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DESIGN, OPTIMIZATION AND *IN VITRO* EVALUATION OF NOVEL METHODS FOR THE OBTAINING OF A PLATELET-RICH PLASMA ENRICHED IN PLATELET AND EXTRAPLATELET GROWTH FACTORS

Doctoral Thesis

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Thesis submitted for the Degree of Doctor of Philosophy

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SUMMARY

Considerable advances have been made in the last decades towards the generation of different Platelet-Rich Plasma (PRP) formulations, centrifugation being one of the most widely used methods for PRP obtaining. However, such protocols focus on the concentration of platelets without paying attention to extraplatelet molecules. In this thesis, several methods for the obtaining of a novel PRP enriched in platelet and extraplatelet growth factors have been proposed. The novel PRP could provide better clinical results than those obtained today with the conventional biological therapies.

TABLE OF CONTENTS

ACRONYMS AND CONTRACTIONS

ANOVA: Analysis of variance

APMA: 4-aminophenylmercuric acetate

BDNF: Brain-derived neurotrophic factor

BSA: Bovine serum albumin

CDM: Chondrocyte differentiation medium

CI: Confidence interval

CoC: Cartilage-on-chip

DAPI: 4′,6-diamidino-2-phenylindole

DI: Deionized water

DMEM: Dulbecco´s modified eagle medium

ECM: Cartilage extracellular matrix

ECX: Energy dispersive x-ray technique

EGF: Epidermal growth factor

ELISA: Enzyme-linked immunosorbent assay

FBM: Fibroblast growth basal medium

FGF: Fibroblast growth factor

fPRP: ultrafiltration-based Platelet-Rich Plasma

GF: Growth factor

HBSS: Hank's balance salt solution

hCh: Human chondrocyte

HEAA: Hydroxyethyl acrylamide

HG: Hydrogel

HGF: Hepatocyte growth factor

hPRP: Hydrogel-based Platelet-Rich Plasma

IGF-I: Insulin-like growth factor

IL-4: Interleukin 4

ITS: Insulin transferrin selenium

JoC: Joint-on-chip

LP-PRP: Leukocyte-Poor Platelet-Rich Plasma

LR-PRP: Leukocyte-Rich Platelet-Rich Plasma

LRF: Leukoreduction filter

MBAAm: N, N′-Methylenebis(acrylamide)

MMP: Metalloproteinase

MPC: Microfluidic platelet mediator

MW: Molecular weight

NGF: Nerve growth factor

NHDF: Normal human dermal fibroblast

nPRP: Novel Platelet-Rich Plasma

OA: Osteoarthritis

PBS: Phosphate buffered saline

PC: Platelet concentrate

PDGF: Platelet-derived growth factor

PDMS: Polydimethylsiloxane

PEG: Polyethylene glycol

PLT: Platelet

PPP: Platelet-Poor Plasma

pRBCs: Processed units of packed red blood cells

PRP: Platelet-Rich Plasma

RBC: Red blood cell

RLU: Relative light unit

rPRP: Rotary evaporation-based Platelet-Rich plasma

RT: Room temperature

SEM: Scanning electron microscope

SID: Strong ion difference

sPRP: Standard Platelet-Rich Plasma

TGF-1ß: Transforming growth factor beta 1

TNCB: 2,4,6-trinitro-1-chlorobenzene

TNF-α: Tumour necrosis factor Alpha

TP: Total Protein

UV: Ultraviolet

VEGF: Vascular endothelial growth factor

vWF: Von Willebrand factor

WBC: White blood cell

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Aim and Outline of the Thesis

1.1. General introduction

Platelet-Rich Plasma (PRP) is a biological hemoderivative product obtained from blood in which platelets are present in a higher concentration than basal levels, according to the Spanish Medicines Agency (AEMPS)[1]. PRP is applied in the treatment of different pathologies to promote tissue regeneration and reparation. These pathologies range from musculoskeletal disorders, oral and maxillofacial surgery and dermatology, among others[2,3,4,5]. Due to its easy preparation, its low-cost processing and its autologous nature[6], PRP has gained great value as a treatment in regenerative medicine in the last few years[7].

Its restorative potential is mainly based on platelets, which are anucleate blood elements, and derived from the haematopoietic line via the megakaryocyte, having a key role in haemostasis and thrombosis[8]. Platelet response to tissue damage occurs in several stages, resulting in the release of an arsenal of potential regenerative and mitogenic substances that are involved in wound healing and that play a key role in tissue regeneration[9]. These substances immediately bind to the external surface of cell membranes[10], mediating many of the cellular functions including cell migration, differentiation, cellular cycle, apoptosis and metabolism, as well as proliferation[11–13]. Therefore, platelets are expected to stimulate injured tissues and to regulate local inflammatory processes[10]. These released molecules include growth factors (GFs) and cytokines contained mostly in α-granules inside platelets[9]. In addition to all these compounds derived from platelet activation, PRP also contains extraplatelet biomolecules in plasma with important biological activity that take part in the regulation of the chemotaxis, cellular differentiation and mitogenesis[14]. Moreover, these factors stimulate mesenchymal and epithelial cells to increase the synthesis of collagen and matrix to promote the formation of fibrous connective tissue and scar formation[13].

Therefore, despite the importance of platelets and their derivates, other extraplatelet components must be carefully considered in the therapeutic potential of PRP.

1.2. Aim and chapter overview

At present, the majority of methods to obtain PRP focus on optimizing platelet concentration, with centrifugation being the most commonly used technique[15]. Thus, there are a large number of protocols by which different PRP products with a wide range of platelet, leukocyte and erythrocyte concentrations can be obtained.

However, although current centrifugation-based systems concentrate platelets and their internal content, none of these methods address the concentration or modulation of the abovementioned extraplatelet plasma molecules, overlooking the biological activity they can bring to current PRPs.

Accordingly, the aim of this thesis is the generation of a PRP formulation with an enrichment of not only the platelet GFs but also the extraplatelet elements that could lead to a possible improvement in the therapeutic capacity of PRP. Therefore, three different new PRP production methods based on water evaporation, filtration, and absorption, with the aim of achieving novel PRP (nPRP) enriched in both, platelets and plasma biomolecules are reported, and whose composition and bioactivity was analysed *in vitro* (Figure 1.1).

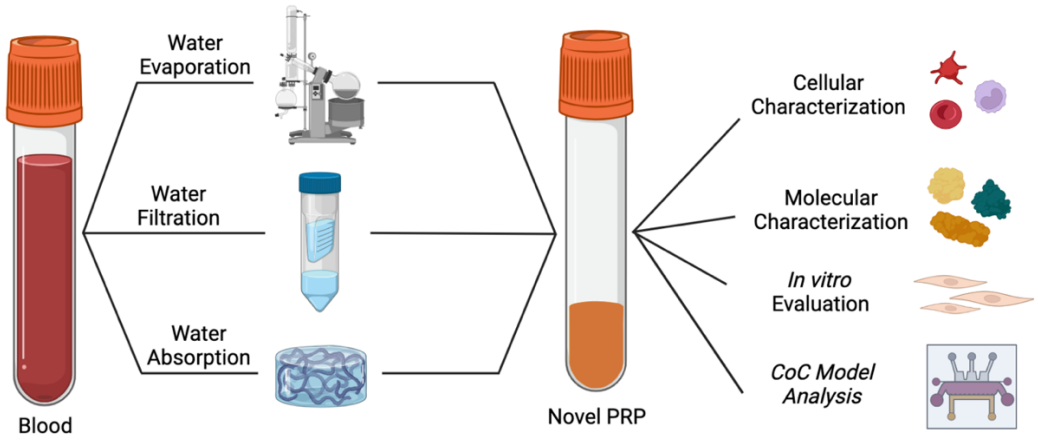

Figure 1.1. Scheme of the work described in this thesis: the development of three different methods for the elimination of the water of the plasma and its cellular and molecular characterization, *in vitro* evaluation, and analysis of the nPRP in a cartilage-on-chip (CoC) platform.

To reach the stated main goals, the following sub-objectives were stablished:

- To develop a method to obtain a nPRP enriched in both, platelet and extraplatelet GFs.
- To characterize the obtained nPRP formulations.
- To carry out the *in vitro* analysis of the nPRP bioactivity.
- To evaluate the suitability of the nPRP method for possible clinical use.
- To validate the effect of the nPRP on a catilage-on-chip osteoarthritic knee model.

1.3. Chapter overview

A detailed overview of each chapter, together with the contributions from research collaborators (where applicable), are given below:

Chapter 2 presents a general overview of the main components of PRP that provides its therapeutical effect and collects the different variations of formulations that exist depending on its application. Above all, this chapter

covers different existing new PRP obtaining methods that attempt to modify the molecular composition of the plasma. Moreover, the Chapter opens a way to explore new techniques to obtain a PRP with specific characteristics that could represent a major leap forward in this kind of treatments.

Chapter 3 reports a novel method for the concentration of extra- and platelet-derived GFs using a rotary evaporator system. A concentrate of platelets and protein is achieved by evaporating half of the volume of the water from the plasma. This nPRP showed a better cell viability when compared to a standard PRP (sPRP).

Chapter 4 describes an ultrafiltration method to obtain a nPRP similar to the one obtained in Chapter 3. Water present in the plasma is removed with a 3KDa filtering unit. The proteins present in the plasma with higher molecular weight (MW) get concentrated and later dissolved in a PRP fraction. This plasma fraction, enriched in platelets and biomolecules, shows superior cell viability compared to sPRP.

Chapter 5 presents the fabrication of an anti-fouling and high-water absorbent hydrogel, together with a method for the absorption of the water from the plasma to obtain a PRP with similar characteristics to the ones obtained in Chapters 3 and 4. Cells showed a higher viability response when incubated with the nPRP compared to a sPRP. Considering the usability of this technique, this system could be suitable for the future development of a commercial system to obtain a nPRP which has shown promising *in vitro* results.

Chapter 6 compares the three previously described methods in order to select the one with the best features in terms of cellular and molecular characterization and their scalability and suitability for clinical use.

Chapter 7 studies the impact of the selected nPRP from Chapter 6 on osteoarthritis (OA) study models, such as 2D primary chondrocytes culture and a CoC OA knee platform. For this purpose, cell viability of primary chondrocytes, expression of collagen type I and II and cytokine release from chondrocyte were analysed. The analysed nPRP showed the ability to enhance chondrocyte cell viability and the potential to improve the collagen II/I ratio, which is indicative of the repair of extracellular matrix degradation in OA. Additionally, it modulated the release of both pro- and anti-inflammatory cytokines, decreasing their levels after exposure. This work was done in collaboration with the group of Prof. Séverine Le Gac at AMBER group, MESA$^+$ institute from the University of Twente, Netherlands.

Finally, **Chapter 8** presents the final remarks and future work of the thesis.

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2

Current Challenges in the Development of Platelet-Rich Plasma-based Therapies

Nowadays, biological therapies are booming and more of these formulations are coming to the market. Platelet-Rich Plasma, or PRP, is one of the most widely used biological therapies due to its ease of obtention and autologous character. Most of the techniques to obtain PRP are focusing on new processes and methods of optimization. However, not enough consideration is being given to modify the molecular components of PRP to generate more effective formulations with the aim of improving PRP treatments. Therefore, this chapter covers different novel PRP obtaining methods that attempt to modify the molecular composition of the plasma.

2.1. Introduction to biological therapies

Biological therapies refer to any type of medical therapy that is derived from living organisms such as humans, animals, or microorganisms. This contrasts with traditional non-biologic pharmaceutical drugs, which are synthesized in a laboratory via chemical processes[1]. The first biological therapy on the market appeared in the 19th century with the discovery of the vaccines[2] and the insulin[3]. Since the 1990s, this number has vastly multiplied being now available to treat a wide variety of medical conditions such as cancer and autoimmune diseases[4]. Table 2.1 shows different classifications of biological therapies employed in the current times. Although these therapies are promising, they all come with different benefits and potential risks[1,5].

Over the last decade, biological products within the framework of regenerative medicine became promising tools in the therapeutic field of different medical specialties such as dentistry, traumatology, sports medicine, aesthetic medicine, or even in different types of surgery as tools to promote tissue repair, among others[6–9].

Table 2.1. Classification of the main biological therapies.

Biological Therapy	Action
Hemoderivative products (PRP and others)	Promote tissue regeneration and reparation for use in applications ranging from the musculoskeletal system to aesthetics and oral medicine[10].
Vaccines	Exploit human immune system to respond to, and remember, encounters with pathogen antigens and protect from catching a disease[11].
Antitoxins	Neutralize unbound exotoxins to eliminate the toxin from the body[12].
Recombinant proteins (insulin or erythropoietin)	Use of recombinant hormones, interferons, interleukins, growth factors, blood clotting factors or enzymes among others for the treating of major diseases such as diabetes, multiple sclerosis, thrombocytopenia, hepatitis, asthma, etc[13].
Recombinant nucleic acids	Inhibit DNA or RNA expression, thereby halting the production of abnormal proteins related to a disease while leaving all other proteins unaffected[14].
Interleukins	Regulate immune responses. They are used as biological response modifiers to boost the immune system in cancer therapy[15].
Monoclonal antibodies	Use in the diagnosis and treatment of many diseases, including some types of cancers. They can be used alone or carrying drugs, toxins, or radioactive substances directly to cancer cells[16].
Stem cell therapies	They are a form of regenerative medicine designed to repair damaged cells within the body by reducing inflammation and modulating the immune system[17].
Gene therapies	Replacement of a faulty gene or add/silence genes in an attempt to cure or help fighting a disease. They are used for treating cancer, heart disease, diabetes, haemophilia or AIDS[18].

The use of these treatments represents a paradigm shift in the way medicine is dealing with injuries and pathologies suffered by the patient: the aim is not to eliminate or replace the affected tissue but to take advantage of biological processes to stimulate tissue repair. Moreover, these techniques also seek to solve the problem from its source, and not only to alleviate the symptoms as is the case of analgesic pharmacology[19]. The versatility of biological products allows to use both conservatively and as an adjuvant to surgical interventions to improve results[20–22]. However, a good knowledge of the technique used in the treatments is necessary to achieve optimal clinical efficacy. It is essential to be clear about the type of product being used, as well as its characteristics, in order to apply it to the right patient and indication, and in the right way[23]. The main biological products for tissue repair can be grouped into cellular products and hemoderivative products, being platelet-rich plasma (PRP), a hemoderivative product, the most used one as cell therapies have certain limitations[24,25]. In fact, current regulatory agencies tend to include cell therapies within the group of so-called *Advanced Therapies*, which have a very restricted use, which is only permitted in certain situations such as clinical research or for compassionate use. For all these reasons, the application of cell therapies is still very limited[26] and their scientific and clinical background is scarcer than that of PRP, which has been widely used in recent years[27].

2.2. Platelet-Rich Plasma

PRP is an autologous biological hemoderivative product within the framework of regenerative medicine, which has gained great value in the last few years due to its easy preparation and low-cost processing. It is applied for the treatment of different pathologies, promoting tissue regeneration and reparation[28]. Its basic principle is to obtain a fraction of blood plasma containing platelets in a higher concentration than that at baseline blood levels[29], according to the Spanish

Medicines Agency[30]. From a pharmacological point of view, it is very difficult to define[31], as PRP has a large number and variety of active substances, often even antagonistic. Indeed, although PRP contains anabolic growth factors (GFs), it also contains many biomolecules that may antagonize its restorative effect on tissue metabolism[32,33]. PRP therapeutic potential relies on the synergistic action of the biomolecules present in both plasma and platelets, and their content, which is the central core of this therapy[34,35].

2.2.1. Platelets

Platelets are anucleate subcellular fragments with a life span of 7 to 10 days and present in blood at a concentration of 150,000-450,000 platelets μL^{-1}. Structurally speaking, platelets have an irregular disc shape of 2 to 5 μm in diameter and 0.5 μm of thickness[29,36]. Megakaryocytes of the bone marrow are the precursors of platelets[37], which after maturation, migrate to the endothelial barrier where project their prolongations releasing the precursors, also known as thrombocytes, that release platelets into the bloodstream[38] (Figure 2.1 A). This discord size fragments are continuously moving toward the edge of blood vessels, being essential to recognize endothelial injuries[36].

Platelets are bounded with an outer plasma membrane containing a large network of receptors that trigger key intracellular signals for the development of their functions[39]. Upon vascular injury, the subendothelium is exposed promoting von Willebrand factor (vWF) to deposit onto the exposed collagen. Platelets get attached at the site of the injury via interactions with these adhesive extracellular molecules. Once adhered, activated platelets aggregate and interact with the polymerizing fibrin network, tissue and other platelets to form a haemostatic plug, which participates in tissue homeostasis and repair[40] (Figure 2.1 B).

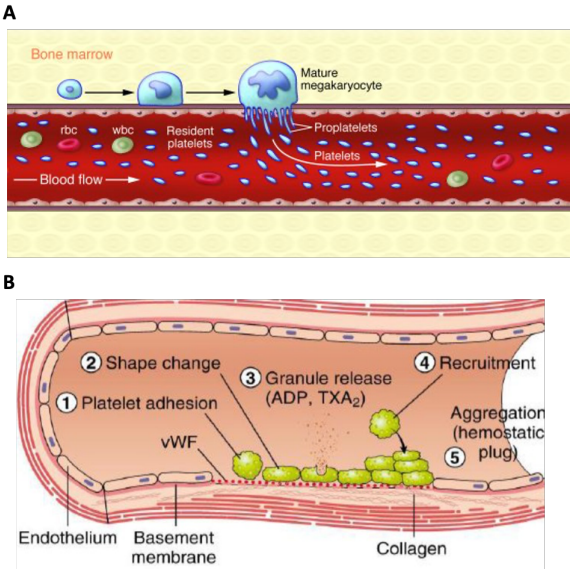

Figure 2.1. Graphical representation of platelet formation and its role in haemostasis. (A) After the maturation of the megakaryocyte in the bone marrow, it arrives to the endothelial barrier and releases its prolongations forming the thrombocytes[41]. **(B)** Platelets, after different signalling pathways, are accumulated in the damaged area creating a plug that participates in the repair of the tissue[42].

As mentioned above, the therapeutic potential of PRP is based on achieving a plasma fraction with a platelet concentration higher than blood levels. Even if there is still controversy to define an optimal platelet concentration, many studies consider a 1.5 – 3 fold increase in the concentration of platelets, the most favourable ones[29,32,43]. Indeed, other studies say that a higher platelet fold could reverse the therapeutical effects of PRP[44].

2.2.2. Molecular content

The internal contents of platelets are stored in different small secretory granules called α-granules, δ-granules (dense bodies) and λ-granules (lysosomal type organelle)[45]. The material presents in these granules is synthesized by the original megakaryocyte, as well as captured by platelets through endocytosis. The α-granules are the most abundant granules and have the highest content of active

biomolecules related to tissue repair. Indeed, they constitute 10% of platelet volume over a number of 50-60 α-granules per platelet[46]. Hundreds of molecules including membrane bound and soluble proteins have been identified inside these organelles, including adhesive proteins, fibrinolytic and coagulation factors, antimicrobial molecules, cytokines and GFs, among others[46]. Essentially, the interaction between these GFs and the surface receptors of the target cells activates the intracellular signalling pathways[47]. Adult mesenchymal stem cells, fibroblasts, osteoblasts, epidermal and endothelial cells express cell membrane receptors, which are specific to each GF[48,49]. It is therefore suggested that the GFs activate several cell types involved in tissue healing, and thus promote healing of soft tissue and regeneration of bone[9]. When platelets activate, it occurs the release of molecules and other elements such as platelet microparticles or exosomes, which have a high anti-fibrotic and anti-inflammatory immunomodulatory effect[50]. Exosomes are small vesicles of 100-400 nm that are involved in cell communication and transport various proteins as well as other biomolecules such as genetic material[36,51–53].

GFs include the Platelet-Derived Growth Factor (PDGF), which is a potent chemotactic factor for several cell types, having an important effect on tissue repair. Another growth factor with a strong presence in platelets is the Transforming Growth Factor Beta (TGF-β), whose effects are diverse and can be of different nature depending on the molecules and cells with which it interacts.

It influences early tissue repair responses, mesenchymal stem cell differentiation processes and the maintaining of cartilage and subchondral bone. Other regulatory factors for tissue repair are the Vascular Endothelial Growth Factor (VEGF), the Epidermal Growth Factor (EGF) or the Fibroblast Growth Factor (FGF), which play key roles in cell migration, proliferation, differentiation, or in angiogenesis. In addition, extraplatelet molecules such as the Insulin-like Growth Factor (IGF-1), or the Hepatocyte Growth Factor (HGF) also play a crucial role in

the tissue repairing process; they are GFs that enhance regenerative processes as well as modulate inflammatory processes. The prominent GFs and their biological functions are detailed in Table 2.2[54–61].

Table 2.2. Biological functions in tissue regeneration of each GF

Growth Factor	Abbreviation	Biological function
Insulin-like Growth Factor	IGF-1	Predominates in plasma, produced by the liver. Promotes cell growth, proliferation, and differentiation.
Transforming Growth Factor β1	TGF-β1	Acts in the early cellular reparative response (migration of cells and angiogenesis) in the wound area. Enhances synthesis of collagen and inhibits osteoclast formation and bone resorption.
Platelet Derived Growth Factor	PDGF	Enhances collagen synthesis, promotes mitosis and chemotaxis of mesenchymal origin cells, proliferation of bone cells, macrophage activation and stimulates vasculogenesis and angiogenesis.
Hepatocyte Growth Factor	HGF	Regulates cell growth, migration, and morphogenesis. Promotes extracellular matrix synthesis and has anti-inflammatory and antifibrotic effects.
Vascular Endothelial GF	VEGF	Is a key mediator in wound healing and the main inducer of angiogenesis due to the stimulation of chemotaxis and proliferation of endothelial cells. Also, it stimulates chemotaxis of macrophages and neutrophils.
Fibroblast GF	FGF	Is a potent inductor of cell proliferation, angiogenesis, and differentiation. Stimulates the growth and differentiation of chondrocytes and osteoblasts. Inhibits osteoclastic actions.
Epidermal GF	EGF	Promotes chemotaxis, mitogenesis and cytokine secretion by epithelial and mesenchymal cells.

PRP is therefore a cocktail of thousands of biomolecules coming from plasma and platelets that regulates haemostasis, coagulation, tissue repair and regeneration, inflammation, cell behaviour and defence against microorganisms, among other

biological processes. All this therapeutic potential depends to a large extent on their composition, which can vary according to the method of obtaining[29] the PRP and the activation process[62].

2.2.3. Methods to obtain PRP

PRP can be formulated in multiple ways with no consensus on a protocol that could be internationally used to standardize and facilitate their formulation[31]. The basis of the preparation method relies on the concept of differential centrifugation protocols[63]. Each component of the whole blood has a different density and, separates into distinct layers when they are spun when using a centrifuge[64]. These layers consist of a lower fraction of erythrocytes, a thin layer of leukocytes or *buffy coat* and, finally, a plasma fraction with platelets at certain concentration depending on the used centrifugation protocol[29].

Centrifugations can be single or double, with a centrifugal force of between 350 and 2000 x g and a centrifugation time of 3 to 15 min[63]. Depending on the parameters used in these centrifugations, the number of platelets may vary. In fact, plasma fractions with lower or higher platelet concentrations can be obtained, or even fractions with platelet concentration gradients. A PRP preparation protocol example is shown in Figure 2.2, the PRGF®-Endoret® method[65], where a platelet concentration gradient is achieved along the plasma fraction and the platelet poor plasma (PPP) fraction has less platelets than the PRP fraction.

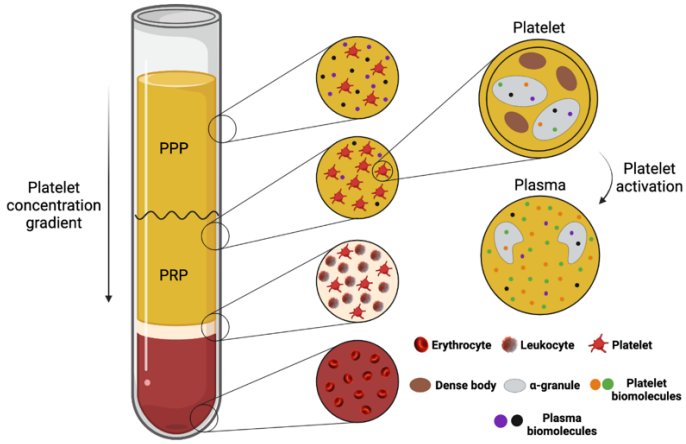

Figure 2.2. Graphical representation of a PRP obtaining method[29] in which a platelet gradient is achieved. Whole blood is separated into three fractions: one full of erythrocytes, another white-coloured fraction called *buffy coat* formed by platelets and leukocytes and finally the plasma area. PRP area represents 2 mL of the total plasma fraction and contains two to three times more platelets than the PPP fraction. After activation of the platelets, all the biomolecules from α-granules are released into the plasma.

2.2.4. PRP types and classification

As previously mentioned, PRP is a plasma fraction with a high concentration of platelets. Moreover, PRP can be modified by adding other elements to its formulation to treat specific pathologies[66]. Therefore, there is a wide range of PRP formulations depending on its use.

The main variables that can condition PRP are the number of platelets[67–69], the presence or absence of erythrocytes[70] and leukocytes[70], the use of PRP in liquid or gel format[71] and, related to this, the way of activating the platelets[62]. This generates many different products under the term PRP that need to be well classified due to a lack of standardization[72]. In response to this, to classify these therapies and become firmly established in the medical-scientific community, Kon *et al*.[73] clearly and simply set out a classification system for PRP. The code is a sequence of six digits grouped in pairs indicating parameters of platelet

composition, purity and activation with the aim of unifying the way PRP is classified for comparison and a more precise definition of the product.

2.2.4.1. Platelet concentration

It is important to note that although PRP can be formulated with different platelet concentrations, a higher number of platelets is not strictly related to a better effect[68].

PPP is the blood fraction with less concentration of platelets comparing with PRP and it does not have the same therapeutic advantages due to the reduction in platelet content, GFs and cytokines[32]. However, that plasma fraction contains the proteins responsible for the coagulation cascade but, according to some studies, PPP is not as effective as PRP[74–76]. Indeed, the effect of platelet concentration was demonstrated by several studies. A platelet ratio below 1.5 times compared to baseline levels showed not to promote bone regeneration, whereas levels between 6 and 11 times higher than baseline revealed an inhibitory response in bone regeneration[77]. However, a platelet ratio of 1.5 to 6 times compared to basal levels has been proven to be effective in numerous treatments[78–80], with the 1.5-3 fold concentration being the most favourable in most pathologies.[81,82]

2.2.4.2. Presence of erythrocytes

Regarding to the presence of erythrocytes in the PRP, the activation of platelets in the presence of red blood cells (RBC) leads to its own degradation processes, such as haemolysis and erythrocytosis, generating products that promote inflammation, cellular stress or even cellular death[70,81], which would hinder the beneficial action of PRP.

2.2.4.3. Leukocyte concentration

When separating plasma from the rest of the blood fractions, leukocyte, or white blood cells (WBCs) layer could be maintained. The Leukocyte-Rich PRP (LR-PRP) includes leukocytes in the autologous blood fraction while the Leukocyte-Poor PRP (LP-PRP) excludes them[32]. The foremost question between these two types of formulations is whether having WBCs helps or affects to the treatment response. Some studies said that the content of leukocytes might play a key role in the antimicrobial response and thus enhancing the release of GFs. WBCs also secrete some proteinases that play an important role during the wound healing process[51,83]. In contrast, other authors stated that the use of leukocytes inhibits the wound healing process by the release of reactive oxygen species (ROS) by neutrophils in the affected tissue area[49]. In fact, the presence of WBCs induces high levels of pro-inflammatory cytokines that may activate the inflammatory and catabolic changes that counters the beneficial effect of GFs in tissue repairing[84]. To be more specific, it was found that LR-PRP activates the transcription factor NF-kB pathway[85]. This factor regulates multiple aspects of innate and adaptive immune functions and serves as a crucial mediator of inflammatory responses. Moreover, it induces the expression of pro-inflammatory genes, including those encoding cytokines and chemokines, being its regulation crucial to prevent inflammation[86].

Overall, there is a lack of adequate studies on the potential positive or negative effects of leukocytes in PRP formulations, so the optimal concentration of WBCs is not clarified yet.

2.2.4.4. Activation methods for platelets

The activation of PRP is the last part of the process of PRP treatment. During platelet activation platelets release their contents, but also triggers the polymerization of fibrinogen into a fibrin mesh[87] keeping biomolecules on it[88], resulting in a controlled release of those molecules while the fibrin mesh is degraded[6,29].

Activation of platelets can be endogenous or through the addition of an exogenous clotting factor, producing different effects which influence the kinetics of GFs[62,89].

Exogenous activation involves physical methods, such as freeze-thaw cycles, or by the addition of certain substances such as calcium chloride or thrombin[89]. The main difference between the addition of $CaCl_2$ and thrombin is that the last, causes rapid aggregation and activation of platelets which leads to a decrease in the amount of GFs. Besides, some GFs have a short half-life and might degrade in minutes to hours if they are not immediately applied in treatment[90,91]. On the other hand, $CaCl_2$ generates a less condensed fibrin matrix, resulting in smaller amounts of thrombin formation and allowing a slower release of GFs over a period of 7 days, enhancing cell migration and wound healing[58,92]. What is more, it avoids the use of exogenous biological elements such as thrombin and the local hypocalcaemia that can be caused by calcium chelating anticoagulants used previously in the blood collection to prepare the PRP[93]. The advantage obtained by combining both agents is the benefit of creating an improved autologous gel or scaffold matrix that could be used in surgical interventions to fill several wound injuries maintaining a gradual GFs release[94].

Regarding the endogenous activation, some methods propose the administration of none activated PRP as platelets are physiologically activated once they enter the body by means of endogenous factors such as calcium in the wound area,

collagen, thrombin, ADP, fibrinogen, von Willebrand factor or serotonin, among others[95].

2.2.4.5. Gel state formulation

Gel formulations provide the damaged tissue with a fibrin matrix that acts as a structural support during the tissue repair process[96]. PRP gels are produced by the addition of thrombin and calcium chloride to the PRP fraction activating platelets and triggering the coagulation cascade, consequently forming a fibrin matrix, which enables the controlled delivery of GFs[32,97].

2.2.5. PRP applications and efficacy

The use of PRP has increased considerably in recent years due to the effectiveness of this treatment in different medical areas. Dermatology is one of the fields in which PRP has been effectively applied[8,98,99], achieving promising results in different areas such as facial rejuvenation, scar and wrinkle reduction and its use in the hair with the aim of reversing alopecia, among other treatments. The field of PRP in dermatology has only been exploited for a few years, therefore more clinical studies are needed to demonstrate a significant improvement over other treatments and its use in new treatments for different pathologies[8].

Over the last few years the use of PRP has been increased in the field of oral and maxillofacial surgery with the purpose of achieving bone regeneration, a rapid wound healing and reduction of bleeding after surgery[100]. During the first years, studies showed that the use of this therapy was beneficial for periodontal regeneration and regeneration of bone around the bone grafts. Nevertheless, future research in this field should be directed toward the implementation of well-designed, adequately powered clinical trials. The results of such trials will

help to elucidate the role of PRP in periodontal and other oral surgical settings[101,102].

The area of traumatology and orthopaedic surgery has generated much interest in the use of PRP for the treatment of certain pathologies[6,9]. One of the applications is the regeneration of bone tissue. Clinical and experimental studies about the osteogenic effect of PRP have shown that GFs enhance gene expression of Type 1 collagen, osteocalcin and osteopontin, thus promoting new bone formation[103]. Although, more studies are needed, according to a study, it has been reported that the use of PRP together with bone grafts resulted in a greater bone density[104].

Tendon and ligament healing were one of the first investigations performed with the PRP. Tendinopathy happens by natural aging and by repetitive stress. The injured tendons heal with scar tissue and that adversely affects its function. Some studies have demonstrated good results as they have shown improved tenocyte proliferation and collagen formation when PRP was applied[103]. Regarding to ligaments, in sports studies the evidence show improved time of healing, reduced pain and reduced time to return to sport[105].

In terms of cartilage damage, PRP induced a high mitogenic response in the chondrocytes enhancing the number of collagen-producing cells and increasing cell apoptosis. All this led to a high expression and synthesis of collagen by chondrocytes[103]. Related to this, osteoarthritis (OA) is a chronic degenerative disease of articular cartilage, thus affecting joint movements. PRP injections for this pathology have shown positive results[106].

Finally, among the therapeutic alternatives to address peripheral nerve injuries, Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF) and PDGF are some of the components of PRP that can enhance nerve regeneration. The biomolecules present in PRP are instrumental agents that act as key drivers

of full functional nerve recovery, promoting processes such as neuroprotection, modulation of inflammation, angiogenesis, enhancement of axonal growth and prevention of denervated muscle atrophy[107].

Theoretically, the use of PRP in orthopaedic treatments has important implications for accelerating and supporting the healing process of the musculoskeletal injuries[108–110]. The versatility of PRP-based products makes them an optimal biomaterial to be applied to the injured area by means of liquid infiltrations, reducing pain and improving the functionality of the joint in degenerative pathologies. These intra-articular infiltrations can be combined with intra-osseous infiltrations to extend the range of action of PRP, covering more joint structures and enhancing their effect[111]. PRP can also be applied in solid form as a membrane during surgery[112]. Thus, the use of PRP in osteochondral focal defects as "scaffolds" for GFs also generates a proliferative, migratory and chondrogenic environment of endogenous mesenchymal progenitor cells that favours the reparative environment[113]. The cellular effects generated as a consequence of the application of PRP to the injured tissue are intended to achieve a clinical result that favours and improves the recovery of the patients.

2.3. Recent methods to obtain PRP focused on platelet concentration

Despite the large expansion of PRP applications in recent years, and the large number of obtaining methods in the market, they are all based on the principle of using different centrifugation programs to separate blood components[31]. Varying the number of centrifugations and parameters, higher or lower platelet yields, WBCs or even platelet concentration gradients can be achieved.

Centrifugation is a standard method that has been used for a long time and it works well to obtain PRP, but it has its limitations. Depending on the number of centrifugations, the samples require more or less manipulation, increasing the waiting time and the risk of platelets settling to the bottom of the tube[114,115]. In addition, depending on the centrifugation force, platelets may be subjected to higher forces that may lead to their activation[116].

Centrifuge-based systems have several disadvantages: are time-consuming, do not concentrate all the biomolecules and it is difficult to control the concentration yield[117,118].

In order to optimize the aforementioned, new methods of obtaining PRP that do not involve centrifugation have been developed in recent years. For this purpose, different techniques have been used depending on the volume to be obtained PRP and the applications, thus creating two classifications: macroscale and microscale systems.

2.3.1. Macroscale systems

Macroscale systems involve any system capable of obtaining 1 mL or higher volumes of PRP. Jacofsky *et al.*[119] developed a single use and disposable platelet concentration device based of a filtration system. They compared the performance of their device with a centrifuge based PRP system. To obtain PRP they used two 55 mL samples of whole blood from donors and transferred it to a 60 mL syringe with 5 mL of citrate dextrose anticoagulant. One of the syringes was centrifuged to obtain 7 mL of PRP and with the other one, they applied the filtration system to obtain the same volume. The filtration process consisted of 3 phases: first, the contents of the syringe were mixed with an aqueous solution and passed through a blood filter to capture the platelets. Finally, another aqueous solution was passed through the filter to release and recover the platelets

in a final volume of 7 mL. The results showed that for the centrifuge method it takes 13.6 ± 0.1 min for the separation and concentration and for the filtered one 8 ± 2 min, being the filtration technique 40 % faster than the centrifugation technique. In addition, the results showed a similar number of concentrated platelets and GF concentrations comparing both systems. In this research, they did not perform any test to find out if platelets were activated during the filtration process. Moreover, they diluted the blood with an aqueous solution modifying the initial composition of the plasma, altering its autologous nature.

Another system carried out by Schmolz *et al*.[120] was the development of a method for the obtaining of a PRP within 2-3 h (Figure 2.3-A). They used a special device, where two separation systems took part: sedimentation and filtration. For the preparation of the PRP, they collected 8 mL of whole blood into a 10 mL syringe with an anti-coagulant and a sedimentation accelerator. The syringe was placed in the special device and was left there for about 60 min to promote sedimentation. After that, the supernatant was moved from the syringe to a second one and after that, 3.5 mL was injected in a specific inlet of the device to make the plasma pass through a membrane filter where the platelets were trapped. Then, another solution was passed through the membrane acting as a washing buffer to remove the anti-coagulant and the sedimentation accelerator. Finally, another solution was passed twice to promote the elution of the platelets' GFs for the obtaining of the PRP solution. In this centrifugation-free study there is no information about the number of platelets that are concentrated but they demonstrate that there are substantial quantities of both GFs, TGF-β and PDGF. Furthermore, the concentration of other important GFs where higher than the one found in the plasma before obtaining the PRP. Conversely, although this system directly releases the content of the platelets, the time needed for it obtaining and the numerous processing steps could present a disadvantage.

Furthermore, as in other methods, the autologous nature of the formulation is altered due to the use of buffers during the PRP obtaining process.

Wu *et al.*[121] developed an ultrasound device to obtain PRP creating standing waves inside a syringe due to the ultrasound waves generated from piezoelectric ceramics. A syringe was filled with 10 mL of whole blood and, subsequently, RBCs accumulate at certain locations of pressure nodes due to the acoustic radiation force (Figure 2.3 B-a). The formed cell cluster have a high sedimentation force, thus separating blood components in different fragments, obtaining 4 mL of PRP within 10 min. This research group demonstrated that the PRP prepared with the ultrasonic device (Figure 2.3 B-b), compared with a commercial product of centrifugal double syringe, achieved a higher platelet recovery rate (%) of 79 ± 9 and 54 ± 10, an RBC removal rate (%) of 99.0 ± 0.1 and 78 ± 10 and a platelet-fold increase of 2.1 ± 0.1 and 1.1 ± 0.2, respectively. In regard to the GFs, they detected similar levels in both methods. Furthermore, no morphological change or platelet activation were found after the use of the ultrasound device.

In another study, Gifford *et al.*[122] reported a portable system for processing whole blood without the need of centrifugation for obtaining PRP (Figure 2.3 C-a). They used half of 500 mL of whole blood for their proposed passive portable system and the other half for the centrifugation process. 1 L blood transfer bag was filled with 250 mL of blood. After that, the bag was placed between two plates with a compression apparatus at a slightly inclined angle of 10° to facilitate the separation of RBCs and WBCs from PRP components. After 150 min of sedimentation, the supernatant PRP was redirected to a microfluidic platelet concentrator with a flow rate of 3.2 mL min^{-1} to obtain PRP and PPP. The entire process for 250 mL took about 3 h (Figure 2.3 C-b). Regarding the data obtained, the passive system obtained 30 mL of PRP with a platelet recovery (%) of 88 ± 4 comparing with the centrifugation which showed a platelet recovery of 96 ± 3. Nevertheless, the passive system removed more WBCs than the centrifugation

method (0.4 ± 0.4 and 5 ± 2, respectively), whereas the centrifugation method obtained higher platelet count (873 ± 146 and 1151 ± 383, respectively). In addition, authors proved that with the passive method there was less platelet activation in contrast to the centrifugation one.

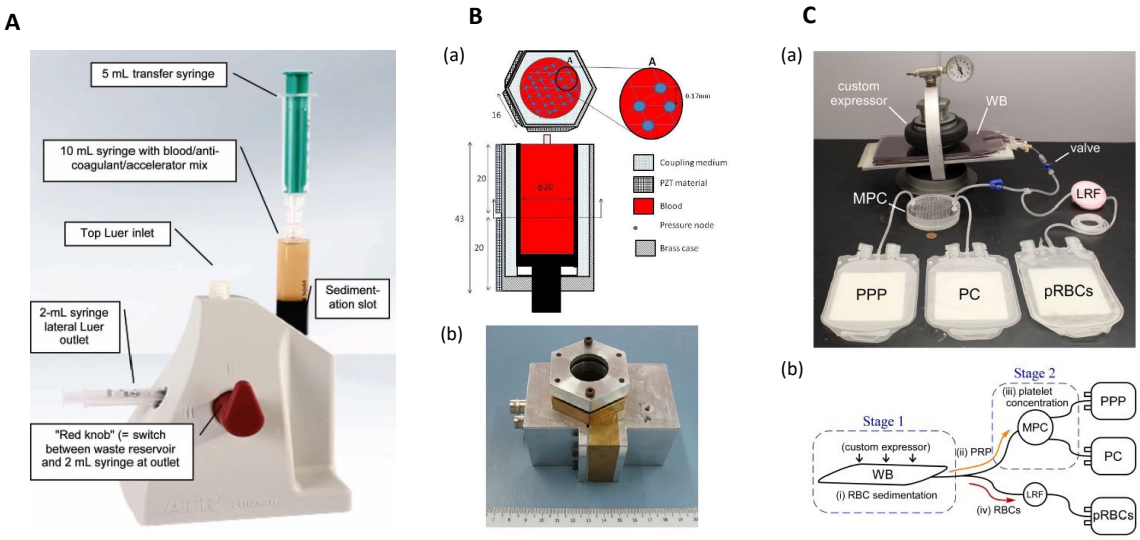

Figure 2.3. Methods to obtain high volumes of PRP. (A) Representation of the sedimentation system. (B) Ultrasound device to obtain PRP. (a) Section of the device where the blood is placed and submitted to ultrasonic waves. (b) Device used for ultrasonic PRP separation. **(C) Portable system to obtain PRP** (a) General set up for the separation of plasma and concentration of platelets. (b) Stages needed to obtain different plasma formulations. Whole blood (WB), microfluidic platelet concentrator (MPC), leukoreduction filter (LRF), platelet poor plasma (PPP), platelet concentrate (PC) and processed units of packed red blood cells (pRBCs).

The main limitations of the methods described above is their long processing time and the need to use diluents or buffers to obtain the PRP, something that is avoided when using centrifugation. The ultrasonic system developed by Wu et al.[121] includes all the necessary characteristics to develop a commercial product but so far it has not been commercialized and nowadays these systems have not replaced those based on centrifugation.

2.3.2. Microscale systems

Microscale systems are those created to obtain lower volumes of PRP (less than 1 mL). Within these types of systems, microfluidics devices designed for the separation of blood components played a very important role in recent years.

Microfluidic technology enables automated and efficient processing of liquid samples. Today is one of the fastest growing fields of research, continuously increasing in economic importance worldwide[123]. The development of autonomous, portable and fully integrated microfluidic devices has the potential to improve process automation, reduce cost for industry and decrease economic and social stress in the healthcare system[124]. There are several microfluidic architectures that allow the separation of plasma and blood cells and depending on the way of separation they can be classified in passive or active systems[125,126]. Aside from these, there are other techniques such as the compact disc (CD) microfluidic based method to separate the plasma[127,128]. Active technologies, based on microelectromechanical systems, use external mechanical forces for fluid control such as dielectrophoresis or acoustophoresis[127,129]. Passive technologies do not include external forces for fluid control as it is promoted by hydrodynamic forces, inertial forces, filtration or sedimentation among others[130]. Regarding the microfluidic systems to obtain PRP, several systems have been developed and each of them make use of the different technologies previously mentioned.

Regarding the hydrodynamic systems, Laxmi *et al*.[118] developed a system whose mechanism of operation consisted of a platelet separation and enrichment polydimethylsiloxane (PDMS) microdevice which involved a combination of biophysical effects (Figure 2.4 A-b). One of these effects, the Fahraeus effect, occurs when blood flows into the microchannel, the deformable RBCs tend to move to the centre of the channel and a cell free area is formed adjacent to the wall of the microchannel. Due to the hydrodynamic interaction with the RBCs

and platelets, these are pushed towards the wall and are conducted to a microchannel bifurcation which is the responsible of platelet concentrate collection (Figure 2.4 A-a). They obtained a system that achieve an 8.7-fold enrichment using undiluted whole blood with a 25.5 % of platelet purity. The flow rate used to obtain that enrichment was of 0.4 mL min^{-1}. Platelet activation test was done obtaining less platelet activation comparing with a conventional centrifugation method. Enrichment and purity of platelets showed opposite trends depending on the flow rates employed. At very low flow rates, the performance decreased, whereas at high flow rates, the quality of the platelets obtained deteriorated.

Dickson *et al*.[131] proposed a platelet separation and enrichment system using a filtration-based microdevice. This filtration system is based on crossflow filtration with two filtering steps (Figure 2.4 B-a). Blood passed continuously through the filters for a continuous separation and enrichment (Figure 2.4 B-b). Due to the pore size of the first filter, RBCs get removed, whereas the second filter separates plasma with no platelets. The obtained PRP was collected while the acellular plasma was recycled together with the rest of the blood and passed again trough the filtering system to obtain the desired platelet concentration. For the device operation, they used undiluted whole blood but to purge air bubbles from the device previous to its use, PBS was injected. During 30 min they stablished a flow rate of 100 μL min^{-1} at a shear rate of 16000 s^{-1} for the experiment, but they envisioned that the study can be transformed into a macroscale device which could obtain 50 mL of platelet enriched solution within 30 min.

The Lab-on-a-disk technique reported by Kim *et al*.[128] consists on a CD format device full of microfluidic channels and chambers (Figure 2.4 C-a). This group proposed a novel system, which consist of a tangential-flow-filtration CD format device that is composed of two integrated track-etched polycarbonate membranes with different pore size for particle filtration (Figure 2.4 C-b). The

disc is composed of several channels and chambers where less than 1 mL of whole blood is added. The disk stars to rotate, and the blood components filter from both filters within 20 min. The first filter removes RBCs and WBCs generating PRP due to the separation of platelet from other cells, while the other removes the residual plasma. The last filter is responsible for the concentration of the platelets in the desired volume (Figure 2.4 C-c). Comparing this with the centrifugation method, the plasma obtained in this way had a 4-fold platelet fold with a purity of 99 % and with minimum WBCs contamination in the sample. Concretely, 30.3 ± 32.4 WBCs per 10^6 platelets were found in the plasma fraction obtained with the disk, while 10260.2 ± 6867.1 WBCs per 10^6 platelets were present in the sample prepared with the manual method. The device worked with non-diluted blood, but a washing buffer and an elution buffer were necessary to perform the separation of blood components and obtain PRP.

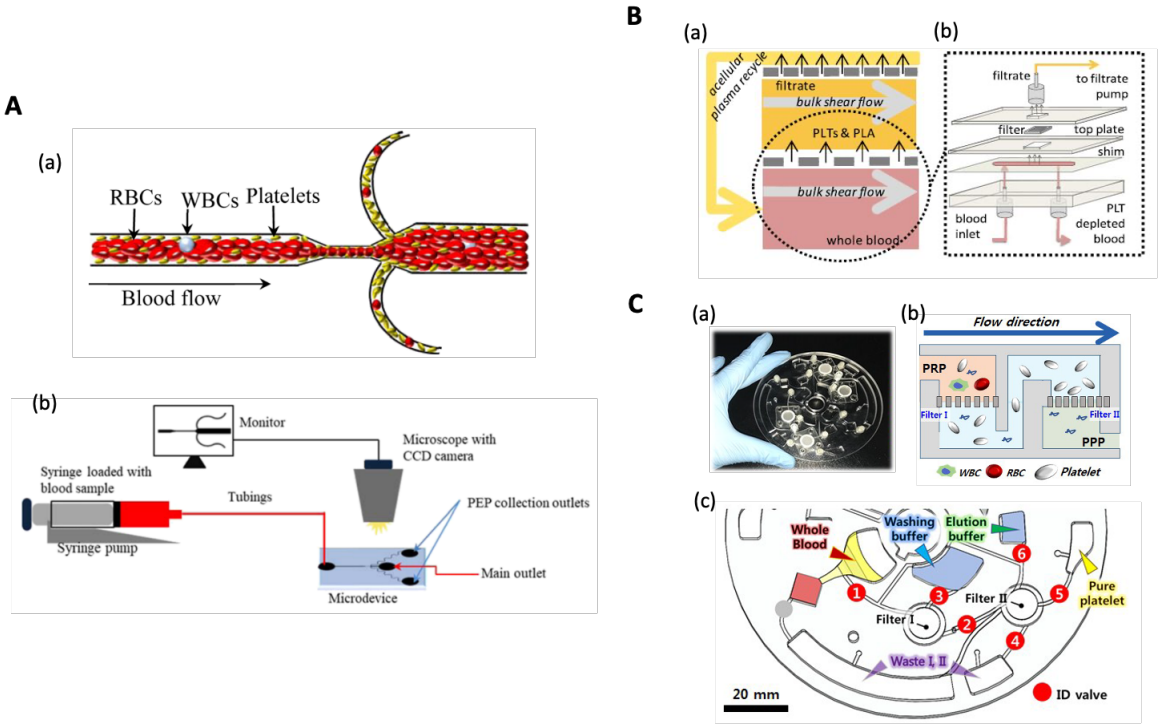

Figure 2.4. Methods to obtain small volumes of PRP. (A) Hydrodynamic system for platelet enrichment. (a) Section of the microfluidic platform. **(b)** Experimental set-up for platelet enrichment. **(B) Microfluidic platform for the**

obtaining of small and high volumes of PRP (a) Representation of the channels where the blood and plasma are passed through the filtering unit. **(b)** Graphical representation of the different parts of the device. **(C) CD format device to obtain PRP. (a)** Microfluidic CD shape platform **(b)** Filtering system for the capturing of blood components and platelet collection. **(c)** Overview of all the components of the CD device for PRP preparation.

In general, centrifuge-based, and non-centrifuge-based methods to obtain PRP focus on varying the platelets concentration and removing the RBCs and WBCs without considering the other molecular components in the plasma. Although microscale systems have the capacity to obtain high volumes of PRP for its application in therapy, their main limitations are the high platelet fold obtained in the final formulation (ranging from 4 to 9 in contrast with 1.5 to 3-fold usually acquired by centrifugation), which is something that is not so clear that it is beneficial for the treatment with PRP.

2.4. New method to obtain PRP focused on the modulation of the molecular composition

In addition to the platelets components, plasma also contains extraplatelet biomolecules and structures such as microvesicles and exosomes[93] with an important biological activity, which are key in processes related to cell communication and signalling[132]. Among the extraplatelet molecules, IGF-1 and HGF are the ones derived to a greater extent from the liver than from platelets[133,134] and take part in the regulation of the chemotaxis, cellular differentiation and mitogenesis[135]. They are also involved in the synthesis of collagen and matrix to promote the formation of fibrous connective tissue and scar formation[136]. A recent publication has shown that an increased concentration of the growth factor IGF-1 in PRP samples is associated with an increased cell viability[137]. As PRP is a concentrate of platelets and not of extraplatelet

biomolecules, these factors are not present at high concentrations. Additionally, there is almost unanimous agreement that IGF-1 declines in serum with increasing age[138–140], being a direct relationship to the effectiveness of PRP, as at elderly ages the effectiveness decreases. HGF is found in PRP formulations in a less concentration as it is present inside platelets but mostly as an extraplatelet molecule. This factor is important as it regulates cell growth, promotes cell migration, extracellular matrix formation and has anti-inflammatory and antifibrotic effects[134,141].

When applying centrifugation methods, platelets are concentrated in a plasma fraction obtaining the PRP, but it is just a plasma fraction enriched on platelets but not on extraplatelet biomolecules. This occurs because the centrifugation method does not work at the molecular level. In fact, obtaining these molecules involves the use of very high-speed centrifugations, called ultracentrifugations[142], that are not compatible with cell viability as platelets are activated[143] and precipitated[144] during the process.

Thus, the efficacy of PRP can be optimized modulating the PRP molecular cocktail, affecting both platelet and plasma biomolecules. The regulation of the molecular balance (Figure 2.5) between the different PRP biomolecules could favour certain biological processes. It is known that tissue repair is provided by the physiologically natural balance/ratio of GFs and other cytokines, which contain anabolic and catabolic functions in supraphysiologic concentrations, directly into the site of injury optimizing the healing environment[49,145,146]. Preserving a natural ratio of these biomolecules may allow the maintaining of the body's homeostatic environment, which theoretically would provide an abundance of healing factors without disrupting *in vivo* functions[67].

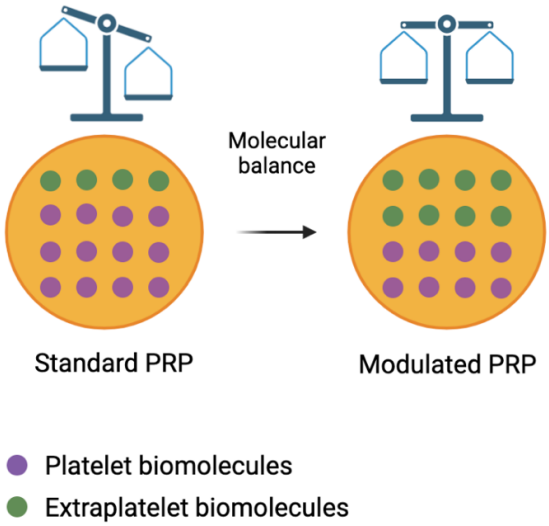

Figure 2.5. Representation of the biomolecule balance for the obtaining of modulated PRP. In standard PRPs, biomolecule imbalance is generated after platelet activation resulting in higher levels of platelet-derived growth factors than extraplatelet ones. Balancing the concentration of those molecules could potentially enhance the effect of the treatment.

2.5. Methods to concentrate the molecular components of PRP

In recent years, aiming to achieve an improvement in the PRP treatments, different PRP formulations have been developed varying the ratio between certain external proteins and platelets secreted proteins after activation.

Several methods could be used to obtain those new PRP formulations, some of them involving microfluidics. For example, a system was developed in which a plasma fraction previously obtained by centrifugation is circulated through a system based on microfluidics[147]. This system generated the evaporation of water from the plasma, dehydrating it and concentrating both platelets and extraplatelet plasma molecules (Figure 2.6 A-a). At the same time, the sample runs to another microfluidic platform in which the concentrated electrolytes were dialyzed (Figure 2.6 A-b). However, this system was designed for very small volumes of PRP and its transfer to volumes applicable to clinical practice made

it unfeasible due to the excessive time involved for sample preparation and to coagulation problems during the evaporation process.

The company Celling Bioscience launched the ART PRP plus kit[148], which has an integrated filter system of nanoporous fibres for the ultrafiltration of proteins from the PRP (Figure 2.6 B). This system can eliminate RBCs and WBCs by the use of a centrifugation and enrich platelets and, in turn, concentrate extraplatelet biomolecules of a molecular size greater than 25 KDa by means of the nanoporous fibres. Among all the proteins present in plasma, its main objective is to concentrate certain proteins such as VEGF, PDG-F, TGF-β, FGF, Alpha-2 Macroglobulin, Interleukin-1 receptor antagonist protein and fibrinogen, not being able to concentrate IGF-1 factor due to its small size molecular weight of 7.65 KDa [133].

The protein-based solution provided from Zimmer Biomet nStride® Autologous Protein Solution Kit is a potential kit for the concentration of extraplatelet molecules (Figure 2.6 C-a). This kit is a cellular concentration system that concentrates anti-inflammatory cytokines and anabolic GFs to significantly reduce pain and promote cartilage health. It generates a type of PRP that, in the last step of its production, concentrates all the proteins using polyacrylamide beads due to the absorption of the water from the plasma[149,150]. However, this system is based on obtaining PRP that contains both leukocytes and erythrocytes, and although WBCs do not seem to negatively influence the properties of PRP, RBCs can be detrimental to, for example, joint structures (Figure 2.6 C-b). Moreover, the final concentration of biomolecules in this system occurs after platelet activation and the release of their contents. This premature release could result in a rapid degradation of the platelet biomolecules before they can act on the target tissue.

A novel therapeutic preparation of a novel protein-based formulation is the Orthokine autologous therapy (Figure 2.6 D). This treatment is based on the production of anti-inflammatory cytokines such us IL-4 and IL-10 and the IL-1βRa, which is an antagonist of the pro-inflammatory cytokine IL-1β. The technique involves culturing the whole blood from the patient in a special syringe that contains $CrSO_4$ surface-treated glass spheres for 24 h at 37 °C in an incubator. The contact of the blood with these beads leads to a fast increase in the concentration of the above mentioned cytokines up to 140 times in the case of the IL-1βRa as $CrSO_4$ initiates the activation of monocytes and release pro and anti-inflammatory cytokines[151]. After 24 h of incubation, blood is centrifuged to collect the plasma fraction to be injected inside the affected area[152].

Last but not least, a new protein solution based in the Orthokine and nStride kits obtaining method from the company Qrem with the name of Qrem cytokine kit[153] was released recently (Figure 2.6 E). This new method consists of a kit composed of two containers. One container is filled with 18 mL of patient's peripheral whole blood while the other is filled with 20 mL. The kit is placed in the device which perform automatically all the processing steps for the obtaining of the final autologous solution. The first steps of the process yield a platelet and leukocyte concentrate in one of the containers while in the other a serum is obtained. This primary serum contains autologous activating elements such as thrombin and calcium, as well as anti-inflammatory cytokines and GFs. At the end of the process, the mixing of both containers occurs, which leads to the activation of the platelets and leukocytes and the formation of a fibrin matrix.

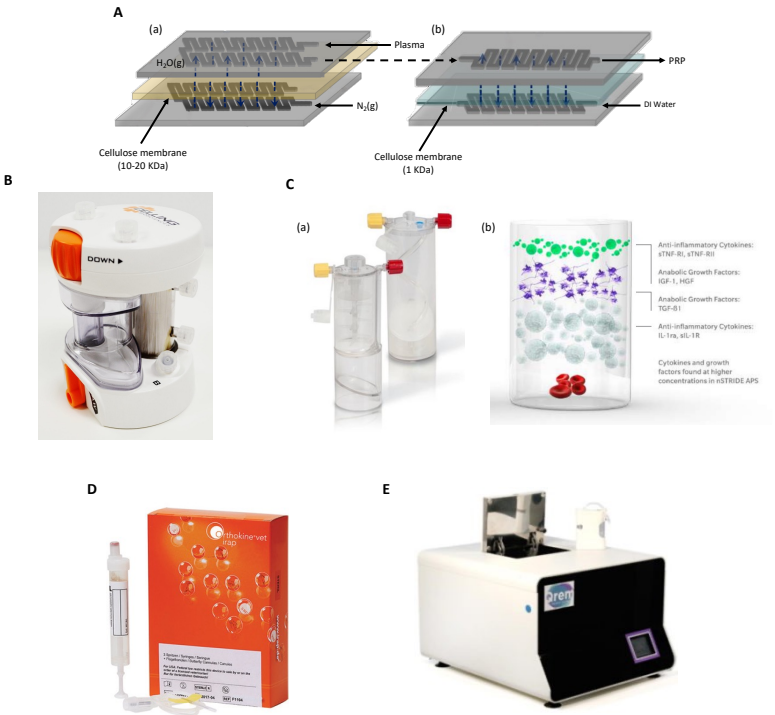

Figure 2.6. Devices to obtain novel formulations of PRP with varying ratios of biomolecules. (A) Microfluidic platform for the evaporation of plasma water content [147]**. (a)** Microfluidic platform for the evaporation of the water from the plasma **(b)** Microfluidic platform for the dialysis of the excess of electrolytes from the plasma **(B) Plasma ultrafiltration system for PRP obtaining. (C) Plasma concentration system by the use of acrylamide hydrogel beads. (a)** Devices used for the separation of blood components and the final concentration of plasma with acrylamide beads. **(b)** Bioactive molecules present in the final plasma **(D) Orthokine protein-based autologous solution. (E) Qrem protein-based autologous solution.** Device and the kit for the obtaining of the protein-based solution are shown.

2.6. Conclusions

PRP is an autologous biological product used in the field of regenerative medicine due to its great capacity to regenerate tissue, its easy preparation and low-cost procedure.

Centrifugation is mainly used to obtain a plasma fraction from whole blood with different concentrations of both platelets and WBCs. Depending on the treatment to be applied, one formulation or another may be produced, applying different

centrifugation programs in which the force, temperature and time can vary. There is no consensus on the ideal formulation of PRP regarding concentration of platelets to ensure a therapeutic effect, although experts recommend 1.5 to 3 times to the basal level of platelets in whole blood.

In order to minimize sample manipulation, reduce processing times and avoid platelet activation novel methods beyond centrifugation has been developed. A major disadvantage of these non-centrifugation-based methods, similarly to the centrifugation-based methods, is that they focus on increasing the concentration of platelets, neglecting the effect of an unbalanced ratio between platelets and soluble extraplatelet factors. This is very relevant because blood plasma contains many biomolecules whose combination can have a synergistic effect potentially making treatments with PRP more effective. Therefore, along with platelets it would be necessary to concentrate plasma biomolecules in order to keep the homeostatic ratio between platelets and extraplatelet biomolecules in PRP.

New methods have been developed over time to produce PRP formulations with increased concentration of platelets and soluble factors, but often the new formulations contain an increased concentration of only selected biomolecules leaving others aside. In fact, the growth factors IGF-1 and HGF are present at high concentration in the extraplatelet environment and are not usually concentrated in conventional PRPs. Some have even kept RBCs and WBCs in their formulations, not being beneficial in many cases.

In short, this Chapter 2 gives a review of novel systems for PRP production. Considering that many devices have focused on the concentration of certain proteins of the plasma without regard to achieving a balance between extraplatelet and platelet biomolecules of the PRP, this review emphasizes the need to investigate new formulations of PRP that can improve the efficacy of the treatment.

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Los capítulos 3, 4 y 5 están sujetos a confidencialidad por el autor

6

Evaluation of the clinical use suitability of the proposed methods to obtain nPRP

This chapter contains the evaluation of the main criteria considered for the choice of the most favourable system for industrialisation. The criteria are mainly based on the cellular and molecular characterisation of the PRPs, as well as their bioactivity on cell lines and adaptability to the market. In the analysis, subjectivity was assumed in the assignment of the scores, although they were based on the results obtained in the previous chapters. The highest scoring system was the one based on hydrogel absorption.

6.1. Elaboration and analysis of a decision table

Previous chapters described three novel methods to obtain a novel Platelet-Rich Plasma (nPRP) with a general objective of concentrating both platelet-derived and extraplatelet biomolecules. Each of the systems is based on the removal of water from the plasma by evaporation, filtration, and absorption to obtain a platelet and extraplatelet biomolecules concentrated plasma. Nevertheless, these systems have certain characteristics that differ from one another and where the final formulations obtained are not identical.

Based on the results obtained with each of the methods, a decision table was made (Table 6.1) to define which of the systems is the most viable for potential clinical use. To that end, different criteria was taken into consideration, such as the capacity to concentrate biomolecules, *in vitro* bioactivity, replicability, scalability and nPRP obtaining time, among others. The choice of the aspects to be taken into account and the scores given are subjective and adjusted to the interests and objectives of the company, since there is no official criterion to determine the score.

Table 6.1. Decision table for the selection of the most viable method for clinical use.

	Criteria	Platelet concentration	Total proteins concentration	Platelet factors concentration	Plasmatic factors concentration	Ion concentration	Platelet activation	Coagulation	Biological activity *in vitro*	Replicability	Scalability	Cleanliness	Obtaining Speed	Total score
	Weight	4	4	5	5	2	2	4	5	4	5	5	3	
	Evaporation	3	3	3	2	1	1	3	2	1	1	1	3	98
	Filtration	2	3	3	3	3	2	3	2	2	3	2	1	118
	Absorption	3	3	3	3	2	2	3	3	2	3	3	3	136
Scoring	*3* *2* *1*	*High* *Medium* *Low*	*High* *Medium* *Low*	*High* *Medium* *Low*	*High* *Medium* *Low*	*Low* *Medium* *High*	*Low* *Medium* *High*	*Normal* *Slow* *No*	*High* *Medium* *Low*	*High* *Medium* *Low*	*High* *Medium* *Low*	*High* *Medium* *Low*	*High* *Medium* *Low*	

Scoring was performed on a scale from 1 to 3, with 3 being the most favourable score for each criterion. In addition, a decision weight was assigned to each criterion on a scale from 1 to 5, with 5 indicating greater relevance in the final decision. Total score for each PRP obtaining method was calculated by the sum of each criterion multiplied by its weight in the final decision.

In terms of platelet concentration capacity, the most efficient methods in concentrating platelets were those based on evaporation and absorption, while the filtration method resulted in a lower concentration capacity. In the case of total protein concentration, all three methods were equally efficient.

Regarding platelet growth factors (GFs), all methods showed the ability to concentrate the Platelet-derived Growth Factor (PDGF), one of the main factors present in platelets. However, not all systems had the ability to concentrate plasma factors equally. Specifically, the evaporation-based system only enriched Insulin-like Growth Factor (IGF-1) but not the Hepatocyte Growth Factor (HGF), whereas the other two methods did. Although the concentration of platelets and total proteins is important in obtaining a PRP, what is essential is the final composition of the PRP, which is reflected in the levels of plasma and platelet GFs. This is why the highest score was given to GFs levels.

Regarding the ion levels present in the final formulations, the filtration-based method was the only one able to maintain physiological electrolyte levels while the evaporation method was the worst. However, this criterion has been given a weight of 2 because, as mentioned in previous chapters, the presence of ions does not have a relevant impact on their bioactivity.

The same applies to platelet activation. Although there are differences between the three systems, where the worst was the evaporation-based one, this has not resulted in a drawback in its bioactivity, although it is unknown if it could have further effects on other processes.

Another important factor in obtaining PRP is the clot generated after its activation. Ensuring that the clot is generated correctly in an acceptable amount of time is necessary to place the product in the affected area for the gradual release of the GFs. For this reason, the weight given to this criterion was a 4 out of 5. With respect to this, the three plasmas obtained by the different methods were able to generate the clot in a similar manner. Although the evaporation method initially presented drawbacks, these were solved by adjusting the pH. This is why the score assigned to each method was the same.

The next criterion, and one of the most relevant, was the bioactivity of the different plasmas. Since this aspect would be one of the most important when deciding about their clinical use, it was assigned the maximum weight. Although all the systems showed greater bioactivity with respect to conventional PRP, the absorption-based system showed the best results. Thus, it was assigned the maximum score over the other two systems.

In addition to the properties and characteristics of the nPRPs, other important factors were the suitability of these obtaining processes for large-scale clinical use. The replicability of each method was evaluated, giving a weight of 4 to this aspect. This issue is affected by the method itself and by the high inter-patient variability. Therefore, it is a factor that is hard to control. Therefore, none of the methods obtained the maximum score and it was the evaporation method that obtained the minimum score, since the conditions of temperature, pressure and time were more difficult to control than in the other methods.

Another important factor is the scalability, which determines the ability to employ these methods on a large scale, and therefore has been assigned the highest score weight. Within this aspect, the ability to use large volumes as well as being able to use a device in the clinic was valued. The least scalable method is the one based on evaporation, since, although it does allow large volumes, the

development of a device suitable for clinical use would be much more complex than the other two methods. In relation to this, another factor to be considered was cleanliness. Similarly, the evaporation-based method scored the lowest because it is difficult to convert it into a closed, single-use system. In this case, the method with the highest score was the absorption-based method. Although the filtration method would allow for a single-use system, this method involves a considerable manipulation and could lead to easier contamination of the final product.

The last aspect considered was the time required to obtain the nPRPs. Although this aspect is important, it is not determinant for the decision, so it was assigned a weight of 3 out of 5. Both the evaporation and the absorption method presented acceptable times for obtaining the PRP in the clinic, since they did not differ much from those required for conventional PRPs. However, the filtration-based method required a longer time to obtain the same product. For this reason, it was given the lowest score, as it cannot be assumed in the clinic.

Based on the decision table elaborated in the previous section, the method with the highest score was the absorption-based method, followed by the filtration-based approach and, in last place, the evaporation-based one.

7

Cartilage-on-chip osteoarthritic model for the determination of the effect of hPRP

This chapter analysed the impact of the hydrogel-based Platelet-Rich plasma on an osteoarthritic 2D human primary chondrocytes culture and a cartilage-on-chip knee platform. For this purpose, cell viability of primary chondrocytes, expression of collagen type I and II and cytokine release from chondrocyte chip cultures were analysed. In this preliminary study, hPRP showed the ability to enhance chondrocyte cell viability and the potential to improve the collagen type II/I ratio expressed by the chondrocytes in the extracellular matrix. Additionally, it modulated the release of both pro- and anti-inflammatory cytokines, decreasing their levels after exposure.

7.1. Introduction

In Chapter 6 it was determined which of the three systems to obtain Platelet-Rich Plasma (PRP) had the most favourable features to be exploited for a possible industrial use. Since the hydrogel-based system scored the highest, it was chosen as a candidate for further studies. The objective of this Chapter is to analyse the effect that hydrogel-based PRP (hPRP) may have in cellular osteoarthritis (OA) models, as PRP is widely used in the field of orthopaedics and specifically in chondrogenic injuries such as OA[1–3].

OA is one of the most common degenerative joint disease and the most prevalent form of arthritis, affecting mostly middle-aged and elderly population[4]. OA is characterized by breakdown of the cartilage, subchondral bone and other structures of the joints, with various degrees of inflammation of the synovium[5]. OA is currently treated with a few pharmacological alternatives. In fact, current treatments focus solely on relieving symptoms and not on tissue repair, eventually implying a total knee arthroplasty[6,7]. However, in recent years biological treatments, such as PRP, have shown efficacy in the management of these cartilage lesions, improving symptomatology, promoting tissue regeneration and significantly delaying the need for joint replacements[8]. However, the preventive effect of PRP in OA and its mechanisms of action are still unknown and there is a knowledge gap on its action at molecular level.

In recent years, a variety of cellular models have been developed to simulate joints, and specifically the knee, to allow the study of OA in a realistic way but avoiding the use of animals[9,10]. These knee models are displayed in joint-on-chip (JoC) models and/or in cartilage-on-chip (CoC) ones. Those models are multi-organ-on-chip platforms that incorporate a range of engineered features to emulate essential aspects and functions of the human joint since cells can be cultured on these chips in a more controlled manner, recreating a human-specific biological environment[11]. They faithfully recapitulate the joint's physiological

responses, being able to revolutionize the research and drug development in rheumatic diseases[12]. In fact, there have been reported *in vitro* models to study and reproduce the pericellular matrix of chondrocytes and characterize their organization in cartilage[13].

A CoC platform could mimic different structures of an OA knee of a patient walking by the use of a micromechanical actuator (Figure 7.1). The use of a mechanical stimulation permits the study of the behaviour of the human primary chondrocytes (hCh) and the evolution of their cartilage extracellular matrix (ECM), what may be useful to understand how the GFs present in the PRP act on the prevention of the OA.

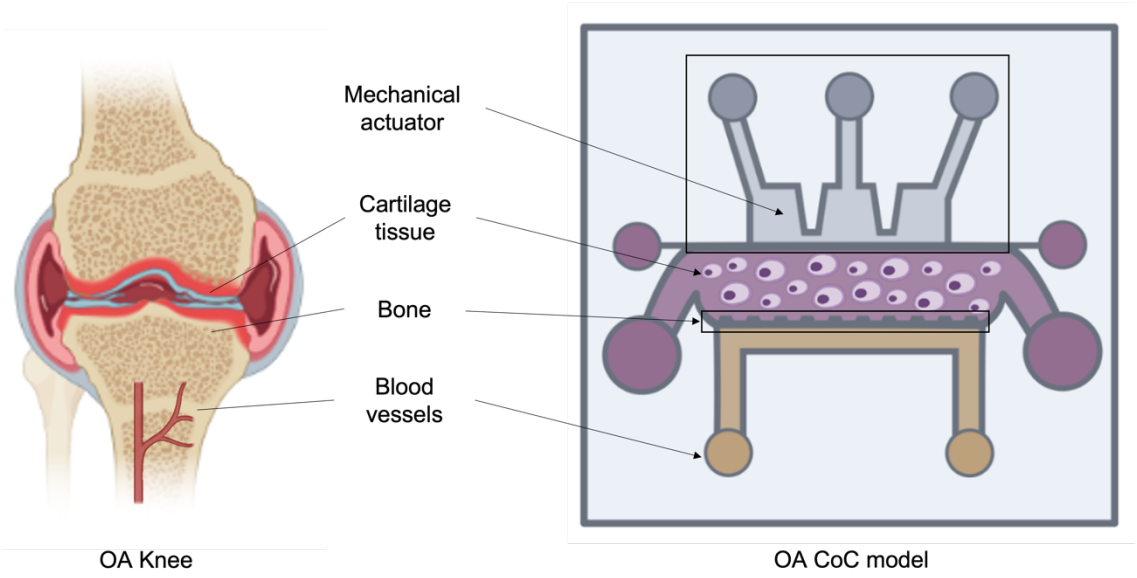

Figure 7.1. Schematic representation of a knee joint. OA cartilage-on-chip model mimics the environment of an OA knee. The CoC has the potential to simulate a patient walking due to the mechanical actuation[14]. A variety of drugs can be tested to see the response of the chondrocytes that simulate the cartilage tissue.

There are certain proteins that change their expression in hCh when these are in an OA environment. For instance, the metalloproteinase-13 (MMP-13), also known as collagenase-3, cleaves the main structural component of the cartilage,

the type II collagen, resulting in the irreversible loss of the cartilage ECM architecture and function[15,16]. The ratio of collagen type II and type I released by the human chondrocytes in the ECM is also affected in OA as, when collagen type II is degraded, collagen type I levels tend to increase for the production of new collagen type II[17]. Thus, the assessment of the ratio of released collagen type I and II could be indicative of a possible state of cartilage degeneration, as occurs in OA.

The analysis of anti-inflammatory and pro-inflammatory cytokines secreted by the hCh could also be a potential indicator of the effectiveness of the PRP treatments. In fact, PRP is known to reduce the pro-inflammatory cytokines[18] and increase the level of anti-inflammatory ones[19].

A CoC device would enable the observation of the cell behaviour and protein expression in a more realistic environment than in a basic *in vitro* 2D or 3D cell culture models because the specific conditions of each pathology are recreated in a more controlled manner. Moreover, it would allow to analyse whether sPRP and hPRP are able to reverse or decrease the expression of certain proteins associated with the development of OA. Additionally, this CoC approach may facilitate new discoveries that could only be achieved by the use of animal models, thus addressing the need to replace, reduce and refine animal experimentation in experimental research.

In the present work, it is reported the effect of a biomechanical stimulus combined with sPRP or hPRP treatment on the chondrocyte's protein production. To that end, the expression of collagen type I and II in the ECM and the examination of anti-inflammatory and pro-inflammatory cytokine levels were measured in CoC containing OA hCh cells.

7.2. Experimental

7.2.1. Isolation and culture of human primary chondrocytes

hCh were isolated from the knee condyles and tibial plateau derived from a total knee replacement surgery. The cartilage pieces were immersed and washed three times in phosphate buffered saline 1X (PBS) with 1% penicillin-streptomycin. Then, the cartilage was cut into 2x2 mm^2 pieces. A solution composed of Hanks' Balanced Salt Solution (HBSS) (Gibco, MT, USA) and collagenase II (Gibco, MT, USA) at 0.1-0.2% was added per gram of cartilage and incubated overnight at 37ºC and 5% CO_2. The following day, the cartilage pieces were filtered by a 40 μm cell strainer so that the remnants of the extracellular matrix of the cartilage remained on the surface of the filter, and the HBSS with the cells fell into the falcon. Two washes with PBS 1X were carried out by centrifugation at 300 x g for 10 minutes. Then, the cellular pellet was resuspended in high glucose and L-glutamine-containing Dulbecco's Modified Eagle Medium (DMEM) (ATCC, VA, USA) + 10% FBS + P/S and placed in a T25 flask. hCh were used at passage 2 for the experimental analyses[20].

7.2.2. Effect of hPRP formulation in osteoarthritic CoC platform

7.2.2.1. Effect of hPRP formulation in osteoarthritis CoC platform

The CoC devices were produced using a silicon wafer previously designed and fabricated by soft lithography in the Nanolab cleanrooms of the MESA+ Institute for Nanotechnology (University of Twente, Enschede, Netherlands)[14]. A mixture of PDMS pre-polymer and curing agent (Sylgard 184, Dow Corning, Midland, MI, USA) with a weight ratio of 20:1 was poured on the mould and cured at 60

°C for 24 h. Three punchers of diameter 1, 1.5 and 2 mm were used to create inlets and outlets of the device. The microfluidic layer was finally assembled to a glass substrate. Bonding step was performed using plasma treatment (Cute, Femto Science, Gyeonggi-do, South Korea). The final CoC devices were placed in an oven at 60 °C during 24 h before their use.

7.2.2.2. Experimental set-up for chip and sample collection

To evaluate the effect of the PRP formulations in the OA model, the experiments were conducted under six different conditions. All chips were seeded with hCh cells and filled with a medium supplemented with 10% PBS, sPRP or hPRP. Three of those conditions were kept in static conditions and the other 3 underwent compression. To serve as static controls, chips were loaded with beads instead of cells and the cell culture medium supplemented with 10% of sPRP or hPRP.

Eight chips were used per condition for controls and samples. Each two chip from each condition represent a replica to obtain sufficient volume (70 μL) to perform the collagen type I and II and cytokine analysis, achieving a total of four replicates. Culture medium was change daily with no perfusion. The duration of the experiment was set to two weeks and different timepoints were selected for the correct performance of the experiment. Figure 7.2 shows the timeline followed for the duration of the experiment.

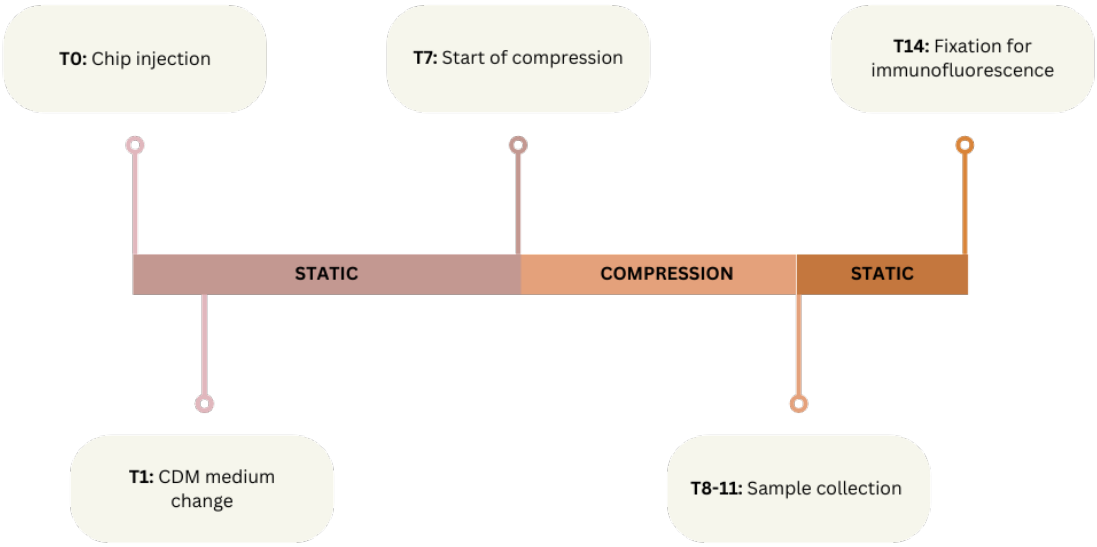

Figure 7.2. Timeline for the experimental set-up. Different timepoints (days) were set for the preparation of the chips, initiation of the compression, sample collection and fixation for the immunofluorescence analysis. Static chips were set in static condition for two weeks, while those that received compression started at T7.

T0: Chip injection

At T0 all the chips were injected with agarose gel together with beads for controls and hCh to test the PRPs. Chips were preheated at 25 °C on a hotplate (Thermo Fisher Scientific, EEUU) to make sure that when agarose is injected, there is no drastic temperature change, and it solidifies uniformly without slipping out of the place.

Agarose 4% ultrapure low melting point (Thermo Fisher Scientific, EEUU) was used to be mixed with the cells for the generation of the agarose gel. Final cell-agarose suspension per condition was done at 1:1 concentration and placed at 37 °C in a heat block (Thermo Fisher Scientific, EEUU) to prevent it from solidifying. Beads for controls and hCh for testing PRPs were seeded at a concentration of 2.75 10^6 mL^{-1}.

Once the agarose-cell mix was prepared, the chip injection was performed. To do that, agarose-cell mix was injected into the corresponding chamber of the CoC chip and filled until the whole chamber was completely full of agarose. After doing so, 70 μL of DMEM medium (Thermo Fisher Scientific, EEUU) was added in the medium chamber by placing pipette tips in the inlet and outlet to prevent evaporation and collect enough volume for further analyses. Once all the chips were filled, they were placed in the incubator with PBS 1X (Gibco, Thermo Fisher Scientific, EEUU) surrounding them to avoid any evaporation of the medium. During the first week of the experiment, all the chips were in static condition to allow adaptation of the chondrocytes.

T1: Medium change

At day 1, all the medium from the chips was replaced with 70 μL chondrocyte differentiation medium (CDM) containing: DMEM with 1% of pen/strep, ITS, L-proline, Asap and sodium pyruvate (Gibco, Thermo Fisher Scientific, EEUU). Every 2-3 days, 10 ng mL^{-1} of TGF-β and $1E^{-7}$ M of dexamethasone was added to the CDM medium. The reason to use CDM from day 1 was to provide chondrocytes with the best environment to ease their production of extracellular matrix.

T7-11: Initiation of the compression

After the first week, the actuation of the devices was started at T7. All the chips for the compression condition were placed in a 3D-printed plate which was connected to a microfluidic pressured based controller pump (Fluigent, France). The plate provided the same pressure to all the chips, which allows the actuation of all the chips at the same time (Figure 7.3).

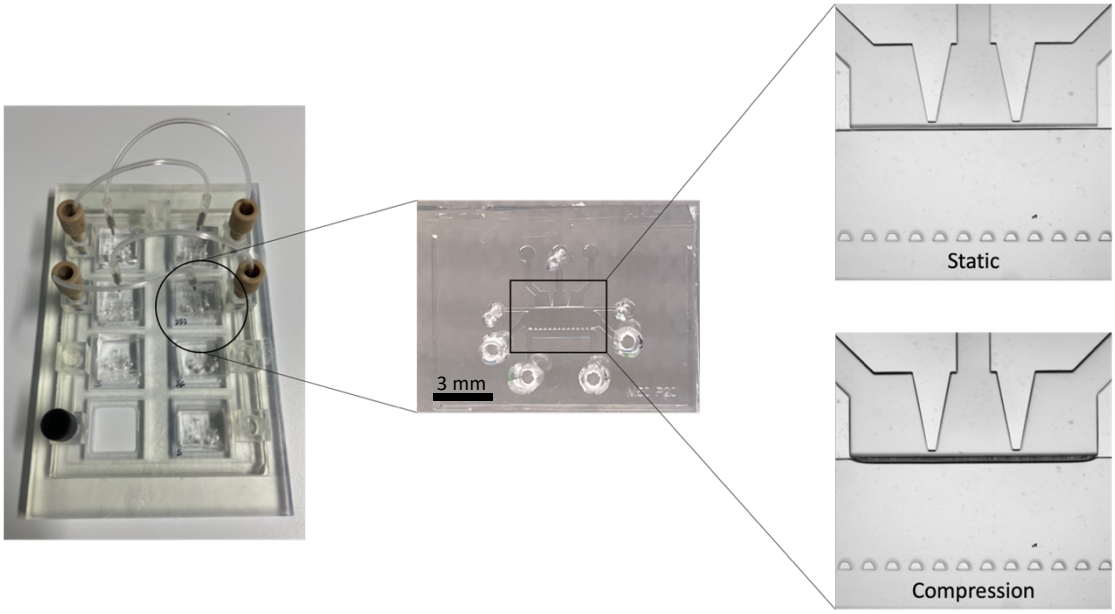

Figure 7.3. 3D-printed platform for the actuation of the chips. Static and compression states of the membrane of the actuation chamber of the chip can be appreciate.

Every day, 1 h of compression was applied to the chips for a total of five days. Fluigent OxyGEN software (Fluigent, France) was used to set the parameters for the actuation of the membrane of the chip. The pump provided a pressure of 300 mBar in a square wave at a frequency of 1 Hz with an amplitude of 150 mBar and a period of 2 s. After each compression, medium samples were collected for further analyses. Subsequently, fresh CDM was added into the chips. 50uL of samples and controls from all the conditions from days 8-11 were collected for future analyses and kept at -80 °C until used.

T14: Fixation for immunofluorescence analysis

At day 14, chips were dissected by peeling their top part and the agarose hydrogels were placed on a PDMS slab. The gels were then washed three times

with PBS 1X and fixed for 20 minutes in 10% Formalin (Sigma Aldrich, Germany). Afterwards, the gels were washed three times with PBS 1X and stored at 4°C until immunofluorescence analysis.

7.2.2.3. Immunofluorescence for collagen I and II expression analysis

7.2.2.3.1. Agarose gel staining

For the immunofluorescence detection of collagen types I and II, staining of the agarose gels previously fixed was done. The staining protocol consisted in washing the gels twice with PBS and permeabilizing them with a 0.3 % Tween 20 (Sigma Aldrich, Germany) solution in PBS for 30 min at room temperature (RT). After that, the gels were washed three times with PBS and blocked using 3 % BSA (Sigma Aldrich, Germany) in PBS for 1 h at RT. Then, primary antibodies for collagen I and collagen II were added to the gels, previously diluted in 3 % BSA with 0.5 % Tween 20. For collagen I, rabbit anti-collagen I polyclonal antibody (Novus Biological) at 1:100 dilution was used while for collagen II, mouse anti-collagen II polyclonal antibody (Novus Biological) at 1:100 concentration was used.

The incubation of the primary antibodies was performed overnight at 4°C. The next step consisted in washing the gels three times, for 5 minutes each, with PBS-T solution. This solution consisted of 0.5 % Tween 20 in PBS. After doing that, a blocking step with 3 % BSA for 1 h was done before the incubation of the secondary antibody for collagen type I for 1h at RT in the dark. Another washing step, equal to the previous one, and a blocking step was done before the incubation of the other secondary antibody for collagen type II. Goat-anti-mouse

AF488 (Invitrogen, Fisher Scientific, EEUU) was used for collagen type II detection, while goat-anti-rabbit AF647 (Invitrogen, Fisher Scientific, EEUU) was used for collagen type I detection. Finally, another washing step and blocking were carried out before the staining of the nucleus of the cells with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Fisher Scientific, EEUU) at a concentration of 1:100 for 15 min at RT. Samples were washed again three times, for 5 min each, and kept in the dark at 4°C until confocal imaging analysis was done.

7.2.2.3.2. Confocal imaging

Confocal imaging was performed for the analysis of the collagen type I and II fluorescence area on the hCh. For the imaging of each agarose gel, six images were taken in the regions around the half height (~125 μm) of the gel (+ or - 30 μm approx.) as shown in the scheme below (Figure 7.4). The six images were always positioned in the same way with respect to the pillar and membrane-sides for each sample. Each image is 425 x 425 μm and the z-height was adjusted slightly to have a decent number of cells in focus. A confocal unit Zeiss LSM 880 (Zeiss, Germany) was used for the imaging.

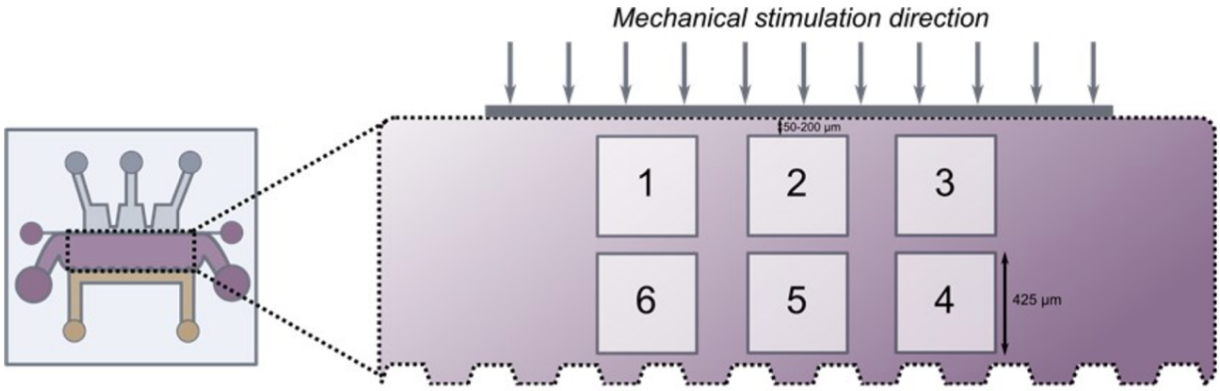

Figure 7.4. Schematic representation of the agarose gel area with hCh. Confocal imaging was done in six different areas of the agarose gel.

7.2.2.4. IL-4 and TNF-α level detection

Anti-inflammatory cytokine IL-4 and pro-inflammatory factor TNF-α expressions were measured. Sample medium from all the conditions at timepoint 11 were used for the analysis. The concentration of the proteins was measured by commercially available milliplex MAP human high sensitivity T cell panel (Sigma-Aldrich, Germany) by a Luminex xMAP analyser (Thermo Fisher Scientific, EEUU).

7.2.2.5. Statistical analysis

The distribution of the samples was assessed by Shapiro-Wilk's normality test. The different variables were determined by the mean and the standard deviation for parametric data, and the median and 95% confidence interval (CI) for non-parametric data. The comparisons were performed by ANOVA and Student's t-test for parametric data, and Kruskal-Wallis and Mann-Whitney U test for non-parametric data. Data were considered statistically significant when $p<0.05$. GraphPad Prism® software (San Diego, CA) was used for the statistical analysis.

7.3. Results and discussion

7.3.1. Viability of primary chondrocytes culture upon exposure to sPRP and hPRP

The bioactivity of each PRP was assessed by measuring its ability to promote cell proliferation in hCh prior to the evaluation of the hPRP in the CoC platform. To do so, these cells were incubated in the presence of either sPRP or hPRP for 96h after which a luminescence-based technique (Real Time-Glo MT Cell Viability, Promega, Fitchburg, USA) was used to determine viability at that time. The results

showed that cells incubated with hPRP presented higher levels of viability compared to those incubated with sPRP ($p < 0.01$) (Figure 7.5).

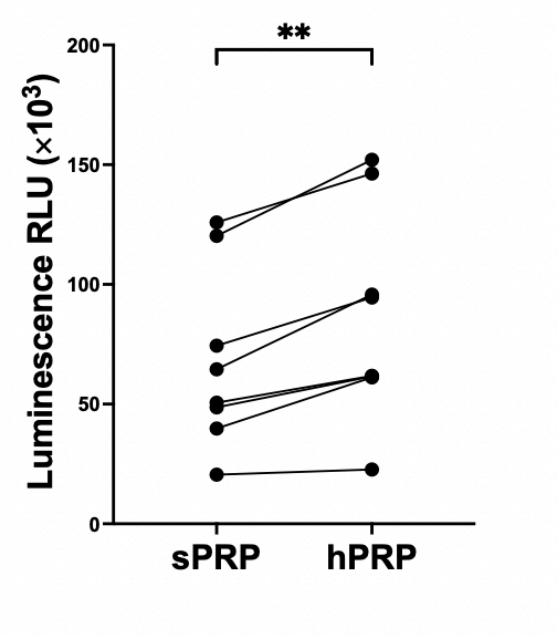

Figure 7.5. Cellular viability in hCh. The viability levels of hCh incubated with sPRP and hPRP are expressed as relative lights units (RLU) and each point represents a different donor (n=8). Statistical analysis was calculated by t-test (** $p < 0.01$).

Overall, hPRP had a positive impact on *in vitro* cell culture. This was confirmed in primary chondrocytes as it did in normal human dermal fibroblast (NHDF) cells in Chapter 5. This could imply an increased efficiency in tissue regeneration for cartilage lesions. Since OA affects the different tissues that comprise the joints such as cartilage, subchondral bone and others, it would be interesting to study the response of other cell types such as synoviocytes, synovial fibroblasts, synovial mesenchymal cells, immune system cells, osteoblasts etc[21,22].

7.3.2. Immunofluorescence analysis for collagen II/I detection in hCh in CoC platform

The expression of collagen type I and II produced by the hCh in the ECM upon PRP treatment with both PRP formulations was measured by immunofluorescence at day 14. To do so, the agarose gels of serum free, sPRP and hPRP conditions were fixed and stained for the analysis of the ratio of fluorescence area of collagen II/I. The results showed that hCh treated with both PRPs in static and compression tend to have a higher expression of collagen II than collagen I. The only significant increase in collagen II was observed between the serum free condition and hPRP when the device was set in static. When comparing the devices in static and compression, there was a tendency to increase collagen II in the case of serum free and hPRP (Figure 7.6 A-B).

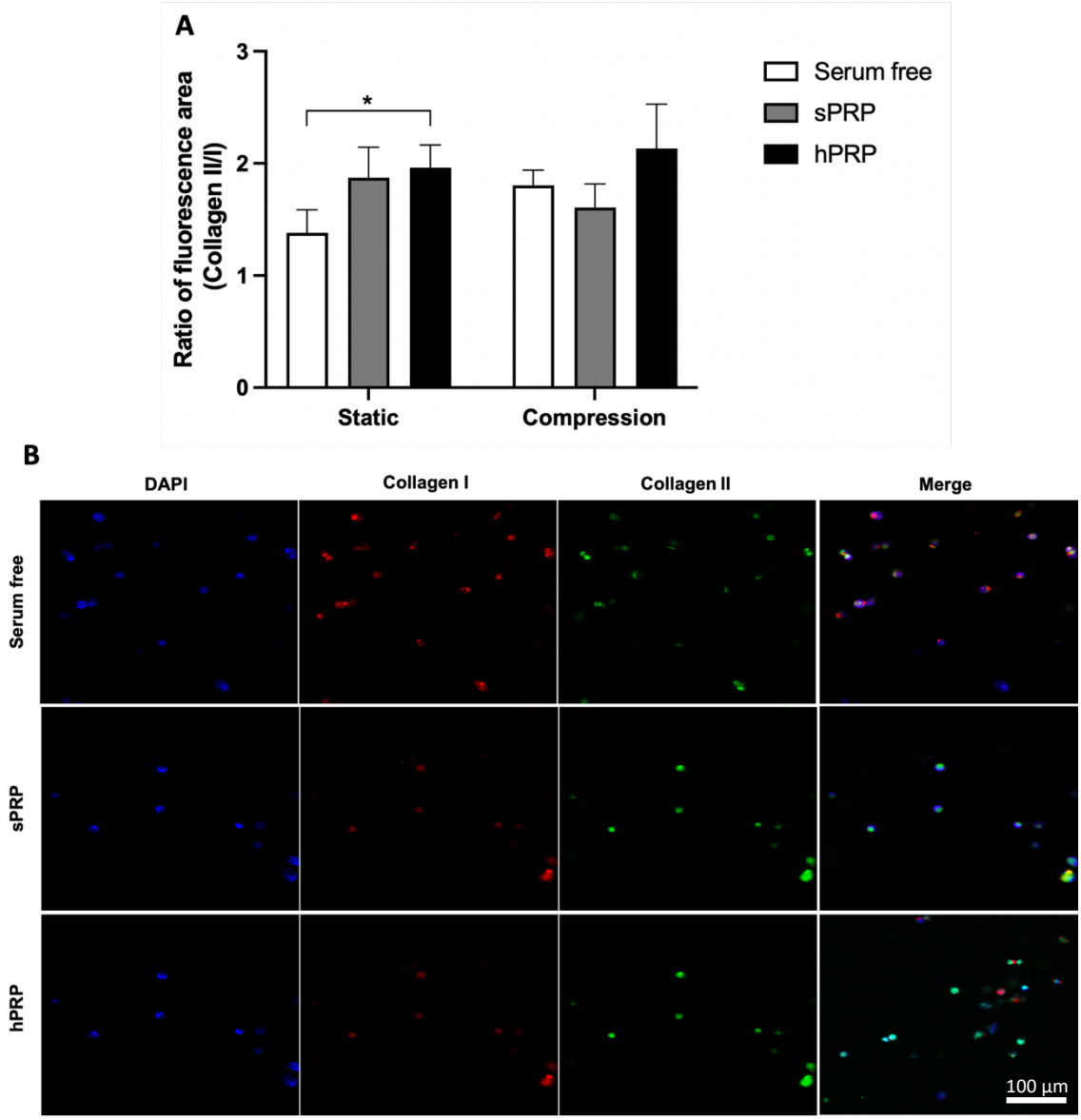

Figure 7.6. Immunofluorescence analysis for collagen type I and II detection. **(A)** Ratio of fluorescence area of collagen II/I in static and compression condition. **(B)** Collagen I and II expression was measured in the agarose gel of hCh cells by immunofluorescence after culturing the cells in serum free, sPRP and hPRP medium. Nuclei are presented in blue (DAPI), collagen I in green (AF488) and collagen II in red (AF647). For the analyses 3 chips per condition were used. Statistical analysis was calculated by two-way ANOVA (* $p < 0.05$).

The first sign of degeneration in OA is an increase in collagen type I[23] and a decrease of collagen II[24]. This is associated with increased cleavage of the collagen by collagenases[25,26] and with an increase in the synthesis of matrix molecules, including collagen II[27] and aggrecan[28,29]. Those synthetised molecules are often damaged[26,29], hindering the cartilage matrix repair. Moreover, hCh are not able to remove and repair the damaged tissue as the articular cartilage is avascular[30,31]. The significant increase in collagen II/I ratio in hPRP compared to serum free condition, and the observed similar tendency with sPRP, suggests that the PRPs could potentially promote collagen II production.

Interestingly, it would be worthwhile to observe the production of collagen II with respect to collagen I and see if it is maintained over time or if it increases. For this it would be necessary to extend the contact time of the medium with the PRPs together with the hCh. In addition, the fact that the observed differences overall did not reach statistical significance could be related to the conditioning time of the chondrocytes to the medium. It would be interesting to increase the incubation time prior to the experiment to allow them to behave at normal physiological rates. On the other hand, the contact time with the medium should be increased in order to observe greater differences in the levels of collagen I and II[32,33].

7.3.3. Pro- and anti- inflammatory cytokine analysis

Interleukin 4 (IL-4) cytokine is well known for its anti-inflammatory effect activity and its chondroprotective effects. *In vivo*, this cytokine inhibits the matrix metalloproteinases (MMP) which are involved in the cleavage of collagen type II[34] and prevents the apoptosis of chondrocytes[35]. Regarding the pro-inflammatory cytokine Tumour Necrosis Factor alpha (TNF-α), it is mainly

synthesized in the synovial chondrocyte cells[36] and it is directly related with the induction of the expression of inflammatory factors in OA .

After subjecting the chips to compression for 5 days, the concentration of the anti-inflammatory IL-4 and pro-inflammatory TNF-α cytokines in the culture medium was analysed with the objective to examine the capacity of sPRP and hPRP to induce an increase or decrease of the expression of these cytokines. For this purpose, the samples were analysed at day 11 for all the conditions. Regarding the detection of IL-4 and TNF-α, Figures 7.7 A and C show the levels of these cytokines in both sPRP and hPRP medium with no cells. As expected, hPRP contained statistical higher concentration of this cytokines as this formulation has double concentration of all the proteins present in the plasma (*$p < 0.05$ and ** $p < 0.01$, respectively). Results showed that unsupplemented (serum free) cells showed basal levels of IL-4. These levels decreased significantly when the cells were treated with sPRP, both in static and compression (***$p < 0.001$ and **$p < 0.01$, respectively). hPRP also promoted the reduction of IL-4 levels, although to a lesser extent than sPRP. These data were statistically significant only in static (**$p < 0.01$). Although the trend was the same for static and compression, the differences were more pronounced in static (Figure 7.7 B).

A similar pattern was observed for TNF-α as for IL-4. However, in this case, in static the reduction in TNF-α levels produced by sPRP and hPRP was statistically significant (***$p < 0.001$ and **$p < 0.01$, respectively). Additionally, statistically significant differences were observed between sPRP and hPRP (* $p < 0.05$), with sPRP showing a greater ability to reduce TNF-α levels. In compression, sPRP significantly reduced TNF-α levels (***$p < 0.001$), while hPRP did not (Figure 7.7 D).

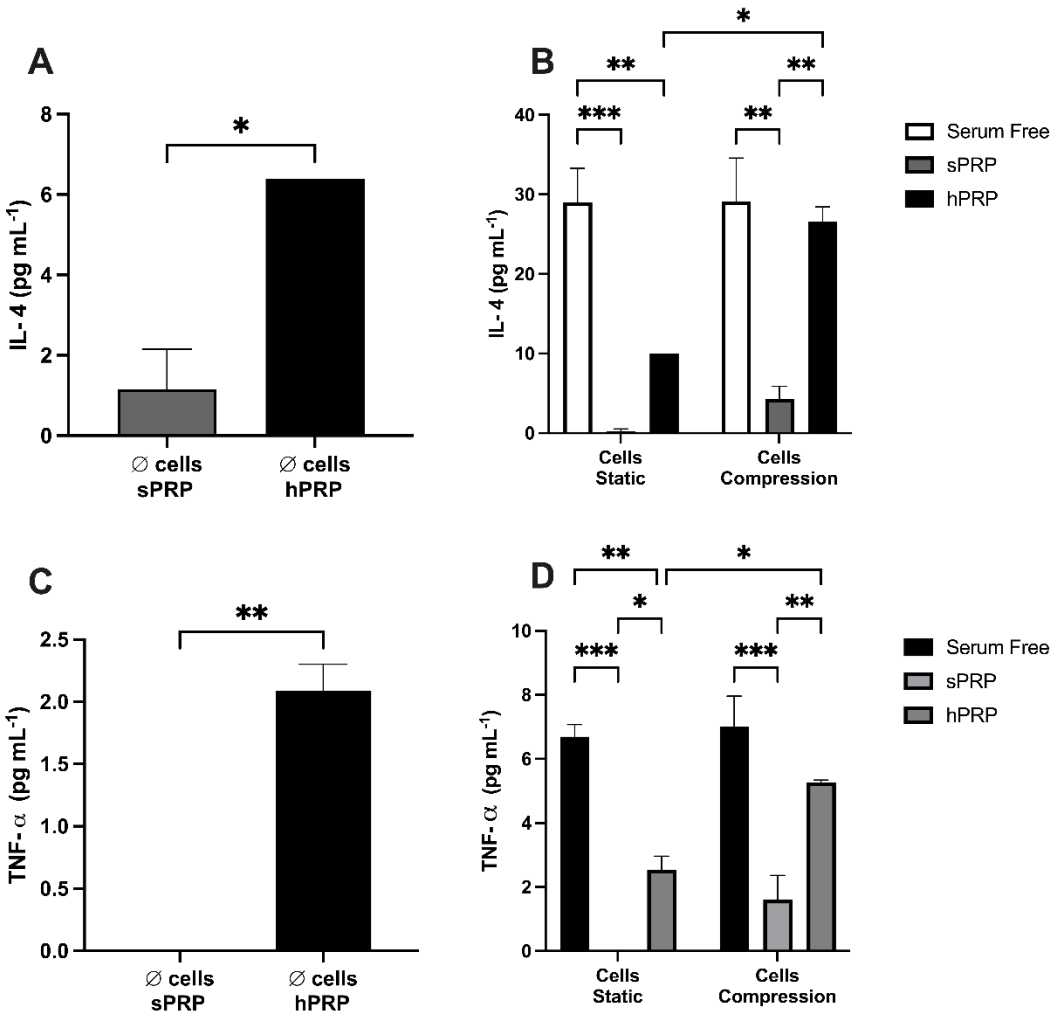

Figure 7.7. Anti-inflammatory IL-4 and pro-inflammatory TNF-α cytokine quantification. (A) Levels of IL-4 cytokine in medium supplemented with sPRP and hPRP with no contact with cells. **(B)** IL-4 expression when medium supplemented with PBS, sPRP and hPRP were put in contact with the hCh in static and compression. **(C)** Levels of TNF-α cytokine in medium supplemented with sPRP and hPRP with no contact with cells. **(D)** TNF-α expression when medium supplemented with PBS, sPRP and hPRP were put in contact with the hCh in static and compression. For the analyses volume from one replica (two chips) per condition were used. Statistical analysis was calculated by two-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Overall, these results demonstrate that sPRP has a greater capacity to reduce interleukin levels than hPRP, regardless of whether the interleukins are anti- or pro-inflammatory. In addition, compression was also shown to play an important

role in cytokine regulation, as the levels of interleukins became closer to basal levels when stimulated. These results were contrary to what was expected as IL-4 and TNF-α have opposing roles in the regulation of inflammation and therefore the actions on chondrocytes would be expected to be opposite.

One of the main problems occurred during this assay was the low signal levels in the detection of the molecules. This may be mainly due to the low number of cells seeded on the agarose gel inside the chips, which is one of the main limitations of the model. Although this cell density was valid for previous studies[14], in view of the low values measured in these samples, it would be necessary to increase the number of cells to enhance the sensitivity of the measurements. In addition, to enhance the signal, it may be beneficial to increase both the contact time of the supplemented medium with the cells to possibly increase the secretion of the proteins. On the other hand, optimizing the compression time on the chip is determinant for the model to resemble the mechanical forces generated by walking. In fact, the behaviour of the hCh could be affected by these stimuli[14]. A model that could be more realistic to the mechanical forces generated by the knee would be a model using multidirectional forces[14].

For all these reasons, it could be concluded that the CoC model used with these conditions is not adequate to draw conclusions. Moreover, these results are the outcome of a single replicate from a single patient, as they are preliminary results of a work that would cover a larger number of patients and replicates.

7.4. Conclusions

hPRP was able to improve the cell viability of primary chondrocytes, compared to standard PRP, in the same line as in other cell types such as skin fibroblasts.

Supplementation with hPRP enriched medium tend to increase the ratio of intracellular expression of collagen II/I in both static and compression conditions, suggesting a possible mitigation of the degradation of the ECM in OA.

According to the CoC platform, both mechanical stimulation and biological stimulation, generated by the PRPs, have an impact on the release and modulation of cytokines. However, the limitations of the model described above, prevent from understanding in depth the influence of each of the PRPs and mechanical stimulation, and whether the same trend would be observed in different donors.

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8

Conclusions and Future Work

8.1. Conclusions and Future Work

Considerable advances have been made in the last decades towards the generation of different PRP obtaining methods, although centrifugation is currently the most widely used. However, all these systems focus on platelet concentration but not on the enrichment of extraplatelet biomolecules.

Advances in the development of a method for the obtaining of a novel PRP with an equilibrium in platelet and plasmatic GFs would boost the elaboration of new commercial kits able to obtain better clinical results than those obtained today with the conventional biological therapies.

The conclusions of the present work, based on the initial objectives, are as follows:

- The methods based on evaporation, by means of a rotary evaporator, filtration, using ultrafiltration units, and absorption, with antifouling hydrogels, are valid for the removal of water from the plasma and the generation of a novel PRP.
- All three methods are capable of generating a PRP enriched in platelets, proteins, platelet factor PDGF and extraplatelet factor IGF-1, although only the filtration and absorption methods are able to concentrate the extraplatelet factor HGF. Although the concentrations of ions in the PRP may vary, as in the case of absorption and evaporation methods,

coagulation occurs normally, with pH adjustment being essential for the evaporation technique.

- The three methods produce a PRP with improved *in vitro* bioactivity compared to a standard PRP, showing increased viability of dermal fibroblasts, when treated with the novel PRP.
- Based on the decision table, the method with the greatest potential for scalability for its clinical use is the hydrogel-based method, which presents good biomolecule enrichment, increased *in vitro* properties and high manufacture feasibility.
- Primary human chondrocytes treated with hPRP in the 2D model showed higher cell viability than those incubated with sPRP.
- Although the CoC model showed a tendency to improve the collagen II/I ratio and to vary the levels of cytokines secreted by chondrocytes, it was concluded that this model must be further optimized in order to draw robust conclusions.
- In view of the previous findings, this novel PRP could be a candidate to improve current biological therapies in the field of regenerative medicine.

As future perspectives, considering the potential of the novel PRP in cell viability, it would be interesting to perform other types of analyses. Studies in a cellular environment stimulated to produce inflammation and observe the effects of the

new plasma as well as the ability to reduce reactive oxygen species (ROS) would be interesting to perform. In addition, it would be remarkable to analyse the effects of the novel PRP on the chondrogenesis process and observe if this new formulation accelerates the capacity of formation and development of cartilage.

In addition, all the analyses performed so far have been in an *in vitro* environment. However, to see the real impact of this new formulation on tissue regeneration, a *in vivo* study or even a clinical trial would be necessary.