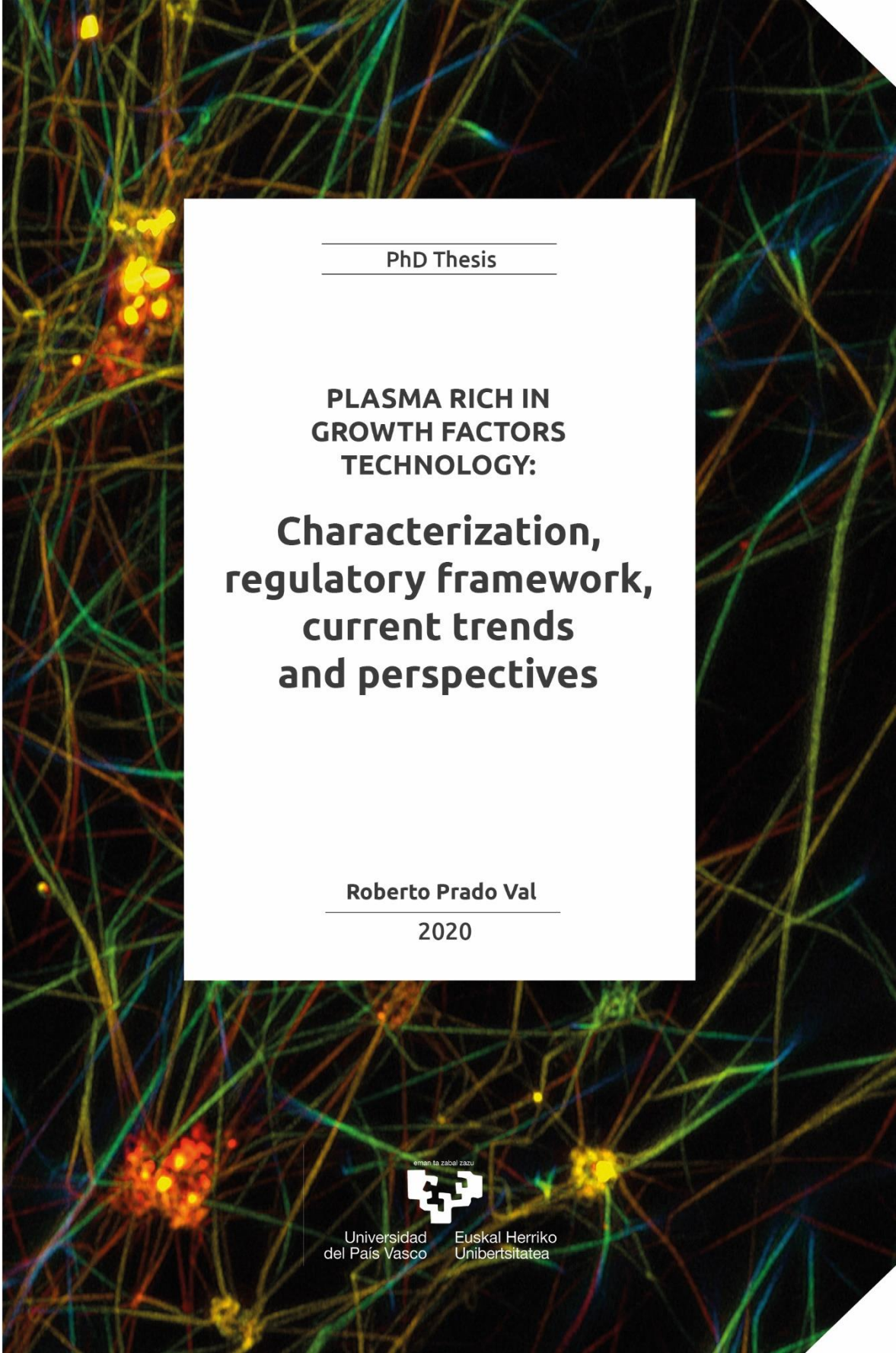


PhD Thesis

PLASMA RICH IN GROWTH FACTORS TECHNOLOGY:

Characterization, regulatory framework, current trends and perspectives

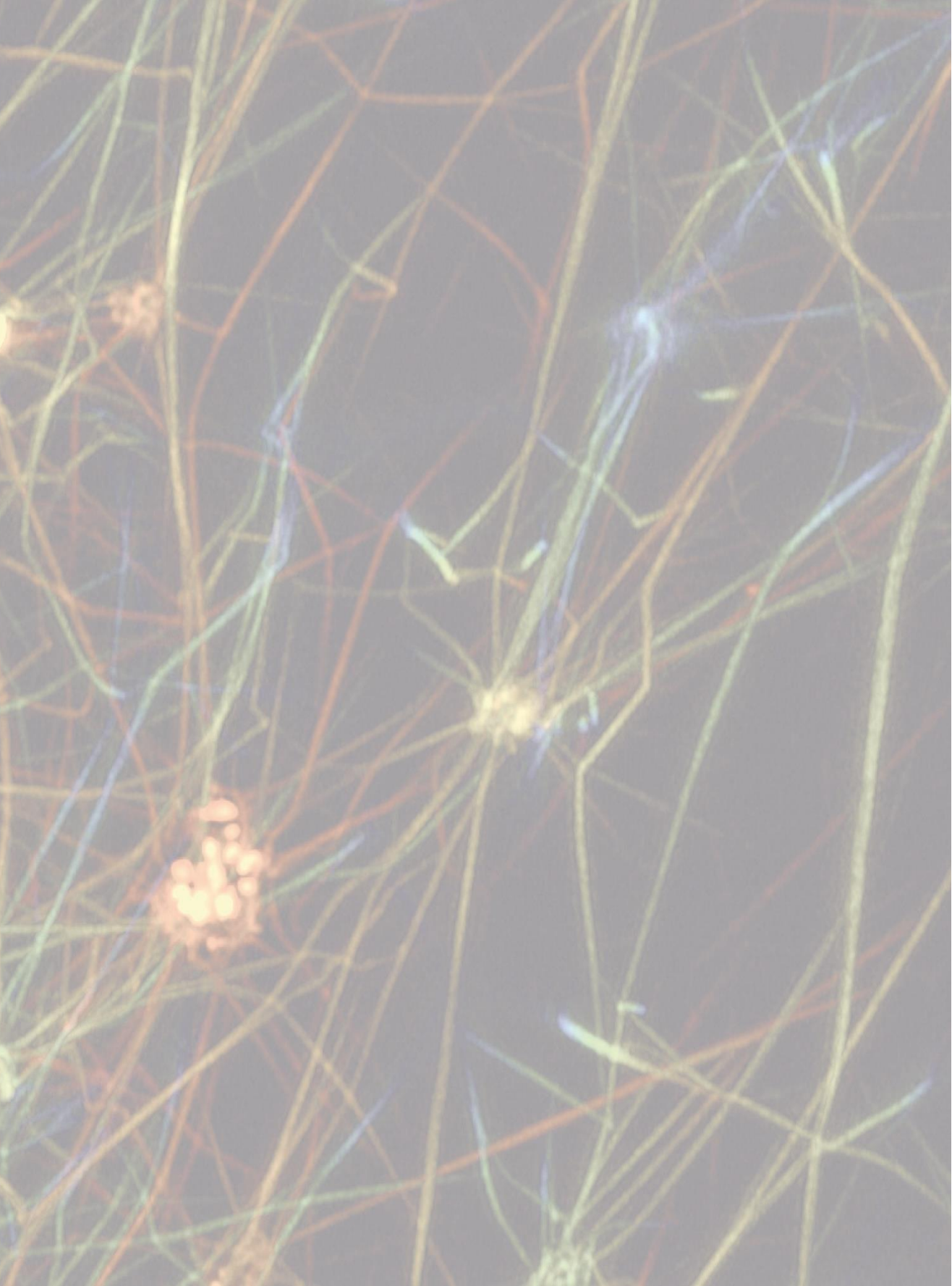
Roberto Prado Val

2020

eman ta zabal zazu

Universidad del País Vasco

Euskal Herriko Unibertsitatea



eman ta zabal zazu

Universidad del País Vasco

Euskal Herriko Unibertsitatea

Facultad De Farmacia

Departamento de Farmacia y Ciencias de los Alimentos

Plasma Rich in Growth Factors technology: characterization, regulatory framework, current trends and perspectives

PhD Thesis

Roberto Prado Val

2020

uirmi University Institute for Regenerative Medicine and Oral Implantology

A mis padres y a mi hermana

A Elizabeth

A David y Celia

En primer lugar, me gustaría agradecer la realización de esta tesis doctoral a mis dos directores, Eduardo y Gorka. Gracias Eduardo por darme la oportunidad de formar parte de tu grupo de investigación en aquel lejano año 2006… Gracias por confiar en mí y permitirme formar parte del equipo "de la intuición a la evidencia". Gracias por tantas intuiciones que me han permitido estos años buscar evidencias. Gorka, gracias por tu guía y tus consejos, gracias por confiar en mí y ordenar mis ideas. He aprendido mucho de ambos.

Gracias a todos los coautores de los artículos que se incluyen en esta tesis doctoral.

Tengo pocos recuerdos de pequeño, pero uno de los que todavía no se ha borrado es el de haber querido ser…inventor. Quizás ser científico es lo más parecido. "Érase una vez…El cuerpo humano"… fue una serie de dibujos animados que despertaba en aquellos momentos mi curiosidad, mis ganas de saber y aprender. No podía imaginar que el capítulo 6 sobre las plaquetas, formaría parte de mi vida investigadora.

Escribir unos agradecimientos es siempre una tarea complica, mezcla de añoranza y melancolía, mezcla de presente y de cualquier tiempo pasado fue mejor. Son muchas las personas que he conocido en mi periplo investigador y muchas las que han influido en su resultado. Seguro ($p<0.005$) que me dejo a muchas. Gracias a los olvidados.

Quizás, los recuerdos más dulces y amargos de mi carrera investigadora fueron los de los primeros años. Años sin canas, plenos de ilusión. Gracias Agus (*Homo heidelbergensis*) por todos aquellos infinitos momentos compartidos: los cafés, el florentino, la tarjeta rota el primer día, civilization y nibbles bubles, el moco verde,…Gracias por tus sabios consejos y reflexiones. Gracias también a Agus (*Homo climate mutatio*) mi gurú del cambio climático. Espero que no tuitees esto. Y gracias Bingen (*Homo callamelus*) por aquella técnica del caramelo. A todos, gracias por seguir ahí año tras año.

Mi siguiente estación se encontraba físicamente cerca, pero a años luz evolutivamente hablando. Gracias Profesor Arechaga por acogerme en el

Servicio de Microscopía y en su laboratorio, lo cual me permitió abandonar el lado oscuro. Gracias a Jon y a Ricardo. Aprendí (y sigo aprendiendo) muchos secretos de la microscopia (Apertura numérica, Köhler, O.61,...) pero sobre todo gracias por los consejos, gracias por ese "Garbage in, garbage out". Gracias también a Alex.

Y llegó aquel día de invierno en el que sin saberlo me convertiría en nómada. En aquel momento no podía imaginar que sería casi medio millón de kilómetros los recorridos en el nuevo proyecto vitoriano. Mis inicios en BTI fueron en Miñano. Conservo imborrables recuerdos de aquella etapa. Gracias a todos. A los que ya no están y a los presentes. Especialmente, gracias a Sara. Nunca sabré si estás en regulatorio o en calidad. ¡Perdóname! Aunque tú siempre sabias cuando iba a haber ovejas... Aquellas mañanas-tardes-noches de quirófano experimental en Miñano me enseñaron mucho y me acercaron al mundo de la traumatología con grandes enseñanzas de Mikel. ¡Gracias Dr Sanchez! Gracias también a Diego y a Pello.

Gracias a las chicas y chicos de la segunda planta del Instituto Anitua. Gracias a audiovisuales y diseño por todas las fotos, dibujos y figura... Gracias ... gracias por esa paciencia infinita cuando veis crecer la versión de la figura hasta números increíbles.

Gracias al personal de La Clínica, gracias por esas extracciones de sangre a deshora. Especialmente gracias a Bea y Eguzkine. Gracias también a Alia, ¡Espero que pronto te toque escribir estas líneas!

Gracias a mis compañeros de laboratorio. A los que ya no están, a los recién llegados y a los más veteranos. En el ecosistema del laboratorio he pasado muchas horas y he aprendido mucho. Gracias Ander por contagiar esa ilusión de juventud. Pero especialmente, me gustaría agradecer a Kiko (alias Dr. Muruzabal) estos años de trabajo en equipo, de ideas peregrinas y no tan peregrinas, de Magneto (no es un nombre de superhéroe), los brainstormings más eficaces, tus consejos científicos, y como no, esos días de "algo se podrá aprovechar"...

¡Gracias Dr Padilla! Espero que la Policía Montada de Canadá no llame a su puerta… Muchas gracias Sabino por esas Master Class que yo haría infinitas. Gracias por esos "evolutivamente hablando". Gracias por tus citas (The aim of science is not to open the door to infinite wisdom but to set a limit to infinite error- Bertolt Brech).

Y al otro lado de este mundo de Matrix de la investigación está mi familia, la familia pequeña y la familia grande.

Gracias a mis padres por haberme hecho como soy. Gracias por haberme apoyado siempre y dado todo sin pedir nada a cambio. Me gustaría ser tan buen padre como vosotros lo sois conmigo. Gracias desde lo más profundo de mi corazón. Gracias a esa hermana, a la que no me parezco en nada, (¿o quizás un poquito sí?) pero que siempre ha estado ahí. Estoy muy orgulloso de ser vuestro hijo y hermano.

¡Como no! Gracias Elizabeth por estar a mi lado en el viaje de la vida. Un viaje complicado si te toca un investigador al que tienes que soportar escribir dos tesis (espero que esta sea la definitiva). Gracias por apoyarme siempre. Gracias por ser como eres. Gracias a los que han llegado hace poco pero son los que más iluminan e ilusionan: gracias a mis hijos David y Celia por recordarme que no hay que perder nunca la curiosidad, que no hay que dejar de sorprenderse en esta vida. Gracias y perdón, a vosotros y a Elizabeth, por los segundos, minutos, horas y días robados en estas cosas de la ciencia. Celia, es lo que tiene tener un padre científico…ya te queda poco para aprender a leer estas líneas (entre tu y yo, aquí falta dibujar un corazón, y sé que a ti se te da mejor que a mí). David Prado Scientist, tú sí que sabes leer, aunque dices que te gusta poco…¡Busca! No pares nuca de decir ese ¡Vamos aita! No sé lo que seréis en un futuro, pero sí sé lo que quiero que seáis, buenas personas.

Seguro que me dejo a alguien. Gracias a los olvidados. Como decía Dory en Buscando a Nemo, "sigue nadando, sigue nadando". Ahora sé por qué lo decía. Quizás era porque "solo los peces muertos nadan a favor de la corriente".

Roberto

"El camino a la próxima cura milagrosa comienza en la ciencia básica"

Juan Carlos Izpisúa

"Si uno quiere ser pintor va a tener que aprender carpintería...

pues necesitará un marco para enmarcar su lienzo"

Foreman, 2011

"To maximise the benefit to society,

you need to not just do research, but do it well"

Doug Altman

List of Abbreviations and Acronyms

2-DE	Two dimensional gel electrophoresis
3D	Three-dimensional
α2M	Alpha2-macroglobulin
AAOS	American Academy of Orthopaedic Surgeons
ADAMTS13	A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13
AEMPS	Spanish Agency of Medicines and Medical Devices
AOSSM	American Orthopaedic Society for Sports Medicine
bFGF	Basic Fibroblastic Growth Factor
BMP2	Bone morphogenetic protein 2
bTG	Beta-thromboglobulin
CONSORT	Consolidated Standards of Reporting Trials
CTGF	Connective tissue growth factor
DDA	Dependent acquisition mode
DTT	Ditiotreitol
EGF	Epithelial Growth Factor
EMA	European Medicines Agency
ENA-78	Epithelial Neutrophil-Activating Peptide 78
EQUATOR	Enhancing the QUAlity and Transparency Of health Research

F1	PRGF Fraction 1
F2	PRGF Fraction 2
FasL	Fas ligand
FBS	Fetal bovine serum
FDA	U.S. Food and Drug Administration
FDR	False discovery rate
FM	Fibroblast medium
FXR/RXR	Farnesoid and retinoid X receptors
GO	Gene Ontology
GRB2	Growth factor receptor-bound protein
GRO-alpha	Growth regulated oncogene- alpha
HCCA	α-Cyano-4-hydroxycinnamic acid
HGF	Hepatocyte Growth Factor
HMGB1	High-mobility group protein 1
HNF4A	Hepatocyte nuclear factor 4 alpha
IAA	Iodoacetamide
IGF-BP	IGF-1 binding protein
IGF-I	Insulin-like Growth Factor type I
IL-6sR	Interleukin-6 soluble receptor
IL-8	Interleukin-8
IPA	Ingenuity Pathways Analysis
ISSCR	International Society for Stem Cell Research

ISTH	International Society on Thrombosis and Haemostasis
LCF	Leukocyte concentration factor
LC-MS	Liquid chromatography–mass spectrometry
L-PRP	Leukocyte-enriched PRP
LXR/RXR	Liver and retinoid X receptors
MALDI TOF	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight
MCP-3	Monocyte chemotactic protein-3
MIBBI	Minimum reporting guidelines for biological and biomedical investigations
MIBO	Minimum Information for studies evaluating Biologics in Orthopaedics
MIP-1	Macrophage inflammatory protein-1
MMP	Matrix metalloproteinases
MPV	Mean platelet volume
n.d.	Not determined
NAP-2	Neutrophil-activating protein-2
NF-κB	Nuclear factor κB
ObM	Osteoblast cell medium
ORS	Orthopaedic Research Society
PAI-1	Plasminogen activator inhibitor-1
PAW	Platelets, Activation and WBC Classification
PBS	Phosphate buffered saline
PCF	Platelet concentration factor

PDGF	Platelet Derived Growth Factor
PF4	Platelet factor 4
PFP	platelet-free plasma
PMA	Premarket Approval
PMN	Polymorphonuclear neutrophils
P-PRP	Pure-PRP
PRGF	Plasma Rich in Growth Factors
PRP	Platelet-rich plasma
PTD2	Plasma transfer device
PTM	Post-translational modification
PY	Platelet yield
RANTES	Regulated upon Activation - Normal T-cell Expressed, and Secreted
RCT	Randomized clinical trial
SDF-1	Stromal cell-derived factor-1
SEM	Scanning electron microscopy
SSC	Scientific and Standardization Committee
STAR	Structured, transparent and accessible reporting
TACE	Tumor necrosis factor-alpha-converting enzyme
TAFI	Thrombin activatable fibrinolysis inhibitor
TEM	Transmission electron microscopy
TF9	Fractionation tube
TFA	Trifluoroacetic acid

TFPI	Tissue factor pathway inhibitor
TGF-β1	Transforming Growth Factor β type 1
TIMP	Tissue inhibitor of metalloprotease
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TSP-1	Thrombospondin-1
uPA	Urokinase plasminogen activator
VEGF	Vascular Endothelial Growth Factor
vWF	Von Willebrand factor
WADA	World Anti-Doping Agency
WBC	White blood cells

Index

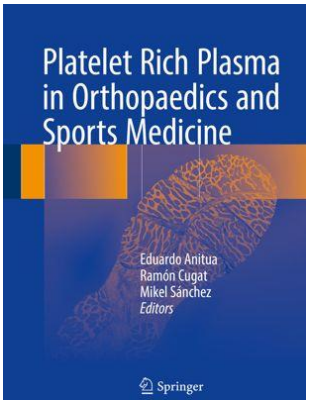
anticoagulant
Medicine
molecular
analysis
Sports
approach
VEGF
medicinal
leukocytes
Platelet
patients
Prado
efficacy
cells
system
proinflammatory
kinetics
study
released
Platelet-rich
physiological
engineering
increased
Platelet-Rich
human
PRP
measured
incubated
cartilage
Nurden
injuries
Proteomic
cytokine
published
repair
recovery
tissue
donor
Spain
trials
IGF-1
HGF
thesis
leukocyte
properties
protein
preparations
Release
stem
guidelines
fractions
factors
Drug
granules
compare
required
Blood
report
Cell
AEMPS
statistically
growth
matrices
migration
erythrocytes
supernatant
plaquetas
activator
platelets
Implementation
Research
reporting
European
regulatory
healing
formulations
significant
scaffold
therapeutic
molecules
scientific
PR
plas
fib
prot

Allogeneic
Anitua
scaffold
protocol
blood
Platelet
GF
Plasma
clinical
Science
samples
release
therapy
treatment
PLASMA
activation
enrichment
regenerative
therapies
scaffolds
network
products
fraction
allogeneic
function
retraction
medicine
product
PRP
References
research
activated
framework
Platelets
applications
procedures
classification
experimental
compared
enriched
three-dimensional
tubes
role
wound
regulation
metabolism
components
process
matrix
pathways
results
mechanisms
bone
clot
injury
Factor
patient
pathway
doctoral
BTI
Journal
contains
PDGF-AB
structure
natural
Discussion
developed
tecnología
functional
Medical
culture
muscle
citrate
Leukocyte
Protein
calcium
chronic
device
treat
EGF
figure
assays
studied
protocolo
immune
reduced
review
representative
influence
included
variability
leucocitos
characterize
methods
fibroblast
collected
analyzed
damaged
individuals
Addressing
inflammatory
aggregation
concentrations
fractionation
effect
Tissue
CONSORT
membrane
formulation
safety
test
dose
step
surgery
reported
article
strategies
healthy
showing
produced
comparison
centrifuged
musculoskeletal
orthopaedics
biology
knowledge
receptor
signaling
soluble
Autologous
Comparison
culture
composition
diseases
objectives
Andia
Orthopaedics

List of original publications

This Thesis is based on the following seven publications, numbered throughout the text from [1] to [7], which have been used in the different chapters that compose it:

[1] Anitua, E., **Prado, R.**, Nurden, A.T., Nurden, P. (2018) Characterization of Plasma Rich in Growth Factors (PRGF): components and formulations. In Platelet Rich Plasma in Orthopaedics and Sports Medicine (Anitua, E., Cugat, R. and Sánchez, M., eds), pp. 29-45, Springer International Publishing

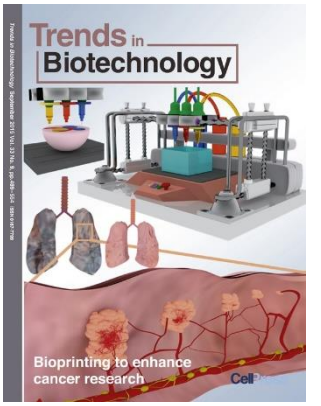


[2] Anitua, E., **Prado, R.**, Azkargorta, M., Rodriguez-Suarez, E., Iloro, I., Casado-Vela, J., Elortza, F., Orive, G. (2015) High-throughput proteomic characterization of plasma rich in growth factors (PRGF-Endoret)-derived fibrin clot interactome. J Tissue Eng Regen Med. 9, E1-12

[3] Anitua, E., Zalduendo, M.M., **Prado, R.**, Alkhraisat, M.H., Orive, G. (2015) Morphogen and proinflammatory cytokine release kinetics from PRGF-Endoret fibrin scaffolds: evaluation of the effect of leukocyte inclusion. *J Biomed Mater Res A.* 103, 1011-1020

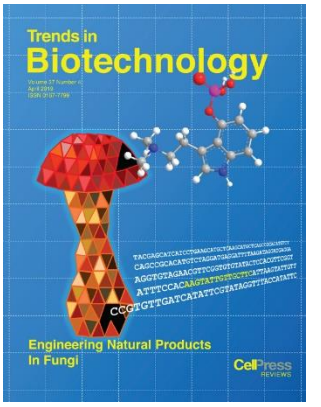
[4] Anitua, E., **Prado, R.**, Troya, M., Zalduendo, M., de la Fuente, M., Pino, A., Muruzabal, F., Orive, G. (2016) Implementation of a more physiological plasma rich in growth factor (PRGF) protocol: Anticoagulant removal and reduction in activator concentration. *Platelets.* 27, 459-466

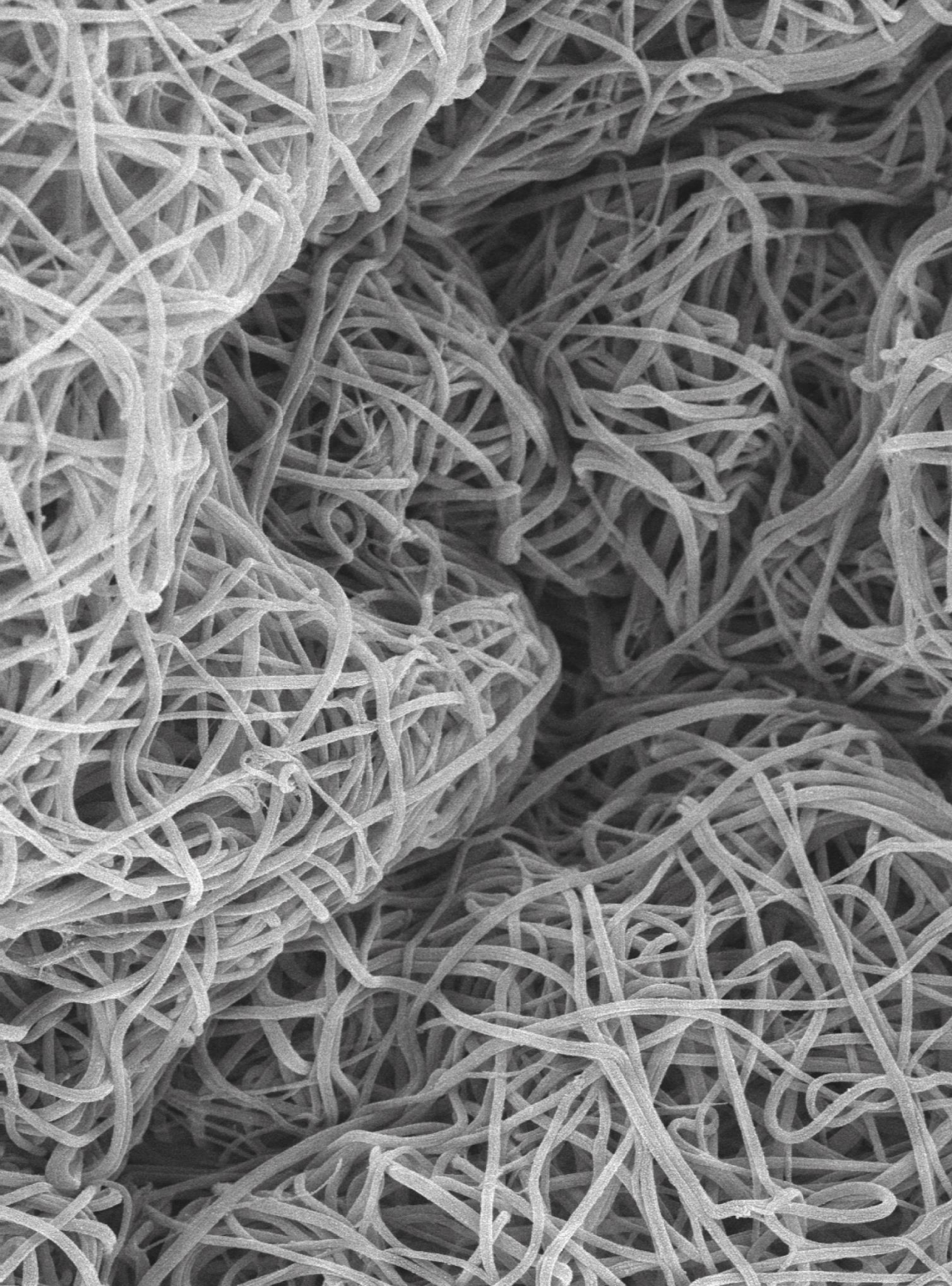
[5] Anitua, E., **Prado, R.**, Orive, G. (2015) Closing regulatory gaps: new ground rules for platelet-rich plasma. Trends Biotechnol. 33, 492-495

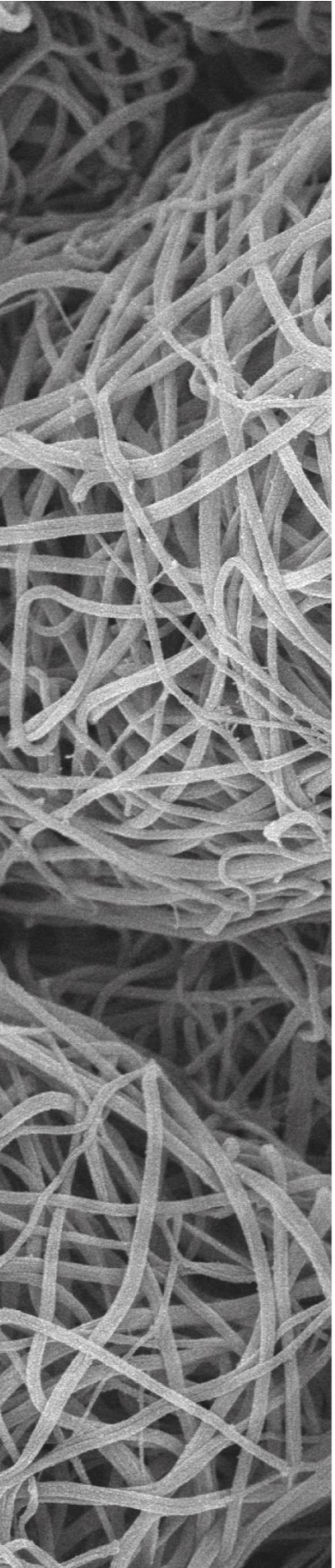


[6] Anitua, E., **Prado, R.**, Orive, G. (2017) Allogeneic Platelet-Rich Plasma: At the Dawn of an Off-the-Shelf Therapy? Trends Biotechnol. 35, 91-93

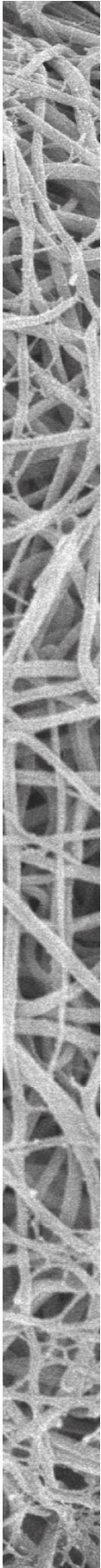
[7] Anitua, E., **Prado, R.** (2019) Addressing Reproducibility in Stem Cell and PRP Therapies. *Trends Biotechnol.* 37, 340-344







Summary. Resumen



1 Resumen

La tecnología de plasma rico en factores de crecimiento, **PRGF** (por sus siglas en inglés, *Plasma Rich in Growth Factors*) es una terapia biológica autóloga que utiliza los propios componentes de la sangre del paciente como agentes terapéuticos para promover la reparación y regeneración de los tejidos dañados. El PRGF se obtiene partiendo de un pequeño volumen de sangre del paciente. Posteriormente, mediante un proceso de centrifugación y fraccionamiento, se selecciona el plasma enriquecido en plaquetas y se descartan los leucocitos y eritrocitos. A partir del plasma enriquecido en plaquetas en forma líquida, y controlando la activación, tanto de las plaquetas como de la cascada de coagulación, se pueden generar múltiples formulaciones: PRGF líquido recién activado, con capacidad de polimerizar *in situ*, sobrenadante de PRGF, coágulo o matriz, y membrana de fibrina. Cada una de ellas posee unas características intrínsecas fundamentales para su aplicación en lesiones concretas. En todos los casos son formulaciones que están diseñadas para optimizar y acelerar la regeneración tisular. EL PRGF actúa como un efectivo agente terapéutico, basándose para su aplicación en los principios de la medicina personalizada, la ingeniería de tejidos y en procedimientos mínimamente invasivos.

La tecnología PRGF ha sido caracterizada en múltiples investigaciones preclínicas, siempre siguiendo los pilares de la investigación científica, y siempre en aras de garantizar la seguridad de los productos aplicados. Esta tesis es el resultado de tres estudios preclínicos *in vitro* de caracterización y optimización del PRGF. Es más, fruto de la evolución del marco regulatorio y de la propia tecnología se realiza un análisis detallado de varios aspectos clave, como son el marco regulatorio del

plasma rico en plaquetas (PRP), su aplicación alogénica y la estandarización de este tipo de terapias biológicas. Se profundizará en estos temas sobre la base de una serie de tres artículos científicos de perspectiva.

Así, esta tesis está basada en 7 publicaciones científicas [1-7] que constituyen una unidad temática. Presenta **tres partes o secciones** claramente distinguibles (Figure 1): una introducción del estado del arte de la tecnología PRGF en forma de revisión [1], tres artículos experimentales [2-4], y seguidamente tres ensayos científicos [5-7].

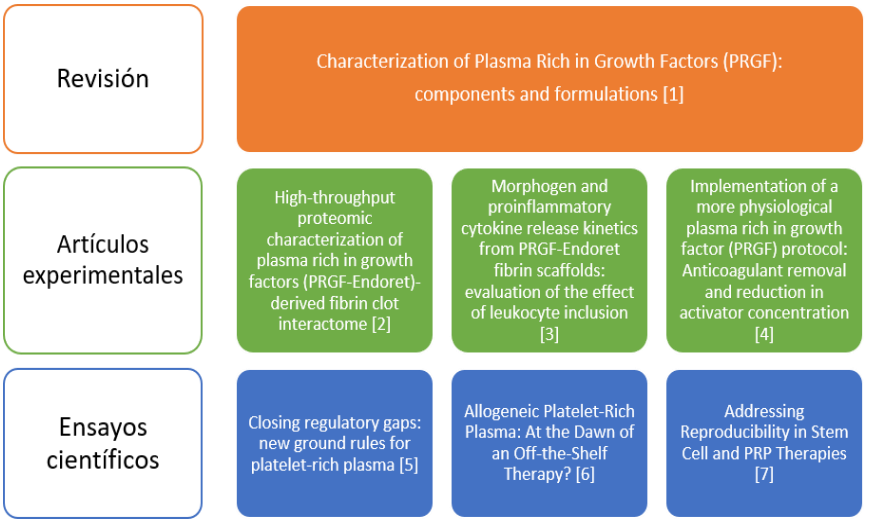


Figura 1. Esquema de las publicaciones científicas incluidas en la presente tesis doctoral. Comprende un artículo introductorio de revisión del estado del arte de la tecnología PRGF, tres artículos experimentales donde se caracteriza y optimiza la tecnología, y finalmente tres ensayos científicos que analizan el marco regulatorio de los derivados de plaquetas, así como los nuevos retos y desafíos.

En el **primer capítulo** de esta tesis se realiza una introducción del estado del arte de la tecnología del PRGF [1] poniendo especial énfasis en el

proceso de obtención, los principales componentes que se potencian y los que se eliminan, así como las moléculas responsables de su eficacia.

En los **capítulos 3, 4 y 5** se muestra el trabajo experimental desarrollado, tanto de caracterización como de optimización de la tecnología PRGF, que ha originado tres artículos científicos experimentales [2-4].

En concreto, en el primer estudio experimental [2] se ha realizado una caracterización proteómica de alto rendimiento de las proteínas que quedan retenidas y adheridas a la matriz o *scaffold* de fibrina de PRGF. El constituyente principal de este coágulo es el andamio de fibrina, pero existe poco conocimiento acerca de otras proteínas que interactúan con este coágulo y que pueden actuar como adyuvantes en el proceso de curación de las heridas. Por ello, el principal objetivo de este primer estudio experimental fue caracterizar las proteínas englobadas y retenidas en dicha matriz. Para ello se empleó un doble enfoque proteómico que combinaba una aproximación 1D-SDS-PAGE seguida de LC-MS / MS, y 2-DE seguida de MALDI-TOF / TOF. Mediante esta aproximación dual se obtuvo una descripción de las proteínas más relevantes que se encontraban en contacto directo con la matriz de fibrina. Posteriormente, las listas de proteínas obtenidas se agruparon en familias y redes según un análisis de ontología genética. En conjunto, se encontró un enriquecimiento de proteínas y familias de proteínas involucradas específicamente en la regeneración de tejidos y en la curación de heridas. Específicamente, entre otras proteínas, se demostró la presencia de trombospondina-1 (TSP-1), vitronectina, fibronectina, alfa-1-antitripsina y alfa-1-antiquimotripsina, todas ellas proteínas con múltiples funciones clave en la regeneración tisular. Después de realizar el análisis de la presencia individual de proteínas, se realizó una clasificación funcional, agrupando las proteínas en familias y redes biológicas. El análisis ontológico de genes de todas las proteínas

identificadas demostró un enriquecimiento significativo de ciertos términos del análisis ontológico que están asociados con la reparación y regeneración de los tejidos, como son, por ejemplo, la respuesta inmune, cicatrización de heridas, angiogénesis, migración y adhesión celular y metabolismo de los lípidos, entre otros.

En una segunda fase se pretendió avanzar más en el conocimiento de la matriz de PRGF y se puso el foco en el conjunto de moléculas liberadas a partir de la matriz de fibrina (**Capítulo 4**). Para ello se determinó la cinética de liberación de distintos morfógenos, o factores de crecimiento, de la matriz de PRGF [3]. De forma simultánea se profundizó en los mecanismos moleculares ligados a la eficacia del PRGF y en especial en el papel que los leucocitos pueden jugar en la composición del PRGF. La presencia de leucocitos en el plasma rico en plaquetas (L-PRP) podría explicar en parte los datos contradictorios de eficacia de las matrices de L-PRP en la regeneración de tejidos. Para tal fin, se comparó una formulación PRGF (sin leucocitos) frente a una formulación rica en leucocitos (L-PRP). Los resultados mostraron que las matrices de fibrina de PRGF eran homogéneas, compactas y acelulares, mientras que las que contenían leucocitos eran heterogéneas, poco compactas y celulares. La incorporación de leucocitos produjo un aumento significativo en el contenido de las citoquinas proinflamatorias IL-1β e IL-16, pero no en la liberación de los morfógenos o factores de crecimiento derivados de las plaquetas. Sorprendentemente, la disponibilidad del VEGF sufrió una disminución importante después de 3 días de incubación en el caso de las matrices de L-PRP. La liberación de citoquinas proinflamatorias en las matrices de PRGF fue muy baja o prácticamente nula. Sin embargo, la inclusión de leucocitos indujo un aumento importante en estas citoquinas. Se pudo observar que las matrices de PRGF se mantuvieron estables durante los ocho días de incubación, al contrario que las de L-PRP. En conjunto, la inclusión de

leucocitos en el PRGF alteró el perfil de liberación de los factores de crecimiento y también aumentó la dosis de citoquinas proinflamatorias, lo cual podría ser uno de los factores que explique los resultados controvertidos que se han descrito en la eficacia de ambas formulaciones.

El siguiente paso, después de realizar una caracterización de las proteínas que quedan en íntimo contacto con la matriz de PRGF [2] y de las que son liberadas al medio [3], fue optimizar el protocolo de obtención del PRGF, reduciendo los niveles de anticoagulante y activador, con el fin de realizar un protocolo más fisiológico (**Capítulo 5**). En este tercer estudio experimental [4] se desarrolló un protocolo enfocado en las tendencias del nuevo marco regulatorio europeo y en el hecho de que la solución anticoagulante en los tubos de extracción de sangre podría ser considerada como medicamento. El protocolo actual (PRGF-A) utiliza tubos de extracción con 0,9 ml de citrato trisódico como anticoagulante y 50 µl de cloruro de calcio / ml de PRGF para activarlo. En contraste, el nuevo protocolo propuesto (PRGF-B), reduce la cantidad de citrato de sodio y cloruro de calcio a 0,4 ml y a 20 µl, respectivamente. Se realizó una batería de ensayos de laboratorio con el fin de comparar el protocolo PRGF-A frente a al PRGF-B, incluyendo la determinación de los parámetros hematológicos críticos, como la concentración de plaquetas y leucocitos, la función plaquetaria, el proceso de obtención del coágulo y su retracción, el contenido de factores de crecimiento y el efecto biológico. Los resultados mostraron que se obtenía un mayor enriquecimiento y recuperación de plaquetas con el nuevo protocolo (PRGF-B). La respuesta al estrés hipotónico de las plaquetas fue también significativamente mejor. De igual forma, se observó una disminución estadísticamente significativa en el estado de activación plaquetaria basal de PRGF-B en comparación con PRGF-A, así como en la duración de la fase *lag* del ensayo de agregación plaquetaria. Otros resultados

positivos de la formulación PRGF-B fueron la reducción de los tiempos de coagulación y retracción y el incremento de la concentración de los cuatro factores de crecimiento analizados (IGF-1, VEGF, PDGF-AB y TGF-β). Por último, el efecto biológico de ambos protocolos de PRGF fue similar, medido como proliferación celular de queratocitos humanos primarios. En base a estos resultados se pudo concluir que el nuevo protocolo de obtención de PRGF (PRGF-B), con una reducción en la cantidad de anticoagulante y activador, presentaba mejores características en la mayoría de los parámetros estudiados.

Después de presentar las evidencias experimentales, los tres siguientes capítulos (**capítulos 6, 7 y 8)** se centran en un análisis teórico de diferentes aspectos relacionados con el PRP y que son esenciales para lograr un avance en la utilización segura y eficaz de este tipo de terapias. Son tres artículos de perspectiva fundamentales en la adecuada translación de las terapias basadas en PRP [5-7].

Así, el **capítulo 6** comienza con un referente teórico y analítico que recoge una exhaustiva revisión del nuevo marco regulatorio del plasma rico en plaquetas introducido por la Agencia Español del Medicamento y Producto Sanitario (AEMPS) en España y de sus potenciales implicaciones [5]. Se analiza la repercusión que ha tenido la consideración del PRP como medicamento de uso humano, y las garantías de seguridad y eficacia que esta clasificación ofrece. Al mismo tiempo se compara la nueva regulación con la existente en otros países. Al igual que cualquier nueva regulación, la clasificación del PRP como medicamento también presenta carencias, incógnitas y retos, los cuales son discutidos tanto en un contexto regulatorio como de su aplicación práctica.

Posteriormente, en el **capítulo 7** se discuten las ventajas e inconvenientes sobre la posibilidad de utilizar PRP no autólogo, o

alogénico, como nueva forma de terapia [6]. Se aporta una muestra representativa de los pocos estudios que han utilizado PRP alogénico y se argumenta a favor de su utilización futura.

Finalmente, en el **capítulo 8** se enfatiza en la necesidad de estandarizar las terapias biológicas, principalmente PRP y células madre, para permitir un avance riguroso de la medicina personalizada con garantías tanto de seguridad como de eficacia [7]. Para ello, y teniendo como pilar fundamental la necesidad de realizar una ciencia reproducible, se propone la utilización de guías estandarizadas para describir los detalles metodológicos de todos los estudios en los que se utilice PRP, tanto a nivel preclínico como clínico.

2 Summary

Plasma Rich in Growth Factors (PRGF) technology is an autologous biological therapy that uses the patient's own blood components as therapeutic agents to promote the repair and regeneration of damaged tissues. PRGF is obtained from a small volume of blood from the patient. Subsequently, through a centrifugation and fractionation process, the platelet-enriched plasma is selected and both leukocytes and erythrocytes are discarded. Multiple formulations can be generated from the PRGF in liquid form, controlling activation of both platelets and coagulation cascade: recently activated liquid PRGF, with in situ polymerization capability, PRGF supernatant, clot or scaffold, and fibrin membrane. Each of them has fundamental intrinsic characteristics for its application in specific injuries. In all cases they are formulations that are designed to optimize and accelerate tissue regeneration. PRGF acts as an effective therapeutic agent, based on the principles of personalized medicine, tissue engineering and minimally invasive procedures.

PRGF technology has been characterized in multiple preclinical research, always following the mainstays of scientific research, and in pursuit of to ensure the safety of the products applied. This thesis is the result of three preclinical *in vitro* studies of PRGF characterization and optimization. Moreover, due to the evolution of the regulatory framework and the technology itself, a detailed analysis of several key aspects is carried out, such as the regulatory framework for platelet-rich plasma (PRP), the allogeneic application and the standardization of this type of biological therapies. These topics will be deepened on the basis of a series of three scientific essays.

Thus, this thesis is based on 7 scientific publications [1-7] that constitute a thematic unit. It presents **three** clearly distinguishable **parts or sections:** an introduction of the state of the art of PRGF technology in a scientific review format [1], three experimental articles [2-4], and thereafter three scientific essays [5-7]. The Figure 2 shows schematically the thesis outline.

In the **first chapter** of this thesis an introduction of the state of the art of PRGF technology is made [1]. It describes the PRGF obtaining process, the main components that are concentred and those that are removed, as well as the molecules responsible for their effectiveness.

Chapters 3, 4 and 5 shows the experimental work developed, both characterization and optimization of PRGF technology, which has originated three experimental scientific articles [2-4].

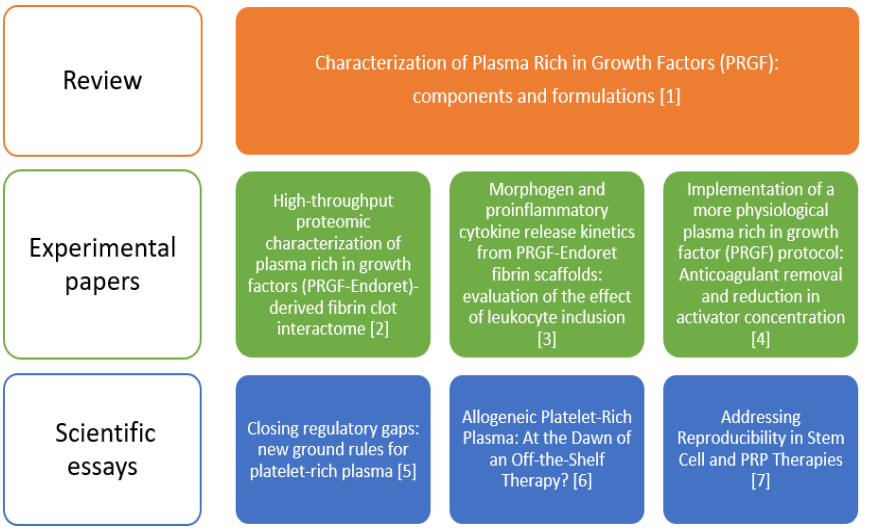


Figure 2. Outline of the scientific publications included in this doctoral thesis. It comprises an introductory article reviewing the state of the art of PRGF technology, three experimental articles where this technology is characterized and optimized, and finally three scientific assays that explore the platelet-rich plasma regulatory framework and the new challenges and trends.

Thus, the first experimental study [2] has conducted a high-performance proteomic characterization of proteins that are retained and bonded to the PRGF fibrin *scaffold*. The main constituent of this clot is the fibrin scaffold, but there is little knowledge about other proteins that interact with this clot and can act as adjuvants in the wound healing process. Therefore, the main objective of this first experimental study was to characterize the proteins enclosed and retained in that matrix. To do this, a double proteomic approach was used: a 1D-SDS-PAGE approximation followed by LC-MS/MS, and 2-DE followed by MALDI-TOF/TOF. This dual approach obtained a description of the most relevant proteins that were in close contact with the fibrin matrix. Subsequently, the lists of proteins obtained were grouped into families and networks according to a genetic ontology analysis. Altogether, an

enrichment of proteins and protein families specifically involved in tissue regeneration and wound healing was found. Specifically, the double proteomic approach detected the presence of thrombospondin-1 (TSP-1), vitronectin, fibronectin, alpha-1-antitrypsin and alpha-1-anticymotrypsin, among other, all of them proteins with multiple key functions in tissue regeneration. After carrying out the analysis of the individual presence of proteins, a functional classification was performed, grouping proteins into families and biological networks. Ontological analysis of genes of all identified proteins demonstrated a significant enrichment of certain terms of ontological analysis that were associated with tissue repair and regeneration, such as immune response, wound healing, angiogenesis, cell migration and adhesion, and lipid metabolism, among others.

In a second phase, the aim was to make further progress in understanding the PRGF matrix and focused on observing the molecules released from the fibrin matrix (**Chapter 4**). For this purpose, the release kinetics of different morphogens, or growth factors, of the PRGF scaffold were determined [3]. At the same time, it was intended to confirm the absence of pro-inflammatory mediators in PRGF. Simultaneously, the *in vitro* effect of the inclusion of leukocytes in PRGF was studied in order to deepen the molecular mechanisms of the efficacy of PRGF. The presence of leukocytes in platelet-rich plasma (L-PRP) could partly explain contradictory data on the effectiveness of L-PRP matrices in tissue regeneration. For this purpose, a PRGF formulation (leukocyte-free) was compared with a leukocyte-rich formulation (L-PRP). The results showed that PRGF fibrin matrices were homogeneous, compact and acellular, while those containing leukocytes were heterogeneous, loose and cellular. The incorporation of leukocytes produced a significant increase in the content of the pro-inflammatory cytokines IL-1 and IL-16, but not in the release of morphogens or

platelet-derived growth factors. Surprisingly, in L-PRP matrices the availability of VEGF suffered a significant decrease after 3 days of incubation. The release of pro-inflammatory cytokines in PRGF matrices was almost absent, or was very low; however, the inclusion of leukocytes induced a significant increase in these cytokines. On the other hand, it was observed that PRGF matrices remained stable during the eight days of incubation, unlike those of L-PRP. Overall, the inclusion of leukocytes in PRGF altered the release profile of growth factors and also increased the dose of pro-inflammatory cytokines, which could be one of the factors explaining the controversial results that have been described in the efficacy of both formulations.

The next step, after performing a characterization of the proteins that remain in close contact with the PRGF matrix [2] and those that are released in the medium [3] was to optimize the PRGF obtaining protocol by reducing the levels of anticoagulant and activator, in order to perform a more physiological protocol (**Chapter 5**). In this third experimental study [4] a protocol focused on trends in the new European regulatory framework and the fact that the anticoagulant solution in blood draw tubes could be considered as a medicinal product. The current protocol (PRGF-A) uses extraction tubes with 0.9 ml of trisodium citrate as an anticoagulant and 50 µl of calcium chloride / ml of PRGF to activate it. In contrast, the new protocol (PRGF-B) reduces the amount of sodium citrate and calcium chloride to 0.4 ml and to 20 µl, respectively. A series of laboratory tests were performed to compare PRGF-A versus PRGF-B, including the determination of critical hematological parameters such as platelet and leukocyte concentration, platelet function, the process for obtaining the clot and its retraction, the growth factor content, and the biological effect.

The results showed that greater platelet enrichment and recovery were obtained with the new protocol (PRGF-B). The response to hypotonic stress of platelets was also significantly better. Furthermore, a statistically significant decrease was observed in the basal platelet activation state of PRGF-B compared to PRGF-A, as well as in the length of the lag phase of the platelet aggregation assay. Other positive results of PRGF-B formulation were the reduction of coagulation and retraction time and the increase in the concentration of the four growth factors analyzed (IGF-1, VEGF, PDGF-AB and TGF-β). Finally, the biological effect of both PRGF protocols was similar, measured as cellular proliferation of primary human keratocytes. On the basis of these results, it could be concluded that the new PRGF obtaining protocol (PRGF-B), with a reduction in the amount of anticoagulant and activator, presented improved characteristics for most of the parameters studied.

After reporting the experimental evidence, the next three chapters (**chapters 6, 7 and 8**) focus on a theoretical analysis of several aspects related to the PRP that are essential to achieve progress in the safe and effective use of this type of therapy. These three articles are fundamental in the successful translation of PRP-based therapies [5-7].

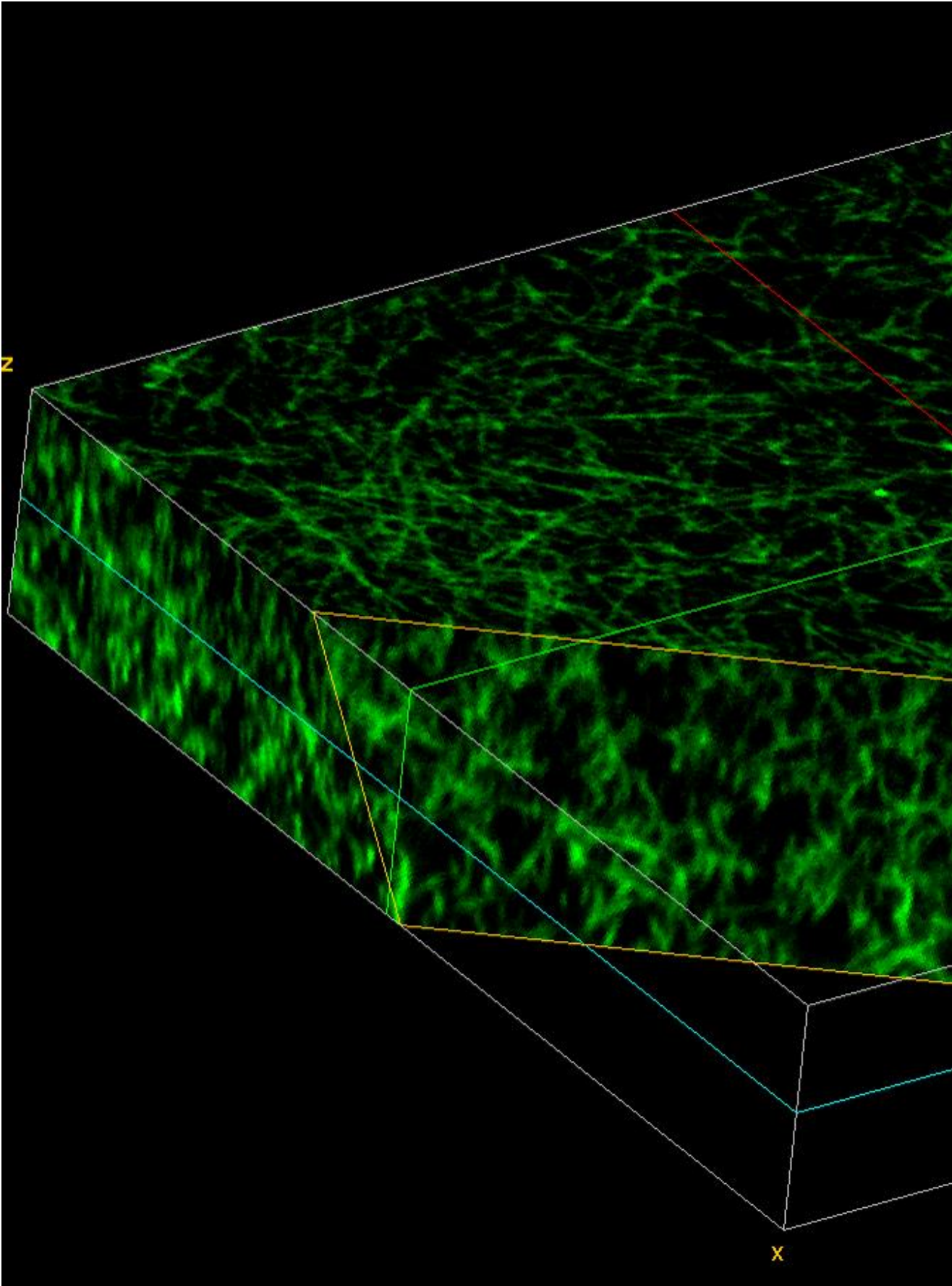
Thus, **Chapter 6** begins with a theoretical and analytical reference that includes an exhaustive review of the new regulatory framework of platelet-rich plasma introduced by the Spanish Agency of Medicines and Medical Devices (AEMPS) in Spain and its potential implications [5].

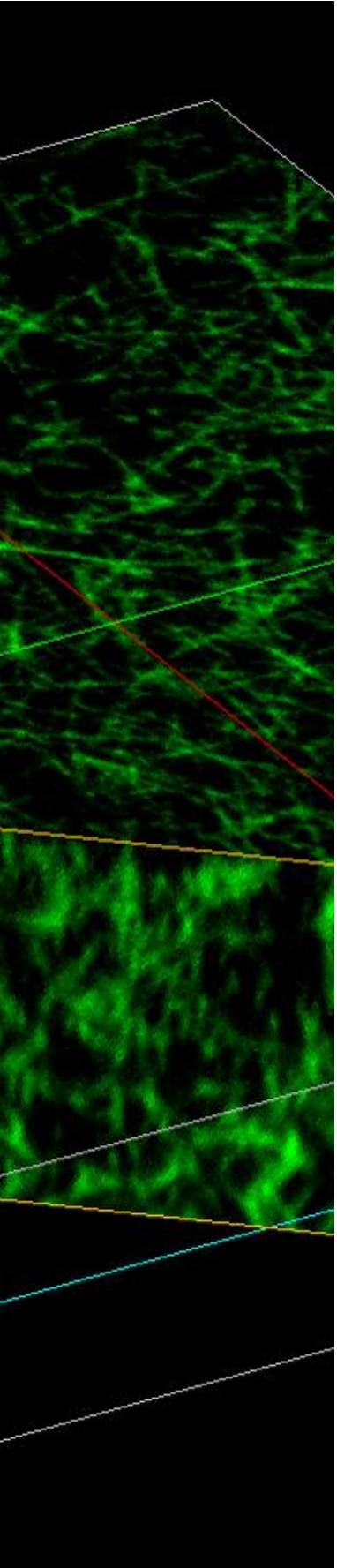
The impact of the new status of the PRP as a medicinal product for human use is analyzed, as well as the guarantees of safety and efficacy that this new classification offers. At the same time the new regulation is compared with the existing one in other countries. As with any new regulation, the classification of the PRP as a medicinal product also presents weaknesses, uncertainties and challenges, all of which are

discussed both in a regulatory framework and in terms of its practical application.

Afterwards, in **Chapter 7,** the advantages and disadvantages of the use of non-autologous or allogeneic PRP as a new type of therapy are discussed [6]. A representative selection of the few studies that have used allogeneic PRP is provided and a rationale is presented for its future use.

Finally, **Chapter 8** emphasizes the need to standardize biological procedures, mainly PRP and stem cells, to allow a strict advancement of personalized medicine with guarantees of both safety and efficacy [7]. For this purpose, and with the need for reproducible science as a fundamental principle, we propose the use of standardized guidelines to describe the methodological details of all studies in which PRP is used, both at a preclinical and clinical level.


Z
X



Chapter 1

Introduction. Characterization of Plasma Rich in Growth Factors (PRGF): components and formulations

Chapter published In *Platelet Rich Plasma in Orthopaedics and Sports Medicine*, Springer International Publishing [1]

Abstract

Platelet-rich Plasma (PRP) is a set of autologous platelet products used to reduce pain and speed up recovery from injury, as the same time that tries to keep the tissue function. The basic rationale is to mimic the natural ways of healing, bringing to the injury site a set of molecules that will accelerate the functional recovery of the tissue trying to regenerate the tissue. Among the jungle of products in this field, Plasma Rich in Growth Factors (PRGF), the Endoret technology, is a pioneering autologous regenerative technology with multiple therapeutic potentials, present in at least four different formulations, depending on the coagulation and activation degree. PRGF-Endoret technology is safe and has multiple applications and potentials.

1 Potential of plasma rich in growth factors (PRGF-Endoret): mimicking the natural healing

The increasing number of musculoskeletal injuries has produced an increasing number and improvement of different treatments of these lesions, especially in the search for minimally invasive procedures or adjuvants [8-10]. One of these cutting-edge technologies is the use of Plasma Rich in Growth Factors (PRGF-Endoret) [8]. This biological treatment mimics the natural ways of wound healing [11] trying to optimise and reduce healing times. This is achieved driving to the injury site the whole protein array of PRGF that will be involved in the repair of damaged tissues. In this way, all the bioactive molecules (including growth factors and other proteins) necessary for tissue repair are locally released.

The tissue repair process occurs naturally in a staged fashion [12] and includes removal of dead cells, proliferation, migration of cells to the injury site, production of new vascular structures, etc. The organisation of all these elements influences the healing of a given injury, preventing fibrotic elements that cause that tissue loss of functional capacity [13, 14]. Growth factors play an important role coordinating the whole process in an orchestrated fashion in all tissues of the musculoskeletal system, including muscle [15], tendon [16], bone [17, 18], cartilage [19]. Growth factors act on other tissues as well, including skin [20], oral soft tissue [21, 22], cornea [23] among others.

PRGF-Endoret technology mimics the natural healing mechanisms, but with two special features: trying to avoid loss of functionality (fibrous tissue) and shortening healing times. This is achieved in part adjusting the PRGF-Endoret formulation and dosage to the type of tissue and injury.

PRGF-Endoret therapy accelerates and improves tissue healing by local delivery of autologous bioactive molecules and contributing with a first line provisional scaffold [8]. This autologous toolbox consists in the use of platelets as a reservoir and vehicle of a large repertoire of proteins [24, 25]. Recently, a proteomic dissection of PRGF scaffold has been performed [2]. In this research, we studied the proteins that remain most closely bound to the fibrin network and that were therefore retained by the mesh itself rather than being released into the supernatant. The high-throughput proteomic techniques used in this characterization allowed us to produce a catalogue of these proteins and subsequently classify them into families on the basis of their function and gene ontology. The results of this process showed that the fibrin network is enriched in proteins specifically involved in tissue regeneration and wound healing. Amongst others, it was found enrichment in certain lipoproteins involved in regenerative processes, particularly by delaying degradation (fibrinolysis) of the fibrin network, thereby extending the controlled release of other molecules. Similarly, an important family of proteins involved in the acute phase reaction was also found to be enriched in this study. These proteins form the first line of defence in the immune system [2].

In the last decade, several systems have been developed to produce a biologically active product, both commercial and homemade, but they differ in the presence of white blood cells, growth factors concentration, and architecture of fibrin scaffold [26-30]. The different PRP commercial systems can be certified for various medical applications, but the therapeutic outcome will depend on the type of platelet-rich plasma used and the employed dosage. Establishing a proper classification of PRPs and identifying the biological differences among them is absolutely necessary to understand some of the

controversial results obtained with these types of technologies so far [31].

One of the most relevant and controversial issues is the presence of leukocytes in the platelet-rich plasma. In order to distinctly define the PRGF technology, and thus be able to compare with other PRPs, PRGF can be categorised according to three of the most cited classifications that have been proposed for PRPs. The first and most widely used [32] classifies PRGF as pure-PRP (P-PRP) since it does not contain WBC. The PRGF is classified as type 4-B (Minimal WBCs, activated with $CaCl_2$, and platelet concentration below 5x) as proposed by others [33] for sports medicine classification. Finally, PRGF would fit in the P2-x-Bβ category (platelet count greater than baseline levels to 750,000 platelets/μL, exogenous activation with $CaCl_2$, with WBC -and specifically neutrophils- below to baseline levels) according to the PAW (platelets, activation and WBC) classification [34].

2 Understanding the properties of platelet-rich plasma products

Several key biological mediators are present in a PRP. The more studied growth factors contained in platelet-rich plasma that are important during tissue repair include IGF-I (Insulin-like Growth Factor type I), TGF-β1 (Transforming Growth Factor β type 1), PDGF (Platelet Derived Growth Factor), HGF (Hepatocyte Growth Factor), VEGF (Vascular Endothelial Growth Factor), EGF (Epithelial Growth Factor) and bFGF (basic Fibroblastic Growth Factor) among others (Table 1) [35, 36]. Some of them (IGF-I and HGF) are plasmatic proteins, and their concentration does not depend on the platelet enrichment. However, most of the growth factors are indeed platelet proteins, both synthesised and

adsorbed, and thus their quantity does depend on the platelet concentration.

Classification	Protein	Biological effects
Adhesive proteins	vWF propeptide, Fibrinogen, Fibronectin, Vitronectin, TSP-1, laminin-8 (alpha4- and alpha5-laminin subunits), signal peptide-CUB-EGF domain containing protein 1 (SCUBE 1)	Cell contact interactions, homeostasis and clotting, and extracellular matrix composition
Clotting factors and associated proteins	FactorV/Va, FactorXI-like protein, multimerin, protein S, high-molecular weight kininogen, antithrombin III, tissue factor pathway inhibitor (TFPI)1	Thrombin production and its regulation
Fibrinolytic factors and associated proteins	PAI-1, uPA, alpha2-antiplasmin, histidine-rich glycoprotein, α2M	Plasmin production and vascular modelling
Proteases and anti-proteases	TIMPs 1–4, metalloprotease-1, -2, -4, -9, ADAMTS13, TACE, protease nexin-2, C1 inhibitor, serpin proteinase inhibitor 8, alpha1-antitrypsin	Angiogenesis, vascular modelling, regulation of coagulation, and regulation of cellular behaviour
Growth factors	PDGF, TGF-beta1 and -beta2, EGF, IGF-1, VEGF (A and C), bFGF (FGF-2), HGF, Bone morphogenetic protein (BMP)-2, -4, -6, CTGF	Chemotaxis, cell proliferation and differentiation, and angiogenesis

Chemokines, cytokines and others	RANTES, IL-8, MIP-1 alpha, ENA-78, MCP-3, GRO-alpha, angiopoietin-1, IGF-BP3, IL-6sR, PF4, bTG, platelet basic protein, NAP-2, connective tissue-activating peptide III, HMGB1, FasL, LIGHT protein, TRAIL, SDF-1 alpha, endostatin-l, osteonectin-1, bone sialoprotein	Regulation of angiogenesis, vascular modelling, cellular interactions, and bone formation
Anti-microbial proteins	Thrombocidins, defensins	Bactericidal and fungicidal properties
Others	Chondroitin 4-sulfate, albumin, immunoglobulins, disabled-2, semaphorin 3A, Prion protein (PrPC)	

Table 1. Platelet protein classification and their biological role. A set of proteins present in platelets and its physiological role in the regeneration of tissues is shown. Reproduced with permission from the reference [37].

To understand the properties of platelet-rich plasma products, it is necessary to detail the different roles of molecules that contain:

- IGF-I: This protein circulates in plasma as a complex with binding proteins (IGFBP). This determines the bioavailability and regulates the interaction between this IGF-I and its receptor [38, 39]. IGF-I is involved in keratinocyte migration and wound healing [40, 41], stimulates bone matrix formation and maintenance [42] by promoting preosteoblast proliferation [43, 44], and also is involved in striated muscle myogenesis [45]. Furthermore, knockout mice for IGF-IR in muscle exhibited

impaired muscle regeneration and deficient myoblast differentiation[46]. Recently, It has been observed that IGF-1 promote tissue repair of skeletal muscle without scar tissue formation by increasing fibre size and muscle size hypertrophy [47]. Also, and related to this, IGF-1 is considered a potent enhancer of tissue regeneration, and its overexpression in muscle injury leads to hastened resolution of the inflammatory phase [48].

- TGF-β1: The role of TGF- β family proteins in wound healing has been recently reviewed [49]. TGF-β has different effects, depending on the tissue and the cell type [13]. The release and posterior bioactivation of latent TGF-β contributes to the early cellular reparative responses, such as migration of cells and neovascularization and angiogenesis [50] into the wound area. In bone, TGF-β1 induces osteogenic differentiation of mesenchymal cells of the bone marrow, upregulating osteoblast differentiation markers [51]. TGF- β plays a crucial role in maintaining homoeostasis of both articular cartilage and subchondral bone [52].
- PDGF: This growth factor is a mitogen and chemotactic factor for all cells of mesenchymal origin [53]. It is important in the repair of joint tissue, including cartilage and meniscus [54, 55]. Bone is also a target of PDGF, influencing its metabolism and acting in repair mechanisms [56, 57], including the recruitment of pericytes to stabilise new blood vessels [58].
- HGF: Also called scatter factor, it regulates cell growth, migration and morphogenesis [59] and plays an important role in wound-healing through an epithelial-mesenchymal interaction [60]. HGF modulates central inflammatory and immune events that are common to many diseases and organ

systems [61]. The antifibrotic effect of HGF has been shown in various tissues [62, 63], through induction of Smad7, and thus regulates the myofibroblast phenotype, allowing the initial contraction of the wound, but making the myofibroblast to gradual disappear [64].

- VEGF: This growth factor is a key mediator in wound healing [65] and the main inducer of angiogenesis since it stimulates chemotaxis and proliferation of endothelial cells [66]. This protein is crucial in the sprouting of new capillaries from preexisting vasculature, mainly initiated by hypoxia in ischemic tissue [67]. Also, VEGF is involved in the regulation of many organ homeostases, such as brain, heart, kidney, or liver [68], and its role may be crucial in cell-mediated tissue regeneration [69].
- EGF: This protein promotes chemotaxis and mitogenesis in epithelial and mesenchymal cells [70, 71] by acting on the regeneration of multiple tissues. It has an important role in skin, cornea, gastrointestinal tract and nervous system [72-76].
- bFGF: This factor, also called FGF-2, is a potent inductor of cell proliferation, angiogenesis and differentiation [77, 78]. Its role in the repair process has been observed in several tissues [79], including bone [80-82], tendon [83, 84], and periodontal tissue [85-87].

Growth factors classically promote several important functions in the regenerative milieu: they are able to stimulate cell proliferation (mitosis), cellular migration (chemotaxis), differentiation (morphogenic effect), angiogenesis, and the combination of several of these effects. These peptides exert the above-mentioned functions in the local environment, close to the site of the application.

However, it is difficult to dissect the contribution of each molecule contained in platelet-rich plasma and examine its effect separately, since many have multiple effects, some of which overlap with others. Also, many molecules are activated in the presence of others, such as TGF-β, which is in a latent state [88] and becomes functional after proteolytic activation or in the presence of other molecules, such as thrombospondin-1 [89] or various integrins.

The idea that platelet-rich plasma contains only factors that stimulate angiogenesis and proliferation would be a little simplistic. In fact, another important property of the PRP is the bacteriostatic effect [90]. These antibacterial effects were observed against *Staphylococcus aureus* and *Escherichia coli* [91]. Classically, these properties have been shown in leukocyte-enriched platelet-rich plasma. However, recently these antimicrobial properties have been evidenced in PRGF-Endoret [92], which by definition has no white cells. Specifically, PRGF-Endoret has bacteriostatic effect against Staphylococcal strains. Moreover, the addition of leukocytes to the PRGF-Endoret preparation did not yield greater bacteriostatic potential than it already had. This data raise questions about the role that leukocytes may play in a platelet-rich plasma preparation, since they do not improve the bacteriostatic properties but, on the contrary, they might significantly increase the presence of pro-inflammatory molecules.

Platelet-rich products act also as anti-inflammatory mediators by blocking monocyte chemotactic protein-1 (MCP-1), released from monocytes, and lipoxin A4 production [93]. HGF in PRP inhibits NF-kB, a key nuclear factor implicated in inflammatory responses, by activation of its inhibitor (ikBα). In this same study, it was also observed that PRP reduced the chemotaxis of the monocytic line U937 [94]. In addition,

serotonin, a neurotransmitter and hormone present in platelets, has been reported to directly mediate liver regeneration [95].

3 PRGF-Endoret: A pioneering technology

For almost two decades our research group has characterised this technology and has studied its therapeutic potential in tissue repair and wound healing [8]. PRGF-Endoret contains a moderated platelet concentration, a two-third fold increase compared to peripheral blood, a dosage shown to induce optimal biological benefit [96]. In fact, lower platelet concentrations can lead to suboptimal effects, whereas higher concentrations might have an inhibitory effect [97]. PRGF-Endoret does not contain leukocytes, and activation is performed only with $CaCl_2$.

The process to produce PRGF-Endoret is easy, fast and reproducible (Figure 3). Blood collection is performed in tubes containing sodium citrate as anticoagulant. Thus, platelets are well preserved. Subsequently, centrifugation is achieved in a specifically designed centrifuge (PRGF System V). The centrifuge has specific parameters to maximise the production of platelets and keep the plasma leukocyte-free. Three typical layers are obtained after centrifugation: (i) a yellowish top layer, the plasma, which contains a gradient of platelets, with maximum concentration of those platelets above the buffy coat; (ii) the leukocyte layer, or *buffy coat*, is located below of plasma layer; and (iii) the bottom layer, that is the layer containing the red cells.

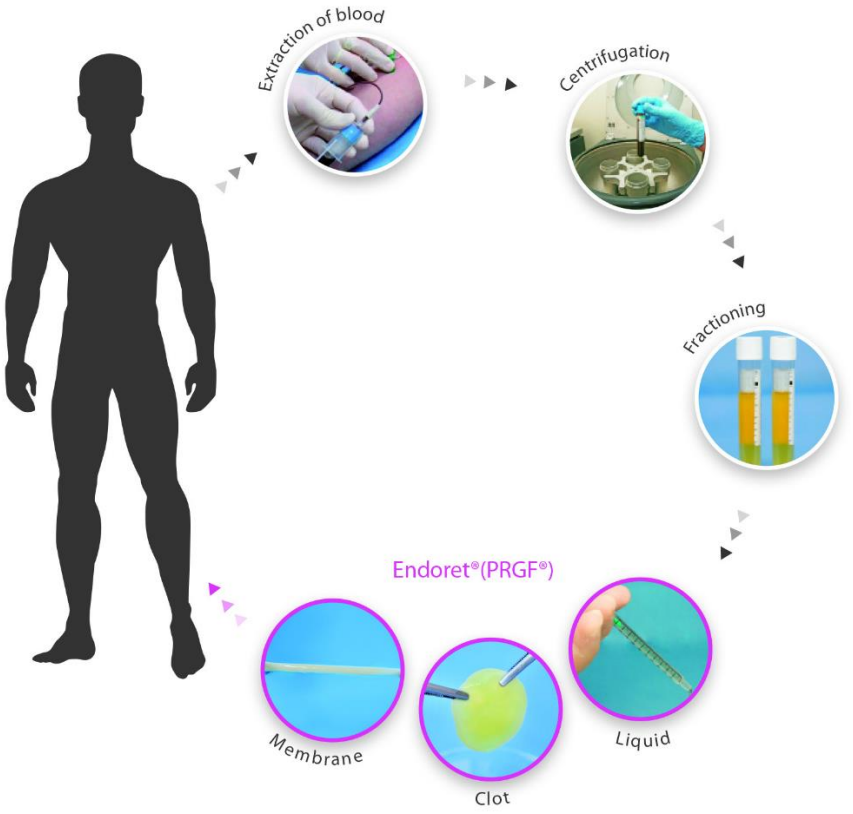


Figure 3. PRGF-Endoret technology overview. PRGF-Endoret aids in the preparation of different autologous therapeutic formulations from patient's own blood.

Regarding the plasma volume, it is possible to empirically differentiate between two different fractions, depending on the respective concentration of platelets. The upper fraction will contain a similar number of platelets than peripheral blood whereas the lower fraction will contain 2 to 3-fold the concentration of platelets compared with blood. However, depending on the application, as in the case of PRGF eye drops, it is possible to collect the entire PRGF column without

performing two fractions [98]. The basic characteristics of PRGF (whole plasma column) are shown in Table 2.

N=30	Whole blood-B	PRGF-B
Leukocytes (x 10^3/µL)	6.1 ± 1.4	0.3 ± 0.2
Erythrocytes (x 10^6/µL)	4.78 ± 0.41	0.01 ± 0.01
Platelets (x 10^3/µL)	235 ± 41	517 ± 107
Leukocyte concentration factor (LCF)	-	0.05 ± 0.03
Platelet concentration factor (PCF)	-	2.2 ± 0.2
Platelet yield (%)	-	66 ± 7

Table 2. Summary of the characterization of whole blood and PRGF samples from thirty donors. The values for PRGF correspond to the whole plasma column. Leukocyte, platelet and erythrocyte concentration was measured in whole blood and PRGF. Leukocyte and platelet concentration factor (enrichment as fold increase) relative to the level of peripheral blood (LCF and PCF) and platelet yield (%) are also indicated. Data are expressed as mean ± SD. Reproduced with permission from reference [4].

With the aim of collecting these plasma fractions from PRGF-Endoret technology, we have recently developed an optimised device, the plasma transfer device (PTD2) (Figure 4).

Figure 4. The plasma transfer device 2 (PTD2) is a disposable and sterile aspiration system that allows the fractionation of PRGF. The device contains an ergonomic button that allows fine control of the suction flow. The suction is performed by the vacuum containing in the fractionation tube (TF9). The aspiration needle is a blunt needle to prevent accidental stab injuries. In this way, PRGF-Endoret is obtained directly in a fractionation tube, in which it can be directly activated with calcium chloride. It is possible to perform the whole procedure without opening the extraction tubes, using an adapter needle.

The PTD2 is a disposable and sterile aspiration system that allows separating the different fractions obtained after centrifugation. In contrast to the traditional pipetting system, the PTD2 system is faster, avoiding intermediate pipetting steps. In addition, the plasma transfer device does not require maintenance of the pipetting system. Depending on clinical needs, the fractionation can be made in one or two fractions, achieving higher volume - lower concentration of platelets (a single fraction), or lower volume - higher concentration of platelets (two fractions, F1 and F2). After fractionation, PRGF-Endoret can be activated in a controlled way by the addition of $CaCl_2$, providing a clot that mimics its natural structure. Moreover, the coagulation is conducted at a speed that allows controlling the whole process. Activation with $CaCl_2$ avoids the use the use of exogenous bovine thrombin, a source of possible immunological reactions [99-101]. Recently, the PRGF obtaining protocol has been improved [4] in order to reduce both the amount of anticoagulant and activator: the new blood extraction tubes (TB9) contain 400 µL of trisodium citrate as anticoagulant, and the new ratio of PRGF Activator would be 20 µL of calcium chloride / mL PRGF.

Another important feature of the PRGF-Endoret technology, when compared with other platelet-rich plasma systems, is the absence of leukocytes, which categorises it in a safe and homogeneous, because the values of leukocytes are highly variable between donors [102], and within the same donor are highly dependent on small perturbation of the body homeostasis. In addition, polymorphonuclear neutrophils (PMN) contain molecules designed to kill microorganisms, but can seriously damage the body tissues. For example, PMNs are important producers of matrix metalloproteinases (MMP), mainly MMP-8 and MMP-9, which can hamper the regeneration of damaged tissue. PMNs also produce free radicals, reactive oxygen species and nitrogen, which can destroy not only microorganisms but surrounding cells [103]. Of

special concern would be to avoid leukocytes if muscle regeneration is required, as *in vivo* PMNs increase muscle damage [104] and do not provide extra functionality. Therefore, it is recommended to use leukocyte-free platelet-rich plasma in infiltrations of damaged muscle [105].

4 PRGF-Endoret technology: A versatile toolbox with multiple formulations

A key point that distinguishes the PRGF-Endoret technology from other platelet-rich plasma products is its versatility. Four different formulations (Figure 5) with therapeutic potential are obtained from the patient's blood, depending on the coagulation and activation degree of the samples. These formulations may be used for different therapeutic purposes:

1) *PRGF-Endoret scaffold*. This three-dimensional matrix encloses autologous growth factors, both plasma and platelet proteins. This scaffold can be used in various applications, such as the treatment of ulcers [106, 107], wound closure and tissue engineering [108]. The three-dimensional structure of the fibrin mesh (Figure 6) allows cell proliferation, since, as mentioned above, it contains factors necessary for growth and migration of cells. In addition, this formulation can be combined with other materials [109], such as autologous bone, demineralized freeze-dried bovine bone, collagen among others, tuning the resulting characteristics of the scaffold [108].

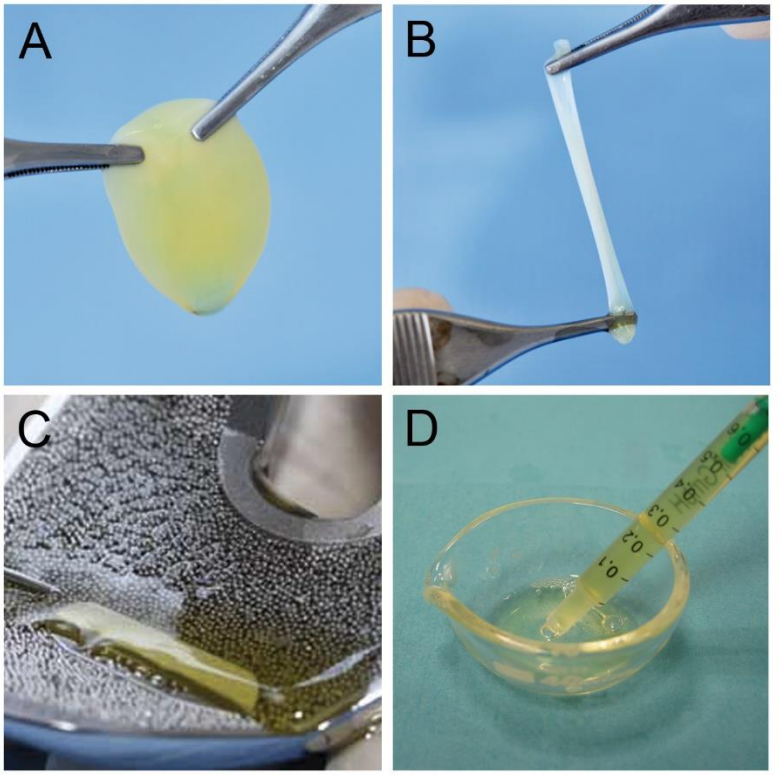


Figure 5. PRGF-Endoret technology formulations: (A) three-dimensional clot or scaffold, (B) elastic and dense autologous fibrin membrane. (C) liquid formulation activated at the moment and deposited on the implant surface, and (D) the PRGF supernatant, ideal as eye drops or cell culture supplement.

1) Liquid PRGF-Endoret, activated at the time of use, is used in intra-articular [110-112] and intraosseous [113-115] injections, surgery [116-118], treatment of skin disorders [106, 107, 119], and implant surface bioactivation by producing a biologically active layer on the titanium surfaces [120, 121].

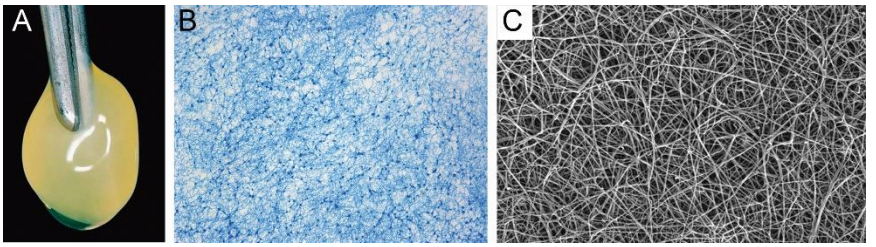
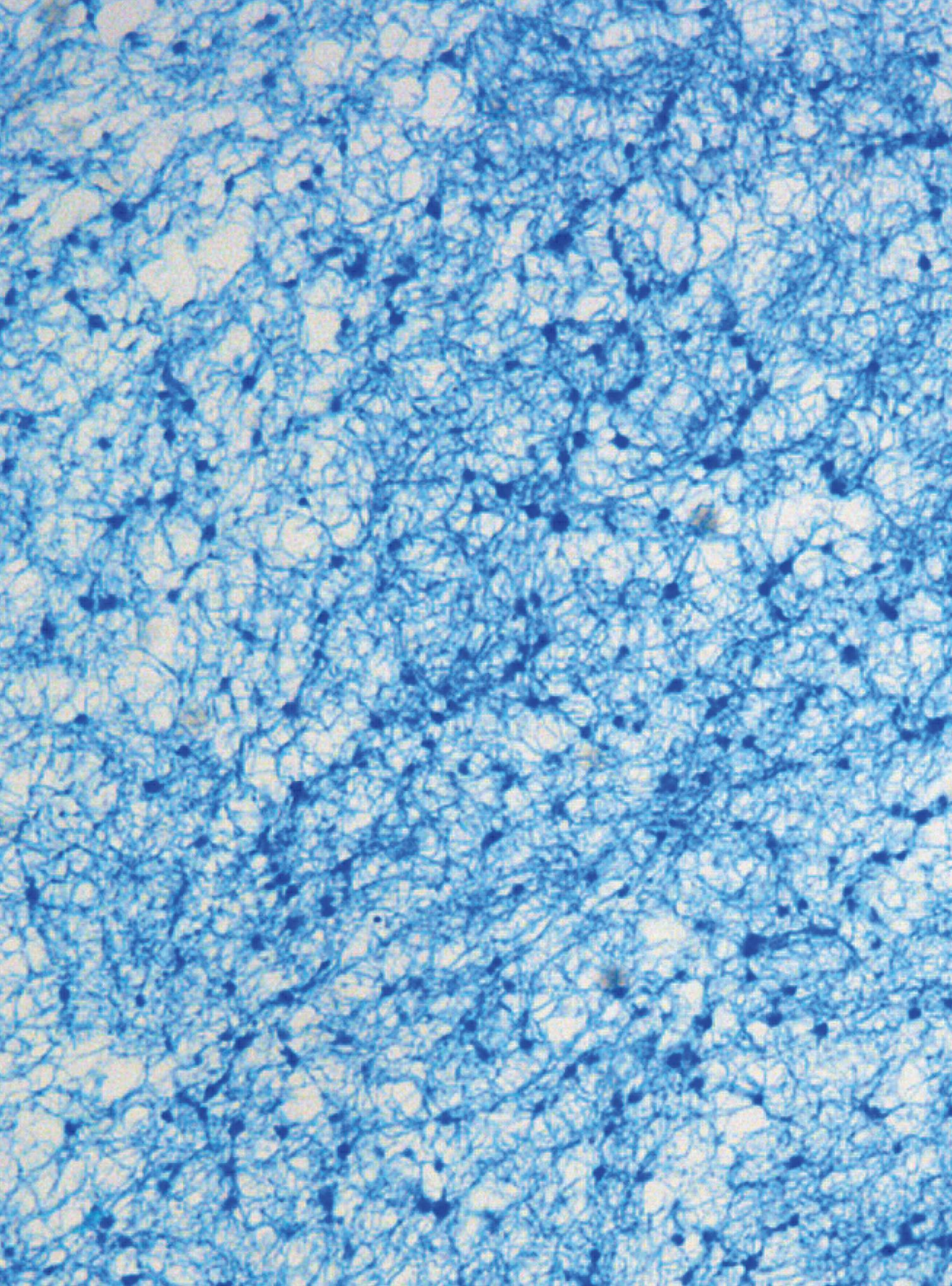


Figure 6. Three-dimensional structure of PRGF-Endoret clot or scaffold. (A) PRGF scaffold observed with the naked eye. (B) Optical microscopy reveals a 3D network of fibrin with platelet aggregates scattered throughout the network (May-Grunwald-Giemsa staining, original magnification x 400). (C) Closer inspection reveals regular and interconnected intact fibrin strands in a leukocyte-free plasma rich in growth factors (PRGF)-Endoret scaffold (original magnification x 3500). Adapted, with permission, from Anitua et al, Trends Biotechnol. 2013;31:364-74.

2) The PRGF-Endoret supernatant contains plasma proteins and platelet releasate and can be used as eye drops treatment for dry eye disease [122] and other corneal defects [123, 124]. Both in basic research studies and applied areas, this formulation can be used to supplement the cell culture medium [108, 125].
3) Autologous fibrin membrane. At the end of the process of coagulation, fibrin scaffold retracts [126]. At that stage, the fibrin membrane can be shaped with tweezers or similar instruments to obtain an elastic, dense and suturable membrane. It is an excellent tool to seal the post-extraction tooth sockets [127-129] and to promote the full epithelialization of other soft tissues [130].

The autologous platelet products have a high therapeutic potential and can be used in various formulations and in various fields of medicine and tissue engineering. At present, there are over forty of these products with different characteristics, in terms of enrichment of platelets, presence of leukocytes, activator type, and final volume among others.

This great variability makes it difficult to standardise protocols and compare results. Furthermore, this large variability can engender confusion among clinicians and researchers [131]. It is, therefore, necessary to reach a consensus and better definition of each product. Our research team has spent more than 20 years developing this technology, which makes PRGF-Endoret one of the best characterised autologous platelet-rich plasma, with multiple and growing therapeutic applications, as result of a continuous research translation to the clinic setting.



Chapter 2

Aim and Specific objectives.

Propósito y Objetivos concretos.

1 Aim

There is a growing need to find both effective and minimally invasive treatments in all areas of medicine. Plasma rich in growth factors (PRGF-Endoret) technology arises in order to address these unmet medical needs. With a standardized procedure, a platelet-rich concentrate is obtained from a small volume of blood, with plasma proteins and free of leukocytes and erythrocytes.

The objective of this doctoral thesis was twofold; on the one hand, to carry out a comprehensive characterization of PRGF technology that serves as a basis to unravel the molecular mechanisms that underpin its effectiveness and allow its optimization. On the other hand, to conduct a detailed analysis of the regulatory framework and future trends in the use of this technology.

2 Specific objectives

The specific objectives of this doctoral thesis are the following:

1. Conduct a high-performance proteomic characterization of the PRGF-derived fibrin clot.

2. Determine the release kinetics of morphogens and proinflammatory cytokines from PRGF scaffolds and assess whether the presence of leukocytes modifies it.

3. Characterize a new and more physiological PRGF preparation protocol and compare it with the previous one.

4. Analyse the new regulatory framework of the PRGF and the rest of platelet-rich plasmas (PRP) after the resolution of the Spanish Agency of Medicines and Medical Devices (AEMPS).

5. Investigate new non-autologous commercial therapeutic strategies, such as the application of allogeneic PRP, and assess its advantages over the autologous approach.

6. Strengthen the need to maintain standardized guidelines in the presentation of methods that describe biological therapies, such as treatment with PRP or stem cells.

3 Propósito

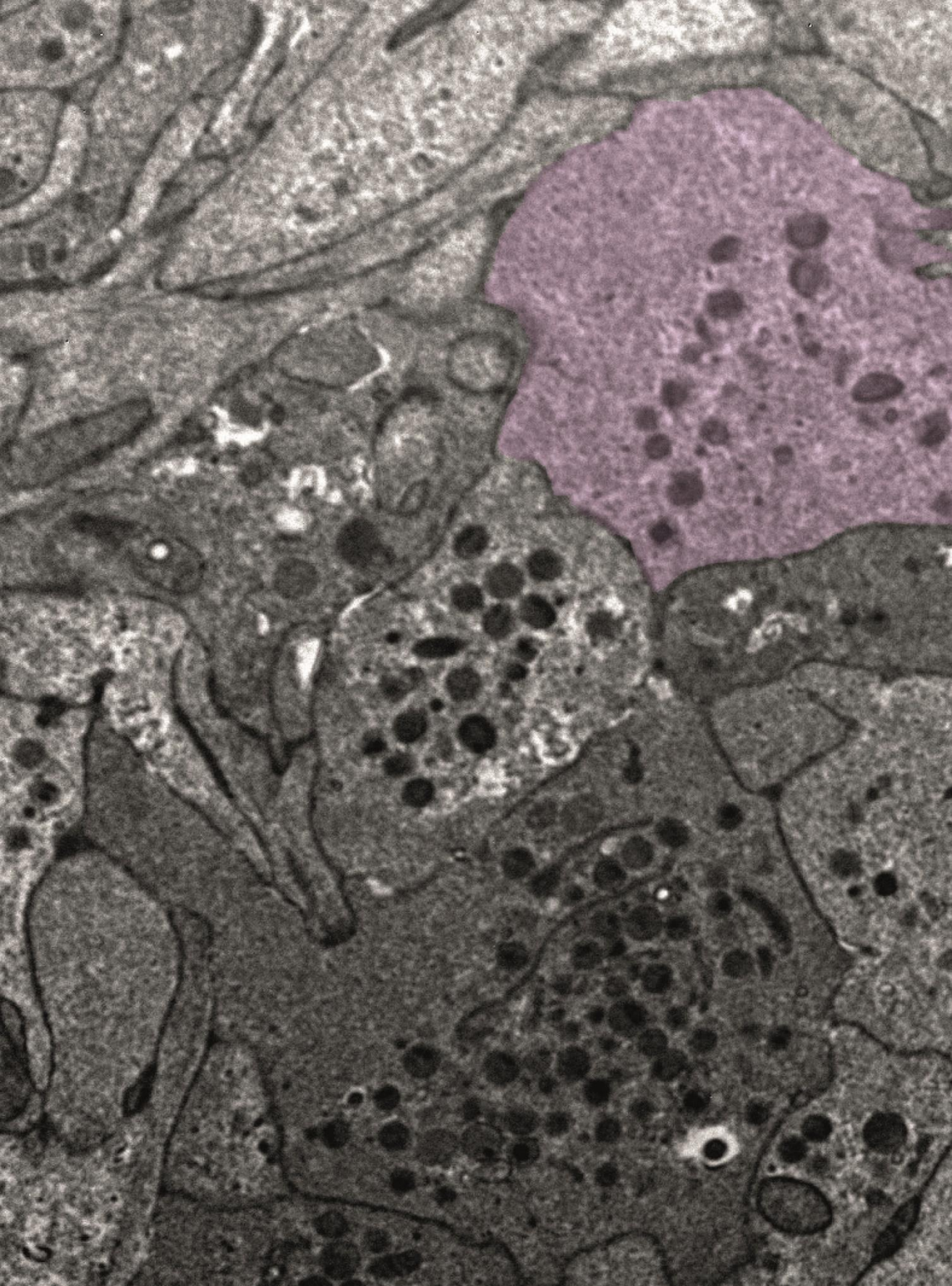
Existe una necesidad cada vez mayor de encontrar tratamientos mínimamente invasivos en todas las áreas de la medicina. Con el fin de cubrir estas necesidades médicas no resueltas surge la tecnología basada en el plasma rico en factores de crecimiento, *plasma rich in growth factors* (PRGF-Endoret). A partir de un pequeño volumen de sangre se obtiene un concentrado rico en plaquetas, con proteínas del plasma y libre de leucocitos y eritrocitos.

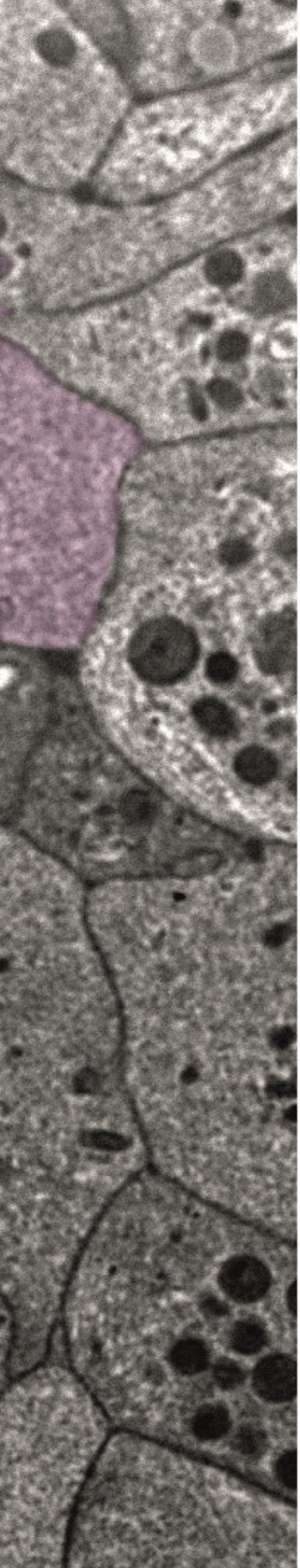
El objetivo de esta tesis doctoral fue doble; por un lado, realizar una caracterización detallada de la tecnología del PRGF que sirva como base para desentrañar los mecanismos moleculares que fundamentan su eficacia y permitan su optimización. Por otro lado, realizar un análisis exhaustivo del marco regulatorio y las tendencias futuras de la utilización de dicha tecnología.

4 Objetivos específicos

Los objetivos específicos de la presente tesis doctoral son los siguientes:

1. Realizar una caracterización proteómica de alto rendimiento del coágulo derivado de PRGF.
2. Determinar la cinética de liberación de morfógenos y citoquinas proinflamatorias de las matrices de PRGF y evaluar si la presencia de leucocitos la modifica.
3. Caracterizar un nuevo protocolo de preparación de PRGF más fisiológico y compararlo con el anterior.
4. Analizar el nuevo marco regulatorio del PRGF y del resto de plasmas ricos en plaquetas (PRP) tras la resolución de la Agencia Española del Medicamento y Producto Sanitario (AEMPS).
5. Investigar nuevas estrategias terapéuticas comerciales no autólogas, como la aplicación del PRP alogénico, y valorar sus ventajas sobre la estrategia autóloga.
6. Reforzar la necesidad de mantener directrices estandarizadas en la presentación de los métodos que describen terapias biológicas, como el tratamiento con PRP o las células madre.





Chapter 3

High-throughput proteomic characterization of plasma rich in growth factors (PRGF-Endoret)-derived fibrin clot interactome

Experimental article published In Journal of Tissue Engineering and Regenerative Medicine [2]

Abstract

Plasma rich in growth factors (PRGF®-Endoret®) is an autologous technology that contains a set of proteins specifically addressed for wound healing and tissue regeneration. The scaffold formed by using this technology is a clot mainly composed of fibrin protein forming a three-dimensional macroscopic network. This biomaterial is easily obtained by biotechnological means from blood and can be used in a range of situations to help wound healing and tissue regeneration. Although the main constituent of this clot is the fibrin scaffold, little is known about other proteins interacting in this clot that may act as adjuvants in the healing process. The aim of this study was to characterize the proteins enclosed by PRGF-Endoret scaffold using a double proteomic approach that combines 1D-SDS-PAGE approach followed by LC-MS/MS, and 2-DE followed by MALDI-TOF/TOF. The results presented here provide a description of the catalogue of key proteins in close contact with the fibrin scaffold. The obtained lists of proteins were grouped into families and networks according to gene ontology. Taken together, it has been found an enrichment of both proteins and protein families specifically involved in tissue regeneration and wound healing.

1 Introduction

Tissue regeneration is a complex process necessary for the healing and repairing after tissue damage [132]. Many proteins, including cytokines and growth factors are involved in this process, but the successful restoration of tissue functions relies on an orchestrated sequence of steps rather than on their isolate action. Therefore, the controlled spatiotemporal release of bioactive factors into injured tissue results a crucial event in tissue therapy [133].

Platelets are anucleate cell fragments mainly involved in the formation of blood scaffolds for the healing of tissue injures. Platelets work as biological dikes preventing blood loss from the organism, but they also induce and coordinate the healing process itself [24, 134]. Once activated, platelets secrete numerous proteins [25, 135], including growth factors, such as PDGF, VEGF or HGF among others, that drive tissue regeneration mechanisms in a staggered way [36]. Platelets, therefore, are potential deliverers of growth factors with multiple applications in tissue engineering [136].

"Plasma rich in growth factors" (PRGF®-Endoret®) is one of the pioneering and leading technologies for the use of autologous platelets with healing purposes [137-139]. PRGF-Endoret is easily obtained from the patient to be treated. Briefly, blood from the patient is extracted and centrifuged so that platelet-enriched and leukocyte-free plasma is isolated from the rest of blood constituents. Then, the platelet-enriched fraction is treated with $CaCl_2$ so that fibrin formation is activated. Fibrin forms a dense and solid clot in which platelets and proteins are embedded. Therefore, this activated fibrin scaffold becomes a growth factor-rich sponge ready to be used for grafting, alone or combined with cells. Thanks to these autologous grafts, PRGF-Endoret-treated patients

get benefited of a faster and more reliable regeneration of their injuries. Its autologous nature, easy and low-cost obtainment, and its versatility make PRGF-Endoret one of the most interesting preparations for wound healing and tissue engineering [140]. Characterization of the protein constituent of these PRGF-Endoret scaffolds could help to understand in deep how this regenerative therapy works, unravelling the molecular mechanisms [141], and thus getting optimized clinical treatment, both in formulations as in the dosage.

With this aim, PRGF-Endoret-derived fibrin scaffolds were processed in order to get rid of the most abundant proteins, while maintaining those in close contact with the fibrin network; thus, the scaffold interactome was characterized following two strategies: A 1D-SDS-PAGE approach followed by LC-MS/MS protein identification, and a 2-DE approach followed by MALDI-TOF/TOF protein identification. These high throughput technologies have been shown to address the resolution of proteomes of complex samples [142-145].

Our results provide a description of the catalogue of proteins enclosed by the fibrin scaffold, and underscore the gain of information obtained by the dual-strategy approaching followed in this work. Moreover, this catalogue is analyzed from a functional viewpoint, in the context of biological processes, pathways, and molecular networks, in search of protein families involved in the regenerative potential of the PRGF-Endoret.

To the best of our knowledge this paper presents, for the first time, a high-throughput proteomic analysis of the fibrin clot interacting proteins of an autologous platelet-enriched product, the PRGF-Endoret technology.

2 Materials and methods

2.1 Donors

Human venous whole blood from three healthy volunteers was withdrawn into 9 mL vacuum tubes containing 3.8 % (w/v) sodium citrate. None of the donors had taken medication in the last week that could alter the haematological parameters. The three donors (BTI researchers) agreed with the experimental protocol and their informed consent was obtained. This study was performed following the principles of the Declaration of Helsinki.

2.2 PRGF-Endoret protocol

The whole blood samples were processed according to the PRGF-Endoret protocol [92]. Briefly, three blood tubes from each donor were centrifuged for 8 min at 580 g (centrifuge PRGF system IV–BTI, Spain). After centrifugation, a gradient of platelets in the plasma was obtained. A Plasma Transfer Device (PTD) (BTI Biotechnology Institute, Vitoria, Spain) was used for fractionation of the plasma, and thus obtain three fractions, with different concentrations of platelets, named F1, F2, and F3. F3 fraction is defined as the one millilitre located just above the *buffy coat*, and is most enriched in platelets. F2 is the immediately above millilitre, and is intermediate in platelet concentration. And finally, F1 is the rest of the plasma column, with variable volume depending on the hematocrit, and is the fraction with a lower concentration of platelets (Figure 7 A).

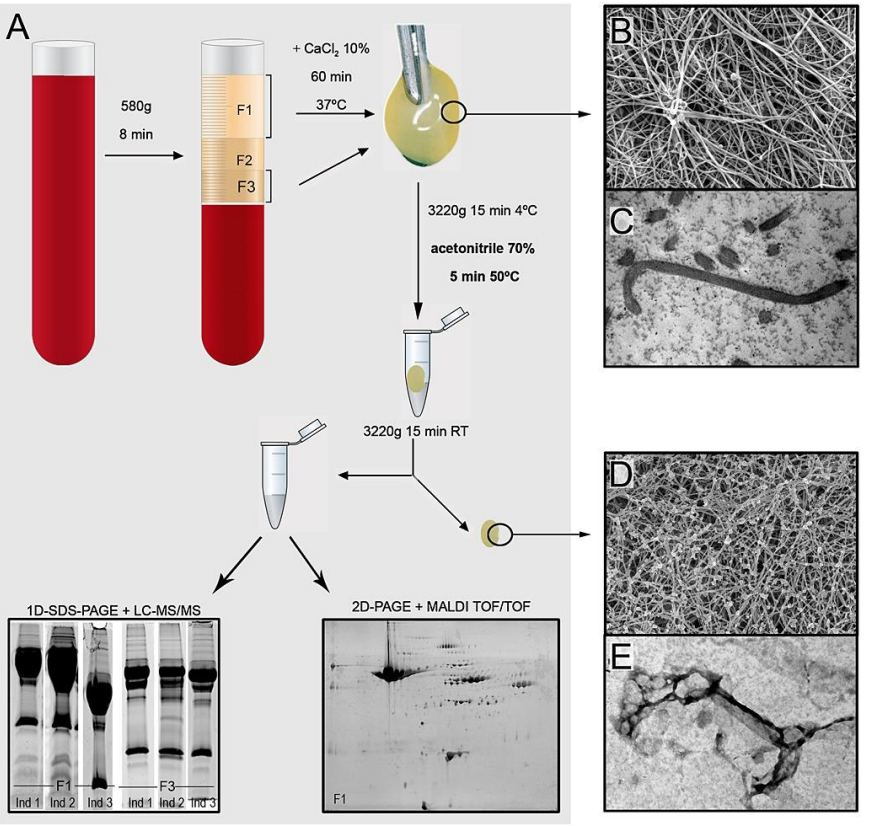


Figure 7. Summary of the process of preparation, characterization and strategy for analysis of the different samples. (A) Schematic illustration of the most important steps in the preparation of samples and subsequent analysis. After centrifugation and fractionation, F1 and F3 fractions were processed independently. After extraction with acetonitrile, samples were processed and analyzed following the dual approach commented (see materials and methods section for further details). Microscopic characterization of the PRGF-Endoret 3D scaffolds prior (B and C) and post (D and E) protein extraction. Original magnifications for SEM (B and D) are x5000 and for TEM (C and E) are x28000.

2.3 Sample preparation protocol

For each donor, fraction 1 from the three tubes was collected in a single tube. The same was done for the F3 fraction. The F2 fractions of the three donors were discarded. Therefore, six independent samples were obtained. The samples were activated in glass tubes adding calcium chloride at a final concentration of 22.8 mM and incubating at 37 °C for 1 h. After clot formation and subsequent retraction, the releasates were discarded and the samples centrifuged at 3220 g for 15 min at 4 °C. Then, the supernatants were discarded and the clots were incubated with 3 mL of 70 % acetonitrile (Sigma) in water for 5 min at 50 °C. Acetonitrile has been already successfully used to disrupt protein-protein interactions in serum proteins as described by *Tirumalai et al* [146]. After incubation, the samples were centrifuged again at 3220 g for 15 min at room temperature and the supernatant collected and frozen at -80 °C to prior subsequent analysis. Each sample was assayed in triplicate.

2.4 PRGF-Endoret characterization

2.4.1 Hematologic variables: Platelets and leukocytes

The platelet, leukocyte and erythrocyte count, in both peripheral blood and PRGF-Endoret (for each of the phases) previous to the activation, was determined using a standard hematological analyzer (ABX MICROS 60, Horiba Medical, Montpellier, France). The platelet enrichment factor was calculated compared to the average platelet concentration in peripheral blood.

2.4.2 Three-dimensional structure of fibrin net

Scanning (SEM) and transmission (TEM) electron microscopy techniques were used for morphological analysis of fibrin clots, prior and post

protein extraction. Each clot was prepared in triplicate. Briefly, the clots were rinsed with PBS, fixed with 2 % glutaraldehyde in 0.1 M cacodylate buffer for 4 h and washed 3 times in cacodylate-sucrose buffer (0.1 M cacodylate, 6.5 % sucrose, pH 7.4). Then, the samples were post-fixed with osmium tetroxide (1 % OsO_4 in 0.1 M cacodylate) for 1 h and washed in 0.1 M cacodylate, and finally dehydrated through ascending alcohol concentrations. At this point, samples were split and independently processed for SEM and TEM. For SEM, the samples were critical point dried (Tousimis Autosamdri 814) and sputter-coated with 5 nm of gold (Edwards E306A) before examination (Hitachi S-4800). For TEM studies, the samples were incubated in propylene oxide for 1 h and then in increasing concentrations of resin Epon, and finally embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM208S electron microscope.

2.5 SDS-PAGE analysis

F1 and F3 samples from three different individuals were dried in a RVC2 25 speedvac concentrator (Christ), and further resuspended in a buffer containing 50 mM Tris pH 6.8, 5 % glycerol, 1.67 % -mercaptoethanol, 1.67 % SDS, and 0.0062 % bromophenol blue. Protein from each individual was boiled for 5 min and resolved independently in 12.5 % acrylamide gels using a Mini-Protean II electrophoresis cell (Bio-Rad). A constant voltage of 150 V was applied for 45 min for this purpose. The gel was fixed in a solution containing 10 % acetic acid, 40 % ethanol for 30 min, and stained overnight in SYPRO Ruby (Bio-Rad). Gels were then washed in a solution containing 10 % ethanol and 7 % acetic acid for 30 min, and the gel image was acquired using a Typhoon Trio scanner (GE Healthcare). Each lane was cut into 18 consecutive pieces and subjected to tryptic digestion followed by LC-MS/MS analysis (View Supplementary Figure S1 for workflow).

2.6 Two dimensional gel electrophoresis (2-DE)

F1 dried samples from three different individuals were resuspended in a buffer containing 7 M urea, 2M thiourea, 4 % CHAPS and 50 mM Tris, and pooled. Prior to the IEF, DTT and pH 4-7 IPG buffer (GE Healthcare) were added to a final concentration of 65 mM and 1 %, respectively. IEF was performed on IPG strips (pH 4–7, 24 cm) on a Multiphor unit (Bio-Rad). Strips were passively rehydrated for 14 hours. The IEF protocol was as follows: two hours at 100 V, two hours at 300 V, 300–1000 V linear gradient for two hours, two at 1000 V, 1000–3000 V linear gradient for one hour, one hour at 3000 V, 3000–5000 V linear gradient for one hour, 5000 V for five h, and 8000 V for four h, up to a total of 60000 Vh. After the first dimension, the strips were incubated in equilibration solution (6 M urea, 2 % SDS, 40 mM Tris-HCl pH 8.8, 10 % glycerol) containing 2 % DTT for 15 minutes, and then were transferred to equilibration solution containing 2.5 % iodoacetamide (IAA). Separation in the second dimension was performed using 12.5 % self-cast acrylamide gels in an Ettan Dalt system (GE Healthcare).

Gels were then stained with SYPRO Ruby (Bio-Rad) following the same steps described in the previous section. Protein patterns were digitized using a Typhoon Trio scanner (GE Healthcare), and gel images were loaded in REDFIN software (Ludesi). Spots were automatically detected using this software, and faint spots (<100 average normalized volume) were filtered out with a volume-based spot filter. A picklist with the spots passing this filter was generated. Spots were picked using the Ettan Dalt Spot Picker (GE Healthcare) and subjected to tryptic digestion followed by MALDI-TOF/TOF analysis (Supplementary Figure S1 for workflow).

2.7 Tryptic digestion

Both gel slices and gel spots were reduced and alkylated prior to their digestion with trypsin. For this purpose, gels were washed and incubated with DTT (10 mM in 50 mM ammonium bicarbonate, 30 µl) at 56 °C for 20 min, followed by an incubation in IAA (50 mM in 50 mM ammonium bicarbonate, 30 µl) for another 20 min in the dark. Then, spots were washed in ammonium bicarbonate, dried by adding excess of acetonitrile and incubated with trypsin (12.5 µg/ml in 50 mM ammonium bicarbonate, 10 µl) for 20 min on ice. After rehydration, the trypsin supernatant was discarded, spots were covered with 50 mM ammonium bicarbonate, and incubated overnight at 37 °C. After digestion, supernatant was collected and acidic peptides were further extracted with trifluoroacetic acid (TFA) 0.1 % and pooled with previous digestion released peptides. Samples were dried out in a RVC2 25 speedvac concentrator (Christ).

2.8 LC-MS/MS analysis

Dried peptides were resuspended in 0.1 % aqueous formic acid (FA), desalted on a Symmetry C18 trapping cartridge (Waters) and further separated on an analytical column (Atlantis C18, 75 µm id x 20 cm, Waters) with an integrated electrospray ionization emitter tip (SilicaTips for Micromass ZSpray NanoFlow, 10 µm diameter, New Objective). Peptides were eluted at a flow rate of 250 nL/min from the analytical column directly to electrospray ionization emitter tip by using a 60 min gradient from 3 to 30 % solvent B (solvent A: 1 % aqueous FA and solvent B: 100 % ACN, 1 % FA).

Data were acquired in a QToF Premier mass spectrometer (Waters, Manchester, UK) in data dependent acquisition mode (DDA). A full scan mass spectrum (m/z: 300-1600) was followed by MS/MS (m/z: 50-1995)

in the 5 most abundant multi-charged ions (+2 and +3) every 4 s. Argon was used as the collision gas. Collision energies varied as a function of the m/z and charge state of each peptide. Dynamic exclusion was incorporated for 30 s. A scan of the reference compound (Glufibrinopeptide B) was acquired every ten scans of the analyte through the whole run.

Raw data was processed using ProteinLynx Global Server v2.2.5 (Waters). The resulting pkl file was searched against SwissProt sequence database (v 57.15) with Human (20294 sequences) selected taxonomy using Mascot as search engine. Carbamidomethyl cysteine was chosen as fixed modification. Oxidation of methionine was chosen as variable modifications. A peptide mass tolerance of 10 ppm and 0.1 Da of fragment mass tolerance and three miss cleavages were allowed. A stringent false discovery rate (FDR) of 1 % was chosen as filter for performing the analysis.

2.9 MALDI-TOF/TOF analysis

Dried peptides were resuspended in 0.1 % TFA, desalted using in-house Poros R2+R3 micro-columns, eluted in α-Cyano-4-hydroxycinnamic acid (HCCA) (prepared in 10:30 acetonitrile:TFA 0.1 %), and spotted on a MALDI Ground Steel 384 plate (Bruker Daltonics). The spots were allowed to dry out prior to the analysis. MS and MS/MS analysis were performed on an Autoflex III Smartbeam TOF/TOF (Bruker), equiped with a LIFT and a reflectron. Peptide ionization was carried out using 200 Hz pulses of a 360 nm solid-state laser. Mass resolution was kept above 7500 for the entire mass window. 1400 scans were carried out for the PMF analysis, and the parental ions were selected manually. MS/MS analysis was performed using 400 scans for the parental ions and 1600 scans for the fragments.

Raw data was processed using FlexAnalysis v3.0 (Bruker Daltonics). The resulting mass lists were generated by BioTools v2.1 (Bruker Daltonics) and the database searches were performed by Mascot v 2.1 (Matrix Science) search engine against SwissProt sequence database (v 57.15). Carbamidomethyl was chosen as fixed modification. No variable modifications were allowed. Peptide mass tolerance of 30 ppm and 0.7 Da of fragment mass tolerance were chosen along with up to one missed cleavages.

2.10 Functional analysis

2.10.1 Gene Ontology study

Gene Ontology (GO) term enrichment was examined to further interpret the identified protein sets. Protein Center software (Proxeon) was used for this purpose. F1 LC-MS/MS, F1 2DE, and F3 LC-MS/MS datasets were characterized and compared among them in order to assess differences in their biological functions. Enrichment of particular GO Terms in our datasets was determined by comparing the frequencies obtained against a selected background (IPI Human database, http://www.ebi.ac.uk/IPI/IPIhuman.html). Significantly overrepresented GO terms (Benjamini-corrected FDR p value<0.05) were only considered for discussion.

2.10.2 Network and Pathway mapping

Ingenuity Pathways Analysis (IPA, Ingenuity® Systems, www.ingenuity.com) was used in order to examine the characteristic molecular interactions and pathways associated to the proteins identified. IPA Network Analysis uses computational algorithms to identify local networks that are particularly enriched in data sets. Such local networks are algorithmically generated based on their connectivity

and ordered by score. This score reflects their relevance based on a p-value, which determines the likelihood that a molecule is assigned to a network by random chance alone. These networks contain the most highly connected focus proteins which, in turn, have specific interactions with other proteins in the network. For this analysis, Ingenuity Knowledge Base (genes only) was used and only experimentally observed descriptions and direct relations were considered.

Canonical Pathways Analysis was used to identify the pathways from the IPA library that were most significantly correlated to our data set. The significance and interest of the association between the data set and a canonical pathway was determined by means of a Fisher's exact test, which defines the probability of observing a correlation by chance alone, and by the ratio between the number of proteins present in our dataset that map to a certain pathway and the total number of proteins of that pathway. Only pathways that met the $p<0.05$ cut-off were considered for further analysis.

3 Results

3.1 Microscopic and haematological characterization of PRGF-Endoret

The haematological results were consistent with the standardized protocol for obtaining PRGF-Endoret, considering the biological variability of the three samples (Table 3). All samples were free of leukocytes ($\leq 0.2 \times 10^3/\mu L$) and erythrocytes ($\leq 0.05 \times 10^6/\mu L$). The content of platelets, concentrations above baseline peripheral blood, was between 1.0 and 1.3 fold (for F1) and between 2.3 and 3.7 fold increase for the most enriched fraction, F3. Also, the typical platelet size gradient between the different plasma fractions was obtained. Thus, platelets

with larger average size (MPV) were located in the closer fraction to the buffy coat (F3).

Typical SEM images of the PRGF-Endoret scaffold were obtained prior and after protein extraction with acetonitrile. In all cases prior to protein extraction, there was a consistent network of fibrin and platelet aggregates scattered throughout the network of fibrin (Figure 7 B). In TEM, it has been observed intact fibrin strands (Figure 7 C). However, after treatment with acetonitrile and protein extraction, integrity of the scaffold was altered. Due to dehydration caused by the acetonitrile treatment, the volume of fibrin scaffold was reduced.

Donor	Sample	Leukocytes ($x10^3/\mu L$)	Erythrocytes ($x10^6/\mu L$)	Platelets ($x10^3/\mu L$)	MPV (μm^3)	Platelet Enrichment
1	Blood	5.3	5.1	227	7.9	1.0
	F1	0.1	0.01	231	7.0	1.0
	F2	0.1	0.01	385	7.5	1.7
	F3	0.2	0.05	769	8.4	3.4
2	Blood	9.3	5.0	276	7.7	1.0
	F1	0.1	0.02	359	7.2	1.3
	F2	0.1	0.02	471	7.4	1.7
	F3	0.1	0.02	633	7.6	2.3
3	Blood	7.0	4.8	297	7.4	1.0
	F1	0.1	0.02	380	6.6	1.3
	F2	0.1	0.02	690	7.0	2.3
	F3	0.2	0.03	1088	7.5	3.7

Table 3. Hematologic features of the three donors. Enrichment of platelets (fold increase) compared to its baseline peripheral blood is indicated for each fraction. (MPV, Mean platelet volume).

This shrinking caused alterations in the microscopic structure of the clot, and fibrin fibers showed abnormalities in both its external (Figure 7 D) and internal (Figure 7 E) structures. The combination of organic solvent together with heating during incubation allowed the extraction of many proteins present in the fibrin scaffold and released into the supernatant.

3.2 Proteomic Characterization of PRGF-Endoret

3.2.1 1D SDS-PAGE followed by LC-MS/MS analysis (1D LC-MS/MS)

The six samples were processed independently, resulting in 6 lanes in the SDS-PAGE gel (Figure 8 A). Protein content comparison was performed by lanes. After tryptic digestion of the bands and further LC-MS/MS analysis, 122, 189 and 170 proteins were identified in F1 extracts, and 168, 184 and 146 proteins in F3 extracts from each individual (Figure 8 B and C). Overall, 254 different proteins were found in fraction F1 and 271 in fraction F3. Only proteins identified in 2 out of 3 donors were considered for further analysis. As a result, 136 proteins were considered for fraction F1, and 142 for fraction F3 (supplementary table S1). As shown in Figure 8 D, both fractions shared 111 proteins (supplementary table S1), reflecting a high similarity in their protein content.

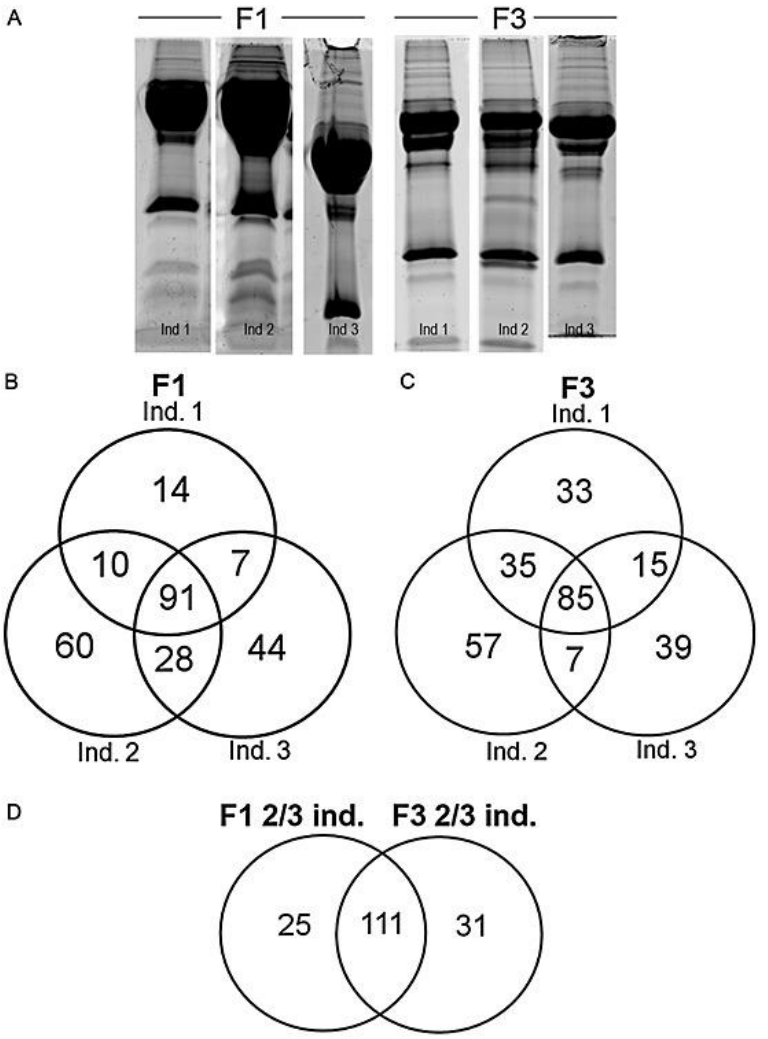


Figure 8. F1 and F3 fractions analysis using SDS-PAGE LC-MS/MS technology. (A) Protein extracts from F1 and F3 fractions resolved by SDS-PAGE. Protein coming from the three different individuals (Ind. 1-3) was independently run and processed. Venn diagrams depicting the proteins identified by LC-MS/MS in F1 (B) and F3 (C) extracts. A decoy search-based 1% FDR cutoff was set for peptide identification. Proteins identified in at least 2 out of 3 individuals were only considered for further analysis. (D) Overlap between these proteins identified in at least 2 out of 3 F1 and F3 extracts. Supplementary data section provides the full list of identified proteins.

3.2.2 2-DE MALDI TOF/TOF analysis

F1 fraction extracted from three donors was pooled and resolved in a 2-DE gel. Detected spots were picked and further analysed by MALDI TOF/TOF (Figure 9 A). 362 spots were detected; of these 136 were identified (Supplementary Figure S2). A total of 49 different proteins were identified in the successfully processed 136 spots (Supplementary table S1).

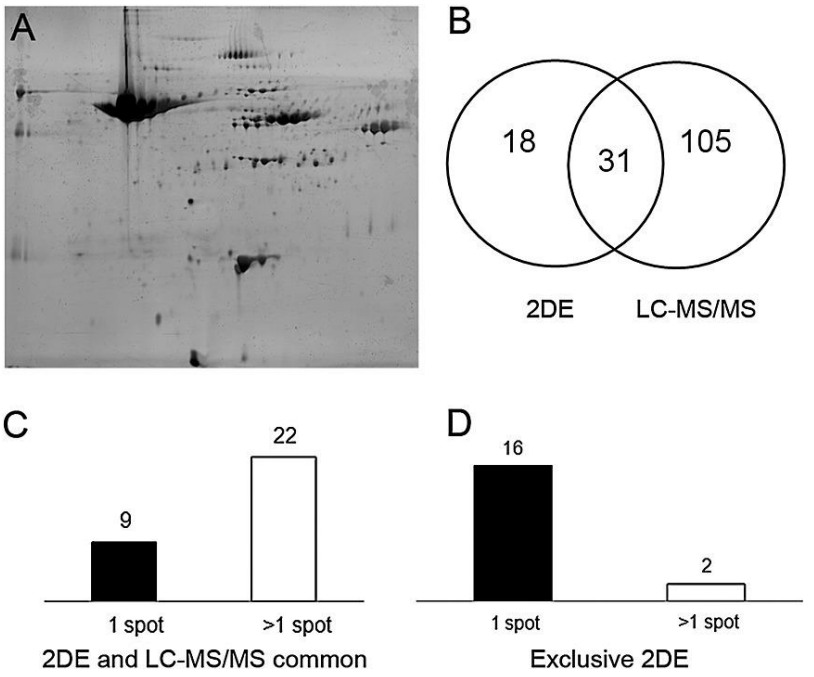


Figure 9. F1 fraction analysis by means of 2D SDS PAGE MALDI TOF/TOF and comparison with the LC-MS/MS study. (A) 2-DE gel of a representative F1 protein extract. Protein from 3 individuals was pooled and analyzed by 2-DE as described in the materials and methods section. (B) Venn diagram showing overlap between proteins identified by 2-DE and LC-MS/MS in F1 extract. Spot distribution of the F1 proteins identified in both 2-DE and LC-MS/MS approaches (C) and 2-DE-exclusive proteins (D). Supplementary data section provides the full catalogue of identified proteins.

As expected, an LC-MS/MS-based approach was able to identify more F1 PRGF-Endoret derived fibrin clot-interacting proteins than the 2D-based approach. Sixty-nine percent of the identified proteins were exclusive of the LC-MS/MS approach, whereas 2-DE-MALDI TOF/TOF reported 11 % exclusive identifications (Figure 9 B and Supplementary table S1).

Twenty-two out of the thirty-one (71 %) overlapped proteins between both approaches appeared in more than one spot in the 2-DE experiment (Figure 9 C and Supplementary figure S3), suggesting some kind of modification. Post-translational modifications, such as phosphorylation, enzymatic cleavage or alternative splicing may help understand this complex expression pattern. In this regard, it has been found proteins like alpha-1 antitrypsin (4 spots), alpha-1 antichymotrypsin (4 spots), kininogen-1 (8 spots), ceruloplasmin (9 spots), among others (see supplementary figure S3) that show the typical isoelectric point shift which is usually related to a post-translational modification (PTM) conferring a net charge variation of the protein. Instead, transthyretrin was identified in three spots with different molecular weight: spot numbers 16, 7 and 239 (see supplementary figure S2). On the other hand, only 2 out of the 18 proteins exclusively identified in 2-DE (11.1 %) were identified in more than one spot in the 2-DE gel (Figure 9 D and Supplementary figure S3). Supplementary table S1 shows an overview of all identified proteins by the dual proteomic strategy providing the details of proteomic analysis.

3.3 Functional analysis: Gene Ontology and pathways analysis.

3.3.1 Gene Ontology study

Clustering of the proteins identified following 1D LC-MS/MS and 2-DE-MALDI-TOF/TOF strategies was performed according to GO hierarchy

[147]. When compared, no significant difference in the GO terms comprised by F1 and F3 was noted (data not shown). A GO term-enrichment analysis of the proteins identified in F1 fraction following both approaches was performed. For this purpose, frequency of the GO terms comprised in F1 fraction was compared against a background (IPI database), and significantly enriched terms (FDR corrected p values <0.05) were considered (Figure 10). Interesting processes such as blood coagulation (8.5 times), immune response (5.1 times), platelet activation (12.5 times), lipoprotein metabolic process (13.6 times), wound healing (7.7 times), cell migration (3.2 times), cell adhesion (2.2 times), and regulation of angiogenesis (8.9 times) were detected among the significantly enriched GO terms (Supplementary Table S2).

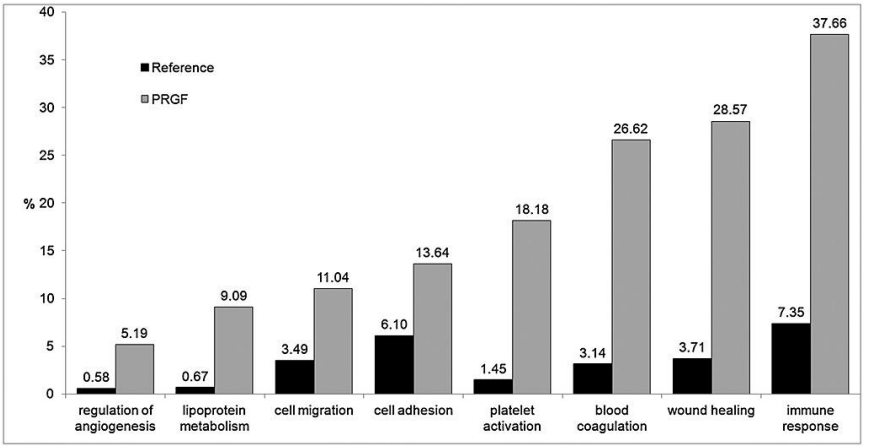


Figure 10. Gene ontology analysis of proteins identified in F1 extract. GO term distribution was compared against a background database (IPI Human) in order to determine which biological processes were significantly enriched in our dataset. This approach offers a classification of fibrin close contact proteins according to their biological function.

3.3.2 Ingenuity Pathways Analysis

The analysis of protein networks clustered F1 proteins in three major networks: amino acid metabolism, molecular transport and small molecule biochemistry (score 21, Figure 11 - upper network), lipid metabolism, molecular transport and small molecule biochemistry (score 44, Figure 11 - middle network), and cell signaling, inflammatory response and lipid metabolism (score 26, Figure 11 - bottom network).

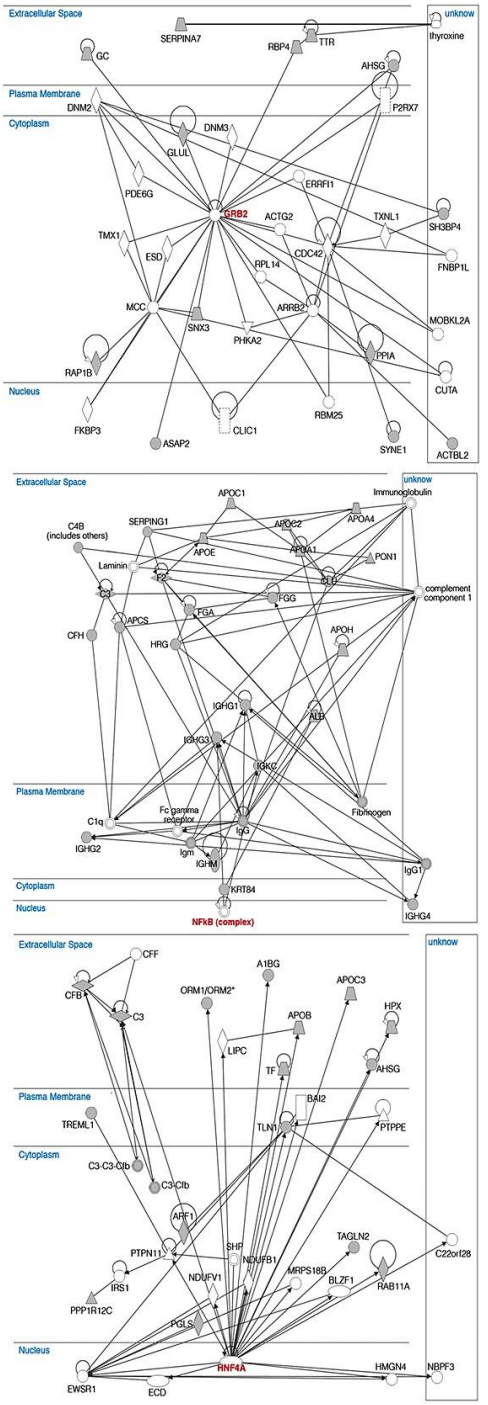


Figure 11. Network analysis results of all proteins identified in F1 fraction. The top networks that were found were amino acid metabolism, molecular transport and small molecule biochemistry (upper network); lipid metabolism, molecular transport and small molecule biochemistry (middle network); and cell signalling, inflammatory response and lipid metabolism (bottom network). The identified proteins in our dataset are indicated in dark gray colour.

Each network converged to a main protein. Growth factor receptor-bound protein (GRB2), nuclear factor κB (NF-κB) and hepatocyte nuclear factor 4 alpha (HNF4A) were the main nodes for upper, middle and bottom network, respectively.

A canonical pathway analysis of F1 was also performed, and 30 pathways were significantly represented in our dataset ($p<0.05$) (Supplementary Figure S4). The ten most representative of these pathways were selected, based on the number of matched proteins from our dataset. These representative pathways were found to be mainly related to the body's response to aggression, and the hematological and immune system, including acute phase response signaling, coagulation system, primary immunodeficiency signaling, intrinsic and extrinsic prothrombin activation pathways, regulation of actin-based motility by rho, complement system, Liver and retinoid X receptors (LXR/RXR) activation, farnesoid and retinoid X receptors (FXR/RXR) activation and systemic lupus erythematosus signaling (Figure 12). Among these, acute phase response signalling (Supplementary Figure S5) was the most relevant and interesting canonical pathway, with a log (p-value) of 30.9 and a ratio of 0.17 (30 out of 178 proteins). Regarding the information provided by IPA, 13 of these mapped proteins have been shown to be increased, 10 decreased, and 5 remained unaffected in plasma during acute phase response.

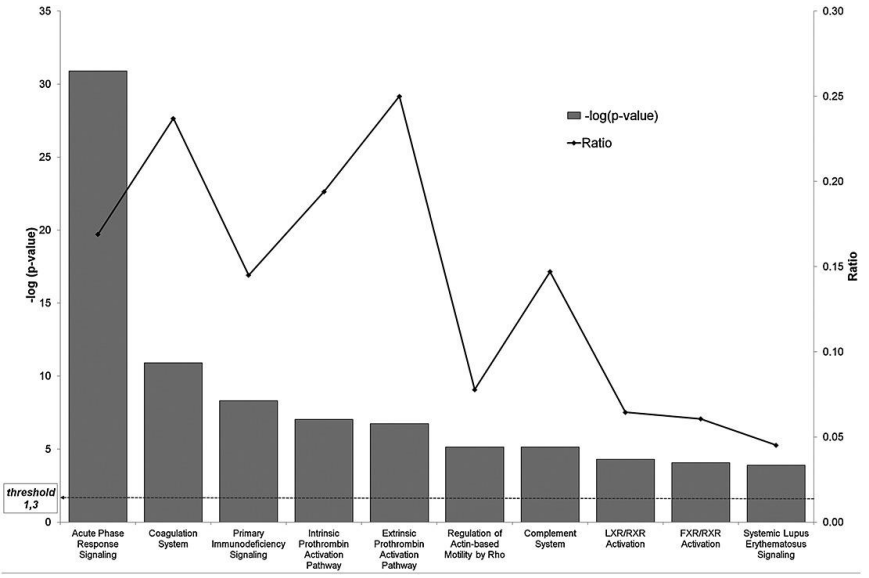


Figure 12. Canonical pathways analysis results of all proteins identified in F1 fraction. The most important ten canonical pathways are shown. The chart displays the significance threshold (>1.3 = p <0.05) from which our dataset and the pathways are not associated randomly (Fisher's exact test). It also shows the ratio of proteins present in PRGF-Endoret that are included in a particular pathway (identified proteins / proteins in the pathway).

4 Discussion

The present study has characterized the proteins most closely linked to the scaffold of the fibrin network formed through the PRGF-Endoret technology. The enrichment of these proteins was performed by treatment of the scaffold with the polar solvent acetonitrile, which helped protein extraction, followed by a dual high-throughput proteomic approach.

The first step of this paper has been to conduct a characterization of PRGF-Endoret, based both in basic hematological parameters and electron microscopy. The enrichment in platelet proteins, the presence of all plasma proteins and the absence of both leukocytes and

erythrocytes are hallmarks of this autologous formulation [131, 138], features that are confirmed in our study. The main difference between F1 and F3 fractions in terms of composition is the concentration of platelet proteins, such as PDGF, VEGF, EGF among others, which is higher in the F3 [141]. However, the three-dimensional structure of the fibrin scaffold is conditioned by the presence and concentration of platelets [96, 148], since high levels of platelet yield more compact three-dimensional matrix, caused by the self-contraction of platelets. Thus, in this research it has also been explored whether the proteins that bind closely to fibrin scaffold change depending on the PRGF-Endoret clinically used fractions (F1 vs. F3).

As expected, the LC-MS/MS-based platform was shown to be more powerful for the characterization of these protein extracts. 136 and 142 different proteins were identified in F1 and F3 extracts of at least 2 out of 3 individuals analyzed, respectively. On the other hand, 136 spots belonging to 49 different proteins were successfully identified in the 2-DE-based F1 analysis. Of the proteins identified in the F1 extract analysis, 31 proteins overlapped between both approaches, 105 were LC-MS/MS exclusive, and 18 were 2-DE exclusive.

Protein modifications, such as PTMs (phosphorylation, methylation, acetylation, glycosylation, sulfonation etc.), alternative splicing, or protein cleavage, may give rise to the generation of different forms of a certain protein that differ in their Mw and/or pI. As a result, these modified versions of a protein may resolve in different parts of a 2-DE gel, giving rise to a population of spots that reflects this heterogeneity. Therefore, the fact that 49 proteins were identified in 136 spots reveals that the proteome of the PRGF-Endoret might be highly modified. Whereas 71 % of the overlapped proteins between 2-DE and LC-MS based approaches were identified in more than one spot, only 11% of the 2-DE-exclusive proteins showed such expression pattern. This result

may reflect differences in protein abundance rather than modification, due to the fact that abundant proteins (and their modified forms) might be easier detected and identified by both approaches than scarce ones. Taken together, these results underscore the ability of 2-DE for the detection of different protein forms and, hence, for increasing the information that can be obtained about the scaffold under study. Anyway, the thorough characterization of these modifications may need a bigger effort and the use of specific proteomic approaches, although such analysis is beyond the scope of this study.

In spite of their differential clot-forming and healing capabilities, the high overlap between the proteins identified in F1 and F3, and the lack of differences in the GO Terms comprised by them suggests that, at this detection level, both F1 and F3 extracts have essentially the same molecular properties. Enrichment and analysis of scarce proteins may help unravel the differences in the protein composition of these two extracts. For this purpose, more starting material and further proteomic fractionation methods, such as affinity-based specific depletion of the most abundant proteins, would be required.

Several of the identified proteins have been shown to be involved in different mechanisms underlying tissue regeneration. Among these, alpha-1-antitrypsin and alpha-1-antichymotrypsin, and more broadly the eight serpins identified in this study, all have a clear role in protecting against proteolytic enzymes [149]. Thus, alpha-1-antitrypsin is the main contributor to protection against enzymes of polymorphonuclear neutrophils [150], and alpha-1-antichymotrypsin has an important role in skin regeneration and chronic wounds [151-153]. Another important protein that has been identified is thrombospondin-1 (TSP-1). This multifunctional glycoprotein, which forms a noncovalent complex with fibrin [154], has antiangiogenic effects [155], playing an important role in the modulation of neovascularization of damaged tissues. In addition,

TSP-1 is involved in the delay of fibrinolysis [156], helping to increase the half-life of fibrin and so extending the controlled release of contained bioactive molecules. Furthermore, a recent study by Gelse *et al* [157] concludes that TSP-1 is involved in cartilage repair, protecting it from excessive ossification. Another protein that is also associated with fibrin is vitronectin [158, 159], which like TSP-1 also participates in fibrinolysis delay [160]. Fibronectin is another relevant protein present in intimate contact with fibrin. This multifaceted molecule [161] is involved in both the migration of cells to the provisional fibrin matrix [162] and in their survival [163]. Moreover, this protein is capable of binding to several growth factors [164], increasing over time the bioavailability of these key proteins.

Not only a catalogue of proteins has been created, but a functional classification analysis has also been made, grouping proteins into families and biological networks. Gene ontology analysis of all the proteins identified in these experiments showed a significant enrichment of certain GO terms associated with tissue repair and regeneration, such as immune response, wound healing, angiogenesis, migration, adhesion, and lipid metabolism among others. A deeper characterization of the proteins involved in these processes may shed light into the mechanisms by which PRGF-Endoret helps the healing process.

The most significantly represented canonical pathway was acute phase response signalling pathway. Acute phase proteins [165] are a group of proteins synthesized in the liver as part of an early defence system of the organism, the innate immune system. These proteins vary their concentration in response to tissue damage, whether there is inflammation, trauma or burns. At last, this response seeks to restore homeostasis, that is, tissue integrity and its function [166]. PRGF-

Endoret is highly enriched in these proteins that cooperate in the early stages of tissue repair.

Strikingly, a high enrichment of a group of proteins related to lipid metabolism was described (more than thirteen-fold over the reference). In addition, two out of three top generated networks are associated with lipid metabolism, especially with the HNF4A. Moreover, two of the ten most important canonical pathways are related to lipids, such as LXR / RXR and FXR / RXR activation pathways. The potential role of some lipids in tissue repair and wound healing had previously been reported [167, 168] and even its involvement in osteogenesis has also been suggested [169]. Moreover, in vitro studies have also shown positive effects of lipoproteins in the migration and proliferation of several cell types [170-172]. Another possible positive effect of the presence of lipoproteins in close contact with the fibrin matrix would be a delayed fibrinolysis, which will cause the release of all molecules and growth factors to be more sustained, thus being carried out for a longer time [173, 174]. The findings are in clear agreement with the recent proteomic study carried out by von Zychlinski and collaborators [175], which correlates lipoproteins and wound healing networks.

Several studies have shown the effect of PRGF-Endoret in pain relief and inflammation reduction [176, 177]. It has recently been shown that platelet-rich plasma induces this effect by an HGF-mediated inhibition of NF-kB pathway [94, 178]. In this study, a strong network of interconnected proteins, linked to NF-kB pathway, was established. Future studies should elucidate whether these proteins are involved in reducing inflammation.

Our results suggest that many of the low and very low abundance fibrin clot interacting proteins may not have been detected in the present study. Nevertheless, by using the presented protein extraction method, gel images allow us to estimate that the detection of very abundant

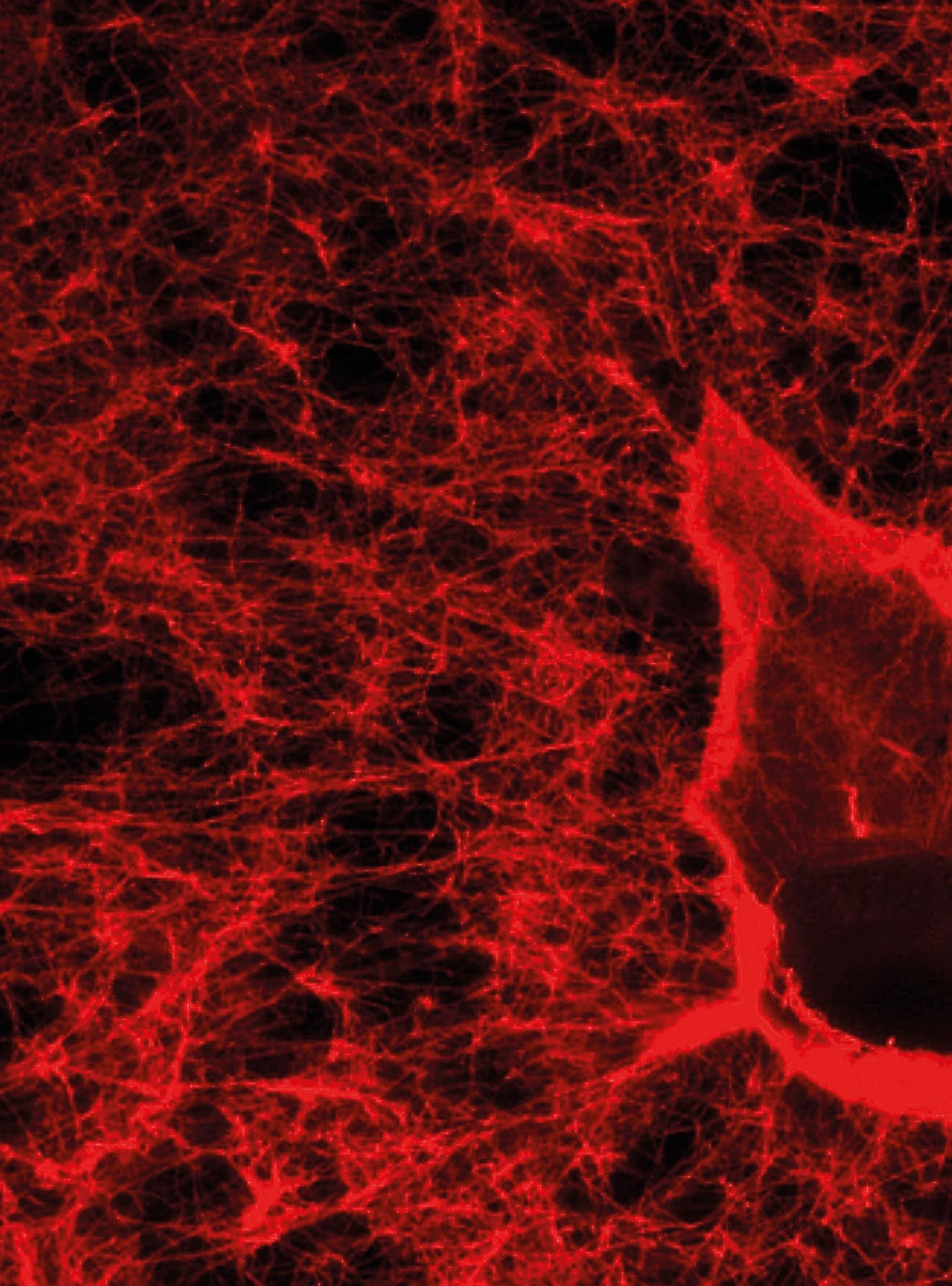
proteins from plasma (e.g. albumin, immunoglobulins, etc.) is relatively low, allowing to detect medium and low abundant proteins in this study. As mentioned above, future proteomics assays will be needed to deepen the complete catalogue of all enriched proteins present in PRGF-Endoret, not only those that are in close contact with fibrin. Although this preclinical *in vitro* study has no direct clinical applications, it does aim to shed light on the molecular mechanisms underlying the clinical benefits provided by the PRGF-Endoret technology.

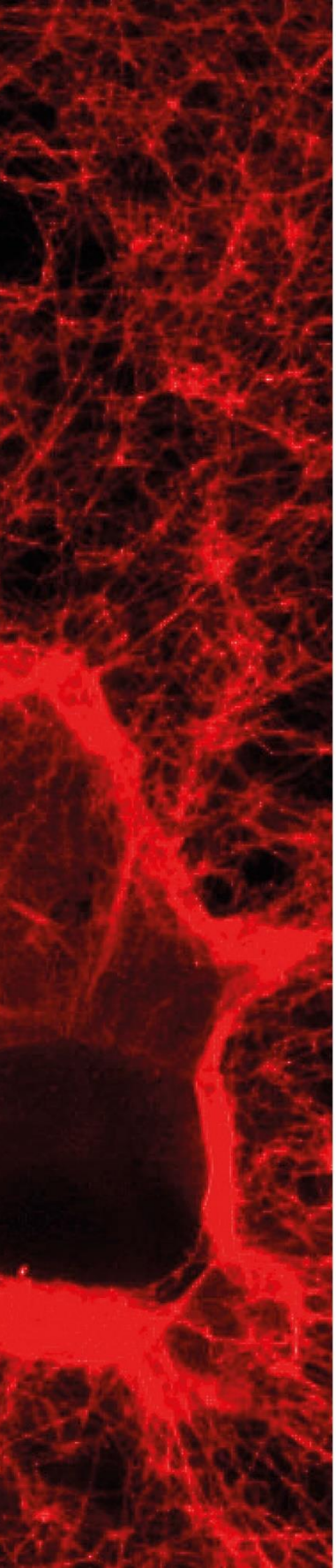
5 Conclusions

PRGF-Endoret technology offers an autologous three-dimensional matrix of fibrin intended for tissue engineering and cell transplantation. This scaffold is known to be enriched in a certain type of proteins, such as growth factors. Nevertheless, it also comprises a rich and diverse cocktail of proteins, specifically enriched to foster an optimal environment of tissue repair.

In the present work, a high-throughput characterization of the proteins more closely linked to the scaffold has been performed. Both proteomic approaches showed complementary results. Resolving the extracted protein in SDS-PAGE followed by LC MS/MS analysis allowed us to identify a high number of scaffold-interacting proteins. On the other hand, the 2-DE approach followed by MALDI TOF/TOF analysis rendered information about the heterogeneity of the identified proteins. Moreover, the integration of the obtained data by gene ontology and pathways analyses provides a deeper understanding of the different proteins involved in tissue regeneration.

Overall, this study provides new data on the close fibrin PRGF-Endoret interactome. The presented information will help for further studies on the way to unravel the subjacent molecular mechanisms of this autologous scaffold in tissue regeneration.





Chapter 4

Morphogen and proinflammatory cytokine release kinetics from PRGF-Endoret fibrin scaffolds: evaluation of the effect of leukocyte inclusion

Experimental article published In

Journal of Biomedical Materials Research Part A [3]

Abstract

The potential influence of leukocyte incorporation in the kinetic release of growth factors from platelet-rich plasma (PRP) may explain the conflicting efficiency of leukocyte platelet-rich plasma (L-PRP) scaffolds in tissue regeneration. To assess this hypothesis, leukocyte-free (PRGF-Endoret) and L-PRP fibrin scaffolds were prepared, and both morphogen and pro-inflammatory cytokine release kinetics were analyzed. Clots were incubated with culture medium to monitor protein release over 8 days. Furthermore, the different fibrin scaffolds were morphologically characterized. Results show that leukocyte-free fibrin matrices were homogenous while leukocyte-containing ones were heterogeneous, loose and cellular. Leukocyte incorporation produced a significant increase in the contents of pro-inflammatory cytokines IL-1β and IL-16 but not in the platelet-derived growth factors release (< 1.5 fold). Surprisingly, the availability of VEGF suffered an important decrease after 3 days of incubation in the case of L-PRP matrices. While the release of pro-inflammatory cytokines was almost absent or very low from PRGF-Endoret, the inclusion of leukocytes induced a major increase in these cytokines, which was characterized by the presence of a latent period. The PRGF-Endoret matrices were stable during the eight days of incubation. The inclusion of leukocytes alters the growth factors release profile and also increased the dose of proinflammatory cytokines.

1 Introduction

Different strategies have been developed for the stimulation of tissue regeneration and function recovery of injured tissue. These strategies vary from the use of biomaterials to the application of tissue engineering technology. Recently, a biological approach to provide pleiotropic morphogens and a biodegradable three-dimensional (3D) fibrin scaffold to the injured tissue has been proposed [2, 179]. This approach consists of the use of blood-derived biomaterials to create different therapeutic formulations that adapt to the needs of various biomedical fields.

At present, many types of musculoskeletal injuries are treated with this autologous technique, such as tendinopathies [180, 181], ligament and tendon ruptures [182, 183], joint diseases [112, 177, 184] and muscle injuries [185, 186], among others. A recent review has also highlighted the therapeutic applications of plasma rich in growth factors (PRGF-Endoret), an autologous and specific pure-PRP (P-PRP), in the regeneration of hard and soft tissues in oral and maxillofacial surgery, the treatment of chronic ulcers, and in the development of tissue-engineered approaches [141, 187].

PRGF-Endoret technology is characterized by a moderate platelet concentration, absence of leukocytes (P-PRP), and the use of calcium chloride for platelet activation [37, 179]. The absence of leukocytes in this formulation is supported by the fact that they synthesize matrix metalloproteinases (MMP), oxygen and nitrogen reactive species (free radicals), and catabolic cytokines, which may not be an optimal milieu for the regeneration of damaged tissue [26, 103, 188, 189]. Additionally, in several clinical studies leukocyte-enriched PRPs (L-PRPs) have not

improved the clinical outcomes in comparison to placebo or no treatment [190-193].

Interestingly, plasma rich in growth factors once activated can be viewed as a local protein and morphogens delivery system as it creates a fibrin scaffold enriched in biologically active molecules [2] that are then progressively released. However, the kinetic release process of protein release from this fibrin scaffold is fairly unknown as it is the potential impact of having leukocytes and pro-inflammatory cytokines in this fibrin matrix.

To address this, the release of platelet derived growth factors (PDGF-AB, TGF-β1, VEGF, EGF), plasma growth factors (HGF, IGF-I) and pro-inflammatory cytokines (IL-1β, IL-16) over 8 days has been studied. Furthermore, structural analysis of the fibrin hydrogel has been performed to highlight differences in the architecture of leukocyte-free and leukocyte-enriched platelet hydrogels.

2 Material and methods

2.1 Preparation of platelet-rich plasma formulations

Human venous whole blood from three healthy volunteers was withdrawn into 22 vacuum tubes of 9 mL containing 3.8 % (w/v) sodium citrate as anticoagulant. None of the donors had taken medication in the last week that could alter the haematological parameters. This study was conducted following the ethical principles for medical research contained in the Declaration of Helsinki amended in 2008.
All samples were processed immediately after collection. For each donor, half of the tubes were processed according to the PRGF-Endoret protocol and the other half were used to prepare L-PRP. In brief,

according to the PRGF-Endoret protocol [37], 11 blood tubes from each donor were centrifuged for 8 min at 580 g (centrifuge PRGF-Endoret system IV, BTI Biotechnology Institute, Vitoria, Spain). PRGF-Endoret was composed of two fractions: fraction 1 (F1) had a similar platelet concentration to the peripheral blood while fraction 2 (F2) had 2-3 fold higher platelet concentration than the peripheral blood. For this study, (Figure 13) the 2 mL of plasma just above the buffy coat (F2) was collected to prepare the leukocyte-free platelet-rich plasma (PRGF-Endoret hydrogel).

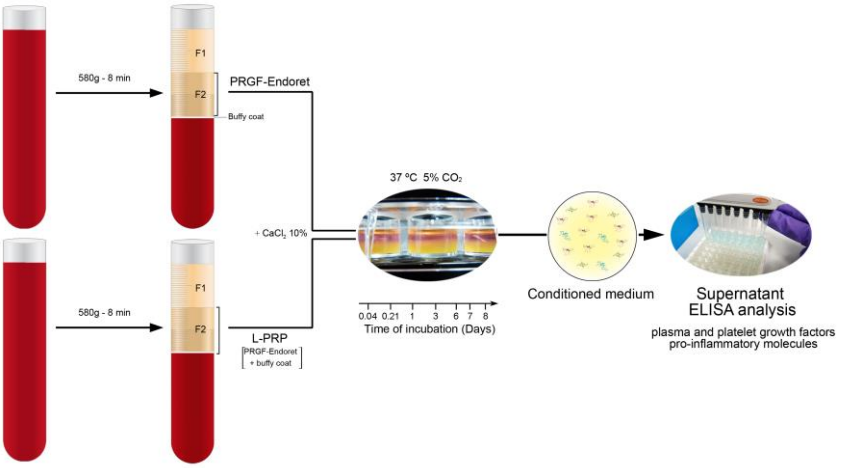


Figure 13. Graphical representation of sample preparation process. After blood centrifugation F1 and F2 PRGF-Endoret can be obtained over the buffy coat. To prepare L-PRP white cells must be collected with F2 fraction. Contrarily, the buffy coat is avoided in the obtaining of F2 PRGF-Endoret. The experimental design is also represented.

For obtaining L-PRP, the same protocol was followed with the other half of tubes, but in addition to the 2 mL of plasma, the buffy coat was also collected.

2.2 Characterization of platelet-rich plasma formulations

The number of platelets and leukocytes was determined in peripheral blood, PRGF-Endoret and L-PRP, using a standard haematological analyzer (ABX MICROS 60, Horiba Medical, Montpelier, France).
In order to characterize the 3D-structure of the gels by conventional microscopy and scanning electron microscopy (SEM) techniques, one aliquot of each type of platelet-rich plasma was activated with 10% $CaCl_2$ (PRGF-Endoret activator, BTI Biotechnology Institute, Vitoria, Spain).

For optical microscopy analysis samples were fixed in formaldehyde 4% at room temperature, dehydrated in a graded series of alcohols and embedded in paraffin. Next, 5-µm-thick sections were cut, stained with hematoxylin and eosin (H&E), and observed under microscopy (DMLB, Leica Microsystems, Wetzlar, Germany) equipped with a digital camera (DFC300FX, Leica Microsystems, Wetzlar, Germany). For SEM evaluation, samples were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer. Then, samples were post-fixed with osmium tetroxide (1 % OsO_4 in 0.1 M cacodylate) and finally dehydrated through ascending alcohol concentrations. Next, the hydrogels were subjected to critical point drying (Autosamdri 814, Tousimis, Rockville, USA) and sputter-coated with 5 nm of gold (E306A, Edwards, Crawley, UK) before examination in a electron microscope (S-4800, Hitachi, Japan). The 3D-structure of both types of matrix was qualitatively evaluated by two independent observers in terms of the presence of cells and structure of the fibrin network.

2.3 Characterization of the release of morphogens and pro-inflammatory cytokines

The characterization of the bioactive release from hydrogels was performed according to Anitua et al [138]. Briefly, 55 µL of 10 % calcium chloride solution was added to 1.1 mL of liquid PRP into a 12-well culture plates to form the hydrogels. After clot formation, 1.6 mL of osteoblast cell medium without growth supplements (ObM) (ScienCell Research Laboratories, Carlsbad, California, USA) was added. Samples were then maintained in a cell incubator at 37 ºC and 100 % humidity. The incubation medium was collected after 1, 5 and 24 hours and 3, 6, 7 and 8 days of incubation. The experiments were performed in triplicates for each hydrogel and for each time point. After each period of incubation, the incubation medium was centrifuged at 400 g during 10 min at room temperature. The supernatant obtained was distributed in aliquots and stored at -80 ºC until use.

Quantification of PDGF-AB, TGF-β1, VEGF, HGF, IGF-I, EGF, IL-1β and IL-16 was performed using available ELISA kits and according to the manufacturer's protocol (Invitrogen Corporation, Camarillo, California, USA, for IL-1β and R&D Systems Inc, Minneapolis, Minnesota, USA for the rest of molecules).

2.4 Study of remaining adherent cells

For samples incubated during 8 days, the plate surface was observed through phase-contrast microscopy (DM IRB, Leica Microsystems) after removing both the fibrin matrix and supernatant. In case that cells are detected, nuclear staining with Hoechst 33342 (Molecular Probes-Invitrogen, Grand Iland, NY, USA) was performed, and microphotographs taken with a digital camera (DFC300 FX, Leica Microsystems).

2.5 Statistical analysis

Shapiro-wilk test was applied to verify if the data followed a normal distribution. Then paired t student test was selected to analyze the statistical significance of the differences between PRGF-Endoret and L-PRP hydrogels at each time point and for each measured protein. Non parametric Wilcoxon test was selected if the data did not follow a normal distribution. The statistical significance was set at $p < 0.05$. All the statistical analyses were performed using the SPSS v15.0 for Windows statistical software package (SPSS Inc., Chicago, IL, USA).

3 Results

3.1 Characterization of plasma rich in growth factor preparations

The platelet concentration obtained in PRGF-Endoret hydrogel is shown in Table 4. Platelet concentrations were 434 x 10^3 platelets/μL, 562 x 10^3 platelets /μL and 279 x 10^3 platelets /μL for donors 1, 2 and 3; while the enrichment with respect to the peripheral blood was 3.0, 2.6 and 2.0-fold, respectively. Table 4 showed that platelet concentrations in the L-PRP hydrogel for donors 1, 2 and 3 were 449, 650 and 349 x 10^3 platelets/μL, accounting for an enrichment factor of 3.1, 3.0 and 2.5, respectively.

Cell type	Donor 1		Donor 2		Donor 3	
	PRGF	L-PRP	PRGF	L-PRP	PRGF	L-PRP
Platelets (x10^3/μl)	434 (3.0x)	449 (3.1x)	562 (2.6x)	650 (3.0x)	279 (2.0x)	349 (2.5x)
Leukocytes (x10^3/μl)	0.3 (0.05x)	5.7 (0.97x)	0.2 (0.05x)	2.2 (0.52x)	0.1 (0.02x)	1.1 (0.2x)
Lymphocytes (x10^2/μl)	2.90	44.75	1.78	17.36	n.d.	9.75
Monocytes (x10^2/μl)	0.07	5.76	0.21	3.50	n.d.	0.89
Granulocytes (x10^2/μl)	0.03	6.50	0.01	1.14	n.d.	0.36
Erythrocytes (x10^6/μl)	0.04	0.06	0.02	0.02	0.00	0.01

Table 4. Platelet, leukocyte and erythrocyte measurements in PRGF-Endoret and L-PRP from three donors. The change relative to the values of peripheral blood is indicated in brackets. (n.d.: not determined).

In relation to the white blood cells, PRGF-Endoret fibrin hydrogels of the three donors were almost leukocyte free, with a content less than 0.3 x 10^3 leukocytes/μL (Table 4). In contrast, L-PRP hydrogels from donors 1, 2 and 3 had values of 5.7, 2.2 and 1.1 x 10^3 leukocytes/μL, respectively. Thus, the leukocyte-containing preparation (L-PRP) protocol resulted in a leukocyte-enrichment of PRGF-Endoret by a factor of 19, 11, and 11, respectively, when compared with leukocyte-free PRGF-Endoret.

A morphological characterization of the three-dimensional structure of fibrin scaffolds of both PRGF-Endoret and L-PRP was performed. The photonic microscopy revealed the virtual absence of leukocytes and erythrocytes in the fibrin scaffold prepared from PRGF-Endoret (Figure 14 A).

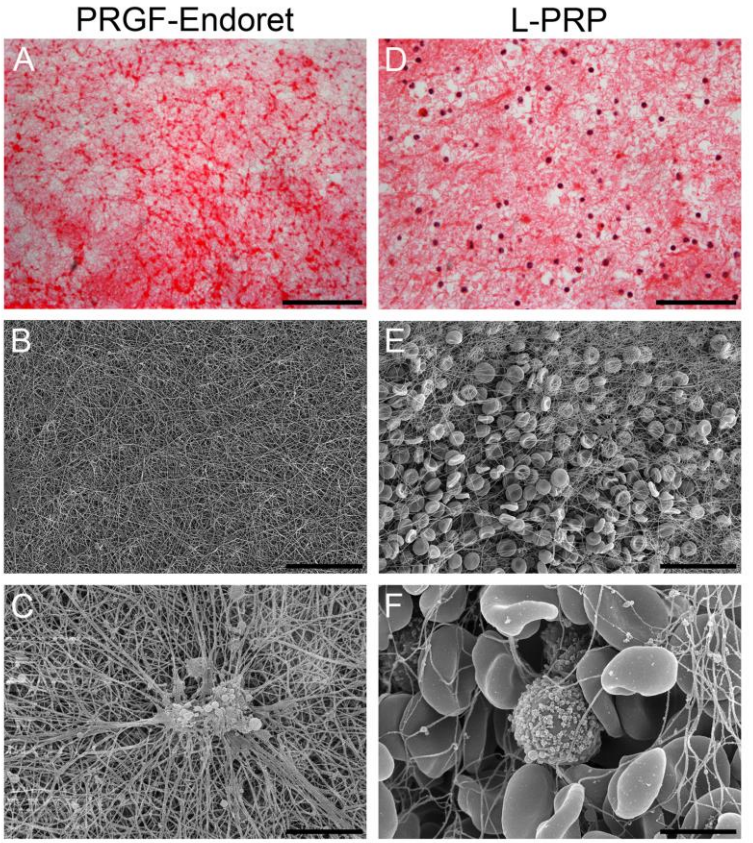


Figure 14. Microscopic characterization of the fibrin scaffold obtained from PRGF-Endoret (A-C) and from L-PRP (D-F) scaffolds. Samples were observed by staining with H & E (A and D) and by SEM at low (B and E) and high (C and F) magnifications. Scale bars: A and D, 50 µm; B and E, 25 µm; C and F, 5 µm.

SEM analysis showed the presence of a consistent and homogeneous network of fibrin (Figure 14 B) with the absence of cellular elements that might disrupt the mesh. At higher magnification, only platelet aggregates were observed scattered throughout the fibrin (Figure 14 C). In contrast, the presence of leukocytes and erythrocytes was evident in the fibrin prepared from L-PRP (Figure 14 D). In the SEM study the

disturbance of the fibrin network was evident. The presence of erythrocytes and scattered leukocytes (Figure 14 E) resulted in an irregular three-dimensional network (Figure 14 F).

3.2 Characterization of the release of morphogens and pro-inflammatory cytokines

Growth factor release from PRGF-Endoret fibrin scaffolds was monitored during an observational period of 8 days.

3.2.1 Release of platelet derived growth factors

The released dose of PDGF-AB from PRGF-Endoret fibrin scaffold varied between donors being the highest for donor 2 followed by donor 1 (Figure 15). PRGF-Endoret fibrin scaffold from donor 1 released about 5201, 5377 and 6948 pg/mL of PDGF-AB after 1, 5 and 24 hours of incubation, respectively. This amount was 5729, 7093 and 8916 pg/mL for donor 2 and 2354, 2818, and 3596 pg/mL for donor 3. A steady state release was observed after 3 days, reaching an average value of 7845 ± 69.58, 9611.5 ± 245.06 and 4146.5 ± 195.64 pg/ml, for donors 1, 2 and 3 (Figure 15).

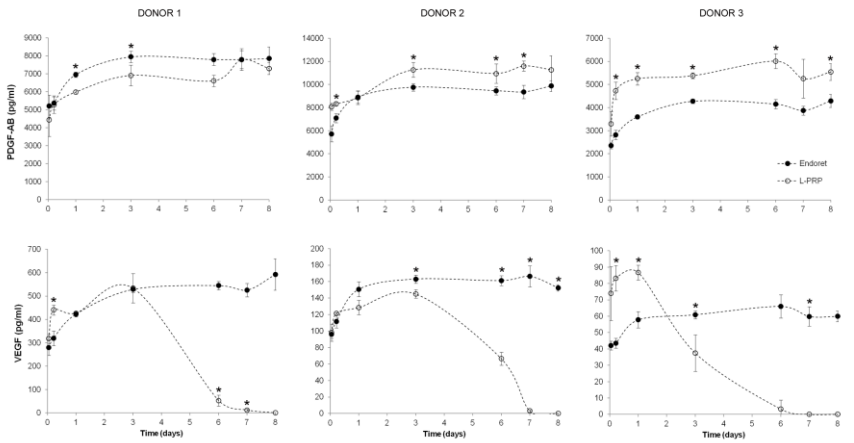


Figure 15. PDGF-AB and VEGF release from fibrin scaffolds obtained from PRGF-Endoret and from L-PRP during an observation period of 8 days. *: $p \leq 0.05$

PDGF-AB release from L-PRP fibrin scaffolds showed a similar pattern that those obtained from PRGF-Endoret (Figure 15). The dose of PDGF-AB released was the highest for donor 2 and the lowest for donor 3. In addition, a steady state release was reached after 3 days of incubation at an average value of 7148 ± 510.2, 11272 ± 266.4 and 5547 ± 332.1 pg/mL was obtained for donors 1, 2 and 3, respectively (Figure 15).

Interestingly, the release of VEGF was markedly different between the two types of fibrin scaffolds (Figure 15). PRGF-Endoret fibrin scaffolds showed a VEGF release profile similar to that of PDGF-AB. It was characterized by a burst release during the first 24 hours and a steady state release after 3 days of incubation (Figure 15). VEGF released from fibrin scaffold in donor 1 was the highest, reaching 279.61, 319.45 and 422.65 pg/mL during the first three time points of the observation period. A plateau region was reached at average values of 547.89 ± 31.04, 160.77 ± 5.92 and 61.70 ± 2.95 pg/mL for donors 1, 2 and 3, respectively.

The incorporation of leukocytes in the scaffold provoked a modification on the VEGF release (Figure 15). Thus, the release of VEGF was increasing during the first 24 hours (for donor 3) and the first 3 days (for donors 1 and 2), after which a progressive decrease in the amount of VEGF in the incubation medium was observed. VEGF was almost absent from the incubation medium after 7 days of incubation (Figure 15).

Figure 16 shows the results of TGF-β1 and EGF release from PRGF-Endoret and L-PRP fibrin scaffolds. PRGF-Endoret fibrin scaffolds released TGF-β1 at an increasing dose during the first 6 days of incubation before reaching a steady state release. After 24 hours, TGF-β1 release reached to 12650, 13267, and 6980 pg/mL of TGF-β1 for donors 1, 2 and 3, respectively. This amount increased up to 19200, 19333 and 9361 pg/mL for donors 1, 2 and 3 respectively after 6 days of incubation.

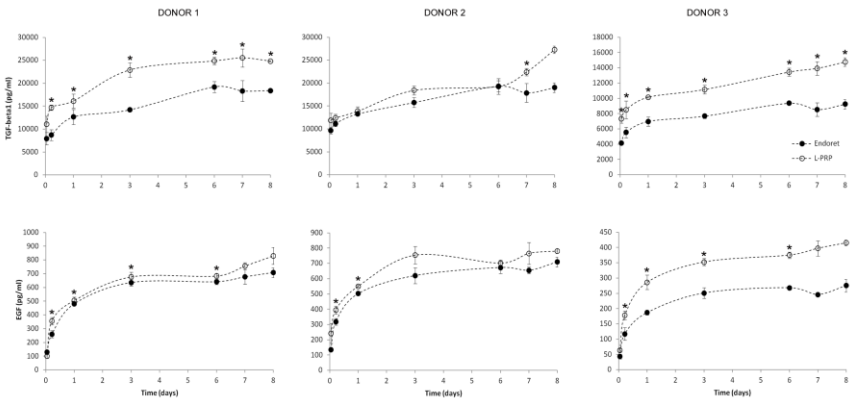


Figure 16. TGF-β1 and EGF release from PRGF-Endoret and L-PRP fibrin scaffolds during an observation period of 8 days. *: $p \leq 0.05$

Leukocyte enrichment of the fibrin scaffold increased the amount of TGF-β1 released to the incubation medium (Figure 16). This increase was continuous during the observation period for donors 2 and 3 while a

steady state release was reached for donor 1. This increase was statistically significant for donor 1 (except at 1 hour) and donor 3, but was not statistically significant for donor 2.

In the case of EGF release, PRGF-Endoret fibrin scaffolds from the three donors showed a similar release profile (Figure 16). Leukocyte inclusion did not significantly alter EGF release from fibrin scaffolds in donors 1 and 2 (Figure 16). The increase in EGF released was statistically significant at 5 hours and days 1, 3 and 6 for donor 1. In the case of donor 2, these differences were only statistically significant at 5 hours and day 1, meanwhile the increase was significant at 5 hours, and days 1, 3, 6 and 8 for donor 3.

3.2.2 Release of plasma derived growth factors

Release of plasmatic growth factors (IGF-I and HGF) from both types of fibrin scaffolds was analyzed. Figure 17 shows that the highest IGF-I released was obtained in PRGF-Endoret fibrin scaffolds of donor 1 while similar amount was delivered in the case of patients 2 and 3. The peak of IGF-I release was observed at day 3, being of 50080, 32990 and 30570 pg/mL for donors 1, 2 and 3, respectively. A steady state release was then obtained with values ranging from 20517 to 44333 pg/mL (Figure 17). Results show that the dose of IGF-I released was significantly higher in PRGF-Endoret fibrin scaffolds at day 1 for donors 1 and 2. Meanwhile, these differences were significantly higher in L-PRP fibrin scaffolds at 1 hour and day 8 for donor 2 and at days 1 and 7 for donor 3. Lastly, inclusion of leukocytes in the fibrin matrix significantly increased the dose of HGF released in all the donors (Figure 17).

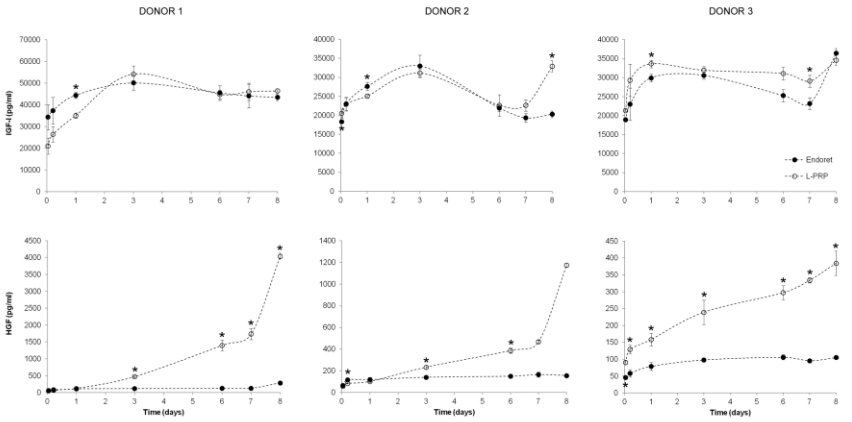


Figure 17. IGF-I and HGF release from PRGF-Endoret and L-PRP fibrin scaffolds during an observation period of 8 days. *: $p \leq 0.05$

3.2.3 Release of pro-inflammatory cytokines

The release of pro-inflammatory cytokines from the fibrin scaffolds was characterized by following the release of IL-1β and IL-16. Figure 18 shows that the IL-1β was absent from the incubation medium of PRGF-Endoret hydrogels throughout the observation period for the three donors. Meanwhile, leukocyte inclusion into the fibrin scaffolds significantly increased the release of IL-1β. Interestingly, the IL-1β profile indicated the presence of latent period. The latter occurred during the first 5 hours of incubation (Figure 18). Then, the released dose of IL-1β was 5.49, 6.55, 7.57, 9.24 and 5.78 pg/mL after 1, 3, 6, 7 and 8 days of incubation for donor 1, respectively. These amounts were about 12.27, 15.19, 23.01, 19.92 and 8.06 pg/mL for donor 2 and 43.08, 55.40, 52.92, 49.37 and 170.94 pg/mL for donor 3.

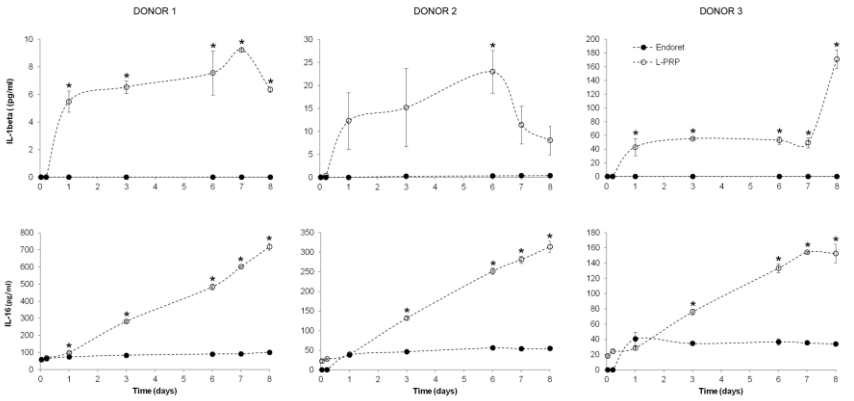


Figure 18. Pro-inflammatory cytokine (IL-1β and IL-16) release from autologous fibrin scaffolds (PRGF-Endoret and L-PRP) during an observation period of 8 days. *: $p \leq 0.05$

Similarly, inclusion of leukocytes significantly enhanced the dose of IL-16. However, the release profile of IL-16 was different among donors of L-PRP. The rate of IL-16 released was about 87, 41 and 21 pg/mL per day for donors 1, 2 and 3, respectively. The released dose after 8 days of incubation was about 719, 314 and 152 pg/mL, respectively.

3.3 Study of remaining adherent cells

None of the PRGF-Endoret wells had adherent cells, however all L-PRP samples contained cells adhered to the cell culture well. These cells had a morphology consistent with macrophage-derived multinucleated giant cells as shown in Hoechst 33342 staining (Figure 19 A) and in more detail (Figure 19 B).

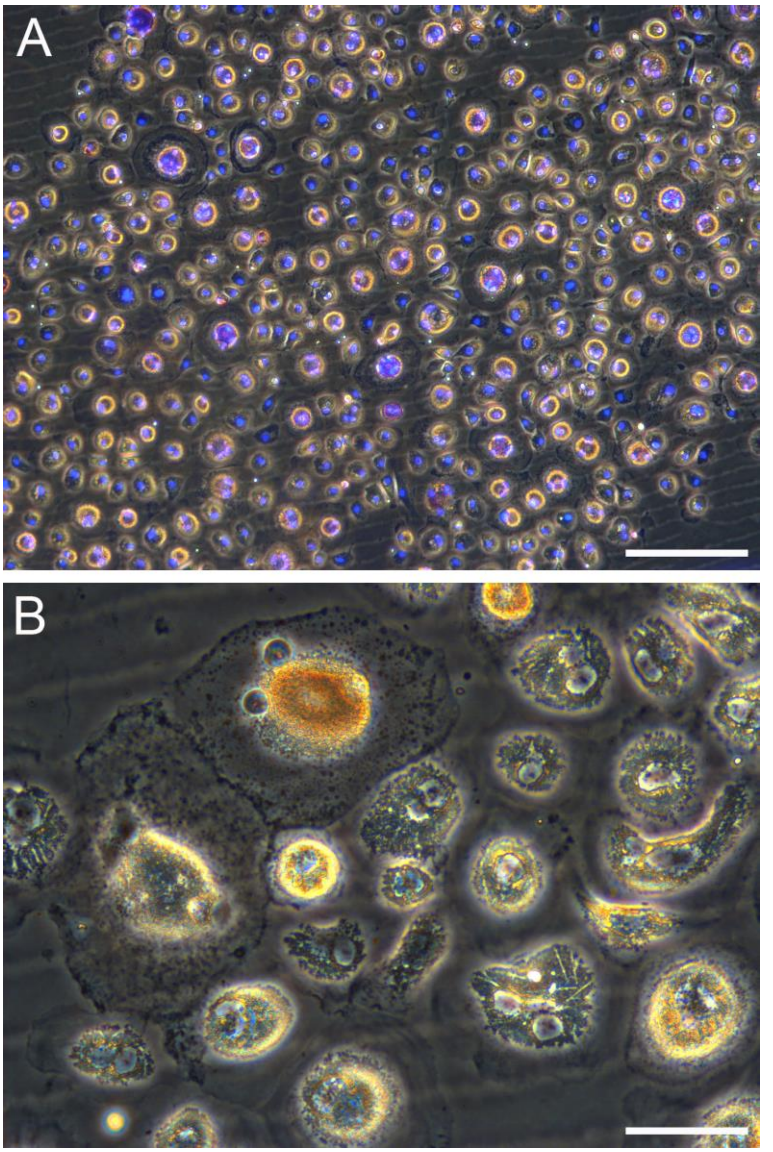


Figure 19. Images of cells adhered to the cell-culture well after removing a fibrin (L-PRP) after 8 days of incubation. (A) Photomicrograph at low magnification (phase contrast combined with Hoechst 33342 nuclear staining). Note the presence of multinucleate cells. (B) High-magnification image showing a detail of the cells found in the culture well. Scale bars: A, 200 µm; B, 50 µm

4 Discussion

The cellular components of PRP products are a differentiating factor that may influence the clinical effectiveness of their administration for the treatment of a disease and/or the regeneration of damaged tissue. Several studies support the use of P-PRP in a large number of pathologies of the musculoskeletal system [188, 194-196]. Other clinical studies have reported the occurrence of adverse effects after the administration L-PRP [195, 197]. These adverse effects are related to the capability of leukocytes to trigger immunological response and increase the concentration of pro-inflammatory cytokines [195, 197]. Besides, this cellular composition can affect the structure of fibrin network produced by the polymerization of fibrinogen into a cross-linked fiber mesh [141].

In this study, the structural characterization of fibrin mesh has clearly shown a more heterogeneous network due to the incorporation of leukocytes and particularly erythrocytes into the mesh, and it has also shown lesser fiber density throughout the network. These morphological modifications may reduce the clot strength and affect the viscoelastic properties of fibrin scaffold [198, 199]. Gersh *et al* have shown that erythrocytes incorporation into fibrin clot at 5-10 % (vol/vol) caused heterogeneity in the fiber network while higher concentration of erythrocytes resulted in loose arrangement of fibers around the cells [199]. Erythrocytes were also capable to modify the viscoelastic properties of the fibrin clot by increase the ratio of viscous modulus (G'') to elastic modulus (G') [199].

Recently, it has been suggested that fibrin scaffold of PRGF-Endoret was dissolved after five days of incubation in a culture medium, and that signs of damage were observable at day 3 [200]. However, the results of the present study conclude that the stability of PRGF-Endoret fibrin

scaffolds was maintained for more than eight days of incubation. Previously, it was reported that 3D PRGF-Endoret fibrin scaffolds cultured with tenocytes were stable for up to six days [201]. On the other hand, it has been shown that the addition of polymorphonuclear (PMN) leukocytes to PRP worsens its viscoelastic properties [202].

The increase in growth factors release from L-PRP fibrin scaffolds could be related to the higher platelet enrichment in L-PRP and the leukocyte contribution in the synthesis/release of certain growth factors, such as TGF-β [203]. This increase could be related to the proliferation of leukocytes within the fibrin hydrogel, as it was observed the presence of macrophages adhered to the cell culture well. Furthermore, fused macrophages (macrophage-derived multinucleated giant cells) were noted, probably due to proinflammatory environment present in the L-PRP [204].

HGF release was also enhanced in L-PRP fibrin scaffolds. HGF reservoir on the leukocytes surface is created thanks to lower-affinity/high-capacity binding site of cell surface-associated heparan sulfate [205-207]. This surface pool of HGF on the leukocytes is rapidly released by the stimulation of coagulation factor Xa [208] and thus may partially account to explain the continuous increase in the amount of HGF released to the incubation medium. Furthermore, Grenier A *et al* have nicely showed that both secretory vesicles and gelatinase/specific granules of human blood PMN neutrophils contain a mobilizable stock of pro-HGF that is proteolytically processed to mature HGF by neutrophil serine protease during degranulation [205]. This degranulation process is initiated by stimulating agents of PMNs like the IL-1β [205]. We have found that IL-1β was mainly present in the incubation medium of L-PRP hydrogels and thus this cytokine would stimulate the PMNs to release the HGF from the intracellular granules.

Interestingly, VEGF levels resulted to be significantly higher at day 8 in the PRGF-Endoret fibrin scaffold, a finding that could be explained by a possible VEGF uptake by its soluble receptor (sVEGFR) synthesized by leukocytes [209, 210]. Another plausible hypothesis is that leukocytes release proteases that could either decrease the concentration of growth factors.

One of the most relevant results of this study is the almost absence of pro-inflammatory cytokines in the PRGF-Endoret fibrin scaffolds, and release of these molecules when leukocytes were included. Of particular concern is the presence of IL-1β in L-PRP hydrogel, since this cytokine triggers a strong inflammatory response because IL-1β can recruit more proinflammatory cells to the site of injury [211]. This cytokine also stimulates catabolic protein production, including MMPs, which breaks the extracellular matrix and inhibits both proteoglycan and collagen synthesis [212]. The negative effect of IL-1β has been investigated in various tissues. *In vitro* studies in tendon cells have shown that the combination of stretching and IL-1β presence produces extracellular matrix degradation [213]. Another study indicates that IL-1β induces the synthesis of catabolic mediators by tenocytes [214]. Cartilage is a particularly sensitive tissue to the presence of catabolic mediators such as IL-1β. This molecule is a major pro-inflammatory cytokine involved in the pathogenesis of osteoarthritis [215].

Similarly, the release of IL-16 was significantly increased in the L-PRP fibrin scaffolds and was virtually absent from PRGF-Endoret hydrogels. This cytokine is synthesized and secreted by T and B lymphocytes, monocytes, dendritic cells, eosinophils and mast cells, being chemotactic for all of them [216-218]. It has been suggested that IL-16 can be a key cytokine in both the initiation of inflammation and in its maintenance [219]. High levels of IL-16 have been observed in

rheumatoid arthritis [220, 221], which could suggest the cytokine role in joint destruction.

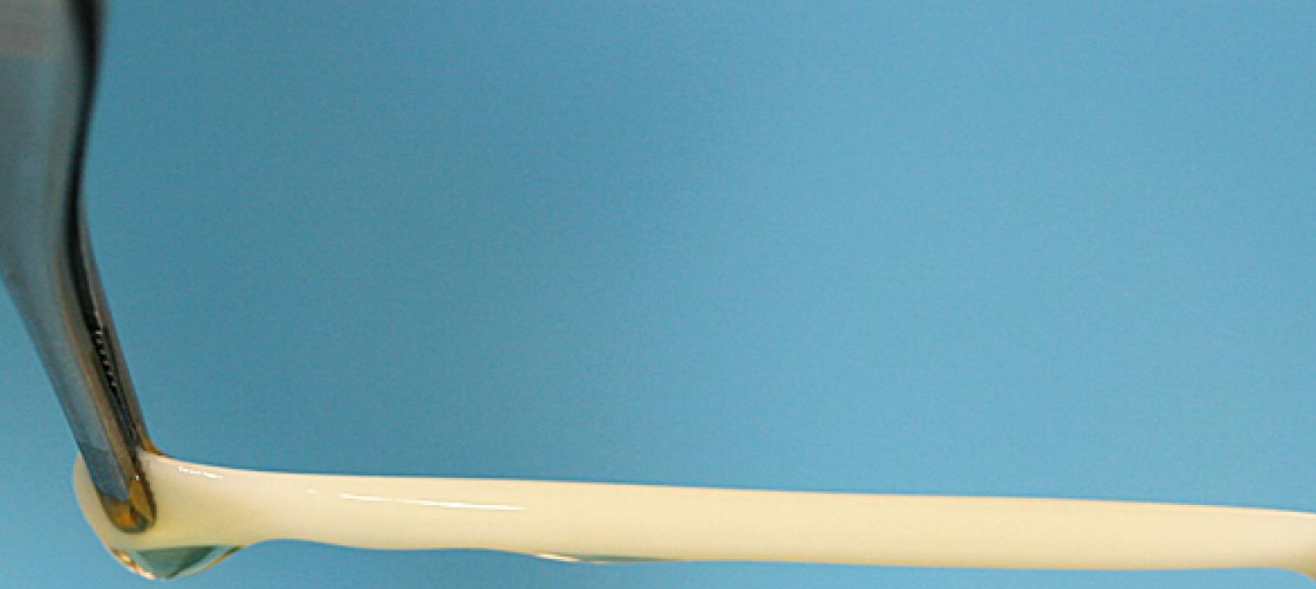
In recent work in equine tendon explants, McCarrel *et al* have elegantly demonstrated that a high concentration of platelets does not counteract the increased amounts of proinflammatory molecules synthesized by leukocytes [188]. It was observed that the optimal regenerative environment with the lowest catabolic gene expression was the leukocyte-reduced PRP. On the other hand, previous *in vitro* studies have shown both the antimicrobial properties of platelet-rich plasma [91, 222], and that the inclusion of leukocytes to PRGF-Endoret does not provide an additional bacteriostatic effect [92]. Few clinical trials have been carried out comparing leukocyte-free PRP versus L-PRP. Filardo *et al* compared the efficacy and safety of intra-articular injections of PRGF-Endoret against an homemade L-PRP in the treatment of osteoarthritis [195]. Both treatments improved the course of the disease, but patients treated with PRGF-Endoret had fewer side effects than those treated with L-PRP, who presented more pain and swelling events [223].

There are some limitations in this *in vitro* study. For example, due to biological variability, the enrichment of leukocytes was not homogeneous in the three donors. Furthermore, commercial L-PRP systems have a leukocyte concentration greater than the one obtained in this study [224], and thus greater differences would be expected when comparing PRGF-Endoret with commercial L-PRPs. It would also be necessary to analyze the biological behaviour of various cell lines with these two kinds of formulations, in terms of proliferation, migration and angiogenesis. Besides these methodological remarks, we should envision the PRP therapy as a biological system that content myriad of balanced bioactive molecules which act in concert with different cell

phenotypes rather than reducing or parsing them to a single cause-effect mechanistic approach.

This study has been designed to quantify the release of aforementioned molecules during eight days. This timing has been chosen in order to get an approximation to an *in vivo* system, because, unlike platelets, leukocytes may continue synthesizing and degranulating different molecules within a fibrin scaffold until this has been degraded.

In summary, the incorporation of leukocytes into PRGF-Endoret fibrin scaffold have resulted in a modification of the architecture of fibrin network and resulted in more heterogeneous and loose mesh. The released amount of growth factors from PRGF-Endoret did not show significant variations in the release profile over time. Overall, leukocyte inclusion into platelet-rich plasma did not enhance the release of platelet derived growth factors, but increased the release of pro-inflammatory cytokines. Further *in vivo* studies are necessary to study the biological significance of these different protein environments in tissue engineering and regenerative medicine.



Chapter 5

Implementation of a more physiological plasma rich in growth factor (PRGF) protocol: Anticoagulant removal and reduction in activator concentration

Experimental article published In *Platelets* [4]

Abstract

Plasma rich in growth factors (PRGF) is a biological therapy that uses patient's own growth factors for promoting tissue regeneration. Given the current European regulatory framework in which anticoagulant solution in blood extraction tubes could be considered as a medicinal product, a new PRGF protocol has been developed. The actual protocol (PRGF-A) and the new one (PRGF-B) have been performed and compared under Good Laboratory Practices. PRGF-A protocol uses extraction tubes with 0.9 mL of trisodium citrate as anticoagulant, and 50 µL of calcium chloride / mL PRGF to activate it. The PRGF-B reduces the amount of sodium citrate and calcium chloride to 0.4 mL and to 20 µL, respectively. Basic hematological parameters, platelet function, the scaffold obtaining process, growth factors content and the biological effect were compared between both PRGF obtaining protocols. *Results:* PRGF-B protocol led to a statistically significant higher enrichment and recovery of platelets regarding to the PRGF-A. Hypotonic stress response by platelets was significantly better in the new protocol. A statistically significant decrease in the basal platelet activation status of PRGF-B compared to PRGF-A was also observed. The duration of the lag phase in the platelet aggregation assay was statistically lower for the PRGF-B protocol. Both the clotting and the clot retraction time were significantly reduced in the B protocol. A higher growth factor concentration was detected in the plasma obtained using the PRGF-B protocol. The new PRGF obtaining protocol, with a reduction in the amount of anticoagulant and activator, has even improved the actual one.

1 Introduction

Platelet-rich-plasma (PRP), and in particular the plasma rich in growth factors (PRGF-Endoret) technology, is a biological therapy that uses patient's own blood proteins as a therapeutic toolbox in the treatment of multiple pathologies [118, 138, 141], as well as in the field of tissue engineering and regenerative medicine [108, 120].

The PRGF technology uses trisodium citrate as blood anticoagulant and calcium chloride to activate the coagulation cascade in a controlled way [37]. Given the current European regulatory framework, Manual on Borderline and Classification in the Community Regulatory Framework for Medical Devices version 1.16 [225] and the need to preserve both additives for a best control of the process, a new PRGF protocol has been developed. According to this framework the anticoagulant solution in the blood extraction tubes could be considered as a medicinal product, and it would lead to more stringent regulations for such products. The actual protocol (referred as PRGF-A in this paper) uses extraction tubes with 0.9 mL of trisodium citrate as anticoagulant, and 50 µL of calcium chloride / mL PRGF to activate it. Conversely, the new protocol (PRGF-B) reduces the amount of sodium citrate and calcium chloride to 0.4 mL and 20 µL, respectively.

In order to study the characteristics of the PRGF obtained with the new protocol and compare them with the current one, it has been designed a battery of assays comprising various aspects of the obtaining process and of the product itself, such as the basic haematological parameters, the study of platelet function, the characterization of the scaffold obtaining process, the determination of key growth factors, and the biological response to the PRGF treatment. These studies represent a

set of assays that would allow us to evaluate and compare PRPs between each other and to determine its properties.

Hence, the objectives of this article are to describe a new protocol for obtaining PRGF and compare it with the current protocol through a set of characterization tests performed under current Good Laboratory Practices. In addition, these assays could serve as a basis for characterizing future platelet-rich plasma formulations.

2 Materials and methods

2.1 Study design and PRGF preparations

The study was performed following the principles established in the Declaration of Helsinki of 1964 as revised in 2013 [226] and was approved by the Ethical Committee of Clinical Research of the University Hospital of Alava (CEIC-HUA; Hospital Universitario de Álava; Vitoria; Spain) under study code BTI-01-IV-15-TUB.

Studies described herein were conducted under current Good Laboratory Practices according to Directive 2004/9/EC and the Royal decree 2043/1994 modified according to the Ministerial Order of 14 April 2000.

After informed consent was given, blood from thirty healthy donors was harvested into two different 9-mL collection tubes containing either 0.9 mL (PRGF-A) or 0.4 mL (PRGF-B) of 3.8% (wt/v) sodium citrate (see Figure 20). The platelet and leukocytes concentration and the volume of whole blood were measured in both types of tubes (PRGF-A and PRGF-B). Subsequently, blood samples were centrifuged at 580 g for 8 minutes at room temperature in the PRGF-Endoret System IV centrifuge (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain). Afterwards, the

whole plasma column over the buffy coat was collected using Endoret kit (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain) avoiding the layer containing leukocytes. At this time, several assays were carried out to characterize the PRGF obtained through each protocol and to evaluate the functionality of platelets contained in it. To characterize the PRGF samples from both protocols, the pH levels, the collected PRGF volume and the platelets, erythrocytes and leukocytes concentrations were measured in the PRGF samples obtained from all donors (Figure 20). As soon as the characterization assays were completed, platelet aggregation, platelet activation and hypotonic stress assays were performed in 15 of the 30 donors to check the platelets´ functionality. Furthermore, the PRGF samples (PRGF-A and B) from 15 of 30 donors were activated with PRGF-Endoret Activator (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain) at a rate of 50 µL and 20 µL per 1 mL of PRGF for PRGF A and B protocols respectively. Both activated samples were then incubated at 37 °C and the clotting and retraction time were measured for each PRGF sample until complete clot retraction. Finally, the PRGF supernatants obtained from 10 of 30 donors were collected after total clot retraction, sterile-filtered, aliquoted and stored at -80 °C until their use in order to check the PRGF-A and -B biological activity. For this purpose, the proliferative potential on human keratocyte cells and the growth factors content of both PRGF supernatants were analyzed (Figure 20).

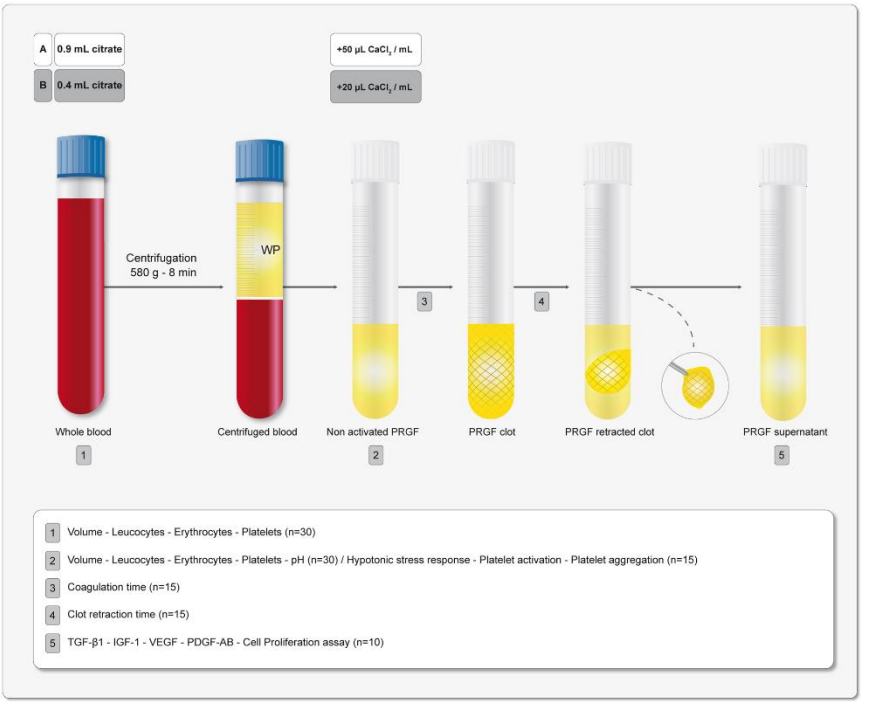


Figure 20. Experimental design and PRGF preparation workflow with A and B protocols. The differences between the two protocols (volume of anticoagulant and activator) are highlighted. The measurements performed at each step of the procedures are indicated with boxed numbers (one to five) and the number of donors (n) assayed in each phase of the study is also indicated (WP, whole plasma).

2.2 Hematology parameters

Platelets, erythrocytes and leukocytes concentration was analyzed with a hematology analyzer (Micros 60, Horiba ABX, Montpellier, France) in whole blood and PRGF samples from all donors. At the same time, the volume of whole blood and PRGF samples was estimated using the graduated scale of the tube.

Platelet and leukocyte concentration factor (PCF and LCF respectively) and platelet yield (PY) were calculated as follows to characterize and compare the plasma rich in growth factors obtained by each protocol (PRGF-A and -B):

i. The platelet concentration factor (PCF), or platelet enrichment, was calculated as the ratio of the platelet count in PRGF to the platelet count in whole blood:

$$PCF = \frac{Platelet\ count\ in\ PRGF}{Platelet\ count\ in\ whole\ blood}$$

ii. The leukocyte concentration factor (LCF), or leukocyte enrichment is similar to above but for the leukocyte parameter:

$$PCF = \frac{Platelet\ count\ in\ PRGF}{Platelet\ count\ in\ whole\ blood}$$

iii. The platelet yield (PY), or platelet recovery, was derived from the percentage of the ratio of the platelet count in the PRGF times the volume of the platelet rich plasma to the platelet count in whole blood times the volume of the whole blood:

$$PY = \frac{Platelet\ count\ in\ PRGF * Volume\ of\ PRGF}{Platelet\ count\ in\ whole\ blood * Volume\ of\ whole\ blood} * 100$$

2.3 pH determination

pH determination was carried out in all plasma samples obtained from both protocols. pH was measured with a pH-meter GLP 21+ (Crison, Barcelona, Spain).

2.4 Hypotonic stress response

The ability to recover platelets´ resting volume after hypotonic environment exposure was tested following the method of *Farrugia et al* [227], to analyze the membrane integrity of platelets obtained from the different PRGF protocols. Distilled water was used as hypotonic medium while phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO) was used as isotonic buffer. Platelet-rich plasma was briefly added to both hypotonic and isotonic environments in 2:1 ratio and light transmittance was measured twice at time point 0 and 10 minutes, using a microplate reader with a filter for wavelength at 610 nm. Values of hypotonic stress response were reported as percent of recovery (reversal %) of platelet volume assuming isotonic buffer values as 100% of recovery.

2.5 Platelet activation

Measurement of translocated P-selectin was used as a method to test the platelet functionality. P-selectin, also called CD62P, GMP-140 or PADGEM, is stored in α-granules of unactivated platelets. After platelet activation through agonists such as thrombin, collagen and ADP, the inner walls of these granules are exposed on the outside of the cells presenting the CD62P protein [228]. The platelet responsiveness to ADP in the presence of U46619 (stable synthetic analog of the endoperoxide prostaglandin PGH2) was evaluated for freshly obtained PRGF

preparations, for the two protocols broadly described before. For that purpose, CD41 and CD62P antibodies that recognize platelet membrane constitutive glycoprotein GpIIb and translocated p-selectin, respectively, were used.

For each donor and for each protocol one aliquot was assayed in stimulation condition and the other one in rest condition. For that purpose, 10 µL of PRGF, 5 µL of anti-CD41-FITC and 5 µL of anti-CD62P-PE (BD Biosciences, San Jose, CA) were added in the test tubes. Afterwards, 11 µL of the stimulating mixture (containing 5 µM of ADP (Sigma-Aldrich, St. Louis, MO) and 2.8 µM of U46619 (Cayman Chemical, Ann Arbor, Michigan)) in PBS were dispensed into the tubes corresponding to stimulating condition. The stimulating mixture was replaced by the same volume of PBS for the tubes used to measure the platelet activation at rest. In both cases, the remaining volume up to 110 µL was completed with PBS. Appropriate controls for flow cytometric technique were used. Finally, samples were incubated for 15 minutes at room temperature in the dark and then were fixed with 400 µL of freshly prepared 1.25% formaldehyde in PBS. A Gallios flow cytometer (Beckman-Coulter, High Wycombe, UK) was used to analyze events.

2.6 Platelet aggregation

Platelet aggregation was performed on a four-channel light transmittance aggregometer (APACT 4004 model, Labitec, Ahrensburg, Germany) with a computer-based curve analysis (APACT LPC-Software, Labitec, Ahrensburg, Germany) following the method developed by Born [229]. First, for each donor, platelet-free plasma (PFP) was produced by centrifugation of PRGF-A and PRGF-B at 1000 g for 1 minute and it was used to adjust to 300 x 10^3 platelets/µL the platelet concentration of PRGF preparations. Then, PFP was set as 100% of

aggregation and PRGF as 0%. Subsequently, 20 µL of collagen I at 100 µg/µL (Hart Biologicals, Hartlepool, UK) was added to 180 µL of PRGF-adjusted preparations. Finally, the aggregation reaction began in an aggregometer cuvette stirring at 1000 rpm and 37 °C. Aggregation curves (normalized optical density at 740 nm versus time) were monitored for 300 seconds and maximal aggregation (%) and lag phase (seconds) values were automatically generated by the software.

2.7 Determination of coagulation time and clot retraction time

For the measurement of coagulation and retraction time, one aliquot of 2 mL of PRGF from each protocol was dispensed into TF9 tubes (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain) in order to be activated with $CaCl_2$ (PRGF-Endoret activator) and trigger the coagulation cascade. The concentration of $CaCl_2$ was 50 µL/mL PRGF-A, and 20 µL/mL for PRGF-B. Subsequently, the activated PRGF was incubated in the Plasmaterm oven (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain) at 37 °C for 1 hour, and was carefully monitored during 60 minutes to determine both the coagulation and retraction times. The whole process was photographed at several time points. The coagulation time was determined by the Lee-White test tube method modified [230] and the clot retraction time was defined as the time in which no changes were found between two consecutive photographs [231].

2.8 Growth factors content

Briefly, PRGF activator (BTI Biotechnology Institute, SL, Miñano, Spain) was added to the obtained plasma fraction and was incubated at 37 °C for 1 hour. The released supernatant was collected after centrifugation

at 1000 g for 10 minutes and stored at -80°C until use. The amount of several growth factors was compared between the aforementioned protocols by commercially available Enzyme-linked immunosorbent assay kits (ELISA) (R&D Systems, Minneapolis, MN): Transforming growth factor β1 (TGFβ1), Insulin like growth factor -1 (IGF-1), Vascular endothelial growth factor (VEGF) and Platelet derived growth factor - AB (PDGF-AB). Measurements were assayed in triplicate.

2.9 Cell Proliferation assay

2.9.1 Cell culture

Primary human keratocytes (termed HK) (ScienCell Research Laboratories, San Diego, CA, USA), were cultured according to manufacturer's instructions. Briefly, cells were cultured at 37 °C and 5% CO_2 atmosphere in complete fibroblast medium (FM) consisting of basal fibroblast medium supplemented with Fibroblast Growth Supplement, 2% fetal bovine serum and antibiotics (ScienCell Research Laboratories, San Diego, CA, USA) until confluence. Then, they were detached with animal origin-free trypsin-like enzyme (TrypLE Select, Gibco-Invitrogen, Grand Island, NY, USA). Cell viability was assessed by trypan blue dye exclusion method. Cells in passage 3 were used in proliferation assays.

2.9.2 Cell proliferation

Cells were seeded at a density of 10000 cells per cm^2 on 96 well optical bottom black microplates in basal fibroblast medium supplemented with either 20% (v/v) of PRGF-A or PRGF-B from each of the ten donors or basal fibroblast medium with 0.1% fetal bovine serum as a control of non-stimulation. Treatments were assayed in quintuplicate. The study period was 72 h. The proliferation of cultured cells was quantified using the CYQUANT Cell Proliferation Assay (Invitrogen, Carlsbad, CA, USA).

Briefly, medium was removed and wells were carefully washed with PBS to avoid detaching the cell monolayers; then, microplates were frozen at –80 °C for efficient cell lysis in the CyQUANT assay. After thawing the plates at room temperature, samples were incubated with RNase A (1.35 Ku/mL) diluted in cell lysis buffer during 1 hour at room temperature. Then 2x CyQUANT GR dye/cell lysis buffer was added to each sample well and mixed gently. After 5 minutes of light protected room temperature incubation, the fluorescence was measured using a fluorescence microplate reader (Twinkle LB 970, Berthold Technologies). To correlate fluorescence units with DNA amount, a DNA standard curve ranging from 7.8 to 500 ng/mL was included in all fluorescence quantifications.

2.10 Statistical analysis

For all statistical analysis, the sample normality was checked with the test of Kolmogorov and Smirnov. A t-test for paired samples was used if values followed a normal distribution with homogeneous variances. Otherwise, the non-parametric Wilcoxon matched-pairs signed-rank test was applied. All values were expressed as mean ± standard deviation (SD). Differences were considered statistically significant when a value of $p<0.05$ was obtained. Statistical analyses were performed using GraphPad InStat software (version 3.1; GraphPad Software, La Jolla, CA, USA).

3 Results

Blood samples were obtained from 30 healthy donors. The mean age of the donors included in the study was 41 ± 8 years with an age range between 26–62 years including 22 males and 8 females. The donors

recruited for the study were systemically healthy and they were not taking medications.

3.1 Hematology parameters

Table 5 shows the most relevant hematological parameters of both whole blood and PRGF, as well as the other calculated parameters described in the Methods section. Results showed a platelet concentration factor (PCF) of 1.8 ± 0.3 and 2.2 ± 0.2 for PRGF-A and PRGF-B protocol, respectively. PRGF-B protocol achieved a significant increase ($p<0.0001$) of PCF with respect to PRGF-A. Similarly, platelet yield (PY) was significantly increased ($p<0.0001$) when the B protocol was used to obtain PRGF. In summary, PRGF-B led to a 66% of PY while it was 60% for PRGF-A. No statistically significant differences in the LCF were found ($p>0.05$).

	Whole blood-A	**Whole blood-B**	**PRGF-A**	**PRGF-B**
Leukocytes (x 10^3/µL)	5.8 ± 1.3	6.1 ± 1.4	0.3 ± 0.2	0.3 ± 0.2
Erythrocytes (x 10^6/µL)	4.51 ± 0.40	4.78 ± 0.41	0.01 ± 0.00	0.01 ± 0.01
Platelets (x 10^3/µL)	256 ± 51	235 ± 41	419 ± 105	517 ± 107
Leukocyte concentration factor (LCF)	-	-	0.05 ± 0.04	0.05 ± 0.03
Platelet concentration factor (PCF)	-	-	1.8 ± 0.3	2.2 ± 0.2
Platelet yield (%)	-	-	60 ± 9	66 ± 7

Table 5. Characterization of whole blood and PRGF samples from the thirty donors included in this study. Leukocyte, platelet and erythrocyte concentration was measured in whole blood and PRGF. Leukocyte and platelet concentration factor (enrichment as fold increase) relative to the level of peripheral blood (LCF and PCF) and platelet yield (%) are also indicated. Data are expressed as mean ± SD.

3.2 pH determination

No statistically significant differences were observed in the mean pH values obtained for each plasma preparation protocol. The values were 7.61 ± 0.08 and 7.64 ± 0.09 for PRGF-A and B, respectively.

3.3 Hypotonic stress response

The platelet volume recovery of the different PRGF preparations was tested as an indicator of cell membrane integrity. PRGF-B formulation showed statistically ($p<0.01$) higher reversal percentages (76.1 ± 11.2%) when compared with PRGF-A protocol (67.1 ± 11.4%) (Figure 21 A).

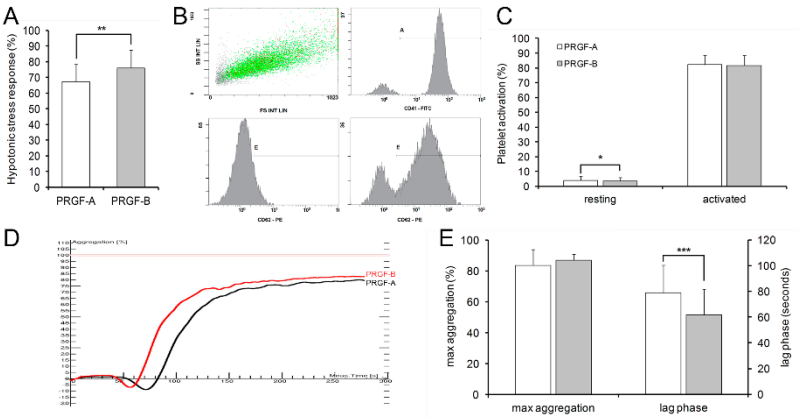


Figure 21. Platelet function assays. (A) Hypotonic stress response. (B) Flow cytometry platelet activation representative images are shown: the upper-left panel represents a typical forward versus side scatter histogram (FS/SS) for platelets, the upper-right panel shows an histogram with the constitutive platelet marker CD41, the lower-left displays a histogram showing no-activation (resting) for CD62 (P-Selectin), and the lower-right panel is a representative histogram of ADP-activated sample marked for CD62. (C) Chart representing the mean values for platelet activation in both PRGF protocols. (D) Typical platelet aggregation monophasic responses for PRGF-A and B protocols. (E) Quantitative aggregation data, maximal aggregation and lag phase for both protocols. ** indicates $p < 0.01$ and *** indicates $p < 0.0001$. (A, C and E data are expressed as mean ± SD).

3.4 Platelet activation

Typical images were obtained in the flow cytometric analysis (Figure 21 B). The percentage of platelet activation at rest differs significantly ($p=0.0302$) between the two protocols evaluated, with an increase in the percentage of activated platelets for the PRGF-A samples (3.88% ± 2.72% *versus* 3.48% ± 2.53% for PRGF-A *versus* PRGF-B protocols) (Figure 21 C). No statistically significant differences were found in the response to ADP stimulation. In this case, the percentages of platelet activation found were 82.15 % ± 6.23 % and 81.67 % ± 6.91 % for A and B protocols, respectively (Figure 21 C).

3.5 Platelet aggregation

Platelet aggregation in response to collagen displayed a long lag phase with a typical monophasic response (Figure 21 D). Maximal aggregation values were similar between the two protocols (83.58% ± 10.25% for PRGF-A versus 86.93% ± 3.84% for PRGF-B), while statistically significant differences ($p<0.0001$) were found regarding the duration of the lag phase (Figure 21 E). This duration was lower for the PRGF-B protocol (61.79 sec ± 19.95 sec) than for the PRGF-A one 78.80 sec ± 21.65 sec).

3.6 Clotting and retraction time

The whole coagulation and retraction process was monitored and photographed. In Figure 22, representative images of the beginning of the process (non-activated), a fully clotted, and a retracted sample are shown. After PRGF activation, the mean clotting time (time until complete gelation) was 8.7 minutes for PRGF-B while it was 17.5 minutes for the PRGF-A, showing a significant clotting time reduction ($p<0.0001$) (Figure 22).

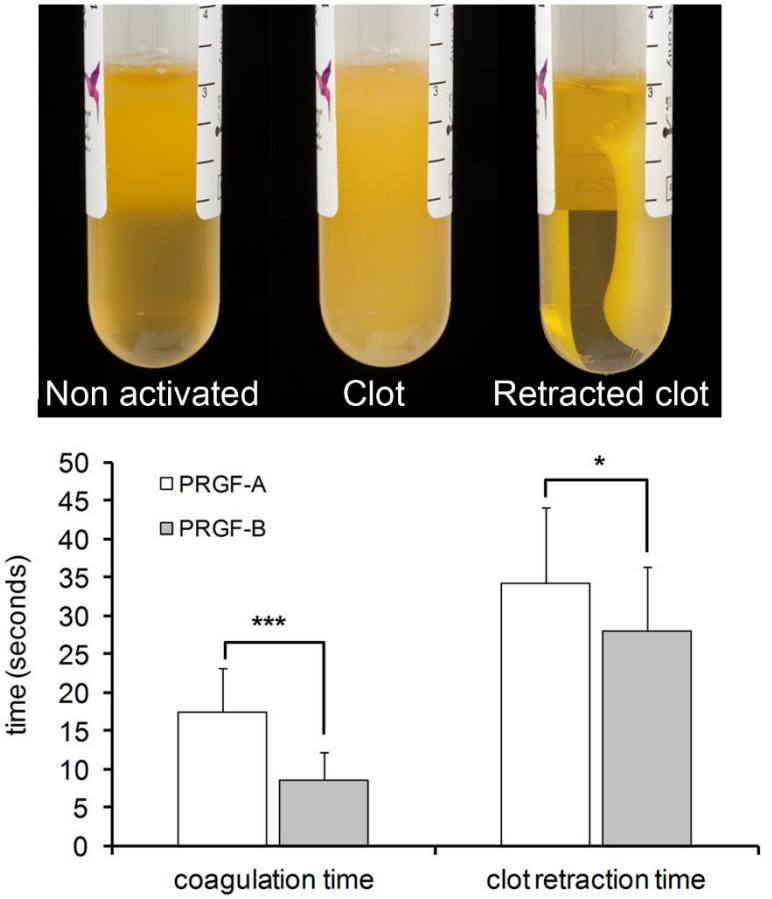


Figure 22. Clotting and retraction time analysis. Representative images of the coagulation process are shown. The mean coagulation and the retraction time are shown in the graphic (as mean ± SD). * indicates $p < 0.05$ and *** indicates $p < 0.0001$.

Subsequently, the mean clot retraction time was measured for each sample, and this period was also significantly reduced ($p<0.05$) for PRGF-B protocol with respect to PRGF-A (28.0 minutes and 34.3 minutes, respectively) (Figure 22). Hence, the PRGF-B protocol reduced the overall duration (clotting + retraction time) in a 30%, showing a

statistically significant reduction ($p<0.05$) of the overall duration between PRGF-B and PRGF-A protocols.

3.7 Growth factors content

PRGF-B protocol showed statistically significant higher levels for each growth factor tested compared to the PRGF-A one. An increase of 12%, 30%, 45% and 27% was found for IGF-1, VEGF, PDGF-AB and TGF-β respectively. ($p<0.05$ for TGF-β1, $p<0.01$ for PDGF-AB and $p<0.001$ for IGF-1 and VEGF) (Figure 23).

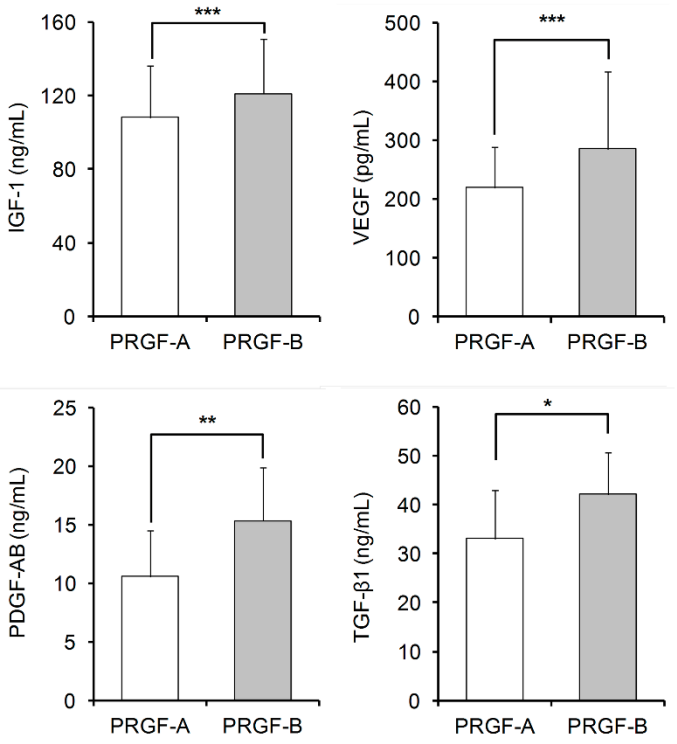


Figure 23. Growth factor concentration analysis in PRGF-A and B. Transforming growth factor β1 (TGFβ1), Insulin like growth factor -1 (IGF-1), Vascular endothelial growth factor (VEGF) and Platelet derived growth factor -AB (PDGF-AB) were assayed in triplicate. The mean ± SD concentration are represented. * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$.

3.8 Cell Proliferation assay

Two representative photomicrographs of keratocytes cultured with each PRGF protocol are shown in Figure 24. Both PRGF preparations (20% PRGF-A or B) stimulated significantly the proliferation of HK cells when compared with the control. Nevertheless, there was no difference ($p>0.05$) between the proliferation induced by PRGF-A (279±51 ng DNA/mL) or by PRGF-B (306±40 ng DNA/mL) (Figure 24).

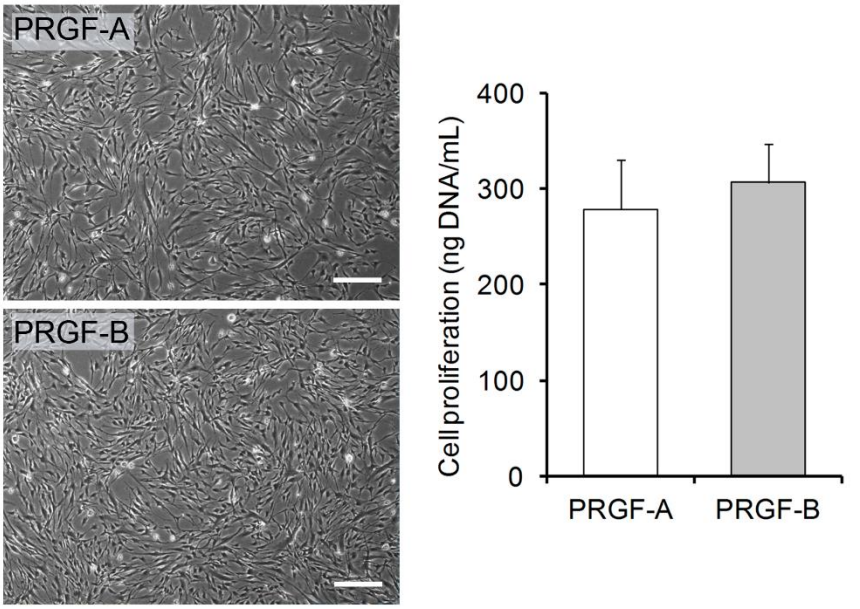


Figure 24. Biological response to PRGF (PRGF-A versus PRGF-B). Representative images of primary human keratocytes (HK) supplemented with PRGF-A and PRGF-B (Scale bars for both micrographs: 400 µm). The cell proliferation quantitative analysis (ng DNA/mL) shows no differences between both protocols.

4 Discussion

The amount of trisodium citrate (3.8% w/v) has been reduced by 55% in the PRGF-B with respect to the current protocol (PRGF-A). This reduction does not affect the properties of anticoagulation, since the amount of trisodium citrate (3.8%) present in the extraction tubes is in excess in relation to whole blood calcium [232], providing a chelation of all calcium from the extracted blood. Therefore, the whole blood in the PRGF-B is diluted with the anticoagulant a 5.5% less than in the PRGF-A. The increase in the blood/anticoagulant ratio produced a statistically significant platelet enrichment and recovery of PRGF-B over PRGF-A. However, in both protocols the buffy coat was smoothly excluded, yielding a leukocyte count close to zero, thus enabling PRGF obtained by the new protocol to remain in the same categories that the former protocol, in terms of the different PRP classifications [110].

No significant differences were found regarding the pH, being around 7.6, a physiological value [233] that does not need further buffer addition. In any case, pH remained above 6, which is a positive factor ~~critical~~ for an adequate platelet function [234].

The new PRGF protocol with reduced anticoagulant improves several platelet function parameters. Platelet volume recovery after hypotonic stress is a metabolic process which can be reduced in damaged or stored platelets [235]. The relationship between the hypotonic stress response and *in vivo* platelet viability was already demonstrated in the seventies [236]. Both protocols have good recovery rate, but protocol B increases existing viability by almost 10%. Basal values of platelet activation assayed by flow cytometry are minimal for both PRGF protocols, and even less than other five PRP systems [237], indicating that none of the two PRGF obtaining protocols (A or B) activate prematurely the

platelets. A slight, but statistically significant decrease in the basal platelet activation status of PRGF- B compared to PRGF-A was observed. When the PRGF is activated with ADP, an increased expression of P-selectin was demonstrated, as an indicator that platelets are functional and thus able to release the entire contents of their granules. No differences between the two protocols were found for this parameter. Thus, ADP-dependent activation was always greater than 80%, in line with previous studies conducted by our research team [238]. Similarly to the other platelet function assays, platelet aggregation assay also indicated that the PRGF obtaining protocols do not affect the ability of platelet aggregation, since in both protocols maximum aggregation was higher than 80% [239]. Moreover, the PRGF-B protocol shortened the lag phase compared to the PRGF-A one. This may be relevant from a clinical point of view as it may shorten the time needed to obtain PRGF, maintaining its therapeutic potential. The new PRGF protocol has also reduced both the time of clotting and the required time to retract that clot, so it can be used quickly and efficiently to produce a fibrin membrane.

Growth factors are the active ingredients of the PRGF. In this study we have measured four of them, three platelet-derived (VEGF, PDGF-AB and TGF-β) and one plasma-derived (IGF-1). Systematically, these four molecules showed a higher concentration in PRGF-B compared to PRGF-A. Not only can be explained this increase by the lower dilution of the blood in the extraction tube for PRGF-B protocol, but also by an increased concentration of platelets in PRGF-B and a different ratio of $CaCl_2$ in the activation of PRGF. Another plausible explanation is that the growth factors release kinetics of PRGF-B was accelerated, since both the coagulation and retraction processes have a greater speed with this new protocol. However, despite the increase of growth factors concentration in PRGF-B, no differences in cell proliferation of HK cells

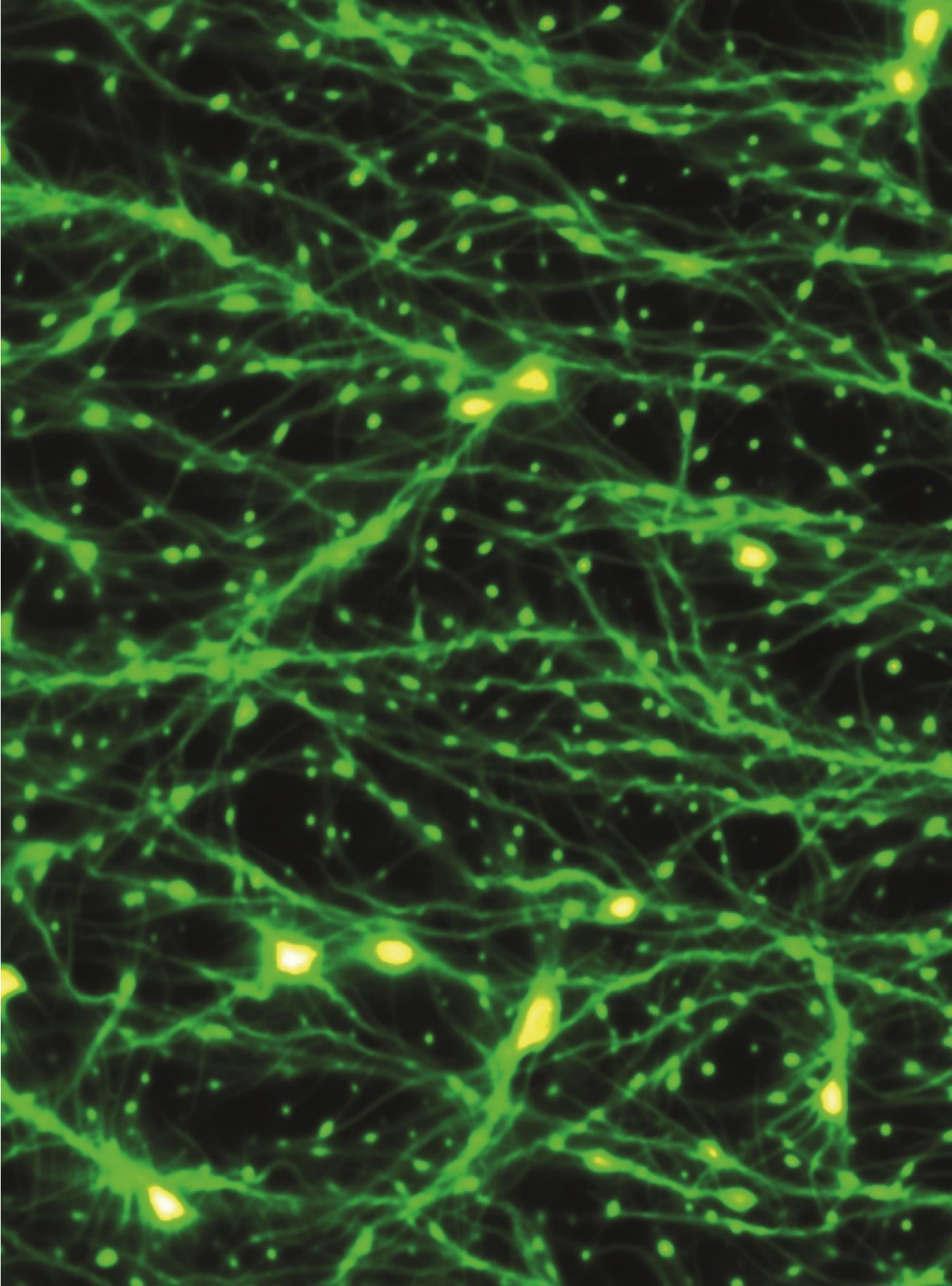
were observed. This fact could be explained in several ways. Firstly, the increased concentration of growth factors in PRGF-B may not be relevant from a biological standpoint to stimulate cell proliferation in this cell lineage [240], argument supported by previous studies where the proliferative dose-dependent response can typically be adjusted to a Gaussian distribution function [241]. Secondly, other proteins different from the ones studied herein could be involved in the regulation of cell proliferation in this cell type.

Currently, the international regulatory bodies are demanding to regulate the biological therapies, including PRP. Specifically, the European Union has classified the blood extraction tubes with anticoagulant as a Class II medical device, but the anticoagulant could be considered in future as a medicinal product. The latter would imply that extraction tubes become a Class III medical device if the manufacturer does not prove that anticoagulant is totally removed [225]. The inclusion in the class III category would involve more stringent regulation. In the case of PRGF, in the activation step, trisodium citrate reacts with $CaCl_2$ to form calcium citrate. In this way, trisodium citrate is totally removed with $CaCl_2$ activation. Calcium citrate is harmless at the concentration that is present in the PRGF [242]. Moreover, different countries are actively regulating PRP therapies, even as a human use medicinal product, which makes the regulatory framework even more restrictive [5].

One of the strengths of this study is the use of paired samples for both protocols (single-donor model) which, in addition to conferring greater statistical power, minimizes the biological variability. However, this study has also several drawbacks. On the one hand, only the process of obtaining the clot has been studied and not its characteristics, such as clot strength, or the ability to retain other biomaterials. Regarding the

biological response, it has been only observed the effect of PRGF in one cell type; therefore, further studies are needed to observe the effect in other cell types.

In conclusion, we have described a new protocol for obtaining PRGF, in which both the amount of anticoagulant and activator have been reduced. Additionally, we have characterized this new protocol in comparison with the actual one, confirming an improvement in some of the characteristics of PRGF.



Chapter 6

Regulatory framework. Closing regulatory gaps: new ground rules for platelet-rich plasma

Perspective article published In *Trends in Biotechnology* [5]

Abstract

The Spanish-Agency-of-Medicines-and-Medical-Devices has drawn up a comprehensive report and resolution, which regulates for the first time the use of platelet-rich plasma as a human use medicinal product. This regulatory framework offers emerging challenges to adapt the use of platelet-rich plasma to the new requirements of safety and efficacy. The heterogeneity of the different products can hinder their regulation, which today differs substantially in the different worldwide regulatory frameworks.

1 Background on platelet-rich plasma therapies

Recent advances in cell biology and engineering, aided by new discoveries in other fields are making possible new therapeutic strategies in regenerative medicine. For example, platelet-rich plasma (PRP) therapies are being increasingly used in different medical fields ranging from dentistry to dermatology, traumatology and more recently ophthalmology and have an estimated global market of $45 million in 2009, projected to grow to $120 million in 2016 [243]. PRP therapies are all based on the preparation and use of plasma obtained from peripheral blood that has been enriched for platelets above the physiological level [244]. Platelets contain a relevant number of bioactive molecules, including growth factors that play an important role in the healing of injured tissue. Additionally, the plasma fraction contains a pool of molecules that contribute in a relevant way the regeneration of tissues, such as fibronectin and vitronectin [2]. PRP technology can also be used in *ex vivo* procedures for expansion of stem cells, replacing fetal bovine serum (FBS) as a culture supplement [108], as demonstrated in a recent clinical trial involving the expansion of adipose-derived stem cells [245].

However, there is no single standard as to the preparation or composition of PRP, which has led to a lack of consensus on the very definition of the term. There are differences between the different commercial PRP kits regarding amount of platelet enrichment, presence of leukocytes and erythrocytes, and activation mode. On this basis, the different types of PRP may thus yield different clinical outputs. Therefore, trying to regulate PRP technology will prove to be quite challenging.

2 Classifying platelet-rich plasma as a medicinal product in Spain

Recently, the Spanish Agency of Medicines and Medical Devices (AEMPS) has drawn up a comprehensive report [246] and a subsequent resolution [247] which regulates for the first time the use of platelet-rich plasma as a human use medicinal product. In order to establish this classification, the AEMPS has considered, among other elements, the composition of platelet-rich plasma, its mechanism of action and medical guidelines, along with the definition of medicinal product given at both Spanish [248] and European level [249]: "*any substance or combination of substances presented as having properties for treating or preventing disease in human beings; or any substance or combination of substances which may be used in or administered to human beings either with a view to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or to making a medical diagnosis*".

The AEMPS has clarified that although it is a medicinal product for human use, PRP obtention does not imply an industrial processing and therefore the PRP cannot be considered as an industrially produced medicinal product. On the other hand, the AEMPS has framed the PRP outside the category of advanced-therapy medicinal products, according to the definition given by the European Union [250]. From a regulatory perspective, excluding PRP therapy from the category of advanced therapies could clearly facilitate, from the regulatory viewpoint, its use both intraoperatively and on an outpatient basis.

For the AEMPS, there are three direct consequences of the inclusion of the platelet-rich plasma in the category of human use medicinal product. First, according to Article 5 of Directive 2001/83/EC of 6 November 2001

[249] referring to these types of products, the use of PRP must only be prescribed by a doctor, dentist or podiatrist, within their respective competencies, and not by any other healthcare or non-healthcare professional. Second, only doctors, dentists and podiatrists with the appropriate qualifications and experience in the treatment may prescribe PRP therapy. These physicians must always use the appropriate equipment and instruments, and can only be used in authorised healthcare facilities and centres in accordance with the current regional government regulations. And third, as with any other medicinal products available only on prescription, advertising it to the general public is prohibited.

The AEMPS report indicates that a case-by-case authorization is not required. However, a physician who prescribes the treatment must ensure regulatory compliance with the competent inspection authorities. This legal framework enables the use of PRP therapies with all the guarantees, providing guarantees of quality, efficacy, traceability and pharmacovigilance (view Box 1 section).

3 Emerging challenges

There are still several key challenges in the development and evolution of the regulation of PRP products in Spain. It will be necessary to have a list of approved medical devices and the medical applications for which their use is authorized. It should be noted that all systems for obtaining PRP are different and that their different composition may influence the clinical output. Thus, the presence of leukocytes in the plasma, the platelet enrichment, and the method of activation and release of growth factors will differ for each system. Although the systems can be grouped by any of the proposed classifications [110], it is difficult to generalize clinical results for one particular system to the rest of them.

Following the principles of the Spanish new regulations, the approval of a PRP commercial system for the treatment of a given pathology, does not mean that automatically all PRP systems may be authorized to treat this disease. This is due to the fact that the characteristics of others PRPs could differ from those ones of the approved PRP, and therefore it would affect the clinical outcome of the treatment. On the one hand, the regulatory process would be hampered, becoming a slow and costly process, but on the other hand, this would increase the guarantees of security and efficacy. This may sound confusing when it comes to biological products such as PRP, but it is of usual application in conventional medicinal products. For example, if a medicinal product contains two active ingredients, such as amoxicillin and clavulanic acid, both active ingredients can be present in different concentration ratio into two or more different medicinal products, so it is quite possible that the prescriptions, namely indications, dosage schedules and even side effects, can also differ between them. Nowadays it is difficult, almost impossible, to characterize all active ingredients in PRP, which can also be different in concentration for each individual. However, certain PRP key parameters can be characterized, such as platelet and leukocyte enrichment and some growth factors, which are the main changing parameters among commercial PRP formulations. Therefore, approval for each PRP type should be applied for, on the basis of their safety and efficacy. Thus, it would be important to define weather the approval for a specific indication is granted either individually for each commercial system, or for each family of systems that share common characteristics (key parameters).

The exact therapeutic mechanism of action of PRP is not well defined, and all active molecules involved in its regenerative potential have not been identified. This multifactorial complex mechanism of action, would require the development of Potency Tests for PRP therapies as a key

step to strengthen its efficacy, similar to those that can be developed for cellular therapy [251] or tissue engineering products [252].

Another noteworthy aspect that should not be forgotten is that like a conventional medicinal product, the dose and administration schedule may influence the clinical outcome. For example, in the treatment of knee osteoarthritis, it is not the same to perform a single PRP infiltration than to perform three of them on a bi-weekly basis [110], as is not the same to infiltrate a pure-PRP formulation or a leukocyte-enriched PRP in terms of adverse events such as pain and swelling [195]. These are important questions that should be considered by the AEMPS and other regulatory bodies when assessing the efficacy of particular PRP formulation.

Currently, the administration of PRP in the United States (U.S.) is considered a medical procedure and therefore is not subject to regulation by the U.S. Food and Drug Administration (FDA). However, the devices used in the preparation of PRP are subject to regulation by the FDA premarket approval process. A 510(k) is a premarket submission made to demonstrate that the device to be marketed is at least as safe and effective, that is, substantially equivalent, to a legally marketed device "*predicate*" (21 CFR 807.92(a)(3)) that is not subject to Premarket Approval (PMA) [253]. Currently the FDA has cleared over forty PRP systems (as of Mar 6, 2015), the great majority of which are intended to be mixed with autograft and/or allograft bone prior to application to an orthopedic site, though there are also some systems intended for use at the point-of-care for the treatment of ulcers and wounds of all types. According to FDA regulations, any use not authorized is considered *off-label* and its prescription should be considered under the responsibility of the physician who carried the procedure out. Currently, FDA does not

include PRP therapies within 21 CFR 1271 regulation of Human cells, tissues, and cellular and tissue-based products [254].

At the European level, there are no real common rules for PRP therapies that are applicable to all member countries. The European Medicines Agency (EMA) has certain regulations, but each state has the authority to independently regulate PRP therapy. Unlike in the USA, there is no registry of authorized PRP systems.

In addition, several other challenges exist. Will the FDA and EMA develop any regulations in their respective spheres establishing the use of PRP in the same way that Spanish Agency of Medicines and Medical Devices has done? Will this type of regulation be possible in the United States, in Europe, or globally? On the one hand, it could adversely affect the free medical practice, but on the other hand, it would reinforce the guarantees of safety and efficacy in patients.

Will allogeneic PRP be considered a medicinal product in the future? What should the regulatory requirements be for translational applications? For example, in the veterinary field, specifically in the treatment of horses, allogeneic commercial kits have been developed with proven non-immunogenicity. It is expected that for humans use, controls similar to those existing in blood banks would be established to prevent disease transmission.

4 Concluding remarks and future perspectives

Commending the initiative of the AEMPS, we deem that the advances in PRP therapy require a multidisciplinary approach with solid foundations in preclinical research and conducting high quality randomized clinical trials. Additionally, an appropriate regulatory framework must be put in place to ensure both patient safety and the consolidation of existing

treatments and the development of future applications. As in the case of industrial “classical” medicines, we propose standardized protocols and tight efficacy and safety requirements (clinical trials) for each type of PRP product, that to take into account the way the product is elaborated may significantly affect its final biological and therapeutic outcomes.

5 Box 1. Guarantees required for the use of platelet-rich plasma in Spain.

The guarantees required for the use of PRP therapies in Spain are the following [246, 247]:

- *Guarantees of quality*

As for any other medicinal product, it will be necessary to establish minimum quality guarantees throughout the manufacturing process. The AEMPS and the different regional regulatory agencies will agree to carry out a common policy on this issue. The regulation also differentiates between "open technique" and disposable kits (closed systems). The first will require more comprehensive quality controls and inspections. Disposable kits must always be medical device CE marked, indicating that they comply with European directives and should be used following the manufacturer's instructions,

- *Guarantees of efficacy*

The AEMPS recognizes that PRP is used in several areas of medicine, but the agency has no clear evidence of efficacy in all of them. Therefore, the AEMPS will create a report including applications for which there is clear evidence of efficacy, classifying the different applications of PRP in three categories depending on the available evidence: pathologies in which there is sufficient evidence to recommend the treatment, those

that have shown that the benefit-risk balance is negative and will not be recommended for use, and those requiring further evidence.

- *Guarantees of traceability*

One of the particular characteristics of this medicine is its biological origin, and therefore the possibility of transmission of disease must be present, despite its autologous character. Prescribing physician will have to adopt the specific control, supervision and traceability measures which prevent the transmission of infectious diseases. In this aspect, the Spanish legislation has several royal decrees on blood donation, autologous donation and autotransfusion, which must be taken into account in ensuring the safety of the procedure for obtaining PRP.

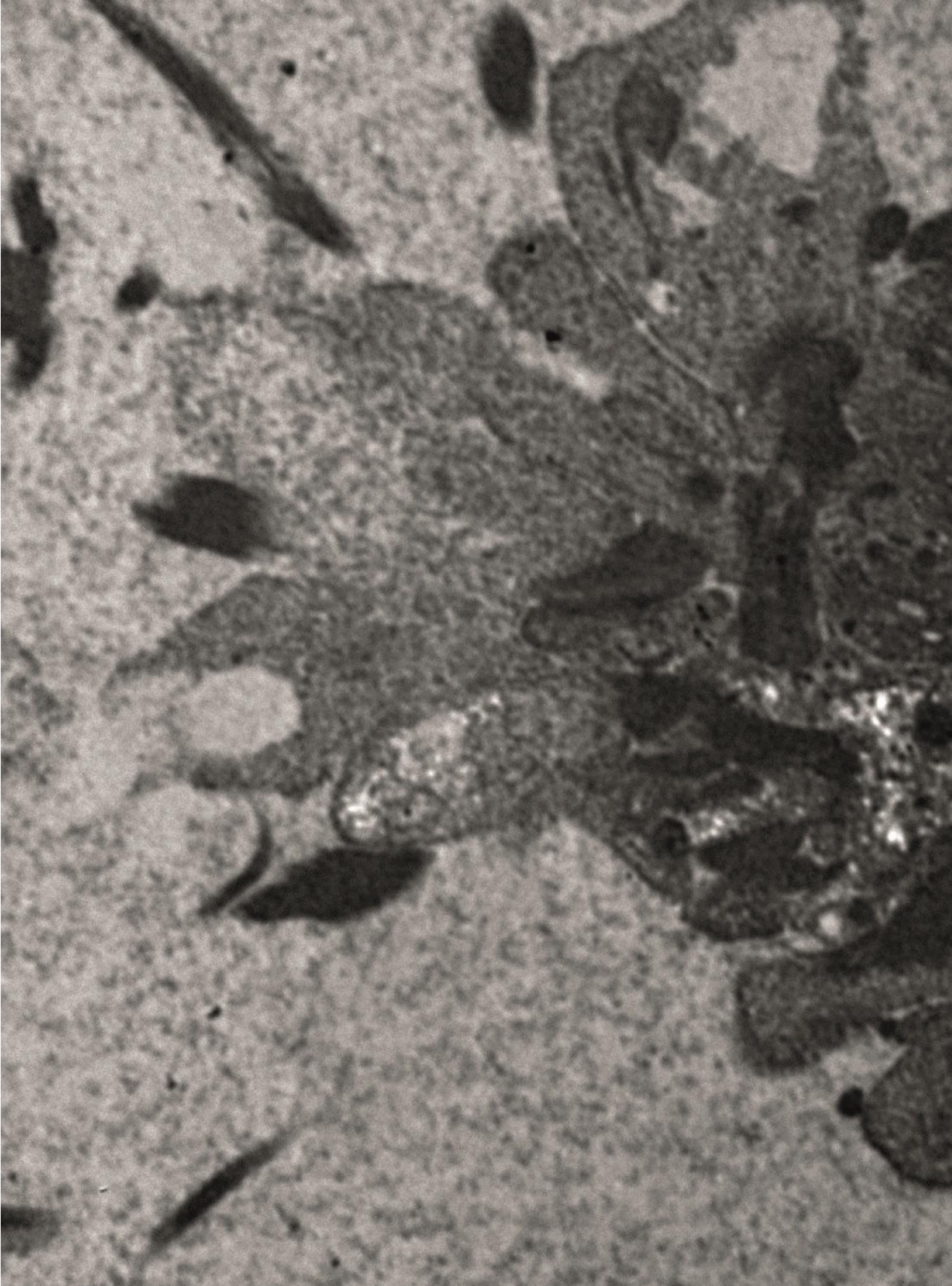
- *Guarantees of pharmacovigilance*

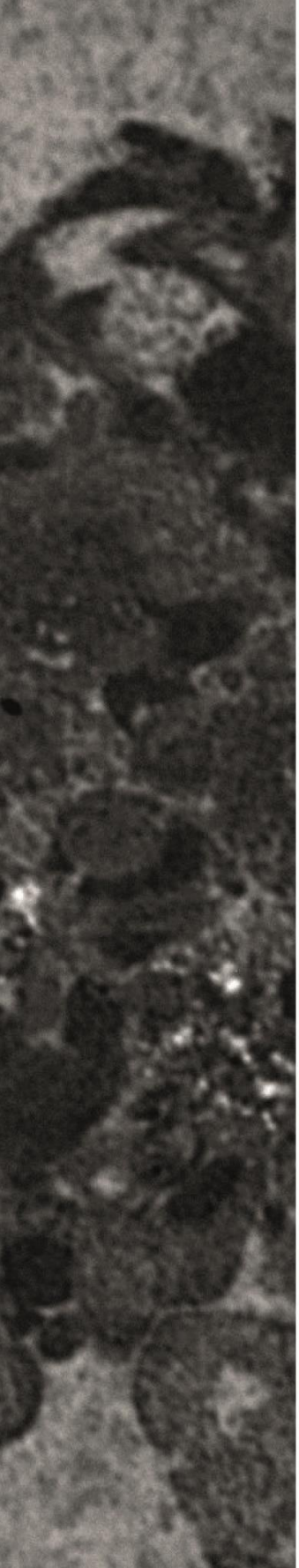
Another highlight of the AEMPS report is the duty to promptly notify to the competent pharmacovigilance authority of each regional government any suspected adverse reactions result of treatment with platelet-rich plasma. Thereby, it would be ensured in the same way as for a conventional medicinal product the rapid location of defective or contaminated lots, significantly increasing treatment biosecurity by establishing a registry of adverse events.

- *Guarantees of information*

All medicinal products must have a summary in which the characteristics of the medicine are detailed, and a package leaflet with basic information and instructions for the patient. However due to particular characteristics, PRP has no a registered summary of product characteristics. In spite of this and according to the new regulation, the patient should receive a minimum information before any treatment, ensuring that the product meets the quality requirements including

recognized efficacy, the pros and cons compared to other treatments, and any potential risks and/or side effects.





Chapter 7

Allogeneic platelet-rich plasma: at the dawn of an off-the-shelf therapy?

Perspective article published In *Trends in Biotechnology* [6]

Abstract

A new therapeutic tool is emerging based on a biomimetic strategy: platelet-rich plasma (PRP) technology. Allogeneic off-the-shelf PRP represents a safe and cost-effective treatment of osteoarthritis and related diseases. This breakthrough opens the door to improved treatments and opportunities but also to new challenges with multiple uncertainties.

1 Allogeneic platelet-rich plasma: at the dawn of an off-the-shelf therapy?

The use of autologous platelet-rich plasma (PRP) therapies has spread extensively in all fields of medicine, but especially in the treatment of musculoskeletal conditions, like osteoarthritis [114]. Currently, PRP technology is also used for *ex vivo* expansion and subsequent transplantation of stem cells in a clinical setting [108].This biologically inspired therapeutic approach uses the patient's own blood (Figure 25 A) to obtain a plasma volume enriched in platelets, known as PRP, to be applied in the patient's injury in order to relieve pain and speed up the recovery time.

Some limitations of PRP therapies include inter-individual variability; large blood quantities required in some cases; diabetic patients; and difficult treatment of neonates, infants, the elderly, or immunocompromised or debilitated patients, so treating certain patients can be challenging or even impossible. However, recent preclinical evidence, together with recent trials, may open up new avenues for PRP therapies.

Bottegoni *et al.* [255] recently published an article describing the treatment of 60 patients diagnosed with osteoarthritis of the knee with homologous (allogeneic) PRP therapy, or PRP obtained from donors in a non-autologous manner (Figure 25 B).

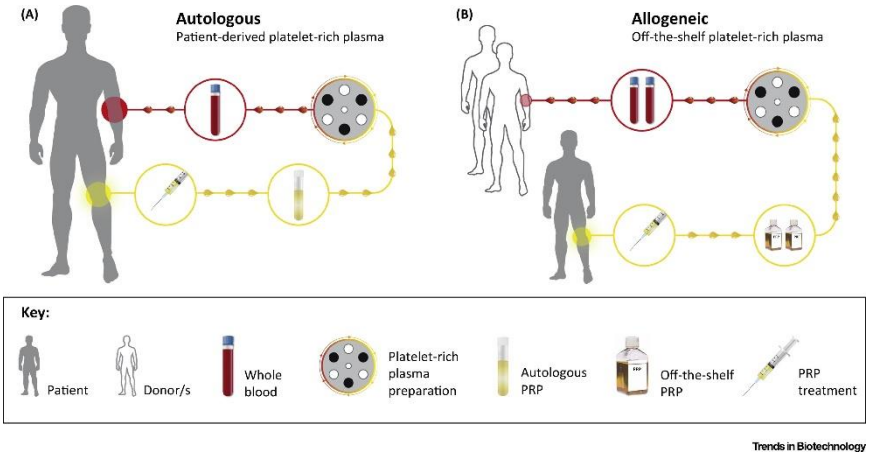


Figure 25. Comparison of the two different procedures of platelet-rich plasma (PRP) preparation. (A) Briefly, peripheral venous blood from the own patient is withdrawn into tubes. After that, the blood is centrifuged and the PRP is obtained through the separation from other blood components. PRP can subsequently be applied in form of different formulations with therapeutic potential for the treatment of the patient's pathology, such as osteoarthritis of the knee as shown in the figure. (B) By contrast, the allogeneic PRP is obtained from the peripheral blood of one or more healthy donors. Later, the off-the self PRP is prepared and preserved until its application in a patient. A same batch of this type of PRP could be used to treat a single patient over time or to treat several patients. The legend is shown at the lower part of the figure.

This is the first description in the scientific literature of knee osteoarthritis treatment carried out with non-autologous PRP. Interestingly, the authors of this study demonstrate that allogeneic PRP is safe, so it can be used in those patients in whom the use of autologous PRP would not be indicated, such as older patients with hematological disorders. In addition, despite being an initial pilot clinical study, the authors demonstrate that PRP therapy is effective in the short term (two and six months post-treatment), reducing pain and recovering articular function as assessed by three broadly used different clinical scores (IKDC, KOOS and EQ VAS scores) that evaluate the course of knee injury and treatment outcomes.

This is not, however, the first pioneering application of allogeneic PRP to treat musculoskeletal conditions. In two previous studies, non-autologous therapies combined with bone grafts were applied to treat disorders of the musculoskeletal system, such as long bone defects. The first study [256] reported the treatment of one single patient, whereas in the second study, a series of nine consecutive patients were treated [257]. Treatment with allogeneic PRP has also been carried out in other fields of medicine such as dermatology, ophthalmology, and oral and maxillofacial surgery.

We would like to make a recommendation concerning the terminology to use when referring to PRP obtained from donors, as we believe that is more appropriate to use the term "allogeneic" than the term "homologous," which could be misleading [258]. *Allogeneic* is defined as the transfer of cells, tissues, or organs between genetically different individuals within the same species. Conversely, *homologous* is used to broadly describe correspondence in structure, origin, and position [258]. The latter is in line with the FDA definition of homologous use for cell therapies [259]: namely, the origin of the cells could be autologous or allogeneic, while the intended use could be homologous or non-homologous.

In 2010, a panel of international experts published a consensus paper on the use of PRP in sports medicine [244], in which one of the topics discussed was the possible effect of PRP as athletic performance-enhancing substance (ergogenic effect) and its situation in the context of anti-doping regulation. The World Anti-Doping Agency (WADA) had included intramuscular infiltrations of PRP in its 2010 list of prohibited substances. However, other routes of administration were permitted, as long as they were declared. WADA's concern was based on the possible stimulation of muscle satellite cells with subsequent muscle growth and

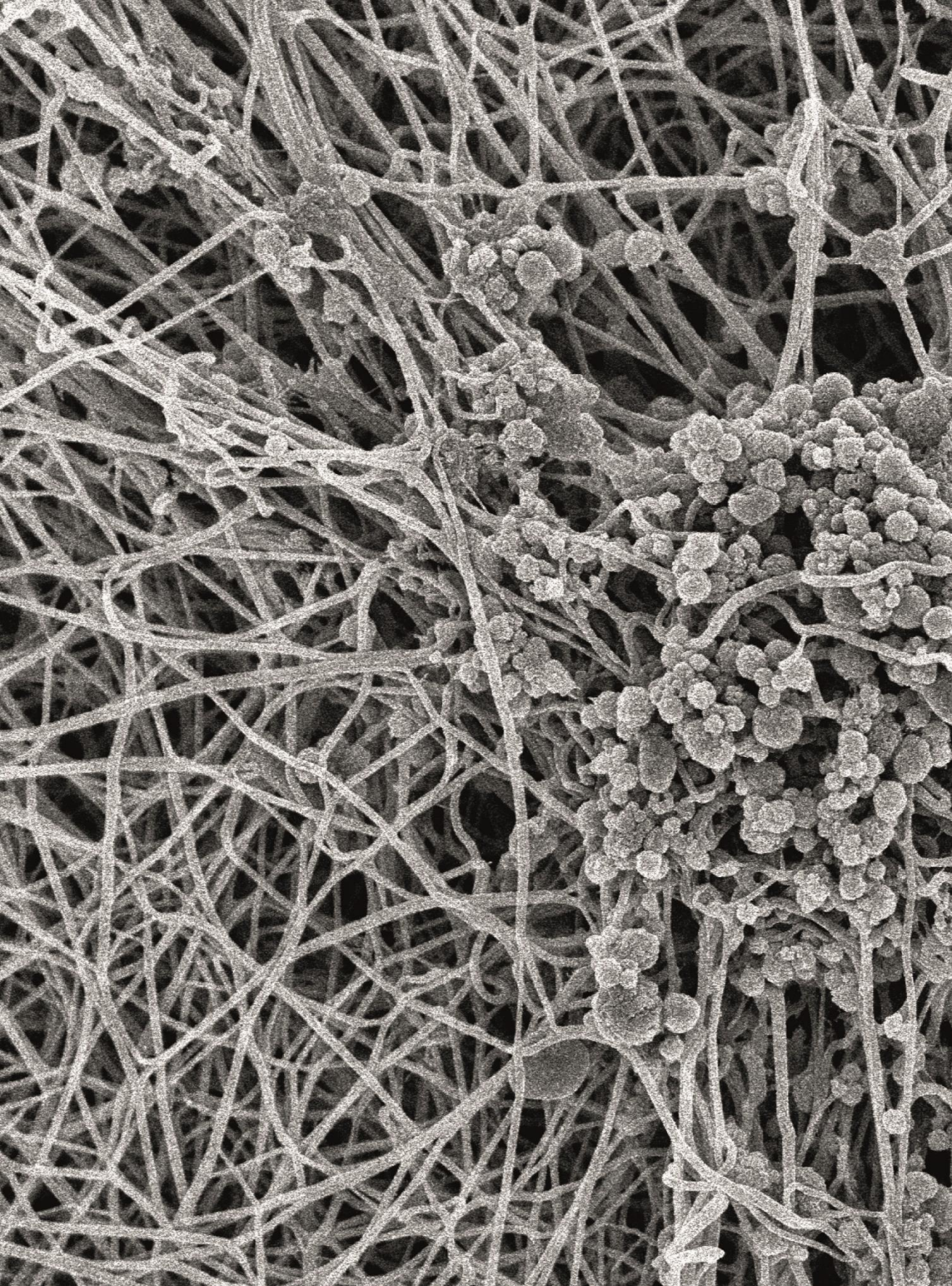
increased muscle capacity beyond simple regeneration. Later, the WADA lifted the ban in the 2011 list, and since then, the use of PRP by all routes of administration has been allowed (https://www.wada-ama.org/en/questions-answers/prohibited-list#item-380). However, the WADA does not differentiate between the use of autologous and allogeneic PRP. Allogeneic PRP opens a challenging debate, given that the last few years have seen an explosion in our understanding and use of blood-derived PRPs. Furthermore, in the current state of scientific knowledge, autologous PRP has not demonstrated any potential for performance enhancement beyond a therapeutic effect, while allogeneic PRP has not been studied from an ergogenic point of view.

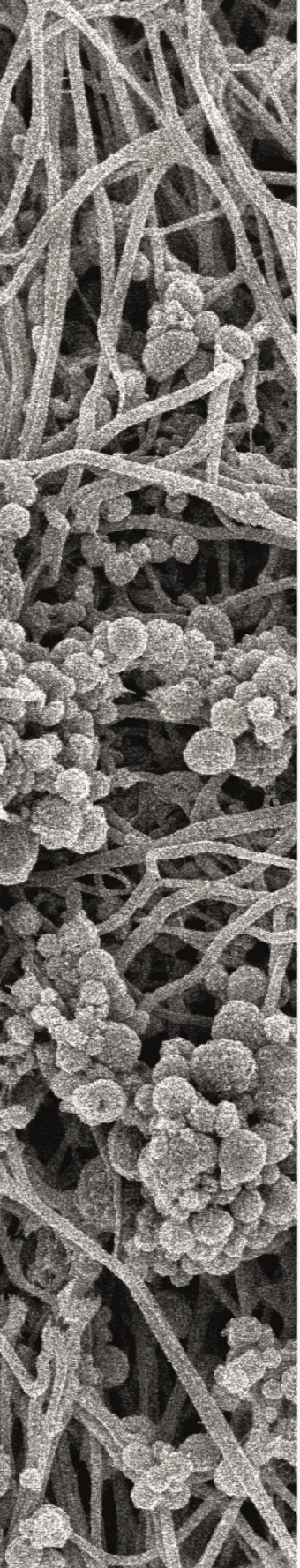
Some parameters that have historically not been taken into account, including age [260] and exercise [261] among others, can alter the balance of growth factors present in PRP. Hypothetically, it would be possible to postulate that injections of allogeneic PRP of selected donors could accelerate the regeneration of tissues more than autologous PRP can. Moreover, non-responders to such autologous therapies might otherwise be responders to off-the-shelf PRP. This idea is nothing new and is based on the parabiotic experiments conducted for more than 150 years ago but it is in the last two decades when major expectations and advances have been carried out. In such experiments, two animals share a common bloodstream [262] providing a unique opportunity to study the effect of the blood of a young animal in and old one. Surprisingly, researchers found improvements in the brain, heart and muscles of old animals that had been joined to young ones, which clearly implies that there should be circulating molecules in the blood of young animals responsible for this improvement in the old animals.

Another factor that presents some uncertainty is whether the presence of leukocytes in allogeneic PRP (L-PRP) could be detrimental so that only

leukocyte-free PRP (P-PRP) could be used as an allogeneic PRP. To the best of our knowledge, there is only one comprehensive description [263] of the immunogenicity of an allogeneic PRP infiltration in the bone and muscle of rabbits. No significant immunological reaction was observed by analyzing changes in the peripheral blood lymphocyte population ($CD4^+/CD8^+$) and the muscle morphology at the infiltration site.

Overall, we have seen that it is feasible to use an allogeneic PRP in the treatment of musculoskeletal disorders such as osteoarthritis, but many questions are yet to be resolved, ranging from the issue of terminology to potential doping to leukocyte inclusion in the formulations. True development of off-the-shelf PRP could represent a safe alternative, and perhaps a more effective one than autologous PRP. Progress in understanding the molecular mechanisms of action of PRP, and the development of PRP potency tests, could contribute to the development of a highly effective allogeneic PRP, not only for difficult situations but for patients in general.





Chapter 8

Addressing reproducibility in stem cell and PRP therapies

Perspective article published In *Trends in Biotechnology* [7]

Abstract

Stem cell and platelet-rich plasma therapies are promising breakthroughs in the treatment of multiple conditions. However, the lack of reproducibility due to inaccurately reporting the protocols used to obtain these biologics can hinder their therapeutic progress. Developing a reporting guideline for biological therapies is required to address this issue.

1 Reporting guidelines and reproducibility crisis

Proper reporting in clinical trials is gaining momentum in an era of reproducibility crisis, one of the biggest challenges facing the scientific community today [264]. The Consolidated Standards of Reporting Trials (CONSORT) statement published in 1996, and the CONSORT 2010 update, are key milestones for increasing the transparency and reproducibility of clinical trials [265]. The creation of the EQUATOR (Enhancing the QUAlity and Transparency Of health Research) Network was the first coordinated effort to systematically tackle the problems of inadequate reporting on a worldwide scale, thus extending the use of reporting guidelines to all areas of research in health sciences. The use of reporting guidelines has contributed to improve science as well as to increase the quality of research methods. For example, Cell Press has introduced a new format for reporting methods [266] called "STAR Methods" (structured, transparent and accessible reporting) in order to improve transparency and reproducibility, in consonance with the methodology of CONSORT and other reporting guidelines of the EQUATOR network.

2 Stem cells and platelet-rich plasma (PRP) as paradigm of biological therapies

New therapies, often experimental, are emerging in this era of reproducibility crisis, and create a new scenario of contradictory results, mainly due to their biological origin and to the great variability in the protocols used to obtain biological therapeutics. Stem cell [267] and platelet-rich plasma [8] therapies are the most promising ones, both in terms of number of registered clinical trials and by clinical impact. However, there is no agreement on their composition, dosage and administration schedule. Furthermore, many of the clinical studies

evaluating their efficacy and safety fail to report the methodological details that allow their replication and contrast with other studies.

We believe that to overcome these limitations and to fuel the advancement of stem cell and PRP treatments, a new reporting guideline for biological therapies should be implemented for adequate methodology reporting, as contemporary biological methods are not completely covered by the CONSORT statement [265] nor by any of its extensions. Likewise, this new reporting guideline would be appropriate for preclinical research.

It is the time to adapt current reporting guidelines to new ground rules that establish stem cell and PRP therapies, similar to others that have been developed for specific interventions, such as acupuncture interventions, non-pharmacologic treatments, or Chinese herbal medicine formulas [268].

3 Transparency and reproducibility in biological therapies

To the best of our knowledge, there are currently two worthwhile initiatives that seek to increase the transparency and reproducibility of stem cell and PRP therapies that could serve as keystones for the development of the new reporting guideline.

The first one, led by the International Society for Stem Cell Research (ISSCR), focuses on stem cell research and clinical translation [269]. Recommendation 3.3.6.3 in this detailed guide states that all randomized clinical trials (RCT) should be reported according to the CONSORT statement. This action, together with prior registration of all clinical trials, aims to increase the transparency and reproducibility of stem cell therapies. However, the CONSORT guidelines are not fully

adapted to all items that should be reported in this type of therapy, since it does not include key methodological details, such as the obtaining protocol or the administration protocol.

The second initiative was developed by a group of experts attending two symposia organized by the American Academy of Orthopaedic Surgeons/Orthopaedic Research Society (AAOS/ORS) and by the American Orthopaedic Society for Sports Medicine (AOSSM). These new recommendations, recently published by Murray and colleagues [270], will provide the basis for the "Minimum Information for studies evaluating Biologics in Orthopaedics" (MIBO) which should accompany any clinical study in this area. We encourage the use of MIBO. The MIBO philosophy is similar to any reporting guideline. It provides two independent and well elaborated checklists, one with 25 items for stem cell and another with 23 for PRP therapies. Both checklists are structured in different sections that correspond to the study workflow: study design, patient details, injury description, intervention, specific biological details of each procedure, delivery, postoperative care and outcomes including side effects. The core of both checklists is specific biological detail together with the delivery or administration of stem cells or PRP. This core had a pivotal role in the development of reporting guidelines not only for clinical trials, but also for *in vitro* and i*n vivo* preclinical studies.

4 Accurate characterisation is required

The accurate characterization of the products to be applied, either stem cells (Box 1 section) or PRP (Box 2 section), is one of the most relevant points to increase the reproducibility of studies. In the same way as the guidelines of the EQUATOR network, MIBO should continuously evolve and include items not currently listed, such as uses of allogeneic PRP [6]

and customized PRP, PRP classification, potency assays, determination of the chromosomal stability for stem cells, different culture supplements used in *ex vivo* expansion, or concomitant treatment (stem cell + PRP injection). MIBO recommendations could represent a turning point for adequate reporting of stem cell and PRP in clinical trials in orthopaedics with the aim of improving transparency and reproducibility.

One example of an opportunity for stronger recommendations is a systematic review recently published by Pas and colleagues [271] evaluating the efficacy of stem cell injections in knee osteoarthritis. The authors conclude that there is a high risk of bias in the studies evaluated and do not recommend this type of therapy for knee osteoarthritis. Among the recommendations made for new studies is to standardize the methodology and report the results appropriately following the CONSORT statement. However, making this statement by itself does not provide sufficient detail to achieve reproducibility in stem cell and PRP therapies.

More recently, on behalf of the Platelet Physiology Subcommittee of the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH), Harrison [272] highlighted that there is insufficient data for evidence-based guidelines development of PRP treatment. One of the causes of this inability is inadequate reporting of data on preparing and characterizing PRP. Only approximately 10% of the clinical studies in which PRP was used to treat musculoskeletal injuries adequately reported the protocol used to obtain the PRP, which implies that replication and comparison to other studies are almost impossible to achieve [273].

5 Concluding remarks and future perspective

The purpose of this paper is twofold: both to draw attention to the lack of transparency and reproducibility reporting both preclinical and clinical trials with stem cells and PRP, and to stimulate discussion and collaboration between stakeholders (authors, reviewers, journal editors, readers, funding bodies and policy makers) in order to develop a new reporting guideline for biological therapies. These ground rules should follow the premises of the EQUATOR network and the philosophy of the foundry reporting guidelines, such as the minimum reporting guidelines for biological and biomedical investigations (MIBBI) and their subsequent evolution to FAIRsharing [274, 275]. MIBO is a key starting point to building the foundations of a new reporting guideline, but at the same time it should also be broader than MIBO and be adopted for all medical and surgical specialties and not just for orthopaedics. Biological therapies are cross-cutting tools of all clinical specialties, and not only of orthopaedics. The new reporting guideline for biological therapies would work as a module or extension, both for clinical studies (as a CONSORT extension) and for preclinical research *in vivo* and *in vitro*.

Each product that is applied to the patient should carry its "barcode", its biological signature, in which all the necessary data and metadata [275] are described in a standardized and accurate fashion: origin, obtaining protocol, administration and all the relevant characteristics that may be involved in their safety and effectiveness. Only in this way it would be possible to compare between different products.

Patients deserve safe and effective treatments as in any field of medicine, which is accomplished in part by improving the transparency and reproducibility of the publications describing these treatments. Biological therapies present great potential, but their progress and

widespread as well-established medical practices may depend to a great extent on proper reporting of results. All of these suggest that there is a compelling need for a new reporting guideline for biological therapies, mainly stem cells or PRP. Consensus among all key stakeholders involved is essential to foster these therapeutics.

6 Box 1. Stem cell therapies.

Advances in stem cell therapies have raised expectations in the treatment of multiple conditions [267]. However, the clinical reality is not in accordance with their theoretical potential, due in part to the lack of standardization of this type of biological therapy. Therefore, it is necessary an adequate reporting of all the parameters involved in the obtaining, *ex vivo* procedures and administration of the stem cells (Figure 26).

The obtaining protocol must collect the relevant data that allow the entire procedure to be replicated, beginning with the origin of the donor tissue, since the cell populations that it contains may present different proliferative ability and differentiation potential, giving rise to different clinical responses.

Stem cells have to be expanded *in vitro* if they are needed in a clinically relevant number for many pathologies. Their heterogeneity has a marked influence on the properties of the expanded cells, since during the *ex vivo* expansion procedures a clonal selection is produced that could result in loss of proliferative capacities. It has also been shown that the choice of stem cell culture supplement, for example fetal bovine serum (FBS) or platelet derivatives, can originate different responses and influence the reproducibility of the process. According to good cell culture practices it is recommended not to use xenogeneic components, such as FBS, in order to reduce immunogenic risks.

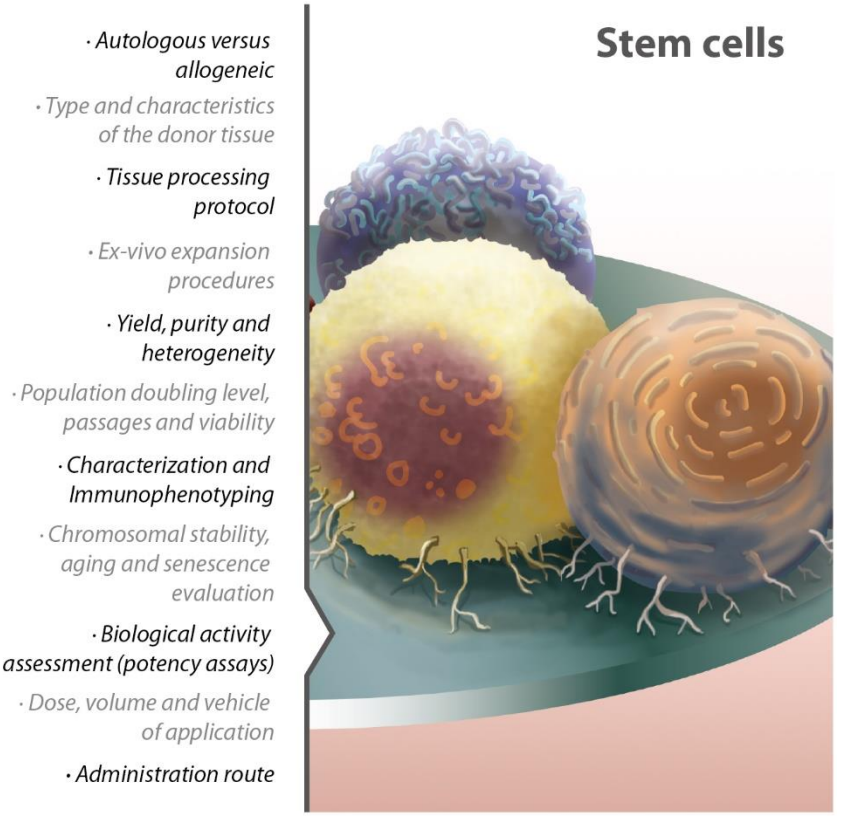


Figure 26. Key parameters in the proper reporting of stem cell therapies. Stem cells can be isolated from various tissues by multiple purification protocols. This diversity makes the comparison between trials a challenge. This figure describes critical parameters that should be reported for an adequate characterization, including those of MIBO (Murray et al, 2017) and other additional items.

One of the main concerns of *ex vivo* expansion processes is the possibility of spontaneous and uncontrolled transformation of stem cells due to their intrinsic potential. Therefore, it is critical to control and adequately report the whole *ex vivo* expansion process. It is key to specify the different parameters that guarantee that these cells are safe before treating the patient, such as yield, purity, heterogeneity, population doubling level, number of passages and viability. One

consistent observation is that the greater the time of in *vitro* expansion, the greater the risk of spontaneous transformation, whereby it is necessary to perform the characterization and the immunophenotyping immediately before the use of stem cells [276].

A critical point is the importance of developing and reporting robust potency assays that measure stem cell attributes that guarantee both quality and consistency and finally predict clinical efficacy.

Finally, it is essential to adequately define the administration route of stem cells, local or systemic, since this election can condition the efficient homing to injured organs and thereof drastically affect the clinical efficacy.

7 Box 2. Platelet-rich plasma therapy.

Platelet-rich plasma (PRP) is broadly defined as a blood derivative with a supraphysiological platelet concentration that is used to regenerate damaged tissues (Figure 27). Traditionally PRP has been obtained from autologous blood, however there is a growing trend towards to use of allogeneic off-the-shelf PRP in certain situations, but nevertheless the risk of blood-borne pathogens and alloimmunization must be minimized by viral detection and/or inactivation.

In addition to the characterization of blood safety, in all cases it would be necessary to carry out and report a basic blood count of the whole blood, in order to compare it with the PRP and thus determine if the obtaining protocol has been carried out accurately. With this performance characterization we would obtain the values of platelet enrichment and recovery with respect to the basal levels of peripheral blood.

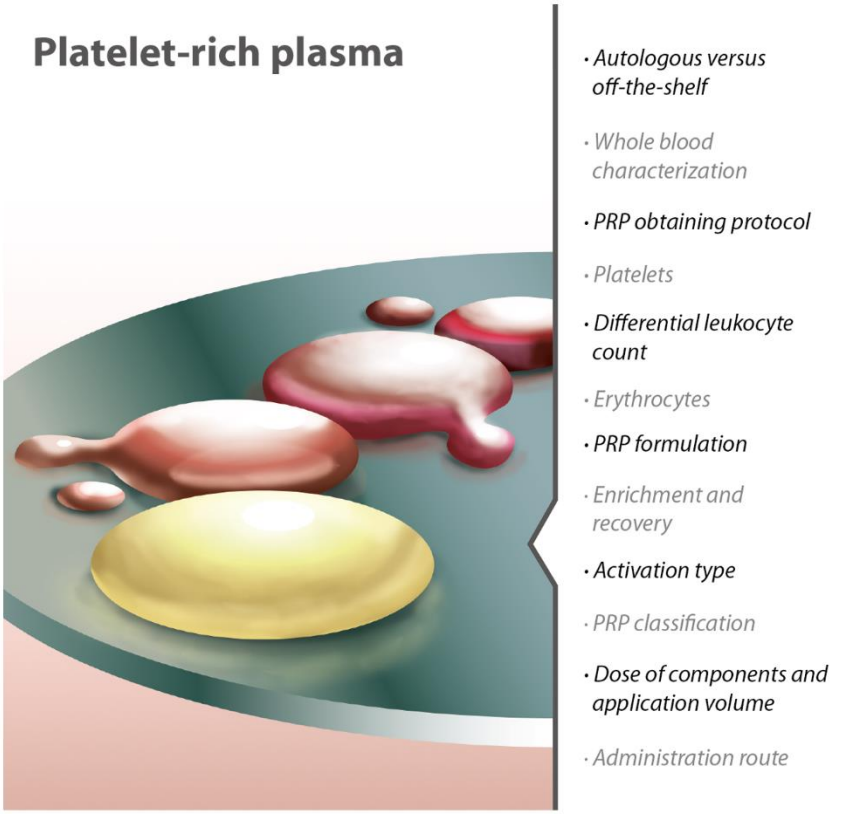


Figure 27. Core attributes in adequate reporting of platelet-rich plasma (PRP) therapies. The use of PRP as autologous biological therapy has reached unexpected limits in recent years, both in the number of clinical trials and in the obtaining systems (Padilla et al, 20171). However, the clinical outcomes are contradictory, mainly due to the variability of the formulations and the inadequate reporting thereof. The development of appropriate reporting guidelines that include the obtaining protocols is crucial to increase the reproducibility of this biological treatment (Murray et al, 2017).

Proper reporting of the PRP obtaining protocol (anticoagulant type, time and g-force of centrifugation/s or storage) is essential, since variations in it can generate different products in terms of enrichment, concentration, or purity of its components. Both viability and function

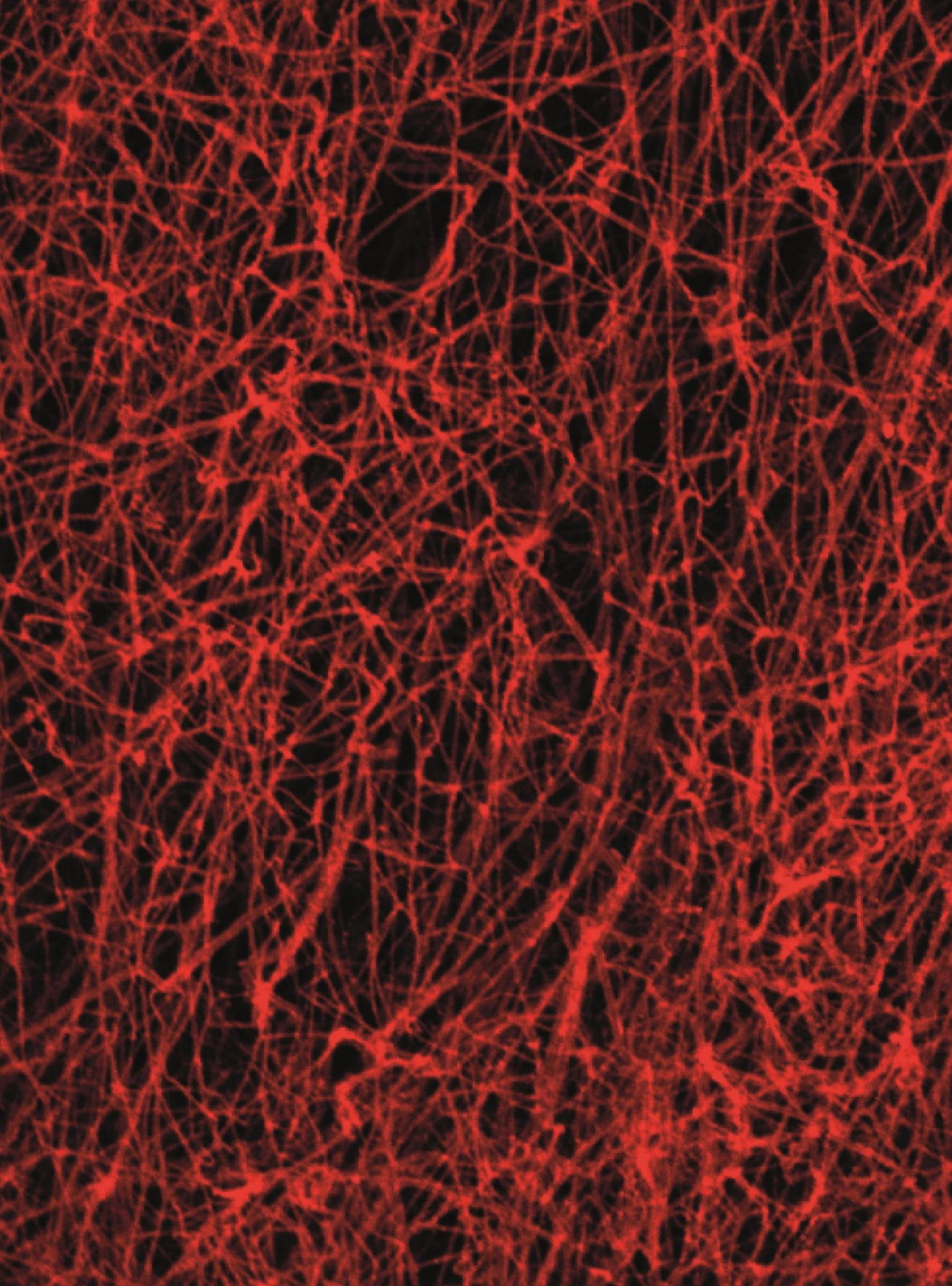
of platelets could also be compromised, leading to reduce the clinical efficacy.

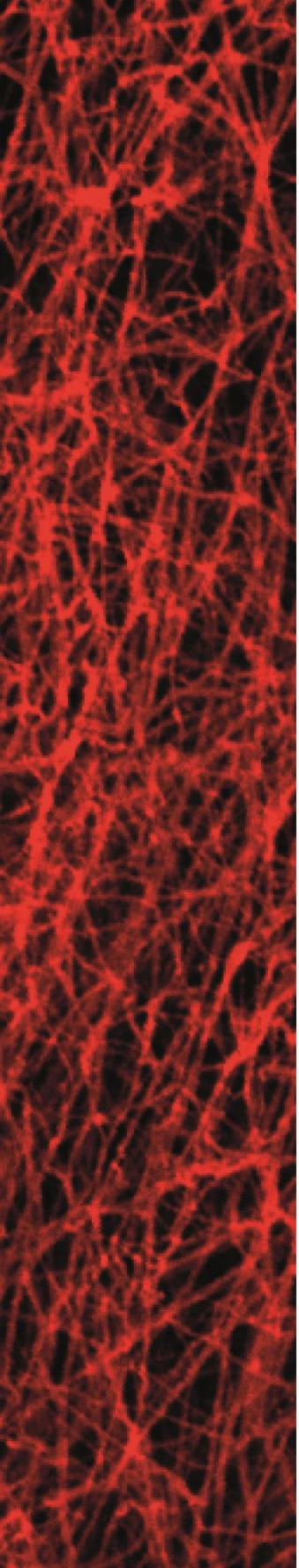
PRP always contains a supraphysiological concentration of platelets, however some formulations may be enriched in leukocytes (or in some of their subpopulations) and indirectly in erythrocytes. The presence of any of these elements modulates both the type and quantity of growth factors and cytokines present in the PRP and therefore the clinical outcome.

On the other hand, the type of activation of platelets (freeze-thaw, calcium chloride, thrombin, etc) can condition the growth factors release of platelet granules.

It is worth noting that in light of the great variability of issues that condition both the efficacy and the safety of the product, we consider that classifying the PRP is a key point in order to be able to compare different protocols.

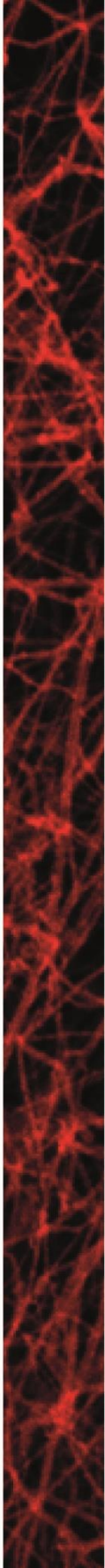
Finally, as in the case of any other medicinal product, a detailed report of the dose, route and schedule of administration is necessary. PRPs are complex multimolecular biological products, so the performance of both pharmacokinetic and pharmacodynamic studies is a challenge. Even thought PRP is applied locally in the injured tissue, its therapeutic action depends on both the receptor tissue and the type of formulation. The bioavailability and release kinetics of growth factors are conditioned by the matrix or scaffold that contains them. For example, the growth factors are retained in the fibrin matrix and released gradually over the time, unlike if they are administered in its liquid form without scaffold resulting in a fast release or burst.





Chapter 9

Discussion



1 Discussion

Regenerative medicine, a field in constant evolution that comprises a multidisciplinary area of research and clinical applications, aims to repair tissues, or even organs, and restore their function [277]. The phenomenon of an aging population and the increase in chronic and degenerative diseases is a challenge that we face today and requires a new strategy that allows the rapid regeneration and replacement of damaged tissues [278]. Without doubt, innovation in surgical techniques and transplants has been a milestone in the history of regenerative medicine (Figure 28), but the true revolution has emerged from the knowledge of the biological mechanisms that dictate the processes of tissue repair and regeneration [279]. Thus, we have moved from a twentieth century medicine in which symptoms were treated to a twenty-first century approach in which diseases are cured. However, the current use of organ and tissue transplantation to treat injuries and losses of organs and tissues suffers from a limited supply of donors and in some cases even from immune complications [280]. These hurdles can be overcome through the use of regenerative medicine and tissue engineering strategies. A further step is not only to develop new regenerative strategies, but to ensure that these are minimally invasive and cost effective in order to reach the greatest number of patients [281].

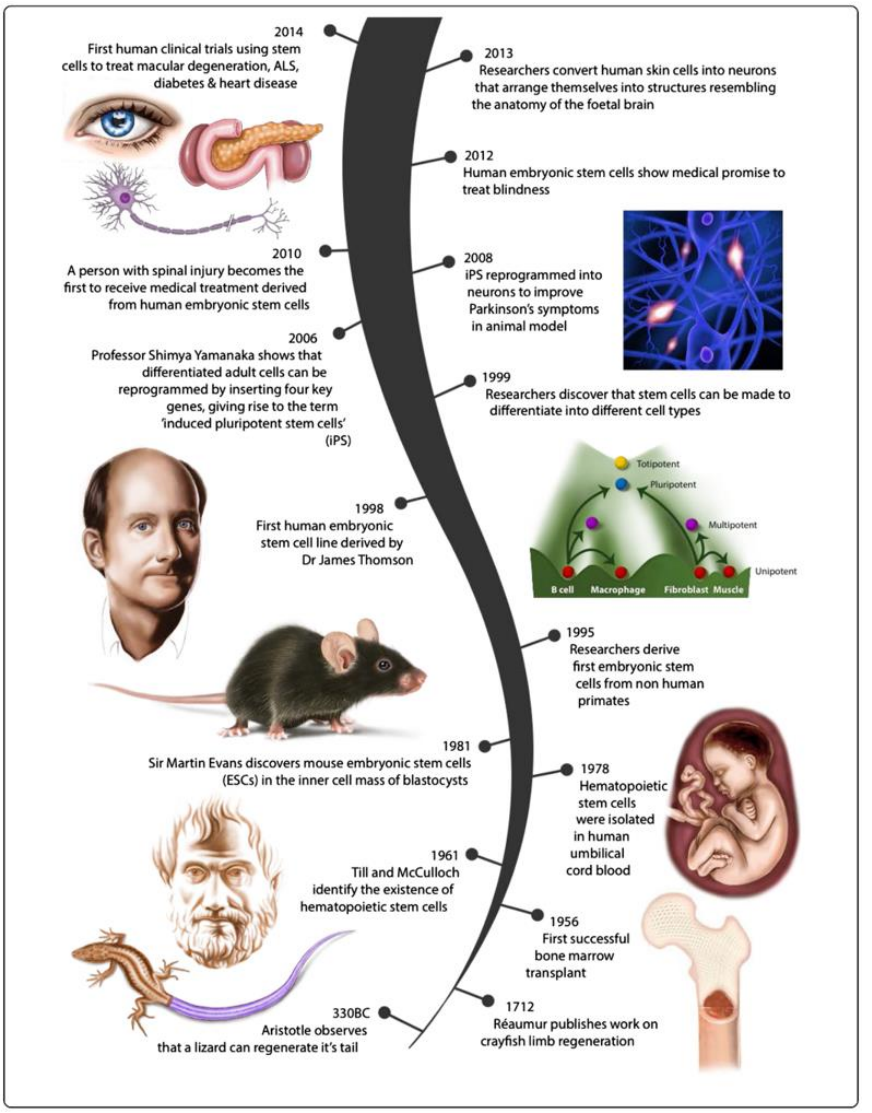


Figure 28. Summary of some of the main milestones in the field of regenerative medicine. Figure from Jessop et al 2016 (BMC Medicine 14:115).

To overcome these unmet medical needs, clinicians have a poweful tool based on the use of blood derivatives in tissue regeneration, based on mimicking natural wound healing processes as a therapeutic strategy

[282]. This approach is based on the role of some of the blood components in tissue regeneration in order to restore tissue functionality through regeneration rather than simple repair (Figure 29).

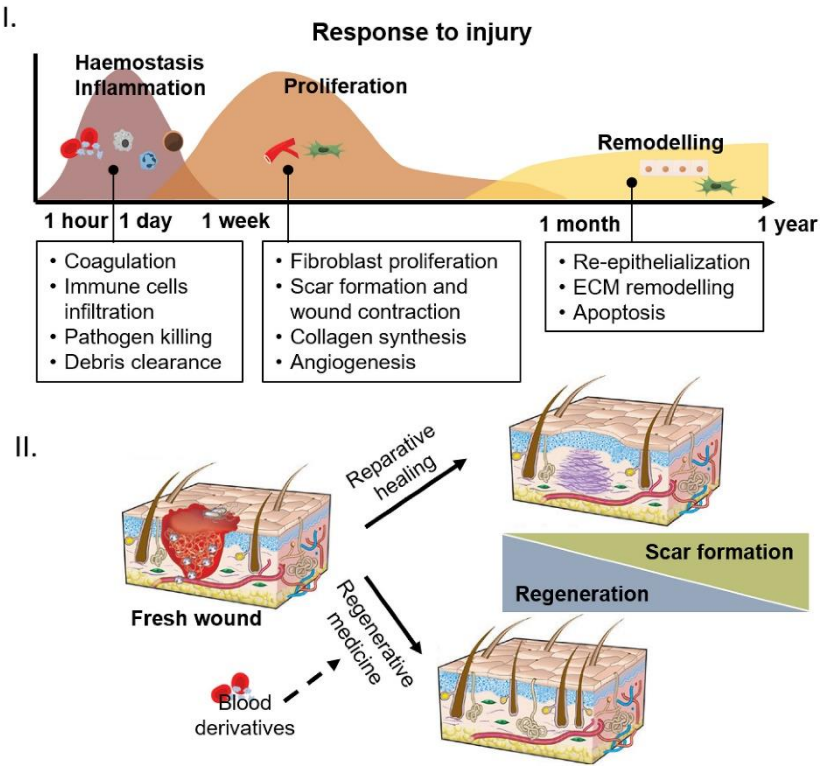


Figure 29. Overview of the natural phases of wound healing versus regenerative medicine therapy approach. (I) The processes of wound healing occur in an orchestrated manner in a spatial and temporal response to injury. (II) The balance of these events plays an important role in the switch between tissue repair and tissue regeneration after injury. Blood derivatives are expected to positively modify the outcome by attempting to generate functional tissue. Figure from Mendes et al 2018 (Adv Drug Deliv Rev 129:376-393)

Blood derivatives, usually known as platelet-rich plasma, use the body's own mechanisms to promote the regeneration of damaged tissue. This

aid consists of the controlled release of multiple molecules with regenerative capacity at the site of the injury with the ultimate goal of mimicking and accelerating the body's own natural regeneration processes. As we have learned from this hypothesis, platelets are the main actors in this optimization of the natural process of wound healing, as they present several hundreds of molecules in their granules, which are released at the site of the injury after their activation (Figure 30) [125].

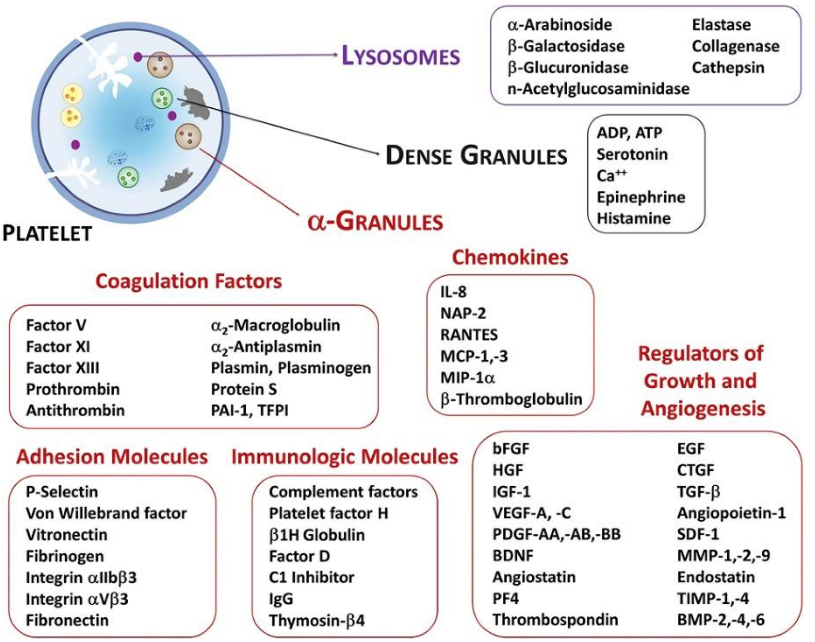


Figure 30. Platelet granules store a myriad of bioactive molecules. Dense granules, alpha granules and lysosomes contain several hundred powerful substances such as lysosomal enzymes, coagulation factors, immune and adhesion molecules, chemokines and growth factors among others, all of which are focused on the defense against aggression and on promoting tissue regeneration. Figure from Burnouf et al 2016 (Biomaterials 76:371-387).

However, there are contradictory results in the clinical efficacy of platelet-rich plasma, mainly due to the different formulations that are

used in clinical practice. There are multiple variations in the composition and enrichment of the various components, such as platelets, leukocytes, erythrocytes or plasma proteins [30, 283, 284]. Within the jungle of all these different platelet-rich plasma products, plasma rich in growth factors - PRGF is a technology with more than two decades of evolution that obtains several therapeutic formulations from a small volume of blood, producing a plasma that is free of both leukocytes and erythrocytes, and that moderately concentrates the platelets [1]. To ensure both the safety and the clinical efficacy of PRGF, it is necessary to know in detail its composition and mechanism of action. For any medicinal product with a defined composition it is relatively easy to perform. However, in the case of biological products such as PRGF, the accurate characterization presents a great challenge.

Taking this context into account, the experimental work of the current doctoral thesis focused on the systematic and detailed characterization of PRGF and its posterior optimization (Figure 31).

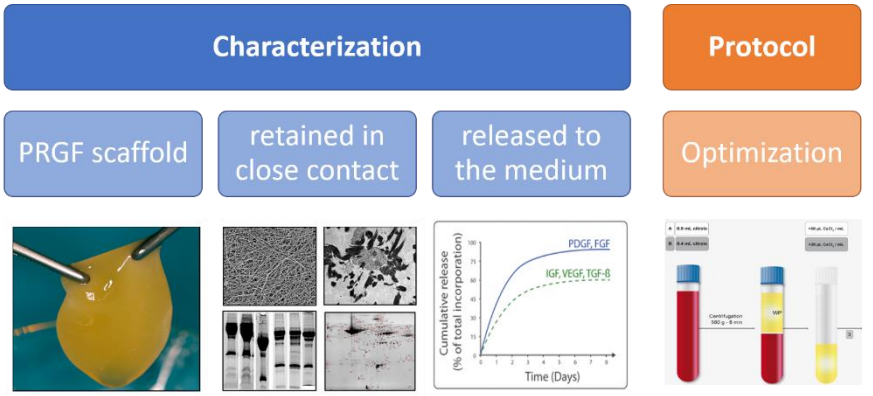


Figure 31. Summary of the experimental work of this thesis, both characterization and optimization of the PRGF technology.

In a first step, we aimed to perform a detailed proteomic characterization of the biomolecules that are retained in close contact with the fibrin network of the PRGF clot [2]. For that purpose, we carried out a high-performance proteomic characterization of proteins that are retained in the PRGF-fibrin scaffold. As it is known, the main constituent of this clot is the fibrin network, but there is little knowledge about other proteins that interact with this scaffold and can act as adjuvants in the wound healing process. In the course of the fibrin polymerization process (Figure 32), multiple molecules are trapped inside it, mainly growth factors released from the platelet degranulation and different molecules from the plasma [285].

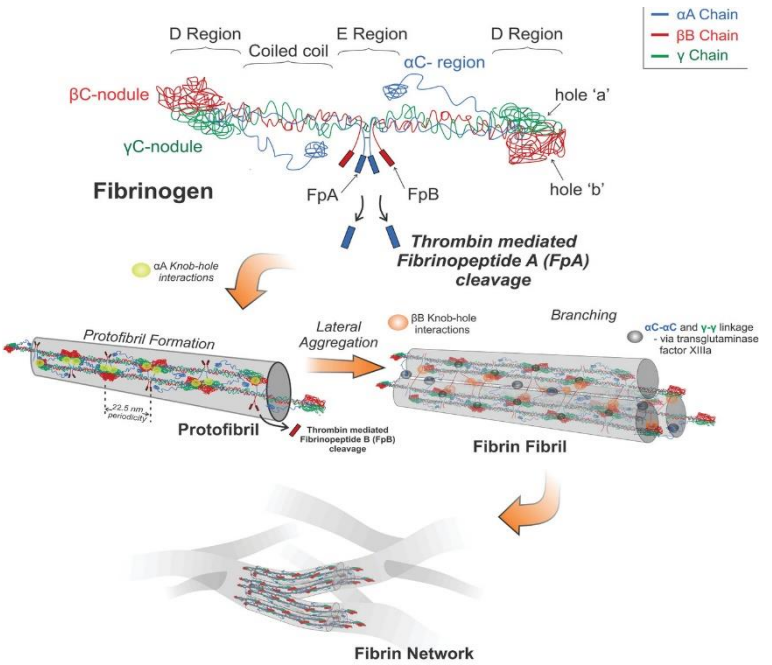


Figure 32. Overview of the conversion of the soluble fibrinogen molecule into the insoluble fibrin. Once the fibrin is polymerized, the fibrils branch and interconnect to form a fibrin network. This is the final step in the natural coagulation cascade that PRGF technology mimics. Figure from Roberts et al 2020 (Macromol Biosci 20: e1900283).

All these proteins bind to the heparan sulfate proteoglycan domains of fibrin through a crosslinking reaction that functionalizes fibrin during the fibrinogen polymerization with multiple cell-signalling molecules related to wound healing [286].

A double proteomic approach was performed in order to characterize the proteins enclosed and retained in the PRGF scaffold. On the one hand, a 1D-SDS-PAGE approximation followed by LC-MS/MS, and on the other 2-DE followed by MALDI-TOF/TOF (Appendix I. Supplementary Figure S1). Through this dual approach, an in-depth description of the most relevant proteins that were in close contact with the fibrin matrix was obtained. Specifically, we detected the presence of TSP-1, vitronectin, fibronectin, alpha-1-antitrypsin and alpha-1-anticymotrypsin, among others, all of them proteins of relevant interest in wound healing. Afterwards, a ontological analysis of genes of all identified proteins was carried out, showing a significant enrichment of certain terms of ontological analysis that were associated with tissue repair and regeneration, such as immune response, wound healing, angiogenesis, cell migration and adhesion, and lipid metabolism, among others.

Since the pioneering publication of this proteomic study [2], and to the best of our knowledge, only two studies have reported a detailed proteomic characterization of the PRP [287, 288]. Longo et al [287] have compared and characterized the proteins of the platelet gel releasate from adult peripheral and cord blood, finding globally similar results, even though the methodology was different from our dual approach. Recently, Lee et al [288] have published a proteomic study in which they describe the proteins present in PRP obtained through two different commercial systems. The results are similar for both, and also to those obtained with PRGF, that is, an enrichment of proteins related to wound

healing. Unfortunately, this study does not explore the differences between the commercial PRP systems with and without leukocytes.

The next goal was to characterize the molecules that are released by the PRGF clot. For this purpose, we studied the release kinetics of several growth factors and proinflammatory molecules, in comparison with a formulation that not only contains platelets but also leukocytes and erythrocytes [3]. The biological mechanism by which PRGF exerts its therapeutic effect is partly determined by the release of the molecules it contains, including growth factors, into the microenvironment surrounding the area of application. In this way, we analyse the release kinetics of six of the main growth factors (PDGF-AB, TGF-β1, VEGF, EGF, HGF and IGF-I) involved in the tissue regeneration process [3]. For this purpose, PRGF scaffolds were incubated for 8 days at 37 ºC. Altogether, the results showed the existence of an initial release phase with a high percentage of the content of each molecule released during approximately the first 24 hours (initial burst effect). Afterwards, a second phase of slow and sustained release followed (controlled release), in such a way that after 8 days the PRGF matrix retained approximately 30% of the total amount of the growth factors.

The next stage of this study was to compare this release pattern with the one obtained if leukocytes were included in the formulation (L-PRP) [3]. We also studied the release of two proinflammatory molecules, IL-1β and IL-16, in the PRGF and L-PRP matrices at different study times. Both scaffolds showed similar release profiles for all of the growth factors analysed, with the exception of VEGF. Specifically, a drastic decrease of VEGF in the culture medium was observed after three days, until it disappeared after seven or eight days. The removal of VEGF is probably due to a possible uptake of this molecule by its soluble receptor synthesized by leukocytes or to the destruction by proteases

released from them. It has recently been described that leukocytes have the ability to regulate VEGF signalling by releasing both VEGF and its soluble antagonistic VEGF-1 receptor [289]. In any case, this fact highlights the negative role of leukocyte inclusion in the PRP, since their presence would translate into a potentially lower angiogenic response. Another relevant result of this research was the demonstration of the absence of inflammatory cytokines in the PRGF fibrin matrix compared to a high release observed in the case of those prepared with leukocytes (L-PRP) [3]. With all these data, in addition to contributing to the characterization of PRGF's active ingredients, we have set the groundwork for trying to unravel the molecular mechanisms responsible for its clinical effectiveness and the differences with other formulations that contain leukocytes.

After conducting a comprehensive characterization, the next stage of this doctoral thesis was to optimize the protocol for obtaining PRGF and compare it with the former one [4]. As with any medical procedure, PRGF is a constantly evolving technology, and therefore it is necessary to adapt it to the new scientific evidence and the current regulatory framework. The new PRGF obtaining protocol consist of reducing the levels of anticoagulant and activator, in order to perform a more physiological protocol. The current protocol (PRGF-A) uses extraction tubes with 0.9 ml of trisodium citrate as an anticoagulant and 50 µl of calcium chloride / ml of PRGF to activate it. In contrast, the new protocol (PRGF-B) reduces the amount of sodium citrate and calcium chloride to 0.4 ml and to 20 µl, respectively. No parameter of those studied had worse results in the new protocol. Furthermore, most of the results of new protocol were improved. When it was proposed to optimize the PRGF obtaining protocol, the need to reduce the concentration of both anticoagulant and activator was taken into account, but maintaining the necessary amounts to prevent spontaneous coagulation of the blood,

while allowing controlled activation with calcium chloride when required by the clinician.

Once the PRGF protocol had been characterized and optimized, we proposed to take another step forward and perform a theoretical analysis of three key points in the development of therapies with the blood derivatives, namely, the new PRP regulatory framework in Spain, the possibility of using allogeneic PRP as therapeutics, and the requirement for standardization of biological therapies such as PRP.

In relation to the new regulatory framework of the PRP in Spain, an exhaustive review has been carried out of the new regulatory framework for platelet-rich plasma introduced by the Spanish Agency for Medicines and Healthcare Products (AEMPS) in Spain [5]. The implications of considering PRP as a medicinal product for human use and the guarantees of safety and efficacy offered by this classification have been discussed. In addition, the gaps and limitations of these rules have been analysed [5]. Unfortunately, the AEMPS has not moved forward with this regulation, as the indications for the use of PRP in different pathologies based on current scientific evidence remain to be developed.

We later focused on the possibility of applying PRP from a donor to treat a different patient, i.e. to perform an allogeneic therapy with PRP [6]. The advantages and disadvantages were presented, and it was clearly concluded that the development of off-the-shelf PRP is crucial and it is postulated as a huge market niche. The development of long-term preservation techniques, such as freezing or freeze-drying, may be required for adequate translation to the market and at the same time to compete with the autologous option. Such strategies are been recently tested on small groups of patients, such as freeze-dried PRP for the treatment of osteoarthritis [290].

The appropriate reporting of the methods of obtaining and applying the PRP is another essential issue and it has become clear in the previous chapter of this thesis [7]. In fact, Murray et al [291] recently publishes a very illustrative infographic (Figure 33) in which the most important points of MIBO, both for PRP and stem cell therapy, are summarized graphically.

Figure 33. Infographic that summarizes the minimum information for studies evaluating biologics in orthopaedics (MIBO). There are some parameters common to all biological therapies and others specific to PRP or stem cell therapies. MIBO is focused on sports medicine and orthopaedics. Figure from Murray et al 2019 (Br J Sports Med 53: 974-975).

In line with the aforementioned, the only journal entirely dedicated to the platelet world, "*Platelets*" journal, has defined a new policy for the publication of articles with PRP, both preclinical and clinical [292]. These guidelines aim to adequately report the methods in which PRP are obtained and applied, i.e. basically the items originally described by Murray et al [270] in the MIBO statement for studies related to traumatology and orthopaedic surgery, and that recently Anitua et al [7] have proposed to extend to all therapeutic areas in which PRP are applied. This is a relevant example of how an accurate standardization and reporting of the PRP obtaining methods is going to be implemented, not only in orthopaedics, but in all medical specialties, as advocated in our publication [7].

PRGF technology has been applied for more than twenty years for the treatment of multiple pathologies (Figure 34) [285]. This technology is a pioneer among blood derivatives [139]. Its application began in the field of oral and maxillofacial surgery [137], and later extended to other areas. Traumatology and orthopaedic surgery was the next medical specialty [8]. In fact, the first scientific publication using PRP in traumatology was the treatment of a cartilage avulsion with PRGF in 2003 [293].

Later, other areas such as ophthalmology [98] or dermatology [294], have been developing specific procedures in their field to treat different pathologies with PRGF. For all these reasons, it is necessary to perfectly translate the advances produced in the development of PRGF. However, this translation must be bidirectional [295], in order to optimize the formulations applied to the patients, who are ultimately the ones who benefit.

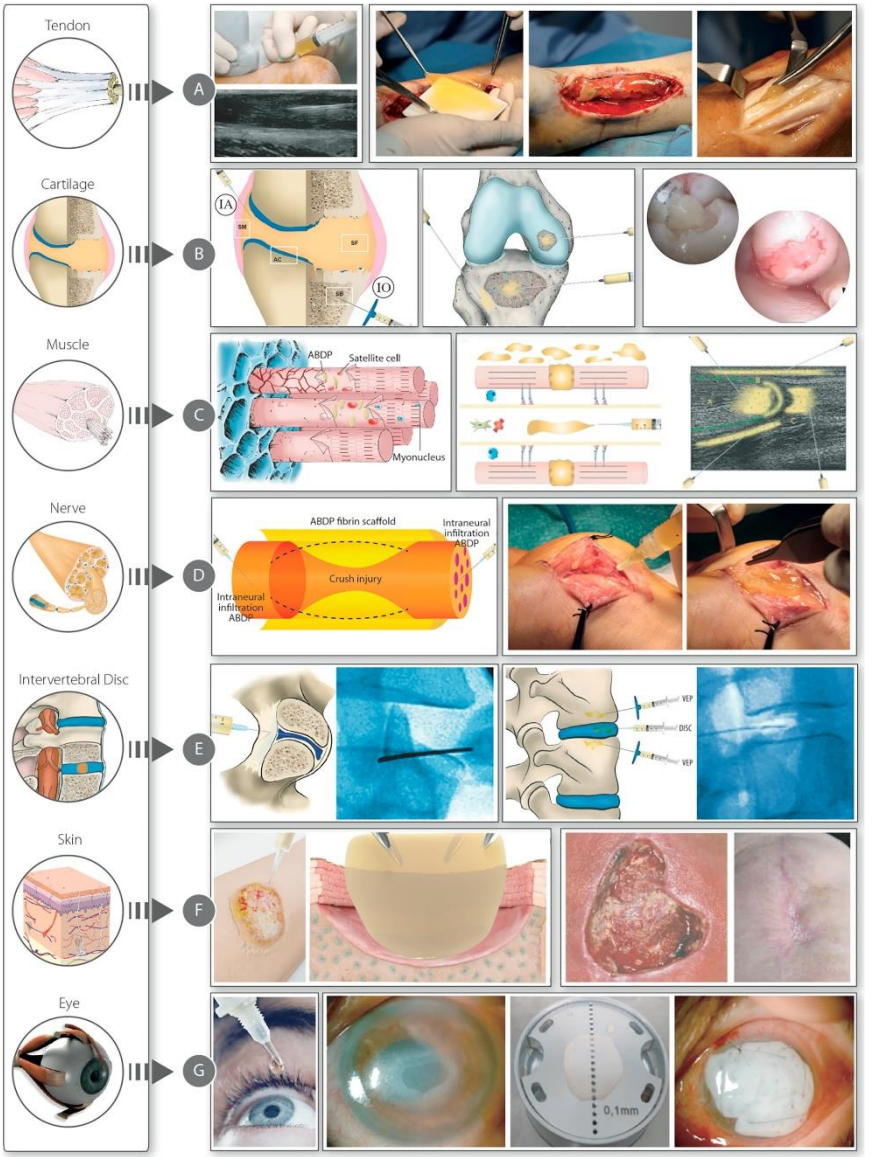
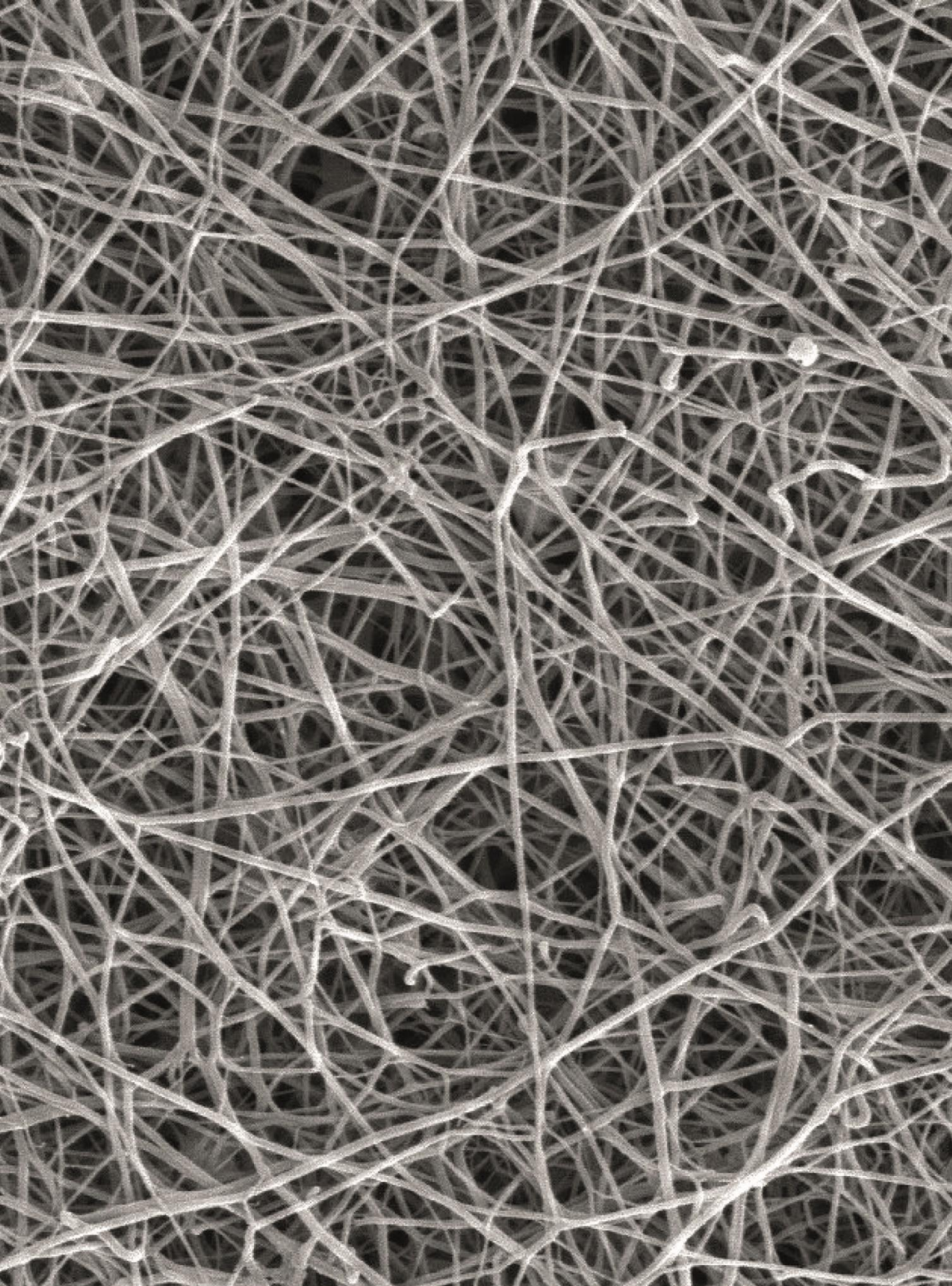


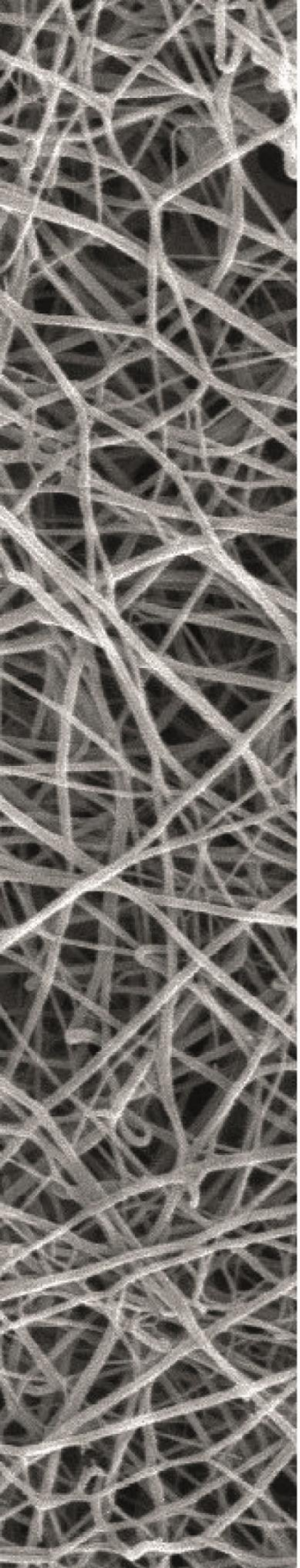
Figure 34. Summary of the application of PRGF technology in the treatment of various pathologies (A) chronic tendinopathies, (B) severe knee osteoarthritis and osteochondral injuries, (C) muscle tears, (D) peripheral nervous system injuries, (E) chronic low back pain, (F) ulcers and skin burns, and (G) ocular surface injuries. Figure from Anitua et al 2019 (Biomaterials 192;440-460).

As already mentioned, in this translation from the bench to the bedside it is essential to perform an appropriate PRGF characterization, to attempt to guarantee and optimize its clinical effectiveness. Once the active ingredients, the biomolecules that are present, have been described, it is necessary to determine their contribution to clinical outcome.

For this purpose, one of the challenges that will arise in the future is the development of potency assays for PRGF [296]. The progress of other types of biological therapies, such as stem cell treatment, has been enhanced by the development of these types of assays [297]. It is therefore necessary to move forward and develop potency assays that not only allow us to evaluate the PRGF activity in certain pathologies, but also to optimize the different formulations, customizing the PRGF technology in function not only of the pathology, but even of its stage. To date there are few preclinical and clinical studies that evaluate the customization of PRP formulations, both including and eliminating molecules. One of the most attractive approaches is the removal of TGF-β from the PRP, in order to try to attenuate possible fibrosis in muscle regeneration [298]. If we focus on additive strategies, a supplementation of bone morphogenetic protein 2 (BMP2) could be helpful in stimulating bone regeneration [299], or with basic fibroblast growth factor (bFGF) to stimulate epithelial regeneration [300]. PRGF contains all these biomolecules, but enhancing or silencing some of them, it would be possible to improve the clinical outcome.

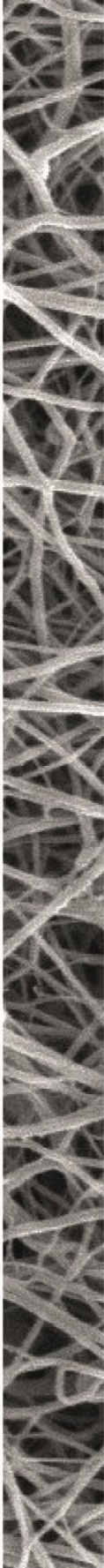
We are at the dawn of a new type of personalized medicine, and the results and reflections provided in this doctoral thesis are a starting point for the expansion of PRGF technology in a way that is customized to each patient, both in a safe and effective manner.





Chapter 10

Conclusions - Conclusiones



1 Conclusions

Following the specific objectives of this doctoral thesis, the conclusions obtained are the following:

1. A high-performance proteomic characterization of the PRGF-derived scaffold has been performed, identifying proteins that are bound in close contact with the fibrin network, and that are not released to the supernatant, but remain retained in the mesh itself. These proteins have been classified into families based on their function and gene ontology. It has been observed that PRGF's fibrin network is enriched in proteins specifically involved in tissue regeneration and wound healing.

2. The release kinetics of morphogens and pro-inflammatory cytokines was determined in PRGF scaffolds for eight days. Furthermore, it was observed that the inclusion of leukocytes in PRGF did not increase the release of growth factors, but it did increase the presence of IL-1β and IL-16, two pro-inflammatory cytokines that were practically absent in leukocyte-free PRGF matrices.

3. A new PRGF obtaining protocol has been developed in which both the concentration of the anticoagulant (sodium citrate) and the activator (calcium chloride) are reduced. In this way, a double objective has been achieved: to develop a more physiological protocol that adapts to the new regulatory requirements and, on the other hand, to improve some of the

characteristics of the obtained PRGF, such as increasing platelet enrichment, decreasing coagulation and retraction time, or increasing the concentration of growth factors.

4. The new regulatory framework for PRPs, including PRGF technology, has been analysed after the AEMPS resolution that considered the application of the PRP as a medicine for human use, but not for industrial production or advanced therapy. This resolution is positive in order to guarantee the adequate use of PRP therapies; however, this new regulation leaves relevant issues to be clarified in the future.

5. The use of allogeneic PRP has multiple advantages over autologous treatment and is a strong candidate for widespread its use in the short term. However, there is currently little clinical experience, so more studies are needed to confirm both its efficacy and safety.

6. It has been observed that the lack of reproducibility in the PRP obtaining protocols is a limiting factor in the progress of PRP biological therapies. Thus, it is recommended to make a detailed characterization of the product to be applied. In addition, the methods used must be described in an appropriate and unified fashion following the guidelines in line with those established by the EQUATOR network, and more specifically by MIBO. Therefore, it is necessary the development of a new guide to inform and publish, both for preclinical and clinical research.

2 Conclusiones

Siguiendo los objetivos específicos de esta tesis doctoral, las conclusiones obtenidas son las siguientes:

1. Se ha realizado una caracterización proteómica de alto rendimiento del coagulo derivado de PRGF, identificándose proteínas que están unidas de forma muy estrecha a la red de fibrina, y que no son liberadas en el sobrenadante, sino que quedan retenidas en la propia malla. Esta serie de proteínas ha sido clasificada en familias en base a su función y su ontología génica. Se ha observado que la red de fibrina de PRGF está enriquecida en proteínas específicamente involucradas en la regeneración de los tejidos y en la curación de heridas.

2. Se determinó la cinética de liberación de morfógenos y citoquinas proinflamatorias en las matrices de PRGF durante ocho días. Además, se observó que la inclusión de leucocitos en el PRGF no aumentó la liberación de factores de crecimiento, pero sí la presencia de IL-1β y and IL-16, dos citoquinas proinflamatorias que se encontraban prácticamente ausentes en las matrices de PRGF libres de leucocitos.

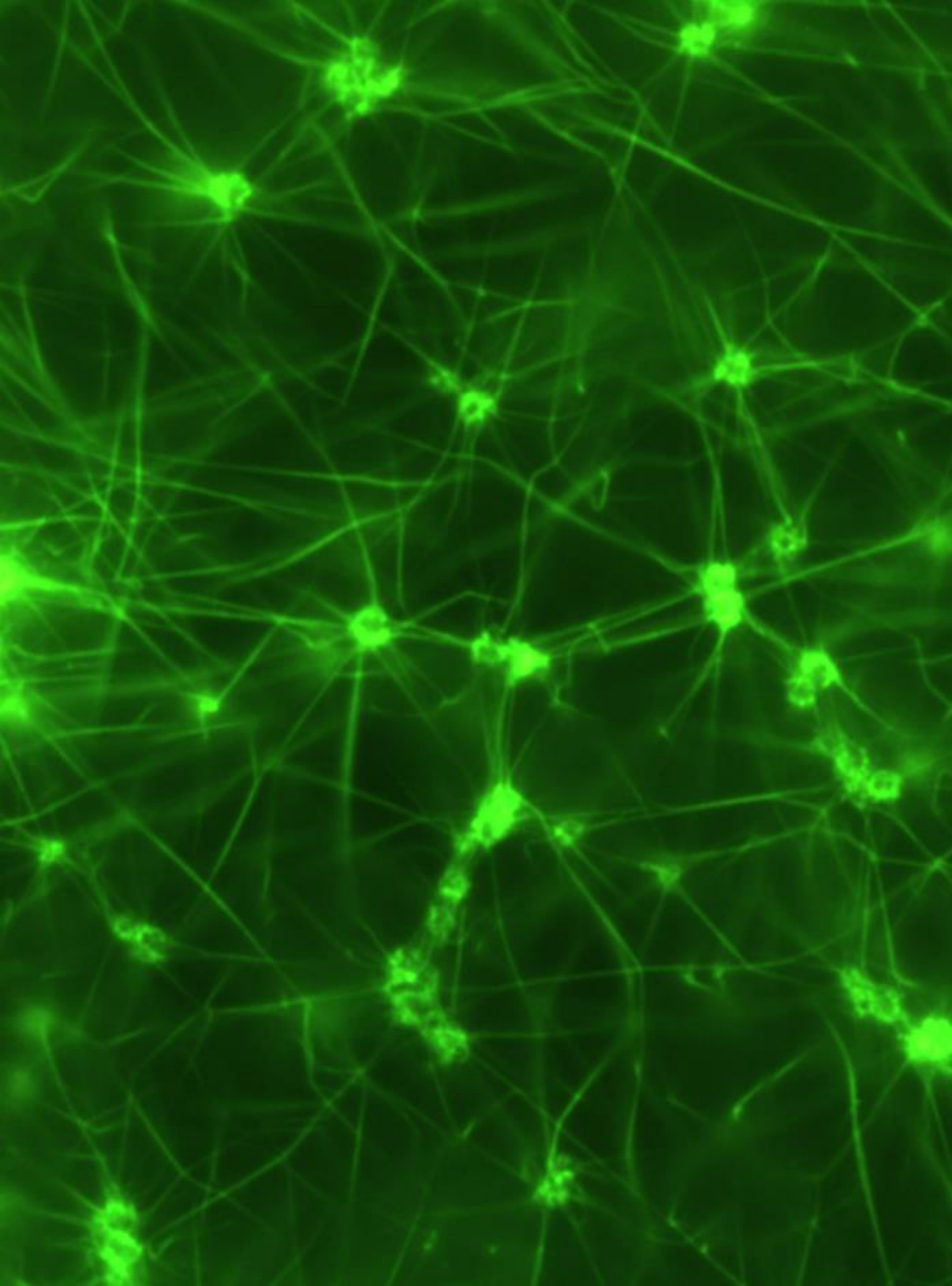
3. Se ha desarrollado un nuevo protocolo de preparación de PRGF en el que se reduce tanto la concentración del anticoagulante (citrato sódico) como del activador (cloruro cálcico). De esta forma se ha logrado cumplir un doble objetivo: lograr un protocolo más fisiológico que se adapta a las nuevas exigencias regulatorias y, por otro lado, mejorar algunas de las características del PRGF obtenido, como aumentar el enriquecimiento en plaquetas, disminuir el tiempo de

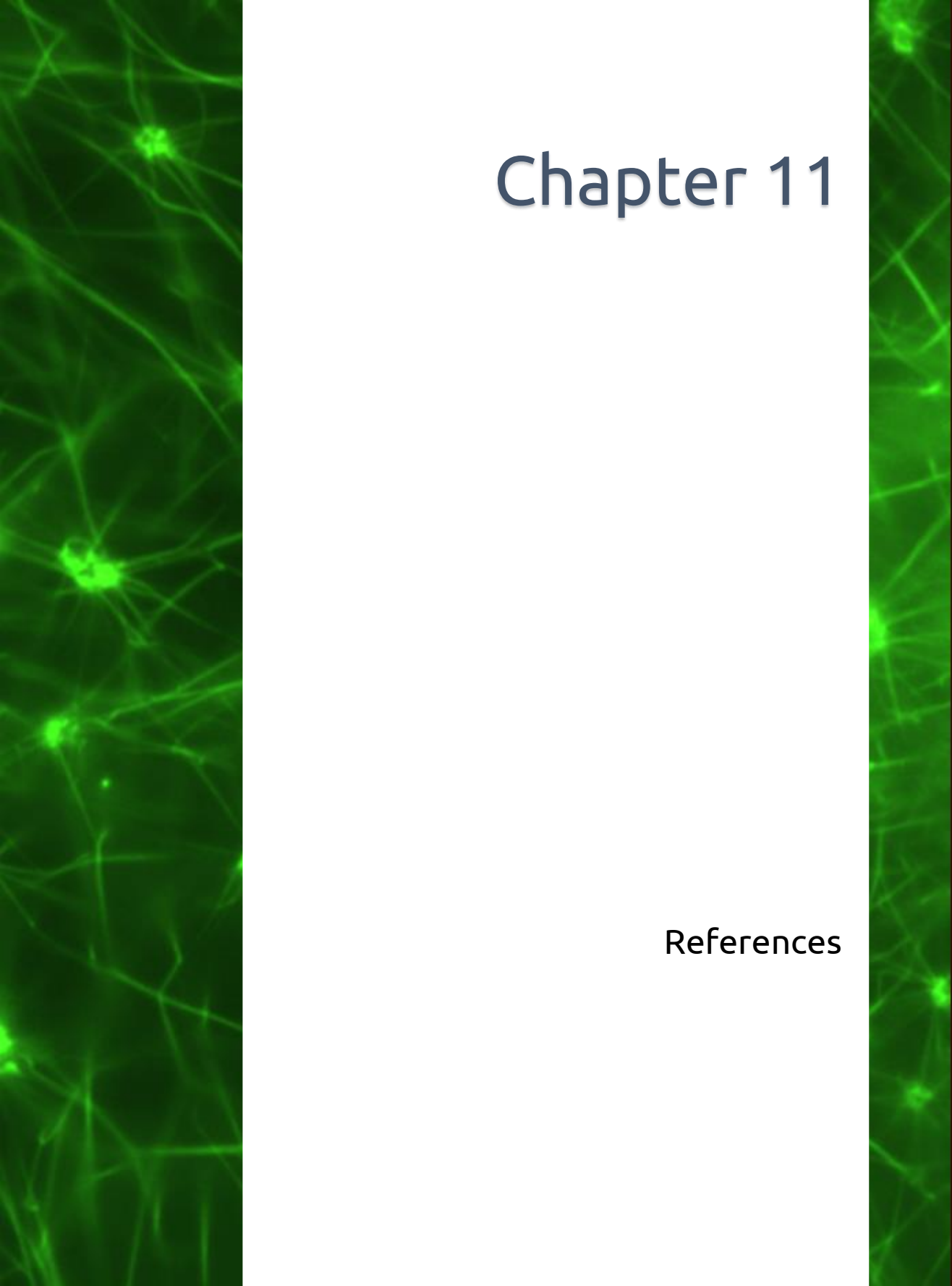
coagulación y retracción, o aumentar la concentración de factores de crecimiento.

4. Se ha analizado el nuevo marco regulatorio de los PRPs, incluyendo al PRGF, tras la resolución de AEMPS en la que se consideraba la aplicación del PRP como un medicamento de uso humano, pero no de producción industrial ni de terapia avanzada. Esta disposición, si bien es positiva para garantizar la adecuada utilización de las terapias de PRP, deja cuestiones relevantes que deberán de ser aclaradas en el futuro.

5. La utilización de PRP alogénico presenta múltiples ventajas sobre el tratamiento autólogo y se postula como firme candidato en terapias con PRP en un futuro cercano. Sin embargo, actualmente existe poca experiencia clínica, por lo que son necesarios más estudios que confirmen tanto su eficacia como su seguridad.

6. Se ha observado que la falta de reproducibilidad en los protocolos de obtención del PRP es un factor limitante en el avance de este tipo de terapias biológicas. Por ello, se recomienda realizar una caracterización detallada del producto a aplicar. Además, los métodos empleados han de ser descritos de forma adecuada y unificada siguiendo unas directrices en la línea de las que establece la red EQUATOR, y más concretamente de MIBO. Por ello, es necesario el desarrollo de una nueva guía para informar y publicar, tanto para investigación preclínica como clínica.





Chapter 11

References

References

1 Anitua, E., Prado, R., Nurden, A.T., Nurden, P. (2018) Characterization of Plasma Rich in Growth Factors (PRGF): components and formulations. In *Platelet Rich Plasma in Orthopaedics and Sports Medicine* (Anitua, E., Cugat, R. and Sánchez, M., eds), pp. 29-45, Springer International Publishing

2 Anitua, E., Prado, R., Azkargorta, M., Rodriguez-Suarez, E., Iloro, I., Casado-Vela, J., Elortza, F., Orive, G. (2015) High-throughput proteomic characterization of plasma rich in growth factors (PRGF-Endoret)-derived fibrin clot interactome. *J Tissue Eng Regen Med.* 9, E1-E12

3 Anitua, E., Zalduendo, M.M., Prado, R., Alkhraisat, M.H., Orive, G. (2015) Morphogen and proinflammatory cytokine release kinetics from PRGF-Endoret fibrin scaffolds: evaluation of the effect of leukocyte inclusion. *J Biomed Mater Res A.* 103, 1011-1020

4 Anitua, E., Prado, R., Troya, M., Zalduendo, M., de la Fuente, M., Pino, A., Muruzabal, F., Orive, G. (2016) Implementation of a more physiological plasma rich in growth factor (PRGF) protocol: Anticoagulant removal and reduction in activator concentration. *Platelets.* 27, 459-466

5 Anitua, E., Prado, R., Orive, G. (2015) Closing regulatory gaps: new ground rules for platelet-rich plasma. *Trends Biotechnol.* 33, 492-495

6 Anitua, E., Prado, R., Orive, G. (2017) Allogeneic Platelet-Rich Plasma: At the Dawn of an Off-the-Shelf Therapy? *Trends Biotechnol.* 35, 91-93

7 Anitua, E., Prado, R. (2019) Addressing Reproducibility in Stem Cell and PRP Therapies. *Trends Biotechnol.* 37, 340-344

8 Padilla, S., Sanchez, M., Orive, G., Anitua, E. (2017) Human-Based Biological and Biomimetic Autologous Therapies for Musculoskeletal Tissue Regeneration. *Trends Biotechnol.* 35, 192-202

9 Sánchez, M., Garate, A., Delgado, D., Padilla, S. (2017) Platelet-rich plasma, an adjuvant biological therapy to assist peripheral nerve repair. *Neural Regeneration Research.* 12, 47-52

10 O'Cearbhaill, E.D., Ng, K.S., Karp, J.M. (2014) Emerging medical devices for minimally invasive cell therapy. *Mayo Clin Proc.* 89, 259-273

11 Eming, S.A., Martin, P., Tomic-Canic, M. (2014) Wound repair and regeneration: mechanisms, signaling, and translation. *Sci Transl Med.* 6, 265sr266

12 Singer, A.J., Clark, R.A.F. (1999) Cutaneous wound healing. *New England Journal of Medicine.* 341, 738-746

13 Beanes, S.R., Dang, C., Soo, C., Ting, K. (2003) Skin repair and scar formation: The central role of TGF-β. *Expert Reviews in Molecular Medicine.* 5

14 Satish, L., Kathju, S. (2010) Cellular and molecular characteristics of scarless versus fibrotic wound healing. *Dermatology Research and Practice.* 2010

15 Karalaki, M., Fili, S., Philippou, A., Koutsilieris, M. (2009) Muscle regeneration: Cellular and molecular events. *In Vivo.* 23, 779-796

16 Muller, S.A., Todorov, A., Heisterbach, P.E., Martin, I., Majewski, M. (2015) Tendon healing: an overview of physiology, biology, and pathology of tendon healing and systematic review of state of the art in tendon bioengineering. *Knee Surg Sports Traumatol Arthrosc.* 23, 2097-2105

17 Soucacos, P.N., Johnson, E.O., Babis, G. (2008) An update on recent advances in bone regeneration. *Injury.* 39

18 Deschaseaux, F., Sensébé, L., Heymann, D. (2009) Mechanisms of bone repair and regeneration. *Trends in Molecular Medicine.* 15, 417-429

19 Fortier, L.A., Barker, J.U., Strauss, E.J., McCarrel, T.M., Cole, B.J. (2011) The role of growth factors in cartilage repair. *Clinical Orthopaedics and Related Research.* 469, 2706-2715

20 Barrientos, S., Stojadinovic, O., Golinko, M.S., Brem, H., Tomic-Canic, M. (2008) Growth factors and cytokines in wound healing. *Wound Repair and Regeneration.* 16, 585-601

21 Dereka, X.E., Markopoulou, C.E., Vrotsos, I.A. (2006) Role of growth factors on periodontal repair. *Growth Factors.* 24, 260-267

22 Smith, P.C., Martinez, C., Caceres, M., Martinez, J. (2015) Research on growth factors in periodontology. *Periodontol 2000.* 67, 234-250

23 Klenkler, B., Sheardown, H., Jones, L. (2007) Growth factors in the tear film: role in tissue maintenance, wound healing, and ocular pathology. *Ocul Surf.* 5, 228-239

24 Nurden, A.T., Nurden, P., Sanchez, M., Andia, I., Anitua, E. (2008) Platelets and wound healing. *Frontiers in Bioscience.* 13, 3532-3548

25 Blair, P., Flaumenhaft, R. (2009) Platelet α-granules: Basic biology and clinical correlates. *Blood Reviews.* 23, 177-189

26 Sundman, E.A., Cole, B.J., Fortier, L.A. (2011) Growth factor and catabolic cytokine concentrations are influenced by the cellular composition of platelet-rich plasma. *American Journal of Sports Medicine.* 39, 2135-2140

27 Nishiyama, K., Okudera, T., Watanabe, T., Isobe, K., Suzuki, M., Masuki, H., Okudera, H., Uematsu, K., Nakata, K., Kawase, T. (2016) Basic characteristics of plasma rich in growth factors (PRGF): blood cell components and biological effects. *Clinical and Experimental Dental Research.* 2, 96-103

28 Pifer, M.A., Maerz, T., Baker, K.C., Anderson, K. (2014) Matrix metalloproteinase content and activity in low-platelet, low-leukocyte and high-platelet, high-leukocyte platelet rich plasma (PRP) and the

biologic response to PRP by human ligament fibroblasts. *Am J Sports Med.* 42, 1211-1218

29 Osterman, C., McCarthy, M.B., Cote, M.P., Beitzel, K., Bradley, J., Polkowski, G., Mazzocca, A.D. (2015) Platelet-Rich Plasma Increases Anti-inflammatory Markers in a Human Coculture Model for Osteoarthritis. *Am J Sports Med.* 43, 1474-1484

30 Braun, H.J., Kim, H.J., Chu, C.R., Dragoo, J.L. (2014) The effect of platelet-rich plasma formulations and blood products on human synoviocytes: implications for intra-articular injury and therapy. *Am J Sports Med.* 42, 1204-1210

31 Castillo, T.N., Pouliot, M.A., Hyeon Joo, K., Dragoo, J.L. (2011) Comparison of growth factor and platelet concentration from commercial platelet-rich plasma separation systems. *American Journal of Sports Medicine.* 39, 266-271

32 Dohan Ehrenfest, D.M., Rasmusson, L., Albrektsson, T. (2009) Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leucocyte- and platelet-rich fibrin (L-PRF). *Trends Biotechnol.* 27, 158-167

33 Mishra, A., Harmon, K., Woodall, J. (2011) Sports medicine applications of platelet rich plasma. *Curr Pharm Biotechnol.*

34 DeLong, J.M., Russell, R.P., Mazzocca, A.D. (2012) Platelet-rich plasma: the PAW classification system. *Arthroscopy.* 28, 998-1009

35 Alsousou, J., Thompson, M., Hulley, P., Noble, A., Willett, K. (2009) The biology of platelet-rich plasma and its application in trauma and orthopaedic surgery: A review of the literature. *Journal of Bone and Joint Surgery - Series B.* 91, 987-996

36 Mazzucco, L., Borzini, P., Gope, R. (2010) Platelet-derived factors involved in tissue repair-from signal to function. *Transfusion Medicine Reviews.* 24, 218-234

37 Anitua, E., Prado, R., Sánchez, M., Orive, G. (2012) Platelet-Rich Plasma: Preparation and Formulation. *Oper Tech Orthop.* 22, 25-32

38 Clemmons, D.R. (1989) Structural and functional analysis of insulin-like growth factors. *British Medical Bulletin.* 45, 465-480

39 Yakar, S., Courtland, H.W., Clemmons, D. (2010) IGF-1 and bone: New discoveries from mouse models. *Journal of Bone and Mineral Research.* 25, 2267-2276

40 Ando, Y., Jensen, P.J. (1993) Epidermal growth factor and insulin-like growth factor I enhance keratinocyte migration. *Journal of Investigative Dermatology.* 100, 633-639

41 Haase, I., Evans, R., Pofahl, R., Watt, F.M. (2003) Regulation of keratinocyte shape, migration and wound epithelialization by IGF-1 and EGF-dependent signalling pathways. *Journal of Cell Science.* 116, 3227-3238

42 Govoni, G.E. (2011) Insulin-like growth factor-I molecular pathways in osteoblasts: Potential targets for pharmacological manipulation. *Curr Mol Pharmacol.*

43 Bikle, D.D., Harris, J., Halloran, B.P., Roberts, C.T., Leroith, D., Morey-Holton, E. (1994) Expression of the genes for insulin-like growth factors and their receptors in bone during skeletal growth. *American Journal of Physiology - Endocrinology and Metabolism.* 267

44 Meinel, L., Zoidis, E., Zapf, J., Hassa, P., Hottiger, M.O., Auer, J.A., Schneider, R., Gander, B., Luginbuehl, V., Bettschart-Wolfisberger, R., Illi, O.E., Merkle, H.P., Von Rechenberg, B. (2003) Localized insulin-like growth factor I delivery to enhance new bone formation. *Bone.* 33, 660-672

45 Florini, J.R., Ewton, D.Z., Coolican, S.A. (1996) Growth hormone and the insulin-like growth factor system in myogenesis. *Endocrine Reviews.* 17, 481-517

46 Heron-Milhavet, L., Mamaeva, D., Leroith, D., Lamb, N.J., Fernandez, A. (2010) Impaired muscle regeneration and myoblast differentiation in mice with a muscle-specific KO of IGF-IR. *Journal of Cellular Physiology.* 225, 1-6

47 Gallego-Colon, E., Villalba, M., Tonkin, J., Cruz, F., Bernal, J.A., Jimenez-Borregureo, L.J., Schneider, M.D., Lara-Pezzi, E., Rosenthal, N. (2016) Intravenous delivery of adeno-associated virus 9-encoded IGF-1Ea propeptide improves post-infarct cardiac remodelling. *Npj Regenerative Medicine*. 1, 16001

48 Tonkin, J., Temmerman, L., Sampson, R.D., Gallego-Colon, E., Barberi, L., Bilbao, D., Schneider, M.D., Musaro, A., Rosenthal, N. (2015) Monocyte/Macrophage-derived IGF-1 Orchestrates Murine Skeletal Muscle Regeneration and Modulates Autocrine Polarization. *Mol Ther.* 23, 1189-1200

49 Douglas, H.E. (2010) TGF-ß in wound healing: A review. *Journal of Wound Care.* 19, 403-406

50 Yang, E.Y., Moses, H.L. (1990) Transforming growth factor β1-induced changes in cell migration, proliferation, and angiogenesis in the chicken chorioallantoic membrane. *Journal of Cell Biology.* 111, 731-741

51 Zhao, L., Jiang, S., Hantash, B.M. (2010) Transforming growth factor β1 induces osteogenic differentiation of murine bone marrow stromal cells. *Tissue Engineering - Part A.* 16, 725-733

52 Zhen, G., Cao, X. (2014) Targeting TGFbeta signaling in subchondral bone and articular cartilage homeostasis. *Trends Pharmacol Sci.* 35, 227-236

53 Chen, P.H., Chen, X., He, X. (2013) Platelet-derived growth factors and their receptors: Structural and functional perspectives. *Biochim Biophys Acta.* 1834, 2176-2186

54 Schmidt, M.B., Chen, E.H., Lynch, S.E. (2006) A review of the effects of insulin-like growth factor and platelet derived growth factor on in vivo cartilage healing and repair. *Osteoarthritis and Cartilage.* 14, 403-412

55 Tumia, N.S., Johnstone, A.J. (2009) Platelet derived growth factor-AB enhances knee meniscal cell activity in vitro. *Knee.* 16, 73-76

56 Canalis, E., Varghese, S., McCarthy, T.L., Centrella, M. (1992) Role of platelet derived growth factor in bone cell function. *Growth regulation.* 2, 151-155

57 Hock, J.M., Canalis, E. (1994) Platelet-derived growth factor enhances bone cell replication, but not differentiated function of osteoblasts. *Endocrinology.* 134, 1423-1428

58 Caplan, A.I., Correa, D. (2011) PDGF in bone formation and regeneration: New insights into a novel mechanism involving MSCs. *Journal of Orthopaedic Research.* 29, 1795-1803

59 Nakamura, T. (1991) Structure and function of hepatocyte growth factor. *Progress in Growth Factor Research.* 3, 67-85

60 Matsumoto, K., Nakamura, T. (1997) Hepatocyte growth factor (HGF) as a tissue organizer for organogenesis and regeneration. *Biochemical and Biophysical Research Communications.* 239, 639-644

61 Molnarfi, N., Benkhoucha, M., Funakoshi, H., Nakamura, T., Lalive, P.H. (2015) Hepatocyte growth factor: A regulator of inflammation and autoimmunity. *Autoimmun Rev.* 14, 293-303

62 Mizuno, S., Kurosawa, T., Matsumoto, K., Mizuno-Horikawa, Y., Okamoto, M., Nakamura, T. (1998) Hepatocyte growth factor prevents renal fibrosis and dysfunction in a mouse model of chronic renal disease. *Journal of Clinical Investigation.* 101, 1827-1834

63 Shang, J., Deguchi, K., Ohta, Y., Liu, N., Zhang, X., Tian, F., Yamashita, T., Ikeda, Y., Matsuura, T., Funakoshi, H., Nakamura, T., Abe, K. (2011) Strong neurogenesis, angiogenesis, synaptogenesis, and antifibrosis of hepatocyte growth factor in rats brain after transient middle cerebral artery occlusion. *Journal of Neuroscience Research.* 89, 86-95

64 Shukla, M.N., Rose, J.L., Ray, R., Lathrop, K.L., Ray, A., Ray, P. (2009) Hepatocyte growth factor inhibits epithelial to myofibroblast transition in lung cells via Smad7. *American Journal of Respiratory Cell and Molecular Biology.* 40, 643-653

65 Bao, P., Kodra, A., Tomic-Canic, M., Golinko, M.S., Ehrlich, H.P., Brem, H. (2009) The Role of Vascular Endothelial Growth Factor in Wound Healing. *Journal of Surgical Research.* 153, 347-358

66 Ferrara, N., Gerber, H.P. (2001) The role of vascular endothelial growth factor in angiogenesis. *Acta Haematologica.* 106, 148-156

67 Jazwa, A., Florczyk, U., Grochot-Przeczek, A., Krist, B., Loboda, A., Jozkowicz, A., Dulak, J. (2016) Limb ischemia and vessel regeneration: Is there a role for VEGF? *Vascul Pharmacol.* 86, 18-30

68 Luo, J., Xiong, Y., Han, X., Lu, Y. (2011) VEGF non-angiogenic functions in adult organ homeostasis: Therapeutic implications. *Journal of Molecular Medicine.* 89, 635-645

69 Song, S.Y., Chung, H.M., Sung, J.H. (2010) The pivotal role of VEGF in adipose-derived-stem-cell-mediated regeneration. *Expert Opinion on Biological Therapy.* 10, 1529-1537

70 Carpenter, G., Cohen, S. (1990) Epidermal growth factor. *Journal of Biological Chemistry.* 265, 7709-7712

71 Kurten, R.C., Chowdhury, P., Sanders Jr, R.C., Pittman, L.M., Sessions, L.W., Chambers, T.C., Lyle, C.S., Schnackenberg, B.J., Jones, S.M. (2005) Coordinating epidermal growth factor-induced motility promotes efficient wound closure. *American Journal of Physiology - Cell Physiology.* 288

72 Schultz, G., Clark, W., Rotatori, D.S. (1991) EGF and TGF-α in wound healing and repair. *Journal of Cellular Biochemistry.* 45, 346-352

73 Schultz, G., Chegini, N., Grant, M., Khaw, P., MacKay, S. (1992) EFFECTS OF GROWTH FACTORS ON CORNEAL WOUND HEALING. *Acta Ophthalmologica.* 70, 60-66

74 Wong, R.W.C., Guillaud, L. (2004) The role of epidermal growth factor and its receptors in mammalian CNS. *Cytokine and Growth Factor Reviews.* 15, 147-156

75 Xian, C.J., Zhou, X.F. (2004) EGF family of growth factors: Essential roles and functional redundancy in the nerve system. *Frontiers in Bioscience.* 9, 85-92

76 Berlanga-Acosta, J., Gavilondo-Cowley, J., López-Saura, P., González-López, T., Castro-Santana, M.D., López-Mola, E., Guillén-Nieto, G., Herrera-Martinez, L. (2009) Epidermal growth factor in clinical practice - A review of its biological actions, clinical indications and safety implications. *International Wound Journal.* 6, 331-346

77 Klagsbrun, M. (1989) The fibroblast growth factor family: Structural and biological properties. *Progress in Growth Factor Research.* 1, 207-235

78 Yun, Y.R., Won, J.E., Jeon, E., Lee, S., Kang, W., Jo, H., Jang, J.H., Shin, U.S., Kim, H.W. (2010) Fibroblast growth factors: Biology, function, and application for tissue regeneration. *J Tissue Eng.* 2010

79 Beenken, A., Mohammadi, M. (2009) The FGF family: biology, pathophysiology and therapy. *Nat Rev Drug Discov.* 8, 235-253

80 Tabata, Y., Yamada, K., Hong, L., Miyamoto, S., Hashimoto, N., Ikada, Y. (1999) Skull bone regeneration in primates in response to basic fibroblast growth factor. *Journal of Neurosurgery.* 91, 851-856

81 Song, K., Rao, N.J., Chen, M.L., Huang, Z.J., Cao, Y.G. (2011) Enhanced bone regeneration with sequential delivery of basic fibroblast growth factor and sonic hedgehog. *Injury.* 42, 796-802

82 Kempen, D.H.R., Creemers, L.B., Alblas, J., Lu, L., Verbout, A.J., Yaszemski, M.J., Dhert, W.J.A. (2010) Growth Factor interactions in bone regeneration. *Tissue Engineering - Part B: Reviews.* 16, 551-566

83 Chang, J., Most, D., Thunder, R., Mehrara, B., Longaker, M.T., Lineaweaver, W.C. (1998) Molecular studies in flexor tendon wound healing: The role of basic fibroblast growth factor gene expression. *Journal of Hand Surgery.* 23, 1052-1058

84 Thomopoulos, S., Das, R., Sakiyama-Elbert, S., Silva, M.J., Charlton, N., Gelberman, R.H. (2010) BFGF and PDGF-BB for tendon

repair: Controlled release and biologic activity by tendon fibroblasts in vitro. *Annals of Biomedical Engineering.* 38, 225-234

85 Shimabukuro, Y., Ichikawa, T., Terashima, Y., Iwayama, T., Oohara, H., Kajikawa, T., Kobayashi, R., Terashima, H., Takedachi, M., Terakura, M., Hashikawa, T., Yamada, S., Murakami, S. (2008) Basic fibroblast growth factor regulates expression of heparan sulfate in human periodontal ligament cells. *Matrix Biology.* 27, 232-241

86 Shirakata, Y., Taniyama, K., Yoshimoto, T., Miyamoto, M., Takeuchi, N., Matsuyama, T., Noguchi, K. (2010) Regenerative effect of basic fibroblast growth factor on periodontal healing in two-wall intrabony defects in dogs. *Journal of Clinical Periodontology.* 37, 374-381

87 Murakami, S. (2011) Periodontal tissue regeneration by signaling molecule(s): What role does basic fibroblast growth factor (FGF-2) have in periodontal therapy? *Periodontology 2000.* 56, 188-208

88 Annes, J.P., Munger, J.S., Rifkin, D.B. (2003) Making sense of latent TGFβ activation. *Journal of Cell Science.* 116, 217-224

89 Belotti, D., Capelli, C., Resovi, A., Introna, M., Taraboletti, G. (2016) Thrombospondin-1 promotes mesenchymal stromal cell functions via TGFbeta and in cooperation with PDGF. *Matrix Biol.*

90 Mariani, E., Filardo, G., Canella, V., Berlingeri, A., Bielli, A., Cattini, L., Landini, M.P., Kon, E., Marcacci, M., Facchini, A. (2014) Platelet-rich plasma affects bacterial growth in vitro. *Cytotherapy.* 16, 1294-1304

91 Bielecki, T.M., Gazdzik, T.S., Arendt, J., Szczepanski, T., Krol, W., Wielkoszynski, T. (2007) Antibacterial effect of autologous platelet gel enriched with growth factors and other active substances: an in vitro study. *J Bone Joint Surg Br.* 89, 417-420

92 Anitua, E., Alonso, R., Girbau, C., Aguirre, J.J., Muruzabal, F., Orive, G. (2012) Antibacterial effect of plasma rich in growth factors (PRGF(R)-Endoret(R)) against Staphylococcus aureus and Staphylococcus epidermidis strains. *Clin Exp Dermatol.* 37, 652-657

93 El-Sharkawy, H., Kantarci, A., Deady, J., Hasturk, H., Liu, H., Alshahat, M., Van Dyke, T.E. (2007) Platelet-rich plasma: Growth factors and pro- and anti-inflammatory properties. *Journal of Periodontology.* 78, 661-669

94 Bendinelli, P., Matteucci, E., Dogliotti, G., Corsi, M.M., Banfi, G., Maroni, P., Desiderio, M.A. (2010) Molecular basis of anti-inflammatory action of platelet-rich plasma on human chondrocytes: Mechanisms of NF-κB inhibition via HGF. *Journal of Cellular Physiology.* 225, 757-766

95 Lesurtel, M., Graf, R., Aleil, B., Walther, D.J., Tian, Y., Jochum, W., Gachet, C., Bader, M., Clavien, P.A. (2006) Platelet-derived serotonin mediates liver regeneration. *Science.* 312, 104-107

96 Anitua, E., Sánchez, M., Zalduendo, M.M., De La Fuente, M., Prado, R., Orive, G., Andía, I. (2009) Fibroblastic response to treatment with different preparations rich in growth factors. *Cell Proliferation.* 42, 162-170

97 Weibrich, G., Hansen, T., Kleis, W., Buch, R., Hitzler, W.E. (2004) Effect of platelet concentration in platelet-rich plasma on peri-implant bone regeneration. *Bone.* 34, 665-671

98 Anitua, E., Muruzabal, F., de la Fuente, M., Merayo, J., Duran, J., Orive, G. (2016) Plasma Rich in Growth Factors for the Treatment of Ocular Surface Diseases. *Curr Eye Res.* 41, 875-882

99 Zehnder, J.L., Leung, L.L.K. (1990) Development of antibodies to thrombin and factor V with recurrent bleeding in a patient exposed to topical bovine thrombin. *Blood.* 76, 2011-2016

100 Landesberg, R., Moses, M., Karpatkin, M. (1998) Risks of using platelet rich plasma gel [2]. *Journal of Oral and Maxillofacial Surgery.* 56, 1116-1117

101 Diesen, D.L., Lawson, J.H. (2008) Bovine thrombin: History, use, and risk in the surgical patient. *Vascular.* 16, S29-S36

102 Weibrich, G., Kleis, W.K.G., Hitzler, W.E., Hafner, G. (2005) Comparison of the platelet concentrate collection system with the

plasma-rich-in-growth-factors kit to produce platelet-rich plasma: A technical report. *International Journal of Oral and Maxillofacial Implants.* 20, 118-123

103 Scott, A., Khan, K.M., Roberts, C.R., Cook, J.L., Duronio, V. (2004) What do we mean by the term "inflammation"? A contemporary basic science update for sports medicine. *British Journal of Sports Medicine.* 38, 372-380

104 Tidball, J.G. (2005) Inflammatory processes in muscle injury and repair. *Am J Physiol Regul Integr Comp Physiol.* 288, R345-353

105 Harmon, K.G. (2010) Muscle injuries and PRP: What does the science say? *British Journal of Sports Medicine.* 44, 616-617

106 Orcajo, B., Muruzabal, F., Isasmendi, M.C., Gutierrez, N., Sanchez, M., Orive, G., Anitua, E. (2011) The use of plasma rich in growth factors (PRGF-Endoret) in the treatment of a severe mal perforant ulcer in the foot of a person with diabetes. *Diabetes Res Clin Pract.* 93, e65-e67

107 Anitua, E., Aguirre, J.J., Algorta, J., Ayerdi, E., Cabezas, A.I., Orive, G., Andia, I. (2008) Effectiveness of autologous preparation rich in growth factors for the treatment of chronic cutaneous ulcers. *Journal of Biomedical Materials Research - Part B Applied Biomaterials.* 84, 415-421

108 Anitua, E., Prado, R., Orive, G. (2013) Endogenous morphogens and fibrin bioscaffolds for stem cell therapeutics. *Trends Biotechnol.* 31, 364-374

109 Anitua, E., Prado, R., Orive, G. (2009) A lateral approach for sinus elevation using PRGF technology. *Clin Implant Dent Relat Res.* 11 Suppl 1, e23-31

110 Anitua, E., Sanchez, M., Aguirre, J.J., Prado, R., Padilla, S., Orive, G. (2014) Efficacy and safety of plasma rich in growth factors intra-articular infiltrations in the treatment of knee osteoarthritis. *Arthroscopy.* 30, 1006-1017

111 Vaquerizo, V., Plasencia, M.A., Arribas, I., Seijas, R., Padilla, S., Orive, G., Anitua, E. (2013) Comparison of Intra-Articular Injections of

Plasma Rich in Growth Factors (PRGF-Endoret) Versus Durolane Hyaluronic Acid in the Treatment of Patients With Symptomatic Osteoarthritis: A Randomized Controlled Trial. *Arthroscopy.* 29, 1635-1643

112 Sanchez, M., Fiz, N., Azofra, J., Usabiaga, J., Aduriz Recalde, E., Garcia Gutierrez, A., Albillos, J., Garate, R., Aguirre, J.J., Padilla, S., Orive, G., Anitua, E. (2012) A randomized clinical trial evaluating plasma rich in growth factors (PRGF-Endoret) versus hyaluronic acid in the short-term treatment of symptomatic knee osteoarthritis. *Arthroscopy.* 28, 1070-1078

113 Sanchez, M., Fiz, N., Guadilla, J., Padilla, S., Anitua, E., Sanchez, P., Delgado, D. (2014) Intraosseous infiltration of platelet-rich plasma for severe knee osteoarthritis. *Arthrosc Tech.* 3, e713-717

114 Sanchez, M., Anitua, E., Delgado, D., Sanchez, P., Prado, R., Goiriena, J.J., Prosper, F., Orive, G., Padilla, S. (2016) A new strategy to tackle severe knee osteoarthritis: Combination of intra-articular and intraosseous injections of Platelet Rich Plasma. *Expert Opin Biol Ther.* 16, 627–643

115 Sanchez, M., Delgado, D., Sanchez, P., Muinos-Lopez, E., Paiva, B., Granero-Molto, F., Prosper, F., Pompei, O., Perez, J.C., Azofra, J., Padilla, S., Fiz, N. (2016) Combination of Intra-Articular and Intraosseous Injections of Platelet Rich Plasma for Severe Knee Osteoarthritis: A Pilot Study. *Biomed Res Int.* 2016, 4868613

116 Sanchez, M., Anitua, E., Cugat, R., Azofra, J., Guadilla, J., Seijas, R., Andia, I. (2009) Nonunions treated with autologous preparation rich in growth factors. *Journal of Orthopaedic Trauma.* 23, 52-59

117 Seijas, R., Santana-Suárez, R.Y., García-Balletbó, M., Cuscó, X., Ares, O., Cugat, R. (2010) Delayed union of the clavicle treated with plasma rich in growth factors. *Acta Orthopaedica Belgica.* 76, 689-693

118 Sanchez, M., Delgado, D., Sanchez, P., Fiz, N., Azofra, J., Orive, G., Anitua, E., Padilla, S. (2014) Platelet rich plasma and knee surgery. *Biomed Res Int.* 2014, 890630

119 Aguirre, J., Anitua, E., Francisco, S., Cabezas, A., Orive, G., Algorta, J. (2015) Efficacy and safety of plasma rich in growth factors in the treatment of venous ulcers: a randomized clinical trial controlled with conventional treatment. *Clinical Dermatology.* 3, 13-20

120 Anitua, E., Tejero, R., Alkhraisat, M.H., Orive, G. (2013) Platelet-rich plasma to improve the bio-functionality of biomaterials. *BioDrugs.* 27, 97-111

121 Tejero, R., Anitua, E., Orive, G. (2014) Toward the biomimetic implant surface: Biopolymers on titanium-based implants for bone regeneration. *Progress in Polymer Science.* 39, 1406-1447

122 Merayo-Lloves, J., Sanchez-Avila, R.M., Riestra, A.C., Anitua, E., Begona, L., Orive, G., Fernandez-Vega, L. (2016) Safety and Efficacy of Autologous Plasma Rich in Growth Factors Eye Drops for the Treatment of Evaporative Dry Eye. *Ophthalmic Res.* 56, 68-73

123 Merayo-Lloves, J., Sanchez, R.M., Riestra, A.C., Anitua, E., Begona, L., Orive, G., Fernandez-Vega, L. (2015) Autologous Plasma Rich in Growth Factors Eyedrops in Refractory Cases of Ocular Surface Disorders. *Ophthalmic Res.* 55, 53-61

124 López-Plandolit, S., Morales, M.C., Freire, V., Etxebarría, J., Durán, J.A. (2010) Plasma rich in growth factors as a therapeutic agent for persistent corneal epithelial defects. *Cornea.* 29, 843-848

125 Burnouf, T., Strunk, D., Koh, M.B., Schallmoser, K. (2016) Human platelet lysate: Replacing fetal bovine serum as a gold standard for human cell propagation? *Biomaterials.* 76, 371-387

126 Heemskerk, J.W., Mattheij, N.J., Cosemans, J.M. (2013) Platelet-based coagulation: different populations, different functions. *J Thromb Haemost.* 11, 2-16

127 Rosano, G., Taschieri, S., Del Fabbro, M. (2013) Immediate postextraction implant placement using plasma rich in growth factors technology in maxillary premolar region: a new strategy for soft tissue management. *J Oral Implantol.* 39, 98-102

128 Taschieri, S., Lolato, A., Ofer, M., Testori, T., Francetti, L., Del Fabbro, M. (2017) Immediate post-extraction implants with or without pure platelet-rich plasma: a 5-year follow-up study. *Oral Maxillofac Surg.* 21, 147-157

129 Mozzati, M., Gallesio, G., Gassino, G., Palomba, A., Bergamasco, L. (2014) Can plasma rich in growth factors improve healing in patients who underwent radiotherapy for head and neck cancer? A split-mouth study. *J Craniofac Surg.* 25, 938-943

130 Sánchez, M., Anitua, E., Cole, A., Da Silva, A., Azofra, J., Andia, I. (2009) Management of post-surgical Achilles tendon complications with a preparation rich in growth factors: A study of two-cases. *Injury Extra.* 40, 11-15

131 Anitua, E., Sánchez, M., Prado, R., Orive, G. (2011) The P makes the difference in plasma rich in growth factors (PRGF) technology. *Platelets.* 22, 473-474

132 Werner, S., Grose, R. (2003) Regulation of wound healing by growth factors and cytokines. *Physiol Rev.* 83, 835-870

133 Anitua, E., Sanchez, M., Orive, G., Andia, I. (2008) Delivering growth factors for therapeutics. *Trends Pharmacol Sci.* 29, 37-41

134 Anitua, E., Andia, I., Ardanza, B., Nurden, P., Nurden, A.T. (2004) Autologous platelets as a source of proteins for healing and tissue regeneration. *Thromb Haemost.* 91, 4-15

135 McNicol, A., Israels, S.J. (1999) Platelet dense granules: structure, function and implications for haemostasis. *Thromb Res.* 95, 1-18

136 Chen, F.M., Zhang, M., Wu, Z.F. (2010) Toward delivery of multiple growth factors in tissue engineering. *Biomaterials.* 31, 6279-6308

137 Anitua, E. (1999) Plasma rich in growth factors: preliminary results of use in the preparation of future sites for implants. *Int J Oral Maxillofac Implants.* 14, 529-535

138 Anitua, E., Sanchez, M., Orive, G., Andia, I. (2007) The potential impact of the preparation rich in growth factors (PRGF) in different medical fields. *Biomaterials.* 28, 4551-4560

139 Anitua, E., Sanchez, M., Prado, R., Orive, G. (2011) Plasma rich in growth factors: the pioneering autologous technology for tissue regeneration. *J Biomed Mater Res A.* 97, 536

140 Anitua, E., Sanchez, M., Orive, G. (2010) Potential of endogenous regenerative technology for in situ regenerative medicine. *Adv Drug Deliv Rev.* 62, 741-752

141 Anitua, E., Alkhraisat, M.H., Orive, G. (2012) Perspectives and challenges in regenerative medicine using plasma rich in growth factors. *J Control Release.* 157, 29-38

142 Anderson, N.L., Anderson, N.G. (2002) The human plasma proteome: history, character, and diagnostic prospects. *Mol Cell Proteomics.* 1, 845-867

143 Aebersold, R., Mann, M. (2003) Mass spectrometry-based proteomics. *Nature.* 422, 198-207

144 Steen, H., Mann, M. (2004) The ABC's (and XYZ's) of peptide sequencing. *Nat Rev Mol Cell Biol.* 5, 699-711

145 Casado-Vela, J., Rodriguez-Suarez, E., Iloro, I., Ametzazurra, A., Alkorta, N., Garcia-Velasco, J.A., Matorras, R., Prieto, B., Gonzalez, S., Nagore, D., Simon, L., Elortza, F. (2009) Comprehensive proteomic analysis of human endometrial fluid aspirate. *J Proteome Res.* 8, 4622-4632

146 Tirumalai, R.S., Chan, K.C., Prieto, D.A., Issaq, H.J., Conrads, T.P., Veenstra, T.D. (2003) Characterization of the low molecular weight human serum proteome. *Mol Cell Proteomics.* 2, 1096-1103

147 Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., Harris, M.A., Hill, D.P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J.C., Richardson, J.E., Ringwald, M., Rubin, G.M., Sherlock, G. (2000) Gene ontology: tool

for the unification of biology. The Gene Ontology Consortium. *Nat Genet.* 25, 25-29

148 Wolberg, A.S. (2010) Plasma and cellular contributions to fibrin network formation, structure and stability. *Haemophilia.* 16 Suppl 3, 7-12

149 Law, R.H., Zhang, Q., McGowan, S., Buckle, A.M., Silverman, G.A., Wong, W., Rosado, C.J., Langendorf, C.G., Pike, R.N., Bird, P.I., Whisstock, J.C. (2006) An overview of the serpin superfamily. *Genome Biol.* 7, 216

150 Kalsheker, N. (1989) Alpha 1-antitrypsin: structure, function and molecular biology of the gene. *Biosci Rep.* 9, 129-138

151 Han, Y.P., Yan, C., Garner, W.L. (2008) Proteolytic activation of matrix metalloproteinase-9 in skin wound healing is inhibited by alpha-1-antichymotrypsin. *J Invest Dermatol.* 128, 2334-2342

152 Reiss, M.J., Han, Y.P., Garner, W.L. (2009) Alpha1-antichymotrypsin activity correlates with and may modulate matrix metalloproteinase-9 in human acute wounds. *Wound Repair Regen.* 17, 418-426

153 Hoffmann, D.C., Textoris, C., Oehme, F., Klaassen, T., Goppelt, A., Romer, A., Fugmann, B., Davidson, J.M., Werner, S., Krieg, T., Eming, S.A. (2011) Pivotal role for alpha1-antichymotrypsin in skin repair. *J Biol Chem.* 286, 28889-28901

154 Weisel, J.W. (2005) Fibrinogen and fibrin. *Adv Protein Chem.* 70, 247-299

155 Adams, J.C., Lawler, J. (2004) The thrombospondins. *Int J Biochem Cell Biol.* 36, 961-968

156 Mosher, D.F., Misenheimer, T.M., Stenflo, J., Hogg, P.J. (1992) Modulation of fibrinolysis by thrombospondin. *Ann N Y Acad Sci.* 667, 64-69

157 Gelse, K., Klinger, P., Koch, M., Surmann-Schmitt, C., von der Mark, K., Swoboda, B., Hennig, F.F., Gusinde, J. (2011) Thrombospondin-1 prevents excessive ossification in cartilage repair tissue induced by osteogenic protein-1. *Tissue Eng Part A.* 17, 2101-2112

158 Podor, T.J., Campbell, S., Chindemi, P., Foulon, D.M., Farrell, D.H., Walton, P.D., Weitz, J.I., Peterson, C.B. (2002) Incorporation of vitronectin into fibrin clots. Evidence for a binding interaction between vitronectin and gamma A/gamma' fibrinogen. *J Biol Chem.* 277, 7520-7528

159 Preissner, K.T., Reuning, U. (2011) Vitronectin in vascular context: facets of a multitalented matricellular protein. *Semin Thromb Hemost.* 37, 408-424

160 Podor, T.J., Peterson, C.B., Lawrence, D.A., Stefansson, S., Shaughnessy, S.G., Foulon, D.M., Butcher, M., Weitz, J.I. (2000) Type 1 plasminogen activator inhibitor binds to fibrin via vitronectin. *J Biol Chem.* 275, 19788-19794

161 Pankov, R., Yamada, K.M. (2002) Fibronectin at a glance. *J Cell Sci.* 115, 3861-3863

162 Greiling, D., Clark, R.A. (1997) Fibronectin provides a conduit for fibroblast transmigration from collagenous stroma into fibrin clot provisional matrix. *J Cell Sci.* 110 (Pt 7), 861-870

163 Lin, F., Ren, X.D., Pan, Z., Macri, L., Zong, W.X., Tonnesen, M.G., Rafailovich, M., Bar-Sagi, D., Clark, R.A. (2011) Fibronectin growth factor-binding domains are required for fibroblast survival. *J Invest Dermatol.* 131, 84-98

164 Macri, L., Silverstein, D., Clark, R.A. (2007) Growth factor binding to the pericellular matrix and its importance in tissue engineering. *Adv Drug Deliv Rev.* 59, 1366-1381

165 Gabay, C., Kushner, I. (1999) Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med.* 340, 448-454

166 Ebersole, J.L., Cappelli, D. (2000) Acute-phase reactants in infections and inflammatory diseases. *Periodontol 2000.* 23, 19-49

167 Yano, Y., Seishima, M., Tokoro, Y., Noma, A. (1998) Stimulatory effects of lipoprotein(a) and low-density lipoprotein on human umbilical vein endothelial cell migration and proliferation are partially mediated by fibroblast growth factor-2. *Biochim Biophys Acta.* 1393, 26-34

168 Bulat, N., Waeber, G., Widmann, C. (2009) LDLs stimulate p38 MAPKs and wound healing through SR-BI independently of Ras and PI3 kinase. *J Lipid Res.* 50, 81-89

169 Ogawa, T., Nishimura, I. (2006) Genes differentially expressed in titanium implant healing. *J Dent Res.* 85, 566-570

170 Yano, Y., Shimokawa, K., Okada, Y., Noma, A. (1997) Immunolocalization of lipoprotein(a) in wounded tissues. *J Histochem Cytochem.* 45, 559-568

171 Woodley, D.T., Fan, J., Cheng, C.F., Li, Y., Chen, M., Bu, G., Li, W. (2009) Participation of the lipoprotein receptor LRP1 in hypoxia-HSP90alpha autocrine signaling to promote keratinocyte migration. *J Cell Sci.* 122, 1495-1498

172 Dobreva, I., Waeber, G., Mooser, V., James, R.W., Widmann, C. (2003) LDLs induce fibroblast spreading independently of the LDL receptor via activation of the p38 MAPK pathway. *J Lipid Res.* 44, 2382-2390

173 Angles-Cano, E., de la Pena Diaz, A., Loyau, S. (2001) Inhibition of fibrinolysis by lipoprotein(a). *Ann N Y Acad Sci.* 936, 261-275

174 Hasumi, K., Yamamichi, S., Harada, T. (2010) Small-molecule modulators of zymogen activation in the fibrinolytic and coagulation systems. *FEBS J.* 277, 3675-3687

175 von Zychlinski, A., Kleffmann, T., Williams, M.J., McCormick, S.P. (2011) Proteomics of Lipoprotein(a) identifies a protein complement associated with response to wounding. *J Proteomics.* 74, 2881-2891

176 Mozzati, M., Martinasso, G., Pol, R., Polastri, C., Cristiano, A., Muzio, G., Canuto, R. (2010) The impact of plasma rich in growth factors on clinical and biological factors involved in healing processes after third molar extraction. *J Biomed Mater Res A.* 95, 741-746

177 Wang-Saegusa, A., Cugat, R., Ares, O., Seijas, R., Cusco, X., Garcia-Balletbo, M. (2011) Infiltration of plasma rich in growth factors for osteoarthritis of the knee short-term effects on function and quality of life. *Arch Orthop Trauma Surg.* 131, 311-317

178 van Buul, G.M., Koevoet, W.L., Kops, N., Bos, P.K., Verhaar, J.A., Weinans, H., Bernsen, M.R., van Osch, G.J. (2011) Platelet-rich plasma releasate inhibits inflammatory processes in osteoarthritic chondrocytes. *Am J Sports Med.* 39, 2362-2370

179 Anitua, E., Zalduendo, M.M., Alkhraisat, M.H., Orive, G. (2013) Release kinetics of platelet-derived and plasma-derived growth factors from autologous plasma rich in growth factors. *Ann Anat.* 195, 461-466

180 Del Buono, A., Papalia, R., Denaro, V., Maccauro, G., Maffulli, N. (2011) Platelet rich plasma and tendinopathy: state of the art. *Int J Immunopathol Pharmacol.* 24, 79-83

181 Mishra, A., Pavelko, T. (2006) Treatment of chronic elbow tendinosis with buffered platelet-rich plasma. *Am J Sports Med.* 34, 1774-1778

182 Sanchez, M., Anitua, E., Azofra, J., Andia, I., Padilla, S., Mujika, I. (2007) Comparison of surgically repaired Achilles tendon tears using platelet-rich fibrin matrices. *Am J Sports Med.* 35, 245-251

183 Sanchez, M., Anitua, E., Azofra, J., Prado, R., Muruzabal, F., Andia, I. (2010) Ligamentization of tendon grafts treated with an endogenous preparation rich in growth factors: gross morphology and histology. *Arthroscopy.* 26, 470-480

184 Andia, I., Sanchez, M., Maffulli, N. (2012) Joint pathology and platelet-rich plasma therapies. *Expert Opin Biol Ther.* 12, 7-22

185 Andia, I., Sanchez, M., Maffulli, N. (2011) Platelet rich plasma therapies for sports muscle injuries: any evidence behind clinical practice? *Expert Opin Biol Ther.* 11, 509-518

186 Hamilton, B., Knez, W., Eirale, C., Chalabi, H. (2010) Platelet enriched plasma for acute muscle injury. *Acta Orthop Belg.* 76, 443-448

187 Sanchez, M., Anitua, E., Orive, G., Mujika, I., Andia, I. (2009) Platelet-rich therapies in the treatment of orthopaedic sport injuries. *Sports Med.* 39, 345-354

188 McCarrel, T.M., Minas, T., Fortier, L.A. (2012) Optimization of leukocyte concentration in platelet-rich plasma for the treatment of tendinopathy. *J Bone Joint Surg Am.* 94, e1431-1438

189 Schnabel, L.V., Mohammed, H.O., Miller, B.J., McDermott, W.G., Jacobson, M.S., Santangelo, K.S., Fortier, L.A. (2007) Platelet rich plasma (PRP) enhances anabolic gene expression patterns in flexor digitorum superficialis tendons. *J Orthop Res.* 25, 230-240

190 de Jonge, S., de Vos, R.J., Weir, A., van Schie, H.T., Bierma-Zeinstra, S.M., Verhaar, J.A., Weinans, H., Tol, J.L. (2011) One-year follow-up of platelet-rich plasma treatment in chronic Achilles tendinopathy: a double-blind randomized placebo-controlled trial. *Am J Sports Med.* 39, 1623-1629

191 de Vos, R.J., Weir, A., van Schie, H.T., Bierma-Zeinstra, S.M., Verhaar, J.A., Weinans, H., Tol, J.L. (2010) Platelet-rich plasma injection for chronic Achilles tendinopathy: a randomized controlled trial. *JAMA.* 303, 144-149

192 Horstmann, W.G., Slappendel, R., van Hellemondt, G.G., Wymenga, A.W., Jack, N., Everts, P.A. (2011) Autologous platelet gel in total knee arthroplasty: a prospective randomized study. *Knee Surg Sports Traumatol Arthrosc.* 19, 115-121

193 Peerbooms, J.C., de Wolf, G.S., Colaris, J.W., Bruijn, D.J., Verhaar, J.A. (2009) No positive effect of autologous platelet gel after total knee arthroplasty. *Acta Orthop.* 80, 557-562

194 Dragoo, J.L., Braun, H.J., Durham, J.L., Ridley, B.A., Odegaard, J.I., Luong, R., Arnoczky, S.P. (2012) Comparison of the acute inflammatory response of two commercial platelet-rich plasma systems in healthy rabbit tendons. *Am J Sports Med.* 40, 1274-1281

195 Filardo, G., Kon, E., Pereira Ruiz, M.T., Vaccaro, F., Guitaldi, R., Di Martino, A., Cenacchi, A., Fornasari, P.M., Marcacci, M. (2012) Platelet-rich plasma intra-articular injections for cartilage degeneration and osteoarthritis: single- versus double-spinning approach. *Knee Surg Sports Traumatol Arthrosc.* 20, 2082-2091

196 Kisiday, J.D., McIlwraith, C.W., Rodkey, W.G., Frisbie, D.D., Steadman, J.R. (2012) Effects of Platelet-Rich Plasma Composition on Anabolic and Catabolic Activities in Equine Cartilage and Meniscal Explants. *Cartilage.* 3, 245-254

197 Bielecki, T., Cieslik-Bielecka, A., Å»elawski, M., Mikusek, W. A side-effect induced by the combination of a demineralized freeze-dried bone allograft and leucocyte and platelet-rich plasma during treatment for large bone cysts: A 4-year follow-up clinical study. *Transfusion and Apheresis Science.* 47, 133-138

198 Bochsen, L., Johansson, P.I., Kristensen, A.T., Daugaard, G., Ostrowski, S.R. (2011) The influence of platelets, plasma and red blood cells on functional haemostatic assays. *Blood Coagul Fibrinolysis.* 22, 167-175

199 Gersh, K.C., Nagaswami, C., Weisel, J.W. (2009) Fibrin network structure and clot mechanical properties are altered by incorporation of erythrocytes. *Thromb Haemost.* 102, 1169-1175

200 Dohan Ehrenfest, D.M., Bielecki, T., Jimbo, R., Barbe, G., Del Corso, M., Inchingolo, F., Sammartino, G. (2012) Do the fibrin architecture and leukocyte content influence the growth factor release of platelet concentrates? An evidence-based answer comparing a pure platelet-rich plasma (P-PRP) gel and a leukocyte- and platelet-rich fibrin (L-PRF). *Curr Pharm Biotechnol.* 13, 1145-1152

201 Anitua, E., Sanchez, M., Nurden, A.T., Zalduendo, M., de la Fuente, M., Orive, G., Azofra, J., Andia, I. (2006) Autologous fibrin

matrices: a potential source of biological mediators that modulate tendon cell activities. *J Biomed Mater Res A.* 77, 285-293

202 Perrin, J., Morlon, L., Vigneron, C., Marchand-Arvier, M. (2008) Influence of polymorphonuclear leukocytes on the plasma clot formation as evaluated by thromboelastometry (ROTEM). *Thromb Res.* 121, 647-652

203 Letterio, J.J., Roberts, A.B. (1998) Regulation of immune responses by TGF-beta. *Annu Rev Immunol.* 16, 137-161

204 Helming, L., Gordon, S. (2007) The molecular basis of macrophage fusion. *Immunobiology.* 212, 785-793

205 Grenier, A., Chollet-Martin, S., Crestani, B., Delarche, C., El Benna, J., Boutten, A., Andrieu, V., Durand, G., Gougerot-Pocidalo, M.A., Aubier, M., Dehoux, M. (2002) Presence of a mobilizable intracellular pool of hepatocyte growth factor in human polymorphonuclear neutrophils. *Blood.* 99, 2997-3004

206 Schuppan, D., Schmid, M., Somasundaram, R., Ackermann, R., Ruehl, M., Nakamura, T., Riecken, E.O. (1998) Collagens in the liver extracellular matrix bind hepatocyte growth factor. *Gastroenterology.* 114, 139-152

207 Taipale, J., Keski-Oja, J. (1997) Growth factors in the extracellular matrix. *Faseb J.* 11, 51-59

208 Ohnishi, T., Kakimoto, K., Bandow, K., Lowenstein, C.J., Daikuhara, Y., Matsuguchi, T. (2006) Mature hepatocyte growth factor/scatter factor on the surface of human granulocytes is released by a mechanism involving activated factor Xa. *J Immunol.* 176, 6945-6953

209 Kishuku, M., Nishioka, Y., Abe, S., Kishi, J., Ogino, H., Aono, Y., Azuma, M., Kinoshita, K., Batmunkh, R., Makino, H., Ranjan, P., Minakuchi, K., Sone, S. (2009) Expression of soluble vascular endothelial growth factor receptor-1 in human monocyte-derived mature dendritic cells contributes to their antiangiogenic property. *J Immunol.* 183, 8176-8185

210 Wu, F.T., Stefanini, M.O., Mac Gabhann, F., Kontos, C.D., Annex, B.H., Popel, A.S. (2010) A systems biology perspective on sVEGFR1: its biological function, pathogenic role and therapeutic use. *J Cell Mol Med.* 14, 528-552

211 Gabay, C., Lamacchia, C., Palmer, G. (2010) IL-1 pathways in inflammation and human diseases. *Nat Rev Rheumatol.* 6, 232-241

212 Daheshia, M., Yao, J.Q. (2008) The interleukin 1beta pathway in the pathogenesis of osteoarthritis. *J Rheumatol.* 35, 2306-2312

213 Archambault, J., Tsuzaki, M., Herzog, W., Banes, A.J. (2002) Stretch and interleukin-1beta induce matrix metalloproteinases in rabbit tendon cells in vitro. *J Orthop Res.* 20, 36-39

214 Tsuzaki, M., Guyton, G., Garrett, W., Archambault, J.M., Herzog, W., Almekinders, L., Bynum, D., Yang, X., Banes, A.J. (2003) IL-1 beta induces COX2, MMP-1, -3 and -13, ADAMTS-4, IL-1 beta and IL-6 in human tendon cells. *J Orthop Res.* 21, 256-264

215 Goldring, M.B. (2000) Osteoarthritis and cartilage: the role of cytokines. *Curr Rheumatol Rep.* 2, 459-465

216 Center, D.M., Kornfeld, H., Cruikshank, W.W. (1997) Interleukin-16. *Int J Biochem Cell Biol.* 29, 1231-1234

217 Cruikshank, W.W., Kornfeld, H., Center, D.M. (2000) Interleukin-16. *J Leukoc Biol.* 67, 757-766

218 Ludwiczek, O., Kaser, A., Koch, R.O., Vogel, W., Cruikshank, W.W., Tilg, H. (2001) Activation of caspase-3 by interferon alpha causes interleukin-16 secretion but fails to modulate activation induced cell death. *Eur Cytokine Netw.* 12, 478-486

219 Conti, P., Kempuraj, D., Kandere, K., Di Gioacchino, M., Reale, M., Barbacane, R.C., Castellani, M.L., Mortari, U., Boucher, W., Letourneau, R., Theoharides, T.C. (2002) Interleukin-16 network in inflammation and allergy. *Allergy Asthma Proc.* 23, 103-108

220 Blaschke, S., Schulz, H., Schwarz, G., Blaschke, V., Muller, G.A., Reuss-Borst, M. (2001) Interleukin 16 expression in relation to disease activity in rheumatoid arthritis. *J Rheumatol.* 28, 12-21

221 Lard, L.R., Roep, B.O., Toes, R.E., Huizinga, T.W. (2004) Enhanced concentrations of interleukin 16 are associated with joint destruction in patients with rheumatoid arthritis. *J Rheumatol.* 31, 35-39

222 Moojen, D.J., Everts, P.A., Schure, R.M., Overdevest, E.P., van Zundert, A., Knape, J.T., Castelein, R.M., Creemers, L.B., Dhert, W.J. (2008) Antimicrobial activity of platelet-leukocyte gel against Staphylococcus aureus. *J Orthop Res.* 26, 404-410

223 Anitua, E., Sanchez, M., Prado, R., Orive, G. (2014) The type of platelet-rich plasma may influence the safety of the approach. *Knee Surg Sports Traumatol Arthrosc.* 22, 1708-1709

224 Wasterlain, A.S., Braun, H.J., Dragoo, J.L. (2012) Contents and Formulations of Platelet-Rich Plasma. *Oper Tech Orthop.* 22, 33-42

225 (2014) Manual on borderline and classification in the Community Regulatory framework for medical devices. Version 1.16. http://ec.europa.eu/health/medical-devices/files/wg_minutes_member_lists/borderline_manual_ol_en.pdf

226 (2013) World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. *Jama.* 310, 2191-2194

227 Farrugia, A., Hughes, C., Douglas, S., Neal, M., James, J. (1989) Microtitre plate measurement of platelet response to hypotonic stress. *J Clin Pathol.* 42, 1298-1301

228 Goodall, A.H., Appleby, J. (2004) Flow-cytometric analysis of platelet-membrane glycoprotein expression and platelet activation. *Methods Mol Biol.* 272, 225-253

229 Born, G.V., Cross, M.J. (1963) The Aggregation of Blood Platelets. *J Physiol.* 168, 178-195

230 Wang, S., Gao, Z., Chen, X., Lian, X., Zhu, H., Zheng, J., Sun, L. (2008) The anticoagulant ability of ferulic acid and its applications for improving the blood compatibility of silk fibroin. *Biomed Mater.* 3, 044106

231 Lian, L., Suzuki, A., Hayes, V., Saha, S., Han, X., Xu, T., Yates, J.R., 3rd, Poncz, M., Kashina, A., Abrams, C.S. (2014) Loss of ATE1-mediated arginylation leads to impaired platelet myosin phosphorylation, clot retraction, and in vivo thrombosis formation. *Haematologica.* 99, 554-560

232 Bjoraker, D.G., Ketcham, T.R. (1981) 3.8% sodium citrate (1:9) is an inadequate anticoagulant for rabbit blood with high calcium. *Thromb Res.* 24, 505-508

233 Ganong, W.F., Barrett, K.E. (2005) General Principles & Energy Production in Medical Physiology. In *Review of Medical Physiology* (Ganong, W.F., ed), pp. 1-30, McGraw-Hill Medical . New York

234 Dumont, L.J., AuBuchon, J.P., Gulliksson, H., Slichter, S.J., Elfath, M.D., Holme, S., Murphy, J.R., Rose, L.E., Popovsky, M.A., Murphy, S. (2006) In vitro pH effects on in vivo recovery and survival of platelets: an analysis by the BEST Collaborative. *Transfusion.* 46, 1300-1305

235 Summerer, M.H., Genco, P.V., Katz, A.J. (1978) The response of human platelets to hypotonic stress: direct measurement of volume change. *Ann Clin Lab Sci.* 8, 447-452

236 Kim, B.K., Baldini, M.G. (1974) The platelet response to hypotonic shock. Its value as an indicator of platelet viability after storage. *Transfusion.* 14, 130-138

237 Magalon, J., Bausset, O., Serratrice, N., Giraudo, L., Aboudou, H., Veran, J., Magalon, G., Dignat-Georges, F., Sabatier, F. (2014) Characterization and Comparison of 5 Platelet-Rich Plasma Preparations in a Single-Donor Model. *Arthroscopy: The Journal of Arthroscopic & Related Surgery.* 30, 629-638

238 Anitua, E., Troya, M., Zalduendo, M.M., Orive, G. (2014) The effect of different drugs on the preparation and biological outcomes of plasma rich in growth factors. *Ann Anat.* 196, 423-429

239 Zhou, L., Schmaier, A.H. (2005) Platelet aggregation testing in platelet-rich plasma: description of procedures with the aim to develop standards in the field. *Am J Clin Pathol.* 123, 172-183

240 Anitua, E., Sanchez, M., Merayo-Lloves, J., De la Fuente, M., Muruzabal, F., Orive, G. (2011) Plasma rich in growth factors (PRGF-Endoret) stimulates proliferation and migration of primary keratocytes and conjunctival fibroblasts and inhibits and reverts TGF-beta1-Induced myodifferentiation. *Invest Ophthalmol Vis Sci.* 52, 6066-6073

241 Anitua, E., Pelacho, B., Prado, R., Aguirre, J.J., Sanchez, M., Padilla, S., Aranguren, X.L., Abizanda, G., Collantes, M., Hernandez, M., Perez-Ruiz, A., Penuelas, I., Orive, G., Prosper, F. (2015) Infiltration of plasma rich in growth factors enhances in vivo angiogenesis and improves reperfusion and tissue remodeling after severe hind limb ischemia. *J Control Release.* 202, 31-39

242 Hamm, L.L. (1990) Renal handling of citrate. *Kidney Int.* 38, 728-735

243 Dhillon, R.S., Schwarz, E.M., Maloney, M.D. (2012) Platelet-rich plasma therapy - future or trend? *Arthritis Res Ther.* 14, 219

244 Engebretsen, L., Steffen, K., Alsousou, J., Anitua, E., Bachl, N., Devilee, R., Everts, P., Hamilton, B., Huard, J., Jenoure, P., Kelberine, F., Kon, E., Maffulli, N., Matheson, G., Mei-Dan, O., Menetrey, J., Philippon, M., Randelli, P., Schamasch, P., Schwellnus, M., Vernec, A., Verrall, G. (2010) IOC consensus paper on the use of platelet-rich plasma in sports medicine. *Br J Sports Med.* 44, 1072-1081

245 Kolle, S.F., Fischer-Nielsen, A., Mathiasen, A.B., Elberg, J.J., Oliveri, R.S., Glovinski, P.V., Kastrup, J., Kirchhoff, M., Rasmussen, B.S., Talman, M.L., Thomsen, C., Dickmeiss, E., Drzewiecki, K.T. (2013) Enrichment of autologous fat grafts with ex-vivo expanded adipose tissue-derived stem cells for graft survival: a randomised placebo-controlled trial. *Lancet.* 382, 1113-1120

246 Spanish Agency of Medicines and Medical. Devices (2013) REPORT/V1/23052013. The Spanish Agency of Medicines and Health Care Products' report on the use of Platelet-Rich Plasma. Informe de la Agencia Española de Medicamentos y Productos Sanitarios sobre el uso

de Plasma Rico en Plaquetas. (http://www.aemps.gob.es/medicamentosUsoHumano/medSituacionesEspeciales/docs/PRP-AEMPS-DEF-mayo13.pdf)

247 Spanish Agency of Medicines and Medical. Devices (2013) Resolution establishing the classification of non-replacement therapeutic use of autologous plasma and its fractions, components or derivatives as a medicinal product for human use to meet specialised needs. Resolución por la que se establece la clasificación del uso terapéutico no sustitutivo del plasma autólogo y sus fracciones, componentes o derivados, como medicamentos de uso humano para atender necesidades especiales. (http://www.aemps.gob.es/legislacion/espana/medicamentosUsoHumano/docs/medEspeciales/resolucion-PRP.pdf)

248 Official State Gazette (2006) Law 29/2006, of July 26th, on the guarantees and rational use of medicines and healthcare products (BOE 178, of July 26th). (http://www.boe.es/buscar/pdf/2006/BOE-A-2006-13554-consolidado.pdf)

249 Official Journal of the European Communities (2001) Directive 2001/83/EC of the European parliament and of the council of 6 November 2001 on the community code relating to medicinal products for human use. http://ec.europa.eu/health/files/eudralex/vol-1/dir_2001_83_cons/dir2001_83_cons_20081230_en.pdf

250 Official Journal of the European Union (2007) EC/1394/2007 of the European Parliament and of the Council of 13 November 2007 on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004. (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:324:0121:0137:en:PDF)

251 Bravery, C.A., Carmen, J., Fong, T., Oprea, W., Hoogendoorn, K.H., Woda, J., Burger, S.R., Rowley, J.A., Bonyhadi, M.L., Van't Hof, W. (2013) Potency assay development for cellular therapy products: an ISCT review of the requirements and experiences in the industry. *Cytotherapy*. 15, 9-19

252 Guthrie, K., Bruce, A., Sangha, N., Rivera, E., Basu, J. (2013) Potency evaluation of tissue engineered and regenerative medicine products. *Trends Biotechnol*. 31, 505-514

253 US Food Drug Administration (FDA). CFR-Code of Federal Regulations Title 21, Part 807: Establishment registration and device listing for manufacturers and initial importers of devices. (http://www.gpo.gov/fdsys/pkg/CFR-2008-title21-vol8/pdf/CFR-2008-title21-vol8-part807.pdf)

254 US Food Drug Administration (FDA). CFR-Code of Federal Regulations Title 21, Part 1271: Human cells, tissues, and cellular and tissue-based products. (http://www.gpo.gov/fdsys/pkg/CFR-2012-title21-vol8/pdf/CFR-2012-title21-vol8-part1271.pdf)

255 Bottegoni, C., Dei Giudici, L., Salvemini, S., Chiurazzi, E., Bencivenga, R., Gigante, A. (2016) Homologous platelet-rich plasma for the treatment of knee osteoarthritis in selected elderly patients: an open-label, uncontrolled, pilot study. *Ther Adv Musculoskelet Dis.* 8, 35-41

256 Smrke, D., Gubina, B., Domanovic, D., Rozman, P. (2007) Allogeneic platelet gel with autologous cancellous bone graft for the treatment of a large bone defect. *Eur Surg Res.* 39, 170-174

257 Gubina, B., Rozman, P., Biscevic, M., Domanovic, D., Smrke, D. (2014) The influence of allogeneic platelet gel on the morphology of human long bones. *Coll Antropol.* 38, 865-870

258 Walker, R.H. (1992) Is it homologous or is it allogeneic? *Transfusion.* 32, 397

259 Knoepfler, P.S. (2015) From bench to FDA to bedside: US regulatory trends for new stem cell therapies. *Adv Drug Deliv Rev.* 82-83, 192-196

260 Dragoo, J.L., Korotkova, T., Wasterlain, A.S., Pouliot, M.A., Kim, H.J., Golish, S.R. (2012) Age-Related Changes of Chondrogenic Growth Factors in Platelet-Rich Plasma. *Oper Tech Orthop.* 22, 49-55

261 Hamilton, B., Tol, J.L., Knez, W., Chalabi, H. (2015) Exercise and the platelet activator calcium chloride both influence the growth factor content of platelet-rich plasma (PRP): overlooked biochemical factors that could influence PRP treatment. *Br J Sports Med.* 49, 957-960

262 Scudellari, M. (2015) Ageing research: Blood to blood. *Nature.* 517, 426-429

263 Zhang, Z.Y., Huang, A.W., Fan, J.J., Wei, K., Jin, D., Chen, B., Li, D., Bi, L., Wang, J., Pei, G. (2013) The potential use of allogeneic platelet-rich plasma for large bone defect treatment: immunogenicity and defect healing efficacy. *Cell Transplant.* 22, 175-187

264 Munafò, M.R., Nosek, B.A., Bishop, D.V.M., Button, K.S., Chambers, C.D., Percie du Sert, N., Simonsohn, U., Wagenmakers, E.-J., Ware, J.J., Ioannidis, J.P.A. (2017) A manifesto for reproducible science. *Nat Hum Behav.* 1, 0021

265 (2010) Consort 2010. *The Lancet.* 375, 1136

266 Marcus, E., whole Cell, t. (2016) A STAR Is Born. *Cell.* 166, 1059-1060

267 Trounson, A., McDonald, C. (2015) Stem Cell Therapies in Clinical Trials: Progress and Challenges. *Cell Stem Cell.* 17, 11-22

268 Linde, K., Brinkhaus, B. (2017) Randomized Trials of Chinese Herbal Medicine: A New Extension of the CONSORT Statement. *Ann Intern Med.* 167, 133-134

269 Kimmelman, J., Hyun, I., Benvenisty, N., Caulfield, T., Heslop, H.E., Murry, C.E., Sipp, D., Studer, L., Sugarman, J., Daley, G.Q. (2016) Policy: Global standards for stem-cell research. *Nature.* 533, 311-313

270 Murray, I.R., Geeslin, A.G., Goudie, E.B., Petrigliano, F.A., LaPrade, R.F. (2017) Minimum Information for Studies Evaluating Biologics in Orthopaedics (MIBO): Platelet-Rich Plasma and Mesenchymal Stem Cells. *J Bone Joint Surg Am.* 99, 809-819

271 Pas, H.I., Winters, M., Haisma, H.J., Koenis, M.J., Tol, J.L., Moen, M.H. (2017) Stem cell injections in knee osteoarthritis: a systematic review of the literature. *Br J Sports Med.* 51, 1125-1133

272 Harrison, P., Subcommittee on Platelet, P. (2018) The use of platelets in regenerative medicine and proposal for a new classification system: guidance from the SSC of the ISTH. *J Thromb Haemost.* 16, 1895-1900

273 Chahla, J., Cinque, M.E., Piuzzi, N.S., Mannava, S., Geeslin, A.G., Murray, I.R., Dornan, G.J., Muschler, G.F., LaPrade, R.F. (2017) A Call for Standardization in Platelet-Rich Plasma Preparation Protocols and Composition Reporting: A Systematic Review of the Clinical Orthopaedic Literature. *J Bone Joint Surg Am.* 99, 1769-1779

274 Sansone, S.A., McQuilton, P., Rocca-Serra, P., Gonzalez-Beltran, A., Izzo, M., Lister, A., Thurston, M. (2018) FAIRsharing: working with and for the community to describe and link data standards, repositories and policies. *bioRxiv* DOI: 10.1101/245183 (https://doi.org/10.1101/245183

https://www.biorxiv.org/content/biorxiv/early/2018/09/26/245183.full.pdf)

275 Taylor, C.F., Field, D., Sansone, S.A., Aerts, J., Apweiler, R., Ashburner, M., Ball, C.A., Binz, P.A., Bogue, M., Booth, T., Brazma, A., Brinkman, R.R., Michael Clark, A., Deutsch, E.W., Fiehn, O., Fostel, J., Ghazal, P., Gibson, F., Gray, T., Grimes, G., Hancock, J.M., Hardy, N.W., Hermjakob, H., Julian, R.K., Jr., Kane, M., Kettner, C., Kinsinger, C., Kolker, E., Kuiper, M., Le Novere, N., Leebens-Mack, J., Lewis, S.E., Lord, P., Mallon, A.M., Marthandan, N., Masuya, H., McNally, R., Mehrle, A., Morrison, N., Orchard, S., Quackenbush, J., Reecy, J.M., Robertson, D.G., Rocca-Serra, P., Rodriguez, H., Rosenfelder, H., Santoyo-Lopez, J., Scheuermann, R.H., Schober, D., Smith, B., Snape, J., Stoeckert, C.J., Jr., Tipton, K., Sterk, P., Untergasser, A., Vandesompele, J., Wiemann, S. (2008) Promoting coherent minimum reporting guidelines for biological and biomedical investigations: the MIBBI project. *Nat Biotechnol.* 26, 889-896

276 Pamies, D., Bal-Price, A., Simeonov, A., Tagle, D., Allen, D., Gerhold, D., Yin, D., Pistollato, F., Inutsuka, T., Sullivan, K., Stacey, G., Salem, H., Leist, M., Daneshian, M., Vemuri, M.C., McFarland, R., Coecke, S., Fitzpatrick, S.C., Lakshmipathy, U., Mack, A., Wang, W.B., Yamazaki, D., Sekino, Y., Kanda, Y., Smirnova, L., Hartung, T. (2017) Good Cell Culture Practice for stem cells and stem-cell-derived models. *ALTEX.* 34, 95-132

277 Mao, A.S., Mooney, D.J. (2015) Regenerative medicine: Current therapies and future directions. *Proceedings of the National Academy of Sciences.* 112, 14452-14459

278 Maresova, P., Javanmardi, E., Barakovic, S., Barakovic Husic, J., Tomsone, S., Krejcar, O., Kuca, K. (2019) Consequences of chronic diseases and other limitations associated with old age – a scoping review. *BMC Public Health.* 19, 1431

279 Jessop, Z.M., Al-Sabah, A., Francis, W.R., Whitaker, I.S. (2016) Transforming healthcare through regenerative medicine. *BMC Medicine.* 14, 115

280 Orlando, G., Soker, S., Stratta, R.J., Atala, A. (2013) Will Regenerative Medicine Replace Transplantation? *Cold Spring Harbor Perspectives in Medicine.* 3

281 Ashammakhi, N., Ahadian, S., Darabi, M.A., El Tahchi, M., Lee, J., Suthiwanich, K., Sheikhi, A., Dokmeci, M.R., Oklu, R., Khademhosseini, A. (2019) Minimally Invasive and Regenerative Therapeutics. *Advanced Materials.* 31, 1804041

282 Mendes, B.B., Gomez-Florit, M., Babo, P.S., Domingues, R.M., Reis, R.L., Gomes, M.E. (2018) Blood derivatives awaken in regenerative medicine strategies to modulate wound healing. *Adv Drug Deliv Rev.* 129, 376-393

283 Fitzpatrick, J., Bulsara, M.K., McCrory, P.R., Richardson, M.D., Zheng, M.H. (2017) Analysis of Platelet-Rich Plasma Extraction: Variations in Platelet and Blood Components Between 4 Common Commercial Kits. *Orthop J Sports Med.* 5, 2325967116675272

284 Oudelaar, B.W., Peerbooms, J.C., Huis In 't Veld, R., Vochteloo, A.J.H. (2018) Concentrations of Blood Components in Commercial Platelet-Rich Plasma Separation Systems: A Review of the Literature. *Am J Sports Med.* 363546517746112

285 Anitua, E., Nurden, P., Prado, R., Nurden, A.T., Padilla, S. (2019) Autologous fibrin scaffolds: When platelet- and plasma-derived biomolecules meet fibrin. *Biomaterials.* 192, 440-460

286 Martino, M.M., Briquez, P.S., Ranga, A., Lutolf, M.P., Hubbell, J.A. (2013) Heparin-binding domain of fibrin(ogen) binds growth factors and promotes tissue repair when incorporated within a synthetic matrix. *Proc Natl Acad Sci U S A.* 110, 4563-4568

287 Longo, V., Rebulla, P., Pupella, S., Zolla, L., Rinalducci, S. (2016) Proteomic characterization of platelet gel releasate from adult peripheral and cord blood. *Proteomics Clin Appl.* 10, 870-882

288 Lee, H.W., Choi, K.H., Kim, J.Y., Kim, K.O., Haotian, B., Yuxuan, L., Noh, K.C. (2020) Proteomic Classification and Identification of Proteins Related to Tissue Healing of Platelet-Rich Plasma. *Clin Orthop Surg.* 12, 120-129

289 Hoeres, T., Wilhelm, M., Smetak, M., Holzmann, E., Schulze-Tanzil, G., Birkmann, J. (2018) Immune cells regulate VEGF signalling via release of VEGF and antagonistic soluble VEGF receptor-1. *Clin Exp Immunol.* 192, 54-67

290 Shirata, T., Kato, Y. (2019) Can intra-articular injection of freeze-dried platelet-derived factor concentrate regenerate articular cartilage in the knee joint? *Regen Ther.* 11, 5-7

291 Murray, I.R., Murray, A.D., Geeslin, A.G., Goudie, E.B., White, T.O., Petrigliano, F.A., LaPrade, R.F. (2019) Infographic: we need minimum reporting standards for biologics. *Br J Sports Med.* 53, 974-975

292 Harrison, P., Alsousou, J. (2020) Studies on platelet rich plasma - new editorial policy for "Platelets". *Platelets.* 31, 281-282

293 Sanchez, M., Azofra, J., Anitua, E., Andia, I., Padilla, S., Santisteban, J., Mujika, I. (2003) Plasma rich in growth factors to treat an articular cartilage avulsion: a case report. *Med Sci Sports Exerc.* 35, 1648-1652

294 Anitua, E., Pino, A., Orive, G. (2017) Opening new horizons in regenerative dermatology using platelet-based autologous therapies. *Int J Dermatol.* 56, 247-251

295 Jia, X. (2016) Translational Medicine: Creating the Crucial Bidirectional Bridge between Bench and Bedside. *Int J Mol Sci.* 17, 1918

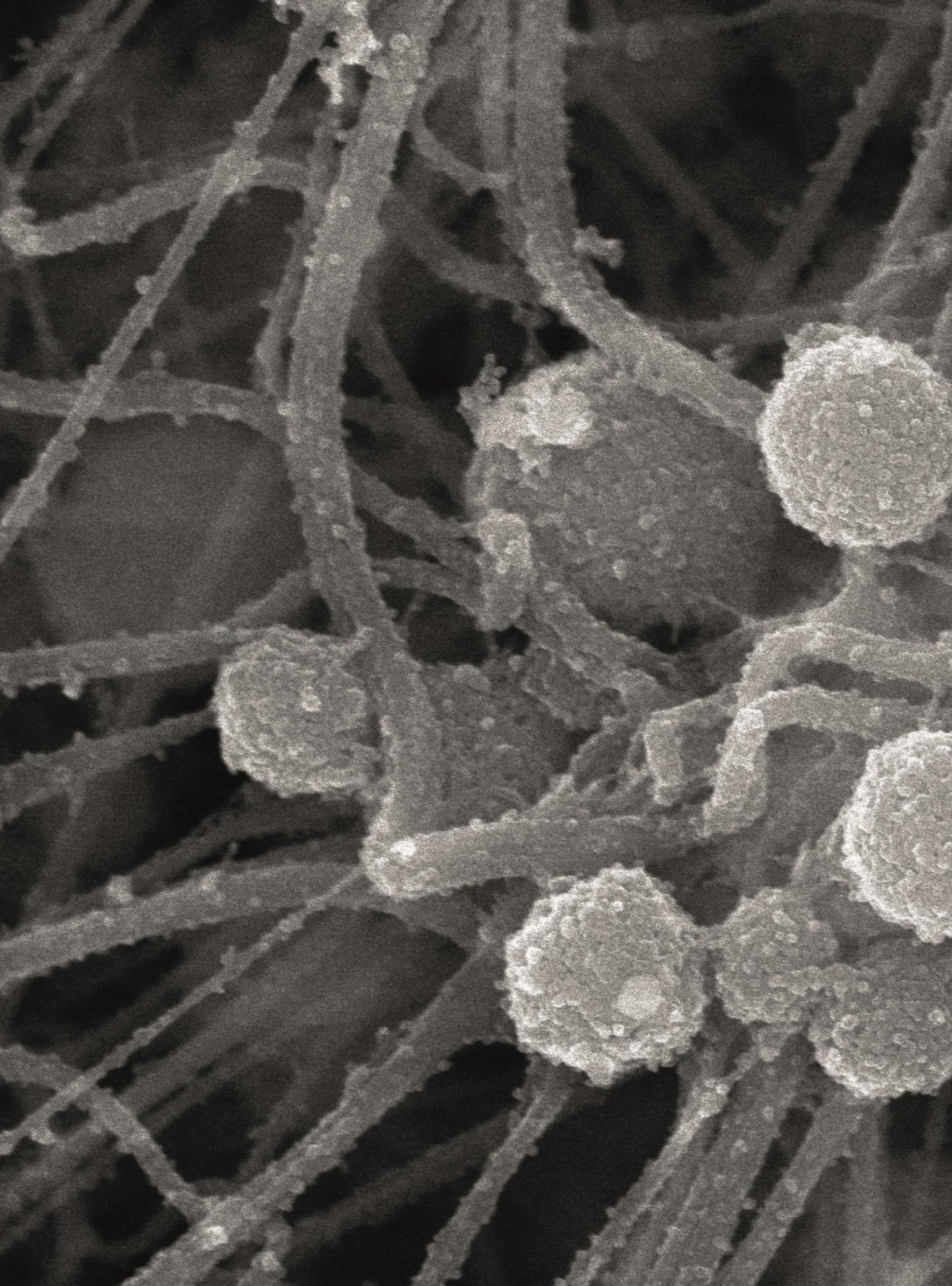
296 Anitua, E., Prado, R., Orive, G. (2016) PRP Therapies-Is It Time for Potency Assays? Letter to the Editor. *Am J Sports Med.* 44, NP63-NP64

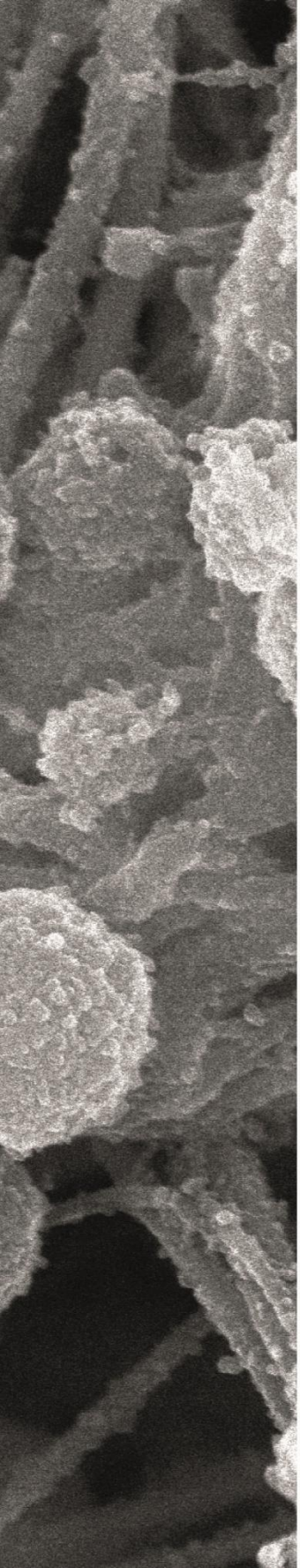
297 Robb, K.P., Fitzgerald, J.C., Barry, F., Viswanathan, S. (2019) Mesenchymal stromal cell therapy: progress in manufacturing and assessments of potency. *Cytotherapy.* 21, 289-306

298 Li, H., Hicks, J.J., Wang, L., Oyster, N., Philippon, M.J., Hurwitz, S., Hogan, M.V., Huard, J. (2016) Customized platelet-rich plasma with transforming growth factor beta1 neutralization antibody to reduce fibrosis in skeletal muscle. *Biomaterials.* 87, 147-156

299 Park, J.H., Kim, J.W., Kim, S.J. (2017) Does the Addition of Bone Morphogenetic Protein 2 to Platelet-Rich Fibrin Improve Healing After Treatment for Medication-Related Osteonecrosis of the Jaw? *J Oral Maxillofac Surg.* 75, 1176-1184

300 Kamakura, T., Kataoka, J., Maeda, K., Teramachi, H., Mihara, H., Miyata, K., Ooi, K., Sasaki, N., Kobayashi, M., Ito, K. (2015) Platelet-Rich Plasma with Basic Fibroblast Growth Factor for Treatment of Wrinkles and Depressed Areas of the Skin. *Plast Reconstr Surg.* 136, 931-939



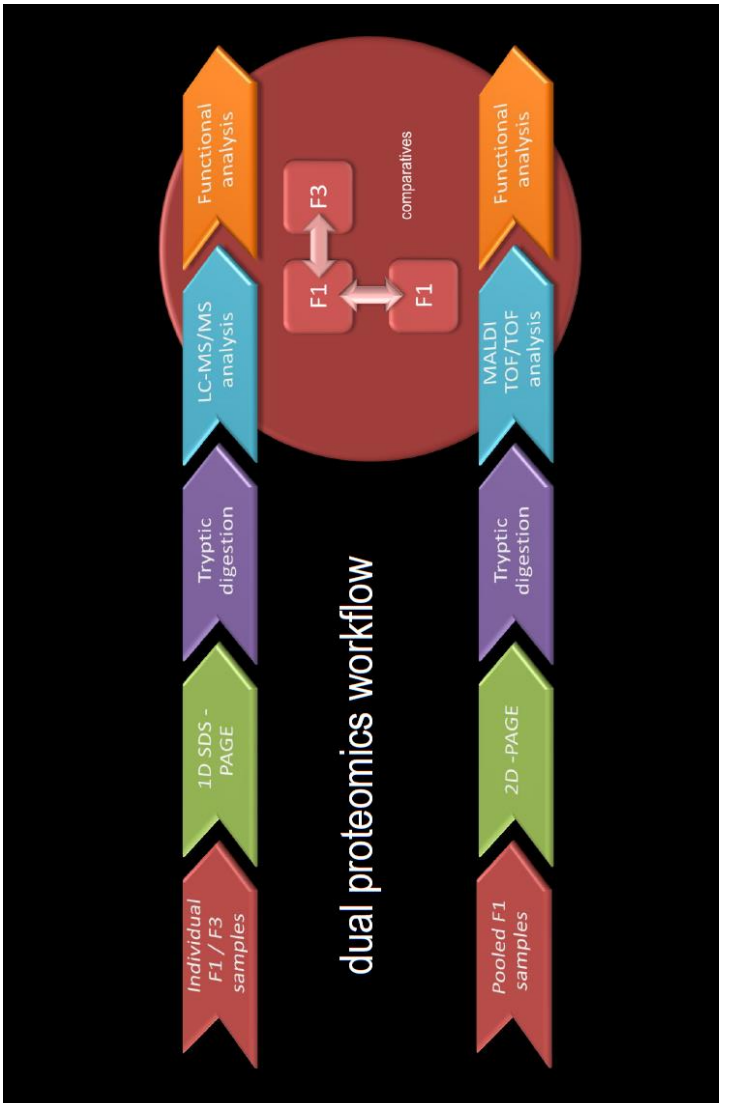


Chapter 12

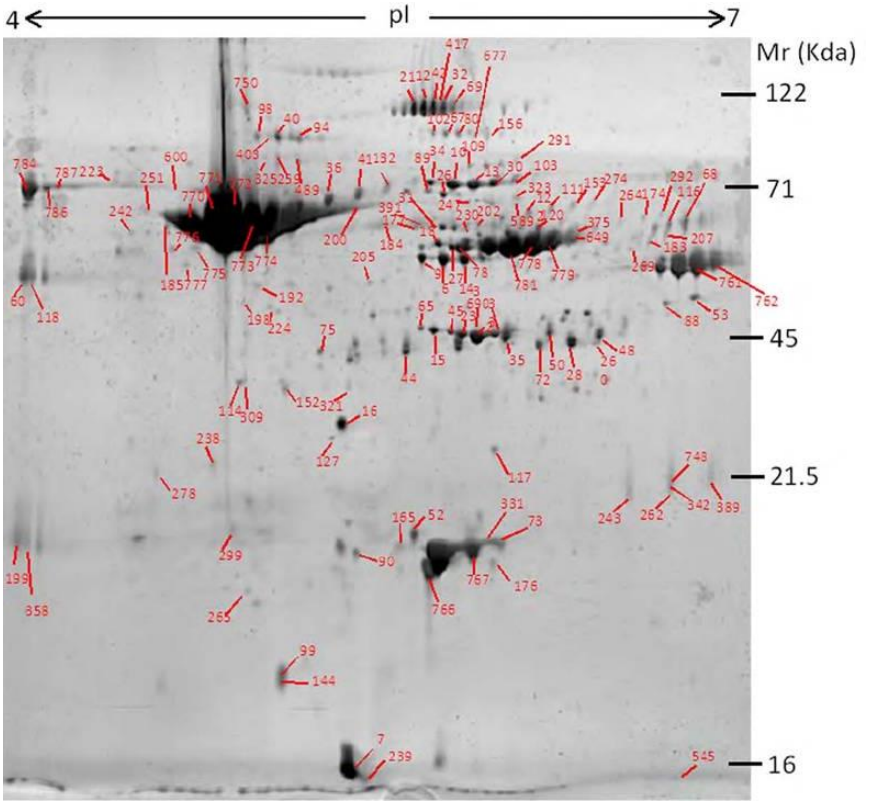
Appendices

1 Appendix I. Supplementary Information for Anitua, E., et al. (2015) High-throughput proteomic characterization of plasma rich in growth factors (PRGF-Endoret)-derived fibrin clot interactome. *J Tissue Eng Regen Med.* 9, E1-E12 [2].

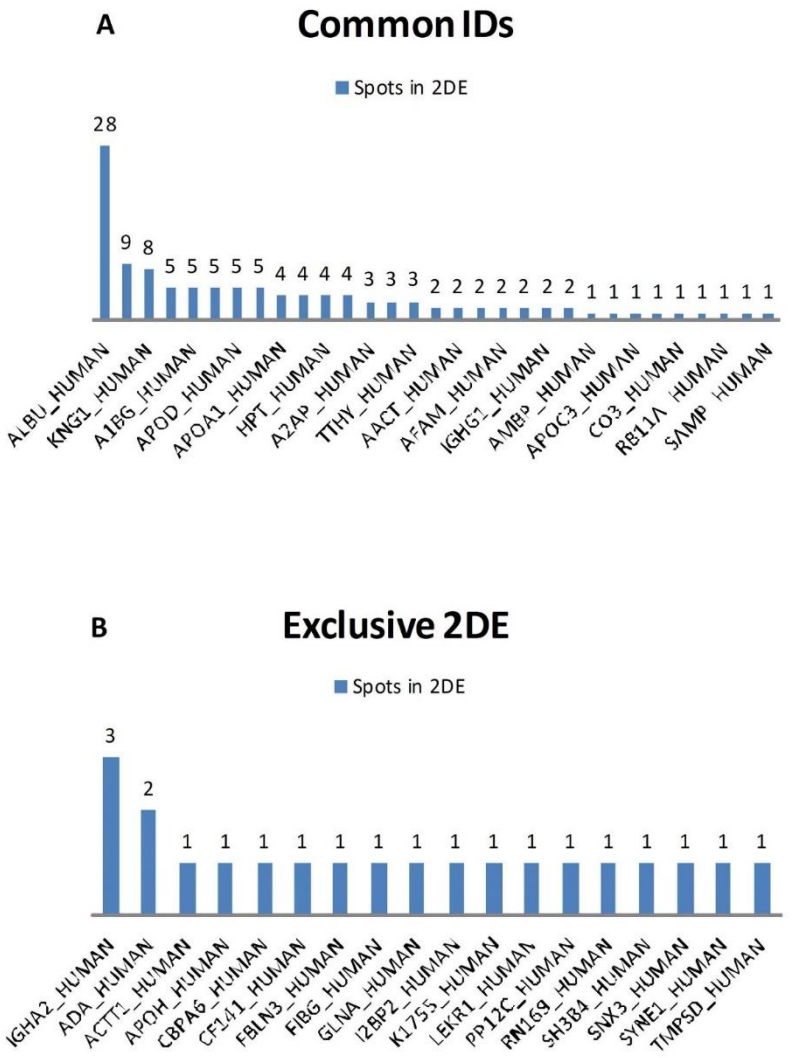
Supplementary Figure S1



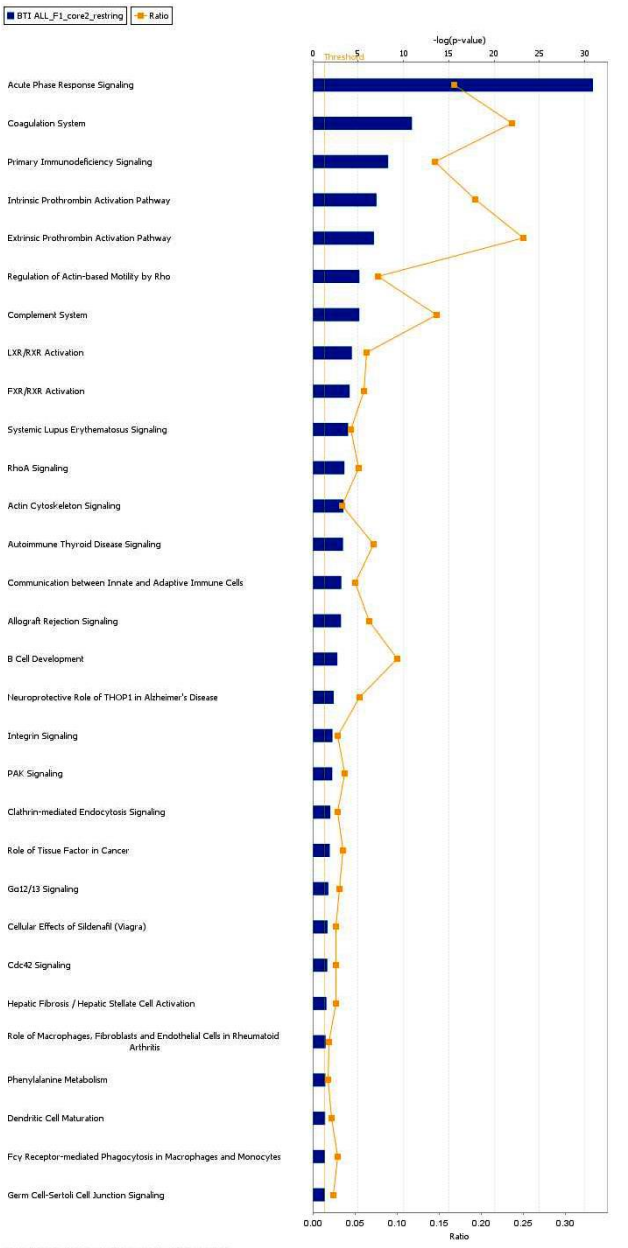
Supplementary Figure S2



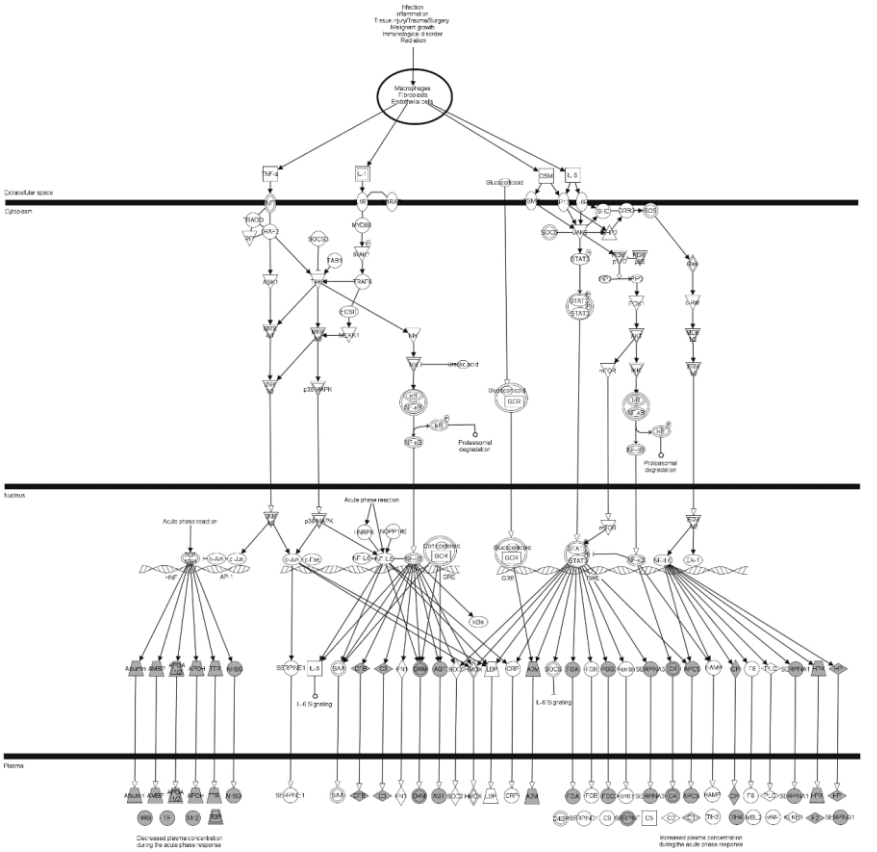
Supplementary figure S3



Supplementary figure S4



Supplementary figure S5



2 Appendix II. Acknowledgements to the Editorials.

Authors would like to thank the Editorials for granting permission to reuse their previously published articles in this thesis:

The links to the final published versions are the following:

Springer Nature

Anitua, E., Prado, R., Nurden, A.T., Nurden, P. (2018) Characterization of Plasma Rich in Growth Factors (PRGF): components and formulations. In *Platelet Rich Plasma in Orthopaedics and Sports Medicine* (Anitua, E., Cugat, R. and Sánchez, M., eds), pp. 29-45, Springer International Publishing
https://link.springer.com/chapter/10.1007/978-3-319-63730-3_2

John Wiley and Sons

Anitua, E., Prado, R., Azkargorta, M., Rodriguez-Suarez, E., Iloro, I., Casado-Vela, J., Elortza, F., Orive, G. (2015) High-throughput proteomic characterization of plasma rich in growth factors (PRGF-Endoret)-derived fibrin clot interactome. *J Tissue Eng Regen Med.* 9, E1-E12
https://onlinelibrary.wiley.com/doi/abs/10.1002/term.1721

Anitua, E., Zalduendo, M.M., Prado, R., Alkhraisat, M.H., Orive, G. (2015) Morphogen and proinflammatory cytokine release kinetics from PRGF-Endoret fibrin scaffolds: evaluation of the effect of leukocyte inclusion. *J Biomed Mater Res A.* 103, 1011-1020
https://onlinelibrary.wiley.com/doi/abs/10.1002/jbm.a.35244

Taylor & Francis

Anitua, E., Prado, R., Troya, M., Zalduendo, M., de la Fuente, M., Pino, A., Muruzabal, F., Orive, G. (2016) Implementation of a more physiological plasma rich in growth factor (PRGF) protocol: Anticoagulant removal and reduction in activator concentration. *Platelets.* 27, 459-466
https://www.tandfonline.com/doi/full/10.3109/09537104.2016.1143921

ELSEVIER

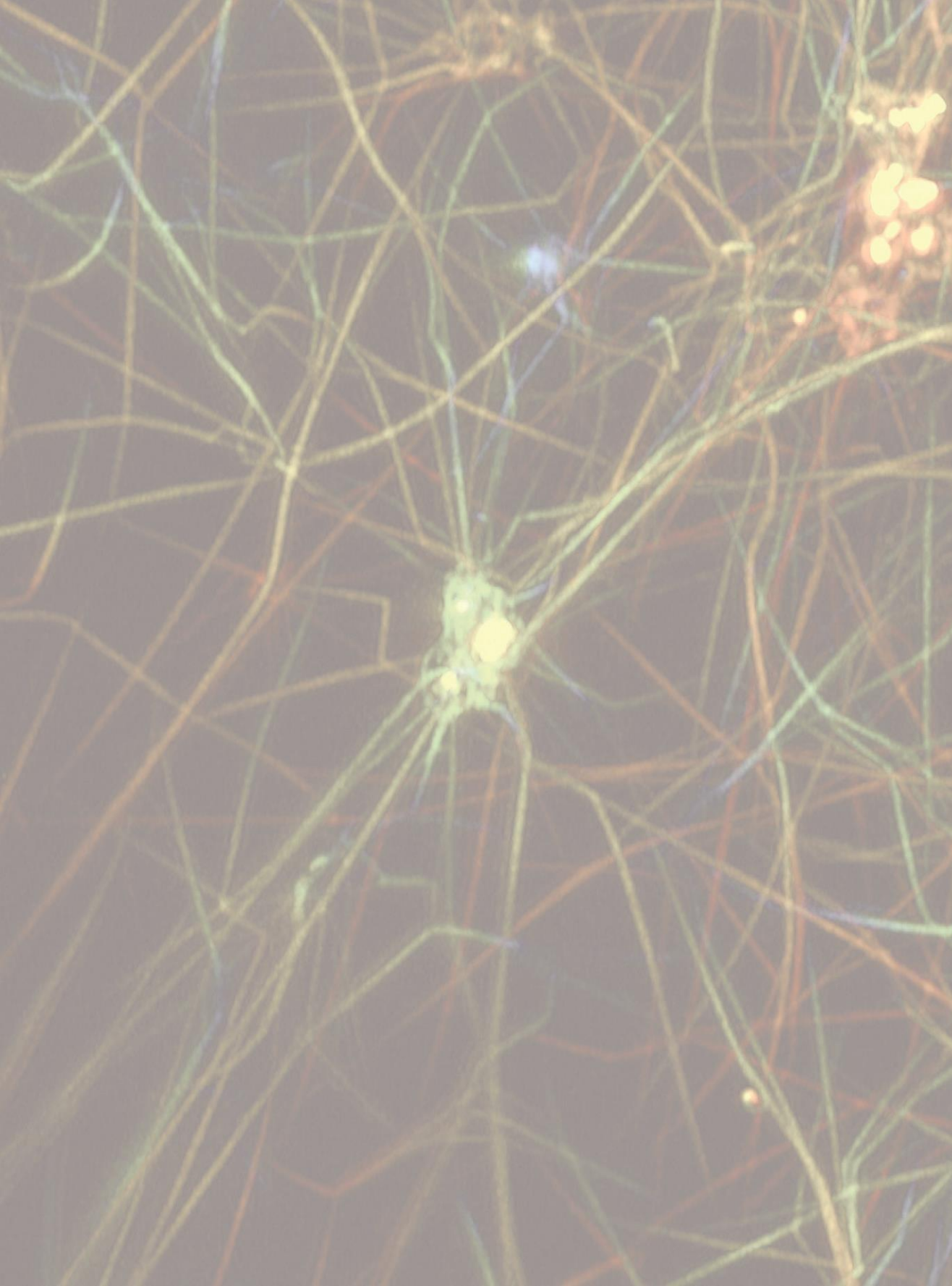
Anitua, E., Prado, R., Orive, G. (2015) Closing regulatory gaps: new ground rules for platelet-rich plasma. *Trends Biotechnol.* 33, 492-495
https://www.sciencedirect.com/science/article/abs/pii/S0167779915001523

Anitua, E., Prado, R., Orive, G. (2017) Allogeneic Platelet-Rich Plasma: At the Dawn of an Off-the-Shelf Therapy? *Trends Biotechnol.* 35, 91-93
https://www.sciencedirect.com/science/article/abs/pii/S0167779916302025

Anitua, E., Prado, R. (2019) Addressing Reproducibility in Stem Cell and PRP Therapies. *Trends Biotechnol.* 37, 340-344
https://www.sciencedirect.com/science/article/abs/pii/S0167779918303202

3 Appendix III. List of figures

4 Appendix IV. List of tables



eman ta zabal zazu

Universidad del País Vasco
Euskal Herriko Unibertsitatea

uirmi
University Institute
for Regenerative Medicine
and Oral Implantology