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MINI-REVIEW

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Bone marrow aspirate clot: A technical complication or a smart approach for musculoskeletal tissue regeneration?

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One of the methods employed to improve healing of damaged tissues is the use of cellular based therapies. A number of regenerative medicine based strategies, from in vitro expanded mesenchymal stem cells (MSCs) to "one-step" procedures using bone marrow (BM) in toto (BM aspirate; BMA) or BM concentrate (BMC), have been developed. Recently, orthopedic researchers focused their attention on the clinical therapeutic potential of BMC and BMA for musculoskeletal regeneration. BMA is reported as an excellent source of cells and growth factors. However, the quality of BM harvest and aspirate is extremely technique-dependent and, due to the presence of megakaryocytes and platelets, BMA is prone to clot. BMA clot formation is usually considered a complication hampering the procedures on both BMC preparation and MSC expansion. Therefore, different protocols have been developed to avoid and/or degrade clots. However, from a biological point of view there is a strong rationale for the use of BMA clot for tissue engineering strategies. This descriptive systematic literature review summarizes preclinical and clinical studies dealing the use of BMA clot for orthopedic procedures and provided some evidence supporting its use as a cell based therapy for cartilage and bone regeneration. Despite these results, there are still few preclinical and clinical studies that carefully evaluate the safety and efficacy of BMA clot in orthopedic procedures. Thus, implementing biological knowledge and both preclinical and clinical studies could help researchers and clinicians to understand if BMA clots can really be considered a possible therapeutic tool.

KEYWORDS
bone marrow clot, bone regeneration, cartilage regeneration, clinical studies, preclinical studies

1 | INTRODUCTION

The constant aging population and the increase of degenerative, non-traumatic, and traumatic diseases affecting the musculoskeletal system have become main socioeconomic issues. One of the methods able to improve healing of damaged tissue is the use of cellular based therapies. Expanded, cultured mesenchymal stem cells (MSCs), bone marrow concentrate and bone marrow in toto, or bone marrow aspirate (BMA), represent the main strategies developed and used for tissue regeneration. However, the potential of these treatments must to be confirmed by reliable clinical data, and specific studies should be designed in order to identify the best cell sources, manipulation, and

delivery techniques, as well as pathology and disease phase indications (Veronesi et al., 2013). BMA has been identified as an excellent source of cells and growth factors and it has been used with success both for bone, cartilage, and soft-tissue healing (Fortier et al., 2010; Gangji, De Maertelaer, & Hauzeur, 2011; Veronesi et al., 2016). Bone marrow (BM), and consequently BMA, contains mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), endothelial progenitor cells, and other progenitor cells, together with growth factors, including bone morphogenetic proteins (BMP), platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), and IL-1 receptor antagonist. In addition, the transplant of the whole BMA, by transferring to the lesion site the entire regenerative potential present in the BM environment, allows to perform the entire procedure directly in the operating room, without the need of additional laboratory stage, thus allowing the transplantation to be done in "one step," reducing costs and risks and not requiring a GMP facility. Although only a limited number of large clinical series have been performed, they appear to indicate that the use of BMA is a promising technique demonstrating increased regenerative potential by the addition of marrow elements (Veronesi et al., 2013).

The main site of BM aspiration is the iliac crest that allows to collect a considerable amount of tissue (Malempati, Joshi, Lai, Braner, & Tegtmeyer, 2009). The quality of local iliac harvest and aspirate is extremely technique-dependent, and failures are typically due to inconsistent aspiration techniques (Watson, 2005). Moreover, the quality of the aspirate is reduced by the increasing volume of BM withdrawn. In fact, while the first 4–5 ml of BMA contains MSCs of high quality, withdrawal of bigger volumes leads to dilution of the aspirate with peripheral blood (Cuthbert et al., 2012). In BMA, MSCs represent only a small percentage of the total cell pool (Veyrat-Masson et al., 2007), thus for most tissue engineering or therapeutic applications they require to be expanded by in vitro culture (Wang, Qu, & Zhao, 2012). In addition, the quality of the in vitro culture mainly depends on the initial cell pool, that is, cell diversity and high starting number (Lazarus, Haynesworth, Gerson, Rosenthal, & Caplan, 1995). On the other hand, MSCs from low quality samples require longer time in culture and extended in vitro passages in order to reach the cells number needed. In both cases, extended in vitro passages are cause of cell senescence and can result in loss of differentiation potential (Bertolo et al., 2016). In addition, because of the presence of megakaryocytes and platelets, BMA is prone to clotting, even after the addition of anticoagulants (Wang, Li, Guo, & Guo, 2015). During the process of coagulation, a complex cascade of chemical reactions occurs involving the conversion of fibrinogen into fibrin fibers that enmesh platelets, cells, and plasma to form a clot (Adams & Bird, 2009). Indeed, there would be a number of reasons to believe that the inclusion of a BMA clot to a lesion site might have relevant biological effects. The formation of a BMA clot will comprise degranulation of platelets (Palta, Saroa, & Palta, 2014). This will deliver many osteotropic cytokines and growth factors into the lesion site. Main bioactive factors released in this process include, but are not limited to, PDGF, epidermal growth factor (EGF), fibroblast growth factors (FGF), and TGF-β (Muschler et al., 2003; Palta et al., 2014). In addition, fibrinolytic activity that occurs during the first few days within a clot may provide an additional source for angiogenic factors (fibrin split products) and this would be particularly important during the early stages of graft incorporation (Muschler et al., 2003). Finally, the clot could also give a higher stability to the graft site in comparison to un-clotted BMA.

In our laboratory, we routinely sample BMA from different sources (BM, adipose tissue, synovial fluid, blood . . . etc) from both humans and animals (Della Bella et al., 2017; Pagani et al., 2017; Veronesi, Pagani, Della Bella, Giavaresi, & Fini, 2014, Veronesi et al., 2015). Recently, we started to sample and evaluate BMA from the vertebral bodies of patients undergoing spine surgery (unpublished). Aim of this activity is to characterize vertebral BMA for future applications in spinal regenerative medicine, with the advantage that additional work will have to be performed only in the clinical scenario. Normally, although samples are mixed with anticoagulant immediately after withdrawal, many samples are at least in part clotted when they arrive in the laboratory for processing, after about 15–20 min. Obviously, at this stage, re-sampling to replace clotted specimens would need an additional intervention under local or general anesthesia, and this is clearly unfeasible. Therefore, in order to maximize cell yield we tried to culture both un-clotted BMA and clotted BMA (mechanically cut) and, unexpectedly, after 15 days of culture we observed an higher growth kinetics of MSCs derived from clotted compared to un-clotted BMA (Figure 1). In addition, in the past we had observed the same phenomenon also for rabbit BMA derived from the iliac crest

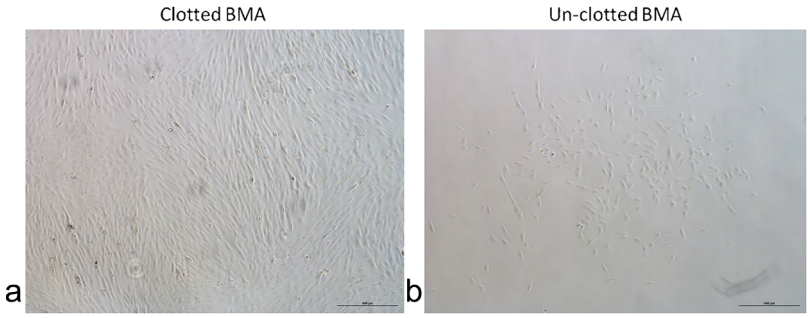

FIGURE 1 a) clotted BMA and b) un-clotted BMA, in normoxic condition, after 15 days of culture

(unpublished results). Therefore, these results suggest that clotted BMA might be an even more efficient source of MSCs than unclotted BMA, and that BMA clot could be entirely transplanted to the lesion site alone or in association to scaffolds, thus providing an alternative sources for tissue engineering applications. Nevertheless, contrary to our results, two in vitro studies (Schlaefli et al., 2015; Wang et al., 2015) analyzing BMA, clotted BMA, mechanically cut BMA clots, and BMA clots treated with urokinase, showed that the average number of colony-forming unit-fibroblast and the number of attached cells in urokinase treated samples was comparable to that of un-clotted samples, which was significantly higher than those of mechanically-cut clots and BMA clots. However, these studies showed that the expression of unique surface markers and the differentiation capacity of cells from all the groups were characteristic of MSCs (Schlaefli et al., 2015; Wang et al., 2015). Despite, these studies strongly suggested urokinase digestion as a good method to treat the clots to isolate MSCs, it is important to underline that marrow coagulation was not physiological but it was mimed by the addition of thrombin (at a final concentration of 1 U/ml) to BMA aliquots. Thrombin generation percentage represents the most important determinant of the final clotting structure (Wolberg, Monroe, Roberts, & Hoffman, 2003), therefore, it is crucial to evaluate what really happen in the hemostatic process.

Despite the clinical use of BMA for orthopedic procedures, very little is known about the use of BMA clot. In fact, even if numerous studies use BMA clot for cartilage microfracture procedure, merely as a consequence of the procedure itself (www.pubmed.org search for "cartilage microfracture" from April 2007 to April 2017, original research articles in English, gave 568 articles, 424 of which on humans, and 144 on animals), almost none of them analyzed and evaluated the biological functions and the therapeutic potential of the BMA clot itself. In addition, for all the other orthopedic procedures the information concerning the use of BMA clot in preclinical models but also in the operative scenario is scarce. Thus, the purpose of this descriptive systematic literature review was to analyze current pre-clinical (both in vitro and in vivo) and clinical studies that employed BMA clots for orthopedic procedures. We think it is very important that researchers and clinicians are familiar with the advantages and/or disadvantages of using BMA clot in clinical setting, because a critical issue for the translation of cell-based therapies in humans is an application which ensures local stable and viable cells and, to date, there is still a lack of standardized systems for clinical use, thus novel "patient-oriented" techniques should be set-up.

2 | MOTIVATIONS

2.1 | Why a systematic review?

We believed necessary to draw a descriptive systematic literature review of current pre-clinical and clinical studies that employed BMA clot in order to understand if clotted BMA might provide a novel, effective, and alternative strategy to the use of already existing tissue engineering approaches, that is, isolated MSCs, BMC, and whole unclotted BMA, thus providing researchers and clinicians with a starting point with strong foundations for improvement. More in detail, we want to organize the knowledge accumulated in nearly 20 years of research, learn from previous research which used BMA clot, and build a foundation for its pre-clinical and clinical use since there is always the need of new and emerging strategies for advances in the field of tissue engineering.

3 | METHODS

3.1 | Descriptive systematic literature review

Our descriptive literature review involved a systematic search that was carried out, according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement, in three databases (www.pubmed.org, www.scopus.com, www.webofknowledge.com). In order to evaluate ongoing clinical studies, the www.clinicaltrials.gov website was also checked. The keywords were: (bone marrow aspirate clot OR bone marrow aspirate clots OR bone marrow clot OR bone marrow clots OR clot derived from bone marrow OR clots derived from bone marrow OR bone marrow derived clot OR bone marrow derived clots OR clotted bone morrow). We sought to identify studies where BM clots for orthopedic procedures were employed. Publications from 1997 to 2017 (original research articles in English) were included. All the papers found have been screened to identify clinical and preclinical studies. A public reference manager ("www.mendeley.com") was used to delete duplicate articles. Two reviewers working independently (FS and DC) screened titles and abstracts in addition to the full publications against the pre-specified criteria. Any disagreements were resolved through discussion until a consensus was reached, or with the involvement of a third reviewer (MF). Reference lists of the selected articles were also screened to obtain further studies for this review.

4 | RESULTS AND DISCUSSION

An initial literature search performed using the previously mentioned key words retrieved 1,121 references (Figure 2). A total of 271 articles were identified using www.pubmed.org, 446 articles using www.scopus.com, and 404 articles were found in www.webofknowledge.com. The resulting references were selected for supplementary analysis based on title and abstracts and were considered eligible. References were submitted to a public reference manager (Mendeley 1.14, "www.mendeley.com") to eliminate duplicate articles. Sixty-six complete articles were then reviewed to establish whether the publication met the inclusion criteria, and nine articles were recognized eligible for review considering publications from March 1997 to March 2017. Of these nine articles one was a clinical study, two in vivo, three in vitro, and three both in vitro and in vivo studies. In addition, using the same keywords, 404 additional articles were identified from the website www.clinicaltrials.gov but only three of them met the inclusion criteria. We did not perform meta-analyses of the selected studies, but reported the results in a descriptive fashion.

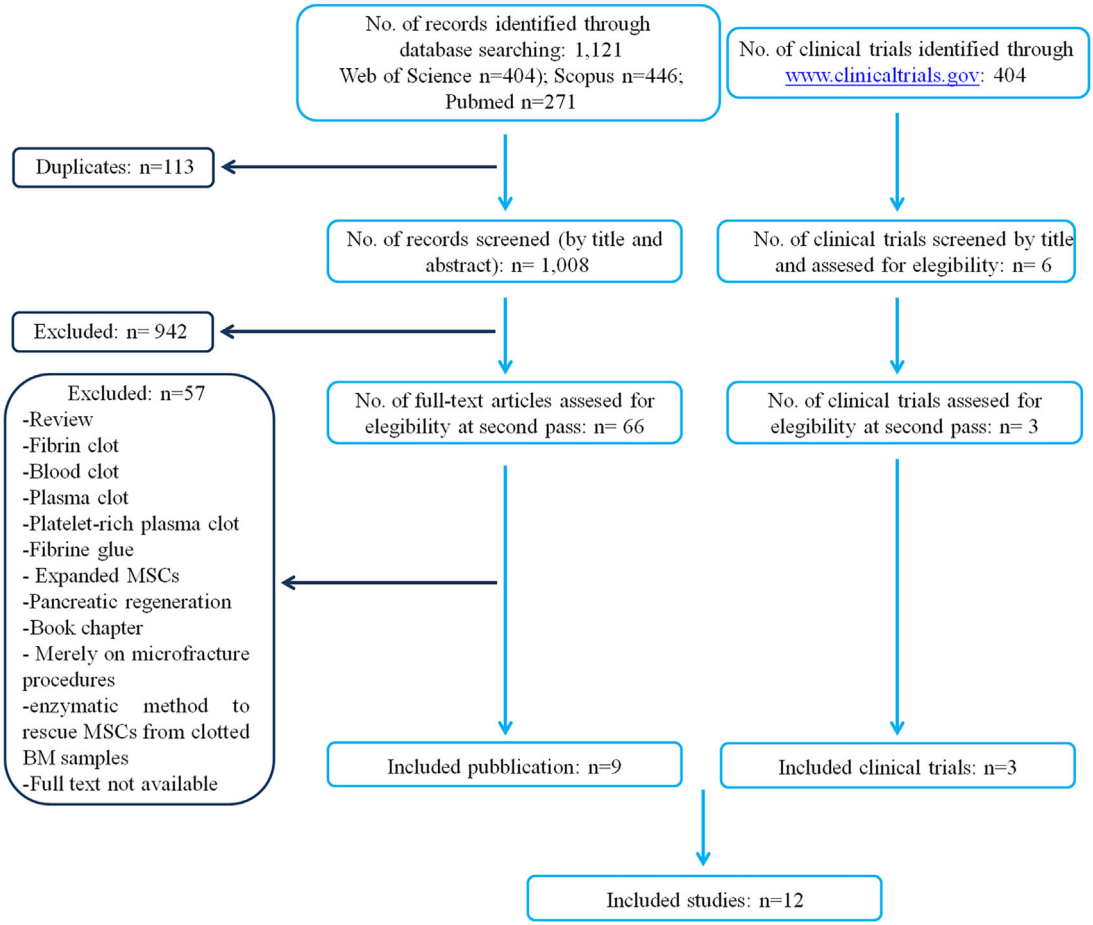

FIGURE 2 Systematic literature review flow diagram. Flow of information through different phases of the systematic review

4.1 | BMA clot for cartilage regeneration

Due to the poor in vivo regenerative capability of cartilage, articular cartilage defect is a great challenge in the field of orthopedic surgery. There are several cartilage repair techniques, including microfracture, joint irrigation or debridement, osteochondral grafting, and autologous chondrocyte implantation. As it is a simple, convenient, and relatively inexpensive, microfracture has become the preferred clinical treatment; however, only few studies on microfracture focused their attention on the analyses of BMA clot formed as a consequence of these procedures (Table 1).

A study on surgical technique and rehabilitation for the treatment of chondral defects demonstrated that BMA clots on the surface of microfracture lesions provide an optimal environment for cartilaginous tissue repair (Steadman, Rodkey, & Rodrigo, 2001). The released marrow elements, MSCs, growth factors, and other healing proteins, form a surgically induced clot that provided an enriched environment for new tissue formation. However, in this study the authors also underlined that this technique results in repaired tissue with low mechanical strength. Thus, in order to increase mechanical strength, (2014, 2015), showed that the BMA-derived mesenchymal stem cells-derived extracellular matrix (ECM) (BMSC-dECM) scaffold, manufactured using a freeze-drying method, favored chondrogenesis of marrow clots, obtained by microholes created in the trochlear groove of rabbits, following microfracture in vitro. Samples included a culture of the marrow clot alone, a culture of the marrow clot with TGF-β3, a culture of the composite of BMSC-dECM scaffold and marrow clot alone, and a culture of the composite with TGF-β3. The study showed that the volume of cultivated tissues in each group was reduced at 1 and 2 weeks, suggesting that clot retraction could play an important role in this process. The volume of the culture of the marrow clot alone gradually shrank over time and finally disappeared after 8 weeks. Thus, it is possible to believe that the degradation of certain components of the marrow clots may have resulted in the lack of a suitable microenvironment for adhesion, proliferation, and secretion of ECM. Enhanced cartilage-like matrix deposition of glycosaminoglycan and type II collagen were seen in cultures of marrow clot with TGF-β3 and in cultures of composite with TGF-β3. In addition, these cultures also show that the expression of chondrogenic genes, such as COL2, ACAN, and SOX9, was gradually up-regulated. BMSC-dECM/marrow clot composite scaffold promotes retention, attachment, and proliferation of cells from the marrow clot, and thus stabilize marrow clot to support chondrogenesis. In addition to these in vitro studies on BMA clot and scaffold, Yao et al. (2015) reported, in a pre-clinical in vitro and in vivo study (2015), the potential advantages of a BMA clot in promoting ECM scaffold chondrogenic regeneration. They realized a

TABLE 1 Published preclinical (in vitro and in vivo) studies on BM clot for orthopedic procedures

Tissue type	Type of study	Bone marrow clot source	Other biological adjuvant	Scaffold material	Experimental time (weeks)	Experimental design	Main outcome	Reference
Cartilage	Clinical	Surgically induced clot	None	None	None	Microfractures into the subchondral bone plate for the treatment of chondral defects.	BM clots on the surface of the microfracture lesions provide an optimal environment for cartilaginous tissue repair.	Steadman et al. (2001)
Cartilage	In vitro	Rabbit iliac crest	Transforming growth factor-β3 (TGF-β3)	Bone marrow-derived mesenchymal stem cells-derived extracellular matrix (ECM) (BMSC-dECM).	1, 2, 4, and 8	Culture of BM clot alone, culture of BM clot with TGF-β3, culture of BMSC-dECM + BM clot alone, culture of BMSC-dECM + TGF-β3.	In culture of BM clot + TGF-β3 and BMSC-dECM + TGF-β3 there was an enhanced cartilage-like matrix deposition of glycosaminoglycan and type II collagen, and upregulation of COL2, ACAN, and SOX9.	Wei et al. (2014, 2015)
Cartilage	In vitro and in vivo (nude mice)	Rabbit iliac crest	TGF-β3	3D porous polycaprolactone (PCL)-hydroxyapatite (HA) + BM clot and MSCs.	4	In vitro: PCL-HA + BM clot and PCL-HA + MSCs. In vivo: PCL-HA + BM clot and PCL-HA + MSCs in vivo subcutaneously implanted into the back of nude mice.	In vitro: PCL-HA + BM clot showed significant improvements in cell adhesion, proliferation, and chondrogenic differentiation in comparison to PCL-HA + MSCs In vivo: PCL-HA + BM clot showed a superior performance in DNA content, Sox9 and Runx2, cells number, ECM accumulation and greater stability, in comparison to PCL-HA + MSCs.	Yao et al. (2015)
Cartilage	In vitro and in vivo (rabbit)	Rabbit iliac crest	Adenoviral vectors containing cDNA for green fluorescent protein	Collagen–glycosaminoglycan matrices preloaded with adenoviral vectors	In vitro: 3 In vivo: 6	In vitro: culture of BM clots and cellular differentiation and transgenic expression In vivo: Collagen–glycosaminoglycan matrices preloaded with adenoviral vectors implanted into a rabbit femoral condyles lesions.	Clotted BMA provided a superior medium for gene delivery to osteochondral defects.	Pascher et al. (2004)
Cartilage	In vitro and in vivo (sheep)	Sheep iliac crest	Adenoviral vectors containing cDNA for green fluorescent protein or TGF-β1	BMA clot transduced with adenoviral vectors containing cDNA for green fluorescent protein or TGF-β1	In vitro: 3 In vivo: 24	In vitro: BM clot transduced with green fluorescent protein (Ad.GFP) In vivo: BM clot, BM clot genetically modified with Ad.GFP, BM clot genetically modified Ad.TGF-b1 implanted into a partial-thickness defect in a femoral condyle.	BM clot transduced with Ad.GFP showed a high number of fluorescent cells throughout the coagulate over 21 days of culture. BM clot formed in the absence of Ad.GFP confirmed the specificity of fluorescence signal. Repaired tissue from TGF-treated defects showed significantly higher amounts of	Ivkovic et al. (2010)

(Continues)

TABLE 1 (Continued)

Tissue type	Type of study	Bone marrow clot source	Other biological adjuvant	Scaffold material	Experimental time (weeks)	Experimental design	Main outcome	Reference
							collagen II.	
Bone	In vivo (dogs)	Dog proximal humerus	None	Mineralized and demineralized cancellous bone matrix.	12	1) cancellous bone matrix enriched with bone marrow cells, 2) cancellous bone matrix plus BM clot and 3) cancellous bone matrix enriched with bone marrow cells plus BM clot implanted in a posterior segmental spinal fusion model.	The union score, fusion volume and fusion area, for the enriched bone matrix plus BM clot composite were superior to the enriched bone matrix alone and the bone matrix plus BM clot.	Muschler et al. (2003)
Bone	In vivo (dogs)	Dog proximal humerus	BMP-7	Osteogenic Protein-1 (OP-1) device.	4	The OP-1 device was implanted in a cancellous metaphyseal femoral defect combined with either clotted blood or BMA.	Increasing the local population of cells and connective tissue progenitors the BM clot enhances the performance of OP-1 device in terms of bone formation.	Takigami et al. (2007)
Bone	In vitro and in vivo (rabbits)	Rabbit trochanter of femur	None	Porous β-tricalcium phosphate (β-TCP) scaffold.	2	Incorporation of gentamicin into the porous β-tricalcium phosphate (β-TCP) scaffold using clotted blood or BMA.	Impregnating gentamicin in a TCP scaffold using clotted blood or BMA as a binder significantly slows drug release as compared to simple aqueous preparations over the course of 3–5 days. Modified tests simulating a restricted diffusion path with clotted blood or tissue surrounding the implant produced release profiles that extend for up to 2 weeks.	Silverman et al. (2007)

new 3D porous polycaprolactone (PCL)-hydroxyapatite (HA) scaffold combined with BMA clot and with bone marrow stem cell. In vitro studies demonstrated that scaffolds combined with BMA clot showed significant improvements in cell adhesion, proliferation, and chondrogenic differentiation. In addition, 4 weeks after in vivo subcutaneous implantation in nude mice, revealed that scaffolds combined with BMA clot showed a superior performance in DNA content, Sox9, and Runx2 expression, cells number and ECM accumulation, in comparison to scaffolds combined with bone marrow stem cell. Importantly, there were no significant differences in mechanics between BMA clot combined scaffolds and BM stem cell seeded scaffolds during in vitro culture and after in vivo implantation. This result showed that the use of BMA clot does not impact negatively on the mechanics of the scaffold and that BMA clot proved to be a highly efficient, reliable, and a simple new method to improve scaffolds biological performance (Yao et al., 2015).

Differently from the above mentioned works, two preclinical studies used an experimental approach exploring the use of gene therapy to repair cartilage defects using clotted BMA (Ivkovic et al., 2010; Pascher et al., 2004). Pascher et al. (2004) using a collagen–glycosaminoglycan matrice preloaded with adenoviral vectors containing various marker genes, in rabbit femoral condyles lesions, found that clotted BMA provided a superior medium for gene delivery to osteochondral defects. Subsequently, Ivkovic et al. (2010) used the same experimental approach in an ovine model where autologous BMA clot transduced with adenoviral vectors, containing cDNA for green fluorescent protein or TGF-β1, was implanted into the femoral condyle. The study showed that genetically modified BMA clots were sufficient to facilitate articular cartilage repair and were able to generate similarly high levels of transgenic expression with better containment of the vector within the defect. The matrix formed from the clot was completely natural, native to the host. These approaches provided all necessary ingredients for successful cartilage repair: transduced mononuclear cells secrete signals that stimulate mesenchymal progenitors to differentiate toward the chondrogenic lineage, and the BMA clot itself provides a natural autologous three-dimensional scaffold to be used for containment of cells and vectors within the defect. Therefore, these studies, suggested that clot formed from BMA may be useful as means of gene delivery to cartilage and perhaps also other musculoskeletal tissues.

4.2 | BMA clot for bone regeneration

Bones can undergo remodeling to adapt to mechanical stress, maintain bone health and repairing small injuries. In particular, osteoclasts and osteoblasts are specialized bone cells responsible for the bone resorption and formation, respectively. However, these mechanisms are not able to repair large bone defects. The "gold standard" procedure for bone defects repair is the transplantation of an autologous bone graft. This however has also several drawbacks, including limited availability, additional surgical site and time, and donor site morbidity. Despite recent advances in the development of novel strategies for bone tissue engineering, most approaches remain inferior to natural bone grafts in their regenerative potential. Therefore, there is an unmet need for new methods able to stimulate bone formation (Table 1). To reach this aim Muschler et al. (2003) evaluated a cancellous bone matrix enriched with BM cells, a cancellous bone matrix plus BMA clot and a cancellous bone matrix enriched with BM cells plus BMA clot in a canine posterior segmental spinal fusion model. The study revealed that the addition of a BMA clot to an enriched cell-matrix composite graft results in significant improvements in graft performance in terms of union score, fusion volume, fusion area, and mechanical testing. A cellular composite graft that contained 50% to 70% more nucleated cells and more than twice the number of osteogenic cells (Enriched Bone Matrix) was inferior to a graft containing fewer cells and progenitors but included the clot environment in the graft site (Bone Matrix Plus BMA Clot). Only when the enriched cell population was combined with a BMA clot to deliver 2½ times the number of marrow cells and more than three times the number of osteogenic cells (Enriched Bone Matrix Plus BMA Clot), results were significantly better than either technique alone. Differently, using an Osteogenic Protein-1 (OP-1) device in a canine cancellous metaphyseal femoral defect, Takigami et al. (2007) evaluated the addition of BMA rather than local blood. In each case, the sample was mixed with the OP-1 device and allowed to clot. Ex-vivo analyses showed that bone formation was increased in animals treated with clotted BMA, suggesting that increasing the local population of cells and connective tissue progenitors using BMA clot can enhance the performance of the OP-1 device.

A different study was carried out by Silverman, Lukashova, Herman, Lane, and Boskey (2007) who developed an in vitro method able to incorporate gentamicin into the porous β-tricalcium phosphate (β-TCP) scaffold by trapping it in clotted BMA, in order to try to prevent and/or treat osteomyelitis. The methods simulated the release in a surgical site and showed long release profiles, with significant amounts of antibiotic being released for up to 2 weeks. Therefore, the use of clotted BMA to slow drug release from a porous implant seems to be an attractive approach to drug incorporation, as it may allow the role of MSCs from new bone to be preserved.

4.3 | Clinical trials on BMA clot

On March 2017, the ongoing clinical trials on BM clots found through www.clinicaltrials.gov web site were 404. Most of them (401) were excluded because the objective of the trials was not related to orthopedics (i.e., anemia, cancer, ventricular dysfunction, fungal infections, polycythemia, etc). The remaining three trials were interventional studies, where the presence of the clot was related to microfracture procedures; two of them were phase III studies, while the other was a proof of concept trial (Table 2). All trials had a minimum follow-up of 12 months but none of them was completed. In detail, the trials evaluated the surgical procedure which creates a BMA clot for the treatment of cartilage defect in association to different scaffolds (i.e., synovial brush; NOVOCART®3D, BST-CarGel). In addition, one of these trials evaluated not only the safety, feasibility, and efficacy of a novel medical device (a synovial brush) in association with the BMA

TABLE 2 List of clinical trials involving BM clot for orthopedic procedures (from clinicaltrials.gov)

Tissue type	Type of study	Clinical condition	Scaffold material	Experimental time (months)	Study arms	Primary Outcome Measures	Status	ClinicalTrials.gov Identifier
Cartilage	Phase III	Defect of articular cartilage cartilage injury osteoarthritis, knee.	Synovial brush	12	Control: conventional microfracture treatment; Experimental: microfracture for the treatment of isolated cartilage defects in combination with arthroscopic synovial brushing.	The mean change in the number of MSCs present in the knee pre- and post- microfracture/ microfracture plus arthroscopic synovial brushing.	Recruiting participants.	NCT02696876
Cartilage	Phase III	Articular cartilage of the femoral condyle.	NOVOCART®3D.	24	Control: conventional microfracture treatment; Experimental NOVOCART 3D.	Pain and function.	Recruiting participants.	NCT01957722
Cartilage	Proof of concept	Disorder of hip region.	BST-CarGel.	12	Control: conventional microfracture treatment; Experimental: BST-CarGel.	Safety (any potential side effects).	Not yet open for participant recruitment.	NCT02540200

clot, but also a procedure (arthroscopic synovial brushing) able to increase the number of autologous MSCs in the clot. This is a novel surgical procedure using a device that has been shown to release MSCs from the synovium in vitro. One trial treated hip disorders while the other two treated knee osteoarthritis, but study arms, type of control group, surgical procedures, clinical approaches, and follow-up evaluations were similar. Each trial was different from the other for patients number (i.e., 20, 50, 233) and lesions size (<2 cm^2, >2 cm^2, from 2 to 6 cm^2). In addition, since none of the trials had been completed, the information available was not always clear. Therefore, at this moment none of the trials could provide useful information about the use of BMA clot for orthopedic procedures.

5 | CONCLUSION AND FUTURE PROSPECTIVE

In the past few years, basic and preclinical research literature clearly indicates the use of BMA, also in combination with various scaffolds and growth factors, for musculoskeletal diseases. However, with the rapidly growing number of tissue engineering techniques, we have seen the need for performing this descriptive systematic literature review on an alternative and little known approach based on the use of BM clot. Since, the formation of BMA clot involves degranulation of platelets leading to the delivery of key cytokines and growth factors that are attracted to, and target bone, we thought that the use of a BMA clot could represent a valid strategy able to facilitate and accelerate orthopedic procedures. Moreover, fibrinolytic activity occurring within a clot could provide an additional source of angiogenic factors, that is, fibrin split products. BMA clot also may serve as a scaffold in which transplanted osteogenic cells and other cells within the site can attach and migrate. Moreover, as shown by Silverman et al. (2007), the clot, having specific characteristics, could facilitate release and/or targeted delivery of antibiotics and drugs. The use of controlled drug release BM clot would provide an important adjunctive route to administer antibiotics and drugs for the treatment of several orthopedic diseases. However, a complete preclinical characterization of this system are needed to validate the potential clinical utility of this drug delivery method.

In this review, therapeutic strategies on the use of BMA clot were employed exclusively for the enhancement of cartilage and bone regeneration and mainly in preclinical studies. Scaffold and bone grafts were always associated with BMA clot in all preclinical studies and also in clinical settings (tissue engineering strategy). Despite the released marrow elements, MSCs, growth factors, and other healing proteins, which provided an enriched environment for new tissue formation, the clot alone results in a repaired tissue with low mechanical strength. However, the use of growth factors and other osteoinductive factors, as well as gene therapy, were also taken into consideration.

We found that preclinical research in this review were carried out in small, medium, and large animal models showing the potential use of BMA clot in tissue engineering strategies for orthopedic procedures.

All authors concluded that BMA clot proved to be an efficient, reliable, and a simple method able to improve the scaffold biological performance. Based on these preclinical data, it would seem that BMA clot with its complex environment of many cell types is able to perform the necessary physiological functions to achieve, facilitate, and accelerate cartilage and bone tissue regeneration. However, none of these studies was able to give a detailed elucidation about the fate of BMA clot and their elements when they were added to a scaffold. In addition, despite the success demonstrated in the animal models, many barriers remain before this therapy can be translated into clinical setting. In fact, this review underlines that there are few and basic clinical trials and all of them have shown the potential use of BMA clot for cartilage microfracture procedures, merely as a consequence of the procedure itself. Additionally, no clinical studies were found concerning the use of BMA clot for bone regeneration or other cartilage related therapies.

Thus, in addition to the need to have a greater number of preclinical and clinical studies able to confirm the potential use of BMA clot as an alternative strategy for tissue engineering approaches, numerous other critical existing limitations include the need to have a better knowledge of its biological action, to understand its biological role and its possible benefits in comparison to the already existing tissue engineering approaches.

In conclusion, and as reported in Figure 3, starting from iliac crest BMA, many ways can be followed to treat patients with bone and cartilage diseases not healing with traditional approaches. In the laboratory, in vitro MSCs isolation, proliferation, expansion; in the operating room, BMC, and BMA preparation. There is also often the need of a proper responsive scaffold to give proper biomechanical properties and, if necessary, also an additive or synergic biologic effect. In this scenario, the great biological potential of BMA clot may deserve more attention. In our opinion, since bone marrow contains a complex environment of many cells types, the use of BMA clot may represent a biological approach able to reduce the costs and some drawbacks linked to BM concentration and expansion. In addition, other advantages concerning the use of BMA clot included, but are not limited to, the elimination of the use of fetal calf serum originated from cell expansion, the elimination of the possible reversibility of the differentiated state, the problem relative to in vivo cells survival and to integration with pre-existing bone. BMA clot could be used directly on the lesion sites but it could be a better source for cell isolation. It is now necessary to implement these preliminary observations on cell recruitment from BMA clot and to evaluate the function of BMA clot with more preclinical research and clinical studies.

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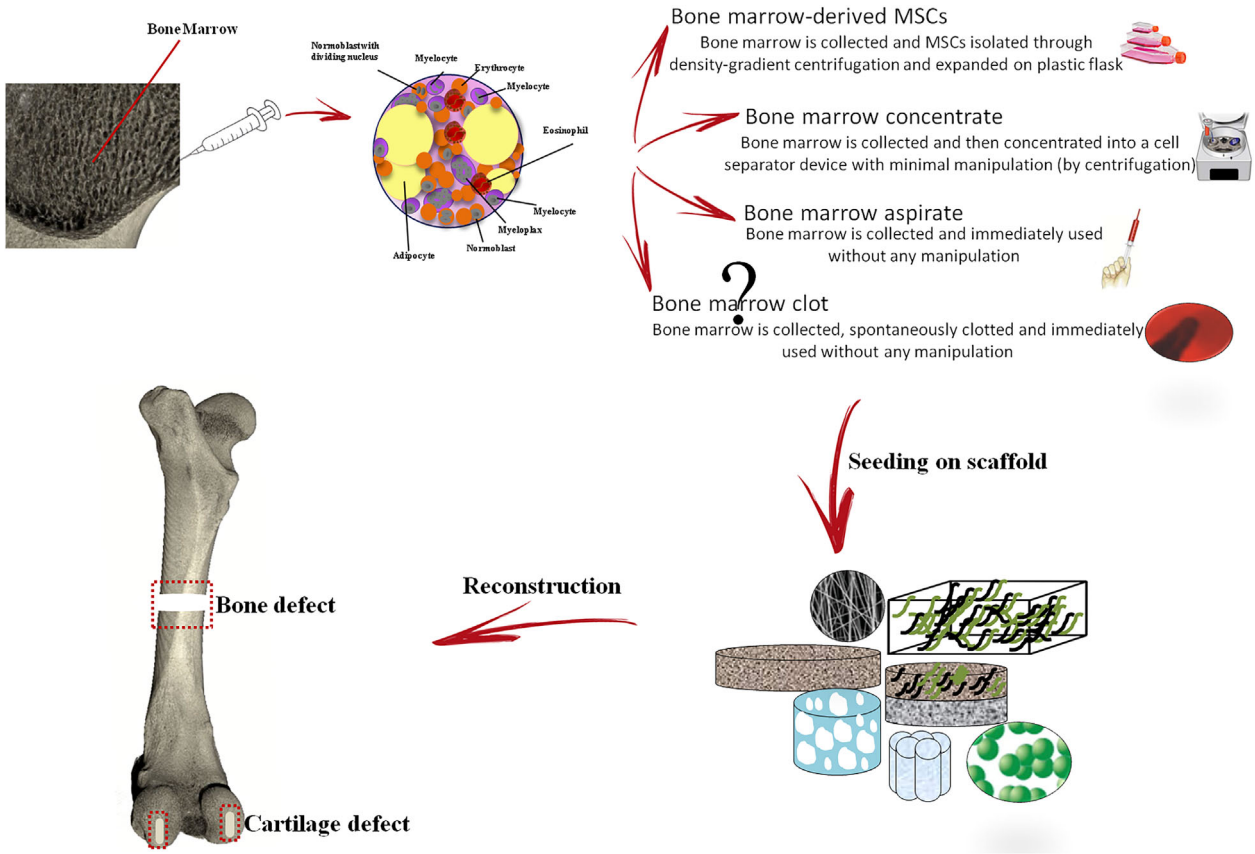

FIGURE 3 Schematic illustration of cell-based approaches in tissue engineering for orthopedic

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