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# Immunobiocompatibility of metallic-ion doped bioactive glasses and titanium-based implants for musculoskeletal regeneration

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## Contents

## SUMMARY

Musculoskeletal disorders (MSDs) are conditions that can impair the body's movement through injury or pain in tissues of the musculoskeletal system, such as muscles, bones, and joints. Among the most common MSDs are osteoarthritis (OA), rheumatoid arthritis (RA), tendinitis, carpal tunnel syndrome, fibromyalgia, low back pain and bone fractures. Risk factors for MSDs include occupation, lifestyle and family history. However, considering that the risk to develop MSDs greatly increases with age, its prevalence is increasing along with the average life expectancy. Therefore, its burden on the patients and healthcare systems worldwide is expected to rise continuously in the following decades.

Nowadays, the treatment of MSDs heavily relies both on drug administration that proves ineffective in most cases and on surgery procedures including biomaterial implantation, which can represent a short-term solution since it does not attempt to treat the disease as a whole, instead only replacing the most affected tissue at the moment. Considering this, the need for new therapies is evident and the approach of **PREMUROSA** (Precision medicine for musculoskeletal regeneration, prosthetics, and active ageing) focuses on personalized medicine to overcome the current drawbacks in MSD treatment. In the case of this thesis, I worked with two types of optimized biomaterials: metallic-ion doped bioactive glasses and titanium-based discs.

Biomaterial implantation generally has two major risks, namely the development of implant-associated infections and hostile immune response. One possible approach to counteract this could be incorporating metallic ions with known antimicrobial properties into the surface of bioactive glasses, which can not only prevent infection, but should also modulate the immune response and enhance other biological processes. Silver, copper and tellurium were selected for their therapeutic effect; specifically, Ag, Cu and Te exhibit antibacterial properties, Ag and Cu have also a pro-angiogenic role and Te exerts an antioxidant effect.

Regarding the titanium-based discs, a patented chemical treatment was applied, which has been previously shown to enhance the deposition of hydroxyapatite upon soaking in simulated body fluid (SBF), while also succeeding in impeding bacterial colonization, whilst not

affecting osteoblast adhesion. The protocol we used consisted in surface acid etching using hydrofluoric acid, which removes the native oxide layer, and then submerging the discs in hydrogen peroxide to confer a controlled oxidized layer with free hydroxyl radicals, promoting protein adsorption.

Currently, almost half of early implant failures are due to inflammation. With that, it is essential to ensure that novel and optimized implants do not exacerbate the natural inflammatory response. In this thesis, the impact of culturing immune cells in contact with metallic-ion doped bioactive glasses and chemically treated titanium-based discs has been evaluated, focusing on their cytokine profile and T-cell phenotype.

Our results regarding the silver doping revealed high toxicity in PBMCs, while tellurium did not impact cell viability but led to highly variable inter-donor T-cell response, particularly in the Tregs subset. Interestingly, copper influenced T effector differentiation, causing a switch from Th17 to Th1 cells, while its undoped control was shown to impact other immune cells like macrophages and eosinophils through overexpression of cytokines (IL-5, IL-13) and chemokines (MCP-1/CCL2, MIP-1β/CCL4).

On the other hand, titanium-based discs did not impact PBMC viability. However, the polished untreated control showed an increased percentage of Th2 cells, while decreasing the Th17 one. The same condition also led to dysregulation of PBMCs' cytokine profile, while treating the discs through acid etching restored T-cell phenotype and cytokine secretion to values similar to the basal condition.

Once assessed the immunobiocompatibility of the tested biomaterials, the next task evaluated the impact of copper-doped bioactive glasses and chemically treated discs on the bone microenvironment, focusing on mesenchymal stromal/stem cells (MSCs) that are fibroblast-like adult multipotent progenitor cells that possess the ability to self-renew and differentiate into several cell types, including into osteoblasts and chondrocytes. Many authors have shown the potential of MSCs to resolve impaired bone regeneration conditions, mainly resorting to *in vivo* animal models. In an attempt to increase treatment efficacy, scientists have also opted for combining MSCs with biomaterials. This coupling ideally promotes MSCs' local delivery and

viability, while stimulating osteogenesis. Among the most used biomaterials there are the ceramics, polymers, composites, nanoparticles, graphene and metals, such as titanium. However, several drawbacks such as ethical issues, immunological rejection, costs, low quantity of cells harvested, among others, still need to be overcome in order to apply MSC transplantation approaches as a therapy. Therefore, our focus shifted towards the byproducts of MSCs, namely Extracellular Vesicles (EVs), since they can mimic the effect of their parental cells, refraining from some of the adverse effects of MSCs.

Our results underline the importance of surface topography and composition in shaping MSC behaviour. Chemically treated titanium discs were able to increase the metabolic activity of MSCs, potentially promoting osteointegration *in vivo*, while bioactive glasses require pre-treatment, to remove the initial ion burst release, and a protein-rich coating, to enhance cell adhesion. Proteomic studies revealed that MSC, cultured in contact with these optimized biomaterials, release EVs with different cargos. In particular, copper-doped bioactive glasses secrete EVs more enriched in pro-angiogenic factors, compared with its undoped control. However, our findings suggest minimal impact of biomaterials on MSC paracrine signalling through EVs, as supplementation with physiological MSC-EV doses had little effect on immune and endothelial cells.

## SOMMARIO

I disturbi muscoloscheletrici (DSM) comprendono tutte le condizioni in cui il movimento viene compromesso a causa del danno o del dolore dei tessuti del sistema muscoloscheletrico, comprendenti muscoli, ossa e articolazioni. Tra i DSM più comuni ci sono l'osteoartrite (AO), l'artrite reumatoide (AR), le tendiniti, la sindrome del tunnel carpale, la fibromialgia, la lombalgia e le fratture ossee. I fattori di rischio per lo sviluppo di DSM includono il lavoro, lo stile di vita e la familiarità. Considerando che il rischio di sviluppare un DSM aumenta considerevolmente con l'età, la sua prevalenza aumenta di pari passo con l'aspettativa di vita media. Pertanto, nei prossimi decenni si prevede un impatto socio-economico crescente sui pazienti e sui sistemi sanitari a livello mondiale.

Al giorno d'oggi, il trattamento dei DSM si basa sulla somministrazione di farmaci, che nella maggior parte dei casi si rivelano inefficaci. La seconda linea è rappresentata da procedure chirurgiche, tra le quali l'impianto di biomateriali. Essi rappresentano una soluzione a breve termine poiché non trattano le cause della malattia, ma si limitano a sostituire il tessuto più colpito al momento. Considerando ciò, vi è la necessità di nuove terapie e l'approccio del progetto PREMUROSA (Precision medicine for musculoskeletal regeneration, prosthetics, and active ageing), per superare gli attuali inconvenienti nel trattamento dei DSM, fonda il suo approccio sulla medicina personalizzata.

L'impianto di biomateriali presenta generalmente due rischi principali: da un lato lo sviluppo di infezioni dell'impianto e dall'altra una risposta immunitaria avversa. Un possibile approccio per contrastare questo fenomeno potrebbe essere quello di incorporare ioni metallici con note proprietà antimicrobiche nella superficie dei vetri bioattivi, che non solo possono prevenire l'infezione, ma potrebbero anche modulare la risposta immunitaria e migliorare altri processi biologici. Argento (Ag), rame (Cu) e tellurio (Te) sono stati selezionati per il loro effetto terapeutico; nello specifico, Ag, Cu e Te presentano proprietà antibatteriche, Ag e Cu hanno anche un ruolo pro-angiogenico e Te esercita un effetto antiossidante.

In questa tesi ho lavorato con due tipi di biomateriali ottimizzati: vetri bioattivi supplementati con ioni metallici e dischi a base di titanio.

Per quanto riguarda i dischi a base di titanio è stato applicato un trattamento chimico brevettato, che migliora la deposizione di idrossiapatite dopo l'immersione in simulated body fluid (SBF), riuscendo anche a impedire la colonizzazione batterica, pur non influenzando l'adesione degli osteoblasti. Il protocollo che abbiamo utilizzato consisteva nell'uso superficiale dell'acido fluoridrico, che rimuove lo strato di ossido nativo, e successivamente nell'immersione dei dischi in perossido di idrogeno per conferire uno strato ossidato sotto controllo con radicali idrossilici liberi, promuovendo l'adsorbimento delle proteine.

Circa il 50% dei fallimenti precoci degli impianti è dovuta a infiammazione. Perciò, è essenziale garantire che i nuovi e ottimizzati impianti non aggravino la naturale risposta infiammatoria. In questa tesi, è stato valutato l'impatto della coltura di leucociti (PBMC) a contatto con vetri bioattivi supplementati con ioni metallici e dischi a base di titanio trattati chimicamente, concentrandosi sul loro profilo di citochine e sul fenotipo dei linfociti T.

I nostri risultati riguardanti la funzionalizzazione con ioni Ag hanno rivelato un'elevata tossicità per i PBMC, mentre il tellurio (Te) non ha avuto alcun impatto sulla vitalità cellulare, ma ha portato ad una variabilità nella risposta dei linfociti T fra donatori molto variabile, in particolare nel sottogruppo dei linfociti T regolatori. È interessante notare che il rame (Cu) ha causato uno switch dai linfociti Th17 a linfociti Th1, mentre il materiale di controllo, non supplementato, ha un impatto su altri leucociti come macrofagi ed eosinofili attraverso la sovraespressione di citochine (IL-5, IL-13) e chemochine (MCP-1/CCL2, MIP -1β/CCL4).

I dischi a base di titanio non hanno influito sulla vitalità dei PBMC. Tuttavia, il controllo non trattato ha aumentato la percentuale dei linfociti Th2, diminuendo quella dei Th17. La stessa condizione ha portato anche alla deregolazione delle citochine prodotte dai PBMC, mentre il trattamento dei dischi mediante "acid etching" ha ripristinato il fenotipo dei linfociti T e la secrezione delle citochine a valori simili alla condizione basale.

Una volta valutata l'immunobiocompatibilità dei biomateriali testati, è stato testato l'impatto dei vetri bioattivi supplementati con rame e dei dischi trattati chimicamente, sulle cellule mesenchimali stromali/staminali (MSC) del microambiente osseo. Esse sono cellule progenitrici adulte multipotenti simili ai fibroblasti che possiedono la capacità di autorinnovarsi

e differenziarsi in diversi tipi cellulari, inclusi osteoblasti e condrociti. Molti autori hanno dimostrato il potenziale delle MSC nel risolvere condizioni di ridotta rigenerazione ossea, ricorrendo principalmente a modelli animali in vivo. Nel tentativo di aumentare l'efficacia del trattamento, gli scienziati hanno anche optato per la combinazione contenente cellule MSC e biomateriali. Questo accoppiamento promuove idealmente la vitalità e il rilascio locale delle MSC, stimolando al contempo l'osteogenesi. Tra i biomateriali più utilizzati ci sono le ceramiche, i polimeri, i compositi, le nanoparticelle, il grafene e i metalli, come il titanio. Tuttavia, diversi inconvenienti che includono questioni etiche, rigetto immunologico, costi, bassa quantità di cellule raccolte, devono ancora essere superati per poter utilizzare il trapianto di MSC a fini terapeutici. Pertanto, la nostra attenzione si è spostata verso i sottoprodotti delle MSC, vale a dire le vescicole extracellulari (EV), poiché possono stimolare l'effetto prodotto dalle loro cellule parentali, senza però avere alcuni degli effetti avversi delle MSC.

I nostri risultati mostrano l'importanza della topografia e della composizione della superficie del biomateriale utilizzato, nel comportamento delle MSC. I dischi di titanio trattati chimicamente sono stati in grado di aumentare l'attività metabolica delle MSC, quindi ciò promuoverebbe l'osteointegrazione in vivo. I vetri bioattivi, invece, richiedono un pretrattamento per rimuovere il rilascio iniziale di ioni e un rivestimento ricco di proteine per migliorare l'adesione cellulare. Studi proteomici hanno rivelato che le colture di MSC a contatto con questi biomateriali ottimizzati rilasciano EV con molecole diverse. In particolare, i vetri bioattivi supplementati con rame secernono EV maggiormente arricchite in fattori pro-angiogenici, se confrontate al controllo non supplementato. I nostri risultati tuttavia, suggeriscono un impatto minimo dei biomateriali sulla segnalazione paracrina delle MSC attraverso gli EV, poiché l'integrazione con dosi fisiologiche di MSC-EV ha avuto scarso effetto sulle cellule immunitarie ed endoteliali.

# INTRODUCTION – PART 1

## GENERAL OVERVIEW OF THE PREMUROSA PROJECT AND KEY CONCEPTS

This thesis was developed within the scope of the PREMUROSA project (Marie Skłodowska-Curie Actions Innovative Training Networks (MSCA ITN) funded by the European Union's Horizon 2020 research and innovation programme – grant agreement number 860462). The PREMUROSA project, as its full name indicates, is focused on "Precision medicine for musculoskeletal regeneration, prosthetics and active ageing". Although its main goal as an ITN is to train young researchers, providing the necessary training in interdisciplinary fields and acquiring both scientific and soft skills, PREMUROSA also declares four main scientific objectives, depicted in **figure 1**.

More specifically, within this project the aims were to:

- Study the role and interactions between materials, extracellular matrix, cells and tissues involved in musculoskeletal regeneration, considering patients' specific characteristics;
- Generate novel *in vitro* strategies to test medical devices mimicking clinical-pathological conditions, such as osteoarthritis, leading to a more personalized clinical approach;
- Create next-generation *in silico* models and decision support systems through machine learning and artificial intelligence techniques;
- Develop a multiparametric "omic" profiling able to categorize patients and predict healing and regeneration responses.

Naturally, the tasks within this project were divided into thirteen early stage researchers (ESRs), each one in charge with more specific duties. Therefore, the content of this thesis represents the study of ESR9 about the role of extracellular vesicles (EVs) as biomarkers or mediators of stem cells differentiation, wound healing and tissue regeneration around biomaterials.

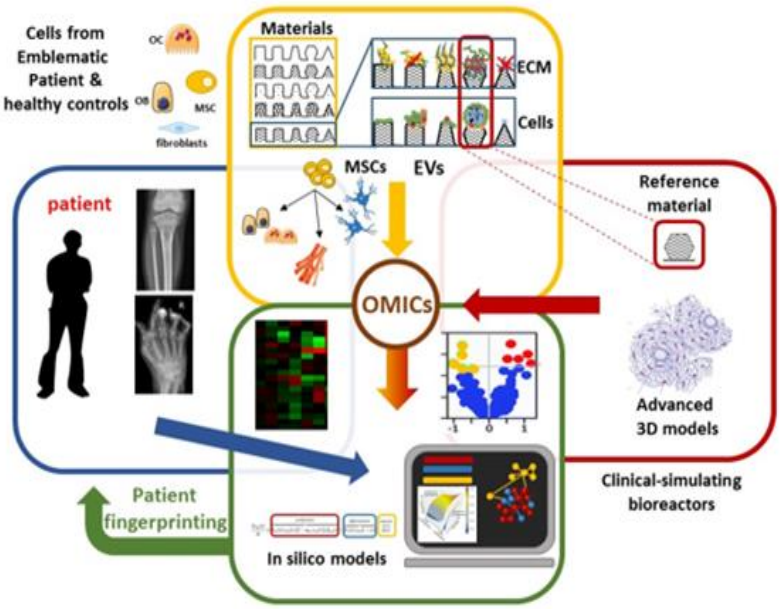


**Figure 1**. General overview of PREMUROSA's scientific objectives. The project proposed to use musculoskeletal disorder patient-derived cells to test optimized biomaterials, with the goal of integrating the obtained *omic* data for the development of novel in silico models and decision support systems. This will aid the establishment of a *patient fingerprint*, which can aid the diagnosis and response to implantation in a personalized manner. Adapted from PREMUROSA's website.

The goal of my PhD program has been to explore the combined therapeutic potential of modified biomaterials, mesenchymal stem/stromal cells (MSCs) and MSC-derived EVs to enhance wound healing and tissue regeneration in the context of MSDs, considering the interaction of the immune response to the biomaterials. We used titanium-based alloys (Ti-6Al-4V with extra low interstitials), treated with a patented surface treatment protocol developed by Politecnico of Turin (POLITO) (EP2214732B1) [1] and bioactive glasses supplemented with silver, copper or tellurium ions, to improve the material osteogenic properties and confer an antibacterial effect.

In particular, we aimed at:

- Defining a literature overview on topics such as EVs, MSCs, biomaterials, musculoskeletal diseases (MSDs) and approaches to tackle them, focusing on the promising role of EVs, I made a revision of the literature and I published a review article (***paper 1***) [2].

- Evaluating the immunobiocompatibility of the different Titanium-based and bioactive glass discs, focusing on T-cell response through immunophenotyping and evaluation of the cytokine secretion (***paper 2 and manuscript 3 in preparation***);
- Assessing and optimising the osteogenic properties of the tested biomaterials, focusing on the behaviour of MSCs in terms of adhesion, proliferation and differentiation *(**manuscript 4 in preparation***);
- Exploring the impact of selected biomaterials on MSCs' paracrine effects, specifically in regards to the EVs content and modulatory role in processes associated with biomaterials implantation in MSC patients, such as immune reaction and angiogenesis (***manuscript 4 in preparation*****)**

**PAPER 1**

cells

MDPI

*Review*

# Extracellular Vesicles in Musculoskeletal Regeneration: Modulating the Therapy of the Future

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**Abstract:** Tissue regeneration is a hot topic in health sciences, particularly because effective therapies promoting the healing of several cell types are lacking, specifically those of the musculoskeletal system. Mesenchymal Stem/Stromal Cells (MSCs) have been identified as crucial players in bone homeostasis, and are considered a promising therapy for diseases such as osteoarthritis (OA) and Rheumatoid Arthritis (RA). However, some known drawbacks limit their use, particularly ethical issues and immunological rejections. Thus, MSCs byproducts, namely Extracellular Vesicles (EVs), are emerging as potential solutions to overcome some of the issues of the original cells. EVs can be modulated by either cellular preconditioning or vesicle engineering, and thus represent a plastic tool to be implemented in regenerative medicine. Further, the use of biomaterials is important to improve EV delivery and indirectly to modulate their content and secretion. This review aims to connect the dots among MSCs, EVs, and biomaterials, in the context of musculoskeletal diseases.

**Keywords:** regenerative medicine; mesenchymal stem/stromal cells; extracellular vesicles; biomaterials



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## 1. Clinical Background

Musculoskeletal disorders (MSDs) are conditions that can limit bodily movement through injury or pain in tissues of the musculoskeletal system, such as muscles, bones, and joints. The most common diseases are osteoarthritis (OA), rheumatoid arthritis (RA), neck and low back pain, tendinitis, carpal tunnel syndrome, fibromyalgia, and bone fractures [1–3]. Risk factors include occupation, lifestyle, and family history. However, since the risk of developing MSDs greatly increases with age, their prevalence increases with the rising average life expectancy [1]. The severity of MSDs ranges from mild pain and discomfort, which interferes with everyday activities, to total impairment of movement. For this reason, early diagnosis and treatment may help ease symptoms and improve long-term quality of life [4,5]. In particular, OA is defined as a chronic joint inflammatory disease that affects all joint tissues of the musculoskeletal system, impacting mainly the hip, hand and knee articulations [6], involving tissues such as the infrapatellar fat pad and meniscus [7,8]. Patients bearing OA usually show synovial inflammation, calcified ligaments, subchondral bone sclerosis, osteophyte formation, and cartilage deterioration [9]. It is estimated that around 250 million people worldwide are suffering from OA [6]. Conversely, RA is an autoimmune inflammatory disorder characterized by synovial joint inflammation and swelling, estimated to affect up to 1% of the population worldwide [10]. Both these MSDs are common causes of permanent disability among the elderly population [11].

There is no cure for MSDs OA and RA; the only available clinical options aim to reduce the impact of the most common symptoms caused by inflammation, especially pain [5,12,13]. These conditions typically progress towards permanent joint damage,

and treatments are intended to slow disease evolution. Currently, non-steroidal anti-inflammatory drugs (NSAIDs) are frequently administered to manage the overall disabilities caused OA [5]. This strategy can in some severe cases be coupled with surgery to replace severely damaged joints. However, considering the multiple complications for the patient associated with this approach, as well as its ineffectiveness in restoring tissue function and movement, the need for novel and effective therapies is clear [5].

New approaches to regenerating musculoskeletal tissues are now emerging. One rapidly-growing strategy to treat damaged tissue, and diseases such as OA and RA, is regenerative therapy; this has the advantage of being a multifactorial approach that combines biomaterial design mimicking the natural extracellular matrix (ECM) of the tissues, these materials being loaded with autologous cells, bioactive molecules and growth factors to stimulate and improve the function of the target tissue [14].

Immunoengineering is a branch of regenerative medicine that focuses on the immune system and that aims to modify the cellular response in order to facilitate tissue reconstruction [15]. In particular, joint diseases such as OA and RA have been targeted with biomaterials and anti-cytokine treatments as a potential innovative therapy [16]. Considering the lack of effectiveness of the currently administered anti-inflammatory drugs in both OA and RA, the current task for researchers and clinicians in the musculoskeletal regeneration field, is to develop and implement more effective therapies. This may occur through the use of biomaterials and the employment of genetic engineering in order to modulate cell behavior.

Within this scenario, the review explores the current research trends regarding tissue regeneration in MSDs, focusing on Extracellular Vesicles (EVs) as future strategies for musculoskeletal system repair through cell therapy, biomaterial science, and immunoengineering. It focuses on the byproducts of Mesenchymal Stem/Stromal Cells (MSCs), more specifically EVs.

## 2. MSCs: Function and Mechanism of Action upon Tissue Damage

MSCs or multipotent mesenchymal stromal cells, as recommended by the International Society for Cellular Therapy (ISCT) [17], are fibroblast-like adult multipotent progenitor cells that possess the ability to self-renew and differentiate in vitro into several cell types [18], mainly into osteoblasts, adipocytes and chondrocytes. MSCs can be harvested from numerous tissues, including bone marrow (BM-MSCs) and other parts of the bone (periosteum and endosteum), adipose tissue (AT-MSCs) such as the infrapatellar fat pad (IFP-MSCs), umbilical cord (UC-MSCs), peripheral blood, and oral tissues [19–23] (Figure 1A).

Oryan et al. have reviewed and grouped the advantages and disadvantages of each type of MSCs, and report that although AT-MSCs achieve higher cell viability, BM-MSCs are more stable in culture and more prone to differentiate into osteoblasts, thus being preferable for transplantation in cases of bone fractures [20]. The therapeutic delivery of BM-MSCs at bone fracture sites is a strategy worthy of consideration, owing to their osteogenic and chondrogenic capabilities [19].

In physiological conditions, MSCs naturally migrate to the site of bone injury/damage, where osteogenesis—i.e., differentiation into osteoblasts (cells capable of forming bone)—occurs [24]. This transition from MSCs into osteoblasts begins with the commitment towards the osteogenic/chondrogenic lineages through the Wingless-int (Wnt) pathway [25]. During this process, there is a natural upregulation of several transcription factors including Distal-less homeobox 5 (DLX5), Osterix (OSX), and Runt-related transcription factor 2 (RUNX2) [25–27] aimed at triggering the differentiation cascade. In turn, this leads to the overexpression of typical osteoblast-related proteins, namely alkaline phosphatase (ALP) that promotes collagen type I (COL1A1) deposition, essential for proper bone formation [28].

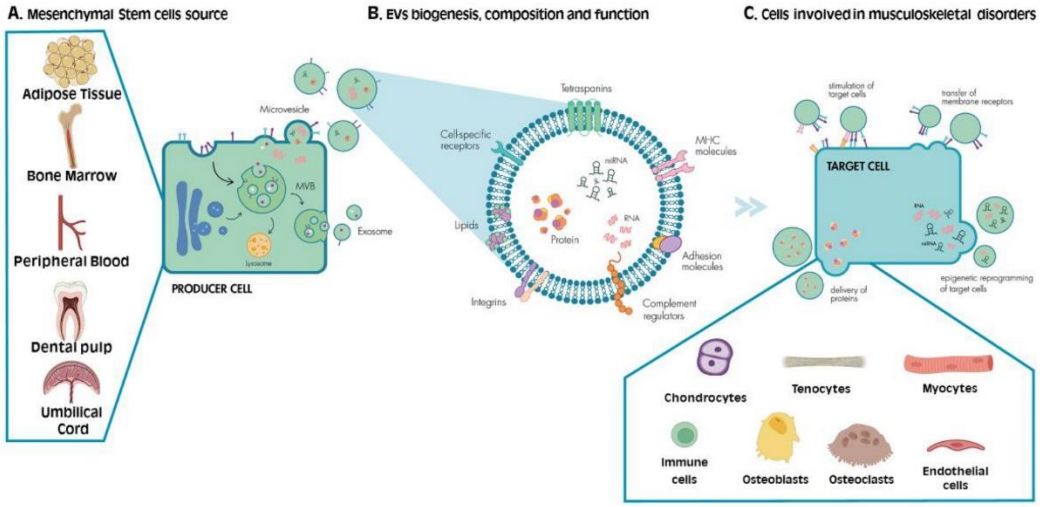


**Figure 1.** Cell-to-cell Communication via EVs in the Musculoskeletal System. (**A**) MSCs can be found in the bone marrow, adipose tissue, dental pulp, peripheral blood, umbilical cord, and among other tissues. (**B**) MSCs release EVs containing proteins, lipids, and nucleic acids (DNA and miRNA) to the surrounding environment. EVs can be formed either through plasma membrane budding (microvesicles) or through an endosomal route (exosomes). (**C**) They express surface markers that interact with the membrane receptors of the target cells (chondrocytes, myocytes, osteoclasts, osteoblasts, immune cells, tenocytes, among others) impacting the vesicle uptake and cargo delivery or directly stimulating and/or reprogramming the target cell. Created with BioRender.com.

After bone damage/fracture, the inflammation process starts [29], with an increased secretion of chemotactic factors and cytokines that mediate bone regeneration. Both innate (macrophages) and adaptive (lymphocytes) immunity act upon injury by phagocyting necrotic tissues and secreting growth factors and cytokines, which collectively contribute to bone repair [20].

In this phase, the MSCs' secretome can promote polarization of macrophages into the M2 anti-inflammatory and the M1 pro-regenerative phenotypes, through the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the signal transducer and activator of transcription 3 (STAT3) pathways [30–32]. In addition, the expression of tumor necrosis factor-alpha (TNF-α) can be reduced through MSCs transplantation; since high levels of TNF-α have a proapoptotic effect in osteoblasts, this should improve bone formation [33]. TNF-α also inhibits the expression of RUNX2 and Osterix, suggesting MSCs could facilitate the host osteoblast formation by restoring normal levels of the two essential transcription factors in osteogenesis [34]. Further, MSCs have been reported to contribute to blood vessel formation, known to be crucial in bone formation and repair [20]. In summary, MSCs are promising for therapeutic applications due to their immunomodulatory, angiogenic, cell recruitment, antiapoptotic, and differentiation effects [20,24].

In MSDs, such as OA disease, MSCs play a pivotal role in immunomodulation, modifying their phenotype in response to molecules produced by damaged tissues, both in acute and chronic phases of the disease [35]. Zhao et al. reported that MSCs are able to regulate macrophages polarization, promoting the healing process. This process brings an overall improvement of inflammation with an increase in interleukin -10 (IL-10) levels and a decrease of IL-12 and IL-1β and a contemporaneous augmentation of the phagocytes' activity [35]. Clinically, MSCs demonstrated to be effective in reducing pain for 5 years after joint injection of MSCs in OA patients [5].

Various bone injuries and anomalies reduce the natural healing process, pointing up the importance of developing new strategies for bone regeneration [36]. MSCs have thus emerged as a promising candidate therapy for MSDs [36]. MSCs can be delivered by systemic injection, as used to treat osteoporosis [37], by local/direct injection, as in the case of nonunion fractures [38–40] or administered with hydrogels or scaffolds, such as decellularized ECM, as in the case of large bone defects [20].

Much research has studied how MSCs resolve impaired bone regeneration conditions, mainly resorting to in vivo animal models [41]. A first study attempted to inject allogeneic BM-MSCs into rats, locally or systemically, to promote fracture healing [37]. Although both treatments showed significant improvement in tissue healing, without triggering an adverse immune response, the authors point out that, in cases of deeper multiple fractures, as expected in osteoporotic patients, the systemic injection might be more beneficial [37]. Other researchers have focused on this approach, paying special attention to MSCs' migration and homing for effective correction of bone lesions, with promising results [42–44]. Regarding nonunion fractures, various studies have resorted to MSCs to stimulate bone regeneration, all with fairly positive results, healing times ranging from 4 weeks to 10 months (reviewed by Fayaz et al. [45]).

Lastly, in an attempt to increase treatment efficacy, MSCs have been combined with biomaterials. These scaffolds ideally promote MSCs' local delivery and viability, while stimulating osteogenesis [24]. Freitas et al. reviewed the properties, advantages, and disadvantages of current materials in this field: ceramic biomaterials—commonly consisting of calcium phosphate (CaP) in the form of hydroxyapatite (HA), β-tricalcium phosphate (β-TCP), or a combination of both, known as biphasic calcium phosphate (BCP); polymers, both natural and synthetic; composites, i.e., a mix of polymeric biomaterials and ceramics; and nanoparticles (NP) [24]. Additionally, graphene and metals, such as titanium and tantalum, are also valid options thanks to their biocompatibility, and their stimulation of proliferation and differentiation [46]. MSCs are already being implemented in clinical trials, in oral and maxillofacial surgery [47–49] and in large bone defects [20,50].

Currently, nearly 400 clinical trials focused on using MSCs to treat pathologies related to multiple organs and cell lineages have been completed, and many more are listed as ongoing (http://www.clinicaltrials.gov/ (accessed on 21 December 2021)). These studies focus on a wide range of diseases, including autoimmune diseases such as RA [51], type 1 and 2 diabetes mellitus [52], multiple sclerosis [53] and systemic lupus erythematosus [54], as well as OA [55], graft versus host disease [56], chronic kidney disease [57], idiopathic pulmonary fibrosis [58], cirrhosis [59], acute myocardial infarction [60] and COVID-19 [61].

Some clinical trials report improvements in OA treatment through the use of MSCs [55]. Centeno et al. combined BM-MSCs with bone marrow aspirates and platelet lysates, improving chondral and meniscus volume while also greatly reducing pain in 60% of patients, and reducing to one-tenth the need for replacement surgery [62]. There are also several active and competed clinical studies regarding RA. For example, Wang et al. administered UC-MSCs intravenously to patients affected by RA ($4 \times 10^4$ cells/injection), together with the common disease-modifying anti-rheumatic drugs (DMARDs) treatment, at intervals of 3, 6 or 8 months. They report an improvement in quality of life and reduced joint swelling and pain, compared with control groups (treated with DMARDs alone), assessed via the disease activity score 28 (DAS28) parameter and the Health Assessment Questionnaire (HAQ) [63]. Using a different approach, Park et al. opted for altering the dosage of UC-MSCs in a single intravenous injection. In this study, patients who received the higher concentration of cells ($1 \times 10^8$ cells) showed lower DAS28 and visual analog scale (VAS) scores, indicative of therapy efficacy [64]. Of note, clinical trials using MSCs as a potential treatment for RA have been considered safe, no toxicity or severe adverse effects being reported thus far [51].

However, drawbacks including ethical issues, immunological rejection, costs, and low quantity of cells harvested, still need to be overcome in order to apply MSCs' transplantation as a therapy [20,65]. It is also reported that MSCs can cause serious secondary effects. In one

such case unwanted differentiation after transplantation into the brain of a child with Ataxia Telangiectasia caused tumors [66]. In order to counteract some of the above problems, the use of MSCs secretome, and more specifically of MSC-derived EVs, has increasingly been employed over recent years [67].

Interestingly, MSCs' secretome can mimic most of the effects of the cells themselves [67]: it contains paracrine products of MSCs' metabolism that promote wound healing by increasing proliferation and differentiation [68] and exerts immunomodulation [69], cell recruitment [70], pro-angiogenic [69] and pro-survival [71] functions, indicating that this cell-free approach may represent an alternative therapy [67]. This thanks to its composition, essentially comprising growth factors (GF) (Epidermal-GF (EGF), Fibroblast- GF(FGF), Hepatocyte-GF (HGF), Insulin-like-GF (IGF), Platelet-Derived-GF (PDGF), interleukins (IL-6, -8, -10), matrix metallopeptidases (MMP )-1, -2, -3, -7) and MMP inhibitors (TIMP-1, -2), angiogenic factors (Vascular-Endothelial-GF(VEGF), Angiogenic Factors Angiopoietin (ANG), chemoattracting proteins (Chemokine (C-C motif) ligand 5 CCL5/RANTES, monocyte chemoattractant protein 1 (MCP-1), adhesion molecules (intercellular adhesion molecule(ICAM), vascular cell adhesion molecule (VCAM), and immunoregulators (Transforming Growth Factor-β (TGF-β), Indoleamine 2,3-dioxygenase (IDO) [14,54]. These molecules are either free, outside cell boundaries, or encapsulated in EVs [67].

## 3. EVs: Biogenesis and Function

Studies focusing on cell-to-cell communication have employed body fluids, for example saliva, urine, blood, and breast milk, to show that all cell types can release lipidic bilayer vesicles, now generically labeled as EVs [72,73]. It is known that EVs naturally carry proteins, lipids, and nucleic acids, also possessing the potential to be bioengineered as drug delivery systems [74,75] (Figure 1B). Depending on both EVs cargo and the target cell type, EVs can influence various cellular processes, more specifically proliferation, differentiation, senescence, and apoptosis, while also impacting immunomodulation, blood coagulation, and angiogenesis [76,77].

Currently, the International Society of Extracellular Vesicles (ISEV) recommends making a distinction among three main types of EVs, based on size and release mechanism: apoptotic bodies, microvesicles, and exosomes [73]. Apoptotic bodies are the largest of these, ranging from 50 nm to 5 µm. They form by blebbing of the plasma membrane in cases of programmed cell death [78]. Microvesicles (MVs) are shed from budding of the plasma membrane, and their size is between 50 nm and 1 µm. Lastly, exosomes are typically less than 150 nm in diameter, and are the result of the fusion of multivesicular bodies (MVBs) with the plasma membrane, releasing intraluminal vesicles (ILVs) into the surrounding microenvironment, hence the term exosomes [73].

Exosome biogenesis starts with the formation of early endosomes, through endocytosis of extracellular components [75]. Budding of the membrane to capture proteins, lipids, and other molecules causes the formation of ILVs, small bodies the size of exosomes, that collect inside endosomes, leading to their maturation into MVBs [75]. ILVs are mainly created by action of the endosomal sorting complex required for transport (ESCRT), a family of four protein complexes, ESCRT-0, -I, -II and -III. MVBs can be degraded by fusing with lysosomes, or may in turn join the plasma membrane, so that their content is released outside the cellular compartment [75]. Some other proteins, specifically those frequently used as exosome markers, also contribute to exosome biogenesis, including CD63, MHC class II, tumor Susceptibility 101 (TSG101), ALG-2-interacting protein X (ALIX), syndecan, syntenin and Heat Shock Protein 70 (HSP70) [75].

Cells under various stimuli can increase or decrease EVs secretion in vitro [72,75]. In particular cases, irradiation [79], hypoxia [80] and other chemical stresses [81,82] may cause cells to boost EVs production and release. Whether this is a way to expel unwanted molecules produced under these stress conditions, or whether it is a signal to neighboring or distant cells, is still a matter of debate [73].

After release, EVs can be incorporated by target cells by direct fusion with the plasma membrane or by endocytosis, either through pinocytosis or by phagocytosis, dependently or independently of clathrin or caveolin, or lipid-raft mediated [75,83]. In general, it is thought that this mechanism is dependent on the recognition of specific surface markers on the vesicle membrane by the cellular membrane [75,84]: tetraspanins present on the EVs surface, such as CD9 and CD81, have a known positive effect in cell adhesion and in viral/parasitic internalization, which may explain their role as vesicle membrane proteins [83,85]. Other protein families have been associated with the EV uptake mechanisms, including integrins (αv, β3 and Lymphocyte function-associated antigen 1(LFA-1 [85]), proteoglycans (HSPGs [86]) and lectins (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin(DC-SIGN) [87] and DEC-205 [88]), since using antibodies against these molecules heavily impacts vesicle internalization in vitro [83]. It is also considered that the preferential EV uptake method may be associated with the cell type and its physiological state [89]. While some cases of specific/preferential uptake of EVs by a certain cell type have been described [87,90], there is still no consensus as to whether the internalization process is indeed cell-type specific [83].

On their surface, EVs can also display several molecules that directly affect a target cell without the need for uptake. Immunoglobulins, complement proteins, coagulation factors, cytokines, enzymes, and DNA have been detected in association with the vesicle membrane [91]. In the context of RA, Cloutier et al. demonstrated that synovial fluid contains platelet-derived EVs displaying immunoglobulins, antigens, and complement proteins as immune complexes capable of exerting a pro-inflammatory signal, in the presence of neutrophils [92]. Lastly, EVs can also function as antigen-presenting vehicles, as shown by Raposo et al., who reported that murine and human B lymphocytes release vesicles containing MHC class II molecules that can activate a specific T cell response [93]. Antigen-presenting EVs can also stimulate both T cells and macrophages phenotype shift towards Tregs and M2, respectively, which diminishes the length of the inflammatory phase in musculoskeletal regeneration [94].

Immune cells were shown to be able to transfer membrane receptors to other cells. For example, leukocyte-derived EVs can transfer monocyte/macrophage tissue factor to platelets [95]. Conversely, platelet-specific adhesion molecules can be relocated to hematopoietic cells through the action of EVs [96]. Other functions of EVs include epigenetic reprogramming of the target cells by altering the DNA methylation patterns, histone modifications and non-coding RNA post-transcriptional editing [97] (Figure 1C).

## 4. MSC-Derived EVs as Novel Therapies: Advantages and Disadvantages

Recent studies have also compared the ability of MSC-derived EVs to mimic the effect of their parent cells [98], and have concluded that MSC-EVs can exert similar effects to MSCs, in terms of injury repair and tissue regeneration [99–102], anti-inflammatory profile [103], cell proliferation and migration [102,104], and promoting collagen synthesis [105] and angiogenesis [102,104,105]. This indicates that MSC-EVs can replace the original cells as a therapeutic tool, eliminating some of the adverse effects of MSCs [98,106]. Indeed, the low immunogenicity and the low toxicity of EVs compared with MSCs are an important advantage of using these vehicles, together with their offering more stable storage, high stability in circulation, easier large-scale production, and superior biocompatibility [107,108]. Additionally, in contrast with MSCs, EVs can penetrate the blood-brain barrier, thus overcoming the first pass effect typical of some drug treatments [108].

MSCs produce EVs that express chemokines receptors able to facilitate targeting with several cell types also in damaged tissues [98]. Furthermore, new technologies, such as decorating EVs surface with binding proteins, are being developed, in order to increase the delivery efficiency of EVs to the target cells, improving cell-to-cell communication [98,109]. These strategies could be very useful to modulate the cellular communication in the musculoskeletal field.

For example, regarding musculoskeletal regeneration, Qin et al. evaluated the impact of MSC-EVs in osteoblasts' biological processes: in this study, culturing human osteoblasts (hFOB 1.19 cell line) with MSC-EVs led to a similar degree of differentiation as exposing the cells to fresh osteoblast culture medium [110]. In addition, the expression of osteogenesis-related genes such as ALP, osteocalcin (OCN), osteopontin (OPN) and RUNX2 in the EVs-treated condition was similar to the hFOBs culture in a commercially available complete osteogenesis media [110]. Conversely, MSC-EVs also impair osteoclast formation in vivo, as reported by Hu et al., in an osteoporotic mouse model [111]. The authors injected human UC-MSC-derived EVs through the tail vein of the osteoporotic mice and noted a decrease in osteoclast number on the trabecular bone surface, coupled with increased number of osteoblasts, compared to the control group [111].

Furthermore, it has been reported in a rat osteochondral defect model that MSC-EVs promote cellular proliferation and migration in both cartilage and synovium, while also stimulating ECM deposition [112]. Additionally, rats treated with MSC-EVs exhibited an increase in M2 macrophage infiltration in cartilage and synovium, accompanied by a reduction of pro-inflammatory cytokines' expression and M1 polarization, which leads to a regenerative immune profile [112]. On another study, muscle tissue regeneration was achieved by Nakamura et al.: the authors reported that in vitro culture of C2C12 cells (mouse myoblast cell line) with MSC-EVs promotes cell proliferation and differentiation, through the increase of nuclei number, fusion index and myogenic markers expression [113]. Moreover, the same authors show that in a mouse model of cardiotoxin-induced muscle injury, MSC-EVs locally administered at the injury site reduce fibrosis and increase angiogenesis, hence improving muscle regeneration [113]. Lastly, Chen et al. tested the ability of EVs to repair tendon damage. In a study performed in rabbits that underwent Achilles tendon repair surgery, the group administered with AT-MSC-EVs evidenced greater tenocyte proliferation and migration, together with mechanical stress resistance improvement of the tendon, compared to the control group [114] (Figure 1C).

However, therapies with EVs must be approached with caution, since their effects have yet to be fully characterized. Information currently available has led researchers to disagree about the tumor induction capacity of MSC-EVs. Some data indicates that MSC-EVs can inhibit tumor cell proliferation and induce dormancy and apoptosis [115–117]; conversely, MSC-EVs are also reported to confer drug resistance and support cancer cell growth and metastatization [118,119] (reviewed by Zhang et al. [120]).

Further, there is no protocol standardization regarding MSC culture and EV isolation in the clinical field. The commonly employed use of fetal bovine serum (FBS) in MSC expansion may cause the unwanted co-isolation of bovine EVs together with MSC-EVs [121], whereas completely removing the serum can impact EV quantity and cargo [122]. Using different EV isolation techniques—ultracentrifugation, differential centrifugation, commercial isolation kits, size-exclusion chromatography, iodixanol density gradient [123], among others—with differing yields and purification efficiencies may also contribute to the disparity of results reported in the literature [98]. Indeed, these different approaches may lead to false conclusions, since bovine-EVs from FBS (and other contaminants) may mask the effects of the target EVs. As an example, FBS-EVs have been associated with increased migration of A549 epithelial adenocarcinoma cell line, compared with EVs that are FBS "depleted" by extended centrifugation, with an estimated removal of 95% of FBS-EVs [121].

## 5. Impact of Biomaterials in MSC-Derived Extracellular Vesicles

As mentioned above concerning MSCs, the delivery of EVs is also an important factor to be taken into consideration. The so-called 'Naked EVs' administration, i.e., without any coating, can lead to unspecific binding to non-target organs, which in turn causes side effects and reduces the concentration at the injury site, hampering treatment efficacy [124]. Conjugation with biomaterials is thus considered a viable strategy to ensure correct targeting, preservation, and controlled release of the treatment [125]. Currently,

the most widely studied coadjutants in EV delivery are hydrogels and scaffolds through vesicle encapsulation [125].

Hydrogels are cross-linked polymer chain networks with hydrophilic properties, containing up to 90% water [126]. These materials have the capability to expand by absorbing biological fluids, enabling EVs to be entrapped and providing controlled delivery [125]. Promising results have been reported using encapsulated MSC-EVs in hydrogels to promote regeneration. Mardpour et al. achieved constant EV release and resistance to degradation by using polyethylene glycol (PEG) hydrogels, in a rat hepatic regeneration model. The study showed both the feasibility of the delivery method and the pro-regenerative effect of MSC-EVs on injured liver [127]. A study by Qin et al. opted for a commercial hydrogel—HyStem-HP—to deliver EVs to promote osteogenesis in a critical size bone defect rat model, and reported that the system was responsible for bone formation enhancement [110]. This technique has also been shown efficient in cartilage regeneration, by combining EVs with a 3D device made of cartilage ECM and gelatin methacrylate hydrogel [128], and with the vesicles incorporated into a Photoinduced Imine Crosslinking (PIC) hydrogel glue [129].

Conversely, 3D polylactide (PLA) scaffolds have been investigated as potential enhancers of bone regeneration, combined with MSCs and MSC-EVs in an in vivo rat model. The study took human gingival MSCs (hGMSCs) and established several combinations to test the effects of the PLA scaffold, hGMSC-EVs and polyethyleneimine (PEI)-engineered EVs [130]. The results showed that all combinations improved bone healing, particularly the treatments with hGMSCs + 3D PLA with either hGMSC-EVs or PEI-EVs; these showed upregulation of the osteogenesis-related genes RUNX2 and Bone Morphogenic Protein (BMP) 2/4 and higher staining for Alizarin Red, a marker for calcium deposition and ECM mineralization, commonly used to evaluate osteogenesis [130]. In another study, MSC-EVs were loaded into collagen I/III sponges and employed in a rat periodontal defect model. The approach was found to promote periodontal tissue regeneration and formation of alveolar bone in vivo, with no evidence of adverse effects [131]. Further, when combined with poly (lactic-co-glycolic acid) supplemented with poly-dopamine (PLGA/pDA), adipose-derived stem cell-EVs can modulate the migration and homing of BM-MSCs to injury sites, thus increasing tissue healing [132]. A number of studies also evidence the advantages of using MSC-EVs coupled with hydrogels or scaffolds as potential therapeutic tools for skeletal regeneration [125].

Lastly, used in combination with EVs ceramics have also shown interesting results regarding bone regeneration. In general, these biomaterials possess similar compositions to the inorganic portion of bones, specifically in terms of calcium and phosphate content. Ceramics thus represent useful options for bone replacement, considering their biocompatibility, osteoconduction, and bone-cell pro-survival enhancement ability [133]. For example, β-TCP has been extensively studied in the musculoskeletal regeneration field for its elevated resorption rate and promotion of MSCs and osteoblasts' proliferation [134]. However, the addition of EVs derived from human induced pluripotent stem cells (iPSC) differentiated into MSCs, actively improved the biomaterial's osteoinductive activity. This combination strongly promoted MSCs' migration, proliferation and osteogenic properties [135], revealing the important role of EVs in regenerative medicine. On that basis, EVs can also be modulated to carry specific molecules, either natural or synthetic, by cell preconditioning or engineering [136] (Figure 2).

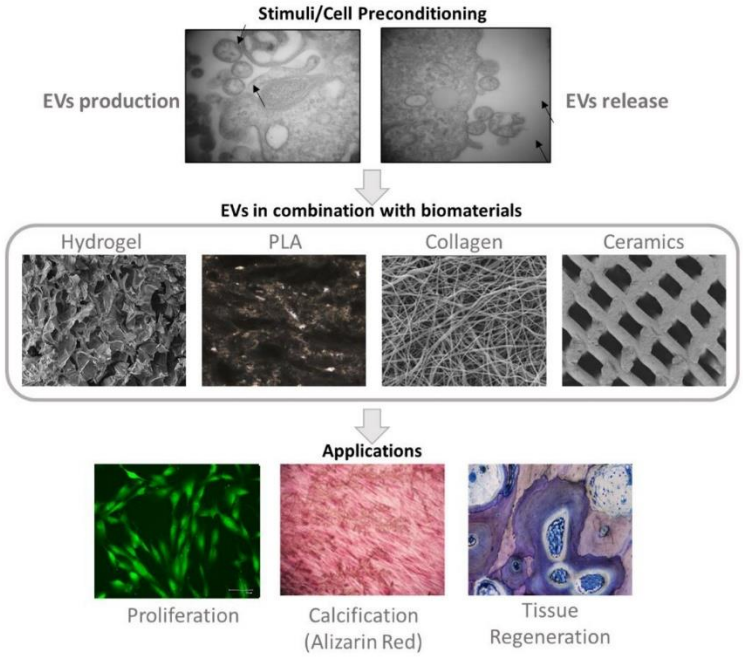


**Figure 2.** Engineering EVs and Their Applications. Cell preconditioning/engineering may increase the production and secretion of EVs, while also modulating its content in a controlled manner. These vesicles can then be delivered in combination with scaffold/biomaterials to ensure correct targeting, by conferring protection to the EVs and allowing a controlled release of their content. Thus, this strategy can lead to an improvement in tissue regeneration, through stimulation of several cell processes including proliferation, calcification and differentiation. The images depicting EVs production and release were obtained by Transmission Electron Microscopy (TEM); PLA and tissue regeneration images were obtained by the ground sections method in brightfield imaging; hydrogel, collagen and ceramics images were obtained by Scanning Electron Microscopy (SEM); the image representing proliferation was captured by confocal microscopy; the Alizarin Red staining used to detect calcification was captured in brightfield microscopy. All images belong to the authors.

## 6. MSC-Derived EVs Modulation through Cell Preconditioning

Preconditioning consists of exposing parent cells to specific stimuli, to enforce the expression and release of different molecules [67,125]. The focus of this review is on enhancing MSCs' regeneration potential by modulating the EV cargo through in vitro cell culturing in a 3D environment, under hypoxia, supplemented with pharmacological agents or inflammatory cytokines (reviewed in [67]). For instance, MSCs cultured in hypoxia (1% $O_2$) or anoxia (0% $O_2$) have produced EVs more suited for acute myocardial infarction treatment, in in vivo rat [102] and mouse [137] models, respectively. The former study cultured BM-MSCs in hypoxia for 72 h and, after EVs injection in rats, reported increased blood flow recovery and cardiac performance, together with a reduction in infarct size, compared with controls [102]. In the latter study, MSCs were cultured overnight in glucose deprivation and then preconditioned with two cycles of anoxia and reoxygenation. Microarray analysis revealed that miR-22 was upregulated in preconditioned-MSC-EVs that, upon injection, reduced apoptosis and enhanced cardiac function after infarction [137].

Regarding the musculoskeletal system, MSCs' preconditioning with dimethyloxaloylglycine (DMOG) was assessed in a critical-sized calvaria-defect rat model [138], a preclinical model frequently used to experimentally evaluate bone regeneration [139]. BM-MSCs were cultured with supplementation of 1000 µM DMOG for 48 h, after which the authors isolated

exosomes from the culture supernatants. Then, exosomes from non-preconditioned and preconditioned MSCs were injected in the rat model, in conjugation with hydroxyapatite (HA) scaffolds. Bone regeneration in vivo was increased when rats were treated with scaffolds embedded with EVs from MSCs preconditioned with low doses of DMOG [138]. The authors demonstrated that the mechanism of action might be related to the ability of EVs released by DMOG-pretreated MSCs to enhance angiogenesis of human umbilical vein endothelial cells (HUVECs), through the downregulation of Phosphatase and tensin homolog (PTEN), which in turn activates the Akt and mammalian target of rapamycin (mTOR) (AKT/mTOR) pathway [138]. A different study used TNF-α as preconditioning—referred to as priming in the study—for AT-MSCs [140]. Lu et al. cultured the cells for 72 h with 1 ng/mL of TNF-α and evaluated the effects of the isolated EVs in human primary osteoblast-like cells (HOBs). This specific preconditioning was found to increase the pro-osteogenic and proliferative induction by EVs in HOBs, thus representing a potential alternate therapy for bone regeneration [140].

## 7. MSC-Derived EVs Modulation through Engineering

Being natural carriers, capable of avoiding immune responses, and having good stability and integrity in the blood, EVs represent an interesting approach as delivery systems. Engineered EVs can offer a combined effect between their natural cargo and externally incorporated drugs or biological material, while also directing the therapy to the intended target, thanks to the specific recognition of surface proteins by receptor cells. Current techniques focus on modifying specific portions of EVs, before and/or after their isolation: cargo editing can enhance or transform the biological function of EVs; surface and membrane editing aims to alter the expression of markers to make the vesicles traceable and/or to change their target cells, while also affecting physical and chemical properties such as solubility [141]. Loading methods with high efficiency include extrusion, sonication, and saponin-assisted loading [141]. In this case, miRNA-loaded EVs were shown to promote cartilage preservation. In OA cartilage, miR-320 expression is decreased and in concomitance an increase of MMP-13 expression was observed. Moreover, the encapsulation of miR-320 seems to regulate MMP-13, and it could be a promising strategy for cartilage protection [142]. However, some protocols can cause membrane deformation or aggregation of particles or molecules [141]. An extensive view of advantages and disadvantages is provided in the review by Man et al. [141].

Further, this semi-synthetic system is flanked by the fully synthetic approach. Nanovesicles mimicking EVs may be developed either from cultured cells (cell-derived nanovesicles—CDNs) or from individual molecules (EV-inspired liposomes—EVLs) [141,143]. The most common technique for obtaining CDNs consists of sonicating whole cells to create particles similar to vesicles, ranging in size from 50 to 200 nm; this technique affords higher quantities of particles compared to conventional EV isolation protocols [143,144]. Depending on their origin, CDNs possess the membrane composition of the parent cells, which eliminates future steps of functionalization necessary for artificial vehicles. In addition, CDNs are more stable and less toxic than synthetic, non-cell-based vehicles [143,145]. Jo et al. found that CDNs derived from embryonic stem cells can stimulate the proliferation of mouse MSCs [146]. CDNs are also reported to be effective in pathogenic situations, when injected in mice with sepsis: they inhibit common symptoms such as hypothermia and eye exudates, by upregulating IL-10. CDNs were detected throughout the whole body, namely in the lungs, kidneys, and liver [147].

EVLs are synthetic particles created to provide the minimal required element for functional delivery systems. Thus, this technology allows the controlled production of pure and specific nanoparticles, without any biological contaminants [143,148]. This "bottom-up" technology is a valid alternative to other commercial agents used for drug delivery and transfection, since it presents better storage stability, an anti-aggregation effect, and reduced toxicity [143,149]. In fact, dendritic cell (DC) derived-EV-based EVLs, containing major histocompatibility complex (MHC) Class I molecules to mimic the antigen display

role to cytotoxic T cells, have been developed. These vesicles were traceable in both in vitro and in vivo settings, and contributed to adhesion and cell activation [150]. EVLs have also been used to deliver an anti-VEGF small interfering-RNA to target the A549 cell line. These EV-mimics enter A549 cells through direct fusion, and show similar uptake and efficiency compared to commercial Lipofectamine 2000 and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [149]. Functionalized liposomes can also be used to create scaffolds for bone regeneration, considering their features such as thermo-responsiveness, adhesiveness, bone targeting and osteoconductivity [151].

## 8. EVs Derived from MSC Differentiation

Although the multi-lineage differentiation capacity of MSCs has long been established, the biological functions of the secretome of osteoblasts, adipocytes, and chondrocytes, derived from MSC differentiation, have received little attention. However, some reported properties of the vesicles released by these cells may show potential for their establishment as therapeutic agents, particularly in the bone regeneration field.

Little is known about the direct role of adipocyte-derived EVs (Adi-EVs) in musculoskeletal processes. However, a potential role of these vesicles in bone regeneration may be inferred, considering their impact on inflammation, a key part of the healing process. Kranendonk et al. have demonstrated that Adi-EVs exert immunomodulatory effects on monocytes, aiding their differentiation into macrophages with both pro- and anti-inflammatory phenotypes [152]. Indeed, characterization of Adi-EVs reveals the presence of several cytokines, including adiponectin, TNF-α, retinol binding protein 4 (RBP4), macrophage colony-stimulating factor (M-CSF) and in particular macrophage migration inhibitory factor (MIF). This, in turn, when in contact with monocytes, induces their differentiation into adipose tissue macrophages (ATM) with their typical mixed profile, secreting both pro-inflammatory—IL-6, TNF-α and macrophage inflammatory protein-1-alpha (MIP-1α)—and anti-inflammatory proteins—IL-10 [152].

Obesity has been reported to severely impact Adi-EVs' properties. Zhang et al. showed that Adi-EVs favor macrophage polarization into the M1 pro-inflammatory profile, using an obesity rat model. They cultured bone-marrow-derived macrophages with Adi-microvesicles from obese and standard diet mice. The results showed an increase in the M1 phenotype vs. controls, as assessed by both quantitative real-time polymerase chain reaction (qRT-PCR)—through upregulation of M1 markers TNF-α, inducible nitric oxide synthase (iNOS), and IL-12—and flow cytometry—by increased number of CD11+ cells [153]. Conversely, M2 macrophage polarization is suppressed by Adi-EVs of high-fat-diet mice, likely due to the exosomal transfer of miR-34a, which represses macrophage expression of Krüppel-like factor 4 (Klf4) [154], a transcription factor essential in the macrophage shift to the anti-inflammatory phenotype [155].

With regard to chondrocytes, there are some evidences pointing to their potential use in musculoskeletal regeneration. Chen et al. injected alginate/cartilage progenitor cell (CPC) constructs into mice, along with either chondrocyte-EVs (CD-EVs) or BM-MSC-EVs, and found that treatment with CD-EVs improved CPC migration, proliferation, and matrix formation, by upregulating SRY-Box Transcription Factor 9 (SOX-9) and collagen type II levels, while inhibiting angiogenesis. Animals treated with CD-EVs showed more favorable results than those receiving BM-MSC-EVs in terms of chondrogenesis [156]. Another study corroborated these findings by combining CD-EVs with UC-MSCs for articular cartilage repair. The study verified that CD-EVs also increased expression of the aforementioned proteins in UC-MSCs in vitro, among other proteins related to chondrocyte maturation, and enhanced knee defect healing in a rabbit model by activating autophagy [157]. However, in an OA setting, OA-derived CD-EVs may lead to IL-1β production by macrophages and negatively impact cartilage degradation and synovitis, which means that the use of autologous CD-EVs may not be suited for OA treatment [158].

Lastly, osteoblast-derived EVs (OB-EVs) have been tested with the goal of bone regeneration. Using osteogenic differentiated pre-osteoblast MC3T3-E1 cell line-derived EVs,

Cui et al. induced MSC osteogenesis in vitro by modulating their microRNA profile. The authors expect these changes to impact several pathways connected with osteoblast differentiation and function, namely the Wnt, insulin, TGF-β, and calcium signaling pathways. The results also showed an increase in β-catenin, an important transcription coactivator of the Wnt pathway, through the upregulation of Ctnnb1, its encoding gene, and the inhibition of Axin1, a negative regulator [159]. The involvement of different microRNAs in osteogenesis has indeed been reported [160,161], although their delivery by OB-EVs remains largely unexplored. However, osteoblast activity can be impaired through miR-214-3p transfer from osteoclasts (bone-resorption cells) through EVs, which inhibit the bone formation process [162]. This osteoblast/osteoclast interaction by EVs also involves other players: RANKL (Receptor activator of nuclear factor kappa-B ligand) is a TNF family member that binds to RANK receptors (Receptor Activator of Nuclear Factor kappa-B) in osteoclast precursor cells to stimulate their differentiation into mature osteoclasts [163]. Cappariello et al. have shown that OB-EVs possess RANKL on their surface, and when in culture with osteoclasts, cell function, size, number of nuclei, and metabolic activity all increase. In RANKL$^{-/-}$ mice, which are also commonly without expression of osteoclast marker Tartrate-resistant acid phosphatase (TRAcP), injection of OB-EVs leads to the emergence of TRAcP-positive cells, in a directly proportionate manner [164]. Osteoclast activity was also successfully inhibited in vivo, by loading OB-EVs with zolendronate and dasatinib [164]. In the light of current information, it is considered that the use of OB-EVs, either naïve or with drug incorporation, may be a promising treatment for bone related diseases—such as osteoporosis—and cancer [165].

## 9. Conclusions and Future Perspectives

MSCs have been widely studied as therapeutic tools for their ability to stimulate renewal and differentiation into specialized connective tissues (bone, cartilage, adipose tissue, muscles), as well as their capability in immunomodulation, and inducing angiogenesis, cell recruitment, differentiation, and apoptosis inhibition [18–23]. However, due to some ethical, economic, and biological disadvantages, the use of MSC-derived EVs is being increasingly studied as a replacement for the use of MSCs in regenerative medicine. These particles maintain the properties of the parent cells while avoiding some of the known drawbacks associated with cell usage. Therefore, EVs are involved in numerous physiological and pathophysiological processes: in MSDs, EVs foster tissue regeneration through the delivery of factors capable of exerting mitogenic, angiogenic and immunomodulatory effects. As explored in this review, MSCs-EVs can interact with several cell types involved in MSDs, namely osteoblasts, osteoclasts, chondrocytes, myocytes, tenocytes, immune cells, and vascular endothelial cells, being able to modulate their cellular processes towards a pro-regenerative phenotype. Despite this, in order to consider EVs as clinical options, isolation methods must be standardized, and functional knowledge regarding different cellular origin must be enhanced. EVs modulation, either by cell preconditioning or engineering, is becoming a hot topic in molecular biology that allows for more control in terms of content, mechanism of action, and yield, although standardization is lacking. The strategies to couple biomaterials and EVs can also have a big impact as a future therapy, for the two technologies can enhance each other's properties and together represent an ideal approach for tissue healing. Lastly, using EVs derived from cell lineages that differentiate from MSCs may offer a more specific approach as targeted/personalized medicine, although focus on this field is still in its early days.

This review has explored the potential of MSC-EVs—and EVs originated from MSC-derived cell types—in several MSDs. In conclusion, the promising in vitro and in vivo studies indicate that the development of new therapies using MSC-EVs and EV modulation/engineering techniques, most notably in conjugation with biomaterials, will in the future erupt into the field of musculoskeletal regenerative medicine.

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## References

1. Cieza, A.; Causey, K.; Kamenov, K.; Hanson, S.W.; Chatterji, S.; Vos, T. Global estimates of the need for rehabilitation based on the Global Burden of Disease study 2019: A systematic analysis for the Global Burden of Disease Study 2019. *Lancet* **2020**, *396*, 2006–2017. [CrossRef]
2. Creed, F. A review of the incidence and risk factors for fibromyalgia and chronic widespread pain in population-based studies. *Pain* **2020**, *161*, 1169–1176. [CrossRef]
3. Tadjerbashi, K.; Åkesson, A.; Atroshi, I. Incidence of referred carpal tunnel syndrome and carpal tunnel release surgery in the general population: Increase over time and regional variations. *J. Orthop. Surg.* **2019**, *27*, 1–5. [CrossRef] [PubMed]
4. Almutairi, K.; Nossent, J.; Preen, D.; Keen, H.; Inderjeeth, C. The global prevalence of rheumatoid arthritis: A meta-analysis based on a systematic review. *Rheumatol. Int.* **2021**, *41*, 863–877. [CrossRef] [PubMed]
5. Abramoff, B.; Caldera, F.E. Osteoarthritis: Pathology, diagnosis, and treatment options. *Med. Clin. N. Am.* **2020**, *104*, 293–311. [CrossRef] [PubMed]
6. Hunter, D.J.; Bierma-Zeinstra, S. Osteoarthritis. *Lancet* **2019**, *393*, 1745–1759. [CrossRef]
7. Zeng, N.; Yan, Z.-P.; Chen, X.-Y.; Ni, G.-X. Infrapatellar Fat Pad and Knee Osteoarthritis. *Aging Dis.* **2020**, *11*, 1317–1328. [CrossRef]
8. Englund, M.; Roemer, F.; Hayashi, D.; Crema, M.D.; Guermazi, A. Meniscus pathology, osteoarthritis and the treatment controversy. *Nat. Rev. Rheumatol.* **2012**, *8*, 412–419. [CrossRef] [PubMed]
9. Mianehsaz, E.; Mirzaei, H.R.; Mahjoubin-Tehran, M.; Rezaee, A.; Sahebnasagh, R.; Pourhanifeh, M.H.; Mirzaei, H.; Hamblin, M.R. Mesenchymal stem cell-derived exosomes: A new therapeutic approach to osteoarthritis? *Stem Cell Res. Ther.* **2019**, *10*, 340. [CrossRef] [PubMed]
10. Guo, Q.; Wang, Y.; Xu, D.; Nossent, J.; Pavlos, N.; Xu, J. Rheumatoid arthritis: Pathological mechanisms and modern pharmacologic therapies. *Bone Res.* **2018**, *6*, 15. [CrossRef] [PubMed]
11. Minetto, M.A.; Giannini, A.; McConnell, R.; Busso, C.; Torre, G.; Massazza, G. Common Musculoskeletal Disorders in the Elderly: The Star Triad. *J. Clin. Med.* **2020**, *9*, 1216. [CrossRef] [PubMed]
12. Messina, O.D.; Wilman, M.V.; Neira, L.F.V. Nutrition, osteoarthritis and cartilage metabolism. *Aging Clin. Exp. Res.* **2019**, *31*, 807–813. [CrossRef] [PubMed]
13. Bullock, J.; Rizvi, S.A.; Saleh, A.M.; Ahmed, S.S.; Do, D.P.; Ansari, R.A.; Ahmed, J. Rheumatoid Arthritis: A Brief Overview of the Treatment. *Med. Princ. Pr.* **2018**, *27*, 501–507. [CrossRef] [PubMed]
14. Pina, S.; Ribeiro, V.P.; Marques, C.F.; Maia, F.R.; Silva, T.H.; Reis, R.L.; Oliveira, J.M. Scaffolding Strategies for Tissue Engineering and Regenerative Medicine Applications. *Materials* **2019**, *12*, 1824. [CrossRef]
15. Green, J.J. Immunoengineering has arrived. *J. Biomed. Mater. Res. Part A* **2021**, *109*, 397–403. [CrossRef]
16. Klimak, M.; Nims, R.J.; Pferdehirt, L.; Collins, K.H.; Harasymowicz, N.S.; Oswald, S.J.; Setton, L.A.; Guilak, F. Immunoengineering the next generation of arthritis therapies. *Acta Biomater.* **2021**, *133*, 74–86. [CrossRef]
17. Horwitz, E.M.; Le Blanc, K.; Dominici, M.; Mueller, I.; Slaper-Cortenbach, I.; Marini, F.C.; Deans, R.J.; Krause, D.S.; Keating, A.; International Society for Cellular Therapy. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. *Cytotherapy* **2005**, *7*, 393–395. [CrossRef] [PubMed]
18. Ullah, I.; Subbarao, R.B.; Rho, G.J. Human mesenchymal stem cells—Current trends and future prospective. *Biosci. Rep.* **2015**, *35*, e00191. [CrossRef] [PubMed]
19. Knight, M.N.; Hankenson, K.D. Mesenchymal Stem Cells in Bone Regeneration. *Adv. Wound Care* **2013**, *2*, 306–316. [CrossRef] [PubMed]
20. Oryan, A.; Kamali, A.; Moshiri, A.; Eslaminejad, M.B. Role of Mesenchymal Stem Cells in Bone Regenerative Medicine: What Is the Evidence? *Cells Tissues Organs* **2017**, *204*, 59–83. [CrossRef] [PubMed]
21. Barry, F.P.; Murphy, M. Mesenchymal stem cells: Clinical applications and biological characterization. *Int. J. Biochem. Cell Biol.* **2004**, *36*, 568–584. [CrossRef]

22. Gugliandolo, A.; Fonticoli, L.; Trubiani, O.; Rajan, T.; Marconi, G.; Bramanti, P.; Mazzon, E.; Pizzicannella, J.; Diomede, F. Oral Bone Tissue Regeneration: Mesenchymal Stem Cells, Secretome, and Biomaterials. *Int. J. Mol. Sci.* **2021**, *22*, 5236. [CrossRef] [PubMed]
23. Zhong, Y.-C.; Wang, S.-C.; Han, Y.-H.; Wen, Y. Recent Advance in Source, Property, Differentiation, and Applications of Infrapatellar Fat Pad Adipose-Derived Stem Cells. *Stem Cells Int.* **2020**, *2020*, 2560174. [CrossRef] [PubMed]
24. Freitas, J.; Santos, S.G.; Gonçalves, R.M.; Teixeira, J.H.; Barbosa, M.A.; Almeida, M.I. Genetically Engineered-MSC Therapies for Non-unions, Delayed Unions and Critical-size Bone Defects. *Int. J. Mol. Sci.* **2019**, *20*, 3430. [CrossRef]
25. Rucci, N. Molecular biology of bone remodelling. *Clin. Cases Miner. Bone Metab.* **2008**, *5*, 49–56. [PubMed]
26. Marks, S.C.; Popoff, S.N. Bone cell biology: The regulation of development, structure, and function in the skeleton. *Am. J. Anat.* **1988**, *183*, 1–44. [CrossRef] [PubMed]
27. Ducy, P.; Zhang, R.; Geoffroy, V.; Ridall, A.L.; Karsenty, G. Osf2/Cbfa1: A Transcriptional Activator of Osteoblast Differentiation. *Cell* **1997**, *89*, 747–754. [CrossRef]
28. Fakhry, E.H.M. Molecular mechanisms of mesenchymal stem cell differentiation towards osteoblasts. *World J. Stem Cells* **2013**, *5*, 136–148. [CrossRef] [PubMed]
29. Loi, F.; Córdova, L.A.; Pajarinen, J.; Lin, T.; Yao, Z.; Goodman, S.B. Inflammation, fracture and bone repair. *Bone* **2016**, *86*, 119–130. [CrossRef] [PubMed]
30. Abumaree, M.H.; Al Jumah, M.A.; Kalionis, B.; Jawdat, D.; Al Khaldi, A.; Abomaray, F.; Fatani, A.S.; Chamley, L.; Knawy, B.A. Human Placental Mesenchymal Stem Cells (pMSCs) Play a Role as Immune Suppressive Cells by Shifting Macrophage Differentiation from Inflammatory M1 to Anti-inflammatory M2 Macrophages. *Stem Cell Rev. Rep.* **2013**, *9*, 620–641. [CrossRef] [PubMed]
31. Gao, S.; Mao, F.; Zhang, B.; Zhang, L.; Zhang, X.; Wang, M.; Yan, Y.; Yang, T.; Zhang, J.; Zhu, W.; et al. Mouse bone marrow-derived mesenchymal stem cells induce macrophage M2 polarization through the nuclear factor-κB and signal transducer and activator of transcription 3 pathways. *Exp. Biol. Med.* **2014**, *239*, 366–375. [CrossRef]
32. Waterman, R.S.; Tomchuck, S.L.; Henkle, S.L.; Betancourt, A.M. A New Mesenchymal Stem Cell (MSC) Paradigm: Polarization into a Pro-Inflammatory MSC1 or an Immunosuppressive MSC2 Phenotype. *PLoS ONE* **2010**, *5*, e10088. [CrossRef] [PubMed]
33. Kovach, T.K.; Dighe, A.S.; Lobo, P.I.; Cui, Q. Interactions between MSCs and Immune Cells: Implications for Bone Healing. *J. Immunol. Res.* **2015**, *2015*, 752510. [CrossRef]
34. Nanes, M.S. Tumor necrosis factor-α: Molecular and cellular mechanisms in skeletal pathology. *Gene* **2003**, *321*, 1–15. [CrossRef]
35. Zhao, X.; Zhao, Y.; Sun, X.; Xing, Y.; Wang, X.; Yang, Q. Immunomodulation of MSCs and MSC-Derived Extracellular Vesicles in Osteoarthritis. *Front. Bioeng. Biotechnol.* **2020**, *8*, 8. [CrossRef]
36. Viganò, M.; Sansone, V.; D'Agostino, M.C.; Romeo, P.; Orfei, C.P.; De Girolamo, L. Mesenchymal stem cells as therapeutic target of biophysical stimulation for the treatment of musculoskeletal disorders. *J. Orthop. Surg. Res.* **2016**, *11*, 163. [CrossRef] [PubMed]
37. Huang, S.; Xu, L.; Zhang, Y.; Sun, Y.; Li, G. Systemic and Local Administration of Allogeneic Bone Marrow-Derived Mesenchymal Stem Cells Promotes Fracture Healing in Rats. *Cell Transplant.* **2015**, *24*, 2643–2655. [CrossRef]
38. Connolly, J.F. Clinical Use of Marrow Osteoprogenitor Cells to Stimulate Osteogenesis. *Clin. Orthop. Relat. Res.* **1998**, *355S*, S257–S266. [CrossRef] [PubMed]
39. Janowski, M.; Lyczek, A.; Engels, C.; Xu, J.; Lukomska, B.; Bulte, J.W.M.; Walczak, P. Cell Size and Velocity of Injection are Major Determinants of the Safety of Intracarotid Stem Cell Transplantation. *Br. J. Pharmacol.* **2013**, *33*, 921–927. [CrossRef]
40. Hernigou, P.; Beaujean, F. Treatment of Osteonecrosis With Autologous Bone Marrow Grafting. *Clin. Orthop. Relat. Res.* **2002**, *405*, 14–23. [CrossRef]
41. Su, P.; Tian, Y.; Yang, C.; Ma, X.; Wang, X.; Pei, J.; Qian, A. Mesenchymal Stem Cell Migration during Bone Formation and Bone Diseases Therapy. *Int. J. Mol. Sci.* **2018**, *19*, 2343. [CrossRef] [PubMed]
42. Granero-Moltó, F.; Weis, J.A.; Miga, M.I.; Landis, B.; Myers, T.J.; O'Rear, L.; Longobardi, L.; Jansen, E.D.; Mortlock, D.P.; Spagnoli, A. Regenerative Effects of Transplanted Mesenchymal Stem Cells in Fracture Healing. *Stem Cells* **2009**, *27*, 1887–1898. [CrossRef]
43. Lien, C.-Y.; Ho, K.C.-Y.; Lee, O.K.; Blunn, G.W.; Su, Y. Restoration of Bone Mass and Strength in Glucocorticoid-Treated Mice by Systemic Transplantation of CXCR4 and Cbfa-1 Co-Expressing Mesenchymal Stem Cells. *J. Bone Miner. Res.* **2009**, *24*, 837–848. [CrossRef] [PubMed]
44. Rapp, A.; Bindl, R.; Heilmann, A.; Erbacher, A.; Müller, I.; Brenner, R.; Ignatius, A. Systemic mesenchymal stem cell administration enhances bone formation in fracture repair but not load-induced bone formation. *Eur. Cells Mater.* **2015**, *29*, 22–34. [CrossRef]
45. Fayaz, H.C.; Giannoudis, P.V.; Vrahas, M.S.; Smith, R.M.; Moran, C.; Pape, H.C.; Krettek, C.; Jupiter, J.B. The role of stem cells in fracture healing and nonunion. *Int. Orthop.* **2011**, *35*, 1587–1597. [CrossRef]
46. Hiew, V.V.; Simat, S.F.B.; Teoh, P.L. The advancement of biomaterials in regulating stem cell fate. *Stem Cell Rev. Rep.* **2018**, *14*, 43–57. [CrossRef]
47. Kaigler, D.; Pagni, G.; Park, C.H.; Braun, T.M.; Holman, L.A.; Yi, E.; Tarle, S.A.; Bartel, R.L.; Giannobile, W.V. Stem Cell Therapy for Craniofacial Bone Regeneration: A Randomized, Controlled Feasibility Trial. *Cell Transplant.* **2013**, *22*, 767–777. [CrossRef]
48. Rickert, D.; Sauerbier, S.; Nagursky, H.; Menne, D.; Vissink, A.; Raghoebar, G.M. Maxillary sinus floor elevation with bovine bone mineral combined with either autogenous bone or autogenous stem cells: A prospective randomized clinical trial. *Clin. Oral Implant. Res.* **2010**, *22*, 251–258. [CrossRef] [PubMed]

49. Gimbel, M.; Ashley, R.K.; Sisodia, M.; Gabbay, J.S.; Wasson, K.L.; Heller, J.; Wilson, L.; Kawamoto, H.K.; Bradley, J.P. Repair of Alveolar Cleft Defects. *J. Craniofacial Surg.* **2007**, *18*, 895–901. [CrossRef]
50. Liebergall, M.; Schroeder, J.; Mosheiff, R.; Gazit, Z.; Yoram, Z.; Rasooly, L.; Daskal, A.; Khoury, A.; Weil, Y.; Beyth, S. Stem Cell–based Therapy for Prevention of Delayed Fracture Union: A Randomized and Prospective Preliminary Study. *Mol. Ther.* **2013**, *21*, 1631–1638. [CrossRef]
51. Lopez-Santalla, M.; Fernandez-Perez, R.; Garin, M.I. Mesenchymal Stem/Stromal Cells for Rheumatoid Arthritis Treatment: An Update on Clinical Applications. *Cells* **2020**, *9*, 1852. [CrossRef]
52. Cho, J.; D'Antuono, M.; Glicksman, M.; Wang, J.; Jonklaas, J. A review of clinical trials: Mesenchymal stem cell transplant therapy in type 1 and type 2 diabetes mellitus. *Am. J. Stem Cells* **2018**, *7*, 82–93. [PubMed]
53. Dulamea, A. Mesenchymal stem cells in multiple sclerosis—translation to clinical trials. *J. Med. Life* **2015**, *8*, 24–27.
54. Zhou, T.; Li, H.-Y.; Liao, C.; Lin, W.; Lin, S. Clinical Efficacy and Safety of Mesenchymal Stem Cells for Systemic Lupus Erythematosus. *Stem Cells Int.* **2020**, *2020*, 6518508. [CrossRef]
55. Freitag, J.; Bates, D.; Boyd, R.; Shah, K.; Barnard, A.; Huguenin, L.; Tenen, A. Mesenchymal stem cell therapy in the treatment of osteoarthritis: Reparative pathways, safety and efficacy—A review. *BMC Musculoskelet. Disord.* **2016**, *17*, 230. [CrossRef] [PubMed]
56. Godoy, J.A.P.; Paiva, R.M.A.; Souza, A.M.; Kondo, A.T.; Kutner, J.M.; Okamoto, O.K. Clinical Translation of Mesenchymal Stromal Cell Therapy for Graft Versus Host Disease. *Front. Cell Dev. Biol.* **2019**, *7*, 255. [CrossRef] [PubMed]
57. Chung, B.H. Use of mesenchymal stem cells for chronic kidney disease. *Kidney Res. Clin. Pr.* **2019**, *38*, 131–134. [CrossRef] [PubMed]
58. Tzouvelekis, A.; Toonkel, R.; Karampitsakos, T.; Medapalli, K.; Ninou, I.; Aidinis, V.; Bouros, D.; Glassberg, M.K. Mesenchymal Stem Cells for the Treatment of Idiopathic Pulmonary Fibrosis. *Front. Med.* **2018**, *5*, 142. [CrossRef]
59. Tsuchiya, A.; Takeuchi, S.; Watanabe, T.; Yoshida, T.; Nojiri, S.; Ogawa, M.; Terai, S. Mesenchymal stem cell therapies for liver cirrhosis: MSCs as "conducting cells" for improvement of liver fibrosis and regeneration. *Inflamm. Regen.* **2019**, *39*, 18. [CrossRef] [PubMed]
60. Chen, Z.; Chen, L.; Zeng, C.; Wang, W.E. Functionally Improved Mesenchymal Stem Cells to Better Treat Myocardial Infarction. *Stem Cells Int.* **2018**, *2018*, 7045245. [CrossRef]
61. Meng, F.; Xu, R.; Wang, S.; Xu, Z.; Zhang, C.; Li, Y.; Yang, T.; Shi, L.; Fu, J.; Jiang, T.; et al. Human umbilical cord-derived mesenchymal stem cell therapy in patients with COVID-19: A phase 1 clinical trial. *Signal Transduct. Target. Ther.* **2020**, *5*, 172. [CrossRef]
62. Centeno, C.J.; Schultz, J.R.; Cheever, M.; Freeman, M.; Faulkner, S.; Robinson, B.; Hanson, R. Safety and Complications Reporting Update on the Re-Implantation of Culture-Expanded Mesenchymal Stem Cells Using Autologous Platelet Lysate Technique. *Curr. Stem Cell Res. Ther.* **2011**, *6*, 368–378. [CrossRef]
63. Wang, L.; Wang, L.; Cong, X.; Liu, G.; Zhou, J.; Bai, B.; Li, Y.; Bai, W.; Li, M.; Ji, H.; et al. Human Umbilical Cord Mesenchymal Stem Cell Therapy for Patients with Active Rheumatoid Arthritis: Safety and Efficacy. *Stem Cells Dev.* **2013**, *22*, 3192–3202. [CrossRef] [PubMed]
64. Park, E.H.; Lim, H.; Lee, S.; Roh, K.; Seo, K.; Kang, K.; Shin, K. Intravenous Infusion of Umbilical Cord Blood-Derived Mesenchymal Stem Cells in Rheumatoid Arthritis: A Phase Ia Clinical Trial. *Stem Cells Transl. Med.* **2018**, *7*, 636–642. [CrossRef]
65. Hipp, J.; Atala, A. Tissue engineering, stem cells, cloning, and parthenogenesis: New paradigms for therapy. *J. Exp. Clin. Assist. Reprod.* **2004**, *1*, 3. [CrossRef] [PubMed]
66. Amariglio, N.; Hirshberg, A.; Scheithauer, B.W.; Cohen, Y.; Loewenthal, R.; Trakhtenbrot, L.; Paz, N.; Koren-Michowitz, M.; Waldman, D.; Leider-Trejo, L.; et al. Donor-Derived Brain Tumor Following Neural Stem Cell Transplantation in an Ataxia Telangiectasia Patient. *PLoS Med.* **2009**, *6*, e1000029. [CrossRef] [PubMed]
67. Ferreira, J.R.; Teixeira, G.Q.; Santos, S.; Barbosa, M.; Almeida-Porada, G.; Gonçalves, R.M. Mesenchymal Stromal Cell Secretome: Influencing Therapeutic Potential by Cellular Pre-conditioning. *Front. Immunol.* **2018**, *9*, 2837. [CrossRef] [PubMed]
68. Teixeira, F.G.; Carvalho, M.M.; Neves-Carvalho, A.; Panchalingam, K.M.; Behie, L.A.; Pinto, L.; Sousa, N.; Salgado, A.J. Secretome of Mesenchymal Progenitors from the Umbilical Cord Acts as Modulator of Neural/Glial Proliferation and Differentiation. *Stem Cell Rev. Rep.* **2014**, *11*, 288–297. [CrossRef] [PubMed]
69. Teng, X.; Chen, L.; Chen, W.; Yang, J.; Yang, Z.; Shen, Z. Mesenchymal Stem Cell-Derived Exosomes Improve the Microenvironment of Infarcted Myocardium Contributing to Angiogenesis and Anti-Inflammation. *Cell. Physiol. Biochem.* **2015**, *37*, 2415–2424. [CrossRef]
70. Chen, L.; Tredget, E.E.; Wu, P.Y.G.; Wu, Y. Paracrine Factors of Mesenchymal Stem Cells Recruit Macrophages and Endothelial Lineage Cells and Enhance Wound Healing. *PLoS ONE* **2008**, *3*, e1886. [CrossRef]
71. Li, T.; Yan, Y.; Wang, B.; Qian, H.; Zhang, X.; Shen, L.; Wang, M.; Zhou, Y.; Zhu, W.; Li, W.; et al. Exosomes Derived from Human Umbilical Cord Mesenchymal Stem Cells Alleviate Liver Fibrosis. *Stem Cells Dev.* **2013**, *22*, 845–854. [CrossRef]
72. Yáñez-Mó, M.; Siljander, P.R.-M.; Andreu, Z.; Zavec, A.B.; Borràs, F.E.; Buzas, E.I.; Buzas, K.; Casal, E.; Cappello, F.; Carvalho, J.; et al. Biological properties of extracellular vesicles and their physiological functions. *J. Extracell. Vesicles* **2015**, *4*, 27066. [CrossRef] [PubMed]
73. Hessvik, N.P.; Llorente, A. Current knowledge on exosome biogenesis and release. *Cell. Mol. Life Sci.* **2018**, *75*, 193–208. [CrossRef] [PubMed]

74. Ludwig, A.-K.; Giebel, B. Exosomes: Small vesicles participating in intercellular communication. *Int. J. Biochem. Cell Biol.* **2012**, *44*, 11–15. [CrossRef]
75. Colombo, M.; Raposo, G.; Théry, C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu. Rev. Cell Dev. Biol.* **2014**, *30*, 255–289. [CrossRef]
76. Van Niel, G.; D'Angelo, G.; Raposo, G. Shedding light on the cell biology of extracellular vesicles. *Nat. Rev. Mol. Cell Biol.* **2018**, *19*, 213–228. [CrossRef] [PubMed]
77. Maione, F.; Cappellano, G.; Bellan, M.; Raineri, D.; Chiocchetti, A. Chicken-or-egg question: Which came first, extracellular vesicles or autoimmune diseases? *J. Leukoc. Biol.* **2020**, *108*, 601–616. [CrossRef]
78. Vader, P.; Mol, E.A.; Pasterkamp, G.; Schiffelers, R.M. Extracellular vesicles for drug delivery. *Adv. Drug Deliv. Rev.* **2016**, *106*, 148–156. [CrossRef]
79. Lehmann, B.; Paine, M.S.; Brooks, A.M.; McCubrey, J.; Renegar, R.H.; Wang, R.; Terrian, D.M. Senescence-Associated Exosome Release from Human Prostate Cancer Cells. *Cancer Res.* **2008**, *68*, 7864–7871. [CrossRef]
80. King, H.W.; Michael, M.Z.; Gleadle, J.M. Hypoxic enhancement of exosome release by breast cancer cells. *BMC Cancer* **2012**, *12*, 421. [CrossRef]
81. Xiao, X.; Yu, S.; Li, S.; Wu, J.; Ma, R.; Cao, H.; Zhu, Y.; Feng, J. Exosomes: Decreased sensitivity of lung cancer A549 cells to cisplatin. *PLoS ONE* **2014**, *9*, e89534. [CrossRef]
82. Kanemoto, S.; Nitani, R.; Murakami, T.; Kaneko, M.; Asada, R.; Matsuhisa, K.; Saito, A.; Imaizumi, K. Multivesicular body formation enhancement and exosome release during endoplasmic reticulum stress. *Biochem. Biophys. Res. Commun.* **2016**, *480*, 166–172. [CrossRef] [PubMed]
83. Mulcahy, L.A.; Pink, R.C.; Carter, D.R.F. Routes and mechanisms of extracellular vesicle uptake. *J. Extracell. Vesicles* **2014**, *3*, 24641. [CrossRef] [PubMed]
84. Tian, T.; Zhu, Y.-L.; Zhou, Y.-Y.; Liang, G.-F.; Wang, Y.-Y.; Hu, F.-H.; Xiao, Z.-D. Exosome Uptake through Clathrin-mediated Endocytosis and Macropinocytosis and Mediating miR-21 Delivery. *J. Biol. Chem.* **2014**, *289*, 22258–22267. [CrossRef]
85. Morelli, A.E.; Larregina, A.T.; Shufesky, W.J.; Sullivan, M.L.G.; Stolz, D.B.; Papworth, G.D.; Zahorchak, A.F.; Logar, A.J.; Wang, Z.; Watkins, S.C.; et al. Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. *Blood* **2004**, *104*, 3257–3266. [CrossRef] [PubMed]
86. Christianson, H.C.; Svensson, K.J.; van Kuppevelt, T.H.; Li, J.-P.; Belting, M. Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 17380–17385. [CrossRef] [PubMed]
87. Näslund, T.I.; Paquin-Proulx, D.; Paredes, P.T.; Vallhov, H.; Sandberg, J.K.; Gabrielsson, S. Exosomes from breast milk inhibit HIV-1 infection of dendritic cells and subsequent viral transfer to $CD4^+$ T cells. *AIDS* **2014**, *28*, 171–180. [CrossRef]
88. Hao, S.; Bai, O.; Li, F.; Yuan, J.; Laferte, S.; Xiang, J. Mature dendritic cells pulsed with exosomes stimulate efficient cytotoxic T-lymphocyte responses and antitumour immunity. *Immunology* **2007**, *120*, 90–102. [CrossRef]
89. Abels, E.R.; Breakefield, X.O. Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake. *Cell. Mol. Neurobiol.* **2016**, *36*, 301–312. [CrossRef]
90. Rana, S.; Yue, S.; Stadel, D.; Zöller, M. Toward tailored exosomes: The exosomal tetraspanin web contributes to target cell selection. *Int. J. Biochem. Cell Biol.* **2012**, *44*, 1574–1584. [CrossRef]
91. Buzás, E.I.; Tóth, E.Á.; Sódar, B.W.; Szabó-Taylor, K.É. Molecular interactions at the surface of extracellular vesicles. *Semin. Immunopathol.* **2018**, *40*, 453–464. [CrossRef] [PubMed]
92. Cloutier, N.; Tan, S.; Boudreau, L.H.; Cramb, C.; Subbaiah, R.; Lahey, L.; Albert, A.; Shnayder, R.; Gobezie, R.; Nigrovic, P.A.; et al. The exposure of autoantigens by microparticles underlies the formation of potent inflammatory components: The microparticle-associated immune complexes. *EMBO Mol. Med.* **2012**, *5*, 235–249. [CrossRef] [PubMed]
93. Raposo, G.; Nijman, H.W.; Stoorvogel, W.; Liejendekker, R.; Harding, C.V.; Melief, C.J.; Geuze, H.J. B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med.* **1996**, *183*, 1161–1172. [CrossRef] [PubMed]
94. Roefs, M.T.; Sluijter, J.P.; Vader, P. Extracellular Vesicle-Associated Proteins in Tissue Repair. *Trends Cell Biol.* **2020**, *30*, 990–1013. [CrossRef] [PubMed]
95. Del Conde, I.; Shrimpton, C.N.; Thiagarajan, P.; Lopéz, J.A. Tissue-factor–bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. *Blood* **2005**, *106*, 1604–1611. [CrossRef]
96. Janowska-Wieczorek, A.; Majka, M.; Kijowski, J.; Baj-Krzyworzeka, M.; Reca, R.; Turner, A.R.; Ratajczak, J.; Emerson, S.G.; Kowalska, M.A.; Ratajczak, M.Z. Platelet-derived microparticles bind to hematopoietic stem/progenitor cells and enhance their engraftment. *Blood* **2001**, *98*, 3143–3149. [CrossRef]
97. Qian, Z.; Shen, Q.; Yang, X.; Qiu, Y.; Zhang, W. The Role of Extracellular Vesicles: An Epigenetic View of the Cancer Microenvironment. *BioMed Res. Int.* **2015**, *2015*, 649161. [CrossRef]
98. Gowen, A.; Shahjin, F.; Chand, S.; Odegaard, K.E.; Yelamanchili, S.V. Mesenchymal Stem Cell-Derived Extracellular Vesicles: Challenges in Clinical Applications. *Front. Cell Dev. Biol.* **2020**, *8*, 149. [CrossRef]
99. Bruno, S.; Grange, C.; Deregibus, M.C.; Calogero, R.A.; Saviozzi, S.; Collino, F.; Morando, L.; Busca, A.; Falda, M.; Bussolati, B.; et al. Mesenchymal Stem Cell-Derived Microvesicles Protect Against Acute Tubular Injury. *J. Am. Soc. Nephrol.* **2009**, *20*, 1053–1067. [CrossRef]

100. Lai, R.C.; Arslan, F.; Lee, M.M.; Sze, N.S.K.; Choo, A.; Chen, T.S.; Salto-Tellez, M.; Timmers, L.; Lee, C.N.; El Oakley, R.M.; et al. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res.* **2010**, *4*, 214–222. [CrossRef]
101. Shao, L.; Zhang, Y.; Lan, B.; Wang, J.; Zhang, Z.; Zhang, L.; Xiao, P.; Meng, Q.; Geng, Y.-J.; Yu, X.-Y.; et al. MiRNA-Sequence Indicates That Mesenchymal Stem Cells and Exosomes Have Similar Mechanism to Enhance Cardiac Repair. *BioMed Res. Int.* **2017**, *2017*, 4150705. [CrossRef]
102. Bian, S.; Zhang, L.; Duan, L.; Wang, X.; Min, Y.; Yu, H. Extracellular vesicles derived from human bone marrow mesenchymal stem cells promote angiogenesis in a rat myocardial infarction model. *J. Mol. Med.* **2014**, *92*, 387–397. [CrossRef]
103. Lee, C.; Mitsialis, S.A.; Aslam, M.; Vitali, S.H.; Vergadi, E.; Konstantinou, G.; Sdrimas, K.; Fernandez-Gonzalez, A.; Kourembanas, S. Exosomes Mediate the Cytoprotective Action of Mesenchymal Stromal Cells on Hypoxia-Induced Pulmonary Hypertension. *Circulation* **2012**, *126*, 2601–2611. [CrossRef] [PubMed]
104. Shabbir, A.; Cox, A.; Rodriguez-Menocal, L.; Salgado, M.; Van Badiavas, E. Mesenchymal Stem Cell Exosomes Induce Proliferation and Migration of Normal and Chronic Wound Fibroblasts, and Enhance Angiogenesis In Vitro. *Stem Cells Dev.* **2015**, *24*, 1635–1647. [CrossRef] [PubMed]
105. Zhang, J.; Guan, J.; Niu, X.; Shangchun, G.; Guo, S.; Li, Q.; Xie, Z.; Zhang, C.; Wang, Y. Exosomes released from human induced pluripotent stem cells-derived MSCs facilitate cutaneous wound healing by promoting collagen synthesis and angiogenesis. *J. Transl. Med.* **2015**, *13*, 49. [CrossRef] [PubMed]
106. Nawaz, M.; Fatima, F.; Vallabhaneni, K.C.; Penfornis, P.; Valadi, H.; Ekström, K.; Kholia, S.; Whitt, J.D.; Fernandes, J.D.; Pochampally, R.; et al. Extracellular Vesicles: Evolving Factors in Stem Cell Biology. *Stem Cells Int.* **2016**, *2016*, 1–17. [CrossRef] [PubMed]
107. Fang, S.-B.; Zhang, H.-Y.; Meng, X.-C.; Wang, C.; He, B.-X.; Peng, Y.-Q.; Xu, Z.-B.; Fan, X.-L.; Wu, Z.-J.; Wu, Z.-C.; et al. Small extracellular vesicles derived from human MSCs prevent allergic airway inflammation via immunomodulation on pulmonary macrophages. *Cell Death Dis.* **2020**, *11*, 409. [CrossRef] [PubMed]
108. Bang, O.Y.; Kim, E.H. Mesenchymal Stem Cell-Derived Extracellular Vesicle Therapy for Stroke: Challenges and Progress. *Front. Neurol.* **2019**, *10*, 211. [CrossRef]
109. Antes, T.J.; Middleton, R.C.; Luther, K.M.; Ijichi, T.; Peck, K.A.; Liu, W.J.; Valle, J.; Echavez, A.K.; Marbán, E. Targeting extracellular vesicles to injured tissue using membrane cloaking and surface display. *J. Nanobiotechnol.* **2018**, *16*, 61. [CrossRef]
110. Qin, Y.; Wang, L.; Gao, Z.; Chen, G.; Zhang, C. Bone marrow stromal/stem cell-derived extracellular vesicles regulate osteoblast activity and differentiation in vitro and promote bone regeneration in vivo. *Sci. Rep.* **2016**, *6*, 21961. [CrossRef]
111. Hu, Y.; Zhang, Y.; Ni, C.-Y.; Chen, C.-Y.; Rao, S.-S.; Yin, H.; Huang, J.; Tan, Y.-J.; Wang, Z.-X.; Cao, J.; et al. Human umbilical cord mesenchymal stromal cells-derived extracellular vesicles exert potent bone protective effects by CLEC11A-mediated regulation of bone metabolism. *Theranostics* **2020**, *10*, 2293–2308. [CrossRef]
112. Zhang, S.; Chuah, S.J.; Lai, R.C.; Hui, J.H.P.; Lim, S.K.; Toh, W.S. MSC exosomes mediate cartilage repair by enhancing proliferation, attenuating apoptosis and modulating immune reactivity. *Biomaterials* **2018**, *156*, 16–27. [CrossRef] [PubMed]
113. Nakamura, Y.; Miyaki, S.; Ishitobi, H.; Matsuyama, S.; Nakasa, T.; Kamei, N.; Akimoto, T.; Higashi, Y.; Ochi, M. Mesenchymal-stem-cell-derived exosomes accelerate skeletal muscle regeneration. *FEBS Lett.* **2015**, *589*, 1257–1265. [CrossRef] [PubMed]
114. Chen, S.-H.; Chen, Z.-Y.; Lin, Y.-H.; Chen, S.-H.; Chou, P.-Y.; Kao, H.-K.; Lin, F.-H. Extracellular Vesicles of Adipose-Derived Stem Cells Promote the Healing of Traumatized Achilles Tendons. *Int. J. Mol. Sci.* **2021**, *22*, 12373. [CrossRef] [PubMed]
115. Bruno, S.; Collino, F.; Deregibus, M.C.; Grange, C.; Tetta, C.; Camussi, G. Microvesicles Derived from Human Bone Marrow Mesenchymal Stem Cells Inhibit Tumor Growth. *Stem Cells Dev.* **2013**, *22*, 758–771. [CrossRef] [PubMed]
116. Roccaro, A.M.; Sacco, A.; Maiso, P.; Azab, A.K.; Tai, Y.-T.; Reagan, M.; Azab, F.; Flores, L.M.; Campigotto, F.; Weller, E.; et al. BM Mesenchymal Stromal Cell–Derived Exosomes Facilitate Multiple Myeloma Progression. *J. Clin. Investig.* **2013**, *123*, 1542–1555. [CrossRef]
117. Ono, M.; Kosaka, N.; Tominaga, N.; Yoshioka, Y.; Takeshita, F.; Takahashi, R.-U.; Yoshida, M.; Tsuda, H.; Tamura, K.; Ochiya, T. Exosomes from bone marrow mesenchymal stem cells contain a microRNA that promotes dormancy in metastatic breast cancer cells. *Sci. Signal.* **2014**, *7*, ra63. [CrossRef]
118. Du, T.; Ju, G.; Wu, S.; Cheng, Z.; Cheng, J.; Zou, X.; Zhang, G.; Miao, S.; Liu, G.; Zhu, Y. Microvesicles Derived from Human Wharton's Jelly Mesenchymal Stem Cells Promote Human Renal Cancer Cell Growth and Aggressiveness through Induction of Hepatocyte Growth Factor. *PLoS ONE* **2014**, *9*, e96836. [CrossRef]
119. Wang, J.; Hendrix, A.; Hernot, S.; Lemaire, M.; De Bruyne, E.; Van Valckenborgh, E.; Lahoutte, T.; De Wever, O.; Vanderkerken, K.; Menu, E. Bone marrow stromal cell–derived exosomes as communicators in drug resistance in multiple myeloma cells. *Blood* **2014**, *124*, 555–566. [CrossRef]
120. Zhang, X.; Tu, H.; Yang, Y.; Fang, L.; Wu, Q.; Li, J. Mesenchymal Stem Cell-Derived Extracellular Vesicles: Roles in Tumor Growth, Progression, and Drug Resistance. *Stem Cells Int.* **2017**, *2017*, 1758139. [CrossRef]
121. Shelke, G.; Lässer, C.; Gho, Y.S.; Lötvall, J. Importance of exosome depletion protocols to eliminate functional and RNA-containing extracellular vesicles from fetal bovine serum. *J. Extracell. Vesicles* **2014**, *3*, 24783. [CrossRef] [PubMed]
122. Li, J.; Lee, Y.; Johansson, H.; Mäger, I.; Vader, P.; Nordin, J.; Wiklander, O.P.B.; Lehtiö, J.; Wood, M.J.A.; El Andaloussi, S. Serum-free culture alters the quantity and protein composition of neuroblastoma-derived extracellular vesicles. *J. Extracell. Vesicles* **2015**, *4*, 26883. [CrossRef] [PubMed]

123. Onódi, Z.; Pelyhe, C.; Nagy, C.T.; Brenner, G.B.; Almási, L.; Kittel, Á.; Manček-Keber, M.; Ferdinandy, P.; Buzás, E.I.; Giricz, Z. Isolation of High-Purity Extracellular Vesicles by the Combination of Iodixanol Density Gradient Ultracentrifugation and Bind-Elute Chromatography From Blood Plasma. *Front. Physiol.* **2018**, *9*, 1479. [CrossRef]
124. Yan, H.-C.; Yu, T.-T.; Li, J.; Qiao, Y.-Q.; Wang, L.-C.; Zhang, T.; Li, Q.; Zhou, Y.-H.; Liu, D.-W. The Delivery of Extracellular Vesicles Loaded in Biomaterial Scaffolds for Bone Regeneration. *Front. Bioeng. Biotechnol.* **2020**, *8*, 1–12. [CrossRef] [PubMed]
125. Zhao, A.G.; Shah, K.; Cromer, B.; Sumer, H. Mesenchymal Stem Cell-Derived Extracellular Vesicles and Their Therapeutic Potential. *Stem Cells Int.* **2020**, *2020*, 8825771. [CrossRef]
126. Warren, D.S.; Sutherland, S.P.H.; Kao, J.Y.; Weal, G.; Mackay, S.M. The Preparation and Simple Analysis of a Clay Nanoparticle Composite Hydrogel. *J. Chem. Educ.* **2017**, *94*, 1772–1779. [CrossRef]
127. Mardpour, S.; Ghanian, M.H.; Sadeghi-Abandansari, H.; Mardpour, S.; Nazari, A.; Shekari, F.; Baharvand, H. Hydrogel-Mediated Sustained Systemic Delivery of Mesenchymal Stem Cell-Derived Extracellular Vesicles Improves Hepatic Regeneration in Chronic Liver Failure. *ACS Appl. Mater. Interfaces* **2019**, *11*, 37421–37433. [CrossRef]
128. Chen, P.; Zheng, L.; Wang, Y.; Tao, M.; Xie, Z.; Xia, C.; Gu, C.; Chen, J.; Qiu, P.; Mei, S.; et al. Desktop-stereolithography 3D printing of a radially oriented extracellular matrix/mesenchymal stem cell exosome bioink for osteochondral defect regeneration. *Theranostics* **2019**, *9*, 2439–2459. [CrossRef]
129. Liu, X.; Yang, Y.; Li, Y.; Niu, X.; Zhao, B.; Wang, Y.; Bao, C.; Xie, Z.; Lin, Q.; Zhu, L. Integration of stem cell-derived exosomes with in situ hydrogel glue as a promising tissue patch for articular cartilage regeneration. *Nanoscale* **2017**, *9*, 4430–4438. [CrossRef]
130. Diomede, F.; Gugliandolo, A.; Cardelli, P.; Merciaro, I.; Ettorre, V.; Traini, T.; Bedini, R.; Scionti, D.; Bramanti, A.; Nanci, A.; et al. Three-dimensional printed PLA scaffold and human gingival stem cell-derived extracellular vesicles: A new tool for bone defect repair. *Stem Cell Res. Ther.* **2018**, *9*, 104. [CrossRef]
131. Chew, J.R.J.; Chuah, S.J.; Teo, K.Y.W.; Zhang, S.; Lai, R.C.; Fu, J.H.; Lim, L.P.; Lim, S.K.; Toh, W.S. Mesenchymal stem cell exosomes enhance periodontal ligament cell functions and promote periodontal regeneration. *Acta Biomater.* **2019**, *89*, 252–264. [CrossRef] [PubMed]
132. Li, W.; Liu, Y.; Zhang, P.; Tang, Y.; Zhou, M.; Jiang, W.; Zhang, X.; Wu, G.; Zhou, Y. Tissue-Engineered Bone Immobilized with Human Adipose Stem Cells-Derived Exosomes Promotes Bone Regeneration. *ACS Appl. Mater. Interfaces* **2018**, *10*, 5240–5254. [CrossRef]
133. Ribas, R.G.; Schatkoski, V.M.; Montanheiro, T.L.D.A.; Menezes, B.; Stegemann, C.; Leite, D.; Thim, G.P. Current advances in bone tissue engineering concerning ceramic and bioglass scaffolds: A review. *Ceram. Int.* **2019**, *45*, 21051–21061. [CrossRef]
134. Jiwoon, J.; Jun, H.K.; Jung, H.S.; Nathaniel, S.H.; Chan, Y.H. Bioactive calcium phosphate materials and applications in bone regeneration. *Biomater. Res.* **2019**, *23*, 4. [CrossRef]
135. Zhang, J.; Liu, X.; Li, H.; Chen, C.; Hu, B.; Niu, X.; Li, Q.; Zhao, B.; Xie, Z.; Wang, Y. Exosomes/tricalcium phosphate combination scaffolds can enhance bone regeneration by activating the PI3K/Akt signaling pathway. *Stem Cell Res. Ther.* **2016**, *7*, 136. [CrossRef]
136. Ramasubramanian, L.; Kumar, P.; Wang, A. Engineering Extracellular Vesicles as Nanotherapeutics for Regenerative Medicine. *Biomolecules* **2019**, *10*, 48. [CrossRef]
137. Feng, Y.; Huang, W.; Wani, M.; Yu, X.; Ashraf, M. Ischemic Preconditioning Potentiates the Protective Effect of Stem Cells through Secretion of Exosomes by Targeting Mecp2 via miR-22. *PLoS ONE* **2014**, *9*, e88685. [CrossRef]
138. Liang, B.; Liang, J.-M.; Ding, J.-N.; Xu, J.; Xu, J.-G.; Chai, Y.-M. Dimethyloxaloylglycine-stimulated human bone marrow mesenchymal stem cell-derived exosomes enhance bone regeneration through angiogenesis by targeting the AKT/mTOR pathway. *Stem Cell Res. Ther.* **2019**, *10*, 335. [CrossRef] [PubMed]
139. Vajgel, A.; Mardas, N.; Farias, B.C.; Petrie, A.; Cimões, R.; Donos, N. A systematic review on the critical size defect model. *Clin. Oral Implant. Res.* **2013**, *25*, 879–893. [CrossRef]
140. Lu, Z.; Chen, Y.; Dunstan, C.; Roohani-Esfahani, S.; Zreiqat, H. Priming Adipose Stem Cells with Tumor Necrosis Factor-Alpha Preconditioning Potentiates Their Exosome Efficacy for Bone Regeneration. *Tissue Eng. Part A* **2017**, *23*, 1212–1220. [CrossRef] [PubMed]
141. García-Manrique, P.; Matos, M.; Gutiérrez, G.; Pazos, C.; Blanco-López, M.C. Therapeutic biomaterials based on extracellular vesicles: Classification of bio-engineering and mimetic preparation routes. *J. Extracell. Vesicles* **2018**, *7*, 1422676. [CrossRef] [PubMed]
142. Meng, F.; Zhang, Z.; Chen, W.; Huang, G.; He, A.; Hou, C.; Long, Y.; Yang, Z.; Liao, W. MicroRNA-320 regulates matrix metalloproteinase-13 expression in chondrogenesis and interleukin-1β-induced chondrocyte responses. *Osteoarthr. Cartil.* **2016**, *24*, 932–941. [CrossRef] [PubMed]
143. Man, K.; Brunet, M.Y.; Jones, M.-C.; Cox, S.C. Engineered Extracellular Vesicles: Tailored-Made Nanomaterials for Medical Applications. *Nanomaterials* **2020**, *10*, 1838. [CrossRef] [PubMed]
144. Ilahibaks, N.; Lei, Z.; Mol, E.A.; Deshantri, A.K.; Jiang, L.; Schiffelers, R.M.; Vader, P.; Sluijter, J.P. Biofabrication of Cell-Derived Nanovesicles: A Potential Alternative to Extracellular Vesicles for Regenerative Medicine. *Cells* **2019**, *8*, 1509. [CrossRef]
145. Antimisiaris, S.G.; Mourtas, S.; Marazioti, A. Exosomes and Exosome-Inspired Vesicles for Targeted Drug Delivery. *Pharmaceutics* **2018**, *10*, 218. [CrossRef]
146. Jo, W.; Jeong, D.; Kim, J.; Park, J. Self-Renewal of Bone Marrow Stem Cells by Nanovesicles Engineered from Embryonic Stem Cells. *Adv. Healthc. Mater.* **2016**, *5*, 3148–3156. [CrossRef]

147. Park, K.-S.; Svennerholm, K.; Shelke, G.; Bandeira, E.; Lässer, C.; Jang, S.C.; Chandode, R.; Gribonika, I.; Lötvall, J. Mesenchymal stromal cell-derived nanovesicles ameliorate bacterial outer membrane vesicle-induced sepsis via IL-10. *Stem Cell Res. Ther.* **2019**, *10*, 231. [CrossRef]
148. Wagner, A.; Vorauer-Uhl, K. Liposome Technology for Industrial Purposes. *J. Drug Deliv.* **2011**, *2011*, 591325. [CrossRef]
149. Lu, M.; Zhao, X.; Xing, H.; Xun, Z.; Zhu, S.; Lang, L.; Yang, T.; Cai, C.; Wang, D.; Ding, P. Comparison of exosome-mimicking liposomes with conventional liposomes for intracellular delivery of siRNA. *Int. J. Pharm.* **2018**, *550*, 100–113. [CrossRef]
150. De La Peña, H.; Madrigal, A.; Rusakiewicz, S.; Bencsik, M.; Cave, G.W.; Selman, A.; Rees, R.C.; Travers, P.J.; Dodi, I.A. Artificial exosomes as tools for basic and clinical immunology. *J. Immunol. Methods* **2009**, *344*, 121–132. [CrossRef]
151. Kang, M.; Lee, C.-S.; Lee, M. Bioactive Scaffolds Integrated with Liposomal or Extracellular Vesicles for Bone Regeneration. *Bioengineering* **2021**, *8*, 137. [CrossRef]
152. Kranendonk, M.E.; Visseren, F.L.; Van Balkom, B.W.; Nolte-'t Hoen, E.N.M.; Van Herwaarden, J.A.; De Jager, W.; Schipper, H.S.; Brenkman, A.B.; Verhaar, M.C.; Wauben, M.H.; et al. Human adipocyte extracellular vesicles in reciprocal signaling between adipocytes and macrophages. *Obesity* **2014**, *22*, 1296–1308. [CrossRef] [PubMed]
153. Zhang, Y.; Mei, H.; Chang, X.; Chen, F.; Zhu, Y.; Han, X. Adipocyte-derived microvesicles from obese mice induce M1 macrophage phenotype through secreted miR-155. *J. Mol. Cell Biol.* **2016**, *8*, 505–517. [CrossRef] [PubMed]
154. Pan, Y.; Hui, X.; Hoo, R.L.C.; Ye, D.; Chan, C.Y.C.; Feng, T.; Wang, Y.; Lam, K.S.L.; Xu, A. Adipocyte-secreted exosomal microRNA-34a inhibits M2 macrophage polarization to promote obesity-induced adipose inflammation. *J. Clin. Investig.* **2019**, *129*, 834–849. [CrossRef] [PubMed]
155. Liao, X.; Sharma, N.; Kapadia, F.; Zhou, G.; Lu, Y.; Hong, H.; Paruchuri, K.; Mahabeleshwar, G.H.; Dalmas, E.; Venteclef, N.; et al. Krüppel-like factor 4 regulates macrophage polarization. *J. Clin. Investig.* **2011**, *121*, 2736–2749. [CrossRef]
156. Chen, Y.; Xue, K.; Zhang, X.; Zheng, Z.; Liu, K. Exosomes derived from mature chondrocytes facilitate subcutaneous stable ectopic chondrogenesis of cartilage progenitor cells. *Stem Cell Res. Ther.* **2018**, *9*, 318. [CrossRef]
157. Ma, K.; Zhu, B.; Wang, Z.; Cai, P.; He, M.; Ye, D.; Yan, G.; Zheng, L.; Yang, L.; Zhao, J. Articular chondrocyte-derived extracellular vesicles promote cartilage differentiation of human umbilical cord mesenchymal stem cells by activation of autophagy. *J. Nanobiotechnology* **2020**, *18*, 163. [CrossRef]
158. Ni, Z.; Kuang, L.; Chen, H.; Xie, Y.; Zhang, B.; Ouyang, J.; Wu, J.; Zhou, S.; Chen, L.; Su, N.; et al. The exosome-like vesicles from osteoarthritic chondrocyte enhanced mature IL-1β production of macrophages and aggravated synovitis in osteoarthritis. *Cell Death Dis.* **2019**, *10*, 522. [CrossRef]
159. Cui, Y.; Luan, J.; Li, H.; Zhou, X.; Han, J. Exosomes derived from mineralizing osteoblasts promote ST2 cell osteogenic differentiation by alteration of microRNA expression. *FEBS Lett.* **2016**, *590*, 185–192. [CrossRef]
160. Wei, J.; Li, H.; Wang, S.; Li, T.; Fan, J.; Liang, X.; Li, J.; Han, Q.; Zhu, L.; Fan, L.; et al. let-7 Enhances Osteogenesis and Bone Formation While Repressing Adipogenesis of Human Stromal/Mesenchymal Stem Cells by Regulating HMGA2. *Stem Cells Dev.* **2014**, *23*, 1452–1463. [CrossRef]
161. Zhang, Y.; Xie, R.-L.; Croce, C.M.; Stein, J.L.; Lian, J.B.; van Wijnen, A.J.; Stein, G.S. A program of microRNAs controls osteogenic lineage progression by targeting transcription factor Runx2. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 9863–9868. [CrossRef]
162. Li, D.; Liu, J.; Guo, B.; Liang, C.; Dang, L.; Lu, C.; He, X.; Cheung, H.Y.-S.; Xu, L.; Lu, C.; et al. Osteoclast-derived exosomal miR-214-3p inhibits osteoblastic bone formation. *Nat. Commun.* **2016**, *7*, 10872. [CrossRef] [PubMed]
163. Kong, Y.-Y.; Yoshida, H.; Sarosi, I.; Tan, H.-L.; Timms, E.; Capparelli, C.; Morony, S.; Oliveira-Dos-Santos, A.J.; Van, G.; Itie, A.; et al. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nat. Cell Biol.* **1999**, *397*, 315–323. [CrossRef] [PubMed]
164. Cappariello, A.; Loftus, A.; Muraca, M.; Maurizi, A.; Rucci, N.; Teti, A. Osteoblast-Derived Extracellular Vesicles Are Biological Tools for the Delivery of Active Molecules to Bone. *J. Bone Miner. Res.* **2018**, *33*, 517–533. [CrossRef] [PubMed]
165. Liu, M.; Sun, Y.; Zhang, Q. Emerging Role of Extracellular Vesicles in Bone Remodeling. *J. Dent. Res.* **2018**, *97*, 859–868. [CrossRef]

## INTRODUCTION – PART 2
## BIOMATERIALS AND IMMUNE RESPONSE

Biomaterials are designed and engineered to interact with biological systems for medical applications, where they can be used to substitute or enhance biological tissues or support specific bodily functions [3]. It is crucial that these biomaterials are biocompatible and neither evocate a substantial immune response nor induce cytotoxicity upon implantation. Furthermore, these must keep their function, either just as structural support, as promoters of tissue healing or drug delivery tools.

Importantly, it has to be considered that biomaterial implantation will lead to an inflammatory response from the host. This response is physiological, but it can evolve into a state of excessive extracellular matrix (ECM) deposition [4] leading to fibrosis which induces the tissue around the biomaterial to harden or scar, impairing its proper repair and perpetuating chronic inflammation [5,6]. Several cell types are involved in fibrosis, and depending on alterations of the immune system the outcome will vary. The innate immune system acts primarily, through the recruitment of cells belonging to the myeloid lineage such as granulocytes and monocytes/macrophages, but also natural killer (NK) and lymphocytes, from lymphoid one [6]. In a physiologically balanced context, the seemingly opposite effects exerted by the different subsets of the immune system guarantee an effective tissue healing; however, excessive secretion of proinflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) by macrophages [7,8] can exacerbate collagen production in fibroblasts, leading to increased ECM deposition [6]. On the other hand, NK cells produce interferon-γ (IFN-γ) which is an important inhibitor of the transforming growth factor-β (TGF-β), both pro-fibrotic and anti-inflammatory cytokine mostly produced by monocytes/macrophages, but also eosinophils [4,6].

Interestingly, macrophages are known for their duality: they can polarize towards a more proinflammatory profile, named M1, or instead exert an anti-inflammatory effect, in the case of M2 [9]. Naturally, the effector cytokines released by these subsets will differ and therefore lead to contrasting outcomes. TGF-β is typically associated with the M2 phenotype, together with IL-4, IL-10 and IL-13, while M1 macrophages are linked with TNF-α and IFN-γ. Furthermore, macrophages are also responsible for the production of matrix metalloproteinases (MMPs),

which cleave collagen, but also of the tissue inhibitors of MMPs, i.e. TIMPs. Considering this, it is believed that the presence of M1 macrophages favours ECM degradation while M2 cells promote ECM deposition and stabilization, thus exacerbating fibrosis [6].

Despite the usual focus on fibroblasts and macrophages for evaluating biomaterial compatibility, the role of T cells in tissue regeneration has been increasingly explored [10,11]. Naïve $CD4^+$ T cells leave the thymus into the circulatory system until they interact with their specific target antigen, bound to major histocompatibility complex (MHC) class II molecules on the surface of an antigen-presenting cell (APC), through their T-cell receptor (TCR) After this first signal, the second step is initiated with the interaction of CD28 molecule, a receptor present on T cells, with costimulatory molecules on APCs, specifically CD80 or CD86. Consequently, the binding of the inducible T-cell costimulator (ICOS) present on activated T cells with ICOS-ligand (ICOSL) contributes to their maintenance. On the opposite, co-inhibitory receptors such as CD152 (CTLA-4) negatively regulate the immune response upon binding to the same ligands as CD28. The third necessary signal is mediated by cytokines and guides the polarization of differentiated T helper (Th) cells towards several subsets, in which the most common are Th1, Th2, and to a lesser extent Th17 [12].

Within the context of fibrosis, it is important to consider the intricate role of T cell subsets in managing the resolution of inflammation. Similarly to the above mentioned contrasting effects of macrophages, an imbalance in T cell homeostasis can severely affect the process of fibrosis upon biomaterial implantation. On the one hand, Th1 cells commonly secrete IFN-γ and TNF-α, exerting a type 1 pro-inflammatory response, as M1 macrophages do; on the other hand, Th2 secrete several cytokines belonging to the type 2 anti-inflammatory response, such as IL-4 and IL-13, previously mentioned in association with M2 macrophages [6]. Additionally, Th17 cells are known for the expression of IL-17, but also IL-21 which leads to an overexpression of IL-4 and IL-13 cytokines in macrophages [4,6]. Lastly, regulatory T cells (Tregs), derived from $CD4^+$ naïve T cells, release TGF-β and IL-10, which are anti-inflammatory cytokines [6,12]. With this, it becomes apparent the pressing need to broaden the biomaterials' immunobiocompatibility studies to also include T-cell phenotyping.

Among the several types of biomaterials used on the orthopaedic field, the most commonly used are as following: **metals** – mainly titanium and stainless steel; **bioactive glasses**; **polymers** – synthetically developed such as polyethylene and polyurethane or natural alternatives as collagen and hyaluronic acid; **ceramics** – most commonly hydroxyapatite; and **composites**, representing a combination of multiple different materials [13].

Metals are widely used as load bearing implants and some internal fixation devices. Their high yield, fatigue and shear strength as well as plentiful hardness, ductility and fracture toughness along with biocompatibility advocate in favour of metal implants as an appropriate option for dental and orthopaedic devices. On the other hand, there can be some drawbacks due to reaction of body enzymes and acids on the implant surface that leads to the release of ions from the metallic surface. These ions can cause toxicity and bone resorption that may lead to implant failure and surrounding tissue damage [14,15]. The most common types of metallic biomaterials in use are Stainless-steel (SS), Titanium-based alloys, Cobalt-based alloys, Shape memory alloys (SMAs), and Magnesium-based alloys [16].

Because of the excellent biocompatibility, titanium and its alloys are widely used in different biomedical applications as per the scope of this project. Implant rejection is minimal when titanium and its alloys are used due to their high capability for osseointegration induction, tissue adhesion and growth [17]. A possible explanation for its biocompatibility is the production of an oxide layer that can provide chemical bonding through various electron interactions. This passive oxide coating formed on the surface provides titanium an excellent resistance to corrosion [18]. Furthermore, studies have shown that titanium surface wettability highly favours the adhesion and proliferation of osteoblasts [18,19]. In general, several other physical properties such as the low electronic conductivity, high corrosion resistance, high mechanical resistance, hemodynamic at physiological pH and low ion formation tendency in aqueous environment can contribute to its high biocompatibility [20].

In addition, ceramics are also commonly used for musculoskeletal regeneration. Damaged or diseased parts of the musculoskeletal system as well as dental abnormalities can be replaced by using bioceramics since they have been modified for load-bearing purposes like bone grafts

and cement, hip acetabular cups and dental implants [21,22]. Bioceramics have exceptional biocompatibility, corrosion resistance, hard crisp surface and osteoconductivity – i.e. the ability of bone-forming cells in the grafting area to move across a scaffold and slowly replace it with new bone over time – but they are less flexible and extremely rigid, making them less useful for clinical application in tissue engineering [21,22].

Bioceramics can be bioinert, bioactive or bioresorbable [23]. When implanted, bioinert materials like alumina and zirconia do not show much interaction with the tissue. They are usually used as structural support implants like bone devices and femoral heads because they are quite resistant to corrosion and are resistant to cracking. On the other hand, bioactive ceramics can directly interact with the living surrounding tissue and show convincing effects on wound healing after implantation as in case of bioactive glasses and hydroxyapatite (HA). Due to their low friability, they are usually used in dental abnormalities and small bone fillings [22]. Bioresorbable ceramics gradually degrade and resorb in the host tissue after implantation, leaving the new tissue free of fabricated material. Tricalcium phosphate (TCP) is an example of bioresorbable ceramics which is used to repair bone defects. It has high surface area, high porosity with low grain size and low crystallinity which help to enhance the resorption rate [24]. Bioceramics of different properties like crystal size and morphology can be prepared by different methods including hydrolysis, sol-gel synthesis, wet precipitation, hydrothermal synthesis, mechanochemical synthesis, microwave processing and spray drying methods. For the synthesis of homogeneous material and for better control of parameters like pH, presence of additives and temperature, wet precipitation method is preferred [25].

Even though there are many types of bioceramics being used in different biomedical applications, bioactive glasses represent an interesting option due to their excellent biocompatibility and bioactivity. They are able to form a bond with mineralized bone tissue in the physiological body environment. Over fifty years ago, Larry Hench introduced the bioactive glasses more specifically Bioglass® 45S5, the first commercially available glass for medical use [26,27]. The composition of most of the bioactive glasses is based on sodium dioxide, calcium oxide, phosphorous and silica. It means that by altering or combining these basic elements,

different types of bioactive glasses can be prepared possessing specific properties such as bone forming efficiency, degradability, antibacterial properties and even soft tissue regeneration and wound healing [28–30]. Osteoconductivity is another feature shown by bioactive glasses along with some crystalline ceramics like HA and tricalcium phosphate (TCP). They make a bond with the bone without any fibrous connective tissue interface [26,31].

When the bioactive glass is implanted, it releases its main ions (calcium, sodium, phosphate and silica) forming carbonated hydroxyapatite (HCA), a bone-like mineral coating through an ion exchange reaction between the glass surface and the surrounding tissue and fluids [30]. This apatite layer improves cellular adhesion and proliferation of osteogenic cells and it is gradually replaced by bone over time [31,32]. Conversely, excessive ion release may lead to undesired toxicity, therefore affecting cell viability and metabolic activity, which in turn can impair the tissue healing process. To counteract this effect, pre-treating the bioactive glasses prior to entering in contact with cells, namely by incubating the material in cell culture medium or buffer [33].

Overall, biomaterial engineering is rapidly developing alternative materials with custom-designed properties, with the goal of improving patients' health and well-being. Considering the immune response generated upon material implantation, biomaterial design is nowadays aiming at controlling and modulating the inflammation, which is required to promote tissue healing. This contributes to a better implant integration and prevents chronic inflammation and rejection reactions, which can cause implants to fail [34,35].

## AIM

The AIM of this part of my thesis was to evaluate the T-cell (immunophenotyping) and cytokine release of PBMCs cultured on different ion doped glasses, as described in the published ***paper 2*** [36]**.**

## PAPER 2 – Human T-Cell Responses to Metallic Ion-Doped Bioactive Glasses

As previously introduced, biomaterials have been commonly used to replace damaged tissues. In particular, bioactive glasses represent the promising bone substitutes due to their intrinsic osteogenic properties. Importantly, biomaterial implantation frequently leads to the development of implant-associated infections and adverse immune responses. For those reasons, incorporating metallic ions with known antimicrobial properties can be a valid alternative to counteract infection, while also modulating the immune response.

In this paper, we evaluated the viability, phenotypical changes and cytokine expression of peripheral blood mononuclear cells (PBMCs) cultured in contact with metallic ion-doped bioactive glasses for 48 hours. Due to their reported therapeutic effect, silver, copper and tellurium were chosen as supplements to the silica-based bioactive glasses, previously developed by POLITO [37–41]. In particular, all three elements exhibit antibacterial properties [37,42,43]. Moreover, both silver and copper can promote angiogenesis [44,45], while tellurium might be used as an antioxidizing agent [38]. In summary, our results demonstrated that the presence of silver severely decreased cell viability; copper increased the Th1 cell percentage while decreasing that of the Th17 subset; lastly, tellurium had no effect in either cell viability or immune response, assessed by multiparametric flow cytometry. To evaluate more deeply the effect of immune response on the ion-doped bioactive glasses, a multiplex cytokines assay was performed. Results showed that IL-5 levels were lowered upon culture with the copper-doped discs, compared with its undoped control; IL-10 levels tended to be diminished in the same doped glass, compared with the basal condition without biomaterial, while in the undoped condition a lower expression of IL-13 and increased MCP-1 and MIP-1β secretions were detected. Overall, we speculated that the Th1/Th17 shift, together with the specific cytokine expression suggested that T-cells could

cross-activate other cell types, more specifically macrophages and eosinophils, in response to the copper-doped bioactive glasses.

**PAPER 2**

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*Article*

# Human T-Cell Responses to Metallic Ion-Doped Bioactive Glasses

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**Abstract:** Biomaterials are extensively used as replacements for damaged tissue with bioactive glasses standing out as bone substitutes for their intrinsic osteogenic properties. However, biomaterial implantation has the following risks: the development of implant-associated infections and adverse immune responses. Thus, incorporating metallic ions with known antimicrobial properties can prevent infection, but should also modulate the immune response. Therefore, we selected silver, copper and tellurium as doping for bioactive glasses and evaluated the immunophenotype and cytokine profile of human T-cells cultured on top of these discs. Results showed that silver significantly decreased cell viability, copper increased the T helper (Th)-1 cell percentage while decreasing that of Th17, while tellurium did not affect either cell viability or immune response, as evaluated via multiparametric flow cytometry. Multiplex cytokines assay showed that IL-5 levels were decreased in the copper-doped discs, compared with its undoped control, while IL-10 tended to be lower in the doped glass, compared with the control (plastic) while undoped condition showed lower expression of IL-13 and increased MCP-1 and MIP-1β secretion. Overall, we hypothesized that the Th1/Th17 shift, and specific cytokine expression indicated that T-cells might cross-activate other cell types, potentially macrophages and eosinophils, in response to the scaffolds.

**Keywords:** bioactive glasses; multiparametric flow cytometry; immunobiocompatibility; metallic ion doping; tissue regeneration; inflammation



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## 1. Introduction

Musculoskeletal disorders (MSDs) are conditions that restrict movement of the body, causing injury and pain in tissues belonging to the musculoskeletal system, including muscles, bones and joints [1,2]. Among the most prevalent MSDs are osteoarthritis, rheumatoid arthritis, low back pain and bone fractures, generally associated with osteoporosis in the elderly population [1,3]. In fact, with the increase in the average life expectancy observed in the last century, there has been a steady rise in the incidence of MSDs [2]. Individuals affected by MSDs experience a spectrum of pain and discomfort, which depending on the severity of the symptoms, can range from a slight interference with the daily activities to complete movement impairment. Therefore, there is a pressing need for novel therapies that can alleviate symptoms and improve the quality of life of MSD patients [1].

Biomaterials are designed and engineered to interact with biological systems for medical purposes. These materials play a crucial role in various fields, such as medicine, biotechnology and tissue engineering, where they can be used to replace or enhance natural biological structures or support specific functions within the body [4]. Biomaterials should be immunobiocompatible, meaning that they do not elicit a significant immune response or cytotoxicity when interacting with an organism, but also functional, whether they are functioning as a structural support, as promotors of tissue regeneration or vehicles for drug delivery. Among the different types of biomaterials on the orthopedic field, the most commonly used include the following: metals, more specifically titanium and stainless steel; polymers, either synthetic like polyethylene and polyurethane or natural such as collagen and hyaluronic acid; ceramics, for example, hydroxyapatite; bioactive glasses; and composites, which are the result of the combination of at least two different materials. Depending on their properties, these biomaterials can be used as implants, drug carriers for controlled delivery, artificial tissues/organs or diagnostic devices [5].

Damaged or diseased parts of the musculoskeletal system as well as dental abnormalities can be replaced by bioceramics since they have been modified for load-bearing purposes like bone grafts and cement, hip acetabular cups and dental implants [6,7]. Bioceramics and bioactive glasses have exceptional biocompatibility, corrosion resistance, a hard, crisp surface and osteoconductivity, i.e., the ability of bone-forming cells in the grafting area to migrate across a scaffold and gradually replace it with new bone tissue over time. Furthermore, they can directly interact with the living surrounding tissue and show convincing effects on wound healing after implantation, as in the cases of bioactive glasses and hydroxyapatite (HA). Due to their low friability, they are usually used in dental abnormalities and small bone fillings [7].

Among the described materials, bioactive glasses represent an interesting option due to their excellent biocompatibility and bioactivity. They are able to form a bond with mineralized bone tissue in the physiological body environment by creating a calcium phosphate layer on their surface [8]. Over fifty years ago, Larry Hench introduced bioactive glasses, more specifically Bioglass® 45S5, the first commercially available glass for medical use [9,10]. The composition of most bioactive glasses is based on silica, sodium oxide, calcium oxide and phosphorous pentoxide. This composition allows for the alteration or combination of these basic elements, enabling the creation of different types of bioactive glasses with specific properties such as bone forming efficiency, degradability, antibacterial properties and even soft tissue regeneration and wound healing [11–13]. When the bioactive glass is implanted, it releases its main ions (calcium, sodium, phosphate and silica) to form carbonated hydroxyapatite (HCA), a bone-like mineral coating, through an ion exchange reaction between the glass surface and the surrounding tissue and fluids [13]. This apatite layer improves cellular adhesion and proliferation of osteogenic cells and it is gradually replaced by bone over time [14,15]. Conversely, excessive ion release may lead to undesired toxicity, therefore affecting cell viability and metabolic activity, which in turn can impair the tissue healing process. To counteract this effect, pre-treating the bioactive glasses prior to entering into contact with cells by incubating the material in cell culture medium or buffer can be a successful strategy [16].

In summary, advancements in biomaterials science have led to the development of increasingly sophisticated materials with tailored properties, enabling innovative solutions in healthcare and biotechnology. Researchers continually explore new biomaterials and their applications to improve patient outcomes and quality of life. However, in a living tissue, when a material is implanted, there will always be a physiologic immune response which represents the first step of tissue repair. Nowadays, biomaterials are being designed considering this immune response and modulating it for improving implant integration, avoiding the chronic inflammatory and foreign body reactions that may lead to the loss of function [17,18].

Fibroblasts and macrophages have been traditionally used for evaluating biomaterial compatibility, such as in the case of silicone breast implants [19]. In the specific case

of musculoskeletal regeneration, cytotoxicity is commonly evaluated on mesenchymal stem/stromal cells (MSCs) and osteoblasts [20], while the role of T-cells in tissue regeneration has only recently been explored; as of today, the reliable data on the role of T-cells are scarce [19,21]. The activation of the immune response requires three signals. Signal 1 is mediated by the binding of the T-cell receptor (TCR) to major histocompatibility complex (MHC) class molecules on antigen-presenting cells (APC); signal 2 is mediated by the engagement of co-stimulatory molecules such as B7.1 (CD80) and B7.2 (CD86) and lastly, cytokines drive the polarization of differentiated T helper (Th) cells towards several subsets, such as T helper (Th)-1, Th2, and to a lesser extent Th17 (signal 3) [22].

T-cells represent up to 70% of peripheral blood mononuclear cells (PBMCs) [23] and are able to modulate bone healing and osteogenesis through cytokine and growth factors secretion [21]. In fact, in vivo experiments showed that T-cell depletion directly impairs the osteoinduction process [24], particularly affecting the deposition of collagen and osteoblast organization [25]. In vitro, conditioned media of $CD4^+$ Th lymphocytes can promote mesenchymal stem/stromal cell (MSC) mineralization [26].

Overall, the aim of this work was to elucidate how the interaction of T-cells with several metal-doped silica-based bioactive glasses, more specifically silver, copper and tellurium, could affect cell viability, T-cell immunophenotype and cytokine secretion. Silver and copper were introduced in the bioactive composition using the ion-exchange process, while tellurium was inserted together with the starting reactants during the glass synthesis via the melt and quenching process. These elements were selected since they have a therapeutic effect; Ag, Cu and Te possess antibacterial properties [27–29], Ag and Cu also have a pro-angiogenic effect [30,31] and Te possesses antioxidant properties [32]. However, the amount must be carefully tailored to avoid cytotoxic effects.

## 2. Results

Extensive physicochemical surface characterization of the doped glasses were previously reported by the authors [27,32–35]. Table 1 compares the most relevant properties of the glasses, regarding the content of the doped metal obtained via energy-dispersive X-ray spectroscopy (EDS) analysis, and the amount of doped metal ion leaching both in simulated body fluid (SBF) and in a cell medium obtained via inductively coupled plasma (ICP) spectroscopy, either combined via optical emission spectroscopy (OES) or mass spectrometry (MS).

**Table 1.** Summary of the properties of the doped glasses [33,35]; * α-MEM without added serum, 5% antibiotics, ** DMEM High Glucose, 1% L-Glutamine, 1% antibiotics. None of the used media contained any cells.

| | **AgSBA2** | **CuSBA3** | **STe5** |
|---|---|---|---|
| Doping method | The surface of the glass (Ion exchange in aqueous solution of $AgNO_3$) | The surface of the glass (Ion exchange in aqueous solution of Cu $(CO_2CH_3)_2$) | The bulk of the glass ($TeO_2$ in the glass network as an oxide) |
| Doped element content at-% (EDS) | 0.7 ± 0.36 | 8.4 ± 0.18 | 3.4 ± 0.08 |
| Doped ion leaching after 3 days in cell medium | 7.9 ± 1.4 ppm * (ICP-OES) | 11.0 ± 2.4 ppm * (ICP-OES) | 5.6 ± 0.3 ppm ** (ICP-OES) |
| Doped ion leaching after 3 days in Simulated Body Fluid (SBF) | 0.37 ± 0.13 ppm (ICP-MS) | 0.14 ± 0.04 ppm (ICP-MS) | 0.21 ± 0.07 ppm (ICP-MS) |

### *2.1. Silver, but Not Copper or Tellurium Ion Doping Induces Apoptosis of PBMCs*

Ions released from biomaterials upon contact with cell culture media may exert a toxic effect on immune cells. We evaluated the viability of PBMCs via flow cytometry after 48 h of culture, as seen in Figure 1a, using a fixable dye that can only be internalized by cells with permeable membranes, indicative of dead cells. In fact, we observed a reduction

of approximately 50%, on average, of PBMC viability when in contact with the silver-doped bioactive glass (Figure 1b), therefore, this formulation was excluded from the further analysis. No significant differences were observed between the formulations containing copper and tellurium, along with their corresponding controls, when compared to the basal condition lacking any bioactive glass disc.

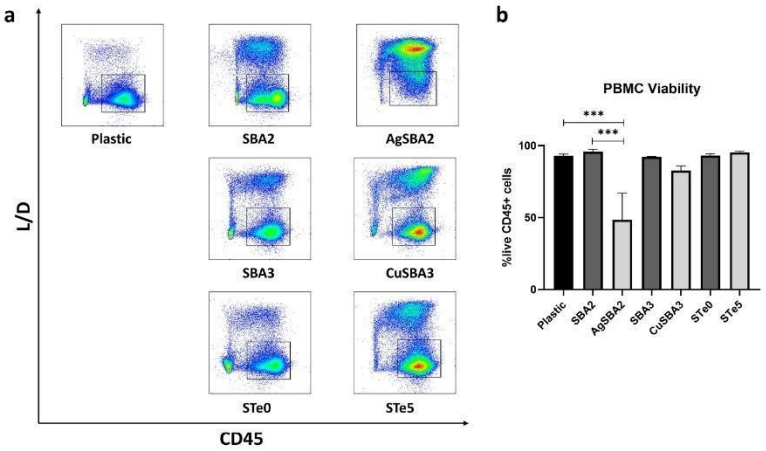


**Figure 1.** PBMC viability assay via flow cytometry. (**a**) Gating strategy: Viable lymphocytes were gated as negative for BD Horizon™ Fixable Viability Stain 780 ($L/D^-$) and $CD45^+$. (**b**) Bar graph representation of flow cytometry results are shown as average ± SEM. (n = 4). Ordinary one-way ANOVA with Tukey's post-hoc correction test was used. *** $p < 0.001$.

### *2.2. Immunophenotyping Reveals Th1/Th17 Shift Linked with Copper but Not Tellurium-Doping*

To assess the immune response to the metal ion doping, PBMCs were cultured on top of bioactive glasses. In particular, the immunophenotype of T lymphocytes was evaluated via multiparametric flow cytometry, as depicted in Figure 2. The employed gating strategy (Supplementary Figure S1) allowed for the detection of several subsets of T lymphocytes, namely $CD4^+$ T helper (Th) and $CD8^+$ cytotoxic T-cells, further specified as naïve, effector, effector memory (EM) or terminally differentiated EM (TEMRA), based on the expression of CD45RA and CD197, regulatory T-cells (Tregs), $CD25^+CD127^-$, as well as Th1, Th2 and Th17, identified by their differential expression of CD183, CD194 and CD196 markers.

Results showed that T-cells cultured in contact with copper-doped bioactive glass discs exhibited an increased frequency of Th1 population, paralleled by a decrease in Th17 cells, compared with the negative control and to a lesser extent with its undoped counterpart. On the other hand, the tellurium-doped glass did not exhibit any statistically significant differences, although large standard deviations were verified in several subsets, more noticeably in the Treg population.

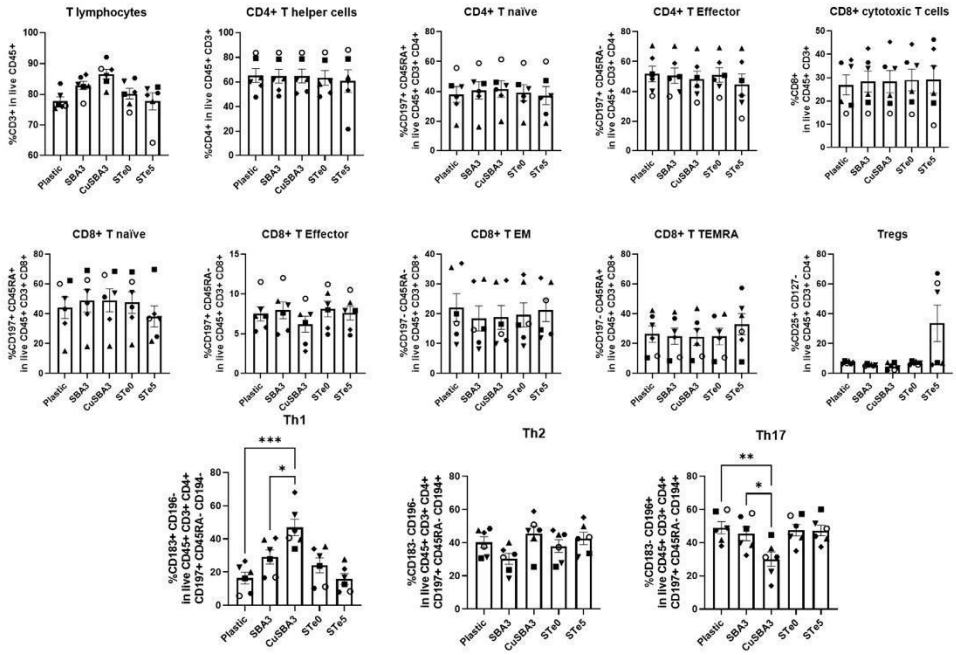


**Figure 2.** Immunophenotype of T-cells cultured in contact with bioactive glass discs assessed via multiparametric flow cytometry. Graphs represent the percentages of immune cells after 48 h culture without biomaterial (cell culture plate plastic—control), in contact with copper-doped (CuSBA3) and tellurium-enriched discs (STe5) and their respective undoped controls (SBA3 and STe0, respectively). Data are shown as average ± SEM, (n = 6). Each symbol represents a different donor. According to the data normality (Shapiro–Wilk test), ordinary one-way ANOVA (with Tukey's post-hoc correction) or Kruskal–Wallis test (with Dunn's post-hoc correction) were used. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

### *2.3. Cytokine Profile Response to Ion-Doped Bioactive Glasses*

Considering the phenotypical variances observed during PBMC culture related exclusively to the copper-doped glass, in order to verify if the Th1/Th17 shift was paralleled by differential cytokine secretion, a further cytokine quantification ELISA was performed using a commercial multiplex kit. This comprised seventeen cytokines released from macrophage or T-cells exclusively, or by both cell populations (Figure 3). Among these, levels of GM-CSF and IL-4 were close to, or below the lower limit of quantification and thus, were not suitable for statistical comparison.

We found a significantly reduced concentration of IL-5 in the supernatant of PBMCs cultured in contact with the copper-doped glasses condition, compared with its undoped counterpart. Copper-doped glasses also showed a trend in the decrease in IL-10 cytokine secretion in comparison with the control (plastic). Furthermore, IL-13 levels were significantly decreased in the undoped condition in comparison with plastic, while MCP-1 and MIP-1β cytokines were significantly increased. These findings indicated that the copper-doped bioactive glass exhibited a more similar cytokine profile to the plastic rather than to its respective undoped bioactive glass discs.

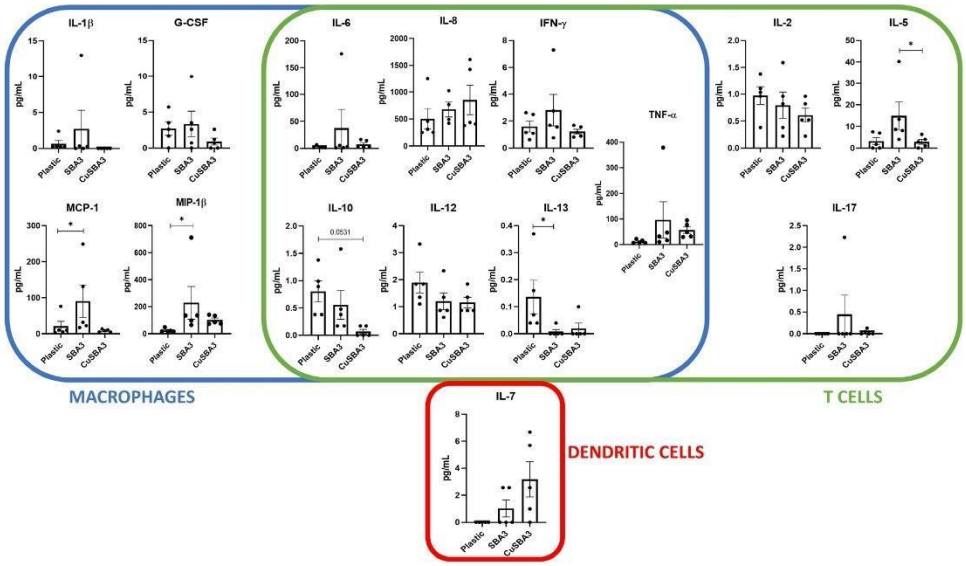


**Figure 3.** Cytokine expression levels of supernatants of PBMC culture in contact with copper-doped bioactive glass discs (CuSBA3) and its respective control (SBA3). Data are shown as average ± SEM, (n = 5). According to the data normality (Shapiro–Wilk test), RM one-way ANOVA (with Bonferroni post-hoc correction) or the Friedman test (with Dunn's post-hoc correction) were used. * $p < 0.05$.

## 3. Discussion

Bioactive glasses commonly release their ions to the surrounding microenvironment. This opens the possibility of incorporating biologically active ions into their composition or onto their surface, which upon release, may promote specific biological functions such as cell proliferation or angiogenesis, or confer novel properties to the bioactive glass, for example an antimicrobial effect [36]. Thus, silver, copper and tellurium have been used as doping ions on the surface of bioactive glass discs. For Ag- and Cu-doped glass, the ion doping was performed via ion exchange which introduced the ions only on to the glass surface, while in the case of STe5, tellurium was part of the bulk glass network. These ions have already been studied in the context of their antimicrobial [27–29] and pro-angiogenic effects [30,31], as well as in bone tissue regeneration and cancer [37–39]. Nevertheless, the data regarding the impact of these formulations on T-cells are scarce. Considering this, and to extend the knowledge provided by previous studies on biofilm formation [27,28,32], we evaluated any effects on viability, T-cell immunophenotype and cytokine release of PBMCs cultured in contact with ion-doped bioactive glasses.

The ionic release test was performed using inductively coupled plasma-optical emission spectrometry (ICP-OES) and data on doped ion-leaching in both cell culture medium and simulated body fluid was added to Table 1. Regarding the doping ions' bio-assimilation, it was reported that blood cells like erythrocytes and macrophages are capable of assimilating $Cu^{2+}$ through copper transporter 1 (CTR1) [40]. Also, silver ions ($Ag^{+}$) seem to be captured by immune cells, such as neutrophils and macrophages, and exert similar effects as silver nanoparticles, specifically in the formation of neutrophils extracellular traps (NETs) and intracellular reactive oxygen species (ROS) [41]. Lastly, tellurite ($TeO_3^{2-}$) which releases $Te^{4+}$ and represents the most abundant form of tellurium in nature, has been shown not only to bind hemoglobin in erythrocytes but also to react with glutathione and to lead to ROS formation in leukocytes [42]. The cytotoxicity of the released ions ($Ag^{+}$, $Cu^{2+}$, $Te^{4+}$) on different cell lines was investigated in previous reports [27–31], showing a different

behavior based on the used method (indirect or direct). Any observed cytotoxic effects, in particular for copper, do not seem to arise from the dissolution products or specific ion concentrations in the medium, but rather from a burst release and contact toxicity with the doped glass surfaces. A recent publication by some of the coauthors used copper-doped bioactive glasses manufactured with the same methodology and evaluated its cytotoxicity using human adipose tissue-derived stem cells (hASCs) [33]. Indirect culture of hASCs with the conditioned media of CuSBA3 discs, soaked for 24 h in α-MEM supplemented with 5% human serum and 1% antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin), did not affect cell viability, while direct contact with CuSBA3 led to extreme cytotoxicity. In accordance, when fibronectin was incorporated onto the surface of CuSBA3, ASCs cytocompatibility remained low, since the coating provided support for cell attachment but it did not prevent the direct contact between cells and bioactive glass disc. On the other hand, allowing the excessive burst ion release prior to the cell seeding through a 24 h pre-incubation in α-MEM rendered CuSBA3 cytocompatible [33]. In our study, we tested PBMCs directly cultured on the top of bioactive glasses. These cells mostly comprised non-adherent cells, therefore they tended not to be in direct contact with the bioactive glass discs. Given that, our data on PBMC viability are in line with the prior report regarding the indirect assays performed with adherent hASCs [33], in which the concentration of ions in the solution, about 10,000 µg/L, did not significantly impact cell viability. Overall, the reported cytotoxicity of copper-doped bioactive glasses was likely due to the contact but not necessarily to the concentration in solution.

In our study, we report the highly toxic effect of silver on PBMCs, as demonstrated by significantly reduced viability of PBMCs. Previous studies showed that the elementary silver in the solution, not doped in any biomaterial, exhibits cytotoxicity in a dose-dependent manner [43]. While generally considered an element with low toxicity [44], there are some clinical data indicating that the exposure to silver may represent the primary cause responsible for damage in cornea, liver, kidney and neurological tissues [45], as well as causing leukopenia [46] and chronic heart inflammation [47]. Moreover, in vitro studies presented some drawbacks of its use, due to a notable impairment of fibroblast [48] and keratinocyte growth [49]. For the above reasons, we opted to focus our attention on the copper- and tellurium-doped formulations for further analyses.

Delving into the T-cell ion-induced phenotype, the main focus of our study, we found that both the tellurium-doped bioactive glass and its undoped control exhibited no discernible impact on the immunophenotype of T-cells. However, we did observe interindividual variability, commonly present when using primary cells, particularly evident in certain subsets, as shown by the high standard deviations detected. In this case, PBMC culture in contact with the tellurium-doped bioactive glass revealed that three out of six donors had a high frequency of Tregs (54.5–67.1%) while the other three donors exhibited a much lower frequency (5.8–7.1%). Also in other subsets, such as $CD4^+$ T helper and $CD8^+$ T TEMRA, albeit less noticeable, we still verified that the culture with the tellurium-doped bioactive glass caused a more variable effect on PBMC phenotype than the other conditions, which might indicate that the response to this element was highly subject-dependent. Although material implantation generally elicits a response by the host, recent biomaterial engineering approaches search to not only modulate it in order to minimize side effects, such as chronic inflammation and foreign body reaction, but also to attempt to improve desirable biological processes, for instance osteointegration [18,50]. Previous reports evidencing the protective effect of the tellurium doping against oxidative stress coupled with our findings reporting no significant alterations in T-cell phenotype, can indicate that the tellurium-doped bioactive glass can be an interesting alternative to the currently used biomaterials for implantation [32].

On the other hand, culturing PBMCs in contact with the copper-doped bioactive glass led to a significant increase in Th1 cells, accompanied by a decrease in Th17 cells, compared with both its undoped counterpart (ordinary one-way ANOVA with Tukey's post-hoc correction; Th1: SBA3 vs. CuSBA3, $p$-value = 0.0332; Th17: SBA3 vs. CuSBA3,

*p*-value = 0.0498) and plastic (ordinary one-way ANOVA with Tukey's post-hoc correction; Th1: CuSBA3 vs. Plastic, *p*-value = 0.0001; Th17: CuSBA3 vs. Plastic, *p*-value = 0.0097). Both cell subsets were derived from the polarization of naïve T-cells, typically exacerbated upon viral and bacterial infections, inducing cell-mediated immunity, mainly by stimulating antibody secretion from B-cells. In addition, both Th1 and Th17 cells are known for their pro-inflammatory phenotype due to their effector cytokine releases. However, their biogenesis and role in the immune system are different. While Th1 cells are generated in the presence of IL-12, IL-18 and IFN-γ cytokines, Th17 requires IL-6, IL-23 and TGF-β for its polarization. Furthermore, Th1 mainly produce IFN-γ and TNF-α, while Th17 cells generally release IL-17A, IL-17F and IL-22. Consequently, their functions are also distinguishable. Th1 are able to enhance APC activity and $CD8^+$ T-cells/macrophage activation, protect against intracellular pathogens and participate in delayed type hypersensitivity, while Th17 acts on fungal and extracellular bacterial infections. Interestingly, Th subsets can cross-regulate each other, meaning that the secreted products of one cell type can stimulate the polarization of $CD4^+$ naïve cells into another specific subset. Finally, Th cells are known to be highly plastic; Th17 cells are considered less stable and can for example differentiate into Th1 cells given the appropriate environmental setup [22]. As reviewed by Adusei et al., Th subsets are capable of modulating the processes of fibrosis and tissue regeneration through differential cytokine secretion [51]. T-cells were studied on fibrotic tissue developed upon silicone implantation where an increase in $CD4^+$ T-cells was detected in the capsular tissue, more notably, Tregs. Moreover, Tregs were more prominent in patients with milder symptoms compared to more severe cases, showing also a more suppressive effect in vitro. Additionally, the authors showed an increase secretion of IL-6, IL-8, IL-17, IFN-γ and TGF-β1 by immune cells present in the capsular tissue, indicative of a pro-inflammatory environment sustained by Th1 and Th17 cells [52].

The direct correlation between copper-doping in bioactive glasses and Th subsets is still ill-defined. However, understanding the complex interactions between the different constituents of the immune system can help elucidating this question. Professional APCs, such as dendritic cells (DCs) and macrophages, are responsible for T-cell activation, therefore any modulatory effect on these players can influence the state of Th cells [22]. Dey et al. reported that the addition of copper oxide nanoparticles to lymphocytes or macrophages in vitro leads to an increase in TNF-α, IFN-γ and IL-12 production; the latter two directly promote the polarization of $CD4^+$ T-cells towards the Th1 phenotype [53]. Although we have not detected any significant changes regarding the aforementioned cytokines, it should be noted that we have evaluated the cytokine levels at 48 h instead of the 24 h used by the authors. Additionally, the physicochemical properties of nanoparticles also differ from the bulk materials, which may lead to slightly diverse biological reactions [54]. Nevertheless, the increase in the percentage of Th1 cells upon culture in contact with the copper-doped bioactive glass in our setting might indicate a similar pro-inflammatory effect of the copper doping.

Schuhladen et al. have evaluated the effect of increasing concentrations of copper-doped bioactive glass nanoparticles (Cu-BG-NPs) on murine DCs' phenotype and function. The authors found that the conditioned media containing the ionic dissolution products of Cu-BG-NPs significantly reduced the expression of CD80 and CD86, the two ligands of CD28, which are essential for T-cell activation. Coupling the phenotype results with the cytokine expression evaluation, the authors concluded that higher concentrations of copper led to a decrease in the secretion of various cytokines such as IL-6 [55]. As previously mentioned, IL-6 is one key factor for the polarization of Th17 cells, therefore its copper-induced reduction might lead to a lower frequency of Th17 cells, which is in line with our results.

Interestingly, the cytokine evaluation of supernatants from PBMC culture with the copper-doped bioactive glass revealed a distinct pattern, different to what had been previously described. IL-5 was revealed as the only cytokine being significantly modulated between the copper-doped and its undoped control. Within the immune system, IL-5 is commonly produced by Th2, innate lymphoid cells type 2 (ILC2), mast cells, natural killer

cells and eosinophils, acting particularly on eosinophil and B-cell growth [56,57]. In our setting, this cytokine appeared to be downmodulated in the copper-doped bioactive glass, achieving values similar to the negative control. Strikingly, another member of its family typically associated with Th2 response [58], namely IL-13, showed an inverse pattern, being under expressed in the undoped control in comparison with both other conditions. Notably, the proportion of Th2 cells did not change according to our immunophenotype results, even though their biological processes could be differentially modulated, therefore resulting in diverse secreted products.

Although often associated, even potentially acting upon some of the same molecules such as the signal transducer and activator of transcription 6 (STAT6), IL-5 and IL-13 are markedly different. As reviewed by Wu et al., IL-5 is mostly responsible for eosinophils' biological processes and survival, while IL-13 impacts more directly the B-cells and the Th2 subset [59]. Comparing with our results, the overexpression of IL-5 accompanied by a downregulation of IL-13 might lead to IL-5-mediated eosinophil activation, produced by Th2 cells. Therefore, it would be relevant to clarify through in vivo testing the role of eosinophils in this context, considering that the tested PBMC fraction should contain only a residual percentage of these cells. In fact, the formation of eosinophilic clusters had been reported in mice after six weeks of bioactive glass implantation, which the authors considered a sign of a possible allergic reaction [60]. Overall, we consider that in the SBA3 condition, the IL-5 likely produced by Th2 cells will or could favor eosinophil activation instead of B-cells, due to the lack of IL-13.

Conversely, two chemokines were upregulated in the undoped bioactive glass, more specifically MCP-1/CCL2 and MIP-1β/CCL4, which are responsible for immune cell recruitment. Both are mostly produced by cells from the myeloid lineage, i.e., monocytes, macrophages and dendritic cells, although they can also be released by T-cells [61,62]. These cytokines have been already linked with the immune response to biomaterials, being secreted by neutrophils [63], macrophages [64] and T-cells [65] (reviewed in [18]). The fact that these pro-inflammatory chemoattractant molecules were significantly more present in the undoped condition might favor macrophage polarization into M1 phenotype, which can be responsible for balancing the inflammatory microenvironment [66], acting as a counterpart of the activated eosinophils.

Of note, no significant differences were found at the cytokine level between the copper-doped condition and the basal condition, without any bioactive glass, although in the case of IL-10, a trend of reduced production upon PBMC culture with the copper-doped discs was observed. The impact of this cytokine in fibrosis seems a paradox. While it is commonly linked to a type 2 response [66], it may also play a role in preventing or reducing the effects of fibrosis [67], therefore further research on this topic is needed (reviewed in [68]).

Our study presents some limitations. Macrophages are among the first cell types to interact with implants, therefore being the main focus of numerous studies that explore the impact of implants in the immune system. The multiplex ELISA panel used in this study allowed for an overview of the cytokines released by PBMCs due to the contact with the bioactive glasses, it did not permit the direct association of the cytokine profile with a specific subset, such as macrophages. Nevertheless, among the cytokines evaluated in PBMC cultures, two were mainly ascribed to macrophages, such as MCP-1 and MIP-1β. Even though the literature already describes the modulation of cytokine release in macrophages by metal ion-doped bioactive glasses, including copper [69–73], it would be of interest to complete the analysis of T-cell immunophenotyping with their interaction with macrophages by polarizing them in vitro. Second, we had to take into account our sample size and the observed variability of donors in their response to the different biomaterial. This variability reflected the diverse genetic backgrounds, immune statuses, and physiological conditions of individual donors that may affect the response. Third, our experimental design investigated the effects of each biomaterial in a static way, though in vivo, these biomaterials are aimed at repairing and reconstructing the defective bone which is a dynamic tissue and subjected to mechanical stress. Lastly, the tissue microen-

vironment could also induce the release of ions from each biomaterial by affecting the T-cell/macrophages interaction, even by modulating macrophage polarization, shifting them towards an anti-inflammatory profile [74].

Overall, the fact that the cytokine profile of the copper-doped bioactive glass was similar to the plastic control, together with the favoring of the Th1 response according to the immunophenotyping assay, indicates that copper-doping might be a valid strategy to prevent fibrotic tissue formation.

## 4. Materials and Methods

### 4.1. Bioactive Glasses Preparation

In the present study, silica-based bioactive glasses were used as bulk discs. These materials were prepared and characterized as previously reported [27,32–35]. Briefly, the composition of SBA2 and SBA3 (undoped controls) are shown in Table 2.

**Table 2.** Composition of SBA2 and SBA3 control bioactive glasses.

| Components | SBA2 (% mol) | SBA3 (% mol) |
|---|---|---|
| $SiO_2$ | 48 | 48 |
| $Na_2O$ | 18 | 26 |
| CaO | 30 | 22 |
| $P_2O_5$ | 3 | 3 |
| $B_2O_3$ | 0.43 | 0.43 |
| $Al_2O_3$ | 0.57 | 0.57 |

SBA2 and SBA3 were prepared via the melt and quenching process. The reactants were melted in a platinum crucible at 1450 °C for 1 h Subsequently, the melt was cooled in a brass mold to obtain glass bars, which were then annealed at 500 °C for 13 h and cut into slices of 2 mm thickness and about 1 cm in diameter. Then, the slices were polished with SiC abrasive papers up to 1200 grit to level the surfaces. Lastly, the introduction of silver ($Ag^+$) and copper ($Cu^{2+}$) ions onto the surface of SBA2 and SBA3, respectively, was achieved through the ion-exchange process. The discs were submerged in an aqueous solution of either $AgNO_3$ (30 mM) or $Cu(CH_3COO)_2$ (1 mM) for 1 h, at 37 °C.

In the case of Te-doped glass (STe5), tellurium was directly introduced into the composition of the bioactive glass (named STe0) as a substitute for silica, as reported in Table 3.

**Table 3.** Composition of the STe0 control bioactive glass and STe5-doped bioactive glass.

| Components | STe0 (% mol) | STe5 (% mol) |
|---|---|---|
| $SiO_2$ | 48.6 | 43.6 |
| $Na_2O$ | 16.7 | 16.7 |
| CaO | 34.2 | 34.2 |
| $P_2O_5$ | 0.5 | 0.5 |
| $TeO_2$ | 0.0 | 5.0 |

STe0 and STe5 were also prepared via the melt and quenching process. In this case, the reactants were melted in a platinum crucible at 1500 °C for 1 h, and then cooled in a brass mold to obtain glass bars, that were annealed at 550 °C for 13 h. These bars were cut into slices of similar dimensions as the previous discs and polished as before. All the aforementioned samples were sterilized by heating to 100 °C for 3 h.

### 4.2. Ion Release in Simulated Body Fluid (SBF) and Cell Medium

The ICP analyses summarized in Table 1, except for STe5, were previously published and reported [33,35]. The glass samples of STe5 were subjected to in vitro bioactivity tests by soaking them in simulated body fluid (SBF). The SBF was prepared using the protocol developed by Kokubo et al. [75]. Polished glass discs were immersed in 50 mL

of SBF for fixed periods (1, 3, 7, 14, 28 days, here reported only 3-day timepoint) with five replicate samples of each glass per time point. Samples were maintained at 37 °C in an incubating shaker with an orbital speed of 120 rpm to simulate the physiological fluid flow. Solution at each time point was collected and the cumulative ion release for each sample was calculated by adding the ion release value at the selected time point to the previous ones. In the case of Te-ion release in cell medium (DMEM high glucose medium (Euroclone, Pero, Italy), supplemented additionally by penicillin/streptomycin and L-Glutamine (1% of both), STe5 specimens were soaked in the medium without FBS, 1.5 mL per disk in triplicates. Samples were maintained in the solution for 3 days, at 37 °C, 5% $CO_2$ incubation. From both SBF and cell medium collected samples, the Te-ion release was determined via an inductively coupled plasma mass spectrometer (ICP-MS, iCAPTM Q, Thermo Fisher Scientific, Waltham, MA, USA).

### *4.3. Blood Specimen Collection*

Peripheral blood was obtained from six healthy adult donors (25–45 years old) in cooperation with the Hospital Maggiore della Carità, Novara, Italy. From each donor, 10 mL of peripheral blood was withdrawn into lithium heparin collection tubes and immediately processed. The study was approved by the local ethics committee (prot. n. 675/CE).

### *4.4. Peripheral Blood Mononuclear Cells (PBMCs) Isolation*

PBMCs were isolated from heparinized blood samples collected from healthy donors. The blood samples were mixed with equal amounts of phosphate buffer saline (PBS 1×) and were carefully overlaid on top of a density gradient isolation solution, Lympholyte-H (Cedarlane®, Burlington, ON, Canada). After centrifugation, the cell ring at the interface was collected, washed with PBS 1X, and cells were counted.

### *4.5. Assessment of Cell Viability via Flow Cytometry*

PBMCs from four healthy adult donors were cultured in RPMI 1640, supplemented with 10% (*v*/*v*) heat-inactivated FBS, 100 U/mL penicillin/streptomycin, and 100 µg/mL gentamicin (Life technologies, Carlsbad, CA, USA), at 37 °C and 5% $CO_2$. A total of $1 \times 10^6$ fresh cells/mL were seeded onto sterile discs of bioactive glasses (or in wells without bioactive glasses—negative control) for 48 h, keeping the polished side upwards. Afterwards, the media was removed, and cells were collected and washed with PBS-EDTA 2 mM. The cells were stained with a viability dye (BD Horizon™ Fixable Viability Stain 780 (Becton and Dickinson, Franklin Lakes, NJ, USA)) for 15 min, at 4 °C, to distinguish live and dead cells. After washing with PBS-EDTA, Human BD™ Fc block solution was added to block the non-specific binding of immunoglobulin to Fc receptors. Subsequently, cells were incubated with antiCD45 BUV395 mAb (clone: HI30), a pan-marker for all leukocytes. Lastly, the cells were washed and resuspended in PBS-EDTA for acquisition using a BD FACSymphony™ A5 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The samples were then analyzed using the BD FACSDIVA™ software version 9.0.

### *4.6. Immunobiocompatibility Assay*

PBMCs from six healthy adult donors were cultured as previously described. After media removal, cells were washed with PBS-EDTA 2 mM and stained with a viability dye (BD Horizon™ Fixable Viability Stain 780) for 15 min, at 4 °C. Cells were then washed with PBS-EDTA and Human BD™ Fc block solution was added. Antigen surface staining was performed by adding an antibody mix containing mouse antiCD3 BUV496 monoclonal antibody (mAb) (clone: UCHT1), antiCD4 BUV737 mAb (clone: SK3), antiCD8 BUV805 mAb (clone: SK1), antiCD25 APC-R700 mAb (clone: 2A3), antiCD45 BUV395 mAb (clone: HI30), antiCD127 BV786 mAb (clone: HIL-7R-M21), antiCD45RA BUV563 mAb (clone: HI100), antiCD183 APC mAb (clone: IC6), antiCD194 PE-CF594 mAb (clone: 1G1), antiCD196 BV480 mAb (clone: 11A9) and antiCD197 BV711 mAb (clone: 150503) in BD Horizon™ Brilliant Stain Buffer for 20 min, at 4 °C. Lastly, the cells were washed and resuspended in

PBS-EDTA for acquisition using a BD FACSymphony™ A5 flow cytometer. Data were then analyzed using the BD FACSDIVA™ software version 9.0. All reagents were purchased from Becton and Dickinson (Franklin Lakes, NJ, USA).

### 4.7. Enzyme-Linked Immunosorbent Assay (ELISA)

PBMCs from five healthy adult donors were cultured as previously described. Cell culture supernatants were collected after 48 h and cytokine levels were quantified using the Bio-Plex Pro Human Cytokine 17-plex Assay according to manufacturer's instructions (Bio-Rad, Hercules, CA, USA). This assay allows for the detection of a wide array of cytokines, specifically: G-CSF, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17A, MCP-1, MIP-1β and TNF-α. The plate was run on a Bio-Plex 200 instrument (Bio-Rad, Hercules, CA, USA). The reported concentrations and detection limits were obtained through the standard curves generated by the kit's standards, using the weighted 5PL curve fitting procedure in Bio-Plex Software Manager ™ version 6.2. Values under the lower limit of quantification (LLOQ) were extrapolated based on the 5PL logistic curve, as previously reported [76].

### 4.8. Statistical Analysis

Data were analyzed using one-way ANOVA, Friedman or Kruskal–Wallis test with post-hoc correction, according to the sample's normality, calculated using the D'Agostino–Pearson test. $p$-value below 0.05 was considered statistically significant. Statistical analyses were performed with GraphPad Instat software (Prism 8 version 8.4.3) (GraphPad Software, San Diego, CA, USA).

## 5. Conclusions

Although bioactive glasses have been used especially for hard tissue regeneration during the last decades, the complete evaluation of immune reaction towards these biomaterials is often lacking. Our approach targeted T-cells that can be responsible for many regulatory functions in the organism including inflammation, which is essential for tissue regeneration. Our findings showed not only that metal ion doping can cause the apoptosis of immune cells and modulate the expression of certain subsets of T-cells in vitro, but it can also impact the cytokine release. Our study reported the highly toxic effect of silver-doping on PBMCs, comparable to the known dose-dependent cytotoxicity of this element in solution, indicating that this formulation required further optimization before being used in in vivo studies. Even though tellurium-enriched bioactive glass did not notably affect PBMC viability, the presence of tellurium elicited a highly variable T-cell response among individuals, most notably within the Treg subset. Additional research is necessary to investigate the distinct immune responses of each individual to the presence of this ion. In regards to the copper-doping, we postulated that the Th17 to Th1 switch, together with the alteration in cytokines such as IL-5 and IL-13 and the chemokines MCP-1/CCL2 and MIP-1β/CCL4, can modulate the immune response to bioactive glass implantation through cross-activation of cell types other than T lymphocytes, such as macrophages and possibly eosinophils. More importantly, the incorporation of copper on the surface of the bioactive glass greatly brought back the cytokine expression to the basal condition without biomaterial by improving its immunobiocompatibility.

Further studies are also needed to evaluate the effect of copper-doped bioactive glass in in vivo settings, where all the relevant players in the inflammatory response associated with tissue regeneration are present.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms25084501/s1.

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**Data Availability Statement:** The authors declare that the data supporting the findings of this study are included within the article or Supplementary Materials and are available from the corresponding author upon reasonable request.

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## References

1. Abreu, H.; Canciani, E.; Raineri, D.; Cappellano, G.; Rimondini, L.; Chiocchetti, A. Extracellular Vesicles in Musculoskeletal Regeneration: Modulating the Therapy of the Future. *Cells* **2022**, *11*, 43. [CrossRef] [PubMed]
2. Cieza, A.; Causey, K.; Kamenov, K.; Hanson, S.W.; Chatterji, S.; Vos, T. Global Estimates of the Need for Rehabilitation Based on the Global Burden of Disease Study 2019: A Systematic Analysis for the Global Burden of Disease Study 2019. *Lancet* **2020**, *396*, 2006–2017. [CrossRef]
3. Shen, Y.; Huang, X.; Wu, J.; Lin, X.; Zhou, X.; Zhu, Z.; Pan, X.; Xu, J.; Qiao, J.; Zhang, T.; et al. The Global Burden of Osteoporosis, Low Bone Mass, and Its Related Fracture in 204 Countries and Territories, 1990–2019. *Front. Endocrinol.* **2022**, *13*, 882241. [CrossRef]
4. Agrawal, R.; Kumar, A.; Mohammed, M.K.A.; Singh, S. Biomaterial Types, Properties, Medical Applications, and Other Factors: A Recent Review. *J. Zhejiang Univ. Sci. A* **2023**, *24*, 1027–1042. [CrossRef]
5. Freitas, J.; Santos, S.G.; Gonçalves, R.M.; Teixeira, J.H.; Barbosa, M.A.; Almeida, M.I. Genetically Engineered-MSC Therapies for Non-Unions, Delayed Unions and Critical-Size Bone Defects. *Int. J. Mol. Sci.* **2019**, *20*, 3430. [CrossRef]
6. Salinas, A.J.; Vallet-Regí, M. Bioactive Ceramics: From Bone Grafts to Tissue Engineering. *RSC Adv.* **2013**, *3*, 11116–11131. [CrossRef]
7. Pina, S.; Reis, R.L.; Oliveira, J.M. Ceramic biomaterials for tissue engineering. In *Fundamental Biomaterials: Ceramics*; Chapter 4; Elsevier: Maryland Heights, MO, USA, 2018.
8. Negut, I.; Ristoscu, C. Bioactive Glasses for Soft and Hard Tissue Healing Applications—A Short Review. *Appl. Sci.* **2023**, *13*, 6151. [CrossRef]
9. Hench, L.L.; Splinter, R.J.; Allen, W.C.; Greenlee, T.K. Bonding Mechanisms at the Interface of Ceramic Prosthetic Materials. *J. Biomed. Mater. Res.* **1971**, *5*, 117–141. [CrossRef]
10. Hench, L.L. The Story of Bioglass®. *J. Mater. Sci. Mater. Med.* **2006**, *17*, 967–980. [CrossRef]
11. Ali, S.; Farooq, I.; Iqbal, K. A Review of the Effect of Various Ions on the Properties and the Clinical Applications of Novel Bioactive Glasses in Medicine and Dentistry. *Saudi Dent. J.* **2014**, *26*, 1–5. [CrossRef]
12. Miguez-Pacheco, V.; Hench, L.L.; Boccaccini, A.R. Bioactive Glasses beyond Bone and Teeth: Emerging Applications in Contact with Soft Tissues. *Acta Biomater.* **2015**, *13*, 1–15. [CrossRef] [PubMed]
13. Cannio, M.; Bellucci, D.; Roether, J.A.; Boccaccini, D.N.; Cannillo, V. Bioactive Glass Applications: A Literature Review of Human Clinical Trials. *Materials* **2021**, *14*, 5440. [CrossRef] [PubMed]
14. Kaur, G.; Kumar, V.; Baino, F.; Mauro, J.C.; Pickrell, G.; Evans, I.; Bretcanu, O. Mechanical Properties of Bioactive Glasses, Ceramics, Glass-Ceramics and Composites: State-of-the-Art Review and Future Challenges. *Mater. Sci. Eng. C* **2019**, *104*, 109895. [CrossRef] [PubMed]
15. Kaur, G.; Pickrell, G.; Sriranganathan, N.; Kumar, V.; Homa, D. Review and the State of the Art: Sol–Gel and Melt Quenched Bioactive Glasses for Tissue Engineering. *J. Biomed. Mater. Res.—Part B Appl. Biomater.* **2016**, *104*, 1248–1275. [CrossRef] [PubMed]

16. Ciraldo, F.E.; Boccardi, E.; Melli, V.; Westhauser, F.; Boccaccini, A.R. Tackling Bioactive Glass Excessive in Vitro Bioreactivity: Preconditioning Approaches for Cell Culture Tests. *Acta Biomater.* **2018**, *75*, 3–10. [CrossRef] [PubMed]
17. Williams, D.F. On the Mechanisms of Biocompatibility. *Biomaterials* **2008**, *29*, 2941–2953. [CrossRef] [PubMed]
18. Franz, S.; Rammelt, S.; Scharnweber, D.; Simon, J.C. Immune Responses to Implants—A Review of the Implications for the Design of Immunomodulatory Biomaterials. *Biomaterials* **2011**, *32*, 6692–6709. [CrossRef] [PubMed]
19. Cappellano, G.; Ploner, C.; Lobenwein, S.; Sopper, S.; Hoertnagl, P.; Mayerl, C.; Wick, N.; Pierer, G.; Wick, G.; Wolfram, D. Immunophenotypic Characterization of Human T Cells after in Vitro Exposure to Different Silicone Breast Implant Surfaces. *PLoS ONE* **2018**, *13*, e0192108. [CrossRef] [PubMed]
20. Chraniuk, M.; Panasiuk, M.; Hovhannisyan, L.; Żołędowska, S.; Nidzworski, D.; Ciołek, L.; Woźniak, A.; Jaegermann, Z.; Biernat, M.; Gromadzka, B. The Preliminary Assessment of New Biomaterials Necessitates a Comparison of Direct and Indirect Cytotoxicity Methodological Approaches. *Polymers* **2022**, *14*, 4522. [CrossRef]
21. Zheng, K.; Niu, W.; Lei, B.; Boccaccini, A.R. Immunomodulatory Bioactive Glasses for Tissue Regeneration. *Acta Biomater.* **2021**, *133*, 168–186. [CrossRef]
22. Owen, J.A.; Punt, J.; Stranford, S.A.; Jones, P.P.; Kuby, J. *Kuby Immunology*, 8th ed.; W.H. Freeman: New York, NY, USA, 2019.
23. Lozano-Ojalvo, D.; López-Fandiño, R.; López-Expósito, I. PBMC-derived T cells. In *The Impact of Food Bioactives on Health: In Vitro and Ex Vivo Models*; Chapter 16; Springer: Cham, Switzerland, 2015.
24. Zhao, Z.; Zhao, Q.; Gu, B.; Yin, C.; Shen, K.; Tang, H.; Xia, H.; Zhang, X.; Zhao, Y.; Yang, X.; et al. Minimally Invasive Implantation and Decreased Inflammation Reduce Osteoinduction of Biomaterial. *Theranostics* **2020**, *10*, 3533. [CrossRef]
25. Khassawna, T.E.; Serra, A.; Bucher, C.H.; Petersen, A.; Schlundt, C.; Könnecke, I.; Malhan, D.; Wendler, S.; Schell, H.; Volk, H.D.; et al. T Lymphocytes Influence the Mineralization Process of Bone. *Front. Immunol.* **2017**, *8*, 562. [CrossRef] [PubMed]
26. Grassi, F.; Cattini, L.; Gambari, L.; Manferdini, C.; Piacentini, A.; Gabusi, E.; Facchini, A.; Lisignoli, G. T Cell Subsets Differently Regulate Osteogenic Differentiation of Human Mesenchymal Stromal Cells in Vitro. *J. Tissue Eng. Regen. Med.* **2016**, *10*, 305–314. [CrossRef] [PubMed]
27. Cochis, A.; Barberi, J.; Ferraris, S.; Miola, M.; Rimondini, L.; Vernè, E.; Yamaguchi, S.; Spriano, S. Competitive Surface Colonization of Antibacterial and Bioactive Materials Doped with Strontium and/or Silver Ions. *Nanomaterials* **2020**, *10*, 120. [CrossRef] [PubMed]
28. Miola, M.; Verné, E. Bioactive and Antibacterial Glass Powders Doped with Copper by Ion-Exchange in Aqueous Solutions. *Materials* **2016**, *9*, 405. [CrossRef] [PubMed]
29. Tang, A.; Ren, Q.; Wu, Y.; Wu, C.; Cheng, Y. Investigation into the Antibacterial Mechanism of Biogenic Tellurium Nanoparticles and Precursor Tellurite. *Int. J. Mol. Sci.* **2022**, *23*, 11697. [CrossRef] [PubMed]
30. Kang, K.; Lim, D.H.; Choi, I.H.; Kang, T.; Lee, K.; Moon, E.Y.; Yang, Y.; Lee, M.S.; Lim, J.S. Vascular Tube Formation and Angiogenesis Induced by Polyvinylpyrrolidone-Coated Silver Nanoparticles. *Toxicol. Lett.* **2011**, *205*, 227–234. [CrossRef] [PubMed]
31. Xie, H.; Kang, Y. Role of Copper in Angiogenesis and Its Medicinal Implications. *Curr. Med. Chem.* **2009**, *16*, 1304–1314. [CrossRef] [PubMed]
32. Miola, M.; Massera, J.; Cochis, A.; Kumar, A.; Rimondini, L.; Vernè, E. Tellurium: A New Active Element for Innovative Multifunctional Bioactive Glasses. *Mater. Sci. Eng. C* **2021**, *123*, 111957. [CrossRef]
33. Lallukka, M.; Houaoui, A.; Miola, M.; Miettinen, S.; Massera, J.; Verné, E. In Vitro Cytocompatibility of Antibacterial Silver and Copper-Doped Bioactive Glasses. *Ceram. Int.* **2023**, *49*, 36044–36055. [CrossRef]
34. Ferraris, S.; Yamaguchi, S.; Barbani, N.; Cazzola, M.; Cristallini, C.; Miola, M.; Vernè, E.; Spriano, S. Bioactive Materials: In Vitro Investigation of Different Mechanisms of Hydroxyapatite Precipitation. *Acta Biomater.* **2020**, *102*, 468–480. [CrossRef]
35. Lallukka, M.; Miola, M.; Najmi, Z.; Cochis, A.; Spriano, S.; Rimondini, L.; Verné, E. Cu-Doped Bioactive Glass with Enhanced in Vitro Bioactivity and Antibacterial Properties. *Ceram. Int.* **2024**, *50*, 5091–5103. [CrossRef]
36. Mehrabi, T.; Mesgar, A.S.; Mohammadi, Z. Bioactive Glasses: A Promising Therapeutic Ion Release Strategy for Enhancing Wound Healing. *ACS Biomater. Sci. Eng.* **2020**, *6*, 5399–5430. [CrossRef]
37. Pajares-Chamorro, N.; Wagley, Y.; Maduka, C.V.; Youngstrom, D.W.; Yeger, A.; Badylak, S.F.; Hammer, N.D.; Hankenson, K.; Chatzistavrou, X. Silver-Doped Bioactive Glass Particles for in Vivo Bone Tissue Regeneration and Enhanced Methicillin-Resistant Staphylococcus Aureus (MRSA) Inhibition. *Mater. Sci. Eng. C* **2021**, *120*, 111693. [CrossRef]
38. Bari, A.; Bloise, N.; Fiorilli, S.; Novajra, G.; Vallet-Regí, M.; Bruni, G.; Torres-Pardo, A.; González-Calbet, J.M.; Visai, L.; Vitale-Brovarone, C. Copper-Containing Mesoporous Bioactive Glass Nanoparticles as Multifunctional Agent for Bone Regeneration. *Acta Biomater.* **2017**, *55*, 493–504. [CrossRef] [PubMed]
39. Zhang, Y.; Hu, M.; Zhang, W.; Zhang, X. Construction of Tellurium-Doped Mesoporous Bioactive Glass Nanoparticles for Bone Cancer Therapy by Promoting ROS-Mediated Apoptosis and Antibacterial Activity. *J. Colloid Interface Sci.* **2022**, *610*, 719–730. [CrossRef] [PubMed]
40. Collins, J.F.; Prohaska, J.R.; Knutson, M.D. Metabolic Crossroads of Iron and Copper. *Nutr. Rev.* **2010**, *68*, 133–147. [CrossRef] [PubMed]
41. Haase, H.; Fahmi, A.; Mahltig, B. Impact of Silver Nanoparticles and Silver Ions on Innate Immune Cells. *J. Biomed. Nanotechnol.* **2014**, *10*, 1146–1156. [CrossRef]

42. Sandoval, J.M.; Verrax, J.; Vásquez, C.C.; Calderon, P.B. A Comparative Study of Tellurite Toxicity in Normal and Cancer Cells. *Mol. Cell. Toxicol.* **2012**, *8*, 327–334. [CrossRef]
43. Hardes, J.; Streitburger, A.; Ahrens, H.; Nusselt, T.; Gebert, C.; Winkelmann, W.; Battmann, A.; Gosheger, G. The Influence of Elementary Silver versus Titanium on Osteoblasts Behaviour in Vitro Using Human Osteosarcoma Cell Lines. *Sarcoma* **2007**, *2007*, 026539. [CrossRef]
44. Gosheger, G.; Hardes, J.; Ahrens, H.; Streitburger, A.; Buerger, H.; Erren, M.; Gunsel, A.; Kemper, F.H.; Winkelmann, W.; Von Eiff, C. Silver-Coated Megaendoprostheses in a Rabbit Model—An Analysis of the Infection Rate and Toxicological Side Effects. *Biomaterials* **2004**, *25*, 5547–5556. [CrossRef]
45. Wan, A.T.; Conyers, R.A.J.; Coombs, C.J.; Masterton, J.P. Determination of Silver in Blood, Urine, and Tissues of Volunteers and Burn Patients. *Clin. Chem.* **1991**, *37*, 1683–1687. [CrossRef]
46. Choban, P.S.; Marshall, W.J. Leukopenia Secondary to Silver Sulfadiazine: Frequency, Characteristics and Clinical Consequences. *Am. Surg.* **1987**, *53*, 515–517.
47. Tozzi, P.; Al-Darweesh, A.; Vogt, P.; Stumpe, F. Silver-Coated Prosthetic Heart Valve: A Double-Bladed Weapon. *Eur. J. Cardio-Thoracic Surg.* **2001**, *19*, 729–731. [CrossRef]
48. McCauley, R.L.; Linares, H.A.; Pelligrini, V.; Herndon, D.N.; Robson, M.C.; Heggers, J.P. In Vitro Toxicity of Topical Antimicrobial Agents to Human Fibroblasts. *J. Surg. Res.* **1989**, *46*, 267–274. [CrossRef]
49. Kuroyanagi, Y.; Kim, E.; Shioya, N. Evaluation of a Synthetic Wound Dressing Capable of Releasing Silver Sulfadiazine. *J. Burn Care Rehabil.* **1991**, *12*, 106–115. [CrossRef] [PubMed]
50. Dhinasekaran, D.; Kumar, A. Fabrication of bioactive structures from sol-gel derived bioactive glass. In *Bioactive Glasses and Glass-Ceramics: Fundamentals and Applications*; Chapter 6; Wiley: Hoboken, NJ, USA, 2022.
51. Adusei, K.M.; Ngo, T.B.; Sadtler, K. T Lymphocytes as Critical Mediators in Tissue Regeneration, Fibrosis, and the Foreign Body Response. *Acta Biomater.* **2021**, *133*, 17–33. [CrossRef] [PubMed]
52. Wolfram, D.; Rabensteiner, E.; Grundtman, C.; Böck, G.; Mayerl, C.; Parson, W.; Almanzar, G.; Hasenöhrl, C.; Piza-Katzer, H.; Wick, G. T Regulatory Cells and TH17 Cells in Peri-Silicone Implant Capsular Fibrosis. *Plast. Reconstr. Surg.* **2012**, *129*, 327e–337e. [CrossRef] [PubMed]
53. Dey, A.; Manna, S.; Kumar, S.; Chattopadhyay, S.; Saha, B.; Roy, S. Immunostimulatory Effect of Chitosan Conjugated Green Copper Oxide Nanoparticles in Tumor Immunotherapy. *Cytokine* **2020**, *127*, 154958. [CrossRef]
54. Huang, C.; Chen, X.; Xue, Z.; Wang, T. Effect of Structure: A New Insight into Nanoparticle Assemblies from Inanimate to Animate. *Sci. Adv.* **2020**, *6*, eaba1321. [CrossRef]
55. Schuhladen, K.; Stich, L.; Schmidt, J.; Steinkasserer, A.; Boccaccini, A.R.; Zinser, E. Cu, Zn Doped Borate Bioactive Glasses: Antibacterial Efficacy and Dose-Dependent: In Vitro Modulation of Murine Dendritic Cells. *Biomater. Sci.* **2020**, *8*, 2143–2155. [CrossRef] [PubMed]
56. Takatsu, K. Interleukin-5 and IL-5 Receptor in Health and Diseases. *Proc. Japan Acad. Ser. B Phys. Biol. Sci.* **2011**, *87*, 463–485. [CrossRef] [PubMed]
57. Pelaia, C.; Paoletti, G.; Puggioni, F.; Racca, F.; Pelaia, G.; Canonica, G.W.; Heffler, E. Interleukin-5 in the Pathophysiology of Severe Asthma. *Front. Physiol.* **2019**, *10*, 1514. [CrossRef] [PubMed]
58. Marone, G.; Granata, F.; Pucino, V.; Pecoraro, A.; Heffler, E.; Loffredo, S.; Scadding, G.W.; Varricchi, G. The Intriguing Role of Interleukin 13 in the Pathophysiology of Asthma. *Front. Pharmacol.* **2019**, *10*, 01387. [CrossRef] [PubMed]
59. Wu, A.Y.; Sur, S.; Grant, J.A.; Tripple, J.W. Interleukin-4/Interleukin-13 versus Interleukin-5: A Comparison of Molecular Targets in Biologic Therapy for the Treatment of Severe Asthma. *Curr. Opin. Allergy Clin. Immunol.* **2019**, *19*, 30–37. [CrossRef] [PubMed]
60. Asikainen, A.J.; Hagström, J.; Sorsa, T.; Noponen, J.; Kellomäki, M.; Juuti, H.; Lindqvist, C.; Hietanen, J.; Suuronen, R. Soft Tissue Reactions to Bioactive Glass 13-93 Combined with Chitosan. *J. Biomed. Mater. Res.—Part A* **2007**, *83*, 530–537. [CrossRef] [PubMed]
61. Gschwandtner, M.; Derler, R.; Midwood, K.S. More Than Just Attractive: How CCL2 Influences Myeloid Cell Behavior Beyond Chemotaxis. *Front. Immunol.* **2019**, *10*, 491100. [CrossRef] [PubMed]
62. Chen, R.; Ma, L.; Jiang, C.; Zhang, S. Expression and Potential Role of CCL4 in CD8+T Cells in NSCLC. *Clin. Transl. Oncol.* **2022**, *24*, 2420–2431. [CrossRef] [PubMed]
63. Kobayashi, S.D.; Voyich, J.M.; Burlak, C.; DeLeo, F.R. Neutrophils in the Innate Immune Response. *Arch. Immunol. Ther. Exp.* **2005**, *53*, 505.
64. Jones, J.A.; Chang, D.T.; Meyerson, H.; Colton, E.; Il, K.K.; Matsuda, T.; Anderson, J.M. Proteomic Analysis and Quantification of Cytokines and Chemokines from Biomaterial Surface-Adherent Macrophages and Foreign Body Giant Cells. *J. Biomed. Mater. Res.—Part A* **2007**, *83*, 585–596. [CrossRef]
65. Chang, D.T.; Jones, J.A.; Meyerson, H.; Colton, E.; Il, K.K.; Matsuda, T.; Anderson, J.M. Lymphocyte/Macrophage Interactions: Biomaterial Surface-Dependent Cytokine, Chemokine, and Matrix Protein Production. *J. Biomed. Mater. Res.—Part A* **2008**, *87*, 676–687. [CrossRef] [PubMed]
66. Wick, G.; Grundtman, C.; Mayerl, C.; Wimpissinger, T.F.; Feichtinger, J.; Zelger, B.; Sgonc, R.; Wolfram, D. The Immunology of Fibrosis. *Annu. Rev. Immunol.* **2013**, *31*, 107–135. [CrossRef] [PubMed]
67. Love, R.J.; Jones, K.S. Biomaterials, Fibrosis, and the Use of Drug Delivery Systems in Future Antifibrotic Strategies. *Crit. Rev. Biomed. Eng.* **2009**, *37*, 259–281. [CrossRef] [PubMed]

68. Steen, E.H.; Wang, X.; Balaji, S.; Butte, M.J.; Bollyky, P.L.; Keswani, S.G. The Role of the Anti-Inflammatory Cytokine Interleukin-10 in Tissue Fibrosis. *Adv. Wound Care* **2020**, *9*, 184–198. [CrossRef] [PubMed]
69. Varmette, E.A.; Nowalk, J.R.; Flick, L.M.; Hall, M.M. Abrogation of the Inflammatory Response in LPS-Stimulated RAW 264.7 Murine Macrophages by Zn- and Cu-Doped Bioactive Sol-Gel Glasses. *J. Biomed. Mater. Res.—Part A* **2009**, *90*, 317–325. [CrossRef] [PubMed]
70. Barbeck, M.; Alkildani, S.; Mandlule, A.; Radenković, M.; Najman, S.; Stojanović, S.; Jung, O.; Ren, Y.; Cai, B.; Görke, O.; et al. In Vivo Analysis of the Immune Response to Strontium- and Copper-Doped Bioglass. *In Vivo* **2022**, *36*, 2149–2165. [CrossRef] [PubMed]
71. Weng, L.; Boda, S.K.; Teusink, M.J.; Shuler, F.D.; Li, X.; Xie, J. Binary Doping of Strontium and Copper Enhancing Osteogenesis and Angiogenesis of Bioactive Glass Nanofibers While Suppressing Osteoclast Activity. *ACS Appl. Mater. Interfaces* **2017**, *9*, 24484–24496. [CrossRef] [PubMed]
72. Lin, R.; Deng, C.; Li, X.; Liu, Y.; Zhang, M.; Qin, C.; Yao, Q.; Wang, L.; Wu, C. Copper-Incorporated Bioactive Glass-Ceramics Inducing Anti-Inflammatory Phenotype and Regeneration of Cartilage/Bone Interface. *Theranostics* **2019**, *9*, 6300. [CrossRef] [PubMed]
73. Rau, J.V.; Curcio, M.; Raucci, M.G.; Barbaro, K.; Fasolino, I.; Teghil, R.; Ambrosio, L.; De Bonis, A.; Boccaccini, A.R. Cu-Releasing Bioactive Glass Coatings and Their in Vitro Properties. *ACS Appl. Mater. Interfaces* **2019**, *11*, 5812–5820. [CrossRef]
74. Chen, Y.; Guan, M.; Ren, R.; Gao, C.; Cheng, H.; Li, Y.; Gao, B.; Wei, Y.; Fu, J.; Sun, J.; et al. Improved Immunoregulation of Ultra-Low-Dose Silver Nanoparticle-Loaded $TiO_2$ Nanotubes via M2 Macrophage Polarization by Regulating GLUT1 and Autophagy. *Int. J. Nanomedicine* **2020**, *15*, 2011–2026. [CrossRef]
75. Kokubo, T.; Takadama, H. How Useful Is SBF in Predicting in Vivo Bone Bioactivity? *Biomaterials* **2006**, *27*, 2907–2915. [CrossRef] [PubMed]
76. Breen, E.J.; Tan, W.; Khan, A. The Statistical Value of Raw Fluorescence Signal in Luminex XMAP Based Multiplex Immunoassays. *Sci. Rep.* **2016**, *6*, 26996. [CrossRef] [PubMed]

**AIM**

The AIM of this part of my thesis was to evaluate T-cell phenotype and cytokine release of PBMCs cultured on different Titanium-based discs, as described in the manuscript 3 (in preparation)**.**

## Manuscript in preparation 3 – Evaluation of the immune response of peripheral blood mononuclear cells cultured on titanium-based discs

### Introduction

With the increased incidence and burden of musculoskeletal disorders, the development of improved biomaterials to be used as implants is of paramount importance [36,46]. Currently, ceramics or polymers cannot replace metallic biomaterials in all applications, due to the importance of mechanical strength and toughness in ensuring safety. Features such as high yield, fatigue and shear strength as well as plentiful hardness, ductility and fracture toughness along with biocompatibility advocate in favour of metal implants as an appropriate option for dental and orthopaedic devices. On the other hand, there can be some drawbacks due to reaction of body enzymes and acids on the implant surface that leads to the release of ions from the metallic surface. These ions can cause toxicity and bone resorption that may lead to implant failure and surrounding tissue damage [14,15].

The most common types of metallic biomaterials in use are Stainless-steel (SS), Titanium-based alloys, Cobalt-based alloys, Shape memory alloys (SMAs), and Magnesium-based alloys [16]. In particular, titanium and its alloys are widely used in different biomedical applications [1]. The choice for titanium generally implies low implant rejection due to their high osseointegration induction, tissue adhesion and growth [17]. The natural production of an oxide layer on the surface of titanium implants confers resistance to corrosion and is able to reduce ion release, contributing to its biocompatibility [1,19]. However, due to being generally inert, the oxidized surface can lead to the formation of fibrotic tissue, which can impair osteointegration [1,47].

Upon implantation, biomaterials swiftly become coated with proteins such as fibronectin, vitronectin, albumin, complement, etc., which adhere to the surface [48]. This prompts activated platelets to release chemoattractants that guide the migration of macrophages to the wound site, where they adhere to the biomaterial surface through integrin-mediated interactions with

the adsorbed proteins [49]. Lymphocytes also appear at the implant site [50], and they can also adhere to the surface and interact with macrophages. Consequently, the subsequent activation and modulation of lymphocyte function play a crucial role as an early element in the overall wound healing process following biomaterial implantation.

To complement the excellent mechanical properties of titanium with a more bioactive effect, surface modifications have been attempted, through techniques such as acid-etching, grit-blasting, particle sintering, plasma spray coating and micro-patterning [1,51]. In fact, the creation of a rough structure instead of a polished surface, greatly favours protein adsorption, osteoblast adhesion and implant stability [52]. Many options are already available in the market, but their manufacturing methods may leave traces of harmful metal ions and other contaminants, which elicit immune reactions and impair osteointegration [52,53].

A recently optimized alternative to the currently used titanium-based implants has been developed through surface etching in diluted hydrofluoric acid, used to remove the native oxide layer, and then submerged in hydrogen peroxide to generate controlled oxidation [35]. This creates a nanoporous titanium oxide layer with free hydroxyl radicals, ideal for protein adsorption, which also possesses a high resistance to scratching [1,54]. This treatment has shown to be effective in the deposition of hydroxyapatite upon soaking in simulated body fluid (SBF), but also in impeding bacterial colonization, whilst not affecting osteoblast adhesion [40,55].

Considering that around half of early implant failures are due to inflammation [56,57], it is essential to guarantee that any novel implant does not elicit an exacerbated inflammatory response. Therefore, we evaluated immune cell viability and cytokine production, as well as T-cell phenotype changes, induced by culture of peripheral blood mononuclear cells (PBMCs) in contact with titanium-based discs treated by acid etching and comparing with its respective untreated control. We found that although cell viability remains high in both conditions, untreated discs cause an increase in the percentage of Th2 cells, accompanied by a decrease in Th17 ones. Furthermore, several pro- and anti-inflammatory cytokines were increased in the supernatant of PBMCs cultured on top of the untreated discs, compared with both CT and basal conditions, while only a slight increasing trend was observed between CT and the basal condition.

These findings show that polishing may affect the immune response to untreated titanium, while CT could mitigate inflammation by improving its biocompatibility.

## Material and methods

### *Titanium-based discs (Ti-6Al-4V ELI)*

Titanium-based biomaterials, specifically the Ti-6Al-4V alloy with extra low interstitial (ELI) content, were used. These materials were prepared in the Department of Applied Science and Technology, Institute of Materials Physics and Engineering, POLITO. Ti6Al4V–ELI discs have 2 mm of thickness and 1 $cm^2$ area. The samples were polished with papers of 320, 600 and 800 grit attached to a sample holder and then polished manually with papers of 1000, 2500 and 4000 grit. The samples were then washed with acetone in the ultrasonication bath for 10 min at 40 $^oC$. Then, they were rinsed with distilled water twice in the same condition and put to dry. Lastly, the sterilization was performed through autoclaving at 120 $^oC$ for 20 minutes. These samples were subsequently referred to as “Polished”.

### *Chemical treatment (CT) – mimeTi*

Attempting to improve osteointegration, we applied a patented surface treatment developed by POLITO (EP2214732B1) to the titanium alloys, that consists in a thermochemical process that combines acid attack with surface oxidation [1]. The samples were briefly soaked in 0,5 M hydrofluoric acid (HF) to remove the native oxide layer, followed by a control oxidation step in $H_2O_2$ with agitation for 2 hours at 60 °C. Lastly, the samples were rinsed with water, and then air dried and sterilized using UV for 1 hour. This treatment confers a hydroxyl-enriched micro- and nanotextured oxide layer, intended to enhance apatite deposition and reduce bacterial adhesion [1,58]. These samples will be subsequently referred to as “Chemically Treated” or simply “CT”.

### *Blood specimen collection*

Peripheral blood was obtained from seven healthy adult donors (25-45 years old) in cooperation with the Hospital Maggiore della Carità, Novara, Italy. From each donor, 10 mL of peripheral blood was withdrawn into lithium heparin collection tubes and immediately processed. The study was approved by the local ethics committee (prot. n. 675/CE).

***Peripheral Blood Mononuclear Cells (PBMCs) isolation***

PBMCs were isolated from heparinized blood samples collected from healthy donors. The blood samples were mixed with equal amounts of phosphate buffer saline (PBS 1X) and were carefully overlaid on top of a density gradient isolation solution – Lympholyte-H (Cedarlane®, Ontario, Canada). After centrifugation, the cell ring at the interface was collected, washed with PBS 1X, and cells were counted.

***Apoptosis assay***

To assess the effect of the Titanium-based discs on cell apoptosis, the Annexin V Apoptosis Detection Kit was used, substituting propidium iodide (PI) with 7-Aminoactinomycin D for its higher stability and specificity for DNA [59]. For this, PBMCs from three healthy adult donors were cultured in RPMI 1640, supplemented with 10 % (v/v) heat-inactivated FBS, 100 U/mL penicillin/streptomycin, and 100 µg/mL gentamicin (Life technologies, CA, USA), at 37 °C and 5 % $CO_2$. $1x10^6$ fresh cells/mL were seeded onto chemically treated or untreated Ti6Al4V–ELI discs for 48 h. Cells were harvested, washed with PBS, and stained in accordance with manufacturer's protocol. The stained samples were then acquired on FACSymphony™ A5 Cell Analyzer (Becton and Dickinson, NJ, USA) and analyzed using BD FACSDiva software (Version 9.0., Becton and Dickinson, NJ, USA).

***Immunobiocompatibility assay***

PBMCs from seven healthy adult donors were cultured as previously described. After media removal, cells were washed with PBS-EDTA 2mM and stained with a viability dye – BD Horizon™ Fixable Viability Stain 780 – for 15 min, at 4 °C. Cells were then washed with PBS-EDTA and Human BD™ Fc block solution was added. Antigen surface staining was performed by adding an antibody mix containing mouse anti-CD3 BUV496 monoclonal antibody (mAb) (clone: UCHT1), anti-CD4 BUV737 mAb (clone: SK3), anti-CD8 BUV805 mAb (clone: SK1), anti-CD25 APC-R700 mAb (clone: 2A3), anti-CD45 BUV395 mAb (clone: HI30), anti-CD127 BV786 mAb (clone: HIL-7R-M21), anti-CD45RA BUV563 mAb (clone: HI100), anti-CD183 APC mAb (clone: IC6), anti-CD194 PE-CF594 mAb (clone: 1G1), anti-CD196 BV480 mAb (clone: 11A9) and anti-CD197 BV711 mAb (clone: 150503) in BD Horizon™ Brilliant Stain Buffer for 20 min, at 4 °C,. Lastly, the cells were

washed and resuspended in PBS-EDTA for acquisition using a BD FACSymphony™ A5 flow cytometer. Data were then analyzed using the BD FACSDIVA™ software (Version 9.0., Becton and Dickinson, NJ, USA). All reagents were purchased from Becton and Dickinson (NJ, USA).

***Enzyme-Linked Immunosorbent Assay (ELISA)***

PBMCs from five healthy adult donors were cultured as previously described. Cell culture supernatants were collected after 48 h and cytokine levels were quantified using the Bio-Plex Pro Human Cytokine 17-plex Assay according to manufacturer's instructions (Bio-Rad, CA, USA). This assay allows for the detection of a wide array of cytokines, specifically: G-CSF, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17A, MCP-1, MIP-1β and TNF-α. The plate was run on a Bio-Plex 200 instrument (Bio-Rad, CA, USA). The reported concentrations and detection limits were obtained through the standard curves generated by the kit's standards, using the weighted 5PL curve fitting procedure in Bio-Plex Software Manager ™. Values under the lower limit of quantification (LLOQ) were extrapolated based on the 5PL logistic curve, as previously reported [60].

***Statistical analysis***

Data were analyzed using one-way ANOVA, Friedman or Kruskal-Wallis test with post hoc correction, according to the sample's normality, calculated using D'Agostino-Pearson test. p-value below 0.05 was considered statistically significant. Statistical analyses were performed with GraphPad Instat software (Prism 8 version 8.4.3) (GraphPad Software, San Diego, CA, USA).

## Results

### *Polished untreated and CT treated titanium-based discs do not affect viability of human PBMC*

PBMCs were cultured in contact with treated and untreated Ti6Al4V-ELI discs, and their cell viability was evaluated by flow cytometry. Firstly, we observed changes in the physical parameters when culturing PBMCs on titanium-based discs. Considering the size and complexity (FSC vs SSC) parameters, we found a population with smaller size (highlighted in red in **figure 1A**) in Ti6Al4V–ELI polished samples, that does not exist in the control (no biomaterial) or CT conditions. This effect was already observed at 24h (data not shown) but it was exacerbated at 48h. This could be because ions released from biomaterials upon contact with cell culture media might have a toxic effect on immune cells. As shown in **Figure 1B**, we adapted a specific kit that allows for the distinction between live, early apoptotic and late apoptotic cells. Specifically, Annexin V binds phosphatidylserine (PS), the main component of cell membranes which is typically not exposed in live cells, with complete integer cell membranes. Once apoptosis is induced, PS is translocated towards the external of the cell, thus being able to bind Annexin V, which due to being conjugated with FITC, becomes detectable through flow cytometry. Instead, the inclusion of 7-AAD, a non-permeable DNA intercalator, can only be present in late apoptotic cells. This is because only when the membrane loses its integrity later on the apoptotic phase, 7-AAD can penetrate the intracellular compartment and bind DNA in the nucleus [61]. Altogether, the viability of the immune cells was not significantly affected upon culture with titanium-based discs up to 48 h, evidencing that although some cells become smaller, they are not undergoing apoptosis.

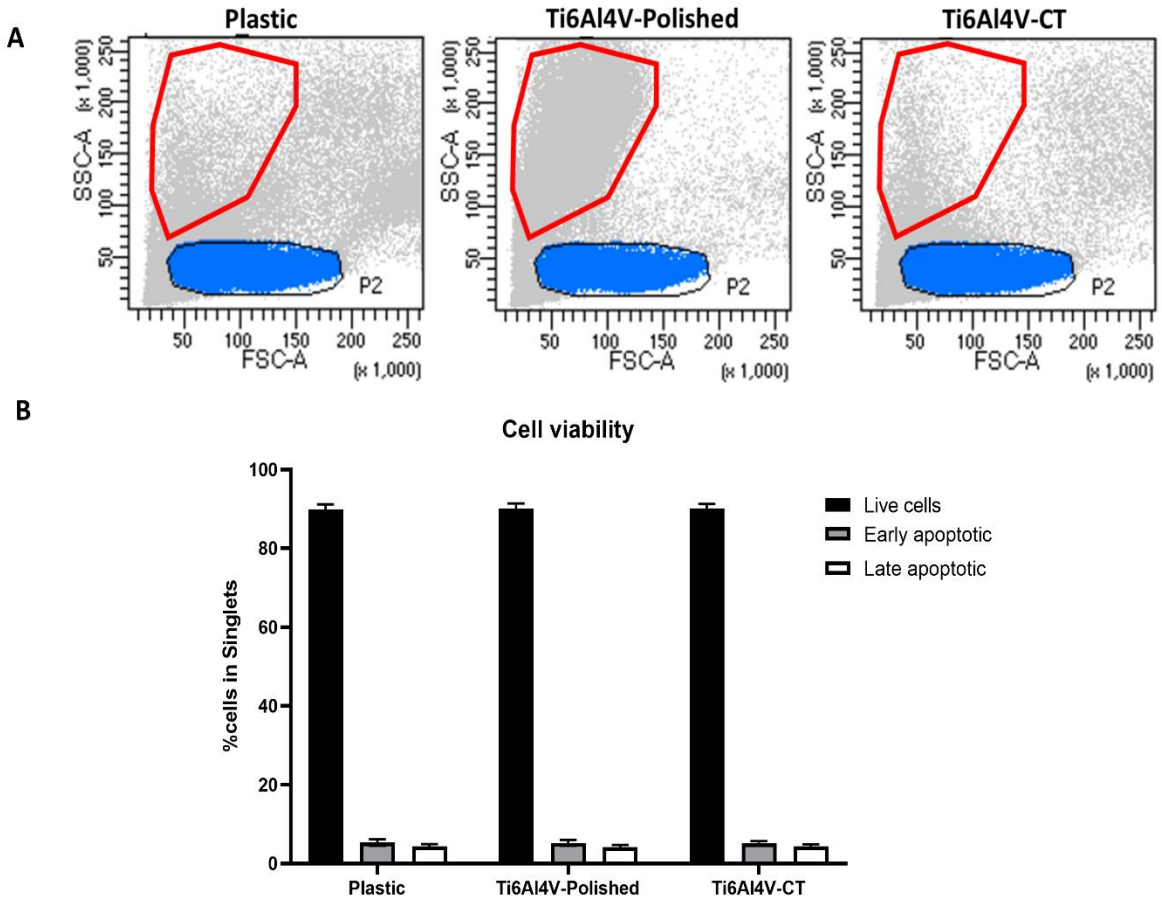



**Figure 1.** A) Comparison between size (Forward scatter – FSC) and complexity (side scatter – SSC) of PBMCs cultured in contact with either untreated (polished) or acid etched (CT), compared with the control (Plastic – no biomaterial); B) Apoptosis assay of PBMCs cultured in contact with Ti6Al4V-ELI discs, through Annexin V / 7-AAD assay. Bar graphs represent the percentages of live cells, identified as $AnnV^-/7\text{-}AAD^-$, cells in early apoptosis, as $AnnV^+/7\text{-}AAD^-$, and cells in late apoptosis, as $AnnV^+/7\text{-}AAD^+$.

### *Multiparametric flow cytometry reveals a Th2/Th17 shift on polished untreated Titanium-based discs*

The immunophenoptying of PBMCs was performed upon their culture with polished or CT Ti6Al4V-ELI discs, allowing for the distinction of several subsets of $CD4^+$ T helper and $CD8^+$ cytotoxic T cells. Although the tested biomaterials did not impact cell viability, we found a significant increase in the frequencies of Th2 cells, accompanied by the downregulation of Th17 cells, in the Ti6Al4V–ELI polished condition, when compared to the control (plastic, no biomaterial) or the CT discs, as shown in **Figure 2**.

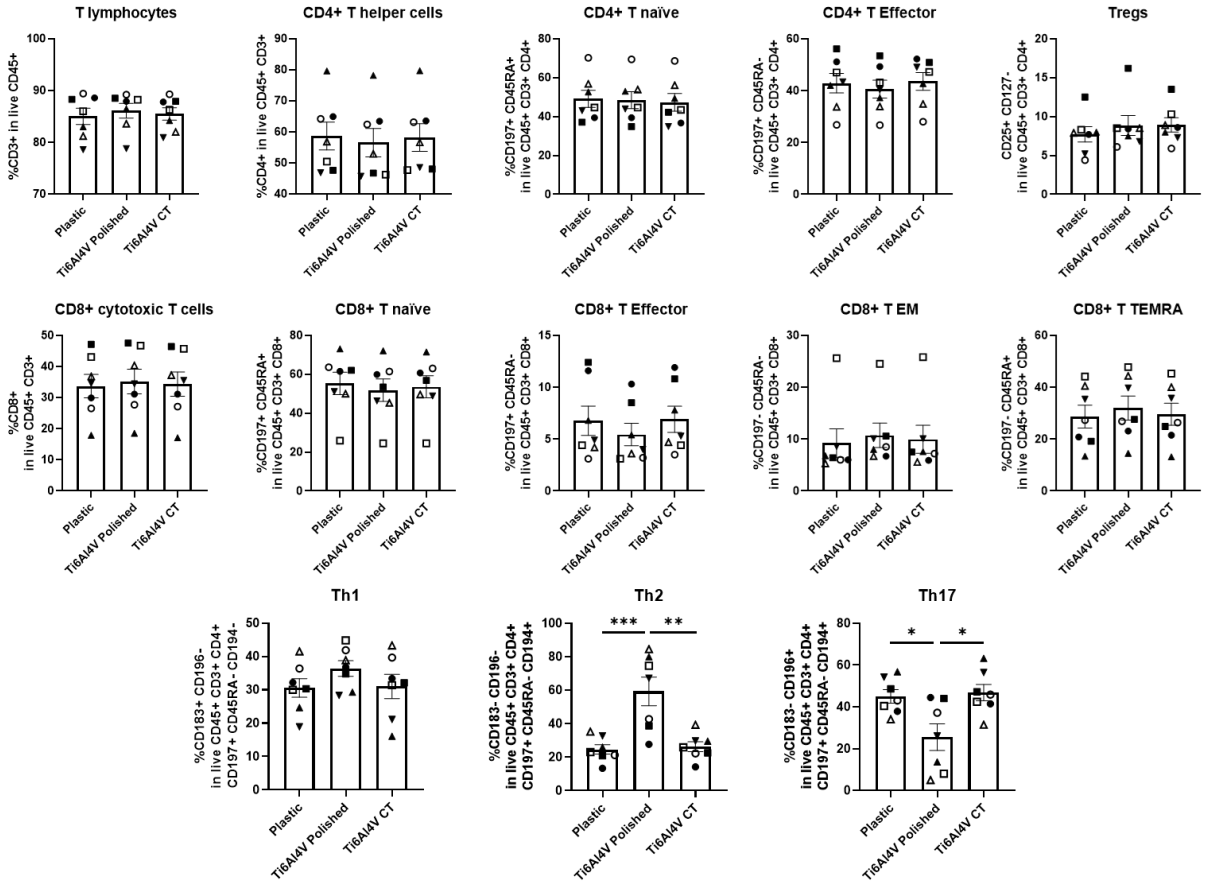



**Figure 2. Immunophenotype of T cells cultured in contact with Ti6Al4V-ELI discs assessed through multiparametric flow cytometry.** Scatter bar with plot graphs represent the percentages of immune cells after 48 h culture without biomaterial (cell culture plate plastic – control), in culture with Ti6Al4V-ELI discs treated with acid etching (chemical treatment – CT) and its respective untreated discs (polished). Data are shown as average ± SEM, (n=7). Each symbol represents a different donor. According to the data normality (Shapiro-Wilk test), ordinary One-way ANOVA (with Tukey's post-hoc correction) or Kruskal-Wallis test (with Dunn's post-hoc correction) were used. *p<0.05, **p<0.01, ***p<0.001

Interestingly, we observed that the chemical treatment employed on the Ti6Al4V-ELI discs appears to revert the T-cell phenotype to a profile resembling the basal condition, where no biomaterial is present.

### *Polished untreated Ti6Al4V-ELI exacerbates PBMC cytokine-based responses*

Due to these phenotypic differences found upon PBMC culture in contact with the different conditions, we questioned if polishing could also influence the secretion of both pro- and anti-inflammatory cytokines, evaluated by ELISA on the supernatants of PBMCs cultured in contact with the titanium-based discs. Overall, among the conditions, the expression of GM-CSF, IL-4 and IL-7 cytokines were below the limit of detection of the kit. Although there were no

detectable differences between CT and the polished discs, several cytokines were upregulated upon PBMC culture in contact with the polished discs in comparison with the basal control, more specifically G-CSF, IL-1β, IFN-γ, MIP-1β, IL-2, IL-6, and TNF-α. Particularly, in the polished condition the expression of IL-8 was over the upper limit of quantification, which may indicate a significant increase in the secretion of this cytokine, compared to the control and CT conditions (**Figure 3**).

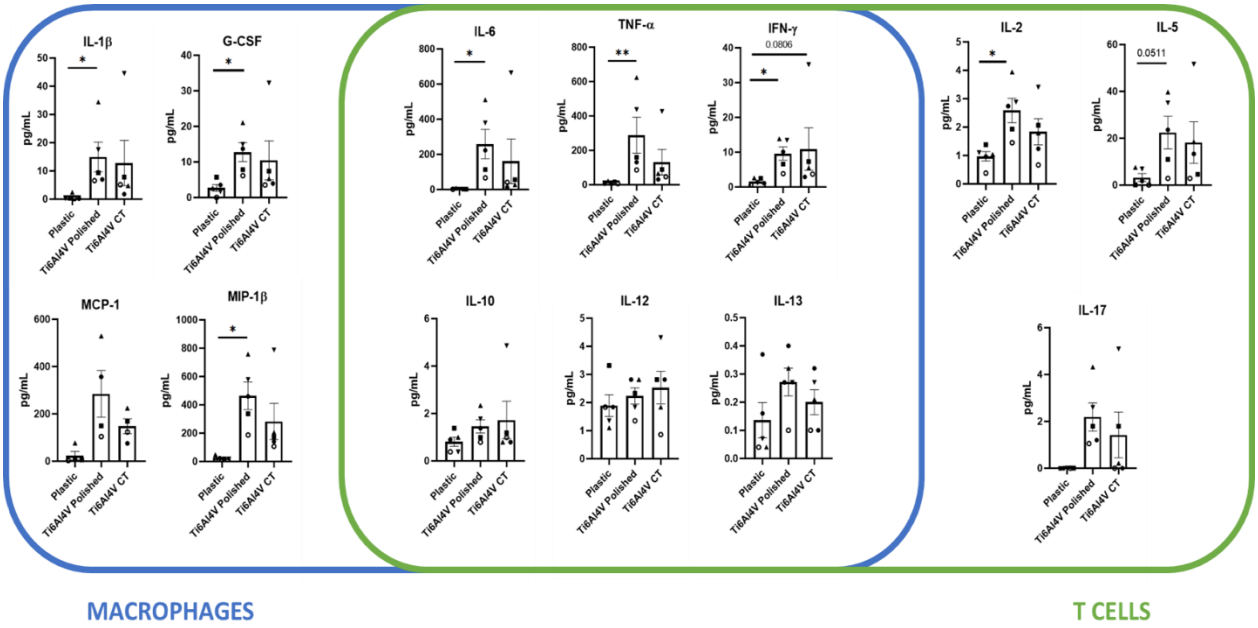



**Figure 3.** Cytokine expression levels of supernatants of PBMC after 48 h culture without biomaterial (cell culture plate plastic – control), in culture with Ti6Al4V-ELI discs treated with acid etching (chemical treatment – CT) and its respective untreated discs (ploished). Data are shown as average ± SEM, (n=5). According to the data normality (Shapiro-Wilk test), RM One-way ANOVA (with Bonferroni post-hoc correction) or Friedman test (with Dunn's post-hoc correction) were used. *p<0.05, **p<0.01.

## Discussion

Several studies have investigated the biocompatibility of titanium and its alloys in the context of orthopedic and dental implants [62]. Research has shown that titanium implants promote favourable biological responses, including minimal inflammatory reactions and the formation of a stable interface with the surrounding tissues [63].

In orthopedic applications, titanium and its alloys are used to produce joint replacements, bone plates, and screws among the others. The mechanical properties of titanium, including its high strength and fatigue resistance, make it an ideal material for withstanding the load-bearing requirements of orthopedic implants [64]. Furthermore, the biocompatibility of titanium allows for long-term implant stability and reduces the risk of adverse reactions within the body. Similarly, in dental implantology, these biomaterials are widely used for the fabrication of dental implants. The osseointegration capability of titanium allows for the direct structural and functional connection between the dental implant and the surrounding bone, providing stability and support for dental prostheses [65]. Additionally, the corrosion resistance of titanium ensures the longevity of dental implants within the oral environment [66].

Polishing plays a crucial role during the finalization and refinement of titanium-based products, since it removes surface irregularities and contaminants [67], resulting in a smoother surface and corrosion resistance, as well as promoting osseointegration and minimizing the risk of bacterial adhesion [66]. Several studies have investigated the impact of polishing on human epithelial cells [68], adipose stromal cells [69], and bone marrow-derived cells [70]. Okubo et al showed that the number of attached cells after 24h of incubation exhibited a 60% decrease on polished surfaces, by compromising not only the attachment but also the retention of human epithelial cells [68]. Jung et al reported that the polished-titanium discs may alter the expression of cellular matrix and focal adhesion genes [69]. Silva et al showed that adherent cells differentiate into osteogenic lineage on polished surfaces, though exhibiting a low proliferation activity [70].

In the present work we aimed at investigating the influence of Ti6Al4V-ELI polished and CT discs on the initial cell responses and behaviour of human PBMCs, by evaluating any effects on cell viability, T-cell immunophenotype and cytokine release.

Our findings showed that both polished and CT Ti6Al4V-ELI discs did not affect cell viability of lymphocytes, while polishing altered the size of the cells that became smaller compared to the control and CT titanium discs. Our results align with those of Okubo et al. who reported that the size and perimeter of human epithelial cells were significantly reduced on titanium polished surfaces [68], and they were restored upon ultraviolet light treatment. These authors concluded that this phenomenon is attributed to the adverse effects of the silicon polishing of titanium surfaces. We hypothesize that the smaller cells observed on polished discs could correspond to monocytes, natural killer cells, and dendritic cells, which typically exhibit greater Forward Scatter (FSC) than lymphocytes (gating P2, blue, Figure 1). These cells were possibly more sensitive to ionic stress or entering apoptosis upon contact with the Ti6Al4V–ELI discs without the chemical treatment. The polishing of titanium surfaces can significantly impact the release of ions in addition to the surface roughness. The release of ions from polished titanium surfaces is a critical consideration, as it can have implications for biocompatibility and the clinical performance of titanium implants [71]. While polishing reduces surface roughness, it can also induce the release of nanoparticles [72]. Wang et al reported that titanium particles *in vitro* may induce apoptosis of human mesenchymal stem cells [73].

Both polished and CT titanium discs did not affect cell viability or induce apoptosis of lymphocytes. Nevertheless, we observed that polished titanium discs increased the percentage of Th2 cells while reducing Th17 cells, compared to both CT discs and plastic. However, both immune subsets were restored to levels like plastic when cultured with CT discs. In our recent work we also observed a decreased in the percentage of Th17 cells in copper-doped bioactive glasses [36], which was balanced by the increase of Th1 cells, together with the alteration in cytokines such as IL-5 and IL-13 and the chemokines MCP-1/CCL2 and MIP-1β/CCL4. We concluded that bioactive glasses may possibly modulate the immune response through cross-activation of cell types other than T lymphocytes, such as macrophages and possibly eosinophils [36]. Th1 cells are associated with cell-mediated immunity and the production of pro-inflammatory cytokines, while Th2 cells are involved in humoral immunity and the secretion of anti-inflammatory cytokines [74]. Th17 cells are characterized by the production of IL-17 cytokine and provide host protection against microbes that Th1 or Th2 immunity are not well suited for,

such as extracellular bacteria and some fungi [75]. The finding that polishing titanium discs may decrease the percentage of Th17 cells could, in part, explain the failure of titanium implants due to the development of bacterial infection [76]. Among the cytokines that we have evaluated in the supernatant of cultured PBMCs, we did not observe any differences in IL-17 levels between polished and CT Ti6Al4V-ELI discs. However, there was a trend towards an increase of IL-17 levels in both polished and CT titanium discs compared to plastic, though the percentage of Th17 cell was reduced, this finding would suggest that other cell types may be responsible for IL-17 secretion. In fact, IL-17 can be also secreted by γδ T cells or NKT cells: among PBMCs, the former generally account for 1 to 5% [77], while the latter between 5-15% [78].

We observed that IL-2 was upregulated in polished discs compared to plastic and it showed a slight increase compared to CT discs as well. IL-2 possesses dual and contrasting functions, i.e. it plays a role in both the initiation and resolution of inflammatory immune responses. IL-2 inhibits the development of Th17 cells [79] by also signalling via STAT5 [80]. We speculate that the decrease of Th17 cells, seen in polished discs, could be ascribed to IL-2 resulted then increased.

Furthermore, we observed that polishing may also influence the release of other cytokines/chemokines. Regarding macrophages, G-CSF, IL-1β, and MIP-1β, were significantly upregulated in polished and with a trend in CT discs in comparison with plastic. G-CSF is a key regulator of neutrophils and it contributes to protecting the host against infection. Conversely, G-CSF can play a deleterious role in inflammatory diseases [81]. IL-1β is a pro-inflammatory cytokine involved in several biologic processes, such as immune regulation, connective tissue metabolism, and inflammation among the others [82]. As well as G-CSF, IL-1β also plays a protective role against the formation of bacterial biofilms, primarily by directly influencing the immune response to infections [83]. MIP-1β/CCL4 is a chemokine which plays a crucial role in the recruitment of immune cells: our findings would suggest that both polished and CT titanium discs may promote the attachment of cells onto the surface, favouring the osseointegration [84].

On the T cells side, we found an increase of IL-5 levels in polished discs in comparison to plastic. These results align with the increase of Th2 cells, since IL-5 is produced by Th2 cells as

well as innate immune ones with its main effects targeting eosinophil proliferation and promoting B-cell growth [85].

Lastly, we found that both polished and CT discs induced the secretion of IFN-$\gamma$ and TNF-$\alpha$ cytokines. IFN-$\gamma$ levels were increased in both polished and CT titanium discs, while TNF-$\alpha$ levels were increased only in polished discs in comparison with plastic. Both cytokines have a role in the fibrotic process, leading to the polarization of M1 macrophage (pro-inflammatory) and to the migration of fibroblasts to the biomaterial generating fibrosis [86], which would ensure implant integration around the surrounding tissue.

In conclusion, it is important to consider that the polishing process may not only alter the surface topography of biomaterial, but also impact the initial response of immune cells, which refers to the immediate reaction of the body's immune system upon contact with it. This includes processes such as inflammation, immune cell recruitment, and cytokine release among the others, which are pivotal in determining the biocompatibility and suitability of the biomaterial for its intended application. Understanding this initial response is essential for assessing the potential immunogenicity and overall compatibility of the biomaterial within the biological environment. CT of titanium discs may offer a solution to mitigate the effects observed with polished titanium discs, thus holding significant relevance in the context of biomedical applications.

## AIM

The last part of my thesis, that are part of **manuscript 4 in preparation** was aimed at:

i) assessing and optimising the osteogenic properties of the tested biomaterials, focusing on the behaviour of MSCs in terms of adhesion, proliferation and differentiation;

*ii)* exploring the impact of selected biomaterials on MSCs' paracrine effects, specifically in regards to the EVs content and modulatory role in processes associated with biomaterials implantation in MSC patients, such as immune reaction and angiogenesis.

## Manuscript in preparation 4 – Evaluation of the impact of biomaterials for musculoskeletal regeneration on Mesenchymal Stem Cells and its paracrine effects

### Introduction

Regenerative medicine represents a multidisciplinary route able to combine biomaterial engineering, through the optimization of biocompatible materials that reproduce the properties of the extracellular matrix (ECM), with cell engineering, which by using autologous cells can lead to a personalized therapy approach [2]. The most used cells for regeneration of the musculoskeletal system are mesenchymal stem cells (MSCs), that are adult multipotent progenitor cells with fibroblast-like shape that can self-renew as well as differentiate *in vitro* into osteoblasts and chondrocytes, among other cell types [87]. Unfortunately, several drawbacks are associated with direct MSC administration in patients, such as ethical issues, high cost of production, immunological reaction (in the case of non-autologous transplantation) and the low quantity of cells harvested, along with the reported undesirable secondary effects. Therefore, the alternative, currently being explored, regards MSCs' secretome, more specifically, extracellular vesicles (EVs) released by MSCs [88].

Despite all the reported evidence of the effectiveness of using MSC-EVs as potential treatment options for curing musculoskeletal disorders, the lack of standardized protocols in the EV isolation and MSC culture still impairs the translation of this field of research to the clinic [2]. However, once able to overcome this obstacle, the strategy to combine biomaterials, MSCs and

EVs in regenerative medicine would positively impact the future of musculoskeletal regeneration, since the two technologies may enhance each other's properties and represent an ideal approach for tissue healing.

Several studies evaluated the direct effects of implants on bone-related cells, such as MSCs or osteoblasts [89,90]. However, very few assessed the impact of these biomaterials in cell preconditioning, which will affect the communication and recruitment of other cell types to the implant site. With that, we sought to investigate if culturing MSCs in contact with metal-ion doped bioactive glasses or acid-etched titanium-based discs could be modulated to the extent of producing EVs with different protein cargo. These EVs could possibly serve as immunomodulatory agents or promoters of several biological processes involved with tissue healing, such as angiogenesis.

While the CT titanium-based discs seem to immediately promote MSC adhesion, bioactive glasses required a previous incubation with cell culture media, to provoke the initial burst ion release, as well as a coating with FBS, to create a protein layer facilitating cell adhesion. After ensuring the presence of MSCs on top of the discs, EVs were isolated and their effects were tested by using two different approaches. First, due to the possibility of the titanium-based discs altering immune cells' phenotype, we evaluated if that effect could also be modulated in a paracrine manner by EVs derived from MSCs.

Secondly, considering the well documented pro-angiogenic effect of copper, we aimed to investigate if MSCs cultured in contact with Cu-doped bioactive glass discs could modulate the network formation process of endothelial cells *in vitro* through EVs.

## Material and methods

### *Mesenchymal Stem/Stromal Cells (MSCs) isolation*

Mesenchymal stem/stromal cells (MSCs) were isolated from bone marrow samples of six donors affected by osteoarthritis. The samples were thoroughly mixed with 5-10 mL of Dulbecco's Modified Essential Medium (DMEM) and passed through a 100 μm cell strainer into a 50 mL Falcon tube to remove any debris. Then, the cell mix was carefully overlaid in Lympholyte® and centrifuged at 1100 x *g,* for 30 minutes, with minimum acceleration and no brake. Then, the ring present in the interface was collected and washed with DMEM, centrifuging at 900 x *g,* for 10 minutes. Then, cells were counted and plated at a concentration of 180 000 cells/cm$^2$ in MSC growth medium (DMEM with low glucose supplemented with 10% heat-inactivated FBS, 1% P/S and 0,5% G) using T75 flasks. The cells were incubated at 37$^o$C and medium was changed twice per week until the cells reached approximately 80 % confluence. For expansion, cells were treated with trypsin, counted and transferred to T75 tissue culture flask at a concentration of 2000 cells/cm$^2$. Isolated cells were characterized according to the guidelines provided by the International Society for Cell & Gene Therapy (ISCT) [87]. MSCs between passages 3-6 were used for the following experiments.

### *MSCs surface markers' characterization by flow cytometry*

MSCs were trypsinized, harvested and washed with PBS 1X before being resuspended in BD Horizon™ Fixable Viability Stain 780 in PBS 1X (1:5000 dilution) and incubated for 15 minutes. Then, after washing with PBS-EDTA 2 mM, the cells were incubated with a monoclonal antibody (mAb) anti-CD14-BV650, mAb anti-CD19-BUV615, mAb anti-CD34-BB700, mAb anti-CD45-BUV395, mAb anti-CD73-BV421, mAb anti-CD90-APC, mAb anti-CD105-PE, and mAb anti-HLA-DR-BV605 (all from BD Biosciences), for 20 min, at 4°C and protected from light. Lastly, the samples were washed and resuspended in PBS-EDTA 2 mM, promptly acquired in a BD FACSymphony™ A5 flow cytometer and later analysed with BD FACSDIVA™ software (Version 9.0., Becton and Dickinson, NJ, USA), using unstained cells as negative controls.

### *MSCs osteogenic differentiation*

Osteogenesis was induced for 28 days by culturing 10 000 cells/well for 28 days in 6-well plates in the presence of DMEM with 10% heat-inactivated FBS, 1% P/S, and 0,5% G (basal media),

supplemented with ascorbic acid $5x10^{-5}$, dexamethasone $10^{-7}$ M and β-glycerophosphate $10^{-2}$ M (all supplements from Sigma-Aldrich) (osteogenic differentiation induction media). At the end point of culture, Alizarin Red S staining was performed, after fixation of the cells overnight with 4% (w/v) paraformaldehyde (PFA), to assess the progression of osteogenesis.

### *Alizarin Red S staining*

Deposition of calcium deposits and matrix mineralization (stained red) was assessed through the Alizarin Red S staining. Briefly, the freshly Alizarin Red S solution was prepared through mixing 0,1 g of Alizarin Red S powder (Sigma-Aldrich) with 10 mL of ethanol 2% (v/v). Fixed cells were washed once with ddH2O and incubated with the staining solution for 10 min, at RT, protected from light. Then, the solution was removed, and the cells washed with ddH2O until the water ran clear. Calcium deposits were visualized under an inverted microscope (Leica).

### *MSCs adipogenic differentiation*

Differentiation of MSCs into adipocytes was achieved throughout a 28-days culturing of 10 000 cells/well in 6-well plates, either in basal conditions, only with DMEM supplemented with 10% heat-inactivated FBS, 1% P/S, and 0,5% G, or with the addition of dexamethasone $10^{-4}$ M, insulin 10 µg/mL, 3-isobutyl-1-methylxanthine $5x10^{-4}$ M (IBMX) and indomethacin $10^{-4}$ M (all from Sigma-Aldrich). To confirm that the adipogenic differentiation successfully occurred, the cells were fixed with 4% (v/v) PFA for further staining with Oil Red O (Sigma-Aldrich) solution at days 21 and 28.

### *Oil Red O staining*

Oil Red O staining was performed in order to qualitatively analyse adipocytic differentiation and to distinguish preadipocytes from adipocytes (where the lipid droplets were stained red). The lipid staining solution was prepared through the addition of 30 mg of Oil Red O powder to 10 mL of 99% (v/v) isopropanol and later diluted in ddH2O in a 3:2 proportion. Fixed cells are washed once with ddH2O and then incubated with 60% (v/v) isopropanol, for 5 min. Subsequently, isopropanol was discarded, and the freshly prepared Oil Red O solution was added to each well, followed by a 5 min incubation, at RT, protected from light. Afterwards, the solution

was discarded, and the cells washed twice with ddH2O, for better visualization of the stained lipids under an inverted microscope (Leica).

***MSC viability assays***

MSCs were tested for cell viability and proliferation using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay and Calcein AM/Propidium Iodide (PI) staining.

For the MTT assay, sterile bioactive glass and titanium discs were placed in a 48-well plate keeping the polished side upwards. Cells were seeded onto each biomaterial surface at a concentration of 5000 cells/well. After 3 and 7 days, the cell viability was assessed. At each time point, the media was removed carefully and 450 µL of fresh media without FBS was added. 50 µL of freshly prepared MTT solution were added and the plates were incubated for 4 hours. In this assay, the yellow tetrazolium salt is reduced to purple insoluble formazan crystals by metabolic activity of viable cells containing NAD(P)H-depending oxidoreductase enzyme. 200 µL dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. All the media was collected from the wells and transferred to another 96-wells plate. Optical density (OD) was measured at 570 nm by spectrometer (Tecan Spark Multimode Microplate Reader).

Calcein/PI staining was another technique used to analyse cell viability. Calcein-AM, an acetoxymethyl ester of calcein, is highly lipophilic and cell membrane permeable. The calcein generated from Calcein-AM by esterase in a viable cell emits a strong green fluorescence. Therefore, Calcein-AM only stains viable cells. On the other hand, PI, a nucleus staining dye, cannot pass through a viable cell membrane. It reaches the nucleus by passing through disordered areas of dead cell membrane and intercalates with the DNA double helix of the cell to emit red fluorescence. Sterile biomaterial discs of bioactive glasses and titanium were placed in a 48-well plate keeping the polished side upwards. MSCs were seeded onto each biomaterial surface at a concentration of 5000 cells/well and incubated for 24 hours at 37 $^{o}$C. Media was discarded and the discs were washed with PBS carefully. 1 mL of calcein/PI staining solution was added at a concentration of 1,5 µL calcein/mL of PI and incubated. After incubation, the biomaterial discs were washed with PBS without disturbing the adhered cells and were

transferred upside down to a 6-well plate. The discs were covered with PBS and observed under a fluorescent microscope (Leica THUNDER Imager 3D Live Cell).

***EVs production, isolation and characterization***

For EV production, cells were cultured in DMEM (for MSCs) or RPMI (for PBMCs). In both cases, the media was supplemented with 1% (v/v) P/S, 0,5 % (v/v) gentamicin, and with EV-free FBS 10% (v/v), to avoid contamination of the sample with bovine EVs. Then, EVs were isolated by differential centrifugation: firstly, the supernatant was collected into a 15 mL Falcon tube and centrifuged at 300 x *g*, for 5 min, at RT, to allow complete cell removal; then, the supernatant was sequentially centrifuged at 2 000 x *g*, for 10 min, to remove cell debris. Afterward, the supernatant was recovered and centrifuged at 100 000 x *g*, for 2 h, at 4 °C in a MED Sorvall WX+ ultracentrifuge. Lastly, the pellet was resuspended in PBS 1x and analysed by Nanoparticle Tracking Analysis (NTA).

NTA analysis was performed using a Nanosight NS300 (Malvern Panalytical) and samples were diluted up to 10X in PBS 1X, according to the concentration of particles. For quality control, only samples containing between 20-120 particles/frame and a ratio of valid particles/total particles greater than 0,2 were considered.

***MSC-derived EVs' effect on T-cell activation***

Briefly, MSCs from 3 healthy donors and 3 patients with MSD were seeded on top of Titanium-based discs, either polished (control) or chemically treated (mimeTi) through a patented acid etching protocol (EP2214732B1), previously described [1]. After 7 days, the supernatants were collected and pooled together and EVs were isolated and quantified through ultracentrifugation and NTA, respectively. Then, $5x10^8$ particles of either condition (healthy vs MSD) were used to supplement $5x10^5$ PBMC (as Del Fattore et al. [91]) and supplemented with phytohaemagglutinin (PHA) at a concentration of 2 µg/mL. The use of a suboptimal concentration was required since the overstimulation with the typical maximum quantity impedes the appreciation of the immunomodulatory effects of MSC-EVs [91]. After 48 hours, their immune profile was analyzed through flow cytometry, focusing on the activation of T cells, using the immunophenotyping panel previously described in this thesis.

***Proteomic analysis***

EVs were lysed with RIPA buffer and sonicated. Proteins were then precipitated with cold acetone and resuspended. Proteins were then reduced in 25 µL of 100 mM NH4HCO3 with 2.5 µL of 200 mM DTT (Merck) at 60°C for 45 minutes and next alkylated with 10 µL 200 mM iodoacetamide (Merck) for 1 hour at RT in dark conditions. Iodoacetamide excess was removed by the addition of 200 mM DTT. The digests were dried by Speed Vacuum and then desalted [92]. Digested peptides were analyzed on an Ultimate 3000 RSLC nano coupled directly to an Orbitrap Exploris 480 with a High-Field Asymmetric Waveform Ion Mobility Spectrometry System (FAIMSpro) (all Thermo Fisher Scientific). Samples were injected onto a reversed-phase C18 column (15 cm × 75 µm i.d., Thermo Fisher Scientific) and eluted with a gradient of 6% to 95% mobile phase B over 80 min by applying a flow rate of 300 nL/min, followed by an equilibration with 6% mobile phase B for 8 min. MS scans were performed in the range of m/z 375–1200 at a resolution of 120,000 (at m/z = 200). MS/MS scans were performed choosing a resolution of 15,000; normalized collision energy of 30%; isolation window of 2 m/z; and dynamic exclusion of 45 s. Two different FAIMS compensation voltages were applied (−45 V and −60 V), with a cycle time of 1.5 s per voltage. FAIMS was operated in standard resolution mode with a static carrier gas flow of 4.6 L/min. The acquired raw MS data files were processed and analyzed using Proteome Discoverer with Chimerys (v3.0.0.757, Thermo Fisher Scientific). SequestHT was used as a search engine and the following parameters were chosen. Database: Homo sapiens (Uniprot, downloaded on 01-02-2018) enzyme: trypsin; max. missed cleavage sites: 2; static modifications: carbamidomethyl (C); dynamic modifications: oxidation (M); precursor mass tolerance: 10 ppm; fragment mass tolerance: 0.02 Da. Only peptides and proteins with FDR value < 0.01 were reported. Abundance of identified peptides was determined by label-free quantification (LFQ) using match between runs. Statistical analyses and t-test were performed on protein abundances using MetaboAnalyst software (https://www.metaboanalyst.ca/). Modulated proteins were analyzed through Database for Annotation, Visualization and Integrated Discovery (DAVID) (version 6.8) (http://david.abcc.ncifcrf.gov/) and IPA (Ingenuity Pathway Analysis, QIAGEN).

***Angiogenic effect of MSCs-derived EVs cultured in contact with bioactive glasses***

The effects of MSC-EVs on angiogenesis was evaluated through a tube formation assay using a µ-Slide Angiogenesis system (ibidi®). Commercially available Human Umbilical Vein Endothelial Cells (HUVEC) were used for this assay. Briefly, each well was coated with 10 µL of Matrigel and left to polymerize at 37 ºC, for 1 h, in a humidity chamber. Meanwhile, HUVEC previously cultured in M199 medium with 10% FBS, 1% P/S, 90 pg/mL Heparin and 450 µg/mL Endothelial Cell Growth Supplement cocktail (ECGS) were detached using Trypsin (0,25%); then the cells were counted and resuspended in Endothelial Growth Medium (EGM-2, Promocell) at the density of $2{,}5x10^5$ cells/mL. After polymerization, $1x10^4$ cells (40 µL) were seeded per each well and the slide was incubated for 15 minutes to allow the cells to attach to the Matrigel. Lastly, 10 µL of EVs derived from a pool of MSCs isolated from healthy donors or patients affected by musculoskeletal disorders, cultured in contact with CuSBA3 and the respective controls (SBA3 and plastic) were added to each well. This amount corresponds to double the concentration obtained from the isolation step. Brightfield images were acquired every 30 min using a Leica DMI6000 Timelapse motorized inverted epifluorescence microscope, for a total of 7 h. Network formation was evaluated through FIJI software [93], using the Angiogenesis Analyzer plugin [94], specific for the identification of typical features of the pseudo vascular organization.

## Results

### *Establishment of the protocol for isolation of MSCs*

In order to isolate MSCs we optimized the protocol as described in the materials and methods section. Through density gradient isolation, MSCs were isolated from bone marrow samples of patients affected by musculoskeletal diseases and later characterized according to the guidelines of the International Society for Cell & Gene Therapy (ISCT) [87]. As seen in **figure 1**, these cells adhere to plastic, display the surface markers CD73, CD90 and CD105 and are negative for the expression of CD14, CD19, CD34, CD45 and HLA-DR markers.

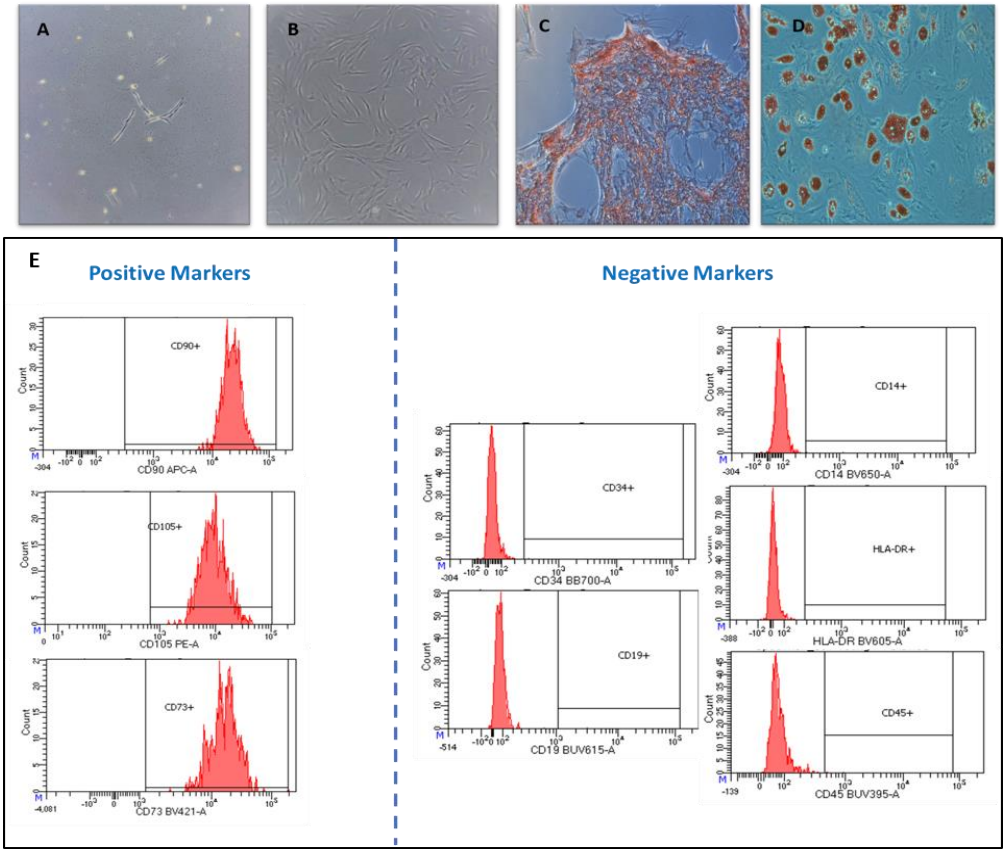



**Figure 1.** Mesenchymal Stem/Stromal Cell characterization. A – Cells adherent to plastic 3 days post-isolation; B – Cells adherent to plastic 10 days after isolation; C – Alizarin Red S staining to confirm mineralization (red) typical of osteogenic differentiation; D – Oil Red O staining to confirm lipid droplet formation (red) typical of adipogenic differentiation; E – Characterization of MSC surface markers by flow cytometry – cells were stained with CD73-BV421, CD90-APC, CD105-PE (positive markers), CD14-BV650, CD19-BUV615, CD34-BB700, CD45-BUV395, and HLA-DR-BV605.

Additionally, they also differentiate into the osteogenic and adipogenic lineage, and despite not being yet characterized according to their chondrogenic potential.

***CT discs and pre-coated bioactive glasses increase metabolic activity of MSCs***

We evaluated how the different biomaterials affected MSCs, more specifically in terms of their metabolic activity. MTT assay was performed at both 3- and 7-days during culture on top of the biomaterials, for a broader indirect view on the possible proliferation enhancement of the discs. In fact, focusing on the titanium-based discs it is possible to see that the polished samples show less metabolic activity compared with the control (no biomaterial) at both timepoints (**Figure 2**).

Regarding the discs that underwent the chemical treatment, we found that at 3 days Ti6Al4V polished discs have a significantly reduced metabolic activity compared to the control (plastic), and a trend in their decrease in comparison with polished ones. These findings were later confirmed at day 7.

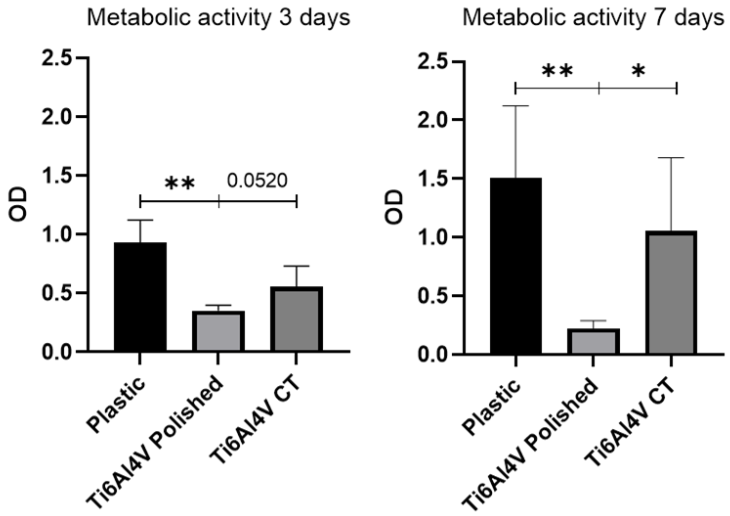



**Figure 2**. MTT assay at days 3 and 7 of MSC culture in contact with the titanium-based discs. Data are shown as average ± SEM. (n=3). According to the samples' normality (Shapiro-Wilk test), ordinary One-way ANOVA (with Tukey's post-hoc correction) or Kruskal-Wallis test (with Dunn's post-hoc correction) were used. *p<0.05, **p<0.01.

In what concerns the bioactive glasses, the results were substantially different. All the tested formulations showed low metabolic activity at both timepoints compared to the plastic (**Figure 3A**). We then asked if the low metabolic activity could be ascribed to a reduced cell viability. Thus, we stained these cells with Calcein/PI and as shown in **Figure 3B** plastic (control) revealed the highest percentage of live cells (green), while among the tested bioactive glasses, the STe0 was the one allowing for more cell viability. The other formulations showed especially low number of cells attached, together with high cell death (red), represented in the figure by

the AgSBA2 panel. These findings suggested the need to optimize the culture conditions when using the bioactive glass discs to favour their attachment.

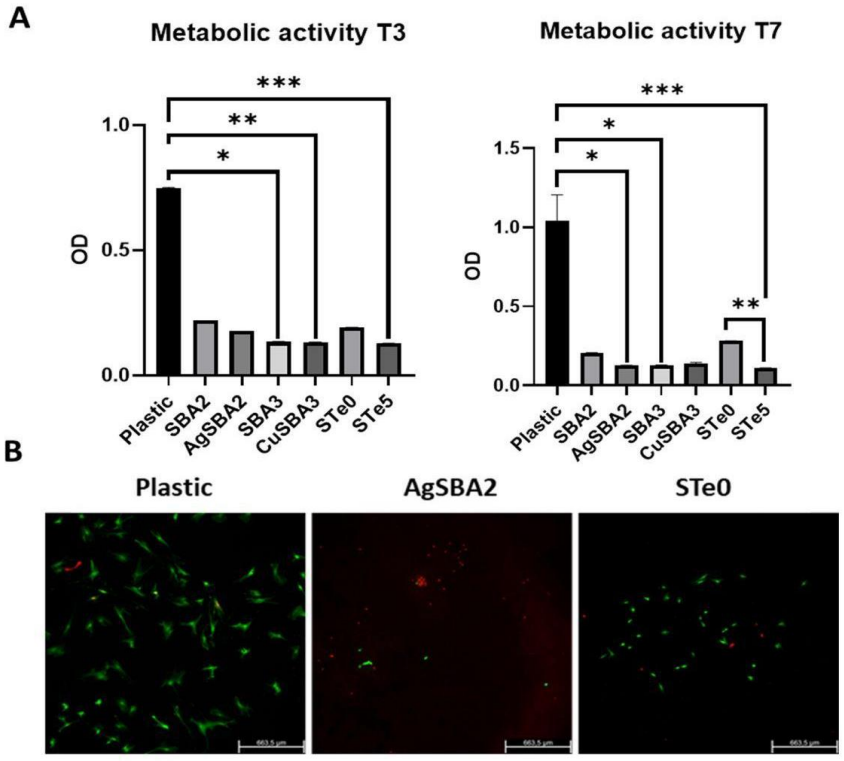



**Figure 3**. Metabolic activity and cell viability of MSCs cultured in contact with bioactive glasses. A – MTT assay at day 3 and 7 of MSC culture in contact with the bioactive glass discs. B – Calcein/PI staining. Green shows live cells and red shows dead cells. Images were acquired using Leica Thunder microscope. Magnification 200X. n=2

Based on the findings above, we pre-incubated the bio glasses discs with the media, as reported in literature [33], and coated with FBS, in order to promote protein coating and facilitate cell adhesion. We found that without pre-incubation there was a visually higher percentage of cell death, considering the majority of the cells appear red by action of PI (**Figure 4**). This is counteracted with 24h pre-incubation, and it is even more improved when the disks are then incubated with serum for 2h (Figure 4). With regard to the FBS coating, we observed an increase (up to 3-fold) of the metabolic activity at both time points considered (**Figure 5**).

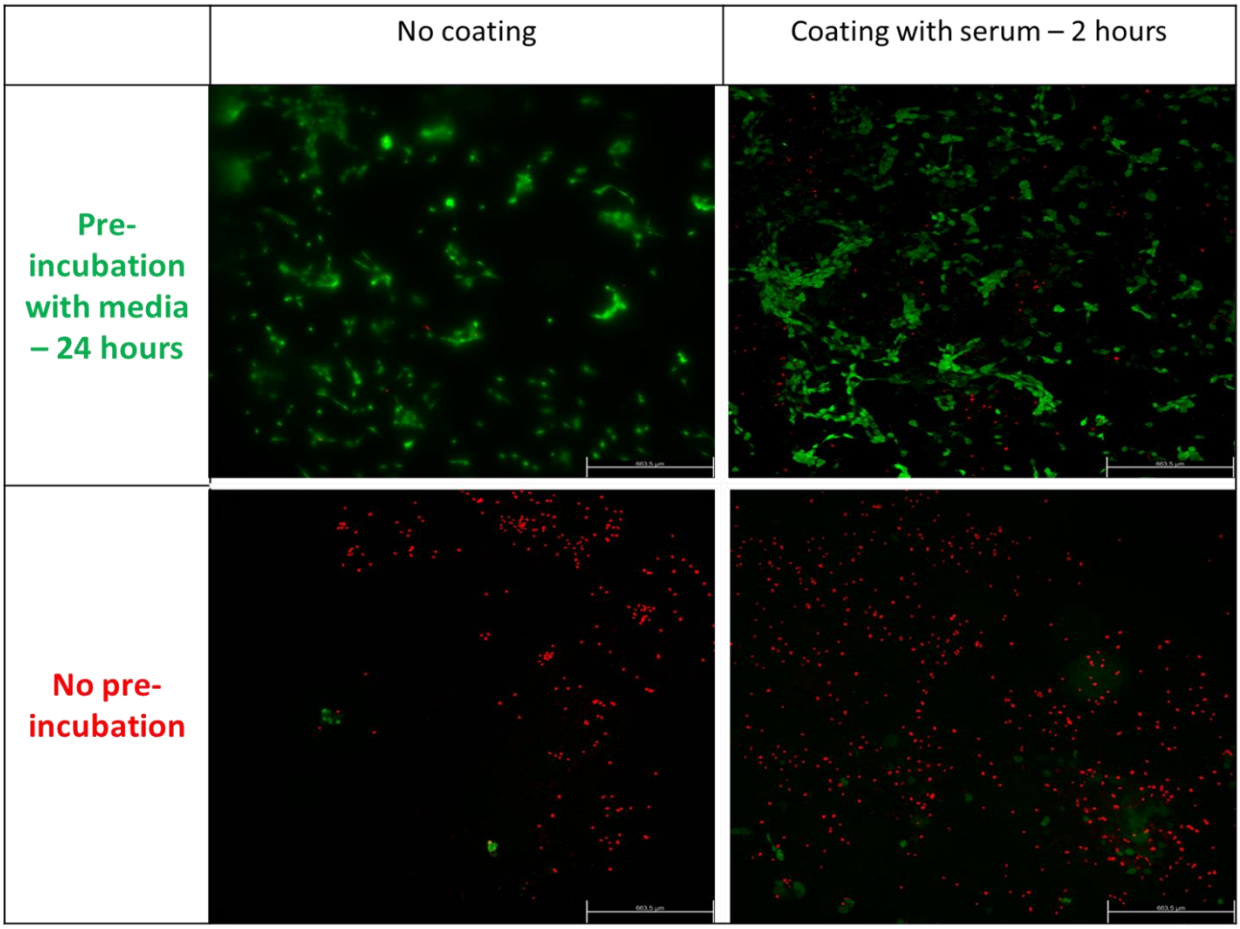



**Figure 4.** Calcein/PI staining of MSCs cultured in contact with SBA2 (representative of the results with all disks). Green shows live cells and red shows dead cells. Images were acquires using Leica Thunder microscope. Magnification 200X.

*MSC-EVs do not affect the phenotyping of T cells*

We attempted to evaluate if a physiologically relevant amount of EVs derived from MSCs cultured in contact with titanium-based discs could modulate the T-cell phenotype. Attesting for the low immunogenicity of EVs, unmatched donors were used, and it is apparent that no significant immune response derived from incompatibility was noted in any of the unstimulated conditions (without PHA but supplemented with EVs). We found that from this experimental setup also there were no significant differences on the immune profile, when comparing MSC-EVs from healthy donors and MSD patients with the control condition without EVs (**Figure 6**). Also, we did not detect changes on T cell subsets between EVs derived from MSCs cultured in

contact with polished or chemically treated discs or without any biomaterial. These results would suggest these preconditioned MSC-EVs are not immunogenic.

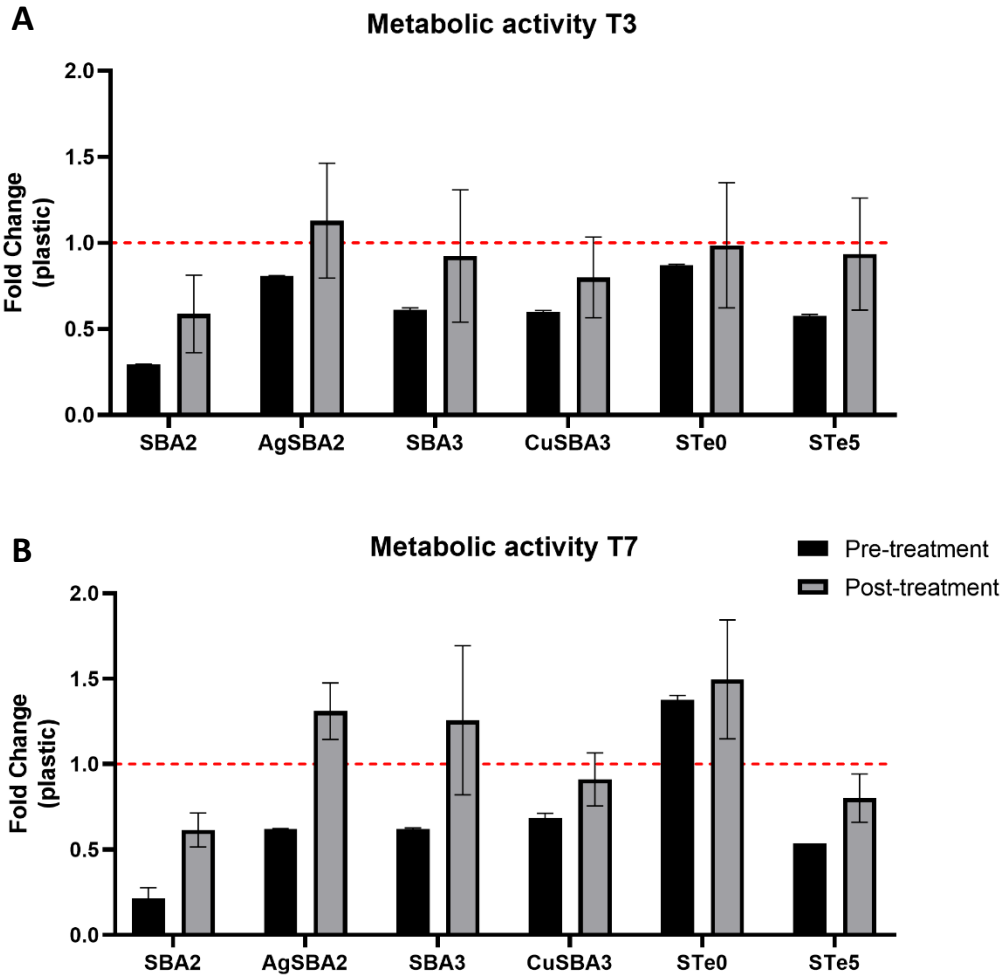
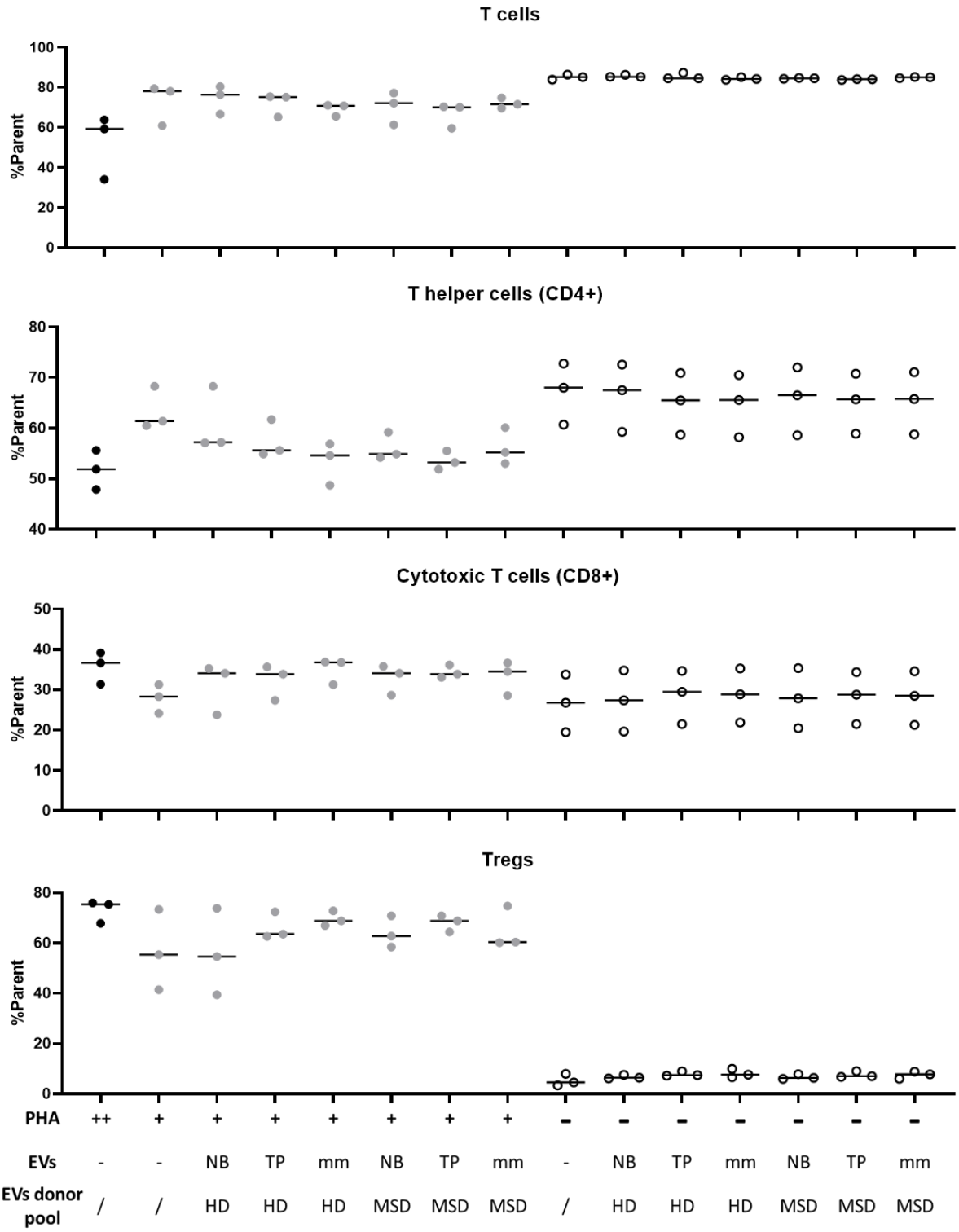



**Figure 5.** Metabolic activity and cell viability of MSCs cultured in contact with bioactive glasses. MTT assay at day 3 and 7 of MSC culture in contact with the bioactive glass discs: A – Before coating B – After coating. n=3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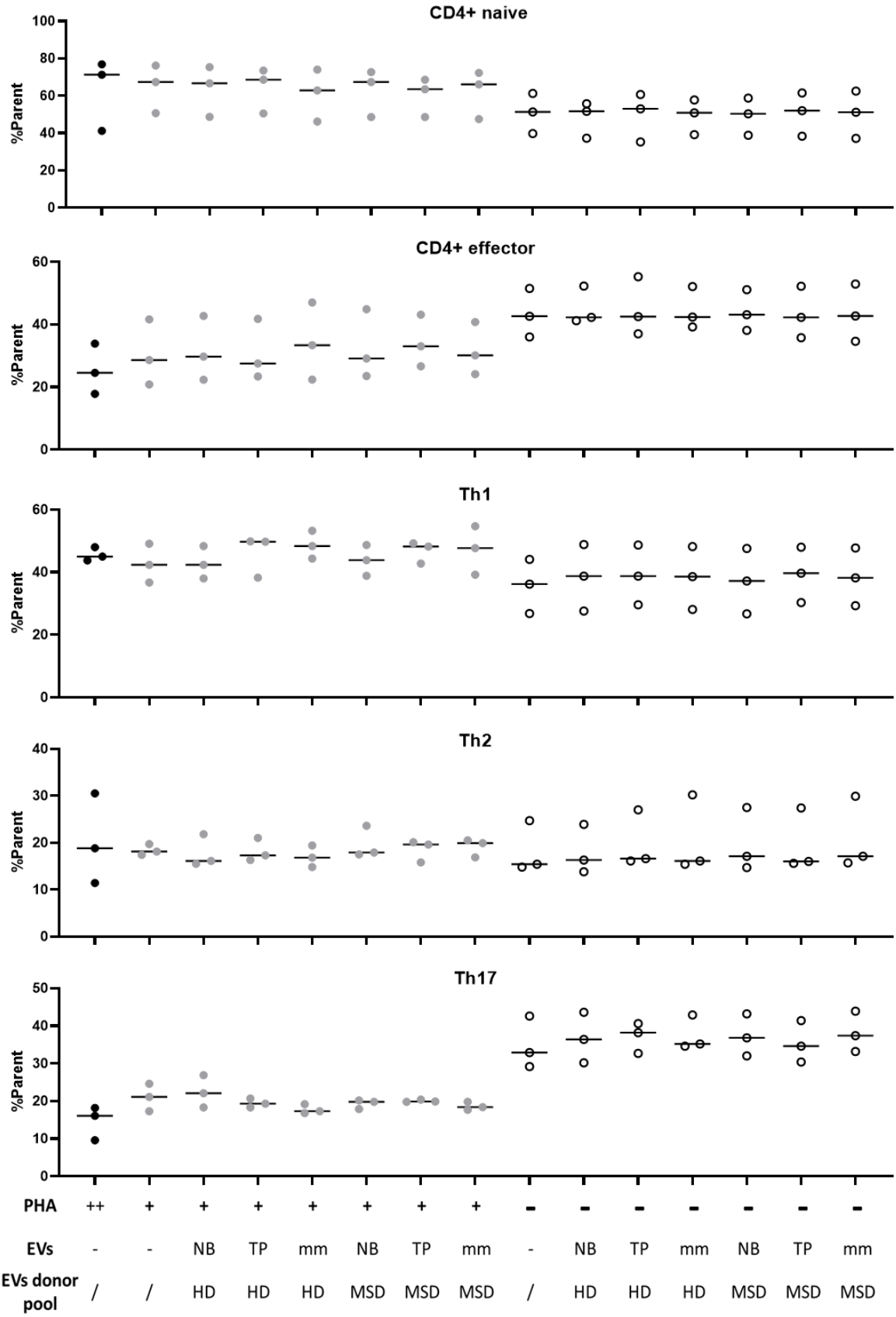
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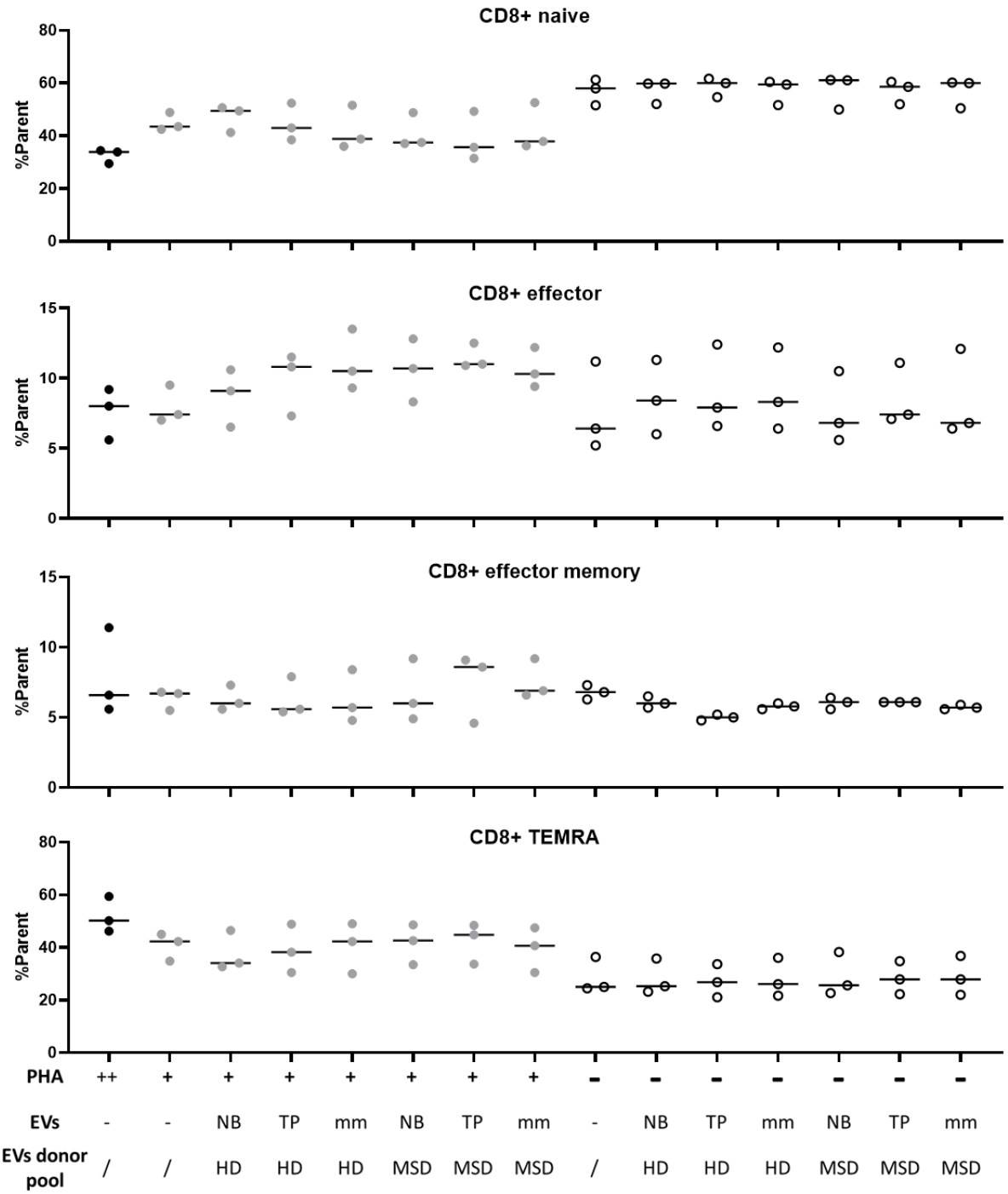



**Figure 6**. T cell immunophenotyping following stimulation with MSC-EVs for 48 hours, PHA stimulation served as positive control.Anti-CD45 human antibody (mAb) (leucocytes), anti-CD3 mAb (T cells), anti-CD4 mAb (T helper cells), anti-CD8 mAb (cytotoxic T cells) anti-CD45RA and anti-CD197 mAbs (naïve, central memory (CM), effector memory cells (EM), and terminally differentiated EM cells), anti-CD25 and anti-CD127 mAbs (regulatory T cells, Tregs), anti-CD183, anti-CD194 and anti-CD196 mAbs (Th1, Th2, and Th17). The samples were acquired using FACSymphony™ A5 (BD Biosciences) flow cytometer and data were analyzed using FACSDIVA software (BD Biosciences). NB – No biomaterial; TP – Titanium Polished; mm – mimeTi HD – Healthy donors; MSD – Patients affected with

musculoskeletal diseases; MSC-EVs correspond to a pool of 3 HD or MSD; PBMCs were obtained from 3 unmatched HD.

***Copper doped glass upregulated angiogenesis, endothelial and epithelial EVs proteins***

To evaluate the effect of culturing MSCs on bioactive glasses on their modulation properties through paracrine signalling, we assessed the protein content of the released EVs by shotgun proteomics. **Table 1** shows the number of proteins significantly different between each bioactive glass and their respective controls, both the undoped glass and the condition without any biomaterial.

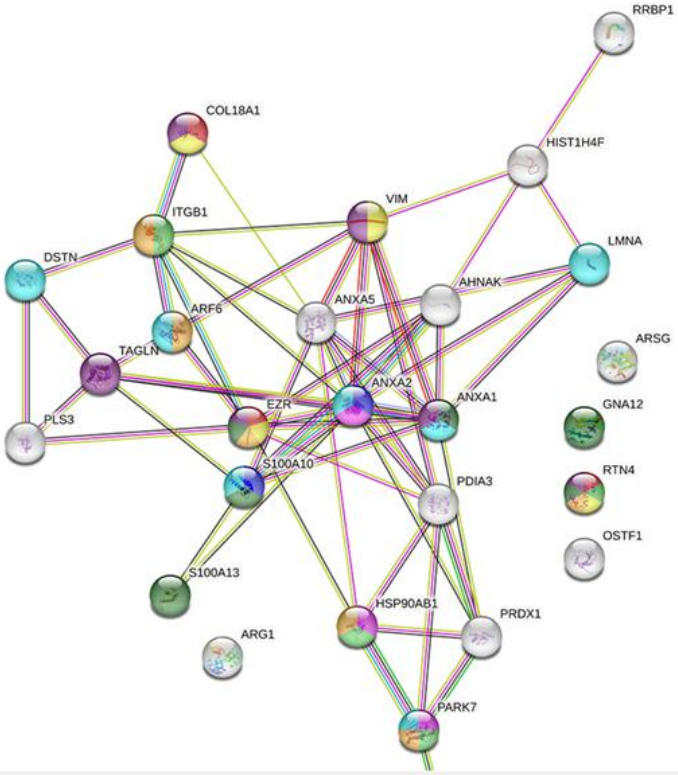
**Table 1 -** Quantification of proteins significantly altered in EVs from MSCs cultured in contact with bioactive glasses

| Doped bioactive glass | Compared with | Number of proteins significantly altered |
|---|---|---|
| **AgSBA2** | Plastic | 260 |
| | SBA2 | 14 |
| **CuSBA3** | Plastic | 261 |
| | SBA3 | 29 |
| **STe5** | Plastic | 261 |
| | STe0 | 79 |

According to the classical approach using DAVID as a tool to associate the altered proteins with specific biological processes [95], we found that among the tested BGs the copper-doped formulation (CuSBA3) revealed a particular tendency for the upregulation of proteins related to angiogenesis as well as endothelial and epithelial cell development and differentiation (**Figure 7)**. Therefore, a functional assay of network formation, using commercial endothelial cells was set up to assess the impact *in vitro* of the upregulated proteins carried by EVs.

P-value shows how likely the observed differences in protein expression could have occurred due to random chance alone. In the case of small p-values (typically below a predetermined significance level, often denoted as $\alpha$, such as 0.05), statistics suggest that the observed differences are unlikely to be due to chance and are more likely due to a natural effect. A small p-value does not prove a significant difference between the groups being compared; it

simply indicates that the observed data is unlikely under the assumption of the null hypothesis (no difference in protein expression between the control and treatment groups).




**Biological Process (Gene Ontology)**

| description | count in network | strength | false discovery rate |
|---|---|---|---|
| Methylglyoxal catabolic process to lactate | 2 of 3 | 2.68 | 0.0195 |
| Membrane raft assembly | 2 of 10 | 2.16 | 0.0366 |
| Positive regulation of vesicle fusion | 2 of 12 | 2.08 | 0.0423 |
| Negative regulation of type 2 immune response | 2 of 14 | 2.02 | 0.0430 |
| Endothelial cell development | 3 of 48 | 1.66 | 0.0260 |
| Positive regulation of protein localization to plasma membr... | 3 of 62 | 1.54 | 0.0317 |
| Cellular response to hydrogen peroxide | 3 of 71 | 1.49 | 0.0396 |
| Negative regulation of cellular protein catabolic process | 3 of 89 | 1.39 | 0.0494 |
| Cellular response to reactive oxygen species | 4 of 133 | 1.34 | 0.0253 |
| Regulation of cell shape | 4 of 153 | 1.28 | 0.0260 |
| Establishment or maintenance of cell polarity | 5 of 197 | 1.26 | 0.0167 |
| Positive regulation of protein kinase B signaling | 4 of 170 | 1.23 | 0.0304 |
| Positive regulation of binding | 4 of 175 | 1.22 | 0.0322 |
| Epithelial cell development | 4 of 195 | 1.17 | 0.0412 |
| Regulation of cellular protein catabolic process | 5 of 275 | 1.12 | 0.0253 |
| Response to drug | 5 of 281 | 1.11 | 0.0253 |
| Positive regulation of cellular protein localization | 6 of 342 | 1.1 | 0.0167 |
| Supramolecular fiber organization | 7 of 480 | 1.02 | 0.0104 |
| Regulation of cellular component size | 5 of 383 | 0.98 | 0.0423 |
| Regulation of cellular protein localization | 7 of 568 | 0.95 | 0.0167 |
| Regulation of cell morphogenesis | 6 of 498 | 0.94 | 0.0260 |
| Actin filament-based process | 7 of 592 | 0.93 | 0.0179 |
| Actin cytoskeleton organization | 6 of 516 | 0.93 | 0.0260 |
| Response to inorganic substance | 6 of 538 | 0.91 | 0.0297 |
| Positive regulation of organelle organization | 7 of 633 | 0.9 | 0.0195 |
| Leukocyte mediated immunity | 6 of 641 | 0.83 | 0.0430 |
| Negative regulation of multicellular organismal process | 11 of 1231 | 0.81 | 0.0041 |
| Epithelial cell differentiation | 6 of 673 | 0.81 | 0.0494 |
| Leukocyte activation | 8 of 929 | 0.8 | 0.0210 |
| Biological adhesion | 8 of 931 | 0.79 | 0.0210 |

**Figure 7.** Gene ontology representation of the proteins significantly upregulated in EVs derived from MSCs cultured in contact with the copper-doped glass (CuSBA3), compared with his undoped control (SBA3).

Additionally, p-values are subject to interpretation and can be influenced by factors such as sample size and experimental design.

Therefore, more recently, other alternatives to p-value are starting to be explored. In collaboration with another PREMUROSA ESR, a new machine learning method has been developed to identify extremely changing proteins in a more accurate manner. By log-transforming the data, clustering all values, selecting the inliers, and calculating the Euclidean distance, i.e. measure of how many standard deviations away a particular point is from the mean of a distribution after adjusting for correlation among variables, several extremely changing proteins were detected. This strategy has recently been published [96] but being a core part of the tasks to be performed by another ESR, it will not be explored further in this manuscript.

***MSC-derived EVs promote angiogenesis in vitro***

It has been reported that MSCs can exert potent paracrine modulatory effects over several cell types [97], including endothelial cells [98]. For that reason, and attending to the potential of copper ions in terms of pro-angiogenesis signalling [45], we investigated if EVs produced by MSCs cultured in contact with copper-doped bioactive glass discs can enhance the vessel network formation of HUVEC. Also, we questioned if the origin of the cells, i.e. healthy donors or patients affected by OA, impacted at all the outcome of the angiogenic process.

During the 7-hour timelapse, we found that HUVEC were able to reorganize across the matrix and the speed and efficacy of the network formation was evaluated through ImageJ, using a specific plugin designed for the identification of typical structures formed during the angiogenesis process (**Figure 8**).

Parameters like length and/or number of several structures such as extremities, nodes, meshes, segments, branches, junctions and pieces were evaluated, at three different timepoints: at the start of the timelapse upon stimulation with MSC-EVs (T0), at the end of the timelapse (T7) and in an intermediate timepoint (T3,5). **Figure 8** shows the evident morphological differences in terms of cellular organization, visible through brightfield microscopy at the beginning of the timelapse and at the endpoint (7 hours).

Upon image analysis, the results obtained from the timelapse were graphically organized as shown in **Figure 9**. According to the data obtained, there is a general decrease of isolated structures, namely branches and segments, from the initial timepoint to the endpoint, accompanied by an increase in mesh size and area, which is expected in a successful network formation assay. However, no statistically significant differences were detected between the tested conditions at any time point considered.

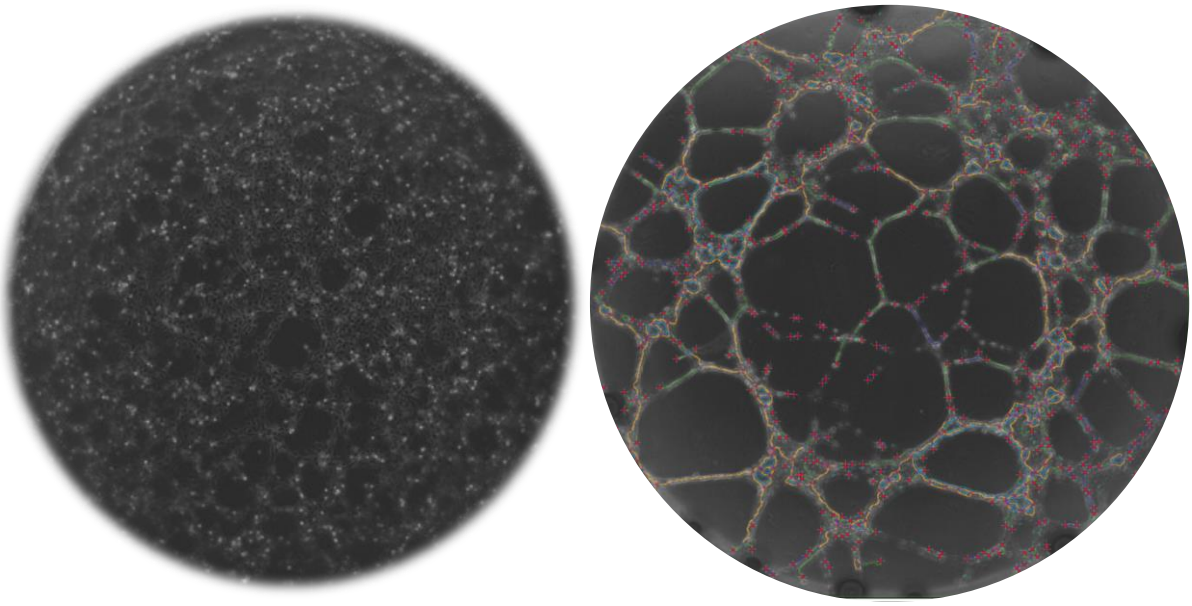


**Figure 8**. Angiogenesis assay. Visual representation of cell organization immediately after HUVEC attachment to Matrigel (left) and 7 hours after (right). Brightfield images acquired at 100X magnification using a Leica DMI6000 Timelapse microscope. Colour code created by Angiogenesis Analyzer plugin: green = branches; cyan = twigs; magenta = segments; orange = master segments; blue sky = meshes; red surrounded by blue = nodes surrounded by junctions' symbol; junctions surrounded by red = master junctions; blue = isolated elements; cyan = small isolated elements; red surrounded by yellow = extremities.

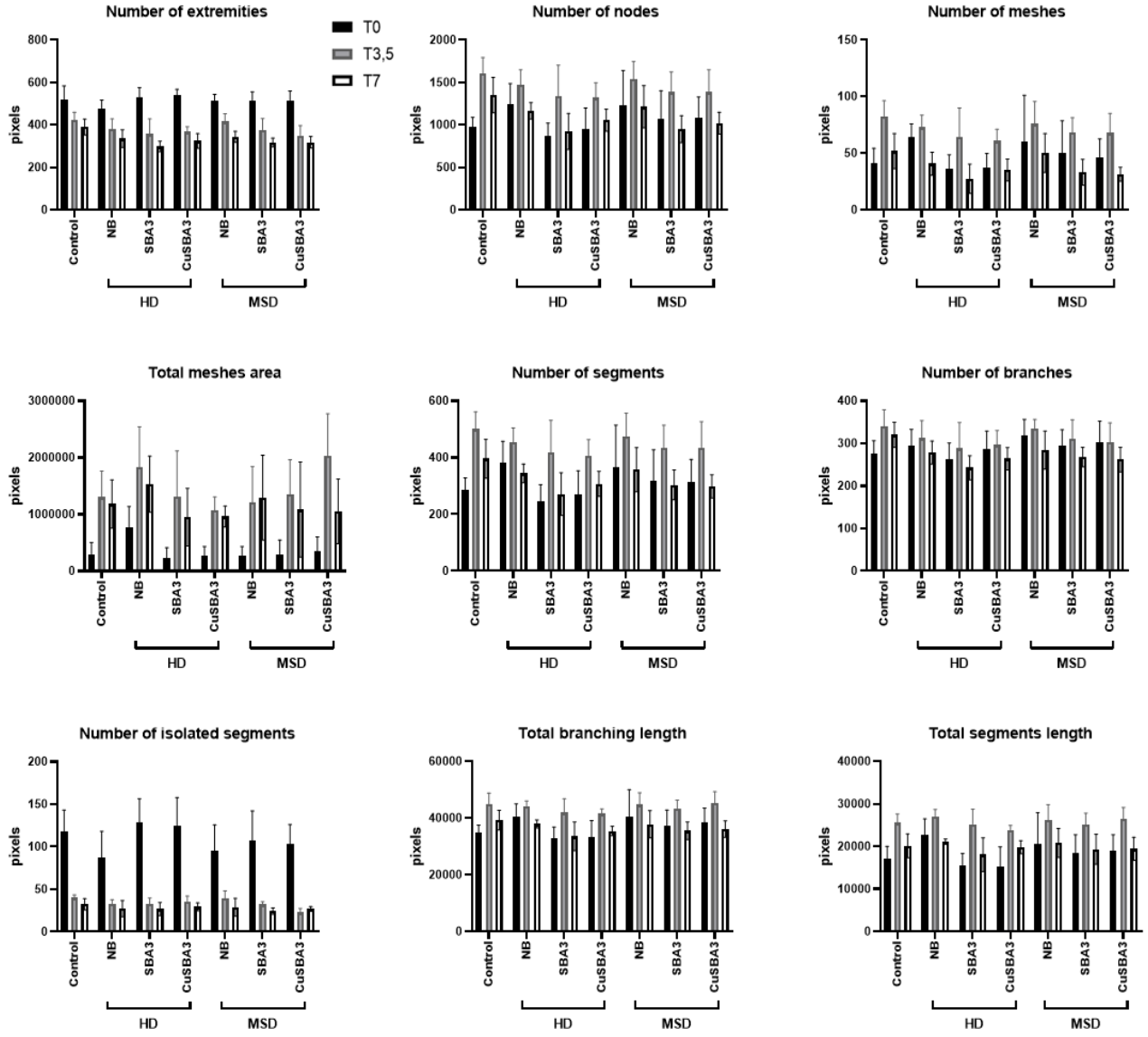



**Figure 9.** Graphical representation of the structures automatically detected by the Angiogenesis Analyzer plugin. Analysed structures include extremities, nodes, meshes, branches, segments, junctions and pieces. HD – Healthy donors; MSD – Patients affected by musculoskeletal disorders; NB – No Biomaterial (control condition) Data are shown as average ± SEM. (n=3). Ordinary one-way ANOVA or Kruskal-Wallis multiple comparisons test were used.

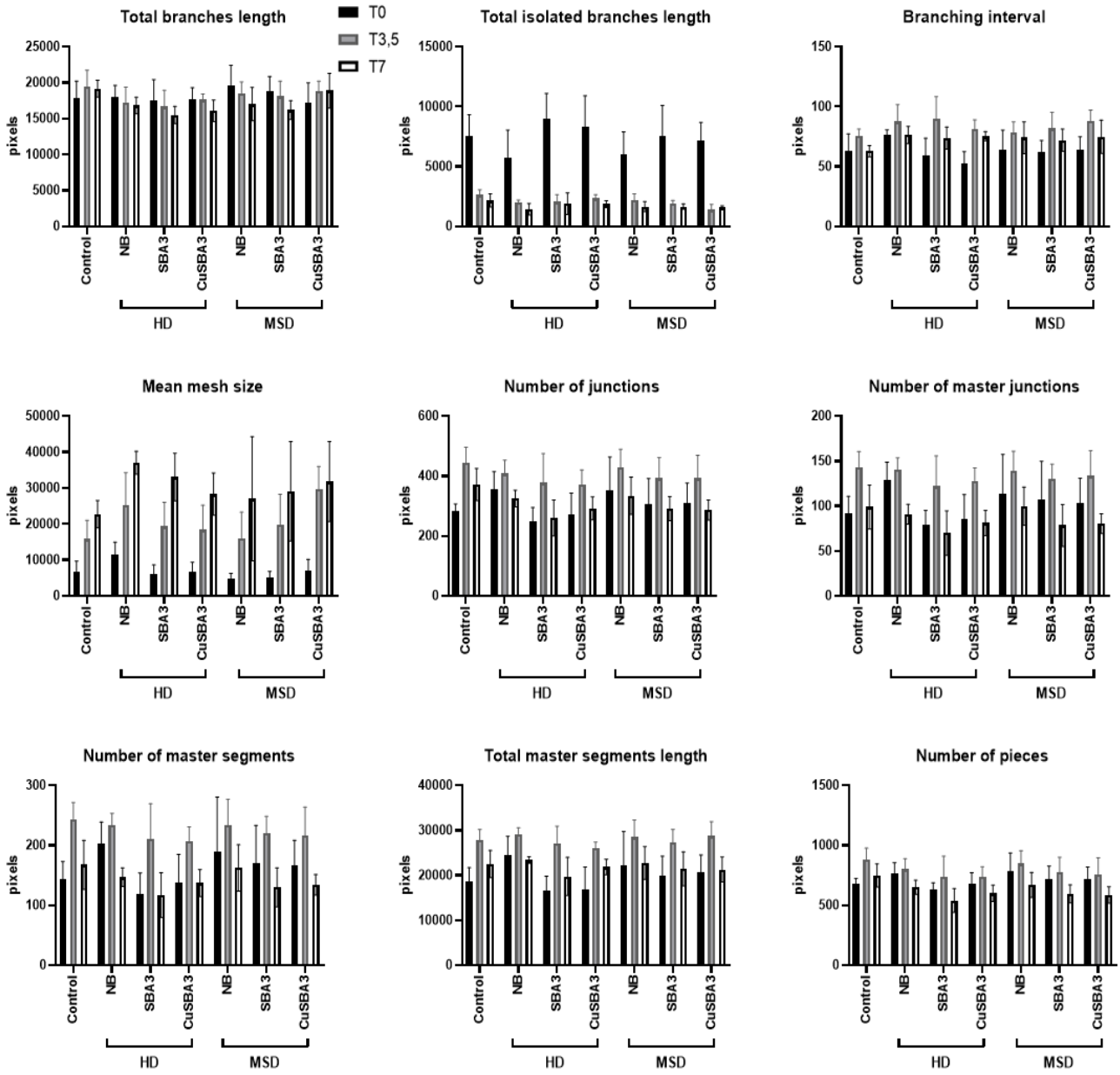



**Figure 9 (cont.).** Graphical representation of the structures automatically detected by the Angiogenesis Analyzer plugin. Analysed structures include extremities, nodes, meshes, branches, segments, junctions and pieces. HD – Healthy donors; MSD – Patients affected by musculoskeletal disorders; NB – No Biomaterial (control condition) Data are shown as average ± SEM. (n=3). Ordinary one-way ANOVA or Kruskal-Wallis multiple comparisons test were used.

## Discussion

Many materials have been used over the last decades as bone substitutes with various outcomes, therefore the actual era of implant design focuses also on optimizing current options besides developing entirely novel alternatives [99]. In this sense, applying chemical treatments, coatings, or other types of surface modifications can greatly improve the bioactive properties of an existing biomaterial [1]. In our study, we employed a patented chemical treatment, consisting on an acid etching approach that creates micro- and nano-texture, as well as confers a negative charge to the surface of titanium-based discs, which is intended to facilitate cell adhesion and osteointegration. The physical characterization which demonstrates the effectiveness of the treatment in modifying the surface topology has been published previously [1].

Our results revealed that MSCs cultured on top of titanium CT discs for 7 days have significantly higher metabolic activity than cells cultured on a polished surface (**Figure 2**). This is in line with several reports showing that adherent cells are able to attach and proliferate more in acid-etched rough surfaces. In particular, rat-derived MSCs attachment, proliferation and differentiation was improved upon culture in sandblasted and acid-etched (SLA) samples treated with $H_2O_2$ [100]. Osteoblasts also benefit from SLA treatment, attaching faster on rougher surfaces [101].

On the case of bioactive glasses, the modifications applied, i.e. the metallic-ion supplementation, did not significantly impact the surface roughness. In fact, due to the polished surface of the discs, it appears that MSCs attachment, viability and metabolic activity were severely impaired (**Figure 3**). Furthermore, the nature of bioactive glasses differs from metallic discs, such as Ti6Al4V-ELI here utilized, in the sense that the formers are much more bioreactive, meaning that they release their ions much faster into the surrounding microenvironment. A common method to overcome this phenomenon, proposed by Ciraldo et al, consists in pre-soaking the bioactive glasses in cell culture media. Furthermore, considering the low cell attachment in polished surface, we opted also to coat the discs with FBS, creating a layer of high molecular weight proteins that can favour cell adhesion [102]. In fact, we could verify that the pre-treatment with both cell culture media and FBS greatly improved MSC metabolic activity (**Figure 4**), allowing their culture and further studies on their EVs content.

It has been extensively reported that MSCs exert an immunomodulatory role in a paracrine manner, which is linked with the release of EVs [103]. MSC-EVs can in fact reproduce the effects of MSCs, particularly in injury repair and tissue regeneration [104], controlling inflammation [105], promoting cell proliferation and migration [106], and stimulating collagen synthesis [107] as well as angiogenesis [106–108]. Due to their reduced immunogenicity and toxicity compared to MSCs, EV administration represent a promising therapeutic tool instead of their parent cells [109,110].

Noteworthy, the culture conditions to which MSCs are subjected will impact the quantity and the effect of the secreted EVs [111]. For example, both hypoxia [112] and 3D culture [113] systems have been studied as means to increase the number of EVs produced and expression of anti-inflammatory factors such as stanniiocalcin-1, while reducing the production of pro-inflammatory proteins [114]. Similarly, chemical stimulation, such as LPS and thrombin, can lead to the release of MSC-EVs with a stronger anti-inflammatory profile [115,116].

Taking this into consideration, we sought to investigate if EVs released by MSCs cultured in contact with titanium-based discs could exert any immunomodulatory effect on PBMCs. After 48 hours of culture with MSC-EVs, PBMCs' immune profile was analyzed through flow cytometry, focusing on the activation of T lymphocytes, in accordance to previous data obtained within the scope of the project on the immunobiocompatibility of the tested biomaterials.

Results show no significant differences on the immune phenotype, when comparing the culture with MSC-EVs from healthy donors and MSD patients with the control condition without EVs (**Figure 6**). Also, no changes were detected between EVs derived from MSCs cultured in contact with polished or chemically treated discs or without any biomaterial.

Although numerous authors report the effects of MSC-EVs in inflammatory and autoimmune diseases, using both *in vitro* and *in vivo* models (as reviewed by Yang et al [117], Dabrowska et al [118], and Harrell et al [119]), the experimental parameters such as method of EV isolation, number/quantity of particles used per cell, and duration of the EV stimulus vary immensely, which evidences the need for optimization and standardization of such protocols. Nevertheless, studies show that MSCs and MSC-EVs inhibit T-cell proliferation, as well as

inflammatory Th1, T follicular helper (Tfh) and DC differentiation, while promoting Th2 and Treg function, typically immunosuppressive cell types [120–126], which we were not able to detect in our experiment.

Chen et al evaluated *in vitro* the effect of MSC-derived exosomes testing up to 20 μg of EVs for each $5x10^5$ PBMCs stimulated with 5 μg/ml Concanavalin A. The authors report that the EVs treatment does not impact PBMC proliferation up to five days, however an increase in PBMC apoptosis, Th2 and Treg differentiation was detected, accompanied by a decrease in the frequency of Th1 and Th17 cells, in a dose dependent manner [127]. Other authors used EVs released by $1x10^5$ MSCs throughout 48 hours of culture to stimulate $1x10^6$ PBMCs and concluded that the addition of EVs promotes Treg proliferation and immunosuppressive ability through the increase of the production of the anti-inflammatory cytokines IL-10 and TGF-β1 [128]. Pachler et al. report that T-cell proliferation due to PHA stimulation can be inhibited by MSC-EVs in a dose-dependent manner comparable to MSCs after 4 days, when the administered EVs are 10-fold greater in quantity that the parental cell number [129]. Overall, although direct culture of PBMCs with titanium-based discs can modulate T-cell phenotype, it appears that MSC-EVs resulting from preconditioning with these biomaterials do not exert a similar effect. However, exploring this interaction in a more physiologically relevant environment such as a dynamic bioreactor or an *in vivo* setting would help clarify over time the real impact of titanium implants in MSC behaviour and immune reaction.

Given the fact that MSC-EVs from culture with titanium-based discs did not alter PBMC phenotype, coupled with the promising proteomic results on the content of EVs secreted by MSCs cultured in contact with copper-doped bioactive glasses, a different approach was considered for these biomaterials. Proteomic results show an upregulation of several proteins involved on endothelial and epithelial cell development, indicative of an enrichment of angiogenesis-related proteins contained in EVs secreted by MSCs cultured in contact with copper-doped bioactive glasses (**Figure 7**). Pro-angiogenic properties of copper are already well documented [45]; moreover, MSCs *per se* contribute to the angiogenesis process, both by differentiating into endothelial cells, but also in a paracrine manner by secreting pro-angiogenic

growth factors and microRNAs [130,131]. Given that, we investigated if these EVs could promote a faster network formation by endothelial cells, using commercial HUVEC as a model.

Using a physiologically relevant EVs amount – double the concentration produced by MSCs in 7 days – it seems that the supplementation of the culture media with EVs form preconditioned MSCs does not affect the swiftness or effectiveness of HUVECs' reorganization (**Figure 9**). Several studies have reported that MSC-EVs can enhance tube formation in HUVEC, using similar assays to the one presented in this manuscript. Liang et al showed that adipose-derived stem cells (ASCs) can be internalized by endothelial cells, promoting the process of angiogenesis both *in vivo* and *in vitro*. In this case, the authors propose that the inherent mechanism in play regards the enrichment of exosomal miR-125a, which in turn downregulates the delta like canonical Notch ligand 4 (DLL4), leading to increased branch count and tube length [132]. Also, the Wnt/β-catenin pathway was shown to be involved in angiogenesis enhancement through EVs transfer. Exosomes from umbilical cord-derived MSCs promoted not only the proliferation and migration of endothelial cells, but also their tube formation capacity, in a dose dependant manner. Moreover, it was detected an increase in the expression and nuclear translocation of β-catenin in endothelial cells treated with MSC-EVs. Lastly, it was also demonstrated that the knock-down of Wnt blocked the nuclear translocation of β-catenin, and consequently the formation of longer tubes was impaired [133]. In another study, preconditioning ASCs with platelet-derived growth factor (PDGF) modulated the generation of EVs able to increase tube formation of human microvascular endothelial cells (HMEC) *in vitro* (supplementing with $10^{11}$ EVs/mL), while also stimulating angiogenesis *in vivo* in severe combined immunodeficient (SCID) mice. The authors attribute this effect to the increased amount of pro-angiogenic factors, mainly CD117/c-KIT and stem cell factor (SCF), in PDGF-stimulated ASC-EVs [134]. Overall, the common point among all these studies that differs from ours is the amount of EVs used to promote the tube formation. The original assays hereby described in this manuscript were performed considering the proportional amount of EVs produced by MSCs cultured on top of 1$cm^2$ discs. Although the experimental scale-up of the set up would be logistically difficult, which can be perceived as a limitation of our study, we believe that by stimulating PBMCs or endothelial cells with EVs at a similar concentration as obtained from MSC culture, we can more

accurately mimic the microenvironment surrounding the implant. While the concentration of EVs in plasma, regardless of cell origin, is estimated to be around $10^{10}$ EVs/mL, in other fluids it is expected to be up to 10-fold lower [135,136]. *In vitro*, it is expected to have less than 1 µg of EVs protein per mL of supernatant [137]. With that, although many studies that apply much higher amounts of EVs proteins can unravel scientifically relevant discoveries, they do not accurately represent the physiological environment. In our case, we demonstrated that, although proteomic analysis confirms the potential of copper to activate angiogenic-related pathways in MSCs – which also reflects on their secreted EVs content – the EVs amount released over 7 days might not be enough to significantly promote blood vessel formation. However, these results still open the possibility to couple the implantation of copper-doped bioactive glasses with the supplementation with a substantially higher concentration of MSC-EVs, albeit additional *in vivo* studies would be required to understand the required amount to be used.

In summary, these results highlight the importance of surface topography and composition on MSCs adhesion, viability and metabolic activity. While increased roughness and a negatively charged surface on titanium-based discs is preferred for osteointegration, bioactive glasses essentially require the removal of excessively released ions prior to cell culture, benefiting also from a simple protein coating to favour cell adhesion. Furthermore, while distinct biomaterials can differentially modulate cells in direct contact, it can also alter the secreted EVs content, therefore affecting cell communication. However, results on the effect of the supplementation with a physiological amount of MSC-EVs on PBMCs and endothelial cells reveals that the impact of biomaterials on MSCs paracrine signalling is minimal.

## CONCLUSIONS AND FUTURE PERSPECTIVES

Our study investigated the immune response to bioactive glasses doped with different metal ions, focusing on T cells critical for regulating inflammation during tissue regeneration. While previous research on bioactive glasses has primarily focused on hard tissue repair, the immune response is often overlooked. The evaluation of the immune response to biomaterials is a crucial aspect in regenerative medicine. When biomaterials are introduced into the body, they interact with the immune system, initiating responses that can significantly impact the outcome of the implant, determining its success or failure.

Our findings revealed that metal doping can influence T cell subsets, cytokine release, and even cell death *in vitro*. In particular, silver doping displayed high toxicity similar to its known dose-dependent cytotoxic effects while in soluble ionic form, suggesting a need for reformulation before further studies. This could be achieved either by modifying the manufacturing protocol to lower the original concentration incorporated on the discs' surface or by pre-incubating for a prolonged period to eliminate the initial ion burst. Tellurium, on the other hand, had minimal impact on cell viability but caused variable T-cell responses between individuals, mostly in Tregs. This highlights the need for a previous assessment of the immune response to tellurium on each patient when considering tellurium-doped bioactive glasses as a potential implant alternative. In the case of copper doping, it may not only promote a switch from Th17 to Th1 cells, but also potentially influence other immune cells like macrophages and eosinophils through changes in cytokine (IL-5, IL-13) and chemokine (MCP-1/CCL2, MIP-1β/CCL4) secretion. Importantly, while the undoped condition led to the increase of the aforementioned factors, copper doping seemed to normalize these cytokine levels to a healthy baseline, suggesting enhanced biocompatibility. Further *in vivo* studies are crucial to confirm these findings in a more complex environment where all the players involved in tissue regeneration and inflammation are present.

Titanium is indeed a well-known biomaterial, highly regarded for its exceptional biocompatibility and mechanical properties. Titanium polishing aimed at refining its by removing of surface imperfections and improving the functional properties of titanium components.

This process can induce the release of nanoparticles that might induce an immune response. This initial response, encompassing inflammation, immune cell recruitment, and cytokine release, is crucial for determining the discs' biocompatibility. Here we report that even though there was no impact on PBMC viability, polishing increases the percentage of Th2 cells while simultaneously decreasing the Th17 one. The same condition also significantly unbalanced PBMCs' cytokine profile toward a pro-inflammatory state, while the chemical treatment of the discs through acid etching reversed all the alterations, seen in T-cell phenotype and cytokine secretion to values similar to the basal condition. Thus, this approach emerged as a promising strategy to potentially mitigate the negative effects observed with polishing, paving the way for improved biocompatibility in biomedical applications.

Lastly, this study also underlines the critical role of surface topography and composition in influencing MSC behaviour. Rougher, negatively charged titanium discs promote osteointegration, while bioactive glasses benefit from pre-treatment to remove excess ions and potentially a simple protein coating to enhance cell adhesion. Interestingly, while biomaterials directly affect attached cells, they can also indirectly influence communication via changes in the content of secreted EVs. However, our findings suggest minimal impact of biomaterials on MSC paracrine signaling through EVs, as supplementation with physiological MSC-EV doses had little effect on immune and endothelial cells. This indicates that relying on the modulatory effects of MSC-EVs directly produced at the implant site might not be sufficient for the improvement of tissue regeneration. However, the enrichment in pro-angiogenic proteins contained inside MSC-EVs upon culture with copper-doped discs suggests that using substantially higher amounts of these EVs as supplemental therapy after copper-doped bioactive glass implantation might aid the bone healing process through enhancing blood vessel formation.

Overall, although our results suggest that copper-doped bioactive glasses and acid etched Ti6Al4V-ELI discs can be more effective alternatives, able to control the immune response while also modulating MSCs biological processes, such as EVs content or cell metabolic activity, a more complete picture on the impact of these biomaterials on all cells belonging to the bone microenvironment is needed. *In vivo* studies could help understand how osteoblasts/osteoclasts interact with the implants, when subjected to the immune microenvironment created by not only

T cells but also monocytes, macrophages and granulocytes. Additionally, while some *in vitro* data on biofilm formation have already been described elsewhere, *in vivo* studies would also contribute to understanding if these modifications also help reducing the chance of infection upon implantation.

## ANNEX – Publications during my PhD

**Paper 5 –** Cappellano, G.; **Abreu, H.**; Raineri, D.; Scotti, L.; Castello, L.; Vaschetto, R.; Chiocchetti, A. High Levels of Circulating Osteopontin in Inflammatory Lung Disease Regardless of Sars-CoV-2 Infection. *EMBO Mol. Med.* **2021**, *13*, doi:10.15252/emmm.202114124.

**Paper 6 –** Cappellano, G.; **Abreu, H.**; Casale, C.; Dianzani, U.; Chiocchetti, A. Nano-Microparticle Platforms in Developing next-Generation Vaccines. *Vaccines* **2021**, *9*, doi:10.3390/vaccines9060606.

**Paper 7 –** Moura, S.R.†; **Abreu, H.**†; Cunha, C.; Ribeiro-Machado, C.; Oliveira, C.; Barbosa, M.A.; Marques, H.; Almeida, M.I. Circulating Micrornas Correlate with Multiple Myeloma and Skeletal Osteolytic Lesions. *Cancers (Basel).* **2021**, *13*, doi:10.3390/cancers13215258.

**Paper 8 –** Raineri, D.; **Abreu, H.**; Vilardo, B.; Kustrimovic, N.; Venegoni, C.; Cappellano, G.; Chiocchetti, A. Deep Flow Cytometry Unveils Distinct Immune Cell Subsets in Inducible T Cell Co-Stimulator Ligand (ICOSL)- and ICOS-Knockout Mice during Experimental Autoimmune Encephalomyelitis. *Int. J. Mol. Sci.* **2024**, *25*, doi:10.3390/ijms25052509.

**Paper 9 –** Nascimben, M.; **Abreu, H.**; Manfredi, M.; Cappellano, G.; Chiocchetti, A.; Rimondini, L. Extracellular Vesicle Protein Expression in Doped Bioactive Glasses: Further Insights Applying Anomaly Detection. *Int. J. Mol. Sci.* **2024**, *25*, doi:10.3390/ijms25063560.

**Paper 10 –** Kovrlija, I.; Menshikh, K.; **Abreu, H.**; Cochis, A.; Rimondini, L.; Marsan, O.; Rey, C.; Combes, C.; Locs, J.; Loca, D. Challenging Applicability of ISO 10993-5 for Calcium Phosphate Biomaterials Evaluation: Towards More Accurate in Vitro Cytotoxicity Assessment. *Biomater. Adv.* **2024**, *160*, doi:10.1016/j.bioadv.2024.213866.

**Paper 11 –** Stolfi, F.†; **Abreu, H.**†; Sinella, R.; Nembrini, S.; Centonze, S.; Landra, V.; Brasso, C.; Cappelano, G.; Rocca, P.; Chiocchetti, A. Omics Approaches Open New Horizons in Major Depressive Disorder: From Biomarkers to Precision Medicine. *Front. Psychiatry* **2024**, *15*, doi: 10.3389/fpsyt.2024.1422939.

## BIBLIOGRAPHY


1. Ferraris, S.; Spriano, S.; Pan, G.; Venturello, A.; Bianchi, C.L.; Chiesa, R.; Faga, M.G.; Maina, G.; Vernè, E. Surface Modification of Ti-6Al-4V Alloy for Biomineralization and Specific Biological Response: Part I, Inorganic Modification. *J. Mater. Sci. Mater. Med.* **2011**, *22*, 533–545, doi:10.1007/s10856-011-4246-2.

2. Abreu, H.; Canciani, E.; Raineri, D.; Cappellano, G.; Rimondini, L.; Chiocchetti, A. Extracellular Vesicles in Musculoskeletal Regeneration: Modulating the Therapy of the Future. *Cells* **2022**, *11*, doi:10.3390/cells11010043.

3. Agrawal, R.; Kumar, A.; Mohammed, M.K.A.; Singh, S. Biomaterial Types, Properties, Medical Applications, and Other Factors: A Recent Review. *J. Zhejiang Univ. Sci. A* **2023**, doi:10.1631/jzus.A2200403.

4. Love, R.J.; Jones, K.S. Biomaterials, Fibrosis, and the Use of Drug Delivery Systems in Future Antifibrotic Strategies. *Crit. Rev. Biomed. Eng.* **2009**, *37*, doi:10.1615/CritRevBiomedEng.v37.i3.20.

5. Davidson, M.D.; Burdick, J.A.; Wells, R.G. Engineered Biomaterial Platforms to Study Fibrosis. *Adv. Healthc. Mater.* **2020**, *9*, doi:10.1002/adhm.201901682.

6. Wick, G.; Grundtman, C.; Mayerl, C.; Wimpissinger, T.F.; Feichtinger, J.; Zelger, B.; Sgonc, R.; Wolfram, D. The Immunology of Fibrosis. *Annu. Rev. Immunol.* **2013**, *31*, doi:10.1146/annurev-immunol-032712-095937.

7. Munitz, A.; Levi-Schaffer, F. Eosinophils: “New” Roles for “Old” Cells. *Allergy Eur. J. Allergy Clin. Immunol.* **2004**, *59*, doi:10.1111/j.1398-9995.2003.00442.x.

8. Wehling-henricks, M.; Sokolow, S.; Lee, J.J.; Myung, K.H.; Villalta, S.A.; Tidball, J.G. Major Basic Protein-1 Promotes Fibrosis of Dystrophic Muscle and Attenuates the Cellular Immune Response in Muscular Dystrophy. *Hum. Mol. Genet.* **2008**, *17*, doi:10.1093/hmg/ddn129.

9. Braga, T.T.; Agudelo, J.S.H.; Camara, N.O.S. Macrophages during the Fibrotic Process: M2 as Friend and Foe. *Front. Immunol.* **2015**, *6*, doi:10.3389/fimmu.2015.00602.

10. Cappellano, G.; Ploner, C.; Lobenwein, S.; Sopper, S.; Hoertnagl, P.; Mayerl, C.; Wick, N.; Pierer, G.; Wick, G.; Wolfram, D. Immunophenotypic Characterization of Human T Cells after in Vitro Exposure to Different Silicone Breast Implant Surfaces. *PLoS One* **2018**, *13*, doi:10.1371/journal.pone.0192108.

11. Zheng, K.; Niu, W.; Lei, B.; Boccaccini, A.R. Immunomodulatory Bioactive Glasses for Tissue Regeneration. *Acta Biomater.* **2021**, *133*, doi:10.1016/j.actbio.2021.08.023.

12. Owen, J.A.; Punt, J.; Stranford, S.A.; Jones, P.P.; Kuby, J. *Kuby Immunology*; 8th Editio.; W.H. Freeman: New York, 2019;

13. Freitas, J.; Santos, S.G.; Gonçalves, R.M.; Teixeira, J.H.; Barbosa, M.A.; Almeida, M.I. Genetically Engineered-MSC Therapies for Non-Unions, Delayed Unions and Critical-Size Bone Defects. *Int. J. Mol. Sci.* **2019**, *20*, 3430, doi:10.3390/ijms20143430.

14. Katti, K.S. Biomaterials in Total Joint Replacement. *Colloids Surfaces B Biointerfaces* **2004**, *39*, doi:10.1016/j.colsurfb.2003.12.002.

15. Davis, R.; Singh, A.; Jackson, M.J.; Coelho, R.T.; Prakash, D.; Charalambous, C.P.; Ahmed, W.; da Silva, L.R.R.; Lawrence, A.A. A Comprehensive Review on Metallic Implant Biomaterials and Their Subtractive Manufacturing. *Int. J. Adv. Manuf. Technol.* **2022**, *120*, doi:10.1007/s00170-022-08770-8.

16. Tanzi, M.C.; Farè, S.; Candiani, G. *Foundations of Biomaterials Engineering*; 2019;

17. Petersen, R. Titanium Implant Osseointegration Problems with Alternate Solutions Using Epoxy/Carbon-Fiber-Reinforced Composite. *Metals (Basel).* **2014**, *4*, doi:10.3390/met4040549.

18. Torrisi, L. Structural Investigations on Laser Deposited Hydroxyapatite Films. *Thin Solid Films* **1994**, *237*, doi:10.1016/0040-6090(94)90229-1.

19. Torrisi, L.; Scolaro, C. Treatment Techniques on Biocompatible Titanium to Modify the Surface Wetting Properties. *Biomed. Mater. Eng.* **2017**, *28*, doi:10.3233/BME-171687.

20. Ju, Y.; Wang, M.; Wang, Y.; Wang, S.; Fu, C. Electrical Properties of Amorphous Titanium Oxide Thin Films for Bolometric Application. *Adv. Condens. Matter Phys.* **2013**, *2013*, doi:10.1155/2013/365475.

21. Salinas, A.J.; Vallet-Regí, M. Bioactive Ceramics: From Bone Grafts to Tissue Engineering. *RSC Adv.* **2013**, *3*, doi:10.1039/c3ra00166k.

22. Pina, S.; Reis, R.L.; Oliveira, J.M. Ceramic biomaterials for tissue engineering. In *Fundamental Biomaterials: Ceramics*; 2018.

23. Roy, M.; Bandyopadhyay, A.; Bose, S. Ceramics in Bone Grafts and Coated Implants. In *Materials and Devices for Bone Disorders*; 2017.

24. Duan, R.; Barbieri, D.; De Groot, F.; De Bruijn, J.D.; Yuan, H. Modulating Bone Regeneration in Rabbit Condyle Defects with Three Surface-Structured Tricalcium Phosphate Ceramics. *ACS Biomater. Sci. Eng.* **2018**, *4*, doi:10.1021/acsbiomaterials.8b00630.

25. Fomin, A.S.; Barinov, S.M.; Ievlev, V.M.; Smirnov, V. V.; Mikhailov, B.P.; Belonogov, E.K.; Drozdova, N.A. Nanocrystalline Hydroxyapatite Ceramics Produced by Low-Temperature Sintering after High-Pressure Treatment. *Dokl. Chem.* **2008**, *418*, doi:10.1134/S0012500808010084.

26. Hench, L.L.; Splinter, R.J.; Allen, W.C.; Greenlee, T.K. Bonding Mechanisms at the

Interface of Ceramic Prosthetic Materials. *J. Biomed. Mater. Res.* **1971**, *5*, doi:10.1002/jbm.820050611.

27. Hench, L.L. The Story of Bioglass®. *J. Mater. Sci. Mater. Med.* **2006**, *17*, doi:10.1007/s10856-006-0432-z.

28. Ali, S.; Farooq, I.; Iqbal, K. A Review of the Effect of Various Ions on the Properties and the Clinical Applications of Novel Bioactive Glasses in Medicine and Dentistry. *Saudi Dent. J.* **2014**, *26*, doi:10.1016/j.sdentj.2013.12.001.

29. Miguez-Pacheco, V.; Hench, L.L.; Boccaccini, A.R. Bioactive Glasses beyond Bone and Teeth: Emerging Applications in Contact with Soft Tissues. *Acta Biomater.* **2015**, *13*, doi:10.1016/j.actbio.2014.11.004.

30. Cannio, M.; Bellucci, D.; Roether, J.A.; Boccaccini, D.N.; Cannillo, V. Bioactive Glass Applications: A Literature Review of Human Clinical Trials. *Materials (Basel).* **2021**, *14*, doi:10.3390/ma14185440.

31. Kaur, G.; Kumar, V.; Baino, F.; Mauro, J.C.; Pickrell, G.; Evans, I.; Bretcanu, O. Mechanical Properties of Bioactive Glasses, Ceramics, Glass-Ceramics and Composites: State-of-the-Art Review and Future Challenges. *Mater. Sci. Eng. C* **2019**, *104*, doi:10.1016/j.msec.2019.109895.

32. Kaur, G.; Pickrell, G.; Sriranganathan, N.; Kumar, V.; Homa, D. Review and the State of the Art: Sol–Gel and Melt Quenched Bioactive Glasses for Tissue Engineering. *J. Biomed. Mater. Res. - Part B Appl. Biomater.* **2016**, *104*, doi:10.1002/jbm.b.33443.

33. Ciraldo, F.E.; Boccardi, E.; Melli, V.; Westhauser, F.; Boccaccini, A.R. Tackling Bioactive Glass Excessive in Vitro Bioreactivity: Preconditioning Approaches for Cell Culture Tests. *Acta Biomater.* **2018**, *75*, doi:10.1016/j.actbio.2018.05.019.

34. Williams, D.F. On the Mechanisms of Biocompatibility. *Biomaterials* **2008**, *29*, doi:10.1016/j.biomaterials.2008.04.023.

35. Franz, S.; Rammelt, S.; Scharnweber, D.; Simon, J.C. Immune Responses to Implants - A Review of the Implications for the Design of Immunomodulatory Biomaterials. *Biomaterials* **2011**, *32*, doi:10.1016/j.biomaterials.2011.05.078.

36. Abreu, H.; Lallukka, M.; Miola, M.; Spriano, S.; Vern, E.; Raineri, D.; Leigheb, M.; Ronga, M.; Cappellano, G.; Chiocchetti, A. Human T-Cell Responses to Metallic Ion-Doped Bioactive Glasses. *Int. J. Mol. Sci.* **2024**, *8*, 4501.

37. Cochis, A.; Barberi, J.; Ferraris, S.; Miola, M.; Rimondini, L.; Vernè, E.; Yamaguchi, S.; Spriano, S. Competitive Surface Colonization of Antibacterial and Bioactive Materials Doped with Strontium and/or Silver Ions. *Nanomaterials* **2020**, *10*, doi:10.3390/nano10010120.

38. Miola, M.; Massera, J.; Cochis, A.; Kumar, A.; Rimondini, L.; Vernè, E. Tellurium: A New

Active Element for Innovative Multifunctional Bioactive Glasses. *Mater. Sci. Eng. C* **2021**, *123*, doi:10.1016/j.msec.2021.111957.

39. Lallukka, M.; Houaoui, A.; Miola, M.; Miettinen, S.; Massera, J.; Verné, E. In Vitro Cytocompatibility of Antibacterial Silver and Copper-Doped Bioactive Glasses. *Ceram. Int.* **2023**, *49*, doi:10.1016/j.ceramint.2023.08.284.

40. Ferraris, S.; Yamaguchi, S.; Barbani, N.; Cazzola, M.; Cristallini, C.; Miola, M.; Vernè, E.; Spriano, S. Bioactive Materials: In Vitro Investigation of Different Mechanisms of Hydroxyapatite Precipitation. *Acta Biomater.* **2020**, *102*, doi:10.1016/j.actbio.2019.11.024.

41. Lallukka, M.; Miola, M.; Najmi, Z.; Cochis, A.; Spriano, S.; Rimondini, L.; Verné, E. Cu-Doped Bioactive Glass with Enhanced in Vitro Bioactivity and Antibacterial Properties. *Ceram. Int.* **2024**, *50*, 5091–5103, doi:10.1016/j.ceramint.2023.11.253.

42. Miola, M.; Verné, E. Bioactive and Antibacterial Glass Powders Doped with Copper by Ion-Exchange in Aqueous Solutions. *Materials (Basel).* **2016**, *9*, doi:10.3390/ma9060405.

43. Tang, A.; Ren, Q.; Wu, Y.; Wu, C.; Cheng, Y. Investigation into the Antibacterial Mechanism of Biogenic Tellurium Nanoparticles and Precursor Tellurite. *Int. J. Mol. Sci.* **2022**, *23*, doi:10.3390/ijms231911697.

44. Kang, K.; Lim, D.H.; Choi, I.H.; Kang, T.; Lee, K.; Moon, E.Y.; Yang, Y.; Lee, M.S.; Lim, J.S. Vascular Tube Formation and Angiogenesis Induced by Polyvinylpyrrolidone-Coated Silver Nanoparticles. *Toxicol. Lett.* **2011**, *205*, doi:10.1016/j.toxlet.2011.05.1033.

45. Xie, H.; Kang, Y. Role of Copper in Angiogenesis and Its Medicinal Implications. *Curr. Med. Chem.* **2009**, *16*, doi:10.2174/092986709787846622.

46. Cieza, A.; Causey, K.; Kamenov, K.; Hanson, S.W.; Chatterji, S.; Vos, T. Global Estimates of the Need for Rehabilitation Based on the Global Burden of Disease Study 2019: A Systematic Analysis for the Global Burden of Disease Study 2019. *Lancet* **2020**, *396*, doi:10.1016/S0140-6736(20)32340-0.

47. Ratner, B.D. Replacing and Renewing: Synthetic Materials, Biomimetics, and Tissue Engineering in Implant Dentistry. *J. Dent. Educ.* **2001**, *65*, doi:10.1002/j.0022-0337.2001.65.12.tb03493.x.

48. Pitchai, M.S.; Ipe, D.S.; Hamlet, S. The Effect of Titanium Surface Topography on Adherent Macrophage Integrin and Cytokine Expression. *J. Funct. Biomater.* **2023**, *14*, doi:10.3390/jfb14040211.

49. Abaricia, J.O.; Farzad, N.; Heath, T.J.; Simmons, J.; Morandini, L.; Olivares-Navarrete, R. Control of Innate Immune Response by Biomaterial Surface Topography, Energy, and Stiffness. *Acta Biomater.* **2021**, *133*, doi:10.1016/j.actbio.2021.04.021.

50. Anderson, J.M. Biological Responses to Materials. *Annu. Rev. Mater. Sci.* **2001**, *31*,

doi:10.1146/annurev.matsci.31.1.81.

51. D'Esposito, V.; Sammartino, J.C.; Formisano, P.; Parascandolo, A.; Liguoro, D.; Adamo, D.; Sammartino, G.; Marenzi, G. Effect of Different Titanium Dental Implant Surfaces on Human Adipose Mesenchymal Stem Cell Behavior. An in Vitro Comparative Study. *Appl. Sci.* **2021**, *11*, doi:10.3390/app11146353.

52. Le Guehennec, L.; Lopez-Heredia, M.A.; Enkel, B.; Weiss, P.; Amouriq, Y.; Layrolle, P. Osteoblastic Cell Behaviour on Different Titanium Implant Surfaces. *Acta Biomater.* **2008**, *4*, doi:10.1016/j.actbio.2007.12.002.

53. Stricker, A.; Bergfeldt, T.; Fretwurst, T.; Addison, O.; Schmelzeisen, R.; Rothweiler, R.; Nelson, K.; Gross, C. Impurities in Commercial Titanium Dental Implants – A Mass and Optical Emission Spectrometry Elemental Analysis. *Dent. Mater.* **2022**, *38*, doi:10.1016/j.dental.2022.06.028.

54. Ferraris, S.; Yamaguchi, S.; Barbani, N.; Cristallini, C.; Gautier di Confiengo, G.; Barberi, J.; Cazzola, M.; Miola, M.; Vernè, E.; Spriano, S. The Mechanical and Chemical Stability of the Interfaces in Bioactive Materials: The Substrate-Bioactive Surface Layer and Hydroxyapatite-Bioactive Surface Layer Interfaces. *Mater. Sci. Eng. C* **2020**, *116*, doi:10.1016/j.msec.2020.111238.

55. Ferraris, S.; Cochis, A.; Cazzola, M.; Tortello, M.; Scalia, A.; Spriano, S.; Rimondini, L. Cytocompatible and Anti-Bacterial Adhesion Nanotextured Titanium Oxide Layer on Titanium Surfaces for Dental and Orthopedic Implants. *Front. Bioeng. Biotechnol.* **2019**, *7*, doi:10.3389/fbioe.2019.00103.

56. Gamna, F.; Yamaguchi, S.; Cochis, A.; Ferraris, S.; Kumar, A.; Rimondini, L.; Spriano, S. Conferring Antioxidant Activity to an Antibacterial and Bioactive Titanium Surface through the Grafting of a Natural Extract. *Nanomaterials* **2023**, *13*, doi:10.3390/nano13030479.

57. Sun, Y.; Li, Y.; Wu, B.; Wang, J.; Lu, X.; Qu, S.; Weng, J.; Feng, B. Biological Responses to M13 Bacteriophage Modified Titanium Surfaces in Vitro. *Acta Biomater.* **2017**, *58*, doi:10.1016/j.actbio.2017.06.019.

58. Alessandra Gobbo, V.; Lallukka, M.; Gamna, F.; Prato, M.; Vitale, A.; Ferraris, S.; Najmi, Z.; Cochis, A.; Rimondini, L.; Massera, J.; et al. Functionalization of a Chemically Treated Ti6Al4V-ELI Alloy with Nisin for Antibacterial Purposes. *Appl. Surf. Sci.* **2023**, *620*, doi:10.1016/j.apsusc.2023.156820.

59. Falzone, N.; Huyser, C.; Franken, D.R. Comparison between Propidium Iodide and 7-Amino-Actinomycin-D for Viability Assessment during Flow Cytometric Analyses of the Human Sperm Acrosome. *Andrologia* **2010**, *42*, doi:10.1111/j.1439-0272.2009.00949.x.

60. Breen, E.J.; Tan, W.; Khan, A. The Statistical Value of Raw Fluorescence Signal in Luminex XMAP Based Multiplex Immunoassays. *Sci. Rep.* **2016**, *6*, doi:10.1038/srep26996.

61. Zimmermann, M.; Meyer, N. Annexin V/7-AAD Staining in Keratinocytes. *Methods Mol. Biol.* **2011**, *740*, doi:10.1007/978-1-61779-108-6_8.

62. Verma, R.P. Titanium Based Biomaterial for Bone Implants: A Mini Review. *Mater. Today Proc.* **2020**, *26*, doi:10.1016/j.matpr.2020.02.649.

63. Stich, T.; Alagboso, F.; Křenek, T.; Kovářík, T.; Alt, V.; Docheva, D. Implant-Bone-Interface: Reviewing the Impact of Titanium Surface Modifications on Osteogenic Processes in Vitro and in Vivo. *Bioeng. Transl. Med.* **2022**, *7*, doi:10.1002/btm2.10239.

64. Al-Shalawi, F.D.; Mohamed Ariff, A.H.; Jung, D.W.; Mohd Ariffin, M.K.A.; Seng Kim, C.L.; Brabazon, D.; Al-Osaimi, M.O. Biomaterials as Implants in the Orthopedic Field for Regenerative Medicine: Metal versus Synthetic Polymers. *Polymers (Basel).* **2023**, *15*, doi:10.3390/polym15122601.

65. Nicholson, J.W. Titanium Alloys for Dental Implants: A Review. *Prosthesis* **2020**, *2*, doi:10.3390/prosthesis2020011.

66. Kowalski, J.; Rylska, D.; Januszewicz, B.; Konieczny, B.; Cichomski, M.; Matinlinna, J.P.; Radwanski, M.; Sokolowski, J.; Lukomska-Szymanska, M. Corrosion Resistance of Titanium Dental Implant Abutments: Comparative Analysis and Surface Characterization. *Materials (Basel).* **2023**, *16*, doi:10.3390/ma16206624.

67. Barbour, M.E.; O'Sullivan, D.J.; Jenkinson, H.F.; Jagger, D.C. The Effects of Polishing Methods on Surface Morphology, Roughness and Bacterial Colonisation of Titanium Abutments. *J. Mater. Sci. Mater. Med.* **2007**, *18*, doi:10.1007/s10856-007-0141-2.

68. Okubo, T.; Ikeda, T.; Saruta, J.; Tsukimura, N.; Hirota, M.; Ogawa, T. Compromised Epithelial Cell Attachment after Polishing Titanium Surface and Its Restoration by UV Treatment. *Materials (Basel).* **2020**, *13*, doi:10.3390/ma13183946.

69. Jung, S.; Bohner, L.; Hanisch, M.; Kleinheinz, J.; Sielker, S. Influence of Implant Material and Surface on Mode and Strength of Cell/Matrix Attachment of Human Adipose Derived Stromal Cell. *Int. J. Mol. Sci.* **2020**, *21*, doi:10.3390/ijms21114110.

70. Silva, T.S.N.; Machado, D.C.; Viezzer, C.; Silva, A.N.; De Oliveira, M.G. Effect of Titanium Surface Roughness on Human Bone Marrow Cell Proliferation and Differentiation. An Experimental Study. *Acta Cir. Bras.* **2009**, *24*, doi:10.1590/S0102-86502009000300007.

71. Zhang, P.; Zou, Z.; Pang, M.; Wang, Z. Preparation and Performance Testing of Green Non-Toxic Polishing Solution for Medical Titanium Alloy. *ECS J. Solid State Sci. Technol.* **2024**, *13*, doi:10.1149/2162-8777/ad15a5.

72. Song, X.; Wang, X.; Wang, S.; Liu, S.; Ge, S. Experimental Study on Ultra-Precision Polishing of Ti-6al-4v by Ultraviolet-Induced Nanoparticle Colloid Jet Machining. *Materials (Basel).* **2021**, *14*, doi:10.3390/ma14175014.

73. Wang, M.L.; Tuli, R.; Manner, P.A.; Sharkey, P.F.; Hall, D.J.; Tuan, R.S. Direct and Indirect

Induction of Apoptosis in Human Mesenchymal Stem Cells in Response to Titanium Particles. *J. Orthop. Res.* **2003**, *21*, doi:10.1016/S0736-0266(02)00241-3.

74. Berger, A. Th1 and Th2 Responses: What Are They? *BMJ* **2000**, *321*, doi:10.1136/bmj.321.7258.424.

75. Tesmer, L.A.; Lundy, S.K.; Sarkar, S.; Fox, D.A. Th17 Cells in Human Disease. *Immunol. Rev.* **2008**, *223*, doi:10.1111/j.1600-065X.2008.00628.x.

76. Souza, J.G.S.; Bertolini, M.M.; Costa, R.C.; Nagay, B.E.; Dongari-Bagtzoglou, A.; Barão, V.A.R. Targeting Implant-Associated Infections: Titanium Surface Loaded with Antimicrobial. *iScience* **2021**, *24*, doi:10.1016/j.isci.2020.102008.

77. Rimailho, L.; Faria, C.; Domagala, M.; Laurent, C.; Bezombes, C.; Poupot, M. Γδ T Cells in Immunotherapies for B-Cell Malignancies. *Front. Immunol.* **2023**, *14*, doi:10.3389/fimmu.2023.1200003.

78. Kokordelis, P.; Krämer, B.; Boesecke, C.; Voigt, E.; Ingiliz, P.; Glässner, A.; Wolter, F.; Srassburg, C.P.; Spengler, U.; Rockstroh, J.K.; et al. CD3(+)CD56(+) Natural Killer-like T Cells Display Anti-HCV Activity but Are Functionally Impaired in HIV(+) Patients with Acute Hepatitis C. *J. Acquir. Immune Defic. Syndr.* **2015**, *70*, doi:10.1097/QAI.0000000000000793.

79. Hoyer, K.K.; Dooms, H.; Barron, L.; Abbas, A.K. Interleukin-2 in the Development and Control of Inflammatory Disease. *Immunol. Rev.* **2008**, *226*, doi:10.1111/j.1600-065X.2008.00697.x.

80. Laurence, A.; Tato, C.M.; Davidson, T.S.; Kanno, Y.; Chen, Z.; Yao, Z.; Blank, R.B.B.; Meylan, F.; Siegel, R.; Hennighausen, L.; et al. Interleukin-2 Signaling via STAT5 Constrains T Helper 17 Cell Generation. *Immunity* **2007**, *26*, doi:10.1016/j.immuni.2007.02.009.

81. Martin, K.R.; Wong, H.L.; Witko-Sarsat, V.; Wicks, I.P. G-CSF – A Double Edge Sword in Neutrophil Mediated Immunity. *Semin. Immunol.* **2021**, *54*, doi:10.1016/j.smim.2021.101516.

82. Lopez-Castejon, G.; Brough, D. Understanding the Mechanism of IL-1β Secretion. *Cytokine Growth Factor Rev.* **2011**, *22*, doi:10.1016/j.cytogfr.2011.10.001.

83. Carta, S.; Tassi, S.; Semino, C.; Fossati, G.; Mascagni, P.; Dinarello, C.A.; Rubartelli, A. Histone Deacetylase Inhibitors Prevent Exocytosis of Interleukin-1β- Containing Secretory Lysosomes: Role of Microtubules. *Blood* **2006**, *108*, doi:10.1182/blood-2006-03-014126.

84. Gschwandtner, M.; Derler, R.; Midwood, K.S. More Than Just Attractive: How CCL2 Influences Myeloid Cell Behavior Beyond Chemotaxis. *Front. Immunol.* **2019**, *10*, doi:10.3389/fimmu.2019.02759.

85. Takatsu, K. Interleukin-5 and IL-5 Receptor in Health and Diseases. *Proc. Japan Acad. Ser. B Phys. Biol. Sci.* **2011**, *87*, doi:10.2183/pjab.87.463.

86. Yanez, M.; Blanchette, J.; Jabbarzadeh, E. Modulation of Inflammatory Response to Implanted Biomaterials Using Natural Compounds. *Curr. Pharm. Des.* **2017**, *23*, doi:10.2174/1381612823666170510124348.

87. Dominici, M.; Le Blanc, K.; Mueller, I.; Slaper-Cortenbach, I.; Marini, F.C.; Krause, D.S.; Deans, R.J.; Keating, A.; Prockop, D.J.; Horwitz, E.M. Minimal Criteria for Defining Multipotent Mesenchymal Stromal Cells. The International Society for Cellular Therapy Position Statement. *Cytotherapy* **2006**, doi:10.1080/14653240600855905.

88. Ferreira, J.R.; Teixeira, G.Q.; Santos, S.G.; Barbosa, M.A.; Almeida-Porada, G.; Gonçalves, R.M. Mesenchymal Stromal Cell Secretome: Influencing Therapeutic Potential by Cellular Pre-Conditioning. *Front. Immunol.* **2018**, doi:10.3389/fimmu.2018.02837.

89. Li, H.; Huang, J.; Wang, Y.; Chen, Z.; Li, X.; Wei, Q.; Liu, X.; Wang, Z.; Wen, B.; Zhao, Y.; et al. Nanoscale Modification of Titanium Implants Improves Behaviors of Bone Mesenchymal Stem Cells and Osteogenesis in Vivo. *Oxid. Med. Cell. Longev.* **2022**, *2022*, doi:10.1155/2022/2235335.

90. Park, J.W.; Tsutsumi, Y.; Park, E.K. Osteogenic Differentiation of Human Mesenchymal Stem Cells Modulated by Surface Manganese Chemistry in SLA Titanium Implants. *Biomed Res. Int.* **2022**, *2022*, doi:10.1155/2022/5339090.

91. Fattore, A. Del; Luciano, R.; Pascucci, L.; Goffredo, B.M.; Giorda, E.; Scapaticci, M.; Fierabracci, A.; Muraca, M. Immunoregulatory Effects of Mesenchymal Stem Cell-Derived Extracellular Vesicles on T Lymphocytes. *Cell Transplant.* **2015**, *24*, doi:10.3727/096368915X687543.

92. Manfredi, M.; Martinotti, S.; Gosetti, F.; Ranzato, E.; Marengo, E. The Secretome Signature of Malignant Mesothelioma Cell Lines. *J. Proteomics* **2016**, *145*, doi:10.1016/j.jprot.2016.02.021.

93. Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; et al. Fiji: An Open-Source Platform for Biological-Image Analysis. *Nat. Methods* **2012**, *9*, doi:10.1038/nmeth.2019.

94. Carpentier, G.; Berndt, S.; Ferratge, S.; Rasband, W.; Cuendet, M.; Uzan, G.; Albanese, P. Angiogenesis Analyzer for ImageJ — A Comparative Morphometric Analysis of "Endothelial Tube Formation Assay" and "Fibrin Bead Assay." *Sci. Rep.* **2020**, *10*, doi:10.1038/s41598-020-67289-8.

95. Schmidt, A.; Forne, I.; Imhof, A. Bioinformatic Analysis of Proteomics Data. *BMC Syst. Biol.* **2014**, *8*, doi:10.1186/1752-0509-8-S2-S3.

96. Nascimben, M.; Abreu, H.; Manfredi, M.; Cappellano, G.; Chiocchetti, A.; Rimondini, L. Extracellular Vesicle Protein Expression in Doped Bioactive Glasses: Further Insights Applying Anomaly Detection. *Int. J. Mol. Sci.* **2024**, *25*, doi:10.3390/ijms25063560.

97. Harrell, C.R.; Djonov, V.; Volarevic, V. The Cross-Talk between Mesenchymal Stem Cells and Immune Cells in Tissue Repair and Regeneration. *Int. J. Mol. Sci.* **2021**, *22*, doi:10.3390/ijms22052472.

98. Sierra-Parraga, J.M.; Merino, A.; Eijken, M.; Leuvenink, H.; Ploeg, R.; Møller, B.K.; Jespersen, B.; Baan, C.C.; Hoogduijn, M.J. Reparative Effect of Mesenchymal Stromal Cells on Endothelial Cells after Hypoxic and Inflammatory Injury. *Stem Cell Res. Ther.* **2020**, *11*, doi:10.1186/s13287-020-01869-3.

99. Ong, K.L.; Yun, B.M.; White, J.B. New Biomaterials for Orthopedic Implants. *Orthop. Res. Rev.* **2015**, *7*, doi:10.2147/ORR.S63437.

100. Xiu, P.; Jia, Z.; Lv, J.; Yin, C.; Cheng, Y.; Zhang, K.; Song, C.; Leng, H.; Zheng, Y.; Cai, H.; et al. Tailored Surface Treatment of 3D Printed Porous Ti6Al4V by Microarc Oxidation for Enhanced Osseointegration via Optimized Bone In-Growth Patterns and Interlocked Bone/Implant Interface. *ACS Appl. Mater. Interfaces* **2016**, *8*, doi:10.1021/acsami.6b05893.

101. Keller, J.C.; Schneider, G.B.; Stanford, C.M.; Kellogg, B. Effects of Implant Microtopography on Osteoblast Cell Attachment. *Implant Dent.* **2003**, *12*, doi:10.1097/01.ID.0000058309.77613.87.

102. El-Ghannam, A.; Ducheyne, P.; Shapiro, I.M. Effect of Serum Proteins on Osteoblast Adhesion to Surface-Modified Bioactive Glass and Hydroxyapatite. *J. Orthop. Res.* **1999**, *17*, doi:10.1002/jor.1100170307.

103. Liu, H.; Li, R.; Liu, T.; Yang, L.; Yin, G.; Xie, Q. Immunomodulatory Effects of Mesenchymal Stem Cells and Mesenchymal Stem Cell-Derived Extracellular Vesicles in Rheumatoid Arthritis. *Front. Immunol.* **2020**, *11*, 1–7, doi:10.3389/fimmu.2020.01912.

104. Lai, R.C.; Arslan, F.; Lee, M.M.; Sze, N.S.K.; Choo, A.; Chen, T.S.; Salto-Tellez, M.; Timmers, L.; Lee, C.N.; El Oakley, R.M.; et al. Exosome Secreted by MSC Reduces Myocardial Ischemia/Reperfusion Injury. *Stem Cell Res.* **2010**, doi:10.1016/j.scr.2009.12.003.

105. Lee, C.; Mitsialis, S.A.; Aslam, M.; Vitali, S.H.; Vergadi, E.; Konstantinou, G.; Sdrimas, K.; Fernandez-Gonzalez, A.; Kourembanas, S. Exosomes Mediate the Cytoprotective Action of Mesenchymal Stromal Cells on Hypoxia-Induced Pulmonary Hypertension. *Circulation* **2012**, doi:10.1161/CIRCULATIONAHA.112.114173.

106. Shabbir, A.; Cox, A.; Rodriguez-Menocal, L.; Salgado, M.; Van Badiavas, E. Mesenchymal Stem Cell Exosomes Induce Proliferation and Migration of Normal and Chronic Wound Fibroblasts, and Enhance Angiogenesis in Vitro. *Stem Cells Dev.* **2015**, doi:10.1089/scd.2014.0316.

107. Zhang, J.; Guan, J.; Niu, X.; Hu, G.; Guo, S.; Li, Q.; Xie, Z.; Zhang, C.; Wang, Y. Exosomes Released from Human Induced Pluripotent Stem Cells-Derived MSCs Facilitate Cutaneous Wound Healing by Promoting Collagen Synthesis and Angiogenesis. *J. Transl. Med.* **2015**,

doi:10.1186/s12967-015-0417-0.

108. Bian, S.; Zhang, L.; Duan, L.; Wang, X.; Min, Y.; Yu, H. Extracellular Vesicles Derived from Human Bone Marrow Mesenchymal Stem Cells Promote Angiogenesis in a Rat Myocardial Infarction Model. *J. Mol. Med.* **2014**, doi:10.1007/s00109-013-1110-5.

109. Fang, S. Bin; Zhang, H.Y.; Meng, X.C.; Wang, C.; He, B.X.; Peng, Y.Q.; Xu, Z. Bin; Fan, X.L.; Wu, Z.J.; Wu, Z.C.; et al. Small Extracellular Vesicles Derived from Human MSCs Prevent Allergic Airway Inflammation via Immunomodulation on Pulmonary Macrophages. *Cell Death Dis.* **2020**, doi:10.1038/s41419-020-2606-x.

110. Bang, O.Y.; Kim, E.H. Mesenchymal Stem Cell-Derived Extracellular Vesicle Therapy for Stroke: Challenges and Progress. *Front. Neurol.* **2019**, doi:10.3389/fneur.2019.00211.

111. Qian, X.; An, N.; Ren, Y.; Yang, C.; Zhang, X.; Li, L. Immunosuppressive Effects of Mesenchymal Stem Cells-Derived Exosomes. *Stem Cell Rev. Reports* **2021**, *17*, doi:10.1007/s12015-020-10040-7.

112. Showalter, M.R.; Wancewicz, B.; Fiehn, O.; Archard, J.A.; Clayton, S.; Wagner, J.; Deng, P.; Halmai, J.; Fink, K.D.; Bauer, G.; et al. Primed Mesenchymal Stem Cells Package Exosomes with Metabolites Associated with Immunomodulation. *Biochem. Biophys. Res. Commun.* **2019**, *512*, doi:10.1016/j.bbrc.2019.03.119.

113. Miceli, V.; Pampalone, M.; Vella, S.; Carreca, A.P.; Amico, G.; Conaldi, P.G. Comparison of Immunosuppressive and Angiogenic Properties of Human Amnion-Derived Mesenchymal Stem Cells between 2D and 3D Culture Systems. *Stem Cells Int.* **2019**, *2019*, doi:10.1155/2019/7486279.

114. Bartosh, T.J.; Ylöstalo, J.H.; Mohammadipoor, A.; Bazhanov, N.; Coble, K.; Claypool, K.; Lee, R.H.; Choi, H.; Prockop, D.J. Aggregation of Human Mesenchymal Stromal Cells (MSCs) into 3D Spheroids Enhances Their Antiinflammatory Properties. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, doi:10.1073/pnas.1008117107.

115. Shahir, M.; Mahmoud Hashemi, S.; Asadirad, A.; Varahram, M.; Kazempour-Dizaji, M.; Folkerts, G.; Garssen, J.; Adcock, I.; Mortaz, E. Effect of Mesenchymal Stem Cell-Derived Exosomes on the Induction of Mouse Tolerogenic Dendritic Cells. *J. Cell. Physiol.* **2020**, *235*, doi:10.1002/jcp.29601.

116. Sung, D.K.; Sung, S.I.; Ahn, S.Y.; Chang, Y.S.; Park, W.S. Thrombin Preconditioning Boosts Biogenesis of Extracellular Vesicles from Mesenchymal Stem Cells and Enriches Their Cargo Contents via Protease-Activated Receptor-Mediated Signaling Pathways. *Int. J. Mol. Sci.* **2019**, *20*, doi:10.3390/ijms20122899.

117. Yang, C.; Sun, J.; Tian, Y.; Li, H.; Zhang, L.; Yang, J.; Wang, J.; Zhang, J.; Yan, S.; Xu, D. Immunomodulatory Effect of MSCs and MSCs-Derived Extracellular Vesicles in Systemic Lupus Erythematosus. *Front. Immunol.* **2021**, *12*, 1–13, doi:10.3389/fimmu.2021.714832.

118. Dabrowska, S.; Andrzejewska, A.; Janowski, M.; Lukomska, B. Immunomodulatory and Regenerative Effects of Mesenchymal Stem Cells and Extracellular Vesicles: Therapeutic Outlook for Inflammatory and Degenerative Diseases. *Front. Immunol.* **2021**, *11*, doi:10.3389/fimmu.2020.591065.

119. Harrell, C.R.; Jovicic, N.; Djonov, V.; Arsenijevic, N.; Volarevic, V. Mesenchymal Stem Cell-Derived Exosomes and Other Extracellular Vesicles as New Remedies in the Therapy of Inflammatory Diseases. *Cells* **2019**, *8*, doi:10.3390/cells8121605.

120. Yu, Y.; Valderrama, A.V.; Han, Z.; Uzan, G.; Naserian, S.; Oberlin, E. Human Fetal Liver MSCs Are More Effective than Adult Bone Marrow MSCs for Their Immunosuppressive, Immunomodulatory, and Foxp3+ T Reg Induction Capacity. *Stem Cell Res. Ther.* **2021**, *12*, doi:10.1186/s13287-021-02176-1.

121. Franco da Cunha, F.; Andrade-Oliveira, V.; Candido de Almeida, D.; Borges da Silva, T.; Naffah de Souza Breda, C.; Costa Cruz, M.; Faquim-Mauro, E.L.; Antonio Cenedeze, M.; Ioshie Hiyane, M.; Pacheco-Silva, A.; et al. Extracellular Vesicles Isolated from Mesenchymal Stromal Cells Modulate CD4+ T Lymphocytes Toward a Regulatory Profile. *Cells* **2020**, *9*, doi:10.3390/cells9041059.

122. Bolandi, Z.; Mokhberian, N.; Eftekhary, M.; Sharifi, K.; Soudi, S.; Ghanbarian, H.; Hashemi, S.M. Adipose Derived Mesenchymal Stem Cell Exosomes Loaded with MiR-10a Promote the Differentiation of Th17 and Treg from Naive CD4+ T Cell. *Life Sci.* **2020**, *259*, doi:10.1016/j.lfs.2020.118218.

123. Zhang, Z.; Huang, S.; Wu, S.; Qi, J.; Li, W.; Liu, S.; Cong, Y.; Chen, H.; Lu, L.; Shi, S.; et al. Clearance of Apoptotic Cells by Mesenchymal Stem Cells Contributes to Immunosuppression via PGE2. *EBioMedicine* **2019**, *45*, doi:10.1016/j.ebiom.2019.06.016.

124. Wang, D.; Feng, X.; Lu, L.; Konkel, J.E.; Zhang, H.; Chen, Z.; Li, X.; Gao, X.; Lu, L.; Shi, S.; et al. A CD8 T Cell/Indoleamine 2,3-Dioxygenase Axis Is Required for Mesenchymal Stem Cell Suppression of Human Systemic Lupus Erythematosus. *Arthritis Rheumatol.* **2014**, *66*, doi:10.1002/art.38674.

125. Lee, S.; Kim, S.; Chung, H.; Moon, J.H.; Kang, S.J.; Park, C.G. Mesenchymal Stem Cell-Derived Exosomes Suppress Proliferation of T Cells by Inducing Cell Cycle Arrest through P27kip1/Cdk2 Signaling. *Immunol. Lett.* **2020**, *225*, doi:10.1016/j.imlet.2020.06.006.

126. Qiu, G.; Zheng, G.; Ge, M.; Wang, J.; Huang, R.; Shu, Q.; Xu, J. Mesenchymal Stem Cell-Derived Extracellular Vesicles Affect Disease Outcomes via Transfer of MicroRNAs. *Stem Cell Res. Ther.* **2018**, *9*, doi:10.1186/s13287-018-1069-9.

127. Chen, W.; Huang, Y.; Han, J.; Yu, L.; Li, Y.; Lu, Z.; Li, H.; Liu, Z.; Shi, C.; Duan, F.; et al. Immunomodulatory Effects of Mesenchymal Stromal Cells-Derived Exosome. *Immunol. Res.* **2016**, *64*, 831–840, doi:10.1007/s12026-016-8798-6.

128. Du, Y.M.; Zhuansun, Y.X.; Chen, R.; Lin, L.; Lin, Y.; Li, J. guo Mesenchymal Stem Cell

Exosomes Promote Immunosuppression of Regulatory T Cells in Asthma. *Exp. Cell Res.* **2018**, *363*, doi:10.1016/j.yexcr.2017.12.021.

129. Pachler, K.; Ketterl, N.; Desgeorges, A.; Dunai, Z.A.; Laner-Plamberger, S.; Streif, D.; Strunk, D.; Rohde, E.; Gimona, M. An in Vitro Potency Assay for Monitoring the Immunomodulatory Potential of Stromal Cell-Derived Extracellular Vesicles. *Int. J. Mol. Sci.* **2017**, *18*, doi:10.3390/ijms18071413.

130. Watt, S.M.; Gullo, F.; Van Der Garde, M.; Markeson, D.; Camicia, R.; Khoo, C.P.; Zwaginga, J.J. The Angiogenic Properties of Mesenchymal Stem/Stromal Cells and Their Therapeutic Potential. *Br. Med. Bull.* **2013**, doi:10.1093/bmb/ldt031.

131. Tao, H.; Han, Z.; Han, Z.C.; Li, Z. Proangiogenic Features of Mesenchymal Stem Cells and Their Therapeutic Applications. *Stem Cells Int.* **2016**, *2016*, doi:10.1155/2016/1314709.

132. Liang, X.; Zhang, L.; Wang, S.; Han, Q.; Zhao, R.C. Exosomes Secreted by Mesenchymal Stem Cells Promote Endothelial Cell Angiogenesis by Transferring MiR-125a. *J. Cell Sci.* **2016**, *129*, doi:10.1242/jcs.170373.

133. Zhang, B.; Wu, X.; Zhang, X.; Sun, Y.; Yan, Y.; Shi, H.; Zhu, Y.; Wu, L.; Pan, Z.; Zhu, W.; et al. Human Umbilical Cord Mesenchymal Stem Cell Exosomes Enhance Angiogenesis Through the Wnt4/β-Catenin Pathway. *Stem Cells Transl. Med.* **2015**, *4*, doi:10.5966/sctm.2014-0267.

134. Lopatina, T.; Bruno, S.; Tetta, C.; Kalinina, N.; Porta, M.; Camussi, G. Platelet-Derived Growth Factor Regulates the Secretion of Extracellular Vesicles by Adipose Mesenchymal Stem Cells and Enhances Their Angiogenic Potential. *Cell Commun. Signal.* **2014**, *12*, doi:10.1186/1478-811X-12-26.

135. Johnsen, K.B.; Gudbergsson, J.M.; Andresen, T.L.; Simonsen, J.B. What Is the Blood Concentration of Extracellular Vesicles? Implications for the Use of Extracellular Vesicles as Blood-Borne Biomarkers of Cancer. *Biochim. Biophys. Acta - Rev. Cancer* **2019**, *1871*, doi:10.1016/j.bbcan.2018.11.006.

136. Auber, M.; Svenningsen, P. An Estimate of Extracellular Vesicle Secretion Rates of Human Blood Cells. *J. Extracell. Biol.* **2022**, *1*, doi:10.1002/jex2.46.

137. Syromiatnikova, V.; Prokopeva, A.; Gomzikova, M. Methods of the Large-Scale Production of Extracellular Vesicles. *Int. J. Mol. Sci.* **2022**, *23*, doi:10.3390/ijms231810522.