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PH.D. THESIS

IDENTIFICATION OF PREDICTIVE FACTORS IN PLATELET-RICH PLASMA AND MESENCHYMAL STEM CELLS FOR REGENERATIVE MEDICINE APPLICATION

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“Amor che tutto move”
Alla mia famiglia, che mi ha insegnato il valore del sacrificio e della dedizione,
e al mio fidanzato, che con il suo supporto incondizionato mi ha dato la forza di superare ogni ostacolo.

Sommario

ABSTRACT

Regenerative medicine represents a promising medical science field, focusing on repairing, replacing or regenerating damaged tissues and organs. Among the most promising strategies are Autologous Platelet Concentrates (APCs), widely used for the infiltrative treatment of Knee Osteoarthritis (OA) to improve tissue healing and relieve pain, and Bone Marrow Aspirate Concentrates (BMACs), for the treatment of Critical Limb Ischemia (CLI).

APCs have demonstrated potential in enhancing tissue healing by promoting cell proliferation, angiogenesis, and collagen synthesis, making them valuable in musculoskeletal injuries, chronic wounds, and cartilage repair. BMACs, a source of mesenchymal stem cells and hematopoietic progenitors, offer significant regenerative capabilities, particularly in orthopedic and cartilage repair, through cell differentiation and tissue remodeling.

My doctoral thesis focuses on two studies. In both cases, a panel of cytokines, chemokines, and growth factors (GFs) was determined to identify biomarkers predictive of clinical outcome in grade I OA and CLI patients.

In Study 1, the Numeric Pain Rating Scale (NPRS) was evaluated as a clinical readout before and after APC infiltrations. Lower white blood cell count (WBC) and higher Monocyte-chemoattractant Protein-1 levels in peripheral blood (PB) were associated with APC-induced pain relief. Platelet-derived Growth Factor (PDGF) levels in APC were significantly higher in OA patients displaying larger NPRS reduction, independently of platelet count. Finally, simultaneous determination of PDGF, Vascular Endothelial Growth Factor, and Macrophage Inflammatory Protein-1α in APC discriminated OA patients with very poor and no response. Thus, platelet-released GFs rather than platelet count may predict clinical outcomes in grade 1 knee OA.

In Study 2, bone marrow (BM) samples were collected before and after cell concentration with the Sepax-2 instrument. In parallel, peripheral blood (PB) samples were collected at baseline (T0). Treadmill Test and Transcutaneous partial pressure of oxygen were evaluated as clinical outcomes. POST-SEPAX samples showed a statistically significant increase in cell count and viability compared to PRE-SEPAX samples. Inflammatory cytokines such as IL-1ra, IL-8, IL-10 and growth factors such as bFGF, gCSF, and PDGF were found at higher concentrations in BM compared to PB. In bone marrow

POST-SEPAX, among the released factors, IL-2 and TNF-α concentrations, negatively correlated with TpcO2.

This study confirmed the useful application of stem cell therapy in improving pain and revascularization in patients affected by CLI and promoted soluble factors, such as IL-2 TNF-α, as prognostic factors in the management of pathology.

1. BACKGROUND

Regenerative medicine is an emerging interdisciplinary field of research and clinical applications focused on the repair, replacement, or regeneration of cells, tissues, or organs to restore impaired function resulting from defects, disease, or trauma. This field integrates multiple biotechnological approaches, including growth factors, stem cell therapies, tissue engineering, and cellular therapies, advancing beyond traditional methods of transplantation and tissue replacement[1].

Historically, therapeutic strategies were limited to autologous and allogenic transplants: in the first case, they are constrained by the availability of donor tissue from the patients, while allogeneic transplants are associated with significant drawbacks, including high costs, the risk of immune rejection, and the need for lifelong immunosuppressive treatment. Consequently, recent research has increasingly focused on novel and less invasive strategies to achieve tissue regeneration, with the goal of overcoming these limitations[2,3].

The gold standard in regenerative medicine focuses on restoring impaired anatomical structures and inducing self-renewal processes within damaged tissues. This differs from traditional medical practices, which typically focus on replacing damaged structures rather than promoting their regeneration. The targets of regenerative medicine and the conditions to which it is applicable are broad: degenerative articular cartilage, ischemic, chronic degenerative and metabolic diseases, which lead to organ failure. Furthermore, regenerative medicine is increasingly focused on prevention and early intervention, emphasizing not only the treatment of existing diseases but also the prophylactic restoration of tissue function before permanent damage occurs. This approach offers the potential to mitigate the onset of chronic degenerative diseases and prevent the need for more invasive interventions later in life[2].

1.1 Soluble Factors in Tissue Regeneration

The success of the wound healing process depends on growth factors (GFs), cytokines, and chemokines, which are involved in a complex network of signaling pathways, regulating cellular processes essential for tissue repair[4]. New strategies in regenerative medicine focus on understanding and exploiting the therapeutic potential of autologous growth factors. These peptide molecules are involved in stimulating proliferation, chemotaxis, migration and wound healing.

1.1.1 Growth Factors

Growth Factors (GFs) are soluble, diffusible, polypeptidic macromolecules which are produced by a great variety of cells. GFs are multifunctional and participate in tissue repair and healing. They regulate key processes, such as mitogenesis, chemotaxis, cellular differentiation and metabolism[3]. Their biological activity is mediated through paracrine, autocrine, or endocrine signaling mechanisms, wherein they bind to specific receptors on target cells, initiating a cascade of intracellular signaling events. These signaling cascades regulate a wide array of cellular functions, including gene expression, cell cycle progression, and extracellular matrix remodeling[5]. A deeper understanding of the molecular interactions governing growth factors activity has paved the way for the development of targeted regenerative therapies aimed at enhancing wound healing and tissue regeneration, with the potential to improve clinical outcomes and accelerate recovery [4,6].

1.1.2 Cytokines

Cytokines are soluble proteins with low molecular weight, that play a critical role in cell signaling within the immune system and other physiological processes. They are produced by various cell types, including immune cells (macrophages, lymphocytes, and dendritic cells), endothelial cells, and fibroblasts, in response to infections, inflammation, or injury. Cytokines mediate and regulate immune responses, inflammation, hematopoiesis, and tissue repair by binding to specific receptors on target cells [7]. They can be classified into several families based on their structure and function, including interleukins (ILs), interferons (IFNs), tumor necrosis factors (TNFs), and chemokines [8].

1.2 Autologous Platelet Concentrates (APCs)

Platelets represent a natural reservoir of soluble mediators, including cytokines and growth factors located within the α-granules. Platelets are involved in maintaining vascular integrity by sensing and responding to endothelial damage and have additional roles in wound healing and repair as well as activation of inflammatory and immune response [9]. Platelet activation may occur at the site of injury with the release of bioactive molecules, which synergistically act to promote tissue repair processes and to modulate immune and inflammatory responses [10].

Autologous Platelet Concentrates (APCs) can be defined as preparations derived from specific centrifugation of the patient's whole blood. The result is a biomaterial containing high concentrations of platelets, as well as leukocytes and GFs, such as PDGF, TGF-β1 and - β2, VEGF, FGF, and insulin-like growth factor-1 (IGF-1)[3,11]. The efficacy of APCs in promoting wound healing and tissue regeneration has been the subject of considerable scientific interest in recent years. Platelet concentrates may be beneficial for tissues with limited blood supply, slow cell turnover, and limited extracellular matrix repair, due to their ability to facilitate the recruitment, proliferation, and maturation of cells involved in the regeneration of tendons, ligaments, bones, and cartilage[12].

APCs have several applications in the medical field, including stimulation of tissue regeneration in orthopedics, dentistry, plastic surgery, ulcer healing, repair of musculoskeletal injuries and treatment of osteoarthritis. The main advantages of using platelet derivatives are their autologous nature and the simplicity of collection and preparation. They can be classified into several different generations based on their characteristics and the preparation methods required [13].

1.2.1 Platelet Rich Plasma (PRP)

The first generation of platelet concentrates includes Platelet Rich Plasma (PRP). PRP is derived from the patient's whole blood and is obtained by a two-stage centrifugation process: separation and concentration[3] (Figure 1).

For the separation phase, the whole blood is collected in a sterile tube containing a citrate-phosphate-dextrose anticoagulant solution and centrifuged at 5600 rpm. At the end, a three-layer separation is obtained [14]:

- The first layer contains platelet-poor plasma (PPP) and represents 40% of the total volume;
- The middle layer is the buffy coat (BC) and contains platelets and leukocytes, making up only 5% of the total volume;
- The bottom layer is made of red blood cells (RBCs) and represents 55% of the total volume.

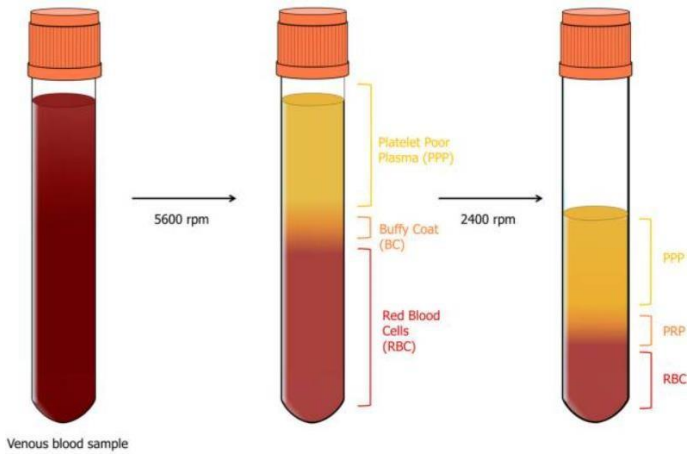

Figure 1. PRP preparation scheme. Venus blood is collected in a sterile tube containing a citrate–phosphate–dextrose anticoagulant solution and is centrifuged first at 5600 rpm. Three layers are formed: PPP, BC, and RBC. PPP, BC, and a small proportion of RBC are transferred to another tube without anticoagulant. This is then subjected to centrifugation at 2400 rpm, which leads to the formation of three distinct layers: residual RBC at the bottom, PPP at the top, and an intermediate layer known as PRP. To obtain "activated" PRP, the platelets are first resuspended in an appropriate volume of plasma, and then a mixture of bovine thrombin and calcium chloride is added to activate the platelets[3].

PPP and BC are aspirated with a sterile syringe and transferred into another tube without anticoagulant, which is then centrifuged at 2400 rpm. This second step concentrates the platelets at the bottom of the tube and produces three distinct layers: residual RBC, PPP at the top, representing 80% of the total volume, and an intermediate layer of buffy coat, known as PRP[3,14]. PRP is then prepared by

resuspending the platelets in an adequate volume of plasma (2-4 mL), then a mixture containing bovine thrombin and calcium chloride is added to activate the platelets, resulting in 'activated' PRP. The PRP appears as a gel due to the incorporation of the platelets into a fibrin network and only then is it ready for use. The concentration of platelets and growth factors in PRP is 3–5 times higher than in peripheral blood[15].

Based on the platelet and leukocyte content, PRP can be further divided into two categories: leukocyte-poor (or pure) platelet-rich plasma (P-PRP) and leukocyte- and platelet-rich plasma (L-PRP). In order, P-PRP has a low-density fibrin network, a low or absent leukocyte content, and platelets may be damaged during preparation. L-PRP is characterized by a high concentration of leukocytes and platelets, while the fibrin network remains relatively lose[3,16].

PRP has been shown to contain numerous growth factors involved in tissue repair, but it has a significant drawback: its preparation requires the use of anticoagulants and bovine thrombin to induce fibrin polymerization, which can interfere with physiological healing process[17].

1.2.2 Platelet Rich Fibrin (PRF)

The second generation of platelet concentrates is platelet-rich fibrin (PRF), which was introduced by Choukroun in 2000 as an alternative to PRP[14]. Venous blood samples of 10 mL are taken without anticoagulants and centrifuged at 3000 rpm for 10 min (Figure 2). At the end of this process, three layers are obtained: the lower layer consisting of RBC, the intermediate layer containing the fibrin clot, known as PRF, and the upper layer of plasma, PPP[18].

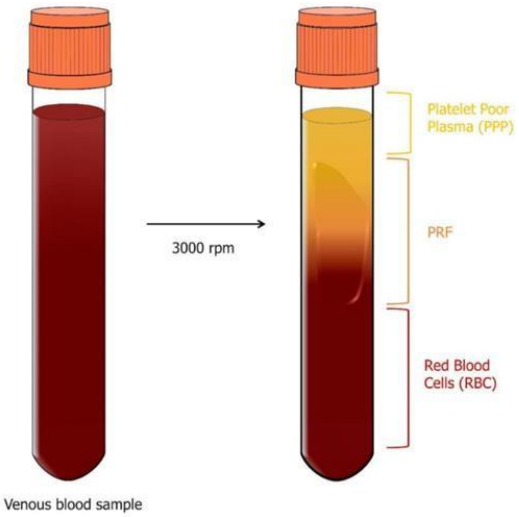

Figure 2. PRF preparation scheme. Venous blood samples of 10 mL are collected without the use of anticoagulants and then centrifuged at 3000 rpm for 10 min. At the conclusion of this process, three distinct layers are obtained: the bottom layer composed of RBC, the middle layer containing the fibrin clot, referred to as PRF, and the top layer consisting of plasma, PPP.

In the absence of anticoagulants, the platelets are activated, and clotting factors are released. Initially, fibrinogen is concentrated in the upper part of the tube before exogenous thrombin converts it to fibrin, which is then localized in the center of the tube between the red cell layer and the acellular plasma layer[14,19]. The cellular component of the PRF includes platelets, leukocytes, macrophages, granulocytes, neutrophils, and red blood cells. Platelets contribute to the initial phase of healing, while other cell populations participate in the anti-inflammatory phase.

1.2.3 Concentrated Growth Factors (CGFs)

The third and latest generation of platelet derivatives are Concentrated Growth Factors (CGFs)[20]. These are obtained by centrifuging the blood sample (8 mL) at alternating speeds for 13 min (2 min at 2700 rpm, 4 min at 2400 rpm, 4 min at 2700 rpm, and 3 min at 3000 rpm) using a specific centrifuge, the Medifuge MF200 (Figure 3). At the end, three different layers can be observed in the sample[21]: on upper layer of plasma, PPP, a middle layer containing fibrin and growth factors, CGFs, and a lower layer of RBC. The middle layer, CGF, can be further subdivided into the upper white part, the lower red part, and the intermediate BC.

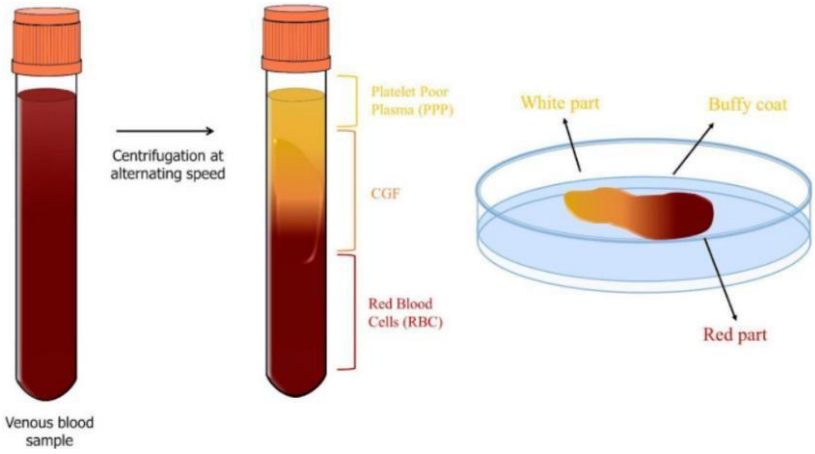

Figure 3. Scheme of CGF preparation. CGFs are obtained by centrifuging the blood sample at alternating speeds for a total of 13 minutes: 2 minutes at 2700 rpm, 4 minutes at 2400 rpm, 4 minutes at 2700 rpm and 3 minutes at 3000 rpm. After this procedure, the sample separates into three distinct layers: the top layer consists of plasma, PPP, the middle layer contains CGFs, and the bottom layer consists of RBCs. The CGFs can be further divided into three parts: the upper white part, the lower red part and the middle BC.

The process of alternating-speed centrifugations results in the formation of a denser fibrin matrix containing more growth factors and cells compared to PRF and PRP[18,22]. The fibrin scaffold acts as a dense network containing platelets, leukocytes, and CD34+ cells[23]. Growth factors that are more abundant in CGFs include PDGF, TGF-β1, FGF-b, VEGF, IGF-1, EGF, and BMP. These are trapped in the fibrin matrix and released gradually, ensuring that regenerative properties are controlled and sustained over time, as well as reducing the risk of an inflammatory response associated with excessive concentrations of growth factors[3].

1.3 Mesenchymal Stem Cells for Tissue Regeneration

Recently, regenerative medicine has focused on research into minimally invasive techniques to regenerate damaged tissues. A breakthrough in this direction has been the use of stem cell therapy, which aims to regenerate damaged tissues and organs with new cells derived from stem cell transplantation[3,24]. Numerous studies have investigated Mesenchymal Stem Cells (MSCs) for tissue regeneration, including bone, cartilage meniscus, tendons, ligaments, both in vitro and in vivo[24]. Although stem cell therapy represents a novel approach to tissue regeneration, its clinical application is hampered by challenges, such as the limited survival and differentiation capacity of transplanted cells. In addition, MSC therapies involve collection, isolation, and in

vitro expansion of these cells to ultimately enable their transplantation. This clinical use is also hardened by the potential contamination of the cell culture, the overall duration of the whole process, and the high costs involved[25] (Figure 4).

MSCs are an ideal cell source for tissue regeneration. They are located in almost all tissues, including bone marrow, adipose tissue, and synovium, and are easily isolated. MSCs can differentiate into almost any end-stage cellular lineage to enable their seeding in specific scaffolds. Their immunological properties, including anti-inflammatory, immunoregulatory, and immunosuppressive capacities, contribute to their potential role as immune tolerant agents[24].

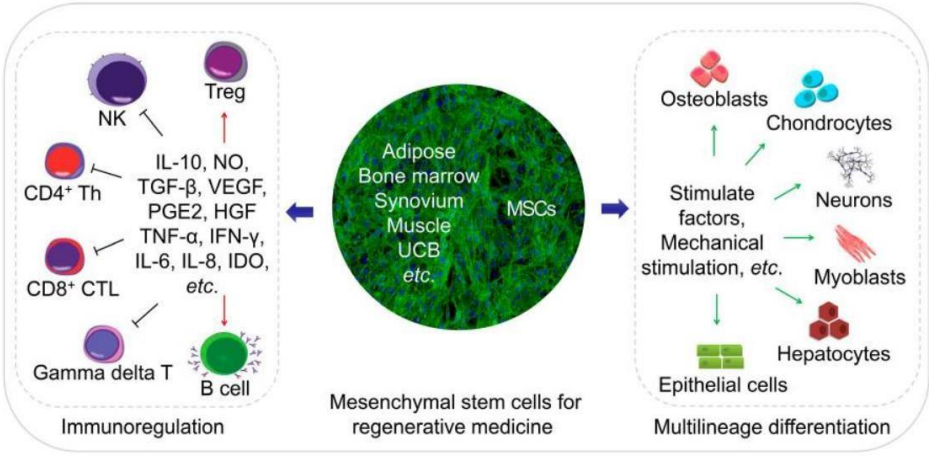

Figure 4. Schematic diagram of regenerative medicine based on Mesenchymal Stem Cells (MSCs). The MSCs can be easily extracted from varies tissues, and the multilineage differentiation and immunoregulatory properties of MSCs make them an ideal cell therapeutic candidate[25].

MSCs show a strong propensity to improve tissue damage in response to injury and disease. Recent studies have suggested the capacity of MSCs to secrete soluble factors that alter the tissue microenvironment. They can enhance regeneration ability of injured tissues, stimulate proliferation and differentiation of endogenous stem-like progenitors, decrease inflammatory and immune reactions[26,27].

1.4 Endothelial Progenitor Cells for Tissue Regeneration

The role of Endothelial Progenitor Cells (EPCs) in regenerative medicine is increasingly recognized as crucial in tissue repair and vascular regeneration. EPCs plays a pivotal role in both neovascularization and tissue homeostasis by contributing to the repair of damaged endothelium and promoting angiogenesis. EPCs are primarily mobilized

from the bone marrow and peripheral blood in response to tissue injury or ischemia, where they home to sites of vascular damage, incorporating into existing blood vessels or facilitating the formation of new capillaries[28]. Their function is mediated through a combination of mechanisms including paracrine signaling, direct cellular differentiation, and integration with endothelial cells already present at the site of injury[29] (Figure 5).

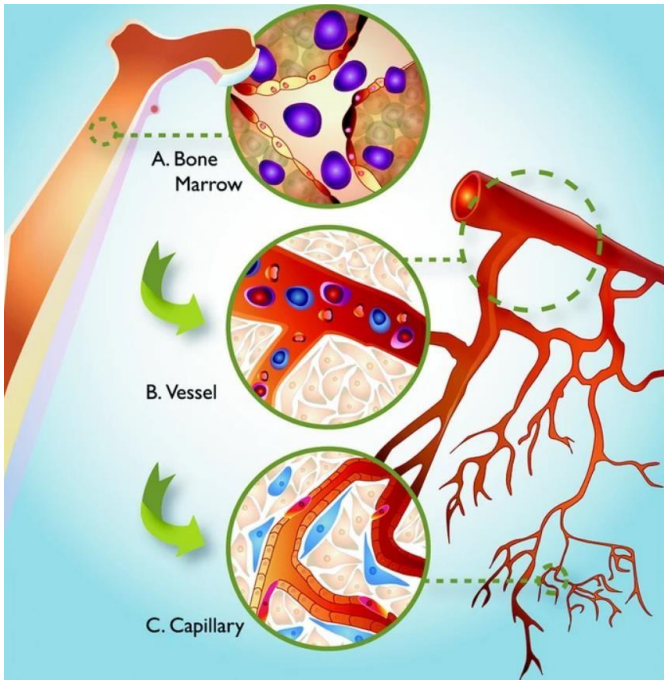

Figure 5. EPCs are bone marrow-derived cells that contribute to postnatal angiogenesis. EPCs (seen in purple at A) can be mobilized from the bone marrow stroma by various growth factors and exit through sinusoidal vessels. Once in the bloodstream (B), EPCs begin to differentiate (indicated by the color change). Subsequently, these cells home to sites of angiogenesis in capillary beds (C) and attach to the endothelium[29].

In regenerative medicine, EPCs are being explored for their potential therapeutic applications in a variety of conditions such as cardiovascular diseases, diabetic ulcers, and wound healing, where endothelial dysfunction and inadequate blood supply are major limiting factors. Moreover, the use of EPCs in tissue-engineering approaches holds promise for developing bioengineered vascular grafts, improving the success of organ transplants, and enhancing the efficacy of other regenerative therapies by improving tissue perfusion and oxygenation. Despite their therapeutic potential, several challenges remain, including the need for better understanding of EPC biology, their long-term functional stability post-transplantation, and strategies to enhance

their efficacy in vivo. Nonetheless, ongoing advancements in stem cell biology and bioengineering may help overcome these limitations, positioning EPCs as a key component in the future of regenerative medicine[30].

1.5 Polymeric Scaffold

Scaffolds are artificial structures capable of supporting the formation of three-dimensional tissue. Their use in the orthopaedic field appears to be a powerful weapon for treating numerous diseases[31]. Polymeric materials and films significantly impact regenerative medicine applications used as antimicrobial films and scaffolds (cell expansion or a transplant). Polymers are organic materials composed of long chains of atoms joined by covalent bonds that can be naturally derived or synthetic. The ideal polymer for tissue engineering should be mechanically stable, biocompatible and bioactive, and biodegradable. Mechanical properties are crucial in the development of new biopolymers. A scaffold should provide the stiffness of the tissue origin, ensuring its native mechanical properties; considering that different tissues require specific mechanical characteristics, due to multicellular composition, it is ideal to create tunable polymeric matrices that allow mechanical changes inside of the construct through time[32,33] (Figure 6).

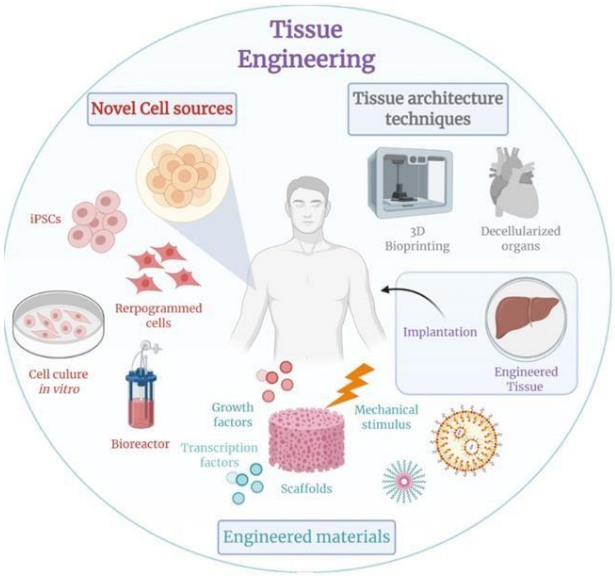

Figure 6. The emerging field of tissue engineering aims to recover injured tissues by consolidating cells from thehost body or donor's body with exceptionally porous scaffold biomaterials that can act as a template for tissue recovery, tocontrol the development of new tissue. Signals, cells, and scaffolds are triad components of tissue engineering that combineto produce functional tissue and organs (https://commons.wikimedia.org/wiki/File:Tissue_Engineering.png)

Biocompatibility means cells or tissue can survive interacting with polymer, but not necessarily for them to duplicate or differentiate. Some biocompatible polymers support cell viability, but cells are not able to self-duplicate, differentiate, or migrate. This results in cell death within short period of time. In this context, polymeric material must be both biocompatible and bioactive. When a polymer is bioactive, in general, it is biocompatible and allows cell attachment, migration, differentiation, proliferation, and the cell can perform its biological functions[34].

Last, biomaterial should be biodegradable in a short (one month for soft tissue, or salivary glands) or a long time (six months to one year for hard tissue like bone). In soft tissue, this will allow the host to reabsorb the material once an artificially regenerated tissue or organoid has been implanted, driving the interaction of artificial and host environments. For hard tissue such as bone, the calcification process takes time, thus the polymeric scaffold should provide mechanical support for a longer period, ensuring the host could accept the new implant and allowing host cells to interact with the new, engineered graft[35].

2. STUDY 1

Platelet-derived growth factor as biomarker of clinical outcome for autologous platelet concentrate therapy in Osteoarthritis

2.1 Cartilage tissue

Articular cartilage is a specialized connective tissue of mesenchymal origin, devoid of blood and lymphatic vessels, that lines the bone inside the joints. Cartilage consists of cells called chondrocytes, which are embedded in an extracellular matrix (ECM), consisting of collagen fibres, H2O, hyaluronic acid, and proteoglycans. This tissue plays a crucial role in providing structural support and facilitating smooth joint movement. There are three main types of cartilage—hyaline cartilage, fibrocartilage, and elastic cartilage—each with distinct structural and functional properties[36]. Hyaline cartilage, the most common type, forms the articular surfaces of joints, the respiratory tract, and the fetal skeleton. Fibrocartilage, characterized by dense collagen fibers, provides tensile strength and is found in intervertebral discs and menisci. Elastic cartilage contains elastin fibers and contributes to the flexibility of structures like the ear[37–39] (Figure 7).

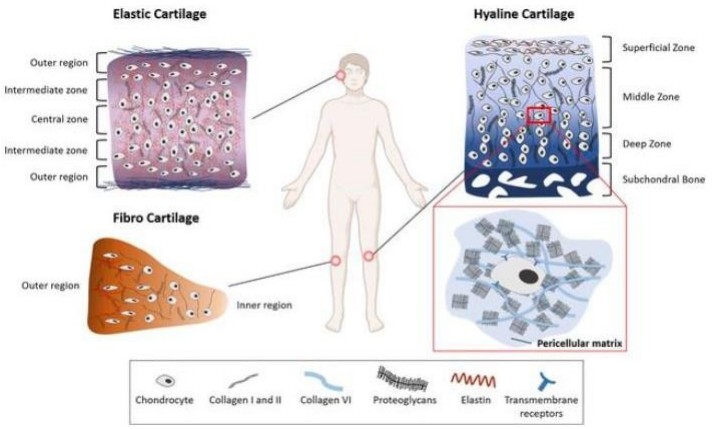

Figure 7. Extracellular matrix heterogeneity (ECM) of different cartilage types. Elastic cartilage and hyaline articular cartilage are arranged in different zones with the chondrocyte shape becoming smaller and flatter towards the outer region and superficial zone, respectively. Proteoglycan (PG) concentration articular cartilage in the knee joint increases with depth of the tissue. In meniscal cartilage (fibro cartilage) the PG concentration is highest in the outer region. The pericellular matrix surrounds the chondrocytes and contains a high amount of PGs such as aggrecan and perlecan, and collagen type VI in articular and meniscal cartilage[38].

In adult joints, the tissue exhibits a complex organization consisting of superficial, medial and deep zones, an intricate and abundant extracellular matrix, composed of numerous molecules and macromolecules, and a subchondral junction that is important for physical stability and bonding to the underlying bone. The main ECM components are type II collagen and type X collagen organized in fibrils providing tensile strength, and aggrecan organized in multimeric superstructures providing elasticity. These macromolecules establish the basic and fundamental biomechanical property of articular cartilage: resilience[40] (Figure 8).

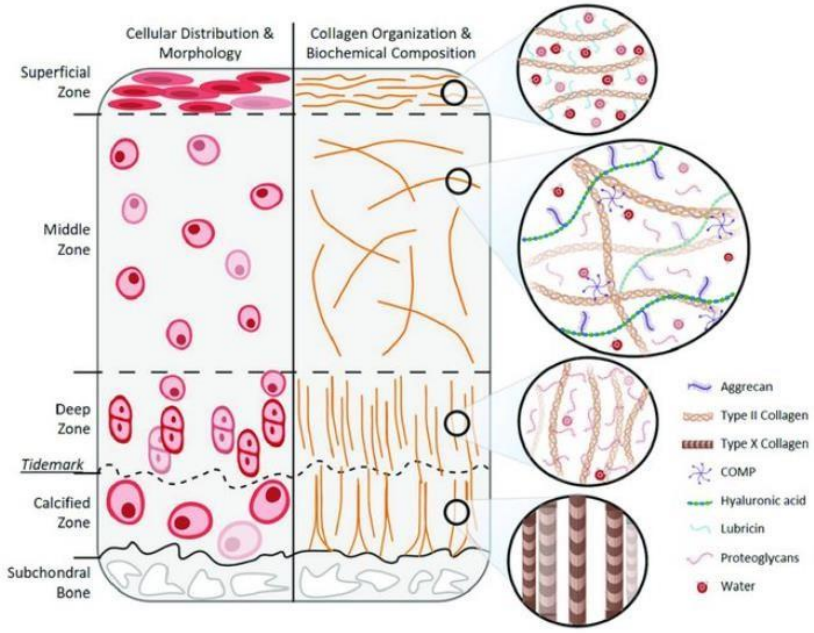

Figure 8. Hyaline cartilage structure and biochemical composition. Schematic representation of hyaline cartilage zonal structure and variable cellular distribution, morphology, collagen organization, and biochemical composition[37].

Cartilage has limited regenerative capacity, which presents challenges in treating injuries and degenerative conditions, such as Osteoarthritis (OA)[41,42]. Understanding the complex biology of cartilage, maintenance, and response to injury, is crucial for advancing therapeutic approaches in regenerative medicine and tissue engineering[37].

2.2 Osteoarthritis - Epidemiology and Treatment

Articular cartilage is tissue matrix-rich, hypocellular, and avasculare. This structure enables it to function as a lubricating and load-bearing surface in the joints. Injury to cartilage often progresses from the articular surface to the subchondral bone, leading

to the development of degenerative joint diseases such as Osteoarthritis (OA). OA is a degenerative joint disease characterized by the progressive deterioration of articular cartilage, subchondral bone remodeling, and synovial inflammation. It is the most common form of arthritis, primarily affecting weight-bearing joints such as the knee, hip, and spine, although it can involve any joint. The pathophysiology of OA is multifactorial, involving mechanical, biochemical, and genetic factors. Cartilage breakdown occurs due to an imbalance between anabolic and catabolic processes within the joint, where increased protease activity, particularly by matrix metalloproteinases (MMPs) and aggrecanases, leads to the degradation of extracellular matrix components. OA is the most common cause of mobility loss, severely affects quality of life, work productivity and cost of health care, and is the most prevalent form of musculoskeletal disease worldwide. Clinical symptoms include activity limitation and pain[43,44] (Figure 9).

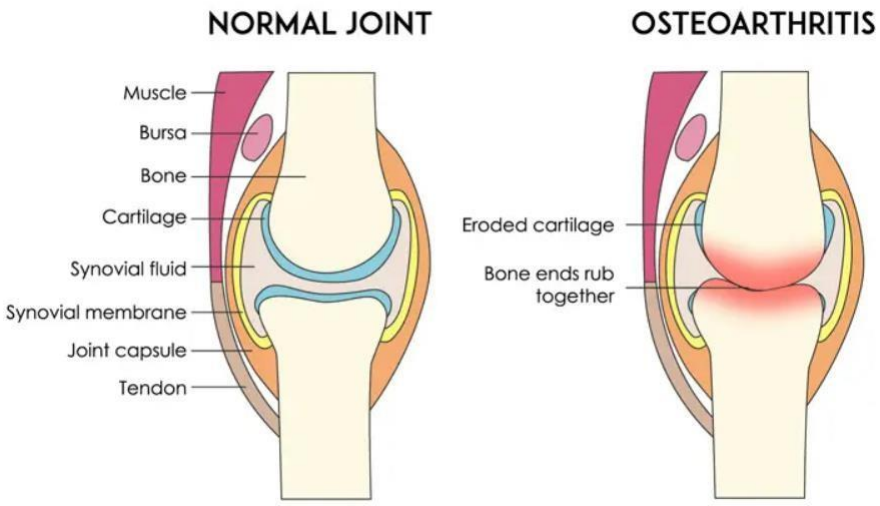

Figure 9. Structural comparison of normal joint with osteoarthritic knee. Healthy knee joint with soft bone surface, normal meniscus and articular cartilage. In osteoarthritic knee, osteophytes, bone cysts and sclerosis occurred in subchondral bones. Fibrillated cartilage, meniscal tear, and inflammation of synovial membrane are other abnormalities could be seen in osteoarthritic knee joint.

Concurrently, inflammatory cytokines play a significant role in amplifying joint inflammation and promoting cartilage degradation. In the early stages of OA, the cartilage undergoes softening, fibrillation, and loss of proteoglycan content. Over time, the cartilage thins and becomes less able to withstand mechanical stress, leading to subchondral bone sclerosis, the formation of osteophytes, and synovial

membrane thickening. These structural changes contribute to pain, stiffness, and impaired joint function. Diagnosis is typically based on clinical symptoms, radiographic imaging, and in some cases, laboratory tests. Radiographic findings may include joint space narrowing, osteophyte formation, and subchondral sclerosis[45]. In patients with OA, several clinical trials have been carried out using biological products, including platelet concentrates and cell therapies, by means of infiltrative treatment[46,47](Figure 10).

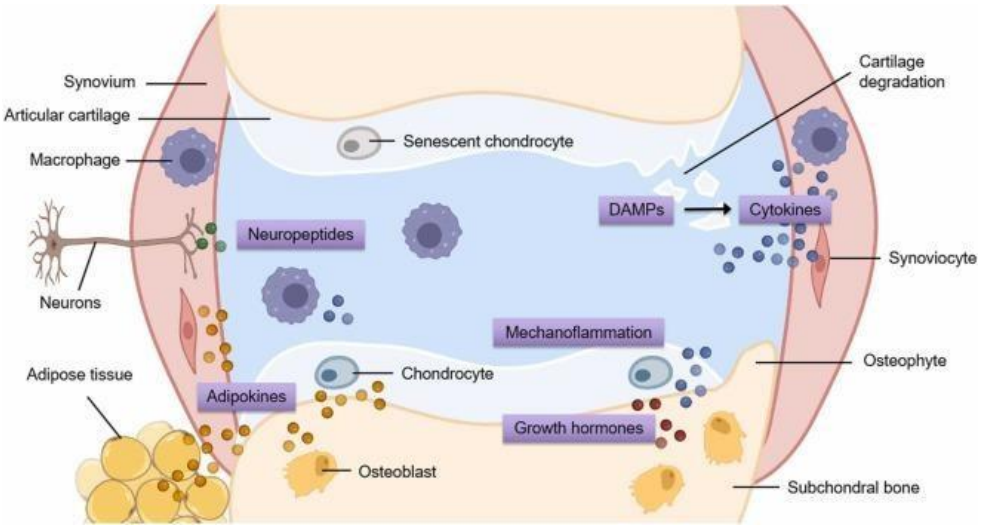

Figure 10. Overview of the inflammatory mediators in OA. The interplay between the different pro-inflammatory mediators and mechanisms in the different tissues of the OA joint[46].

These are valuable therapeutic agents, with main advantages of reduced immune reactions, ease of collection and preparation, and relatively low costs. Pharmacologic treatment may include nonsteroidal anti-inflammatory drugs, and intra-articular corticosteroid, hyaluronic acid injection. In more advanced cases, surgical interventions such as joint replacement may be considered.

2.2.1 Knee Osteoarthritis

Knee osteoarthritis (KOA), one of the most common types of OA, is mainly characterized by cartilage and subchondral bone impairment. In addition, meniscus and ligament injuries and synovitis could be associated. Furthermore, inflammation is evidenced as a trigger factor in KOA pathogenesis and its association is directly related to the severity of pain[48].

The Kellgren-Lawrence classification determines the progression of KOA into five grades, which have been determined as follows: grade 0, absolute absence of radiological features; grade 1, beginning of osteophyte formation and doubtful joint space narrowing; grade 2, definite osteophytosis and possibility of observing joint space narrowing; grade 3, multiple osteophytes, sclerosis, joint space narrowing and likelihood of bone deformation; grade 4, giant osteophyte, intense sclerosis and joint space narrowing, bone deformation, particularly in the femoral head[49](Figure 11).

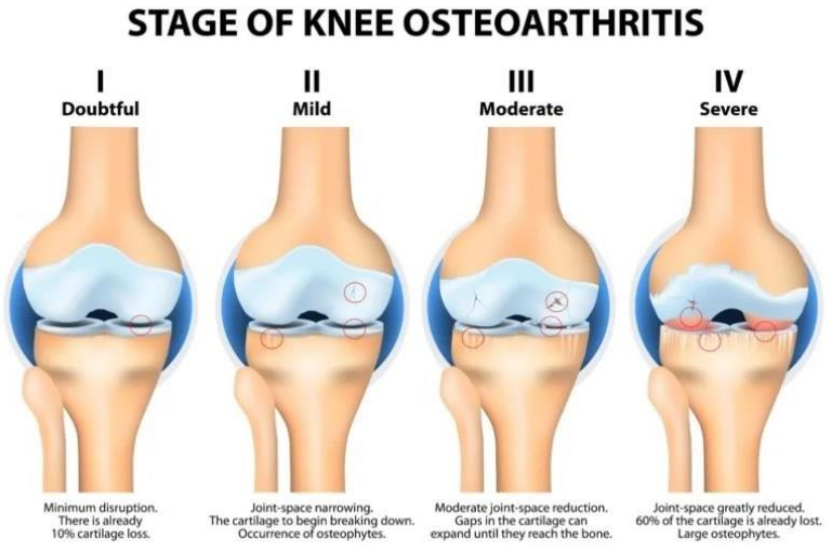

Figure 11. Kellgren-Lawrence (KL) classification system. The KL classification system classifies degrees of severity according to two crucial aspects of OA: the extent of joint space narrowing and the degree of osteophyte formation.

The rising burden of this chronic degenerative disease and increased demands for efficient treatment for KOA have highlighted regenerative medicine as a promising approach[48]. Regenerative medicine represents novel therapeutic modalities for KOA such as autologous platelet concentrates (APCs), monoclonal antibodies, and cell-based therapies with different cells[50].

3. AIM OF THE STUDY

Degenerative joint cartilage diseases have a strong social impact, significantly affecting quality of life. In recent decades, several non-invasive and quite successful solutions have been proposed for the treatment of pain by improving joint function. Traditional clinical treatments, which include palliative and reparative methods, have shown some improvement in pain reduction and are also widely used in the treatment of Osteoarthritis (OA).

OA is a chronic disease affecting the joint and its tissues, primarily leading to progressive damage to articular cartilage and, subsequently, to the subchondral bone and surrounding synovial structures. OA is a disabling condition with increasing incidence and prevalence in the worldwide[51]. Clinical trials on the use of biological products, such as Autologous Platelet Concentrates (APCs) and cell therapies, through infiltrative treatment are effective in patients with OA. Platelet concentrates are autologous products, obtained by centrifugation from patients peripheral blood samples. The rationale for the use of these therapies is the presence of cytokines, chemokines, growth factors and other biologically active substances with immunomodulatory, angiogenic and pain-relieving functions[52,53].

The aim of this study is to evaluate whether the determination of soluble factors in peripheral blood samples and autologous platelet concentrates could identify predictive biomarkers for clinical outcomes in patients with OA.

4. MATERIAL AND METHODS

4.1 Population Enrolment

The study population included 51 patients with knee grade I OA, recruited at ASL Napoli 2 Nord. The inclusion criteria for APC infiltration therapy were as follows: age > 18 years; presence of pain, spasm, or functional disability with failure of conservative treatment for at least 3 months but less than 5 years. The exclusion criteria were inflammatory or autoimmune diseases, pregnancy, and bleeding disorders. APC infiltration into the articular cavity was performed at enrolment (T0) and after 30 days (T1). For each patient, biometrical, biochemical (glycaemia, creatininemia, alanine-amino-transferase, total and HDL cholesterolemia, triglyceridemia, total protidemia, ferritinemia, C-Reactive protein), and clinical data were collected at outpatient hospital admission before APC infiltration (T0), and follow-up biochemical and clinical data were collected at T1, before the second APC infiltration, and at 120-days (T2). Numeric Pain Rating Scale (NPRS) scores were also recorded at T0, T1, and T2. The NPRS is a one-dimensional, 11-point scale that assesses pain intensity in adults. It consists of a horizontal line, ranging from 0 to 10, corresponding to "no pain" and "worst pain imaginable[54].

The investigations were carried out in accordance with the guidelines of the Declaration of Helsinki of 1975, revised in 2013. Informed consent was obtained from all patients prior to the procedure. The study protocol was approved by the ethics committee “Università Federico II – AORN A. Cardarelli” (prot. N. 172/2023).

4.2 Specimen Collection

Peripheral Blood (PB) and APC samples were collected from each patient at T0 and T1. APC was obtained by centrifugation of venous blood samples using an automated and standardized cell separator (IMPACT – Plasmaconcept). After obtaining the samples, 3 ml was infiltrated intra-articularly at the site of the injured cartilage and an aliquot (500 ml) was collected for further analysis. Samples were then centrifuged at 3.000 g for 10 minutes and stored at -80°C until assay was performed.

4.3 IMPACT – Plasmaconcept

The IMPACT platform is a CE-certified medical device that enables the fully automated production of blood components for non-transfusion use, separating fractions from blood to be used for a specific therapy. IMPACT's innovative technology enables the production of different concentrations of PRP and cytokines.

The various IMPACT programmes have been scientifically tested with specific parameters for each therapeutic target (https://ngmed.it/prodottingmed/impact-plasmaconcept/).

For the kit preparation, the starting point is always the patient's venous whole blood. The separation of the desired blood fractions is controlled by the optical sensor, which sits in the centre of the rotor – one sensor for each rotor unit. The two sensors work independently, so you can process two blood samples from two different patients at the same time.

Depending on the therapeutic objective, different cellular components are separated, at different concentrations. The IMPACT system makes it possible to produce different PRP formulations: Leukocyte-rich or leukocyte-poor variants can be prepared, which can also be further concentrated. In this way, an individualised and patient-specific therapy can be realised according to the indications. Ready-to-use IMPACT sets are available for the preparation of various PRP formulations.

For the APC we have:

- rAPC "Red" APC: Leukocyte-rich, erythrocyte-poor
- wAPC "White" APC: Leukocyte-poor, erythrocyte-free

IMPACT APC is a platelet concentrate optimized for maximum platelet yield. The final volume used for evaluations is 1ml of APC concentrate. The operator has the possibility to change the final volume himself between 1ml to 3 ml.

4.4 Determination of Cytokines, Chemokines, and Growth Factors

PB and APC samples were screened for the concentrations of interleukin (IL)-1ra, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, Eotaxin,

basic Fibroblast Growth Factor (bFGF), Granulocyte-Colony Stimulating Factor (G-CSF), Granulocyte and Macrophage-Colony Stimulating Factor (GM-CSF), Interferon-γ (IFN-γ), Interferon-γ inducible Protein 10 (IP-10), Monocyte Chemoattractant Protein-1 (MCP-1), Macrophage Inflammatory Protein-1 (MIP-1) α, MIP-1β, Platelet-Derived Growth Factor (PDGF) bb, C-C motif Chemokine ligand 5 (CCL5)/RANTES, Tumor Necrosis Factor (TNF) α, and Vascular Endothelial Growth Factor (VEGF) using the Bio-Plex Pro Human Cytokine GrpI Panel 27-Plex kit for a Multiplex Elisa (cat. No. M500KCAF0Y)[55].

The method is based on the principle of ELISA, an immunochemical method for detecting and quantifying concentrations of analytes in the biological sample of interest. Besides being a highly specific method, it is also very sensitive, reproducible and automated. It requires enzymes, which can mark the antibody or antigen and allow its detection when the specific antigen-antibody binding takes place. The Bio-plex can be considered an evolution of the ELISA, as it is capable of analysing up to one hundred analytes simultaneously, in a short time and with a high throughput. Indeed, it is possible to quantify multiple cytokines in a single plate in 3 hours, using only 12.5 µl of serum. This allows a complete cytokine profile to be obtained, limiting the amount of sample used, reducing preparation time, and maximising throughput.

The magnetic bead-based assay was performed on a Bio-Plex 200 analyzer with Bio-Rad Bio-Plex Manager (Bio-Rad, Hercules, CA, United States). All the values obtained were included within the detection limits indicated by the manufacturer (bio-rad.com/Bio-Plex/AnalyteGuide)[56,57].

4.5 Statistical Analysis

Statistical Analyses were performed using the R statistical platform (https://www.R-project.org/) in RStudio GUI software, version 4.1.2, and GraphPad Prism 8.4.2 software (GraphPad Software Inc., La Jolla, CA).

Continuous variables were described as mean ± standard deviation or median InterQuartile Range (IQR, 25^{th} to 75^{th} percentile). Shapiro-Wilk normality test was used to evaluate whether the continuous data were normally distributed and,

according to the results, a Welch's two-tailed t-test for independent samples (for parametric data) or a Mann–Whitney U-test (for non-parametric data) was used for unpaired comparisons. Paired comparisons were performed using the paired t-test (for normally distributed data) or Wilcoxon matched-pairs signed-rank test (for non-normally distributed data). Fold change tests were analyzed with either a one-sample t-test (for normally distributed data) or Wilcoxon signed-rank test, with a hypothetical value set at 1. NPRS progression throughout the study was analyzed with a mixed effect analysis, Geisser-Greenhouse correction, and Tukey's post hoc multiple comparisons test. Categorical variables were reported as counts of occurrences (percentages) and were compared using Fisher's exact test. Correlations among continuous variables were assessed using non-parametric Spearman's correlation. Receiver operating characteristic (ROC) curves were constructed using the 95% CI De Long's method. The ROC curve for the combined effect of multiple continuous parameters was evaluated by generating a multivariate logistic regression model and combining each prediction value with their relative outcomes. All p-values <0.05 were considered statistically significant.

4.5.1 Shapiro-Wilk test

The Shapiro-Wilk test was used to check normality by comparing two alternative estimators of variance; through this test, we assessed whether the continuous data were normally distributed, and the results showed that the values were expressed as median and interquartile.

4.5.2 Mann Whitney U Test

The Mann-Whitney test allows to check whether two statistical samples come from the same population. We used this test to determine significant differences between two independent samples. The null hypothesis in the Mann-Whitney test (MW) is that the two samples are drawn from a single population, and therefore for this reason their probability distributions are equal. The alternative hypothesis is that one of the samples is larger in a stochastic way. This requires that the two samples be statistically independent and that the observations are at least continuous or discrete ordinals. The assumptions of the MW test are:

- The two samples under study by the test are mutually independent and the observations within each sample are independent.

- The observations are comparable (for example, for any two observations, it is possible to establish whether they are equal or whether on the contrary, which of the two is greater).

This test is used to make comparisons on the medians of two subgroups of observations, and therefore constitutes the non-parametric counterpart of the independent-sampled t-test[58].

4.5.3 Wilcoxon test

The Wilcoxon signed-rank test is a non-parametric statistical hypothesis test used either to test the location of a set of samples or to compare the locations of two populations using a set of matched samples. When applied to test the location of a set of samples, it serves the same purpose as the one-sample Student's t-test. On a set of matched samples, it is a paired difference test like the paired Student's t-test (also known as the "t-test for matched pairs" or "t-test for dependent samples"). Unlike Student's t-test, The Wilcoxon signed-rank test does not assume that the differences between paired samples are normally distributed[59].

4.5.4 ROC curve

A tool for assessing the accuracy of a diagnostic test, based on sensitivity and specificity, is provided by the ROC (Receiver Operating Characteristic) curve, a statistical technique to identify the optimal threshold value (best cut-off), i.e. the test value that maximises the difference between true positives and false positives. To obtain valid results from ROC curves, it is essential that the presence/absence of a specific disease is ascertained by means of a gold standard. A summary indicator, which provides a concise and objective measure of the efficacy or accuracy of a test, is the area under the ROC curve, denoted AUC (Area Under the Curve)[60].

ROC curves can be compared with each other by means of an appropriate statistical test available in almost all commercially available software. The ROC curve is constructed by considering all possible test values and for each of these, the

proportion of true positives (sensitivity) and the proportion of false positives are calculated. Sensitivity, specificity, negative predictive power and positive predictive power classify individuals as affected or unaffected by a specific disease on the basis of a predefined test value (threshold value). A diagnostic test with an area under the curve ≥ 80%, whose values are between 0.5 and 1.0, is considered adequate. For the interpretation of the values of the area under the ROC curve, it is possible to consider the classification proposed by Swets (1998)[61]:

- AUC = 0.5 the uninformative test

- 0.5 < AUC ≤ 0.7 inaccurate test

- 0.7 < AUC ≤ 0.9 moderately accurate test

- 0.9 < AUC < 1.0 highly accurate test

- AUC = 1.0 perfect test

5. RESULTS

5.1 Patients Phenotyping

51 consecutive patients with grade I OA eligible for APC infiltration therapy were recruited for this study. Forty patients were female (78.43%). The population had an overall mean age of 67.22 years and a body mass index (BMI) of 28.4. Nineteen patients (37.25%) were obese (BMI > 30 Kg/m2), and nine (17.65%) had Type-2 Diabetes (Table 1). Blood cell and platelet counts, glycemia, and C-reactive protein, ferritin, AST, and ALT levels were within the expected ranges at T0, T1, and T2 (Table 2).

Table 1: Biometrical and clinical phenotyping of the recruited population (N= 51) Continuous data are reported as Mean ± SD or Median [IQR], while categorical variables are reported as count (%).

Sex (Females)	40 (78.43%)
Age (Years)	67.22 ± 10.21
BMI (Kg/m^2)	28.4 ± 5.17
Obesity	19 (37.25%)
Diabetes	9 (17.65%)
Smoke	15 (29.41%)

Table 2: Biochemical phenotyping for the recruited population, stratified for Females and Males groups. Data are reported as Median [IQR].

	T0	T1	T2
RBC (10^9/l)			
Males	5.08 [4.85; 5.38]	5.08 [4.86; 5.39]	5.24 [4.44; 5.58]
Females	4.5 [4.29; 4.76]	4.48 [4.28; 4.59]	4.55 [4.32; 4.83]
WBC (10^{12}/l)			
Males	7.97 [6.35; 9.65]	7.73 [6.58; 8.38]	7.85 [7.08; 8.5]
Females	6.75 [5.64; 8.05]	6.59 [5.09; 7.8]	7.05 [5.64; 7.9]
PLT (10^9/l)	247 [198; 278]	239 [184; 263]	235 [195.5; 281.5]
Glycaemia (mg/dl)	93 [84; 107]	91 [81.5; 105]	92 [79; 97]
CRP (mg/l)	0.145 [0.07; 0.42]	0.14 [0.08; 0.25]	0.17 [0.1; 0.26]
Ferritin (ng/ml)			
Males	105 [67; 155]	167 [112; 241.5]	163 [92; 291]
Females	43 [22.25; 75.5]	33 [13; 63]	24 [10; 61]
AST (ng/ml)			
Males	22 [16; 24.5]	24 [19.65; 26]	20 [18; 27.5]
Females	18 [15; 21.75]	16 [15; 19]	17 [15; 24]
ALT (ng/ml)			
Males	22 [16; 24.5]	24 [20; 51]	14 [13.5; 35.5]
Females	17 [14.25; 21]	17 [13; 20]	15 [11; 19]

At enrolment (T0), the mean Numeric Pain Rating Scale (NPRS) score was 8.12 ± 1.6, indicating severe pain. Upon 30 days (T1) from APC infiltration, NPRS score was 6.64 ± 1.93 (paired p-value <0.001); the reduction was more pronounced upon 120 days (T2), with a mean value of 5.08 ± 2.01 (paired p-value <0.001) (Figure 12).

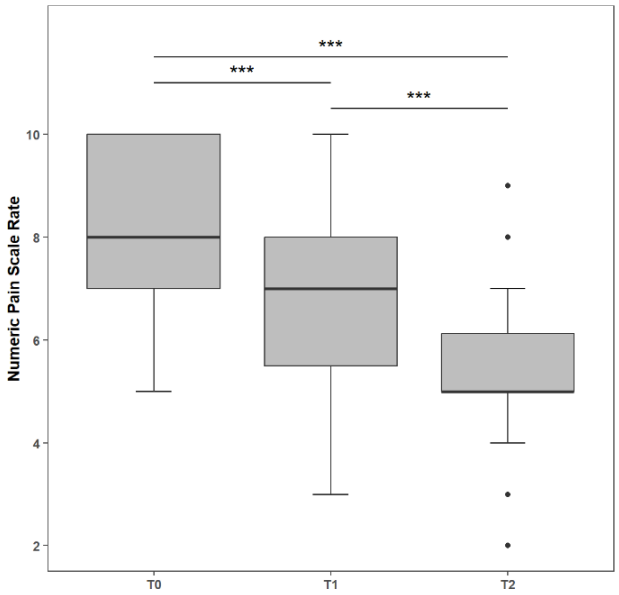

Figure 12. Numeric Pain Rating Scale distribution. NPRS was recorded at the enrolment (T0), after 30 days (T1) and 120 days (T2). Box plots denote median and 25th to 75th percentiles (boxes), while whiskers represent the Tukey interval. Asterisks denote statistical significance (*: p<0.001).**

5.2 Cytokines and growth factors screening in PB and APC

PB and APC samples were collected, and the concentrations of cytokines and GFs were determined. All tested factors, except for interleukin (IL)-7 and IL-15, were detectable in both PB and APC. IL-4, IL-5, IL-10, IL-13, Platelet-Derived Growth Factor (PDGF), C-C motif Chemokine Ligand 5 (CCL5/RANTES), and Vascular Endothelial Growth Factor (VEGF) increased by more than 1.5-fold in APC compared with PB (Table 3). No significant difference was detected between cytokine and GF levels in APC at T0 compared to T1 (Table 4).

Table 3: PB and APC screening for Cytokines, Chemokines and Growth factors at T0. Results are reported as median [IQR] and expressed as pg/ml. Emboldened p-values are statistically significant (p<0.05).

Marker	T0 PB	T0 APC	Paired p-val	Fold Change	
				Est (95% CI)	p-val
IL-1β	0.71 [0.42; 1.30]	0.79 [0.57; 1.15]	0.713	1.31 (1.08 – 1.54)	**0.009**
IL-1ra	165 [139; 274]	206 [165; 293]	0.187	1.34 (1.07 – 1.6)	**0.013**
IL-2	2.55 [1.55; 4.01]	3.04 [2.05; 4.72]	0.531	2.25 (0.402 – 4.11)	0.158
IL-4	1.91 [1.29; 2.68]	2.43 [1.91; 3.2]	**<0.001**	1.79 (1.32 – 2.25)	**0.001**
IL-5	34.5 [20.4: 42.1]	43.6 [43.6; 54]	0.054	2.07 (1.11 – 3.03)	**0.03**
IL-6	0.6 [0.17; 1.48]	1.19 [0.46; 2.38]	0.147	9.90 (-2.48 – 21.3)	0.148
IL-8	5.88 [4.4; 8.87]	6.87 [5.88; 8.37]	0.138	1.19 (1.03 – 1.35)	**0.022**
IL-9	267 [209; 329]	329 [284; 379]	**<0.001**	1.31 (1.19 – 1.43)	**<0.001**
IL-10	3.01 [1.5; 4.98]	4.98 [3.51; 7.9]	**<0.001**	2.57 (1.95 – 3.19)	**<0.001**
IL-12	12.5 [7.63; 17.3]	26.8 [17.3; 41]	**<0.001**	8.65 (-2.3 – 19.6)	0.166
IL-13	1.91 [1.59; 3.64]	2.65 [2.16; 4.47]	**<0.001**	1.7 (1.41 – 1.99)	**<0.001**
IL-17	23.5 [16.4; 30]	29.1 [25.7; 32.5]	**<0.001**	1.34 (1.2 – 1.49)	**<0.001**
Eotaxin	55.9 [37.9; 77.9]	49 [33.1; 65.7]	0.11	0.953 (0.843 – 1.06)	0.388
bFGF	14.9 [12.8; 16.8]	20.6 [16.8; 25.1]	**<0.001**	1.43 (1.27 – 1.59)	**<0.001**
G-CSF	93.2 [65.8; 135]	109 [87.9; 151]	**0.004**	1.26 (1.13 – 1.38)	**<0.001**
GM-CSF	1.9 [1.14; 2.9]	3.51 [2.25; 5.18]	**<0.001**	4.03 (0.73 – 7.34)	0.071
IFN-γ	7.55 [5.48; 9.63]	9.63 [8.07; 12.5]	**<0.001**	1.41 (1.26 – 1.56)	**<0.001**
IP-10	255 [181; 440]	230 [155; 332]	**<0.001**	0.882 (0.826 – 0.939)	**<0.001**
MCP-1	16.5 [11; 26.6]	15.7 [12.6; 24.5]	0.058	1.15 (1.02 – 1.28)	**0.026**
MIP-1α	1.44 [1.11; 1.86]	1.73 [1.44; 2.12]	**<0.001**	1.27 (1.15 – 1.39)	**<0.001**
MIP-1β	105 [92.6; 133]	132 [108; 149]	**<0.001**	1.21 (1.12 – 1.3)	**<0.001**
PDGF	170.74 [92.03; 242.91]	423.22 [232.01; 688.56]	**<0.001**	3.96 (2.91 – 5.01)	**<0.001**
RANTES	1461 [1171; 2270]	2823 [2201; 4399]	**<0.001**	1.97 (1.68 – 2.25)	**<0.001**
TNF-α	107 [89; 136]	140 [119; 174]	**<0.001**	1.37 (1.25 – 1.48)	**<0.001**
VEGF	106 [77.7; 152]	148 [68.7; 202]	**0.026**	1.77 (1.33 – 2.21)	**0.001**

Table 4: APC screening for Cytokines, Chemokines and Growth factors at both T0 and T1. Results are indicated as median [IQR] and expressed as pg/ml. p-values in bold are considered as statistically significant (p<0.05).

Marker	T0 APC	T1 APC	Paired p-val	Fold Change	
				Est (95% CI)	p-val
IL-1β	0.79 [0.57; 1.15]	0.71 [0.57; 1.01]	0.11	1.06 (0.72 – 1.39)	0.737
IL-1ra	206 [165; 293]	206 [165; 260]	0.121	0.92 (0.79 – 1.05)	0.233
IL-2	3.04 [2.05; 4.72]	2.55 [0.903; 6.96]	0.713	1.16 (0.96 – 1.36)	0.121
IL-4	2.43 [1.91; 3.2]	2.68 [2.05; 3.40]	0.789	1.16 (-0.17 – 2.50)	0.755
IL-5	43.6 [43.6; 54]	45.1 [29.9; 70.1]	0.424	1.25 (0.99 – 1.51)	0.056
IL-8	6.87 [5.88; 8.37]	7.87 [5.88; 8.87]	0.35	1.14 (0.81 – 1.91)	0.136
IL-9	329 [284; 379]	292 [274; 311]	0.964	1.10 (0.92 – 1.29)	0.259
IL-10	4.98 [3.51; 7.9]	4.98 [4.12; 9.46]	0.898	1.04 (0.95 – 1.12)	0.421
IL-12	26.8 [17.3; 41]	22.1 [14.9; 36.3]	0.863	1.28 (0.90 – 1.66)	0.144
IL-13	2.65 [2.16; 4.47]	3.12 [2.16; 4.91]	0.701	1.30 (0.83 – 1.77)	0.205
IL-17	29.1 [25.7; 32.5]	29.4 [23.5; 34.1]	0.882	1.12 (0.85 – 1.38)	0.378
Eotaxin	49 [33.1; 65.7]	41.2 [33.2; 58.2]	0.982	1.07 (0.92 – 1.23)	0.331
bFGF	20.6 [16.8; 25.1]	18.8 [16.8; 24.3]	0.247	1.14 (0.99 – 1.30)	0.063
G-CSF	109 [87.9; 151]	119 [93.2; 140]	0.999	1.13 (0.92 – 1.34)	0.215
GM-CSF	3.51 [2.25; 5.18]	3.8 [2.9; 5.05]	0.757	1.06 (0.91 – 1.22)	0.418
IFN-γ	9.63 [8.07; 12.5]	8.93 [8.24; 12.1]	0.682	2.93 (-0.45 – 6.32)	0.251
IP-10	230 [155; 332]	168 [132; 246]	0.782	1.06 (0.88 – 1.24)	0.497
MCP-1	15.7 [12.6; 24.5]	13 [10.2; 19.4]	0.112	0.95 (0.81 – 1.10)	0.491
MIP-1α	1.73 [1.44; 2.12]	1.86 [1.59; 1.99]	0.915	1.09 (0.91 – 1.27)	0.319
MIP-1β	132 [108; 149]	118 [103; 128]	0.782	1.08 (0.93 – 1.24)	0.274
PDGF	423.22 [232.01; 688.56]	331 [143;488]	0.639	1.05 (0.95 – 1.14)	0.325
RANTES	2823 [2201; 4399]	2367 [1836; 2532]	0.221	1.03 (0.85 – 1.20)	0.762
TNF-α	140 [119; 174]	121 [106; 132]	0.925	1.03 (0.93 – 1.14)	0.519
VEGF	148 [68.7; 202]	141 [96.5; 186]	0.683	1.89 (0.66 – 3.11)	0.147

In APC, a correlation matrix displayed a cluster of PDGF, RANTES/CCL5, and VEGF, which were mainly representative of platelet-released factors (54). An additional

cluster included IL-4, IL-5, IL-10, IL-17, basic Fibroblast Growth Factor (bFGF), Interferon-γ (IFN-γ), and Macrophage Inflammatory Protein-1 (MIP-1) α (Figure 13 A). In PB at T0, MIP-1α was negatively correlated with patients' NPRS (ρ = -0.306, p = 0.029) and positively correlated with WBC count (ρ = 0.455, p = 0.001). Moreover, a negative correlation between the WBC count and NPRS was detected (ρ: -0.419, p-val = 0.003) (Figure 13 B). No other significant correlations were observed.

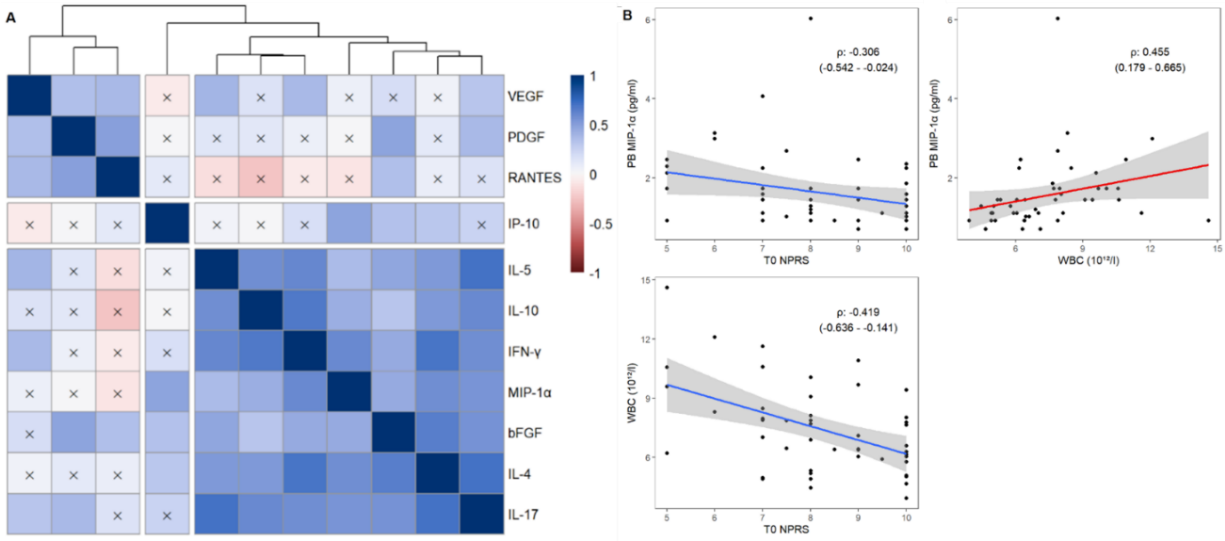

Figure 13. Correlation Analysis in PB and APC. (A) APC correlation matrix and hierarchical clustering. Spearman's correlation coefficients for APC cytokine and growth factor scaled measurements at T0 are visualized by tile-color intensity (according to the legend on the right). ρ-values closer to 1 show a positive correlation; ρ-values closer to −1 show a negative correlation; ρ-values closer to 0 denote the absence of a correlation among the considered variables. All values not labeled with a black X are statistically significant (p-value <0.05). Dendrograms on top of the matrix show the hierarchical clustering. (B) Correlations among biochemical data in peripheral blood and NPRS. Scatter plots represent the patients' distributions: T0 NPRS and PB MIP-1α (top left), T0 NPRS and WBC (bottom left); T0 WBC and PB MIP-1α (top right). For each plot, a linear regression is reported, with 95% confidence interval marked in grey, as well as the ρ Spearman correlation value with the relative 95% CI. Every correlation reported is statistically significant (p<0.05).

5.3 Relationship between early NPRS variation and biochemical parameters

The population was then stratified according to the variation in NPRS score from T0 to T1 (indicative of an early response to therapy). Patients with an NPRS reduction above the mean (ΔT0-T1:1.48, Table 5) were labeled as early responders (n= 28), while patients with an NPRS reduction lower than the mean were labeled as poor early responders (n= 23). Basal (T0) NPRS was significantly higher in early responders

compared to their counterparts (8.71 ± 1.46 vs 7.39 ± 1.48, respectively; p-val: 0.003). The WBC count was lower in early responders than in poor early responders (6.3 [5.33; 7.88] vs. 8.05 [6.57; 10.4]; p = 0.005) (Table 6).

Table 5: Numeric Pain Scale Rate (NPRS) score. NPRS is reported as Mean ± Standard Deviation for the three time points. Mean Differences throughout times are reported as Mean (95% CI). p<0.005 were considered statistically significant.

	T0	T1	T2
NPRS	8.12 ± 1.6	6.64 ± 1.93	5.08 ± 2.01
		ΔT0-T1	**ΔT1-T2**
Mean (95% CI)	-	1.48 (0.82 – 2.13)	1.55 (0.798 – 2.32)
Paired p-val		**<0.001**	**<0.001**
			ΔT0-T2
Mean (95% CI)	-	-	3.04 (2.35 – 3.73)
Paired p-val			**<0.001**

Table 6: Biometrical, biochemical and clinical phenotyping for early responders and poor early responders at T1. PB and APC cytokines are reported as pg/ml. p-values in bold denote statistical significance (p<0.05).

	Poor Early Responders (n: 23)	Early Responders (n: 28)	p-val
Sex (Females)	17 (73.91%)	23 (82.14%)	0.514
Age (years)	67.7 ± 8.27	66.9 ± 11.7	0.778
Weight (Kg)	78 [68; 88]	77 [65; 80]	0.159
Height (m)	1.65 [1.60; 1.69]	1.60 [1.59; 1.70]	0.199
BMI (Kg/m^2)	27.3 [24.2; 32.4]	28.1 [24.6; 30.9]	0.919
Obese	10 (43.48%)	9 (32.14%)	0.563
Diabetes	4 (17.39%)	5 (17.86%)	0.999
Smoke	6 (26.09%)	10 (35.71%)	0.552
T0 NPRS	7.39 ± 1.48	8.71 ± 1.46	**0.003**
RBC (10^9/l)	4.73 [4.30; 5.01]	4.61 [4.12; 4.96]	0.356
WBC (10^{12}/l)	8.05 [6.57; 10.4]	6.3 [5.33; 7.88]	**0.005**
PLT (10^9/l)	248 [202; 275]	244 [192; 290]	0.655
PB MCP-1	11.83 [10.23; 16.46]	20.16 [12.03; 25.52]	**0.012**
APC PDGF	274.9 [155.47; 446.52]	486.6 [335.7; 688.56]	**0.009**
APC RANTES	2462 [2128; 3101]	3554 [2375; 4888]	**0.023**
APC VEGF	139.65 [72.67; 163.4]	171.06 [114.48; 235.79]	**0.027**

Moreover, early responders had significantly higher levels of Monocyte Chemoattractant Protein-1 (MCP-1) in PB at T0 than poor early responders (p = 0.012; Figure 14 A). Interestingly, early responders displayed significantly higher values of PDGF (p = 0.009), RANTES/CCL5 (p = 0.023), and VEGF (p = 0.027) in APC than poor responders (Figure 14 B-D, Table 6).

ROC curves for both MCP1 in PB (AUC, 0.751; 95% CI, 0.563 – 0.867; p = 0.005; Figure 14 A) and PDGF, RANTES/CCL5, and VEGF in APC (PDGF AUC: 0.726, p = 0.004, RANTES AUC: 0.686, p = 0.014; VEGF AUC: 0.679, p = 0.025; Figure 14 B-D) displayed a significant accuracy for early response to APC treatment with respect to NPRS score variation. No other biometric, biochemical, or clinical data showed significant differences between the two groups.

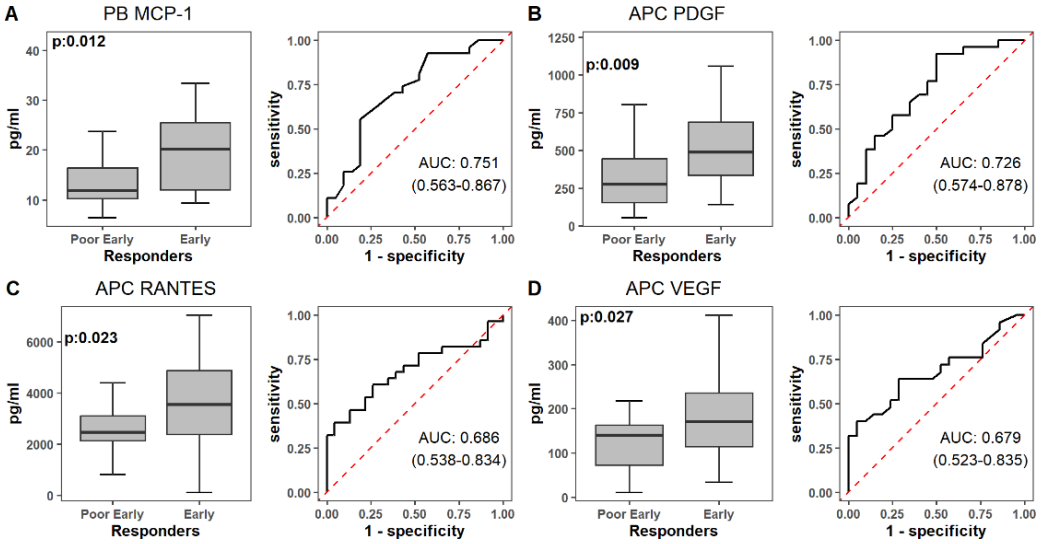

Figure 14. Soluble factors in PB and APC of early responders vs poor early responders. Boxplots depict concentrations at T0 of MCP-1 in PB (A) and of PDGF (B), RANTES (C) and VEGF (D) in APC for early responders and poor early responders. Concentrations are expressed as pg/ml. For each molecule, the relative ROC Curve for discriminating among the two groups is shown. The figure reports only those factors displaying statistical significance (p<0.05).

5.4 Relationship between long-term NPRS variation and biochemical parameters.

Patients were then stratified according to NPRS reduction from T0 to T2 (mean ΔT0-T2: 3.04; 25th percentile: ≤ 2.0; 75th percentile: ≥ 5.0); thus, 12 patients were classified as overall low responders, 23 as mid responders, and 16 as high responders. Again, a larger decrease was observed in individuals with higher NPRS (p-val <0.001) and lower

WBC count (p: 0.015) at T0 (Table 7). The concentration of PDGF in APC was higher in high and mid compared to low responders (p-val: 0.004; Figure 15). No other significant difference among groups was found in PB and APC.

Table 7: Biometrical, Biochemical and Clinical phenotyping for Responders and Non-Responders at T2. Peripheral Blood and APC cytokines are reported as pg/ml. p-values in bold denote statistical significance (p<0.05).

	Low Responders (n: 12)	Mid Responders (n: 23)	High Responders (n: 16)	p-val
Sex (Females)	10 (83.3%)	15 (65.2%)	15 (93.8%)	0.092
Age (years)	68 ± 12.3	65.82 ± 7.35	68.6 ± 12.3	0.679
Weight (Kg)	79 [70.5; 85.75]	80 [69; 83.5]	70 [65; 73.5]	0.095
Height (m)	1.62 [1.6; 1.67]	1.67 [1.6; 1.71]	1.6 [1.6; 1.68]	0.263
BMI (Kg/m^2)	29.24 [25.36; 33.45]	29.43 [26.26; 31.05]	26.44 [24.23; 27.73]	0.183
Obese	6 (50%)	(47.8%)	(12.5%)	0.062
Diabetes	4 (33.3%)	3 (13%)	2 (12.5%)	0.265
Smoke	2 (16.7%)	9 (39.1%)	4 (25%)	0.344
T0 NPRS	6.54 ± 1.34	8.46 ± 1.32	8.81 ± 1.39	**<0.001**
RBC (10^9/l)	4.61 [4.26; 4.78]	4.62 [4.28; 5.13]	4.7 [4.39; 4.99]	0.554
WBC (10^{12}/l)	9.09 [6.54; 10.31]	7.65 [6.39; 8.48]	6.15 [5.43; 6.78]	**0.015**
PLT (10^9/l)	265 [250.25; 280.5]	236.5 [202; 274.25]	244 [186.5; 278.5]	0.452
APC PDGF	155.5 [141.1; 469.8]	411.1 [281.6; 526.6]	494 [340.9; 652.5]	**0.004**

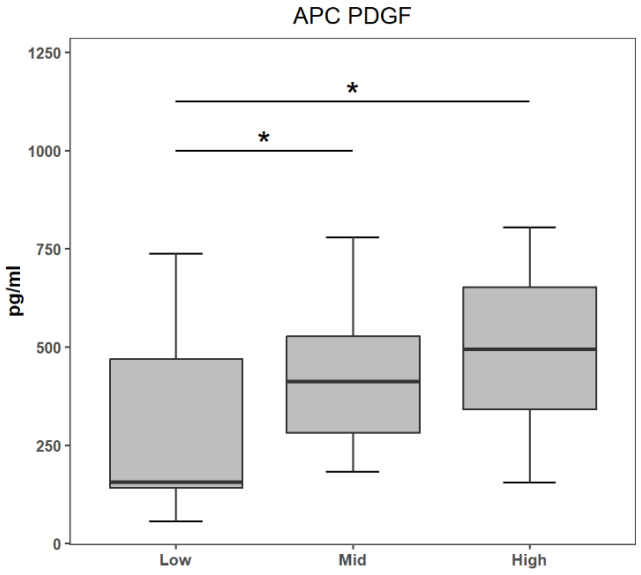

Figure 15. Soluble factors in APC in overall responders. Boxplots denote PDGF concentrations in APC at T0 for patients displaying Low, Mid and High response to therapy at T2. PDGF concentration in APC is expressed as pg/ml. Asterisks denote statistical significance (*: p<0.05).

5.5 Comparison between optimal responders and poor responders

Of the fifty-one total patients, eleven (21.57%) showed NPRS reduction at both T1 and T2 (optimal responders), while eight (15.69%) displayed slight to no-NPRS reduction (never responders). These two groups did not display significant difference in biometrical and clinical data other than basal NPRS, which was higher in optimal responders (p-val: 0.004). The WBC count was lower in optimal responders, compared to never responders (p-val: 0.008). No other significantly different biochemical feature was found in PB of the two groups. Intriguingly, in APC, optimal responders displayed slightly lower levels of MIP-1α (p-val: 0.033), and 2.5- and 2.4-fold higher levels of PDGF (p-val: 0.027) and VEGF (p-val: 0.032), respectively, compared to never responders. These markers also displayed a significant predictive value in discriminating optimal responders from never responders (MIP-1α AUC: 0.801, p-val: 0.006; PDGF AUC: 0.793, p-val: 0.011; VEGF AUC: 0.781, p-val: 0.013) (Figure 16 A, Table 8). Thus, the combined effect of MIP-1α, PDGF and VEGF resulted in a higher predictive power in discriminating optimal from never responders (AUC: 0.931, p-val< 0.001) (Figure 16 B).

Table 8: Biometrical, Biochemical and Clinical phenotyping for optimal responders and never responders through T1 and T2. PB and APC cytokines are reported as pg/ml. p-values in bold denote statistical significance (p<0.05).

	Never Responders (n: 8)	Optimal Responder (n: 11)	p-val
Sex (Females, %)	F: 6 (75%)	F: 10 (90.91%)	0.546
Age (years)	66.25 ± 7.27	65.64 ± 12.08	0.806
Weight (Kg)	79 [71.75; 94.25]	72 [65.75; 79]	0.163
Height (m)	1.64 [1.62; 1.69]	1.6 [1.53; 1.64]	0.587
BMI (Kg/m^2)	30.48 [25.32; 34.28]	28.51 [26.66; 30.5]	0.485
Obese	5 (62.5%)	3 (30%)	0.342
Diabetes	2 (25%)	1 (9.09%)	0.546
Smoke	1 (12.5%)	2 (18.18%)	0.999
T0 NPRS	6.62 ± 1.41	9.05 ± 1.56	**0.004**
RBC (10^9/l)	4.42 [4.22; 4.75]	4.5 [4.06; 4.9]	0.707
WBC (10^{12}/l)	9.83 [9.22; 10.44]	5.91 [4.84; 6.19]	**0.008**
PLT (10^9/l)	248 [245; 257]	205 [171; 258]	0.571
APC MIP-1α	2.12 [1.85; 2.22]	1.59 [1.16; 1.79]	**0.033**
APC PDGF	189.79 [115.02; 400.77]	467.86 [358.21; 670.54]	**0.027**
APC VEGF	76.67 [42.57; 152.43]	181.74 [143.64; 238.35]	**0.032**

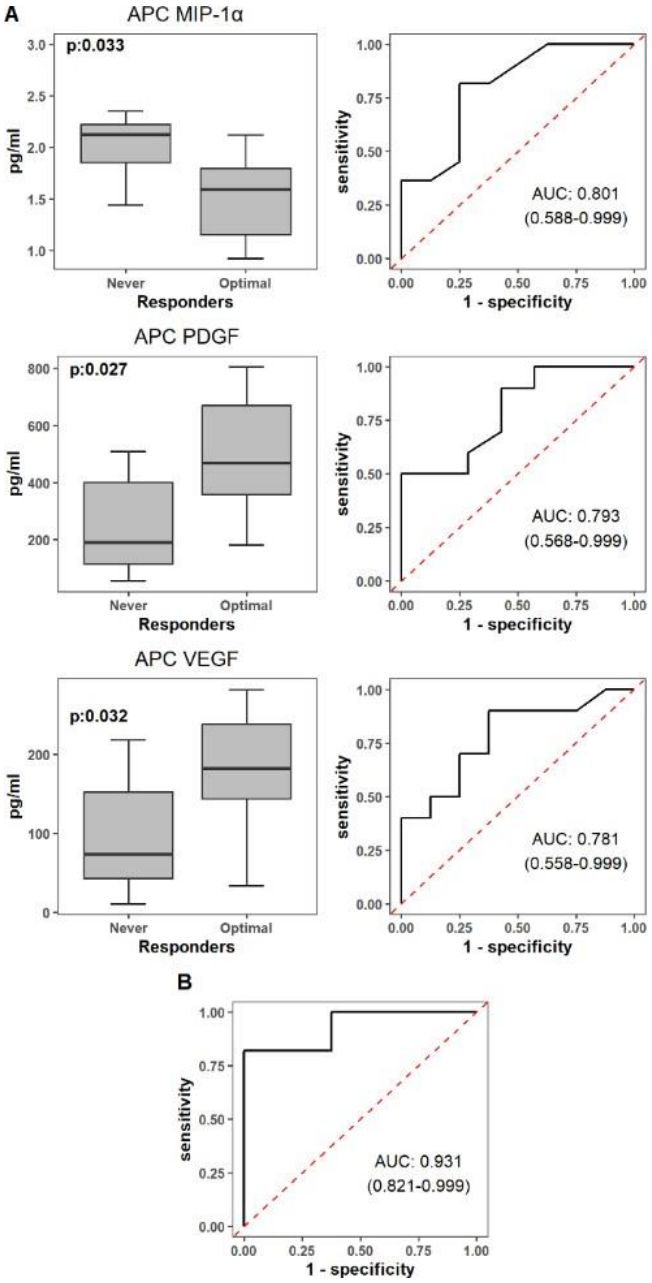

Figure 16. Soluble factors in APC in optimal vs never responders. (A) Boxplots depict concentrations of MIP-1α, PDGF, and VEGF in APC (B) for optimal responders and never responders. Concentrations are expressed as pg/ml. For each molecule, the relative ROC Curve for discriminating among the two groups is shown. The figure reports only those factors displaying statistical significance (p<0.05). (B) ROC Curve for the multivariable model including APC MIP-1α, APC PDGF and APC VEGF (p<0.05).

6. DISCUSSION

Osteoarthritis (OA) is a chronic disease with multifactorial pathogenesis, that affects the joint and its tissues, primarily causing progressive damage to articular cartilage and, subsequently, to the subchondral bone and surrounding synovial structures. OA is a disabling condition with increasing incidence and prevalence in the general population. Several joints can be involved, most notably the hip and knee. The epidemiology and pathogenesis of hip and knee OA have been studied more extensively than in other joints, and increasing interest has been devoted to the study of molecular mechanisms leading to cartilage damage because of their central role in the pathogenesis of OA. The risk factors most frequently associated with knee OA are ageing, genetic predisposition and obesity. It is crucial to understand that OA is the end stage of numerous alterations that overcome the compensatory mechanisms that limit damage to articular cartilage, as the reactive capacity of cartilage tissue in the face of injury is limited. In this scenario, inflammatory processes and activation of the immune system play a crucial role[51].

The use of platelet concentrates to enhance tissue regeneration and wound healing has been largely exploited in the last two decades for the repair of articular cartilage injuries[3,53,62]. The rationale for the use of platelets is their ability to exert specific effects on inflammation, proliferation, and differentiation of chondrocytes by releasing a large number of cytokines, chemokines, and GFs[63]. Their infiltration into articular cavities may allow the enrichment of soluble mediators in tissues with limited blood supply and slow cell turnover. Effective pain relief and improvement of quality of life have also been documented following intra-articular injection of platelet concentrates[64,65].

We observed a progressive decrease in pain after APC treatment in patients with grade I OA. Pain relief was detected one month after the first APC administration and was further enhanced following the second injection, as measured after four months. Nevertheless, the degree of response to APC varied among the patients, and they

were classified based on NPRS variation. The highest degree of response was observed in the patients with the highest initial NPRS scores. Interestingly, however, an early response was also associated with a low WBC count and low MIP-1a levels in PB, suggesting that systemic inflammation could be a detrimental factor for the APC response. Indeed, MIP-1α plays a major role in the development of systemic inflammation and may interfere with organ repair process[66]. Surprisingly, we also found that MCP-1 levels were higher in peripheral blood of patients displaying better early response (as recorded 1 month after injection) to APC treatment. MCP-1 is one of the key chemokines that regulates the migration and infiltration of monocytes/macrophages. High circulating MCP-1 levels may contribute to monocyte recruitment into healing tissues. However, MCP-1 role in cartilage repair requires further investigation[56].

To assess whether predictive factors for APC response could be identified, we measured the concentrations of GFs and cytokines in APC. The concentrations of PDGF, VEGF, and RANTES/CCL5 were positively associated with early response to APC. Notably, a hierarchical cluster of PDGF, VEGF, and RANTES/CCL5 was detected in platelet concentrates, which was consistent with the degranulation pattern and release of healing mediators[56,67,68].

When the overall response was evaluated four months after intra-articular injections of APC, better outcomes were achieved in the presence of higher concentrations of PDGF and VEGF, and lower concentrations of MIP-1α. Interestingly, no association was found with platelet count, suggesting that growth factor release, rather than platelet number, is relevant to clinical outcomes. There is still controversy regarding the most effective platelet concentration for *in vivo* applications[69].

In vitro studies have shown conflicting results in terms of cell proliferation or other regenerative effects[70]. Some reports have shown that high concentrations of platelets are most beneficial, while others have provided evidence that very high concentrations of platelet-rich plasma (PRP) could be counterproductive, with a potential risk of cell death. We have previously shown that distinct effects on

individual cell types may be due to the release of different amounts of specific GFs, including PDGF and VEGF[67].

The functional relevance of PDGF has been largely addressed and is widely accepted. Indeed, PDGF plays a pivotal role in cell proliferation and tissue repair[71]. Moreover, PDGF has recently been shown to attenuate OA development by inhibiting inflammation and enhancing cell proliferation via the JAK2/STAT3, PI3K/AKT, and p38 signaling pathways[72]. Consistently, PDGF has been used in functionalized scaffolds to promote the recruitment of synovial mesenchymal stem cells for osteochondral repair[73].

Cartilage lacks blood and lymphatic supply. Normal articular cartilage resists vascular invasion due to its matrix composition and the production of diffusible angiogenesis inhibitors[73,74]. Hence, the action of VEGF on cartilage repair is still not well defined. Undoubtedly, VEGF is a relevant factor for tissue repair and regeneration [75,76]. However, previous studies have shown that angiogenesis blockade promotes cartilage repair in animal models. VEGF-attenuated PRP, by means of VEGF-binding microspheres, had no effect on PRP-induced chondrogenic differentiation of stem cells *in vitro* and improved therapeutic effect on cartilage repair in rats[77]. On the other hand, VEGF may exert non-angiogenic actions[78]. It has also been described that VEGF may be involved in macrophage recruitment and M2 polarization,which may contribute to relieving intra-articular inflammatory burden. Nevertheless, VEGF release by APC might represent a surrogate marker of platelet degranulation and, in addition to being a relevant biomarker of clinical outcome, might have a neutral or even inhibitory effect on chondropathic pain[79].

Finally, our data revealed that the presence of MIP-1α in both PB and APC might be detrimental to clinical outcomes. Low MIP-1α levels in APC of optimal responders may be due to low systemic inflammation in patients. Therefore, it is conceivable that favorable clinical outcomes are facilitated by the transfer of low levels of inflammatory cytokines into the articular cavity.

In conclusion, PDGF in platelet concentrates is a highly reproducible predictive biomarker of APC-induced pain reduction in individuals with grade I OA. High levels of PDGF, but not platelet counts, are associated with better clinical outcomes. The simultaneous determination of PDGF, VEGF, and MIP-1α in platelet concentrates may confer higher accuracy in predicting clinical outcomes.

7. STUDY 2

Functional interaction between mesenchymal stem cells and soluble factors for bone marrow aspirate concentrate therapy in Critical Limb Ischemia

7.1 Peripheral Arterial Disease

Peripheral Arterial Disease (PAD) is a vascular disease, characterized by reduced perfusion or obstruction of peripheral arteries, most frequently in the legs. This condition is caused by the accumulation of atherosclerotic plaques (deposits of fat, cholesterol, and other substances) in the arterial walls, resulting in reduced blood flow. PAD can cause various symptoms, including claudication (pain, cramping, or fatigue in the legs during physical activity), ischemia, wounds that do not heal and, in severe cases, gangrene. The main risk factors for PAD are smoking, diabetes mellitus, hypertension, hyperlipidaemia, and a history of cardiovascular disease[80] (Figure 17).

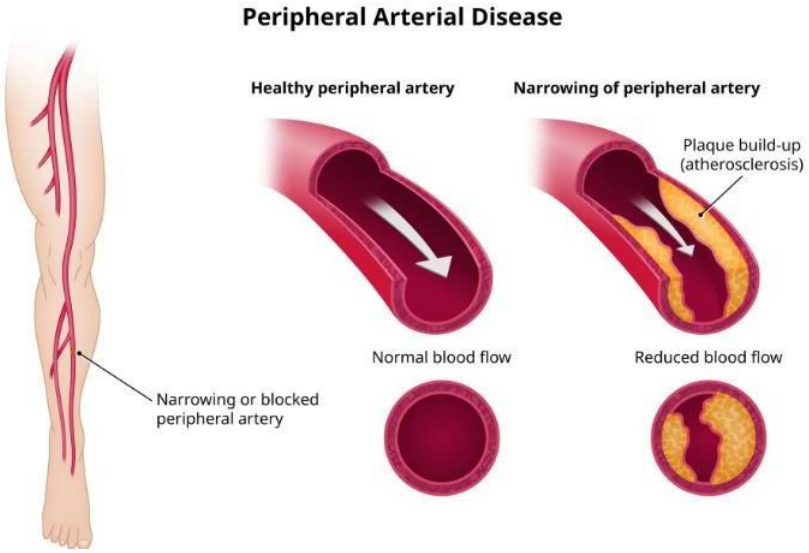

Figure 17. Peripheral Arterial Disease (PAD). PAD is a chronic condition where narrowed arteries reduce blood circulation to the limbs and is usually caused by a buildup of fatty deposits in arteries. PAD occurs when your limbs such as your legs do not receive enough blood flow. This in turn causes symptoms such as cramping, or leg pain when walking (claudication).

Individuals with PAD have a significantly higher risk of stroke and myocardial infarction due to the common atherosclerotic process. Treatment strategies include lifestyle modifications (such as smoking cessation, exercise and dietary changes), pharmacotherapy to manage underlying risk factors, and revascularisation procedures (such as angioplasty or bypass surgery) for more advanced cases. PAD can be a debilitating condition if not managed properly, resulting in impaired mobility and increased morbidity. The prevalence of the disease in the population is 5% and increases most in adults. In individuals over the age of 70, it can be as high as 20%. From an aetiological point of view, it is classified as:

- in 90-95%, obstructive arteriopathies of a degenerative and fibrotic type on an atherosclerotic basis, accumulation of cholesterol in the walls of the arteries with loss of elasticity

- in the remaining 5% of cases, obstructive arteriopathies of a phlogistic, or inflammatory type.

Most patients with PAD have mild or even no complaints. In other cases, they may present with necrosis or gangrene of the limb. This depends on the severity of the disease and thus the degree of occlusion. The most common symptom is claudicatio intermittens, acute pain, numbness, weakness and fatigue in the muscle groups of the lower limbs, especially at the calf level. This manifestation occurs following more or less intense exercise and resolves after a few minutes of rest[81].

The clinical manifestations of the disease can vary widely, patients with PAD are classified into different stages, according to the Fontaine (or Leriche-Fontaine) classification (Figure 18).

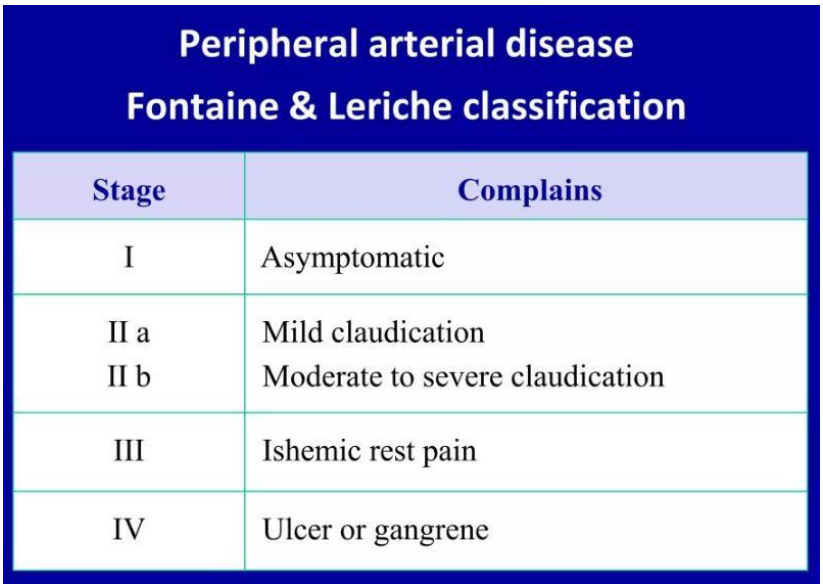

Figure 18. Schematic representation of the stages of PAD according to Leriche-Fontaine's classification.

Currently, a different classification is preferred, giving importance to the timing of surgery:

- moderate ischemia, a condition in which the patient may present the main symptoms in more or less severe forms;

- severe ischemia, a condition in which the patient risks amputation, following necrosis or gangrene of the limb, and therefore surgery will be necessary for possible revascularisation.

7.2 Critical Limb Ischemia

Critical Limb Ischemia (CLI) is an advanced manifestation of PAD, characterized by the presence of rest pain, intermittent claudication (IC), and trophic lesions, such as ulcers and/or gangrene, due to a severe compromise of blood flow to the affected extremity. Unfortunately, to date, the most probable outcome of CLI, in the absence of significant hemodynamic improvements, is represented by a high rate of mortality and amputation[82] (Figure 19).

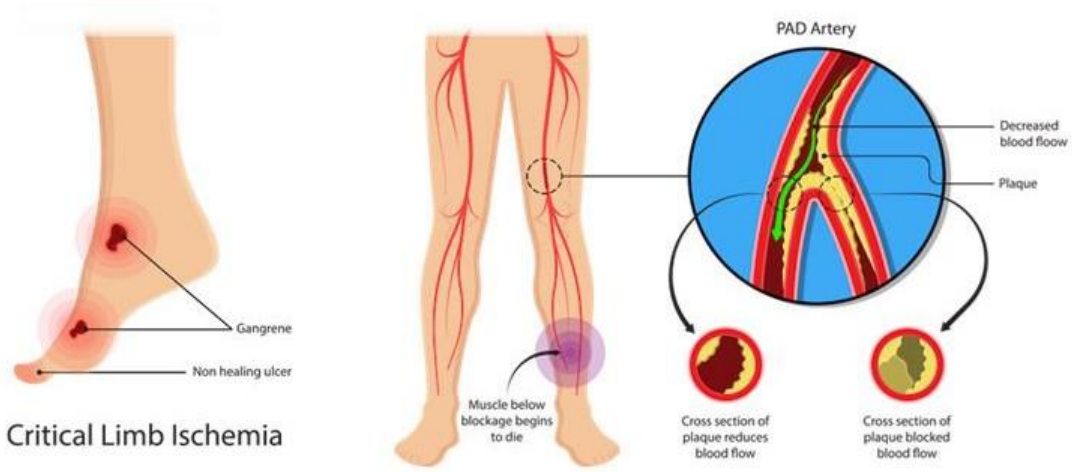

Figure 19. Critical Limb Ischemia (CLI). CLI is typically associated with advanced atherosclerosis and can result in major complications, including limb amputation if left untreated. It occurs when there is a substantial blockage of the arteries, leading to insufficient oxygen and nutrient supply to the tissues, often resulting in rest pain, non-healing ulcers, or gangrene.

The incidence of CLI is largely underestimated, since the data of severe arteriopathy affecting lower limbs are based on extrapolations deriving from the evolution of intermittent claudication[83].

7.2.1 Risk factors of CLI

Risk factors of CLI are different and include atherosclerosis, cigarette smoking, diabetes mellitus, hypertension and hyperhomocysteinemia. Atherosclerosis accounts for more than 90% of cases of PAD[84]. The chances of developing PAD in smokers compared to non-smokers are doubled and the severity of the disease is directly related to the number of smoked cigarettes and the length of time they smoke. In addition, smoking triples the risk of worsening arterial disease and doubles the risk of amputation[85]. In subjects with diabetes mellitus, CLI shows a triple frequency compared to the corresponding non-diabetic arterial disease and has a 15 times higher amputation rate. Interestingly, hypertension is associated with CLI although weaker than coronary artery disease[86]. Among the most recent risk factors of CLI, elevated homocysteine levels have been associated with a 2-3 times greater risk of developing PAD, and approximately 30-40% of patients with CLI have high levels of homocysteine[87].

7.2.2 Symptoms, Diagnosis and Treatment

The typical symptom of the CLI at rest is a constant burning sensation or numbness in the ankle or foot in the absence of activity. Elevating the extremity increases symptoms while placing the limb in a dependent position provides relief. Intermittent claudication, a cramping pain in the muscles of the lower limbs downstream of the vascular lesion occurs during exercise, such as walking, and is relieved by a short period of rest. This symptom is due to the discrepancy between the oxygen demand and oxygen supply by the muscle and has a negative impact on various aspects of quality of life[82].

Progression of CLI induces the onset of pain even at rest, during the hours of night sleep, where the supine position exacerbates an already compromised hemodynamic situation. In a minority of patients, clinical overview can evolve towards critical ischemia, with the possible appearance of trophic lesions and gangrene due to severe skin hypoxia. This condition may require in the most serious forms surgical revascularization or amputation of the affected limb. Among patients with claudication followed for 5 years, about 75% remain stable, 20% develop an aggravation of claudication and 5% have critical ischemia of the lower limbs. The risk of amputation in patients with claudication is about 1% per year. Amputation is much more frequent once resting pain symptoms or tissue ulcerations become manifest. Diagnosis of CLI is usually based on the anamnesis and physical examination, that allow to identify signs and symptoms of this diseases. However, to achieve the correct diagnosis it is necessary to perform several other clinical tests. For this purpose, non-invasive techniques are used including the measurement of the Ankle-Brachial Index (ABI), consisting in the systolic pressure at the ankle, divided by the systolic pressure at the arm, that represents the ratio of ankle-to-brachial systolic blood pressure. Normal values measured at rest are between 1.30 and 0.91; values between 0.90 and 0.40 indicates a mild to moderate degree, values below 0.40 indicate a severe level of illness. Values over 1.30 are an indication of incompressibility of the vessels probably due to calcinosis[80].

The ABI can be falsely high in the presence of stiffened ankle arteries related to medial artery calcification, a condition mostly observed in patients with diabetes or chronic kidney disease[88]. When required, especially in anticipation of surgery, the execution of other more sophisticated radiological imaging techniques such as computed tomography angiography (CTA), magnetic resonance angiography (MRA), or the more invasive angiography may be indicated. For evaluating the indication of revascularization in patients with PAD, both CTA and MRA are considered as appropriate imaging tests. The sensitivity and specificity of multidetector CTA compared with angiography is ≈ 90% for detecting PAD[89].

CTA uses iodinated contrast and ionizing radiation to visualize pathology from the aorta to the lower extremity. The scan times take a few seconds, but diagnosis can be difficult in small tibial vessels with calcification and multiple occlusions. The sensitivity and specificity of MRA in detecting PAD with stenosis >50% is the same as CTA, 90% to 100%. MRA has several advantages in diagnosing PAD over CTA. Indeed, MRA requires no radiation, and calcium does not interfere with the diagnosis. Another advantage of MRA is that it allows for hemodynamic measurements[90].

Further investigations that can be carried out are the evaluation of microcirculation through the monitoring of the transcutaneous oxygen and carbon dioxide tensions (TcpO2 and TcpCO2). This technique is a non-invasive method for patients who need continuous monitoring of oxygen and carbon dioxide with minimal blood draws and can quantify the level of local muscle oxygenation in the arteriopathic patient during the execution of an incremental protocol on the treadmill and for the study of oxygen consumption at rest[91].

Diagnostic angiography, the gold standard for preoperative imaging, requires less contrast than CTA, provides optimal resolution for distinguishing distal lesions, and facilitates immediate endovascular treatment. However, catheter-based angiography is invasive and not without complications[82].

The prognosis of patients affected by CLI is particularly severe due to the high mortality and morbidity rate, already evident within a few months of follow-up from

the diagnosis of CLI. Indeed, both the European Consensus and subsequently by the Trans-Atlantic Inter-Society Consensus (TASC) indicated that about 20-25% of the patients have a risk of major amputations and mortality already within one year of diagnosis. In addition, an Italian multicenter study of 1.560 patients with no option CLI showed a rate of amputations greater than 12% within one year of diagnosis, while the mortality rate was 13% within six months, 22% within 1 year and 31.6% within two years[92].

Therapeutic treatments of CLI patients depend on the severity of the disease. They should predict the progression of the atherosclerotic process, prevent morbidity and mortality from cardiovascular events, improve walking performance and quality of life and in multiple severe stages also suggest the best choice between the different surgical revascularization techniques. The reduction of the atherosclerotic and the cardiovascular risks should include the elimination of cigarette smoking and the monitoring of glycemia, blood pressure and dyslipidemia. Among the drugs specifically indicated for PAD, it seems recommended the only the use of antiplatelet agents, in particular aspirin and alternatively clopidogrel[93].

The main treatment of CLI in patients with salvageable limbs is revascularization to improve distal perfusion. Revascularization includes various techniques, such as dilatation and recanalization of an artery (percutaneous transluminal angioplasty or PTA) or traditional surgical techniques such as thromboendarterectomy or bypass of obstructed arteries. Recourse to surgery is justified in more advanced stages of the disease, while in intermediate stages it should be considered for situations of symptomatic failure or failure of comprehensive rehabilitation and pharmacotherapy[94].

Despite the rapid development of interventional techniques, therapeutic options for patients with CLI remain limited: approximately 40% of patients with CLI are not eligible for surgical or endovascular revascularization (patients with chronic critical limb ischemia without the possibility of revascularization). Therefore, new diagnostic and therapeutic options for revascularization are of utmost importance.

7.3 New Therapeutic Approaches

Among possible new therapeutic approaches to treat patients with CLI are cell biological therapies, including gene therapy and cell-mediated therapy. Gene therapy is a potentially usable treatment for patients with CLI. It has been observed that, in some patients, treated by transfection of the plasmid DNA-VEGF at scalar doses, after one year of follow-up, there was an improvement in clinical conditions, such as: reduction in pain, increase in ABI, neoformation of collateral vessels and an increase in distal flow. These studies have also shown that the use of VEGF has limitations, as the clinical use of growth factors can lead to the development of pre-existing tumours, the worsening of diabetic retinopathy and the induction of angiogenesis within the stable atheromasic plaque, which could lead to dangerous instability[42] (Figure 20).

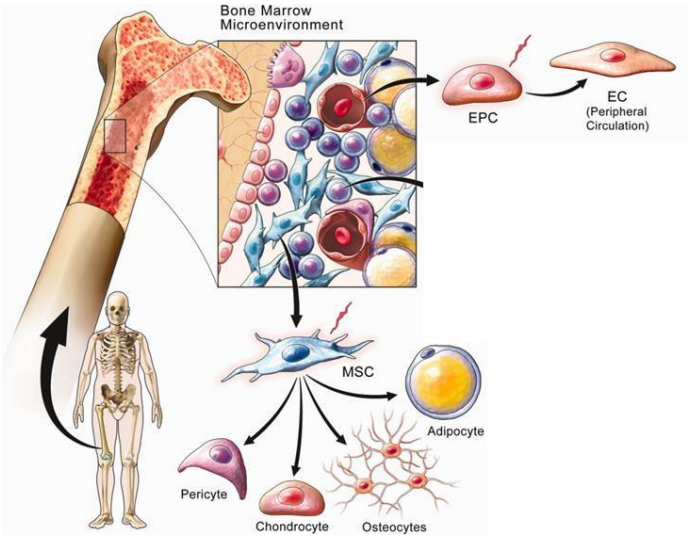

Figure 20. Pro-vascular progenitor cells in patients with CLI - Depletion of pro-vascular progenitor cells in patients with critical limb ischemia. The human bone marrow is a rich reservoir of progenitor cells that coordinate blood vessel repair. Circulating, vessel-resident endothelial precursor cells serve as the building blocks of blood vessels and inosculate into the vessel wall during vasculogenesis. MSCs generate pericytes that surround the vessels and smooth muscle cells that stabilise the newly formed vessels and secrete trophic factors that recruit accessory cells (M2 macrophages) involved in activating arteriogenic remodelling and perfusion of collateral vessels (96).

Cell-mediated therapy is based on the use of Endothelial Progenitor Cells (EPCs) or Mesenchymal Stem Cells (MSCs), derived from bone marrow and peripheral blood, for endothelial regeneration and neo-angiogenesis after tissue ischemia, and thus have been identified as a new potential therapeutic target in CLI[95].

7.4 Endothelial Progenitor Cells (EPCs)

Until a few years ago it was thought that the vascularization of ischemic tissues in adults was restricted to the migration and proliferation of mature endothelial cells, a process known as "angiogenesis"[96]. However, a population of mononuclear cells (MC), including endothelial progenitor cells (EPCs), has been identified in both bone marrow and peripheral blood. The first descriptions of EPCs were by Asahara et al. in 1997, who showed that, even in adults, bone-marrow-derived hematopoietic progenitor cells (PCs) can give rise to endothelial cells (ECs) that contribute to active neovascularization in ischemic tissues[28]. These cells were termed EPCs, and since their discovery intense effort has been focused on defining the role of EPCs in the regeneration of injured endothelium, neovascularization of ischemic tissue and cancer angiogenesis. Other evidence has suggested that particular stimuli (e.g., growth factors) or pathological conditions (e.g., ischemia) are able to mobilize EPCs, which are cells circulating in small quantities in the blood stream and are capable of implanting themselves in physiological and pathological neovascularization sites or in damaged tissues and then, to differentiate into endothelial cells. This process is known as “vasculogenesis” [95] (Figure 21).

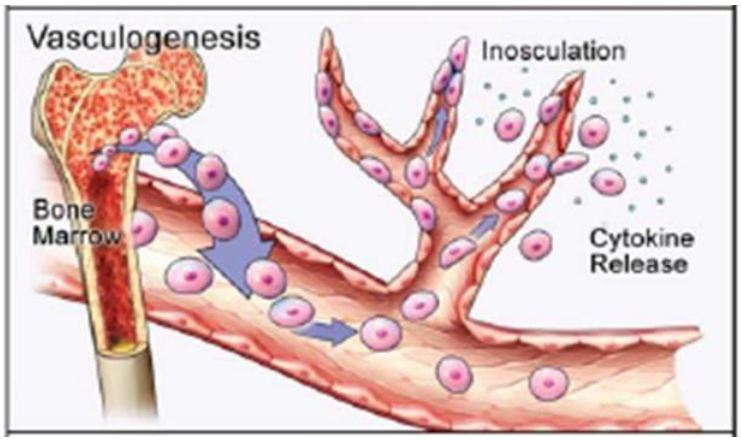

Figure 21. Mechanism of blood vessel regeneration. Vasculogenesis is the process by which new blood vessels are formed de novo from endothelial progenitor cells during early embryonic development. It primarily occurs in the mesodermal layer, where mesodermal cells differentiate into angioblasts, which then organize into vascular structures. Vasculogenesis is a critical process in establishing the initial vascular network before angiogenesis, the formation of new vessels from pre-existing ones, takes over during later stages of development.

Studies, conducted both in animals and in humans, have shown that EPCs are able to improve the function of ischemic tissues, both by inducing and modulating vasculogenesis and angiogenesis, in areas with reduced oxygen supply, and by stimulating the re-endothelialization of damaged blood vessels[97].

EPCs express some markers including CD34, vascular endothelial growth factor receptor-2 (VEGF-R2) and CD133. Upon release, EPCs begin to lose the expression of CD133, and acquire the expression of markers characterizing endothelial cells, particularly CD31, which plays an important role in the interaction between adjacent endothelial cells, VE-Cadherin, involved in the organization of new vessels and vWF, a multimeric protein that mediates platelet adhesion closer to endothelial damage[97]. The release of EPCs into the circulation from the bone marrow is regulated by a large variety of molecules such as the endothelial nitric oxide synthase (eNOS). Tissue ischemia stimulates the production of factors such as VEGF, which by acting on its KDR receptor, activates the phosphorylation of eNOS via Akt. The production of nitric oxide (NO) is essential for the activation of MMP-9, a key enzyme, for the mobilization of stem and progenitor cells, including the haemangioblast[98]; eNOS-mediated NO production is also under the control of the adrenergic system, which through the α2 and β adrenergic receptors (βARs) modulates the release of NO, causing endothelial vasodilation[99].

Several evidence has indicated that CLI risk factors play a negative role in EPC. Indeed, smoking is the major independent predictor for the reduction of EPC levels, while a reduced migration is mainly related to high blood pressure. Hyperglycemia significantly affects the function of EPCs, reducing their survival and altering their functioning[100].

7.5 Mesenchymal Stem Cells (MSCs)

Mesenchymal Stem Cells (MSCs) represent an important group of cells used in vascular regeneration. They are stromal cells with the ability of self-renewal and differentiation into various cell types such as chondrocytes, osteoblasts, adipocytes, and myoblasts. These cells were discovered more than 40 years ago by Friedenstein A.J. et al., which described a population of bone marrow cells similar in appearance

to fibroblasts, capable *in vitro* of differentiating into typical cells of the mesenchymal line, using specific media[101]. Several studies have demonstrated the ability of these cells to differentiate both *in vitro* than *in vivo*, into neuronal cells, hepatocytes, skeletal muscle cells, cardiac cells. Human mesenchymal stem cells were initially isolated from the bone marrow (hMSCs) and subsequently identified in other tissues as well, such as muscle, connective tissue, adipose tissue and various fetal tissues[102] (Figure 22). Amniotic fluid and placenta are also rich in MSCs [101 103].

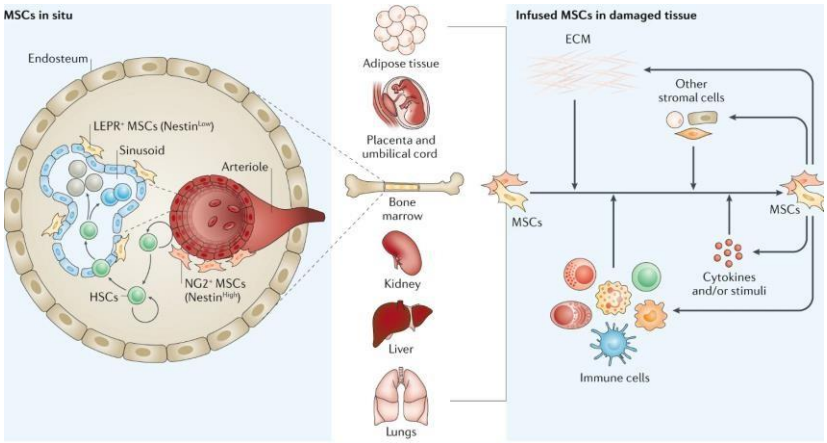

Figure 22. Isolation and characterization of MSCs – MSCs have been successfully isolated from multiple tissues, such as bone marrow, adipose tissue, umbilical cord, placenta, kidney, liver and lung. Analyses to date indicate that the tissue from which the MSCs originate affects their ability to differentiate, their secretome and their immunoregulatory potential as well as their reparative ability. Both endogenous and infused MSCs actively migrate to injured tissues. Through crosstalk with the components of inflammatory niches, such as extracellular matrix (ECM), immune cells, inflammatory factors and other stromal cells, MSCs facilitate the process of tissue repair by rectifying aberrant immune responses and promoting tissue regeneration (right panel)[102].

Adult MSCs isolated from bone marrow aspirates are characterized by a heterogeneous morphology and represent the ideal candidates for the development of future cellular and gene therapies for several reasons:

- they are easily obtainable from adult tissues and therefore their use does not present ethical problems, unlike embryonic stem cells;

- they show poor immunogenicity as they possess a superficial phenotype poorly recognized by T cells (absence of MHC II) with consequent poor rejection reaction in autologous and allogeneic transplants[104];

- they are able to migrate to damaged tissues and inflammation sites

- they can be rapidly expanded in vitro and grown over several passages, without losing the phenotype or differentiation capacity.

In addition, MSCs placed in culture are easily identifiable as they form colonies of adherent cells characterized by the typical fibroblastoid morphology. MSCs can be induced to differentiate through the use of specific media or several tissue factors, such as basic fibroblast growth factor (bFGF) or heparin-binding epidermal growth factor-like growth factor (hb-EGF), which seem able not only to increase their proliferation, but also to interfere with the differentiation capacity, keeping them in the state of multipotentiality[105,106]. Unfortunately, to date, it is difficult to determine with certainty the role of these multipotent stem cells *in vivo*, a role that cannot be predicted from *in vitro* culture. For this reason, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposes minimal criteria to define human MSC. They must be plastic-adherent when maintained in standard culture conditions. MSC must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules, and MSC must differentiate to osteoblasts, adipocytes and chondroblasts *in vitro*[107].

The therapeutic potential of MSCs transplantation for the treatment of ischemic conditions such as coronary artery disease, peripheral arterial disease, and stroke has been explored in animal models and early-phase clinical trials. The mechanism of the therapeutic effect of MSC transplantation in ischemic disease has been postulated to be due to paracrine, immunomodulatory, and differentiation effects[108]. MSCs are reported to promote angiogenesis because of their capacity to induce ECs proliferation, migration, and tube formation, while decreasing apoptosis and fibrosis. Furthermore, MSCs support neoangiogenesis, releasing soluble factors that contribute to stimulating angiogenesis. These cells are thought to improve hind limb ischemia by secreting cytokines that regulate macrophage differentiation to M2, an anti-inflammatory phenotype[109].

Despite promising pre-clinical studies in animal models, transplantation of bone marrow-derived cell populations in patients with CLI has shown partial benefit

preventing limb amputation[95]. Thus, careful preclinical evaluation of emerging concepts and technologies are critical for the expedited development of cell therapy trials for CLI.

8. AIM OF THE STUDY

Critical limb ischemia (CLI) is the most advanced form of peripheral arterial disease and has serious clinical implications such as rest pain, intermittent claudication, and trophic lesions. Smokers, as well as patients with type 2 diabetes and cardiovascular diseases, are the most affected and have a high risk of amputation. Therapy can be pharmacological, but when the used drugs fail, it is required a surgical approach. In particular, the goals of surgical revascularization are to provide an adequate flow in the periphery to alleviate ischemic pain at rest or promote the healing of trophic lesions. However, the treatment and management for CLI patients must not only aim to rescue the limb through a peripheral revascularization intervention, but also to optimize medical therapy and to promote tissue repair and regeneration.

Recent studies have highlighted the role of endothelial progenitor cells (EPCs), or mesenchymal stem cells (MSCs) derived from bone marrow or peripheral blood in restoring tissue response. CLI patients not amenable to surgical or endovascular revascularization showed a clear improvement in the disease after treatment with cell concentrates extracted from autologous bone marrow. These improvements are evidenced by increased blood flow, increased tissue oxygen saturation, and significantly reduced pain. Furthermore, amputation has been avoided in 75% of cases.

The main purpose of this study has been to identify possible predictive biomarkers presumably released by stem cells derived from bone marrow for the therapy of no option critical ischemia in patients who have no other therapeutic options.

9. MATERIAL AND METHODS

9.1 Population Enrolment

The study included 22 patients from Vascular Surgery of Casa di Cura Villa dei Fiori - Acerra, affected by Critical Limb Ischemia (CLI). The inclusion criteria were: diagnosis of CLI in which the infiltration of bone marrow concentrate was expected; age> 18 years; ability to understand the treatment and acceptance of informed consent; patients in whom at least one of the following diagnostic criteria was found:

- occlusion of the ankle artery with absolute pressure <50 mmHg or ABI <0.4

- toe pressure <40 mmHg

- Initial non-hemodynamic macroangiopathic lesions (stage I)

- Intermittent claudication (stage II)

- arterial disease with pain at rest (stage III)

- vasculopathy with the presence of trophic lesions and / or gangrene (stage VI)

- tcpO2 <35% in ambient air.

The patient enrolled in therapy with the following drugs had to show at least one month of stability of the relative reference hematochemical parameters: Plavix/aspirin, TAO, statins, anti-hypertensive; Hct> = 28%, GB <= 14000/mmc, Plt> = 50000/mmc, INR <= 1.6 or PTT <1.5 x control (to avoid bleeding) NB patients on Coumadin therapy had to an INR <1.6 at the moment enrollment.

The exclusion criteria were: life expectancy <6 months for other diseases; history of hematological diseases (Non-Hodgkin's Lymphomas, Hodgkin's Lymphomas, Myelodysplastic Syndromes) which prevent bone marrow transplantation; chronic renal failure during the dialysis treatment; malignant neoplasms or changes in the following tests: PSA (age > 45 years), chest x-ray (age > 50 years), mammography (age > 40 years), PAP test (age > 21 years); decompensated Diabetes Mellitus (glycosylated hemoglobin > 10%); high anesthetic risk (ASA class IV, V); ischemic injury that puts the patient's life at risk and requires immediate amputation; complete occlusion of the

iliac axis not treated surgically or endovascularly, ipsilateral to the side to be treated; extensive necrosis of the lower limb to be treated or other conditions that make limb amputation inevitable; clinically active infection treated with antibiotics within 7 days of enrollment; treatment with immunosuppressive drugs (including Prednisone > 5 mg/day); women who were pregnant or breastfeeding.

Personal, anamnestic, and biochemical data were collected at the admission in hospital (T0) and after 30 days (T1) (glycaemia, creatininemia, alanine-amino-transferase, total and HDL cholesterolemia, triglyceridemia, total protidemia, ferritinemia, C-Reactive protein). Measurements of height, weight and abdominal circumference were taken. In all subjects, a peripheral blood sample and an aliquot of bone marrow aspirate were collected before and after cell separation with SEPAX-2 instrument. The bone marrow samples (2 mL of pre-concentration bone marrow aspirate and 2 mL of post-concentration bone marrow aspirate) have been used for:

- determination of the percentage of mesenchymal stem cells (MSCs) and endothelial progenitors (EPCs) by flow cytometry analysis (FACS) of specific surface antigens.

- the isolation and *in vitro* culture of MSC and EPC cells population.

- the determination of a panel of cytokines, chemokines, and growth factors by ELISA in Multiplex.

The study was approved by the local Ethics Committee. All procedures performed in the study were in accordance with the ethical standards of the institutional or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards and conformed to the Declaration of Helsinki on human research. All patients included in the study gave written informed consent after receiving an accurate explanation of the study protocol and of the potential risks related to the procedures adopted by the study.

9.2 Specimen collection – Sepax® System

Bone marrow samples were collected during surgery employing Sepax-2 technology. The Sepax® system equipment, combined with the kits for each application protocol, is a fully automated cell separation technology, with the CE ed approval by the American FDA. The Sepax cell separator is a medical device that processes blood or other biological matrixes, and its own components through a closed system, therefore sterile, safe, and operator independent. The Sepax operation is based on centrifugation, density gradient separation and extraction for optical detection using the latest generation of high-quality sensors frequency. The equipment also has the unique ability to automatically process small volume cells products. Sepax® technology is patented by Biosafe SA, protected by patents EUR 0912250 and US 6123655, and GMP-compatible. The application software included in the device has been developed specifically to manage processes of volume reduction and automated cell separation, ensuring their total safety. The Sepax system works in combination with dedicated, exclusive disposable separation kits Biosafe SA production. The Sepax disposable kit, an integral part of the Sepax system, offers a system closed and sterile circuit in which the cellular product is separated into its various components.

The software protocols represent an integral part of the Sepax system. The ReadyCell protocol is designed to isolate the fraction of total nucleated cells (TNC, Total Nucleated Cells) from bone marrow blood (30ml to 220ml) in a small final volume of 7-20 ml, with a processing time of about 20 minutes. The procedure involves the withdrawal of the medullary blood; the connection of the bag to the disposable kit (CS-470.1); the installation of the kit on the Sepax equipment; the automatic procedure with the priming and filtration phase; and finally, the cells are collected in a final vial present in the disposable kit. The disposable kit CS-470.1 is designed to process the bone marrow, with the aim of obtaining TNC fraction with a high cell concentration, placing the product final in a vial.

9.3 Cell isolation and growth

An aliquot (800 µL) of Peripheral Blood (PB) and Bone Marrow Aspirate samples were used for the isolation and characterization of mononuclear cells. For each sample, 8 mL of erythrocyte lysis solution (BD Pharm Lyse™ 10X) were added at room temperature and incubated for 15 minutes in the dark. The peripheral blood mononuclear cell (PBMC) fraction containing stem cells was obtained by centrifugation at 200 g for 5 min. After having carefully aspirated the supernatant, 1X PBS containing 1% heat-inactivated fetal bovine serum and 0,1% sodium azide (PBS-FBS) was added to each sample that was next centrifugated at 200 g for 5 min. Then, supernatant was carefully aspirated, and the pellet was resuspended in 1mL of PBS 1X. Then the cell count was performed using Trypan Blue dye (1:1) to assess the viability of the samples. For growth evaluation, cells were counted with the TC10TM automated cell counter (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol and divided equally in two aliquots that were plated in T-25 flasks in regular Dulbecco's modified Eagle's medium (DMEM)-F12 (1:1) supplemented with 20% fetal bovine serum, 2-mM glutamine, 100-unit/ml penicillin, and 100-unit/ml streptomycin for MSC isolation (Lonza Group Ltd, Basel, Switzerland) and EGM-2 Endothelial Medium BulletKit supplemented with 2% fetal bovine serum and specific growth factor (human Epidermal growth factor hEGF, vascular endothelial growth factor VEGF, R3-Insulin-like growth factor R3-IGF-1, Ascorbic acid, Hydrocortisone, human Fibroblast growth factor-beta hFGF-β, Heparin) for EPCs isolation (Lonza Group Ltd, Basel, Switzerland).

9.4 Flow cytometry analysis

Percentage and expression of endothelial and mesenchymal markers on isolated cells were analyzed by flow cytometry. Total BM and PB samples were suspended in 100µl phosphate-buffered saline (PBS) for analysis. Cells were incubated with PE-conjugated anti-CD73 antibody, FITC-conjugated anti-CD90 antibody, APC-conjugated anti-CD34 antibody, PE-conjugated anti-CD133 antibody and PE Vio770-conjugated anti-CD45 antibody as well as dye/isotype-matched antibodies (all from BD Biosciences, San Jose, CA, USA) in the dark for 20 min at 4C. Afterward, unbound antibodies were washed out and the samples were centrifuged at 200 g for 5 minutes. Then, the

supernatant was carefully aspirated, the pellet was resuspended in 400µl PBS and each sample processed by a BD LSR Fortessa (BD Biosciences, San Jose, CA, USA) and analyzed using BD FACS Diva software.

9.5 Determination of cytokines, chemokines, and growth factors

Samples obtained after 10.000 g for 10 minutes centrifugation were stored at -80°C until the assay was performed. Samples were screened for the concentration of interleukin (IL)-1ra, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, basic Fibroblast Growth Factor (FGF), Eotaxin, Granulocyte- Colony Stimulating Factor (G-CSF), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), Interferon-γ (IFN-γ), Interferon-γ inducible Protein 10 (IP-10), Monocyte Chemoattractant Protein-1 (MCP-1), Macrophage Inflammatory Protein-1 (MIP-1) α, MIP-1β, C-C motif Chemokine ligand 5 (CCL5)/RANTES, TNF-α, Platelet derived Growth Factor (PDGF-BB) and Vascular Endothelial Growth Factor (VEGF) by using the Bioplex multiplex Human Cytokine, Chemokine and Growth factor kit (cat. n. M500KCAF0Y, Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol, and as previously described in "Material and Methods - Study 1".

9.6 Statistical analysis

Statistical Analysis were performed using both R software platform (http://www.R-project.org/) and GraphPad Prism 8.4.2 software (GraphPad Software Inc., La Jolla, Ca).

The D'Agostino-Pearson normality test was performed to check whether continuous variables followed the normal (or gaussian) distributions model. According to the results obtained from the normality test, to check the differences among two continuous variables, t-test for normal distributions and Mann Whitney U test for non-normal distributions were performed. Simultaneously, paired t-test and Wilcoxon test were used for paired comparisons among two distributions. Comparisons between more than two variables were made using the Tukey corrected ANOVA test or the Kruskal-Wallis test with Dunn's post hoc adjustment. The correlations between two variables were obtained by either Pearson correlation test

or Spearman rho ranking test. To assess the Odds Ratio for variables, univariate logistic regression models were used.

All p-values <0.05 are considered statistically significant.

10. RESULTS

10.1 Patients Phenotyping

22 patients with CLI were recruited for this study. Clinical characteristics of the study population are reported in Table 9. Twenty patients were male (90.9%). The population had an overall mean age of 68.09 and a body mass index (BMI) of 26.9. Seven patients (31.8%) were smokers, and ten (45.5%) had Type-2 Diabetes.

Table 9. Clinical phenotyping of the population enrolled (N=22). Age and BMI are expressed as mean ± SD. Smoker and Diabetic patients are indicated as number and percentage.

Parameters [unit]	Total Population (N=22)
Age [years]	68.09 ± 11.04
Gender	M (90.9%)
BMI	26.93 ± 3.31
Smoker; Yes	7 (31.8%)
Diabetic; Yes	10 (45.5%)

Samples of bone marrow (BM) aspirate before cell separation (PRE-SEPAX), after cell separation (POST-SEPAX), and peripheral blood (PB) at baseline (T0) were collected for each patient.

10.2 Isolation and characterization of Bone Marrow Cells

BM and PB samples were isolated to collect mononucleated cells. After isolation, the obtained pellet was counted to assess cell number. Total POST-SEPAX cells increased by around 2.5-fold compared to total PRE-SEPAX cells, demonstrating the efficacy of SEPAX-2 instrument to obtaining the fraction of total nucleated cells with a higher cell concentration (Figure 23).

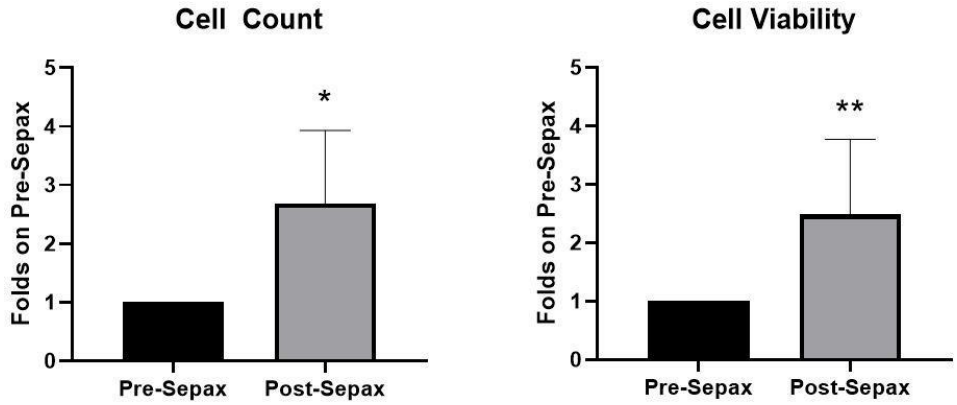

Figure 23. Total Bone Marrow cells count and viability. An aliquot of bone marrow aspirate collected before (PRE-SEPAX) and after cell separation with SEPAX-2 instrument (POST-SEPAX) were isolated. Total BM cells were counted, and the results were reported as fold over basal. The graph on the right reported the increase in viability in POST-SEPAX samples around 2.5-fold compared to PRE-SEPAX samples. Data in the graphs represent the mean ± SD of patients enrolled in the study. Asterisk denotes statistically significant differences (*p<0.05; **p<0.01).

Total BM samples and PB samples were analyzed to assess the presence of the different stem cell populations. In detail, the erythrocyte were lysed, and the mononucleated cells were incubated with the following antibodies: anti CD34, anti CD133 and anti CD45 for EPCs identification and anti CD90, anti CD73, anti CD45 for MSCs identification. The population was identified by analyzing the cellular physical parameters, therefore the side scatter (SSC) versus forward scatter (FSC). Then, it was determined the positive and negative population for the markers of interest. Briefly, for EPCs, the CD45dim population was selected, and next was defined as the percentage of this population that was simultaneously positive for CD133 and CD34. In addition, for MSCs, the population that was simultaneously negative for CD45 and positive for CD73 and CD90 was selected. The results show that the fraction of EPCs and MscS were statistically increased in BM samples compared to peripheral blood. Conversely, there were no differences in MSCs concentration between BM and PB cells (Table bottom 10).

10.3 Cytokines and growth factors screening in bone marrow PRE-SEPAX, POST-SEPAX and PB

Next, aliquots of bone marrow PRE-SEPAX, POST-SEPAX, and PB collected at baseline (T0) were used to evaluate a panel of growth factors, cytokines and chemokines such

as IL-1β (interleukin 1beta), IL-1rα, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, Eotaxin, FGF basic (fibroblast growth factor), G-CSF (granulocyte colony stimulating factor), GM-CSF (granulocyte macrophage- colony stimulating factor), IFN-γ (interferon gamma), IP-10 (interferon-gamma-inducible protein-10), MCP-1 (monocyte chemoattractant protein-1), MIP-1α, MIP-1β (macrophage migration inhibitory factor-1 alpha and 1 beta), PDGF-BB (platelet-derived growth factor), RANTES, TNF-α and VEGF (vascular endothelial growth factor) by a multiplexed ELISA assay (Bioplex). The results in Table 10 show that bone marrow had a significant increase in IL-1ra, bFGF, gCSF, IFNγ, MCP-1, MIP-1α, MIP-1β and PDGF compared to the peripheral blood sample at baseline. Notably, these inflammatory molecules have a significant correlation between systemic and bone marrow levels. In addition, EPC and MSC cells correlate positively between PB and BM levels (Table bottom 10).

Marker	Peripheral Blood	Bone Marrow	P-val	R (95% CI)	P-val
IL-1β	1.77 [1.03; 3.49]	1.55 [0.74; 8.75]	0.369	0.528 (0.008 – 0.824)	**0.035**
IL-1ra	805.28 [563.29; 1101]	1248 [805.28; 3214]	**0.028**	0.554 (0.113 – 0.813)	**0.011**
IL-2	2.61 [1.33; 5.38]	5.43 [4.05; 6.29]	0.144	0.546 (0.02 – 0.848)	**0.043**
IL-4	2.05 [1.51; 2.96]	2.23 [1.45; 3.57]	0.416	-0.02 (-0.459 – 0.426)	0.932
IL-5	25.89 [26.89; 52.17]	75.38 [60.17; 100.37]	0.068	-0.396 (-0.983 – 0.912)	0.604
IL-6	1.93 [1.42; 2.84]	7.48 [2.73; 21.95]	0.328	-0.408 (-0.888 – 0.498)	0.364
IL-7	15.25 [7.65; 28.91]	24.65 [15.23; 49.06]	0.296	0.620 (-0.579 – 0.971)	0.265
IL-8	8.97 [5.91; 18.37]	16.37 [9.4; 69.98]	0.252	0.787 (0.478 – 0.923)	**<0.001**
IL-9	361.08 [244.49; 440.81]	370.09 [224.96; 437.08]	0.168	0.493 (0.065 – 0.768)	**0.027**
IL-10	3.01 [1,27; 3.98]	6.04 [4.04; 7.54]	0.201	-0.461 (-0.768 – -0.016)	**0.047**
IL-12	1.41 [1.41; 5.33]	2.65 [0.76; 5.14]	0.777	0.736 (0.141 – 0.941)	**0.024**
IL-13	1.25 [0.57; 1.97]	0.88 [0.47; 1.18]	0.601	0.263 (-0.311 – 0.696)	0.364
IL-15	94.17 [44.31; 118.8]	256.39 [229.9; 291.18]	0.559	-0.02 (-0.887 – 0.877)	0.974
IL-17	9.47 [7.63; 16.47]	11.42 [8.49; 16.55]	0.162	0.464 (0.027 – 0.752)	**0.039**
Eotaxin	31.4 [25.44; 42.71]	101.69 [73.15; 195.55]	0.758	-0.173 (-0.572 – 0.292)	0.466
bFGF	38.05 [27.49; 42.02]	47.34 [32.97; 66.25]	**0.041**	0.082 (-0.374 – 0.506)	0.731
gCSF	56.44 [46.58; 74.8]	92.05 [62.27; 118.13]	**0.006**	0.068 (-0.386 – 0.496)	0.774
gmCSF	1.25 [0.37; 2.2]	2.68 [1.97; 4.23]	0.105	0.031 (-0.689 – 0.720)	0.942
IFNγ	8.88 [6.69; 11.24]	18.55 [14.98; 24.25]	**0.029**	0.237 (-0.229 – 0.615)	0.313
IP10	242.26 [185.58; 372.11]	216.16 [155.86; 323.32]	0.968	0.483 (0.051 – 0.762)	**0.031**
MCP-1	20.72 [15.95; 27.07]	130.59 [79.17; 160.73]	**0.03**	-0.082 (-0.506 – 0.374)	0.729
MIP-1α	2.38 [2.1; 2.76]	3.05 [1.9; 5.08]	**0.009**	0.519 (0.068 – 0.794)	**0.019**
MIP-1β	215.69 [184.83; 242.21]	227.84 [194.28; 251.71]	**0.009**	0.114 (-0.346 – 0.529)	0.632
PDGF	109.86 [54.2; 252.76]	216.07 [134.22; 331.82]	**0.029**	0.712 (0.394 – 0.878)	**<0.001**
RANTES	2118 [1133; 3883]	8441 [3446; 10958]	0.343	-0.002 (-0.444 – 0.441)	0.993
TNFα	34.64 [32.6; 46.87]	37.46 [32.44; 58.23]	0.662	0.504 (0.079 – 0.774)	**0.023**
EPC (%)	10 [2.5; 17.5]	26.6 [19.38; 35.55]	**<0.001**	0.506 (0.02 – 0.799)	**0.032**
MSC (%)	0.3 [0.17; 0.4]	0.4 [0.12; 0.67]	**0.05**	0.532 (0.012 – 0.826)	**0.034**
CD34+ (%)	2.1 [1.4; 3.2]	2.3 [1.7; 3.9]	0.363	0.498 (-0.109 – 0.835)	*0.083*
CD34/45 (%)	3.1 [1.3; 3.8]	2.2 [1.4; 3.4]	0.506	0.573 (0.017 – 0.867)	**0.041**

Table 10. PB and BM-related cytokines – PB and BM samples were isolated and analyzed by Bioplex assay. The concentration of cytokines, chemokines, and growth factors are expressed in pg/ml, as median values with interquartile ranges for each marker.

Table 11 shows the levels of cytokines and growth factors in the PRE-SEPAX and POST-SEPAX bone marrow samples. IL-1β, IL-1ra, bFGF, gCSF, IFNγ, MCP-1, MIP-1α, MIP-1β,

PDGF and RANTES increased by more 2.5-fold in bone marrow POST-SEPAX compared with PRE-SEPAX.

Marker	Bone Marrow PRE	Bone Marrow POST	BM POST/PRE FC	BM POST/PRE P-val
IL-1β	1.55 [0.74; 8.75]	3.2 [0.82; 6.96]	2.25 (1.18 – 3.33)	**<0.001**
IL-1ra	1248 [805.28; 3214]	5089 [2209; 11403]	5.96 (2.25 – 9.67)	**0.003**
IL-2	5.43 [4.05; 6.29]	5.84 [3.73; 7.88]	1.39 (1.04 – 1.82)	*0.077*
IL-4	2.23 [1.45; 3.57]	2.06 [1.49; 2.85]	1.31 (0.51 – 2.11)	0.424
IL-5	75.38 [60.17; 100.37]	115.29 [61.95; 177.44]	5.21 (-1.29 – 11.72)	0.179
IL-6	7.48 [2.73; 21.95]	6.82 [4.7; 17.37]	1.31 (0.44 – 2.17)	0.439
IL-7	24.65 [15.23; 49.06]	23.98 [16.73; 32.44]	0.86 (-0.14 – 1.87)	0.696
IL-8	16.37 [9.4; 69.98]	76.2 [25.84; 530.57]	14.33 (-6.54 – 35.21)	0.196
IL-9	370.09 [224.96; 437.08]	383.02 [266.03; 463.16]	1.31 (0.94 – 1.68)	*0.092*
IL-10	6.04 [4.04; 7.54]	6.9 [4.74; 9.91]	1.41 (0.97 – 1.85)	*0.07*
IL-12	2.65 [0.76; 5.14]	3.28 [1.41; 5.17]	14.49 (-3.66 – 32.63)	0.131
IL-13	0.88 [0.47; 1.18]	1.54 [0.88; 1.97]	2.49 (0.9 – 4.08)	*0.06*
IL-15	256.39 [229.9; 291.18]	331.46 [210.28; 432.7]	1.23 (0.75 – 1.72)	0.292
IL-17	11.42 [8.49; 16.55]	11.02 [4.5; 15.21]	0.97 (0.61 – 1.34)	0.892
Eotaxin	101.69 [73.15; 195.55]	88.75 [51.13; 189.01]	1.46 (0.39 – 2.54)	0.376
bFGF	47.34 [32.97; 66.25]	53.06 [44.24; 69.34]	1.39 (1.12 – 1.67)	**0.007**
gCSF	92.05 [62.27; 118.13]	135.33 [70.87; 255.84]	1.94 (1.36 – 2.52)	**0.003**
gmCSF	2.68 [1.97; 4.23]	4.24 [2.46; 4.98]	10.25 (-7.97 – 28.47)	0.299
IFNγ	18.55 [14.98; 24.25]	26.67 [19.38; 38.57]	1.65 (1.24 – 2.06)	**0.003**
IP10	216.16 [155.86; 323.32]	201.35 [112.71; 330.03]	1.08 (0.83 – 1.33)	0.504
MCP-1	130.59 [79.17; 160.73]	169.68 [113.22; 210.62]	2.02 (1.07 – 3.51)	**0.02**
MIP-1α	3.05 [1.9; 5.08]	6.11 [3.22; 9.52]	2.23 (1.49 – 2.97)	**0.002**
MIP-1β	227.84 [194.28; 251.71]	291.1 [219.27; 383.6]	1.55 (1.09 – 2.01)	**0.022**
PDGF	216.07 [134.22; 331.82]	399.81 [218.48; 1545]	4.35 (1.34 – 7.36)	**0.007**
RANTES	8441 [3446; 10958]	12866 [7861; 20938]	2.86 (1.33 – 4.39)	**0.019**
TNFα	37.46 [32.44; 58.23]	46.14 [33.72; 55.37]	1.15 (0.88 – 1.42)	0.248

Table 11. PRE-SEPAX and POST-SEPAX related cytokines – PRE-SEPAX and POST-SEPAX samples were isolated and analyzed by Bioplex assay. The concentration of cytokines, chemokines, and growth factors are expressed in pg/ml, as median values with interquartile ranges for each marker. The table also shows the fold change values and the associated p-value. Values in italics indicate trends close to statistical significance.

10.4 Basic evaluation of CLI patients

Diagnosis of CLI cannot be stressed enough as consequences of the high morbidity and mortality associated with the disease process itself. Although diagnosis of CLI is defined by clinical findings, it should be confirmed by other objective examinations including the transcutaneous partial pressure of oxygen (TcpO2) and treadmill test (TT). TT evaluates the patient's ability to ambulate, and the distance after which the patient is forced to stop because of severe pain and muscle cramps. TcpO2 and TT parameters were collected at baseline (T0) and after 30 days at follow-up (T1), to evaluate the efficacy of the infiltration of bone marrow concentrate. The ΔTT was obtained by subtracting ambulation distance at T0 from that at T1. A similar ratio is used for ΔtcpO2. At the end of follow-up patients improved their functions and a significant increase of both ΔTT and ΔtpcO2 was observed at T1 compared to T0 (Table 12).

Table 12. Clinical parameters of patients enrolled in the study. The Treadmill test is expressed as meter walked by patient and $TcpO_2$ is expressed as mmHg. The bold p-value indicates a statistically significant difference between T1 and T0 values ($p < 0.05$).
a = Wilcoxon matched-pairs test.

	T0	T1	ΔT	p-value (test)
TT (m)	990 [10; 1000]	1225 [275; 1500]	750 [245; 950]	**<0.0001[a]**
$tcpO_2$ (mmHg)	33 [15; 48]	34 [30; 64]	27 [7; 34]	**<0.0001[a]**

10.5 Correlation between clinical parameters and release of soluble factors

A correlation between ΔtcpO2 with a panel of 27 different cytokines, chemokines, and growth factors was performed, to verify whether the increase of soluble factors observed in serum at baseline could be a predictor of physical function recovery. In PB, bone marrow PRE-SEPAX and POST-SEPAX samples, inflammatory molecules such as IL-2 (Interleukin 2) and TNF-α (Tumor Necrosis Factor-alpha) negatively correlated with tcpO2 at baseline (IL-2 r: -0.627 p= 0.037; r: -0.449 p= 0.061; r: -0.560 p= 0.013) (TNF- α r: -0.528 p= 0.024; r: -0.507 p= 0.027; r: -0.505 p= 0.027) (Figure 25).

marker	Peripheral Blood		Bone Marrow PRE		Bone Marrow POST	
	r (95% CI)	p-value	r (95% CI)	p-value	r (95% CI)	p-value
IL-2	-0.627 (-0.724 – -0.284)	**0.037**	-0.449 (-0.767 – 0.047)	0.061	-0.560 (-0.821 - -0.106)	**0.013**
TNFα	-0.528 (-0.798 – -0.081)	**0.024**	-0.507 (-0.793 - -0.039)	**0.027**	-0.504 (-0.791 - -0.034)	**0.027**

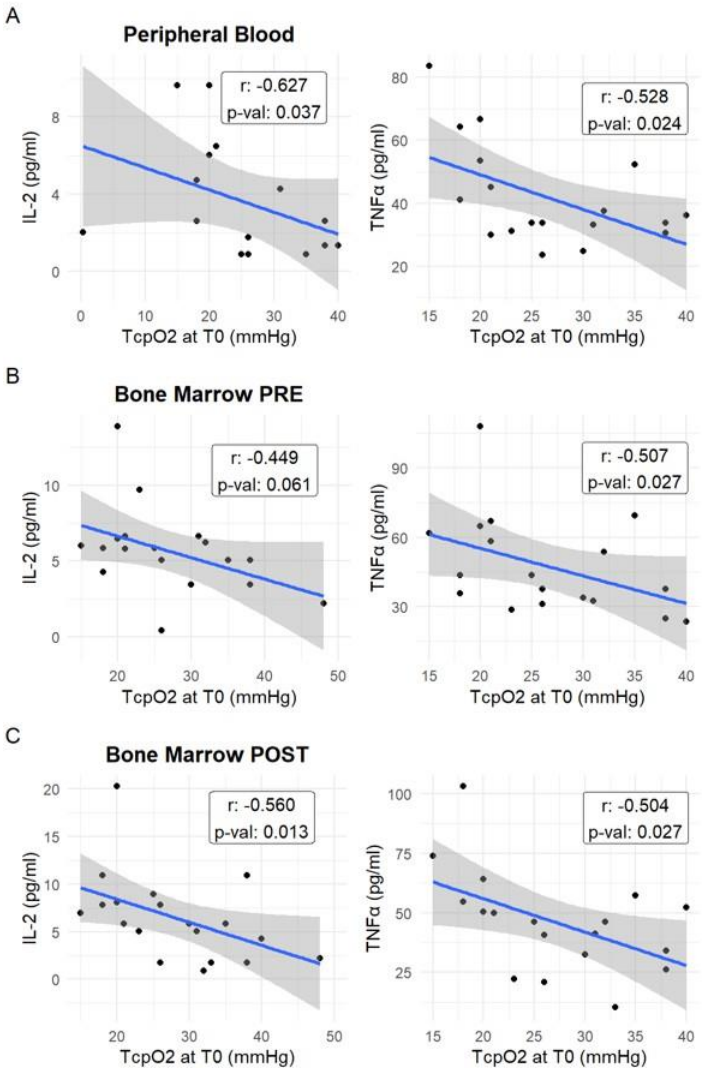

Figure 25. Correlation analysis in PB, PRE-SEPAX and POST-SEPAX samples among clinical parameter $tcpO_2$ and a panel of soluble factors at baseline. For each plot, a linear regression is reported, with a 95% confidence interval marked in grey, as well as ϱ Spearman correlation value with relative 95% CI. Every correlation reported is statistically significant (p<0.05).

Thus, the population was stratified according to the variation in outcome $tcpO_2$ from T0 to T1 ($\Delta TpcO_2$). Patients with $\Delta TpcO_2$ >17mmHg were classified as positive outcome $TcpO_2$. In PB, the concentration of IL-12 and MSC cells were significantly higher (p= 0.026, OR=0.035; p= 0.007 OR=0.05 respectively), compared to negative outcome $tcpO_2$ patients (<17mmHg). In bone marrow POST-SEPAX, IL-9 was a

negative biomarker (p= 0.017, OR= 0.047), while CD34+ (%) was higher in positive outcome $TcpO_2$ patients (Figure 26).

	$\Delta TpcO_2$ < 17mmHg	$\Delta TpcO_2$ > 17mmHg	P-val	OR (95% CI)	P-val
Peripheral Blood					
IL-12	1.41 [0.76; 1.41]	6.46 [3.99; 12.16]	**0.026**	1.31 (1.069 – 1.824)	**0.035**
MSC (%)	0.25 [0.2; 0.3]	0.45 [0.4; 0.57]	**0.007**	1.276 (1.009 – 1.429)	**0.05**
Bone Marrow POST					
IL-9	442.15 [346.63; 469.39]	273.49 [262.36; 281.94]	**0.017**	0.787 (0.581 – 0.957)	**0.047**
CD34+ (%)	1.4 [1; 1.9]	3.9 [2.4; 7]	**0.033**	1.806 (2.860 – 4.346)	**0.046**

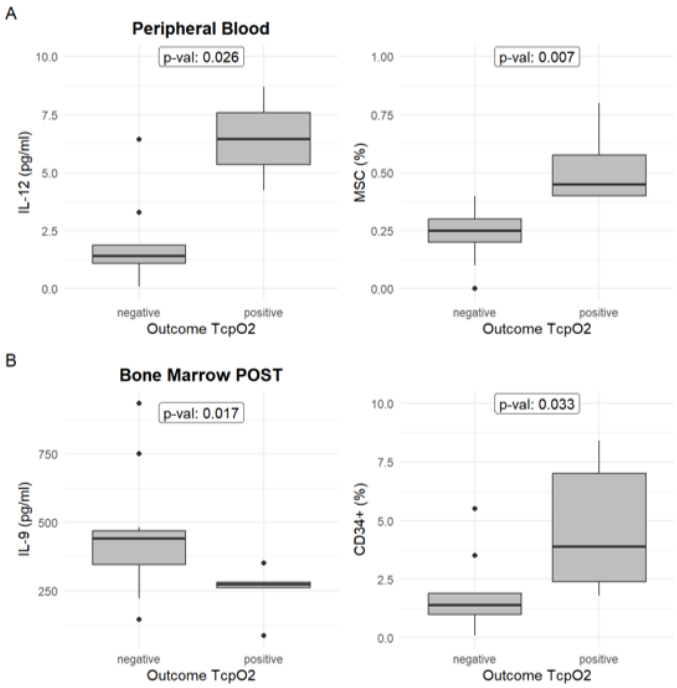

Figure 26. Relationship between $\Delta TpcO_2$soluble factors in PB and bone marrow POST-SEPAX. Population was stratified in negative and positive outcome $\Delta TpcO_2$. Boxplots depict concentrations in PB and bone marrow POST-SEPAX. Concentrations are expressed as pg/mL. The figure reports only those factors displaying statistical significance (p<0.05).

11. DISCUSSION

Critical Limb Ischemia (CLI) is a severe manifestation of peripheral artery disease (PAD), characterized by insufficient blood flow to the lower extremities, resulting in tissue ischemia, pain at rest, and an increased risk of limb loss. CLI has a significant impact on patients' quality of life, with the risk of amputation being one of the most devastating outcomes. Traditional therapeutic options include revascularization procedures, pharmacological interventions, and in some cases, amputation when other treatments fail. However, emerging regenerative therapies, particularly autologous stem cell transplantation, have demonstrated potential in restoring blood flow and promoting tissue regeneration.

CLI is a growing global health concern, particularly in elderly populations and individuals with diabetes mellitus, smoking history, or hypertension. According to the World Health Organization (WHO), the incidence of CLI is expected to increase with the aging population and the rising prevalence of lifestyle-related diseases. In the United States and Europe, CLI affects an estimated 1-2% of adults over the age of 65, with a significant burden on healthcare systems due to the high cost of treatment and long-term care. The global prevalence is even higher in low- and middle-income countries, where access to advanced diagnostic and treatment options is limited. CLI is often diagnosed at advanced stages, leading to increased healthcare costs and the potential for amputations[110,111].

In Italy, CLI is a leading cause of amputation, with an estimated 15,000-20,000 new cases reported annually. The prevalence of CLI is notably high among the elderly and individuals with comorbid conditions, particularly diabetes, which is a major risk factor for the disease. Despite the availability of modern revascularization techniques, including endovascular procedures, the rates of major amputations remain concerning. Approximately 10-15% of CLI patients in Italy require amputation due to non-healing ulcers, gangrene, or failure of revascularization treatments.

The application of autologous bone marrow stem cells in the treatment of CLI represents a promising and minimally invasive approach to improving limb salvage and reducing the risk of amputation.

Studies have shown that the infusion of autologous bone marrow-derived stem cells leads to significant improvements in blood flow, tissue oxygenation, and even clinical outcomes in some CLI patients [112,113].

Bone marrow is a rich source of stem cells, including hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). MSC cells have attracted attention due to their ability to differentiate into various cell types, such as endothelial cells, smooth muscle cells, and adipocytes, which are critical for vascular repair and regeneration. These cells also secrete a range of bioactive molecules, including growth factors, cytokines, and extracellular matrix components, which help modulate inflammation, promote angiogenesis, and facilitate tissue repair[114]. Bone marrow concentrates re-infused to patients include a cell fraction, consisting of MSCs and EPCs. Indeed, these cells can be isolated by the bone marrow and have emerged as potentially useful substrates for neovascularization and tissue repair and bioengineering. To date, it is unclear which cell type and source yields superior benefit on a per cell basis during the CLI. In any cell therapy application, it is essential to identify active cells that mediate beneficial effects. In addition, each patient has a different concentration of cells present in the bone marrow aspirate[115].

Study 2 focused on cellular and soluble components in bone marrow concentrates to identify some possible prognostic biomarkers in patients undergoing stem cell therapy. This study was conducted on 22 CLI patients and for each patient a bone marrow aspirate (BM) sample was collected before (PRE-SEPAX) and after (POST-SEPAX) cell separation with the SEPAX-2 instrument and a peripheral blood (PB) sample at baseline (T0). SEPAX-2 is a medical device, already used in vascular surgery with excellent clinical results, which enables automated cell separation in a sterile and safe manner. After cell isolation, a significant increase in count and viability cell was observed in POST-SEPAX bone marrow aspirates compared to PRE-SEPAX samples, confirming the effectiveness of this technology. As it is unclear which cells and/or molecules are involved in improving patient health, different cell populations in the bone marrow concentrate and PB collected at baseline were characterized by FACS analysis. Next, to verify the possible involvement of the soluble factors released by

the BM and PB samples in the revascularization process, a panel of 27 cytokines, chemokines and growth factors has been evaluated by ELISA in multiplex assay.

In PRE-SEPAX samples, there was a significant increase in IL-1ra, bFGF, gCSF, IFN-γ, MCP-1, MIP-1α, MIP-1β, and PDGF concentration compared to PB. EPC and MSC cells are significantly higher in bone marrow, compared to systemic levels. Finally, we have evaluated the same panel of cytokines, chemokines, and growth factors in bone marrow PRE-SEPAX and POST-SEPAX. IL-1b, IL-1ra, gCSF, IFN-γ, MCP-1, MIP-1α, and PDGF increased by more 2.5-fold after cell separation.

A correlation between the release of soluble factors in serum and bone marrow the transcutaneous partial pressure of oxygen ($TcpO_2$ mmHg) at the baseline has been performed. Results show a negative correlation for both IL-2 and TNF-α concentrations with TcpO2 in serum and bone marrow at T0. These pro-inflammatory cytokines stimulate a series of events that can increase oxygen demand in tissues involved in the immune response, increasing vascular permeability. During acute or chronic inflammation, activated immune cells (such as T-cells, in response to IL-2) and the production of reactive oxygen species (ROS) require more oxygen. However, limited perfusion reduces the ability to respond to this increased oxygen demand, leading to a decrease in $TcpO_2$[116,117].

Finally, $\Delta TcpO_2$ was detected one month after BMAC administration. The degree of response displayed variability among patients, and they were classified based on $TcoO_2$ variation. Outcome ΔTcpo2 positive patients showed a significant systemic increase in IL- 12 and MSC cells. Both parameters might represent positive biomarkers in responders' patients. In bone marrow samples, after separation, a decrease in IL-9 and in addition a significant increase in CD34+ hematopoietic stem cells were detected. These cells are essential for maintaining the balance of the blood cell population and are widely utilized in clinical settings, particularly in stem cell transplantation and gene therapy, where they can be harvested, expanded, and transplanted to treat various blood disorders, such as leukemia, lymphoma, and other hematological malignancies. Additionally, CD34-positive cells are important in regenerative medicine due to their potential to differentiate into other cell types under certain conditions.

In conclusion, different types of cell populations have been identified in bone marrow and peripheral blood collected from CLI patients. This study adds new information on cell-based applications that can improve pro-vascular function and be used as an alternative treatment option for CLI.

12. REFERENCES

1. Regenerative Medicine and the Developing World Heather L Greenwood, Peter A Singer, Gregory P Downey, Douglas K Martin, Halla Thorsteinsdóttir, Abdallah S Daar * Author information Article notes Copyright and License information PMCID: PMC1564180 PMID: 16968130.

2. A proposed definition of regenerative medicine Abdallah S Daar 1, Heather L Greenwood Affiliations Expand PMID: 18038409 DOI: 10.1002/term.20.

3. Progress in Regenerative Medicine: Exploring Autologous Platelet Concentrates and Their Clinical Applications Laura Giannotti 1, Benedetta Di Chiara Stanca 1, Francesco Spedicato 1, Paola Nitti 2, Fabrizio Damiano 1, Christian Demitri 2, Nadia Calabriso 3, Maria Annunziata Carluccio 3, Andrea Palermo 4, Luisa Siculella 1, Eleonora Stanca 1 Affiliations Expand PMID: 37761809 PMCID: PMC10530962 DOI: 10.3390/genes14091669.

4. Growth factors and cytokines in wound healing Stephan Barrientos 1, Olivera Stojadinovic, Michael S Golinko, Harold Brem, Marjana Tomic-Canic Affiliations Expand PMID: 19128254 DOI: 10.1111/j.1524- 475X.2008.00410.x.

5. Infiltration of plasma rich in growth factors for osteoarthritis of the knee short-term effects on function and quality of life Ana Wang-Saegusa 1, Ramón Cugat, Oscar Ares, Roberto Seijas, Xavier Cuscó, Montserrat Garcia- Balletbó Affiliations Expand PMID: 20714903 DOI: 10.1007/s00402-010- 1167-3.

6. Human Platelet-Rich Plasma Regulates Canine Mesenchymal Stem Cell Migration through Aquaporins Alessia Parascandolo 1 2, Michele Francesco Di Tolla 1, Domenico Liguoro 1 2, Manuela Lecce 1, Saverio Misso 3, Fabiana Micieli 4, Maria Rosaria Ambrosio 1 2, Serena Cabaro 1 2, Francesco Beguinot 1 2, Alessandra Pelagalli 5 6, Vittoria D'Esposito 1 2, Pietro Formisano 1 2 Affiliations Expand PMID: 37223543 PMCID: PMC10202607 DOI: 10.1155/2023/8344259.

7. Cytokines: From Clinical Significance to Quantification Chao Liu 1, Dewei Chu 1, Kourosh Kalantar-Zadeh 2, Jacob George 3, Howard A Young 4, Guozhen Liu 5 6 Affiliations Expand PMID: 34114369 PMCID: PMC8336501 DOI: 10.1002/advs.202004433.

8. Inflammatory cytokines in vascular dysfunction and vascular disease Alexander H Sprague 1, Raouf A Khalil Affiliations Expand PMID: 19413999 PMCID: PMC2730638 DOI: 10.1016/j.bcp.2009.04.029.

9. Platelet genomics and proteomics in human health and disease Iain C Macaulay 1, Philippa Carr, Arief Gusnanto, Willem H Ouwehand, Des Fitzgerald, Nicholas A Watkins Affiliations Expand PMID: 16322782 PMCID: PMC1297260 DOI: 10.1172/JCI26885.

10. Platelet-rich plasma preparation for regenerative medicine: optimization and quantification of cytokines and growth factors Paola Romina Amable, Rosana Bizon Vieira Carias, Marcus Vinicius Telles Teixeira, Italo da Cruz Pacheco, Ronaldo José Farias Corrêa do Amaral, José Mauro Granjeiro, Radovan Borojevic PMID: 23759113 PMCID: PMC3706762 DOI: 10.1186/scrt218.

11. Do autologous platelet concentrates (APCs) have a role in intra-oral bone regeneration? A critical review of clinical guidelines on decision-making process Marc Quirynen 1, Sam Siawasch 1, Andy Temmerman 1, Simone Cortellini 1, Rutger Dhondt 1, Wim Teughels 1, Anna B Castro 1 Affiliations Expand PMID: 37845802 DOI: 10.1111/prd.12526.

12. Schär, Michael O. MD1,2; Diaz-Romero, Jose PhD1; Kohl, Sandro MD2; Zumstein, Matthias A. MD2,a; Nesic, Dobrila PhD1. Platelet-rich Concentrates Differentially Release Growth Factors and Induce Cell Migration In Vitro. Clinical Orthopaedics and Related Research 473(5):p 1635-1643, May 2015. | DOI: 10.1007/s11999-015-4192-2.

13. Platelet-Rich Blood Derivatives for Stem Cell-Based Tissue Engineering and Regeneration Elham Masoudi # 1 2, João Ribas # 1 2 3, Gaurav Kaushik 1 2, Jeroen Leijten 1 2, Ali Khademhosseini 1 2 4 5 6 Affiliations Expand PMID: 27047733 PMCID: PMC4817373 DOI: 10.1007/s40778-016-0034-8.

14. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part I: technological concepts and evolution David M Dohan 1, Joseph Choukroun, Antoine Diss, Steve L Dohan, Anthony J J Dohan, Jaafar Mouhyi, Bruno Gogly Affiliations Expand PMID: 16504849 DOI: 10.1016/j.tripleo.2005.07.008.

15. Is Platelet-Rich Plasma a Future Therapy in Pain Management? Nebojsa Nick Knezevic 1, Kenneth D Candido 1, Ravi Desai 2, Alan David Kaye 3 Affiliations Expand PMID: 26614728 DOI: 10.1016/j.mcna.2015.08.014.

16. Comparative evaluation of leukocyte- and platelet-rich plasma and pure platelet-rich plasma for cartilage regeneration Zhengliang Xu 1, Wenjing Yin 1, Yuelei Zhang 1, Xin Qi 1, Yixuan Chen 1, Xuetao Xie 1, Changqing Zhang 1 Affiliations Expand PMID: 28265109 PMCID: PMC5339695 DOI: 10.1038/srep43301.

17. Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leucocyte- and platelet-rich fibrin (L-PRF) David M Dohan Ehrenfest 1, Lars Rasmusson, Tomas Albrektsson Affiliations Expand PMID: 19187989 DOI: 10.1016/j.tibtech.2008.11.009.

18. Use of PRP, PRF and CGF in Periodontal Regeneration and Facial Rejuvenation-A Narrative Review Eitan Mijiritsky 1 2, Haya Drora Assaf 3,

Oren Peleg 1, Maayan Shacham 4, Loredana Cerroni 5, Luca Mangani 5 Affiliations Expand PMID: 33920204 PMCID: PMC8070566 DOI: 10.3390/biology10040317.

19. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part II: platelet-related biologic features David M Dohan 1, Joseph Choukroun, Antoine Diss, Steve L Dohan, Anthony J J Dohan, Jaafar Mouhyi, Bruno Gogly Affiliations Expand PMID: 16504850 DOI: 10.1016/j.tripleo.2005.07.009.

20. Lecture, International academy of implant prosthesis and osteoconnection L Sacco - Lecture, 2006.

21. Analysis of CGF Biomolecules, Structure and Cell Population: Characterization of the Stemness Features of CGF Cells and Osteogenic Potential Eleonora Stanca 1, Nadia Calabriso 2, Laura Giannotti 1, Paola Nitti 3 4, Fabrizio Damiano 1, Benedetta Di Chiara Stanca 1, Maria Annunziata Carluccio 2, Giuseppe Egidio De Benedetto 4, Christian Demitri 3, Andrea Palermo 5, Franco Ferrante 6, Luisa Siculella 1, Alessio Rochira 1 Affiliations Expand PMID: 34445573 PMCID: PMC8396261 DOI: 10.3390/ijms22168867.

22. In vitro and in vivo effects of concentrated growth factor on cells and tissues Fahimeh Tabatabaei 1 2, Zahra Aghamohammadi 1, Lobat Tayebi 2 Affiliations Expand PMID: 32090458 DOI: 10.1002/jbm.a.36906.

23. Growth factors, CD34 positive cells, and fibrin network analysis in concentrated growth factors fraction Luigi Fabrizio Rodella 1, Gaia Favero, Ramon Boninsegna, Barbara Buffoli, Mauro Labanca, Giorgio Scarì, Luigi Sacco, Tiziano Batani, Rita Rezzani Affiliations Expand PMID: 21780251 DOI: 10.1002/jemt.20968.

24. Mesenchymal Stem Cells for Regenerative Medicine Yu Han 1,2,†, Xuezhou Li 1,2,†, Yanbo Zhang 3,*, Yuping Han 4,*, Fei Chang 1,*, Jianxun Ding 2 Author information Article notes Copyright and License information PMCID: PMC6721852 PMID: 31412678.

25. Recent advances in stem cell therapeutics and tissue engineering strategies Seong Gyu Kwon 1, Yang Woo Kwon 1, Tae Wook Lee 1, Gyu Tae Park 1, Jae Ho Kim 1 2 Affiliations Expand PMID: 30598836 PMCID: PMC6299977 DOI: 10.1186/s40824-018-0148-4.

26. Mesenchymal stem cells: a promising candidate in regenerative medicine Ye Chen 1, Jian-Zhong Shao, Li-Xin Xiang, Xue-Jun Dong, Guo- Rong Zhang Affiliations Expand PMID: 18295530 DOI: 10.1016/j.biocel.2008.01.007.

27. Immunoregulatory function of mesenchymal stem cells Antonio Uccelli 1, Lorenzo Moretta, Vito Pistoia Affiliations Expand PMID: 17013987 DOI: 10.1002/eji.200636416.

28. Isolation of putative progenitor endothelial cells for angiogenesis T Asahara 1, T Murohara, A Sullivan, M Silver, R van der Zee, T Li, B Witzenbichler, G Schatteman, J M Isner Affiliations Expand PMID: 9020076 DOI: 10.1126/science.275.5302.964.

29. The Ever-Elusive Endothelial Progenitor Cell: Identities, Functions and Clinical Implications CHAD L. BARBER AND M. LUISA IRUELA-ARISPE Molecular Biology Institute [C.L.B., M.L.I.-A.], Department of Molecular, Cellular and Developmental Biology [M.L.I.-A.], Jonsson Comprehensive Cancer Center [M.L.I.-A.], University of California, Los Angeles, Los Angeles, CA, 90095.

30. Concise Review: Endothelial Progenitor Cells in Regenerative Medicine: Applications and Challenges Mark Seow Khoon Chong 1, Wei Kai Ng 1, Jerry Kok Yen Chan 2 Affiliations Expand PMID: 26956207 PMCID: PMC4798740 DOI: 10.5966/sctm.2015-0227.

31. The role of biomaterials and scaffolds in immune responses in regenerative medicine: macrophage phenotype modulation by biomaterial properties and scaffold architectures Ezgi Antmen 1, Nihal Engin Vrana 2 3, Vasif Hasirci 1 4 5 Affiliations Expand PMID: 34762077 DOI: 10.1039/d1bm00840d Free article.

32. Polymeric Scaffolds for Dental, Oral, and Craniofacial Regenerative Medicine David T Wu 1 2 3 4, Jose G Munguia-Lopez 1 5, Ye Won Cho 2, Xiaolu Ma 1, Vivian Song 1, Zhiyue Zhu 5, Simon D Tran 1 Affiliations Expand PMID: 34834134 PMCID: PMC8621873 DOI: 10.3390/molecules26227043.

33. Sadasivuni K.K., Saha P., Adhikari J., Deshmukh K., Ahamed M.B., Cabibihan J.-J. Recent advances in mechanical properties of biopolymer composites: A review. Polym. Compos. 2020;41:32–59. doi: 10.1002/pc.25356.

34. Song J., Winkeljann B., Lieleg O. Biopolymer-based coatings: Promising strategies to improve the biocompatibility and functionality of materials used in biomedical engineering. Adv. Mater. Interfaces. 2020;7:2000850. doi: 10.1002/admi.202000850.

35. Biodegradable polymer scaffolds for tissue engineering L E Freed 1, G Vunjak-Novakovic, R J Biron, D B Eagles, D C Lesnoy, S K Barlow, R Langer Affiliations Expand PMID: 7764913 DOI: 10.1038/nbt0794-689.

36. The Role of Extracellular Matrix and Hydrogels in Mesenchymal Stem Cell Chondrogenesis and Cartilage Regeneration Magdalena Strecanska 1 2, Lubos Danisovic 1 2, Stanislav Ziaran 1 3, Michaela Cehakova 1 2 Affiliations Expand PMID: 36556431 PMCID: PMC9784885 DOI: 10.3390/life12122066.

37. Articular Cartilage: Structural and Developmental Intricacies and Questions Rebekah S Decker 1, Eiki Koyama 2, Maurizio Pacifici 2

Affiliations Expand •PMID: 26408155 • PMCID: PMC4624030 • DOI: 10.1007/s11914-015-0290-z.

38. Multiscale Strain Transfer in Cartilage Manuela A Boos 1, Shireen R Lamandé 2 3, Kathryn S Stok 1 Affiliations Expand PMID: 35186920 PMCID: PMC8855033 DOI: 10.3389/fcell.2022.795522.

39. Structure and Function of Articular Cartilage Harpal K.Gahunia and Kenneth P.H. Pritzker. in.

40. Current Applications of Mesenchymal Stem Cells for Cartilage Tissue Engineering Lizeth Fuentes-Mera, Alberto Camacho, Nidia K. Moncada- Saucedo and Víctor Peña-Martínez Additional information is available at the end of the chapter http://dx.doi.org/ 10.5772/intechopen.68172.

41. Repair and tissue engineering techniques for articular cartilage Eleftherios A Makris 1, Andreas H Gomoll 2, Konstantinos N Malizos 3, Jerry C Hu 1, Kyriacos A Athanasiou 4 Affiliations Expand PMID: 25247412 PMCID: PMC4629810 DOI: 10.1038/nrrheum.2014.157.

42. Diagnostic and therapeutical algorhythm in lower critical limb ischemia where immediate revascularization procedures are impossible - E. Melillo, M. Nuti, A. Balbarini. Diagnostic and therapeutical algorhythm in lower critical limb ischemia where immediate revascularization procedures are impossible. Trends Med 2006; 6(3):201-234. © 2006 Pharma Project Group srl - review.

43. Origin and function of cartilage stem/progenitor cells in osteoarthritis Yangzi Jiang 1, Rocky S Tuan 1 Author information Article notes Copyright and License information PMCID: PMC5413931 NIHMSID: NIHMS853536 PMID: 25536487.

44. An update on the pathogenesis and epidemiology of osteoarthritis David T Felson 1 Affiliations Expand PMID: 15049520 DOI: 10.1016/S0033- 8389(03)00161-1.

45. Recent Updates of Diagnosis, Pathophysiology, and Treatment on Osteoarthritis of the Knee Sunhee Jang 1,2,†, Kijun Lee 1,2,†, Ji Hyeon Ju 1,2,* Editor: Masato Sato Author information Article notes Copyright and License information PMCID: PMC7961389 PMID: 33807695.

46. Fundamentals of osteoarthritis: Inflammatory mediators in osteoarthritis Author links open overlay panel Astrid De Roover # 1 , Ana Escribano-Núñez # 1 , Silvia Monteagudo , Rik Lories # †.

47. Current Non-Surgical Curative Regenerative Therapies for Knee Osteoarthritis Ali Bahari Golamkaboudi # 1, Elham Vojoudi # 1, Kosar Babaeian Roshani 2, Pejman Porouhan 3, David Houshangi 4, Zahra Barabadi 5 Affiliations Expand PMID: 39145857 DOI: 10.1007/s12015-024- 10768-6.

48. Cartilage regeneration and inflammation modulation in knee osteoarthritis following injection of allogeneic adipose-derived mesenchymal stromal cells: a phase II, triple-blinded, placebo controlled, randomized trial Bahareh Sadri # 1, Mohammad Hassanzadeh # 2, Abolfazl Bagherifard 3, Javad Mohammadi 4, Mehdi Alikhani 1, Kasra Moeinabadi-Bidgoli 1, Hoda Madani 1, Dylana Diaz-Solano 1 5, Shahedeh Karimi 1, Mohammad
Mehrazmay 6, Mehdi Mohammadpour 2, Massoud Vosough 7 Affiliations Expand PMID: 37316949 PMCID: PMC10268462 DOI: 10.1186/s13287-023- 03359-8.

49. Current and novel theranostic modalities for knee osteoarthritis September 2021Sechenov Medical Journal 12(3) DOI: 10.47093/2218-7332.2021.293.03 LicenseCC BY 4.0 Bahareh SadriBahareh SadriShirin NouraeinShirin NouraeinNikoo hossein-khannazerNikoo hossein-khannazerShow all 5 authorsMassoud VosoughMassoud Vosough.

50. The use of PRP injections in the management of knee osteoarthritis Brendan O'Connell 1, Nicholas Martin Wragg 2, Samantha Louise Wilson 3 Affiliations Expand PMID: 30758709 DOI: 10.1007/s00441-019-02996-x.

51. Knee Osteoarthritis: Epidemiology, Pathogenesis, and Mesenchymal Stem Cells: What Else Is New? An Update Riccardo Giorgino 1 2, Domenico Albano 1, Stefano Fusco 3, Giuseppe M Peretti 1 4, Laura Mangiavini 1 4,
Carmelo Messina 1 4 Affiliations Expand PMID: 37047377 PMCID: PMC10094836 DOI: 10.3390/ijms24076405.

52. Platelet-rich plasma use in meniscus repair treatment: a systematic review and meta-analysis of clinical studies Ziquan Li 1 2, Xisheng Weng 3 4 Affiliations Expand PMID: 36209223 PMCID: PMC9548158 DOI: 10.1186/s13018-022-03293-0.

53. Platelet-Rich Plasma: New Performance Understandings and Therapeutic Considerations in 2020 Peter Everts 1, Kentaro Onishi 2,
Prathap Jayaram 3, José Fábio Lana 4, Kenneth Mautner 5 Affiliations Expand PMID: 33096812 PMCID: PMC7589810 DOI: 10.3390/ijms21207794.

54. Clinical importance of changes in chronic pain intensity measured on an 11-point numerical pain rating scale John T Farrar 1, James P Young Jr, Linda LaMoreaux, John L Werth, Michael R Poole Affiliations Expand PMID: 11690728 DOI: 10.1016/S0304-3959(01)00349-9.

55. Lifestyle and Dietary Habits Affect Plasma Levels of Specific Cytokines in Healthy Subjects Vittoria D'Esposito 1 2, Michele Francesco Di Tolla 2, Manuela Lecce 2, Francesco Cavalli 3, Michele Libutti 4, Saverio Misso 5,
Serena Cabaro 1 2, Maria Rosaria Ambrosio 1 2, Alessia Parascandolo 2,
Bianca Covelli 2, Giuseppe Perruolo 2, Mario Sansone 6, Pietro Formisano 1 2 Affiliations Expand PMID: 35811952 PMCID: PMC9270017 DOI: 10.3389/fnut.2022.913176.

56. White cell and platelet content affects the release of bioactive factors in different blood-derived scaffolds S Cabaro 1 2, V D'Esposito 1 2, R Gasparro 3, F Borriello 1 4, F Granata 1 4, G Mosca 1 2, F Passaretti 1 2, J C Sammartino 3, F Beguinot 1 2, G Sammartino 3, P Formisano 1 2, F Riccitiello 3 Affiliations Expand PMID: 28635382 DOI: 10.1080/09537104.2017.1319046.

57. Cytokine signature and COVID-19 prediction models in the two waves of pandemics Serena Cabaro # 1 2, Vittoria D'Esposito # 1 2, Tiziana Di Matola 3, Silvia Sale 3, Michele Cennamo 1, Daniela Terracciano 1, Valentina Parisi 1, Francesco Oriente 1, Giuseppe Portella 1, Francesco Beguinot 1 2, Luigi Atripaldi 3, Mario Sansone 4, Pietro Formisano 5 6 Affiliations Expand PMID: 34675240 PMCID: PMC8531346 DOI: 10.1038/s41598-021-00190-0.

58. Corder GWF, D.I. Nonparametric Statistics: A Step-by-Step Approach. Wiley. 2014.

59. Wilcoxon F. Individual comparisons by ranking methods. Biometrics Bulletin. 1945;1(6):80-3.

60. Diagnostic tests and ROC curves analysis]. | I test diagnostici e l'analisi della curva ROC. D'Arrigo Graziella;Torino Claudia;Zoccali Carmine;Tripepi Giovanni 2011.

61. Ventura, R. (2017). biostatistica . Egea.

62. Platelet-activated serum might have a therapeutic effect on damaged articular cartilage Munetaka Yokoyama 1, Masato Sato 1, Yoshiki Tani 1, Miyuki Yokoyama 1, Mami Kokubo 2, Masayuki Yamato 2, Teruo Okano 2, Joji Mochida 1 Affiliations Expand PMID: 28194878 DOI: 10.1002/term.2238.

63. Pure Platelet and Leukocyte-Platelet-Rich Plasma for Regenerative Medicine in Orthopedics-Time- and Preparation-Dependent Release of Growth Factors and Effects on Synovial Fibroblasts: A Comparative Analysis Erminia Mariani 1 2, Lia Pulsatelli 2, Luca Cattini 2, Paolo Dolzani 2, Elisa Assirelli 3, Annarita Cenacchi 4, Alessandro Di Martino 5, Carla Renata Arciola 1 6, Giuseppe Filardo 7 Affiliations Expand PMID: 36675025 PMCID: PMC9867505 DOI: 10.3390/ijms24021512.

64. Does intraoperative application of leukocyte-poor platelet-rich plasma during arthroscopy for knee degeneration affect postoperative pain, function and quality of life? A 12-month randomized controlled double-blind trial Christian Duif 1, Tobias Vogel, Fatma Topcuoglu, Georgios Spyrou, Christoph von Schulze Pellengahr, Matthias Lahner Affiliations Expand PMID: 25957981 DOI: 10.1007/s00402-015-2227-5.

65. Platelet Concentrates in Musculoskeletal Medicine Erminia Mariani 1 2, Lia Pulsatelli 1 Affiliations Expand PMID: 32079117 PMCID: PMC7072911 DOI: 10.3390/ijms21041328.

66. The role of MIP-1 alpha in the development of systemic inflammatory response and organ injury following trauma hemorrhage Chi-Hsun Hsieh 1, Michael Frink, Ya-Ching Hsieh, Wen-Hong Kan, Jun-Te Hsu, Martin G Schwacha, Mashkoor A Choudhry, Irshad H Chaudry Affiliations Expand PMID: 18684972 DOI: 10.4049/jimmunol.181.4.2806.

67. Growth-promoting action and growth factor release by different platelet derivatives F Passaretti 1, M Tia, V D'Esposito, M De Pascale, M Del Corso, R Sepulveres, D Liguoro, R Valentino, F Beguinot, P Formisano, G Sammartino Affiliations Expand PMID: 23855408 DOI: 10.3109/09537104.2013.809060.

68. Expanding applications of allogeneic platelets, platelet lysates, and platelet extracellular vesicles in cell therapy, regenerative medicine, and targeted drug delivery Thierry Burnouf 1 2 3, Ming-Li Chou 4 5, David J Lundy 4 6, Er-Yuan Chuang 4 6, Ching-Li Tseng 4 6, Hadi Goubran 7 Affiliations Expand PMID: 37704991 PMCID: PMC10500824 DOI: 10.1186/s12929-023-00972-w.

69. Substantial Variability in Platelet-Rich Plasma Composition Is Based on Patient Age and Baseline Platelet Count Luciano Rossi 1, Maximiliano Ranalletta 1, Ignacio Pasqualini 2, Juan Pablo Zicaro 1, Matías Costa Paz 1, Pablo Camino 3, Nicolas S Piuzzi 2 4 Affiliations Expand PMID: 37388884 PMCID: PMC10300586 DOI: 10.1016/j.asmr.2023.03.017.

70. The optimal platelet concentration in platelet-rich plasma for proliferation of human cells in vitro-diversity, biases, and possible basic experimental principles for further research in the field: A review Olav K Straum 1 Affiliations Expand PMID: 33240635 PMCID: PMC7668201 DOI: 10.7717/peerj.10303.

71. A Promising Candidate in Tendon Healing Events-PDGF-BB Yixuan Chen 1, Li Jiang 1, Kexin Lyu 1, Jingwei Lu 1, Longhai Long 2, Xiaoqiang Wang 2, Tianzhu Liu 3, Sen Li 2 Affiliations Expand PMID: 36291727 PMCID: PMC9599567 DOI: 10.3390/biom12101518.

72. Anti-inflammatory and Chondroprotective Effects of Platelet-derived Growth Factor-BB on Osteoarthritis Rat Models Yu Cai 1, Zhengchao Wang 2, Bokai Liao 3, Zhenxing Sun 4, Pengfei Zhu 5 Affiliations Expand PMID: 35640164 DOI: 10.1093/gerona/glac118.

73. Platelet-Derived Growth Factor-Functionalized Scaffolds for the Recruitment of Synovial Mesenchymal Stem Cells for Osteochondral Repair Yuan Luo 1 2, Xiaodong Cao 2, Junfeng Chen 2, Jianwei Gu 2, Hao Yu 1, Junying Sun 1, Jun Zou 1 Affiliations Expand PMID: 35126525 PMCID: PMC8813289 DOI: 10.1155/2022/2190447.

74. The resistance of certain tissues to invasion: penetrability of explanted tissues by vascularized mesenchyme R Eisenstein, N Sorgente, L W Soble, A Miller, K E Kuettner PMID: 4129060 PMCID: PMC1904085.

75. An appraisal of vascular endothelial growth factor (VEGF): the dynamic molecule of wound healing and its current clinical applications Aakansha Giri Goswami 1, Somprakas Basu 1, Farhanul Huda 1, Jayanti Pant 2, Amrita Ghosh Kar 3, Tuhina Banerjee 4, Vijay Kumar Shukla 5
Affiliations Expand PMID: 35584274 DOI: 10.1080/08977194.2022.2074843.

76. Vascular endothelial growth factor (VEGF) delivery approaches in regenerative medicine Nima Beheshtizadeh 1, Maliheh Gharibshahian 2, Mohammad Bayati 3, Reza Maleki 4, Hannah Strachan 5, Sarah Doughty 5, Lobat Tayebi 5 Affiliations Expand PMID: 37562236 DOI: 10.1016/j.biopha.2023.115301.

77. VEGF-attenuated platelet-rich plasma improves therapeutic effect on cartilage repair Jae Sung Lee 1, Ping Guo 2, Katarina Klett 2, MacGregor Hall 3, Krishna Sinha 3, Sudheer Ravuri 2, Johnny Huard 2, William L
Murphy 1 4 5 Affiliations Expand PMID: 35348136 PMCID: PMC9622215 DOI: 10.1039/d1bm01873f.

78. Old Player-New Tricks: Non Angiogenic Effects of the VEGF/VEGFR Pathway in Cancer Panagiotis Ntellas 1 2, Leonidas Mavroeidis 1 2, Stefania
Gkoura 1 2, Ioanna Gazouli 1 2, Anna-Lea Amylidi 1 2, Alexandra Papadaki 1 2, George Zarkavelis 1 2, Davide Mauri 1 2, Georgia Karpathiou 3, Evangelos Kolettas 4 5, Anna Batistatou 6, George Pentheroudakis 1 2 Affiliations Expand PMID: 33121034 PMCID: PMC7692709 DOI: 10.3390/cancers12113145.

79. VEGF may contribute to macrophage recruitment and M2 polarization in the decidua Karen C Wheeler 1, Manoj K Jena 1 2, Bhola S Pradhan 1, Neha Nayak 1, Subhendu Das 1, Chaur-Dong Hsu 1, David S Wheeler 3, Kang Chen 1, Nihar R Nayak 1 Affiliations Expand PMID: 29324807 PMCID: PMC5764356 DOI: 10.1371/journal.pone.0191040.

80. Lower Extremity Peripheral Artery Disease: Contemporary Epidemiology, Management Gaps, and Future Directions: A Scientific Statement From the American Heart Association Michael H Criqui, Kunihiro Matsushita, Victor Aboyans, Connie N Hess, Caitlin W Hicks, Tak W Kwan, Mary M McDermott, Sanjay Misra, Francisco Ujueta; American Heart Association Council on Epidemiology and Prevention; Council on Arteriosclerosis, Thrombosis and Vascular Biology; Council on Cardiovascular Radiology and Intervention; Council on Lifestyle and Cardiometabolic Health; Council on Peripheral Vascular Disease; and Stroke Council PMID: 34315230 PMCID: PMC9847212 DOI: 10.1161/CIR.0000000000001005.

81. Management of peripheral arterial disease and intermittent claudication R M Schainfeld 1 Affiliations Expand PMID: 11757887.

82. Lower extremity critical limb ischemia: A review of clinical features and management Scott R Levin 1, Nkiruka Arinze 1, Jeffrey J Siracuse 2 Affiliations Expand PMID: 31005554 DOI: 10.1016/j.tcm.2019.04.002.

83. Intermittent claudication: prevalence and risk factors W G Hughson, J I Mann, A Garrod PMID: 647301 PMCID: PMC1604804 DOI: 10.1136/bmj.1.6124.1379.

84. Update on peripheral artery disease: Epidemiology and evidence-based facts Jun Shu 1, Gaetano Santulli 2 Affiliations Expand PMID: 29843915 PMCID: PMC6113064 DOI: 10.1016/j.atherosclerosis.2018.05.033.

85. Epidemiology and Risk of Amputation in Patients With Diabetes Mellitus and Peripheral Artery Disease J Aaron Barnes 1, Mark A Eid 1, Mark A Creager 1, Philip P Goodney 1 Affiliations Expand PMID: 32580632 PMCID: PMC7377955 DOI: 10.1161/ATVBAHA.120.314595.

86. Epidemiology of Peripheral Artery Disease and Polyvascular Disease Aaron W Aday 1, Kunihiro Matsushita 2 3 Affiliations Expand PMID: 34110907 PMCID: PMC8202714 DOI: 10.1161/CIRCRESAHA.121.318535.

87. Relationship between serum homocysteine, fibrinogen, lipoprotein-a level, and peripheral arterial disease: a dose-response meta-analysis Hecheng Wang # 1, Pengpeng Wu # 1, Deying Jiang 2, Hao Zhang 1, Jian Zhang 3, Yu Zong 1, Yanshuo Han 4 Affiliations Expand PMID: 36411481 PMCID: PMC9677707 DOI: 10.1186/s40001-022-00870-1.

88. Advances in Revascularization for Peripheral Artery Disease: Revascularization in PAD Joshua A Beckman 1, Peter A Schneider 2, Michael S Conte 2 Affiliations Expand PMID: 34110904 DOI: 10.1161/CIRCRESAHA.121.318261.

89. The role of dynamic contrast-enhanced MRI in evaluation of percutaneous transluminal angioplasty outcome in patients with critical limb ischemia Nikolaos Galanakis 1, Thomas G Maris 2, Nikolaos Kontopodis 3, Christos V Ioannou 3, Konstantinos Tsetis 1, Apostolos Karantanas 1, Dimitrios Tsetis 4 Affiliations Expand PMID: 32516699 DOI: 10.1016/j.ejrad.2020.109081.

90. Epidemiology of peripheral artery disease Michael H Criqui 1, Victor Aboyans 2 Affiliations Expand PMID: 25908725 DOI: 10.1161/CIRCRESAHA.116.303849.

91. Novel transcutaneous sensor combining optical tcPO2 and electrochemical tcPCO2 monitoring with reflectance pulse oximetry Willem van Weteringen 1, Tom G Goos 2 3, Tanja van Essen 2, Christoph

Ellenberger 4, Josef Hayoz 4, Rogier C J de Jonge 2 5, Irwin K M Reiss 2, Peter M Schumacher 4 Affiliations Expand PMID: 31741291 PMCID: PMC6994448 DOI: 10.1007/s11517-019-02067-x.

92. Long-term mortality and its predictors in patients with critical leg ischaemia. The I.C.A.I. Group (Gruppo di Studio dell'Ischemia Cronica Critica degli Arti Inferiori). The Study Group of Criticial Chronic Ischemia of the Lower Exremities No authors listed PMID: 9314849.

93. Antithrombotic Therapy for Symptomatic Peripheral Arterial Disease: A Systematic Review and Network Meta-Analysis Loes H Willems 1, Dominique P M S M Maas 2, Kees Kramers 2 3 4, Michel M P J Reijnen 5, Niels P Riksen 2, Hugo Ten Cate 6 7, Rozemarijn J van der Vijver-Coppen 8, Gert J de Borst 9, Barend M E Mees 10, Clark J Zeebregts 11, Gerjon Hannink 12, Michiel C Warlé 8 Affiliations Expand PMID: 35997941 PMCID: PMC9499921 DOI: 10.1007/s40265-022-01756-6.

94. Quality of life in patients with no-option critical limb ischemia underlines the need for new effective treatment Ralf W Sprengers 1, Martin Teraa, Frans L Moll, G Ardine de Wit, Yolanda van der Graaf, Marianne C Verhaar; JUVENTAS Study Group; SMART Study Group Collaborators, Affiliations Expand PMID: 20598482 DOI: 10.1016/j.jvs.2010.04.057.

95. Concise Review: Cell Therapy for Critical Limb Ischemia: An Integrated Review of Preclinical and Clinical Studies Mohammad Qadura 1 2, Daniella C Terenzi 1 2, Subodh Verma 2 3, Mohammed Al-Omran 1 2, David A Hess 1 2 4 5 Affiliations Expand PMID: 29226477 DOI: 10.1002/stem.2751.

96. Angiogenesis in cancer, vascular, rheumatoid and other disease J Folkman 1 Affiliations Expand PMID: 7584949 DOI: 10.1038/nm0195-27.

97. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors M Peichev 1, A J Naiyer, D Pereira, Z Zhu, W J Lane, M Williams, M C Oz, D J Hicklin, L Witte, M A Moore, S Rafii Affiliations Expand PMID: 10648408.

98. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells Alexandra Aicher 1, Christopher Heeschen, Christiane Mildner-Rihm, Carmen Urbich, Christian Ihling, Katja Technau- Ihling, Andreas M Zeiher, Stefanie Dimmeler Affiliations Expand PMID: 14556003 DOI: 10.1038/nm948.

99. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand Beate Heissig 1, Koichi Hattori, Sergio Dias, Matthias Friedrich, Barbara Ferris, Neil R Hackett, Ronald G Crystal, Peter Besmer, David Lyden, Malcolm A S Moore, Zena Werb, Shahin Rafii Affiliations Expand PMID: 12062105 PMCID: PMC2826110 DOI: 10.1016/s0092-8674(02)00754-7.

100. Hyperglycemia reduces survival and impairs function of circulating blood-derived progenitor cells Nicolle Kränkel 1, Volker Adams, Axel Linke, Stephan Gielen, Sandra Erbs, Karsten Lenk, Gerhard Schuler, Rainer Hambrecht Affiliations Expand PMID: 15662022 DOI: 10.1161/01.ATV.0000156401.04325.8f.

101. Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta Pieternella S In 't Anker 1, Sicco A Scherjon, Carin Kleijburg- van der Keur, Godelieve M J S de Groot-Swings, Frans H J Claas, Willem E Fibbe, Humphrey H H Kanhai Affiliations Expand PMID: 15579651 DOI: 10.1634/stemcells.2004-0058.

102. Immunoregulatory mechanisms of mesenchymal stem and stromal cells in inflammatory diseases Yufang Shi 1 2, Yu Wang 3, Qing Li 3, Keli Liu 3, Jianquan Hou 4, Changshun Shao 4, Ying Wang 5 Affiliations Expand PMID: 29895977 DOI: 10.1038/s41581-018-0023-5.

103. Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation Pieternella S In 't Anker, Sicco A Scherjon, Carin Kleijburg-van der Keur, Willy A Noort, Frans H J Claas, Roelof Willemze, Willem E Fibbe, Humphrey H H Kanhai PMID: 12900350 DOI: 10.1182/blood-2003-04-1291.

104. Immunologic properties of human fetal mesenchymal stem cells Cecilia Götherström 1, Olle Ringdén, Charlotte Tammik, Eva Zetterberg, Magnus Westgren, Katarina Le Blanc Affiliations Expand PMID: 14749666 DOI: 10.1016/j.ajog.2003.07.022.

105. Biology of mesenchymal stem cells Ippokratis Pountos 1, Peter V Giannoudis Affiliations Expand PMID: 16188553 DOI: 10.1016/j.injury.2005.07.028.

106. HB-EGF/HER-1 signaling in bone marrow mesenchymal stem cells: inducing cell expansion and reversibly preventing multilineage differentiation Mauro Krampera 1, Annalisa Pasini, Antonella Rigo, Maria Teresa Scupoli, Cristina Tecchio, Giorgio Malpeli, Aldo Scarpa, Francesco Dazzi, Giovanni Pizzolo, Fabrizio Vinante Affiliations Expand PMID: 15755902 DOI: 10.1182/blood-2004-09-3645.

107. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement M Dominici 1, K Le Blanc, I Mueller, I Slaper-Cortenbach, Fc Marini, Ds Krause, Rj Deans, A Keating, Dj Prockop, Em Horwitz Affiliations Expand PMID: 16923606 DOI: 10.1080/14653240600855905.

108. Therapeutic potential for mesenchymal stem cell transplantation in critical limb ischemia Aaron Liew, Timothy O'Brien PMID: 22846185 PMCID: PMC3580466 DOI: 10.1186/scrt119.

109. Identification of the initial molecular changes in response to circulating angiogenic cells-mediated therapy in critical limb ischemia Lucia Beltran- Camacho 1 2, Margarita Jimenez-Palomares 1 2, Marta Rojas-Torres 1 2, Ismael Sanchez-Gomar 1 2, Antonio Rosal-Vela 1 2, Sara Eslava-Alcon 1 2, Mª Carmen Perez-Segura 1, Ana Serrano 1, Borja Antequera-González 1 2, Jose Angel Alonso-Piñero 1 2, Almudena González-Rovira 1 2, Mª Jesús Extremera-García 1 2, Manuel Rodriguez-Piñero 3, Rafael Moreno-Luna 4, Martin Røssel Larsen 5, Mª Carmen Durán-Ruiz 6 7 Affiliations Expand PMID: 32143690 PMCID: PMC7060566 DOI: 10.1186/s13287-020-01591-0.

110. Global vascular guidelines on the management of chronic limb-threatening ischemia Michael S Conte 1, Andrew W Bradbury 2, Philippe Kolh 3, John V White 4, Florian Dick 5, Robert Fitridge 6, Joseph L Mills 7, Jean-Baptiste Ricco 8, Kalkunte R Suresh 9, M Hassan Murad 10; GVG Writing Group Collaborators, Affiliations Expand PMID: 31159978 PMCID: PMC8365864 DOI: 10.1016/j.jvs.2019.02.016.

111. The burden of critical limb ischemia: a review of recent literature Steve Duff 1, Michael S Mafilios 2, Prajakta Bhounsule 3, James T Hasegawa 3 Affiliations Expand PMID: 31308682 PMCID: PMC6617560 DOI: 10.2147/VHRM.S209241.

112. Characteristics of responders to autologous bone marrow cell therapy for no-option critical limb ischemia Juraj Madaric 1 2, Andrej Klepanec 3, Martina Valachovicova 4, Martin Mistrik 5, Maria Bucova 6, Ingrid Olejarova 3, Roman Necpal 3, Terezia Madaricova 3, Ludovit Paulis 7, Ivan Vulev 3 4 Affiliations Expand PMID: 27530339 PMCID: PMC4987968 DOI: 10.1186/s13287-016-0379-z.

113. Characterization of mesenchymal stem cells of 'no-options' patients with critical limb ischemia treated by autologous bone marrow mononuclear cells Cestmir Altaner 1, Veronika Altanerova, Marina Cihova, Lubica Hunakova, Katarina Kaiserova, Andrej Klepanec, Ivan Vulev, Juraj Madaric Affiliations Expand PMID: 24069226 PMCID: PMC3771988 DOI: 10.1371/journal.pone.0073722.

114. Therapeutic angiogenesis by autologous adipose-derived regenerative cells: comparison with bone marrow mononuclear cells Changning Hao 1, Satoshi Shintani 2, Yuuki Shimizu 1, Kazuhisa Kondo 1, Masakazu Ishii 1, Hongxian Wu 1, Toyoaki Murohara 1 Affiliations Expand PMID: 25063790 DOI: 10.1152/ajpheart.00310.2014.

115. Novel autologous cell therapy in ischemic limb disease through growth factor secretion by cultured adipose tissue-derived stromal cells Hironori Nakagami 1, Kazuhisa Maeda, Ryuichi Morishita, Sota Iguchi, Tomoyuki Nishikawa, Yoichi Takami, Yasushi Kikuchi, Yukihiro Saito, Katsuto Tamai, Toshio Ogihara, Yasufumi Kaneda Affiliations Expand PMID: 16224047 DOI: 10.1161/01.ATV.0000190701.92007.6d.

116. Compared to Intermittant Claudication Critical Limb Ischemia Is Associated with Elevated Levels of Cytokines Juho Jalkanen 1, Mikael Maksimow 2, Maija Hollmén 2, Sirpa Jalkanen 2, Harri Hakovirta 1 Affiliations Expand PMID: 27611073 PMCID: PMC5017674 DOI: 10.1371/journal.pone.0162353.

117. Higher Levels of Cytokines in Patients with Chronic Limb-Threatening Ischemia Joana Ferreira 1, Susana Roque 2, Adhemar Longatto-Filho 3, Julieta Afonso 2, Alexandre Carneiro 4, Isabel Vila 5, Cristina Silva 5, Cristina Cunha 5, Amílcar Mesquita 6, Jorge Cotter 7, Margarida Correia-Neves 2, Armando Mansilha 8, Pedro Cunha 7 Affiliations Expand PMID: 38821475 DOI: 10.1016/j.avsg.2024.02.033.

Al FormiLab

Eccoci qui giunti al capolinea,
siamo agli sgoccioli del mio percorso di dottorato e tante cose sono cambiate dagli ultimi ringraziamenti (vedi tesi magistrale), a partire dalla fine del Covid19 e questa volta l'abbraccio non ce lo toglie nessuno!
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Dottorando	**Dr.ssa Romano Serena**
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