Chapter

The Rationale of Autologously Prepared Bone Marrow Aspirate Concentrate for use in Regenerative Medicine Applications

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Abstract

Autologously prepared bone marrow aspirate concentrates, have the potential to play an adjunctive role in various patient pathologies that have not been able to heal with conventional treatment modalities. The use of bone marrow aspirate (BMA) and concentrates in regenerative medicine treatment plans and clinical applications is based on the fact that bone marrow cells, including progenitor and nucleated cells, platelets, and other cytokines, support in tissue healing and tissue regenerative processes. The use of concentrated BMA cells focuses primarily on mesenchymal stem cells (MSCs), with the ability to self-renew and differentiate into multiple cell types. Concentrated bone marrow cells can be retrieved from harvested BMA and ensuing minimal manipulative cell processing techniques, executed at point of care (POC). The application of bone marrow biological therapies may offer solutions in musculoskeletal pathologies, spinal disorders, chronic wound care, and critical limb ischemia (CLI), to effectively change the local microenvironment to support in tissue healing and facilitate tissue regeneration. This chapter will address the cellular content of bone marrow tissue, harvesting and preparation techniques, and discuss the biological characteristics of individual marrow cells, their inter-connectivity, and deliberate on the effects of BMA concentration.

Keywords: regenerative medicine, bone marrow aspiration, niche microenvironment, bone marrow concentrate, centrifugation, hematopoietic stem cells, mesenchymal stem cells, differentiation, immunomodulation

1. Introduction

The objectives of regenerative medicine applications are to support the body to form new functional tissues to replace degenerative or defective ones and to provide therapeutic treatment for conditions where conventional therapies are inadequate. The human body has an endogenous system of regeneration through stem cells,

as they are found almost in every type of tissue. Regenerative medicine treatment options using autologous stem cells can be safely executed by well-trained physicians at point of care (POC). This review is not meant to be exhaustive, but our aims are to shed light on the bone marrow progenitor and stem cell mechanisms and highlight present and future applications of autologous bone marrow-derived stem cells in this exciting new regenerative medicine discipline.

In this chapter a definition is provided on embryotic and non-embryotic stem cells, followed by an intensive review of non-embryotic autologous adult stem cells. The use of allogeneic MSCs, the fabrication of engineered constructs by seeding of natural or synthetic scaffolds with cells, released from autologous tissues will not be presented in this chapter, as only relatively few of these cell-based approaches have entered the clinical arena. In particular, we deliberate on the biology and clinical application of mesenchymal stem cells originating from freshly harvested bone marrow. We portray on the techniques of a marrow harvesting procedure using ultrasound and fluoroscopic techniques. Explicit scientific information is provided on the bone marrow aspirate cellular content, their specific biological functions and intercellular interactions, as these, among others, contribute to tissue regeneration following clinical regenerative medicine applications. Furthermore, we underline the differences between bone marrow aspirate and, centrifugated, bone marrow aspirate concentrate injectates, both prepared at point of care from freshly aspirated marrow.

Finally, a condensed literature review addressing a variety of clinical orthobiological indications, spinal disorders, chronic wounds, and critical limb ischemia is provided. Regenerative medicine technologies, using marrow-derived mesenchymal stem cell-based therapies, as part of the regenerative medicine treatment armamentarium, offer solutions to a number of undeniable clinical conditions that have not been able to adequately result in a solution through the use of medicines or surgeries.

2. What are stem cells?

Becker, McCulloch, and Till first conducted experiments that lead to the discovery of stem cells in 1963. After injecting bone marrow cells into irradiated mice, nodules developed in proportion to the number of bone marrow cells injected, and they concluded that each nodule arose from a single marrow cell. At a later stage, they produced evidence that these cells were capable of endless self-renewal, which is as we know now, a fundamental feature of stem cells [1]. A stem cell is a type of cell that is non-specific/specialized in its function; in contrast, for instance, a heart or brain cell is functionally specific.

Generally, we recognize two types of stem cells, embryonic and non-embryonic, with two defining properties. Firstly, they have the capacity of self-renewal, therefore giving rise to more stem cells. Secondly, they are capable of differentiating into different lineages under appropriate conditions.

Embryonic stem cells (ESCs) are obtained from 5- to 12-day-old embryos, and they are pluripotent and have a high plasticity as they can differentiate into ectoderm, mesoderm, and endoderm layers, whereas non-embryonic stem cells (non-ESCs) are multipotent, and it appears that they are able to form multiple cell lineages which form an entire tissue, usually specific to one germ layer, e.g., adult stem cells [2].

The capability for stem cell potency, in combination with the relative ease to prepare bone marrow stem cell injectates, is an invaluable property for

regenerative medicine cell-based therapies in general and more specifically to treat, e.g. musculoskeletal disorders (MSK-D), chronic wounds, and critical limb ischemia.

2.1 Non-embryotic autologous adult stem cells

Non-ESCs are undifferentiated, and their proliferation potential compared to embryogenic stem cells is limited, as they have lost their pluripotent capability, placing them lower in the stem cell hierarchy. Nonetheless, it has been suggested that non-ESCs maintain their multipotent differentiation potential. Since they are categorized as allogenic products, they are commercially prepared from several allogenic sources, like amniotic fluid, umbilical cord, and Wharton's jelly [3]. In this chapter we will only deliberate on non-embryotic, autologous adult bone marrow aspirate (BMA)-derived progenitor/stem cells and other bone marrow (BM) stromal cells, prepared at POC with dedicated and approved centrifuges for BM concentration.

2.2 Bone marrow-derived stromal cells

Friedenstein and colleagues reported first on the isolation of bone marrow-derived stem cells from BM stroma and incubated it in plastic culture dishes and identified mesenchymal stem cells as colony-forming unit fibroblasts (CFU-Fs) [4]. The BM stroma is made up of a network of fibroblast-like cells and includes a subpopulation of multipotent cells which are able to generate the mesenchyme, known as the mass of tissue, that develops mainly from the mesoderm of the embryo subpopulation. The cells are referred to as mesenchymal stem cells (MSCs) [5]. The *Friedenstein* culture method revealed that MSCs are capable of differentiating into several connective tissue cell types [6], described first by Pittenger and associates [7].

3. Bone marrow anatomical structure

The bone is an organ composed of cortical and trabecular bone, cartilage, and hematopoietic and connective tissues. The bone tissue has an essential role in the structure and protection of the human body. Spongy, or trabecular bone, is composed of a lattice of fine bone plates filled with hematopoietic marrow, fat-containing marrow, and arterial-venous sinusoidal blood vessels. Furthermore, it consists of bone cells at different developmental stages (including pre-osteoblasts, osteoblasts, and osteocytes), collagen fibrils, and calcium and phosphate deposits [8]. Arterial vessels enter the marrow through foramina nutricia and then divide into several arterioles. Small arterioles and capillaries from these vessels span throughout the bone marrow and supply sinusoids, which are interconnected by inter-sinusoidal capillaries [9]. The BM tissue is soft, similar to the peripheral blood, flexible connective tissue comprising the center and the epiphysis of bones, referred to as the BM cavity. In this place a variety of new blood cells are produced and ultimately released to the peripheral circulation.

3.1 Red and yellow bone marrow

We recognize two categories of bone marrow tissue: the red and yellow marrow. Depending on age, the red marrow is replaced by the yellow marrow. In adults, the

red bone marrow is a rich source of bone marrow-derived cells and present in most skeletal system bones of the iliac crest, tibia, spine vertebrae, humerus, calcaneus, ribs, and near point of attachment of long bones of legs and arms. In this well-shielded environment, an estimate of 500 billion cells per day can be produced, in particular erythrocytes, granulocytes, and platelets [10]. Regenerative medicine applications have a focus on the use of the red bone marrow as it contains myeloid and lymphoid stem cells and MSCs. In contrast the yellow marrow consists primarily of fat cells with poor vascularity and is deprived of the multipotential MSCs [11].

3.2 Bone marrow-specific regions

The BM cavity in the trabecular bone is subdivided into four regions: endosteal, sub-endosteal, central, and perisinusoidal regions [12]. In **Figure 1**, the four regions, according to the model of Lambertsen and Weis, have been adopted and modified [13]. In general, the bone marrow consists of a hematopoietic component (parenchyma) and a vascular component (stroma). The parenchyma includes hematopoietic progenitor and hematopoietic stem cells (HSCs), which are localized close to the endosteum and around the blood vessels. BM stroma cells, including endothelial cells, are recognized as multipotential non-hematopoietic progenitor cells, capable of differentiating into various tissues of mesenchymal origin, including osteoblasts, chondrocytes, tenocytes, endothelial cells, myocytes, fibroblasts, nerves, and adipocytes, as verified in in vitro and partially in in vivo research [14, 15]. The bone marrow's microvasculature includes single layers of endothelium arising in sinusoids, where they also contribute in rolling extravasations of leukocytic cells into and out of the BM tissue structures. The function of the vasculature and BM-derived endothelial cells is that they provide a barrier between the BM compartment as a functional and spatial entity from the extra-lymphoid BM section and the peripheral circulation, as described by Kopp et al. [9]. The endothelial cells likewise contribute

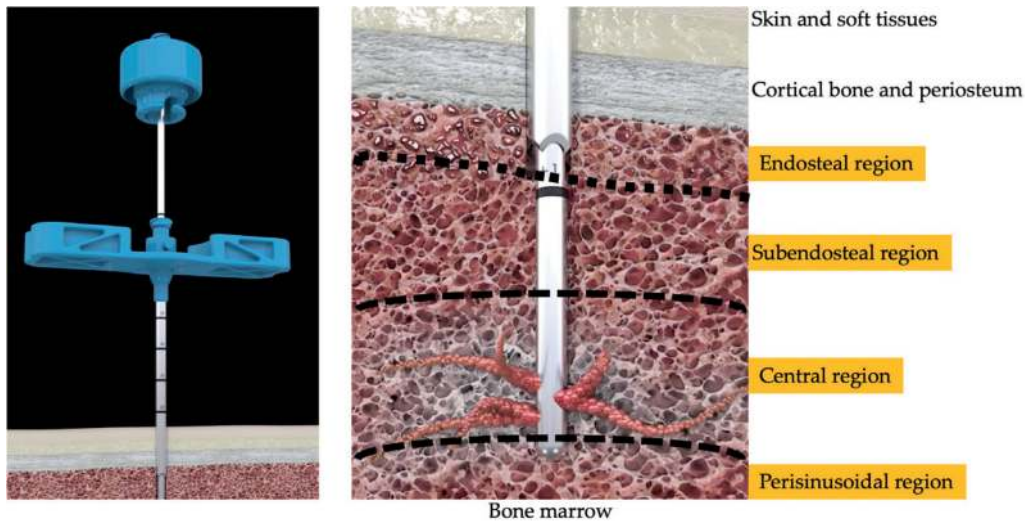

Figure 1.
Bone marrow subdivisions. On the left side, the Aspire introducer (Aspire Bone Marrow Harvesting System™, EmCyte Corporation, Fort Myers, FL, USA) has passed the cortical bone entering the marrow cavity. The harvesting cannula is inserted through the introducer in the marrow cavity. On the right side, a representation of the subdivisions in the bone marrow cavity subdivisions is indicated, showing the endosteal, sub-endosteal, central, and perisinusoidal regions. The endosteal and sub-endosteal regions compose the endosteal niche, harboring the proliferative and quiescent HSC-MSC niches. The marrow tissue is extracted via the side holes of the harvesting cannula (adapted and modified from Lambertsen and Weis [13]).

to tissue regeneration, as endothelial precursor cells are essential in improving vascularization of damaged and degenerative tissue cells by the secretion of pro-angiopoietic factors of invading cells [16].

3.3 Bone marrow niches

A *niche* is defined by anatomy and function. Stem cell niches are defined as specific cellular and molecular microenvironments regulating stem cell and progenitor functions. A niche consists of signaling molecules, intercellular contact, and the interaction between stem cells and their neighboring extracellular matrix (ECM). This three-dimensional microenvironment is thought to control genes and properties that define "stemness," including the control and balance between quiescence, self-renewal, proliferation, and differentiation of diverse cell types. Additionally, the microenvironment provides stem cell autonomous signaling mechanisms [17, 18], and it engages in specific cascades to a stress response [19]. Acquired and prepared BM stem cells from one of the niches and subsequently injected into a totally different microenvironment can potentially differentiate into cell types of this new environment [20]. Zhao et al. used a rat stroke model in which BM-MSCs were transplanted into neural tissues. They demonstrated that MSCs originating from the BM-MSC niche differentiated into neuronal cells after transplantation into the neural microenvironment [21]. Their observation revealed the plasticity potential of BM-MSCs, as well as the possible influence of the recipient niche, as BM-MSCs were capable of dedifferentiation into cells from other cell lineages. Their finding has potentially significant clinical implications for regenerative medicine applications overall. Since autologously prepared MSCs originate from their specific and original BM niche but are used in other cellular tissue types to treat various pathologies, they can be successfully engaged in tissue repair and regeneration through regenerative medicine application techniques. This is a distinctly different approach in the physiological release of newly formed BM cells, because they are retained in the BM cavity until they mature and thereafter released in the vascular peripheral circulation [15].

3.3.1 Hematopoietic and mesenchymal niche

HSC niches are present in various (prenatal) tissues, like the aorta-gonad-mesonephros region and the yolk sac, followed by the placenta, fetal liver, spleen, and bone marrow. Postnatally, the bone marrow is the primary site of HSC presence [22]. The model of the HSC niche was first described by Schofield in 1978 [23], later confirmed by others, to describe the physiologically limited microenvironment in which HSCs, MSCs, and their progenitors reside in the bone cavity where they are enfolded by BM stromal cells [24], covered by the bony structure of the BM cavity. The stem cell niches in bone have been extensively described by Yin and Li, providing insights into the actions of osteoblastic and vascular niches, revealing central roles for numerous signaling and adhesion molecules [25]. A significant portion of these hematopoietic cells is found next to the endosteal bone surface, designating a clear role for osteoblasts in the regulation of HSCs and thus hematopoiesis [26]. Based on flow cytometry research by Kiel et al., HSCs are more likely than other hematopoietic cells to be immediately adjacent to a sinusoid, in the trabecular region of the BM [27]. This location suggests that HSCs and their niche may be directly, or indirectly, regulated by factors present near the bone planes. The HSC niche is comprised of many different niche constituents including osteoclasts, endothelial cells, fibroblasts, adipocytes, and the HSC progenitor cells [28].

3.3.2 Perivascular niche

The BM is highly vascularized, with large central arteries branching into progressively smaller microvessels like arterioles and transitioning into venous sinusoids near the bone (endosteal) surface. Therefore, it has been suggested that HSCs are maintained in a perivascular niche by endothelial or perivascular cells, as they are frequently located adjacent to the blood vessels [29]. These occurrences resulted in the expression of various perivascular mesenchymal cell makers CD146, stromal cell-derived factor-1 (SDF-1) also referred to as CXCL12, and Nestin-GFP, defining the heterogenous BM stroma cell composition [9], including the MSCs that surround the blood vessels [30]. The more perivascular nature of MSC niches was validated by Shi and Gronthos, demonstrating the expression of α-smooth muscle actin (αSMA) at perivascular sites, with the immunohistochemical localization of specific CD marker cells [31]. Mores studies confirmed the presence of MSCs in BM, expressing a Nestin-GFP transgene, localized and attached around the BM blood vessels and part of the perivascular HSC niche [32]. Kunisaki et al. indicated that most HSCs do not only have a perivascular presence, but they are preferentially located in the BM endosteal regions. The endosteal regions contain a complex network of stromal cells as well that have been implicated in HSC maintenance, including arteriolar and venous endothelial cells, pericytes, and chemokine (C-X-C) ligand 12 (CXCL12) reticular cells. Their study also suggested that quiescent HSCs localize preferentially to small arterioles near the endosteum, suggesting that distinct niches may exist for both quiescent and proliferating HSCs [33]. From all these findings, it can be concluded that pericytes are in fact MSCs, because they can differentiate in osteoblasts, chondrocytes, and adipocytes [34].

3.3.3 Megakaryocyte niche

Megakaryocytes (MK) are the precursor cells of blood platelets. BM hematopoietic cells are responsible for platelet production. MK may regulate HSCs indirectly as they are closely associated with BM sinusoidal endothelium, extending cytoplasmic protrusions into the sinusoids to produce platelets. A direct regulation of HSC by MK through signaling of transforming growth factor beta 1 was established, with activation of quiescent HSCs and increased proliferation rate. In the event of a sudden response to systemic stress signaling, fibroblast growth factor-1 as part of the MK growth factor pool will start signaling HSCs and will overshadow the TGF-b1 signaling in order to stimulate high volumes of HSC expansion [35].

3.4 Extracellular matrix

The role and function of the extracellular matrix (ECM) can be defined as key structural-functional components of cell niches, including soluble factors, cell-cell contacts, and cell-matrix adhesions present in these microenvironments. ECM components include fibrillar proteins, with, among others, collagen fibers, fibronectin, and other filamentous network components. The ECM's mechanical stability is provided by collagen [36]. Other significant ECM components supporting the BM niches are glycosaminoglycans and mainly hyaluronic acid via its receptor CD44. The surface marker is also expressed by MSCs and HSCs [37]. Intracellular signaling in the ECM occurs through cytokine and growth factor membrane receptors, similar to the MSC niche. These cytokines and receptor activities contribute to cross

talk between ECM components and MSC niches, provoking cell differentiation. For instance, Djouad et al. demonstrated that the induction of MSC differentiation towards chondrocytes in articular cartilage was induced and/or influenced by molecules from both the MSC niche and the ECM components of this microenvironment, leading to chondrogenic differentiation of MSCs [38]. Other studies suggested that ECM deposited by microvascular endothelial cells enhances MSC endotheliogenesis [39]. In general, no specific ECM components are identified that maintain MSCs in their immature state, as a niche matrix would do. However, it has become clear that the ECM can regulate MSC differentiation on a solitary basis, indicating potential applications for regenerative medicine applications and tissue engineering.

4. Bone marrow aspiration procedures

Exploiting BM preparations at POC seeks to overcome the limitations of ex vivo MSC culturing. Clinicians utilizing regenerative medicine applications have a growing interest in using the concentrated bone marrow products, since it is well acknowledged that BM is a plentiful source of MSCs, progenitors, and other cells residing in the trabecular part of flat and long bones, acquired via appropriately performed BMA procedures [40, 41]. The regenerative medicine market is rapidly growing, with many procedures performed in musculoskeletal, orthopedic, and spinal disorders, wound care management including critical limb ischemia, and tissue engineering [42–45]. Several groups have mentioned some considerations when performing BM harvesting procedures, addressing a variety of factors that have an impact on patient comfort and the quality of the harvested BM. Emphasis was given to procedural safety issues when using harvesting needle systems, level of experience of the operator, the choice for concentration technology and centrifugation devices, and pain management [46]. Autologous regenerative medicine BM-MSC applications may range from a harvesting a low volume of BM and direct, unprocessed, tissue injection to the use of centrifugation protocols to concentrate and filter the BMA prior to injecting it in patients [47].

4.1 Bone marrow harvesting needle systems

Various bone marrow needle harvesting systems are available on the market, each with their own proprietary design characteristics and thus marrow aspiration dynamics when transferring marrow cavity cells through a needle system into collection syringes. In **Figure 2**, three different needle systems are shown. Potentially, different needle design features might affect the quality and viability of the harvested BM tissue, as well as the cellular yields. Therefore, BM needle system features and harvesting dynamics are important considerations when considering BMA procedures. Physicians have been using a variety of harvesting needles during the last decades, including the traditional Jamshidi™ harvesting needle (Ranfac Corporation, Avon, MA, USA). Based on design differences, not every BMA is born equal, and cellular yields, composition, and viability might vary among harvesting devices. For interpretation purposes, some of the cellular difference between two newly developed BMA needle harvesting systems, the Aspire Bone Marrow Harvesting System™ and the Marrow Cellution Bone Marrow Aspiration Device™ (EmCyte Corporation, Fort Myers, FL, USA, and Ranfac Corporation, Avon, MA, USA, respectively) is shown. A significant difference between the two harvesting

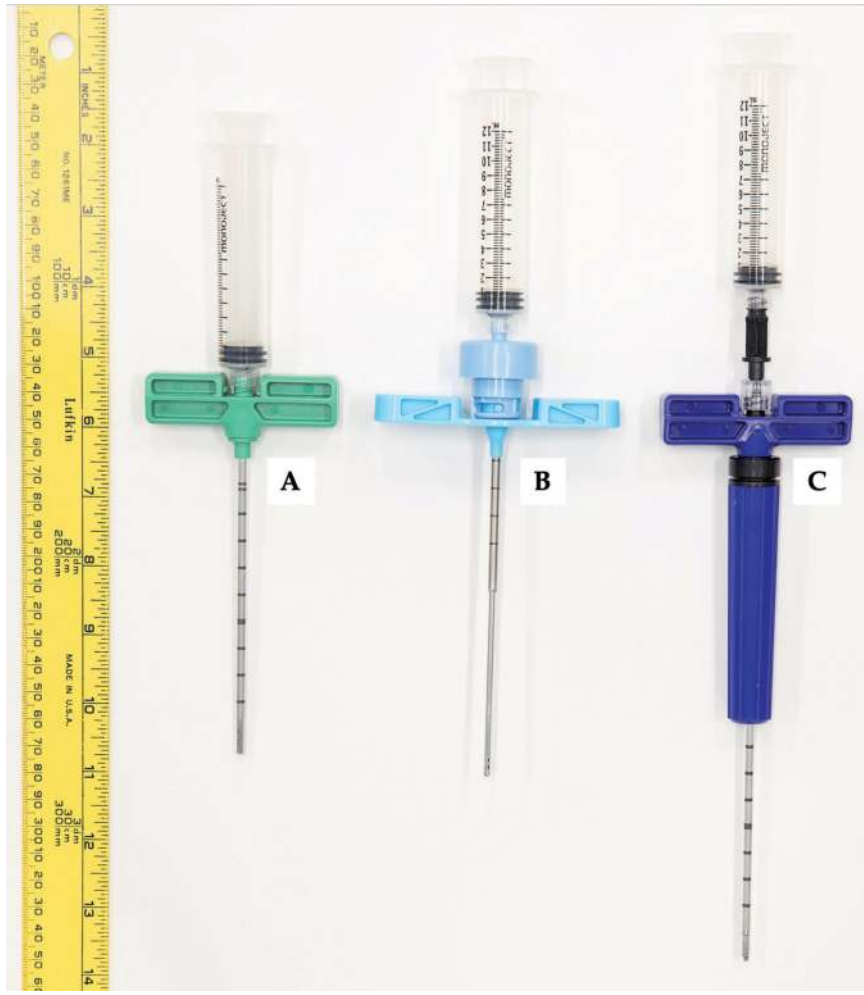

Figure 2.
Bone marrow aspiration devices. (A) Jamshidi™ device (Ranfac Corporation, Avon, MA, USA), with a sharp and open distal tip, allows for more peripheral blood aspiration. (B) Aspire Bone Marrow Harvesting System™ (EmCyte Corporation, Fort Myers, FL, USA) consists of a separate trocar introducer and aspiration needle with a completely closed and blunt tip. The side holes of the aspiration needle are designed to minimize cellular trauma and hemolysis during aspiration. (C) Marrow Cellution Bone Marrow Aspiration Device™ (Ranfac Corporation, Avon, MA, USA) is used as an aspiration device only, to aspirate 10 ml of bone marrow, followed by unfiltered injection.

systems is that the Marrow Cellution device is developed and used by physicians for BMA aspiration with direct injection only, without filtration or processing prior to patient injection [47]. Therefore, these specimens mimic the marrow cavity cellular content and their specific cell concentrations. This includes a red blood cell (RBC) content and hematocrit which is similar to the peripheral blood values. Conditional negative forces occur with the syringe pull during marrow aspiration; this particular BMA injectate can have high plasma-free hemoglobin (PFH) concentrations, which cannot be removed from the injectate. The Aspire™ harvesting system is designed to penetrate the trabecular bone, maintaining a quiescent tissue environment during deployment and collection, contributing to a reduction in tissue activation, plasma-free hemoglobin content, and clotting. The Aspire™ harvesting system is intended to provide a BMA for centrifugation processing, leading in this occasion to the creation of PurePRP SupraPhysiologic BMC® (EmCyte Corporation, Fort Myers, FL, USA). In **Table 1**, comparative laboratory data between the abovementioned needle systems in a bilateral patient harvesting model are disclosed.

Laboratory parameters	BMA-MC 10 ml	BMA-A 10 ml	BMC 11 ml
TNC × 10^6/mL	25.8	31.8	73.7
Platelets × 10^6/mL	117	117	576
CD34+ (HSCs) × 10^5/mL	1.42	1.12	2.51
CFU-F (MSCs) × 10^3/mL	446	1.13	837
Hematocrit %	36.2	36.2	9.8
RBCs × 10^9/mL	4.02	4.08	1.44
PFH mg/dl	913	721	299
Hemolysis %	4.6	3.2	1.6
Cell viability %	94.4	94.4	96.8

BMA-MC, bone marrow aspirate Marrow Cellution System; BMA-A, bone marrow aspirate Aspire System; BMC, bone marrow concentrate; TNC, total nucleated cells; CD34+, hematopoietic stem cell marker/expression in bone marrow; CFU-F, colony-forming unit fibroblast: assay for bone marrow mesenchymal stem cell analysis, MSCs, mesenchymal stem cells. (BMA-MC, Marrow Cellution Device™—Ranfac Corporation, Avon, MA, USA; BMA-A, Aspire Bone Marrow Harvesting System™—EmCyte Corporation, Fort Myers, FL, USA).

Table 1.
Comparative quantification between two different bone marrow aspiration systems and bone marrow concentrate, in a bilateral patient model.

4.2 Large vs. small BMA collection syringes

In theory, a larger-volume BMA collection syringe should produce a stronger negative pressure and therefore harvest more MSCs. However, Hernigou et al. found that the aspiration of only 10–20% of the full syringe volume resulted in a higher MSC concentration in both 10 and 50 ml syringes, indicating that high-quality harvesting of MSCs requires a significant negative pressure in the marrow cavity to liberate MSCs. They also concluded that the collection of MSCs decreased as the syringe was filled, at a lower negative pressure [40]. Therefore, smaller syringes and thus smaller aspiration volumes result in higher MSC concentrations than with larger aspiration volumes [48]. This translates to the physical equation, "Negative Pressure = Pull Force/Plunger Surface Area," resulting in the fact that with the same pull force and a smaller diameter syringe plunger, a higher negative pressure is created [49]. Lately, the authors use 10 ml syringes, employing a fast and intermittent pull technique to collect small volumes from different intra-trabecular sites (**Figure 3**). This is in accordance with a trend towards small-volume HPD aspiration techniques [50]. Another advantage for using 10 ml syringes is that anticoagulation protocols can be better managed. Smaller syringes fill considerably quicker than larger syringes, and smaller syringes can be adequately agitated with the anticoagulant to avoid clotting.

4.3 Image-guided aspiration

In order to perform BM-MSC procedures, a certain volume and quality of marrow tissue are required in order to prepare a bone marrow concentrate (BMC). The aspiration volume is contingent on the processing volume of the BMC concentration system that is being used. It is imperative to locate precisely the donor site, as MSCs are located in the marrow cavity subcortical area and around the blood vessels [19]. The precise delivery of local anesthetics and safe trocar placement are accomplished by using image guidance during aspiration procedure. In the following section, we focus on the posterior super iliac spine (PSIS) sites, as it is the most frequently reported anatomical site for BMA.

Figure 3.
Bone marrow aspiration. After the aspiration needle has been advanced in the marrow cavity, the marrow is extracted using a firm, but a gentle, aspiration pressure is applied to the 10 ml syringe. The aspiration needle is easily rotated to collect marrow from a fresh area.

4.3.1 Ultrasound imaging

When the PSIS is targeted, patients are positioned in the prone position, while avoiding lumbar lordosis. Sonographic assessment using a portable ultrasound system with a 5–2 MHz low-frequency curvilinear transducer is positioned in a transverse plane over the hyperechoic bilateral sacral cornua, with the patient lying prone and the monitor screen in the line of sight of the operator. The probe is then translated contralaterally from the physician, keeping the hyperechoic sacrum visualized. Next, the probe is translated proximally, with the hyperechoic ilium coming into view, while maintaining the hyperechoic sacrum, until the most superficial depth of the ilium is reached, known as the PSIS, contralateral to the examiner [51]. After identification of the PSIS, the most superficial depth is confirmed in both transverse and longitudinal orientation (**Figure 4**). With the probe in the transverse plane at the PSIS, the slope of the iliac wing is noted for correct angulation of the BM trocar, and the most superficial depth of the PSIS is brought under the most medial aspect of the ultrasound probe. Using a sterile marker, a mark and

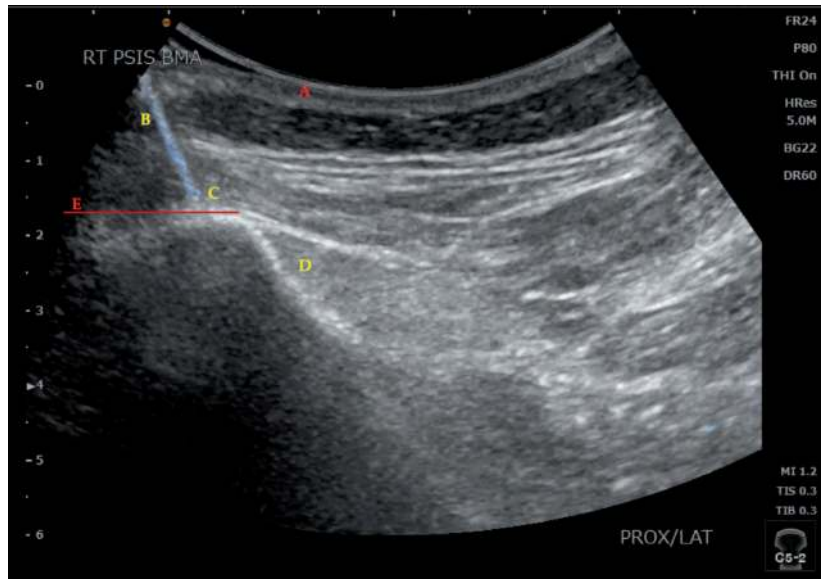

Figure 4.
Ultrasound imaging of the PSIS. With the probe in the transverse plane, the PSIS is confirmed, and the slope (D) of the iliac wing is noted for correct angulation of the BM trocar (B), and the most superficial depth (C) of the PSIS is brought under the most medial aspect of the ultrasound probe. Note: (A) indicates the skin surface, and (E) marks the depth of the PSIS below the skin, in this patient approximately 1.6 cm (courtesy of J. Rothenberg).

directional line is made in both parallel and perpendicular orientations to form an intersection at the most superficial depth of the PSIS. This mark is maintained on the patient during skin preparation prior to the introduction off the BM trocar, and a superficial wheal of local anesthetic is placed at the point of planned trocar skin entry. Following the local antiseptic measures, sterile ultrasound gel is applied at the marked area, and a sterile probe cover is applied to the 5–2 MHz curvilinear array transducer. Typically, a mixture of local anesthetics is injected around the PSIS cortex and periosteal sleeve, under continued sonographic guidance, making sure to "walk off" the PSIS in four directions (superiorly, medially, laterally, and inferiorly), confirmed by sonographic guidance. The trocar is then introduced, using either a manual force that is perpendicular and slightly lateral to the patient, at 9–12 counterclockwise-clockwise rotations, or a mallet. The next steps of the procedure are subject to the implementation of the instructions for use provided by the manufacturer of the aspiration harvesting system.

4.3.2 Fluoroscopic imaging

After proper patient positioning, the fluoroscopic equipment is installed to optimize the positioning for fluoroscopic imaging, using ipsilateral or contralateral oblique beam angulations for viewing the targeted PSIS site. The *perpendicular fluoroscopic approach* requires a beam angle around 15° ipsilateral to the PSIS entering laterally with angulation towards the sacroiliac joint. This angle will view the lateral ilium outer wall, and a needle is directed anteromedially. Fluoroscopic images support in positioning the tip of the trocar above the target area for entering the PSIS. The *parallel fluoroscopic approach* results in viewing down the PSIS table, at a 25° contralateral oblique beam position. This results in a classic view of the "teardrop" (**Figure 5**). Imaging can confirm the entry point into the PSIS table and visualize the angle through the cortex, allowing for safe trocar advancement in the BM cavity, at the tick part of the ilium bone [52]. Using proper fluoroscopic

Figure 5.
Fluoroscopy imaging of the PSIS. General prone position of the patient on a fluoroscopic table for BMA. The parallel fluoroscopic approach *results in viewing down the PSIS table, at a 25° contralateral oblique beam position. This results in a classic view of the "teardrop," referring to the outline of the medial and lateral borders, as shown in the monitor. The tip of a needle (black circle), in the numbed skin, is marking the entry site of the bone marrow trocar to be placed in the marrow cavity, while the physician is on the ipsilateral side of the fluoroscope, viewing the correct position on the monitor (red circle) (courtesy of G. Flanagan II).*

techniques, the parallel approach technique allows for a safe deeper marrow penetration. However, at all times, regardless of the approach, avoid increased manipulation and tissue trauma using the sharp trocar, as this will increase the risk for neurovascular injury, bleeding, tearing of lateral gluteal muscle origins, and post-procedural pain.

4.4 Bone marrow aspiration anatomical sites

As MSCs represent a small population of BM cells [7], it is of critical importance to choose a BMA site that will yield the most MSCs. BM is relatively easy to harvest, largely available, and dispensable. Obviously, it is important that the BMA procedure is performed impeccably to obtain an optimal quality of viable BM tissue [5, 53]. In humans, the most common anatomical location to obtain BM is the iliac crest, but other BMA sites have been utilized (**Table 2**). Recently, McDaniel and co-workers, using a porcine model, reported that all studied anatomical bone marrow harvesting locations contained MSCs but the iliac crest was the most abundant

Anterior superior iliac spine (ASIS)
Posterior superior iliac spine (PSIS)
Proximal tibia
Distal tibia
Distal femur
Proximal humerus
Vertebral body
Calcaneus
Sternum

Table 2.
Bone marrow aspiration sites in humans.

source of MSCs [10]. These findings were confirmed in a clinical study, where MSCs were found in BM acquired from the metaphysis of the distal femur, the proximal tibia, and iliac crest. A similar MSC immunophenotype and differentiation potential (into the bone, fat, and cartilage) were seen in BMA from all sites. However, in their study the concentration of MSCs in the iliac crest was significantly higher than in samples from the distal femur and proximal tibia. More specifically, the literature indicates high yields of BM-MSCs acquired from the posterior superior iliac spine (PSIS) [50, 54]. Noteworthy, the group of Narbona-Carceles commented on the relative easiness and safety of lower extremity aspiration techniques [55].

5. Major type of cells in bone marrow

The literature pronounces BMAs as a heterogenous mix of cells, referring in most instances to MSCs, HSCs, and mononuclear cells. Platelets, megakaryocytes, and RBCs are seldomly mentioned, let be subject to BM research [24].

5.1 Hematopoietic stem cells

The major function of the bone marrow is to generate blood cells. In particular in adults, marrow-derived HSCs are the principle cells of origin of all mature hematopoietic cell phenotypes. HSCs are adult stem cells with extensive self-renewal capabilities and are able to differentiate into specialized blood cells with key roles in some biological activities: control homeostasis balance, immune functions, and response to microorganisms and inflammation. Most HSCs are in a quiescent state within the BM niches. They respond to the signals after the balance of blood cells, or HSC pool, is disturbed from either intrinsic or extrinsic stimuli and signaling processes [56].

5.1.1 Hematopoiesis

Hematopoiesis—the process by which mature blood cells are formed—has been studied intensely for over a century. The vast majority of hematopoiesis occurs in the bone marrow where it must balance enormous production needs. More than 500 billion blood cells are produced every day, with precise regulation of the number of each blood cell type released in the circulation [57]. Hematopoiesis is considered as a pyramidal/hierarchical process with cells of greatest maturation potential or primitiveness sitting at the top of the hierarchy and cells that have undergone terminal differentiation at the bottom. Terminally differentiated blood cells are classified into one of the two major lineages: those derived from myeloid lineages and from lymphoid progenitors. Myeloid cells include erythrocytes, platelets, and cells responsible for cellular immunity, such as macrophages and granulocytes (**Figure 6**). Cells derived from lymphoid progenitors are major participants in coordinating humeral and cellular immunity. Experimental data suggested that HSCs differentiate into hematopoietic progenitor cells that are capable of exponential proliferation as well as continuing the process of differentiation. Alternatively, HSCs are capable of self-replicating activities, which may give rise to one or two identical daughter cells. As a result, HSC activity must be tightly regulated to meet physiologic demands but also to protect HSCs from oncogenic, physical, and chemical damage to occur. The site or physical location that regulates self-renewal, proliferation, and differentiation of HSCs has been discussed in the HSC niche paragraph.

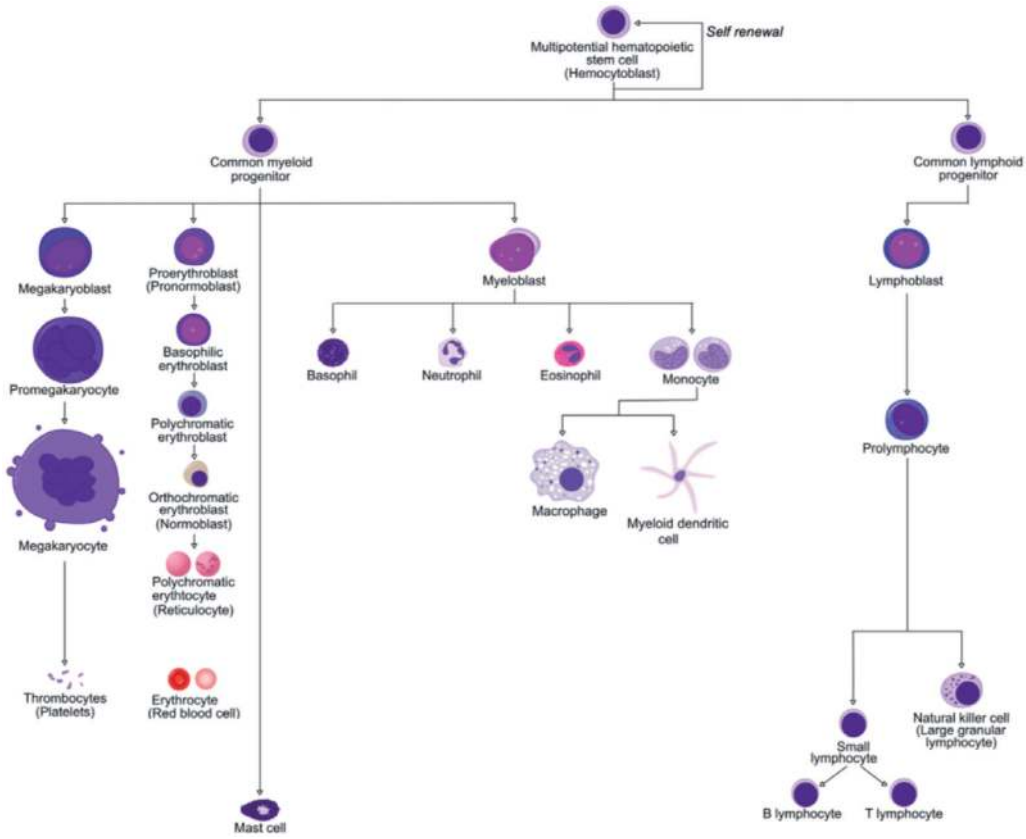

Figure 6.
Hematopoietic stem cell hierarchy. Self-renewing HSCs give rise to common myeloid progenitors and common lymphoid progenitors, producing different types of progenitor cells and ultimately fully differentiated cells. The myeloid progenitors produce granulocyte-macrophage progenitors giving rise to differentiated leukocytic cells and mast cells. The megakaryocyte/erythrocyte progenitors give rise to megakaryocytes, platelets, and erythrocytes. The lymphoid progenitors differentiate ultimately in lymphocytic cell variances.

5.1.2 HSC and angiogenesis

Emerging evidence suggests that BM-derived endothelial cells and HSCs, including their progenitor cells, contribute to tissue vascularization. HSCs deliver specific angiogenetic factors, facilitating the incorporation of endothelial progenitor cells into newly sprouting vessels. Several clinical studies have shown that BM-derived cells contribute to neo-angiogenesis during wound healing [44], critical limb ischemia [45], and postmyocardial infarction [58]. This should contribute to the clinical discussion of the value of BM-derived HSC and vascular progenitor as they are able to contribute to tissue restoration by accelerating tissue vascularization and regeneration [15, 59].

5.2 Mesenchymal stem cells

In recent decades, physicians performing regenerative medicine applications have been more interested in the potential of BM-MSCs than of HSCs. Imaginable reasons for this particular interest in MSCs might be recent published expert opinions: the *in vivo* ability of MSCs to migrate into tissues, their sturdy regenerative and reparative properties, and the MSC-mediated immunomodulatory actions.

These typical characteristics and particular mode of actions enable conceivable BM cell-based treatment options [60, 61]. In particular, MSCs do not express significant histocompatibility complexes and immune-stimulating molecules, leading to graft rejection. Likewise, a rapid development in clinical outcome reporting, with a better understanding of BM tissue molecular biology, improved bone marrow aspiration techniques and, at POC BM concentration and preparation methods, has increased the interest and demand for autologous BM stem and progenitor cell therapies.

5.2.1 MSC isolation procedure from bone marrow aspirates

An effective BM-MSC injection is reliant on the performance of the marrow aspiration procedure, minimizing cellular trauma, while maximizing cellular yields and simultaneously avoiding peripheral RBC infiltration [62]. BM aspiration procedures, and not diagnostics, are routinely performed to collect bone marrow tissue to be processed using dedicated BM-MSC concentration kits for regenerative medicine applications. Kits may include a harvesting needle system and/or BM concentration device (**Figure 7**). These at POC MSC isolation techniques are a streamlined method to concentrate marrow cells, including MSCs, HSCs, and progenitor cells. These MSC centrifugation procedures demand less time and attention than laboratory preparation and culturing methodologies which are technically demanding. Double-spin centrifugation protocols create a layered BMC buffy coat stratum, based on different centrifugal forces that accomplish density cellular separation, as a result of the specific cellular gravity of the individual marrow components, as shown in **Figure 8**. Furthermore, BMA concentration-based technologies provide an economic and clinical/patient advantage when compared to the culturing technologies.

Figure 7.
Bone marrow preparation essential components. In bone marrow concentration and preparation kits, the foremost components are a bone marrow harvesting needle and a concentration device (courtesy of EmCyte Corporation, Fort Myers, FL, USA).

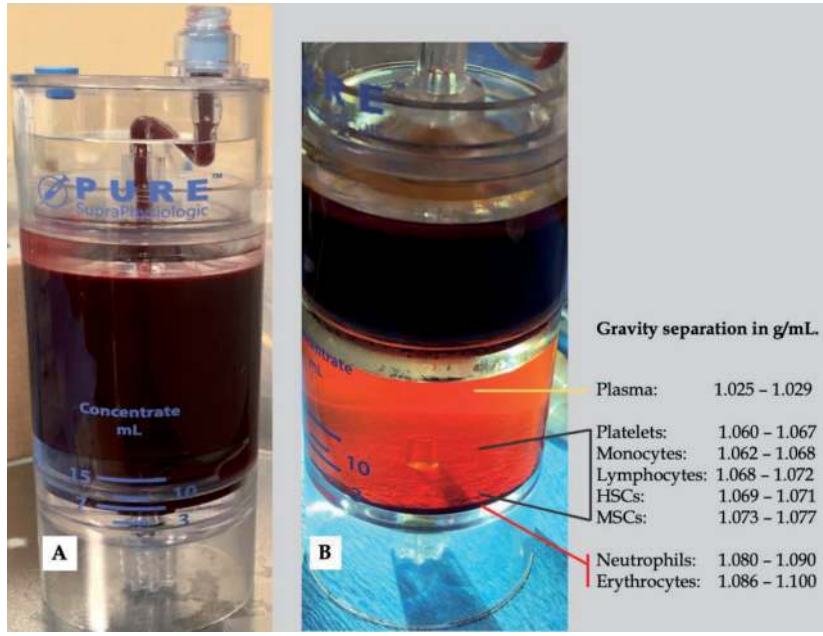

Figure 8.
Bone marrow gravity separation following centrifugation. (A) Bone marrow aspirate in concentration device before centrifugation. In (B), the bone marrow aspirate is concentrated, with a view on the buffy coat stratum (gray layer on top of the RBC layer), referenced by the two black lines. Following a two-step centrifugation protocol, the centrifugal forces achieve density marrow cell separation due to the specific gravities of the individual marrow components.

5.2.2 MSC culturing protocols

Traditionally, BM-MSCs have been separated from other BM cells following strict laboratory cell processing protocols. These cell processing techniques are lengthy procedures, as they cannot be performed at POC, as a same-day procedure. In many parts of the world, clinicians are allowed to use autologous, fresh, and non-cultured BMA and BMC products that are prepared at POC. In the USA, regenerative medicine biological procedures demand the use of the so-called 510-K FDA-approved devices. The use of MSCs following laboratory expansion techniques is facing considerable legislative barriers. Furthermore, the literature has cited potential risks associated with laboratory MSC cell processing techniques, like tumorigenicity [63], genetic instability [64], and immunogenicity [65]. Others raise concern regarding the efficacy and function of cultured MSCs by in vitro culture conditions during the cell passages for cell expansion. Karp and Teo reported on the loss of specific MSC surface receptors functions, negatively affecting chemotaxis [66]. Others have informed on impaired homing abilities and disappearing CXCR4 receptors following cell culturing, when compared to non-cultured BMA, in which high CXCR4 concentrations were measured [67]. Last but not least, laboratory MSC cell culturing methods for regenerative medicine practices require the availability of a specialized and dedicated facility, using strict regulatory protocols which will increase costs [68].

5.2.3 Characterization of bone marrow mesenchymal stem cells

In order to better understand the specifics of MSC cell concentrations, counts, and quality, it's important to understand the differences between laboratory techniques analyzing HSCs and MSCs, as they differ with regard to the specificity

and relevance of the different BM cell types, possibly effecting regenerative medicine therapy outcomes.

5.2.3.1 ISCT criteria

The International Society of Cellular Therapy (ISCT) has developed criteria in order to outline human MSCs for both laboratory-based scientific investigations and for preclinical studies [69]. MSCs are defined as those cells that are able to adhere to plastic and express a number of cell surface markers while undergoing multilineage differentiation [70].

5.2.3.2 Flow cytometry and CD markers

It has been difficult to determine what type of cells is neighboring both MSCs and HSCs and contributes to the regulation of the functional continuation of stem cell, as immunostaining methods are complex procedures to perform. Flow cytometry is a laboratory technique used to detect and measure characteristics of cell/particle populations by measuring their physical and chemical properties. A specific protocol for the identification of dissimilar cell surface molecules is called cluster of differentiation (CD) where monoclonal antibodies (markers) are used to establish positive and negative staining for certain cell types. Specifically for MSC and HSC, explicit CD markers are established to validate BM cellular content, as it is widely accepted that MSC cultures are a heterogenous source of cells with varying self-renewal and differentiation properties [71]. This indicates that there is no single unique indicator for identification. Hence, a panel of both positive and negative protein markers is used to identify the cell surface markers that are expressed by MSC populations, like CD29, VD44, CD51, CD73, CD90, CD105, CD166, and Stro1. While they must be negative for hematopoietic stem cell markers like CD14, CD34, and CD45 [72], some of these markers are included in the minimum ISCT criteria.

5.2.3.3 Colony-forming unit fibroblast assay

In the initial BM monolayer, several hematopoietic oriented cells (macrophages, endothelial cells, and lymphocytes) adhere to plastic [7]. Nevertheless, in culturing conditions only fibroblast-like spindle-shaped cells proliferate and form ultimately CFU-F colonies. These cells are representative of the more highly proliferative cells in MSCs [73]. The CFU-F assay is a different method used to determine the MSC presence in a vial of BM tissue. Unlike a complete blood count test, which is a quantitative blood cell analysis, the CFU-F assay is a laboratory assay in particular for stem and progenitor cell determination (**Figure 9**) [74]. The CFU-F assay is a qualitative indicator of the proliferative and differentiation capability of individual MSC cells within a BMA or BMC sample. The cells are seeded and cultured in a growth medium where they have to adhere to plastic, at 37°C. After 14 days the cultures are evaluated, and CFU-Fs are counted, whereby a minimum of 50 cells per CFU need to be defined.

5.3 MSC capacities

MSCs are multipotent stem cells which can be obtained from various adult tissues, like the BM stroma, adipose tissue, synovium, periosteum, and trabecular bone. Typical features are their ability for self-renewal, defined as sustaining

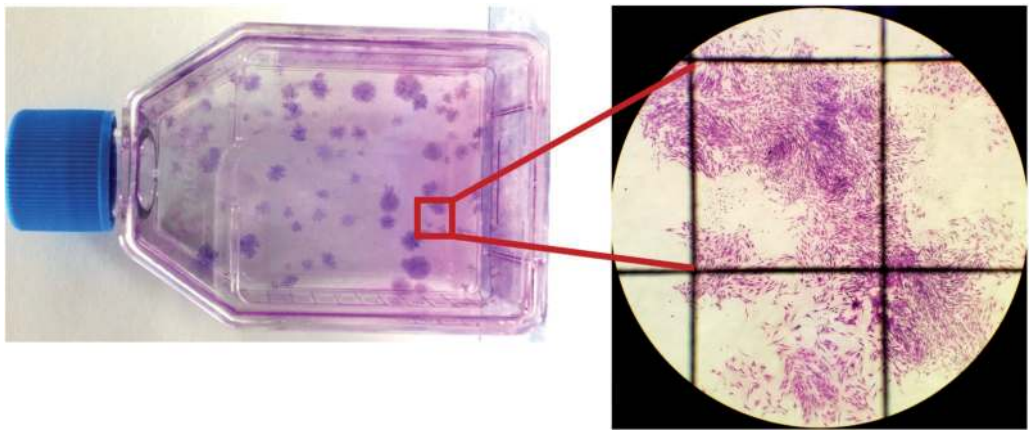

Figure 9.
MSC culturing. A picture of a flask cultured with stained human MSCs. The zoomed-in area is a light micrograph showing the morphology of a MSC colony in a patient treated with BMC. After culturing for 14 days, the MSC count in this example was 1065/mL (picture courtesy of BioSciences Research Associates, Cambridge, MA, USA).

biological pathways and mechanisms to preserve the undifferentiated stem state, and the regulation of lineage-specific differentiation [39]. Although the number of MSCs represents only a small fraction of non-hematopoietic, multipotent cells of the bone marrow (0.001–0.01%), understanding these unique cells has taken great strides forward. Generally, MSCs have developed a great attractiveness for regenerative medicine autologous therapeutic applications and tissue engineering opportunities, because of their multipotentiality and relative ease of isolation from numerous tissues, like BM [75]. MSCs can be also identified as specialized populations of mural cells or pericytes, sharing a niche with HSCs. Under appropriate conditions and an optimal microenvironment, MSCs can differentiate into various mesodermal lineages like osteoblasts, chondrocytes, endothelial cells, adipose tissue, and smooth muscle cells (**Figure 10**) [76]. These MSC proficiencies have led to the use of MSC as a potential strategy for treating various diseases, since they encourage biological processes, for example angiogenesis, cell proliferation, and cell differentiation [77]. Furthermore, they synthesize cytokines and trophic mediators which participate in tissue repair processes, immune modulation, and the regulation of inflammatory processes [78]. Caplan also suggested that the modulation of inflammation is instigated by the suppression of inflammatory T-cell proliferation and inhibition of monocyte and myeloid cell maturation [79]. Based on the above characteristics, it can be assumed that MSCs are capable to institute a regenerative microenvironment at the site of release and improve the various cell recruitment, cell-signaling, and differentiation of endogenous stem cells, with the potential to instigate tissue repair in a variety of disease states.

5.4 MSC immunomodulatory effects

In parallel with their major role as undifferentiated cell reserves, MSCs have immunomodulatory functions which are exerted by direct cell-to-cell contact, secretion of cytokines, and/or by a combination of both mechanisms. MSCs have been shown to exert profound anti-inflammatory and immunomodulatory effects on almost all the cells of the innate and adaptive immune systems via a variety of mechanisms, notably cytokine and chemokine secretion [80]. The immunosuppressive capabilities of MSCs are only exploited when they are exposed to sufficiently high concentrations of pro-inflammatory cytokines, like interferon-gamma (IFN-γ), tissue necrosis factor α, (TNF- α), and interleukins α or ß (IL-1α, IL ß).

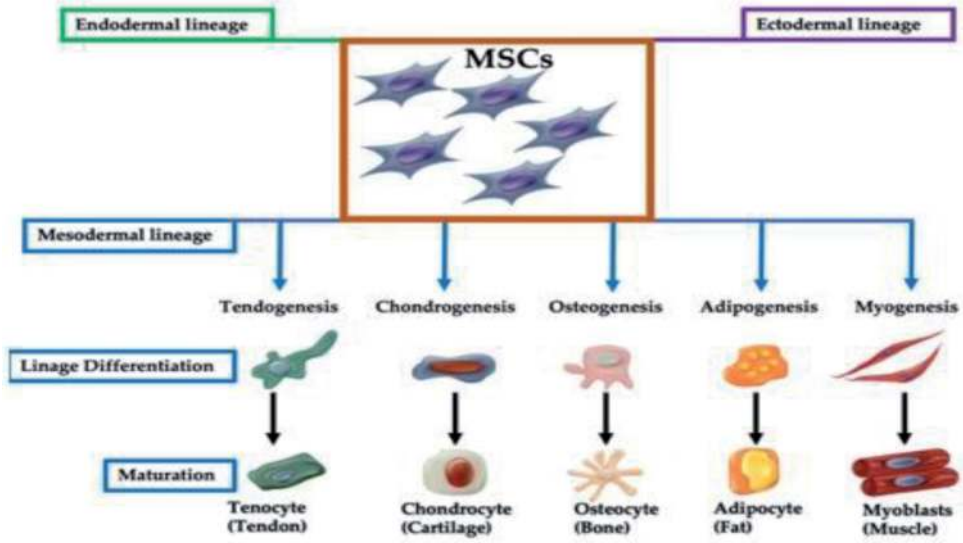

Figure 10.
MSC differentiation potential. MSC differentiation potential into endodermal, ectodermal, and mesodermal lineages. The mesodermal lineage differentiation has been recognized as the most attractive differentiation lineages for regenerative medicine applications, executed at point of care, as these produce osteoblasts, chondrocytes, tenocytes, adipose tissues, and smooth muscle cells.

In order for MSCs to become "immunosuppressants," they need to be triggered by these inflammatory cytokines, and the inflammatory environment is then a crucial factor for MSCs to exert their immunomodulatory effects. These are wielded by blocking apoptosis of native and activated neutrophils, aside from decreasing neutrophils from binding to vascular endothelial cells and the mobilization of neutrophils to the area of damage [81]. Furthermore, MSCs constrain the complement-mediated effects of peripheral blood mononuclear cell proliferation [82], and they limit mast cell degranulation and the secretion of pro-inflammatory cytokines, while at the same time MSCs migrate towards CXCL12 and other chemotactic factors [83]. In **Figure 11** the MSC cell-dependent trophic support mechanisms are shown. Data from Jiang and others suggested that MSCs can block the differentiation of CD34+ cells from BM or blood monocytes into mature dendritic cells by direct contact as well as by secreted paracrine factors [84]. Under their influence, M1 (pro-inflammatory) macrophages are transformed into M2-type cells with an anti-inflammatory phenotype, and the interleukin-10 secreted by them inhibits T-cell proliferation. This immunosuppressive effect related to T-cell proliferation and decrease in cytokine production by MSCs was, among others, confirmed by Sato et al. [85]. However, the mechanisms by which MSCs are mobilized and recruited to damaged sites are not known. In addition, how they survive and differentiate into distinct cell types is still not clear. Once MSCs have been applied to the microenvironment of injured or degenerated tissues, many factors stimulate the release of many growth factors by MSCs; a detailed growth and trophic factor overview is shown in **Table 3**. These growth factors stimulate the development of fibroblasts, endothelial cells, and tissue progenitor cells [86]. It is credible to state that the use of MSCs and their potential in immunomodulation in regenerative medicine applications hold great promise [87].

5.5 MSC growth factor activity

In order for MSCs to differentiate into several cell lineages, the action of specific growth factors and chemical mediators are needed in these processes [88, 89].

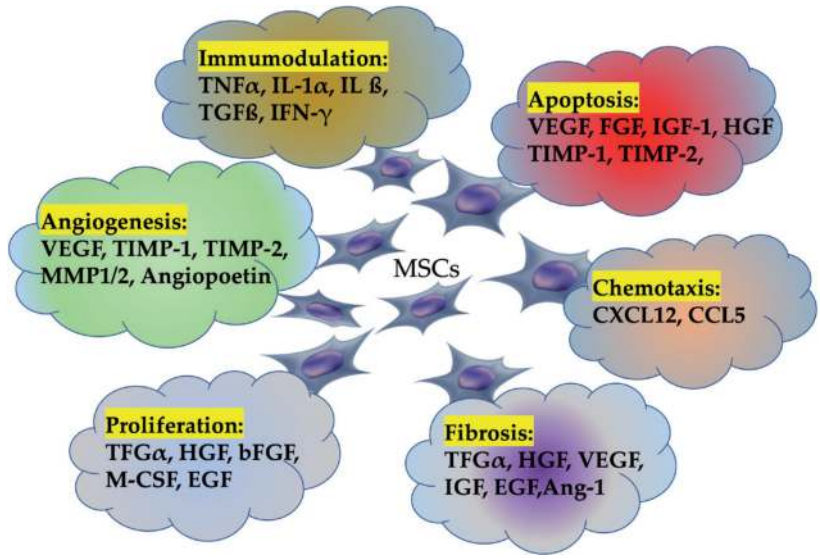

Figure 11.
MSC trophic mechanisms. After bone marrow cell injections, MSCs produce a variety of trophic factors impacting healing cascades by reducing cell apoptosis, fibrosis, and inflammation. Furthermore, by acting on cell proliferation cascades, they contribute to differentiation and mobilization of cells. MSC paracrine trophic factors are potentially important in maintaining endothelial integrity and promoting angiogenesis and the secretion of various growth factors and reparative cytokines.

Growth factor/cytokine	Activity in MSC regenerative repair
Epidermal growth factor	Wound healing
	Tissue regeneration
Fibroblast growth factor	Tissue repair
	Intrinsic stem cell survival
	Tissue regeneration
	Neurogenesis
Hepatocyte growth factor	Vasculogenesis
	Intrinsic neural cell regeneration
Insulin-like growth factor	Wound healing
	Neurogenesis
Keratinocyte growth factor	Wound healing
Platelet-derived growth factor	Tissue repair
Transforming growth factor beta	Wound healing
Vascular endothelial growth factor	Angiogenesis
	Wound healing
Angiopoietin-1	Angiogenesis
	Tissue repair
Erythropoietin	Angiogenesis
Interleukin-8	Wound healing
Stem cell-derived factor-1	Neuroprotective effect
	Wound healing

Table 3.
Growth and trophic factors contributing to MSC tissue regenerative processes.

Once MSCs are mobilized, or after BM tissue injections, they produce a number of trophic factors that impact healing responses. At a local tissue level, they act by reducing cell apoptosis, fibrosis, inflammation, and activation of cascades that lead to cell proliferation and differentiation, mobilization of cells, and an onset of angiogenesis via paracrine and autocrine pathways [90]. Crucial agents involved in these processes include a variety of growth factors. The MSC trophic effects are associated with the secretion of reparative cytokines and growth factors [91], which contribute finally to tissue repair of inflamed and degenerated tissues, retaining positive MSC paracrine effects [92]. Many of the MSC growth factors are generated on the principle of the cell regulating protein nuclear factor-κB activation, after an initial exposure to pro-inflammatory stimuli such as IFN-γ, TNF-α, and IL-1β or even hypoxia [93]. These factors most likely coexist in prepared MSC-containing BM vials and delivered at tissue injury sites. In this situation, MSC growth factors and other cell mediators may have the potential to exert their specific activities via molecular interplays and subsequently promote optimal MSC-associated therapeutic tissue healing, in particular in a highly concentrated environment [94]. The endothelial monolayer barrier function of tissue capillary beds is often disturbed under degenerative and inflamed conditions, allowing for the blood to release proteins and white blood cells, while MSCs produce and release growth factors that affect endothelial cell and subsequently promote the development of tissue progenitor cells and fibroblasts and support tissue regeneration and repair [95]. Some clinicians combine platelet-rich plasma concentrations [96] with BM products in order to have a more biologically active graft, projected to optimize regenerative medicine treatment outcomes. However, it is important to comprehend the detailed mechanisms underlying the inflammation-modulated production of growth factors by MSCs, as this will provide a better perspective for the clinical application of MSCs or their paracrine factors in tissue regeneration.

5.6 MSCs and angiogenesis

MSC paracrine trophic factors are potentially important in maintaining endothelial integrity and promoting angiogenesis through their ability to regulate endothelial cell proliferation and ECM production [97]. Furthermore, endothelial cell permeability is reduced, and MSCs inhibit interactions between leukocytes and endothelial cells [98]. Apart from MSC trophic factors, fibroblasts have fundamental functions in maintaining tissue integrity and promote tissue healing through their secretion of cytokines that support ECM building. These endothelial and angiogenetic capabilities have been demonstrated in clinical studies addressing chronic wound healing [99] and recovery from postmyocardial infarction [100].

5.7 Homing and migration

An enduring problem in the field of cell-based regenerative medicine therapies is the factual delivery of the harvested and prepared cells to the site of injury, a process termed "homing" [101]. One of the major characteristics of MSCs after administration is that they are able to migrate to sites of inflammation and tissue damage, which are typically associated with cytokine outburst [102]. Homing mechanism to degenerated and injured tissue sites are influenced by factors like age, cell viability, the number of available cells (dosage), and the delivery method. Unlike the well-characterized phenomenon of leukocyte homing by de novo, or exogenously delivered (BM) MSCs, is still unclear. Evidently, an increase in leukocyte migration, with induced rolling response to inflamed tissue sites, has been noted by engineered MSCs [103]. For successful cell-based regenerative therapies,

it is critically important for MSCs to control cell adhesion in the ECM of the treated tissue. This will occur through the expression of fibronectin and specific integrin and selectin protein adhesion molecules, which are binding to collagen and fibrin ECM components [102], initiating tissue healing and regeneration through cell adhesion, cell growth, migration, and differentiation [104]. The migration ability of MSCs is further controlled by a wide range of growth factors, acting under the receptor tyrosine kinase signaling principle [105], once more illustrating the importance and presence of platelets and their growth factors in the collected BM vial. Furthermore, the administration of MSCs via various delivery routes (intravenous, intraperitoneal, intra-arterial, in situ injections) seems to have an effect on MSC homing [66].

6. Bone marrow aspirate aspiration and processing

When applying regenerative medicine MSC applications, physicians have a choice to use either a BMA as a regenerative injectate, without any processing steps, or they can harvest a particular BMA volume necessary to produce a BMC, with dedicated devices and centrifuges. Additionally, the differences between a BMA injectate only and a BMA concentrate are discussed.

6.1 BMA-MSC procedural steps

In the freshly aspirated BMA samples, the heterogenous cellular content is pervasively distributed in the vial, as long as clotting is prevented.

6.1.1 Anticoagulation protocol

Prior to a BMA procedure, it is recommended that bone marrow harvesting devices, concentration devices, and all of the processing accessories that will be in contact with BM are subject to a thorough heparin rinsing. Furthermore, several instructions for use advice to leave a volume of anticoagulant in the aspiration syringes and processing device as well, as BM tissue has the potential for rapid clotting. Before a BMC concentration device is loaded for processing, the aspiration syringes volumes are transferred into one consolidating collection syringe and subsequently filtered through a 200u heparin rinsed filter to eliminate particles, fibrin strands, and fat tissue.

6.1.2 Two-step BMA centrifugation protocol

It is our belief that the preparation of a vial of concentrated MSCs is best created by the so-called double-spin protocols, using dedicated and approved disposable concentration devices. BMA centrifugal processing techniques, to produce a viable BM-MSC injectate, are generally accepted methods when executed at POC, because these preparation protocols seek to overcome the limitations of MSC ex vivo cell culturing techniques. In this section we touch on a BMC preparation protocol to produce PurePRP SupraPhysiologic Bone Marrow Concentrate (PureBMC®SP, EmCyte Corporation, Fort Myers, FL, USA). The PureBMC®SP autologous biologic is part of an autologous cellular platform technology, facilitating the preparation of platelet-rich plasma and adipose tissue concentrates. A two-step centrifugation and preparation protocol will concentrate the indispensable BMA cellular content to a BMC. Following a first centrifugation spin, the BMA is sequestered in a BM plasma fraction (BMPF), containing a buffy coat layer and RBCs. The BMPF is

aspirated, immediately followed by a separate collection of 2 ml of RBCs, following the instructions for use of the PureBMC® concentration device. Both volumes are then transferred for a second centrifugal spin cycle to the concentration compartment of the same device. During the second spin, a specific centrifugation protocol is accomplished, leaving the bone marrow cells in a concentrated fashion attached at the bottom of the chamber. Excessive BMPF is manually removed, leaving behind a specific BMC volume for resuspension. The amount of this volume depends on the requirements for clinical applications. Therefore, the BMC injectate volume may vary between 3 and 10 ml, with increased cell concentrations according to this final volume varying between 4- and 10-fold the native concentrations.

6.2 Cellular differences between BMA and BMC injectates

In a BMA injectate, the concentrations of the cells resemble the concentration of the cells that are present in the bone marrow cavity. However, based on aspiration techniques, the number of MSC might be increased. A BMC is a small volume of fluid containing a high concentration of cells extracted from the bone marrow, such as high yields of MSCs (can be measured as CFU-Fs), HSCs, progenitor cells, total nucleated cells, and platelets, at a significant concentration above BMA baseline values. Furthermore, the heterogenous nature of marrow cells is completed by the presence of increased levels of growth factors [106, 107], cytokines like IL-8, and interleukin-1RA [94]. Additionally, in a BMC injectate following a two-step centrifugation procedure, the RBC and plasma-free hemoglobin (PFH) concentrations are significantly decreased when compared to a BMA injectate.

6.2.1 Red blood cells and hemolysis

Throughout the aspiration procedure, RBCs can be damaged as a result of high shear forces [108]. As a consequence, the RBC membrane will start to disintegrate, and hemolysis, with the release of PFH, will occur. Damaged RBCs and free hemoglobin (Hb) lead to the development and release of toxic Hb forms, like free hemin, ferric Hb, and iron [109]. This is of particular concern as PFH and their split products, heme and iron, cannot be cleared, by natural scavenger proteins, when bone marrow injectates are applied in any microenvironment, as these are outside of the blood stream. A graphic representation of the pathophysiological effects and reactions of PFH, leading to various hemolytic-related sequelae and potentially encumbering clinical outcomes, is presented in **Figure 12**.

6.2.2 Comparative laboratory data BMA vs. BMC

In **Table 1** the effects of concentrating BMA to BMC with regard to some of the most important marrow constituents and factors are shown, as discussed in Section 4.1. The data in the table represent a clinical bilateral BMA model, using two different harvesting systems. For both systems, BMA was aspirated in an identical manner, at three different depth levels collecting in total 10 ml of marrow. Furthermore, to compare the cellular differences between a BMC injectate and a BMA injectate (BMA-MC), we collected an additional 40 ml of BMA with the Aspire system, after the first 10 ml. This allowed for a total processing volume of 60 ml to produce BMC. Laboratory analysis resolved that both BMA devices were almost similar with regard to cell viability and numbers. Interestingly, with regard to CFU-Fs, the data are in accordance with Hernigou [40], and the first marrow aspiration provides the highest number of CFU-Fs. However, when comparing the BMA-MC cellular composition (a patient treatment specimen) with the BMC treatment specimen,

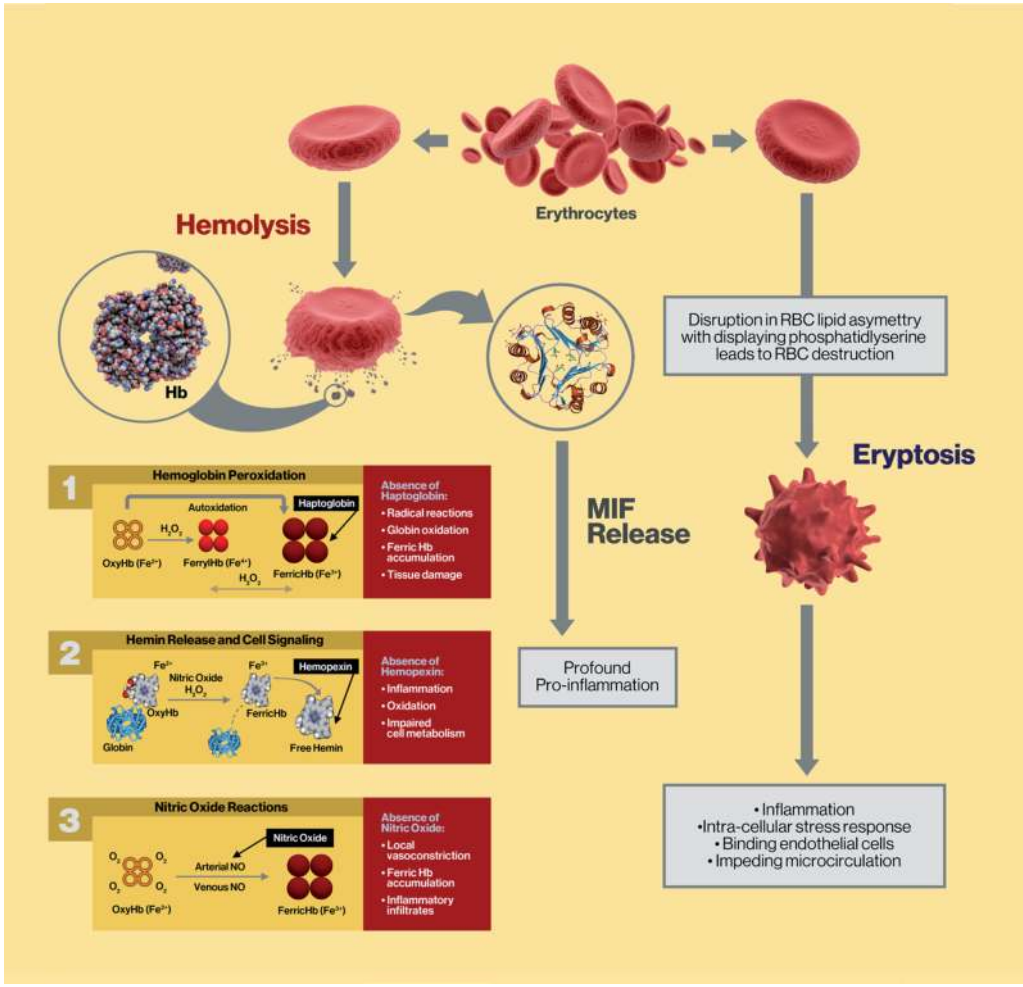

Figure 12.
Pathophysiological effects and reactions of RBCs in BMC vials. In absence of scavengers and compensatory mechanisms, PFH split products can lead to toxic consequences like inflammation and prooxidant effects, endothelial cell dysfunction, and vasoconstriction. Biological treatment specimen, containing high concentrations of RBCs, will lead to RBC cell membrane disruption (eryptosis,) releasing macrophage migration inhibitory factor (MIF) (courtesy of P. Everts and modified from Schaer et al. [109]).

significant differences occur. Centrifugation foremost significantly increases cells who take part in regenerative processes when compared to the BMA-MC product. In contrast the non-regenerative RBCs and PFH concentrations are significantly reduced in the treatment vial, while maintaining a higher cell viability after centrifugation. Our findings, with regard to cellular enrichments comparing a BMA with a BMC, are in agreement with others [41, 110], but not regarding RBC content and PFH. The cell concentrations are not only depending on the centrifugation protocols and the final BMC volume but are contingent of a meticulously executed BMA procedure, maintaining high cell viability, with minimal cell destruction.

6.3 Not every bone marrow concentrate is born equal

Currently, eight BMC harvesting devices are available on the market, producing different formulations of BMC and tissue viabilities and yielding different cellular concentration characteristics [111, 112]. As such, BMC preparations may vary widely regarding HSCs, MSCs, progenitors, platelet growth factors, and RBC content. Given this heterogeneity, the impact of BMC therapies on tissue regeneration may vary greatly. Explicitly, it is important to understand that the BMC

non-stem cell cellular components in the treatment vial might have significant roles regarding behavior and function of MSCs. Recently, the cellular variances were confirmed in a systematic review, evaluating BMC studies in musculoskeletal pathologies [53]. Presumably, as postulated by numerous authors, the variances in BMC cellular compositions have a significant effect on the biological activity and regenerative potency of the treatment specimen, and these inconsistencies impact clinical outcomes [111, 113]. Unfortunately, an exact understanding of the underlying signaling relationships is not completely understood [114].

7. Bone marrow-derived mesenchymal cells in musculoskeletal disorders

The use of autologous BM-derived MSCs for the treatment of a variety of musculoskeletal ailments is progressing significantly. Literature findings demonstrate positive outcomes after regenerative medicine MSC applications, in particular in joints, tendons, and bone, and hold great promise for future MSK-D applications, especially if more research and larger clinical trials are performed, focusing on cell validation processes and elucidating on potential dose responses.

7.1 Knee osteoarthritis

In this section we will present several clinical studies in which autologous, heterogenous BMC was used as a regenerative biologic to treat a variety of musculoskeletal disorders [42, 115]. Studies reporting on similar pathologies using BM-derived/cultured MSCs are not mentioned as it has been reported that these technologies have different biomedical properties and extraction methods [116] and potentially possess new challenges and indications, when compared to at POC prepared fresh autologous BMCs. In clinical settings, BMC has been exploited as an ortho-biological treatment option for a range of indications, like symptomatic focal femoral head osteonecrosis, OA of the knee and hip, focal chondral defects, as well as another MSK-D. The rationale to use autologous BMC in osteoarthritic (OA) joints and other indications is its potential in facilitating anti-inflammatory and anabolic effects after injection. Moreover, the heterogenous BMC cellular assortment is known for its angiogenetic properties, therefore contributing to chondrocyte metabolism and inducing homing of (progenitor) stem cells to the treated areas [117]. Rodriguez-Fotan and co-workers used a two-step BMC preparation protocol in patients with early onset of OA in the knee or hip (Kellgren-Lawrence grades I–II, Tönnis grades I–II, respectively), with BMA aspirated from the anterior iliac crest. After a single BMC injection, 63% of treated patients had improved clinical symptoms at 6-month postinjection. They concluded that the intra-articular BMC injections are safe procedures and no adverse events were reported [118]. In a prospective single-blind, placebo-controlled trial, 25 patients with bilateral knee OA and a median age of 60 years were randomized to receive BMC into one knee and saline placebo into the contralateral knee, thereby utilizing each patient as his/her own control. Safety data, effect of pain relief, knee function (Osteoarthritis Research Society International) measures, and the visual analog scale (VAS) for pain were observed until 6 months after the injections [119]. However, no differences between the two groups were observed. Of interest in this study was that the final injectate composition consisted of a mix of BMC and BMPF suspension. However, the eventual consequences of diluting BMC with BMPF on outcomes were not discussed. In another case series by Kim et al., a more invasive treatment approach was used. BMC was mixed with adipose tissue as a multi-tissue preparation for knee OA injections, in patients with a mean age of 60.7 years. At 9-month follow-up,

all patients showed clinical improvement, with satisfactory results in 70.7% of patients [120]. Remarkably, the authors found that patients with inferior treatment results had a greater severity of OA prior treatment, as they were marked at Kellgren-Lawrence grade IV, suggesting that advanced OA may be more restrained to BMC therapy. The side effects encountered in this study, joint inflammation and pain, were in accordance with data from Rodriguez-Fotan [118]. Recently, a similar (retrospective) study was executed by Mautner and associates. Patients were prospectively treated either with bone marrow aspirate concentrate (BMAC) or micro-fragmented adipose tissue (MFAT) injections, for symptomatic knee OA [121]. The follow-up responses consisted of 76 patients (with 106 knees). The Knee Injury and Osteoarthritis Outcome Score (KOOS) questionnaire, Emory Quality of Life (EQOL) questionnaire, and VAS for pain were compared with baseline scores for all patients, and outcomes between BMAC and MFAT groups were evaluated. Data demonstrated a significant improvement in joint function and VAS pain scores after both MFAT and BMAC injections. No significant difference between the two autologous biological groups was demonstrating that BM- or adipose tissue-derived ortho-biological injections resulted in similar functional improvements.

7.2 Shoulder disorders

Lately, Darrow et al. reported on patients treated for shoulder osteoarthritis or rotator cuff tears ($N = 50$), with either BMC or BMA injections. Patients were grouped in receiving one or two injections [122]. Outcome reports included resting pain, active pain, upper extremity functionality scale, and overall improvement percentage. Data were compared to baseline and between the two groups. All patients had significant posttreatment improvements in resting pain, active pain, and functionality scores, when compared to baseline values. Patients receiving two treatments, average interval duration of 22 days, experienced statistically significant more improvements in active pain than the patients receiving one injection. There were no significant outcome differences between patients with a rotator cuff tear or OA. Unfortunately, no information was provided on the BMA and BMC procedure, and no laboratory validation data were reported.

7.3 Osteonecrosis

Philippe Hernigou, world renowned for treating femoral head osteonecrosis, advocates the use of autologous BMC cell therapies [123]. He described a substantial repair and stabilization of necrotic femoral heads with percutaneous injection of autologous BMC, in combination with surgical core decompression. In a later paper, he reviews three decades of BMC therapies in hip osteonecrosis, emphasizing the quality of the BMC and cellular competence and addressing the effects of BM cell concentrates on the microenvironmental changes within osteonecrotic bone [124]. Other groups reported on prospective randomized clinical trials for femoral head osteonecrosis, comparing surgical decompression alone versus decompression augmented by autologous BMC preparations. The biologics were implanted during the surgical decompression procedure. In one study, patients were evaluated using the Western Ontario and McMaster Universities Osteoarthritis (WOMAC) Index questionnaire, VAS pain index, and MRI. The mean WOMAC and VAS scores in all patients improved significantly ($P < 0.001$). Post-procedural MRIs showed a significant ($P = 0.046$) improvement in patients in whom the surgical procedure was combined with BMC [125]. In a similar study, a significant decrease in pain associated with a functional benefit lasting the entire observation period was observed in the BMC-treated patients. However, no difference in clinical outcomes between

the two study groups was seen during a 2-year follow-up period, with no significant difference between the femoral head survival rate [126]. Importantly, they analyzed the MSC and nuclear cell content of the BMC. There was a significant rise in nuclear cells and CFU-Fs (6.3-fold and 1.5-fold baseline values, respectively). Despite a significant rise in CFU-Fs in the BMC, the total deliverable MSC cell counts were relatively low. This might be related to the design features and specifications of the fully automated, sensor-controlled processing BMC device that was used with a single-step centrifugation protocol.

7.4 Cartilage repair

Awad and associates recently published a meta-analysis on knee cartilage repair [127]. They conducted a systematic review using the PRISMA guidelines and the *Cochrane Handbook for Systematic Reviews of Interventions*. A meta-analysis was conducted to estimate the effect size for function and pain in 724 patients, with a mean age of 44.2 years. In this review, both cultured BM-MSCs and autologous non-cultured BMC were used. All autologous BMC treatment specimens were prepared following a two-step centrifugation method. Their most important meta-analytic finding was that the administration of non-cultured, fresh, BMC significantly reduced pain and improved knee function. This might be induced by the heterogeneous composition of the non-cultured BMC, as all constituents will synergistically foster cartilage regeneration and local pain management. Furthermore, BMC holds a certain volume of autologous plasma which can function as a cellular scaffold with the advantage of a more sustained release, compared to a pure cultured MSC product.

7.5 Tendinopathies

In a retrospective study, Stein et al. used BMC for primary Achilles tendon repairs, following traumatic injuries [128]. The BMC was adjunct to augment the surgical correction. Although the study lacked a control group, at a mean follow-up of 30 months, there were no re-ruptures reported. In a small patient study, centrifuged BMC specimen were injected in patients, refractory to conservative therapies, with clinical and radiological evidence of chronic patellar tendinopathy. Long-term follow-up showed statistically significant improvement in the majority of its reported scores [129]. A series of patients, diagnosed with clinical lateral epicondylitis, were treated with a single-spin BMC protocol. A significant improvement was noted when pre-BMC scores were compared with postinjection scores, at 12-weekpost-intervention. The authors suggested that BMC injections in patients who have failed non-operative treatment, before a surgical intervention, should be considered, and in their belief BMC injections can be developed as second-line conservative treatment in chronic tendinopathy, potentially reversing the degenerative process [130].

8. Bone marrow concentrates in spinal disorders

Degenerative disk disease (DDD) affects the disks that separate the spine bones. Age-related changes can lead to arthritis, disk herniation, or spinal stenosis. Pressure on the spinal cord and nerves may cause pain. DDD is associated with significant morbidity. Conservative treatment options, physical therapy, self-care, medication, and spinal injections are used to manage the symptoms. However, these measures are often not significantly responsive. Surgery has been an option if the

pain is chronic. Nowadays, autologous regenerative applications have been made available to patients as an alternative treatment option.

8.1 Degenerative disk disease

Pettine et al. studied the use of intra-discal BMC injections in patients with DDD [131]. The authors injected 26 symptomatic patients for lumbar diskogenic back pain and disability and evaluated their postinjection outcomes using disability scores, pain scores, and MRIs. At 2-year follow-up, patients experienced significant improvements in disability and pain scores. This group was the first to report on MSC dose-dependent outcome responses. Patients receiving greater concentrations of autologous BM-MSC (expressed as CFU-Fs > 2000/ml) experienced a faster and greater reduction in pain scores. Later, these findings were strengthened with a follow-up study at 36 months, showing similar outcome results [43]. At 5-year follow-up, absolute and percentage reductions in pain and disability scores were sustained, with no adverse events reported through the 5-year follow-up period [132]. The American Society of Interventional Pain Physicians published recently guidelines addressing responsible and safe use of autologous biologics in the management of lower back pain [133]. Their extensive analysis revealed that there is level III evidence for the use of PRP and BM-MSCs. The guidelines also state that, following diagnostic evidence, regenerative therapies should be provided to patients as an independent therapy. If appropriate and indicated, regenerative therapies can be combined with conventional medicinal therapy or in conjunction with physical and behavioral therapy.

8.2 Spinal surgery

Hart et al. informed on a prospective, randomized, and blinded study in patients with lumbar disease the use of BMC mixed with allograft spongiosa chips during surgical posterolateral fusion (PLF) procedures. Patients underwent instrumented lumbar spine PLF procedures [134]. Fusion status and the degree of mineralization were evaluated by two radiologists blinded to patient group affiliation. X-ray examination, in control patients at 12-month follow-up, showed that the bone graft mass fused in none of the cases and, at 24-months, in four cases (10%). In the BMC treatment group, 6 cases (15%) achieved fusion at 12 months and 14 cases (35%) at 24 months. Computed tomography scans showed that 40% of control patients and 80% of BMC-treated patients had evidence of at least a unilateral continuous bridging of the bones between neighboring vertebrae at 24 months, significantly favoring the mixture of spongiosa bone with autologous BMC ($P < 0.05$) as an efficient option to augment PLF healing.

9. Bone marrow concentrates in chronic wounds

Cell-based therapies are an attractive approach for the treatment of recalcitrant chronic wounds. BM-MSCs have been studied as a therapeutic strategy in chronic hard-to-heal wounds [135]. The orchestrated process of wound healing entails cellular and hormonal physiological processes in inflammation, proliferation, collagen matrix formation, and epithelialization which are regulated by various platelet-derived growth factors, such as TGF-b, VEGF, PDGF, granulocyte-macrophage colony-stimulating factor, the interleukin family, EGF, FGF, and TNF-a [44, 105]. In chronic, poor-healing wounds, the activity and effectivity of growth factors and cytokines are often reduced due to a chronically inflamed wound.

Under these conditions the neo-angiogenetic wound healing potential is reduced, resulting in poor or no full wound epithelialization. The rationale for using BMC in these patients is the potential to modulate the immune response and secreting paracrine factors which promote (neo) angiogenesis, thereby providing biological ingredients for wound tissue repair that can jumpstart full wound closure [76, 136]. Optimal wound bed preparation is of the essence in wound healing strategies and encompasses tissue debridement with proper management of the bacterial load. Based on BM-MSC characteristics and their biological activity, MSCs are capable of interacting with resident wound cells to transform resident cells to functional matrix building cells, as described by Balaji et al. [137]. This finding might be of particular importance for dermal rebuilding processes, to stimulate (transplanted) keratinocyte-mediated wound epithelialization.

10. Bone marrow concentrates in critical limb ischemia

Patients with significant, below the knee, vascular diseases and who are, first of all, not eligible for revascularization surgery or endovascular treatments due to several comorbidities or have high operative risk and had multiple failures of revascularization or high rate of re-stenosis, might be suitable candidates for biological cell-based therapy with BM-MSCs. Patients diagnosed with critical limb ischemia (CLI) might also suffer from chronic non-healing wounds, and the estimated amputation and mortality rates are high [138]. The application of regenerative medicine therapies, in particular the use of BM-MSCs protocols, has merged as a treatment option in patients with CLI. In these patients, the justification to use BMC is to promote the regeneration of impaired endothelium and stimulate neo-angiogenesis in ischemic areas [139]. Several varieties of BM-MSC therapies have been studied in CLI patients, ranging from BM-derived mononuclear cells, CD34+ BM cells, to mesenchymal stromal cells. The outcomes of cell-based trials have been encouraging and demonstrated a significant decrease in the rate of amputation [140]. It can be concluded that BM-MSC applications have the potential to modify the natural history of intractable CLI, while high-quality randomized trials are needed [45].

11. Conclusions

Regenerative medicine technologies offer solutions to a number of compelling clinical problems that have not been able to adequately result in a solution through the use of drugs, surgery, or permanent replacement devices. Reviewing the last decades regarding autologous biological therapies, BM-MSCs have gained great interest. The purpose of this chapter was to review specific characteristics of bone marrow tissue and its cellular content, in particular the mesenchymal stem cells. Considerations when performing aspiration techniques and bone marrow concentrate preparations were presented, including explicit roles of hematopoietic and mesenchymal stem cells and other cytokines. Among autologous tissue-based cellular therapies, bone marrow mesenchymal cell therapies have been the most frequently employed and reported on, despite the fact that effects of coadjuvants, dosing, repetitive procedures, etc. are not yet established. Cultured MSC therapeutic interventions require strict procedures and biological license agreements, making them less attractive for same-day regenerative therapies. Using at POC BM-MSC concentrates overcomes these lengthy regulatory processes without the need for mandates. Clinical translation of BM-MSC-based therapies remains a work

in progress, as proper standardization has not yet been recognized [53]. However, in the clinical setting, effective and safe autologous BMA harvesting and preparation of BMC have been reported [42]. More, better, organized randomized clinical trials that are warranted with accurate follow-up data revealing the efficacy of BM-MSC therapy, including laboratory validation of the used products, should be a future goal. Furthermore, proper deliberations should manage the enormous variability aspects, like aspiration techniques, imaging options and procedures, BMC preparation protocols, effect of patient age, as well as tissue disease state. Therapy failures should also be highlighted in order to understand how they impact the therapy outcomes. Ultimately, the adoption of an accepted standard of overall regenerative biological preparations, including critical and ambivalent nuances, is crucial for future regenerative medicine practices.

Conflict of interest

Author P. Everts is also the chief scientific officer of the EmCyte Corporation.

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