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Plasma Rich in Growth Factors to treat Knee Osteoarthritis

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Resumen

La artrosis de rodilla es un proceso patológico inducido mecánicamente, y mediada por un desajuste de citoquinas y enzimas que compromete todo el tejido articular de la rodilla. Esta patología es un proceso degenerativo diferente al que ocurre durante el envejecimiento normal produciendo dolor, pérdida de movilidad y deformación de las zonas afectadas.

La artrosis no es una enfermedad grave porque amenace la supervivencia sino porque disminuye considerablemente la calidad de vida por culpa del dolor y la falta de movimiento, incluso puede llegar a ser incapacitante en fases avanzadas. El problema social que acarrea se agrava a medida que aumentan las expectativas de vida de la población. La edad y la obesidad son los mayores factores de riesgo relacionados con la artrosis; con el envejecimiento de la sociedad y el incremento de las tasas de obesidad aumenta el número de pacientes con artrosis y atenderles debidamente supone un enorme gasto social y sanitario. La frecuencia y la cronicidad de la enfermedad la convierten en un reto para la sanidad y los sistemas sociales de todos los países desarrollados. La organización mundial de la salud estima que el 40% de las personas con más de 70 años tiene artrosis de rodilla, el 80% de pacientes con artrosis presenta limitaciones de movimiento y un 25% de ellos no puede realizar las actividades normales de la vida cotidiana. La prevalencia entre los distintos países es desigual, siendo Europa y EEUU las zonas más castigadas. En países avanzados como EEUU las cifras son alarmantes; la estimaciones apuntan unos 46 millones de enfermos con artrosis, más del 50% de adultos con más de 50 años. Para el año 2030 está cifra puede llegar a los 70 millones. Habiéndose convertido la artrosis en la enfermedad musculoesquelética más común, el reto de los sistemas sanitarios es enorme y el coste que esto supone en un futuro cercano es casi inasumible, siendo un verdadero problema financiero a nivel global.

Aunque en un principio la etiopatogenia de la artrosis se centraba casi exclusivamente en el cartílago, actualmente prevalece la idea de que es una

enfermedad que afecta a toda la articulación: cartílago, hueso subcondral, membrana sinovial, ligamentos, tejido neural, etc. De esta forma, todos los componentes de la articulación son imprescindibles para mantener la homeostasis de ésta, y factores tanto genéticos como adquiridos o ambientales pueden romper este equilibrio. Con el tiempo, los esfuerzos para mantener este equilibrio fallan y se produce la degeneración del cartílago, del hueso subcondral y del resto de componentes de la articulación, convirtiéndose en un problema clínico. Esta degeneración de toda la articulación puede evolucionar más o menos rápido pero hasta el momento no existe curación.

Actualmente ningún tratamiento es capaz de parar la progresión de la artrosis o revertir el daño ocasionado, siendo la colocación de prótesis mediante intervención quirúrgica la única salida para estos pacientes. Los tratamientos conservadores incluyen farmacología oral enfocados en el alivio de los síntomas pero no en frenar la enfermedad. También son habituales los suplementos nutritivos condroprotectores de acción muy lenta como la glucosamina, el sulfato de condroitina o la diacerina. Igualmente a menudo se recomienda una disminución de peso y programas adecuados de ejercicio. En fases más avanzadas de la enfermedad se pueden administrar inyecciones intraarticulares. Por ejemplo, en las crisis dolorosas se introducen corticoides en el interior de la articulación para promover un alivio temporal del dolor. Otro método mínimamente invasivo son las infiltraciones de ácido hialurónico (AH) que es un amortiguador y lubricante natural propio de las articulaciones; así, con las inyecciones de AH se consigue restaurar la viscosidad y elasticidad intraarticular, reduciendo el estrés mecánico que se produce sobre el cartílago. Esta técnica ha demostrado ser eficaz en las etapas tempranas de la enfermedad pero a lo largo del tiempo esa eficacia desaparece. Así pues, los tratamientos actuales se centran en paliar el dolor, y no previenen ni curan la enfermedad, ni tampoco frenan su evolución; por lo tanto, es necesario que los esfuerzos empleados en desarrollar nuevos fármacos se centren en la investigación de tratamientos que modifiquen la evolución de la enfermedad y frenen y reviertan la degeneración producida en el cartílago, en el hueso subcondral y en el resto de la articulación.

En los últimos años ha emergido como alternativa a los tratamientos actuales las infiltraciones de plasmas ricos en plaquetas (PRP). Entre estos destaca el plasma rico en factores de crecimiento (PRGF®-Endoret®), una terapia biológica y autóloga que usa el propio plasma del paciente y los factores de crecimiento derivados de las plaquetas, así como un scaffold de fibrina autóloga con propósitos regenerativos. Se ha visto que varios de estos factores de crecimiento actúan sobre toda la articulación influyendo en el desarrollo de la artrosis; así, ayudan a restaurar la homeostasis de la articulación (TGFβ, PDGF, IGF), tienen efectos inductivos y protectores sobre los condrocitos (VEGF, FGF), y actúan sobre los sinoviocitos de la membrana sinovial estimulando la producción de ácido hialurónico y otras moléculas. Además este coctel de factores también tiene características antinflamatorias (HGF), bacteriostáticas y quimiotácticas que atraen a células madres mesenquimales, las cuales también participarían en la regeneración del cartílago. Todas estas propiedades contribuyen a fomentar un ambiente biológico en la articulación propicio para ralentizar la degeneración del cartílago y aliviar los síntomas clínicos.

Teniendo en cuenta las limitaciones de los tratamientos actuales antes descritos y el potencial terapéutico del PRP, el primer estudio enmarcado de estas tesis fue un ensayo clínico aleatorizado en el que se comparaban las infiltraciones intraarticulares de ácido hialurónico con las infiltraciones intraarticulares de PRP en pacientes con artrosis de rodilla. Para ello 176 pacientes fueron asignados de forma aleatorizado en dos grupos; los pacientes del grupo control (N=89) recibieron tres infiltraciones de ácido hialurónico con una pauta de una por semana durante tres semanas. A los pacientes del grupo experimental (N=87) se les administró tres infiltraciones de PRP con la misma pauta que el grupo control. A los seis meses del tratamiento, los pacientes que fueron tratados con PRP mostraron una mejoría en el dolor significativamente superior al grupo del ácido hialurónico, demostrando que a corto plazo el PRP es más eficaz que el ácido hialurónico en el tratamiento de la artrosis.

Sin embargo, el éxito de este tratamiento no reside sólo en las características propias del PRP, sino también en su correcta aplicación: una mala aplicación

puede llevar a una respuesta ineficaz por parte del paciente. De esta forma, aunque las infiltraciones intraarticulares del PRP han mostrado ser seguras y eficaces, esta forma de infiltración no llega a alcanzar las capas más profundas del hueso subcondral, lo que podría limitar su potencial terapéutico en artrosis más severas y avanzadas.

Recientes estudios ponen de manifiesto la importancia del hueso subcondral en la patogenia de la artrosis, lo que remarca la idea antes descrita de que la artrosis es una enfermedad que afecta a toda la articulación. Cuando se altera la homeostasis debido a estos cambios bioquímicos y biomecánicos, todos los tejidos de la articulación participan en restaurar el equilibrio biológico. Estos esfuerzos para recuperar la homeostasis se traducen en respuestas a nivel celular y de la matriz extracelular en todos los tejidos; así, se producen comunicaciones entre las capas más profundas del hueso subcondral y el cartílago y, por otro lado, entre éste y el líquido sinovial que baña toda la articulación. Esta comunicación hueso-cartílago se ha evidenciado con estudios que demuestran cómo vasos y canales alcanzan el cartílago desde el hueso subcondral, y que además son más abundantes en el cartílago de pacientes con artrosis.

Aceptando la idea de que la artrosis es una patología que afecta a toda la rodilla por completo y que todos los tejidos (líquido sinovial, membrana sinovial y hueso subcondral) son dianas terapéuticas clave para un tratamiento eficaz, el siguiente trabajo de esta tesis describe una nueva administración de PRP que pretende alcanzar también el hueso subcondral. Esta técnica consiste en combinar las infiltraciones intraarticulares con infiltraciones intraóseas de PRP en el cóndilo femoral y la meseta tibial, con la intención de que el PRP estimule biológicamente a las células presentes en el hueso subcondral. Para demostrar la seguridad y eficacia de esta técnica, se realizó un ensayo clínico piloto sobre 14 pacientes que presentaban una artrosis severa y que seguramente la colocación de una prótesis hubiese sido el tratamiento de elección. Estos pacientes recibieron un primer tratamiento que combinaba una infiltración intraarticular PRP y dos infiltración intraóseas (una en el cóndilo femoral y otra en la meseta tibial); en las dos siguientes semanas recibieron dos infiltraciones de PRP

intraarticular, una por semana. A los 6 meses del tratamiento se observó una mejoría significativa del dolor y la función respecto a los valores iniciales, y sin ningún efecto adverso relevante.

En este ensayo piloto, también se estudió el posible efecto biológico de este tipo de administración, mostrando un descenso significativo en el número de células madre mesenquimales a lo largo del tratamiento. Para comprender mejor este efecto, se realizó un último estudio en el que se analizaba la composición celular de los líquidos sinoviales en dos grupos de pacientes; un grupo recibió infiltraciones intraarticulares y a los pacientes del otro grupo se les administró PRP de forma intraósea. En este estudio se observó un descenso significativo en el número de células madre presentes en el líquido sinovial de los pacientes que recibieron infiltraciones intraóseas. Sin embargo, este descenso no se produjo en los pacientes que solo recibieron PRP intraarticular, confirmando la importancia del hueso subcondral en la patogenia y evolución de la artrosis. El descenso celular podría ser debido a la acción biológica del PRP, modulando fenómenos como la inflamación que están relacionados con la presencia de células madre. Sin embargo, serán necesarios más estudios para comprender todas las acciones biológicas que puede desencadenar el PRP en las diferentes células y tejidos.

Por lo tanto, nuestros resultados animan a realizar futuros estudios con el fin de arrojar más luz sobre los mecanismos celulares y moleculares y para dilucidar si la aplicación de PRP administrado de forma tanto intraarticular como intraósea podría conducir a cambios estructurales en los diferentes tejidos. Además, las infiltraciones intraóseas podrían tener una aplicación inmediata en otras patologías como en los edemas óseos, restaurando la homeostasis del hueso subcondral y actuando como tratamiento preventivo en la artrosis de rodilla. Por último, también se necesitarán más estudios para ampliar nuestro conocimiento sobre el líquido sinovial como fuente de células madre mesenquimales y su potencial terapéutico.

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Acknowledgments

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Finally, thanks to everyone who has believed in my ideas, who has supported and loved me. This thesis is dedicated to all of them.

Abstract

Knee osteoarthritis (KOA) is a mechanically induced, cytokine and enzyme-mediated disorder involving the whole joint tissues of the knee. Among the new emerging treatments to address this pathology, mesenchymal stem cells (MSCs) and Platelet Rich Plasma (PRP) stand out. We conducted a multicenter, double-blind clinical trial to evaluate and compare the efficacy and safety of plasma rich in growth factors (PRGF) versus hyaluronic acid (HA), as a short-term treatment for knee pain from osteoarthritis. PRGF showed superior short-term results when compared to HA, with a comparable safety profile, in alleviating symptoms of mild to moderate OA of the knee. However, some patients showed no response to intraarticular infiltration of plasma rich in growth factors, likely due to the fact that this treatment is insufficient to reach the subchondral bone. In order to elucidate this lack of response, we explored a new strategy consisting in performing intraosseous infiltrations of PRP into the subchondral bone besides the conventional intraarticular injection to tackle several knee joint tissues at once. Targeting synovial membrane, synovial fluid, articular cartilage, and subchondral bone with intraarticular injections and intraosseous infiltrations of PRP reduced pain and MSCs in SF, besides significantly improving knee joint function in patients with severe KOA. We set out a third study to assess the suitability of SF as a source of MSCs and their response to the biological mechanisms implicated in the effects of two different treatment modalities of PRP applications on osteoarthritic patients. The synovial fluids of 31 patients, who received PRP using two different techniques, were collected just before and one week after infiltration. SF of osteoarthritic patients contains a resident population of MSCs, which was reduced when PRP was infiltrated into the subchondral bone. The reduction in MSCs in the synovial fluid was further confirmed by the presence of colony forming units (CFU-F). Patients receiving intra-articular infiltration did not show variations in any of the cellular populations by flow cytometry or CFU-F assay.

In summary, PRGF-Endoret is safe and effective therapy in reducing KOA pain. Targeting synovial membrane, synovial fluid, articular cartilage, and subchondral bone with intraarticular injections and intraosseous infiltrations of PRP this autologous biologic reduces pain and MSCs in SF, besides significantly improving knee joint function and may favor MSCs therapeutic effect by decreasing pro-inflammatory processes present in the synovial fluid of OA patients.

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List of Abbreviations

AC.	Articular cartilage
ACL.	Anterior cruciate ligament
ADAMTs.	A disintegrin and metalloproteinase with thrombospondin motifs
ADL.	Function in daily living
ARE.	Antioxidant response element
BDGF.	Brain-derived growth factor
BM.	Bone marrow
BMI.	Body mass index
BMLs.	Bone marrow lesions
BTI.	Biotechnology Institute
Ca CL2.	Calcium chloride
CB1.	Cannabinoid receptor 1
CB2.	Canabinoid receptor 2
CCL-5.	Chemokine C-C motif ligand 5
CFU-F.	Colony forming units
COX-2.	Cyclooxygenase 2
COMP.	Cartilage oligomeric matrix protein
CPCs.	Chondroprogenitor cells
CTGF.	Connective tissue growth factor
CXCR4.	CXC receptor 4
DAMPs.	Damage-associated molecular patterns
ECM.	Extracellular matrix
F1.	Fraction one
F2.	Fraction two
FGF.	Fibroblastic growth factor
GFs.	Growth factors
HA.	Hyaluronic acid
HGF.	Hepatocyte growth factor
HMGB1.	High-mobility group protein B1
IA.	Intraarticular
IFN.	Interferon gamma
IGF.	Insulin-like growth factor
IL-1β.	Interleukin-1β
IL-6.	Interleukin 6
IL-8.	Interleukin 8
IL-10.	Interleukin 10
IO.	Intraosseous
KOA.	Knee osteoarthritis

KOOS. Knee injury and osteoarthritis outcome score
L-PRP. Leukocyte PRP
LWHA. Low molecular-weight hyaluronic acid
M-CSF. Macrophage colony stimulating factor
M1. Proinflammatory macrophages
M2. Trophic macrophages
MMPs. Matrix metalloproteinases
MRI. Magnetic resonance imaginig
MSCs. Mesenchymal stem cells
NF-kβ. Nuclear factor kβ
NGF. Nerve growth factor
NO. Nitric oxide
NrF2. NF-E2-related factor 2
NSAID. Nonsteroidal antiinflammatory drug
OMERAT-OARSI. Outcome measures for rheumatology committee and osteoarthritis research society International standing committee for clinical trials response criteria initiative
PDGF. Platelet-derived growth factor
PGE2. Prostaglandine E2
PM. Periarticular muscles
PMNs. Polymorphonuclear neutrophils
PRGF. Plasma rich in growth factors
PRP. Platelet rich plasma
QOL. Quality of life
RANKL. Receptor activator for Nuclear kB ligand
ROS. Reactive oxygen species
SAMP8 Senescence-accelerated prone mouse
SB. Subchondral bone
SDF-1. Stromal derived growth factor
SF. Synovial fluid
SIRT-1. Sirtuin-1
SM. Synovial membrane
SOX-9. SRY-type high-mobility-group box transcription factor-9
TGFB. Transforming growth factor
TIMP. Tissue inhibitors of metalloproteinases
TLRs. Toll-like receptors
TNFα. Tumor necrosis factor
US. Ultrasound
VAS. Visual Analogue Scale
VEGF. Vascular endothelial growth factor
WOMAC Western Ontario and McMaster Universities osteoarthritis Index questionnaire

List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I. Anitua E, Sánchez M, Orive G, S Padilla. A biological therapy to osteoarthritis treatment using platelet-rich plasma. Expert Opin Biol Ther. 2013 Aug;13(8):1161-72.

II. Sánchez M, Anitua E, Goiriena JJ, Sanchez P, Delgado D, Orive G, Padilla S. A new strategy to tackle severe knee osteoarthritis: Combination of intra-articular and intraosseous injections of Platelet Rich Plasma. Expert Opin Biol Ther. 2016;16(5):627-43

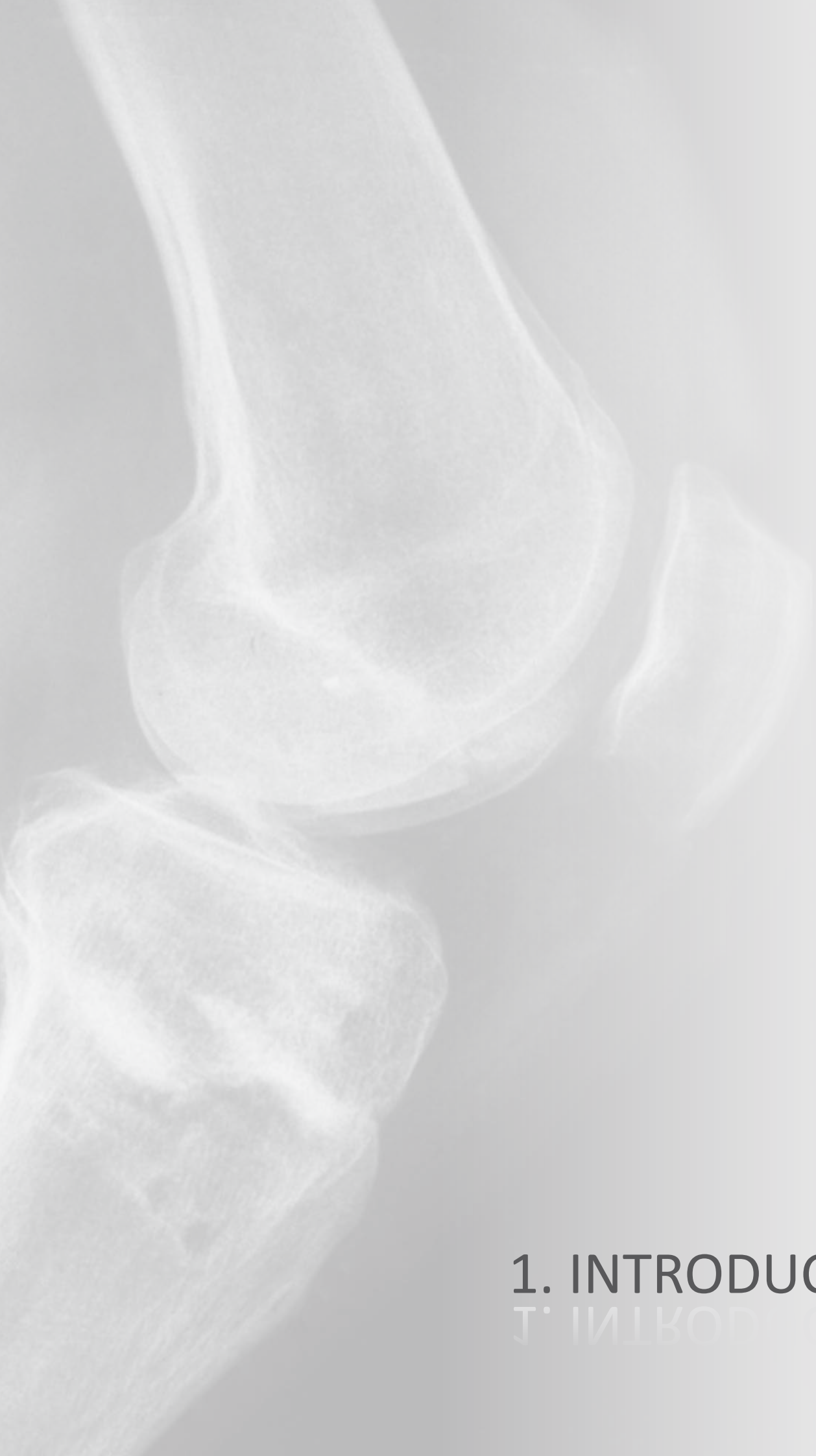
III. Anitua E, Prado R, Sánchez M, Orive G. Platelet-Rich Plasma: Preparation and Formulation. Oper Tech Orthop. 2012;22:25-32 .

IV. Sanchez M, Fiz N, Azofra J, Usabiaga J, Aduriz Recalde E, Garcia Gutierrez A, Albillos J, Gárate R, Aguirre JJ, Padilla S, Orive G, Anitua E. 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: the journal of arthroscopic & related surgery: official publication of the Arthroscopy Association of North America and the International Arthroscopy Association.2012;28:1070-8.

V. Sánchez M, Fiz N, Guadilla J, Padilla S, Anitua E, Sánchez P, Delgado D. Intraosseous Infiltration of Platelet-Rich Plasma for Severe Knee Osteoarthritis. Arthrosc Tech. 2014 Dec 15;3(6):e713-7.

VI. Sánchez M, Delgado D, Sánchez P, Muinos- López E, Paiva B, Granero-Moltó F, Prosper F, Pompei O, Pérez JC, Azofra J, Padilla S, Fiz N. Combination of intra-articular and intraosseous injections of Platelet Rich Plasma for Severe Knee Osteoarthritis: a pilot study. Biomed Res Int. 2016;2016:4868613. doi: 10.1155/2016/4868613. Epub 2016 Jul 4.

VII. Muinos-Lopez E, Delgado D, Sánchez P, Paiva B, Anitua E, Fiz N, Aizpurua B, Guadilla J, Pérez JC, Padilla S, Granero-Moltó F, Prosper F, Sánchez M. Modulation of synovial fluid cell by intraarticular and intraosseous Platelet Rich Plasma administration. Stem Cells Int. 2016;2016:1247950.



1. INTRODUCTION

1. INTRODUCTION (I - II)

1.1. OVERVIEW OF THE KNEE JOINT

Knee joint is a complex biological system composed of articular cartilage (AC), synovial fluid (SF), synovium (SM), menisci, ligaments, subchondral bone (SB), and periarticular muscles (PM) . They all are highly specialized mechano-sensitive and/or load-bearing tissues whose homeostasis relies on the precise interaction between biomolecules and cells when the latter are subjected to physiological loading [200, 221]. From a mechanical viewpoint, the knee is a complex, shock-absorbing interface in which a coordinated and sequentially ordered engagement of the joint´s elements and muscles is required to maintain the physical integrity of anatomical structures and homeostasis of knee tissues. In the functionality of the knee, articular cartilage provides an elastic, gliding, smooth frictionless surface, while SB, a very low viscoelastic structure, together with periarticular muscles and ligaments, act as shock absorber structures, accounting for 30% and 50% of the total absorbing energy and only 1-3% for the AC 30, [88]. Articular cartilage is an avascular tissue that lies functionally sandwiched between the SM and the SB, two highly vascularized and innervated tissues that might well be the triggers of inflammation and make the chondrocytes victims instead of culprit of articular cartilage destruction [29]. Except for articular cartilage, the rest of joint tissues are endowed with chemoreceptors and mechanoreceptors from where the nociceptive stimulus might drive to peripheral pain. As such, the joint can be considered to be an organ [29, 162]. The hyaline cartilage is surprisingly durable and has a very low friction coefficient, thus making it highly resistant to compression and shear forces under physiological conditions. The integrity of this cartilage has a major effect on quality of life as its deterioration or damage markedly restricts mobility due to the resulting pain, thereby limiting autonomy, quality of life, and, eventually, survival. Changes in the degree of physical activity, whether too much (competitive sports) or too little (sedentary lifestyle), together with the appearance of a completely new diet, are a key factor underlying the emergence of new diseases in Western society[117].

1.1.1. Biology of Knee joint tissues

Adult hyaline cartilage is a tissue with no vascular, lymphatic, or nerve component whose only cell element is the chondrocyte, a mesenchymal cell which synthesizes and maintains an abundant protein-rich extracellular matrix (ECM) at a very low remodeling rate[32, 128, 5]. The quantity and orientation

of these two tissue components (chondrocytes and extracellular matrix) vary depending on their location and level of mechanical stress. Water, which is mainly located in the surface regions, with a small quantity being located much deeper, accounts for between 65% and 80% of wet cartilage weight. This water provides lubrication and a low coefficient of sliding friction to the joint surface whereas also participating actively in cartilage nutrition and tissue-based load distribution (Fig. 1). More than 90% of the collagen fibers present are type II—although types VI, IX, and XI are also present—accounting for 10–20% of the wet weight. Their distribution and organization into a network via the different layers provides cartilage with the ability to mainly transfer stress forces. The rest of the joint cartilage (10–20%) consists of decorin, biglycans, and proteoglycans, especially aggrecans. These components bind to hyaluronic acid (synthesized by chondrocytes and synovial fibroblasts) via two molecules, namely chondroitin and keratan sulfate [199]. Chondrocytes account for only 1–5% of the volume of hyaline cartilage, and are quiescence cells, thus they do not divide in physiological conditions. They are responsible for maintaining homeostasis/allostasis in the tissue as a result of their high metabolic activity and ability to respond to mechanical stress and biological stimuli by continually remodeling the ECM, thereby adapting it to its required role. As a functional structure that is highly specialized for the efficient lubrication, distribution, and transmission of mechanical forces in the joint, AC is a stratified tissue. Thus, as can be seen in Fig. 1, its components are organized into four different zones. This arrangement allows the cartilage to transfer the physiological forces to which the joint is subjected.

SM is a specialized mesenchymal soft tissue made up of a lining layer with two distinct types of cells: synoviocytes that are fibroblastic-like cells and secrete lubricin and hyaluronan, and macrophages, although mesenchymal stem cells (MSCs) too have been isolated both in normal and osteoarthritic synovial membrane[81, 84]. MSCs might play an important role as chondroprogenitor cells (CPCs) in the reparative response to articular cartilage damage[87, 190]. Another layer, known as subintima, includes blood and lymphatic vessels associated with terminal nerve nociceptors. The multicellularity and vascularity endow the SM with a highly reactive capacity against what their cells might interpret as an insult or stress (mechanical or biochemical) [187, 193]. Such an insult would trigger an inflammatory defense response in order to preserve or restore joint tissue homeostasis and function[71] (Fig. 1).

Stemming primarily from an ultrafiltrate of plasma and secretions of chondrocytes and synoviocytes, SF is a viscous liquid composed of hyaluronan (HA) and lubricin, cytokines and growth factors, and a minor presence of cells[84]. Synovial fluid plays a crucial role in joint homeostasis through the lubrication of articulating cartilage surface, facilitating the transport of nutrients and waste prod-

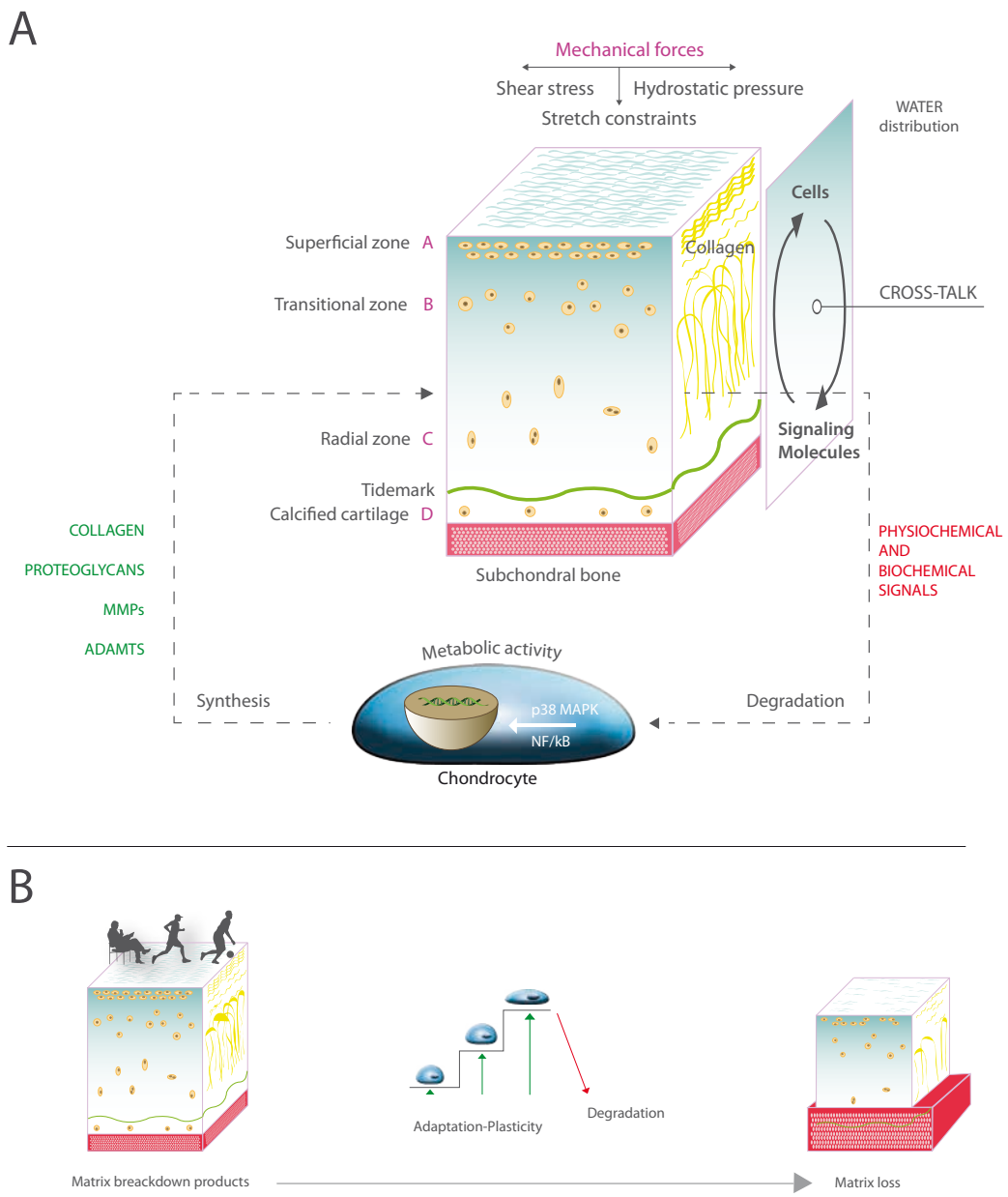


FIG. 1

A. In the complex cartilage-bone-based mechanotransduction system, the mechanical energy applied to the joint is reflected in the extracellular matrix, and subsequently in the chondrocyte nucleus. Joint cell exposure to non physiological stimuli leads to a rupture in the cartilage balance between degradation and synthesis known as cartilage homeostasis. There appears to be a molecular, cellular, and fluid communication between the cartilage and bone.

B. The survival/viability of the chondrocyte is affected to a large extent by the presence of a sufficient (plasticity), but not excessive(degeneration), mechanical stimulus that would inevitably lead to the disarrangement of structures such as the subchondral bone mediated mainly by deregulated chondrocytes, perpetuating a catabolic microenvironment and eventually the joint failure.

ucts, and the communication between different cell populations of the joint[84] where the population of MSCs in normal conditions increases after joint structure-damage and osteoarthritis (OA) [93, 136, 192]. SB is a complex structure at the interface between AC and the rigid skeleton composed of epiphyseal trabecular bone that lies immediately below the calcified cartilage , and consists of two different anatomical entities, one called subchondral or cortical plate which is nonporous and poorly vascularized cortical bone, and the SB which contains bone marrow (fatty) and trabecular bone 35, 88 with blood vessels, sensory nerves, endothelium, osteoblasts, osteoclasts and haematopoietic bone marrow [202]. Together with the AC, it forms the osteochondral functional unit, which undergoes mechanical stresses that trigger adaptive cell responses and establish a crosstalk among them to adjust their architecture to ongoing physical and biochemical challenges [131, 202]. SB play a key role in both shock absorbing by attenuating 30-50 % of the joint load and by providing the deepest layers of articular cartilage with nutrients supply and removal of waste products [88, 127].

1.1.2. Homeostasis and joint-tissue damage

At a biomechanical level, knee components work as a network from which the joint´s functional property as an organ emerges, a property known as dynamic stability, whose equivalent at the tissular and cellular level is termed tissue and cell homeostasis. Such identities do not imply biological constancy but rather dynamic adaptability[33]. The phenotype of chondrocytes, synoviocytes, and osteoblasts is constantly adapting to its dependence on the biochemical, biophysical and mechanical loading features of their microenvironment [97, 144, 177, 221.] Signals and ligands from ECM drive cell responses and tightly fine tune the anabolic/catabolic balance in order to maintain or to adapt their ECM composition to the ongoing mechanical challenges[221,] thereby protecting against the deleterious effect of some supraphysiological stimuli [119]. Although the load-bearing capability of articular cartilage is largely attributable to pericellular, territorial and interterritorial spatial organization of collagens, aggrecans, hyaluronans, and water [199, 216], it is the chondrocyte that senses and distinguishes among unloaded, physiological and hyperphysiologic loading magnitudes, and responds accordingly to them [77, 144, 173, 200, 217] in order to maintain a dynamic homeostasis of ECM by degrading, synthesizing, and reassembling it. In the unloaded cartilage, the chondrocyte shows an inhibition of aggrecans and collagens synthesis and an enhanced matrix degradation mediated by matrix metalloproteinases (MMPs) and A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTs). When subjected to physiologic mechanical stress, chondrocyte responds with an inhibition of IL-1-induced proinflamatory cytokines production and an increased matrix synthesis whereas in the cartilage with hyperphysiologic and injurious strains an increase matrix catabolism, matrix frag-

ments and necrosis cell in addition to the activation of proinflammatory pathways leads to cartilage destruction [96, 144, 173, 200].

Tissue interactions govern most developmental processes, from the very early patterning events of cell differentiation, through a process called morphogenesis and finally growth of the many organs in the embryo. All human synovial joints share the same developmental processes. Formation of the skeleton is no exception, and most of the tissues differentiating in the newly forming limb arise from mesenchymal cells. These cells give rise to the various articular tissues, with the exception of neuronal elements and blood vessels[42, 148]. The regulation of articular cartilage development and homeostatic processes throughout life is carried out under the influence of numerous growth factors and cytokines which act in concert as signaling molecular pathways[71, 73].

1.2. KNEE OSTEOARTHRITIS

Knee osteoarthritis (KOA) is a mechanically induced, cytokine and enzyme-mediated disorder undergoing different phases and phenotypes, with pain as the clinical hallmark of the disease [118]. Knee osteoarthritis encompasses a cluster of degenerative joint conditions with different biochemical, inflammatory, and genetic signatures that generates distinct subtypes, evolving in phases, the severity of whose single resulting phenotype impacts the quality of life of the patient [96]. Estimates suggest that about 46 million patients suffer from OA in developed countries, more than 50% of adults over 50 years; by 2030 this figure may reach 70 million [69]. Despite the enormous effort made to mitigate symptoms, what is lacking is an early disease-modifying therapeutic intervention aimed at preventing the progressive destruction of articular cartilage, or even reversing the initial post-traumatic damage. In this absence of a whole regenerative joint therapy, doctors must resort to joint replacement as the only solution for patients in advanced cases of OA[139].

1.2.1. Joint injury and inflammation

Abnormal mechanical stress and/or biochemical mediators variously stemming from trauma, obesity, lesion or disfunction of knee components, as well as metabolic diseases break knee dynamic stability and trigger biological responses that disrupt the homeostasis of cells and tissues of the joint in a locally, sustained, low-grade inflammatory fashion leading to a matrix degradation (Fig. 2) [25, 187].

In OA synovial joints, chondrocytes, synoviocytes, and osteoblasts respond with an early inflammatory catabolic response when subjected to a microenvironment with either non-physiological magnitudes of mechanical loading or pro-inflammatory cytokines, thereby leading to aggrecans depletion, collagen II cleavage, and disruption of water tissue distribution [173, 199]. The extracellular matrix breakdown of insoluble fibrous structural proteins (collagens, laminins, elastase) and proteoglycans carried out by matrix metalloproteinases (MMP-1 and MMP-3) and aggrecanases (ADAMTS-4 and ADAMTS-5) lead to both a decrease in load-bearing capacity of articular cartilage and a production of fragments which, acting as damage-associated molecular patters (DAMPS) activate pattern-recognition receptors expressed by chondrocytes, synovial macrophages and fibroblasts, and osteoblast to express inflammatory cytokines and to influence cell fates [71, 82, 186] (Fig 2). In the wake of this sterile matrix degradation of articular cartilage, there is a depletion of aggrecans and cleavage of collagen II, which leads to the erosion of cartilage, subsequently altering the nanostiffness of articular cartilage and weakening its load-bearing capacity[30, 199]. Besides the release of matrix-degrading products, the ECM degradation deeply impacts the micromechanical environment of chondrocytes and changes the magnitude of dynamic compressive forces transferred from them to the underlying bone, and these aberrant new sustained (chronic) abnormal forces prompt chondrocytes and osteoblasts to respond with a pro-inflammatory gene expression through activation of the NFkB signalling pathway [79, 144] and increased osteoclastogenesis, thereby increasing bone resorption and sclerosis [174, 177] respectively. Nevertheless, evidence is accumulating about how alterations of subchondral bone induced by mechanical or vascular stresses, might be the start point in the catabolic loop of AC degradation and extend to SM (Fig. 2) [34, 118, 127, 162]. Cartilage is an avascular tissue whose cells rely on synovial fluid and subchondral plate to obtain oxygen and a supply of nutrients, the subchondral bone account source for at least 50% of articular cartilage requirements in oxygen and glucose [88, 127]. Therefore, despite the fact that tracking down the “first pathogenic event” responsible for the initiation of KOA still proves an elusive quest, any induced mechanical or metabolic damage to joint tissues in combination with predetermined influences such as genetic, obesity, and aging, paves the way to initiating a harmful joint environment involving AC, SM, and SCB, and then it is difficult to establish who was first [108]. The neurovascular structures of the knee are rife with nociceptors, which together with synovial fibroblasts and macrophages possess Toll-like receptors (TLRs) [126]. Subchondral bone has always been present in the equation of OA pathogenesis [107, 161], and the hypothesis that changes in the subchondral bone may play a pivotal role in the development and progression of articular cartilage breakdown and might even precede the latter [35, 225] Pelletier 2005 (del 2015????) is increasingly recognized mainly associated with the mounting evidence in the crosstalk or communication between the SB and AC [106, 150, 151, 202, 204] (Figs. 3 and 4)

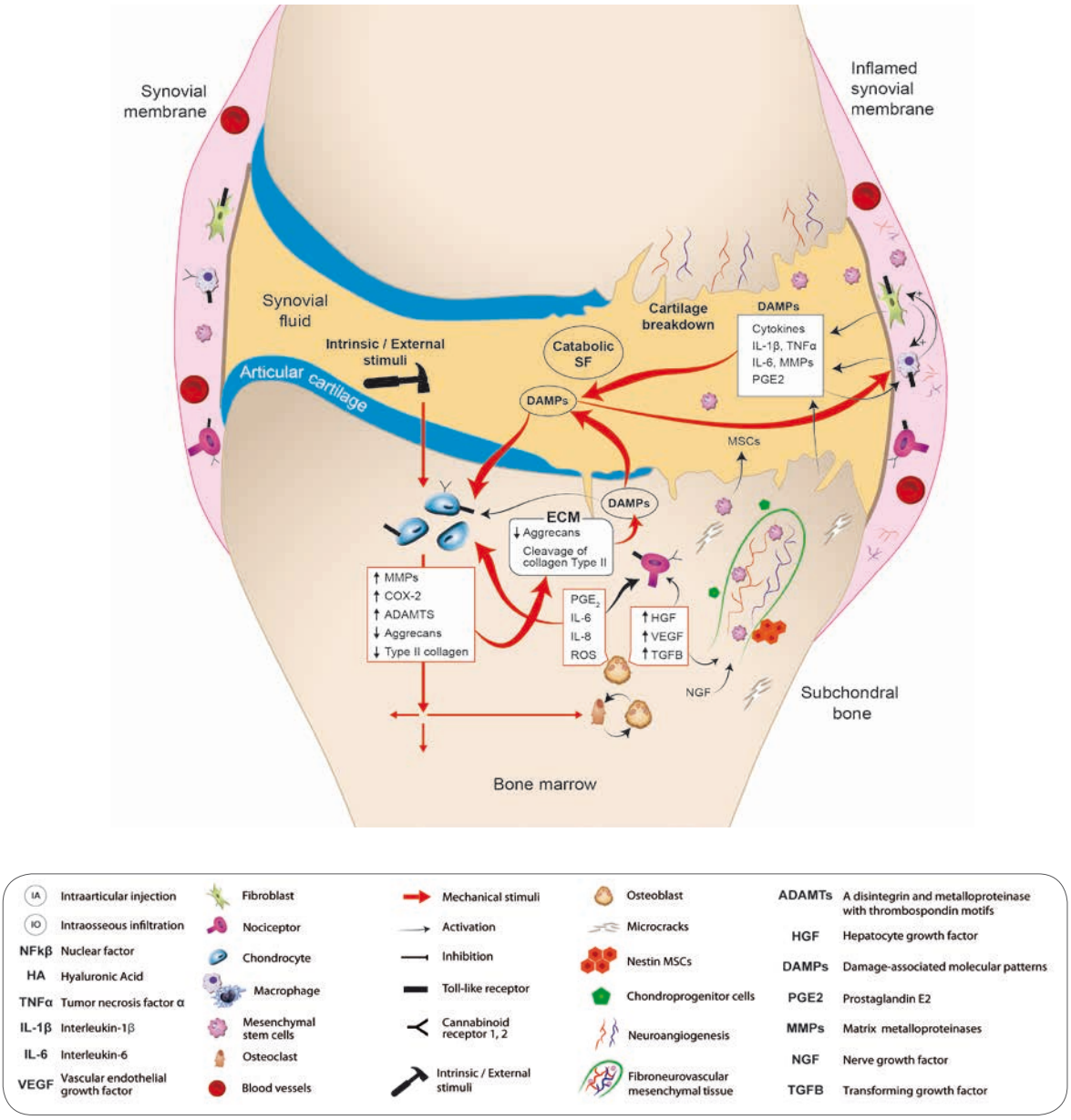
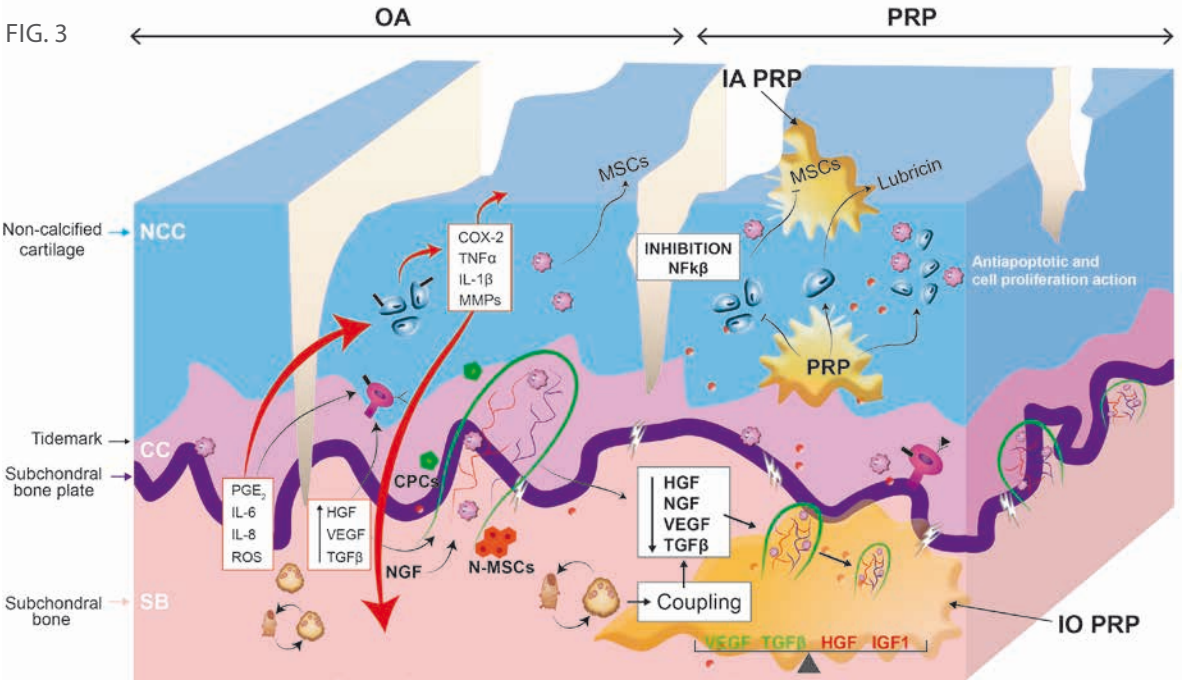


FIG. 2

Abnormal distribution of mechanical loading across joint cartilage breaks the homeostasis of articular cartilage and provokes adaptive or catabolic cell responses, which leads to an increased synthesis of matrix metalloproteinases (MMPs) and aggrecanases (ADAMTS), expression of proinflammatory cytokines and mediators such as interleukin-1B (IL-1B) and cyclooxygenase-2 (COX-2), high levels of reactive oxygen species (ROS), disruption of water tissue distribution, and matrix fragments 30, 70, 72, 199. Proinflammatory cytokines involved in OA, such as IL-1B and TNF-a are major players in the destruction of AC by inhibiting the synthesis of aggrecans and collagen type II while at the same time stimulating the synthesis of MMPs in chondrocytes 96. It has been reported that activation of TLRs of synovial macrophages and fibroblasts, and monocytes by DAMPs present in an inflammatory SF, is an important pathway in promoting synovitis in OA through the NFkB pathway 186, cells that respond with the production of MMP-1, MMP-3, and MMP13, IL-1B, TNFa, and IL-6 among other catabolic mediators, promoting synovitis in OA 96, 186, 193.

FIG. 3



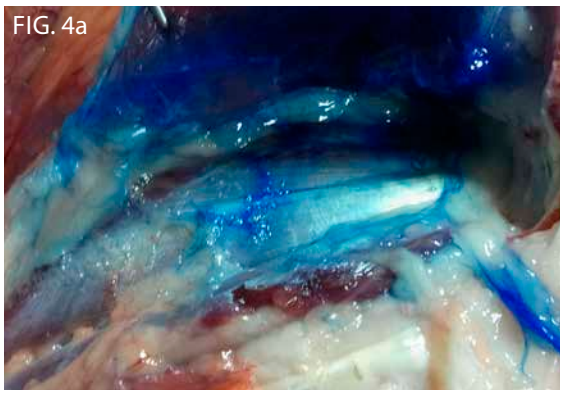


FIG. 3

This schematic drawing illustrates the outside-in (AC-SB) and inside-out (SB-AC) flow of mediators and cells. SB as a point of egress of morphogens and cells, through the channels and vessels breaching the osteochondral junction, partially recruited by the osteoarthritic synovial fluid[106, 202]. This cell invasion of cartilage might be facilitated by the loss of aggrecans, collagen II cleavage, and disruption of water tissue distribution 199 of the AC as well as by the secretion by MSCs of fibrinolytic enzymes [145]. The excessive presence of TGFB1 and VEGF in OA subchondral bone [106, 202, 225] could be a driving factor for changes in osteoblast-osteoclast coupling thereby leading to a bone remodelling imbalance [106, 204, 224], overexpression of NGF, and fibroneurovascular growth, all of which are changes that additionally might well contribute to overlying cartilage degradation [224, 225], pain [35, 131, 202] and an osteoarthritic joint [224, 225].

FIG. 4

Infiltration of activated PRP previously stained with methylene blue performed in sheep`s joint to ascertain its diffusion across the joint. Once the animals were put down and the joint opened, we infiltrated the femoral condyle as well, and took these picture in which first the PRP liquid-to-gel ·3D injectable scaffold had been converted into a matrix-like viscous and malleable structure, which adhered to synovium and covered it , and second it diffused across the condyle (Fig. 4 unpublished data).

1.2.2. Interaction between tissues, cells, and biomolecules at knee joint level

OA is driven primarily by both mechanical stress and inflammatory signals (IL-1β and TNFα) orchestrated by the NF-kβ signaling molecules which have been shown to mediate articular cartilage degradation by upregulation of matrix-degrading MMPs [72, 132]. The activation of the NF-kβ signaling pathway can generate altered states of quiescent chondrocytes thereby pushing chondrocytes to a more differentiated, hypertrophic-like state in an attempt to maintain or restore tissue homeostasis, as well as recapitulating some developmental cell phenotypes [72, 132, 205]. A variety of cells and cell signaling molecules, which dynamically form the structural network of the joint tissues, are extremely well communicated and may use the fluid flow to migrate and reach injured areas mainly attracted by cell signaling factors, biochemical gradients and matrix fragments [66, 98, 206]. Cells from different tissues of the joint but chiefly the quiescent chondrocytes undergo and sense nonphysiological stimuli as an insult, modulating and taking on a different phenotype whose gene expression products (anabolics and catabolics) orchestrate a defense-inflammatory response [72, 185, 207] in a miscued attempt to either maintain the tissue homeostasis and integrity or mimic the repair process (Figs. 1 and 2).

Nevertheless the tissue response turns out to be catabolic, thereby altering the cells' microenvironment and breaking down the extracellular matrix. The response of chondrocytes in the osteoarthritic cartilage is heterogeneous and oriented towards hypoanabolism, which encompasses cell proliferation, apoptosis, and phenotypic alterations. Such a response results in a reactive or hypertrophic chondrocyte phenotype known as deregulated chondrocytes [72, 185, 205] whose catabolic gene-expression causes a net loss of extracellular matrix [185]. Not only chondrocytes but also synovial macrophages and fibroblasts influenced in a paracrine manner take on a pro-inflammatory phenotype [24, 71]. The extracellular matrix which is made up mainly of water, type II collagen and aggrecans, drains away and degenerates as a consequence of the action of catabolic cytokines (TNFα and IL-Iβ), metalloproteinases (MMPs, MMP13), and aggrecanases (ADAMTS). These products are primarily released by chondrocytes, synoviocytes, and mononucleated cells, breaking the collagen and aggrecans down in a slow and relentless degenerative process [72, 185] and thereby giving rise to articular chondrocytes expressing classic hypertrophic markers (characteristic of the growth-plate chondrocytes) and apoptosis [1, 72, 157].

1.2.2.1. Synovial membrane and subchondral bone in OA

In recent years, a great deal of evidence has been accumulating in favour of seeing as decisive the contribution of synovitis and SB on articular cartilage degradation, and on the progression of OA, where AC may after all be the victim, and not the only culprit of catabolic inflammatory cytokines

stemming from SM and SB, and triggered by abnormal mechanical stresses (Fig. 2) [30, 88, 96, 127, 160, 186, 193]. Hence, cartilage integrity is highly dependent on the underlying subchondral bed and vice versa, as well as on a healthy synovium and its product the synovial fluid (Fig. 3) [20, 162].

Evidence has been mounting for the role of synovium inflammation in the pathogenesis and progression of OA [48, 186]. Matrix-degradation products such as fibronectin, tenascin C, high-mobility group protein B1 (HMGB1), and low molecular-weight hyaluronic acid (LWHA) among others within the SF [187, 193] can act as Toll-like receptor (TLR) ligands or damage-associated molecular patterns (DAMPs) and activate TLR-2 and TLR 4 of synovial macrophages and fibroblasts, chondrocytes, and osteoblasts, leading to the activation of the intracellular signaling pathway nuclear factor kappa B (NFkB) (Fig. 2) [132, 186]. The activation of the NFkB signaling pathway mediates the expression of several inflammatory genes and the synthesis of interleukin 1beta (IL-1B), interleukin 6 (IL-6), interleukin 10 (IL-10), nitric oxide (NO), prostaglandine E2 (PGE2), tumor necrosis factor alpha (TNF-a), interferon gama (IFN-j), and nerve growth factor (NGF) among other inflammatory cytokines (Fig. 2 and 5) [71, 72, 96, 132, 186]. Moreover, NFkB transcription factor has been postulated as a functional connection among the mechanobiological, developmental programming and stress-inflammatory responses of AC, SM, and SB, making the NFkB signaling pathway a potential multi-faceted target in OA disease (Fig. 5) [132, 134].

Another pathway involved in OA synovitis is the activation of complement as it has been shown by Wang et al (2011) who reported that the expression and activation of complement is abnormally high in the human OA joint, where the presence of some products of dysregulated cartilage remodeling such as fibromodulin, cartilage oligomeric matrix protein (COMP), and osteoadherin in synovial fluid and membranes might account for this activation [186]. Important clinical features of the inflamed synovium are pain, swelling, and stiffness [193], whereas hystopathological changes are characterized by an uneven, abnormal cell infiltration and an aberrant proliferation of macrophages, fibroblasts, and blood and lymphatic endothelial cells that lead to a neofibroangiogenesis [193]. SM and SB are highly vascularized and innervated tissues endowed with heat receptors, chemoreceptors, and mechanoreceptors from where nociceptive stimuli, coming from a microenvironment undergoing non-physiological mechanical loading and/or pro-inflammatory cytokines and damage-associated molecular patterns (DAMPs), might initially lead to peripheral and eventually both peripheral and neuropathic pain by mechanisms yet to be fully identified [126, 186]. In addition, proinflammatory cytokines may contribute to pain by stimulating hyperalgesia and sensitizing joint nociceptors to other stimuli [186, 193] thereby perpetuating a catabolic vicious circle among SM, AC, and SB (Figs. 2, 3, and 5).

1.2.2.2. Joint Inflammation and Mesenchymal Stem Cells

Aggression and inflammation to AC, SM, menisci, and ligaments has been reported to bring about an increase of MSCs in SF [137, 192], which is commonly interpreted as a tissue response to injury [93, 137] equivalent to the response of migratory chondrogenic progenitor cells from SB to injured cartilage [99,194].

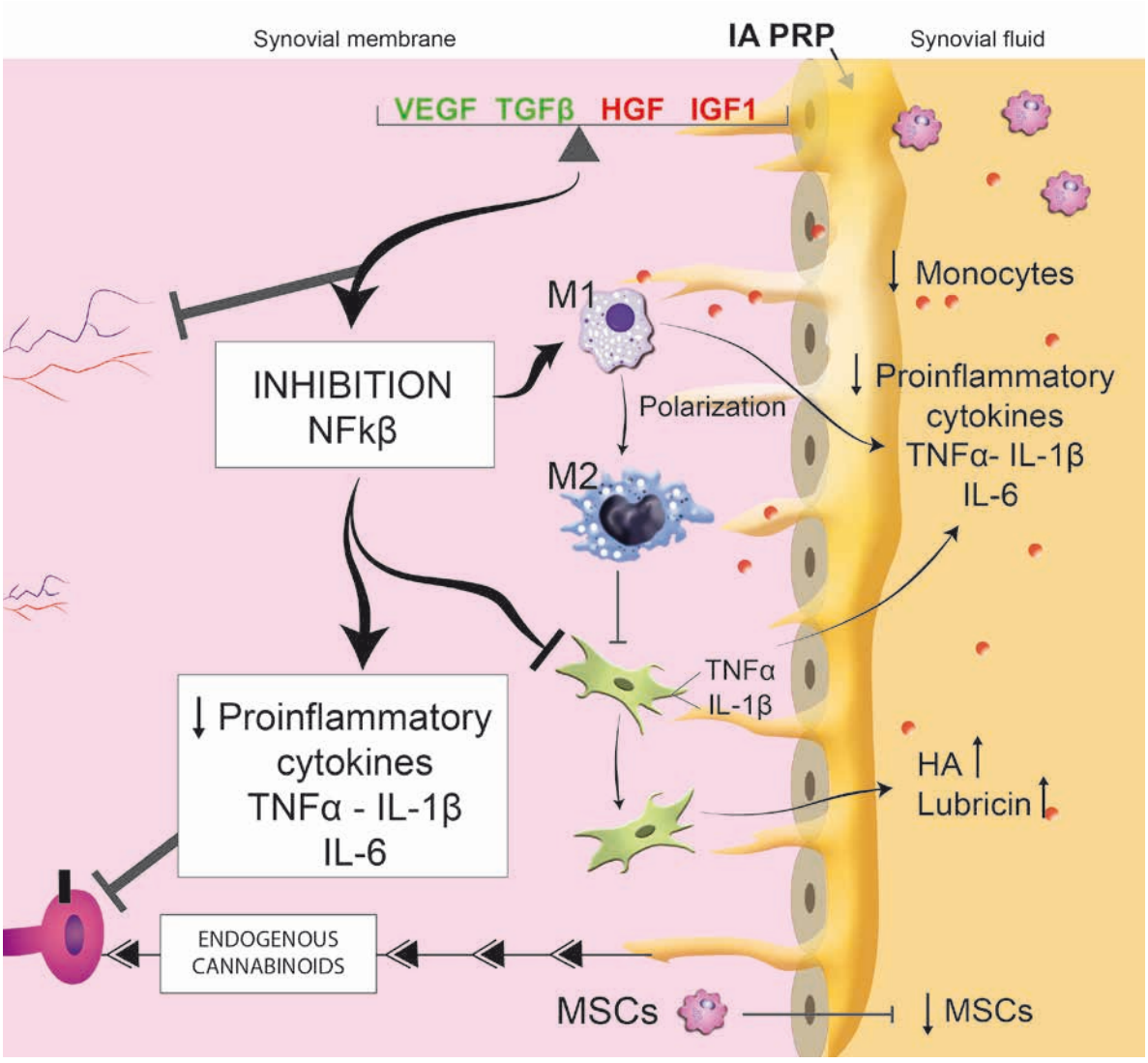


FIG. 5

The repertoire of antiinflammatory responses induced by PRP may break the catabolic loop, and dampen inflammatory response in SM and AC when these cells are exposed to proinflammatory cytokines and to abnormal mechanical stress and DAMPS, which is the significant OA context [70, 118, 186]. This sterile disruption of ECM homeostasis in osteoarthritic joint and an early inflammatory response has been suggested to resemble a chronic injury [187].

Moreover, several studies have reported that the accumulation of SF MSCs increases with the severity of osteoarthritis, joint damage and the disease duration [21, 114, 192]. Healthy human and osteoarthritic cartilage and SF contain a population of cells with characteristics of mesenchymal progenitor cells [93, 158] with migratory and chondrogenic potential [93, 99, 112, 138]. According to these observations, endogenous mesenchymal stem cells have been postulated as a reservoir of repair cells and immunomodulatory drugstore cells to dampen inflammation [195]. Although the source of MSC increase has yet to be determined, the most likely origin may be the SM [93, 192], the breakdown zone of superficial AC [158], and the SB [99, 106, 194, 202]. However, the SB origin of SF MSCs is less likely to occur for as some authors have suggested, the marrow of patients with severe OA is almost depleted in MSCs and the remaining MSCs are functionally deficient [21].

The chondroprogenitor cells (CPCs), with MSC features have a multipotent differentiation capacity towards the chondrogenic lineage [99] and may be the target of the GFs which traffic cell information through the MSCs by their trophic activity [39]. These multipotent cells might offer us the most valuable component when it comes to the repair process, namely, cells [38]. A similar process appears to be responsible for fibrocartilage repair synthesis when the Pridie drilling procedure is carried out in some reconstructive cartilage surgeries. This surgical procedure has presumed that the adult marrow-derived mesenchymal stem cells (MSCs) from the subchondral bone, are able to differentiate into bone, cartilage, muscle, marrow stroma, tendon-ligament, fat and other connective tissues[37]. In addition to the subchondral bone marrow, the synovium is another important source of MSCs in the joints tissues showing a high chondrogenic potential comparable to that of bone marrow-derived MSCs [87, 190].

1.2.2.3. The role of SB in pathophysiology and clinical symptoms of osteoarthritis

Bone, like cartilage, responds to mechanical stress in an intensity-dependent manner and a tight regulation between the sequential processes of deposition and resorption at the same site. These processes are carried out by the coupling of osteoblast and osteoclast metabolic activities [79] and unlike cartilage, when damaged regenerates spontaneously due mainly to its high elevated vascular and cellular network [83]. Evidence is gathering not only about the involvement of bone, and more particularly SB in the development and progression in OA but also about how these SB changes might even precede changes in AC of OA joints [97, 107, 108, 162, 202, 224]. SB has always been present in the equation of OA pathogenesis, and more than 40 years ago, partially inspired by the 1827 proposal by surgeon Dr. P.P. Physick on the SB as an effective shock absorber, Radin et al[161, 162], suggested a cause-effect connection among mechanical loading, subchondral bone sclerosis, and osteoarthritis.

SB is the layer of bone which lies immediately below the calcified cartilage [145], and consists of two different anatomical entities, one called subchondral or cortical plate which is nonporous and poorly vascularized cortical bone, and the SB which contains bone marrow (fatty) and trabecular bone [35, 88]. Together with the AC, it forms the osteochondral functional unit, which undergoes mechanical stresses that trigger adaptive cell responses and establish a crosstalk among them to adjust their architecture to ongoing physical and biochemical challenges [131, 202]. In the functionality of the osteochondral unit, articular cartilage provides an elastic, gliding, smooth frictionless surface, while SB, a very low viscoelastic structure, together with periarticular muscles and ligaments, acts as shock absorber structures, accounting for 30% and 50% of the total absorbing energy and only 1-3% for the AC [30, 88]. Besides the pivotal shock absorbing function, SB is a source of vessels whose perfusion rate enables an important nutritional route for AC but any damage to this microvasculature affects venous bony circulation thereby altering cartilage and chondrocyte function [88, 106, 127].

1.2.2.3.1. SB turnover and structural changes in OA

The osteochondral unit in an OA joint undergoes several structural changes including loss of articular cartilage, development of inflamed synovium, calcified cartilage thickening and tidemark duplication, undermineralization of bone, sclerosis and stiffness of SB, bone marrow lesions (BMLs), cysts, osteophyte, and a localized bone marrow replacement by fibroneurovascular tissue [97, 106, 150, 202].

Despite the high turnover of SB in OA, an uncoupling between bone formation and resorption at the same site leads to an increase in bone volume without a concomitant increase in bone mineralization pattern [79, 97, 108].This SB sclerosis is characterized by an increase of the osteoid volume, and a decrease of calcium bind to collagen fiber, and is associated with a gain of trabecular thickness, loss of trabecular number, and a trabecular network more separated and less interconnected [79, 224]. It has been suggested that sclerotic subchondral bone, localized at subchondral plate, could decrease the load transfer to the underlying bone tissue leading to osteoporotic-like changes [106]. Moreover, SB can undergo microdamage, such as microcracks and clefts, that modify SB stiffness and reduce the shock-absorbing capacity of SB, thereby making chronic a microdamage context and perpetuating an accelerated bone remodelling, which impairs normal mineralization of bone once it has been deposited, most likely by an altered osteoblastic phenotype [35, 36, 106]. Magnetic resonance imaging (MRI) has helped to detect subchondral bone marrow edema-like lesions (BMLs), which have been found to be associated with pain and disease progression in KOA [63], and together with bone attrition, are strong indicators of a structural deterioration in KOA [106]. Several studies paralleling MRI bone marrow edema lesion studies with histological analysis of SB retrieved at the time of joint replacement, re-

vealed microfractures and increased bone remodelling, subchondral ingrowth of fibrovascular tissue and increased vascularity, as well as various types of bone marrow fibrosis [203]. These observations were confirmed in rodent models of OA [130, 202]. The increased activity of osteoclasts in OA cause channels to extend from SB to AC, passing across the calcified tissues into the noncalcified articular cartilage [131]. The neurovascular invasion of those new-formed channels is accompanied by a new fibroneurovascular mesenchymal tissue within the channel along with cells such as macrophages, osteoclasts, osteoblasts, and endothelial cells, which interact to stimulate angiogenesis and growth of sympathetic and sensory nerves [202] and reach the noncalcified cartilage, a finding which has been supported by animal models of OA (Fig. 2 and 3)[202].

1.2.2.3.2. Cellular interactions and molecular crosstalk in osteochondral unit in OA

There is now good evidence that even in a non-diseased joint, naturally occurring pores and holes enable communication between SB and AC via diffusion of small molecules [124, 150, 151]. This communication may be exacerbated by structural changes seen early in the osteochondral unit in OA. The increased osteoclastic activity in the OA subchondral plate[97] may increase the permeability of bone-cartilage interface by inducing channel formation in the tidemark, in addition to the existent aberrant fibroneurovascular tissue and vasculature, and mechanical stress-induced microcracks [35, 202, 204]. Reinforcing this view, Pan et al [151] have demonstrated the diffusion of small-size molecules between SB and AC by utilizing the FLIP method with sodium fluorescein in the distal femur of mice, and this communication is greatly increased in osteoarthritic joints of the mice model [150]. Therefore, the presence of these connections enables an elevated crosstalk among chondrocytes, osteoblasts, osteoclasts and MSCs through biological factors and signalling pathways (Fig. 2 and 3).

Several in vitro and in vivo studies have demonstrated that osteoblasts from sclerotic subchondral bone show an altered phenotype. Westacott et al [215] reported that osteoblasts in OA-affected bone exhibited a different phenotype, whose activity can degrade articular cartilage in vitro. Supporting this observation, [76] Hilial et al [80] reported that osteoblasts from OA subchondral bone have an abnormal metabolism with increased levels of PGE2 and TGFβ (Figs. A and B). Using a co-culture model of OA subchondral bone osteoblasts with chondrocytes, Sanchez et al reported that osteoblasts induced a catabolic response of chondrocytes including a decrease in aggrecan, type II collagen and SOX-9, and an increase of MMP-3 and MMP-13 among other mediators [175, 176]. Moreover, osteoblasts from scletoric subcondral bone have an elevated TGFβ expression [79] and under cyclical compression express proangiogenic factors such as VEGF, FGF, and IL-8 [177]. Hepatocyte growth factor (HGF) is a pleiotropic morphogen present in articular cartilage but produced by osteoarthritic subchondral

bone osteoblats, osteoclasts, and MSCs [74, 75, 198], with likely implications in both the chondrocyte anabolic state and the proliferation of an invasive fibroneurovascular tissue in SB [75, 108, 223], the latter when an uncoupling osteoclast-osteoblast activity may lead to an overexpression of HGF (Figs. 2 and 3) [74]. The excessive presence of TGFB1 and VEGF in OA subchondral bone [202, 225] could be a driving factor for changes in osteoblast-osteoclast coupling thereby leading to a bone remodelling imbalance [106, 224], NGF expression [26], and fibroneurovascular growth changes that additionally might well contribute to overlying cartilage degradation [224, 225], pain [35, 131, 202] and an osteoarthritic joint [224, 225]. In a recent study, Zhen et al. [225] showed that by inhibiting TGF-β signalling in a specific population of MSCs present at the SB (Nestin positive MSCs), the severity of OA was reduced, a change associated with improvement of bone parameters, cartilage structure and joint function without affecting TGFB signalling in AC (Figs. 2 and 3) [225]. In fact, previous studies have shown that the decrease of MSCs in the synovial fluid, in low degree OA, suggests clinical improvement [192]. MSCs from osteoarthritic bone marrow have been reported to be substantially reduced in yield and proliferative activity besides showing a weakened chondrogenic and adipogenic activity and increased osteogenic activity [21]. However, in vitro studies indicate that the inclusion of growth factors, as a supplementary culture medium, can be beneficial in reverting their chondrogenic activity [189].

1.2.3. Current therapeutic approaches to treat knee osteoarthritis

The appropriate treatment of cartilage injuries and OA remains a daunting clinical challenge despite advances in both pharmacological managenment of the pain and inflammation, and advances in the surgical procedures and techniques and, in extremis, OA has been considered a disease with no cure [85]. Despite the enormous effort made to mitigate symptoms, what is lacking is an early disease-modifying therapeutic intervention aimed at preventing the progressive destruction of articular cartilage, or even reversing the initial post-traumatic damage. In this absence of a whole regenerative joint therapy, doctors must resort to joint replacement as the only solution for patients in advanced cases of OA [139]. Among the new emerging treatments to address this pathology, mesenchymal stem cells (MSCs) and Platelet Rich Plasma (PRP) stand out [168]. MSCs present an important therapeutic potential promoting regeneration derived from their proliferative and multipotential properties that could lead to the formation of new chondrocytes and cartilage regeneration, a process that has been observed in promising preclinical studies and clinical trials [65, 91, 167]. However, there are still specificities on this broader treatment that require deeper analysis, probing such questions as which cell sources are more appropriate, whether there may be an influence on therapeutic effectiveness from in vitro expansion, dosage, and the delivery method [143].

Since it is yet to be established which of the joint tissues or structures is the primary driver of knee OA, and therapeutic strategies solely targeting one cell or tissue target may well be proved to fail [92], it is advisable that approaches to treat OA should be aimed at reaching several joint tissues with the purpose of reducing joint inflammation, controlling pain, improving joint functionality, and restoring tissue homeostasis.

1. 3. AN INNOVATIVE BIOLOGICAL APPROACH TO THE TREATMENT OF OSTEOARTHRITIS: PLATELET-RICH PLASMA

1.3.1. The scientific rationale underlying PRPs as therapeutic

1.3.1.1.Platelets as a source of growth factors

Mammal platelets are circulating monitors, trackers and surveyors of the integrity of the vascular system and of the internal milieu as well as carriers of cytokines, chemokines and growth factors, fulfilling the function of coordinators of coagulation, inflammation and repair processes[31, 188]. In addition to these bioactive mediators (α-granules: TGFB, PDGF, VEGF, FGF, EGF, IGF-1, HGF, BMPs, BDNF and dense granules: Histamine, Serotonin, Ca and ATP/ADP) there are other contents in the plasma of PRPs (IGF-1, HGF, fibrinogen, fibronectine and other proteins) which together with adhesive proteins expressed by activated platelets, all play a central role in the cell signaling pathways involved in both tissue injury recognition and in the repair of damaged tissues[31, 146]. Platelets appear to be crucial in post-embryonic morphogenesis in identifying tissue loss or injury, factors that activate platelets thereby releasing by degranulation, growth factors and cytokines which trigger mechanisms to reconstruct structures and restore function mainly by stimulating cell migration and proliferation, regulating angiogenesis, chemoattracting circulating progenitor cells and guiding tissue remodeling[3, 15, 67, 133]. Drawing on these mechanisms and observations made by Crisan and col.[45] and Caplan [37] concerning the immunomodulatory and trophic effects of MSCs[37, 45], it might be possible to suggest a synergy between platelets and MSCs.

Although a universally accepted definition of PRPs in terms of platelet concentration and presence or absence of leukocytes is lacking, PRP products can be depicted as an autologous platelet concentrate within a plasma suspension, and whose composition is determined by the method used to obtain it. Platelet Rich Plasma products include plasma and twofold or more increases in platelet concentrations above baseline levels, and the concentration of leukocytes and erythrocytes varies widely[28, 49]

from a complete absence of products to a high concentration of them. In particular, PRGF is depicted as an endogenous blood-derived product which conveys growth factors, cytokines, and morphogens contained in the platelets as well as fibrinogen and other plasmatic proteins in a biologically balanced aggregate, and managed and delivered in a pharmacological manner[6, 13]. This multifaceted, versatile, biological system is made up of an autologous, balanced blend of plasma with a moderated platelet concentration (a two-third-fold increase compared with peripheral blood) that does not contain leukocytes or erythrocytes. The process of platelet activation and hydrolysis of prothrombin into thrombin is driven by the addition of calcium chloride, simultaneously causing the release of a plethora of growth factors and the polymerization of fibrin[5, 146].

Besides conveying GFs, PRGF provides the damaged tissue with a transient biological scaffold made up of fibrin which stems from the polymerization of fibrinogen, a pleiotropic blood protein that regulates coagulation, inflammation, and tissue regeneration [16, 133, 146]. The three-dimensional network, formed either “in vitro“ as a clot or “in situ” as an extracellular matrix after the intraarticular infiltration over injured areas, contains binding sites for cell adhesion as well as proteins such as thrombospondin-1 (TSP-1),alpha-1-antitrypsin fibronectin, acute phase proteins or proteins related to lipid metabolisms (Fig. 6).

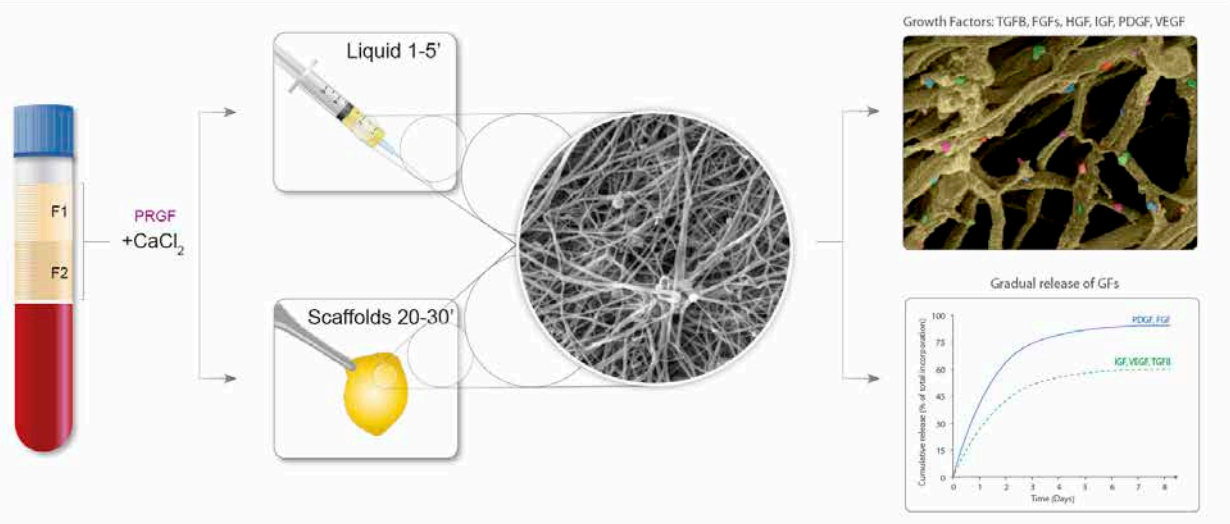


FIG. 6
PRGF preparation process with two formulations for molecular intervention, a liquid injectable scaffold and in vitro formed membrane-scaffold. The generated fibrin matrix is embedded with a pool of growth factors stemmed from activated platelets and plasma. GFs are released in a gradual and sustained manner at the dysfunctional and degenerated sites as the fibrinolytic process takes place.

Since cells that make up and populate musculoskeletal tissues, including chondrocytes are mechano-sensive, in this varied molecular landscape, migratory cells such as MSCs and CPs might adhere and undergo physiological loading, thereby regulating their gene expression and eventually repairing the injured tissue; cells cannot express a physiological phenotype in an empty space. Therefore, after the intraarticular infiltration over the injured areas, a fibrin-scaffold formed "in situ" as an extracellular matrix, serves as a highway for mechanical energy to transit from the environment to the cell, thereby bridging cell-to-cell tissue transition, promoting multi-cellular assembly and providing mechanical support as well as endowing tissues with a suitable microenvironment for biological restoration[13, 146]. Since they are autologous, bio-reabsorbable, bio-compatible, and free of leukocytes and red cells, PRGF scaffolds are the best tailored among all the tissue engineering materials.

1.3.1.2. Growth factors and PRPs in cartilage repair

GFs are biochemical modulators and regulators which are shared with developmental biological processes and will be redeployed for tissue repair after injury[123, 205]. Transforming growth factor-β superfamily (TGFβ) has been shown to play an anabolic role in cartilage repair. In particular, TGFβ1, the major growth factor within PRPs and one of the most important in cartilage regeneration, stimulates both chondrogenesis of synovial lining and bone marrow-derived MSC[62, 105] and chondrocyte synthetic activity with matrix deposition [68]. Moreover, TGFβ1 counteracts the catabolic activity of IL- β1 including the degradation of type II collagen and proteoglycan produced by chondrocytes[50, 159] and increases chondrocyte phenotype expression[196]. Insulin-like growth factor (IGF-1) is another component of PRPs with a potent anabolic effect on articular cartilage metabolism and its presence is required to maintain the integrity of articular cartilage[57]. In addition to positive influence of IGF-1 on the repair of extensive areas of damaged cartilage and protection of the synovial membrane from chronic inflammation[67], IGF-1 is, together with PDGF, a potent chemotactic factor for chondrocytes, which stimulates synthesis of extracellular matrix in human osteoarthritis but does not avoid the matrix catabolism[141]. Moreover, its presence in cartilage enhances the effect of other Growth factors present in articular cartilage[147].

PRP application to cartilage repair is underpinned by a substantial body of evidence in basic science, as well as in preclinical and clinical levels of practice. In vitro, treatment of mature porcine chondrocytes with L-PRP releasate stimulates cell proliferation, and glycosaminoglycan and collagen synthesis[3]. The presence of PRGF releasate without leukocytes on human osteoarthritic synoviocyte cultures enhances the synthesis of Hyaluronic acid (HA) and HGF compared to synoviocytes cultured on

a platelet-poor medium. Moreover, the enhanced secretion of HA and HGF by PRGF was maintained despite the fact that synoviocytes were treated with interleukin-1β[12, 15]. In one proteomic study conducted on human osteoarthritic chondrocytes cultured with different mediums, the PRP-enriched medium showed to be more efficient than other mediums at increasing cell proliferation and reverting and restoring the pattern of gene expression determined in a normal chondrocyte phenotype without undergoing hypertrophy[23, 197]. Bendinelli et al. have reported an important HGF-mediated anti-inflammatory and anabolic effect of platelet-rich plasma on immortalized chondrocytes lineage by attenuating or reducing the transactivating activity of NF-kB[23], a proposal that has been reinforced by the results obtained in osteoarthritic chondrocytes by van Bull et al[209]. (2011). In addition, PRP decreased the expression of COX2 and CXCR4 target genes, whose products might be involved in controling chemotaxis of inflammatory cells such as monocytes thereby reducing local inflammation[23]. Wu et al[219] have shown, using a 3D in vitro model, that the combination of PRP with a collagen matrix (with immortalized human chondrocytes) recovered type II collagen and proteoglycan synthesis which had been inhibited by 3 days of treatment with IL-1β+TNF α, thereby illustrating the protective efficacy of PRP on chondrogenic-specific gene expression such as Col II and AGN[219]. In another recent study, Anitua et al. determined that synovial fibroblast culture incubated with PRGF +HA induced a greater increment in synovial cell migration compared with the response to HA alone[8].

Furthermore, drawing on the aforementioned evidence, some in vivo studies have used PRP in an attempt to restore local hyaline cartilage injuries. When PRP liquid was loaded in microporous poly-lactic-glycolic acid scaffolds and applied on large osteochondral defects in a rabbit model, the neo-chondrogenesis induced showed chondrocyte-like cell and a high ECM synthesis and the defects were totally filled with a repair tissue similar to hyaline cartilage, compared with the control that showed a fibrous tissue repair[200]. The preventive effect of PRP infiltrations delivered in gelatin hydrogel microspheres in a rabbit model has been reported, showing a suppression of histomorphologic signs of the OA progression compared with microspheres containing PPP. Therefore it has been suggested that the treatment of OA might be carried out using a combination of growth factors[67, 125, 205] in an attempt to redress the extracelular matrix through the cells behavior.

1.3.2. PRP as an emergent and promising knee osteoarthritis treatment

Despite important advances made in the development of treatments to reduce pain and inflammation, and in spite of endeavors to develop an efficacious and early disease and structure-modifying therapeutic intervention, the path to osteoarthritis treatment remains elusive.

1.3.2.1. Inflammation and oxidative stress

In vitro and in vivo studies have reported that PRP and GFs within it such as HGF, IGF-1, PDGF, and TGFB, and platelet microparticles have proven to exert an immunomodulatory effect and promote an antiinflammatory environment. HGF and platelet microparticles have been reported to polarize macrophages from M1 to M2 phenotype[44,164, 211]. IGF-I, PDGF, HGF, and PRP releasate modify the inflammatory status of chondrocytes by suppressing the NF-kβ signaling pathway[23, 140, 209] (Fig. 3), which might lead to the decreased presence of IL-B, and TNF-α and other pro-inflammatory cytokines in synovial fluid [60, 186]. Reinforcing this interpretation, Anitua et al reported that LPS-treated osteoblasts and fibroblasts which had been cultured in the presence of releasates obtained from PRP without leukocytes, showed an increased expression of Ikβ-α, an antiinflammatory protein that anchors the transcription factor NF-kβ to the cytoplasma and inhibits its activation, whereas releasates obtained from leukocyte-rich PRP induced a NF-kβ activation[17]. In one recent study, Xie et al[220] reported that PRP attenuated the multiple-cyclic tensile stain mediated MMPs, NO, and PGE2 synthesis in chondrocytes, suggesting that PRP may protect chondrocytes from mechanically induced injury. Connective tissue factor (CTGF), one of the most abundant growth factors released by platelet activation[103] was reported to protect chondrocytes from age-related degenerative changes and from cellular stress, the latter mediated through NF-kβ[89].

On the other hand, synovial fibroblasts from osteoarthritic patients cultured in 20% PRP supernatant produced a significant amount of HGF, even in the presence of IL-1β, which is known to inhibit the NFkβ on macrophages[44] and to mediate the antiinflammatory effects of PRP on fibroblasts[226]. In a recent work, Assirelli et al[19] observed that L-PRP (leukocyte-rich PRP)-treated human synoviocytes sustained a long-term upregulation of IL-β, IL-8 and FGF-2, together with a down-regulation of HGF and TIMP-4 expression, two anti-catabolic mediators in cartilage, the former indicating a proinflammatory and procatabolic response. These observations were not present when the culture medium was obtained by P-PRP (Pure PRP) or PPP (Poor PPP), a notable signal that suggests there is indeed an impact of leukocytes on the biologic effects of PRP. This repertoire of antiinflammatory responses induced by PRP may break the catabolic loop, and dampen inflammatory response in SM and AC when these cells are exposed to proinflammatory cytokines and to abnormal mechanical stress and DAMPS, which is the significant OA context (Figs. 2, 3, and 4)[187]. One cellular process that accentuates the catabolic state of the AC and SB is the oxidative stress resulting from the imbalance between levels of reactive oxygen species (ROS) relative to antioxidant, which is amplified by aging[70, 122]. Osteoblasts cultured in the presence of PRP supernatant showed an up-regulation of NrF2-ARE pathway and subsequent activation of antioxidant response element (ARE), an important mecha-

nism involved in detoxifying ROS and protecting chondrogenic and osteogenic precursor cells[208]. Moreover, intraosseous infiltrations of PRP in mice can revert the decreased expression of SIRT1 in bone-marrow derived stem cells from aged animals, making stem cells more resistent to oxidative stress and maintaining their stemness, suppressing adipogenesis within the bone marrow and improving osteogenesis and bone mineral density[120, 121]. Hence, PRP might additionally play a role as an anti-aging factor by stabilizing AC and protecting SB against oxidative stress[76, 89, 120, 121, 208]. However, as aging is one physiological risk factor for developing OA[122], there are some age-related changes in the composition of PRP, such as the reduction of IGF-1 and PDGF in elderly people, two important chondrogenic mediators[55], that might account for some contradictory outcomes in the application of this therapy.

1.3.2.2. OA and Pain

Pain is considered the clinical hallmark of KOA, and several clinical trials have been conducted to assess the efficacy of intraarticular injections of PRP for both pain and function of the knee. There are several relevant studies demonstrating a significant pain reduction and an improvement in knee joint physical function[210, 212] in patients with KOA treated by 3 weekly infiltrations of PRP[64, 152, 178, 182, 184, 210]. The mechanism/s causing osteoarthritis pain remain yet to be fully identified[126] as do the proposed mechanisms of PRP effectiveness. Two mechanisms might likely link the pain reduction to PRP treatment. The first is the suppression of NFkβ on intraarticular inflamed cells, which leads to the reduction of proinflammatory cytokines that otherwise, might contribute to pain by stimulating hyperalgesia and sensitizing joint nociceptors to other stimuli[186, 193]. The second is the reported significant amount of endogenous cannabinoids within PRP51 that might act as ligands for cannabinoid receptor 1(CB1) and 2 (CB2) of chondrocytes, synovium cells, and bone cells[56, 129, 166] of OA patients, thereby supporting both a pain and inflammation reduction by targeting the endogenous cannabinoid systems (Figs 2, 3, and 4) [51, 56, 129, 166].

1.3.2.3. Trophic and anabolic effects

PRP has been shown to have a consistent in vitro proliferative effect on cultured human chondrocytes in a dose-and time-dependent manner[41, 54, 171, 197] and on rabbit chondrocyte when GFs are delivered in a sustained manner through the upregulation of CB1 and CB2 receptors[114]. Moreover, an in vitro and in vivo anabolic effect of PRP on chondrocytes has been reported by increasing the synthesis of proteoglycan and collagen type II[3, 219] or decreasing catabolism by reducing MMP-13 expression and TNF-α concentration in synoviocyte and cartilage co-cultured systems with PRP media[201]. Another chondroprotective effect is based on the visco-inducing effect of PRP, which stimu-

lates the synthesis of hyaluronic acid and lubricin by synoviocytes and chondrocytes respectively[12, 171, 201], which help restore the SF homeostasis and function (Fig. 7), the latter preventing chondrocyte apoptosis, synovial cell overgrowth, cartilage breakdown, and inhibition of the MSC release and migration[61, 90, 102, 171]. On the other hand, platelet rich plasma obtained by apheresis, and characterized by a low platelet concentration and very few leukocytes has been shown to exert positive effects on migration, proliferation and chondrogenic differentiation of cultured human subchondral mesenchymal progenitor cells [100, 101, 102].

Several soluble morphogens embedded in a fibrin network such as IGF-I and -II, PDGF, SDF-1, TGF-β, CCL5 and fibronectin, among other biomolecules, have been shown to be involved in the recruitment and homing, and in a chondrogenic-differentiation effect of PRP on chondroprogenitor or MSCs from subchondral mesenchymal progenitor cells[90, 102, 104, 165]. Last but not least, uncontrolled angiogenesis and fibroneurovascular tissue proliferation are two histological features of osteoarthritic SM and SB. Despite the fact that PRP contains proangiogenic and profibrotic growth factors (VEGF, FGF, PDGF,and TGFβ) several in vitro and in vivo studies have reported no increase in the level of VEGF and TGFβ[10, 15] nor were tissular fibrosis or an aberrant angiogenesis induced[9-11, 180].

1.3.3. Subchondral bone as a tissue target in OA treatment: Intraosseous infiltration of Platelet-rich plasma

The realization of the biological and mechanical connection between AC and SB has lead to numerous in vivo animal studies that have shown that targeting SB with some drugs can have protective structural effects on cartilage[40]. Blocking or limiting the bone remodelling with alendronate[78], zoladronic acid[109] or improving the microstructure and quality of subchondral bone in osteoarthritic and osteoporotic rabbits with parathyroid hormone[22], prevent cartilage degradation and OA progression. Moreover, Sagar et al[170] reported a reduction in pain behaviour after a subcutaneous treatment with osteoprotegerin in a monosodium iodoacetate (MIA) rat model of OA pain, and Pelletier et al[153] demonstrated that an oral strontium ranelate treatment in an experimental osteoarthritic dog model reduced the progression of structural changes including the subchondral bone. Despite the fact that the translation of these promising observations in preclinical research to human clinical trials has often failed, as indicated by a recent metaanalysis of clinical trial with risedronate in knee osteoarthritis[47], recent clinical trials are raising expectations. For instance, using zoledronic in patients with clinical KOA associated with bone marrow lesions (BMLs) assessed by MRI, Laslett et al[111] reported a beneficial effect on pain and on BML evolution at 6 months. In participants from the osteoarthritis initiative, Laslett et al[111] demonstrated significant pain reduction during the first 3 years

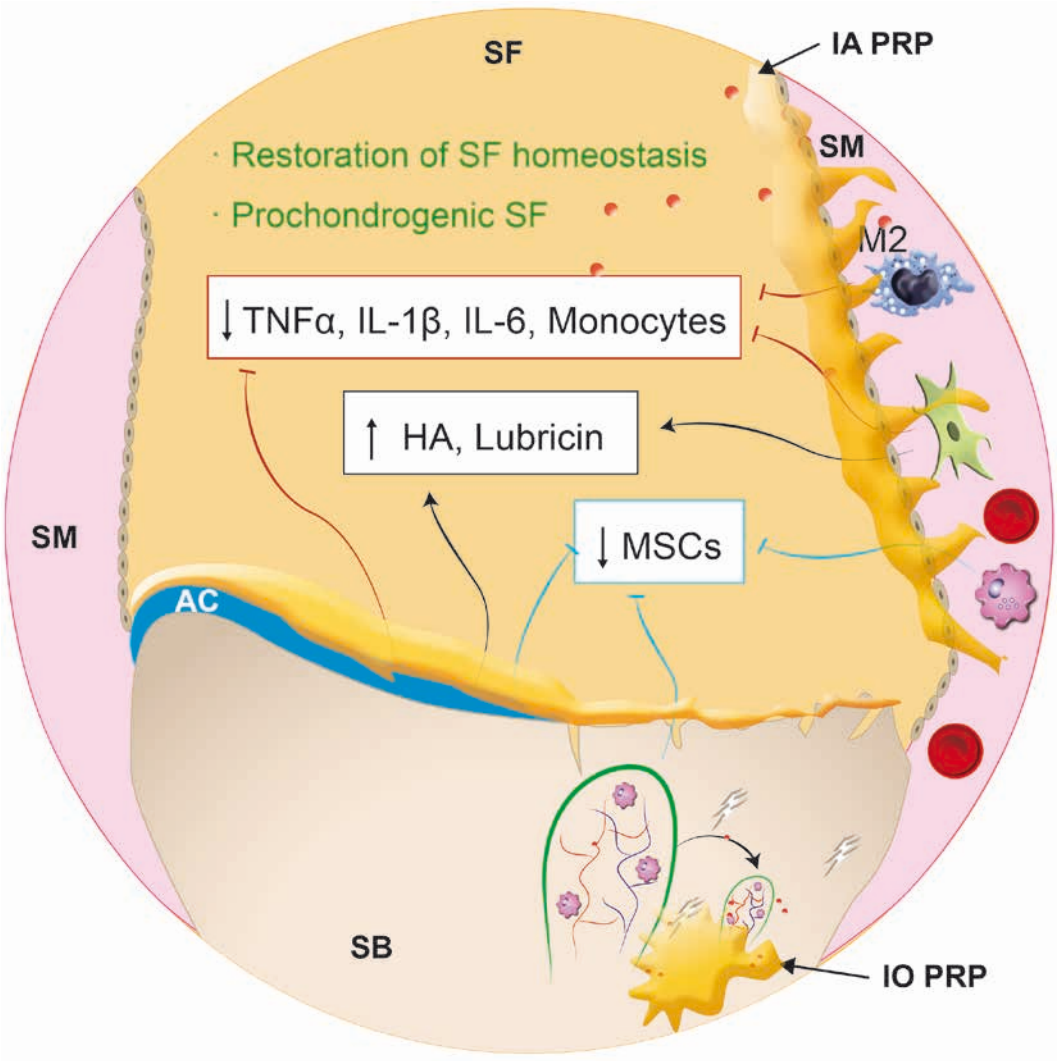
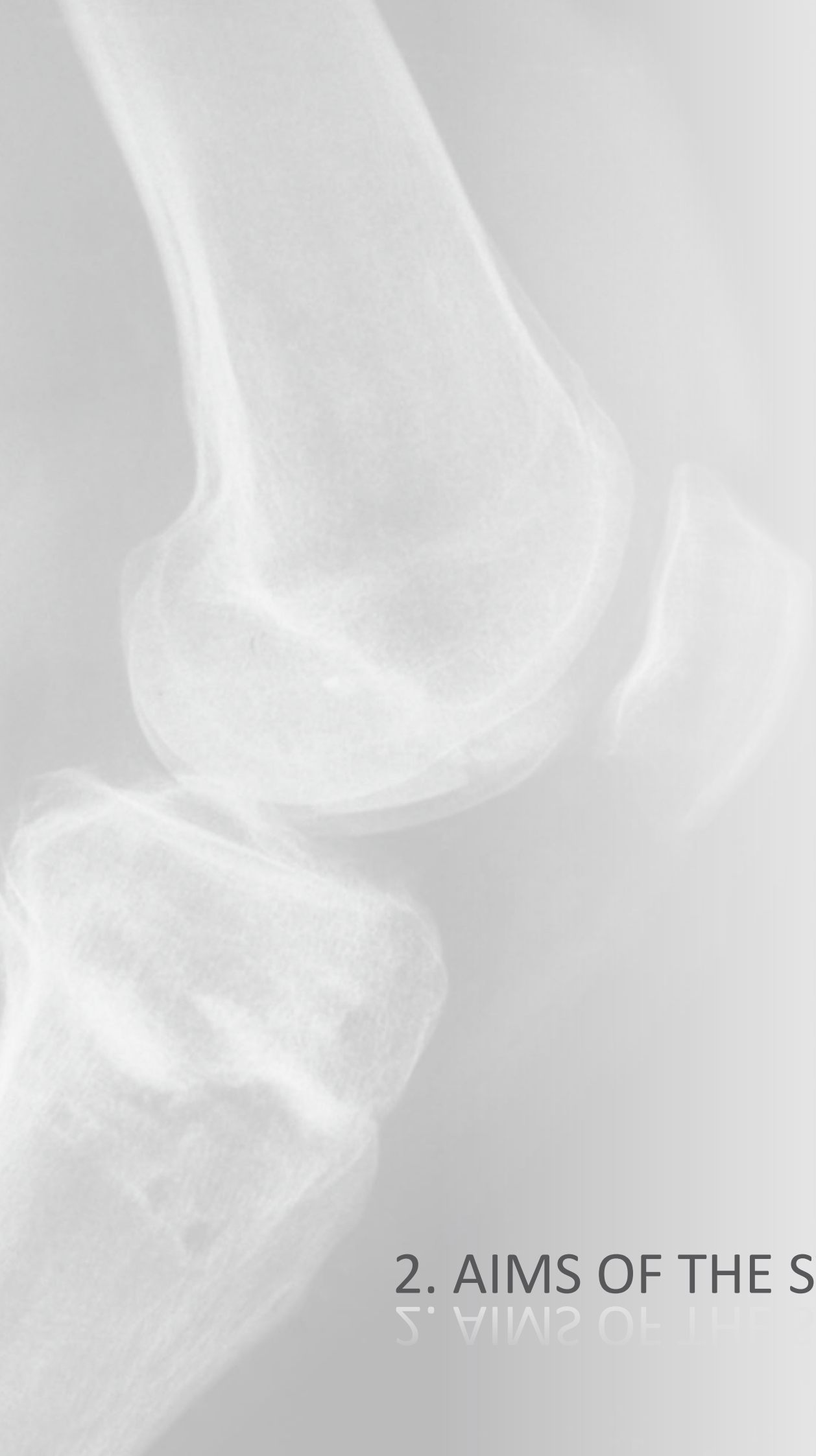


FIG. 7
Intraarticular infiltration of PRP helps restore SF homeostasis by stimulating the synthesis of hyaluronic acid and lubricin by synoviocytes and chondrocytes respectively[12, 171, 201], dampening inflammation and suppressing the concentration chemoattractan cytokines in SF, which might contribute to the inhibition of the MSC release and migration[60, 61, 186]. PRP might favour a homing and chondrogenic-differentiation effect on MSCs of subchondral mesenchymal progenitor cells and SF-MSCs[100, 102, 104, 135].

of treatment with biphosphonates. Two more clinical trials have shown positive structural effects of strontium ranelate on KOA, one improving the joint space narrowing[163] and the other reducing the loss of cartilage volumes concurrent with the decrease of BMLs at 3 years of follow up[154].

Infiltrations of PRP into the BM cavity of femur of young and old ovariectomized-SAMP8 age-related osteoporotic female mice have been reported to up-regulate osteogenesis and down-regulate adipogenesis[121]. The increase of fat tissue mass in BM is correlated with decreased bone mineralization in aged SAMPS8 mice[120, 121], bone demineralization that occurs in osteoarthritic subchondral bone together with cysts[35]. Moreover, improvement of bone mineral density in PRP-treated osteoporotic mice concurred with both histological sections of the bone samples showing more trabecular bone areas and more intense calcium staining and a suppression of bone resorption process as evidenced by the decrease of RANKL transcript[121]. In a trial on 13 healthy volunteers, Philippart et al[156] reported fatigue on the first day as the only clinical adverse effect after a self-stimulation of BM of the iliac crest by injected autologous platelet-rich plasma[156].



2. AIMS OF THE STUDY

2. AIMS OF THE STUDY

The overall outcomes in basic science, preclinical, and clinical studies suggest four synergetic effects of PRP application on the osteoarthritic joint. By modulating gene expression and gene products, PRP may well influence cell behavior which is conducive to maintaining the homeostatic state of the joint tissues thereby reducing pain and improving joint function and motion. (Fig. 8).

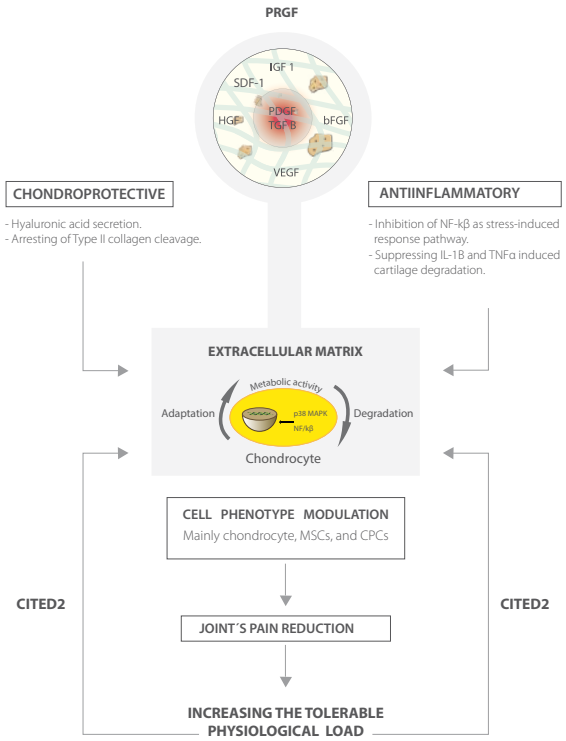
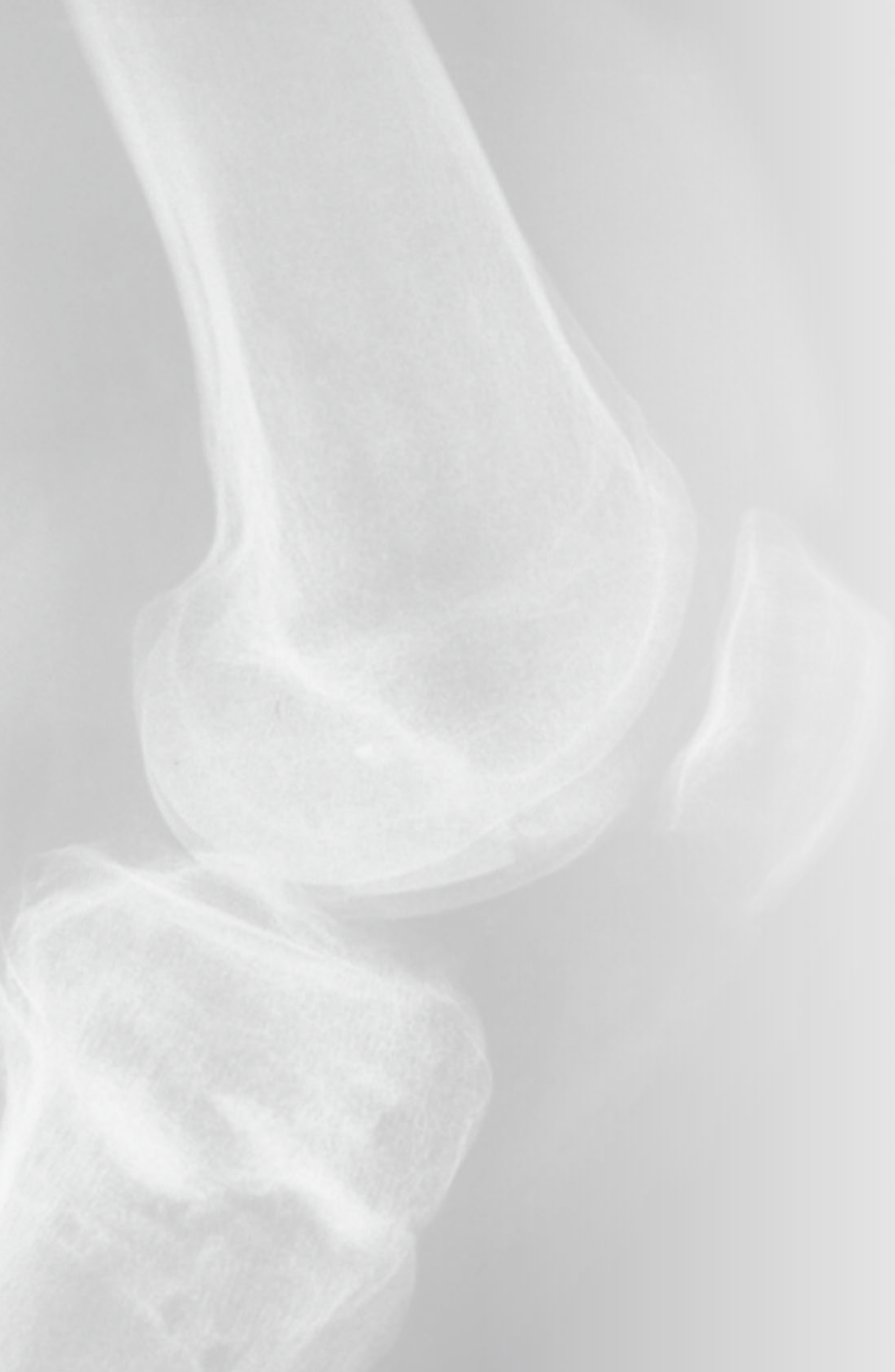


FIG. 8
Four synergetic effects of PRGF application on the osteoarthritic joint proposed by our group.

The aims of this study were:

1. To validate the PRP intraarticular injections as a safe and efficacious treatment for KOA.
2. To assess a novel way of treating severe knee OA by targeting SM, superficial articular cartilage, SF, and SB by combining intraarticular and intraosseous infiltrations of plasma rich in growth factors (PRP).
3. To explore the suitability of SF as a source of MSCs and their response to the biological mechanisms implicated in the effects of two different treatment modalities of PRP applications on OA patients: intraarticular injections targeting the SM, superficial AC, and SF, or combining intraarticular injections and intraosseous infiltrations, the latter reaching as well the SB.



3. METHODS AND RESULTS

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3.1. METHODS

3.1.1 Platelet-rich plasma: Depiction, preparation, and formulation

For almost two decades our research group has characterized this technology and has studied its therapeutic potential in tissue repair and wound healing[14]. PRGF contains a moderated platelet concentration, a two-third fold increase compared to peripheral blood, a dosage shown to induce optimal biological benefit[15]. PRGF does not contain leukocytes, and activation is performed only with $CaCl_2$. The process to produce PRGF is easy, fast and reproducible (Fig 9). 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. The centrifuge has specific parameters to maximize the production of platelets and keep the plasma leukocyte-free. After the centrifugation, three layers are typically obtained: a yellowish top layer, the plasma, which contains a gradient of platelets, with maximum concentration of those platelets above the buffy coat. The leukocyte layer, or buffy coat, is located below the plasma layer. The bottom layer is the layer containing the red cells. 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 to peripheral blood whereas the lower fraction will contain 2 to 3-fold the concentration of platelets compared with blood. 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). After fractionation, PRGF can be activated in a controlled way by the addition of $CaCl_2$, providing a clot that mimics its natural structure. Activation with $CaCl_2$ avoids the use of exogenous bovine thrombin, a source of possible immunological reactions[53, 110, 222]. Another important feature of the PRGF is the absence of leukocytes, which categorizes it as safe and homogeneous, because the values of leukocytes are highly variable between donors[214] 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. Once PRP liquid formulation is activated, plasma fibrinogen polymerizes into a three-dimensional transient fibrin scaffold, which contains heparan sulfate binding domains for growth factors

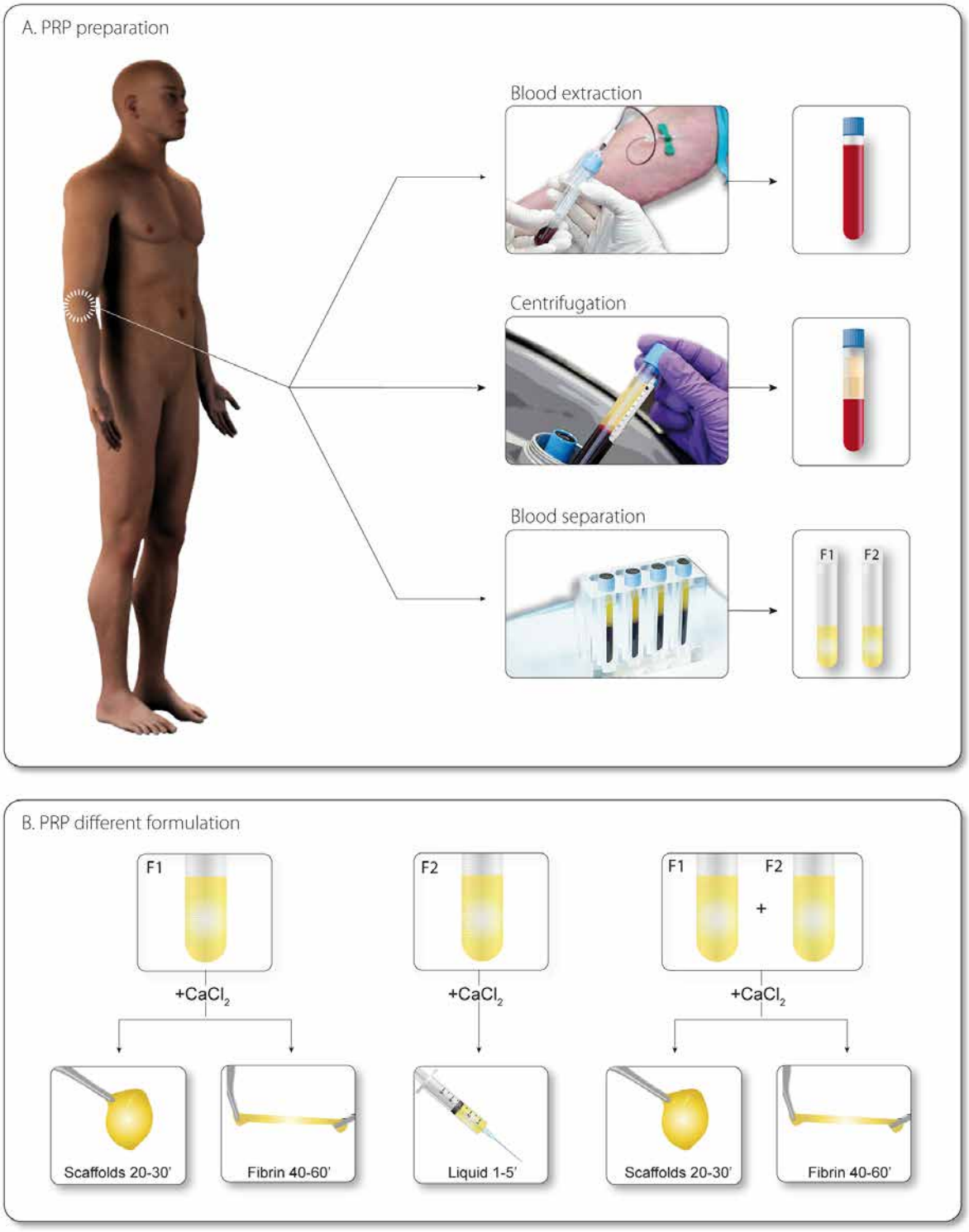


FIG. 9

Platelet Rich Plasma protocol. Obtaining platelet-rich plasma involves the extraction of a small volume of blood from the patient, its centrifugation to fractionate the blood and the separation of platelet-rich fractions (F1 and F2) (A). After activation with calcium chloride PRP fractions can be obtained for various formulations including liquid, clot and membrane (B).

(PDGF, FGF, HGF, BDGF, VEGF, IGF, TGF-B), cytokines (TNF-a, IL-2,3,4,5), chemokines (PF4), ECM components (Fibronectin, thrombospondin, tenascin), cell adhesion (L-selectin, N-CAM), acute phase proteins, and proteins related to lipid metabolism[5, 146]. Once infiltrated into the joint and subchondral bone, this liquid-to-gel ·3D injectable scaffold is converted into a matrix-like viscous and malleable structure, which adheres to SM, AC and SB, and covers them[5, 16, 183] (Figs. 5, 6 and 7). When fibrinolysis begins, a gradual, sustained release of GFs and other biomolecules occurs, in contrast to a bolus delivery modality[7, 27, 135]. Such a gradual yet sustained release of GFs influence cells and mimic the biological repair process[2, 16, 27] (Fig. 6).

Two different formulations 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[149] wound closure and tissue engineering [11]. The three dimensional structure of the fibrin mesh (Fig. 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, such as autologous bone, demineralized freeze-dried bovine bone and collagen, among others, adjusting the resulting characteristics of the scaffold.

2. Liquid PRGF-Endoret, activated at the time of use, is used in intra-articular and intraosseous injections[178, 182, 212], in bone and tendon surgery[179, 181], and treatment of skin disorders and regeneration[149]. Therefore, after the intraarticular or intraosseous infiltration over the injured areas, a fibrin-scaffold formed "in situ" as an extracellular matrix, serves as a highway for mechanical energy to transit from the environment to the cell, thereby bridging cell-to-cell tissue transition, promoting multi-cellular assembly and providing mechanical support as well as endowing tissues with a suitable microenvironment for biological restoration[13, 146]. Since they are autologous, bio-reabsorbable, bio-compatible, and free of leukocytes and red cells, PRGF scaffolds are the best tailored among all the tissue engineering materials (Figs. 3, 5, 6, and 7).

3.1.2. A randomized clinical trial evaluating plasma rich in growth factors (PRGF-Endoret) versus hyaluronic acid in the short-term treatment of symptomatic knee osteoarthritis (IV)

This study was carried out in accordance with the international standards on clinical trials: Real Decreto 223/2004, Declaration of Helsinki in its latest revised version (Tokio, 2004) and Good Clinical Practice Regulations (ICH). The study protocol was reviewed and approved by the Reference Ethics Committee. All patients provided written informed consent before entry into the study.

Patient selection

One hundred and eighty seven patients were initially selected in the study. Patients were considered eligible if they were between 41 to 74 years of age and suffered from osteoarthritis of the knee as diagnosed on the basis of American College of Rheumatology criteria.23 with radiographic confirmation (Allback grade 1-3, on a scale of 1 to 4, with higher numbers indicating more severe signs of the disease).

Recruitment of patients began January 18, 2008, at 3 clinical centers. The recruitment finished November 12, 2009 and the study was completed on Sep 13, 2010. A preliminary assessment of each patient was carried out in the first basal visit by an orthopedic surgeon, 30 days prior to randomization and the medical history was completed. Patients were only included in the study if they met all inclusion / exclusion criteria shown in Table I. Each patient also received a booklet that contained detailed instructions and the Western Ontario and McMaster Universities Osteoarthritis Index WOMAC questionnaire. This booklet had to be completed by the patient and carried along with them in each of the following visits.

TABLE I. Inclusion and exclusion criteria in the PRGF/HA intraarticular infintrations CT

Inclusion criteria	Exclusion criteria
• Male and female patients aged between 40 to 72 years. • Diagnosed with tibiofemoral osteoarthritis of the knee by X-ray. • Joint pain >35 mm on a 0-100 mm visual analogue scale (VAS). • Radiological severity Ahlbäck grade <4. • Body Mass Index (BMI) ranging between 20 and 32. • Possibility for observation during the follow-up period.	• Bilateral knee osteoarthritis requiring infiltration in both knees. • Body Mass Index ≥ 33 • Suffering from polyarticular disease. • Severe mechanical deformity (diaphyseal varus deformity of 4° and valgus of 16°). • Previous arthroscopy within the last year. • Hyaluronic acid intra-articular infiltration within the last 6 months. • Systemic autoinmune reumathoid disease (Conective tissue disease and systemic necrotizing vasculitis). • Glycosylated hemoglobin above 7%. • Blood disorders (thrombopathy, thrombocytopenia, anemia with Hb <9). • Be undergoing immunosuppressive therapy and / or warfarin. • Having undergone treatment with steroids during the 3 months previous to inclusion in the study. • Treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) during the 15 days prior to its inclusion in the study.

Interventions

All patients who met the inclusion criteria (176 of 187 enrolled initially since 11 patients had already been excluded) were scheduled at the first visit and received either of the two active treatments under study depending on the randomization made previously: infiltration of the affected knee with PRGF-Endoret (three injections on a weekly basis) or infiltration of the affected knee with hyaluronic acid (Euflexxa®) (three injections on a weekly basis).

PRP was prepared according to the protocol of PRGF-Endoret technology (BTI Biotechnology Institute, Vitoria-Gasteiz, Spain) already depicted in point 3.1.1., and applied according to the explanation in the point 3.1.3.

Randomization and allocation concealment

A total of three treatment visits were carried out with a weekly periodicity. During these visits the treatment assigned by randomization was delivered. A stratified randomization (one stratum per center) was carried out. Both the evaluators and patients remained blind to the treatments.

All subjects included in the study were identified by patient number after signing informed consent.

Each patient was identified by a numerical code. The correspondence between the number of patients and their treatment was performed using specific software for randomization, keeping that relationship in a sealed envelope. This envelope was not opened until the moment before applying the treatment. Neither the patients nor the evaluators were informed at treatment time. To maintain masking the application area was hidden from view and blood was drawn for all patients to prepare the PRGF-Endoret technology.

Procedures

All subjects underwent blood draw an hour before applying the treatment. Patients were recalled for follow-up visits 1, 2 and 6 months after the last treatment administration. The only permitted medication throughout the clinical trial was acetaminophen. The intake of any type of NSAID was an exclusion criterion. The amount of acetaminophen consumed by each patient in each treatment and follow-up visits was recorded. Acetaminophen consumption was measured by counting the number of empty containers that were previously administered in the previous follow-up visit.

Response was assessed by researchers not involved in the application of treatment. In the data report forms did not make any reference to the treatment applied.

Outcome measures

Efficacy assessments

The primary efficacy outcome was defined as the percentage of patients having a 50% decrease in the summed score for the WOMAC pain subscale from baseline to week 24. This outcome was measured by applying the questionnaire WOMAC compared to baseline therapy on the basis of the criteria of the Outcome Measures for Rheumatology Committee and Osteoarthritis Research Society International Standing Committee for Clinical Trials Response Criteria Initiative (OMERACT-OARSI).
The secondary efficacy outcomes included the scores on the WOMAC subscales for stiffness and physical function, the percentage of OMERAT-OARSI responders and the number of acetaminophen mg per day. The evolution from baseline in overall knee pain after application of the visual analogue scale that ranged from 0 to 100, was determined by the Womac and Lequesne scales.

Safety assessments

The nature, onset, duration, severity, and outcome of all adverse events, as well as any association of an adverse event related to the study medication were assessed and documented at each visit. Indeed, the only permitted medication throughout the clinical trial was acetaminophen. The intake of any type of NSAIDs was an exclusion criteria and a reason to be excluded from the study.
In order to evaluate the safety profile of the treatments, all complications and/or adverse events were recorded with an accountability scale. The use of rescue medication was recorded daily in the patients' diaries.

Sample size calculation

A sample size of 220 patients, 110 subjects per group was estimated to provide at least 90% power to detect differences in the proportions of patients achieving 50% pain improvement compared to PRGF inflitration versus hyaluronic acid, at a 5% level of significance. The sample size was calculated using the exact test with the aim of comparing two proportions by applying the chi-square test assuming that the proportion of patients would achieve an improvement in pain over 50% would be 30% in the experimental group versus to 12% in the control group.

Data analysis

Initially, a descriptive analysis of the sample was performed taking into account the demographic and clinical variables of patients. Quantitative variables (age, BMI) were determined by the mean, SD and range, and for qualitative variables (gender, marital status, education level, physical activity, history, medication type and severity of radiological OA) a frequencies analysis was conducted.

Analysis of the primary outcome measure was conducted according to the intention to treat. The baseline comparability of treatment groups was performed by applying a T student test for quantitative variables and a chi-square analysis for categorical variables. The primary efficacy variable was assessed using a chi-square test. Secondary efficacy variables were evaluated using either a chi-square test for qualitative variables or a T student test for quantitative variables. For all outcomes, a nominal p value of less than 0.05 was considered to indicate statistical significance.

3.1.3. Intraosseous infiltration of Platelet-rich plasma for severe knee osteoarthritis (V)

Patients with grades 3 and 4 of knee tibiofemoral OA based on the Ahlbäck scale are considered candidates for this technique, which consists in one intraarticular infiltration and two PRP intraosseous infiltrations into medial femoral condyle and into the medial tibial plateau (Fig. 10) at the first procedure, and two more intraarticular PRP infiltrations 7, and 14 days after the intraosseous procedure.

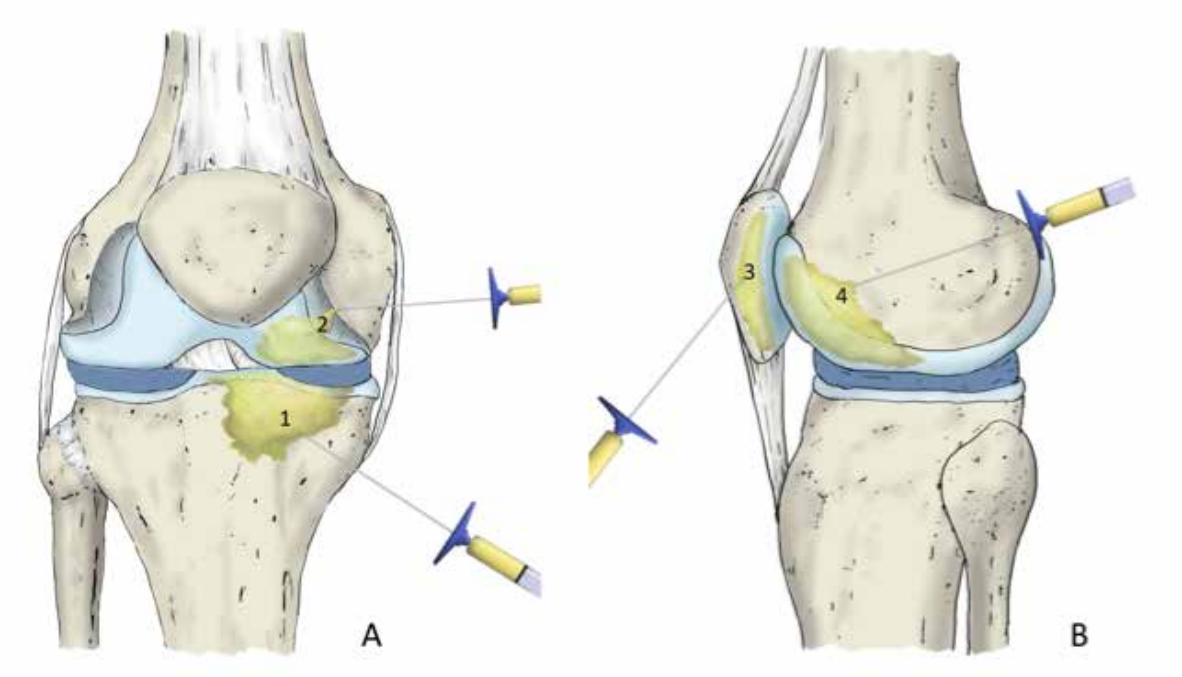


FIG. 10

(A) The platelet-rich plasma (PRP) intraosseous infiltration of a knee with severe femorotibial osteoarthitis is performed into the medial tibial plateau (1) and medial femoral condyle (2). (B) If the patient presents with femoropatellar osteoarthritis, the approach is external and the patella (3) and trochlea (4) are infiltrated. Before these intraosseous infiltrations are performed, conventional knee intra-articular infiltration of PRP is conducted.

Prior to inducing sedation, about 80 ml of venous blood is extracted from the patient in order to prepare the PRP according to PRGF-Endoret technology (Biotechnolgy Institute BTI, Vitoria-Gasteiz, Spain). Sedation is performed by infusing a single dose of normal saline, a single dose of midazolam (0.03-0.05 mg/kg) and fentanyl (3.2 mg/kg), in peripheral vein; single or repeated dose of propofol is also administrated (1-2 mg/kg), depending on the duration of the infiltration. The degree of sedation is 4 or 5 on Richmond Sedation Scale. Patients are monitored by the standards of the American Society of Anesthesiologists. The patient is positioned supine on an operating room table; the infiltration area is prepared with a povidone-iodine solution, covering a region with 10cm proximally and 10cm distally to the infiltration zone. Sterile drapes are placed defining the treatment zone (proximal, distal, medial and lateral).

Once the patient is sedated and prepared, and PRP is obtained, two marks are drawn in the medial region of the knee, one located 2cm proximal and the other located 2cm distal to medial joint line and centered in the midline sagittal plane. Next, a 24G needle is used to anesthetize the area of infiltration, which is introduced through the mark and moved to contact the femoral condyle; without retreating the needle, the periosteum of the femoral condyle is infiltrated with 2 ml of 2% mepivacaine. Then, the needle is withdrawn and moved into contact with the inner face of the tibial plateau (through the other mark) and without retracting the needle, the periosteum of medial tibial plateau is infiltrated with 2 ml of 2% mepivacaine.

Intraarticular infiltration

Intraarticular infiltration is conducted first and with a 21 G needle. The needle penetrate into the joint through the external patellar wing, centered in the central region of the patella in the cranio-caudal plane. Lateralization of the patella during infiltration facilitates this process (Fig. 11 A). After placing the needle into the joint space, synovial fluid arthrocentesis will be done if it is necessary. Once arthrocentesis is carried out, and without removing the needle, 8 ml of PRP is infiltrated. The infiltration is directed into the mind-point area of the femoropatellar region using an external approach in order to prevent infiltration into the synovial membrane, which would cause pain (Fig. 11B).

Intraosseous tibial plateau infiltration

Once the area is anesthetized, PRP is infiltrated into tibial plateau. A 13G trocar used for bone biopsy (CareFusion, San Diego, USA) is introduced into the bone through the mark previously made. The trocar is placed 2 cm distal to the joint line, leaning on the periosteum; then the trocar is introduced

2 cm into the thickness of the medial tibial plateau, following a parallel direction to the articular surface. Once the trocar is placed in the desired position 5 ml of PRP is infiltrated through the trocar (Figs. 11C, 11D).

Intraosseous femoral condyle infiltration

Next, PRP is injected into femoral condyle. A 13G trocar used for bone biopsy is introduced into the bone through the mark previously made. The trocar is placed 2 cm proximal to the joint line, leaning on the periosteum. Then, the trocar is introduced 2 cm into the thickness of the medial femoral condyle (to the middle area of the medial condyle), following a parallel direction to the articular surface of the condyle. Once the trocar is placed in the desired position 5 ml of PRP is infiltrated through the trocar (Figs. 11E, 11F).

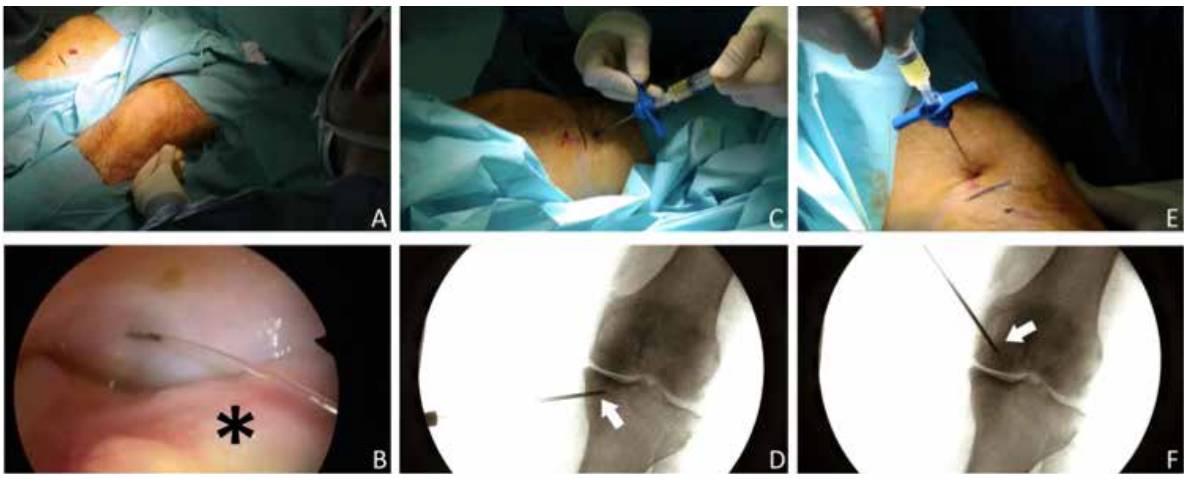


FIG. 11

After the patient is positioned supine on the operating room table, (A) intra-articular infiltration is performed into the joint through the external patellar wing, centered in the central region of the patella in the craniocaudal plane; (B) the infiltration is directed towards the midpoint area of the femoropatellar region using an external approach and preventing infiltration into the synovial membrane (asterisk). (C, D) Intraosseous tibial plateau infiltration is conducted into the medial tibial plateau, just to its middle area. The arrow indicates the trocar. (E, F) Concerning intraosseous femoral condyle infiltration, a trocar (arrows) is applied to the thickness of the medial femoral condyle, as far as the middle area of the medial condyle.

Intraosseous infiltration exploits these communications between cartilage and subchondral bone in order that PRP reaches the deeper layers of cartilage. There is a viscous consistency of PRP and the cellular material of subchondral bone which coagulates and remains in the areas of injured cartilage from which it has come (Fig. 12)

Finally, and after completing the infiltrations and removing the sterile drapes, the skin is cleaned with an alcohol solution, applying wound dressings on infiltration points. After infiltration is completed, the site is iced. In the days following surgery, the patient can bear weight and take analgesics (acetaminophen) as demanded for pain.

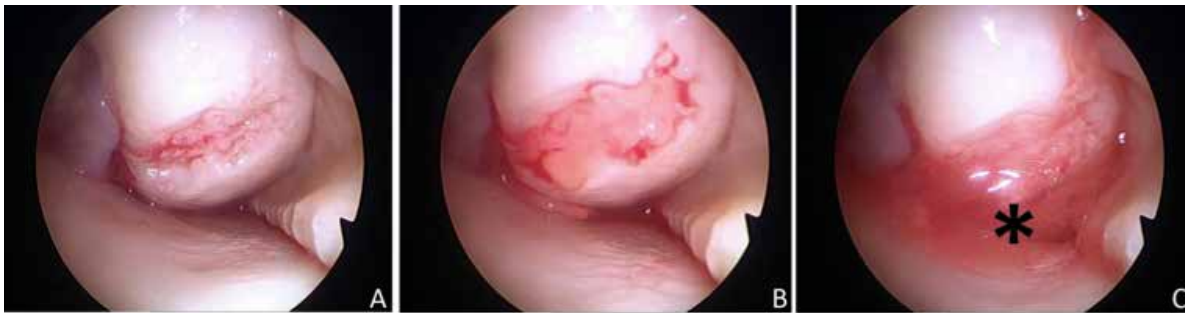


FIG. 12
(A) Communications between cartilage and subchondral bone are more pronounced in degenerated cartilage. (B) The platelet-rich plasma infiltrated into subchondral bone flows through the degenerated zones, and because of its viscous consistency, (C) it remains stuck in the area, creating a transient matrix (asterisk)

3.1.4 Combination of intra-articular and intraosseous injections of Platelet Rich Plasma for Severe Knee Osteoarthritis: a Pilot study (VI)

The study was carried out in accordance with the international standard on clinical trials: Real Decreto 223/2004, Declaration of Helsinki in its latest revised version (Fortaleza, Brazil; 2013), and Good Clinical Practice Regulations (International Conference for Harmonization). The study protocol was reviewed and approved by the Reference Ethics Committee. All patients provided written informed consent before entry into the clinical trial.

Patient Selection

Nineteen patients were initially assessed for eligibility. Patients were considered eligible if they were aged between 40 and 77 years and presented severe knee osteoarthritis according to radiographic confirmation (Ählback grades 3 and 4, on a scale 1 and 4, with higher numbers indicating more severe signs of degree). Finally, 14 patients were enrolled in the study from January 2014. Table II compiles the inclusion and exclusion criteria that patients had to meet in order to be included in this study. The enrollment finished October 29, 2014 and the clinical trial was completed on June 10, 2015.

TABLE II. Inclusion and exclusion criteria in the IA/IO infiltrations CT

Inclusion criteria	Exclusion criteria
Patients of both sexes aged 40 to 77 years	Bilateral knee osteoarthritis that requires infiltration in both knees
Predominant internal tibiofemoral knee osteoarthritis	Values of Body Mass Index > 33
Joint pain above 2.5 VAS points	Polyarticular disease diagnosed
Radiographic severity degree 3 and 4 according to the Ahlbäck scale	Severe mechanical deformity (diaphyseal varus of 4º and valgus of 16º)
Values of Body Mass Index between 20 and 33	Arthroscopy in the last year prior to treatment
Possibility for observation during the follow up period	Intraarticular infiltration of hyaluronic acid in the past 6 months
	Systemic autoimmune rheumatic disease (connective tissue diseases and systemic necrotizing vasculitis)
	Poorly controlled diabetes mellitus (glycosylated hemoglobin above 9%)
	Blood disorders (thrombopathy, thrombocytopenia, anemia with Hb <9)
	Undergoing immunosuppressive therapy and / or warfarin
	Treatment with corticosteroids during the 6 months prior to inclusion in the study

In the first basal visit, an orthopedic surgeon conducted a clinical and radiographic assessment of each patient, including their medical history and a complete blood count. Moreover, the doctor delivered a booklet that contained detailed instructions and the Knee Injury and Osteoarthritis Outcome Score (KOOS) questionnaire, which had to be completed by the patients at the baseline visit and before follow-up visits.

Patients were identified by a code number and scheduled to undergo the experimental procedure, which consisted of three treatments of Platelet Rich Plasma (PRP) on a weekly basis. The first treatment included one PRP intraarticular infiltration and two PRP intraosseous infiltrations (femoral condyle and tibial plateau). The next two treatments were conventional intraarticular injections.

PRP preparation and treatment

PRP was prepared according to the protocol of PRGF-Endoret technology (BTI Biotechnology Institute, Vitoria-Gasteiz, Spain) already depicted in the point 3.1.1., and applied according to the explanation in the point 3.1.3.

Follow up

Patients were called for follow-up visits 2 and 6 months after the last treatment visit in order to conduct clinical evaluation. During these visits, the patient submitted the questionnaires given at baseline. The doctor carried out a clinical examination and an evaluation of pain and function by visual analogue scale (VAS) and Lequesne Index, respectively. Acetaminophen consumption was also controlled.

Clinical Outcomes

The primary efficacy was defined as the decrease in knee pain from the baseline to second month and sixth month (endpoint), according to the KOOS questionnaire. The secondary efficacy outcomes included the other areas of KOOS: Symptoms, Function in daily living (ADL), Function in sport and recreation (Sport/Rec) and knee related Quality of life (QOL). They measured at baseline, two months and six months (endpoint). Furthermore, measurement of VAS and Lequesne Index were also evaluated.

Safety Outcomes

To evaluate the safety of treatment applied, all complications and adverse events were assessed and reported during treatment visits as well as follow up visits. Their nature, onset, duration and severity were documented.

Biological Outcomes

Presence of mesenchymal stem cells (MSC) in synovial fluids before and one week after intraosseous infiltration was evaluated by flow cytometry and cultures of colony-forming cells (CFU-F). Concerning flow cytometry, each sample was immunophenotyped using an 8-color direct immunofluorescence technique. Concentrated cell suspensions were stained with the following combination of monoclonal antibodies (MoAb) [Brilliant violet (BV) 421/orange chrome (OC) 500/fluorescein isothiocyanate (FITC)/phycoerythrin (PE)/peridinin chlorophyll protein-cyanin 5.5 (PerCP-Cy5.5) /PE-cyanin 7 (PE-Cy7)/allophycocyanin (APC)/APCH7]: i) CD105/CD45/CD73/CD271/CD34/CD13/CD90/CD44. Regarding CFU-F assay, collected synovial fluids were diluted in phosphate buffer saline PBS and centrifuged

in order to harvest the cellular content. The sample was used for colony-forming assay (CFU-F) and seeded on a 100 mm diameter culture plate. Seven days after plating colonies were visualized and counted by 0.5% crystal violet staining.

Sample Size Calculation

Power analysis was conducted to estimate the minimum sample size needed to achieve 80% power at a 5% level of significance for the primary outcome measure. An assumed effect size of 10 points (Minimal clinically Important Change, MIC) with a standard deviation (SD) of 12 points was used26. This analysis suggested a minimum of 13 patients, expecting a dropout rate of 0.1 (Fig. 13).

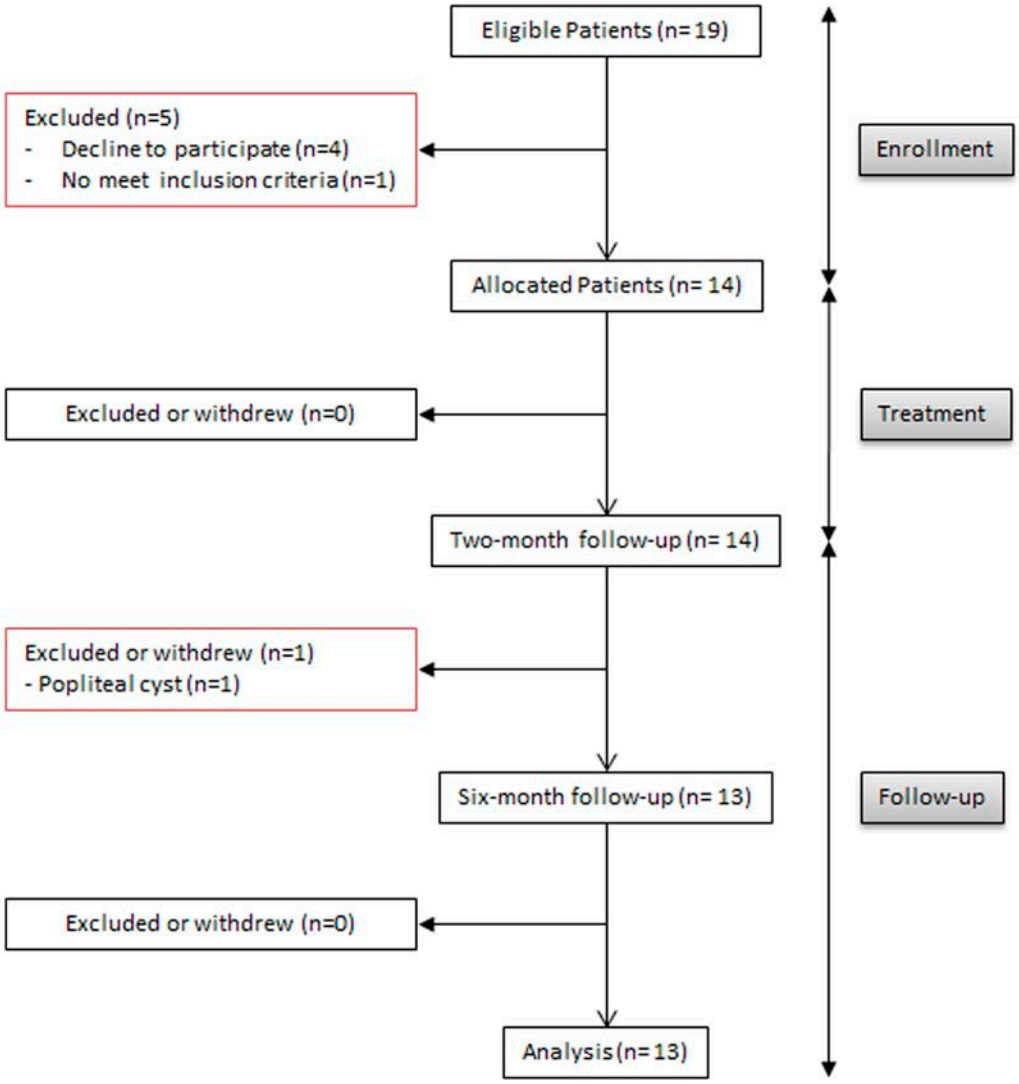


FIG. 13
Randomization of patients of the IA/IO pilot study.

Statistical Analysis

Demographic and medical variables (gender, age and OA grade) were determined by the mean, standard deviation, range and percent. For this study a per protocol analysis was used. Comparisons were performed by Student's t-test for paired-samples parametric data or Wilcoxon signed-rank test for paired-samples non-parametric data, after assessing the normal distribution of the samples by Saphiro-Wilk test. Data were considered statistically significant when p values were less than 0.05. Statistical analysis was performed with SPSS 17.0 (SPSS, Chicago, IL). (Fig. 14).

3.1.5. Modulation of synovial fluid cell contain by intraarticular and intraosseous Platelet Rich Plasma administration (VII)

Treatments and collection of synovial fluids

Synovial fluids were collected from 31 patients with knee OA. Patients were divided into two treatment groups; patients of the IA group received intraarticular infiltrations of PRP (n=14) and patients of the IO group (n=17) were treated with PRP intraarticular infiltrations together with PRP intraosseous injections. The choice of IA or IO modality treatment was made based on the failure of previous pharmacological treatments, namely, the patients who had been medically oriented toward a knee replacement as the only solution for their OA were allocated in the IO group. Synovial fluids were collected from 31 patients, before and after one week of PRP treatment. Patients were allowed to consume acetaminophen.

PRP was prepared according to the protocol of PRGF-Endoret technology (BTI Biotechnology Institute, Vitoria-Gasteiz, Spain) already depicted in the point 3.1.1., and applied according to the explanation in the point 3.1.2.

The institutional review board approved this study, and informed consents were obtained from every patient included in the study.

Multidimensional flow cytometry (MFC) immunophenotyping

Approximately 2-6 mL of arthrocentesis-derived SF of each patient was immunophenotyped using an 8-color direct immunofluorescence technique. After sample centrifugation, 100 µL of the concentrated cell suspension was stained for 15 minutes at room temperature in darkness, with the following combination of monoclonal antibodies (MoAb) [Brilliant violet (BV) 421/orange chrome (OC) 500/fluorescein isothiocyanate (FITC)/phycoerythrin (PE)/peridinin chlorophyll protein-cyanin 5.5 (PerCP-Cy5.5)/ PE-cyanin 7 (PE-Cy7)/ allophycocyanin (APC)/APCH7: i) CD105 / CD45 / CD73 / CD271

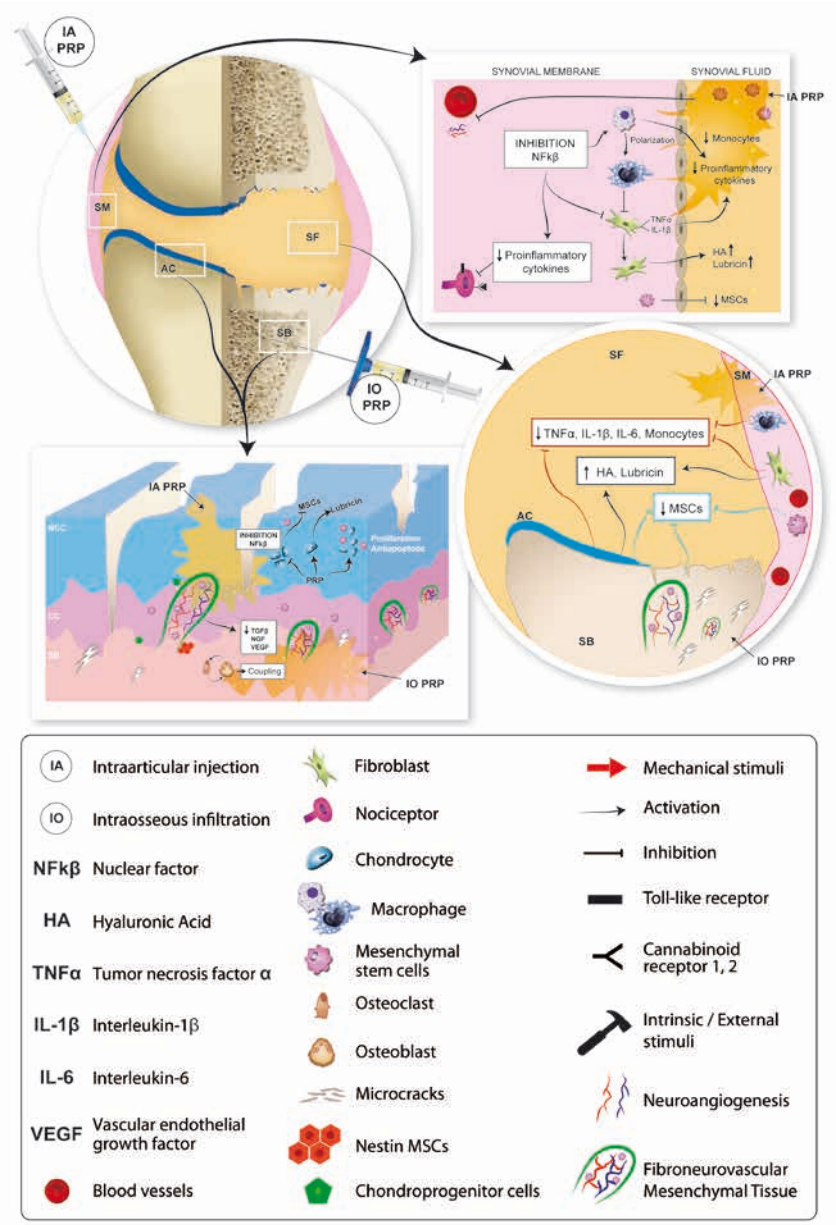


FIG. 14

Depiction of a new strategy to treat severe knee OA by targeting different knee joint structures such as synovial membrane (SM), synovial fluid (SF), articular cartilage (AC) and subchondral bone (SB) with intraarticular injections (IA) and intraosseous infiltrations (IO) of plasma rich in growth factors (PRP) (Sanchez et al 2014). This procedure reduces pain and mesenchymal stem cells (MSCs) in SF, besides significantly improving knee joint function of patients with severe OA. We suggest that various growth factors, cytokines and chemokines trapped in the fibrin network of PRP might inhibit the NFkβ on synovial macrophages, fibroblasts as well as on chondrocytes, thereby dampening the inflammatory response of SM and AC (23,44,140,164). In addition, IO infiltrations in subchondral bone, might buffer the excess of transforming growth factor β (TGFβ) as well as restore hepatocyte growth factor (HGF) activity synthesized by osteoblasts, thereby leading to a new reestablished homeostatic balance between TGFβ-1 and HGF (19,26,225). The buffer effect of PRP on TGFβ-1 signalling pathway in SB might reduce the presence of nestin MSCs in SF, likely associated with the shrinking of fibroneurovascular tissue in the SB, as an antifibrotic mechanism which has already been reported in other cell phenotypes (14,19)

/ CD34 / CD13 / CD90 / CD44. After staining, 2 mL of FACS lysing solution (Becton/Dickinson Biosciences, San Jose, CA) was added. After 5 minutes incubation at room temperature, samples were sequentially centrifuged 5 minutes at 540 g and resuspended in 100 µL of premixed Perfect-COUNT microspheres (Cytognos SL, Salamanca, Spain). Subsequently, data acquisition was performed for around 5.000 nucleated cells per tube in a FACSCantoII flow cytometer (Becton Dickinson Biosciences – BD – San Jose, CA) using the FACSDiva 6.1 software (BD). Monitoring of instrument performance was performed daily using the Cytometer Setup Tracking (CST; BD) and rainbow 8-peak beads (Spherotech, Inc; Lake Forest, IL) after laser stabilization, following the EuroFlow guidelines 25; sample acquisition was systematically performed after longitudinal instrument stability was confirmed. MSCs and residual leukocytes were identified through a boolean gating strategy based on forward scatter, side scatter, and CD45 expression; monocytes were defined on the basis of their relatively higher light scatter properties, CD13 and CD45 bright expression, whereas lymphocytes were identified through low scatter properties and strong CD45 reactivity (Fig. 15). Absolute cell numbers per volume unit were calculated following the manufacturer recommendation.

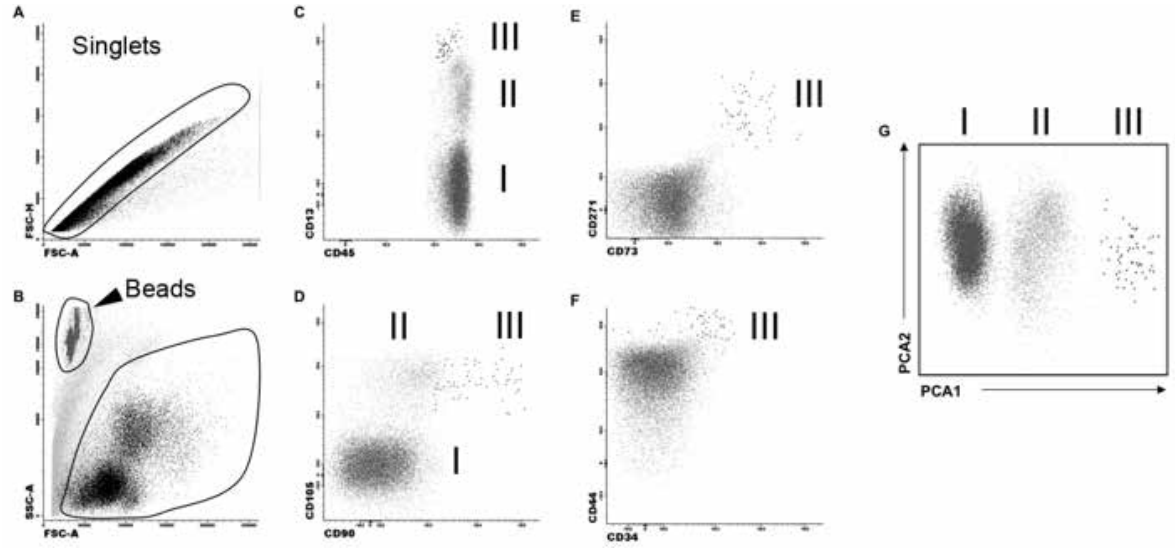


FIG. 15
Identification of MSCs, and White Blood cell in synovial fluid.

MSCs isolation from knee Synovial fluid

Collected SF were diluted in phosphate buffer saline PBS and the cellular content then harvested by centrifugation. One part of each sample was seeded in a 6-well plate under standard cell culture conditions with Dulbecco's Modified Eagle Medium (DMEM; Lonza) supplemented with 20% fetal bovine serum (Gibco), 1% penicillin-streptomycin (P/E) (Gibco) and 1 ng/ml of human recombinant basic fibroblast growth factor (bFGF; R&D systems) (Expansion Medium). The adherent cells were expanded in a humidified 5% CO2 atmosphere at 37 °C and used for further differentiation experiments. The remaining sample was used for colony-forming assay (CFU-F) and seeded on a 100 mm diameter culture plate. Seven days later plating colonies were visible and counted by 0.5% crystal violet staining.

Synovial fluid MSCs differentiation

Mesenchymal lineage differentiation assays were carried out between passages 2 to 4 to confirm the osteogenic, adipogenic and chondrogenic capacity of the cells. For the osteogenic and adipogenic differentiation 8000 cells/cm2 were plated in a 12 well plates. Adipogenic differentiation was induced using DMEM supplemented with 10% FBS, 1 µM Dexamethasone, 0.5 mM 3-Isobutyl-1-methylxanthine, 50 µM Indomethacin. For the osteogenic differentiation cells were cultured in DMEM supplemented with 10% FBS, 50 µg/ml L-(+)-Ascorbic acid, 10 mM β-glicerol Phosphate and 10 nM Dexmethasone. For chondrogenic differentiation, 2.5 x 105 cells were spun-down at 600 g for 10 minutes in polystyrene 15ml conical tubes and incubated with hMSC Chondrogenic Differentiation BulletKit™ Medium (Lonza). Differentiations were achieved at 28 days. Specific histological and immunohistochemistry analyses were done to assess differentiations: Oil Red O for adipogenic differentiation, Alizarin Red for osteogenic differentiation and Toluidine Blue and Type II Collagen immunostaining (anti-type II Collagen Mab, MP Biosystems) for chondrogenic differentiation.

Statistical Analysis

Comparisons were performed by Wilcoxon signed-rank test for non-parametric data and Student t test for parametric data, after assessing the normal distribution of the samples by Saphiro-Wilk test. Data were considered statistically significant when p values were less than 0.05. Statistical analysis was performed with SPSS 17.0 (SPSS, Chicago, IL).

3.2. RESULTS

3.2.1. A randomized clinical trial evaluating plasma rich in growth factors (PRGF-Endoret) versus hyaluronic acid in the short-term treatment of symptomatic knee osteoarthritis (IV)

A total of 187 patients were screened, and 176 underwent randomization (Fig. 16). The most common reason for exclusion included a body-mass index higher than 32 (six patients), the inability to meet radiographic criteria (four patients) and a genu varus deformity of the knee (one patient).

A slightly higher percentage of patients were women (52%), with a mean age of 59.8 and a mean body-mass index of 28. The groups were well balanced in terms of age, gender, body-mass index, percentage of patients suffering from primary arthritis, consumption of analgesics per day, radiographic grade (Ahlbäck scale) and WOMAC and Lequesne scores (Table III). A total of ten patients from the PRGF group and 13 from the hyaluronic group were precluded excluded from the study. The exclusion and withdrawal percentages did not differ significantly among the groups.

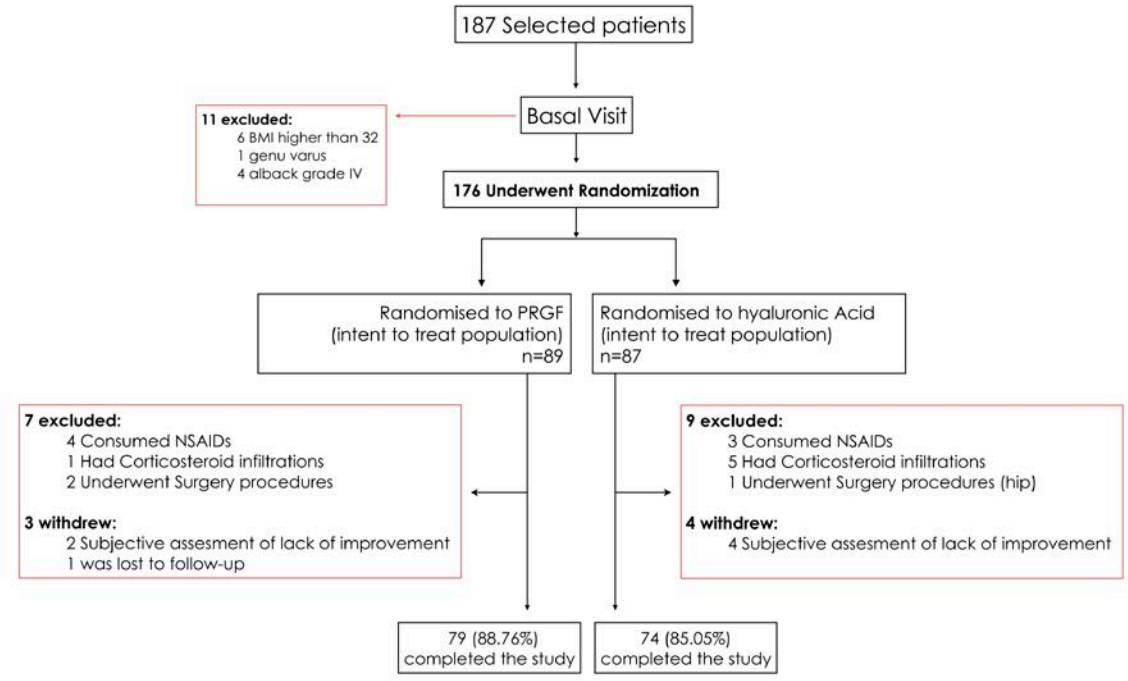


FIG. 16
Randomization of patients of the PRGF/HA clinical trial.

TABLE III. Baseline characteristics of the patients in the PRGF/HA CT.

	PRGF	Hyaluronate	p value
Age (years)	60.5± 7.9	58.9± 8.2	0.198
Sex (% Females)	46 (52%)	45 (52%)	0.996
BMI (Body Mass Index) †	27.9± 2.9	28.2± 2.7	0.590
Primary arthritis	73 (82%)	68 (78%)	0.521
Nº acetaminophen mg/day	2.6±7.1	1.7±5.6	0.631
Ahlbäck Grade ‡			
I	45 (51%)	42 (49%)	0.973
II	32 (36%)	32 (38%)	
III	12 (13%)	11 (13%)	
Normalized Womac score ¥			
Pain subscale	40.4±16	38.4; ±5.6	0.417
Stiffness subscale	41.8±17.3	38.5±18.3	0.233
Physical Function subscale	39.6±16.3	38.8± 17.4	0.755
Global	121.8±44.4	115.6±45.1	0.378
Lequesne index ¶	9.5; ±3.0	9.1±3.2	0.408
No.	89	87	

*Quantitative variables are expressed as mean and SD, except acetaminophen which is expressed as median and range. Qualitative variables are shown as absolute and relative frequencies. Is considered statistically significant at p> = 0.05.

† The body-mass index is the weight in kilograms divided by the square of the height in meters.

‡ Ahlback grade: Grade I: Joint space narrowing (joint space < 3 mm). Grade II: Joint space obliteration. Grade III Minor bone attrition (0–5 mm).

¥ Normalized scores for the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) can range from 0 to 100 for all subscales.

¶Lequesne score is a index of severity for osteoarthirtis of the knee with includes 3 subscales (pain or discomfort; maximum distance walked and activities of daily living). To make the assessment of the severity of gonarthrosis proceeds to the sum of all points being the minimum score of 0 points and the maximum 24. 0: No severity. 1-4: Mild 5-7: Moderate. 8-10: Severe. 11-13: Very severe. ≥ 14: Extremely severe.

Clinical outcomes

Results of primary and secondary outcome measures for the entire study population and each WOMAC pain stratum are summarized in Table IV. Analysis of the primary outcome measure (defined as the percentage of patients having a 50% decrease in the summed score for the WOMAC pain subscale from baseline to week 24) revealed that the rate of response to PRGF-Endoret was significantly higher than the rate of response to hyaluronic acid (Fig. 17). As compared with the rate of response to hyaluronic acid, the rate of response to PRGF-Endoret was 14.1 percentage points higher (95% CI 0.5-27.6; P=0.044). Regarding the secondary outcome measures, the rate of response to PRGF-Endoret was in all the cases higher than the rate of response to hyaluronic acid, although no significant differences were reached.

	PRGF	HA	Proportion dif (95% CI)	p value
Patiens ... no.	89	87		
50% decrease in WOMAC pain score ... no. (%)	34 (38.2)	21 (24.1)	14.1 (0.5 - 27.6)	0,044
OMERAT-OSARSI responders ... no. (%) ‡	47 (52.8)	43 (49.4)	3.4 (-11.4 - 18.1)	0.653
20% decrease in WOMAC pain score ... no (%)	51 (57.3)	46 (52.9)	5.2 (-10.3 - 19.1)	0.555
	PRGF	**HA**	**Mean dif (95% CI)**	**p value**
Normalized WOMAC pain score ¥				
% change from baseline	-35.0±41.6	-21.8±73.1	13.1 (-5.8 - 32.1)	0.172
At end of follow-up	24.1±15.5	26.9±15.8	2.8 (-2.2 - 7.9)	0.265
Normalized WOMAC stiffness score				
% change from baseline	-37.2±40.6	-31.5±41.6	5.6 (-7.7 - 19.0)	0.403
At end of follow-up	25.2±15.4	25.5±17.9	0.3 (-5.0 - 5.7)	0.901
Normalized WOMAC physical function score				
Change from baseline	-33.9±39.0	-29.3±38.8	4.6 (-7.8 - 17.1)	0.465
At end of follow-up	24.8±15.9	25.9±17.2	1.1 (-4.2 - 6.4)	0.682
Normalized WOMAC total score				
% change from baseline	-35.1±38.4	-32.5±31.9	2.7 (-8.7 - 14)	0.642
At end of follow-up	74.0±42.7	78.3±48.1	4.3 (-10.2 - 18.8)	0.561
LEQUESNE index ¶				
% change from baseline	-43.9±34.6	-40.2±39.4	3.7 (-8.1 - 15.5)	0.534
At end of follow-up	5.2±3.4	5.4±3.3	0.2 (-0.9 - 1.3)	0.714
Acetaminophen g/day ... median (range)	0.1 (2.0)	0.1 (2.3)		0.853

*Quantitative variables are expressed as mean and SD, except acetaminophen wich is expressed as median and range. Qualitative variables are shown as absolute and relative frequencies. Is considered statistically significant at p> = 0.05
‡OMERACT–OARSI Outcome Measures in Rheumatology Clinical Trials–Osteoarthritis Research Society, and HAQ Health Assessment Questionnaire.
¥ Normalized scores for the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) can range from 0 to 100 for all subscales.
¶Lequesne score is a index of severity for osteoarthirtis of the knee with includes 3 subscales (pain or discomfort; maximum distance walked and activities of daily living). To make the assessment of the severity of gonarthrosis proceeds to the sum of all points being the minimum score of 0 points and the maximum 24. 0: No severity. 1-4: Mild 5-7: Moderate. 8-10: Severe. 11-13: Very severe. ≥ 14: Extremely severe.

TABLE IV. Results of primary and secondary outcomes in the PRGF/HA intraarticular infiltrations CT

Overall, the rate of use of rescue acetaminophen was low (Table IV). There were not significant differences in the use of acetaminophen between the groups for all randomized patients or within each pain stratum.

Fifty adverse events were reported in 50 patients, 26 in the PRGF-Endoret group and 24 in the hyaluronic acid group (Table IV). Adverse events were generally mild and evenly distributed between the groups (P=0.811). Most of these adverse events (96% in the PRGF-Endoret group and 92% in the hyaluronic acid group) were not related with the type of treatment. The number of patients who withdrew because of adverse events was similar between groups (Fig. 16).

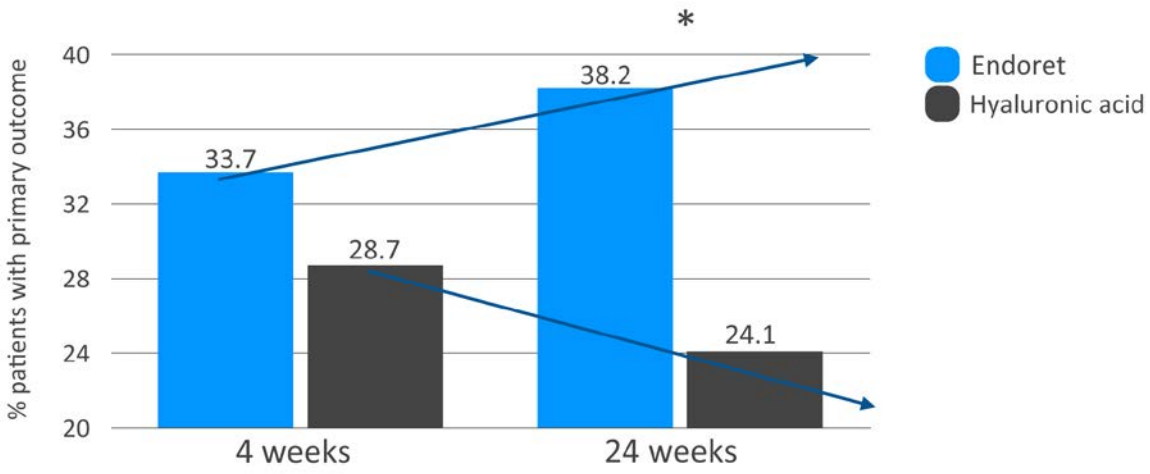


FIG. 17
Comparison of the clinical effect of PRGF and HA intraarticular infiltrations.

One patient who received hyaluronic acid felt numbness in the infiltration area and another patient of this group suffered from itching in the outside area of both thighs. One patient treated with PRGF-Endoret suffered from pain after the third infiltration. All the adverse events disappeared in 48 hours.

3.2.2 Combination of intra-articular and intraosseous injections of Platelet Rich Plasma for Severe Knee Osteoarthritis: a Phase II Clinical Trial (VI)

A total of 19 patients were considered eligible to participate in this study, and 14 patients were finally enrolled (Fig 13). Of the 5 excluded patients, four declined to participate and one presented predominant lateral osteoarthritis. Of the 14 patients who started, 13 completed the study and one

was excluded during the follow up period because of the occurrence of a popliteal cyst.

Nine of the thirteen patients who finished the study were men (69.23%) and four were women (30.77%), with a mean age of 62.23 ± 9.6 years (range: 47-75 years). Nine patients have been diagnosed with OA III (69.23%) and five with OA IV (30.77%), according to Ahlbäck Scale (Table V).

TABLE V. Demographic data, biological and clinical **outcomes**

DEMOGRAPHIC DATA						
	Total: n	Men: n (%)	Women:n (%)	Age: mean + sd (range)	OA III: n (%)	OA IV: n (%)
Patients	13	9 (69.23)	4 (30.77)	62.23 ± 9.6 (47-75)	9 (69.23)	4 (30.77)
BIOLOGICAL OUTCOMES						
		One week post-infiltration:				
	Baseline: mean + sd	mean + sd	p			
MSC/µL	7.98 ± 8.21	4.04 ± 5.36	0.019*			
CFU-F/mL	601.75 ± 312.30	139.19 ± 123.61	0.012*			
CLINICAL OUTCOMES						
	Baseline: mean + sd	**Endpoint: mean + sd**	**p**	**δ: mean + sd (% change)**	**Improved patients: n (%)**	**Patients with MCII[22]: n (%)**
KOOS Pain	61.55 ± 14.11	74.60 ± 19.19	0.008*	13.10 ± 14.89 (24.19 ± 40.07)	11 (84.62)	8 (61.53)
KOOS Symptoms	60.56 ± 17.35	71.70 ± 18.82	0.004*	11.14 ± 11.34 (19.73 ± 25.42)	11 (84.62)	8 (61.53)
KOOS ADL	68.44 ± 14.08	80.86 ± 15.58	0.022*	12.45 ± 17.31 (23.25 ± 38.82)	11 (84.62)	8 (61.53)
KOOS Sport/Rec	29.23 ± 20.29	45.38 ± 22.40	0.017*	11.78 ± 11.54 (76.94 ± 115.23)	10 (76.92)	7 (53.84)
KOOS QOL	28.10 ± 19.75	39.28 ± 16.52	0.012*	14.90 ± 22.03 (66.66 ± 72.64)	11 (84.62)	8 (61.53)
VAS	6.77 ± 1.75	2.88 ± 2.48	<0.001*	-3.88 ± 2.82 (-55.04 ± 38.21)	11 (84.62)	10 (76.92)
Lequesne Index	8.69 ± 2.65	5.77 ± 3.49	0.008*	-2.92 ± 3.35 (-31.18 ± 46.61)	10 (76.92)	

Clinical Outcomes

Table V summarizes results of primary and secondary outcome measures for the entire study population that completed the study. Analysis of the primary outcome measure (as the decrease in knee pain from baseline to week 24, according to the KOOS questionnaire) showed a statistically significant improvement in pain reduction from 61.55±14.11 at baseline to 74.60±19.19, six months after treatment (p=0.008). Eleven patients improved (84.62%), and 8 patients (61.52%) reported minimal clinically important improvement (MCII) (Table V). Depending on the osteoarthritis grade, eight of the 9 patients with grade 3 (88.88%) showed improvement, as did 3 of the 4 with grade 4 (75%).

Regarding secondary outcomes, there was also a statistically significant improvement in all other areas of the KOOS (Symptoms, p=0.004; ADL, p=0.022; Sport/Rec, p=0.017; QOL, p=0.012), as well as VAS score (p<0.001) and Lequesne Index (p=0.008).

The improvement of the patients was observed at two month follow-up, and it was maintained until week 24, when the study ended (Fig. 18). The two patients who did not respond to treatment were indicated for a total knee arthroplasty.

Two adverse events were reported in 2 patients and unrelated to the treatment. One of the patients experienced an episode of fever and the other exacerbation of knee pain three months after treatment. Both events were resolved satisfactorily by oral pharmacology allowed in the study. In addition, one patient withdrew because of a popliteal cyst that was treated by fluid drainage and corticosteroids infiltration.

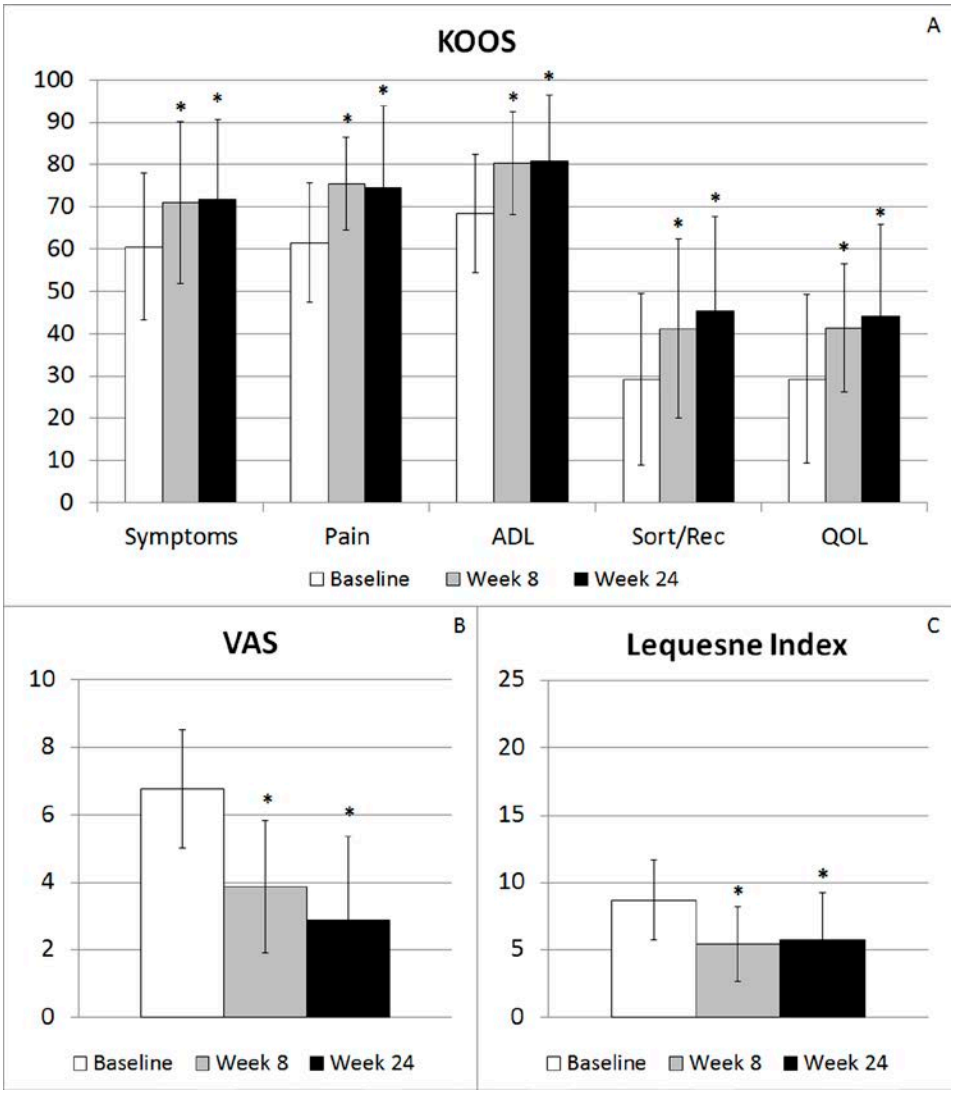


FIG. 18
Follow-up of clinical symptoms after intraosseous infiltrations of PRGF.

Biological Outcomes

Baseline levels of Mesenchymal Stem Cells (MSCs) presented in synovial fluid were 7.98 ± 8.21 MSC/µL, while one week after intraosseous infiltration the values dropped at 4.04 ± 5.36 MSC/µL (p=0.019) (Table V).

Concerning cultures of colony-forming cells (CFU-F), a decrease in the number of CFU-F was also observed one week after infiltration, being the number of CFU-F/mL before and after treatment of 601.75 ± 312.30 and 139.19 ± 123.61, respectively (p=0.012) (Table V).

3.2.3 Modulation of synovial fluid cell contain by intraarticular and intraosseous Platelet Rich Plasma administration (VII)

Characteristic of the patients

The mean age of patients in the IA group was 62.6 ± 11.8 years and the range was 41-77 years. The percentages of patients of this group with osteoarthritis grade II, III and IV according to Ahlbäck scale were 50%, 35.7% and 14.3% respectively. Concerning IO group, the average age of patients was 63.6 ± 11.2 years and the range was 41-80 years. In this group, the percentages of patients classified by Ahlbäck scale were 29.4% for grade II, 47.1% for grade III and 23.5% for grade IV. (Table VI).

TABLE VI. Patients included in the study and their clinical OA grade

	IA group	IO group
Age	62.6±11.8	63.6±11.2
Age range	41-77	41-80
OA grade II (%)	50	29.4
OA grade III (%)	35.7	47.1
OA grade IV (%)	14.3	23.5

Phenotypic characterization of the cell population of synovial fluid

To determine the influence of PRP treatment in the cellularity of the joint the presence of mononucleated cells (MNC) cells and their populations was analyzed in the synovial fluids of both groups, before and after treatment, by flow cytometry as described in material and methods (Fig. 15). Con-

cerning IA group, the concentration of MNC, lymphocytes, monocytes and MSCs in the synovial fluid pre and post treatment did not show significant differences (Table VII).

TABLE VII. Phenotypic characterization of the cell population in synovial fluid of IA group

	Pre treatment	Post treatment	p value
MNC (cells/ml)	237.11±223.32	243.81±193.37	0,32
Lymphocytes (cells/ml)	103.65±125.00	85.38±94.16	0,06
Monocytes (cells/ml)	130.66±101.88	142.62±112.81	0,73
MSCs (cells/ml)	2.60±4.38	1.53±2.51	0,32

MNC, mononuclear cells; MSCs, mesenchymal stem cells

Interestingly, although in the IO group the variations in the concentration of MNC, lymphocytes and monocytes in the synovial fluid were also not significant, MSCs showed a significant decrease after intraosseous treatment (Table VIII).

TABLE VIII. Phenotypic characterization of the cell populations in synovial fluid of IO group

	Pre treatment	Post treatment	p value
MNC (cells/ml)	441.92±371.87	354.82±411.44	0,38
Lymphocytes(cells/ml)	179.83±237.87	184.19±337.00	0,072
Monocytes (cells/ml)	199.37±160.28	119.06±98.47	0,053
MSCs (cells/ml)	7.61±8.68	2.46±3.86	0,01

MNC, mononuclear cells; MSCs, mesenchymal stem cells

Table IX shows the cellular increments (δ) before and after each infiltration, and compares the differences between the two treatments. The decrease in the levels of MSCs observed after intraosseous infiltration of PRP was higher than the decrease after intraarticular treatment (p=0.045).

TABLE IX . Cellular increment of the cell populations in synovial fluid

Cellular Increment (TM)

	Intraarticular	intraosseus	p value
MNC (cells/ml)	109.70±272.66	-91.33±334.47	0.905
Lymphocytes (cells/ml)	-65.04±106.50	42.64±171.96	0.159
Monocytes (cells/ml)	-19.64±156.00	-97.80±147.95	0.280
MSCs (cells/ml)	-1.41±5.38	-6.36±6.64	0.045
CFU-F (CFU/ml)	-6.87±236.79	-266.30±296.79	0.037

MNC, mononuclear cells; MSCs, mesenchymal stem cells; CFU-F, colony forming unit fibroblast

Culturing of colony-forming cells (CFU-F)

To confirm the reduction in MSCs in the SF we assessed the capacity of the MSCs population to sustain clonal growth on plastic surfaces (CFU-F). Consistently with the flow cytometry results, the intraarticular injection of PRP did not result in a significant variation in CFU-F, 332.52 ± 234.96 CFU/mL before treatment to 327.54 ± 223.32 CFU/mL post treatment (p=0.92). Remarkably, in the IO group we found a significant reduction in CFU-F from 477.51 ± 253.44 CFU/mL before intraosseous injections to 222.95 ± 151.36 CFU/mL one week post- infiltration (p<0.01) (Fig. 19). Consistent with the results obtained with the number of MSCs, the decrease in the CFU-F levels after intraosseous infiltration was greater than the decrease after intraarticular injection (p=0.037).

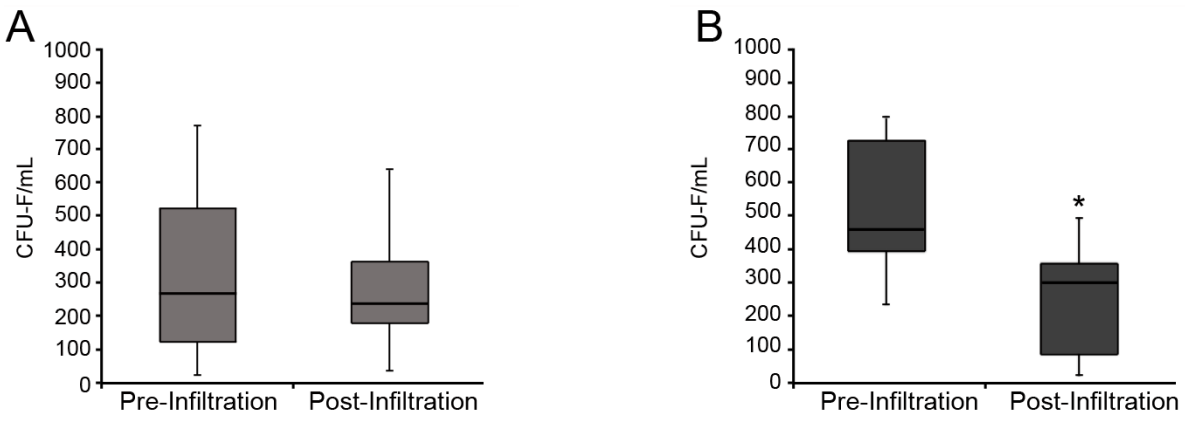


FIG. 19
Culturing of colony-forming cells (CFU-F) before and after intraarticular and intraosseous infiltrations of PRGF.

To confirm the mesenchymal progenitor nature of the CFU-F cells present at the synovial fluid we performed an in vitro multipotency assay by differentiation to the three mesenchymal lineages osteoblast, adipocyte and chondrocyte under defined conditions (Fig. 20). Although only a limited number of assays showed tri-lineage differentiation capacity (7 out of 68 assays, 10%) the majority of the assessed synovial fluid-derived mesenchymal cells showed bi lineage differentiation capacity (51 out of 68, 75%) supporting the mesenchymal nature of the population.

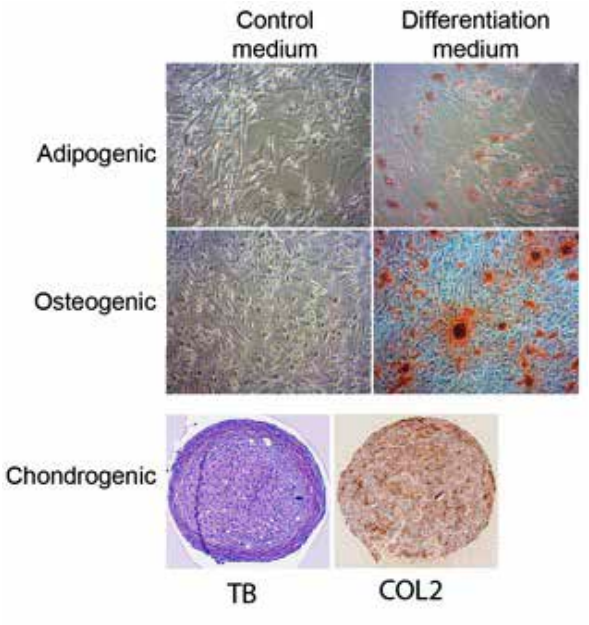
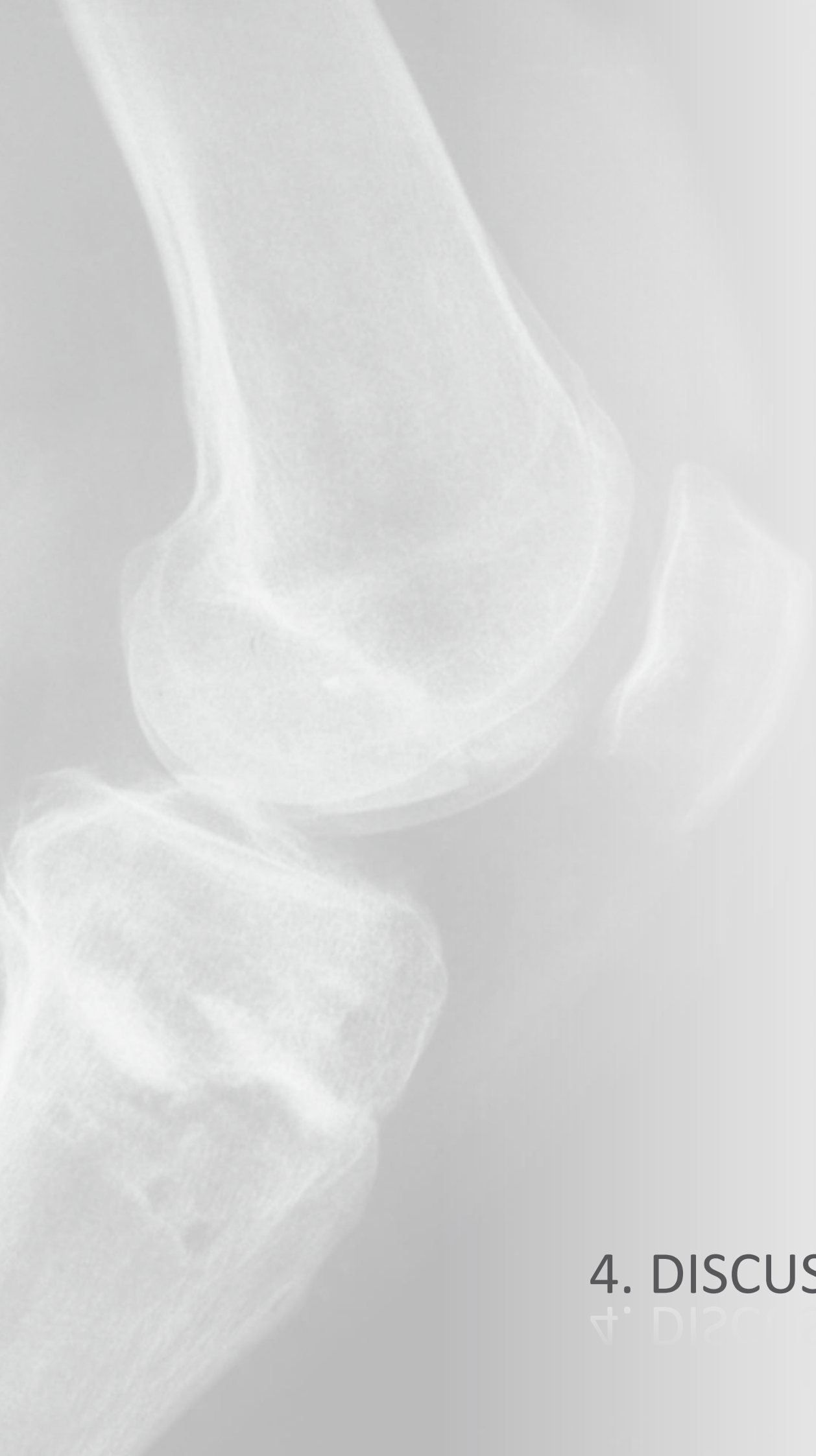


FIG. 20
In vitro multipotency assay of MSCs from osteoarthritic SF.



4. DISCUSSION

DISCUSSION

We conducted, for the first time in the literature, a randomized, double blind, hyaluronic acid-controlled, multicenter trial to evaluate rigorously the efficacy and safety of intra-articular injections of PRGF in the treatment of pain due to osteoarthritis of the knee. Three injections of PRGF, an autologous pool of growth factors and fibrin scaffold biomaterial, resulted in clinically significant reductions in knee pain, stiffness and physical function in patients with knee osteoarthritis. The analysis of the primary outcome showed that PRGF-Endoret was significantly more effective than hyaluronic acid. Clinically meaningful pain relief is in general defined as a reduction in pain intensity of more than 30% from the baseline level[169, 172] and reduction of 50% is considered as high improvement in pain according to the OMERACT-OARSI criteria[155]. In this study, the percentage of patients at the end of follow-up with a primary response to PRGF-Endoret was 38.2 whereas the rate of response to hyaluronic acid was 24.1%. In addition, the rate of response to each treatment followed an opposite pattern, with a substantial improvement of the primary outcome in the PRGF-Endoret group at 24 weeks and a gradual decrease in the case of the hyaluronic acid group. This data may suggest that in addition to the HA action[12], the PRGF-Endoret has other beneficial biological effects on cartilage in the long run. All the secondary outcome measures decreased with both active treatments and no significant differences were found between groups. The pool of growth factors obtained from platelet rich plasma decreases NFκB activation, a major pathway involved in the pathogenesis of OA, which is characterized by a catabolic and inflammatory joint environment[209]. Moreover, the supernatant of autologous proteins also inhibits MMP-13 production by IL-1β and TNFα-stimulated human articular chondrocytes[218]. The majority of the adverse events that were reported by patients were mild in severity. Most of the adverse events were not related with the type of treatment and they were evenly distributed between the groups.

The limitations of this study include the lack of measurement of physical activity levels in patients after applying the treatments, the different experience of physicians in the implementation of PRGF-Endoret treatment, the lack of longitudinal analysis and subgroup analysis for participating centers, the short-term follow-up of 24 weeks, the lack of a placebo group and the exclusion of patients who had the highest degree of severity on radiography (Alback grade 4).

Intraarticular delivery is the conventional modality to deliver PRP in patients with KOA and it has been shown to be safe and efficacious in improving clinical symptoms[64, 152, 178, 182, 184, 210, 212].

This route of drug delivery reaches the SM and the AC, which is sometimes inefficiently targeted by systemic drug delivery. Intraarticular delivery circumvents systemic toxicity and its side effects, offers an excellent bioavailability, and does not present molecular size limitation, in contrast to the systemically delivered molecules entering the joint via capillaries of the subsynovium[59, 95]. Nevertheless, intraarticular therapy faces other challenges when treating chronic nonsystemic sterile-inflammatory conditions as in the case of KOA. One significant challenge is a short joint dwell time of drugs, since the lymphatic drainage clears proteins in a few hours. This is not the case of PRP, since it acts as a dynamic liquid scaffold with a fibrin network from where GFs are gradually released into the tissue[16, 27, 135]. Nevertheless, the increasingly recognized role of SB in the pathophysiology of OA might make the intraarticular route insufficient to tackle all the joint tissues involved in KOA.

Intraosseous delivery strategy for local, prolonged, and sustainable release of GFs has been proven to be efficacious in some musculoskeletal pathology, non-union fractures, osteoporosis, and bone fracture healing among them[35, 97, 108, 202]. Surgical experience in cartilage defect repair over the past 30 years has revealed that only when the subchondral bone is involved through bone marrow stimulating procedures such as transcortical Pridie drilling and microfractures, is there a synthesis of a temporary functionally efficient fibrocartilage tissue, not have been reported serious adverse effects[86]. There is good in vitro and vivo evidence that events in the subchondral bone concur with and have a direct effect on the overlying articular cartilage[22, 34, 79, 127]. Moreover, although the titles and much of the text of Liu et al[120] and Philippart et al [156] papers are not focused on osteoarthitis, these studies shed important light on the role that intraosseous infiltrations of PRP might play in subchondral bone homeostasis by targeting both osteoblast-osteoclast coupling and mesenchymal stem cell responses, as well as in its safety. The combination of intra-articular and intraosseous injections of PRP is an in situ local biological “joint-centric” approach to treat severe KOA addresses the SM, SF and superficial zone of AC by intraarticular injections of PRGF, and deep zones of AC and SB through PRP intraosseous infiltrations[183]. These PRP infiltrations convey a mimetic biomaterial embedded with a pool of growth factors acting as a smart scaffold[43] which might sustain a gradual delivery of growth factors at the dysfunctional and deregulated tissues as a niche therapy.

In light of the aforementioned research and others not mentioned here due to space limitation, and the significant clinical improvement obtained in some patients with KOA, but not in all patients, treated with intraarticular infiltrations of PRP[64, 178, 182, 210], our group arrived at the strategy of combining another drug delivery route, namely, the intraosseous infiltrations[52, 183] combining with intraarticular infiltrations of PRP.

This is the first time that an in situ local biological therapeutic approach was used to treat severe KOA and address the knee joint as a whole by reaching the synovial membrane, synovial fluid and superficial zone of articular cartilage via intraarticular injections of PRP, and deep zones of articular cartilage and subchondral bone through PRGF intraosseous infiltrations resulting in a significant pain reduction and decrease of MSC and CFU-F in synovial fluid with no adverse effects. There are several potential mechanisms by which intraarticular injections and intraosseous infiltrations of plasma rich in growth factors might reduce knee pain. Once the activated PRP is injected into the intraarticular or intraosseous space, plasma fibrinogen is cleaved and fibrin polymerizes in situ as a three-dimensional scaffold adhering to SM, AC, and SCB (Figs. 3, 5, 6, 7, and 14).

Afterwards, the fibrinolysis begins, and the pool of growth factors, cytokines, and other biomolecules trapped into the fibrin network will gradually be delivered over 7-10 days[16]. In vitro and in vivo studies have reported that PRP and growth factors within it such as HGF, IGF-1, and PDGF suppress macrophage, fibroblast, and chondrocyte activation by inhibiting the NFκB pathway[23, 44, 60, 140] and thereby dampening the synovial and articular cartilage inflammatory response[186]. In addition, the significant amount of endogenous cannabinoids within PRP might act as ligands for cannabinoid receptor 1 (CB1) and 2 (CB2) of chondrocyte and synovium cells of OA patients[56, 166] thereby supporting a pain reduction by targeting the endogenous cannabinoid systems[51, 56, 166]. On the other hand, the excessive presence of TGF-β1 and VEGF in OA subchondral bone and articular cartilage[202, 225] could be a driving factor for changes in osteoblast-osteoclast coupling, which lead to a bone remodeling imbalance[106, 224], NGF expression[26, 106], and fibroneurovascular growth, all changes that might well contribute to pain[126, 202]. It is reasonable to speculate that the concurrent presence of, and a balanced ratio between, platelet-secreted TGF-β1 and VEGF, and plasma growth factors such as IGF-1 and HGF[19], all conveyed by PRP intraosseous infiltration, might buffer the excess of TGF-β1 in SB as well as restore HGF activity synthesized by osteoblasts. This new reestablished homeostatic balance between TGF-β1 and HGF[106, 225] would reduce the synthesis of NGF, VEGF and other inflammatory mediators thereby contributing to the reduction of pain and hyperalgesia in severe stages of KOA[26].

The increase in tolerable physical load, assessed by the significant improvement in the secondary efficacy outcomes such as function in daily living (ADL), function in sport and recreation (Sport/Rec) and knee related Quality of life (QOL), might entail a positive chondroprotective and antiinflammatory effect since as several lines of evidence suggest, moderate mechanical loading of joints prevents cartilage degradation by suppressing the activation of NFκ$^{B116, 200}$.

The significant reduction of MSC in SF after treatment with this novel PRP therapy is open to interpretation. Several studies have reported that the accumulation of MSCs in SF increases with the severity of osteoarthritis, joint damage[192], and the disease duration[94, 192]. Although the source of this MSC increase has not yet been determined, the most likely origin of the increased presence of MSC in SF of KOA patients might be the SM[93, 192], the breakdown zone of superficial AC[158], and the SB[106, 202]. By adhering to SM, superficial AC, and SF and by gradually delivering various components such as IGF-1, HGF, PDGF, TGF-β1 and platelet microparticles (PM)[211], intra-articularly injected PRP may influence macrophage M1 polarization towards M2 phenotype [44, 211] as well as modify the inflammatory status of chondrocytes and the superficial zone of AC by suppressing the NFκB signaling pathway[23, 140]. By lowering the concentration of chemoattractant inflammatory cytokines in SF, PRP may well contribute to the inhibition of the MSC release and migration[58, 60, 186]. Another origin for SF MSCs might be the SB as a point of egress, through the channels and vessels breaching the osteochondral junction, partially recruited by the osteoarthritic SF[58, 106, 202]. The buffer effect of PRP on TGF-β1 signaling pathway in SB might reduce the presence of nestin MSCs[225] likely associated with the shrinking of fibroneurovascular tissue of KOA subchondral bone as an antifibrotic mechanism which has already been reported in several cell phenotypes[19]. Moreover, the process of cell homing whereby SF MSCs might be recruited to damaged areas of AC and take part in the in vivo repair of that cartilage might also contribute to MSCs reduction[113], just as the PRP fibrin network, containing fibronectin, IGF-1 and II, PDGF, SDF-1, and TGF-β1 may exert a recruitment, homing, and chondrogenic-differentiation effect on subchondral mesenchymal progenitor cells[100, 102, 219]. This second study has, as well, some limitations. First, a relatively small number of patients were enrolled in the study, all belonging to the same severe KOA phenotype stage. Second, the clinical follow up of 6 months seems to be a short period to draw conclusive clinical indications. Third, a mechanistic account of the significant pain and SF MSCs reduction experienced by the majority of patients is lacking.

In our third study, we carried out two different treatment modalities of PRP applications on OA patient. After the first infiltration of intraarticular and intraosseous treatments, it was observed that cell levels in SF, particularly monocytes and MSCs decreased (Tables VII and VIII), suggesting an anti-inflammatory effect of PRP, a result which is consistent with the clinical improvement reported by Sanchez et al[178, 184] and Vaquerizo et al[210] using three intraarticular administrations of PRP on a weekly basis. Although the decrease regarding inflammatory process in synovial fluid after intraarticularly injected PRP is not statistically significant, this trend should be more pronounced after two more PRP intraarticular injections, which is the total number of applications for this conventional treatment, as significant clinical improvement has been shown in our first CT[182]. However, the decrease in the

concentration of MSCs was statistically significant after combining a single application of intraosseous treatment with intraarticular infiltration (IO group, p=0.01) (Table VIII). This was also confirmed when the levels of CFU-F were analyzed before and after treatment administration (Fig. 19).

It is worth mentioning that the cell population in synovial fluid before the PRP treatment and the degree of OA severity was considerably varied between both groups. The levels of synovial fluid-MSCs (SF-MSCs) in the IA group were very close to healthy population levels and substantially lower than in the IO group. Likewise, the percentage of patients in the IO group with advanced degree of OA (OA grade III and IV) was of 70.6% compared to 50% in the IA group (Tables V and VI)[94]. This observation is in accordance with several studies where the SF-MSCs levels were associated with the severity of osteoarthritis, joint damage and the disease duration[94, 114, 192]. Moreover, in the IO group, the higher level of mononuclear cells (MNC) could well support a deeper involvement of the synovial membrane inflammation in knee OA. Accordingly, MSCs have been postulated as a reservoir of repair cells and their release is interpreted as a tissue response to injury[93, 142, 195].

Several growth factors within PRP have proven to promote an antiinflammatory macrophage phenotype[44, 164, 211], suppress the NF-kβ signaling pathway on synovial fibroblasts and chondrocytes of the superficial zone of articular cartilage[140], and induce the synthesis of hyaluronic acid and lubricin by synoviocytes and chondrocytes respectively, the latter preventing chondrocyte apoptosis, cartilage breakdown, and inhibition of the MSC release and migration[61, 90, 101, 171]. All these modulatory and trophic effects of intraarticularly injected PRP on the synovial membrane, superficial articular cartilage, and synovial fluid combined with the decline of monocytes in the synovial fluid would result in a lower level of pro-inflammatory cytokines and restoration of the joint homeostasis leading to a more favorable synovial fluid environment for chondrogenic differentiation of MSCs[60, 61, 101, 143].

The combination of intraarticular and intraosseous infiltrations targets the three most likely origins of SF-MSCs increase, namely, the synovial membrane, the breakdown zone of superficial articular cartilage and the subchondral bone[93, 106, 142, 183, 192, 202]. When comparing the two treatment groups, the decrease in MSCs and CFU-F after PRP treatment was more pronounced in the IO group (Table VIII). This observation suggests that in the modulation of MSC by PRP, the subchondral bone is an important player and potential target, and might be a MSC egress point through the channels and vessels breaching the osteochondral junction and reaching the cartilage, partially recruited by the osteoarthritic environment of the synovial fluid[58, 106, 202].

There are several potential mechanisms by which intraosseous infiltrations of PRP might account for this significant reduction in MSCs in synovial fluid. The excessive presence of TGF-β1 and VEGF in osteoarthritic subchondral bone may be a driving factor for changes in osteoblast-osteoclast coupling, which lead to a bone remodeling imbalance and fibroneurovascular growth[106, 202, 204, 225]. Moreover, Zhen et al showed that by inhibiting TGF-β signaling in a specific population of MSCs present at the subchondral bone (Nestin positive MSCs) the severity of OA was reduced[225]. In fact, previous studies have shown that the decrease in MSCs in the synovial fluid, in low degree OA, suggest clinical improvement[192]. It is reasonable to speculate that, by administering PRP directly into subchondral bone, the concurrent presence of platelet-secreted TGF-β1 and VEGF as well as plasma growth factors such as IGF-I and HGF, could have a modulatory effect on TGF-β signaling pathway[19]. The reduced presence of MSCs, could likely be associated with the shrinking of fibroneurovascular tissue of OA subchondral bone, an explanation which parallels the antifribrotic mechanism already reported in several cell phenotypes[8, 10, 19].

A further significant component to the SF-MSCs reduction induced by PRP treatment would be the process of cell homing whereby SF-MSCs might be locally recruited to damaged areas of the articular cartilage taking part in the in vivo repair of this tissue, a possibility already reported by Lee et al[112]. This study showed that following humeral-head excision the entire articular surface of a rabbit synovial joint was regenerated by homing of endogenous cell and TGF-β3-infused bioscaffold. The PRP fibrin network, comprised of fibronectin, IGF-I and -II, PDGF, SDF-1, and TGF-β among other biomolecules, may exert recruitment, homing, and a chondrogenic-differentiation effect on subchondral mesenchymal progenitor cells. It has been reported that PRP is rich in fibronectin, a plasma protein incorporated into the fibrin network during the natural polymerization and one of the major factors for the recruitment of mesenchymal progenitor cells[5, 102, 104, 165].

Another interesting aspect in our study is that MSCs can be detected in the synovial fluid of all patients when measured through CFU-F assay. Using flow cytometry analysis prior to treatment, the presence of MSCs was observed in the synovial fluid in 21 of the 31 enrolled patients, representing 67.7% of total. The level of MSCs in these synovial fluids was as low as 5.19±7.15 MSCs/µL. However, the use of this technique to measure fresh synovial fluids without a prior cell expansion cycle can represent a limitation due to the low number of cells[114]. In order to overcome this limitation, the presence of MSCs in those synovial fluids was evaluated by means of culturing on plastic surfaces to determine the presence of colony-forming cells (CFU-F). In this case, CFU-F were found in the synovial fluid of all patients, with an average value of 410.59±246.36 CFU-F/mL. These results are

consistent with those reported in other studies in which the possibility of using synovial fluid as a source of autologous mesenchymal stem cells is demonstrated[93, 94].

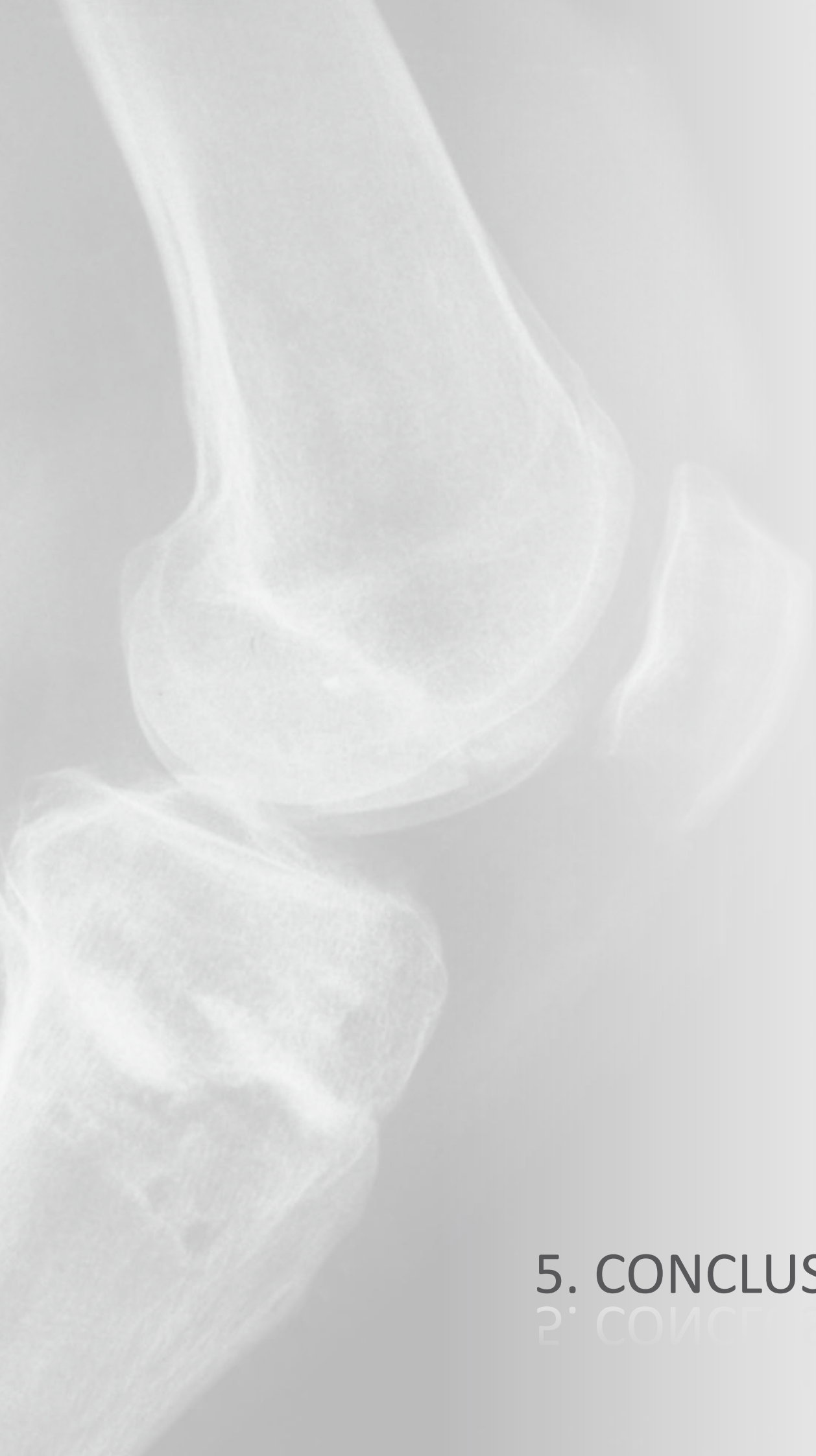
The use of synovial fluid as a source of cells for obtaining MSCs may be a promising alternative for treating diseases related to cartilage degeneration diseases such as OA. Thus, various factors must be considered when deciding the cell source and good environmental conditions for optimal effects[143]. The advantage of using synovial fluid as a cell source over other niches, such as bone marrow or fat tissue, is foremost its easy access. Furthermore, arthrocentesis is usually a necessary step prior to conducting an intraarticular injection of corticosteroids, hyaluronic acid or PRP. Additionally, MSCs present in the synovial fluid may derive from the synovial membrane, a tissue involved in the cartilage repair process[18].

Our hypothesis is that the concurrent presence and a balanced ratio between platelet-secreted TGFB-1 and VEGF, and plasma growth factors such as IGF-1 and HGF[4, 8, 9, 19], all conveyed by PRP intraosseous infiltrations, might reduce or buffer the excess of TGFB in SB and restore HGF activity synthesized by subchondral bone cells. This modulatory effect of PRP on TGFB-1 signaling pathway might shrink the fibroneurovascular tissue that replaces the bone marrow of OA subchondral bone, an explanation which parallels the antifibrotic mechanism already reported to be exerted by the PRP on several cell phenotypes[8, 9, 19]. This new reestablished homeostatic balance between TGFB1 and HGF[204, 225] would reduce the synthesis of NGF, VEGF and other inflammatory mediators thereby contributing as well to modulate the aberrant fibroneurovascular tissue and to alleviate pain and hyperalgesia[191].

Rebuilding a physiological-homeostatic network at knee organ failure tissue level, as is the case of severe KOA, must be an orderly process, which entails a daunting therapeutic task. Knee joint is a paradigm of autonomy and connectedness of their anatomical structures and tissues from which they are made. We propose an innovative approach to the treatment of severe knee osteoarthritis including a combination of intraarticular and intraosseous infiltrations of PRP[52, 183]. This novel therapeutic approach to treat severe KOA adresses the knee joint as a whole by reaching the synovial membrane, synovial fluid and superficial zone of articular cartilage by intraarticular injections of PRGF, and deep zones of articular cartilage and subchondral bone through PRP intraosseous infiltrations resulting in a significant pain reduction and decrease of MSC and CFU-F in synovial fluid with no adverse effects[183]. Our proposal to tackle severe knee OA by considering this condition a failure of an organ[162] and with a "joint-centric" rather than a purely "cartilage-centric" approach is challenging though promising[183].

However we do not forget that " the aim of science is not to open the door to infinite wisdom but to set a limit to infinite error " (Bertolt Brecht), and many questions and uncertainties still persist unanswered in the field of PRPs and inflammation. When the concept of inflammation defined as "a cooperative and amplifying protective multicellular response, orquestrated both locally and remotely, that is intended to eliminated the original insult and their toxic consequences, thereby initiating the repair process", there are some difficulties applying it to tissue damage brought about by mechanical stresses, which is the case of most sterile inflammatory pathologies such the KOA.

In summary, targeting synovial membrane, synovial fluid, articular cartilage, and subchondral bone with intraarticular injections and intraosseous infiltrations of PRP reduces pain and MSCs in SF, besides significantly improving knee joint function in patients with severe knee OA.



5. CONCLUSIONS

CONCLUSIONS

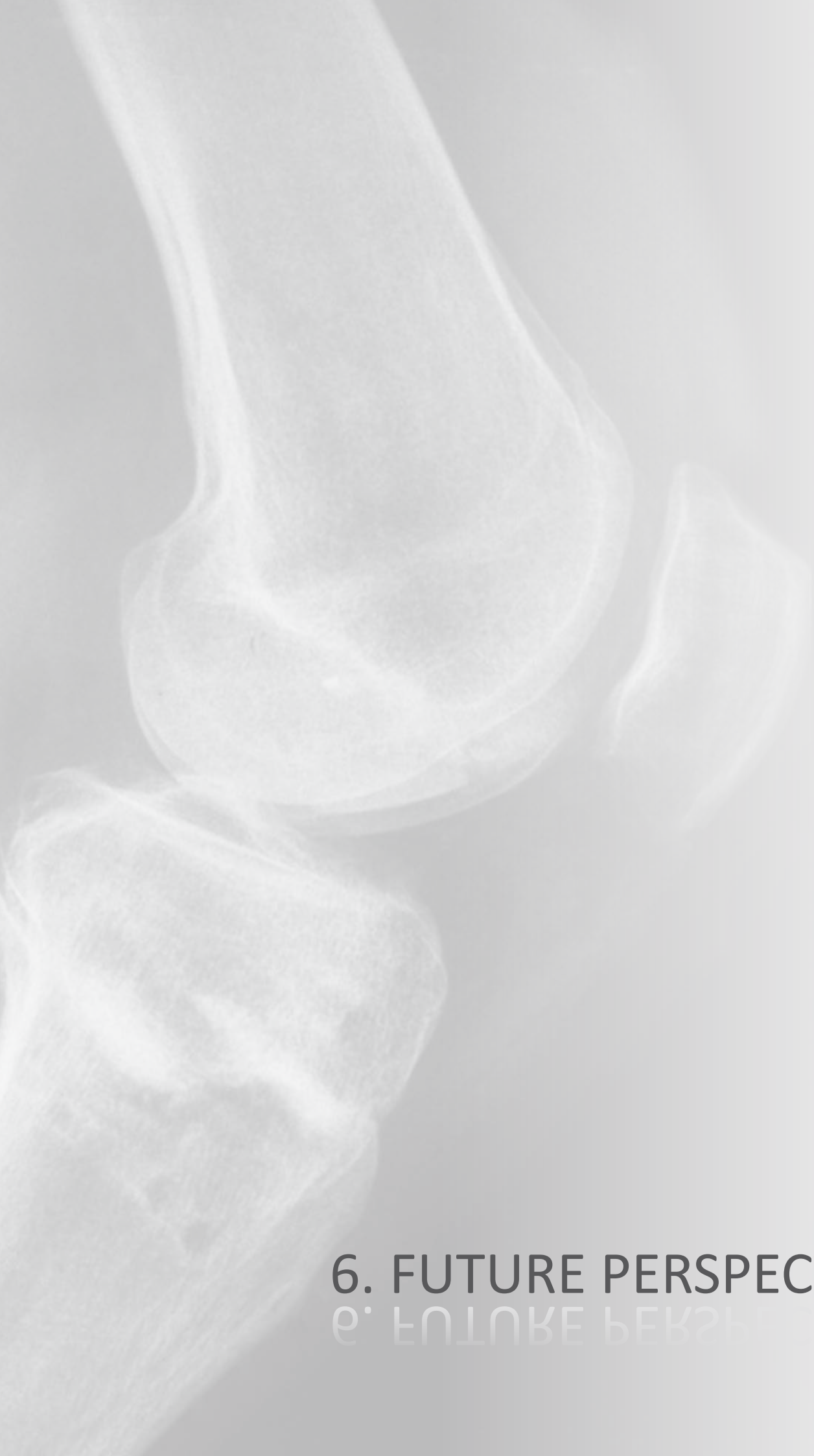
1. PRGF showed superior short-term results when compared with to HA in a randomized controlled trial, with a comparable safety profile, in alleviating symptoms of mild to moderate OA of the knee.

2. PRGF is safe, and effective therapy in reducing pain of KOA patients and it shows more long-lasting beneficial effect than hyaluronic acid.

3. Targeting synovial membrane, synovial fluid, articular cartilage, and subchondral bone with intra- articular injections and intraosseous infiltrations of PRGF is a safe procedure and reduces pain and MSCs in SF, besides significantly improving knee joint function in patients with severe knee OA.

4. MSCs modulation generated by PRGF may be favored by decreasing pro-inflammatory processes present in the synovial fluid of OA patients, and thereby might act as a structure-modifying therapeutic agent.

5. Combination of intraarticular and intraosseous infiltrations of PRGF rebuilds a physiological-homeostatic network at knee organ failure tissue level thereby paving the way for hyaline cartilage-like tissue regeneration.



6. FUTURE PERSPECTIVES

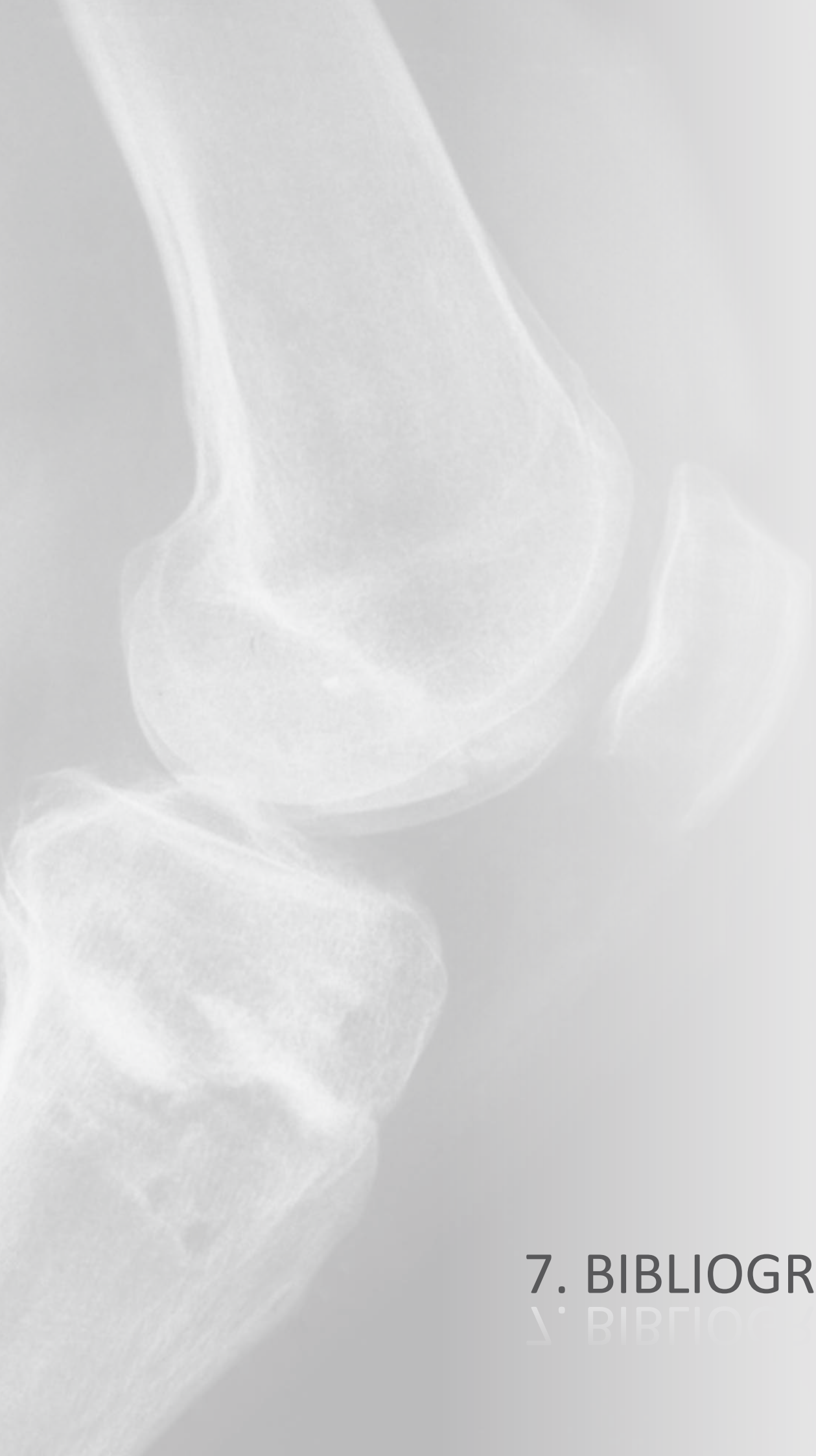
FUTURE PERSPECTIVES

In spite of a wealth of preclinical and clinical publications on PRP, many uncertainties remain regarding the ultimate molecular mechanism/s, the variability in its composition mainly due to the presence/absence of leukocytes, the platelet concentration, the donors age, and the manner in which PRPs are applied to the damaged tissues[213]. The restoration of TGFβ and other extracellular matrix GFs balance by the application of PRP deserves a deeper research and opens the door to explore the analgesic, antiinflammatory and immunomodulatory, and trophic-anabolic effects of PRP through a systems biology approach. In addition, we cannot rule out a systemic effect of intraosseous infiltrations as suggested by studies carried out in animal model, which should be explored. And finally, we still do not know how to combine PRP with rehabilitation programs and exercise in a synergistic application with the goal of full recovery of knee function[117, 221].

Fortunately, there are reasons for optimism. Novel formulations and fabrication methods are likely to help broaden the catalogue of PRGF applications. In addition, 3D bioprinting, may help to control the final properties of the autologous preparations. The exploration of PRGF potential for the ex vivo expansion of mesenchymal stem cells, together with the value of fibrin scaffolds for stem cell handling and transplantation, may also reduce some of the challenges faced in the field. Finally, homologousPRGF may become an alternative to patients whose blood components such as plasma, platelets, or fibrinogen lack several regenerative key inductors.

Our results encourage further studies in order to shed more light on the cellular and molecular mechanisms and to elucidate whether the PRP application in both modalities might lead to structural joint tissue changes as in vitro and preclinical research using this therapy have reported[100-102, 115, 219]. Moreover, intraosseous infiltrations of PRGF may have an immediate application to bone edema lesions, which might facilitate the AC damage, by restoring the homeostasis of subchondral bone thereby acting as a preventative treatment of KOA. Finally, also further studies will be needed in order to increase our knowledge about synovial fluid as source of MSC s and their therapeutic potential.

The successive intra-articular injections of Platelet Rich Plasma in the knee joint of Osteoarthritic patients has shown significantly higher reductions in knee pain and stiffness and improvement in physical function, even compared with Hyaluronic acid (HA). However, PRP has not yet been proven to really modify the overall histology or molecular composition of OA cartilage. In these clinical trials, the surrogate marker for OA amelioration was the pain and physical function. The trials, did not evaluate the influence of PRGF on histological and molecular make-up of Osteoarthritic cartilage. Although PRP therapy opens a new disease-modifying osteoarthritis strategy, we acknowledge that this biological approach may only play a part, albeit, a key part in solving this condition. We must not lose sight of the fact that physical rehabilitation as well as others systemic factors such as nutritional deficiencies, can affect the joint vulnerability.



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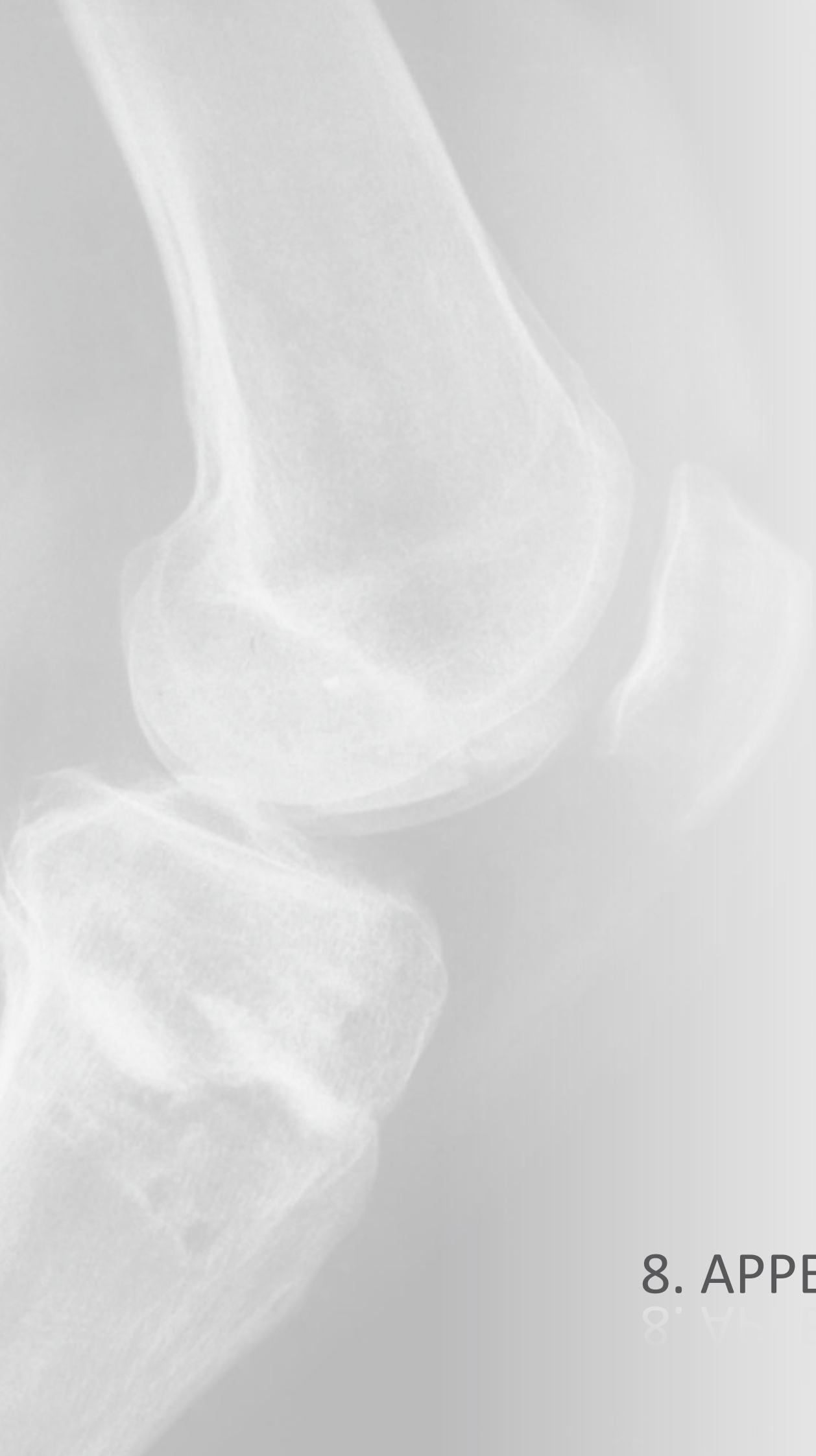

8. APPENDIX

APPENDIX

EXPERT OPINION

A biological therapy to osteoarthritis treatment using platelet-rich plasma

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Introduction: Osteoarthritis (OA) is a degenerative disease affecting the synovial joint. It is caused by cells exposure to non-physiological stimuli, either mechanical or biochemical, and the loss of bone-cartilage homeostasis. Some of these changes, however, may be reversed by the use of single or combined growth factors, suggesting that the treatment of OA could be addressed using a pool of growth factors.

Areas covered: This review addresses current molecular and biological knowledge and implicates the recapitulation of some developmental processes during endochondral ossification in OA aetiology and pathogenesis. Platelets act as carriers of endogenous morphogens that may modulate cell fate and therefore affect joint tissues structure and function. We shed light on the platelet-rich plasma effects on biological level that might drive the osteoarthritic joint's improvement both in structure and function.

Expert opinion: We present the therapeutic potential of plasma rich in growth factors (PRGF-Endoret), an endogenous biological therapy that might modulate the gene expression of cells such as chondrocytes, synoviocytes, macrophages, and mesenchymal stem cells, and thereby influence an anabolic microenvironment of synovial joint which is conducive to maintaining the homeostatic state of the joint's tissues, and hence reduce pain and improve the joint motion.

Keywords: cartilage, growth factors, osteoarthritis, platelet-rich plasma

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1. Introduction

Osteoarthritis (OA) is a mechanically induced disorder that evolves as a heterogeneous, multistage, and degenerative disease provoking the synovial joint failure as an organ. OA is a relatively local disease, for the most part affecting one or two joints, typically knees or hips. The disease represents a family of synovial joint degeneration which alters every component of the tissues involved, from the molecular to the cellular and extracellular levels. Although induced by an insult, either mechanical or biochemical, OA is biochemically mediated and ultimately causes the structural and functional failure of the joint. In addition to the central role that mechanical stress and age play in the onset and progression of OA [1], we conceptualize OA as a multifaceted disease in which systemic (hormonal status, gender, and genetics) and abnormal biomechanical loading on joints (obesity, joint injury, high-intensity and prolonged sports activities) make the joint vulnerable [2]. Another additional risk factor might be mutations in genes whose products make up the extracellular matrix. Although many of the OA-associated genes are involved in the development of the joints, none of them appears to be involved in the hyaline articular cartilage degradation and loss [3] which is considered a central pathological feature of OA [4]. It is important to remember that genes only represent the cells

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Article highlights.

- Osteoarthritis is a degenerative disease that gradually affects all the joint tissues provoking pain and loss of function.
- Developmental biology has shed some light on the osteoarthritis pathogenesis by bringing growth factors into play as cell fate modulators on different joint tissues.
- Growth factors may have the capacity to establish a molecular cross-talk among joint tissues, thereby controlling the pro-inflammatory phenotype of synovial joint's cells and maintaining an anabolic microenvironment.
- Platelet-rich plasma (PRP) products deliver growth factors, cytokines and adhesive proteins as well as other plasma proteins such as fibrinogene, prothrombin, and fibronectin.
- Plasma rich in growth factors (Endoret) application to osteoarthritic joints results in reducing joint pain and improving joint function by restoring tissue homeostasis as indicated by the chondroprotective, anti-inflammatory, and cell-phenotypic modulation effect on joint tissues.
- We are only at the beginning of a new era in which we must optimize PRP procedures at the same time we continue drawing on its healing power and relief in a wide range of medical conditions.

This box summarises key points contained in the article.

potentiality for change and that is the microenvironment's signalling presence that rules cells behaviour [5].

Whereas pain represents the clinical hallmark of the disease coexisting with other clinical features such as stiffness, instability, swelling, crepitus and functional limitation [6,7], it is the degeneration of the joint's tissues and changes in the periarticular bone rearrangement and the hyaline articular cartilage breakdown in particular that constitute the major factors leading to disability and impaired quality of life [8,9]. Due to the aneural feature of cartilage, the source of nociceptive stimuli might well stem from structures which are richly innervated such as synovium, subchondral bone, periostium, and joint capsule, however this relation has yet to be established [1].

In addition to articular cartilage, mature synovial joints have classically been considered to consist of ligaments and fibrous capsules lined with a synovial membrane whose cells exude a lubricating fluid (synovial fluid). The synovium is a specialized mesenchymal soft tissue made up of a lining layer with two distinct types of cells: synoviocytes that are fibroblastic-like and secrete lubricin and hyaluronan, and macrophages, although mesenchymal stem cells (MSCs) too have been isolated both in normal and osteoarthritic human articular cartilage [10,11]. These might play an important role as chondroprogenitor cells in the reparative response to articular cartilage damage [12]. Another layer, known as subintima, includes blood and lymphatic vessels associated with nerve fibres. The multicellularity and vascularity endow the synovium with a highly reactive capacity against what their cells might interpret as an insult or stress (mechanical or biochemical). Such an insult would trigger an inflammatory defence response in order to maintain or restore joint tissue homeostasis and function [13].

2. Bone-cartilage homeostasis disruption

Exposure of the joint cells to non-physiological stimuli, either mechanical or biochemical, leads to a rupture in the cartilage balance between anabolism and catabolism known as cartilage homeostasis [8,9,14]. Although the homeostatic processes within the joint occur at the cellular, tissular and organ level, the behaviour of cells such as chondrocytes, synoviocytes, macrophages and osteocytes is truly responsible for carrying them out [15]. The disarrangement of structures that make up the synovial joints such as hyaline cartilage, synovium, synovial fluid, menisci, and subchondral bone gives rise to the failure of the synovial joint, a key component in the body's motion.

Articular cartilage and the subchondral bone are endowed with different adaptive responses to mechanical load and damage, and this asymmetry might disrupt the homeostasis between them [16]. Several groups have proposed a molecular crosstalk between the bone and cartilage pointing to the subchondral bone reactions to the mechanical stress as the triggering factor in the OA [4,15-17] hence challenging the traditional view of the articular cartilage as an isolated tissue and offering a view of the possible existence of fluid, cell, and molecular communication between the cartilage and the subchondral bone (**Figure 1**) [18,19].

Tissue interactions govern most developmental processes, from the very early patterning events of cell differentiation, through a process called morphogenesis and finally growth of the many organs in the embryo. All human synovial joints share the same developmental processes. Formation of the skeleton is no exception, and most of the tissues differentiating in the newly forming limb arise from mesenchymal cells. These cells give rise to the various articular tissues, with the exception of neuronal elements and blood vessels [20,21]. The regulation of articular cartilage development and homeostatic processes throughout life is carried out under the influence of numerous growth factors and cytokines which act in concert as signalling molecular pathways [22].

3. Current cellular and molecular knowledge about the common signalling molecules and pathways underlying osteoarthritis

A variety of cells and cell signalling molecules which dynamically form the structural network of the joint tissues are extremely well communicated and may use the fluid flow to migrate and reach injured areas mainly attracted by cell signalling factors (growth factors and cytokines), biochemical gradients and matrix fragments [23-25]. Cells from different tissues of the joint but chiefly the quiescent chondrocytes undergo and sense non-physiological stimuli as an insult,

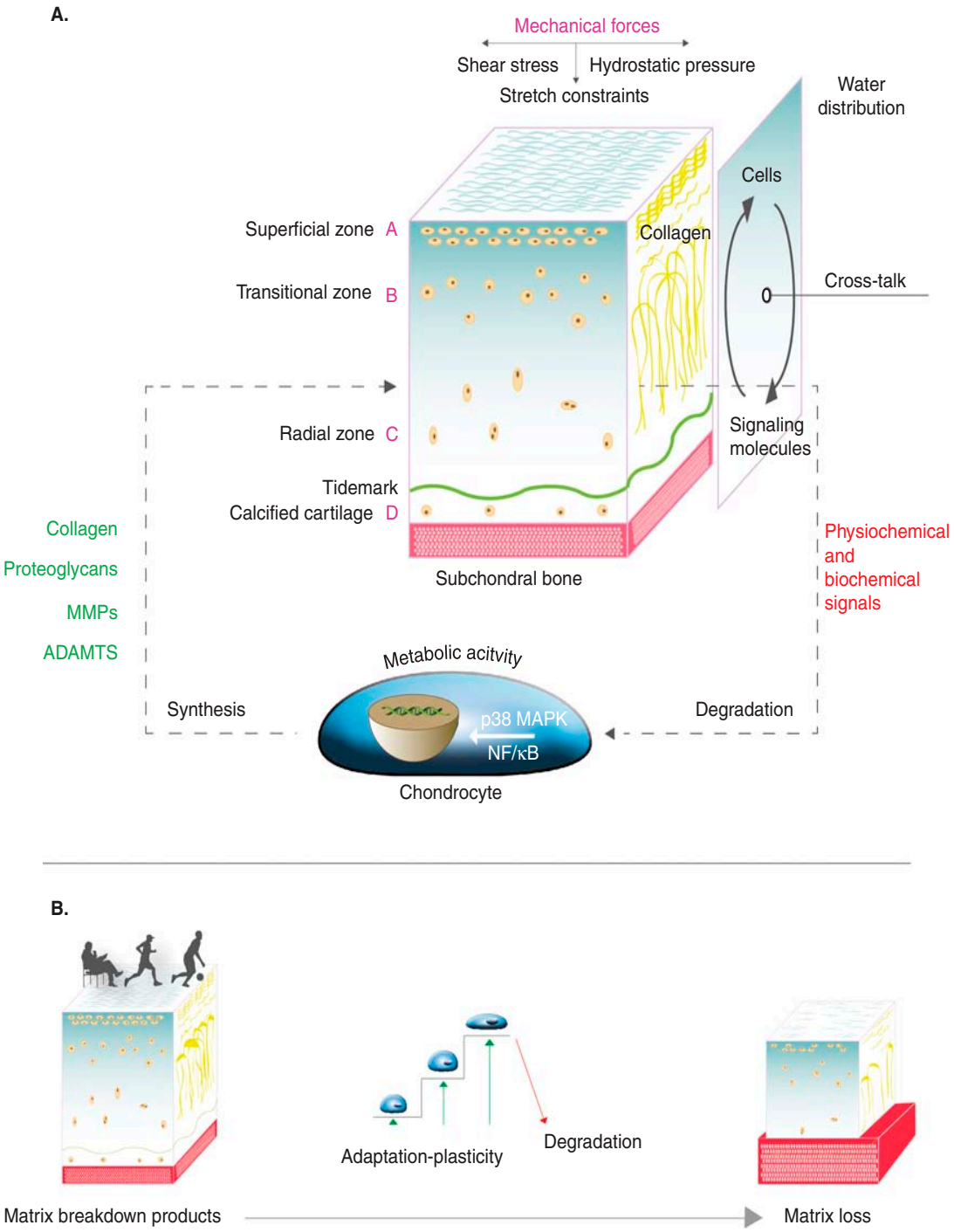

Figure 1. A. In the complex cartilage-bone-based mechanotransduction system, the mechanical energy applied to the joint is reflected on in the extracellular matrix, and subsequently in the chondrocyte nucleus. Joint cells exposure to non-physiological stimuli tips the loss of tissue balance between cartilage degradation and synthesis known as cartilage homeostasis. There appears to be a molecular, cellular, and fluid communication between the cartilage and bone. **B.** The survival/viability of the chondrocyte is affected to a large extent by the presence of a sufficient (plasticity), but not excessive (degeneration), mechanical stimulus that would inevitably lead to the disarrangement of structures such as the subchondral bone mediated mainly by deregulated chondrocytes, perpetuating a catabolic microenvironment and eventually the joint failure.

modulating and taking on a different phenotype whose gene expression products (anabolics and catabolics) orchestrate a defence-inflammatory response [26-28] in a miscued attempt to either maintain the tissue homeostasis and integrity or mimic the repair process. Nevertheless the tissue response turns out to be catabolic, thereby altering the cells' micro-environment and breaking down the extracellular matrix. The response of chondrocytes in the osteoarthritic cartilage

is heterogeneous and oriented towards hypoanabolism, which encompasses cell proliferation, apoptosis, and phenotypic alterations. Such a response results in a reactive or hypertrophic chondrocyte phenotype known as deregulated chondrocytes [26,28-30] whose catabolic gene expression causes a net loss of extracellular matrix [26]. Not only chondrocytes but also macrophages and synoviocytes influenced in a paracrine manner take on a pro-inflammatory phenotype [13,31]. The extracellular matrix which is made up mainly of water, type II collagen and aggrecans drains away and degenerates as a consequence of the action of catabolic cytokines (TNF-α and IL-Iβ), metalloproteinases (MMPs, MMP13), and aggrecanases (ADAMTS). These products are primarily released by chondrocytes, synoviocytes, and mononucleated cells, breaking the collagen and aggrecans down in a slow and relentless degenerative process [9,26,28] and thereby giving rise to articular chondrocytes expressing classic hypertrophic markers (characteristic of the growth-plate chondrocytes) and apoptosis [8,29,30]. This interpretation of biological processes strongly suggests that context matters, and that the extracellular matrix (ECM) in connective tissues, as in bone, muscle, tendon and cartilage, hosts physical–chemical processes which are key to tissue-repair processes such as cell-recruitment and differentiation, and patterning-remodelling.

There is a clear role played by tissue and cellular signalling pathways and networks in osteoarthritic aetiopathogenesis, pathways and networks which are shared in the developmental biological process [29,30,32] and which will be partially redeployed and may, in osteoarthrosis pathogenesis, adopt a role as mediator. At the molecular level the aforementioned biological processes are mediated by a group of highly conserved polypeptides known as growth factors (GFs) which are proteins specialized in signalling cellular pathways and mainly, but not solely, released by local cells such as chondrocytes, macrophages, and synoviocytes. Growth factors modulate the cell's behaviour and shape the structure of the tissues thereby determining their functions [33,34]. In addition to the inflammatory response in the osteoarthritic aetiopathogenesis, mainly led by the local cells in one reparative-reactive attempt of the cartilage, the osteoarthritic joint is the destination of several migratory cells, namely MSCs and chondroprogenic progenitor cells (CPCs) [10,12,35,36] which may come from the subchondral bone through the tidemark into the cartilage tissue. The CPC, with MSC features have a multipotent differentiation capacity towards the chondrogenic lineage [35] and may be the target of the GFs which traffic cell information through the MSCs by their trophic activity [37]. These multipotent cells might offer us the most valuable component when it comes to the repair process, namely, cells [38]. A similar process appears to be responsible for fibrocartilage repair synthesis when the Pridie drilling procedure is carried out in some reconstructive cartilage surgery. This surgical procedure has presumed that the adult marrow-derived mesenchymal stem cells (MSCs) from the subchondral bone are able to differentiate into bone, cartilage, muscle, marrow stroma, tendon-ligament, fat and other connective tissues [39]. In addition to the subchondral bone marrow, the synovium is another important source of MSCs in the joint tissues showing a high chondrogenic potential comparable to that of bone marrow-derived MSCs [12]. These observations are in accordance with insights and clinical experience, suggesting that MSCs are naturally found as perivascular cells or pericytes. Once they have migrated to the injured site, these cells behave not only as proliferative and differentiated cells but also and significantly as immunomodulatory and trophic ones [39,40].

There is growing evidence indicating that in OA, articular chondrocytes expressing classic hypertrophic markers (known as deregulated chondrocytes) [26,28-30] with a catabolic gene expression and extracellular matrix destruction of articular cartilage, resembles that observed in the hypertrophic zone of foetal growth plate during endochondral ossification, a resemblance suggesting that developmental biology might shed some light on the OA pathogenesis [26-30]. OA is driven primarily by both mechanical stress and inflammatory signals (IL-1β and TNF-α) orchestrated by the NF-κβ signalling molecules which have been shown to mediate articular cartilage degradation by upregulation of matrix-degrading MMPs [28,41]. The activation of the NF-κβ signalling pathway can generate altered states of quiescent chondrocytes thereby pushing chondrocytes to a more differentiated, hypertrophic-like state in an attempt to maintain or restore tissue homeostasis, as well as recapitulating some developmental cell phenotypes [28,29,41]. Most of the cellular and molecular changes previously mentioned are also described in the growth plate chondrocyte and may be reversed by the use of single or combined growth factors such as TGFβ-2, FGF-2, IGF-1 or insulin [42,43] combinations that have been shown to produce synergistic effects in preserving chondrocyte homeostasis [44,45].

4. An innovative biological approach to the treatment of osteoarthritis: platelet-rich plasma

The appropriate treatment of cartilage injuries and OA remains a daunting clinical challenge despite advances in both pharmacological management of the pain and inflammation, and advances in the surgical procedures and techniques and, in extremis, OA has been considered a disease with no cure [1]. Although is not within the scope of this article to address the wide range of therapeutic strategies in the treatment of OA, we wish to remark that only a holistic approach could fulfil the goal of clinicians, namely, to control pain, to improve function and to stop the progression of disease [1]. Since the synovial joint is a complex, shock-absorbing interface in which a coordinated and sequentially ordered engagement of the joint's elements and muscles is required to

maintain the physical integrity of anatomical structures and homeostasis of the joint's tissues, every pharmacological and surgical therapy should be assisted by mechanotherapy [46]. In this respect, and as a clinical application of cells mechanotransduction, a rehabilitation program which included the employment of PRGF in a synergistic manner would play a crucial role in both promoting the repair or remodelling of injured tissue and avoiding the degradation of cartilage and atrophy of joint's structures such as bone, periarticular muscles, tendons and ligaments with the goal of full recovery of function [46-48].

One innovative biological approach to the treatment of OA is the application of platelet-rich plasma in intraarticular injections. Although a universally accepted definition of PRPs in terms of platelet concentration and presence or absence of leucocytes is lacking, PRP products can be depicted as an autologous platelet concentrate within a plasma suspension, and whose composition is determined by the method used to obtain it. Platelet-rich plasma products include plasma and twofold or more increases in platelet concentrations above baseline levels, and the concentration of leucocytes and erythrocytes varies widely [49-51] from a complete absence of products to a high concentration of them. In particular, the PRGF is depicted as an endogenous blood-derived product which conveys growth factors, cytokines, and morphogens contained in the platelets as well as fibrinogen and other plasmatic proteins in a biologically balanced aggregate, and managed and delivered in a pharmacological manner [33,52,53]. This multifaceted, versatile, biological system is made up of an autologous, balanced blend of plasma with a moderated platelet concentration (a two- to threefold increase compared with peripheral blood) that does not contain leucocytes. The process of platelet activation and hydrolysis of prothrombin into thrombin is driven by the addition of calcium chloride, simultaneously causing the release of a plethora of growth factors and the polymerization of fibrin [33,53,54]. Once activated the liquid formulation is in the ensuing moments injected as a solution into soft tissues, and due to its local and gradual activation (*in vitro* and *in vivo*) and homogeneous distribution through and interaction with the ECM of different tissues, is converted into a matrix-like viscous and malleable structure [34].

Mammal platelets are circulating monitors, trackers and surveyors of the integrity of the vascular system and of the internal milieu as well as carriers of cytokines, chemokines and growth factors, fulfilling the function of coordinators of coagulation, inflammation and repair processes [55,56]. In addition to these bioactive mediators (α-granules: TGFB, PDGF, VEGF, FGF, EGF, IGF-1, HGF, BMPs, BDNF and dense granules: histamine, serotonin, Ca and ATP/ADP), there are other contents in the plasma of PRPs (IGF-1, HGF, fibrinogen, fibronectine and other proteins) which together with adhesive proteins expressed by activated platelets, all play a central role in the cell signalling pathways involved in both tissue injury recognition and in the repair of damaged tissues (**Table 1**) [33,56]. Platelets appear to be crucial in post-embryonic morphogenesis in identifying tissue loss or injury, factors that activate platelets thereby releasing by degranulation, growth factors and cytokines which trigger mechanisms to reconstruct structures and restore function mainly by stimulating cell migration and proliferation, regulating angiogenesis, chemoattracting circulating progenitor cells and guiding tissue remodelling [54,57-59]. Drawing on these mechanisms and observations made by Crisan and col.2008 and Caplan 2009 concerning the immunomodulatory and trophic effects of MSCs [39,40], it might be possible to suggest a synergy between platelets and MSCs.

Besides conveying GFs, PRGF provides the damaged tissue with a transient biological scaffold made up of fibrin which stems from the polymerization of fibrinogen, a pleiotropic blood protein that regulates coagulation, inflammation, and tissue regeneration [33,54]. The three-dimensional network, formed either "*in vitro*" as a clot or "*in situ*" as an extracellular matrix after the intraarticular infiltration over-injured areas, contains binding sites for cell adhesion as well as proteins such as thrombospondin-1 (TSP-1), alpha-1-antitrypsin fibronectin, acute phase proteins or proteins related to lipid metabolisms. Since cells that make up and populate musculoskeletal tissues, including chondrocytes are mechano-sensitive, in this varied molecular landscape, migratory cells such as MSCs and CPs might adhere and undergo physiological loading, thereby regulating their gene expression and eventually repairing the injured tissue; cells cannot express a physiological phenotype in an empty space. Therefore, after the intraarticular infiltration over the injured areas, a fibrin-scaffold formed "*in situ*" as an extracellular matrix serves as a highway for mechanical energy to transit from the environment to the cell, thereby bridging cell-to-cell tissue transition, promoting multicellular assembly and providing mechanical support as well as endowing tissues with a suitable microenvironment for biological restoration [33,34]. Since they are autologous, bio-reabsorbable, bio-compatible, and free of leucocytes and red cells, PRGF scaffolds are the best tailored among all the tissue engineering materials.

Oral and maxillofacial surgery and implantology, skin ulcers, orthopaedic surgery and bone regeneration as well as repair of injured muscle and tendon are some of the fields in which the application of platelet-rich plasma has consistently demonstrated its safety and successful outcomes in restoring tissue functions [60-64]. Therefore, the platelet-rich plasma application to osteoarthritic joints is intended to trigger and mimic the biological process of tissue healing based primarily on the synergistic influence that growth factors may exert on the joint tissues as they do in articular cartilage development and homeostasis [22], namely, by arresting type II collagen cleavage, reversing the reactive chondrocytic phenotype thereby regaining a healthier phenotype, and repairing articular cartilage [29,44,57,65,66].

Table 1. Primary platelet and plasma contents and their biological functions in tissue regeneration [28,46].

Category	Name or acronym of the molecule	Biological function
Adhesive proteins	VWF + pro-peptide, Fibrinogen (Fg), Fibronectin (Fn), Vitronectin (Vn), Thrombospondin-1,-2 (TSP-1, -2), laminin-8	Cell contact interaction, extracellular matrix composition
Proteases and anti-proteases	Tissue inhibitor of metalloprotease 1-4 (TIMPs 1-4), metalloprotease-1,-2,-4,-9 (MMP-1,-2,-4,-9), ADAMTS13, ADAMS10,17, serpin proteinase inhibitor, platelet inhibitor of FIX, C1 inhibitor, α 1-antitrypsin	Angiogenesis, vascular modelling, regulation of cellular behaviour
Growth and mitogenic factors	Platelet-derived growth factor (PDGF), Transforming growth factor β1and β2 (TGF β1, β2), Epidermal growth factor (EGF), Insulin-like growth factor 1 (IGF-1), Vascular endothelial growth factor A and C (VEGF A, C), Basic fibroblastic growth factor (FGF-2), Hepatocyte growth factor (HGF), Bone morphogenetic protein -2,-4,-6 (BMP-2,-4,-6), CTGF, SCUBE1, IGFBP3	Chemotaxis, cell proliferation and differentiation, angiogenesis
Chemokines, cytokines and others	RANTES, IL-8, MIP-α, ENA-78, MIP-2, MCP-1, MCP-3, SDF-1α, PF4, β-TG, pro-platelet basic protein (PBP), NAP-2, connective-tissue-activating peptide III T, angiopoietin-1, High mobility group box 1 (HMGB1), IL-6sR, endostatin, osteonectin, bonesialoprotein, osteoprotegerin	Regulation of angiogenesis, chemotaxis, vascular modelling, cellular interaction, bone formation
Membrane glycoproteins	alphaIIbbeta 3 (αIIbβ3), alphavbeta3 (αvβ3) PECAM-1, most plasma membrane constituent, receptors for primary agonists, CD63, CD40L, tissue factor, P-selectin, furin, GLUT3, semaphorin 4D, TLT-1, TNF-related apoptosis inducing ligand (TRAIL), syntaxin-2, SANP23	Platelet aggregation and adhesion, endocytosis of proteins, secretion, inflammation, thrombin generation, platelet-leucocyte and platelet–vascular cell interactions
Others	Content of dense granules: ATP/ADP, calcium, serotonin, histamine	Fibrin formation, capillary permeability, vascular local regulation

5. Growth factors and PRPs in cartilage repair

These biochemical modulators and regulators which are shared with developmental biological processes will be redeployed for tissue repair after injury [15,29].

Transforming growth factor-β superfamily (TGFβ) has been shown to play an anabolic role in cartilage repair. In particular, TGFβ1 the major growth factor within PRPs and one of the most important in cartilage regeneration, stimulates both chondrogenesis of synovial lining and bone marrow-derived MSC [67,68] and chondrocyte synthetic activity with matrix deposition [69]. Moreover, TGFβ1 counteracts the catabolic activity of IL-β1 including the degradation of type II collagen and proteoglycan produced by chondrocytes [70,71] and increases chondrocyte phenotype expression [72]. Insulin-like growth factor (IGF-1) is another component of PRPs with a potent anabolic effect on articular cartilage metabolism and its presence is required to maintain the integrity of articular cartilage [73]. In addition to positive influence of IGF-1 on the repair of extensive areas of damaged cartilage and protection of the synovial membrane from chronic inflammation [57], IGF-1 is, together with PDGF, a potent chemotactic factor for chondrocytes, which stimulates synthesis of extracellular matrix in human OA but does not avoid the matrix catabolism [74]. Moreover, its presence in cartilage enhances the effect of other growth factors present in articular cartilage [75].

PRPs application to cartilage repair is underpinned by a substantial body of evidence in basic science, as well as in preclinical and clinical levels of practice. *In vitro*, treatment of mature porcine chondrocytes with L-PRP releasate stimulates cell proliferation, and glycosaminoglycan and collagen synthesis [58]. The presence of PRGF releasate without leucocytes on human osteoarthritic synoviocyte cultures enhances the synthesis of hyaluronic acid (HA) and HGF compared to synoviocytes cultured on a platelet-poor medium. Moreover, the enhanced secretion of HA and HGF by PRGF was maintained despite the fact that synoviocytes were treated with interleukin-1β [59,76]. In one proteomic study conducted on human osteoarthritic chondrocytes cultured with different mediums, the PRP-enriched medium showed to be more efficient than other mediums at increasing cell proliferation and reverting and restoring the pattern of gene expression determined in a normal chondrocyte phenotype without undergoing hypertrophy [77,78]. Bendinelli *et al.* have reported an important HGF-mediated anti-inflammatory and anabolic effect of platelet-rich plasma on immortalized chondrocytes lineage by attenuating or reducing the transactivating activity

of NF-κB [78], a proposal that has been reinforced by the results obtained in osteoarthritic chondrocytes by Van Buul *et al.* [79]. In addition, PRP decreased the expression of COX2 and CXCR4 target genes, whose products might be involved in controlling chemotaxis of inflammatory cells such as monocytes thereby reducing local inflammation [78]. Wu *et al.* [80] have shown, using a 3D *in vitro* model, that the combination of PRP with a collagen matrix (with immortalized human chondrocytes) recovered type II collagen and proteoglycan synthesis which had been inhibited by 3 days of treatment with IL-1β+TNF-α, thereby illustrating the protective efficacy of PRP on chondrogenic-specific gene expression such as Col lI and AGN [80]. In another recent study, Anitua *et al.* determined that synovial fibroblast culture incubated with plasma rich in growth factors (Endoret) + HA induced a greater increment in synovial cell migration compared with the response to HA alone [81].

Furthermore, drawing on the aforementioned evidence, some *in vivo* studies have used PRP in an attempt to restore local hyaline cartilage injuries. When PRP liquid was loaded in microporous poly-lactic-glycolic acid scaffolds and applied on large osteochondral defects in a rabbit model, the neochondrogenesis induced showed chondrocyte-like cell and a high ECM synthesis and the defects were totally filled with a repair tissue similar to hyaline cartilage, compared with the control that showed a fibrous tissue repair [82]. The preventive effect of PRP infiltrations delivered in gelatin hydrogel microspheres in a rabbit model has been reported, showing a suppression of histomorphological signs of the OA progression compared with microspheres containing PPP [83]. Therefore, it has been suggested that the treatment of OA might be carried out using a combination of growth factors [29,57,65] in an attempt to redress the extracellular matrix through the cells behaviour.

6. Conclusions

There is increasing recognition and evidence of a molecular crosstalk between cartilage and subchondral bone which might be harnessed by growth factors delivered from PRPs, thereby counteracting the influence of catabolic gene expression of immature or deregulated chondrocytes on the extracellular matrix, triggered and maintained by mechanical stress. PRGF-Endoret might influence an anabolic microenvironment, containing the right combination of chemical cues, which is conducive to maintaining the homeostatic state of the joints tissues, reducing pain and improving the joint motion, structure, and function.

7. Expert opinion

The potential of endogenous regenerative technology (Endoret) for *in situ* regenerative medicine has yielded positive and promising clinical–surgical outcomes in musculoskeletal system pathologies [34,61,84]. This autologous and biological therapy to cartilage repair is underpinned by a substantial body of evidence in basic science [58,76,80] as well as in preclinical [82,83] and clinical levels of practice [66,85-91].

The successive intraarticular injections of platelet-rich plasma in the knee joint of osteoarthritic patients have shown significantly higher reductions in knee pain and stiffness and improvement in physical function, even compared with hyaluronic acid (HA) [64,82,87] although this product has not yet been proven to really modify the overall histology or molecular composition of OA cartilage. In these clinical trials, the surrogate marker for OA amelioration was the pain. The trials did not evaluate the influence of Endoret on histological and molecular make-up of osteoarthritic cartilage. Although PRPs open a new disease-modifying OA therapy, we acknowledge that this biological approach may only play a part, albeit, a key part in solving this condition. We must not lose sight of the fact that physical rehabilitation as well as other systemic factors such as nutritional deficiencies can affect the joint vulnerability [1]. Healing does not mean "regenerating", and repairing does not mean "recovering the function".

These clinical outcomes have demonstrated that Endoret use is safe as well as efficacious. Taking into account the overall results in basic science, in preclinical and in osteoarthritic patients, we are led to suggest four synergetic effects on the osteoarthritic context (**Figure 2**). First, there is a chondroprotective effect of the synovial joint due to both the hyaluronic acid secretion by synoviocytes [76] and the arresting of type II collagen cleavage by the combination of TGFβ and FGF [29] which contribute to the homeostasis of the articular cartilage. Second, we see an anti-inflammatory effect on human chondrocytes on the basis of the HGF effect both present in PRP and secreted by the synoviocytes [64] inhibiting the intracellular signalling regulator of the inflammatory and stress-induced response [41] pathway NF-κβ [78,79]. Moreover, PRP up-regulates chondrogenic-specific genes and down-regulates the expression of inflammatory molecules on immortalized human articular chondrocyte cell hPi [80]. Third, there is a cell-phenotypic modulation of both chondrocytes which prevent hypertrophic differentiation and maintain them in an arrested state [28-30] and of MSCs and CPCs which promote chondrogenic differentiation once they have migrated from vascular areas (synovium and subchondral bone) [12,17,35,36] towards injured areas under the action of PRP [80], GFs such as TGF-β and IGFs [71,92,93] or FGF-2 [94]. Fourth, by attenuating and reducing the joint's pain [64,86-88,90,95] the physical activity level might improve and increase the physiological load tolerable for the joints. The increased tolerable physical load might entail a chondroprotective effect since it has been proved that moderate mechanical loading [14,48] has an anticatabolic effect on the articular cartilage through either the action of CITED2 [96] or by suppressing NF-κβ activation and, in this manner, it may mediate the anti-inflammatory effect of moderate joint motion [14,48,97]. But not all PRPs are the same, and in a clinical trial conducted by Filardo *et al.* [98] which compared the efficacy and safety of intraarticular injections of Endoret

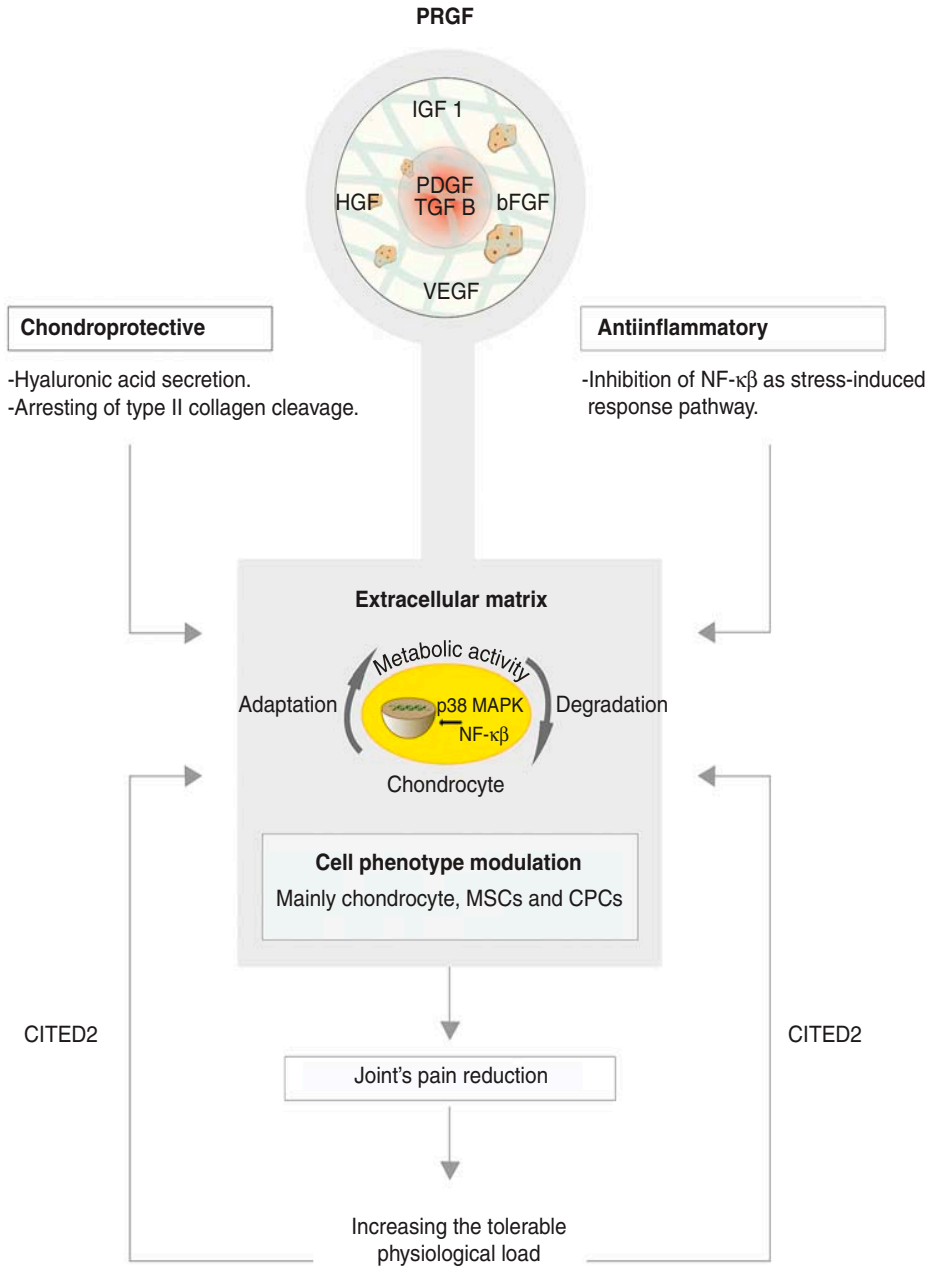

Figure 2. The overall outcomes in basic science, preclinical, and clinical studies suggest four synergetic effects of PRP application on the osteoarthritic joint. By modulating gene expression and gene products, PRP may well influence cells behaviour which are conducive to maintaining the homeostatic state of the joint's tissues thereby reducing pain and improving joint function and motion.

against a leucocyte-PRP in the treatment of OA, patients treated with Endoret had fewer side effects than those treated with leucocyte-PRP whose patients presented more pain and swelling events.

The aforementioned observations emphasize the important role that both growth factors and autologous platelets and plasma products play by providing a storm of signalling factors which are biologically active soluble metabolites, and by regulating a vast range of cellular behaviours both in osteoarthritis and in the articular cartilage repair process. By modulating gene expression and gene products of cells such as chondrocytes, synoviocytes, macrophages, mesenchymal stem cells as well as their cell cycles through epigenetic mechanisms [12,28,80] PRP might influence an anabolic microenvironment, containing the right combination of physical as well as chemical cues, which is conducive to maintaining the homeostatic state of the joint tissues, reducing pain and improving the joint motion, structure, and function.

Platelet-rich plasma therapy draws on the autologous biological system of growth factors and fibrin whose effects on different joint cells and their microenvironments are promising. The biological approach with the application of Endoret

on osteoarthritic joints results in reducing joint pain and improving joint function by restoring tissue homeostasis as indicated by the chondroprotective, anti-inflammatory, and cell-phenotypic modulation effect on joint tissues.

However, there remain some mechanistic and dosage aspects that must be elucidated in order to determine, harness, and optimize the therapeutic potential of platelet-rich plasma products. Although somewhat controversial, one differential element among the various biological compositions of PRPs that might clearly alter its healing potentiality is the leucocyte concentration. Several unanswered questions remain, such as how many infiltrations would be ideal in a first approach, the interval between them, and whether there should be a 1-year anniversary repetition of infiltrations. Due to the heterogeneous composition of PRPs, stemmed from the myriad of methods to obtain them as well as from individual variability, it is difficult to ascertain general guidelines in order to optimize them. We have to acknowledge that we are only in the dawn of biological therapies and PRP products are just in their infancy. The fact that such endogenous PRP therapy acts on a variety of tissues which can be seen as biological systems or networks themselves should not be seen as an absence of accuracy, like a scatter shot, simply because most of the proteins in platelet-rich plasma exert a broad regulatory and pleiotropic function. Indeed, it is only in rather exceptional cases that a specific physiological function can unconditionally and unambiguously be assigned to a given protein as a discrete entity [99]. There seems to be no specific biological factor for each specific cellular function. There are simply biological factors which, in a particular tissue environment, and acting together, induce the expression of cell phenotypes with different cell behaviours [100]. In recognition of the emerging current view of the bone-cartilage as a biological unit [16,17,19,35] mentioned previously in this paper, the articular cartilage should be regarded as only part of the target in the OA treatment. Therefore, in the coming years attempts to harness subchondral bone's source of migratory cell such as MSCs and CPCs [10,12,35,36] and of signalling factors (growth factors and cytokines) must include the subchondral bone as an additional target in the OA treatment with Endoret.

In the light of basic science and clinical studies, we may state that the application of Endoret, in addition to being safe, has been shown to be clinically efficacious in OA treatment although many interesting challenges remain. As knowledge about the regenerative effect of growth factors is growing, their application is being extended, and new challenges arise. We are only at the beginning of a new era in which we must optimize this procedure at the same time we continue drawing on its healing power.

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G Orive and S Padilla designed the proposal. S.P. drafted the first version of the article. E Anitua, M Sánchez and G Orive revised and edited the final version. All authors have reviewed and approved the manuscript.

Declaration of interest

E Anitua, G Orive and S Padilla are scientists at BTI Biotechnology Institute, a biotech company that has developed the technology of plasma rich in growth factors. The remaining authors have no competing interests to declare. No funding bodies had any role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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REVIEW

A new strategy to tackle severe knee osteoarthritis: Combination of intra-articular and intraosseous injections of Platelet Rich Plasma

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ABSTRACT

Introduction: Knee osteoarthritis (KOA) is a mechanically induced, cytokine and enzyme-mediated disorder involving all the joint tissue of the knee. Rebuilding a physiological-homeostatic network at the tissue level following knee organ failure, such as in severe KOA, is a daunting task with therapeutic targets encompassing the articular cartilage, synovium and subchondral bone. Intraarticular infiltration of plasma rich in growth factors (PRP) has emerged as a promising symptomatic approach, although it is insufficient to reach the subchondral bone.

Areas covered: This review addresses current molecular and cellular data in joint homeostasis and osteoarthritis pathophysiology. In particular, it focuses on changes that subchondral bone undergoes in knee osteoarthritis and evaluates recent observations on the crosstalk among articular cartilage, subchondral bone and synovial membrane. In addition, we review some mechanistic aspects that have been proposed and provide the rationale for using PRP intraosseously in KOA.

Expert opinion: The knee joint is a paradigm of autonomy and connectedness of its anatomical structures and tissues from which it is made. We propose an innovative approach to the treatment of severe knee osteoarthritis consisting of a combination of intraarticular and intraosseous infiltrations of PRP, which might offer a new therapeutic tool in KOA therapy.

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1. Introduction

Knee osteoarthritis (KOA) is a mechanically induced, cytokine and enzyme-mediated disorder with different biochemical, inflammatory, and genetic signatures undergoing distinct phases and phenotypes, and encompassing all joint tissues, with pain and inflammation as the clinical and biochemical hallmarks of the disease.[1–3] This complex mechanical organ includes articular cartilage (AC), an avascular hydrated tissue functionally sandwiched between two highly innervated and vascularized tissues, namely, synovial membrane (SM), which produces synovial fluid (SF), and subchondral bone (SB), ligaments, capsule and periarticular muscles (PM).[4] Intraarticular joint tissues are endowed with very distinct load-bearing cellular responses, which are responsible for the organization of their specific extracellular matrix (ECM), which account for the bulk mechanical properties of the tissues in order to transfer, absorb and dissipate the mechanical forces among them in a frictionless and pain-free movement.[4,5]

Subchondral bone has always been present in the equation of the cartilage repair process and osteoarthritis (OA) [6–8] but it has suffered neglect for decades as an important player in the etiopathogenesis of OA.[8,9] There is an increasingly recognized communication between the subchondral bone and articular cartilage based on the changes that the subchondral bone undergoes in patients with severe OA, including microcracks and structural defects, vascularization of channels, nerve growth and a progressive replacement of the subchondral marrow with fibroneurovascular mesenchymal tissue.[10–12] As it is yet to be established precisely which of the joint tissues or structures is the primary driver of KOA, and therapeutic strategies targeting solely one cell or tissue target may well prove to fail [13], it is advisable that approaches to KOA treatment should be aimed at reaching several joint tissues with the objective of reducing joint inflammation, controlling pain, improving joint functionality and restoring the homeostasis of joint tissues.

A biologically inspired therapeutic approach consisting in intraarticular infiltrations of PRP has proven to substantially reduce pain in patients with KOA [14,15] and to improve joint stiffness and physical function.[16] Unlike a single growth-factor-delivered therapeutic strategy in a bolus manner, PRP conveys many bioactive mediators within an autologous fibrin network released gradually, which have been shown to exert

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Article highlights.

- Knee osteoarthritis is a mechanically induced, cytokine- and enzyme-mediated cluster of disorders affecting the whole joint.
- There is an intense molecular and cellular crosstalk among AC, SB, and SM in KOA, which establishes a catabolic loop.
- Any attempt to treat KOA should address the articular cartilage, the synovial membrane, the synovial fluid and subchondral bone as therapeutic targets.
- Platelet rich plasma is a multimolecular and safe therapy, and its clinical benefits might be attributed to trophic-anabolic, antiinflammatory and analgesic effects.
- Intraosseous infiltrations of PRP modulate SB homeostasis by antioxidative stress protection, adipogenesis suppression and improvement in bone mineralization effect.
- The combination of intraarticular and intraosseous injections of PRP might offer a new therapeutic tool to address the knee joint pathology as a whole, by reaching the SM, SF and superficial zone of AC by intraarticular injections, and the deep zones of AC, and SB through PRP intraosseous infiltrations.

This box summarizes key points contained in the article.

positive effects on reestablishing homeostasis of joint tissues through a breadth of actions such as antiinflammatory, immunomodulatory and antioxidative effects [17–24], an analgesic effect [14–16,25], and finally chondroprotective and anabolic-trophic effects.[26–29]

This review will explore some of the recent insights and observations concerning the involvement of subchondral bone in the pathophysiology of osteoarthritis and additionally will highlights the increasing understanding of knee joint homeostasis and the role that PRP therapy could play in the disease-modifying osteoarthritis treatment of the knee.

2. Joint tissue responses to mechanical stimuli: homeostasis, adaptation and inflammation

2.1. Joint homeostasis and mechanical stress

At a biomechanical level, knee components work as a network from which the joint´s functional property as an organ emerges, a property known as dynamic stability, whose equivalent at the tissular and cellular level is termed tissue and cell homeostasis. Such identities do not imply biological constancy but rather dynamic adaptability.[30] The phenotype of chondrocytes, synoviocytes, and osteoblasts is constantly adapting to its dependence on the biochemical, biophysical and mechanical loading features of their microenvironment. [31–34] Signals and ligands from extracellular matrix (ECM) drive cell responses and tightly fine tune the anabolic/catabolic balance in order to maintain or to adapt their ECM composition to the ongoing mechanical challenges,[31] thereby protecting against the deleterious effect of some supraphysiological stimuli.[35] Abnormal mechanical stress and/or biochemical mediators variously stemming from trauma, obesity, lesion or disfunction of knee components, as well as metabolic diseases break knee dynamic stability and trigger biological responses that disrupt the homeostasis of cells and tissues of the joint in a locally, sustained, low-grade inflammatory fashion leading to a matrix degradation (Figure 1).[2,36,37]

In the wake of this sterile matrix degradation of articular cartilage, there is a depletion of aggrecans and cleavage of collagen II, which leads to the erosion of cartilage, subsequently altering the nanostiffness of articular cartilage and weakening its load-bearing capacity.[4,38] Besides the release of matrix-degrading products, the ECM degradation deeply impacts the micromechanical environment of chondrocytes and changes the magnitude of dynamic compressive forces transferred from them to the underlying bone, and these aberrant new sustained (chronic) abnormal forces prompt chondrocytes and osteoblasts to respond with a pro-inflammatory gene expression through activation of the NFkB signaling pathway [32,43] and increased osteoclastogenesis, thereby increasing bone resorption and sclerosis [34,44] respectively. Nevertheless, evidence is accumulating about how alterations of subchondral bone induced by mechanical or vascular stresses might be the start point in the catabolic loop of AC degradation and extend to SM (Figure 1).[1,7,45,46] Cartilage is an avascular tissue whose cells rely on synovial fluid and subchondral plate to obtain oxygen and a supply of nutrients, having the subchondral bone account source for at least 50% of articular cartilage requirements in oxygen and glucose.[46,47]

Therefore, despite the fact that tracking down the 'first pathogenic event' responsible for the initiation of KOA still proves an elusive quest, any induced mechanical or metabolic damage to joint tissues in combination with predetermined influences such as genetic, obesity and aging, paves the way to initiating a harmful joint environment involving AC, SM and SCB, and then it is difficult to establish who was first.[8]

2.2. Synovial membrane and subchondral bone in cartilage homeostasis

In recent years, a great deal of evidence has been accumulating in favour of seeing as decisive, the contribution of synovitis and subchondral bone on articular cartilage degradation, and on the progression of OA, where AC may after all be the victim, and not the culprit of catabolic inflammatory cytokines stemming from synovial membrane and subchondral bone, and triggered by abnormal mechanical stresses.[3,4,41,42,48] Hence, cartilage integrity is highly dependent on the underlying subchondral bed and vice versa, as well as on a healthy synovium and its product the synovial fluid.[7,49]

Evidence in basic science, preclinical and clinical settings has been mounting for the role of synovium inflammation in the pathogenesis and progression of OA.[2,3] Matrix-degradation products such as fibronectin, tenascin C, high-mobility group protein B1 (HMGB1) and low molecular-weight hyaluronic acid (LWHA) among others in the SF [37,42] can act as Toll-like receptor (TLR) ligands or damage-associated molecular patterns (DAMPs) and activate TLR-2 and TLR-4 of synovial macrophages and fibroblasts, chondrocytes and osteoblasts, leading to the activation of the intracellular signaling pathway nuclear factor kappa B (NFkB) (Figure 1).[3,50] The activation of the NFkB signaling pathway mediates the expression of several inflammatory genes and the synthesis of interleukin 1beta (IL-1B), interleukin 6 (IL-6), interleukin 10 (IL-10), nitric oxide (NO), prostaglandine E2 (PGE2), tumor necrosis factor alpha

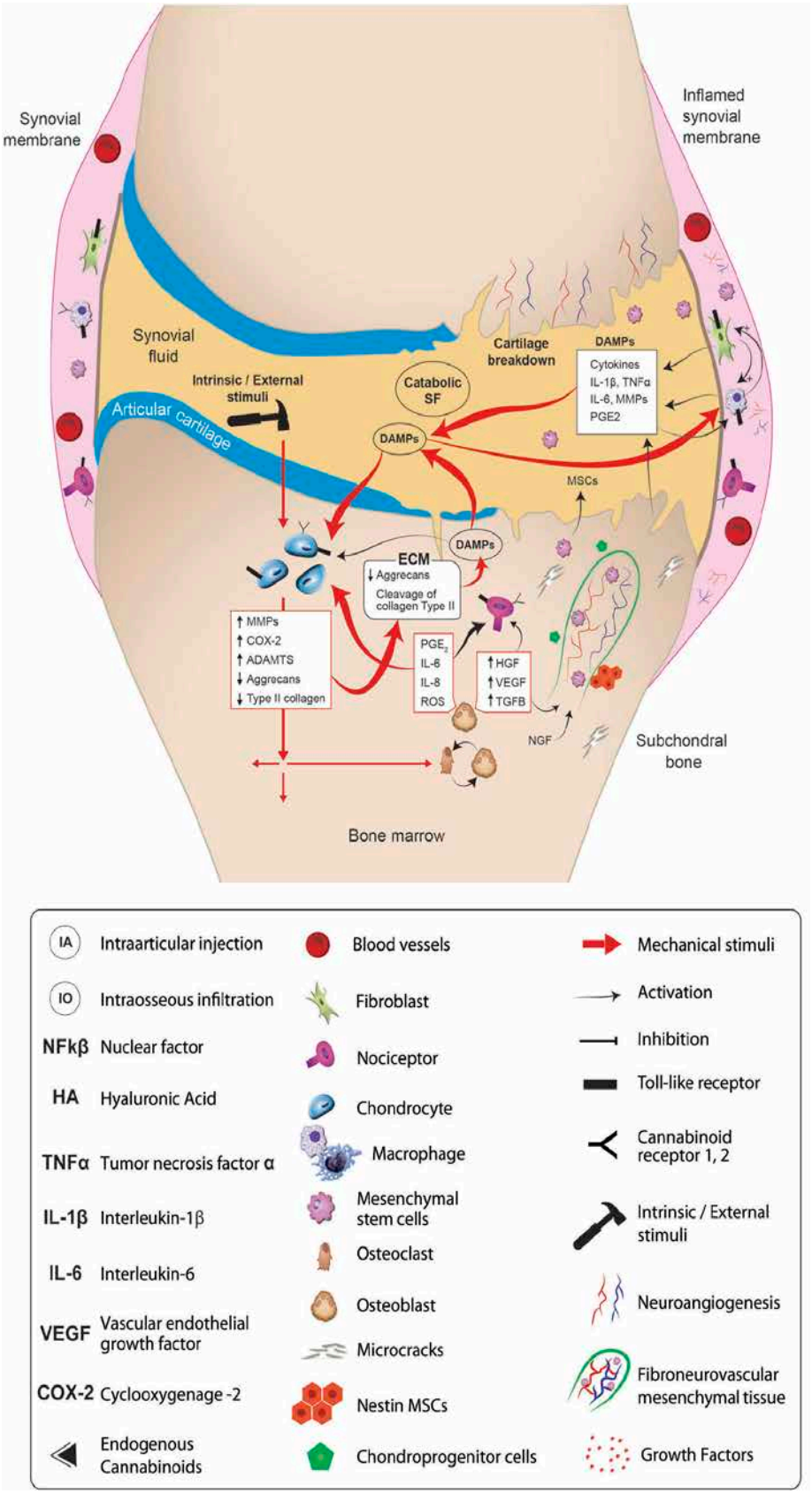

Figure 1. Abnormal distribution of mechanical loading across joint cartilage breaks the homeostasis of articular cartilage and provokes adaptive or catabolic cell responses, which leads to an increased synthesis of matrix metalloproteinases (MMPs) and aggrecanases (ADAMTS), expression of proinflammatory cytokines and mediators such as interleukin-1B (IL-1B) and cyclooxygenase-2 (COX-2), high levels of reactive oxygen species (ROS), disruption of water tissue distribution and matrix fragments.[4,38–40] Proinflammatory cytokines involved in OA, such as IL-1B and TNF-a are major players in the destruction of AC by inhibiting the synthesis of aggrecans and collagen type II while at the same time stimulating the synthesis of MMPs in chondrocytes.[41] It has been reported that activation of TLRs of synovial macrophages and fibroblasts, and monocytes by DAMPs present in an inflammatory SF, is an important pathway in promoting synovitis in OA through the NFkB pathway [3], cells that respond with the production of MMP-1, MMP-3, and MMP13, IL-1B, TNFa and IL-6 among other catabolic mediators, promoting synovitis in OA.[3,41,42]

(TNF-a), interferon gama (IFN-j) and nerve growth factor (NGF) among other inflammatory cytokines (Figure 1).[3,39,41,50,51] Moreover, NFkB transcription factor has been postulated as a functional connection among the mechanobiological, developmental programming and stress-inflammatory responses of AC, SM and SB, making the NFkB signaling pathway a potential multi-faceted target in OA disease.[13,32,50] Another pathway involved in OA synovitis is the activation of complement as it has been shown by Wang et al. [52] who reported that the expression and activation of complement is abnormally high in the human OA joint, where the presence of some products of dysregulated cartilage remodeling such as fibromodulin, cartilage oligomeric matrix protein (COMP), and osteoadherin in synovial fluid and membranes might account for this activation.[3]

Important clinical features of the inflamed synovium (synovitis) are pain, swelling and stiffness,[42] whereas histopathological changes are characterized by an uneven, abnormal cell infiltration and an aberrant proliferation of macrophages, fibroblasts, and blood and lymphatic endothelial cells that lead to a neofibroangiogenesis.[42] SM and SB are highly vascularized and innervated tissues endowed with heat receptors, chemoreceptors and mechanoreceptors from where nociceptive stimuli, coming from a microenvironment undergoing non-physiological mechanical loading and/or pro-inflammatory cytokines and damage-associated molecular patterns (DAMPs), might initially lead to peripheral and eventually both peripheral and neuropathic pain by mechanisms yet to be fully identified.[3,53] In addition, proinflammatory cytokines may contribute to pain by stimulating hyperalgesia and sensitizing joint nociceptors to other stimuli [3,42] thereby perpetuating a catabolic vicious circle among SM, AC and SB.

2.3. Joint inflammation and mesenchymal stem cells

Aggression and inflammation to AC, SM, menisci and ligaments has been reported to bring about an increase of MSCs in SF,[54,55] which is commonly interpreted as a tissue response to injury [56,57] equivalent to the response of migratory chondrogenic progenitor cells from SB to injured cartilage.[58,59] Moreover, several studies have reported that the accumulation of SF MSCs increases with the severity of osteoarthritis, joint damage and the disease duration.[55,60,61] Healthy human and osteoarthritic cartilage and SF contain a population of cells with characteristics of mesenchymal progenitor cells [56,62] with migratory and chondrogenic potential.[56,58] According to these observations, endogenous mesenchymal stem cells have been postulated as a reservoir of repair cells and immunomodulatory drugstore cells to dampen inflammation.[63] Although the source of MSC increase has yet to be determined, the most likely origin may be the SM, [55,56] the breakdown zone of superficial AC,[62] and the SB.[10,12,58,59] However, the SB origin of SF MSCs is less likely to occur for as some authors have suggested, the marrow of patients with severe OA is almost depleted in MSCs and the remaining MSCs are functionally deficient.[60]

Bone, like cartilage, responds to mechanical stress in an intensity-dependent manner and a tight regulation between the sequential processes of deposition and resorption at the same site. These processes are carried out by the coupling of osteoblast and osteoclast-metabolic activities [43] and unlike cartilage, when damaged regenerates spontaneously due mainly to its high elevated vascular and cellular network. Evidence is accumulating not only about the involvement of bone, and more particularly SB in the development and progression in OA but also about how these SB changes might even precede changes in AC of OA joints.[7,8,12,33,64]

3. The role of SB in pathophysiology and clinical symptoms of osteoarthritis

3.1. The subchondral bone-articular cartilage functional unit

Subchondral bone has always been present in the equation of OA pathogenesis and more than 40 years ago, partially inspired by the 1827 proposal by surgeon Dr. P.P. Physick on the SB as an effective shock absorber. Radin et al. [7,65] suggested a cause-effect connection among mechanical loading, subchondral bone sclerosis and osteoarthritis. Subchondral bone is the layer of bone which lies immediately below the calcified cartilage (Figure 2),[66] and consists of two different anatomical entities, one called subchondral or cortical plate which is nonporous and poorly vascularized cortical bone, and the SB which contains bone marrow (fatty) and trabecular bone.[47,67] Together with the AC, it forms the osteochondral functional unit, which undergoes mechanical stresses that trigger adaptive cell responses and establish a crosstalk among them to adjust their architecture to ongoing physical and biochemical challenges.[12,68] In the functionality of the osteochondral unit, articular cartilage provides an elastic, gliding, smooth frictionless surface, while subchondral bone, a very low viscoelastic structure, together with periarticular muscles and ligaments, acts as shock absorber structures, accounting for 30 and 50% of the total absorbing energy and only 1–3% for the AC.[4,47] Besides the pivotal shock absorbing function, SB is a source of vessels whose perfusion rate enables an important nutritional route for AC but any damage to this microvasculature affects venous bony circulation thereby altering cartilage and chondrocyte function.[10,46,47]

3.2. SB turnover and structural changes in OA

The osteochondral unit in an OA joint undergoes several structural changes including loss of articular cartilage, development of inflamed synovium, calcified cartilage thickening and tidemark duplication, undermineralization of bone, sclerosis and stiffness of SB, bone marrow lesions (BMLs), cysts, osteophyte, and a localized bone marrow replacement by fibroneurovascular tissue.[10–12,33]

Despite the high turnover of SB in OA, an uncoupling between bone formation and resorption at the same site leads to an increase in bone volume without a concomitant increase in bone mineralization pattern.[8,33,43] This SCB sclerosis is characterized by an increase of the osteoid volume and a decrease of calcium bind to collagen fiber, and is associated with a gain of trabecular thickness, loss of trabecular number, and a trabecular

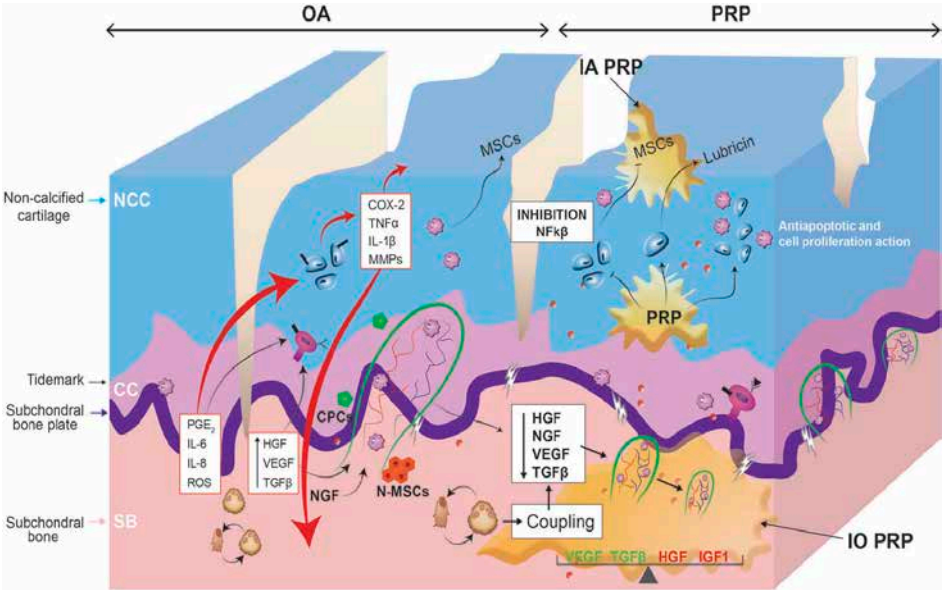

Figure 2. SB. Targeting the osteoarthritic subchondral bone with Intraosseous infiltration of PRP. This schematic drawing illustrates the outside-in (AC-SB) and inside-out (SB-AC) flow of mediators and cells. SB as a point of egress of morphogens and cells, through the channels and vessels breaching the osteochondral junction, partially recruited by the osteoarthritic synovial fluid.[8,12] This cartilage cell invasion might be facilitated by the loss of aggrecans, collagen II cleavage, and disruption of water tissue distribution [38] of the articular cartilage as well as by the secretion by MSCs of fibrinolytic enzymes.[66] The excessive presence of TGFB1 and VEGF in OA subchondral bone [8,12,69] could be a driving factor for changes in osteoblast-osteoclast coupling thereby leading to a bone remodeling imbalance.[8,64,70] NGF expression,[71] and fibroneurovascular growth changes that additionally might well contribute to overlying cartilage degradation, [64,69] pain [12,67,68] and an osteoarthritic joint.[64,69]

network more separated and less interconnected.[43,64] It has been suggested that sclerotic subchondral bone, localized at subchondral plate, could decrease the load transfer to the underlying bone tissue leading to osteoporotic-like changes.[10] Moreover, SB can undergo microdamage, such as microcracks and clefts, that modify SB stiffness and reduce the shock-absorbing capacity of SB, thereby chronifying a microdamage context and perpetuating an accelerated bone remodeling, which impairs normal mineralization of bone once it has been deposited, most likely by a modified osteoblastic phenotype.[10,67,72] Magnetic resonance imaging (MRI) has helped to detect subchondral bone marrow edema-like lesions (BMLs), which have been found to be associated with pain and disease progression in KOA,[73] and together with bone attrition, are strong indicators of a structural deterioration in KOA.[10] Several studies conducted in human knee and hip OA paralleling MRI bone marrow edema lesion studies with histological analysis of SB retrieved at the time of joint replacement, revealed microfractures and increased bone remodeling, subchondral ingrowth of fibrovascular tissue and increased vascularity, as well as various types of bone marrow fibrosis.[73–75] These observations were confirmed in rodent models of OA.[12,76] The increased activity of osteoclasts in OA cause channels to extend from SB to AC, passing across the calcified tissues into the noncalcified articular cartilage.[68] The neurovascular invasion of those newly formed channels is accompanied by a new fibroneurovascular mesenchymal tissue within the channel along with cells such as macrophages, osteoclasts, osteoblasts and endothelial cells, which interact to stimulate angiogenesis and growth of sympathetic and sensory nerves [12] and reach the noncalcified cartilage, a finding which has been supported by animal models of OA (Figure 1).[12]

3.3. Cellular interactions and molecular crosstalk in osteochondral unit in OA

There is now good evidence that even in a non-diseased joint, naturally occurring pores and holes enable communication between SB and AC through diffusion of small molecules. [11,70,77] This communication may be exacerbated by structural changes seen early in the osteochondral unit in OA. The increased osteoclastic activity in the OA subchondral plate [33] may increase the permeability of bone–cartilage interface by inducing channel formation in the tidemark, in addition to the existent aberrant fibroneurovascular tissue and vasculature, and mechanical stress-induced microcracks.[12,67,78] Reinforcing this view, Pan et al. [77] have demonstrated the diffusion of small-size molecules between SB and AC by utilizing the FLIP method (Imaging method based on fluorescence loss, which quantifies diffusivity of small molecules) with sodium fluorescein in the distal femur of mice, and this communication is greatly increased in osteoarthritic joints of the mice model.[11] Therefore, the presence of these connections enables an elevated crosstalk among chondrocytes, osteoblasts, osteoclasts and MSCs through biological factors and signaling pathways.

Several *in vitro* and *in vivo* studies have demonstrated that osteoblasts from sclerotic subchondral bone show an altered phenotype. In an *in vitro* study, Westacott et al. [79] reported that osteoblasts in OA-affected bone exhibited a different phenotype, whose activity can degrade articular cartilage *in vitro*. Supporting this observation,[80] Hilial et al. reported that osteoblasts from OA subchondral bone have an abnormal metabolism with increased levels of PGE2 and TGFβ (Figure 1 and 2). Using a co-culture model of OA subchondral bone osteoblasts with

chondrocytes, Sanchez et al. reported that osteoblasts induced a catabolic response of chondrocytes including a decrease in aggrecan, type II collagen and SOX-9, and an increase of MMP-3 and MMP-13 among other mediators.[81,82] Moreover, osteoblasts from scletoric subcondral bone have an elevated TGFβ expression [43] and under cyclical compression express proangiogenic factors such as VEGF, FGF and IL-8.[34] Hepatocyte growth factor (HGF) is a pleiotropic morphogen present in articular cartilage but produced by osteoarthritic subchondral bone osteoblats, osteoclasts and MSCs,[69,83,84] with likely implications in both the chondrocyte anabolic state and the proliferation of an invasive fibroneurovascular tissue in SB,[10,12,69] the latter when an uncoupling osteoclast–osteoblast activity may lead to an overexpression of HGF (Figure 1 and 2).[83] The excessive presence of TGFB1 and VEGF in OA subchondral bone [12,71] could be a driving factor for changes in osteoblast–osteoclast coupling thereby leading to a bone remodeling imbalance,[10,64] NGF expression,[85] and fibroneurovascular growth changes that additionally might well contribute to overlying cartilage degradation,[64,71] pain [12,67,68] and an osteoarthritic joint.[64,71] In a recent study, Zhen et al. showed that by inhibiting TGF-β signaling in a specific population of MSCs present at the SB (Nestin positive MSCs), the severity of OA was reduced, a change associated with improvement of bone parameters, cartilage structure and joint function without affecting TGFB signaling in AC.[71] In fact, previous studies have shown that the decrease of MSCs in the synovial fluid, in low degree OA, suggests clinical improvement.[55] MSCs from osteoarthritic bone marrow have been reported to be substantially reduced in yield and proliferative activity besides showing a weakened chondrogenic and adipogenic activity and increased osteogenic activity.[60] However, *in vitro* studies indicate that the inclusion of growth factors, as a supplementary culture medium, can be beneficial in reverting their chondrogenic activity.[86]

4. Plasma rich in growth factors as an effective and safe therapeutic approach to treat OA

4.1. PRP as an emergent and promising knee osteoarthritis treatment

Despite important advances made in the development of treatments to reduce pain and inflammation, and in spite of endeavors to develop an efficacious and early disease and structure-modifying therapeutic intervention, the path to osteoarthritis treatment remains elusive. Among the emerging biologic interventions to target the clinical and biochemical hallmarks of OA, namely joint pain and inflammation, platelet rich plasma stands out.[87]

4.2. Platelet-rich plasma preparation and content

4.2.1. What is platelet-rich plasma?

Drawing on the regenerative potential of platelets, plasma biomolecules and fibrin matrix,[88] a plethora of systems to produce PRPs have been developed to enhance the natural regenerative capacity of damaged tissues.[89,90] Platelet rich plasma is an autologous platelet concentrate within a plasma suspension whose cell and plasma composition are determined by the method used to obtain it. PRP products include plasma and twofold or more increases in platelet concentrations above peripheral blood levels and the concentration of leukocytes and erythrocytes varies widely, from a complete absence to a high concentration of them.[89] There is a wide range of PRP products obtained by different blood-spinning preparation protocols (number of centrifugations and centrifugation speeds and time, the type of anticoagulant and platelet activation methods),[89,91,92] and consequently, the different biological effects that necessarily result, produce very distinct clinical outcomes, which produce a confusing picture of efficacy.

4.2.2. Plasma rich in growth factors (PRGF) preparation

PRGF, one of the multiple autologous platelets- and plasma-derived products, which is included in PRPs, is produced as follows. Briefly, peripheral venous blood from the patient is withdrawn into 9 ml tubes containing 3.8% (wt/vol) sodium citrate as anticoagulant. Blood is centrifuged at 580 *g* for 8 min at room temperature. The 2 ml plasma fraction located just above the sedimented red blood cells is collected in a tube without aspirating the buffy coat (F2). The remaining upper volume of plasma is deposited in another tube (F1). The activation of PRGF is carried out by adding calcium chloride (10% wt/vol).[14] Additionally, PRPs can be manufactured by using other standardized or commercial systems whose protocol heavily influences the composition of the final product (platelet concentration, the presence of leukocytes and erythrocytes, the level of platelets activation).[89,93]

4.3. Platelet-rich plasma rationale

Plasma rich in growth factors (PRP) consists of a pool of autologous growth factors (GFs) and other bioactive mediators stemmed from platelets and plasma. Once PRP is activated, plasma fibrinogen polymerizes into a three-dimensional transient fibrin scaffold, which contains heparan sulfate binding domains for growth factors (PDGF, FGF, HGF, BDGF, VEGF, IGF and TGF-B), cytokines (TNF-a, IL-2,3,4,5), chemokines (PF4), ECM components (Fibronectin, thrombospondin and tenascin), cell adhesion (L-selectin and N-CAM), acute phase proteins and proteins related to lipid metabolism.[94,95] By sequestering several growth factors, microparticles, and other biomolecules released from the degranulation of platelets and plasma [95–97] this biocompatible and biodegradable scaffold provides plastic-elastic stiffness and generates growth factor gradients that are essential cues for cell proliferation, differentiation, migration and correct orientation in the nascent tissue.[98] Once infiltrated into the joint and subchondral bone, this liquid-to-gel 3D injectable scaffold is converted into a matrix-like viscous and malleable structure, which adheres to SM, AC and SB and covers them (Sanchez et al. 2014; Figure 3).[99] When fibrinolysis begins, a gradual, sustained release of GFs and other biomolecules occurs, in contrast to a bolus delivery modality.[96,100] Such a gradual yet sustained release of GFs influence on cells, mimics the biological repair process,[96,97,100] which is the topic of a review published in this journal.[101]

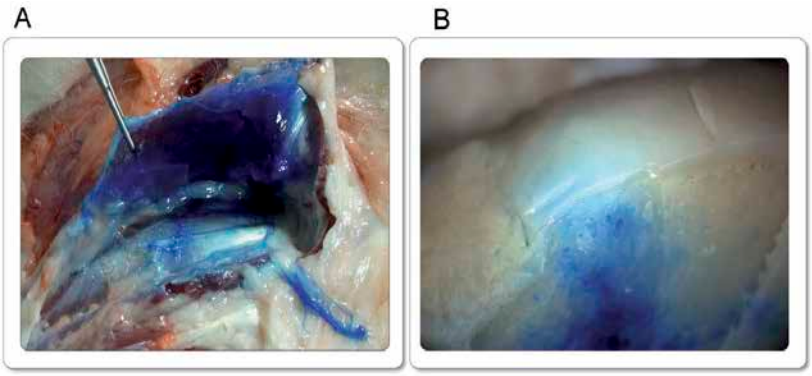

Figure 3. Infiltration of activated PRP previously stained with methylene blue was performed in sheep`s joint to ascertain its diffusion across the joint. Once the animals were put down and the joint opened, we infiltrated the femoral condyle as well and took these picture in which the PRP liquid-to-gel ·3D injectable scaffold was converted into a matrix-like viscous and malleable structure, which adhered to synovium (A) and covered it as it diffused across the condyle (B) (figure 2 unpublished data).

4.4. *Some pitfalls in the application of PRPs on tissue repair*

Despite the care and seriousness with which medical staff may elaborate and apply PRPs in different medical fields, the poor standardization, which mainly pivots around the controversial presence/absence of leukocytes, the modalities of application and the donor-related variabilities, are three elements that contribute to drawing misleading conclusions about the clinical efficacy of PRPs.[90,93,102] In a sterile inflammatory repair scenario such as musculoskeletal injuries including KOA, leukocytes may aggravate tissue damage and promote a proinflammatory microenvironment by releasing TNF-α, IL-6, IFN-γ cytokines, which then induce the over-expression of MMPs, elastase and cathepsin G, as well as reactive oxygen species among others, thereby breaking down the ECM and exacerbating the original lesion.[40,103] Several research groups have highlighted the detrimental effect that the presence of leukocytes within PRP may exert on synoviocytes, chondrocytes, human subchondral MSCs [93,104–106] as well as on clinical symptoms.[15] With regard to the modality of application, PRP cannot be considered a magic bullet applied as a kind of single scatter shot. Rather, a biological approach is most productive with distributed infiltrations: infiltrating several times and including healthy peripheral tissue which surrounds the injury, with the aim of recruiting, activating and mobilizing resident MSCs and influencing macrophages and endothelial cells as well. Finally, the Spanish Agency of Medical Devices (AEMPS) has defined PRP as a human-use medicinal product and framed PRP outside the category of advanced-therapy medicinal products. Therefore PRP therapy can be applied intraoperatively and on an outpatient basis.

There is no doubt that the challenges to fulfill the requirements of safety and efficacy are daunting, and these must be demonstrated by further clinical trials. Moreover, the heterogeneity of PRPs is hindering their regulation and several gaps will be filled in the coming years particularly regarding PRPs medical indications.[107] As the body of research about the regenerative effects of PRPs skyrockets, expansion of their applications is inevitable. Several unanswered questions remain, some regarding molecular mechanisms involved in the clinical benefits and others encompassing aspects of dosage, such as how many injections would be ideal, the interval between them and the suitability of combining PRP with stem cells to enhance the healing power of PRPs.

4.5. *Inflammation and oxidative stress*

In vitro and in vivo studies (Table I) have reported that PRP and GFs within it such as HGF, IGF-1, PDGF and TGFB, and platelet microparticles have proven to exert an immunomodulatory effect and promote an antiinflammatory environment. HGF and platelet microparticles have been reported to polarize macrophages from M1 to M2 phenotype. [20,108,109] IGF-I, PDGF, HG and PRP releasate modify the inflammatory status of chondrocytes by suppressing the NF-kβ signaling pathway [17–19] (Figure 2), which might lead to the decreased presence of IL-β, and TNF-∝ and other pro-inflammatory cytokines in synovial fluid [3,110,111] (Figure 4). Reinforcing this interpretation, Anitua et al. reported that LPS-treated osteoblasts and fibroblasts which had been cultured in the presence of releasates obtained from PRP without leukocytes, showed an increased expression of Ikβ∝, an antiinflammatory protein that anchors the transcription factor NFkβ to the cytoplasma and inhibits its activation, whereas releasates obtained from leukocyte-rich PRP induced a NFkβ activation.[112] In one recent study, Xie et al. [113] reported that PRP attenuated the multiple-cyclic tensile strain mediated MMPs, NO and PGE2 synthesis in chondrocytes, suggesting that PRP may protect chondrocytes from mechanically induced injury. Connective tissue factor (CTGF), one of the most abundant growth factors released by platelet activation [114] was reported to protect chondrocytes from age-related degenerative changes and from cellular stress, the latter mediated through NFkβ.[115] On the other hand, synovial fibroblasts from osteoarthritic patients cultured in 20% PRP supernatant produced a significant amount of HGF, even in the presence of IL-1β, which is known to inhibit the NFkβ on macrophages [20] and to mediate the antiinflammatory effects of PRP on fibroblasts.[57] In a recent work, Assirelli et al. [105] observed that L-PRP (leukocyte-rich PRP)-treated human synoviocytes sustained a long-term upregulation of IL-β, IL-8 and FGF-2, together with a down-regulation of HGF

Table 1. Summary of *in vitro* and *in vivo* effects of platelet-rich plasma and growth factors.

Cell type/animal model	Intervention	Outcome	Reference
Immortalized human chondrocytes	PRP releseate after thrombin and $CaCl_2$ activation and single centrifugation	Reduction of transactivating activity of NFκB, decreased COX-2 and CXCR4 expression	[17]
Human monocytic tumor cell line	PRP releseate after thrombin and $CaCl_2$ activation and single centrifugation	Decreased chemotaxis	[17]
Human osteoarthritic chondrocytes	10% PRP releseate after $CaCl_2$ activation	Decreased IL-1β-related inflammation, inhibition of NFκB activation	[18]
Primary canine chondrocytes	Medium supplemented with HGF and IGF-1	Inhibition of IL-1β-mediated activation of NFκB, decreased apoptosis in chondrocytes	[19]
Mouse bone marrow derived macrophages	Medium supplemented with HGF	Decreased IL-6 production, increased IL-10 production, reduction of transactivating activity of NFκB	[20]
Human osteoarthritic synoviocytes	Autologous conditioned plasma	Decreased TNF-α concentration, decreased MMP-13 expression, increased HAS-2 expression	[21]
Human osteoarthritic chondrocytes	Autologous conditioned plasma	Decreases TNF-α concentration, increased cartilage synthetic activity	[21]
Primary human osteoblast and osteoblast-like cell line	5% and 10% PRP releseate after activation and single centrifugation	Increased antioxidant response element activity, increased Nrf2 accumulation, increases VEGF gene expression	[22]
Human adipose-derived stromal cells	PRP releseate after thrombin activation	Increased cell proliferation, ALP activity and mineralization	[23]
Aged mouse bone marrow stem cells and adipose derived stem cells	PRP activated with bovine thrombin and single centrifugation	Increased cell proliferation, colony formation and osteogenesis, decreased adipogenesis, restoration cell senescence markers, resistance oxidative stress	[24]
Young-senescence-accelerated prone mouse strain (SAMP38) mice	PRP activated with bovine thrombin and single spin; injection into the tibia bone marrow	Delayed mice aging, improved survival and body weight, recovered cellular potential of stem cells	[24]
Human keratinocyte cell line	PRP releseate after freeze-thaw cycle activation and single centrifugation	Increased endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) production	[25]
Mouse model of acute inflammatory pain induced	PRP releseate after freeze-thaw cycle activation and single centrifugation	Reduced nociceptive behavior	[25]
Immortalized human chondrocytes cultured in a collagen scaffold	PRP activated with bovine thrombin and single centrifugation	Decreased IL-1β and TNF-α production, restored collagen type II and chondrogenesis	[26]
Human osteoarthritic chondrocytes	5% PRP releseate after double freeze-thaw cycle activation and single centrifugation	Increased cell proliferation, proteoglycan synthesis, Sox-9 and aggrecan expression, and chondrogenic differentiation proteins production	[27]
Human osteoarthritic synoviocytes	20% PRP and 20% PRP releseate after $CaCl_2$ activation	Increased hyaluronic secretion and HGF production	[28]
Human synoviocytes, chondrocytes and anterior cruciate ligament-derived cells	Autologous conditioned plasma	Increased cell proliferation and superficial zone protein production	[29]
Human subchondral mesenchymal progenitor cells	Different PRP formulations	Modulated chondrogenic differentiation by PRP formulation	[93]
Human type B fibroblast-like synoviocytes	Different PRP formulations	Increased cell death and IL-1 β, IL-6 and TNF-α production by formulations contained leukocytes and red blood cells	[104]
Huma osteoarthritic synovial fibroblasts	Leukocyte-rich PRP	Increased FGF-2, IL-1β and IL-8 production, decreased HGF and TIMP-4 production	[105]
Human osteoarthritic chondrocytes	Different PRP formulations	Stimulated cell proliferation and chondrocyte anabolism by PRP, stimulated catabolic pathway by leukocyte-rich PRP	[106]
Mouse macrophages cell line	Different formulations of human and mouse PRP	Decreased nitric oxide, TNF-α and inducible NO synthase	[108]
Human acute monocytic leukemia THP-1 cells	Platelet-derived micropartilces	Promoted monocytes towards a resident phagocytic phenotype	[109]
Primary human gingival fibroblast and primary human alveolar fibroblast	Leukocyte-rich PRP	Increased NFκB activation, decreased cell proliferation, increased pro-inflammatory cytokines production	[112]
Bovine chondrocytes	PRP releseate after $CaCl_2$ activation and single centrifugation	Increased type II collagen and aggrecan messenger RNA expression, decreased cyclic tensile strain-mediated catabolic and inflammatory response	[113]
Human nasoseptal chondrogenic cells and human bone marrow mesenchymal stromal cells	PRP releseate after $CaCl_2$ activation and single centrifugation	Promoted chondrogenic differentiation and their recommitment	[119]
Rabbit chondrocytes	Pool of rabbit PRP loaded in hydrogel scaffold	Increased cell viability and cannabinoid receptor CB1 and CB2 expression	[120]

(*Continued*)

Table 1. (Continued).

Cell type/animal model	Intervention	Outcome	Reference
Male 4-month-old New Zeland white rabbits with induced articular cartilage defect in the groove of femur	Pool of rabbit PRP loaded in hydrogel scaffold	Enhanced cell proliferation and maturation of joint chondrocytes	[120]
Human cortico-cpongius progenitor cells	PRP releseate after freeze-thaw cycle activation and single centrifugation	Stimulated cell migration, increased cartilage matrix formation, promoted chondrogenic differentiation	[121]
Human subchondral progenitor cells in polyglicol acid-hyaluronan scaffolds	PRP releseate after freeze-thaw cycle activation and single centrifugation	Induced collagen type II and IX, aggrecan and cartilage oligomeric matrix protein expression	[122]
Human tenocytes	Different PRP releseate after $CaCl_2$ activation supplemented with PDGF and TGF-β1	Modulated cell proliferation and collagen type I, HGF and VEGF production by TGF-β1 addition	[123]
Primary human keratocytes and conjunctival fibroblasts	PRP releseate after $CaCl_2$ activation and single centrifugation	Stimulated cell proliferation and migration, inhibited TGF-β1-induced myofibroblast differentiation	[124]
Human tenocytes	PRP releseate after $CaCl_2$ activation and single centrifugation	Stimulated cell proliferation and HGF and VEGF production	[125]
Human tenocytes and synoviocytes	PRP releseate after $CaCl_2$ activation and single centrifugation	Stimulated cell migration	[126]

and TIMP-4 expression, two anti-catabolic mediators in cartilage, the former indicating a proinflammatory and procatabolic response. These observations were not present when the culture medium was obtained by P-PRP (Pure PRP) or PPP (Poor PPP), a notable signal that suggests there is indeed an impact of leukocytes on the biologic effects of PRP. This repertoire of antiinflammatory responses induced by PRP may break the catabolic loop, and dampen inflammatory response in SM and AC when these cells are exposed to proinflammatory cytokines and to abnormal mechanical stress and DAMPS, which is the significant OA context (Figure 2 and 4).[37] One cellular process that accentuates the catabolic state of the AC and SB is the oxidative stress resulting from the imbalance between levels of reactive oxygen species (ROS) relative to antioxidant, which is amplified by aging.[35,116,117] Osteoblasts cultured in the presence of PRP supernatant showed an upregulation of NrF2-ARE pathway and subsequent activation of antioxidant response element (ARE), an important mechanism involved in detoxifying ROS and protecting chondrogenic and osteogenic precursor cells.[22] Moreover, intraosseous infiltrations of PRP in mice can revert the decreased expression of SIRT1 in bone-marrow derived stem cells from aged animals, making stem cells more resistant to oxidative stress and maintaining their stemness, suppressing adipogenesis within the bone marrow and improving osteogenesis and bone mineral density.[23,24] Hence, PRP might additionally play a role as an anti-aging factor by stabilizing AC and protecting SB against oxidative stress.[22–24,115] However, as aging is one physiological risk factor for developing OA,[35,117] there are some age-related changes in the composition of PRP, such as the reduction of IGF-1 and PDGF in elderly people, two important chondrogenic mediators,[118] that might account for some contradictory outcomes in the application of this therapy.

4.6. OA and pain

Pain is considered the clinical hallmark of KOA and several clinical trials have been conducted to assess the efficacy of intraarticular injections of PRP for both pain and function of the knee. There are several relevant studies using the same type of PRP product (PRGF) demonstrating a significant pain reduction and an improvement in knee joint physical function [16] in patients with KOA treated by 3 weekly infiltrations of PRP.[14–16,127] The mechanism/s causing osteoarthritis pain remain yet to be fully identified [53] as do the proposed mechanisms of PRP effectiveness. Two mechanisms might likely link the pain reduction to PRP treatment. The first is the suppression of NFkβ on intraarticular inflamed cells, which leads to the reduction of proinflammatory cytokines that otherwise, might contribute to pain by stimulating hyperalgesia and sensitizing joint nociceptors to other stimuli.[3,42] The second is the reported significant amount of endogenous cannabinoids within PRP [25] that might act as ligands for cannabinoid receptor 1 (CB1) and 2 (CB2) of chondrocytes, synovium cells and bone cells [128] of OA patients, thereby supporting both a pain and inflammation reduction by targeting the endogenous cannabinoid systems (Figure 2 and 4).[25,128]

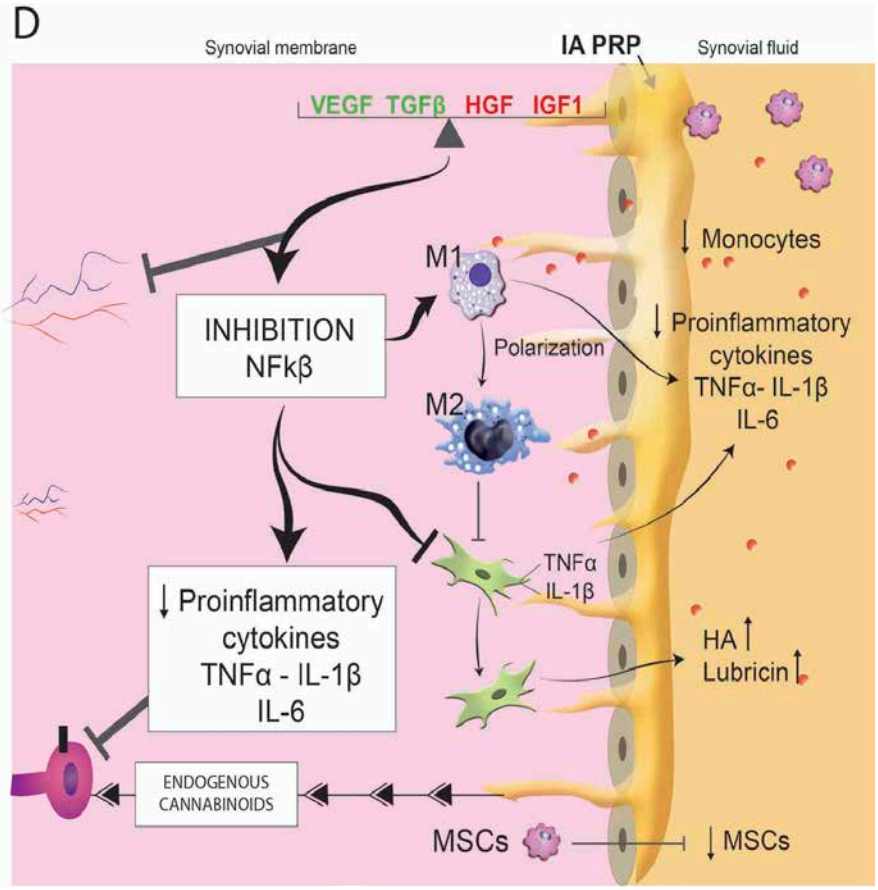

Figure 4. SM. This repertoire of antiinflammatory responses induced by PRP may break the catabolic loop, and dampen inflammatory response in SM and AC when these cells are exposed to proinflammatory cytokines and to abnormal mechanical stress and DAMPS, which is the significant OA context.[1,3,40] This sterile disruption of ECM homeostasis in osteoarthritic joint and an early inflammatory response has been suggested to resemble a chronic injury.[3]

4.7. Trophic and anabolic effects

PRP has been shown to have a consistent *in vitro* proliferative effect on cultured human chondrocytes in a dose-and time-dependent manner [27,29,119] and on rabbit chondrocyte when GFs are delivered in a sustained manner through the upregulation of CB1 and CB2 receptors.[120] Moreover, an *in vitro and in vivo* anabolic effect of PRP on chondrocytes has been reported by increasing the synthesis of proteoglycan and collagen type II [26] or decreasing catabolism by reducing MMP-13 expression and TNF-□ concentration in synoviocyte and cartilage co-cultured systems with PRP media.[21] Another chondroprotective effect is based on the visco-inducing effect of PRP, which stimulates the synthesis of hyaluronic acid and lubricin by synoviocytes and chondrocytes respectively,[21,28,29] which help restore the SF homeostasis and function (Figure 5), the latter preventing chondrocyte apoptosis, synovial cell overgrowth, cartilage breakdown, and inhibition of the MSC release and migration.[29,111,121] On the other hand, platelet rich plasma obtained by apheresis, and characterized by a low platelet concentration and very few leukocytes has been shown to exert positive effects on migration, proliferation and chondrogenic differentiation of cultured human subchondral mesenchymal progenitor cells.[93,121,122] Several soluble morphogens embedded in a fibrin network such as IGF-I and -II, PDGF, SDF-1, TGF-β, CCL5 and fibronectin, among other biomolecules, have been shown to be involved in the recruitment and homing, and in a chondrogenic-differentiation effect of PRP on chondroprogenitor or MSCs from subchondral mesenchymal progenitor cells.[121,129] Last but not least, uncontrolled angiogenesis and fibroneurovascular tissue proliferation are two histological features of osteoarthritic SM and SB. Despite the fact that PRP contains proangiogenic and profibrotic growth factors (VEGF, FGF, PDGF and TGFβ) several *in vitro and in vivo* studies have reported no increase in the level of VEGF and TGFβ [123] nor were tissular fibrosis or an aberrant angiogenesis induced.[123,124,130,131]

5. Targeting subchondral bone as one important tissue in the knee OA treatment

5.1. Subchondral bone as a tissue target in OA treatment

The realization of the biological and mechanical connection between AC and SCB has lead to numerous *in vivo* animal studies that have shown that targeting SB with some drugs can have protective structural effects on cartilage.[9] Blocking or limiting the bone remodeling with alendronate, [132] zoledronic acid [133] or improving the microstructure and quality of subchondral bone in osteoarthritic and osteoporotic rabbits with parathyroid hormone, [9] prevent cartilage degradation and OA progression. Moreover, Sagar et al. [134] reported a reduction in pain behavior after a subcutaneous treatment with osteoprotegerin in a monosodium iodoacetate (MIA) rat model of OA pain, and Pelletier et al. [135] demonstrated that

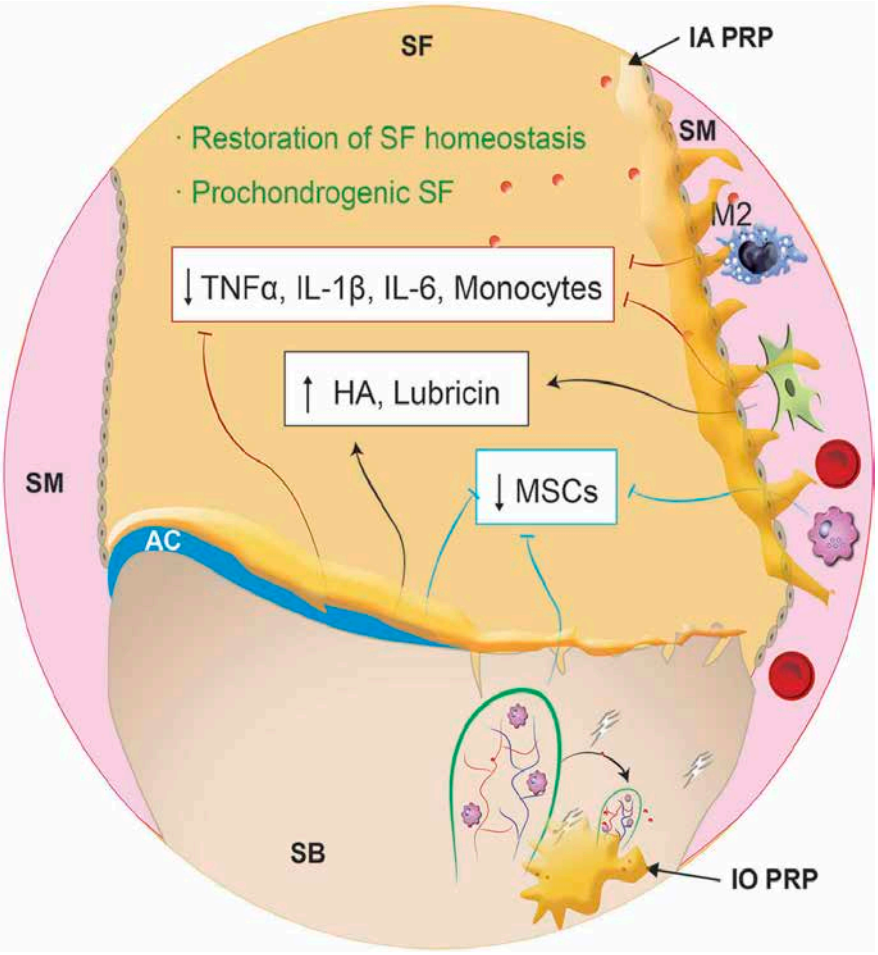

Figure 5. Intraarticular infiltration of PRP helps restore SF homeostasis by stimulating the synthesis of hyaluronic acid and lubricin by synoviocytes and chondrocytes respectively,[21,28,29] dampening inflammation and suppressing the concentration chemoattractan cytokines in SF, which might contribute to the inhibition of the MSC release and migration.[3,95,96] PRP might favour a homing and chondrogenic-differentiation effect on MSCs of subchondral mesenchymal progenitor cells and SF-MSCs.[88,108,111].

an oral strontium ranelate treatment in an experimental osteoarthritic dog model reduced the progression of structural changes including the subchondral bone. Despite the fact that the translation of these promising observations in preclinical research to human clinical trials has often failed, as indicated by a recent metaanalysis of clinical trial with risedronate in knee osteoarthritis,[136] recent clinical trials are raising expectations. For instance, using zoledronic in patients with clinical KOA associated with bone marrow lesions (BMLs) assessed by MRI, Laslett et al. [137] reported a beneficial effect on pain and on BML evolution at 6 months. In participants from the osteoarthritis initiative, Laslett et al. [138] demonstrated significant pain reduction during the first 3 years of treatment with biphosphonates. Two more clinical trials have shown positive structural effects of strontium ranelate on KOA, one improving the joint space narrowing [139] and the other reducing the loss of cartilage volumes concurrent with the decrease of BMLs at 3 years of follow up.[140]

5.2. Intraosseous infiltrations of PRP

Infiltrations of PRP into the BM cavity of femur of young and old ovariectomized-SAMP8 age-related osteoporotic female mice have been reported to up-regulate osteogenesis and down-regulate adipogenesis.[23] The increase of fat tissue mass in BM is correlated with decreased bone mineralization in aged SAMPS8 mice,[23,24] bone demineralization that occurs in osteoarthritic subchondral bone together with cysts.[67] Moreover, improvement of bone mineral density in PRP-treated osteoporotic mice concurred with both histological sections of the bone samples showing more trabecular bone areas and more intense calcium staining and a suppression of bone resorption process as evidenced by the decrease of RANKL transcript.[23] In a trial on 13 healthy volunteers, Philippart et al. [141] reported fatigue on the first day as the only clinical adverse effect after a self-stimulation of BM of the iliac crest by injected autologous platelet-rich plasma.[141] Figure 5 shows the histological analysis of cartilage and SB from a patient suffering from severe KOA who underwent intraosseous infiltrations of PRGF. Eight months later, the patient had not improved clinically and underwent a knee replacement. During the surgery, we took this sample of cartilage and subchondral bone from the femoral condyle in which 5 cc of PRGF had been infiltrated intraosseously. Part of the biopsy showed a good gross appearance, with pearly areas similar to the original hyaline cartilage, though histological study revealed a fibrocartilage repair tissue. Another area showed nearly exposed bone.

In light of the aforementioned research and others not mentioned here because of space limitation, and the significant clinical improvement obtained in some but not all patients

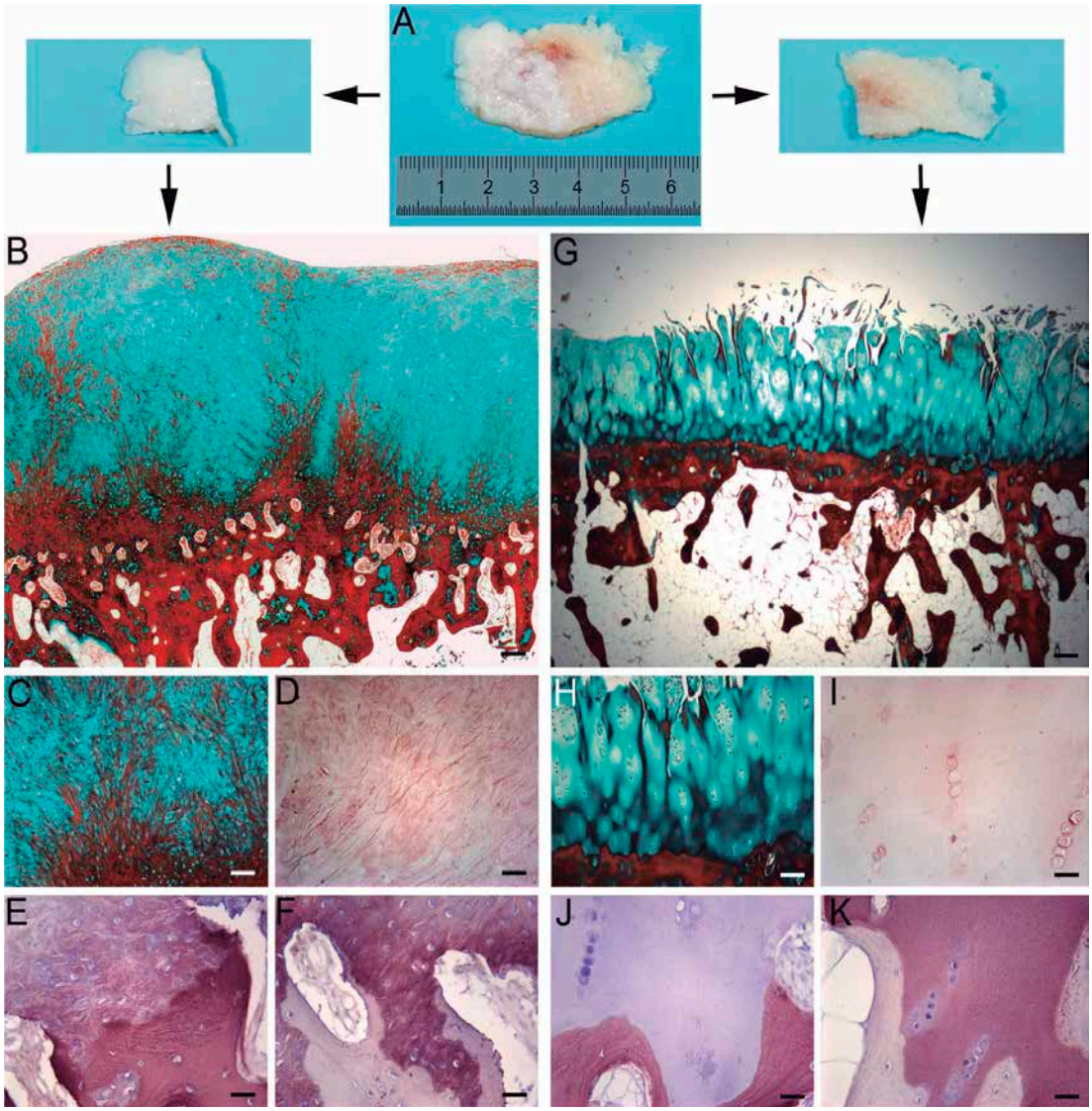

Figure 6 Fibrocartilage repair tissue after intraosseus PRGF infiltrations in the treatment of human knee osteoarthritis: a histological study. (A) Macroscopic morphology of the sample. The sample was divided into two pieces. The fragment on the left-hand corresponds to fibrocartilage repair tissue (B to F) while the right-hand fragment shows osteoarthritic cartilage (G to K). B and G show panoramic images of the sample (Masson's trichrome staining). In photomicrographs C and H, details of the structure of articular cartilage are observed (Masson's trichrome staining). The presence of elastic fibers is demonstrated by Orcein staining (D and I). These fibers can be seen in D, while they are absent in I. An immunohistochemical study was performed to detect the presence of type I (E and J) and type II (F and K) collagen. In all samples (E to K), both subchondral bone (always positive for type I and negative for type II collagens) and cartilage are observed. In fibrocartilage (E and F) both types of reactivity are observed, while in the degenerated cartilage, only type II collagen positivity is shown (K). Histologically, the pearly area (the left-hand side of the sample) is fibrocartilage repair tissue, while the right-hand side of the sample displays an osteoarthritic area with loss of cartilage surface integrity.

with KOA treated with intraarticular infiltrations of PRP [14–16,142]. Our group arrived at the strategy of combining another drug delivery route, namely, the intraosseous infiltrations combined with intraarticular infiltrations of PRP.[99,143]

We have already conducted a phase II clinical trial combining intraarticular and intraosseous infiltrations of PRP for severe KOA. The first treatment included one PRP intraarticular infiltration and two PRP intraosseous infiltrations (in femoral condyle and tibial plateau). The procedure is carried out in the operating room under a 4–5 degree of sedation of the patient. In addition, local anesthesia is conducted into the periosteum of condyle and tibial plateau by injecting 2 ml of 2% mepivacaine. Intraosseous infiltrations are performed with a 13G trocar used for bone biopsy, and the control of trocar placement is facilitated using a fluoroscope.[144] Two more weekly intraarticular infiltrations were performed. After a 6 month follow-up, a significant pain reduction and decrease of MSC and CFU-F in synovial fluid with no adverse effects were reported.[14,99,143] We have been performing intraosseous infiltrations of PRGF since 2003 applying them regularly at the condyle and tibial tunnels in the arthroscopic reconstruction of anterior cruciate ligament, and in osteochondral injuries and osteonecrosis of the hip and knee.[144]

6. Conclusions

There is a substantial and growing body of evidence indicating that subchondral bone is a crucial target, which should be included in KOA therapy. PRP molecular intervention positively influences SB, SF, AC and SM homeostasis, adaptation, and metabolism in addition to reducing joint pain and inflammation, and providing a circuit breaker in KOA, thereby acting as a symptomatic and structure-modifying OA therapy. However, many unanswered questions remain, regarding

molecular mechanisms, dosage aspects and whether combining PRP with stem cells might enhance the efficacy of PRP.

7. Expert opinion

Intraarticular delivery is an alternative modality to deliver PRP in patients with KOA and it has been shown to be safe and efficacious in improving clinical symptoms.[14–16] This route of drug delivery reaches the SM and the AC, which is sometimes inefficiently targeted by systemic drug delivery. Intraarticular delivery circumvents systemic toxicity and its side effects, offers an excellent bioavailability and does not present molecular size limitation, in contrast to the systemically delivered molecules entering the joint through capillaries of the subsynovium.[145,146] Nevertheless, intraarticular therapy faces other challenges when treating chronic nonsystemic sterile-inflammatory conditions as in the case of KOA. One significant challenge is a short joint dwell time of drugs, as the lymphatic drainage clears proteins in a few hours. This is not the case of PRP, as it acts as a dynamic liquid scaffold with a fibrin network from where GFs are gradually released into the tissue.[96,100] Moreover, the increasingly recognized role of SB in the pathophysiology of OA [8,12,33,67] might make the intraarticular route insufficient to tackle all the joint tissues involved in KOA.

Intraosseous delivery strategy for local, prolonged and sustainable release of GFs has been proven to be efficacious in some musculoskeletal pathology, non-union fractures, osteoporosis and bone fracture healing among them.[143,147,148] Over the past 30 years, surgical experience in cartilage defect has revealed that only when the subchondral bone is involved through bone marrow stimulating procedures such as transcortical Pridie drilling and microfractures, is a temporary functional fibrocartilage tissue synthesized, with no serious adverse reported.[5] There is good *in vitro* and *vivo* evidence that events in the subchondral bone concur with and have a direct effect on the overlying articular cartilage.[9,43,45,46] Moreover, although the titles and much of the text of Liu et al. [24] and Philippart et al. [141] papers are not focused on osteoarthitis, these studies shed important light on the role that intraosseous infiltrations of PRP might play in subchondral bone homeostasis by targeting both osteoblast-osteoclast coupling and mesenchymal stem cells responses, as well as in its safety.

The combination of intra-articular and intraosseous injections of PRP is an *in situ* local biological 'joint-centric' approach to treat severe KOA addresses the SM, SF and superficial zone of AC by intraarticular injections of PRGF, and deep zones of AC and SB through PRP intraosseous infiltrations.[99] These PRP infiltrations convey a mimetic biomaterial embedded with a pool of growth factors acting as a smart scaffold [149] which might sustain a gradual delivery of growth factors at the dysfunctional and deregulated tissues as a niche therapy. Rebuilding a physiological-homeostatic network at knee organ failure tissue level, as is the case of severe knee OA, must be an orderly process, which entails a daunting therapeutic task. Our hypothesis is that the concurrent presence and a balanced ratio between platelet-secreted TGFB-1 and VEGF, and plasma growth factors such as IGF-1 and HGF, [105,124–126] all conveyed by PRP intraosseous infiltrations, might reduce or buffer the excess of TGFB in SB and restore HGF activity synthesized by subchondral bone cells. This modulatory effect of PRP on TGFB-1 signaling pathway might shrink the fibroneurovascular tissue that replaces the bone marrow of OA subchondral bone, an explanation which parallels the antifibrotic mechanism already reported to be exerted by the PRP on several cell phenotypes. [105,124,126] This new reestablished homeostatic balance between TGFB1 and HGF [71,78] would reduce the synthesis of NGF, VEGF and other inflammatory mediators thereby contributing as well to modulate the aberrant fibroneurovascular tissue and to alleviate pain and hyperalgesia.[150]

However we do not forget that '*the aim of science is not to open the door to infinite wisdom but to set a limit to infinite error* ' (Bertolt Brecht), and many questions and uncertainties still persist unanswered in the field of PRPs and inflammation. When the concept of inflammation defined as a cooperative and amplifying protective multicellular response, orquestrated both locally and remotely, that is intended to eliminate the original insult and their toxic consequences, thereby initiating the repair process, [30] there are some difficulties applying it to tissue damage brought about by mechanical stresses, which is the case of most sterile inflammation pathologies such the KOA.

In spite of a wealth of preclinical and clinical publications on PRP, many uncertainties remain regarding the ultimate molecular mechanism/s, the variability in its composition mainly because of the presence/absence of leukocytes, the platelet concentration, the donors age and the manner in which PRPs are applied to the damaged tissues.[90] Moreover, we need to delve into the systemic effect that this procedure might entail as few studies on human have been carried out regarding PRP treatments and systemic effects.[151,152]

The restoration of TGFβ and other extracellular matrix GFs balance by the application of PRP deserves a deeper research and opens the door to explore the analgesic, antiinflammatory and immunomodulatory, and trophic-anabolic effects of PRP through a systems biology approach. In addition, we cannot rule out a systemic effect of intraosseous infiltrations as suggested by studies carried out in animal model, which should be explored. And finally, we still do not know how to combine PRP with rehabilitation programs and exercise in a synergistic application with the goal of full recovery of knee function.[31]

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Declaration of interest

E Anitua is the Scientific Director of and S Padilla, R Prado and G Orive are scientists at BTI Biotechnology Institute, a dental implant company that investigates in the fields of oral implantology and PRGF-Endoret technology. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Operative Techniques in
Orthopaedics

Platelet-Rich Plasma: Preparation and Formulation

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Platelet-rich plasma is a set of autologous platelet products used to accelerate recovery from injury. 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 itself, and not to merely repair with scar tissue. Among the jungle of products in this field, PRGF-Endoret (BTI-Biotechnology Institute, Vitoria, Spain) is a pioneering autologous regenerative technology with multiple therapeutic potentials, present in at least 4 different formulations, depending on the coagulation and activation degree of the samples. PRGF-Endoret technology is safe and has multiple applications and potentials.

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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 nonoperative management modalities.[1] One of these cutting-edge technologies is the use of plasma rich in growth factors (PRGF-Endoret).[2] This type of biological treatment mimics the natural ways of wound healing[3] trying to optimize and reduce healing times. This is achieved driving to the injury site the whole protein array of platelet-rich plasma (PRP) that will be involved in the repair of damaged tissues. In this way, all the proteins necessary for tissue repair are released locally.

The process of tissue repair occurs naturally in a staged fashion,[4] and includes removal of dead cells, proliferation, migration of cells to the injury site, production of new vascular structures, and so on. The organization of all these elements influences the healing of a given injury, preventing fibrotic elements that cause loss of functional capacity of that tissue.[5,6] Growth factors play an important role, coordinating the whole process in an orchestrated fashion in all tissues of the musculoskeletal system, including muscle,[7] tendon,[8] bone,[9,10] and cartilage.[11] Growth factors act on other tissues as well, including skin,[12] oral soft tissue,[13,14] cornea,[15] among others.

The technology of PRGF-Endoret mimics the natural healing mechanisms but with 2 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.[16] This autologous toolbox consists in the use of platelets as a reservoir and vehicle of a large repertoire of proteins.[17,18]

In the past 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.[19-23]

For human therapeutic uses, we recommend that only commercial systems are used, although some centers still use homemade products for both basic research and clinical use.

The commercial systems can be certified for various medical applications, but the therapeutic outcome will depend on the type of PRP and the dosage used. Establishing a proper classification of the PRPs and identifying the biological differences

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Table 1 Platelet Protein Classification and Their Biological Role[16]

Classification	Protein	Biological Effects
Adhesive proteins	Von Willebrand factor (vWF) propeptide, Fibrinogen, Fibronectin, Vitronectin, Thrombospondin 1 (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	Factor V/Va, Factor XI-like protein, multimerin, protein S, high-molecular-weight kininogen, antithrombin III, tissue factor pathway inhibitor	Thrombin production and its regulation
Fibrinolytic factors and associated proteins	Plasminogen, Plasminogen activator inhibitor-1 (PAI-1), urokinase plasminogen activator (uPA), alpha2-antiplasmin, histidine-rich glycoprotein, thrombin activatable fibrinolysis inhibitor (TAFI), alpha2-macroglobulin (α2M)	Plasmin production and vascular modeling
Proteases and antiproteases	Tissue inhibitor of metalloprotease 1 -4 (TIMPs 1 -4), metalloprotease-1, -2, -4, -9, A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13), tumor necrosis factor-alpha-converting enzyme (TACE), protease nexin-2, C1 inhibitor, serpin proteinase inhibitor 8, alpha1-antitrypsin	Angiogenesis, vascular modeling, regulation of coagulation, and regulation of cellular behavior
Growth factors	Platelet-derived growth factor, transforming growth factor beta1 and beta2, epithelial growth factor, insulin-like growth factor type I, vascular endothelial growth factor (A and C), basic fibroblastic growth factor (FGF-2), hepatocyte growth factor, Bone morphogenetic protein (BMP)-2, -4, -6, connective tissue growth factor (CTGF)	Chemotaxis, cell proliferation and differentiation, and angiogenesis
Chemokines, cytokines, and others	Regulated upon Activation - Normal T-cell Expressed, and Secreted (RANTES), Interleukin-8 (IL-8), Macrophage inflammatory protein-1 (MIP-1) alpha, Epithelial Neutrophil-Activating Peptide 78 (ENA-78), Monocyte chemotactic protein-3 (MCP-3), Growth regulated oncogene- alpha (GRO-alpha), angiopoietin-1, IGF-1 binding protein 3 (IGF-BP3), interleukin-6 soluble receptor (IL-6sR), Platelet factor 4 (PF4), beta-thromboglobulin (bTG), platelet basic protein, neutrophil-activating protein-2 (NAP-2), connective tissue-activating peptide III, high-mobility group protein 1 (HMGB1), Fas ligand (FasL), Homologous to lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus (HSV) glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes (LIGHT), Tumor necrosis factors (TNF)-related apoptosis-inducing ligand (TRAIL), Stromal cell-derived factor-1 (SDF-1) alpha, endostatin-I, osteonectin-1, bone sialoprotein	Regulation of angiogenesis, vascular modeling, cellular interactions, and bone formation
Antimicrobial proteins	Thrombocidins, Defensins	Bactericidal and fungicidal properties
Others	Chondroitin 4-sulfate, albumin, immunoglobulins, disabled-2, semaphorin 3A, Prion protein (PrPC)	

Table 1 shows a set of proteins present in platelets and its physiological role in the regeneration of tissues.

among them is absolutely necessary to understand some of the controversial results obtained with these types of technologies so far.[21] In terms of composition, different types of plasma rich in platelets differ in their platelet count enrichment (greater or less than 5x), leukocyte content (greater or less than 1x), and whether they are activated or not. On this basis, it can be classified into 8 different types. These variables influence tissue biological response and thus the treatment efficacy.[22]

Understanding the Properties of PRP Products

Several key biological mediators are present in a PRP. The more studied growth factors contained in PRP that are important during tissue repair include insulin-like growth factor type I (IGF-I), transforming growth factor beta type 1 (TGF-β1), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), epithelial growth factor, and basic fibroblastic growth factor among others (Table 1).[24,25] Some of them (IGF-I and HGF) are plasmatic proteins, and their concentrations do not depend on the platelet enrichment. However, most of the growth factors are indeed platelet proteins, both synthesized and adsorbed, and thus, their quantity does depend on the platelet concentration.

To understand the properties of PRP products, it is necessary to detail the different roles of molecules that contain the following:

- IGF-I: This protein circulates in plasma as a complex with binding proteins. This determines the bioavailability and regulates the interaction between this IGF-I and its receptor.[26,27] IGF-I is involved in keratinocyte migration and wound healing,[28,29] stimulates bone matrix formation and maintenance[30] by promoting preosteoblast proliferation,[31,32] and is also involved in striated muscle myogenesis.[33] Furthermore, knockout mice for IGF-I receptor (IGF-IR) in muscle exhibited impaired muscle regeneration and deficient myoblast differentiation.[34]
- TGF-β1: The role of TGF-β family proteins in wound healing has been recently reviewed.[35] TGF-β has different effects, depending on tissue and cell type.[5] The release and posterior bioactivation of latent TGF-β contributes to the early cellular reparative responses, such as migration of cells, neovascularization, and angiogenesis[36] into the wound area. In bone, TGF-β1 induces osteogenic differentiation of mesenchymal cells of the bone marrow, upregulating osteoblast differentiation markers.[37]
- PDGF: This growth factor is a mitogen and chemotactic factor for all cells of mesenchymal origin. It is important in the repair of joint tissue, including cartilage and me-

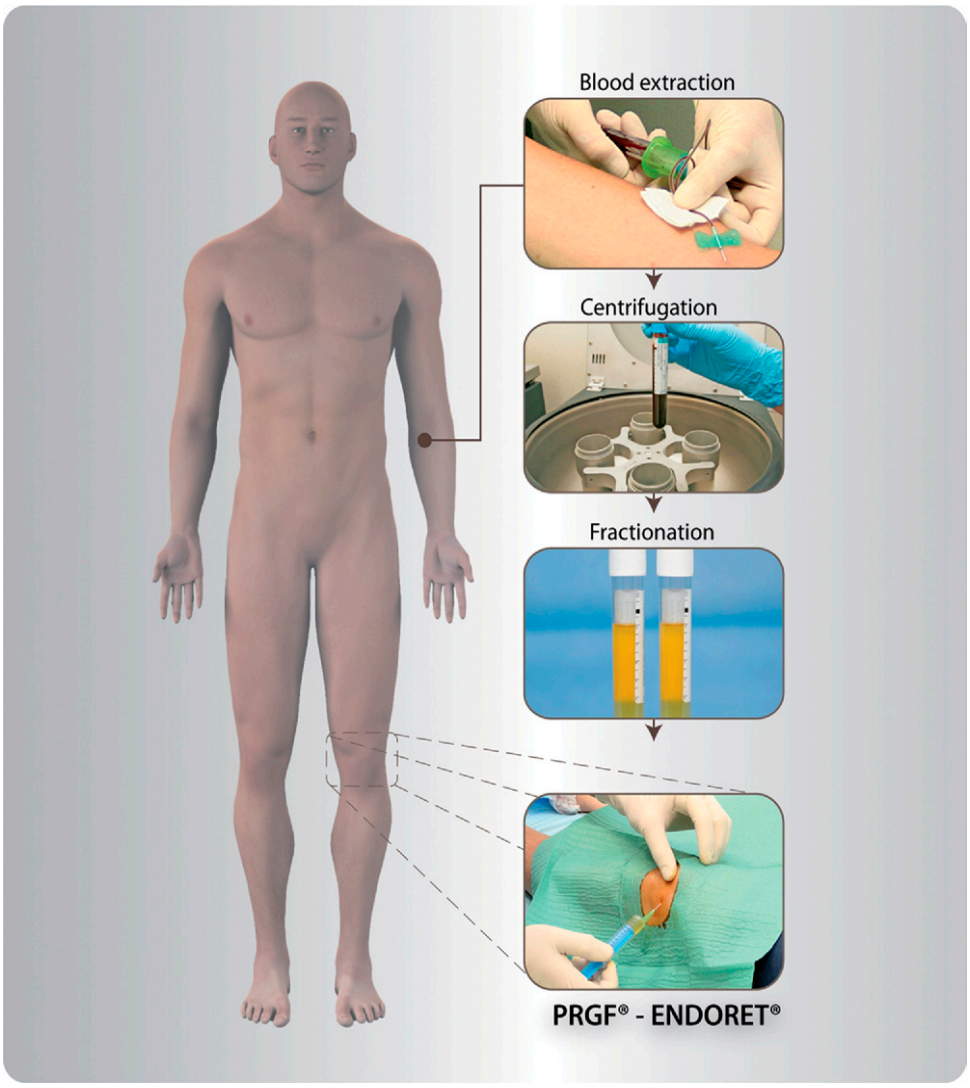

Figure 1 PRGF-Endoret technology overview. PRGF-Endoret aids in the preparation of different autologous therapeutic formulations from patient's own blood.

niscus.[38,39] Bone is also a target of PDGF, influencing its metabolism and acting in repair mechanisms[40,41] including the recruitment of pericytes to stabilize new blood vessels.[42]

- HGF: This growth factor regulates cell growth, migration, and morphogenesis,[43] and plays an important role in wound healing through an epithelial-mesenchymal interaction.[44] The antifibrotic effect of HGF has been shown in various tissues,[45,46] through induction of Smad7, and thus regulates the myofibroblast phenotype, allowing the initial contraction of the wound, but making the myofibroblast to gradually disappear.[47]
- VEGF: This growth factor is a key mediator in wound healing[48] and the main inducer of angiogenesis because it stimulates chemotaxis and proliferation of endothelial cells.[49] Also, VEGF is involved in the regulation of many organ homeostasis, such as brain, heart, kidney, or liver,[50] and its role may be crucial in cell-mediated tissue regeneration.[51]
- Epithelial growth factor: This protein promotes chemotaxis and mitogenesis in epithelial and mesenchymal cells[52,53] by acting on the regeneration of multiple tissues. It has an important role in skin, cornea, gastrointestinal tract, and nervous system.[54-58]
- Basic fibroblastic growth factor: This factor, also called fibroblast growth factor 2, is potent inductor of cell proliferation, angiogenesis, and differentiation.[59,60] Its role in the repair process has been observed in several tissues, including bone,[61-63] tendon,[64,65] and periodontal tissue.[66-68]

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 aforementioned 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 PRP and examine its effect separately, as 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[69] and becomes functional after proteolytic activation or in the presence of other molecules, such as thrombospondin-1 or various integrins.

The idea that PRP 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. These antibacterial effects were observed against *Staphylococcus aureus* and *Escherichia coli*.[70] Classically, these properties have been shown in leuko-enriched PRP. However, recently, these antimicrobial properties have been evidenced in PRGF-Endoret,[71] 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. These data raise questions about the role that leukocytes may play in a PRP preparation because they do not improve the bacteriostatic properties, but, on the contrary, they might significantly increase the presence of proinflammatory molecules.

Platelet-rich products act also as anti-inflammatory mediators by blocking monocyte chemotactic protein-1, released from monocytes, and lipoxin A4 production.[72] HGF in PRP inhibits NF-κB, 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.[73] In addition, serotonin, a neurotransmitter and hormone present in platelets, has been reported to directly mediate liver regeneration.[74]

PRGF-Endoret: A Pioneering Technology

For almost 2 decades, our research group has characterized this technology and has studied its therapeutic potential in tissue repair and wound healing.[75] PRGF-Endoret contains a moderated platelet concentration, a two-third–fold increase compared with peripheral blood, a dosage shown to induce optimal biological benefit.[76] In fact, lower platelet concentrations can lead to suboptimal effects, whereas higher concentrations might have an inhibitory effect.[77] PRGF-Endoret does not contain leukocytes, and activation is performed only with calcium chloride ($CaCl_2$).

The process to produce PRGF-Endoret is easy, fast, and reproducible (Fig. 1). 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 IV, BTI-Biotechnology Institute, Vitoria, Spain). The centrifuge has

Figure 2 The plasma transfer device is a disposable and sterile aspiration system that allows the fractionation of PRGF-Endoret. 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. The user accessible 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.

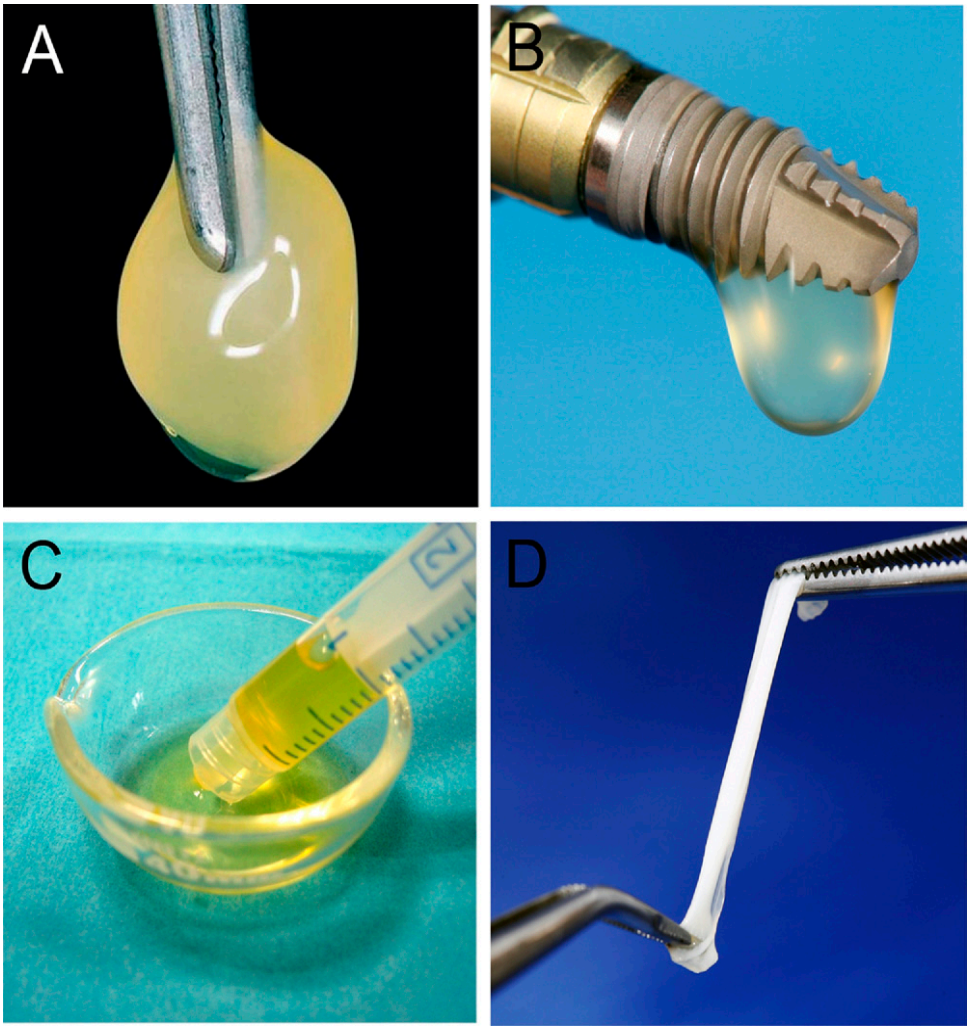

Figure 3 The 4 different formulations of the PRGF-Endoret technology: (A) the 3-dimensional scaffold, (B) the liquid formulation, activated at the moment, on the titanium surface, (C) the PRGF-Endoret supernatant, and (D) the elastic and dense autologous fibrin membrane.

specific parameters to maximize the production of platelets and keep the plasma leukocyte free. After centrifugation, the following 3 typical layers are obtained: a yellowish top layer, the plasma, which contains a gradient of platelets, with maximum concentration of those platelets above the buffy coat; the leukocyte layer, or *buffy coat*, is located below of plasma layer; and the bottom layer is the layer containing the red cells. Regarding the plasma volume, it is possible to empirically differentiate between 2 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.

With the aim of collecting these plasma fractions from PRGF-Endoret technology, we have recently developed an optimized device—the plasma transfer device (PTD) (Fig. 2). The PTD is a disposable and sterile aspiration system that allows separating the different fractions obtained after centrifugation. In contrast to the traditional pipetting system, the PTD system is faster, avoiding intermediate pipetting steps. In addition, the PTD does not require maintenance of the pipetting system. Depending on clinical needs, the fractionation can be made in 1 or 2 fractions, achieving higher volume—lower concentration of platelets (a single fraction) or lower volume—higher concentration of platelets (2 fractions). 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 of exogenous bovine thrombin, a source of possible immunologic reactions.[78-80]

Another important feature of the PRGF-Endoret technology when compared with other PRP systems is the absence of leukocytes, which categorizes it as a safe and homogeneous, because the values of leukocytes are highly variable between donors[81] 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.[82] Of special concern would be to avoid leukocytes if muscle regeneration is required, as in vivo PMNs increase muscle damage[83] and do not provide extra functionality. Therefore, it is recommended to use leukocyte-free PRP in infiltrations of damaged muscle.[84]

PRGF-Endoret Technology: A Versatile Toolbox with Multiple Formulations

A key point that distinguishes the PRGF-Endoret technology from other PRP products is its versatility. Four different formulations (Fig. 3) 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. It is a 3-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,[85,86] wound closure, and tissue engineering.[87] The 3-dimensional structure of the fibrin mesh (Fig. 4) allows cell proliferation because, as mentioned earlier, it contains factors necessary for growth and migration of cells. In addition, this formulation can be combined with other materials,[88] such as autologous bone, demineralized freeze-dried bovine bone, collagen among others, adjusting the resulting characteristics of the scaffold.
2. Liquid PRGF-Endoret, activated at the time of use, is used in intra-articular injections,[89,90] surgery,[91,92] treatment of skin disorders,[85,86] skin regeneration,[93] and implant surface bioactivation by producing a biologically active layer on the titanium surfaces.[94]
3. The PRGF-Endoret supernatant contains plasma proteins and platelet releasate, and can be used as eye-drop treatment for dry eye disease[95] and other corneal defects.[96] In both basic and applied studies, this formulation can be used to supplement the cell culture medium.[76,97,98]
4. Autologous fibrin membrane. At the end of the process of coagulation, fibrin scaffold retracts. 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 postextraction tooth sockets[99] and to promote the full epithelialization of soft tissues.[100]

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 40 of these products with different characteristics, in terms of enrichment of platelets, presence of leukocytes, kind of activator, and final volume among others. This great variability makes it difficult to standardize protocols and compare results. Furthermore, this large variability can engender confusion among clinicians and researchers.[101] It is, therefore, necessary to reach a consensus and better definition of each product. Our research team has spent more than 15 years developing this technology, which makes PRGF-Endoret one of the best characterized autologous PRP, with multiple and growing therapeutic applications, as result of a continuous research translation to the clinic setting.

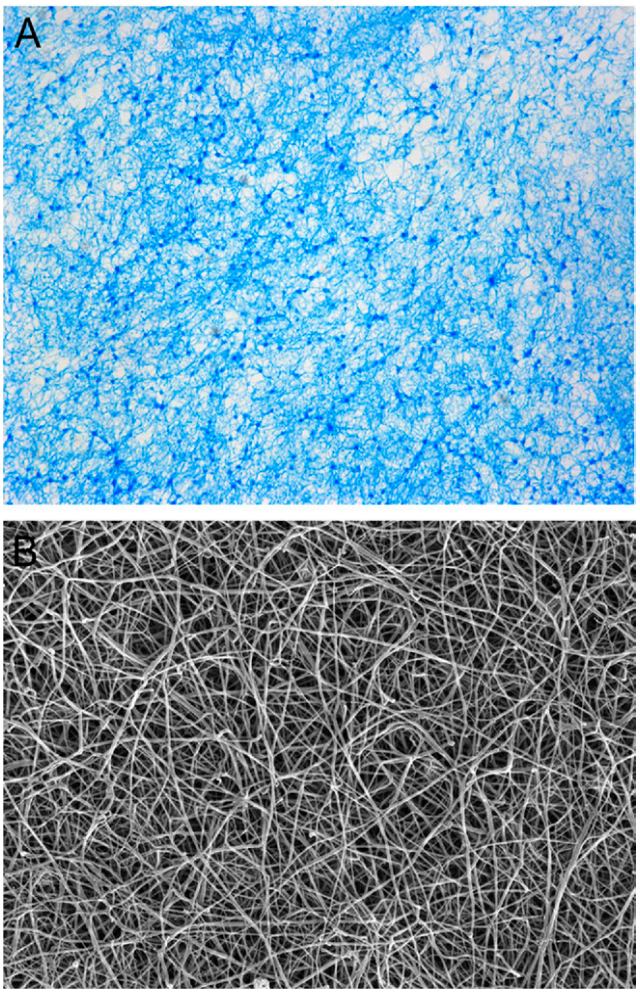

Figure 4 Three-dimensional structure of PRGF-Endoret fibrin network. (A) Network of fibrin and platelet aggregates scattered throughout the network of fibrin. May-Grunwald-Giemsa staining. (B) Detail of the tridimensional structure of the fibrin network. Note the interconnected intact fibrin strands. Scanning electron microscopy. Original magnifications (A, x400; B, x3500).

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A Randomized Clinical Trial Evaluating Plasma Rich in Growth Factors (PRGF-Endoret) Versus Hyaluronic Acid in the Short-Term Treatment of Symptomatic Knee Osteoarthritis

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Purpose: This multicenter, double-blind clinical trial evaluated and compared the efficacy and safety of PRGF-Endoret (BTI Biotechnology Institute, Vitoria-Gasteiz, Spain), an autologous biological therapy for regenerative purposes, versus hyaluronic acid (HA) as a short-term treatment for knee pain from osteoarthritis. **Methods:** We randomly assigned 176 patients with symptomatic knee osteoarthritis to receive infiltrations with PRGF-Endoret or with HA (3 injections on a weekly basis). The primary outcome measure was a 50% decrease in knee pain from baseline to week 24. As secondary outcomes, we also assessed pain, stiffness, and physical function using the Western Ontario and McMaster Universities Osteoarthritis Index; the rate of response using the criteria of the Outcome Measures for Rheumatology Committee and Osteoarthritis Research Society International Standing Committee for Clinical Trials Response Criteria Initiative (OMERACT-OARSI); and safety. **Results:** The mean age of the patients was 59.8 years, and 52% were women. Compared with the rate of response to HA, the rate of response to PRGF-Endoret was 14.1 percentage points higher (95% confidence interval, 0.5 to 27.6; $P = .044$). Regarding the secondary outcome measures, the rate of response to PRGF-Endoret was higher in all cases, although no significant differences were reached. Adverse events were mild and evenly distributed between the groups. **Conclusions:** Plasma rich in growth factors showed superior short-term results when compared with HA in a randomized controlled trial, with a comparable safety profile, in alleviating symptoms of mild to moderate osteoarthritis of the knee. **Level of Evidence:** Level I, randomized controlled multicenter trial.

Osteoarthritis (OA) is an heterogeneous disease that affects the structures of the joints. It has become one of the most common painful conditions affecting adults and the most frequent cause of mobility disability in the United States and Europe.[1] The incidence of OA is rising, influenced by the aging population and the epidemic of obesity.[2] Recent estimates suggest that symptomatic knee OA affects 13% of persons aged 60 years or older and a total of 20 million Americans, a number that is expected to double over the next 2 decades.[3]

Unfortunately, there are currently no agents available that can halt OA progression and reverse any existing damage. Analgesics and nonsteroidal anti-inflammatory drugs (NSAIDs) have suboptimal effectiveness, and there are some concerns regarding their safety, in light of the well-described gastrointestinal and cardiorenal side effects.[4] Current therapeutic approaches focus on developing less invasive procedures and applying them earlier

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in the disease when the structural changes of OA may be prevented or delayed.[5]

Synovial hyaluronic acid (HA) is a high–molecular weight glycosaminoglycan that acts as a fluid shock absorber, protecting cells and the intracellular collagen network from mechanical stress. The purpose of intra-articular injections of HA is to return the lost viscoelasticity to the joint, being frequently applied with some good results,[6] although several contradictory findings have also been reported.[7] Results from a clinical trial involving 306 patients showed that at the 40-month visit, significantly more patients responded to intra-articular injections of HA compared with placebo in the management of knee OA symptoms ($P = .004$).[8] Furthermore, a recent meta-analysis including 54 trials and involving more than 7,500 patients has also provided information about the therapeutic trajectory of HA for knee OA. Interestingly, HA was found to be efficacious by 4 weeks, reaching its peak effectiveness at 8 weeks but exerting a residual detectable effect at 24 weeks.[9]

Recent data support the application of platelet-rich plasma products as an effective and safe method in the treatment of the initial stages of knee OA.[10] Some growth factors present in platelet-rich plasma products, including transforming growth factor β, platelet-derived growth factor, and insulin-like growth factor 1, contribute to the maintenance of a homeostatic balanced status between anabolism and catabolism on the articular cartilage.[11-14] Others such as vascular endothelial growth factor and basic fibroblast growth factor show chondroinductive roles.

Platelet-rich plasma injections showed more and longer efficacy when compared with HA injections in reducing pain and symptoms and recovering articular functions.[15] In an interesting prospective study, Filardo et al.[16] compare, for the first time, the safety and efficacy of 2 different approaches of platelet-rich plasma production in the treatment of knee OA. In particular, they evaluated 2 platelet-rich plasma products prepared following either a single-spinning approach (PRGF-Endoret; BTI Biotechnology Institute, Vitoria-Gasteiz, Spain) or double-spinning approach (homemade leuko–platelet-rich plasma). Results showed that although both treatment groups presented a statistically significant improvement in all the scores evaluated at all follow-up times, significantly more adverse events (involving pain and swelling) were detected in the group treated with the platelet-rich plasma prepared with the double-spinning approach.

Plasma rich in growth factors (PRGF) is an autologous biological therapy based on using the patient's own plasma and platelet-derived growth factors and endogenous fibrin scaffold for regenerative purposes.[17] There has been increasing recognition of the potential role of this autologous cocktail of growth factors in stimulating tendon and synovial cell proliferation, migration, autocrine release of hepatocyte growth factors and HA, and even differentiation of tendon stem cells exclusively into tenocytes.[18-21] An absence or reduction in postsurgical inflammation is a consistent clinical observation associated with the use of this biological approach. A small retrospective cohort study showed that 3 intra-articular injections of PRGF-Endoret at 1-week intervals substantially reduced pain in patients with OA of the knee compared with those treated with HA.[22] In this randomized, double-blind, HA-controlled, multicenter trial, we explored the use of intra-articular injections of PRGF-Endoret as a novel, safe, and efficacious biological approach in the treatment of pain due to OA of the knee. The hypothesis was that PRGF-Endoret would improve pain symptoms compared with HA, possibly through the release of proteins and growth factors, in patients affected by knee degeneration.

METHODS

The study was carried out in accordance with the international standards on clinical trials: Real Decreto 223/2004, Declaration of Helsinki in its latest revised version (Tokyo, Japan; 2004), and Good Clinical Practice Regulations (International Conference for Harmonization). The study protocol was reviewed and approved by the Reference Ethic Committee. All patients provided written informed consent before entry into the study.

Patient Selection

One hundred eighty-seven patients were initially selected in the study. Patients were considered eligible if they were aged between 41 and 74 years and had OA of the knee diagnosed based on American College of Rheumatology criteria[23] with radiographic confirmation (Ahlbäck grades 1 to 3, on a scale of 1 to 4, with higher numbers indicating more severe signs of the disease).

Recruitment of patients began January 18, 2008, at 3 clinical centers. The recruitment finished November 12, 2009, and the study was completed on September 13, 2010. A preliminary assessment of each patient was carried out in the first basal visit by an orthopaedic surgeon, 30 days before randomization, and the

Table 1. *Inclusion and Exclusion Criteria*

Inclusion Criteria	Exclusion Criteria
Male and female patients aged between 40 and 72 yr	Bilateral knee OA requiring infiltration in both knees
Diagnosed with tibiofemoral OA of knee by radiography	BMI ≥33
Joint pain >35 mm on 0- to 100-mm visual analog scale	Suffering from polyarticular disease
Radiologic severity Ahlbäck grade <4	Severe mechanical deformity (diaphyseal varus deformity of 4° and valgus of 16°)
BMI ranging between 20 and 32	Previous arthroscopy within last year
Possibility for observation during follow-up period	HA intra-articular infiltration within last 6 mo
	Systemic autoimmune rheumatoid disease (connective tissue disease and systemic necrotizing vasculitis)
	Glycosylated hemoglobin above 7%
	Blood disorders (thrombopathy, thrombocytopenia, anemia with hemoglobin <9)
	Undergoing immunosuppressive therapy and/or warfarin
	Having undergone treatment with steroids during 3 mo before inclusion in study
	Treatment with NSAIDs during 15 d before its inclusion in study

medical history was completed. Patients were only included in the study if they met all inclusion/exclusion criteria shown in Table 1. Each patient also received a booklet that contained detailed instructions and the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) questionnaire. This booklet had to be completed by the patient and carried along with him or her at each of the following visits.

Interventions

All patients who met the inclusion criteria (176 of 187 enrolled initially because 11 patients had already been excluded) were scheduled at the first visit and received either of the 2 active treatments under study depending on the randomization made previously: infiltration of the affected knee with PRGF-Endoret (3 injections on a weekly basis) or infiltration of the affected knee with HA (Euflexxa; Copenhagen, Denmark) (3 injections on a weekly basis).

To prepare the PRGF-Endoret, at each treatment visit, 36 mL of peripheral blood was extracted from each patient by venipuncture directly into 4 extraction tubes containing 3.8% sodium citrate as anticoagulant. The extracted blood was centrifuged at 580*g* for 8 minutes at room temperature in a BTI Biotechnology Institute system centrifuge. Once the blood tubes were centrifuged, we proceeded to physically separate the plasma fractions by meticulous pipetting and under strictly sterile conditions.

We pipetted only the 2 mL of plasma rich in platelets remaining above the red series and the "buffy coat," avoiding picking up the leukocytes. Before infiltration, all these 2-mL fractions were put together in a single tube (total, 8 mL), with gentle inversion of the tube in a sterile glass container where it would be activated before infiltration, by adding 400 μL of calcium chloride.

Randomization and Allocation Concealment

A total of 3 treatment visits were carried out with a weekly periodicity. During these visits, the treatment assigned by randomization was delivered. A stratified randomization (1 stratum per center) was carried out. Both the evaluators and patients remained blind to the treatments.

All subjects included in the study were identified by a patient number after signing informed consent forms. Each patient was identified by a numerical code. The correspondence between the number of patients and their treatment was performed using specific software for randomization, keeping that relation in a sealed envelope. This envelope was not opened until the moment before applying the treatment. To maintain masking, the application area was hidden from view and blood was drawn for all patients to prepare the PRGF-Endoret.

Procedures

All subjects underwent blood draw an hour before application of the treatment. Patients were recalled for follow-up visits 1, 2, and 6 months after the last treatment administration. The only permitted medication throughout the clinical trial was acetaminophen. The intake of any type of NSAID was an exclusion criterion. The amount of acetaminophen consumed by

each patient for each treatment and at follow-up visits was recorded. Acetaminophen consumption was measured by counting the number of empty containers that were previously administered in the previous follow-up visit.

Response was assessed by researchers not involved in the application of treatment. The data report forms did not make any reference to the treatment applied.

Outcome Measures

Efficacy Assessments: The primary efficacy outcome was defined as the percentage of patients having a 50% decrease in the summed score for the WOMAC pain subscale from baseline to week 24. We measured this outcome by applying the WOMAC questionnaire compared with baseline therapy based on the criteria of the Outcome Measures for Rheumatology Committee and Osteoarthritis Research Society International Standing Committee for Clinical Trials Response Criteria Initiative (OMERACT-OARSI).

The secondary efficacy outcomes included the scores on the WOMAC subscales for stiffness and physical function, the percentage of OMERACT-OARSI responders, and the amount of acetaminophen in milligrams per day. The evolution from baseline in overall knee pain after application of the visual analog scale that ranged from 0 to 100 was determined by the WOMAC and Lequesne scales.

Safety Assessments: The nature, onset, duration, severity, and outcome of all adverse events, as well as any association of an adverse event related to the study medication, were assessed and documented at each visit. Indeed, the only permitted medication throughout the clinical trial was acetaminophen. The intake of any type of NSAIDs was an exclusion criteria and a reason to be excluded from the study.

To evaluate the safety profile of the treatments, all complications and/or adverse events were recorded with an accountability scale. The use of rescue medication was recorded daily in the patients' diaries.

Sample Size Calculation

A sample size of 220 patients, with 110 subjects per group, was estimated to provide at least 90% power to detect differences in the proportions of patients achieving 50% pain improvement with PRGF infiltration versus HA at a 5% level of significance. We calculated the sample size using the exact test with the aim of comparing 2 proportions by applying the χ^2 test assuming that the proportion of patients who would achieve an improvement in pain over 50% would be 30% in the experimental group versus 12% in the control group.

Data Analysis

Initially, we performed a descriptive analysis of the sample, taking into account the demographic and clinical variables of patients. Quantitative variables (age, body mass index [BMI]) were determined by the mean, standard deviation, and range, and for qualitative variables (gender, marital status, education level, physical activity, history, medication type, and severity of radiologic OA), a frequencies analysis was conducted.

Analysis of the primary outcome measure was conducted according to the intention to treat. The baseline comparability of treatment groups was performed by applying a Student t test for quantitative variables and a χ^2 analysis for categorical variables. The primary efficacy variable was assessed using a χ^2 test. Secondary efficacy variables were evaluated using either a χ^2 test for qualitative variables or a Student t test for quantitative variables. For all outcomes, a nominal $P < .05$ was considered to indicate statistical significance.

RESULTS

A total of 187 patients were screened, and 176 underwent randomization (Fig 1). The most common reason for exclusion included a BMI higher than 32 (6 patients), the inability to meet radiographic criteria (4 patients), and a genu varus deformity of the knee (1 patient). A slightly higher percentage of patients were women (52%), with a mean age of 59.8 years and a mean BMI of 28. The groups were well balanced in terms of age, gender, BMI, percentage of patients with primary arthritis, consumption of analgesics per day, radiographic grade (Ahlbäck scale), and WOMAC and Lequesne scores (Table 2). A total of 10 patients from the PRGF group and 13 from the hyaluronic group were excluded from the study. The exclusion and withdrawal percentages did not differ significantly between the groups.

Clinical Outcomes

Results of primary and secondary outcome measures for the entire study population and each WOMAC pain stratum are summarized in Table 3. Analysis of the primary outcome measure (defined as the percentage of patients having a 50% decrease in the summed score for the WOMAC pain subscale

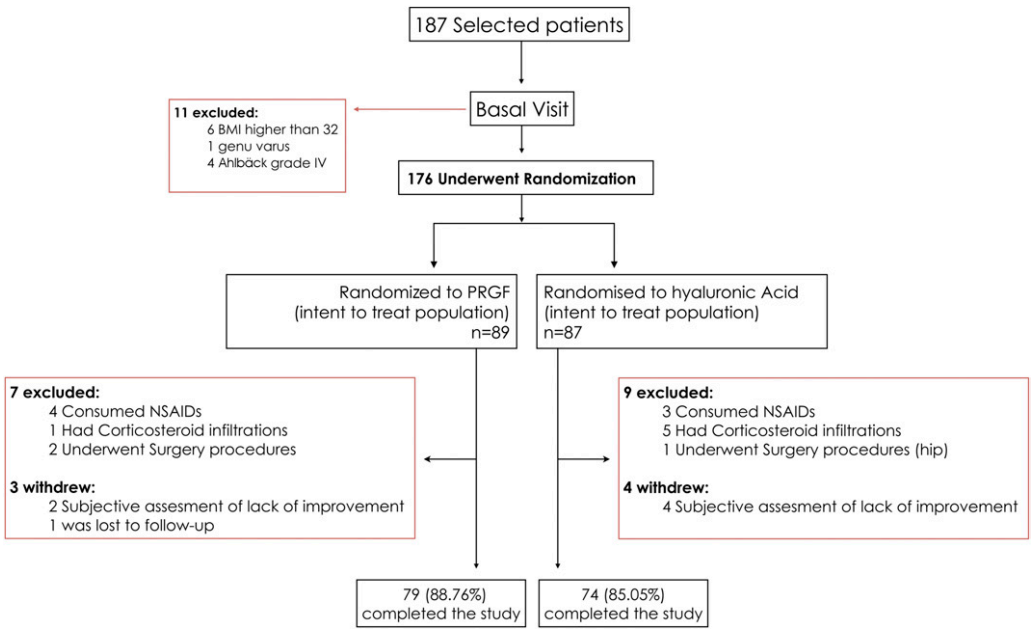

FIGURE 1. Enrollment and outcomes.

from baseline to week 24) showed that the rate of response to PRGF-Endoret was significantly higher than the rate of response to HA. Compared with the rate of response to HA, the rate of response to PRGF-Endoret was 14.1 percentage points higher (95% confidence interval, 0.5 to 27.6; $P = .044$). Regarding the secondary outcome measures, the rate of response to PRGF-Endoret was higher than the rate of response to

TABLE 2. *Baseline Characteristics of Patients*

	PRGF	HA	*P* Value
Age (yr)	60.5 ± 7.9	58.9 ± 8.2	.198
Sex (% female patients)	46 (52)	45 (52)	.996
BMI (kg/m^2)	27.9 ± 2.9	28.2 ± 2.7	.590
Primary arthritis	73 (82%)	68 (78%)	.521
Dose of acetaminophen (mg/d)	2.6 ± 7.1	1.7 ± 5.6	.631
Ahlbäck grade*			
I	45 (51%)	42 (49%)	.973
II	32 (36%)	32 (38%)	
III	12 (13%)	11 (13%)	
Normalized WOMAC score†			
Pain subscale	40.4 ± 16	38.4 ± 5.6	.417
Stiffness subscale	41.8 ± 17.3	38.5 ± 18.3	.233
Physical function subscale	39.6 ± 16.3	38.8 ± 17.4	.755
Global	121.8 ± 44.4	115.6 ± 45.1	.378
Lequesne index‡	9.5 ± 3.0	9.1 ± 3.2	.408
No.	89	87	

NOTE. Quantitative variables are expressed as mean and SD, except acetaminophen, which is expressed as median and range. Qualitative variables are shown as absolute and relative frequencies. $P < .05$ is considered statistically significant.

*Grade I indicates joint space narrowing (joint space <3 mm); grade II, joint space obliteration; and grade III, minor bone attrition (0 to 5 mm).

†Normalized scores for the WOMAC can range from 0 to 100 for all subscales.

‡Lequesne score is an index of severity for OA of the knee that includes 3 subscales (pain or discomfort, maximum distance walked, and activities of daily living). To assess the severity of gonarthrosis, the sum of all points is determined, with a minimum score of 0 and maximum of 24, where 0 indicates no severity, 1 to 4, mild; 5 to 7, moderate; 8 to 10, severe; 11 to 13, very severe; and 14 or greater, extremely severe.

TABLE 3. *Primary and Secondary Outcomes*

	PRGF	HA	Proportion Mean Difference (95% Confidence Interval)* Dif (95% CI)	*P* Value
No. of patients	89	87		
50% decrease in WOMAC pain score [No. (%)]	34 (38.2)	21 (24.1)	14.1 (0.5-27.6)	.044
OMERAT-OSARSI responders [No. (%)]†	47 (52.8)	43 (49.4)	3.4 (−11.4-18.1)	.653
20% decrease in WOMAC pain score [No. (%)]	51 (57.3)	46 (52.9)	5.2 (−10.3-19.1)	.555
Normalized WOMAC pain score‡				
% change from baseline	−35.0 ± 41.6	−21.8 ± 73.1	13.1 (−5.8-32.1)	.172
At end of follow-up	24.1 ± 15.5	26.9 ± 15.8	2.8 (−2.2-7.9)	.265
Normalized WOMAC stiffness score				
% change from baseline	−37.2 ± 40.6	−31.5 ± 41.6	5.6 (−7.7-19.0)	.403
At end of follow-up	25.2 ± 15.4	25.5 ± 17.9	0.3 (−5.0-5.7)	.901
Normalized WOMAC physical function score				
Change from baseline	−33.9 ± 39.0	−29.3 ± 38.8	4.6 (−7.8-17.1)	.465
At end of follow-up	24.8 ± 15.9	25.9 ± 17.2	1.1 (−4.2-6.4)	.682
Normalized WOMAC total score				
% change from baseline	−35.1 ± 38.4	−32.5 ± 31.9	2.7 (−8.7-14)	.642
At end of follow-up	74.0 ± 42.7	78.3 ± 48.1	4.3 (−10.2-18.8)	.561
Lequesne index§				
% change from baseline	−43.9 ± 34.6	−40.2 ± 39.4	3.7 (−8.1-15.5)	.534
At end of follow-up	5.2 ± 3.4	5.4 ± 3.3	0.2 (−0.9-1.3)	.714
Acetaminophen [median (range)] (g/d)	0.1 (2.0)	0.1 (2.3)		.853

NOTE. A primary response was defined as a 50% decrease in the summed score for the pain subscale of the WOMAC. Quantitative variables are expressed as mean and SD, except acetaminophen, which is expressed as median and range. Qualitative variables are shown as absolute and relative frequencies. $P < .05$ is considered statistically significant.

*Mean difference is shown for normalized WOMAC scores and Lequesne index. Otherwise, the proportion difference is shown.

†OMERACT-OARSI Outcome Measures in Rheumatology Clinical Trials-Osteoarthritis Research Society and Health Assessment Questionnaire.

‡Normalized scores for the WOMAC can range from 0 to 100 for all subscales.

§Lequesne score is an index of severity for OA of the knee that includes 3 subscales (pain or discomfort, maximum distance walked, and activities of daily living). To assess the severity of gonarthrosis, the sum of all points is determined, with a minimum score of 0 and maximum of 24, where 0 indicates no severity, 1 to 4, mild; 5 to 7, moderate; 8 to 10, severe; 11 to 13, very severe; and 14 or greater, extremely severe.

HA in all cases, although no significant differences were reached.

Overall, the rate of use of rescue acetaminophen was low (Table 3). There were no significant differences in the use of acetaminophen between the groups for all randomized patients or within each pain stratum.

Fifty adverse events were reported in 50 patients, 26 in the PRGF-Endoret group and 24 in the HA group (Table 4). Adverse events were generally mild and evenly distributed between the groups ($P = .811$). Most of these adverse events (96% in the PRGF-Endoret group and 92% in the HA group) were not related to the type of treatment. The number of patients who withdrew because of adverse events was similar between groups (Fig 1).

One patient who received HA felt numbness in the infiltration area, and another patient in this group had itching on the outside area of both thighs. One patient treated with PRGF-Endoret had pain after the third infiltration. All the adverse events disappeared in 48 hours.

DISCUSSION

We conducted the first randomized, double-blind, HA-controlled, multicenter trial to rigorously evaluate the efficacy and safety of intra-articular injections of PRGF-Endoret in the treatment of pain caused by OA of the knee. Three injections of PRGF-Endoret, an autologous pool of growth factors and fibrin scaffold biomaterial, resulted in clinically significant reductions in knee pain, stiffness, and in improving the physical function in patients with knee OA. The analysis of the primary outcome showed that PRGF-Endoret was significantly more effective than HA. Clinically meaningful pain relief is in general defined

TABLE 4. *Adverse Events*

	Adverse Event	Grade	Relation to the Treatment	Evolution	Serious Adverse Event or Unexpected
HA group					
1	Low back pain	1	Possible	Resolved	No
2	Low back pain	1	Unrelated	Resolved	No
3	Febrile syndrome	1	Unrelated	Resolved	No
4	Left knee surgery	1	Unrelated	Resolved	No
5	Abdominal pain and dizziness	2	Unrelated	Persistent	No
6	Toothache	1	Unrelated	Resolved	No
7	Flu	2	Unrelated	Resolved	No
8	Trauma	1	Unrelated	Resolved	No
9	Knee and hip pain	2	Unrelated	Resolved	Yes
10	Right knee pain	1	Unrelated	Persistent	No
11	Low back pain	2	Unrelated	Resolved	No
12	Toothache	2	Unrelated	Resolved	No
13	Ankle sprain	1	Unrelated	Resolved	—
14	Renal colic	1	Unrelated	Resolved	No
15	Back pain	2	Unrelated	Resolved	No
16	Bronchitis	2	Unrelated	Resolved	No
17	Neck pain	2	Unrelated	Resolved	No
18	Low back pain	3	Unrelated	Resolved	No
19	Itching both outer thighs	1	Unrelated	Resolved	No
20	Headache	2	Highly likely	Resolved	No
21	Low back pain	1	Unrelated	Resolved	No
22	Headache	1	Unrelated	Resolved	No
23	Right knee pain	2	Unrelated	Resolved	No
24	Low back pain	2	Unrelated	Resolved	No
PRGF group					
1	Dizziness	1	Unrelated	Resolved	No
2	Acute knee pain	1	Unrelated	Resolved	No
3	Left hip pain	3	Unrelated	Resolved	—
4	Other knee pain	1	Unrelated	Resolved	No
5	Left knee pain	1	Unrelated	Resolved	No
6	Contracture lumbar	4	Unrelated	Resolved	—
7	Urine infection	1	Unrelated	Resolved	No
8	Low back pain	1	Unrelated	Resolved	No
9	Headache	2	Unrelated	Resolved	No
10	Sciatica	2	Unrelated	Resolved	No
11	Knee trauma during study	3	Unrelated	Resolved	Yes
12	Fall/back pain	2	Unrelated	Resolved	No
13	Pain after third injection	3	Highly likely	Resolved	No
14	Shoulder pain	1	Unrelated	Resolved	No
15	Left knee contusion	1	Unrelated	Resolved	No
16	Right shoulder pain	1	Unrelated	Persistent	No
17	Cold	1	Unrelated	Resolved	No
18	Cold	1	Unrelated	Resolved	No
19	Right knee pain	3	Unrelated	Persistent	No
20	Left knee pain	2	Unrelated	Persistent	No
21	Back pain	1	Unrelated	Resolved	No
22	Headache	1	Unrelated	Resolved	No
23	Cold	1	Unrelated	Resolved	No
24	Coxalgia	1	Unrelated	Resolved	No
25	Right knee pain	1	Unrelated	Persistent	No
26	Right knee pain	1	Unrelated	Persistent	No

as a reduction in pain intensity of more than 30% from the baseline level,[24,25] and a reduction of 50% is considered as high improvement in pain according to the OMERACT-OARSI criteria.[26] In this study the percentage of patients at the end of follow-up with a primary response to PRGF-Endoret was 38.2, whereas

the rate of response to HA was 24.1%. In addition, the rate of response to each treatment followed an opposite pattern, with a substantial improvement of the primary outcome in the PRGF-Endoret group at 24 weeks and a gradual decrease in the case of the HA group. These data may suggest that, in addition to the HA action,[18] the PRGF-Endoret has other beneficial biological effects on cartilage in the long run. All the secondary outcome measures decreased with both active treatments, and no significant differences were found between groups. These results may have important considerations for the medical community.

Mechanical stress and growth factors play a pivotal role in modulating the phenotypic expression of chondrocytes. The pool of growth factors obtained from platelet-rich plasma decreases nuclear factor–κB activation, a major pathway involved in the pathogenesis of OA, which is characterized by a catabolic and inflammatory joint environment.[27] Moreover, the supernatant of autologous proteins also inhibits matrix metalloproteinase 13 production by interleukin 1β– and tumor necrosis factor α–stimulated human articular chondrocytes.[28]

Most of the adverse events that were reported by patients were mild in severity. Most of the adverse events were not related to the type of treatment, and they were evenly distributed between the groups.

The limitations of this study include the lack of measurement of physical activity levels in patients after applying the treatments, the different experience of physicians in the implementation of PRGF-Endoret treatment, the lack of longitudinal analysis and subgroup analysis for participating centers, the short-term follow-up of 24 weeks, the lack of a placebo group, and the exclusion of patients who had the highest degree of severity on radiography (Ahlbäck grade 4). However, our study had a mean score for knee pain on the visual analog scale on the day of randomization of 56 ± 15, and 20% of the patients in our study had a score over 70.

Although several studies have evaluated the potential of PRGF-Endoret[22,29] and other platelet-rich plasma products,[30] our study is the first randomized, controlled, multicenter trial that shows that PRGF-Endoret is safe and effective in the treatment of patients with OA of the knee, with the beneficial effects persisting for 24 weeks. This autologous technology has European Conformity and Food and Drug Administration clearance to be used for the treatment of musculoskeletal injuries.

CONCLUSIONS

PRGF showed superior short-term results when compared with HA in a randomized controlled trial, with a comparable safety profile, in alleviating symptoms of mild to moderate OA of the knee.

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Intraosseous Infiltration of Platelet-Rich Plasma for Severe Knee Osteoarthritis

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Abstract: We describe a new technique of platelet-rich plasma (PRP) infiltration for the treatment of severe knee osteoarthritis. PRP intra-articular infiltration is a promising treatment for knee osteoarthritis, but it still has some limitations in high-degree osteoarthritis. Diagnosis of osteoarthritis is based on clinical and radiographic findings, and patients with grade III or IV knee tibiofemoral osteoarthritis based on the Ahlbäck scale are considered candidates for this technique. The technique consists of performing intraosseous infiltration of PRP into the subchondral bone, which acts on this tissue and consequently on cartilage-bone communication. Although the intraosseous injection hinders the conventional knee intra-articular infiltration, it allows an extension of the range of action of the PRP, which acts directly on the subchondral bone, which is involved in the progression of osteoarthritis. Thus this technique involves a new administration of PRP that can delay knee arthroplasty; moreover, it can be applied for not only severe osteoarthritis but also other pathologies in which the subchondral bone is critical in the etiology, such as necrosis and osteochondral lesions.

Osteoarthritis (OA) is a disease of the synovial joints that evolves with pain, loss of motion, and deformation of affected joints. Initially, the pathogenesis of OA was focused almost exclusively on the cartilage; however, nowadays, it is considered a disease that involves all tissues of the joint, all of which are crucial for maintaining articular homeostasis. Both genetic and acquired or environmental factors can disrupt this anabolic-catabolic balance, resulting in cartilage degeneration, osteophyte formation, and inflammation of the synovial membrane and becoming a clinical problem.[1] Currently, no treatment can stop the progression of OA or reverse the damage, making joint replacement the only solution for these patients. Conservative treatment include oral pharmacologic treatment, such as analgesics, nonsteroidal anti-inflammatory drugs, or symptomatic slow-acting drugs for OA, and intra-articular infiltrations of steroids and hyaluronic acid, focused on relieving the symptoms but not on stopping the disease.[2]

In recent years intra-articular infiltrations of platelet-rich plasma (PRP) have emerged as an alternative to current treatments. This biological therapy uses the patient's own platelets and plasma, which mainly convey fibrin and growth factors as effectors. These growth factors act on the entire joint and may well have an influence on the development of OA; they promote restoration of joint homeostasis, have inductive and protective effects on chondrocytes, and stimulate the production of hyaluronic acid by synoviocytes. All these properties help to promote a generative biological environment and to slow down joint and cartilage degeneration, thereby relieving symptomatology.[3] Several clinical trials showing promising results have been published; however, there are still some doubts about whether this form of administration is able to reach the deeper layers of the cartilage and subchondral bone, thereby possibly limiting the growth factors' therapeutic potential especially in severe OA.[4,5]

In light of recent studies showing the importance of the subchondral bone in the pathogenesis of OA and showing cartilage–subchondral bone communication,[6] we propose a combination of intra-articular and intraosseous injections to treat severe OA. With this combination, it is possible to expand the effective range of PRP by acting not only on the subchondral bone and, consequently, on its cartilage communications but also

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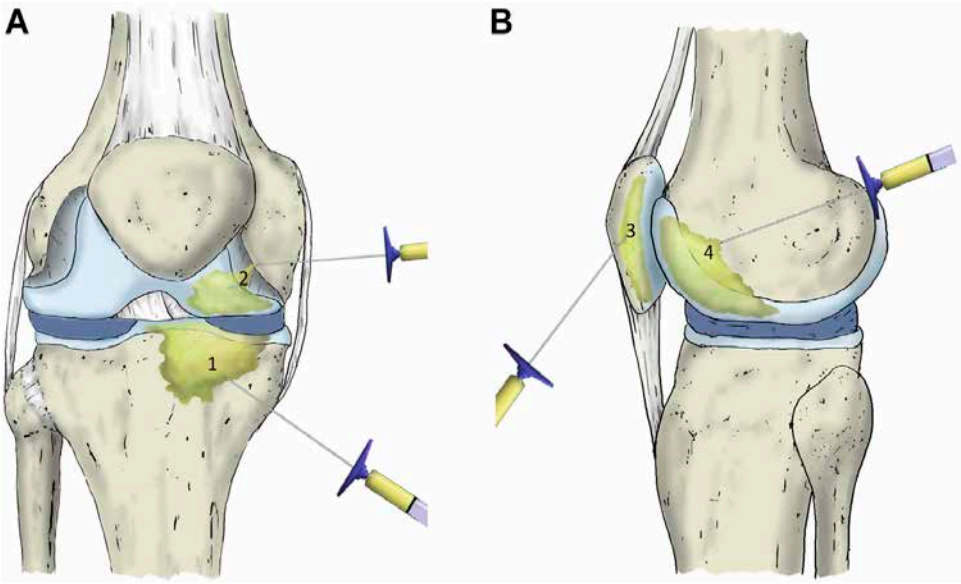

Fig 1. (A) The platelet-rich plasma (PRP) intraosseous infiltration of a knee with severe femorotibial osteoarthritis is performed into the medial tibial plateau (1) and medial femoral condyle (2). (B) If the patient presents with femoropatellar osteoarthritis, the approach is external and the patella (3) and trochlea (4) are infiltrated. Before these intraosseous injections are performed, conventional knee intra-articular infiltration of PRP is conducted.

on mesenchymal stem cells to modulate the affected tissue regeneration.[7]

Surgical Technique

Diagnosis

Diagnosis of OA is based on clinical and radiographic findings. The radiographs used are the weight-bearing anteroposterior view of the knee, the lateral view at 30° of knee flexion, and the axial view at 20° of knee flexion. Patients with grade III or IV knee tibiofemoral OA based on the Ahlbäck scale are considered candidates for our technique, which consists of a PRP intra-articular infiltration and 2 PRP intraosseous infiltrations into the medial femoral condyle and into the medial tibial plateau (Fig 1).

Patient Preparation

Before sedation is induced, about 80 mL of venous blood is extracted from the patient to prepare the PRP according to PRGF-Endoret technology (Biotechnology Institute, Vitoria-Gasteiz, Spain).[4] Sedation is performed by infusing a single dose of normal saline solution, as well as a single dose of midazolam (0.03 to 0.05 mg/kg)

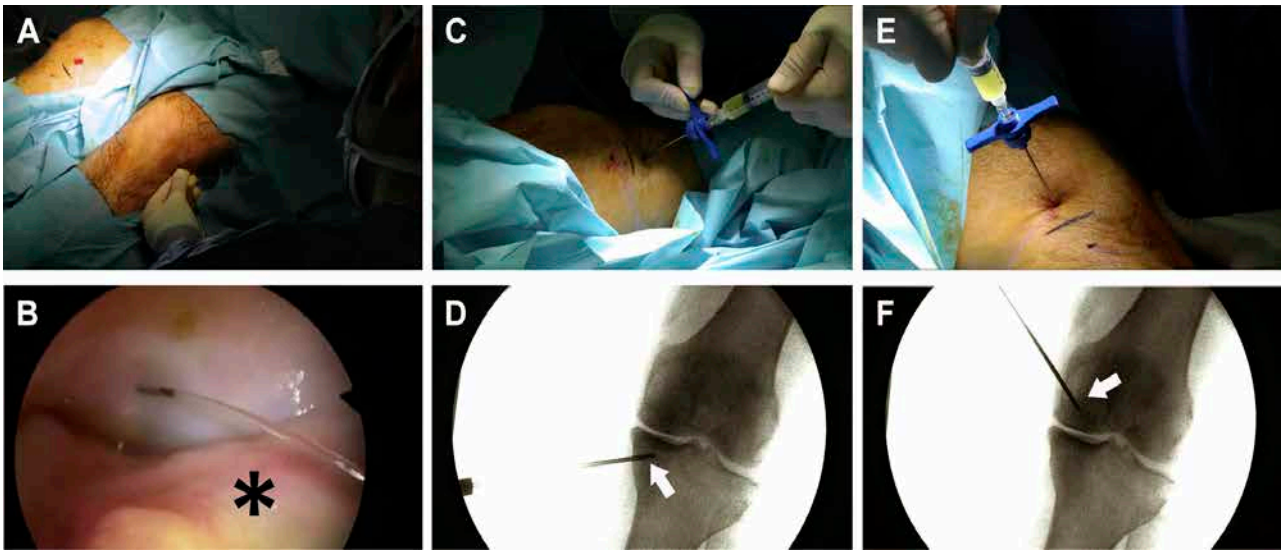

Fig 2. After the patient is positioned supine on the operating room table, (A) intra-articular infiltration is performed into the joint through the external patellar wing, centered in the central region of the patella in the craniocaudal plane; (B) the infiltration is directed into the midpoint area of the femoropatellar region using an external approach and preventing infiltration into the synovial membrane (asterisk). (C, D) Intraosseous tibial plateau infiltration is conducted into the medial tibial plateau, just to its middle area. The arrow indicates the trocar. (E, F) Concerning intraosseous femoral condyle infiltration, a trocar (arrow) is applied to the thickness of the medial femoral condyle, as far as the middle area of the medial condyle.

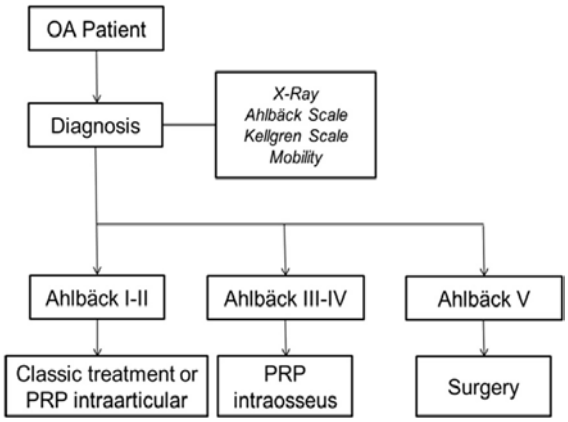

Fig 3. Patients are diagnosed with osteoarthritis (OA) based on physical examination findings and imaging techniques, using scales such as the Ahlbäck scale or Kellgren-Lawrence scale. Depending on the osteoarthritis grade, different treatments can be applied. If the patient presents with Ahlbäck grade I or II, we propose a classic treatment or intra-articular infiltration of platelet-rich plasma (PRP). If the patient presents with grade III or IV, we apply intraosseous infiltration with 2 intra-articular infiltrations in the subsequent weeks. If the patient presents with grade V, he or she undergoes a total knee arthroplasty.

and fentanyl (3.2 mg/kg), in the peripheral vein; a single or repeated dose of propofol is also administered (1 to 2 mg/kg), depending on the duration of the infiltration. The degree of sedation is −4 or −5 on the Richmond Sedation Scale. The patient is monitored according to the standards of the American Society of Anesthesiologists. The patient is positioned supine on the operating room table; the infiltration area is prepared with a povidone-iodine solution, covering a region 10 cm proximal and 10 cm distal to the infiltration zone. Sterile drapes defining the treatment zone (proximal, distal, medial, and lateral) are placed (Video 1).

Once the patient has been sedated and prepared and the PRP has been obtained, 2 marks are drawn in the medial region of the knee, one located 2 cm proximal and the other located 2 cm distal to the medial joint line and centered in the midline sagittal plane. Next, a 24-gauge needle is used to anesthetize the area of infiltration; it is introduced through the mark and moved into contact with the femoral condyle. Without retraction of the needle, the periosteum of the femoral condyle is infiltrated with 2 mL of 2% mepivacaine. Then, the needle is withdrawn and moved into contact with the inner face of the tibial plateau (through the other mark), and without retraction of the needle, the periosteum of the medial tibial plateau is infiltrated with 2 mL of 2% mepivacaine.

Table 1. Benefits

Stimulates subchondral bone
Reaches deeper layers of cartilage
Acts on molecules and mesenchymal stem cells of subchondral bone
Is applicable to high grades of osteoarthritis, necrosis, and osteochondral lesions

Table 2. Technical Pearls

Intra-articular infiltration is performed into the midpoint of the femoropatellar region using an external approach to prevent infiltration into the synovial membrane.
The use of a fluoroscope can facilitate trocar placement.
Although the patient is under sedation, local anesthesia is recommended.
After infiltration, it is advisable to apply ice to the area.

Intra-articular Infiltration

After application of local anesthesia, intra-articular infiltration is conducted first. We penetrate a 21-gauge needle into the joint through the external patellar wing, centered in the central region of the patella in the craniocaudal plane. Lateralization of the patella during infiltration facilitates this process (Fig 2A). After placement of the needle into the joint space, synovial fluid arthrocentesis can be performed if it is necessary. Once arthrocentesis has been carried out and without removal of the needle, 8 mL of PRP is infiltrated. The infiltration is directed into the midpoint area of the femoropatellar region using an external approach to prevent infiltration into the synovial membrane, which would cause pain (Fig 2B).

Intraosseous Tibial Plateau Infiltration

Once the area is anesthetized, PRP is infiltrated into the tibial plateau. A 13-gauge trocar used for bone biopsy (CareFusion, San Diego, CA) is introduced into the bone through the mark previously made. The trocar is placed 2 cm distal to the joint line, leaning on the periosteum; the trocar is then introduced 2 cm into the thickness of the medial tibial plateau (to the middle area of the medial tibial plateau), following a parallel direction to the articular surface. Once the trocar has been placed in the desired position, 5 mL of PRP is infiltrated through the trocar (Fig 2 C and D).

Intraosseous Femoral Condyle Infiltration

Next, PRP is injected into the femoral condyle. A 13-gauge trocar used for bone biopsy is introduced into the bone through the mark previously made. The trocar is placed 2 cm proximal to the joint line, leaning on the periosteum. Then, the trocar is introduced 2 cm into the

Table 3. Pitfalls

The technique requires training and practice; therefore the infiltration time is increased.
The technique requires patient sedation.
In the 48 hours after treatment, the patient has more pain than with conventional infiltration.
Treatment will be more complicated in case of an infection due to infiltration.

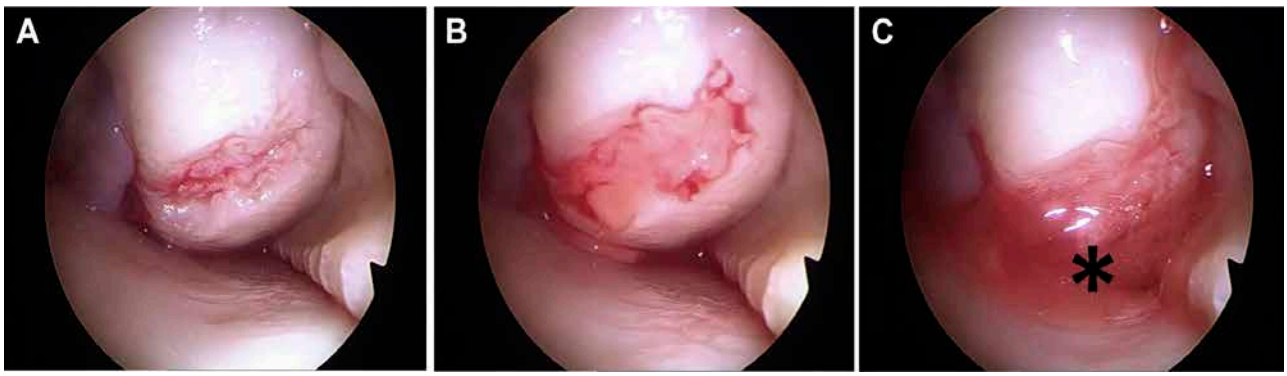

Fig 4. (A) Communications between cartilage and subchondral bone are more pronounced in degenerated cartilage. (B) The platelet-rich plasma infiltrated into subchondral bone flows through the degenerated zones, and because of its viscous consistency, (C) it remains in the area, creating a matrix (asterisk).

thickness of the medial femoral condyle (to the middle area of the medial condyle), following a parallel direction to the articular surface of the condyle. Once the trocar has been placed in the desired position, 5 mL of PRP is infiltrated through the trocar (Fig 2 E and F).

Finally, after completion of the infiltrations and removal of the sterile drapes, the skin is cleaned with an alcohol solution, with application of wound dressings at the infiltration points. After infiltration is completed, ice is applied to the site. In the days after surgery, the patient can bear weight and take analgesics (acetaminophen) as required for pain.

Patellofemoral OA

If patients present with severe patellofemoral OA, the same procedure as described earlier will be performed, but in these cases the local anesthesia and PRP are infiltrated into the patella and into the trochlear zone of the condyle. Both approaches will be conducted in the middle area, from the external side of the knee, with introduction of the trocar 2 cm into the thickness. In such cases 3 mL of PRP is infiltrated into the patella and 5 mL into the condyle.

Discussion

The frequency and chronicity of OA make it a challenge for the health and social systems of all developed countries. In affluent countries such as the United States, the numbers are staggering; estimates suggest that about 46 million patients have OA, with OA in more than 50% of adults older than 50 years. By 2030, this figure may reach 70 million.[8] Current treatments focus exclusively on relieving the symptoms but not on curing the disease, making joint arthroplasty the definitive option for patients.[2] The results obtained with new therapies such as PRP and the use of stem cells are promising but still have some limitations, such as the mode of administration. The most commonly used form of administration is intra-articular injection, which is effective in patients with mild degrees of OA but is not so effective in those with severe OA.[4] With this new administration technique for PRP, in which the intra-articular injection is combined with intraosseous infiltrations, treating patients with higher grades of OA is a possibility, giving them an alternative to knee arthroplasty or at least delaying this more radical intervention (Fig 3).

The main limitation of intraosseous infiltration is related to patient preparation, involving sedation and local anesthesia because of the subchondral bone injection. These factors, in addition to training of the medical team, make this technique take more time and make it more expensive than a conventional intra-articular injection. The pressure increment inside the bone could entail pain 48 hours after treatment, so the patient should be advised of this possibility. Sometimes, the use of fluoroscopic control (FMControl, Vitoria-Gasteiz, Spain) is also necessary for proper administration (Tables 1-3).

The aforementioned disadvantages are not present during intra-articular infiltration, but this form of infiltration does not reach the deeper layers of the cartilage and subchondral bone, thereby limiting its therapeutic potential. Recent studies have shown the importance of the subchondral bone in the pathogenesis of OA, and subchondral bone−cartilage communication has been shown in multiple experiments.[6,9] When homeostasis is disrupted because of biochemical and biomechanical offenders, all the tissues of the joint are involved in restoring biological balance. These efforts to restore homeostasis entail cellular and extracellular matrix responses in all tissues. Thus communications occur between the deeper layers of the subchondral bone and cartilage and, on the other hand, between these and the synovial fluid that surrounds the entire joint. This bone-cartilage communication has been described in studies showing channels that reach the cartilage from the subchondral bone, which are more abundant in the cartilage of patients with OA.[6]

Intraosseous infiltration exploits the communication between the cartilage and subchondral bone such that

PRP reaches the deeper layers of cartilage. There is a viscous consistency of PRP and the cellular material of subchondral bone that coagulates and remains in the areas of injured cartilage from which it has come (Fig 4). In addition, infiltrating PRP directly into the subchondral bone could act on this tissue and its mesenchymal stem cells; these cells would be maintained in the PRP matrix and modulate the repair process of subchondral bone, which has a direct impact on halting the progression of OA.[6] Therefore, with our technique, PRP could achieve a more extensive range of action and, thereby, higher effectiveness and could be useful not only in severe OA but also in other pathologies, such as necrosis of the condyle or tibial plateau, and during surgical treatment of osteochondral lesions.

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Clinical Study

Combination of Intra-Articular and Intraosseous Injections of Platelet Rich Plasma for Severe Knee Osteoarthritis: A Pilot Study

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The aim of this study was to assess a novel approach to treating severe knee osteoarthritis by targeting synovial membrane, superficial articular cartilage, synovial fluid, and subchondral bone by combining intra-articular injections and intraosseous infiltrations of platelet rich plasma. We explored a new strategy consisting of intraosseous infiltrations of platelet rich plasma into the subchondral bone in combination with the conventional intra-articular injection in order to tackle several knee joint tissues simultaneously. We assessed the clinical outcomes through osteoarthritis outcome score (KOOS) and the inflammatory response by quantifying mesenchymal stem cells in synovial fluid. There was a significant pain reduction in the KOOS from baseline (61.55 ± 14.11) to week 24 (74.60 ± 19.19), after treatment ($p = 0.008$), in the secondary outcomes (symptoms, $p = 0.004$; ADL, $p = 0.022$; sport/rec., $p = 0.017$; QOL, $p = 0.012$), as well as VAS score ($p < 0.001$) and Lequesne Index ($p = 0.008$). The presence of mesenchymal stem cells in synovial fluid and colony-forming cells one week after treatment decreased substantially from 7.98 ± 8.21 MSC/μL to 4.04 ± 5.36 MSC/μL ($p = 0.019$) and from 601.75 ± 312.30 to 139.19 ± 123.61 ($p = 0.012$), respectively. Intra-articular injections combined with intraosseous infiltrations of platelet rich plasma reduce pain and mesenchymal stem cells in synovial fluid, besides significantly improving knee joint function in patients with severe knee osteoarthritis. This trial is registered on EudraCT with the number 2013-003982-32.

1. Introduction

Knee osteoarthritis (KOA) is a mechanically induced, cytokine and enzyme-mediated disorder comprising different phases and phenotypes, with pain as the clinical hallmark of the disease [1]. This diarthrodial joint is a complex biological system where articular cartilage (AC), an aneural and avascular tissue, lies functionally sandwiched between two highly vascularized and innervated tissues, namely, synovial membrane (SM), which produces synovial fluid (SF), and subchondral bone (SB), both endowed with heat receptors, chemoreceptors, and mechanoreceptors. Nociceptive stimuli, coming from a microenvironment undergoing nonphysiological mechanical loading and/or proinflammatory cytokines and damage-associated molecular patterns (DAMPS), might initially lead to peripheral and eventually both peripheral and neuropathic pain traits by mechanisms yet to be fully identified [2–4]. Moreover, the aggression

to these tissues causes a surge of mesenchymal stem cells (MSCs) in SF as a part of tissue response to injury [5, 6].

In patients with severe OA, the subchondral bone undergoes changes which include microcracks and structural defects, vascularization of channels, nerve growth, and a progressive replacement of the subchondral marrow with fibroneurovascular mesenchymal tissue changes which underpin the increasingly recognized crosstalk and pathway for direct transport of growth factors such as transforming growth factor B (TGFβ) and nerve growth factor (NGF) and even for cells such as macrophages and MSCs between the subchondral bone and articular cartilage [7–10].

As it is yet to be established which of the joint tissues or structures is the primary driver of KOA and therapeutic strategies that solely target one cell or tissue may well prove to fail, it is advisable that approaches to treating KOA should aim at reaching several joint tissues [11].

In patients with severe KOA, platelet rich plasma (PRP) and many bioactive mediators present in it have been shown to exert positive effects on the homeostasis of joint tissues through chondroprotective, anabolic, anti-inflammatory, and immunomodulatory effects and to substantially reduce pain, relieve joint stiffness, and improve physical function [12–20]. The aim of this study is to assess a novel approach to treating severe KOA, targeting synovial membrane, superficial articular cartilage, synovial fluid, and subchondral bone by combining intra-articular injections and intraosseous infiltrations of PRP. The hypothesis was that the addition of intraosseous injections of PRP directly into the subchondral bone to conventional intra-articular treatment would achieve a positive effect on patients with severe KOA.

2. Patients and Methods

The study was carried out in accordance with the international standard on clinical trials: Real Decreto 223/2004, Declaration of Helsinki in its latest revised version (Fortaleza, Brazil; 2013), and Good Clinical Practice Regulations (International Conference for Harmonization). The study protocol was reviewed and approved by the Reference Ethics Committee. All patients provided written informed consent before entry into the study.

2.1. Patient Selection. Nineteen patients were initially assessed for eligibility. Patients were considered eligible if they were aged between 40 and 77 years and presented severe knee osteoarthritis according to radiographic confirmation (Ahlbäck degrees 3 and 4, on a scale from 1 to 4, with the highest degrees indicating more severe OA). Finally, 14 patients were enrolled in the study from January 2014. The inclusion and exclusion criteria that patients had to meet in order to be included in this study are as follows.

Inclusion criteria are the following:

Patients of both sexes aged 40 to 77 years.

Predominant internal tibiofemoral knee osteoarthritis.

Joint pain above 2.5 VAS points.

Radiographic severity degrees 3 and 4 according to Ahlbäck scale.

Values of body mass index between 20 and 33.

Possibility for observation during the follow-up period.

Exclusion criteria are the following:

Bilateral knee osteoarthritis which requires infiltration in both knees.

Values of body mass index > 33.

Polyarticular disease diagnosed.

Severe mechanical deformity (diaphyseal varus of 4° and valgus of 16°).

Arthroscopy in the last year prior to treatment.

Intra-articular infiltration of hyaluronic acid in the past 6 months.

Systemic autoimmune rheumatic disease (connective tissue diseases and systemic necrotizing vasculitis).

Poorly controlled diabetes mellitus (glycosylated hemoglobin above 9%).

Blood disorders (thrombopathy, thrombocytopenia, and anemia with Hb < 9).

Undergoing immunosuppressive therapy and/or warfarin.

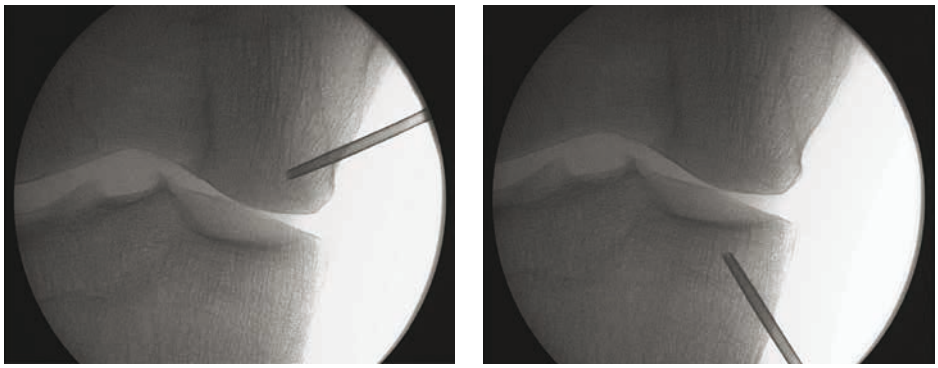
Treatment with corticosteroids during the 6 months prior to inclusion in the study.

The enrolment finished on 29 October 2014 and the pilot study was completed on 10 June 2015.

In the first visit, an orthopedic surgeon conducted a clinical and radiographic assessment of each patient, including their medical history and a complete blood count. Moreover, the doctor delivered a booklet that contained detailed instructions and the knee injury and osteoarthritis outcome score (KOOS) questionnaire, which had to be completed by the patients at the baseline visit and before follow-up visits. Patients were allowed to consume acetaminophen, but it was restricted 48 hours before filling the questionnaires.

Patients were identified by a code number and scheduled to undergo the experimental procedure, which consisted of three treatments of PRP on a weekly basis. The first treatment included one PRP intra-articular infiltration and two PRP intraosseous infiltrations (femoral condyle and tibial plateau). The next two treatments were conventional intra-articular injections.

2.2. PRP Preparation. 90 mL of venous blood was extracted from the patient in order to prepare the PRP and withdrawn into 9 mL tubes containing 3.8% (wt/V) sodium citrate. Blood was centrifuged at 580 g for 8 minutes at room temperature. The 2 mL plasma fraction located just above the sedimented red blood cells, but not including the buffy coat, was collected in a tube and carried to the injection room for use. This plasma fraction preparation contained a moderate concentration of platelets (2 to 3 times the concentration of platelets



(a) (b)

Figure 1: Fluoroscopic images. Intraosseous infiltration into the medial femoral condyle (a) and tibial plateau (b).

compared with peripheral blood, depending on the platelet count and size as well as the hematocrit) and an absence of erythrocytes and leukocytes [21]. To initiate the activation of platelets clotting, calcium chloride (10% wt/V) was added to the liquid PRP aliquots just before injection. All procedures were performed under sterile conditions.

2.3. Treatment. In the patient's first treatment, one PRP intra-articular injection and two PRP intraosseous injections were performed. Under anesthesiologist surveillance, sedation of the patient was induced by infusing a single dose of midazolam (0.03–0.05 mg/kg) and fentanyl (3.2 mg/kg), in a peripheral vein; single or repeated dose of propofol was also administered (1-2 mg/kg), depending on the duration of the infiltration. The degree of sedation was −4 or −5 on Richmond Sedation Scale. The patient was positioned in a supine position on an operating room table and two marks were drawn in the medial region of the knee, one located 2 cm proximal and the other located 2 cm distal to medial joint line; the infiltration area was prepared with a povidone-iodine solution. Local anesthesia was conducted by injecting 2 mL of 2% mepivacaine into the periosteum of condyle and tibial plateau. After evacuating the totality of the synovial fluid, 8 mL of PRP (the first intra-articular infiltration of a series of three) was infiltrated intra-articularly through the mid-point area of the femoropatellar region using a lateral approach in order to reach the joint space after lateralization of the patella. Intraosseous infiltrations were performed with a 13 G trocar used for bone biopsy, which was manually introduced into the bone and inserted 2 cm into the medial tibial plateau and medial femoral condyle. Once the trocars were placed in the desired position, 5 mL of PRP was infiltrated into subchondral bone of each structure. The control of trocar placements was facilitated by using a fluoroscope (Figure 1) [22]. After intraosseous infiltration is completed, ice is applied to the site. In the days after surgery, the patient can bear weight and take analgesics (acetaminophen) as required for pain. It is worth mentioning that the application of intra-articular and intraosseous infiltrations of PRP does not entail any reduction in physical activity and patients resume their daily activities few hours after the procedure is performed.

Two more intra-articular PRP infiltrations were performed 7 and 14 days after the first treatment. Moreover, the synovial fluid evacuated prior to the infiltrations was preserved for analysis.

2.4. Follow-Up. Patients were called for follow-up visits 2 and 6 months after the last treatment visit in order to conduct clinical evaluation. During these visits, the patient submitted the questionnaires given at baseline. A rheumatologist carried out a clinical examination and an evaluation of pain and function by visual analogue scale (VAS) and Lequesne Index, respectively. Acetaminophen consumption was also controlled.

2.5. Clinical Outcomes. The primary outcome was defined as the decrease in knee pain from the baseline to second month and sixth month (endpoint), according to the KOOS questionnaire. Furthermore, measurement of VAS and Lequesne Index was also evaluated; the secondary outcomes included the other areas of KOOS: symptoms, function in daily living (ADL), function in sport and recreation (sport/rec.), and knee related quality of life (QOL).

2.6. Safety Outcomes. To evaluate the safety of treatment, all complications and adverse events were assessed and reported during patient visits. Their nature, onset, duration, and severity were documented.

2.7. Biological Outcomes. Presence of mesenchymal stem cells (MSC) in synovial fluids before and one week after intraosseous infiltration was evaluated by flow cytometry and cultures of colony-forming cells (CFU-F). Concerning flow cytometry, each sample was immunophenotyped using an 8-color direct immunofluorescence technique. Concentrated cell suspensions were stained with the following combination of monoclonal antibodies (MoAb) in order to detect the expression of CD105/CD45/CD73/CD271/CD34/CD13/CD90/CD44: [Brilliant violet (BV) 421/orange chrome (OC) 500/fluorescein isothiocyanate (FITC)/phycoerythrin (PE)/peridinin chlorophyll protein-cyanin 5.5 (PerCP

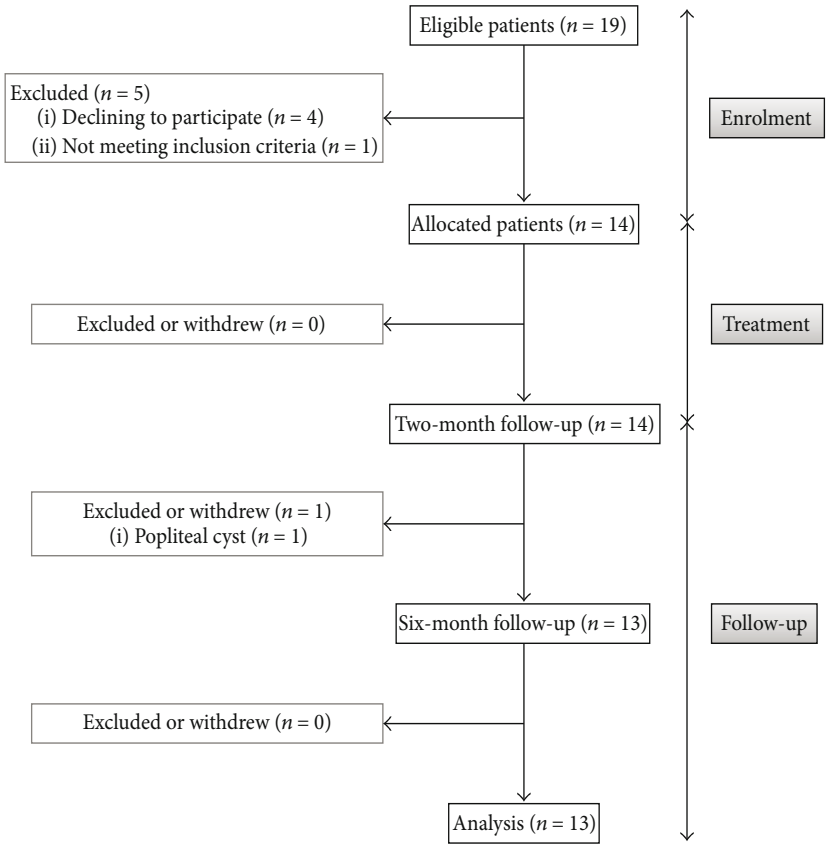


Figure 2: Enrolment and outcomes.

-Cy5.5)/PE-cyanin 7 (PECy7)/allophycocyanin (APC)/APCH7]. Regarding CFU-F assay, collected synovial fluids were diluted in phosphate buffered saline (PBS) and centrifuged in order to harvest the cellular content. The sample was used for colony-forming assay (CFU-F) and seeded on a 100 mm diameter culture plate. Seven days later, plating colonies were noted and counted by 0.5% crystal violet staining.

2.8. Sample Size Calculation. Power analysis was conducted to estimate the minimum sample size needed to achieve 80% power at a 5% level of significance for the primary outcome measures. An assumed effect size of 10 points (minimal clinically important change, MIC) with a standard deviation (SD) of 12 points was used [23]. This analysis suggested a minimum of 13 patients, expecting a dropout rate of 0.1.

2.9. Statistical Analysis. Demographic and medical variables (gender, age, and OA grade) were determined by the mean, standard deviation, range, and percent. For this study, a pair protocol analysis was used. Comparisons were performed by Student's t-test for paired-samples parametric data or Wilcoxon signed-rank test for paired-samples nonparametric data, after assessing the normal distribution of the samples by Shapiro-Wilk test. Data were considered statistically significant when $p < 0.05$. Statistical analysis was performed with SPSS 17.0 (SPSS, Chicago, IL).

3. Results

A total of 19 patients were considered eligible to participate in this study, and 14 patients were finally enrolled (Figure 2). Of the 5 excluded patients, four declined to participate and one presented predominant lateral osteoarthritis. Of the remaining 14 patients, 13 completed the study and one was excluded during the follow-up period due to a popliteal cyst.

Nine of the thirteen patients who finished the study were men and four were women, with a mean age of 62 ± 10 years (range: 47–75 years). Nine patients were diagnosed with OA III and five were diagnosed with OA IV, according to Ahlbäck scale (Table 1).

3.1. Clinical Outcomes. Table 1 summarizes results of primary and secondary outcome measures for the entire population that completed the study. Analysis of the primary outcome measure (as the decrease in knee pain from baseline to week 24, according to the KOOS questionnaire) showed a statistically significant improvement in pain reduction from 61.55 ± 14.11 at baseline to 74.60 ± 19.19 six months after treatment ($p = 0.008$). Eleven patients improved, and 8 patients reported minimal clinically important improvement (MCII) (Table 1). Depending on the osteoarthritis grade, eight of the 9 patients with degree 3 showed improvement as did 3 of the 4 patients with degree 4.

Regarding secondary outcomes, there was also a statistically significant improvement in all other areas of the KOOS

Table 1: Demographic data and biological and clinical outcomes.

Demographic data						
Patients	Total: n	Men: n (%)	Women: n (%)	Age: mean ± SD (range)	OA III: n (%)	OA IV: n (%)
	13	9 (69.23)	4 (30.77)	62.23 ± 9.6 (47–75)	9 (69.23)	4 (30.77)
Biological outcomes						
	Baseline: mean ± SD	One week after infiltration: mean ± SD	p			
MSC/μL	7.98 ± 8.21	4.04 ± 5.36	0.019*			
CFU-F/mL	601.75 ± 312.30	139.19 ± 123.61	0.012*			
Clinical outcomes						
	Baseline: mean ± SD	Endpoint: mean ± SD	p	δ: mean ± SD (% change)	Improved patients: n (%)	Patients with MCII [22]: n (%)
KOOS pain	61.55 ± 14.11	74.60 ± 19.19	0.008*	13.10 ± 14.89 (24.19 ± 40.07)	11 (84.62)	8 (61.53)
KOOS symptoms	60.56 ± 17.35	71.70 ± 18.82	0.004*	11.14 ± 11.34 (19.73 ± 25.42)	11 (84.62)	8 (61.53)
KOOS ADL	68.44 ± 14.08	80.86 ± 15.58	0.022*	12.45 ± 17.31 (23.25 ± 38.82)	11 (84.62)	8 (61.53)
KOOS sport/rec.	29.23 ± 20.29	45.38 ± 22.40	0.017*	11.78 ± 11.54 (76.94 ± 115.23)	10 (76.92)	7 (53.84)
KOOS QOL	28.10 ± 19.75	39.28 ± 16.52	0.012*	14.90 ± 22.03 (66.66 ± 72.64)	11 (84.62)	8 (61.53)
VAS	6.77 ± 1.75	2.88 ± 2.48	<0.001*	−3.88 ± 2.82 (−55.04 ± 38.21)	11 (84.62)	10 (76.92)
Lequesne Index	8.69 ± 2.65	5.77 ± 3.49	0.008*	−2.92 ± 3.35 (−31.18 ± 46.61)	10 (76.92)	

OA: osteoarthritis; MSC: mesenchymal stem cells; CFU-F: cultures of colony-forming cells; VAS: visual analogue scale; KOOS: knee injury and osteoarthritis outcome score; ADL: function in daily living; sport/rec.: function in sport and recreation; QOL: quality of life; δ: difference from baseline. MCII: minimal clinically important improvement; *$p < 0.05$ with respect to basal level.

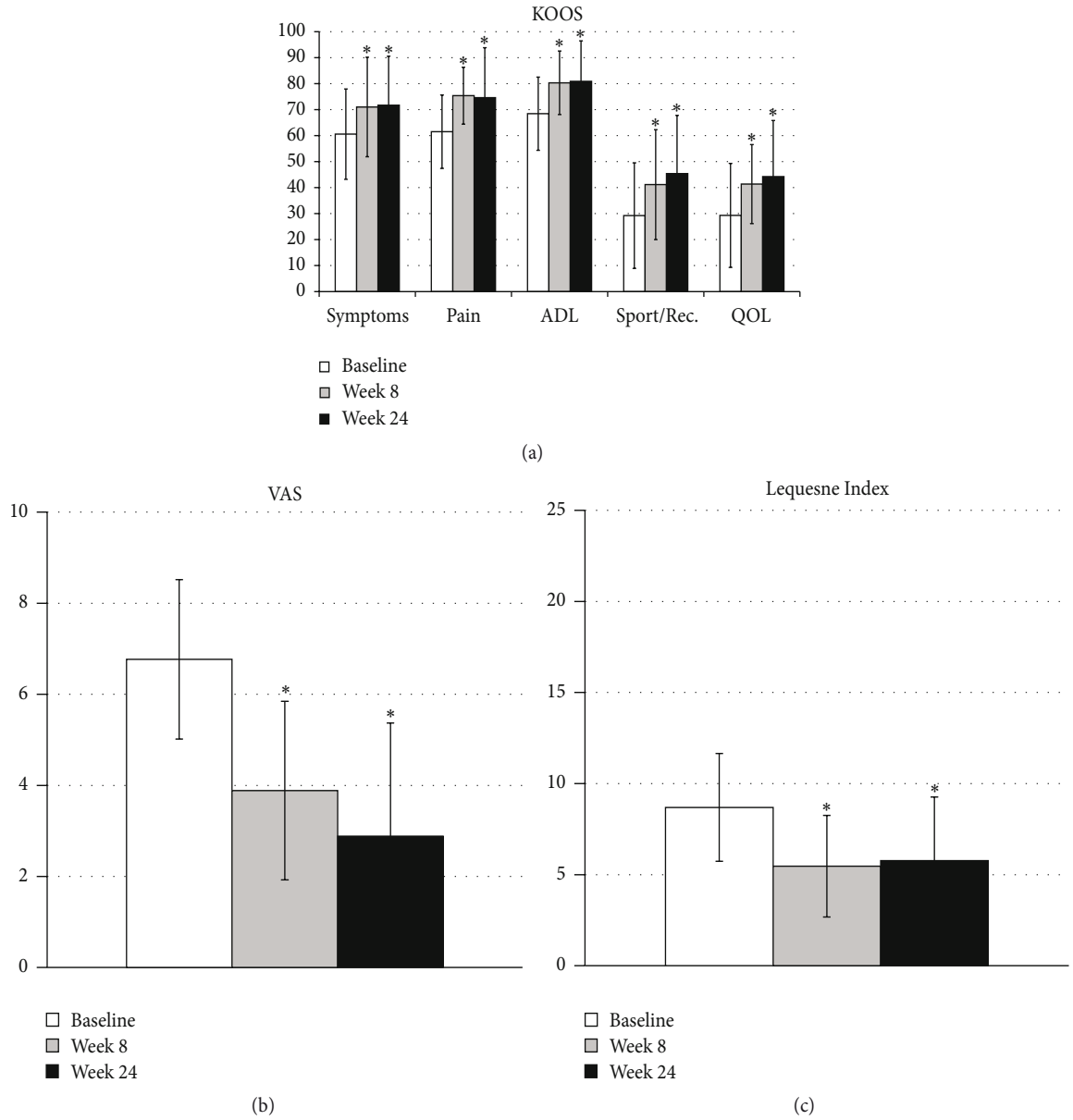


Figure 3: Clinical outcomes. KOOS (a), VAS (b), and Lequesne Index (c) at baseline, 8 weeks after treatment, and 24 months after treatment. ADL: function in daily living; sport/rec.: function in sport and recreation; QOL: quality of life. $^{*}p < 0.05$ with respect to basal level.

(symptoms, $p < 0.004$; ADL, $p < 0.02$; sport/rec., $p < 0.02$; QOL, $p < 0.02$), as well as VAS score ($p < 0.001$) and Lequesne Index ($p = 0.008$).

The improvement of the patients was observed at 8 weeks of follow-up, and it was maintained until week 24, when the study ended (Figure 3). The two patients who did not respond to treatment were indicated for a total knee arthroplasty.

Two patients reported 2 adverse events likely unrelated to the treatment. One of the patients experienced an episode of fever associated with flu episode, and the other reported exacerbation of knee pain three months after the treatment. Both events were mended satisfactorily by oral pharmacological treatment, which was allowed in the study. In addition, one patient was excluded because of a popliteal cyst caused by sports activity which was treated with fluid drainage and corticosteroid infiltration.

3.2. Biological Outcomes. Baseline levels of mesenchymal stem cells (MSCs) presented in synovial fluid were 7.98 ± 8.21 MSC/μL, while one week after intraosseous infiltration the values significantly declined to 4.04 ± 5.36 MSC/μL ($p = 0.019$) (Table 1).

Concerning cultures of colony-forming cells (CFU-F), a substantial reduction in the number of CFU-F was also observed one week after infiltration, namely, the number of CFU-F/mL before and after treatment of 601.75 ± 312.30 and 139.19 ± 123.61, respectively ($p = 0.02$) (Table 1).

4. Discussion

The combination of intra-articular and intraosseous injections of PRP is an *in situ* local biological "joint-centric"

approach to treat severe KOA addressing the SM, SF, and superficial zone of AC by intra-articular injections of PRP and deep zones of AC and SB through PRP intraosseous infiltrations [24]. The significant pain reduction from baseline shown in these results is according to several studies which have shown the substantial pain reduction in patients with KOA treated with intra-articular infiltrations of PRP [20, 25–27]. However, some patients do not respond to this treatment, a result which converges with the severity of osteoarthritis [28–30]. These studies confirmed that patients with advance KOA such as Ahlbäck III type did not improve after intra-articular injections of PRP. Intra-articular drug delivery does not address the subchondral bone as a tissue target, which might be one of the reasons for this absence of response. In this study, we added intraosseous injections for the conventional intra-articular treatment to address the SB as one crucial tissue target in the treatment of severe KOA (Figure 4).

There are several potential mechanisms by which intra-articular injections and intraosseous infiltrations of PRP might reduce knee pain. *In vitro* and *in vivo* studies have reported that PRP and growth factors within it such as HGF, IGF-1, and PDGF suppress macrophage, fibroblast, and chondrocyte activation by inhibiting the NFκB pathway, thereby dampening the synovial and articular cartilage inflammatory response [4, 15–17]. In addition, the significant amount of endogenous cannabinoids within PRP might act as ligands for cannabinoid receptors 1 (CB1) and 2 (CB2) of chondrocyte and synovium cells of OA patients, thereby supporting a pain and inflammation reduction by targeting the endogenous cannabinoid systems [2, 31–34]. On the other hand, the excessive presence of TGFβ1 and VEGF in OA subchondral bone and articular cartilage could be a driving factor for changes in osteoblast-osteoclast coupling [7, 19, 35–37], which leads to a bone remodeling imbalance, NGF expression, and fibroneurovascular growth, all changes which might well contribute to pain [3, 7–9, 33, 35–37]. It is reasonable to speculate that the concurrent presence of, and a balanced ratio between, platelet-secreted TGFβ1 and VEGF and plasma growth factors such as IGF-1 and HGF [37], all conveyed by PRP intraosseous infiltration, might buffer the excess of TGFβ1 in SB as well as restoring HGF activity synthesized by osteoblasts. This new reestablished homeostatic balance between TGFβ1 and HGF would reduce the synthesis of NGF, VEGF, and other inflammatory mediators, thereby contributing to the reduction of pain and hyperalgesia in severe stages of KOA [9, 36].

In this study, patients also showed a significant improvement in the secondary efficacy outcomes such as function in daily living (ADL), function in sport and recreation (sport/rec.), and knee related quality of life (QOL). This increased intolerable physical load might entail a positive chondroprotective and anti-inflammatory effect, since as several lines of evidence suggest, moderate mechanical loading of joints prevents cartilage degradation by suppressing the activation of NFκB [38].

The significant reduction of MSC in SF after treatment with this novel PRP therapy is open to interpretation. Several studies have reported that the accumulation of MSCs in SF increases with the severity of osteoarthritis, joint damage, and the disease duration [39, 40]. Although the source of this MSC increase has not yet been determined, the most likely origin of the increased presence of MSC in SF of KOA patients might be the SM, the breakdown zone of superficial AC, and the SB [6, 7, 9, 39–41]. By adhering to SM, superficial AC, and SF and by gradually delivering various components such as IGF-1, HGF, PDGF, TGF-β1, and platelet microparticles (PM), intra-articularly injected PRP may influence macrophage M1 polarization towards M2 phenotype and modify the inflammatory status of chondrocytes and the superficial zone of AC by suppressing the NFκB signaling pathway [15–17, 42]. By lowering the concentration of chemoattractant inflammatory cytokines in SF, PRP may well contribute to the inhibition of the MSC release and migration [4, 26, 43]. Another origin for SF MSCs might be the SB as a point of egress through the channels and vessels breaching the osteochondral junction, partially recruited by the osteoarthritic SF [7, 9, 43]. The buffer effect of PRP on TGFβ1 signaling pathway in SB might reduce the presence of nestin MSCs likely associated with the shrinking of fibroneurovascular tissue of KOA subchondral bone as an antifibrotic mechanism which has already been reported on several cell phenotypes [36, 37]. Moreover, the process of cell *homing* whereby SF MSCs might be recruited to damaged areas of AC and take part in the *in vivo* repair of that cartilage might also contribute to MSCs reduction [44], just as the PRP fibrin network, containing fibronectin, IGF-1 and IGF-II, PDGF, SDF-1, and TGFβ1 may exert a recruitment, homing, and chondrogenic-differentiation effect on subchondral mesenchymal progenitor cells [14, 45, 46].

This study has some limitations. First, a relatively small number of patients were enrolled in the study with no control group, all belonging to the same severe KOA phenotype stage. Second, the clinical follow-up of 6 months seems to be a short period to draw conclusive clinical indications. Third, an evaluation of patients with X-ray or MRI has been very useful to document eventual changes in the subchondral bone after PRP treatment. Finally, a mechanistic account of the significant pain and SF MSCs reduction experienced by the majority of patients is lacking. The first three limitations are inherent in the nature of the study.

5. Conclusions

In summary, targeting synovial membrane, synovial fluid, articular cartilage, and subchondral bone with intra-articular injections and intraosseous infiltrations of PRP reduces pain and MSCs in SF, besides significantly improving knee joint function in patients with severe knee OA, with no adverse event reported. This work aims to be a first step for further research in this field, both in basic research and in increasingly robust clinical trials.

Ethical Approval

This trial is approved by Clinical Research Ethics Committee of the Basque Country.

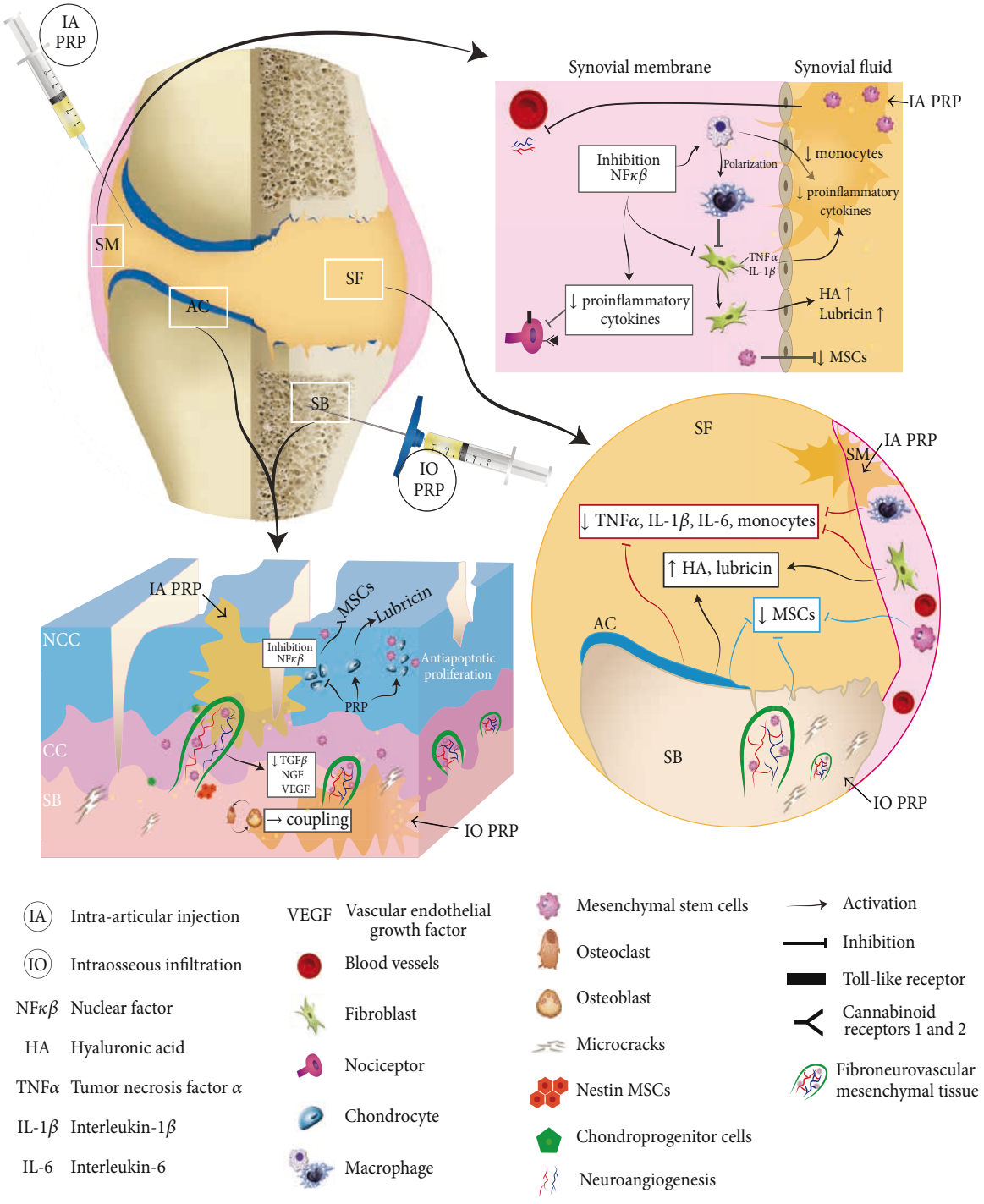


Figure 4: Mechanisms of intra-articular and intraosseous injections of platelet rich plasma. Depiction of a new strategy to treat severe knee OA by targeting different knee joint structures such as synovial membrane (SM), synovial fluid (SF), articular cartilage (AC) with noncalcified cartilage (NCC) and calcified cartilage (CC), and subchondral bone (SB) with intra-articular injections (IA) and intraosseous infiltrations (IO) of platelet rich plasma (PRP) [24]. This procedure reduces pain and mesenchymal stem cells (MSC) in SF, besides significantly improving knee joint function of patients with severe OA. We suggest that various growth factors, cytokines, and chemokines trapped in the fibrin network of PRP might inhibit the NF$\kappa\beta$ on synovial macrophages, fibroblasts as well as on chondrocytes, thereby dampening the inflammatory response of SM and AC [15–18]. In addition, IO in subchondral bone, might buffer the excess of transforming growth factor β1 (TGF-β1) as well as restore hepatocyte growth factor (HGF) activity synthesized by osteoblasts, thereby leading to a new reestablished homeostatic balance between TGF-β1 and HGF [35–37]. The buffer effect of PRP on TGF-β1 signalling pathway in SB might reduce the presence of nestin MSCs in SF, likely associated with the shrinking of fibroneurovascular tissue in the SB, as an antifibrotic mechanism which has already been reported on other cell phenotypes [36, 37].

Competing Interests

Sabino Padilla is scientist at BTI Biotechnology Institute, a dental implant company that investigates the fields of oral implantology and PRGF-Endoret technology. The other authors have no potential competing interests.

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Research Article

Modulation of Synovial Fluid-Derived Mesenchymal Stem Cells by Intra-Articular and Intraosseous Platelet Rich Plasma Administration

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The aim of this study was to evaluate the effect of intra-articular (IA) or a combination of intra-articular and intraosseous (IO) infiltration of Platelet Rich Plasma (PRP) on the cellular content of synovial fluid (SF) of osteoarthritic patients. Thirty-one patients received a single infiltration of PRP either in the IA space ($n = 14$) or in the IA space together with two IO infiltrations, one in the medial femoral condyle and one in the tibial plateau ($n = 17$). SF was collected before and after one week of the infiltration. The presence in the SF of mesenchymal stem cells (MSCs), monocytes, and lymphocytes was determined and quantified by flow cytometry. The number and identity of the MSCs were further confirmed by colony-forming and differentiation assays. PRP infiltration into the subchondral bone (SB) and the IA space induced a reduction in the population of MSCs in the SF. This reduction in MSCs was further confirmed by colony-forming (CFU-F) assay. On the contrary, IA infiltration alone did not cause variations in any of the cellular populations by flow cytometry or CFU-F assay. The SF of osteoarthritic patients contains a population of MSCs that can be modulated by PRP infiltration of the SB compartment.

1. Introduction

Knee osteoarthritis (OA) encompasses a cluster of degenerative joint conditions with different biochemical, inflammatory, and genetic signatures generating distinct subtypes. Evolving in phases, the severity of the resulting phenotype impacts the quality of life of the patient and represents an economic burden and social challenge. Estimates suggest that about 46 million patients suffer from OA in developed countries, more than 50% of adults over 50 years; by 2030, this figure may reach 70 million [1]. It is essential to develop novel treatments that slow or stop the progression of this disease and even reverse the damage. Current treatments such as analgesics, nonsteroidal anti-inflammatory drugs, intra-articular infiltrations of steroids, or hyaluronic acid just relieve the symptoms, and, in advanced cases of OA, joint replacement is the only solution for these patients [2].

The knee joint is a complex biological system composed of synovial fluid (SF), synovial membrane (SM), meniscus, ligaments, subchondral bone (SB), and articular cartilage (AC). AC is an avascular tissue that lies functionally sandwiched between the SM, which generates the SF, and the

SB. Stemming primarily from an ultrafiltrate of plasma and secretions of chondrocytes and synoviocytes, SF is a viscous liquid composed of hyaluronan (HA) and lubricin, cytokines, growth factors, and a minor presence of cells. Aggression and inflammation to intra-articular tissues bring an increase of MSCs in SF [3, 4], which is commonly interpreted as a tissue response to injury [5, 6], equivalent to the response of migratory chondrogenic progenitor cells from SB to injured cartilage [7, 8]. Although the source of MSCs has not been yet clearly determined, the most likely origin might be the SM [4, 5], the breakdown zone of superficial AC, and the SB [6, 9, 10]. Recent findings suggest that the increase in pathological situations of certain molecules such as monocyte chemotactic protein-1, SDF-1, and TGF-β1 could promote the recruitment of MSCs [11, 12].

SB has always been present in the equation of OA pathogenesis [13]. There is an increasingly recognized communication between the SB and AC based on the changes that the SB undergoes in patients with severe OA, including microcracks and structural defects, vascularization of channels, nerve growth, and a progressive replacement of the subchondral marrow with fibroneurovascular mesenchymal tissue [9, 10, 14, 15]. Since the primary driver of knee OA is not yet established between the different joint tissues, therapeutic strategies solely targeting one cell or tissue are prone to fail [16]. Thus, approaches to treat OA should be aimed at reaching several joint tissues with the purpose of reducing joint inflammation, controlling pain, improving joint functionality, and restoring tissue homeostasis.

Among the new emerging treatments to address knee OA, mesenchymal stem cells (MSCs) and Platelet Rich Plasma (PRP) stand out [17], with the scientific rationale for the use of PRP in the treatment of knee OA growing. Intra-articular infiltrations of PRP have proven to substantially reduce pain in patients with knee and hip OA and to improve joint stiffness and physical function [18–21]. PRP and many of the bioactive mediators that contain IGF-I, TGF-β1, HGF, PDGF, VEGF, NGF, BDNF, CTGF, BMPs, Vitronectin, fibronectin, SDF-1, and PF4, among others, have shown positive effects on homeostasis of joint tissues through chondroprotective, anabolic, anti-inflammatory, and immunomodulatory effects [22–26]. Also MSCs hold an important therapeutic potential promoting regeneration, derived from their proliferative and multipotential differentiation properties. MSCs could lead to the formation of new chondrocytes and cartilage regeneration, a process that has been observed in promising preclinical studies and clinical trials [27–29]. However, there are still specificities on this broader treatment that require deeper analysis as to what cell sources are more appropriate, influence on therapeutic effectiveness of *in vitro* expansion, and dosage [30]. We hypothesize that targeting SM, SF, AC, and SB with a combination of intra-articular injections and intraosseous (IO) infiltrations of PRP on severe knee OA [31] could have a deeper biological impact on knee joints tissues and therefore be a more effective treatment than the conventional intra-articular (IA) infiltrations of PRP.

2. Methods

2.1. Treatment Groups and Collection of Synovial Fluids. Patients were divided into two modality treatment groups; patients of the IA modality group received a single IA infiltration of PRP ($n = 14$) and patients of the IO group ($n = 17$) were treated with a combination of one IA infiltration of PRP followed by two PRP IO infiltrations of PRP (one in the tibial plateau and one in the medial femoral condyle). Both groups received two more IA infiltrations of PRP on a weekly basis. SF were collected from 31 patients, before and after the first week of PRP treatment. The choice of IA or IO modality treatment was made based on the failure of previous medical treatments; namely, the patients who had been oriented toward a total knee replacement as the only solution for their OA were allocated in the IO group.

2.2. PRP Preparation. A small volume between 36 and 72 mL of peripheral blood was extracted from each patient into extraction tubes containing 3.8% sodium citrate as anticoagulant. After centrifugation at 580 ×g for 8 minutes, plasma fractions were separated by pipetting under sterile conditions. In each tube, the 2 mL of plasma rich in platelets remaining above the red cells and the "buffy coat" were collected, avoiding picking up the leukocytes, and were put together [31]. This preparation was characterized by containing 2 to 3 times the concentration of platelets compared with peripheral blood and the absence of erythrocytes and leukocytes (BTI Biotechnology Institute, Vitoria-Gasteiz, Spain).

2.3. Procedures. For the IA group, 8 mL of PRP was infiltrated in the joint space. Before infiltration, a 21 G needle was placed into the joint space and SF arthrocentesis was carried out and collected SF were preserved for analysis as pretreatment sample. One week after, another arthrocentesis was carried out to analyze the SF after treatment. For the IO group, a sedation of the patient was induced by infusing a single dose of normal saline, a single dose of midazolam (0.03–0.05 mg/kg), and fentanyl (3.2 mg/kg), in peripheral vein; single or repeated dose of propofol was also administered (1-2 mg/kg), dependent on the duration of the infiltration. The degree of sedation was −4 or −5 on the Richmond Sedation Scale. Local anesthesia was conducted by injecting 2 mL of 2% mepivacaine into the periosteum of the condyle and tibial plateau. As in the case of IA group, an arthrocentesis was carried out to evacuate the totality of SF which was preserved for analysis as pretreatment sample of IO group. PRP was infiltrated into the joint space first (8 mL) and then into SB of the tibial plateau (5 mL) and the femoral condyle (5 mL), using a 13 G bone-biopsy trocar manually introduced into SB; the use of the fluoroscope facilitated trocar placement. It is worth noting that Sánchez et al. have illustrated visual direct evidence that the intraosseously injected PRP was allocated into the SB [32].

The institutional review board approved this study, and informed consents were obtained from every patient included in the study.

2.4. Multidimensional Flow Cytometry (MFC) Immunophenotyping. Approximately 2–6 mL of arthrocentesis-derived SF of each patient was immunophenotyped using an 8-color direct immunofluorescence technique. After sample centrifugation, 100 μL of the concentrated cell suspension was stained for 15 minutes at room temperature in darkness, with the following combination of monoclonal antibodies (MoAb): Brilliant violet (BV) 421/orange chrome (OC) 500/fluorescein isothiocyanate (FITC)/phycoerythrin (PE)/peridinin chlorophyll protein-cyanin 5.5 (PerCP-Cy5.5)/PE-cyanin 7 (PE-Cy7)/allophycocyanin (APC)/APCH7: (i) CD105/CD45/CD73/CD271/CD34/CD13/CD90/CD44. After staining, 2 mL of FACS lysing solution (Becton/Dickinson Biosciences, San Jose, CA) was added. After 5 minutes of incubation at room temperature, samples were sequentially centrifuged for 5 minutes at 540 ×g and resuspended in 100 μL of premixed Perfect-COUNT microspheres (Cytognos SL, Salamanca, Spain). Subsequently, data acquisition was performed for around 5,000 nucleated cells per tube in a FACSCantoII flow cytometer (Becton Dickinson Biosciences (BD), San Jose, CA) using the FACSDiva 6.1 software (BD). Monitoring of instrument performance was performed daily using the Cytometer Setup Tracking (CST; BD) and rainbow 8-peak beads (Spherotech Inc., Lake Forest, IL) after laser stabilization, following the EuroFlow guidelines; sample acquisition was systematically performed after longitudinal instrument stability was confirmed. MSCs and residual leukocytes were identified through a Boolean gating strategy based on forward scatter, side scatter, and CD45 expression; monocytes were defined on the basis of their relatively higher light scatter properties and CD13 and CD45 bright expression, whereas lymphocytes were identified through low scatter properties and strong CD45 reactivity (Figure 1). Absolute cell numbers per volume unit were calculated following the manufacturer's recommendation.

2.5. MSCs Isolation from Knee Synovial Fluid. Collected SF were diluted in phosphate buffer saline (PBS) and the cellular content was then harvested by centrifugation. One part of each sample was seeded in a 6-well plate under standard cell culture conditions with Dulbecco's Modified Eagle Medium (DMEM; Lonza) supplemented with 20% fetal bovine serum (Gibco), 1% penicillin-streptomycin (P/E) (Gibco), and 1 ng/mL of human recombinant basic fibroblast growth factor (bFGF; R&D systems) (Expansion Medium). The adherent cells were expanded in a humidified 5% CO_2 atmosphere at 37°C and used for further differentiation experiments. The remaining sample was used for colony-forming assay (CFU-F) and seeded on a 100 mm diameter culture plate. Seven days later, plating colonies were visible and counted by 0.5% crystal violet staining. It was established that a CFU-F contains more than 10 morphologically homogeneous cells.

2.6. Synovial Fluid MSCs Differentiation. Mesenchymal lineage differentiation assays were carried out as described in Muinos-López et al. 2016 [33]. Briefly, SF-derived cells were assessed between passages 2 and 5 to confirm their osteogenic, adipogenic, and chondrogenic capacity. For osteogenic and adipogenic differentiation, 8000 cells/cm^2 were seeded in 12-well plates. Adipogenic differentiation was induced using DMEM supplemented with 10% FBS, 1 μM Dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 50 μM Indomethacin for 21 days. For the osteogenic differentiation, cells were cultured in DMEM supplemented with 10% FBS, 50 μg/mL L-(+)-ascorbic acid, 10 mM β-glycerol phosphate, and 10 nM Dexamethasone for 21 days. For chondrogenic differentiation, 2.5*E*5 cells were spun-down at 600 ×g for 10 minutes in polystyrene 15 mL conical tubes and incubated with hMSC Chondrogenic Differentiation BulletKit™ Medium (Lonza). Differentiations were analyzed at 28 days. In all differentiation assays, a negative control was included where the cells were maintained with expansion medium (DMEM containing 10% FBS) without induction factors. In all differentiation assays, medium was changed every 2-3 days.

2.7. Histological and Immunohistochemistry Differentiation Analyses. Adipogenic and osteogenic differentiation were assessed by Oil Red O and Alizarin Red staining, respectively. For adipogenic differentiation, after fixation with 4% paraformaldehyde (Panreac) for 10 minutes, cells were rinsed with 60% isopropyl alcohol followed by a 60% solution of Oil Red for 20 minutes to reveal intracellular oil droplets. For osteogenic differentiation, mineral precipitates were revealed with a 2% solution of Alizarin Red, pH 4.2, for 15 minutes at room temperature and washed with deionized water. Chondrogenic differentiation was evaluated by toluidine blue staining and immunohistochemistry (IHC) for type II collagen. Cell pellets were included in paraffin and sectioned, 4 μm thick. Toluidine Blue, 1% (weight/volume) in 1% acetic acid solution, was used to visualize anionic glycoconjugates, proteoglycans (PG), and glycosaminoglycans (GAG). For IHC, sections were hydrated in grade ethanol and subjected to antigen unmasking by sequential 15 min treatments of hyaluronidase (4 mg/mL in PBS) and pepsin (4 mg/mL in 0.01 N HCl solution) at 37°C. Endogenous peroxidase activity was blocked by H_2O_2 treatment (3% H_2O_2 in PBS). Samples were incubated overnight at 4°C with a mouse monoclonal antihuman type II collagen (0.5 μg/mL; Clone II-4CII, MP Biomedicals). Staining was visualized with DAB using EnVision® chromogenic kit (DAKO) according to the manufacturer's instructions.

2.8. Statistical Analysis. Data were determined by the mean and standard deviation. Comparisons were performed by Wilcoxon signed-rank test for nonparametric data and Student's t-test for parametric data, after assessing the normal distribution of the samples by Shapiro-Wilk test. Data were considered statistically significant when p values were less than 0.05. Statistical analysis was performed with SPSS 17.0 (SPSS, Chicago, IL).

3. Results

3.1. Characteristics of the Patients. The mean age of patients in the IA group was 62.6 ± 11.8 years and the range was 41–77 years. The percentages of patients of this group with

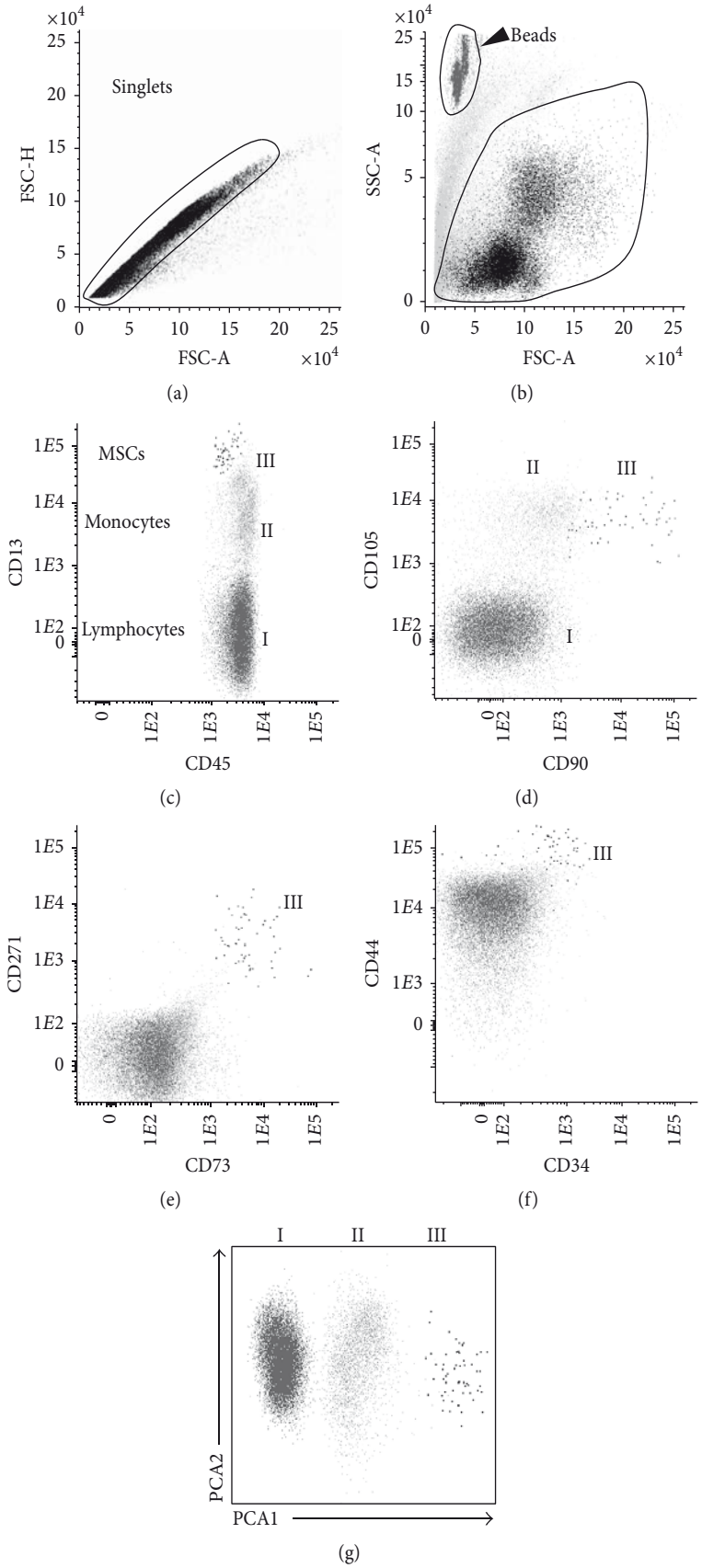

Figure 1: Phenotypic characterization of synovial fluid MSCs. After exclusion of doublets (a) and debris (b), mesenchymal stem cells (MSCs) were identified through a Boolean gating strategy according to their strong reactivity for CD13, CD44, CD73, CD90, and CD105 and intermediate to high levels of CD271 (e-f), in the absence of CD34 (f). Monocytes were defined on the basis of their relatively higher light scatter properties and CD13 and CD45 bright expression, whereas lymphocytes were identified through low scatter properties and strong CD45 reactivity. In panel (g), the automated population separator (APS) graphic representation of the Infinicyt software is shown with the three cell populations phenotypically separated by principal component analysis (PCA). I: lymphocytes; II: monocytes; III: MSCs.

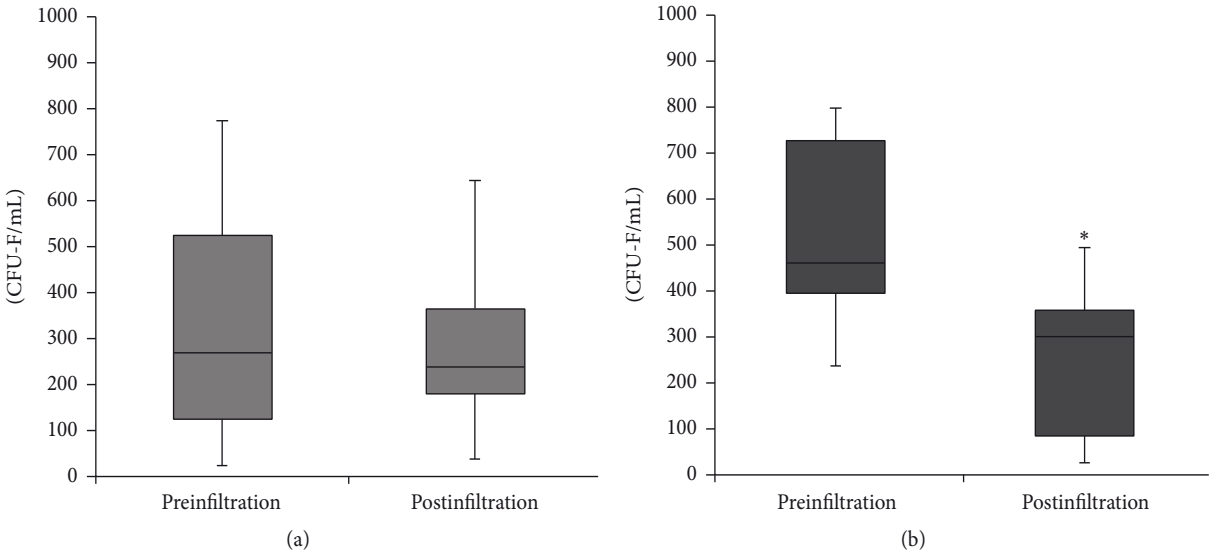

Figure 2: Colony-forming units fibroblast. Levels of colony-forming units fibroblast in the synovial fluids (CFU-F) before (preinfiltration) and one week after (postinfiltration) infiltration of Platelet Rich Plasm (PRP). (a) Intra-articular infiltration of PRP. (b) IO infiltration of PRP. $^{*}p < 0.05$.

Table 1: Patients included in the study and their clinical OA grade.

	IA group	IO group
Age (mean ± SD)	62.6 ± 11.8	63.6 ± 11.2
Age range	41–77	41–80
OA grade II (%)	50	29.4
OA grade III (%)	35.7	47.1
OA grade IV (%)	14.3	23.5

osteoarthritis grades II, III, and IV according to Ahlbäck scale were 50%, 35.7%, and 14.3%, respectively. Regarding the IO group, the average age of patients was 63.6 ± 11.2 years and the range was 41–80 years. In this group, the percentages of patients classified by Ahlbäck scale were 29.4% for grade II, 47.1% for grade III, and 23.5% for grade IV (Table 1).

3.2. Phenotypic Characterization of the Cell Population of Synovial Fluid. To determine the influence of PRP treatment in the cellularity of the joint, the presence of mononucleated cells (MNC) cells and their populations was analyzed in the SF of both groups, before and after treatment, by flow cytometry, as described in Methods (Figure 1).

Regarding the IA group, the concentration of MNC, lymphocytes, monocytes, and MSCs in the SF before and after treatment did not show significant differences (Table 2).

Interestingly, although in the IO group the variations in the concentration of MNC, lymphocytes, and monocytes in the SF were also not significant, MSCs showed a significant decrease after IO treatment (Table 3).

Table 4 shows the cellular increments (δ) before and after each infiltration and compares the differences between the two treatments. The decrease in the levels of MSCs observed after IO infiltration of PRP was higher than the decrease after IA treatment ($p = 0.045$).

3.3. Culturing of Colony-Forming Cells (CFU-F). To confirm the reduction of MSCs in the SF, we assessed the capacity of the MSCs population to sustain clonal growth on plastic surfaces (CFU-F). Consistent with the flow cytometry results, the IA injection of PRP did not result in a significant variation in CFU-F, 332.52 ± 234.96 CFU/mL before treatment to 327.54 ± 223.32 CFU/mL after treatment ($p = 0.92$) (Figure 2(a)). In the IO group, we found a significant reduction in CFU-F from 477.51 ± 253.44 CFU/mL before IO injections to 222.95±151.36 CFU/mL one week after infiltration ($p < 0.01$) (Figure 2(b)). Consistent with the results obtained with the number of MSCs, the decrease in the CFU-F levels after IO infiltration was greater than the decrease after IA injection ($p = 0.037$).

To confirm the mesenchymal progenitor nature of the CFU-F cells present in the SF, we performed an *in vitro* multipotency assay by differentiation to the three mesenchymal lineages osteoblast, adipocyte, and chondrocyte under defined conditions (Figure 3). Although only a limited number of assays showed trilineage differentiation capacity (7 out of 68 assays, 10%), the majority of the assessed synovial fluid-derived mesenchymal cells showed bilineage differentiation capacity (51 out of 68, 75%), with a majority of assays positive for adipogenesis and osteogenesis lineage (97%), supporting the mesenchymal nature of the population.

4. Discussion

In this study, we carried out two different treatment modalities of PRP applications on OA patients. IA group received intra-articular injections of PRP and a combination of intra-articular and intraosseous injections was applied in the IO group in order to address the SB.

One week after administration of IA infiltration, it was observed that MSCs and monocytes level in SF decreased (Table 2). Although this decrease was not significant, it could

Table 2: Phenotypic characterization of the cell population in SF of IA group.

	Pretreatment (mean ± SD)	Posttreatment (mean ± SD)	p value
MNC (cells/mL)	237.11 ± 223.32	243.81 ± 193.37	0.32
Lymphocytes (cells/mL)	103.65 ± 125.00	85.38 ± 94.16	0.06
Monocytes (cells/mL)	130.66 ± 101.88	142.62 ± 112.81	0.73
MSCs (cells/mL)	2.60 ± 4.38	1.53 ± 2.51	0.32

MNC, mononuclear cells; MSCs, mesenchymal stem cells.

Table 3: Phenotypic characterization of the cell populations in SF of IO group.

	Pretreatment (mean ± SD)	Posttreatment (mean ± SD)	p value
MNC (cells/mL)	441.92 ± 371.87	354.82 ± 411.44	0.38
Lymphocytes (cells/mL)	179.83 ± 237.87	184.19 ± 337.00	0.072
Monocytes (cells/mL)	199.37 ± 160.28	119.06 ± 98.47	0.053
MSCs (cells/mL)	7.61 ± 8.68	2.46 ± 3.86	0.01

MNC, mononuclear cells; MSCs, mesenchymal stem cells.

suggest an anti-inflammatory effect of PRP. This trend may be more pronounced after two more PRP IA injections, which would be consistent with the significant clinical improvement reported by Sánchez et al. and Vaquerizo et al. using three IA administrations of PRP on a weekly basis [19, 21]. This conventional modality to deliver PRP in patients results in a liquid-to-gel transition 3D fibrin scaffold. When fibrinolysis degrades this scaffold, growth factors within the fibrin scaffold such as IGF-I, HGF, PDGF, TGF-β1, and platelet microparticles are released gradually. These growth factors have been proven to promote an anti-inflammatory macrophage phenotype [23, 34–36] and suppress the NF-$\kappa\beta$ signaling pathway in synovial fibroblasts and chondrocytes of the superficial zone of AC [24] and induce the synthesis of hyaluronic acid and lubricin by synoviocytes and chondrocytes, respectively, with the latter preventing chondrocyte apoptosis, cartilage breakdown, and inhibition of the MSC release and migration [25, 37–39]. Although the decline of monocytes in the SF was not statistically significant, this fact together with all these modulatory and trophic effects of intra-articularly injected PRP on the SM, superficial AC, and SF could suggest a lower level of proinflammatory cytokines and restoration of the joint homeostasis leading to a more favorable SF environment for chondrogenic differentiation of MSCs [30, 37, 39, 40].

Concerning IO group, levels of monocytes and cells also declined, but in this case decrease in the concentration of MSCs was statistically significant (Table 3). This was also confirmed when the levels of CFU-F were analyzed before and after treatment administration (Figure 2(b)). It is worth mentioning that the MSCs population in SF before the PRP treatment and the degree of OA severity was considerably varied between both groups. The levels of SF-MSCs in the IA group were very close to healthy population levels and substantially lower than in the IO group. Likewise, the percentage of patients in the IO group with advanced degree of OA (OA grades III and IV) was 70.6% compared with 50% in the IA group. This difference between the two groups became similar after application of the IO treatment, which approximated the MSCs level to IA group and the healthy population. This observation is in accordance with several studies where the SF-MSCs levels were associated with the severity of OA, joint damage, and the disease duration [4, 34].

When comparing the two treatment groups, the decrease in MSCs after PRP treatment was more pronounced in the IO group (Table 4). Although the drop in the IO group could be influenced by the higher level of MSC present in this group before treatment, this greater decrease was also observed in the CFU-F, where the baseline difference between groups is not so critical. The influence of arthrocentesis in this cell drop must also be taken into consideration, since a week might not be enough for MSCs to migrate to the IA space. Considering that the SM and the breakdown zone of superficial AC are postulated as the main sources of cells that reach the SF and they are continuously soaked with this fluid, it seems possible that MSCs repopulate the SF in a week [4, 5, 41]. Regarding MSCs migration from SB, and despite the lack of clinical studies that analyze the time needed for this process, *in vitro* studies have shown this migration after 20 hours, so a week seems enough for MSCs to reach the SF from SB [42].

This observation suggests that, in the modulation of MSC by PRP, the SB is an important player and potential tissue target and might be a MSC egress point through the channels and vessels breaching the osteochondral junction and reaching the cartilage, partially recruited by the osteoarthritic environment of the SF [9, 10, 42]. The excessive presence of TGF-β1 and VEGF in osteoarthritic SB may be a driving factor for changes in osteoblast-osteoclast coupling, which lead to a bone remodeling imbalance and fibroneurovascular growth [9, 10, 12, 16]. Moreover, Zhen et al. showed that by inhibiting TGF-β signaling in a specific population of MSCs present at the SB (Nestin positive MSCs) the severity of OA was reduced [12]. In fact, previous studies have shown that the decrease in MSCs in the SF, in low degree OA, suggests clinical improvement [4]. It is reasonable to speculate that, by administering PRP directly into SB, the concurrent presence of platelet-secreted TGF-β1 and VEGF as well as plasma growth factors such as IGF-I and HGF could

Table 4: Cellular increment (δ).

	IA group (mean ± SD)	IO group (mean ± SD)	p value
MNC (cells/mL)	109.70 ± 272.66	−91.33 ± 334.47	0.905
Lymphocytes (cells/mL)	−65.04 ± 106.50	42.64 ± 171.96	0.159
Monocytes (cells/mL)	−19.64 ± 156.00	−97.80 ± 147.95	0.280
MSCs (cells/mL)	−1.41 ± 5.38	−6.36 ± 6.64	0.045
CFU-F (CFU/mL)	−6.87 ± 236.79	−266.30 ± 296.79	0.037

MNC, mononuclear cells; MSCs, mesenchymal stem cells; CFU-F, colony-forming unit fibroblast.

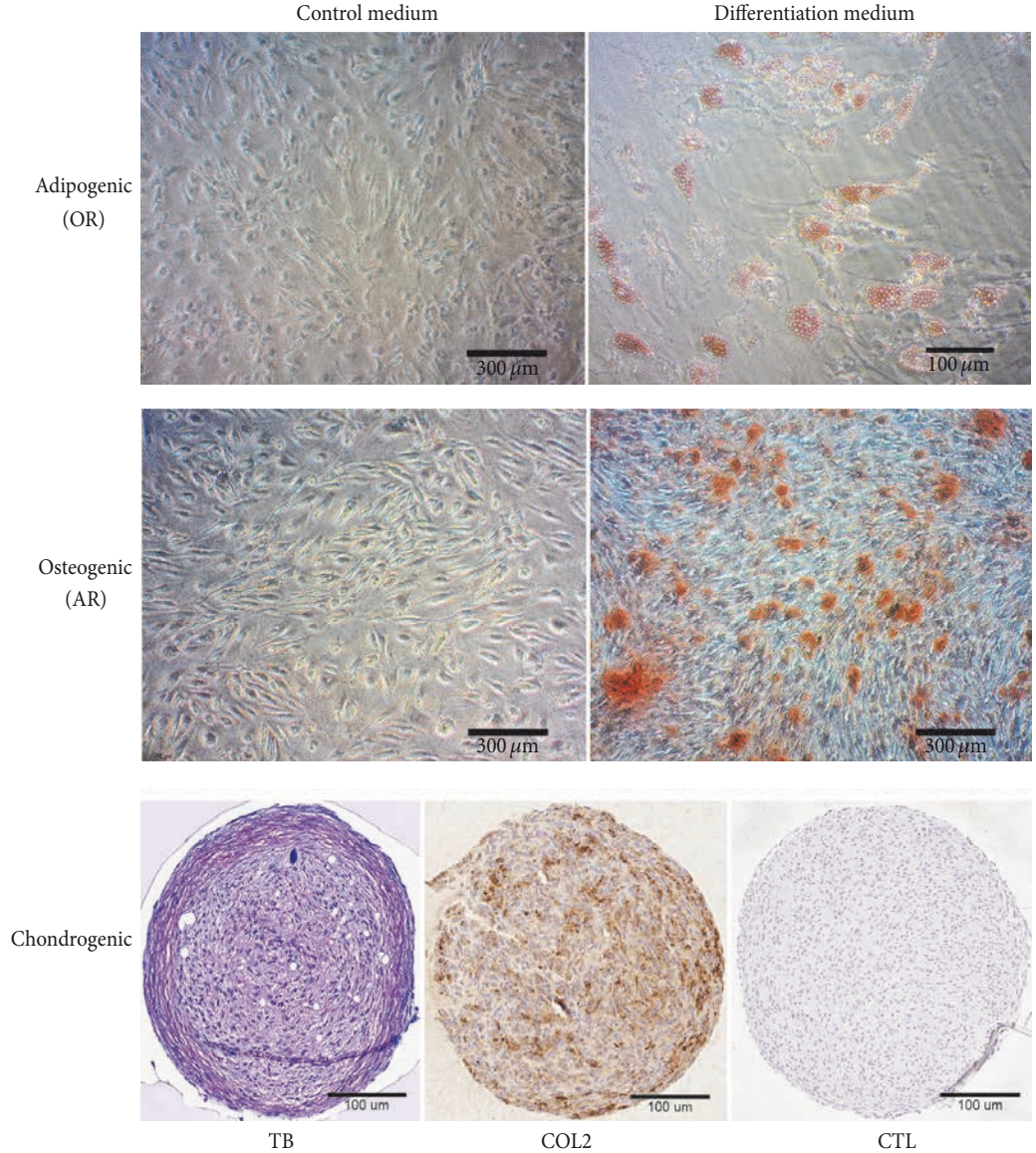

Figure 3: Differentiation assay. *In vitro* differentiation assay of synovial fluid isolated cells to the mesenchymal lineages, adipocytes, osteoblasts, and chondrocytes. Control medium was expansion medium. Adipogenic differentiation was visualized by Oil Red (OR) staining. Osteogenic differentiation was visualized with Alizarin Red (AR) staining. Chondrogenic differentiation was visualized with Toluidine Blue (TB) staining and COL2 and immunohistochemistry using a monoclonal antibody directed to type II collagen. CTL, no primary antibody was added.

have a modulatory effect on TGF-β signaling pathway [12, 43]. This might reduce the presence of MSCs and could likely be associated with the shrinking of fibroneurovascular tissue of OA SB, an explanation which parallels the antifibrotic mechanism already reported in several cell phenotypes [43, 44].

A further significant component to the SF-MSC reduction induced by PRP treatment would be the process of cell *homing* whereby SF-MSCs might be locally recruited to damaged areas of the AC taking part in the *in vivo* repair of this tissue, a possibility already reported by Lee et al. [45]. It has been reported that PRP is rich in fibronectin,

a plasma protein incorporated into the fibrin network during the natural polymerization and one of the major factors for the recruitment of mesenchymal progenitor cells [37, 46–48].

Another interesting aspect in our study is to analyze the SF as suitable source of MSCs. Using flow cytometry analysis prior to treatment, the presence of MSCs was observed in the SF in 21 of the 31 enrolled patients, representing 67.7% in total. The level of MSCs in these SF was as low as 5.19 ± 7.15 MSCs/μL. However, the use of this technique to measure fresh SF without a prior cell expansion cycle can represent a limitation due to the low number of cells [35]. In order to overcome this limitation, the presence of MSCs in those SF was evaluated by means of culturing on plastic surfaces to determine the presence of colony-forming cells (CFU-F). In this case, CFU-Fs were found in the SF of all patients, with an average value of 410.59 ± 246.36 CFU-F/mL. These results are consistent with those reported in other studies in which the possibility of using SF as a source of autologous MSCs is demonstrated [5, 34]. This source of cells for obtaining MSCs may be a promising alternative for treating diseases related to cartilage degeneration diseases such as OA.

Various factors must be considered when deciding the cell source and good environmental conditions for optimal effects [30]. The advantage of using SF as a cell source over other niches, such as bone marrow or fat tissue, is foremost its easy access. Arthrocentesis is usually a necessary step prior to conducting an IA injection of corticosteroids, hyaluronic acid, or PRP. Additionally, MSCs present in the SF may derive from the SM, a tissue involved in the cartilage repair process [49, 50], and their chondrogenic capacity could be increased compared with other types of MSCs [51].

This study has some limitations. First, a relatively small number of samples were analyzed and no data were obtained after second and third infiltrations of both treatments because many patients did not present with knee swelling in their last visits. Second, there is a difficulty in working with synovial fluid, for both its complexity and small volumes obtained. Because of this, a cytokine analysis in order to study the inflammatory process could not be carried out, so the work focused mainly on cellularity. Third, the donor-related variability concerning the amount of platelet-derived and plasmatic growth factors present in the PRP could account for the disparity in biological and clinical outcomes.

5. Conclusions

In summary, targeting different knee joint structures such as SM, AC, and SB with IA and IO infiltrations of PRP reduces the inflammatory environment and MSCs in SF. *In vitro* differentiation assays for SF-MSCs from OA patients showed different grades of multipotency toward the adipocyte, osteoblast, and chondrocyte lineages, although bilineage differentiation capacity was most frequently observed, confirming their identity. MSC modulation generated by PRP may be increased by acting directly on the SB, whose influence is crucial to the pathogenesis of OA. In addition, the use of PRP may favor MSCs therapeutic effect by decreasing proinflammatory processes present in the SF of OA patients. While being promising, a limitation of our study is the considerable intersubject variability; therefore, a larger sample would possibly be necessary to draw more definitive conclusions. Our results encourage further studies in order to shed more light on the cellular and molecular mechanisms and to elucidate whether the PRP application in both modalities might lead to structural joint tissue changes as *in vitro* and preclinical researches using this therapy have reported [26, 39, 52]. Finally, further studies will be needed in order to increase our knowledge about SF as source of MSC and their therapeutic potential.

Competing Interests

Eduardo Anitua and Sabino Padilla are scientists at BTI Biotechnology Institute, a dental implant company that investigates the fields of oral implantology and PRGF-Endoret technology. The other authors have no potential conflict of interests.

Authors' Contributions

Emma Muiños-López and Diego Delgado contributed equally to this work.

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