive control. This image confirms it is possible to differentiate human primary $CD34^+$ cells in order to *ex vivo* produce segmented neutrophils exhibiting a morphology similar to peripheral blood neutrophils.

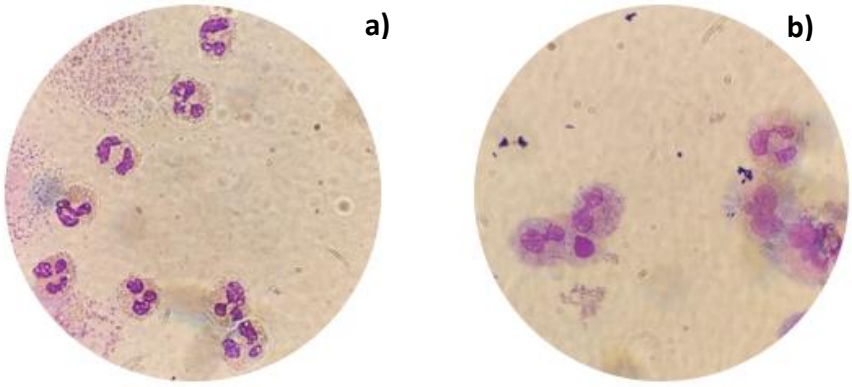


Figure 15. Cytospin slide of peripheral blood and *ex vivo* manufactured neutrophils observed under the microscope at 100x magnification. a) Peripheral blood segmented neutrophils. b) *Ex vivo* manufactured neutrophils at end of the 15 day differentiation protocol.

Culture population characterization from the neutrophils differentiated, from $CD34^+$ cells isolated from fresh MNCs using 2 MS columns, was performed by microscopy. In **Figure 16** a pie chart displaying the proportions of the different population, segmented by cell stage, of the produced neutrophils is presented. The composition of the population, sample number=206 cells counted, showed that 49.5% of the produced cells were neutrophils (35.4% segmented and 14.1% band), 20.4% promyelocytes, 17% myelocytes, and 13.1% metamyelocytes. This population composition is consistent with the drop in the relative expansion curve for the analyzed culture, since 62.6% of the population is composed by post-mitotic cell stages, metamyelocytes, banded and segmented neutrophils.

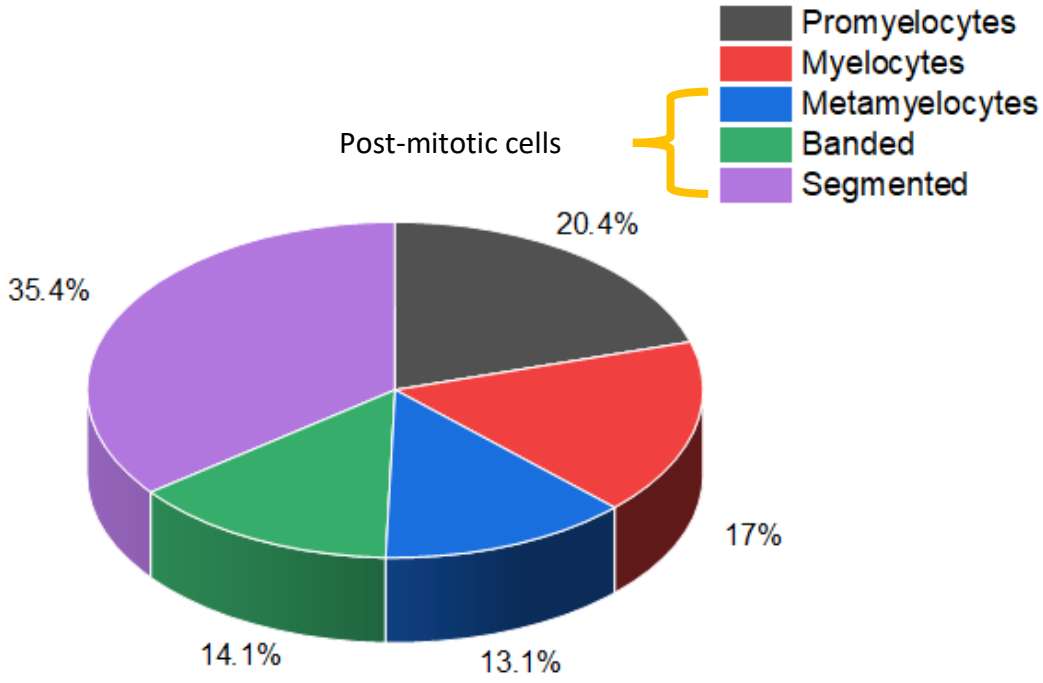


Figure 16. Population composition of the *ex vivo* produced neutrophils differentiated from $CD34^+$ cells isolated from fresh MNCs using 2 MS columns.

3.2.2.2. Neutrophil phenotype assessment by flow cytometry

Neutrophil phenotype was assessed by flow cytometry. To achieve this the co-expression of CD11b and CD15 surface markers was evaluated. CD11b, also known as integrin alpha M, is a trans-membranal protein subunit that binds to CD18 sub unit to form the heterodimer macrophage antigen 1 (MAC-1) (Sapey and Stoclkey, 2009). CD11b expression is associated to neutrophils, monocytes and natural killer cells (Naeim et al., 2013). Regarding CD15, also known as Lewis x antigen, is a cell surface glycan. CD15 is mainly expressed by monocytes, neutrophils, and promyelocyte cell lineage. CD15 expression in neutrophils is associated to degranulation process and phagocytosis (Nakayama et al., 2001; Zeineb et al., 2008). Co-expression of these surface markers corresponds to mature neutrophils,

although CD15 and CD11b expression starts at promyelocyte and myelocyte cell stages respectively. In **Figure 17** the gating strategy followed for the evaluation of neutrophil phenotype is presented.

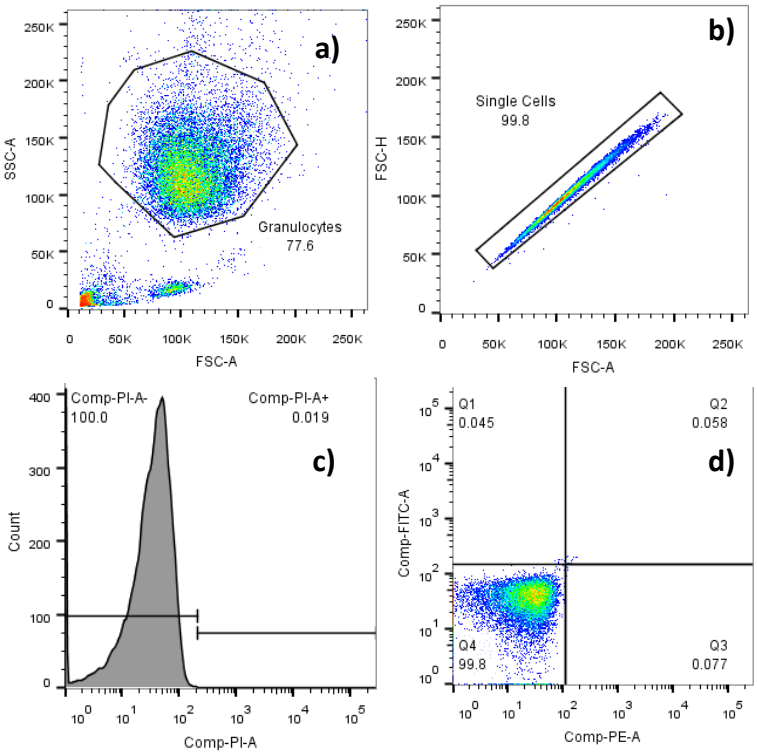


Figure 17. Gating strategy for the phenotype assessment of the *ex vivo* manufactured neutrophils. a) Overall population selection. b) Single events selected for analysis. c) Selection of the negative population for PI. d) Alive unstained population of *ex vivo* manufactured neutrophils.

Figure 18 displays the CD11b and CD15 expression assessment by flow cytometry for the *ex vivo* manufactured neutrophils differentiated from $CD34^+$ cells isolated from thawed MNCs using 1 MS column, UCB1 18.7% purity. Neutrophils isolated from peripheral blood served as positive control. The phenotype analysis from the

cultured cells indicates that 48.6% of the day 15 culture expressed are CD11b+/CD15+. These results suggest that mature neutrophils were successfully produced *ex vivo* from hematopoietic stem cells isolated from UCB. In comparison, 82.4% of the peripheral blood neutrophils expressed the studied markers. Additionally, these results suggest that purity of the $CD34^+$ cell fraction originally cultured does not have a negative impact in the final differentiated phenotype.

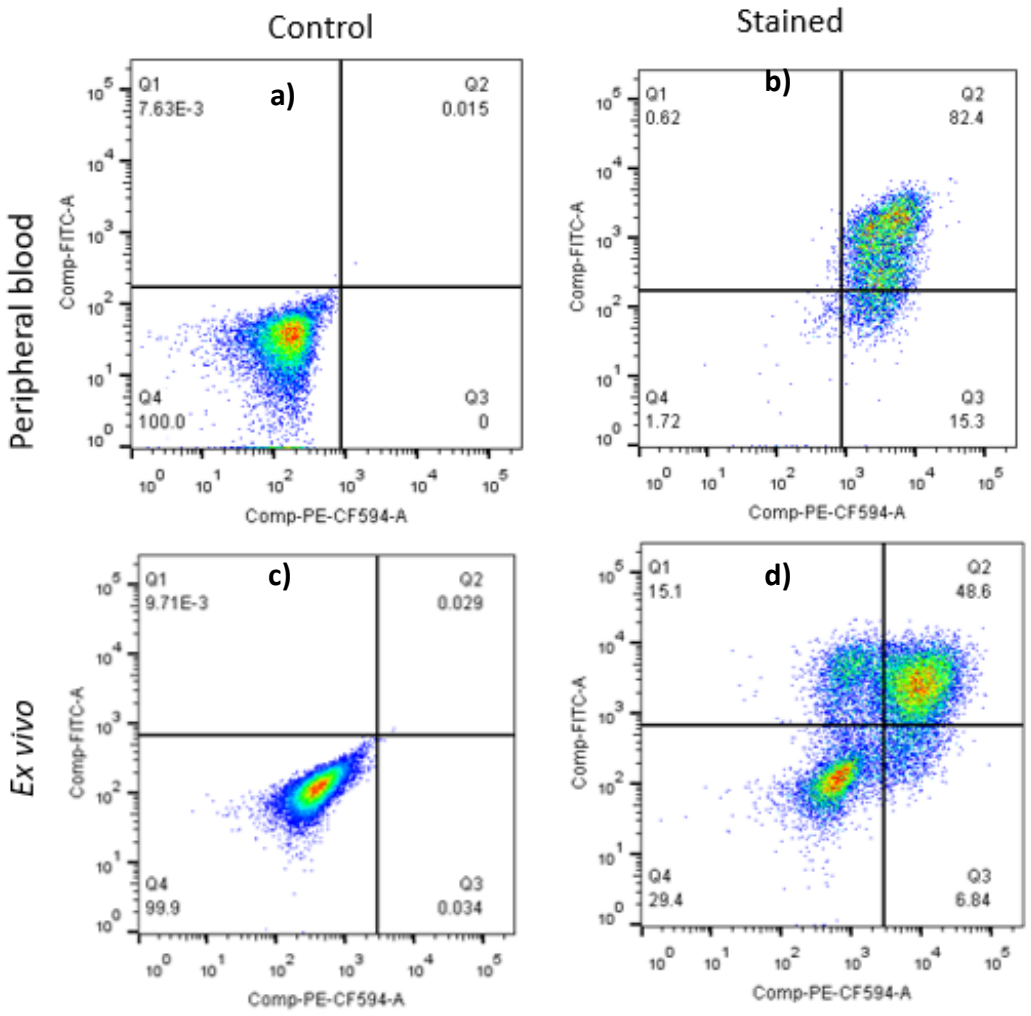


Figure 18. Population composition of peripheral blood and *ex vivo* manufactured neutrophils differentiated from $CD34^+$ cells isolated from thawed MNCs using 1 MS column. a) Unstained peripheral blood neutrophils. b) Peripheral blood neutrophils

stained with FITC and PE fluorochromes. c) Unstained *ex vivo* manufactured neutrophils. d) *Ex vivo* manufactured neutrophils stained with FITC and PE.

Phenotype assessment by analyzing the co-expression of CD11b and CD15 was also performed to the *ex vivo* manufactured neutrophils differentiated from $CD34^+$ cells isolated from fresh MNCs using 2 MS columns, UCB5 95.4% purity. **Figure 19** shows the surface marker expression analysis. Peripheral blood neutrophils served as positive control for this test.

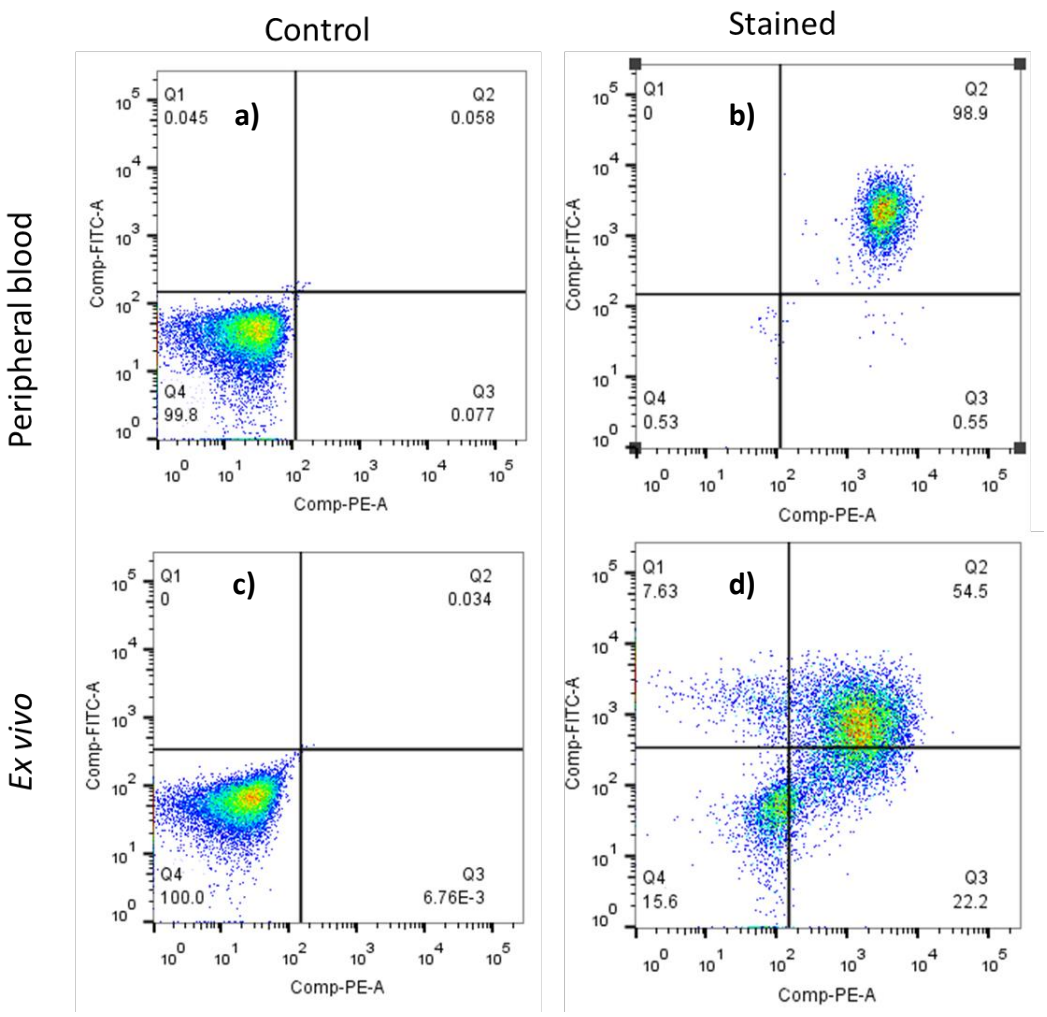


Figure 19. Population composition of peripheral blood and *ex vivo* manufactured neutrophils differentiated from $CD34^+$ cells isolated from fresh MNCs using 2 MS columns. a) Unstained peripheral blood neutrophils. b) Peripheral blood neutrophils stained with FITC and PE fluorochromes. c) Unstained *ex vivo* manufactured neutrophils. d) *Ex vivo* manufactured neutrophils stained with FITC and PE.

The phenotype analysis from the differentiated cells indicates that 54.5% of such population expressed CD11b and CD15. These results indicate that neutrophil-like cells were successfully produced *ex vivo* from hematopoietic stem cells isolated from

UCB. In comparison, 98.9% of the peripheral blood neutrophils expressed the studied markers. The *ex vivo* manufactured neutrophils' mature population, co-expressing CD11b and CD15, is consistent with the mature population determined by microscopy analysis (**Figure 16**). Additionally these results reinforce the theory that purity of the $CD34^+$ cell fraction originally cultured does not have a negative impact in the final differentiated phenotype. Although the purity fraction of both cultures was significantly different, 18.7% for UCB1 and 94.5% for UCB5, both cultures achieved similar percentages for mature neutrophils at the end of the 15 day differentiation culture, 48.6% for UCB1 and 54.5% for UCB5.

Results presented in this section confirm the feasibility of *ex vivo* manufacturing neutrophils from $CD34^+$ cells isolated from UCB. Additionally the produced neutrophils exhibited a morphology similar to neutrophils from PB.

3.2.3. ROS production by *ex vivo* manufactured neutrophils

In order to assess the functionality of the produced neutrophil-like cells, their ability to produce ROS to induce oxidative burst was tested. To measure ROS production neutrophils were activated using PMA, when activated neutrophils breakdown 1, 2, 3-DHR (Rinaldi et al., 2007). Peripheral blood neutrophils were used as positive control. For this assay neutrophils were initially incubated with 1,2,3-DHR and the fluorescence of the inactivated neutrophils was measured by flow cytometry. Fluorescence produced by DHR incubated inactive neutrophils served as control to measure the production of ROS when neutrophils were activated. Posteriorly in order to activate such neutrophils, PMA was added to neutrophils containing DHR. When incubated with neutrophils, PMA simulated protein kinase C, which consequently activates the production of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Karlsson et al., 2000). Hence activated neutrophils produce NADPH, which oxidases DHR into rodahmine-1,2,3, producing fluorescence that can be measured in 530/30 filter of the flow cytometer (Roos and Boer, 2014). **Figure 20** displays the results of the ROS production assay. When activated, *ex vivo* produced neutrophils

were capable to oxidize DHR. This indicates the production NADPH, confirming ROS production. Although PB neutrophils mean fluorescence intensity (MFI) indicated greater production of ROS species than *ex vivo* manufactured neutrophils, the difference may be explained by the composition of both populations. Mature neutrophil population from PB neutrophils was 98.9% contrasting with mature population from *ex vivo* produced neutrophils, 54.5%. This result confirm that the *ex vivo* produced neutrophil-like cells behave like functional neutrophils in the production of ROS.

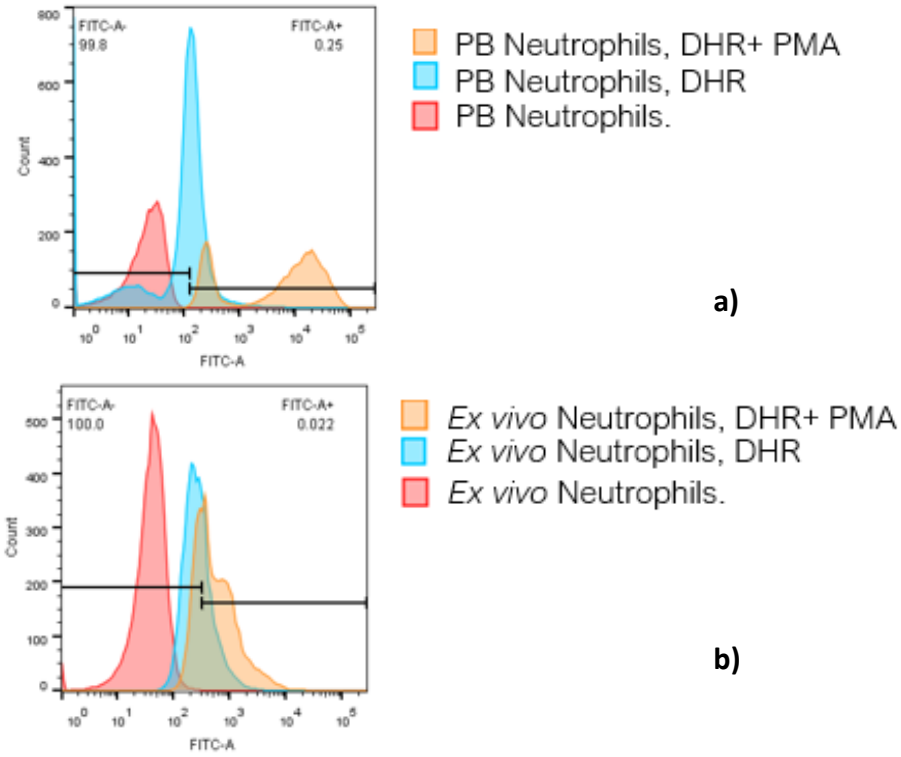


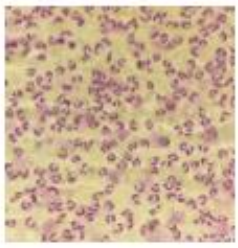
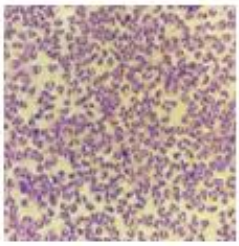
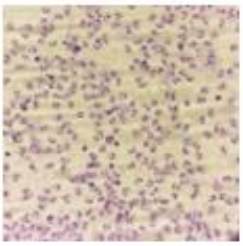
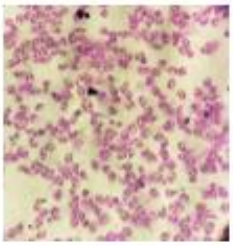
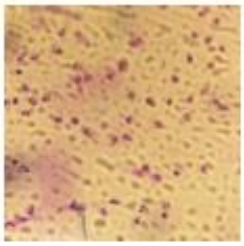
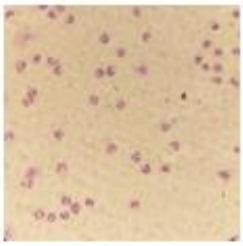
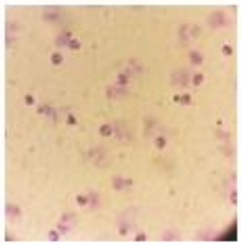
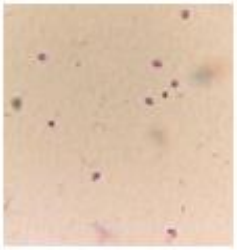
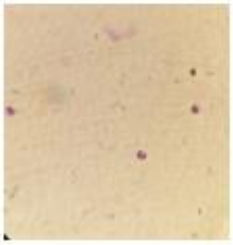
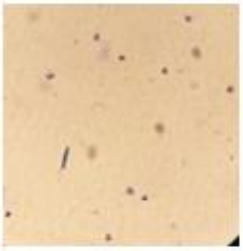
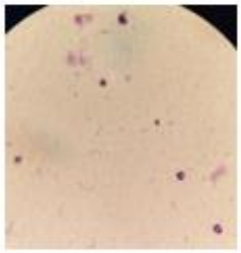
Figure 20. Neutrophil ROS production assay. a) Peripheral blood neutrophils. b) *Ex vivo* manufactured neutrophils.

3.3. Cytospin preparation optimization

An optimization was performed to produce the best quality Cytospin slides. Cytospin slide preparations offer an alternative to cell blocks and smears. The principal

advantage of Cytospin relays in a better preservation and appreciation of cell morphology (Qamar et al., 2018). **Table 10** displays a photography taken from a slide prepared according to various treatments. Cytospin slides with higher resolution resulted in better cell morphology characterization and visualization. Best cell visualization was obtained by loading 100 cells/µL in the Cytospin chamber. Cell concentration of 1,000 cells/ µL produced oversaturated slides, preventing adequate cell appreciation. Whereas cell concentration of 10 cells/ µL produced slides with very low number of cells visible in the optical field. Regarding staining method, Leishman's stain produced clearer slides allowing correct visualization of nucleus, granules, and cytoplasm. Although Wright's stain produced slides with high resolution in nucleus, the cytoplasm was barely visible causing difficulties to observe granules and define cell borders. Finally, the Cytospin centrifuge program with best performance was Program 1 (1,100 RPM for 5 min). This program achieved higher centrifugal forces applied to the cells, resulting in higher resolution and less visual contamination caused by smears. In conclusion the best treatment for producing better quality Cytospin slides was Treatment #6: 100 cell/µL, Program 1(1,100 RPM for 5 min), and Leishman's stain. Therefore, all the cytospin slides shown in the present section were prepared with these conditions.

Table 10. Cytospin preparation slides optimization results observed under the microscope at 40x magnification.

Cell/µl	P1 (1,100 rpm x 5min)		P2 (1,000 rpm x 4min)	
	Wright	Leishman	Wright	Leishman
1,000				
100				
10				

3.4. *In silico* evaluation of cost of goods of using immobilized SCF.

Once a soluble culture for *ex vivo* neutrophil production was established, it was possible to calculate the cost of using SCF for neutrophil differentiation using real data. The maximum number of cells produced using soluble SCF were 32.6 $x10^6$ cells/mL. This cell response served as the standard for *in silico* evaluations were a cell response of 100% is defined as the production of 32.6 $x10^6$ cells/mL. According to the number of cells produced by using soluble SCF, the CoG of soluble SCF to

produce 1 billion cells was calculated at USD$593.94/$10^9$ cells. CoG of soluble SCF is a benchmark, and was used to be compared to the simulated CoG of iSCF.

3.4.1. Sensitivity analysis of the variables

A sensitivity analysis was performed to determine which of the six variables had more impact in CoG according to **Equation 1**. The impact of each of the previously mentioned variables is displayed in **Figure 21.** According to this analysis the three factors with more impact over CoG are [iSCF], cell response, and the cost of acquiring the biotinylated SCF (bSCFcost). In contrast the factors with less impact over CoG are the biotin content per SCF (Biotin:SCF), cost of acquiring the magnetic beads (bead cost), and the immobilization yield.

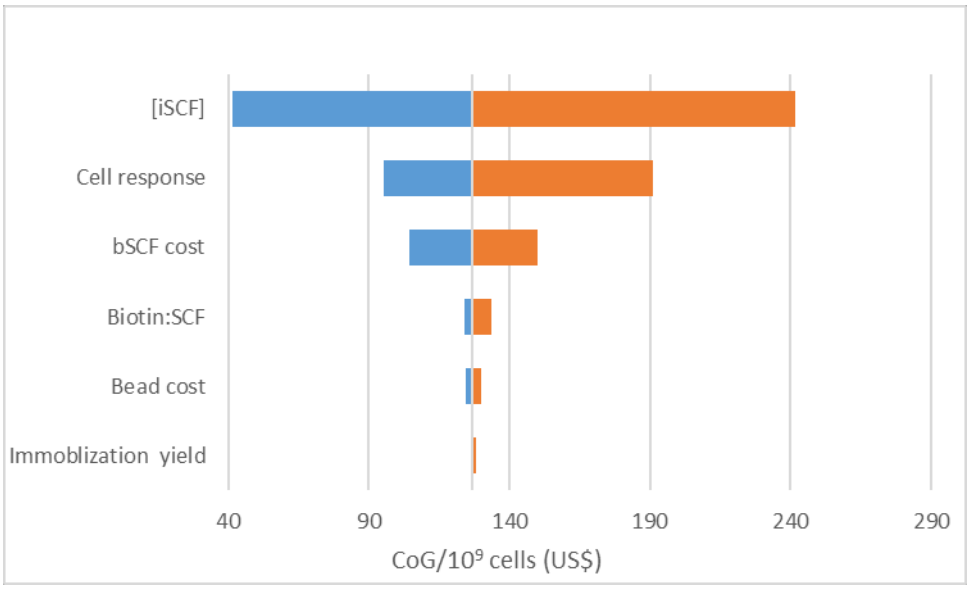


Figure 21. Sensitivity analysis of the impact the variables of interest have in the CoG.

A simulation was performed using the three top ranked variables according to the sensitivity analysis. For this simulation 100 random values between the maximum and minimum ranges of each values were assigned. As result 100 scenarios were

simulated, were the maximum CoG was USD$ 379.11/ 10^9 cells. In contrast the minimum CoG simulated was USD$41.29/ 10^9 cells. According to these data even the maximum CoG of using iSCF is considerably lower than the CoG of soluble SCF (USD$593.94/109 cells). Is important to consider that the acquisition cost of bSCF is lower than the acquisition cost of the SCF used to differentiate the $CD34^+$ cells into neutrophils. Material acquisition costs are determined by the supplier and may vary.

To analyze how the three factors impact the CoG a 3D surface response map and scatter dot cloud plot were generated with the previous data, they are displayed in **Figure 22**. Visual interpretation of **Figure 22** indicates that [iSCF] and cell response have a considerable impact over the CoG. Whereas the bSCF cost, color variable, has less impact over CoG. A linear regression considering [iSCF] and cell response as independent variables and CoG as dependent variable was performed. This regression obtained a correlation coefficient (R^2) of 0.92. These results suggest that concentration of immobilized SCF used for the culture and the number of cells produced by the culture are the principal variables to determine the CoG of using iSCF. In an optimal scenario, low concentrations of iSCF with high cell response will minimize the CoG for iSCF.

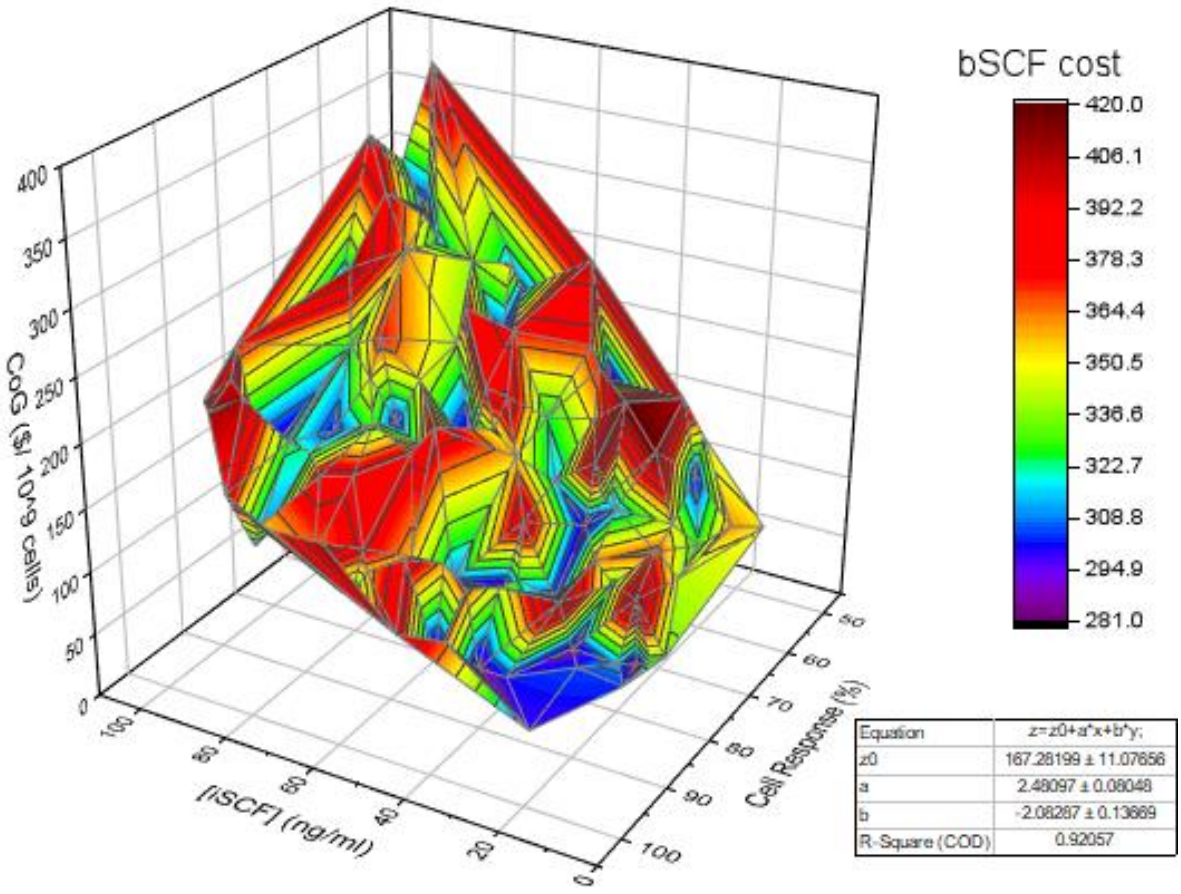


Figure 22. Surface response plot indicating how [iSCF], cell response, and bSCF cost affect the CoG for iSCF.

4. Discussion

Here an initial goal was to identify the impact of cryopreservation on human hematopoietic CD34+ cell yield and purity. The total number of isolated cells and the yield of enriched $CD34^+$ cells per MNCs processed were higher for those samples freshly processed compared to numbers obtained using the cryopreserved samples. In a similar way the purity of the collected $CD34^+$ fraction was almost 5 fold higher for the UCB samples that were processed immediately, on the same day of collection (94.5%), compared to the fractions collected from cryopreserved samples (18.4% and 20.7%). Interestingly, a consistent increment can be observed in the total number of $CD34^+$ cells and the yield of $CD34^+$ cells isolated/MNCs processed in freshly processed cells over cryopreserved cells, this increment was 6.97-fold and 25.3-fold respectively. This information indicates that cryopreservation and post-thaw $CD34^+$ isolation process has a negative impact in the number of cells that may be isolated by the enrichment process, additionally since the purity of the isolated cells is negatively affected the specificity of the process is also affected. The difference between both samples may be due to the loss of cells during the initial process of cryopreserving $CD34^+$ cells. Cryopreservation is associated to induce apoptosis in a significant number of $CD34^+$ cells isolated from UCB (Sparrow et al., 2006). Other isolation process involving cryopreserved human stem cells indicate that post-thaw isolation may affect the viability of human induced pluripotent stem cells (hPSCs) (van den Brink et al., 2020). The results in the present research concur with the results reported by Mata and collaborators (2019), in which a $CD34^+$ cell purity of 89.9% was obtained by freshly processing UCB using 2 MS columns. Cryopreservation may lead to lose up 60% of the initial total cell count. In contrast, different cryopreservation times do not appear to increase the number of $CD34^+$ cells lost (Owen et al., 2012). A different $CD34^+$ isolation process reported a 10% decrease in the purity of the isolated $CD34^+$ cells when the samples were processed after cryopreservation (Koizumi et al., 2000).

One of the specific objectives of the present dissertation was to establish *ex vivo* neutrophil differentiation culture protocols from human $CD34^+$ cells, using soluble growth factors. The results indicate that cells exhibiting a neutrophil phenotype, morphology, and bioactivity as evidenced by ROS production, could be produced by differentiating UCB $CD34^+$ cells in the laboratory. Furthermore, the present results indicate that the purity of the $CD34^+$ cells does not affect the phenotype of the *ex vivo* produced neutrophils. Whereas the final number of cells produced at the end of the culture was significantly lower when cryopreserved cells were used. This may be explained because cryopreserved $CD34^+$ cells exhibit a reduced clonogenic capacity (Zulkafli et al., 2016). Nevertheless the expansion in the number of cells produced by differentiating fresh cells after 15 days was of 3,264.88 ± 545.57, this fold-increase is within the order of magnitude reported by Timmins and collaborators (2009), 5,860 ± 1,890 fold increase, using the same protocol. The CD11b+/CD15 + population composition at day 15 between of both cultures produced was 48.1% for UCB and 54.5% for UCB, these results are consistent to what Timmins et al. (2009) reported, about 50% mature neutrophils, therefore, here we successfully replicated the phenotype obtained by the protocol stablished by Timmins et al., (2009) . More recent neutrophil differentiation protocols are focused in optimizing the expansion at initial stages of the cultures with aim to increase the overall number of cells produced. To confirm neutrophil like phenotype the expression of CD66b was evaluated with flow cytometry, at the end protocol 61.5% of the population was $CD66b^+$. Additionally cell morphology was evaluated by microscopy, the resulting cells exhibited multilobed nuclei (Jie et al., 2017).

The results of the *in silico* evaluations of immobilizing SCF to reduce CoG suggest that the optimal scenario would mainly be affected by two variables: the concentration of iSCF used to supplement the medium and the overall numbers of cells produced after 15 days in culture. Thus, the optimal scenario is composed by a using low concentrations of iSCF and cell response similar to a fold increase of 3,264. Recent research on SCF immobilization suggest that this strategy could

reduce the usage of SCF in a significant way. For example results reported by Worrallo and collaborators (2017) indicate that using iSCF at a concentration of 2.9 ng/mL is possible to produce an expansion cell response similar to the 65% of using soluble SCF at a concentration of 200 ng/mL. Additionally covalent immobilization of SCF may improve the stability of the GF during cell culture, resulting in improved cell proliferation (Mahadik et al., 2015). Both the cell used for cultures as the immobilization techniques used by these authors differ with the one initially stated by the present research. Therefore, the same results would not be expected. The obtained results are promising to considerably reduce the usage of SCF to differentiate $CD34^+$ cells to *ex vivo* produce neutrophils. The remaining four variables considered in the model; cost of acquiring the bSCF, the ratio between biotin and SCF, cost of acquiring the magnetic beads, and the immobilization yield; had a more discrete impact in the CoG. The possibility to reduce the production cost of *ex manufactured* neutrophils is critical to increase the economic feasibility of a cell therapy based on this approach. According to recent studies only 10% of the cell therapies would reach to phase 4 clinical studies. This reduced percentage may be explained by the high production costs as a limiting factor (Heathman et al., 2015).

5. Conclusions

After the analysis of the results obtained in the present research is important to highlight the following concluding remarks. First, freshly processed UCB resulted in purer fractions of $CD34^+$ cells obtained. Thus, a purer fraction will be translated to more cells isolated per CB. Additionally the yield of $CD34^+$ cells obtained per MNC was higher in freshly isolated samples. Therefore, to increase the number of $CD34^+$ is recommended to process UCB samples freshly. Second, it was possible to generate cells *ex vivo* that exhibit neutrophils´ phenotype, morphology, and ROS production by differentiating $CD34^+$ cells. The purity of the cultured $CD34^+$ cells did not have an effect on the phenotype of the obtained cells. In contrast, the purity of the cultured $CD34^+$ cells have proportional effect in the final number of cells obtained.

Those cultures with high purity produced more cells at the end of the differentiation process than those cultures with lower purity. Hence, high purity of $CD34^+$ cells is optimal to increase the final number of cells produced. Finally, according to our model, the most influential factors over the CoG of iSCF are the number of neutrophils *ex vivo* produced and the concentration of iSCF that would be used in the cultures. Optimal scenarios to decrease the CoG involve low concentrations of iSCF and high cell response.

6. Future investigation

The principal limitation for this research was the low number of UCB samples that could be collected and processed, which limit the interpretation of the current results. Future research is required to determine the impact that interpersonal variation between the samples have over the purity of isolated cells, the number of $CD34^+$ cells isolated, and the *ex vivo* produced neutrophils.

Future research also must investigate the process of SCF immobilization. These experiments are required to understand the immobilization kinetics and yield in order to optimize the equation designed to calculate the CoG for iSCF. Similarly, stability test profiles of immobilized SCF are required to evaluate the impact of immobilization would have over the activity, structure, and stability of the immobilized SCF.

Finally, further research is required to evaluate the possibility to immobilize different GFs, possibly onto the same matrix, and evaluate the impact the multiple immobilization would have on the phenotype and the overall number of cells produced. Future research involving immobilized SCF to induce $CD34^+$ cell differentiation to *ex vivo* produce neutrophils may take in consideration to the [iSCF] the quantity of cell produced. In order to reach optimal CoG reduction, low [iSCF] must be used, without reducing in a significant way the maximum number of cells produced.

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Curriculum Vitae

Pedro Antonio Robles Ovalle was born the 9th of April of 1995 in Torreon, Coahuila. He is the firstborn of Dr. Pedro Antonio Robles Trillo and Sandra Luz Ovalle Carrillo. His only sister is Xcaret Alejandra Robles Ovalle. He studied the undergraduate program of Biotechnology Engineering at Tencnologico de Monterrey, Campus Monterrey. He is specialized in molecular biology and was performed an internship at the university technology transfer office as intellectual property and patent passant in the department of biotechnology. He completed his undergraduate studies on May of 2018. On August of 2018 he was admitted in the Master in science specialized in biotechnology program at Tecnologico de Monterrey, Campus Monterrey. As a research member of the Engineered Cell Therapy Group, Pedro published one review paper in a peer-reviewed journal. This work represents the conclusion of his master´s program.

Appendix 1: Immobilization of Growth Factors for Cell Therapy Manufacturing

frontiers

Immobilization of Growth Factors for Cell Therapy Manufacturing

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Author contribution statement

DEO wrote sections 3, 4, 5 and 6 and developed Figure 1. PRO wrote sections 1, 2, 5 and 6. MB and KMD provided initial context and structure for this manuscript, wrote and edited all content. All authors provided critical feedback and approved the final version of the manuscript.

Keywords

cell therapy, growth factor, Immobilization, Stem Cell Factor, cost-of-goods, Cell product manufacturing Growth factor immobilization for cell therapy

Abstract

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Cell therapy products exhibit great therapeutic potential but come with a deterring price tag partly caused by their costly manufacturing processes. The development of strategies that lead to cost-effective cell production is key to expand the reach of cell therapies. Growth factors are critical culture media components required for the maintenance and differentiation of cells in culture and are widely employed in cell therapy manufacturing. However, they are expensive, and their common use in soluble form is often associated with decreased stability and bioactivity. Immobilization has emerged as a possible strategy to optimize growth factor use in cell culture. To date, several immobilization techniques have been reported for attaching growth factors onto a variety of biomaterials, but these have been focused on tissue engineering. This review briefly summarizes the current landscape of cell therapy manufacturing, before describing the types of chemistry that can be used to immobilize growth factors for cell culture. Emphasis is placed to identify strategies that could reduce growth factor usage and enhance bioactivity. Finally, we describe a case study for stem cell factor.

Contribution to the field

Cell therapies exhibit therapeutic potential for a range of life-threatening conditions including leukemias and congenital diseases. However, their expensive production processes translate into high price of sale for patients and health agencies. This bottleneck largely limits the widespread use of these innovative and promising treatments. Developing strategies for their cost-effective production is key to expand the reach of cell therapies. Growth factors are critical components of culture media, required for the maintenance and differentiation of cells in vitro, and have been evidenced as a major cost contributor to cell therapy manufacturing. Growth factor immobilization is emerging as a possible strategy to optimize growth factor use in cell culture which in turn will decrease cell production costs. To date, multiple immobilization techniques have been reported for attaching growth factors onto a variety of biomaterials, but most of these were investigated in the context of tissue engineering and adherent cells. The attached manuscript proposes a comprehensive review of the techniques that can be applied to immobilize growth factors for suspension cell culture and cell therapy manufacturing, detailing the specific chemistry used in each approach, and presenting a case study for Stem Cell Factor.

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Immobilization of Growth Factors for Cell Therapy Manufacturing

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Abstract

Cell therapy products exhibit great therapeutic potential but come with a deterring price tag partly caused by their costly manufacturing processes. The development of strategies that lead to cost-effective cell production is key to expand the reach of cell therapies. Growth factors are critical culture media components required for the maintenance and differentiation of cells in culture and are widely employed in cell therapy manufacturing. However, they are expensive, and their common use in soluble form is often associated with decreased stability and bioactivity. Immobilization has emerged as a possible strategy to optimize growth factor use in cell culture. To date, several immobilization techniques have been reported for attaching growth factors onto a variety of biomaterials, but these have been focused on tissue engineering. This review briefly summarizes the current landscape of cell therapy manufacturing, before describing the types of chemistry that can be used to immobilize growth factors for cell culture. Emphasis is placed to identify strategies that could reduce growth factor usage and enhance bioactivity. Finally, we describe a case study for stem cell factor.

Keywords: Cell therapy, Growth factor, Immobilization, Stem cell factor, Cost-of-goods, Cell product manufacturing.

1. Introduction

Growth factors (GFs) are signaling molecules that influence cell fate upon binding their cognate receptors. Their production is tightly regulated in healthy organisms, to specifically modulate cell physiology (Burgess, 2015). Once a GF binds its receptor, a signal transduction pathway initiates, leading to regulated gene expression, and the modulations in metabolism, protein synthesis, ion fluxes and cytoskeleton organization responsible for the resulting phenotype at the cellular level (Lee et al., 2011). GFs may stimulate various biological pathways such as proliferation, differentiation, and survival (Fortier et al., 2011; Cross and Dexter, 1991). The effect of a GF is strongly influenced by its concentration, the nature of the target cell, the presence of co-stimuli, and the existence of said GF as a soluble molecule, or immobilized, embedded in the extracellular matrix (ECM) (Cross and Dexter, 1991; Pan et al., 2002). For example tumor necrosis factor α (TNF-α) exists either as a soluble or immobilized molecule *in vivo*, which was shown to define its role as selectively supporting or inhibiting tumor growth and survival in various tumor cell lines (Ardestani et al., 2013).

Due to the determinant role of GFs in cell fate, most culture protocols require their addition to culture media, to date mostly as soluble molecules, to produce cells of specific phenotypes for research or for therapeutic use. In this context, the use of GFs represents a major cost contributor in the production of cell therapies (Torres-Acosta et al., 2019; Kirouac and Zandstra, 2008). The recent clinical success of cell therapies, such as chimeric-antigen receptor (CAR) T cell therapies, combined with their costly production and the realization that GFs hugely contribute to this cost, evidenced a strong need to develop strategies to reduce GF usage (Torres-Acosta et al., 2019).

GFs used in soluble form exhibit accelerated depletion from culture media through low stability and bioactivity, which calls for constant replenishing. Immobilizing GFs avoids their internalization post-receptor binding, which may enhance or preserve biological activity (Chen et al., 1997; Ito et al., 2001). Immobilization may allow spatial- and time-controlled presentation to target cells, for example when bound to the surface of magnetic particles. This feature could additionally open a window of opportunity for reuse (Worrallo et al., 2017; Samorezov and Alsberg, 2015; Zandstra et al., 1997). Immobilization of GFs may lead to their use in overall smaller quantities in cell culture, effectively decreasing cell production costs. However, techniques used to immobilize GFs are varied and choosing the most appropriate chemistry for a particular protein under a specific use is not trivial.

To date, the majority of the literature has focused on the production of immobilized GF (iGF) for tissue engineering applications (Reed and Wu, 2014; Z. Wang et al., 2017; Hajimiri et al., 2015; Cabanas-Danés et al., 2014; Lee et al., 2011; Atienza-Roca et al., 2018). Therefore, this review aims to discuss general approaches to immobilize GFs in the context of cell therapy manufacturing. While tissue engineering usually involves the culture of adherent cells, cell therapy production deals with single cell suspensions at very large scale, usually in bioreactors (van den Bos et al., 2014; Pigeau et al., 2018). Here, we summarize the clinical and economic relevance of cell therapies and describe pertinent cell therapy manufacture bioprocesses to evidence areas of opportunity for the use of iGF. Then we describe the chemistry of current relevant strategies used

to immobilize GFs and discuss their selectivity, bioactivity and release. Finally, we present the case of stem cell factor (SCF), a relevant GF used in stem cell research.

2. Cost of cell therapy development and manufacturing: flipside of the coin

In contrast to drugs and bioactive molecules, cells respond dynamically to stimuli and tailor their phenotype *in vivo*, which gives cell therapies a possible curative edge for medical conditions exhibiting poor response rates using conventional medicines (de Wilde et al., 2016). Encouraging clinical trials launched multiple cell therapies as novel alternatives to treat life threatening conditions including neurodegenerative and autoimmune diseases, cancer, congenital diseases, and metabolic disorders (de Wilde et al., 2016; Mason et al., 2011). Between 2004 and 2014, European countries have welcomed testing of 198 cell and gene-based therapies in clinical trials (de Wilde et al., 2016). The significant increase in number of clinical trials of cell-based therapies in recent years highlight the relevance of these treatments in the future of global health care.

The first *ex vivo* stem cell-based therapy to receive regulatory approval, Strimvelis, treats the rare congenital disease adenosine deaminase severe combined immunodeficiency (ADA-SCID) (Stirnadel-Farrant et al., 2018). Strimvelis is based on the modification of autologous $CD34^+$ cells through *ex vivo* retroviral transduction, to express functional adenosine deaminase. Modified cells are infused back into the patient as a one-time injection of ≥ 4 million $CD34^+$ cells/kg, homing to the bone marrow and repopulating healthy blood cells, in theory for the lifetime of the patient (European Medicines Agency, 2016). This therapy has demonstrated a 100% survival rate up to 7 years post-injection (European Medicines Agency 2016). In 2018, Strimvelis treatment cost was €594,000 (Stirnadel-Farrant et al., 2018).

Another example of a current FDA and EMA-approved therapy is Sipuleucel-T. This therapy is aimed to treat castration-resistant prostate cancer. Sipuleucel-T is produced by culturing the patient's own antigen presenting cells (APCs) such as monocytes and others, together with PA2024, a recombinant protein combining the antigen prostatic acid phosphatase to GF granulocyte-macrophage colony stimulating factor (GM-CSF). Exposing APCs to this combination of GF/antigen results in cell activation against cancer antigens, prompting a targeted anti-cancer response in the patient (Pieczonka et al., 2015). In 2011, Sipuleucel-T was commercialized by Dendreon (under the name Provenge), with a dose cost of US$169,206 (Shukla et al., 2019).

While APCs re-educate the immune system of the patient *in vivo* as seen with Sipuleucel-T, it is now possible to engineer *ex vivo* a patient's T lymphocytes to readily recognize and eliminate cancer cells. Tisangenlecleucel (Kymriah) is an FDA approved therapy based on chimeric antigen receptors (CAR) recombinantly expressed within a patient's own T lymphocytes cell membranes. These engineered CAR-T cells have shown success in treating acute lymphoblastic leukemia (ALL) in pediatric patients by targeting the CD19 antigen expressed by malignant B cells (Vairy et al., 2018). Tisangenlecleucel is manufactured by viral transduction of $CD3^+$ lymphocytes enriched post-leukapheresis. Clinical trials evidenced 81% of patients in remission at 3 months follow up, from which 60% showed complete remission (Maude et al., 2018). However, the current

therapy costs US$475,000[1]. A similar CAR T-cell therapy, Axicabtagene ciloleucel commercialized by Kite Pharma under the name Yescarta also targets CD19 together with a CD28 co-stimulation (Jain et al., 2018). Yescarta is produced using retroviral transduction and specifically targets non-pediatric patients with diffuse large B-cell lymphoma and non-Hodgkin lymphoma (European Medicines Agency 2014; Food and Drug Administration and Center for Biologics Evaluation and Research, 2017). The treatment with Yescarta increased 9.5 years life expectancy, versus 2.6 years using conventional chemotherapy treatments. In 2018, Yescarta costed US$522,921 per treatment (Roth et al., 2018).

Despite the superior curative potential of cell therapies, their availability is strongly limited by their price tag. Only 10% of therapies currently in Phase I clinical trial will reach stage 4 (Heathman et al., 2015). In addition to safety and efficacy considerations for pursuing the implementation of a specific cell therapy, high production costs is a known deterrent for manufacturers (Heathman et al., 2015). Technoeconomic and cost-benefit analyses have emerged to determine whether these therapies are rationally implementable. The manufacturing process comprises ~90% of the total investment destined to develop a novel cell therapy (Vormittag et al., 2018). In both autologous and allogenic cell therapy manufacturing, the process comprises all steps post cell-sourcing, including washes, cell activation and proliferation, final product formulation, and quality controls (Vormittag et al., 2018). Amongst the factors that contribute to these manufacture costs, the purchase of materials necessary for cell culture, such as culture medium and supplements, are listed as strong cost contributors (Lipsitz et al., 2017; Torres-Acosta et al., 2019). For example, in the context of induced pluripotent stem cells which are a promising player in the cell therapy field, GFs constitute the majority of essential components promoting cell growth, with 4 out of 6 components of the "essential medium" E8 being GFs (Chen et al., 2011). Efforts are needed to optimize manufacturing processes in order to reduce cost of goods and boost accessibility to patients. One approach is the development and implementation of automation, to reduce labor costs (Heathman et al., 2015). Another alternative is the immobilization of GFs. As immobilization improves GF stability and prevents degradation, immobilized GFs (iGFs) may lead to their use in decreased quantities in comparison with cultures using their soluble counterpart (Worrallo et al., 2017).

Optimizing cell manufacturing is essential to the commercial success of cell therapies. Immobilizing GFs promises to significantly reduce GF usage and consequently, manufacturing costs (Lotz et al., 2013; Worrallo et al., 2017). In addition to economic saving, GF immobilization may induce enhanced signaling, evidenced by increased cell bioactivity (Kitajima et al., 2007; Boucher et al., 2010; Yang et al., 2012; Budiraharjo et al., 2013; Lotz et al., 2013; Kumorek et al., 2015). While to the best of our knowledge GF immobilization is not currently used to produce cell therapies, the immobilization of other media components has been implemented, providing a proof-of-concept for feasibility and implementation. Dynabeads are magnetic polymer particles which surface is covered with covalently immobilized antibodies. In clinically implemented protocols, Dynabeads are used as a culture media supplement to expand T cells through CD3 and CD28 antibody-mediated activation (Neurauter et al., 2007). Implementing cost-effective

[1] Herper, M. (2018). Patient Advocate Says Novartis' $475,000 Breakthrough Should Cost Just $160,000. Forbes U.S. Edition. Available at: https://www.forbes.com/sites/matthewherper/2018/02/08/patient-advocate-says-novartis-475000-breakthrough-should-cost-just-160000/#1cc41805152f

technologies that contribute to enhance and simplify production processes will become critical for cell therapy manufacturing at a relatively low cost (Wang and Rivière, 2016).

3. Methods for GFs immobilization

Current protocols for cell production involve frequent feeding with soluble GFs to control cell proliferation and phenotype. The use of iGFs is an attractive approach for cell production processes, as it offers various advantages. The presentation of GFs in an immobilized form allows receptor binding and activation at the plasma membrane, but prevents GF internalization and intracellular recycling, resulting in sustained cell stimulation (Chen et al., 1997; Ichinose et al., 2006; Rodrigues et al., 2013; Kim et al., 2016b). Of note, some physiological responses are positively regulated through receptor-ligand endocytosis. In such instances where endocytosis is necessary for the desired phenotype to be achieved, soluble GFs should be used (Ceresa, 2011). GF immobilization also improves GF stability, mimics a physiological ECM-bound presentation of ligands often occurring *in vivo,* and enables GF reuse without loss of bioactivity (Mizumachi and Ijima, 2013; Mao et al., 2017; Wang et al., 2020). Production systems incorporating iGFs have been developed for cells cultured in adherence and in suspension with the overall aim to improve the efficiency of culture processes (Rahman et al., 2010; Yang et al., 2012; Lotz et al., 2013; Mao et al., 2017; Worrallo et al., 2017). Usually adherent cells are cultured on surfaces incorporating immobilized GFs, and because of the relative ease of GF immobilization in these conditions, the majority of reports detail such protocols. More recently, cells growing in suspension have been cultured in contact with iGF, using a variety of approaches. For instance, GFs may be immobilized onto magnetic beads, or encapsulated, prior to their addition to the culture media (Lotz et al., 2013; Worrallo et al., 2017). Of note, adherent cells can be cultured in suspension through encapsulation within GF immobilized matrices (Mao et al., 2017). This is highly relevant for cell production processes as culturing cells in suspension increases real estate available in a defined culture vessel. Producing cells in suspension with the incorporation of iGF is an attractive additional optimization over current processes, which we propose to discuss in this review.

Two approaches can be used to immobilize GFs into surfaces: chemical and physical interactions. The former relies on attaching GFs through covalent or non-covalent bonds directly to the substrate surface or to molecules that are used as linkers between the immobilizing surface and the GF. The latter approach involves the entrapment or adsorption of GFs into a substrate, allowing a diffusion-based release. The nature of cells being produced dictates GF requirements, and a single GF or a combination of various GF may be necessary to produce the desired cell phenotype and quantity. Most available reports have focused on immobilizing a single GF to be investigated as soluble or immobilized in a complex medium. Nonetheless, the functionalization of different surfaces with multiple GFs has also been implemented using both chemical and physical approaches (Stefonek-Puccinelli and Masters, 2008; Shah et al., 2011; Banks et al., 2014; Lequoy et al., 2016; Mao et al., 2017; Cheng et al., 2019). Importantly, the synergistic or antagonistic effect that co-immobilized GFs may have on bioactivity strongly depends on the nature of GFs and their concentrations in culture, so that no generalization can be made about the immobilization of more than a single GF (Stefonek-Puccinelli and Masters, 2008; Banks et al., 2014; Mao et al., 2017).

3. Physical immobilization of GFs

Physical immobilization is technically the simplest method to immobilize a GF, and is commonly used for tissue engineering applications, specifically for bone regeneration purposes (Jensen et al., 2015; Ma et al., 2015a; Nyberg et al., 2016; Hettiaratchi et al., 2017; Schumacher et al., 2017). It is commonly achieved by adding a determined number of GF into a polymer matrix before its gelatinization. There are three different approaches for performing GFs physical immobilization (Figure 1). Advantages of GF physical immobilization involve technical accessibility, low cost of reagents and preservation of bioactivity of the iGF. Furthermore, Hydrogels used for GF immobilization by physical immobilization are suitable for cell scaffolding. However, poor spatial distribution and control over release are obtained using this method, explaining current efforts using different methods. Despite these drawbacks, physical immobilization remains a common method for achieving GF immobilization.

3.1. Physical encapsulation

Physical encapsulation is based on the immobilization of the GF into a matrix or scaffold. The interaction between the GF and the selected material for entrapment relies on hydrophobic, hydrophilic-hydrophilic and electrostatic interactions (Lee et al., 2011). A wide variety of GFs have been immobilized using physical encapsulation for tissue engineering applications, including vascular endothelial growth factor (VEGF), GM-CSF, bone morphogenetic protein-2 (BMP-2) and insulin-like growth factor (IGF-1) (Rocha et al., 2008; Ali et al., 2009; Abbah et al., 2012; Hameed et al., 2019). Moreover, this technique enables the culture of non-adherent cells with iGFs, since GFs can be encapsulated and added to suspension culture systems, such as flasks and bioreactors. To our knowledge, only one study has employed this particular immobilization method for the optimization of a cell production process (Lotz et al., 2013). The encapsulation of fibroblast growth factor-2 (FGF-2) into PLGA microspheres improved human embryonic stem cells (hESCs) phenotype and culture conditions. After 72 hours of culture, the levels of FGF-2 released from the microspheres remained almost unchanged. Whereas, the levels of soluble FGF-2 decayed by 90%. In the cultures in which encapsulated FGF-2 was employed, an increase in expression of pluripotency markers was observed. Additionally, stem cell spontaneous differentiation was reduced significantly when cells were cultured with FGF-2 beads instead of soluble FGF-2. The controlled release of FGF-2 decreased the frequency of medium changes necessary to maintain hESCs culture from daily to biweekly feeding. These results demonstrate that the use of encapsulated FGF-2 is an effective method to lower consumable costs and labor required to maintain hESCs.

In another study, Lee et al. (2004) incorporated transforming growth factor-beta-1 (TGF-β1) loaded microspheres into porous chitosan scaffolds to support chondrogenesis (Lee et al., 2004). Encapsulated TGF-β1 promoted cartilage regeneration by significantly promoting chondrocyte adhesion, proliferation and glycosaminoglycan production compared to scaffolds without TGF-β1. Additionally, the chitosan scaffolds facilitated controlled release of TGF-β1, with an initial burst effect that stabilized after 3 days, and by day 7, 44.8% of the initial loading was released. These results indicate that encapsulation is an effective approach to control the release of GFs while maintaining their bioactivity.

Due to its biocompatibility and biomechanical properties, alginate is widely used for GF and cell encapsulation in tissue engineering and regenerative medicine applications (Hwang et al., 2009). Choi et al. (2010) developed a GF delivery system based on microcapsules made of poly(lactic-co-glycolic acid) (PLGA) and alginate (Choi et al., 2010). BMP-2 and dexamethasone were loaded into the microcapsules to facilitate osteogenic differentiation of rate bone marrow stromal cells (BMSCs). PLGA-Alginate microcapsules retained approximately 40% of the loaded GF after 30 days. BMSCs cultured with BMP-2-loaded microcapsules showed greater expression levels of osteogenic markers, such as collagen type I, osteopontin, ALP, and osteocalcin, compared to cells cultured with microcapsules lacking GF. Therefore, PLGA and alginate microcapsules are a delivery system of relevance, able to induce osteogenic differentiation in BMSCs when loaded with a GF. In another study, VEGF was encapsulated in calcium-alginate beads, where loading efficiency could reach 97% under optimized conditions. Further, a constant rate of 6 ng of GF/ml/day could be achieved and sustained for 14 days (Gu et al. 2004).

Nanoparticles have recently gained prominence as advanced drug delivery systems in the biotechnology and pharmaceutical industries. In the context of GF immobilization, nanoparticles can be loaded with GFs, facilitating controlled delivery (Wang et al., 2017). For instance, VEGF was loaded into dermatan sulfate sodium salt-poly-l-lysine (DS-PLL) and gum tragacanth-poly-l-lysine (GT-PLL) nanoparticles with loading efficiencies of 93.1% and 80.2% respectively (Zandi et al., 2020). Nanoparticles -immobilized VEGF induced higher proliferation compared to soluble VEGF in HUVEC culture. Other GFs, including BMP-2, epidermal growth factor (EGF), FGF, insulin-like growth factor-1 (IGF-1) and TGF-β1, have been immobilized to nanoparticles to control spatial presentation in tissue engineering and regenerative medicine (Matsuo et al., 2003; Rajam et al., 2011; Ertan et al., 2013; Wang et al., 2016).

3.2. Surface adsorption

GF immobilization through surface adsorption is one of the simplest methods to generate functionalized matrices for biomedical purposes. It involves the use of a biocompatible matrix monolayer, with a GF attached to it. Depending on the GF and matrix material, the binding of the GF to the matrix will not only depend on electrostatic interactions, but also on hydrophobic ones (Luginbuehl et al., 2004). The adsorption of GF to the matrices may be modified by utilizing different types of materials with particular properties. The properties of materials and solutions used to immobilize the GF can be tuned to control adsorption (King and Krebsbach, 2012). Some of the disadvantages of this approach are low loading efficiency, poor control over release and minimum spatial control (Midy et al., 1998; Budiraharjo et al., 2013; Wang et al., 2017). This approach has been utilized to immobilize different types of GFs, including BMP-2, FGF and FGF-2 (Ziegler et al., 2008; Budiraharjo et al., 2013).

3.3. Layer-by-layer (LbL) immobilization

In order to address poor control over release problem present in surface adsorption, LbL immobilization represents an alternative with improved spatial distribution and control over release the adsorbed GF. LbL is based in the incorporation of several layers of matrix with GF adsorbed on it. This immobilization strategy depends mainly on electrostatic interactions between GFs and oppositely charged electrolytes, although, hydrophobic interactions and hydrogen bonds

are also present (Zhang et al., 2015; Wang et al., 2017). In addition to improving release control and spatial distribution, LbL is a simple and inexpensive technique. The efficiency of LbL does not depend on the GF size/shape, so multiple GFs can be attached by optimizing the architecture design (Gomes et al., 2015).

LbL is widely used for neural and cardiac tissue repair, bone regeneration, and wound healing approaches (Kulkarni et al., 2014; Lynam et al., 2015; Amano et al., 2016; Guduric et al., 2017; Liu et al., 2017; Mandapalli et al., 2017). As an example of this type of immobilization, Naves et al. (2016) created a LbL architecture based on poly(ethylene imine) (PEI) in combination with heparin and chitosan to immobilize acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF), respectively (Naves et al., 2016). The rate of GF release is inversely correlated to the number of layers in the matrix. Interestingly, increasing amounts of adsorbed GF lead to increased stability and lower release, and this phenotype has more impact in architectures of lesser layers. For both aFGF and bFGF a 6-bilayer architecture resulted significantly more stable than a 3-bilayer one. These results coincide with the release profile for bFGF for both 6 and 3 bilayer architecture, where after 14 days 0.4 ng/ml and 0.8 ng/ml of GF were released respectively. Both results indicate that 3 bilayer substrates can release GF faster than a 6 bilayer one, due to its lower stability. Regarding GF *in vitro* bioactivity; NIH 3T3 mouse fibroblast were used in culture. Although proliferation assays showed that both 3 and 6 bilayer substrates induced proliferation, it was the 3-bilayer architecture which significantly enhanced fibroblast proliferation, after the 14-day culture. Final cell count for aFGF 3 bilayer substrate was 1.8 times higher than the response achieved by the aFGF 6 bilayer substrate. These results can be easily explained due to the lower stability for 3 bilayers; hence this architecture will release a greater amount of GF over time, therefore enhancing cell proliferation. Additionally, long-term bioactivity for immobilized aFGF and bFGF was tested. Although cell proliferation was induced, it was not enhanced. Hence, bioactivity is maintained but not increased, this long-term activity can be attributed to the multilayer architecture protecting the GFs and preventing their denaturalization.

In another study, FGF-2 was immobilized into a 5 polyelectrolyte bilayer architecture, and used as substrate for cell culture (Ding et al., 2018). Polyelectrolytes used to adsorb FGF-2 were poly methacrylic acid (PMAA) and poly L-histidine hydrochloride (PLH), forming the resulting FGF-2(PMAA/ PHL)5 architecture. FGF-2 (PMAA/PHL)5 resulted in a constant release of the GF. In consequence fibroblast proliferation was slightly improved, when compared with GF added in a single dose. Kumorek et al. (2015) also utilized a LbL approach for immobilizing FGF-2, utilizing an albumin/heparin (Alb/Hep) 2 bilayer architecture. Before GF adsorption, the LbL was crosslinked covalently with glutaraldehyde, to stabilize the LbL substrate. To asses immobilized GF bioactivity, FGF-2 dependent cells were cultured in tissue culture plates coated with FGF-2 (Alb/Hep) 2 bilayer for 7 days. Results indicated that functionalized FGF-2 (Alb/Hep)2 surfaces enhanced proliferation and cell differentiation. Taken together, the results of the previously mentioned studies demonstrate that LbL is a simple and effective approach to achieve consistent release of GFs, opening the possibility to reduce costs and maintenancec of adherent cells culture.

4.1. Chemical immobilization of GFs

4.1.1. Covalent approaches for GFs tethering

Covalent immobilization of GFs to biomaterials usually engages functional groups in GFs for immobilizing them onto a surface. A major concern is that these functional groups may be near to the GF active site, affecting bioactivity when the GF is immobilized (Leipzig et al., 2010). In this type of immobilization GFs are attached onto the matrix strongly and irreversibly. Covalent binding of GFs to surfaces is required when these cannot be adsorbed onto a substrate surface, or when a gradual release from the substrate is necessary. An overview of covalent immobilization methods is presented in Figure 2.

4.1.1.1. Carbodiimide coupling immobilization

Carbodiimide coupling represents one of the most common methods for covalently attaching GFs onto substrate surfaces. Carbodiimides are cross-linking agents that mediate the reaction between amine groups and carboxylic acids to form amide linkages. Among the several carbodiimide reagents available, 1-ethyl3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) is the most used for bioconjugation processes. The conjugation reaction proceeds by the generation of an active *o*-acylisourea intermediate, which then reacts with a nucleophile such as a primary amine for amide bond formation. Unfortunately, *o*-acylisourea can hydrolyze in aqueous solutions, regenerate the carboxylic groups and fail to bind the target GF, decreasing the reaction efficiency. In order to prevent this reverse reaction, carbodiimides are mostly coupled with N-hydroxysuccinimide (NHS). NHS reacts with *o*-acylisourea and EDC to form a carboxyl-NHS ester, which is more stable in aqueous solutions, minimizing susceptibility to hydrolysis (Hermanson, 2008). In addition to linking GFs to the substrate, EDC may bind substrate molecules between themselves, resulting in a lower GF loading efficiency (Chiu et al., 2011). This problem can be attenuated by performing a step immobilization process: EDC activation of the substrate is performed first, followed by addition of GFs (Chiu et al., 2011).

Carbodiimide coupling may engage the amine groups in the GF lysine residues or N-terminus, as well as the carboxylic groups in the GF aspartate or glutamate residues or C-terminus. Due to this lack of specificity, the possible engagement of bioactive functional groups in bond formation may cause a GF to lose its bioactivity (Nakaji-Hirabayashi et al., 2007). Likewise, the presence of multiple functional groups, causes GF immobilization in a random orientation, which consequently affects epitope availability for the cognate cell receptors (Masters, 2011).

As an example, Psarra et al. (2015) reacted hepatocyte growth factor (HGF) and basic fibroblastic growth factor (bFGF) amine groups with the carboxylic groups on poly(acrylic) acid (PAA) brushes to achieve immobilization through carbodiimide coupling (Psarra et al., 2015). The effect of GF functionalized PAA brushes was investigated on different cell lines. In a human hepatoma cell line (HepG2), covalently immobilized HGF demonstrated higher bioactivity than the soluble GF with a concentration 10 times lower, as well as higher proliferation inhibition. Additionally, the differentiation of mouse embryonic stem cells (mESCs) towards endoderm was evaluated. The immobilized bFGF showed three times higher expression of endoderm differentiation genes in comparison to the control. In another study, the carboxylic groups of either BMP-2 or fibroblast growth factor-2 (FGF-2) were used to form covalent bonds with amine groups of chitosan films and, immobilize each GF using EDC/NHS chemistry for osteogenesis and wound healing (Budiraharjo et al., 2013). For BMP-2 and FGF-2, a loading efficiency of 64 and 50% was obtained, respectively. Regarding bioactivity, immobilized BMP-2 stimulated osteoblasts

proliferation, differentiation and attachment of osteoblasts in a greater extent than the adsorbed GF. While the immobilized FGF-2 induced higher fibroblast attachment, proliferation and collagen synthesis than the adsorbed GF.

4.1.1.2. Thiol coupling immobilization

One strategy in covalent immobilization uses thiol-reactive groups able to couple with thiol-containing molecules. Most of these thiol-reactive groups conjugate free thiol groups by one of two reactions: alkylation or disulfide exchange. Once initiated, these reactions generate either thioether bonds or disulfide bonds, respectively. In general, this technique involves the reaction of free thiols in the GF with thiol-reactive groups in the substrate surface. In GFs, free thiol groups exist in cysteine residues, but may also be introduced chemically or with recombinant technology (Chen et al., 2014; Place et al., 2012; Riahi et al., 2017). Functionalized GFs react with free thiols on the substrate surface, leading to their immobilization.

GFs have been prevalently immobilized through maleimides and, to a lesser extent, through vinyl sulfones, which in addition to reacting with thiol groups can also bind secondary targets such as amine and hydroxyl groups (Rahman et al., 2010; Chen et al., 2014; Zisch et al., 2003; Ichinose et al., 2006; He et al., 2011). Their selectivity depends on the pH of the reaction medium and the nucleophilic characteristic of target groups (Javier et al., 2012; Hermanson, 2008). Zisch *et al.* (2003) used polyethylene glycol (PEG) divinyl sulfone-functionalized hydrogels to immobilize VEGF, an Arginylglycylaspartic acid (RGD) peptide and a metalloproteinase (MMP) substrate peptide (Zisch et al., 2003). Functionalized hydrogels induced angiogenesis, promoted human endothelial cells (HUVECs), adhesion and migration by cell-associated MMPs. Two VEGF variants were selectively immobilized, containing either no reaction site or two unpaired cysteines. Loading efficiency of the variants was surprisingly similar in both cases and close to 80%. This was suggested a result of a huge stoichiometric excess of PEG divinyl-sulfone, inducing the reaction of VEGF amine groups and PEG divinyl-sulfone, incorporating the GF into the hydrogels. Regarding bioactivity, both immobilized VEGF variants showed preserved functionality by promoting migration of HUVECs, inducing angiogenesis and vascularized tissue formation.

VEGF has also been immobilized onto functionalized agarose gels to control blood progenitor cell generation from mESCs aggregates (Rahman et al., 2010). To facilitated binding, VEGF was modified with maleimide reactive groups. To test the efficiency of immobilized VEGF, mESCs aggregates were encapsulated within VEGF immobilized agarose and cultured for 7 days as free-floating aggregates to assess the efficiency of immobilized VEGF in generating the desired phenotype. Immobilized VEGF was 75 times more efficient in inducing of mesodermal markers, brachyury and VEGF receptor 2, than its soluble counterpart by day 4. After 7 days, $CD34^+$ and $CD41^+$ expression, and generation of blood colony forming cells were 108 and 23 times higher. These results suggest that immobilizing GFs in a cell-hydrogel culture system is effective to enhance blood cell production, although large scale implementations remain to be tested.

Other thiol reactive groups, such as allyl ethers and norbornenes, may also be used for immobilizing GFs onto surfaces, as demonstrated with other proteins and peptides (Wittrock et al., 2007; Van Hove et al., 2014). Nevertheless, the selection of a specific reaction should be done on the basis of reaction conditions compatibility with GF functionality. For instance, the presence of

by-products, required pH conditions, the use of organometallic catalysts and stability of the linkages formed should be taken into account (Javier et al., 2012).

4.1.1.3. Light-induced immobilization

Light can induce covalent immobilization of GFs to a substrate. This is based on the use of photoreactive groups, which are converted into highly reactive species covalently binding target molecules upon light exposure (Kawamoto et al., 2018; Hermanson, 2008). Usually, GFs are conjugated to a photoreactive group, then attached to the substrate surface upon exposure to a specific wavelength. Alternatively, substrate surfaces may be functionalized with photoreactive groups, which in turn react with GFs for immobilizing them into the surface. Light-induced immobilization facilitates spatial and temporal control over the immobilization process since the reaction is limited by light exposure parameters. However, light exposure may alter GF bioactivity due to stereochemical alteration, conformational changes, aggregation and fragmentation which are protein specific (Pattison et al., 2012). These drawbacks may be avoided by reducing exposure time and using long-wavelength UV light (Masters, 2011).

Several photoreactive groups are available for functionalizing either GFs or substrate surfaces, including phenyl azide, benzophenone, anthraquinone, diazo compounds, and diazirine derivatives. Of these groups, phenyl azide has an important advantage: a low energy of activation, allowing short light exposure at higher-energy UV wavelengths, which avoids potential damage of photosensitive biomolecules and cells (Hermanson, 2008). When exposed to UV light, phenyl azide groups form nitrene groups, that undergo addition reactions with double bonds, insertion into carbon-hydrogen and nitrogen-hydrogen sites or subsequent ring expansion with amine groups and aliphatic compounds (Kawamoto et al., 2018). As an example of GF attaching via phenyl azide, Stefonek-Puccinelli and Masters (2008) conjugated EGF and IGF-1 to a crosslinker containing a phenyl azide group for immobilizing these GFs to polystyrene plates upon UV radiation (Stefonek-Puccinelli and Masters, 2008). Results showed that when GFs were immobilized individually, they stimulated keratinocyte migration in a greater extent than unmodified polystyrene plates. Interestingly, when both GFs were co-immobilized in the plates, they enhanced migration beyond levels achieved using individually immobilized GFs.

Another photoreactive group efficiently used for GF immobilization is benzophenone. In this approach, surfaces are conjugated with benzophenone, that upon UV light excitation forms a transient diradical that reacts with carbon-hydrogen sites from the GF to form a carbon-carbon covalent bond (Hermanson, 2008; Martin et al., 2011; Gomez et al., 2007). Unlike phenyl azide, unreacted benzophenone can be re-induced to an active state with subsequent UV exposure (Hermanson, 2008). Banks et al. (2014) conjugated photoreactive benzophenone to collagen-glycosaminoglycan (CG) scaffolds for immobilizing BMP-2 and platelet-derived growth factor-BB (PDGF-BB) either individually or together onto the same surface (Banks et al., 2014). Results indicated that the metabolic activity and proliferation of adipose-derived mesenchymal stem cells (ASCs) was impacted by immobilized PDGF-BB but not by BMP-2. Moreover, collagen 1 gene expression was downregulated with immobilized PDGF-BB, however it was strongly increased with the presence of BMP-2 alone or with PDGF-BB.

A commonly used photoreactive group for polymer covalent crosslinking is acrylate. The crosslinking of acrylate containing polymers is initiated by the generation of radicals usually formed from the photo-cleavage of initiator molecules. These radicals react with the unreacted carbon-carbon double bonds of the acrylated biomaterial resulting in covalently crosslinked polyacrylate chains (Bowman and Kloxin, 2008; Lin and Anseth, 2009). The same acrylate groups used for polymers photo-crosslinking are also potential sites for covalently immobilizing GFs as demonstrated with stem cell factor (SCF), basic fibroblastic growth factor (bFGF), VEGF and PDGF-BB (Leslie-Barbick et al., 2009; Mahadik et al., 2015; Saik et al., 2011; DeLong et al., 2005). In this method, the primary amine group of the GF is usually attached to a linker (typically PEG) which contains an acrylate group that in turn reacts with an acrylated substrate for conjugation of the GF.

Another common method for polymer crosslinking, specifically collagen, involves using riboflavin as a photoreactive group. Riboflavin (vitamin B2) is mainly used in ophthalmic and tissue engineering applications to enhance corneal strength and tailor mechanical properties of collagen constructs (Rich et al., 2014; Tirella et al., 2012; Whitcup and Azar, 2017). Recently, it has also been used for crosslinking GFs, such as EGF, bFGF and TGF-β1, onto collagen-based biomaterials (Bertolo et al., 2015; Fernandes-Cunha et al., 2017). During exposure to UV light, oxygen species are released from the carboxylic groups of riboflavin, leading to the generation of light-activated riboflavin and single reactive oxygens. These highly reactive molecules then induce the formation of covalent bonds by reacting with the amino acids from the GF and collagen (Whitcup and Azar 2017; Rich et al., 2014). It has been suggested that possible vulnerable amino acids to photochemical crosslinking using riboflavin include tyrosine, histidine, cysteine and methionine (Rich et al., 2014). Fernandes-Cunha et al. (2017) demonstrated that when EGF is crosslinked to collagen surfaces using riboflavin and blue light exposure, the histidine residues of this GF are engaged in the immobilization process (Fernandes-Cunha et al., 2017). The photo-immobilized EGF maintained its bioactivity by enhancing the proliferation and spreading of corneal epithelial cells (CECs). Additionally, modified surfaces resulted cytocompatible and the photo-crosslinking reaction was not harmful to cells by preserving viability at values near 100%.

A primary advantage of light-induced immobilization over other covalent methods is that allows spatial and temporal control of the immobilization process. This characteristic has been exploited to create patterns of immobilized GFs onto two dimension surfaces and three dimension scaffolds by using UV light and photomasks (Stefonek-Puccinelli and Masters, 2008; Alsop et al., 2014; Banks et al., 2014; Saik et al., 2011). These patterns may provide a specialized effect of GFs in cellular functioning, since *in vivo,* soluble and ECM-bound biomolecules exist in gradient patterns that guide growth, migration, and differentiation of cells in a wide variety of tissues (Keenan and Folch, 2007). For example, Alsop et al. (2014) functionalized CG scaffolds with benzophenone to immobilize VEGF in spatially defined patterns, and evaluate their effect on HUVECs morphology by fluorescent staining (Alsop et al., 2014). The scaffolds with patterned VEGF into geometric designs revealed morphological features of activated HUVECs such as branching, elongation and increased cell-cell contact. Whereas, unmodified scaffolds displayed clumped HUVECs that didn't exhibit an activated morphology. These results indicate that GF patterns may be created by light-induced immobilization for directing cells bioactivity within a biomaterial.

4.1.1.4. Mussel-based immobilization

Mussel-based immobilization is inspired by the ability of marine mussels to attach to wet surfaces by secreting adhesive proteins rich in 3,4-dihydroxyphenylalanine (Dopa) and amine groups (Lee et al., 2007). The catechol side chain in Dopa confers these proteins the ability to bind various types of surface substrates and solidify *in situ* (Kord Forooshani and Lee, 2017). Dopamine, a small molecule containing both catechol and amine groups, is capable of immobilizing biomolecules, such as GFs, to a wide variety of organic and inorganic materials. Under alkaline conditions, dopamine polymerizes and forms adhesive polydopamine films that react with amine and thiol groups via Michael addition or Schiff base reactions (Lee et al., 2007). Polydopamine immobilizes GFs by reacting with amine and thiol groups of the GF and forming covalent bonds.

Yang et al. (2012) demonstrated that polydopamine mediated immobilization can be applied to several different GFs: VEGF, neural growth factor (NGF), bFGF and glial cell line-derived neurotrophic factor (GDNF) (Yang et al., 2012). These GFs were individually immobilized onto polystyrene and PLGA surfaces, and their effect on human adipose stem cells (ADSCs), neural stem cells (NSCs) and HUVECs bioactivity was evaluated. The immobilization of bFGF and VEGF enhanced the proliferation of ADSCs cultured on PLGA surfaces but not on polystyrene surfaces. HUVECs proliferation was enhanced by bFGF and VEGF immobilized onto PLGA surfaces, approximately 2 and 3-fold, respectively. Whereas bFGF and VEGF immobilization onto polystyrene surfaces, increased HUVECs proliferation around 0.5 and 0.7-fold, respectively. In the case of CDNF and NGF functionalized surfaces, differentiation and proliferation of NSCs was enhanced. This study demonstrates that mussel-based immobilization is a technique that allows functional culture of stem cells and primary cells. Other studies have also reported covalent immobilization of GFs onto dopamine treated surfaces for tissue engineering applications (Kang et al., 2013; Poh et al., 2010; Kang et al., 2012; Lai et al., 2011). Since GFs and surfaces do not require complex modification procedures, mussel-based immobilization is considered simpler than other covalent immobilization techniques (Poh et al., 2010).

4.1.1.5. Other covalent methods

Several other chemistries have been used to immobilize GFs covalently onto various surfaces. Plasma treatment involves the introduction of functional groups to surfaces, which can be utilized in various chemistries, such as carbodiimide coupling, to immobilize GFs (Zhang et al., 2012). This particular technique has been used for the immobilization of bone morphogenetic protein-4 (BMP-4), BMP-2, bFGF and FGF-2 (Puleo et al., 2002; Shen et al., 2008; Kokubu et al., 2009; Shen et al., 2009). Silane and imine coupling are other useful techniques for GF immobilization, in which the formed covalent bonds can be hydrolyzed under physiological conditions, allowing control on subsequent release of the GF (Cabanas-Danés et al., 2018).

4.1.2. Non-covalent approaches for GF tethering

A major advantage of using non-covalent immobilization approaches is that in most cases the GF is simply added to target substrates without the need of a prior step of GF modification. Also, the majority of these approaches allow oriented immobilization of the GF because it is possible to select the attachment site of the GF onto the surface. Whereas in most of the covalent approaches, reactive groups are expected to interact with a specific functional group that may be present more

than once in the GF, immobilizing it in a random orientation, which in consequence can affect bioactivity (Nakaji-Hirabayashi et al., 2007; Nakaji-Hirabayashi et al., 2008). Another attractive feature of non-covalent approaches is that most of them are reversible, allowing temporal control of the immobilized GF. An overview of non-covalent approaches is included in Figure 2.

4.1.2.1. ECM-based immobilization

The ECM is a highly dynamic and complex network composed of glycoproteins, proteoglycans, collagen, and glycosaminoglycans. ECM functions are related to cellular processes, serve as a reservoir of GFs, and modulate their bioavailability (Kim et al., 2011). Among the diverse GF-ECM interactions, the largest group involves GFs binding to heparin or heparan sulfate. GFs interact with heparin *in vivo* through specific binding domains, and electrostatic interactions, which take place between negatively charged carboxyl and sulfate groups from heparin and GFs positively charged amino acids (Taipale and Keski-Oja, 1997; Joung et al., 2008). In an attempt to mimic the natural biological environment of GFs, and thus, maintain signaling events that occur *in vivo,* various substrates surfaces have been functionalized with heparin or heparan-sulfate to immobilize a wide variety of GFs (Shin et al., 2015; Ma et al., 2015a; Kim et al., 2016; Kim et al. 2015; Freudenberg et al., 2015; Jha et al., 2015; Kato et al., 2007). Interestingly, Kim et al. (2016) exploited the occurring electrostatic interactions between GFs and heparin to develop an electrochemically based GF release system that disrupts these interactions. This approach demonstrated effective control of bFGF release from heparinized titanium surfaces paired with stable bioactivity.

The attributes of heparin, such as the sulfation pattern and molecular weight, have relevant consequences in bioactivity, loading efficiency and release of immobilized GFs. In this context, Jha et al. (2015) demonstrated that heparin molecular weight and concentration affected the loading and retention of TGF-β1 in hyaluronic acid-based matrices, primarily through greater affinity of TGF-β1 to high molecular weight heparin (Jha et al., 2015). In another study, the sulfation pattern of heparin modulated the release of VEGF from starPEG-heparin hydrogels and influenced VEGF pro-angiogenic action *in vitro* and *in vivo*. The desulfation of heparin resulted in higher release of VEGF, occasioning a higher angiogenic cell response *in vitro* (Freudenberg et al., 2015).

An alternative to substrate surface functionalization with heparin and heparan sulfate is to use other ECM molecules that also interact with GFs *in vivo*, such as collagen, fibrinogen, fibrin, betaglycan, and decorin (Schultz and Wysocki, 2009; Sawicka et al., 2015; Macri et al., 2007; Kato et al., 2007). For instance, Martino et al. (2011) generated a fibrin matrix functionalized with a fibronectin recombinant fragment displaying integrin and fibronectin binding domains to simultaneously control GF and integrin binding (Martino et al., 2011). In this approach, three GFs were immobilized individually: VEGF, PDGF-BB, and BMP-2. *In vitro* experiments showed that fibrin containing immobilized VEGF increased proliferation and migration of endothelial cells about 10% more compared to the soluble GF. Mesenchymal stem cells (MSCs) cultured on fibrin matrices functionalized with BMP-2 didn't had a significant effect on proliferation and migration. Whereas, MSCs cultured on PDGF-BB functionalized matrices showed a 3% increase in proliferation and migration compared to the soluble GF. Finally, fibrin matrices with immobilized PDGF-BB enhanced proliferation around 4%, and migration around 14%. Furthermore, the fibrin

matrices with immobilized GFs enhanced the regenerative effects of GFs *in vivo* in a diabetic mouse model of chronic wounds and in a rat model with calvarial defects. These results suggest that immobilization methods inspired in ECM functional components are able to induce a proper cellular response, as well as improving cell bioactivity and tissue regeneration *in vivo* (Martino et al., 2011).

4.1.2.2. Peptide-based immobilization

Peptides are short oligomers of amino acids synthetized through well-established methods and used in many applications, including the attachment of biomolecules, such as enzymes, proteins, antibodies and GFs, to a wide variety of surfaces (Cabanas-Danés et al., 2013; Crispim et al., 2017; Fu et al., 2011; Jung et al., 2008; Lin et al., 2009; Naffin et al., 2003; Wang et al., 2008). For GF immobilization, surfaces are functionalized with specific binding peptides that interact with a pre-loaded GF through different possible modes, such as hydrophobic interactions, recognition of secondary structure motifs, and electrostatic interactions (Stanfield and Wilson, 1995; Wrighton et al., 1996; Fairbrother et al., 1998; Wang et al., 2008). These peptides are often identified by a screening of several sequences that display different properties and thus, bind to their target (in this case GFs) with varying affinity, specificity, and strength (ten Brummelhuis et al., 2017). Specific binding peptides inspired in naturally occurring binding domains may also be used for GF immobilization. It is important to mention that surface functionalization is not the only approach for peptide-based immobilization of GFs, fusion proteins of GFs with specific binding peptides may also be utilized for this purpose and will be described further on in the genetic fusion section.

As an example of peptide-based immobilization, Wang et al. (2008) attached VEGF to collagen scaffolds by using a modified collagen mimetic peptide (CMP) with multiple anionic charges at the N-terminus, designed to bind collagen by strand invasion, and simultaneously attract VEGF through charge-charge interactions (Wang et al., 2008). Since collagen binding occurs only by contact with melted, single-stranded CMP, two collagen treatments involving either a hot CMP solution (hCMP) or a CMP solution quenched from 80 to 25ºC (qCMP) were evaluated. The loading efficiency of VEGF onto collagen scaffolds treated with hCMP and qCMP was approximately 32 and 20.5%, respectively. Moreover, the collagen scaffolds treated either with hCMP or qCMP after VEGF addition and HUVECs seeding, enhanced tube-like morphology in cells, and activated the integrin-link kinase angiogenic pathway, indicating a biologically relevant cell response to immobilized VEGF.

Besides surface functionalization with specific binding peptides, self-assemble peptide amphiphiles (PAs) have also been used for peptide-based GF immobilization (Stupp et al., 2010; Hsieh et al., 2006; Hosseinkhani et al., 2006). PAs are peptide-based molecules containing a hydrophilic tail and hydrophobic head composed of several amino acids, that mimic surfactant structures (Qiu et al., 2018). In order to confer biological activity to these peptides, they are conjugated to bioactive epitopes that are placed as the hydrophilic tail, since the hydrophobic head must be conserved to ensure the self-assembling process (Qiu et al., 2018; Cui et al., 2010). PAs self-assembly into various nanostructures in aqueous solutions at certain pH, ionic strength and temperature conditions, resulting in exposure of the bioactive epitopes on the nanostructure surface

(Dehsorkhi et al., 2014). Depending on the desired biological response, different epitopes may be conjugated to PAs, particularly for GF immobilization, specific GF binding peptides may be used.

In a pioneering study, PA nanofibers that displayed a high density of TGF-β1 binding epitopes at the surface to immobilize TGF-β1 were employed for use in cartilage regeneration (Stupp et al., 2010). The PA nanofibers containing TGF-β1 binding epitopes (TGFBPA) were mixed with non-bioactive PAs that acted as a filler to ensure adequate epitope binding and GF display. GF release studies showed a 3-fold slower TGF-β1 release after 72 hours when immobilized in TGFBPA scaffolds. *In vitro* experiments demonstrated that TGFBPA scaffolds were able to support MSCs viability and chondrogenic differentiation as well as leading to an increase in gene expression of cartilage markers. The *in vivo* potential of PA scaffolds to promote cartilage regeneration in the presence of bone marrow MSCs was evaluated in adult rabbits with microfractures in the trochlea. Macroscopic and histological evaluation of the defects revealed that in contrast with TGF-β1 alone and filler scaffolds with TGF-β1, TGFBPA scaffolds (with and without TGF-β1) enhanced tissue regeneration. In terms of a modified version of the O'Driscoll 24-point scoring system, TGFBPA scaffolds with and without TGF-β1 had approximately a 1.5-fold higher score, indicating higher quality of the new tissue. Bioactivity showed in TGFBPA scaffolds without exogenous GF was explained as a result of binding events of endogenous TGF-β1 to epitopes present in the scaffold.

4.1.2.3. Coiled-coil interactions

The coiled-coil is an oligomerization domain found in approximately 3-5% of all proteins, involved in a wide range of biological functions (Mason and Arndt, 2004; Truebestein and Leonard, 2016). Examples of coiled-coil-containing proteins include extracellular and motor proteins, such as kinesin, myosin, keratin, and fibrin, as well as transcription factors, such as Jun and Fos (Mason and Arndt, 2004; Goktas et al., 2018). Usually, coiled-coil consists of two to five α-helices, parallel or antiparallel, wound into super-helical structures. The structure of coiled-coil proteins is characterized by a seven amino acid (heptad) repeat, denoted *abcdefg*, that directs folding and dimerization of the helices (Goktas et al., 2018). The positions a and d are hydrophobic amino acids that form the hydrophobic core of the coiled-coil and are packed in a knobs-into-holes arrangement, in which the residue from one helix (knob) packs into a space surrounded by four residues (hole) of the facing helix (Lupas and Gruber, 2005). The amino acids located at positions e and g are oppositely charged polar residues that flank the hydrophobic core and contribute to the coiled-coil structure stability by forming intra-strand salt bridges. Finally, b, c and f are more variable residues, with great relevance for stability (Goktas et al., 2018).

Due to their highly specific and stable interaction, coiled-coil structures have been used in various biomedical and biotechnological applications, including GF immobilization (Apostolovic et al., 2010; Boucher et al., 2010; Riahi et al., 2017; Noel et al., 2016; Murschel et al., 2013; Boucher et al., 2008; Assal et al., 2015). The target GF is expressed as a fusion protein with one coiled-coil strand, while the substrate surface is functionalized with the complementary coiled-coil strand. Although naturally occurring coiled-coils may be used for GF immobilization, most studies appeal to *de novo* coiled-coils because their structure and properties, such as stability, degree of oligomerization, helix orientation, sensitiveness to pH and temperature, and self-assembly, can be controlled by protein/peptide engineering (Apostolovic et al., 2010). For example, De Crescenzo et al. (2003) designed a *de novo* coiled-coil system conformed by two peptides, designated Ecoil

and Kcoil, with varying affinity and stability according to the number of heptads in the Ecoil and Kcoil (De Crescenzo et al., 2003). A five long heptad repeat in both Ecoil and Kcoil demonstrated high affinity and relative rapid association. This coiled-coil pair was used to immobilize EGF in an oriented manner on polyethylene terephthalate (PET) films and promote CECs response (Boucher et al., 2010). The Ecoil peptide was fused to the N-terminus of EGF while the Kcoil peptide was grafted in PET films. The loading efficiency of Ecoil-EGF onto Kcoil-functionalized surfaces was approximately 88%. When compared to adsorbed and soluble EGF, the immobilized GF enhanced CECs adhesion, proliferation and spreading.

4.1.2.4. Genetic fusion

Genetic fusion is another useful technique for GF non-covalent immobilization. It uses recombinant technology to generate fusion proteins of the GF with specific components, such as affinity tags and specific binding peptides (Arnau et al., 2006). Affinity tags show particular affinity to chemical or biological ligands, which are linked on the substrate surface to enable immobilization. As listed in Table 1, a wide variety of affinity tags and their ligands have been used for tethering GFs onto natural and artificial substrates, including: avidin and biotin; antibodies and antigens; histidine and metals; glutathione-s-transferase (GST) and glutathione (GTH); and maltose-binding protein and maltose (Worrallo et al., 2017; Ogiwara et al., 2005; Kato et al., 2005; Kolodziej et al., 2011; Han et al., 2009). In most cases, the interaction between the affinity tag and the ligand is reversible through the addition of competitive agents. Although histidine and biotin belong to the most commonly used affinity tags for immobilization purposes, they will not be described in this section, as their special case is described further on. Of note, GFs can also be immobilized through affinity interactions without the necessity to fuse the GF to a tag. In a pioneering study, an affinity tag-free immobilization strategy was established by exploiting the native sugar lectin-interaction between glycosylated recombinant BMP-2 and concanavalin A (Wang et al., 2020).

Kolodziej et al. (2011) exploited the reversibility of the affinity linkage between GST and GTH to reutilize surfaces where FGF-2 had been previously immobilized (Kolodziej et al., 2011). First, a fusion protein of FGF-2 and GST (FGF-2-GST) was immobilized onto GTH functionalized PEG hydrogel surfaces. Then, FGF-2-GST was released from the hydrogel surfaces upon free GTH addition. The surfaces were completely free from FGF-2-GST and used successfully to bind a second FGF-2-GST. Therefore, GF immobilization through reversible affinity tags may open the possibility for performing iterative immobilization on a single substrate surface, contributing to further decrease of cell culture costs. However, to elucidate the potential application of this approach, the effect of iteratively immobilized surfaces on bioactivity must be investigated.

In a previous section, GF immobilization through peptides was described, however focus was put on surface functionalization with specific binding peptides and not on genetic fusion. In the latter approach, peptide sequences that have an affinity to certain materials or proteins are selected and used for fusion with GFs through recombinant technology. A wide variety of peptides that bind to different substrates including collagen, cellulose, hydroxyapatite, titanium, polystyrene, and beta-tri calcium phosphate, have been used to immobilize GFs (Tada et al., 2014; Ishikawa et al., 2001; Alvarez et al., 2015; Kitajima et al., 2007; Doheny et al., 1999; Kang et al., 2013; Thatikonda et al., 2018). These peptides are derived from combinatorial screens of peptide libraries or based on

naturally occurring binding domains. Although a GF may exhibit affinity to a substrate, avidity of the binding may improve by fusing additional binding domains to the GF. For example, Kitajima et al. (2007) improved the binding affinity of native hepatocyte growth factor (HGF) to collagen 16 times by fusing HGF to a polypeptide derived from the fibrin collagen binding domain (CBD) (Kitajima et al., 2007). In comparison to soluble HGF, the immobilized CBD-HGF promoted HUVECs cell growth for 4 extra days longer, overall yielding 5 times more cells over 10 days. Although recombinant technology is widely used for conjugating GFs to peptides, peptide ligation can also be used for this purpose. To our knowledge, this technology has been used in only example reported to date, in which human bone morphogenetic protein 4 (hBMP4) was attached to hydroxyapatite beads (Sakuragi et al., 2011).

Fusion proteins containing GFs may also include domains to provide additional functionalities. For instance, a fusion protein made of neural growth factor-β (NGF-β) with two functional domains: a factor XIIIa transglutaminase domain (TG) as incorporation site to fibrin matrices, and plasmin substrate domain (P) which provided a cleavage site for the local release of the iGF under cell-activated plasmin (Sakiyama-Elbert et al., 2001). The surface functionalization of fibrin matrices with this fusion protein (TG-P-NGF-β) enhanced neurite extension of neural crest-derived PC12 cells by 50% more compared to native NGF-β. The high bioactivity of TG-P-NGF-β suggested that the iGF was effectively released from the matrices in an active form. In this setting, TG mediated covalent attachment of the fusion protein to the matrices. After thrombin-mediated activation, TG catalyzes the formation of covalent bonds between glutamine and lysine residues in fibrin chains (Corbett et al,. 1997). The TG domain has also been used for the covalent immobilization of VEGF and BMP-2 on fibrin matrices (Schmoekel et al. 2004; Zisch et al., 2001).

4.1.2.5. Biotin-Streptavidin interactions

One of the strongest non-covalent interactions known involves the binding of biotin to avidin or streptavidin. Both of these proteins contain four subunits able to bind biotin with great affinity and specificity (Hermanson, 2008). However, avidin is positively charged and may generate non-specific interactions, while the neutral variant streptavidin does not present this characteristic and may be the preferred alternative (Nguyen et al., 2012). Usually, the immobilized surface is functionalized with either avidin or streptavidin, while biotin is coupled to the molecule of interest. In some instances, it is necessary to test GF immobilization efficiency depending on either the biotin-GF binding or the biotin-streptavidin binding occurring first (Moore et al., 2017). In most approaches, GF immobilization by biotin-streptavidin interactions involves the use of tetrameric streptavidin, which makes the biotin-avidin/streptavidin interaction non-reversible. As an alternative, monomeric streptavidin may be used to produce reversible binding, allowing temporal control of the immobilized GF. Monomeric streptavidin has a reduced affinity for biotin compared to tetrameric streptavidin due to its considerably smaller dissociation constant (Wu et al., 2009). Other approaches to reverse biotin-streptavidin interactions include incubation in high temperature aqueous solutions, development of tetrameric streptavidin mutein with lower affinity for biotin, design of biotin analogs with lower affinity for streptavidin (Holmberg et al., 2005; Ying and Branchaud, 2011; O'Sullivan et al., 2012).

GFs are usually biotinylated using biotin derivatives containing reactive groups that specific for coupling to a particular functional group on the GF. However, the biotin derivative must be

carefully selected to ensure preserved bioactivity of the GF (Hermanson, 2008). Common biotin derivatives for GF modification are the amine-reactive, such as sulfo-NHS-biotin (Shahal et al., 2012; Worrallo et al., 2017; Kim et al., 2016). The NHS ester of this compound reacts with the GF primary amines to form an amide bond and thus, couple with biotin. Some biotinylating reagents may also contain spacer groups, such as the NHS-PEG-biotin, which may improve the binding potential towards avidin or streptavidin by reducing steric hindrance, increase the solubility of the reagent, and increase control over the steric presentation of GFs to target receptors (Hermanson, 2008; Cipolla et al., 2013; Worrallo et al., 2017). It should be highlighted that the use of a spacer group is not limited for biotin-streptavidin based approaches. A spacer group may be attached to a wide variety of affinity ligands or functional groups, such as maleimide, vinyl sulfones and carboxylic acids. GF biotinylation can be achieved using recombinant technology, with GFs expressed as fusion proteins with a biotin label incorporated at the N-terminus (Leipzig et al., 2011; Li et al., 2014).

Recently, Worrallo et al. (2017) developed a potential immobilization strategy for controlling GFs presentation in cell suspension culture platforms (Worrallo et al., 2017). GFs were biotinylated via reaction of NHS on NHS-PEG-biotin with primary amines on lysine residues of GM-CSF, SCF and hematopoietic growth factor thrombopoietin (TPO). Biotinylated GFs were attached to magnetic streptavidin-coated particles. The magnetic properties of these particles allowed temporal control over GFs presentation. Immobilized SCF, GM-CSF, and TPO maintained bioactivity in GF dependent cell lines M-07e and TF-1. Using immobilized GM-CSF (iGM-CSF) permitted a 98.5% decrease in the use of the GF, compared to soluble GM-CSF (sGM-CSF) used over 192 hours of culture. Interestingly, iGM-CSF retained functionality under agitation in a micro-scale stirred tank bioreactor and after short exposure, higher cell growth was obtained relative to sGM-CSF. Taken together, these results demonstrate that this biotin-streptavidin based approach is promising for reducing overall manufacturing costs of GF dependent cell culture systems, such as those being developed for cell therapies, by diminishing GF quantities required to induce a maximum cellular response. Magnetic recollection of iGF post-culture may also open the possibility for GFs recycling in the allogenic setting.

4.1.2.6. Chelator histidine tag interactions

A chelator-based immobilization technique, commonly used for protein purification in chromatographic processes, uses a histidine tag in combination with metal ions immobilized in chelators, such as iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA). Histidine forms coordination bonds with several metal ions, such as Cu^{2+}, Ni^{2+} and Zn^{2+} (Kimple et al., 2013). Based on the chelator and metal ion used, a number of coordination sites are available in the metal ion for interaction with the histidine tag. For example, when Cu^{2+} is chelated by NTA, it has one free site for interaction with histidine residues, whereas Ni^{2+} has three free sites (Hochuli et al., 1987). To immobilize a GF, a histidine tag is placed on either the N-terminus or C-terminus of the GF using recombinant technology, while the substrate surface is functionalized with a chelator and metal ion. The number of histidine residues in histidine tags may vary, however six histidine tags are generally recognized as adequate for yielding high affinity interactions with metal ions. This type of immobilization can be reversed by the addition of metal-chelating agents, such as ethylenediaminetetraacetic acid (EDTA), or competitive agents, such as imidazole (Bornhorst and Falke, 2000).

For example, Kato et al. (2005) immobilized EGF onto culture plates through the linking of a hexahistidine tag to its C-terminus, which binds to metal ions chelated to self-assembled monolayers (SAM) (Kato et al., 2005). Surfaces linked to immobilized EGF (iEGF), allowed cell adhesion and proliferation. In comparison, surfaces with iEGF using carbodiimide coupling, exhibited few aggregated cells. When EGF was immobilized by carbodiimide coupling, cell adhesion was hardly observed, while cells cultured onto EGF chelated surfaces could attach and proliferate (Nakaji-Hirabayashi et al., 2007). This is due to the random orientation of the GF in surfaces with EDC/NHS chemistry mediated immobilization. The intact EGF structure after immobilization by chelation as well as firm immobilization onto the surface was demonstrated. Furthermore, EGF immobilization by EDC/NHS chemistry provoked GF denaturation (Nakaji-Hirabayashi et al., 2008).

5. Case study: Stem Cell Factor immobilization

SCF exists both as a transmembrane and a soluble protein. It is produced by endothelial cells in the hematopoietic stem cell (HSC) niche, and binds to its receptor, CD117 also known as c-kit, on a variety of early hematopoietic cells, promoting their maintenance and proliferation (McNiece and Briddell, 2003). Due to its effect on HSC, soluble SCF (sSCF) has been used to produce a wide variety of therapies based on blood-lineage cells (Timmins et al., 2009; Zonari et al., 2017). In the context of cell therapy manufacture, immobilization could reduce consumable use and labor costs, which could in turn reduce the overall production cost and make cell therapies more accessible. In this context, investigating efficient strategies for SCF immobilization is highly relevant. Several research articles describe immobilizing SCF, and report feasibility and performance on specific cell line expansion processes. However, the economic impact of using this approach for cell therapy production remains to be determined experimentally.

As methods used to immobilize GFs are varied, several aspects must be considered when selecting the appropriate technique for a specific GF. SCF has several functional groups, which can be exploited for attachment to a variety of surfaces. For instance, 22 lysine residues from SCF can be engaged for using covalent methods using amine groups for immobilization, such as carbodiimide coupling and mussel-based immobilization (Jiang, 2000). SCF also contains 5 cysteine residues that can exploited for immobilization based on the use of thiol groups, such as thiol coupling, mussel-based, and light-induced immobilization (Jiang, 2000). Since PEG linkers commonly contain a reactive functional group, a PEG linker may be used to immobilize SCF to surfaces in both covalent and non-covalent methods. For example, PEG-NHS reacts with amine groups in lysine residues from SCF. Paradoxically, as SCF contains more than one amine group, attachment of the linker is not specific to a single site on SCF, which may affect the SCF active site and bioactivity. To avoid this, PEG linked to reactive groups of increased specificity can be selected, such as PEG-CHO and PEG-MAL, both used for specific targeting of N-terminal residues (Agusti et al., 2016).

A pioneering study reporting immobilization of SCF was done by Doheny et al. (1999), in which SCF was fused to a cellulose binding domain using recombinant technology to allow adsorption in a cellulose matrix. Immobilized SCF (iSCF) yielded a 5- to 7-fold increase in cell expansion compared to using similar concentrations of sSCF when culturing murine and human suspension

bone marrow cell lines (Doheny et al., 1999). In a more recent study, Cuchiara et al. (2013) covalently immobilized SCF to PEG hydrogels to culture primary hematopoietic cell populations. A loading efficiency of 80% was achieved and the capacity of iSCF to induce proliferation of murine HSCs remained similar to that of sSCF. However, HSCs spreading was decreased, and cells exhibited a more rounded morphology when iSCF was used compared to sSCF, which is more in accordance to their native physiological state inside the bone marrow (Cuchiara et al., 2013).

Mahadik et al. (2015) immobilized murine SCF to methalcrylamide gelatin (GelMA) hydrogels using PEG-NHS as a linker between the functionalized matrix and SCF (Mahadik et al., 2015). Both sSCF and covalently attached SCF induced similar levels of proliferation in murine HSCs. Retention profiles were performed for covalently attached hydrogels and hydrogels with adsorbed SCF. After 7 days, covalently attached hydrogels retained 80% of the initial concentration of SCF, whereas adsorbed hydrogels only retained 40%. In addition, covalently attached hydrogels induced higher proliferation levels than adsorbed hydrogels. Therefore in the context of HSC *ex vivo* proliferation, SCF covalent attachment may promote improved cell proliferation while stabilizing the GF (Mahadik et al., 2015). In a different study, a non-covalent immobilization approach was used to attach SCF onto magnetic beads using PEGylation of SCF and biotin-streptavidin interactions (Worrallo et al., 2017). Results suggest immobilization-induced stabilization of SCF and possible iSCF dose reduction in cell culture. The SCF-dependent human cell line M-07e exhibited a 65% decrease in viability less when using iSCF at a dose corresponding to only 8% of the sSCF dose. However the cell response to increasing iSCF concentrations was curvilinear and did not reach sSCF response. Interestingly, SCF could be co-immobilized with additional relevant GF such as TPO y GM-CSF, on the surface of magnetic beads at controlled concentrations. This strategy is particular useful if co-signaling is required in specific cell production processes and is currently used to activate T cells with anti-CD3/anti-CD28 for the production of CAR T cell therapies.

Taken together, these results demonstrate differential effects of iSCF on cell proliferation and phenotype depending on the nature of target cell and the immobilization method employed. Overall, iSCF improves cell culture processes, and current results evidence the need for further research into exploiting potential additional benefits, for example the temporal and spatial control over iSCF presentation to cells.

6. Concluding remarks

Cell therapies promise innovative treatments for a variety of life-threatening conditions and the number of clinical trials of cell-based therapies is rapidly increasing. A downfall of these treatments is their high cost of production, which translates into a high selling price hampering their widespread use. Acquisition of materials, especially GFs, is a major contributor to the overall cost of production. GF immobilization has emerged as a strategy to optimize a rational GF usage in cell culture, contributing to reduction of costs.

A wide variety of techniques are available for immobilizing GFs to different biomaterials. Covalent immobilization techniques usually allow a slower release profile of the GF compared to physical approaches. However, they may engage functional groups required for induction of a

cellular response. Of note, if functional groups used for immobilization are present more than once in the GF, immobilization may occur in a random orientation for example, which can affect bioactivity. On the other hand, non-covalent immobilization techniques allow a more specific immobilization because it is possible to select the precise attachment site within the GF sequence in most cases. Furthermore, in most of non-covalent techniques, temporal control is easily achieved, since reversible tags can be used. Physical immobilization techniques typically result in a faster release of the GF, resulting in poor spatial and temporal control over GF presentation to cells, but they do not compromise the availability of functional groups.

In many cases, the techniques presented in this review were specifically applied to immobilize one specific GF. However, to replicate a native cell niche and induce a physiological cellular response, co-signaling with additional GFs is often required. Therefore, immobilization of multiple GFs onto a single surface is highly desirable and represents a relevant field of investigation. Additionally, the combination of multiple techniques may be exploited to tailor the release of GFs and have increased control over their steric presentation to cells.

The impact of GF immobilization on reducing the overall manufacturing cost of cell therapy vary with a number of parameters including the cost of the GF itself, and its specific half-life and loading efficiency. The selected immobilization method also dictates the immobilization support to be used, impacting the cost. Some methods are more sophisticated than others, which demand and increased initial inversion to perform the immobilization. Physical immobilization methods are generally the most cost-effective but entail disadvantages such as lack of control-over-release. Methods involving genetic fusion of an adapter to the GF are more expensive because a recombinant fusion protein must be designed and produced. The cost of the substrate or immobilization surface also factors into final immobilization cost. For instance, magnetic beads are more expensive than a matrix scaffold. In addition to cost, efficiency and specific biological features induced by each of the immobilization methods should be considered when selecting the most appropriate technique to optimize GF usage aiming for overall cost reduction without decreasing cell response. An economical evaluation is critical to evaluate the real impact of GF immobilization in cell manufacturing costs.

It is clear that GF immobilization is a strategy that addresses many of the issues related to the use of soluble GFs. However, most of the immobilization techniques have focused on immobilizing GFs for tissue engineering applications involving cells growing in adherence. The few studies performed on suspended-cells culture systems show promising results by reducing significantly the total amount of GFs required. Further research is needed to elucidate the full potential of GF immobilization to optimize cell production processes, including potential for re-use.

7. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

8. Author Contributions

DEO wrote sections 3, 4, 5 and 6 and developed Figure 1. PRO wrote sections 1, 2, 5 and 6. MB and KMD provided initial context and structure for this manuscript, wrote and edited all content. All authors provided critical feedback and approved the final version of the manuscript.

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Figure Legends

Figure 1. Overview of methods currently in use to perform physical GF immobilization.

Figure 2. Overview of current chemical immobilization strategies. In covalent methods, acrylate crosslinking is representative of the conjugation of an acryloyl GF and PEGDA matrix. While, mussel-based immobilization shows the interaction between the GF and polydopamine coated on the surface.

Tables

Table 1. Genetic engineered binding GFs to natural and artificial substrates.

Substrate		Fused protein	GF	Reference
Natural	Collagen	igG	EGF	(Ogiwara et al., 2005)
	GSH-functionalized nanopatterns	Glutathione-s-transferase	FGF2	(Kolodziej et al., 2011)
	Gelatin and Fibrillar collagen sponges	Fibronectin collagen-binding domain	EGF	(Ishikawa et al., 2001)
	Beta Tricalcium Phosphate (βTCP)	βTCP-binding peptide	EGF	(Alvarez et al., 2015)
	Collagen	Collagen binding domain	HGF	(Kitajima et al., 2007)
	Cellulose	Cellulose-binding domain	SCF	(Doheny et al., 1999)
	Silk coated surfaces	Spider silk protein	bFGF	(Thatikonda et al., 2018)
	Fibrin	Transglutaminase	B-NGF	(Sakiyama-Elbert et al.,

		activity of factor XIIIa		2001)
	Fibrin	Transglutaminase activity of factor XIIIa and plasmin substrate	BMP-2	(Schmoekel et al., 2004)
	Fibrin	Transglutaminase activity of factor XIIIa	VEGF	(Zisch et al., 2001)
Artificial	Gold-coated glass plate	Hexahistidine residues	EGF	(Kato et al., 2005)
	Polystyrene surfaces	Maltose-binding protein	VEGF	(Han et al., 2009)
	Titanium surfaces	Titanium-binding peptides	hEGF	(Tada et al., 2014)
	Hydroxypatite	Statherin active site	EGF	(Kang et al., 2013b)
	Titanium surfaces	Statherin active site	EGF	(Kang et al., 2013b)
	Hydroxypatite	Diphosporylated serines from statherin	hBMP4	(Sakuragi et al., 2011)

Figure 1.TIFF

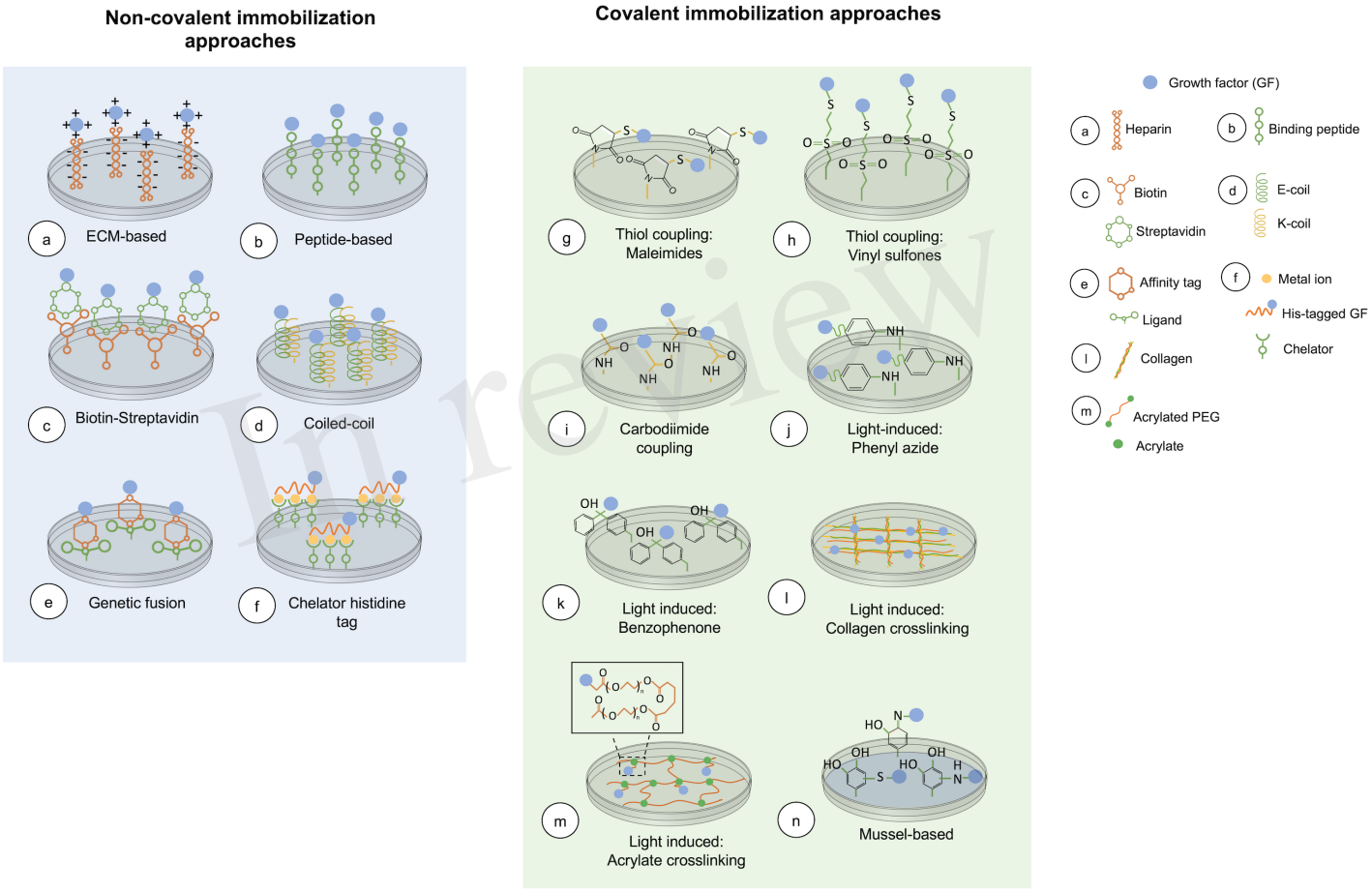
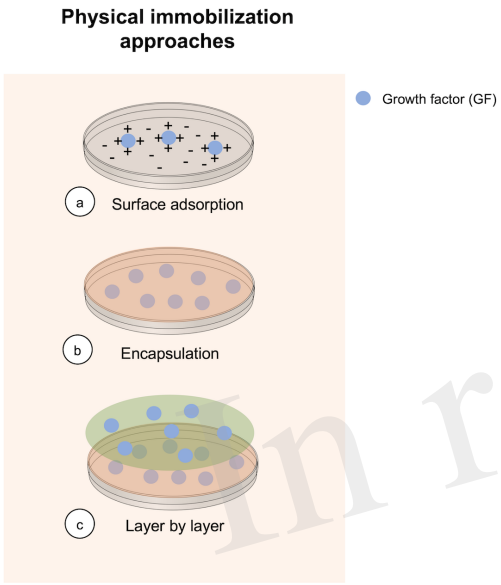


Figure 2.TIFF



Appendix 2: ESTUDIOS DE LA DIFERENCIACIÓN DE CÉLULAS MADRES HEMATOPOYÉTICAS ENRIQUECIDAS DE SANGRE DE CORDÓN UMBILICAL A NEUTRÓFILOS EX VIVO

Protocolo:
ID (Acrónimo): 2018_HSPC_Brunck

Titulado:

ESTUDIOS DE LA DIFERENCIACIÓN DE CÉLULAS MADRES HEMATOPOYÉTICAS ENRIQUECIDAS DE SANGRE DE CORDÓN UMBILICAL A NEUTRÓFILOS EX VIVO

Versión	**2**
Fecha de versión	**Miercoles 5 de Diciembre de 2018**

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- **Índice**

- **LISTA DE ABREVIATURAS**

CD	Cluster of Differentiation
CMH	Celulas Madres Hematopoyeticas
CRISPR/Cas9	Clustered Regularly-Interspaced Short Palindromic Repeats–Caspase 9
G-CSF	Factor Estimulante de Colonia de Granulocitos
GTX	Transfusion de Neutrofilos (Granulocitos)
HPV	Human Papilloma Virus
HSV-TK	Herpes Simplex Virus Thimidine Kinase
MOI	Multiplicity of Infection
NF	Neutropenia Febril
rAAV	Recombinant Adeno-Associated Virus
SCF	Stem Cell Factor
ANC	Absolute neutrophil count
MSC	Mesenchymal Stem Cells
FL	Flt Ligand
TPO	Thrombopoietin
HSPC	Hematopoietic stem and progenitor cells
GM-CSF	Granulocyte-macrophage colony stimulating factor
IL	Interleukin
PBMC	Peripheral blood mononuclear cells

- **SÍNTESIS (Resumen, abstract)**

Título	ESTUDIOS DE LA DIFERENCIACION DE CELULAS MADRES HEMATOPOYETICAS ENRIQUECIDAS DE SANGRE DE CORDÓN UMBILICAL A NEUTRÓFILO EX VIVO
Fase de estudio	Estudios de Laboratorio
Tipo de estudio	Estudios *in vitro* que utilizan tejidos humanos (sangre periférica, sangre de cordón umbilical) que no pueden vincularse con un individuo (= de-identificados).
Clasificación del estudio Según el Reglamento de la Ley General de Salud en Materia de Investigación para la Salud, Artículo N° 17	II. Investigación con riesgo mínimo. Segun la definicion: Estudios prospectivos que emplean el riesgo de datos a través de procedimientos comunes en exámenes

	físicos o psicológicos de diagnósticos o tratamiento rutinarios, entre los que se consideran: pesar al sujeto, pruebas de agudeza auditiva; electrocardiograma, termografía, colección de excretas y secreciones externas, **obtención de placenta durante el parto, colección de líquido amniótico al romperse las membranas**, [...].
Objetivo Principal	Incrementar la cantidad de neutrófilos que se pueden producir ex vivo a partir de células troncales hematopoyéticas CD34+ enriquecidas de la sangre de cordón umbilical humana
Objetivos secundarios	*Objetivo secundario 1:* Establecer y estandarizar los protocolos de diferenciación celular publicados en el artículo de Timmins *y col.,* para producir neutrófilos a partir de células troncales hematopoyéticas CD34+ enriquecidas de sangre de cordón umbilical, en los laboratorios del Centro de Biotecnología FEMSA del Tecnológico de Monterrey, Campus Monterrey. *Objetivo secundario 2:* Transducir células troncales hematopoyéticas CD34+ usando vectores lentivirales para la incorporación del vector CSIV-TRE-RfA-UbC-KT-HPV16 E6/E7 (Kurita et al, 2013), y comprobar la producción de las oncoproteínas E6 y E7 tras inducción con doxiciclina. *Objetivo secundario 3:* Desarrollar neutrófilos *ex vivo* a partir de las células transformadas en el *Objetivo secundario 2*, después de remover el agente inductor, y comparar el rendimiento de neutrófilos con el rendimiento de neutrófilos desarrollados a partir de células troncales hematopoyéticas CD34+ transducidas con un vector control.
Hipótesis Nula	Células troncales hematopoyéticas humanas CD34+ enriquecidas de sangre de cordón umbilical pueden producir una cantidad

	limitada a (en promedio, por cada célula hematopoyética sembrada) 5860 células derivadas de la diferenciación a neutrófilos, incluyendo neutrófilos y sus células progenitoras (Timmins *y col*., Biotechnol Bioeng. 2009).
Hipótesis Alterna	Es posible incrementar el rendimiento actual de neutrófilos desarrollados a partir de células troncales hematopoyéticas humanas CD34+ enriquecidas de sangre de cordón umbilical, usando técnicas de biología sintética y de cultivo celular.
Pregunta de Investigación	¿Es posible incrementar el número de neutrófilos producidos a partir de una cantidad finita de células troncales hematopoyéticas enriquecidas a partir de sangre de cordón umbilical, tras la expresión heteróloga condicional de las oncoproteinas de HPV E6 y E7?
Grupo a investigar	Las células troncales hematopoyéticas serán enriquecidas a partir de sangre de cordón umbilical, sin más requisitos para el grupo de donantes de sangre de cordón umbilical.
"N"	100
Duración aproximada del estudio	4 años a partir de la aprobación del proyecto

● PLANTEAMIENTO DEL PROBLEMA

La **neutropenia** es una condición grave caracterizada por la disminución significativa del nivel de neutrófilos en sangre. Esta condición afecta negativamente la inmunidad del paciente contra patógenos (Crawford, Dale, and Lyman 2004). En consecuencia, infecciones bacterianas y fúngicas son la principal causa de morbilidad y mortalidad en pacientes neutropénicos y septicemia es la principal causa de muerte no atribuible directamente al cáncer en pacientes pediátricos (Zaorsky et al. 2017). La incidencia de la neutropenia en pacientes depende de factores tales como la edad, padecimiento específico del paciente, así como del tratamiento propuesto. Las antraciclinas (por ejemplo, doxorrubicina), taxanos (por ejemplo, docetaxel), alquilantes (por ejemplo, ciclofosfamida) e inhibidores de topoisomerasas (por ejemplo, etopósido) así como gemcitabina y vinorelbina son particularmente mielosupresores y pueden comportar riesgos hasta más del 20% de desarrollar neutropenia y neutropenia febril (NF). Otros factores relacionados con el tratamiento y asociados con un mayor riesgo de NF incluyen antecedentes de quimioterapia y tratamiento con tres o más agentes de quimioterapia (Lyman et al. 2010).

La neutropenia febril se caracteriza por una fiebre >38°C y conteo absoluto de neutrófilos en la sangre periférica <500 células por µl, e incrementa el riesgo de infecciones severas en pacientes.

Además de tener un alto costo para el sistema de Saludo (el costo promedio de hospitalización por episodio de NF en 2009 fue de >US$22,000 (Michels et al. 2012 Pharmacoeconomics. doi: 10.2165/11592980-000000000-00000.), la neutropenia severa y los episodios de NF son los principales impulsores de retrasos y reducciones de la dosis de quimioterapia (Link et al., 2001: Cancer. 2001 Sep 15;92(6):1354-67), los cuales se ha demostrado comprometen los resultados de supervivencia en diversos entornos curativos (Patel et al. 2017 JAMA, doi:10.1001/jamaoncol.2017.1114).

Neutropenia, una consecuencia común de la quimioterapia citotóxica.
Todos los pacientes que han recibido quimioterapia en dosis altas, tienen una etapa obligada de neutropenia, a penas de tratamiento farmacológicos con factor estimulante de colonias de granulocitos, G-CSF por sus siglas en ingles, para reducir este periodo neutropénicos. La quimioterapia es un tratamiento muy común en enfermedades oncológicas y la principal causa de neutropenia. Se estima que en 2008 se registraron 12.4 millones de casos nuevos de cáncer en el mundo, de los cuales 7.6 millones fueron decesos. En países en desarrollo como México, se reportan más de la mitad de casos de cáncer y se estima que el 60% de las muertes ocasionadas por esta enfermedad ocurren en estos países (Accardi, Rosita et al. 2008).

En nuestro país, el cáncer es la segunda causa de mortalidad entre los niños de 4 a 15 años de edad desde el 2010. Entre 2007 y 2012, hubo 35,591 niños tratados por cáncer dentro de 55 institutos mexicanos acreditados por el Seguro Médico Popular, incluyendo pacientes nuevos (14,178) y más de 21,413 pacientes de seguimiento para fines de 2012. Dentro de estas estadísticas, durante 2012 se reporto una incidencia de leucemia fue de 78.1 / 1,000,000 / año. Las leucemias dictan regímenes mielotóxicos que llevan a la neutropenia con riesgos mayores de infección (NF). La prevalencia de leucemia en los seis años del análisis reportado fue del 49.8% (Rivera-Luna et al. 2014). Además, el Registro Epidemiológico de Neoplasias Malignas informa que por cada 100 000 habitantes de la población general existe una incidencia anual de leucemias agudas de 2; de leucemia linfoide aguda de 1.3 y de leucemia mieloide aguda de 0.7. La incidencia de la leucemia linfoide aguda se ha incrementado a 30.9 % en un lapso de 40 años (Crespo-Solis, E. 2010).

La neutropenia febril e infecciones asociadas tienen un importante costo económico y de salud en México y en el Mundo.
Las bacteriemias en los pacientes con cáncer y neutropenia son una causa importante de episodios de NF, estas ocasionan complicaciones graves que pueden llevar a la muerte del paciente.
Solo en los EE. UU., se estima que 60,000 casos de neutropenia relacionados con la quimioterapia requirieron hospitalización en 1999, y que 1 de cada 14 de estos pacientes murió (Caggiano et al. 2005). El costo promedio de hospitalización por neutropenia asociada a la quimioterapia se estima que es de US$ 13,372 (Caggiano et al. 2005) a mas de US$ 22,000 (Michels et al. 2012) por ingreso. En México, pacientes pediátricos neutropénicos con infecciones permanecen hospitalizados por un promedio de 19 días en lugar de 10 días por los pacientes sin infecciones, esto indica un incremento en el tiempo de hospitalización del 100% (Avilés-Robles et al. 2014). Una de las consecuencias de la neutropenia es el retraso del tratamiento citotóxico, lo cual puede asociarse con disminución de su eficacia.

Profilaxis con antimicrobianos y factores estimulantes de colonias de los granulocitos disminuyen riesgos pero son insuficientes para prevenir la neutropenia febril

Lineamientos internacionales prescriben tratamientos profilácticos con antibióticos de amplio espectro para prevenir infecciones en pacientes con duraciones esperadas de neutropenia mayores a 7 días (Flowers et al. 2013; Freifeld et al. 2011). A pesar de cuyas intervenciones, los reportes indican que entre 37 y 67% de pacientes en tratamiento por NF recibieron antibióticos de profilaxis, lo que resalta su eficacidad parcial (Avilés-Robles et al. 2014). El tratamiento con factor estimulante de colonias de granulocitos (G-CSF, por sus siglas en inglés) es uno de los tratamiento profiláctico que existen para prevenir la NF, dicho tratamiento promueve la proliferación de células madres hematopoyéticas y su diferenciación a neutrófilos in vivo. El tratamiento con G-CSF disminuye la duración de la neutropenia y riesgo de NF, sin embargo, algunas instituciones de salud no permiten su uso en pacientes con enfermedades onco-hematológicas debido a posibles interferencias con resultados de aspirados de medula ósea (Iqbal et al. 2016). Además, a pesar de reducir el periodo neutropénico, este tratamiento no lo puede eliminar completamente (tiempo mínimo tras trasplante de medula ósea para tratar leucemia es de 10 días-platica de Magenta Therapeutics en el Congreso Anual de la American Society for Haematology 2018 en San Diego, CA, atendido por la Dra. Brunck).

Transfusión de neutrófilos de donantes en pacientes neutropénicos

La transfusión de neutrófilos es una solución lógica para pacientes neutropénicos, para prevenir, y en casos específicos tratar las infecciones. **Sin embargo, la implementación del proceso y su aval por ensayos clínicos como tratamiento común se han visto obstaculizados por cuestiones prácticas.** Primero, el bajo rendimiento en la colección de neutrófilos de donantes es un proceso fastidioso que necesita la movilización farmacológica del almacenaje de neutrófilos desde la médula ósea hasta la sangre, esto resulta en la necesidad de múltiples donantes por episodio de neutropenia. Segundo, la incapacidad de almacenar sin afectar significativamente la viabilidad de los neutrófilos colectados, además esto se ve afectado debido a la corta vida-media de esas células, esta estrategia de tratamiento implica una colecta constante para satisfacer las necesidades de los pacientes en tratamiento (Gea-Banacloche 2017).

Producción de neutrófilos *ex vivo*

Alternativamente, se ha desarrollado una estrategia experimental que consiste en producir neutrófilos *ex vivo* a partir de células troncales y progenitoras hematopoyéticas (Timmins et al. 2009). Esta estrategia además de resolver problemas de suministro, la generación de células sanguíneas *ex vivo* permite la estandarización de la composición de productos sanguíneos, y a su vez, elimina los riesgos de transmisión de enfermedades infecciosas y la enfermedad de injerto contra huésped.

Cuello de botella y necesidades: descripción y objetivo del proyecto

Si bien actualmente existe un protocolo para producir neutrófilos *ex vivo*, nuestro grupo de investigación considera necesario optimizar su eficiencia biológica, y a su vez su factibilidad financiera, para poder proponer este tipo de terapia en clínicas y hospitales en un futuro a mediano-largo plazo. El presente proyecto propone optimizar el proceso de producción de neutrófilos ex vivo a través de la transducción lentiviral de células troncales hematopoyéticas CD34+ con oncogenes E6 y E7 del virus papiloma humano (HPV por sus siglas en ingles), cual transcripción es condicional a la presencia de doxiciclina. Se ha demostrado recientemente que la producción de proteínas E6 y E7 promueve la expansión de células CD34+ primaria en un proceso de producción

de eritrocitos sin afectar al fenotipo final de los globulos rojos (Trakarnsanga et al, 2017 Nat. Commun. doi: 10.1038/ncomms14750.).

ANTECEDENTES Y JUSTIFICACIÓN

1. Antecedentes

Neutrófilos en la sangre humana, y su papel inmunológico

Los neutrófilos son los leucocitos más abundantes en la circulación sanguínea humana. Estas células reciben este nombre debido a su tinción neutra con la tinción de Wright. También se conocen como neutrófilos polimorfonucleares (PMN, por sus siglas en ingles). Son células redondas de aproximadamente 12-14 μm de diámetro. Están presentes en una concentración (también conocida como cuenta absoluta de neutrófilos o ANC, por sus siglas en ingles) entre 2 $x10^9$ a 7 $x10^9$ por L de sangre, cantidades iguales están marginadas en las paredes de vasos sanguíneos o secuestradas en capilares cerrados. Así, la sangre y la médula ósea forman un conjunto abundante de neutrófilos. Los neutrófilos son las primeras células del sistema inmunológico en llegar a un sitio de infección, por lo tanto, desempeñan un papel fundamental en la eliminación de bacterias y hongos, y juegan un rol importante en la respuesta del huésped contra infecciones y en la homeostasis del sistema inmune.

Los neutrófilos son reclutados a sitios de infección e inflamación por estimulación a través de citosinas liberadas por células infectadas. El núcleo multilobulado contribuye a la elasticidad extrema de la célula, que es importante para que la célula haga un tránsito rápido desde la sangre a través de brechas estrechas en el endotelio.

Una vez llegados a sitios de infección, los neutrófilos tienen la capacidad de engullir bacterias a través de la fagocitosis. Las bacterias engullidas por neutrófilos son degradadas a dentro de vacuolas especiales llamadas fagolisosomas, a través de múltiples procesos incluyendo la acción de enzimas proteolíticas, la acidificación del medio por la producción de especies reactivas de oxigeno, y la secuestración de hierro y cobre, elementos necesarios a la sobrevivencia bacteriana. Los microorganismos reconocidos por neutrófilos como invasores, pero que morfológicamente son demasiados largos para experimentar la fagocitosis (un evento llamado "fagocitosis frustrada"), provocan el inicio de rutas alternativas para su eliminación, como la extrusión de enzimas y compuestos oxidativos en el medio extracelular, y la formación de trampas extracelulares de neutrófilos (NETs, por sus siglas en ingles). Además de contribuir directamente a la eliminación de patógenos, neutrófilos producen citosinas como IL-1beta, IL-8, IL-6, and TGF-beta, las cuales modulan la siguiente fase de la respuesta inmune. Recientemente, se ha descrito una nueva función para los neutrófilos: la presentación de antígenos directamente a linfocitos en órganos linfoides, tal como células presentadoras de antígenos (APC, por sus siglas en ingles).

Producción de neutrófilos *in vivo* en homeostasis y enfermedad

Neutrófilos son producidos a partir de celulas madres hematopoyéticas (CMH) en la medula ósea. Estas células tienen la capacidad de auto-renovarse, así como la capacidad de diferenciarse a todos los tipos de células de la sangre. Se caracterizan por la expresión de marcadores tales como CD34 y CD90 así como también la ausencia de CD38 y CD45RA (Seita and Weissman 2010). En la médula ósea, las células precursoras mieloides maduran a neutrófilos segmentados en aproximadamente 9 días, se estima que la vida media de neutrófilos en la sangre a 6 y 7 horas. Aunque en el tejido no se conoce exactamente y se estima que oscila entre 1 y 4 días. Dado que los neutrófilos maduros tienen una capacidad limitada para regenerar enzimas gastadas, que se

agotan rápidamente por actividad fagociticas, el neutrófilo es por lo tanto incapaz de funcionar continuamente y este es eliminado de la circulación por apoptosis después de un solo episodio de actividad.

En el huésped sano, la producción y eliminación de neutrófilos están equilibradas, lo que da como resultado una concentración bastante constante de neutrófilos en la sangre periférica (Christopher and Link 2007). Cuando ocurre una infección, se generan agentes quimiotácticos que provocan la migración de almacenajes de neutrófilos en la medula ósea al sitio de la infección y la activación de la función defensiva de los neutrófilos; en paralelo se activan células progenitoras de neutrófilos para seguir la respuesta inmune y rellenar el almacenaje de neutrófilos en la medula ósea (Summers et al. 2010; Amulic et al. 2012).

Neutropenia inducida por quimioterapia, y neutropenia febril: definición y consecuencias

La disminución de la concentración de neutrófilos en sangre hasta concentraciones inferiores a 2000 células/mm^3 se conoce como neutropenia y, bajo esta condición, disminuye la respuesta inflamatoria para contener y eliminar las infecciones patogénicas. Cuando la caída de neutrófilos llega a niveles inferiores a 0.5 x10^9/L y se acompaña por una temperatura oral mayor a 38.5 °C, se considera urgencia médica con requerimientos de atención inmediata, ya que es una complicación potencialmente letal. A esta condición se le conoce como neutropenia febril (NF) (Sobrevilla Calvo, Sobrevilla Moreno, and Ochoa Carrillo 2016).

La neutropenia inducida por quimioterapia (NIQ) es el efecto adverso mielotóxico más frecuentemente asociado con quimioterapia citotóxica en pacientes con tumores sólidos. Ciertas características del paciente son un factor fundamental para el riesgo de NIQ, por ejemplo, el factor de riesgo más frecuentemente asociado con neutropenia febril (NF) es la edad mayor de 65 años. Otros factores de riesgo son estadio avanzado del cáncer, episodios previos de NIQ o NF, no recibir factores estimulantes ni antibióticos profilácticos. También, el estado funcional, desnutrición, comorbilidades (incluida la enfermedad renal o cardiaca), leucopenia basal antes del inicio de la quimioterapia y el sexo femenino, aumentan el riesgo de NIQ (Sobrevilla Calvo, Sobrevilla Moreno, and Ochoa Carrillo 2016).

Las consecuencias de la neutropenia incluyen retrasos en el tratamiento citotóxico, lo cual puede disminuir de su eficacia. En caso de NF, con hospitalización, hemocultivos y uso de antibióticos intravenosos, así como de factores estimulantes de colonias de los granulocitos (G-CSF, por sus siglas en ingles). Además, la NF está asociada con incrementos del 15% mortalidad en pacientes en comparación con pacientes oncológicos sin NF (Lyman et al. 2010). En México, pacientes con NF sufren de un incremento significativo de tiempos de hospitalización (Avilés-Robles et al. 2014). El costo promedio de cada episodio de hospitalización por neutropenia febril se estima entre 41,160 pesos (Cantú-Rodríguez et al. 2017), y 129,118 pesos (Morgan-Villela et al. 2009). Para resumir, la neutropenia afecta la calidad de vida de los pacientes e incrementa los costos de la atención médica (Sobrevilla Calvo, Sobrevilla Moreno, and Ochoa Carrillo 2016)

Prevención y tratamientos actuales de la neutropenia febril

Estudios han demostrado que el uso profiláctico de G-CSF puede reducir el riesgo, severidad y duración de la neutropenia y NF (Lyman et al. 2010; Lustberg 2012). Su uso se recomienda en pacientes con riesgos de NF >20%. Las moléculas más estudiadas son filgrastim y pegfilgrastim, con dosis diaria de 5 μg/kg hasta lograr una recuperación de la ANC a lo normal posnadir y de una sola dosis de 6mg por ciclo de tratamiento mielotoxico, respectivamente (Lyman, Lyman, and Agboola 2005). Asimismo, el papel del G-CSF está bien establecido en el trasplante de células progenitoras hematopoyéticas, para movilizarlas y acortar el período de neutropenia después de la citorreducción. No obstante, la neutropenia aún puede persistir por unos 10-15 días, por lo que se propone hacer trasplante de neutrófilos maduros para prevenir o acortar este plazo (Hino et al. 2000).

Desde hace varias décadas se han empleado tratamientos profilácticos con diversos antimicrobianos solos o en sinergia. En 2008, la National Comprehensive Cancer Network de EE. UU. publicó directrices sobre la prevención y el tratamiento de las infecciones relacionadas con el cáncer (Segal et al. 2008). Brevemente, se recomienda fluoroquinolonas de manera profiláctica para grupos de alto riesgo y riesgo intermedio, que en su mayoría comprenden pacientes que reciben dosis altas de quimioterapia y aquellos con neoplasias hematológicas en las que la duración prevista de la neutropenia es superior a 7 días. En Mexico, la profilaxis con fluoroquinolonas no benefició a pacientes con leucemias; en específico no previno eventos febriles ni disminuyó la mortalidad en un estudio realizado en el Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (Ugarte-Torres et al. 2006). Adicionalmente, se reportaron varios informes de la aparición de bacterias resistentes a las fluoroquinolonas en unidades que practican la profilaxis basada en fluoroquinolonas (Kern et al. 2005; Campos 2014; Chong et al. 2014). En consecuencia, considerando el riesgo de resistencias, la profilaxis con antibióticos se propone en pacientes seleccionados.

Transfusión de neutrófilos para prevenir o tratar infecciones en pacientes neutropénicos

Una alternativa lógica a tratamientos farmacológicos para prevenir la neutropenia e infecciones resultantes, es la transfusión de neutrófilos (GTX) obtenidos de donantes (Estcourt et al. 2016; Gea-Banacloche 2017; Price et al. 2015; Kerr et al. 2003). La transfusión de neutrófilos colectados de donantes sanos para tratar a infecciones en pacientes neutropénicos es un tema longevo: múltiples reportes científico describen resultados variables, e inconstancias en protocolos de estudios sin llegar a evidencia clara sobre la eficacia de la terapia. Artículos aislados reporten casos de éxito (Bielorai et al. 2000; Ozsahin et al. 1998), pero el uso de GTX en pacientes neutropénicos aún no ha beneficiado de un acuerdo consensuado de la comunidad científica a través de resultados concluyentes en ensayos clínicos (Gea-Banacloche 2017; Price et al. 2015; Estcourt et al. 2016). Un obstáculo identificado regularmente para proponer ensayos clínicos conclusivos es el bajo rendimiento de neutrófilos colectados de donantes aun con la movilización de almacenajes de neutrófilos a la sangre usando con G-CSF y dexametasona. Aun, se han realizado pequeños estudios clínicos demostrando que la transfusión de células precursoras de neutrófilos expandidas *ex vivo* resulta en períodos menores de neutropenia absoluta (ANC •$0.1x10^9$ neutrófilos/L de sangre) y períodos más cortos de recuperación (McNiece et al. 2000).

Producción *ex vivo* de neutrófilos

Por lo anterior, se ha propuesto la producción *ex vivo* de neutrófilos post-mitóticos alogénicos como método de rutinario de profilaxis en pacientes de alto riesgo (Timmins et al. 2009). Esto

implica una producción masiva de neutrófilos, teniendo como reto lograr la mayor cantidad de neutrófilos maduros a partir de una cantidad finita de células troncales hematopoyéticas.

La manipulación *ex vivo* de este tipo celular se reporta en estudios con protocolos diferentes, buscando 1-la mayor expansión y 2-el fenotipo apropiado al cosechar células. Por ejemplo, se puede co-cultivar CMH con células madres mesenquimales (MSC, por sus siglas en ingles) lo que resulta en una expansión 250 veces mayor al cultivo sin stroma (Li et al. 2007) . Hino *et al* realizaron una metodología que demuestra que las células CD34+ de sangre periférica de adultos pueden expandirse selectivamente *ex vivo* en neutrófilos maduros con funciones similares a las de los neutrófilos de sangre periférica en condiciones normales *in vivo*. Para esto, cultivaron las células CD34+ en presencia de SCF, IL-3, GM-CSF y G-CSF durante siete días. Para la expansión de neutrófilos maduros, las células no adherentes se dividieron en dos grupos para su posterior cultivo con un cocktail diferente de factores de crecimiento, obteniendo en ambos casos una proliferación significativa al día 14. El primer grupo presentó mejores rendimientos: las células estuvieron en presencia de SCF, IL-3, GM-CSF y G-CSF; su aumento fue de 220 veces, un 11% corresponden a neutrófilos maduros. El segundo grupo estuvo en presencia de sólo G-CSF, teniendo un aumento de 130 veces, 51% de neutrófilos maduros (Hino et al. 2000).

Timmins *y col.* implementaron otro protocolo para la expansión de neutrófilos *ex vivo* provenientes de células progenitoras hematopoyéticas CD34+ de sangre de cordón umbilical y de células mononucleares de sangre periférica (PBMC, por sus siglas en inglés). Las células aisladas fueron expandidas en medio libre de suero, con SCF, G-CSF y péptido mimético de tropoyetina. Luego de 15 días de cultivo, se obtuvo una expansión de 5,800 veces para las células de cordón umbilical y de 4000 veces paras las de circulación periférica, con una concentración de neutrófilos maduros de 40% y 60% respectivamente (Timmins et al. 2009). Los rendimientos obtenidos con este protocolo de producción (bifásico, primeramente, en fase estática, seguido por un periodo en biorreactor con agitación) fueron satisfactorios para concluir que el método es viable para la producción a gran escala de neutrófilos.

Estas técnicas de expansión *ex vivo* aún cuentan con la desventaja de trabajar con células finitas, siendo la limitante la obtención de las células para comenzar su expansión. Gracias al avance de las técnicas de biología sintética, se encuentran estudios que respaldan la idea de modificar células progenitoras temporalmente para su inmortalización condicional y así obtener un suministro continuo de estas células.

Recientemente, Trakarnsanga *y col.* lograron la modificación de CMH para su expansión condicional al sobre-expresar las oncoproteinas E6 y E7 de HPV, eso antes de diferenciar las células a eritrocitos y así obtener un suministro inagotable de glóbulos rojos. Para ello, utilizaron células CD34 + de médula ósea adultas y el sistema de expresión Tet-inducible. Proponen que el uso de oncoproteínas virales para crear estas líneas inmortales es poco probable que suponga un riesgo para el uso clínico, porque se elimina su expresión antes de la diferenciación terminal (Trakarnsanga et al. 2017). Proponemos que esta estrategia se puede aplicar para producir neutrófilos, usando un medio de diferenciación a neutrófilos, en lugar de un medio de diferenciación a eritrocitos.

2. Justificación

Justificación
Con base en lo anterior, proponemos estudiar la posibilidad de optimizar un protocolo de cultivo de neutrófilos *ex vivo* para la producción **incrementada** de neutrófilos humanos, para su subsecuente aplicación en una terapia celular para pacientes neutropénicos, a largo plazo (**no considerado dentro de este protocolo**). En México, pacientes con NF sufren de un incremento significativo de tiempos de hospitalización (Avilés-Robles et al. 2014). El costo promedio de cada episodio de hospitalización por neutropenia febril se estima entre 41,160 pesos (Cantú-Rodríguez et al. 2017), y 129,118 pesos (Morgan-Villela et al. 2009).

Este proyecto propone desarrollar una línea celular **condicionalmente inmortalizada** para lograr una producción ilimitada de neutrófilos humanos. Esto, además de resolver problemas de suministro, la generación de células sanguíneas *in vitro* permite la estandarización de la composición de productos sanguíneos, y a su vez, elimina los riesgos de transmisión de enfermedades infecciosas (Motta et al. 2016; Rohde et al. 2014) y la enfermedad de injerto contra huésped (Cid 2017; Kopolovic et al. 2015). Así este proyecto altamente innovador, se contempla como el primer paso para **poder proponer un ensayo clínico con dosis apropiada de neutrófilos y fenotipo estandarizado para contestar la pregunta de manera conclusiva si las GTX previenen o curan la NF en pacientes neutropenicos**, y proponer una terapia celular a pacientes con alto riesgo de neutropenia y NF. Estos datos tienen implicaciones a nivel internacional, ya que como se menciona en los antecedentes del proyecto, no se ha podido establecer de manera conclusiva el efecto de las GTX en pacientes neutropenicos, en ningún ensayo clinico.

PREGUNTA DE INVESTIGACIÓN OBJETIVOS E HIPÓTESIS

3. Pregunta de investigación

¿Es posible incrementar el número de neutrófilos producidos a partir de una cantidad finita de células troncales hematopoyéticas enriquecidas a partir de sangre de cordón umbilical, tras la expresión heteróloga condicional de las oncoproteinas de HPV E6 y E7?

Con este proyecto se contempla responder la pregunta de investigación descrita anteriormente.

4. Objetivo Principal

Incrementar la cantidad de neutrófilos que se pueden producir *ex vivo* a partir de células troncales hematopoyéticas CD34+ enriquecidas de la sangre de cordón umbilical humana.

5. Objetivos Secundarios

Objetivo secundario 1: Establecer y estandarizar los protocolos de diferenciación celular publicados en el artículo de Timmins *y col.,* para producir neutrófilos a partir de células troncales hematopoyéticas CD34+ enriquecidas de sangre de cordón umbilical, en los laboratorios del Centro de Biotecnología FEMSA del Tecnológico de Monterrey, Campus Monterrey.

Objetivo secundario 2: Transducir células troncales hematopoyéticas CD34+ usando vectores lentivirales para la incorporación del vector CSIV-TRE-RfA-UbC-KT-HPV16 E6/E7 (Kurita et al, 2013), y comprobar la producción de las oncoproteínas E6 y E7 tras inducción con doxiciclina.

Objetivo secundario 3: Desarrollar neutrófilos *ex vivo* a partir de las células transformadas en el *Objetivo secundario 2*, después de remover el agente inductor, y comparar el rendimiento de neutrófilos con el rendimiento de neutrófilos desarrollados a partir de células troncales hematopoyéticas CD34+ transducidas con un vector control.

6. Hipótesis

●.6.1 Hipótesis Nula

Células troncales hematopoyéticas humanas CD34+ enriquecidas de sangre de cordón umbilical pueden producir una cantidad limitada a (en promedio, por cada célula hematopoyética sembrada) 5860 células derivadas de la diferenciación a neutrófilos, incluyendo neutrófilos y sus células progenitoras (Timmins *y col*., Biotechnol Bioeng. 2009).

●.6.2 Hipótesis Alterna

Es posible incrementar el rendimiento de neutrófilos desarrollados in vitro a partir de células troncales hematopoyéticas humanas CD34+ enriquecidas de sangre de cordón umbilical (más de 5860 neutrófilos producidos por célula CD34+), a través de la producción condicional de oncoproteinas E6 y E7 desde un vector episomal transducido en la células CD34+ colectadas de sangre de cordón umbilical.

- **DISEÑO DEL ESTUDIO: <u>Nuestro propuesta NO trata de un Ensayo Clínico</u>**
  1. **Descripción del estudio**

     NO APLICA
  2. **Fundamento o Justificación de placebo**

     NO APLICA
  3. **Fundamentos para el diseño del estudio**

     NO APLICA
  4. **Fundamentos para la dosis y el esquema de tratamiento**

     NO APLICA
  5. **Fundamentos para la población de pacientes**

     NO APLICA
  6. **Elección de tratamiento**

     NO APLICA
  7. **Control con placebo**

     NO APLICA
  8. **Fundamento para los factores de estratificación**

     NO APLICA

9. Fundamento para la obtención de muestras de biológicas (tejidos)

NO APLICA

10. Fundamento para muestreo de sangre para medición de los biomarcadores

NO APLICA, las muestras de sangre que contemplamos en este proyecto son para obtener neutrófilos control o CMH (CD34+ positivas enriquecidas de sangre de cordón umbilical)

11. Fundamento para las evaluaciones de los resultados notificados por el paciente

NO APLICA

● MATERIALES Y MÉTODOS

1. Pacientes

El estudio pretende colectar sangre del cordón umbilical de recién nacidos, tras el consentimiento informado previo de los padres.

La sangre de cordón umbilical será procesada dentro de 12 horas para enriquecer las células troncales hematopoyéticas CD34+, las cuales son la materia primaria para iniciar un cultivo celular de proliferación y diferenciación a neutrófilos, con base en el protocolo de Timmins *y col.*

El protocolo presente se basa en la colección de células CD34+, sin pretender estudiar diferencias fenotípicas o cuantitativas en las células enriquecidas de sangre de cordón umbilical entre grupos de pacientes, por lo que cualquier dato de identificación de muestras no es necesario.

Además, se colectaran muestras de sangre periférica de voluntarios sanos *pro re nata* para:
1. Optimizar protocolos de estudio de fenotipo y actividad de neutrófilos
2. Comparar la actividad de neutrófilos producidos *ex vivo* con neutrófilos colectados a partir de sangre periférica (control positivo/estándar de oro).

2. Criterios de Inclusión

Sangre de cordón umbilical: dar a luz a un infante y consentir en participar con la donación de sangre de cordón de forma voluntaria.

Sangre periférica: consentir en participar con la donación de sangre periférica de forma voluntaria.

3. Criterios de Exclusión

Sangre de cordón: haber tenido cualquier tipo de cáncer o leucemia, antes o durante el embarazo.

4. Criterios de Suspensión

No Aplica

5. Visitas

No Aplica

6. EVALUACIONES DEL ESTUDIO

●.6.1 Formas de consentimiento informado y registro de selección

Se obtendrá el consentimiento informado previo (con anticipación, para dar una oportunidad de reflexión), por escrito, por parte de los padres en caso de muestras de sangre de cordon umbilical, o de los donantes de sangre periférica, para incluir sus donaciones de muestras en el estudio.

Los formatos físicos de consentimiento informado se conservarán en la oficina de la Dra. Marion Brunck, dentro de un armario cerrado con llave. Las muestras serán de-identificadas al momento de la colección, es decir que estas muestras serán llamadas únicamente con un código (de acuerdo al tipo de muestra y a la cronología del tiempo de colección, por ejemplo el nombre de la primer muestra de sangre de cordón umbilical= **CU01**). Las hojas de consentimiento informado no podrán ser correlacionada con las muestras ya que el consentimiento informado no se obtendrá el mismo día de la colección de muestra. Los resultados del proyecto serán publicados en una revista internacional indexada "open-access", que podrán consultar gratuitamente los donantes de muestras si lo desean.

●.6.2 Historia clínica y datos demográficos

Se pedirán datos demográficos generales, de-identificados y tiempos de colección, al colectar muestras de sangre de cordón umbilical, a fines de control de calidad y para poder entender cualquier resultado inesperado (por ejemplo, la sangre de cordón umbilical obtenida de un parto prematuro tiene cantidades más altas de células CD34+):

1. Género del donante/del infante recién nacido
2. Edad del donante/semanas de embarazo al nacer el infante
3. Fecha y hora de colección
4. Comentarios adicionales considerados importantes

●.6.3 Exploraciones físicas

NO APLICA

●.6.4 Estudios de laboratorio y gabinete

LABORATORIO DE ANÁLISIS DIAGNÓSTICO-NO APLICA
ESTUDIOS DE INVESTIGACIÓN-VER SECCIÓN METODOLOGÍA ABAJO

●.6.5 Signos vitales

NO APLICA

- **METODOLOGÍA**

1. Metodología de la Investigación

Colección de muestras de sangre

Sangre de cordón umbilical: al recibir consentimiento informado de los padres, la sangre de cordón umbilical será colectada al momento del nacimiento, en tubos de 50mL con citrato de sodio, almacenada a temperatura ambiente y procesada dentro de 24 horas para enriquecimiento de células troncales hematopoyéticas CD34+.

Sangre periférica: tras recibir consentimiento informado, se obtendrá una muestra de sangre periférica (20 mL), de voluntarios sanos en tubos con citrato, para el posterior enriquecimiento de neutrófilos "control", a través de centrifugación sobre gradiente Ficoll-Hypaque (fracción inferior) y lisis de eritrocitos .

Enriquecimiento de células troncales hematopoyéticas CD34+

Células troncales hematopoyéticas CD34+ serán enriquecidas de sangre de cordón umbilical. Brevemente, la extracción del paquete de células mononucleares se llevará a cabo por centrifugación por gradiente con Ficoll-Hypaque (Sigma). Células troncales hematopoyéticas CD34+ serán enriquecidas de las células mononucleares por selección positiva a través de perlas magnéticas conjugadas a anticuerpos monoclonales anti-CD34 (Miltenyi Biotec). Las células troncales hematopoyéticas CD34+ colectadas serán contadas y caracterizadas con citometría de flujo antes y después de almacenar en nitrógeno liquido para su uso posterior en cultivo celular.

Enriquecimiento de neutrófilos de donantes

La sangre periférica será procesada inmediatamente tras su colección. Después de la separación y remoción de células mononucleares por centrifugación por gradiente de Ficoll-Hypaque, los neutrófilos serán enriquecidos por lisis de eritrocitos con un buffer de cloruro amónico. Estos neutrófilos serán usados como control en los ensayos fenotípicos para comparar su fenotipo y actividad con la de los neutrófilos producidos *ex vivo.*

Protocolo de diferenciación de neutrófilos

Se seguirá el protocolo de diferenciación a neutrófilos publicado por Timmins *y col.* Brevemente, cultivos estáticos de células CD34+ se iniciarán a una densidad de 10^4 células / mL en matraces de cultivo celular estándar. El medio de cultivo consiste en StemLine II (Sigma-Aldrich) complementado con factor de células madre (SCF por sus siglas en inglés, Amgen), G-CSF (Amgen) y un péptido mimético de trombopoyetina (custom peptide). Se agregará medio fresco el día 5 y cada 2 dias después del dias 5 y hasta dia 15, por una duración total de 15 días, en incubación a 37 °C y 5% de CO^2.

Ensayos fenotípicos

Estos ensayos se llevarán a cabo a lo largo de la proliferación y expansión celular, y los resultados serán comparados entre neutrofilos obtenidos de sangre periferica (Control), y neutrofilos desarrollados ex vivo (prueba, con y sin expression heterologa de HPV E6/E7).

Se usará citometría de flujo (BD FACS Celesta, en el Centro de Biotecnologia FEMSA) para investigar la expresión de CD34, CD38, CD90, y la expresión de moléculas de linaje tales como CD3, CD2, CD11b, CD14, CD14, CD19, CD56, CD66b, CD235 a lo largo de la diferenciacion

celular. Además se investigará viabilidad (7-AAD), proliferación (Ki-67) y apoptósis (Annexin V) (todos los fluorocromos, BD Pharmingen/Miltenyi Biotec). Para analizar la morfología de neutrófilos, preparaciones para CytoSpin se teñirán con tinción de Leishmans (Sigma), y células se clasificarán de acuerdo con la apariencia tal como se detalla en el Atlas de hematología de Anderson. La expresión de CD15 y CD11b, así como capacidad de producir especies de oxígeno reactivas (CellROX Green Assay, ThermoFisher), y capacidad de fagocitosis (Vybrant Phagocytosis Assay Kit, ThermoFisher) se cuantificarán mediante citometría de flujo (BD FACS Celesta). La habilidad de quimiotaxis a IL-8 será investigada con ensayos Transwell (Corning) como se ha descrito antes (Davidson and Patel 2014). Finalmente, la capacidad de matar a microorganismos de relevancia clínica en pacientes neutropénicos, será investigada *in vitro* a través de la co-incubación de neutrófilos con los microorganismos (aislados de pacientes) obtenidos de la ATCC, por 24h (*Candida albicans, Aspergillus fumigatus, Escherichia coli),* seguida por la lisis de neutrófilos e investigación en placa de números de unidad formadora de colonia.

Producción de Lentivirus

Obtuvimos el plásmido CSIV-TRE-RfA-UbC-KT-HPV16 E6/E7 a través de una colaboración con del Riken Institute (Japón). Para su empacamiento en lentivirus y producción, se obtendrán células HEK293T de la ATCC, y se producirá el lentivirus como sistema de 3 plásmidos, con los plásmidos de empacamiento pCAG-HIVgp y pCMV-VSV-G-RSV-Rev (Riken BioResource Centre, Japón). Brevemente, HEK293T serán cultivadas a confluencia optima, y transfectadas con los plásmidos usando un protocolo básico con polietilenimina (Sigma). El medio de cultivó se cambiará 18h después de la transfección. A partir del día siguiente, títulos de virus serán medidos en el sobrenadante después de diferentes tiempos de incubación para optimizar concentraciones y tiempos de cosecha. Partículas de virus serán concentradas por ultra-centrifugación. Para producir el lentivirus control, se producirá el plásmido CSIV-TRE-fA-UbC-KT, al remover secuencias genómicas de HPV usando enzimas de restricción. Se comprobará la transfección y ensamblaje de partículas viral en HEK293T al medir fluorescencia de Kusabira Orange (integrado al vector inicial) con microscopia de fluorescencia. Para comprobar la producción de las oncoproteinas E6/E7 de manera condicional, se extraerá RNA de HEK293T antes y 24h después de añadir doxiciclina al medio de cultivo, y se medirán producciones diferenciales de cDNA de HPV E6 y E7 por qPCR Tiempo-Real. Finalmente, se lisarán células HEK293T y se investigara la presencia de las proteínas E6/E7 con Western Blot y anticuerpos monoclonales anti-HPV E6 y E7 (Abcam).

Transducción lentiviral de células troncales hematopoyéticas CD34+

Las células troncales hematopoyéticas CD34+ serán transducidas con el lentivirus producido, mediante infección de cultivo como se ha descrito en la literatura (Guenechea et al. 2000). Brevemente, células CD34+ se cultivarán a una densidad de 10,000 células por pozo en placas de 96-pozos (Nunc) en medio de cultivo de diferenciación a neutrófilos, menos los factores de crecimiento G-CSF y trombopoyetina. Después de 24h de adaptación, infecciones con lentivirus se llevaron a cabo usando MOIs entre 12 y 450 durante 24 h de incubación a 37 °C y 5% de CO^2. Después de una infección de 24 h, las células seran cosechadas y se lavaran en medio StemLine II antes de iniciar cultivos de expansión (con doxiciclina, sin G-CSF/trombopoietina en el medio de cultivo) o cultivos de diferenciación a neutrófilos (ver sección:"Protocolo de diferenciación de neutrófilos " arriba).

- Análisis estadístico

Los análisis estadísticos se realizarán utilizando el paquete de software R. Se investigarán diferencias en términos de fenotipo entre neutrófilos de sangre humana y neutrófilos producidos *ex vivo* usando la prueba de t-Student.

1. VARIABLES

- VARIABLES DEL ESTUDIO
  - Genero del cordón, semanas de embarazo al nacer y donar el cordón, edad y genero del donante de sangre movilizada.
  - Cantidades relativas de moléculas de superficie de membrana como por ejemplo CD11b, evidenciadas en Relative Fluorescence Units por citometría de flujo.
  - Cantidades relativas de producción de superoxido, evidenciadas en Relative Fluorescence Units por citometría de flujo.
  - Killing index: Capacidad de eliminar *in vitro* microorganismos de relevancia clínica (*Escherichia coli, Candida albicans*), por conteo manual.
  - La unidad primaria de observación es individual (cada muestra de sangre)
  - El grupo de contraste contiene las muestras de sangre periférica muestreada de voluntarios sanos, a partir de cuales se enriquecerán neutrófilos, a comparar con los neutrófilos desarrollados *ex vivo*.

2. Cuadro de Variables

VARIABLE	DEFINICIÓN CONCEPTUAL	DEFINICIÓN OPERACIONAL	TIPO DE VARIABLE	ESCALA DE MEDICIÓN	VALOR DE VARIABLE
Genero del cordón	Sangre de cordon umibilical puede provenir del nacimiento de un niño o de una niña. Se recordara este información para saber, *post hoc*, si influye en la capacidad de expansion de las CMH procedente de estos.	Valores de la variable se resumirán de manera cuantitativa (numero de neutrófilos resultantes)	Cuantitativa	N/A	Sexo feminino/Masculino
Semanas de embarazo al nacer el donante de	Se recordará este información para saber, *post hoc,* si influye en la capacidad de	Valores de la variable se resumirán de manera cuantitativa	Cuantitativa	N/A	Entre 24-42 semanas

sangre de cordón	expansion de las CMH procedente de estos.	(numero de neutrófilos resultantes)			

3. TÉCNICAS DE ANÁLISIS ESTADÍSTICO

●.3.1 Métodos y modelos de análisis de los datos según tipo de variables

Se investigarán diferencias en términos de fenotipo entre neutrófilos de sangre humana y neutrófilos producidos *ex vivo* usando la prueba de t Student.

●.3.2 Programas a utilizar para análisis de datos.

Los análisis estadísticos se realizarán utilizando el paquete de software R.

● CONSIDERACIONES ÉTICAS

1. Cumplimiento con las leyes y regulaciones

Este estudio se realizará en total conformidad con la guía de la ICH E6 de las Buenas Prácticas Clínicas y con los principios de la Declaración de Helsinki o con las leyes y regulaciones del país (en el cual se lleve a cabo la investigación, lo que le brinde la mayor protección al individuo).

2. Consentimiento informado

Se proporcionará una forma de consentimiento informado de muestra del patrocinador. Si corresponde, se entregará en una traducción certificada del idioma local. El patrocinador o la persona designada debe revisar y aprobar cualquier desviación propuesta de las formas de consentimiento informado de muestra del patrocinador o cualquier forma de consentimiento alternativa que proponga el centro del estudio (en conjunto, las "formas de consentimiento") antes de la presentación al IRB/EC. Las formas de consentimiento aprobadas por el CEI/CE deben proporcionarse al patrocinador a fin de presentarlas ante las autoridades sanitarias de acuerdo con los requisitos locales.

Si aplica, la forma de consentimiento informado contendrá distintas secciones para cualquier procedimiento opcional. El investigador o la persona designada autorizada explicará a cada paciente los objetivos, métodos y posibles riesgos asociados con cada procedimiento opcional. Se les dirá a los pacientes que tienen la libertad de negarse a participar y que pueden retirar su consentimiento en cualquier momento y por cualquier motivo. Se requerirá una firma específica, por separado, para documentar el acuerdo de un paciente para participar en procedimientos opcionales. Los pacientes que se nieguen a participar no proporcionarán una firma por separado.

El paciente o el representante legalmente autorizado deben firmar y fechar las formas de consentimiento antes de su participación en el estudio. El historial de casos o los registros clínicos de cada paciente deben documentar los procesos de consentimiento informado y que el consentimiento informado por escrito se obtuvo antes de la participación en el estudio.

Las formas de consentimiento deberán revisarse cuando haya cambios en los procedimientos del estudio o cuando haya nueva información disponible que pueda afectar la voluntad de la paciente para

participar. Las formas de consentimiento aprobadas por el IRB/EC modificadas finales deben proporcionarse al patrocinador a fin de presentarlas ante las autoridades sanitarias.

Los pacientes deben volver a otorgar su consentimiento con la versión más reciente de las formas de consentimiento (o con un apéndice de información/hallazgos nuevos significativos de acuerdo con las leyes aplicables y las políticas del IRB/EC) durante su participación en el estudio. En el caso de las formas de consentimiento modificadas o actualizadas, el historial de casos o los registros clínicos de cada paciente deben documentar que el proceso de consentimiento informado y el consentimiento informado por escrito se obtuvo usando las formas de consentimiento actualizadas/modificadas para seguir participando en el estudio.

El paciente o el representante legalmente autorizado del paciente **deben recibir una copia de cada forma de consentimiento firmada.** Todas las formas de consentimiento firmadas y fechadas deben permanecer en el archivo del estudio de cada paciente o archivo del centro y deben estar disponibles para que los monitores del estudio las verifiquen en cualquier momento.

3. Comité de Ética

El investigador principal debe presentar este protocolo, formas de consentimiento informado, cualquier información proporcionada al paciente y la información de apoyo pertinente al Comité de Ética en Investigación, quien debe revisarlos y aprobarlos antes de iniciar el estudio. Además, Comité de Ética en Investigación debe aprobar cualquier material de reclutamiento para los pacientes.

El investigador principal es responsable de proporcionar resúmenes escritos del estado del estudio al Comité de Ética en Investigación anualmente, o con más frecuencia, de acuerdo con los requisitos, las políticas y los procedimientos que establezca el Comité de Ética en Investigación. Los investigadores también son responsables de notificar inmediatamente al Comité de Ética en Investigación cualquier enmienda al protocolo.

Además de los requisitos para notificar todos los eventos adversos a las entidades Regulatorias, los investigadores deben cumplir con los requisitos de notificación de los eventos adversos serios a la autoridad sanitaria local y al Comité de Ética en Investigación.

4. Confidencialidad

- Procedimientos para salvaguardar la privacidad de los sujetos que participan en la investigación, así como para conservar los resultados de la investigación.

El investigador principal mantiene estándares de confidencialidad asignando **un código a cada paciente** incluido en el estudio mediante un número de identificación único del paciente. Esto significa que los nombres de los pacientes no se incluyen en los conjuntos de datos que se transmiten.

La información médica del paciente obtenida en este estudio es confidencial y solo puede divulgarse a terceros según lo permite la forma de consentimiento informado (o la autorización por separado para usar y revelar la información personal sobre la salud) firmada por el paciente, a menos que lo permita o lo requiera la ley.

La información médica puede entregarse al médico personal del paciente u otro personal médico adecuado responsable del bienestar del paciente con fines de tratamiento.

Los datos generados en este estudio deben estar disponibles para sus inspecciones previa solicitud por parte de representantes de las autoridades sanitarias nacionales y locales, y el IRB/EC, según corresponda.

- **RIESGOS PREVISIBLES Y PROBABLES**

Sangre de cordón:
La donación de sangre del cordón umbilical suele realizarse después de que se ha dado a luz un bebé y se corta el cordón umbilical. Se considera seguro, aunque en algunos países existe una discusión sobre el momento en que debe cerrarse la vena umbilical. Se seguirán las recomendaciones y protocolos usuales del Dr. Daniel Méndez Lozano, con las prioridades de bienestar y salud de mama y bebé. Así, no se prevé riesgos adicionales a riesgos los usuales de parto.

Sangre periférica de voluntarios sanos:
Donar sangre es un proceso seguro, y la Dra Marion Brunck es capacitada como flebotomista (Mater Medical Research Institute-Australia). Los efectos secundarios menores que podrían ocurrir incluyen: hematomas y dolor al sitio de punción, mareos y náuseas si no le gusta donar sangre.

- **PROTECCIÓN FRENTE AL RIESGO FÍSICO Y/O EMOCIONAL**

Sangre de cordón y sangre periférica de voluntarios sanos: Se informará personalmente a los posibles voluntarios para donar sangre del cordón umbilical o sangre periférica con días de anticipación y se les dará una hoja de información sobre el proyecto de investigación para que ellos se quedan con la información. Los posibles voluntarios tendrán la oportunidad de hacer preguntas sobre el proceso de recolección y la investigación que beneficiará, antes de firmar y devolver la hoja de consentimiento informado. La información personal sobre los posibles voluntarios será desidentificada usando un código único. No prevemos ningún otro desacuerdo no detallado anteriormente.

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Appendix 3: Ethics protocol approval

Tecnológico de Monterrey
Escuela de Medicina

No. de Folio: P000086-2018_HSPC_Brunck-CEIC-CR002

Monterrey, Nuevo León, México a Lunes, 07 de Enero de 2019

Dra. Marion Emilie Geneviève Brunck -
Investigadora Principal
Centro de Biotecnología FEMSA (ITESM) (Campus Mty)
Av. Eugenio Garza Sada # 2501 Sur
Tecnológico CP: 64849
Monterrey
Nuevo León, México

APROBADO
07 ENE 2019

PRESENTE.-

Estimada **Dra. Marion Emilie Geneviève Brunck -**

Por medio de la presente le informamos a Usted que el Comité de Ética en Investigación de la Escuela de Medicina del Instituto Tecnológico y de Estudios Superiores de Monterrey., a revisado la documentación del Protocolo: **2018_HSPC_Brunck** Titulado: **ESTUDIOS DE LA DIFERENCIACION DE CELULAS MADRES HEMATOPOYETICAS ENRIQUECIDAS DE SANGRE DE CORDON UMBILICAL A NEUTRÓFILOS EX VIVO** , que a continuación se enuncia:

1.- Carta de Control de Cambios y Respuestas a Comentarios de los Comités de Ética al Protocolo: 2018 HSPC Brunck Versión 1.0
2.- Protocolo: 2018 HSPC Brunck, Titulado: ESTUDIOS DE LA DIFERENCIACIÓN DE CÉLULAS TRONCALES HEMATOPOYÉTICAS ENRIQUECIDAS DE SANGRE DE CORDÓN UMBILICAL A NEUTRÓFILOS EX VIVO, Versión 2.0, fechado 5 de Diciembre de 2018, Monterrey, N.L., México.
3.- Hoja de Información para Paciente-Donación de Sangre de Cordón Umbilical-Versión 2 Versión fechada 5 de diciembre de 2018, Protocolo: 2018 HSPC Brunck, Titulado: ESTUDIOS DE LA DIFERENCIACION DE CELULAS TRONCALES HEMATOPOYETICAS ENRIQUECIDAS DE SANGRE DE CORDON UMBILICAL A NEUTRÓFILOS EX VIVO
4.- Hoja de Información para Paciente-Donación de Sangre Periférica Versión 2 Versión fechada 5 de diciembre de 2018 Protocolo: 2018 HSPC Brunck, Titulado: ESTUDIOS DE LA DIFERENCIACION DE CELULAS TRONCALES HEMATOPOYETICAS ENRIQUECIDAS DE SANGRE DE CORDON UMBILICAL A NEUTRÓFILOS EX VIVO

Tecnológico de Monterrey Escuela de Medicina
COMITÉ DE ÉTICA EN INVESTIGACIÓN

Le informamos que en esta sesión ordinaria celebrada el Lunes, 07 de Enero de 2019 cumpliendo con los lineamientos de las GCP-ICH y leyes locales vigentes en México; estando presentes 4 integrantes del Comité de Ética en Investigación; los documentos mencionados anteriormente han sido revisados en aspectos metodológicos, técnicos y éticos por **el Comité de Ética en Investigación de la Escuela de Medicina del Instituto Tecnológico y de Estudios Superiores de Monterrey.**, por lo que queda en carácter de:

Aprobado
Vigencia de Aprobación Lunes, 07 de Enero de 2019 a Martes, 07 de Enero de 2020

Sin más por el momento quedo a sus órdenes

Atentamente,

Dr. Federico Ramos Ruiz
Presidente
Comité de Ética en Investigación
"Comité de Ética en Investigación de la Escuela de Medicina del Instituto Tecnológico y de Estudios Superiores de Monterrey."
"No. De registro del Comité de Ética en investigación ante la Comisión Nacional de Bioética CONBIOETICA 19 CEI 011-2016-10-17 "

Federico Ramos Ruiz
Presidente del Comité de Ética en Investigación
I verified this document
10-01-2019T10:38:36

Appendix 4: Hoja de Información para Paciente-Donación de Sangre de Cordón Umbilical

ESTUDIOS DE LA DIFERENCIACION DE CELULAS MADRES HEMATOPOYETICAS ENRIQUECIDAS DE SANGRE DE CORDON UMBILICAL A NEUTRÓFILOS EX VIVO

[Colección de Sangre de Cordón Umbilical para su Uso en el Desarrollo de Nuevas Terapias Celulares]

Hoja de Información para Paciente

Investigador Principal: Dra. Marion E. G. Brunck
Contacto: marion.brunck@itesm.mx

Investigador Medico TecSalud: Dr. Daniel Humberto Méndez Lozano, Ginecología y Obstetricia
Contacto: danielmendez@itesm.mx

Introduccion
Está invitado a participar en este proyecto de investigación que involucra la colección de sangre de cordón umbilical para su uso en el desarrollo de potenciales nuevas terapias celulares. Esto se debe a que dará a luz en un hospital parte de TecSalud.

Esta Hoja de Información del Participante / Formulario de Consentimiento le informa sobre el proyecto de investigación. Saber lo que está involucrado lo ayudará a decidir si desea participar en la investigación. Por favor, lea esta información cuidadosamente. Haga preguntas sobre cualquier cosa que no entienda o sobre la que quiera saber más. Antes de decidir si participar o no, es posible que desee hablar sobre esto con un pariente, amigo o profesional de la salud. La participación en esta investigación es voluntaria. Si no desea participar, no es necesario. Recibirá la mejor atención posible ya sea que participe o no.

Si decide que desea participar en el proyecto de investigación, se le pedirá que firme un formulario de consentimiento. Al firmarlo nos está diciendo que Usted:

- Comprenda lo que ha leído
- Está de acuerdo para participar en el proyecto de investigación con la donación de sangre de cordón umbilical
- Está de acuerdo con los objetivos y actividades de la investigación que se describe.

¿De qué se trata este proyecto de investigación?
La sangre del cordón umbilical, que generalmente se desecha, se recogerá después del parto y las células madre sanguíneas se aislarán de ella. Células madres son progenitoras de células de la sangre que proponemos, a largo plazo, se podrían producir en el laboratorio a fines terapéuticas. Un cuello de botella en la producción de estas células para terapia celular es el numero de células que se pueden producir en el laboratorio. Para incrementar el numero de células que se pueden

producir a partir de las células madres colectadas de sangre de cordón umbilical, proponemos modificar las células madres a través de herramientas de biología molecular, para que se puedan dividir mas rápidamente (y así producir más células). Este proceso de modificación de la biología de las células madres es completamente reversible, y se ha probado recientemente en laboratorios de Inglaterra. Queremos establecer esta prueba de concepto aquí, en México. Las células que pretendemos producir a partir de su donación de células madres NO se usaran a fines terapéuticas, pero a fines de investigación únicamente, y se desecharon al terminar el experimento.

Nuestra investigación utiliza estas células madre para examinar las primeras etapas de la formación de células sanguíneas, de modo que podamos comprender mejor cómo replicar el proceso fuera del cuerpo. Las células del cordón umbilical se cultivarán en el laboratorio de acuerdo con un método específico que permite la producción de una gran cantidad de glóbulos blancos a partir de células madre del cordón umbilical, un proceso que tiene lugar naturalmente dentro del cuerpo. En diversos momentos durante este procedimiento, se tomarán medidas de las diferentes propiedades de las células, lo que proporcionará más información sobre cómo se está produciendo el proceso y cómo podría mejorarse. Esta información puede resultar en la modificación del método para producir más y mejores glóbulos blancos. En última instancia, esperamos fabricar glóbulos blancos útiles para la prevención y el tratamiento de infecciones en pacientes, especialmente aquellos con cáncer o enfermedades de la sangre.

¿La donación de sangre del cordón umbilical lo ayuda a usted o a su bebé?
La investigación realizada no lo ayudará a usted ni a su bebé, en este momento. Sin embargo, esta contribución a nuestro conocimiento de la producción de células sanguíneas puede tener implicaciones para el tratamiento de otros pacientes, especialmente aquellos con cáncer o enfermedades sanguíneas.

¿La investigación revelará información genética sobre mí o mi bebé?
Este proyecto no incluirá ninguna prueba, como pruebas genéticas, que genere información sobre usted o los riesgos de salud futuros de su bebé, o información que sea relevante para la salud de otros miembros de la familia.

¿Hay algún riesgo para mi bebé y para mí si donar sangre de cordón umbilical para este proyecto?
No. No hay riesgo para usted o su bebé. La sangre se recoge del cordón umbilical y la placenta después del parto como parte de la recolección de rutina de muestras de laboratorio. Para proteger su privacidad, la muestra de sangre del cordón umbilical recibirá un código de estudio único y la información relacionada con el donante de sangre del cordón umbilical se conserva de forma anónima. Los datos se usarán solo para esta investigación en particular y no se divulgarán a ninguna otra agencia.

¿Cómo participo en este proyecto?
La participación en este proyecto de investigación es voluntaria. Si no desea participar, no hay ningún problema. Si decide participar, una vez que haya leído y entendido la información provista, nos gustaría que firme este formulario y que se lo entregue al personal del equipo. Puede conservar una copia para su propia referencia.

¿Hay alguna compensación por la participación en este proyecto?
La participación en este proyecto es completamente voluntaria y no se le pagará por donar sangre del cordón umbilical para este proyecto de investigación. Elegir no participar o retirarse de este proyecto no afectará la atención disponible para usted y su bebé.

¿Quién está organizando y financiando la investigación?
Este proyecto de investigación está siendo llevado a cabo por personal y estudiantes de posgrado del Centro de Biotecnología FEMSA, y de la Escuela de Medicina del Tecnológico de Monterrey. La financiación proviene por el momento de una beca de investigación obtenida de la Escuela de Medicina, y estamos aplicando a fondos del gobierno mexicano y fondos internacionales.

¿Quién ha revisado el proyecto de investigación?
Toda investigación con humanos en México es revisada por un grupo independiente de personas llamado Comité de Ética e Investigación. Los aspectos éticos de este proyecto de investigación han sido aprobados por el Comité de Ética e Investigación de la Escuela Nacional de Medicina del Tecnológico de Monterrey.

Este estudio cumple con los lineamientos del proceso de revisión ética del Teconógico de Monterrey y la Declaración de Helsinki sobre Conducta Ética en Investigación Humana.

Confidencialidad
Toda la información cruda se mantendrá confidencial y solo será visitada por los investigadores involucrados en el estudio. Las muestras serán de-identificadas al momento de la colección, es decir que estas muestras serán llamadas únicamente con un código (por ejemplo, la primera muestra de sangre de Cordón Umbilical colectada será llamada CU01), y no podrán ser relacionadas en ningún momento con los donantes. Las hojas de consentimiento informado no podrán ser correlacionada con las muestras ya que el consentimiento informado NO se obtendrá el mismo día que la colección de muestra. Los resultados globales del proyecto serán publicados en una revista internacional indexada "open-access", que podrán consultar gratuitamente los donantes de muestras si lo desean. Es importante destacar que al de-identificar las muestras, no será posible comentar ningún resultado personal a los donantes.

Apreciación
Gracias por participar en este proyecto. En última instancia, se espera que la información de este proyecto conduzca a un nuevo tratamiento clínico para el cáncer, las enfermedades de la sangre y los pacientes trasplantados. Estás haciendo un regalo generoso a la comunidad.

_____

ESTUDIOS DE LA DIFERENCIACION DE CELULAS MADRES HEMATOPOYETICAS ENRIQUECIDAS DE SANGRE DE CORDON UMBILICAL A NEUTRÓFILOS EX VIVO

[Colección de Sangre de Cordón Umbilical para su Uso en el Desarrollo de Nuevas Terapias Celulares]

Hoja de Consentimiento Informado

He leído la hoja de información para el estudio antemencionado y se me ha entregado una copia para guardar.

He tenido la oportunidad de platicar mis dudas a mi entera satisfacción.

Por medio de la presente, doy mi consentimiento para que la sangre del cordón umbilical de mi bebé sea recogida y utilizada en la investigación descrita anteriormente.

No llevo a sabiendas ninguna enfermedad transmitida por la sangre ni tengo ninguna otra condición médica que pueda afectar mi seguridad o la de los investigadores involucrados.

Entiendo que puedo retirar mi consentimiento en cualquier momento sin dar una razón. Entiendo que toda la información personal se mantendrá confidencial.

Nombre y Apellidos: _____

Firma: _____

Fecha: _____

Nombre y Apellidos de Testigo: _____

Firma: _____

Fecha: _____

REVOCACIÓN DEL CONSENTIMIENTO

Al firmar esta parte del formulario de consentimiento, está solicitando la extracción de sus muestras de sangre o las células de este proyecto de investigación en su totalidad, sin prejuicios o repercusiones para usted como donante.

Nombre y apellidos: _____

Firma: _____

Fecha: _____

Appendix 5: Hoja de Información para Paciente-Donación de Sangre Periférica

ESTUDIOS DE LA DIFERENCIACION DE CELULAS MADRES HEMATOPOYETICAS ENRIQUECIDAS DE SANGRE DE CORDON UMBILICAL A NEUTRÓFILOS EX VIVO

[Colección de Sangre Periférica para su Uso en el Desarrollo de Nuevas Terapias Celulares]

Hoja de Información para Paciente

Investigador Principal: Dra. Marion E. G. Brunck
Contacto: marion.brunck@itesm.mx

Investigador Medico TecSalud: Dr. Daniel Humberto Méndez Lozano, Ginecología y Obstetricia
Contacto: danielmendez@itesm.mx

Introducción
Está invitado a participar en este proyecto de investigación que involucra la recolección de sangre periférica para su uso en el desarrollo de potenciales nuevas terapias celulares.

Esta Hoja de Información del Participante / Formulario de Consentimiento le informa sobre el proyecto de investigación. Saber lo que está involucrado lo ayudará a decidir si desea participar en la investigación. Por favor, lea esta información cuidadosamente. Haga preguntas sobre cualquier cosa que no entienda o sobre la que quiera saber más. Antes de decidir si participar o no, es posible que desee hablar sobre esto con un pariente, amigo o profesional de la salud. La participación en esta investigación es voluntaria. Si no desea participar, no es necesario.

Si decide que desea participar en el proyecto de investigación, se le pedirá que firme un formulario de consentimiento. Al firmarlo nos está diciendo que Usted:

- Comprenda lo que ha leído
- Está de acuerdo para participar en el proyecto de investigación con la donación de sangre periférica
- Está de acuerdo con los objetivos y actividades de la investigación que se describe.

¿De qué se trata este proyecto de investigación?
Nuestra investigación utiliza estas células madre extraídas de sangre de cordón umbilical para examinar las primeras etapas de la formación de células sanguíneas, de modo que podamos comprender mejor cómo replicar el proceso fuera del cuerpo. Las células del cordón umbilical se cultivarán en el laboratorio de acuerdo con un método específico que permite la producción de una gran cantidad de glóbulos blancos a partir de células madre del cordón umbilical, un proceso que tiene lugar naturalmente dentro del cuerpo. En diversos momentos durante este procedimiento, se tomarán medidas de las diferentes propiedades de las células, lo que proporcionará más información sobre cómo se está produciendo el proceso y cómo podría mejorarse. Esta

información puede resultar en la modificación del método para producir más y mejores glóbulos blancos. En última instancia, esperamos fabricar glóbulos blancos útiles para la prevención y el tratamiento de infecciones en pacientes, especialmente aquellos con cáncer o enfermedades de la sangre.

Con el fin de determinar la naturaleza de las células desarrolladas en el laboratorio, es necesario compararlas con células sanguíneas normales de donantes adultos sanos (de sangre periférica).

¿Hay algún riesgo para mí?
Se usará una aguja para recolectar sangre de una vena en su brazo. Puede experimentar alguna molestia como resultado, y algunas personas experimentan mareos y desmayos. Si anteriormente ha donado sangre y sabe que tiene tendencia a desmayarse al hacerlo, reconsidere su oferta para actuar como donante. Durante la recolección se recomienda sentarse en una silla si esto le resulta más cómodo. Algunas personas experimentan hematomas en el sitio de inserción de la aguja. Esto puede causar incomodidad adicional y puede ser antiestético. Si en algún momento se siente incómodo y desea que finalice el proceso de cobro, informe a la persona que realiza la recopilación. El uso de sangre para este proyecto de investigación conlleva los mismos riesgos que si estuviera dando sangre en un centro médico para realizar pruebas de diagnóstico. Si bien no es posible para nosotros eliminar estos riesgos, la persona que recolecta su sangre ha recibido entrenamiento profesional en la realización de la recolección de sangre. Una tercera persona estará presente en todo momento para ayudar en caso de una emergencia. Para la seguridad de nuestros investigadores, solicitamos que confirme que no porta a sabiendas ninguna enfermedad transmitida por la sangre. Si tiene una enfermedad transmitida por la sangre, simplemente devuélvanos esta forma incompleta. No es necesario que proporcione una razón para negarse a participar.

¿Cuánta sangre daré?
Solo requerimos cantidades relativamente pequeñas de sangre de una sola colección (20 ml), hasta un máximo de 50 ml. Es posible que se le pida que haga una donación en varias ocasiones, sin embargo, no le pediremos que done sangre más de una vez cada 7 días.

¿Cómo participo en este proyecto?
Una vez que haya leído y entendido la información provista, firme este formulario y entréguelo a la persona que se comunicó con usted para convertirse en donante. Puede conservar una copia para su propia referencia. Le informaremos sobre el momento en que se recolectará su sangre. Esto tendrá lugar en el Centro de Biotecnología FEMSA o en la Escuela de Medicina-Hospital San Jose. Como voluntario, no recibirá ningún tipo de compensación. Apreciamos su contribución a esta importante investigación.

¿Quién ha revisado el proyecto de investigación?
Toda investigación con humanos en México es revisada por un grupo independiente de personas llamado Comité de Ética e Investigación. Los aspectos éticos de este proyecto de investigación han sido aprobados por el Comité de Ética e Investigación de la Escuela Nacional de Medicina del Tecnológico de Monterrey.

Este estudio cumple con los lineamientos del proceso de revisión ética del Teconógico de Monterrey y la Declaración de Helsinki sobre Conducta Ética en Investigación Humana.

Confidencialidad

Toda la información cruda se mantendrá confidencial y solo será visitada por los investigadores involucrados en el estudio. Las muestras serán de-identificadas al momento de la colección, es decir que estas muestras serán llamadas únicamente con un código (por ejemplo, la primer muestra de Sangre Periférica colectada será llamada SP01), y no podrán ser relacionadas en ningún momento con los donantes. Las hojas de consentimiento informado no podrán ser correlacionada con las muestras ya que el consentimiento informado NO se obtendrá el mismo día que la colección de muestra. Los resultados globales del proyecto serán publicados en una revista internacional indexada "open-access", que podrán consultar gratuitamente los donantes de muestras si lo desean. Es importante destacar que al de-identificar las muestras, no será posible comentar ningún resultado personal a los donantes.

Apreciación

Gracias por participar en este proyecto. En última instancia, se espera que la información de este proyecto conduzca a un nuevo tratamiento clínico para el cáncer, las enfermedades de la sangre y los pacientes trasplantados. Estás haciendo un regalo generoso a la comunidad.

ESTUDIOS DE LA DIFERENCIACION DE CELULAS MADRES HEMATOPOYETICAS ENRIQUECIDAS DE SANGRE DE CORDON UMBILICAL A NEUTRÓFILOS EX VIVO

[Colección de Sangre Periférica para su Uso en el Desarrollo de Nuevas Terapias Celulares]

Hoja de Consentimiento Informado

He leído la hoja de información para el estudio antemencionado y se me ha entregado una copia para guardar.

He tenido la oportunidad de platicar mis dudas a mi entera satisfacción.

Por medio de la presente, doy mi consentimiento para dar sangre periférica para su uso en la investigación descrita anteriormente.

No llevo a sabiendas ninguna enfermedad transmitida por la sangre ni tengo ninguna otra condición médica que pueda afectar mi seguridad o la de los investigadores involucrados.

Entiendo que puedo retirar mi consentimiento en cualquier momento sin dar una razón. Entiendo que toda la información personal se mantendrá confidencial.

Nombre y Apellidos: _____

Firma: _____

Fecha: _____

Nombre y Apellidos de Testigo: _____

Firma: _____

Fecha: _____

REVOCACIÓN DEL CONSENTIMIENTO

Al firmar esta parte del formulario de consentimiento, está solicitando la extracción de sus muestras de sangre o las células de este proyecto de investigación en su totalidad, sin prejuicios o repercusiones para usted como donante.

Nombre y apellidos: _____

Firma: _____

Fecha: _____