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RESEARCH

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Posterior iliac crest vs. proximal tibia: distinct sources of anti-inflammatory and regenerative cells with comparable 6-month clinical outcomes in treatment of osteoarthritis

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Abstract

Background Human bone marrow is a source of mesenchymal stem cells (MSCs), other progenitor cells, and factors with anti-inflammatory and regenerative capacity. Though the fraction of MSCs out of the nucleated cells is very small, bone marrow aspirate (BMA) for osteoarthritis (OA) has noteworthy effects. BMA is usually collected from the posterior or anterior iliac crest, and rarely from the proximal tibia. We investigated the clinically beneficial concentration of ex vivo MSCs, derived from BM harvested from the posterior iliac crest and proximal tibia by Marrow Cellution™ Aspiration System, and their phenotypic differences, in comparison to autologous Platelet-Rich Plasma (PRP) treatment prepared with a manual, closed system.

Methods A single-center, parallel, randomized controlled study was designed to investigate the efficacy of BMA from the posterior iliac crest compared to BMA from the proximal tibia, against a control group treated with PRP, in knee OA. Thirty patients with knee OA grade I-IV, according to Kellgren-Lawrence (KL), were distributed into each group. Visual Analog Scale (VAS) and Western Ontario & McMaster Universities Arthritis Index (WOMAC) score were used for clinical outcome evaluation.

Results Data from an intermediate analysis of 6-months follow-up, involving 15 patients in each arm, showed that the posterior iliac crest was significantly more densely populated with mononuclear cells, than the proximal tibia ($p = 0.005$). Flow cytometric analysis on ex vivo BMA showed a significantly greater number of MSCs in the BM-derived from the posterior iliac crest when compared with the proximal tibia ($p < 0.001$), together with a significantly higher number of platelets (PLTs) ($p < 0.001$). Surprisingly, despite these differences in cells number, the improvement in early pain and function scores, after each treatment, were statistically significant within each of the three arms. BM from the proximal tibia showed the highest ΔWOMAC, while BM from the posterior iliac crest showed the highest ΔVAS; however, these differences were not statistically significant across the three arms ($p > 0.05$). A better outcome, in terms of ΔVAS, was observed in patients classified as KL I-II, when treated with BMA from crest ($p < 0.001$) and PRP ($p = 0.004$). Moreover, the effect of BMA treatment on ΔVAS depends on MSCs % only in the Tibia Arm ($r = -0.59$, $p = 0.021$), where we also found a correlation between ΔWOMAC and monocytes ($r = 0.75$, $p = 0.016$).

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Conclusion The results indicate that the iliac crest yields a higher concentration of MSCs compared to the proximal tibia, however both BM, independently of the MSCs concentration, show a beneficial clinical outcome in the treatment of knee OA. Furthermore, BMA is not superior to PRP treatment.

Introduction

Pathophysiology

Osteoarthritis (OA), also known as osteoarthrosis is one of the most common, costly and disabling form of degenerative joint disease, characterized by progressive deterioration and loss of articular cartilage with concomitant structural and functional changes in the entire joint [1, 2]. Indeed, although articular cartilage can tolerate a tremendous amount of intensive and repetitive physical stress, it manifests the inability to heal even a minor injury. In addition to the biomechanical forces that place inappropriate levels of stress on the joints, recent scientific evidence suggests that OA has significant inflammatory and metabolic components as well as environmental and genetic factors [3–5].

Management

Although in the clinical context total knee arthroplasty is proven as an effective solution for severe knee OA, there are little satisfactory results [6]. Accordingly, several non-surgical and non-invasive interventions have been described to treat symptomatic knee OA, including knee joint intra-articular (IA) injections, oral nonsteroidal anti-inflammatory drugs and physical therapy.

Orthobiologics

IA injections include also the autologous PRP treatment [7], which has the potential to alleviate pain and improve function for up to one year in patients with mild-to-moderate knee OA [8]. PRP enhances osteogenesis and accelerates wound healing, thanks to its growth factors. In addition to autologous PRP, in the last years, a great number of studies have evaluated also the potential of Mesenchymal Stromal Cells (MSCs) in cartilage tissue regeneration both in vitro and in animal models as recently reviewed [9]. These studies are also supported by a number of clinical trials which have demonstrated the potential efficacy of MSCs derived from bone marrow (BM), adipose tissue, and umbilical cord blood in the treatment of OA [10]. Human BM is a source of MSCs and other progenitor cells, as well as growth factors and cytokines, that may aid anti-inflammation and regeneration for various tissues, including cartilage and bone [11]. Furthermore, the use of BM-derived cells may bypass the time-consuming and technically difficult process of cell expansion and differentiation, enabling both harvesting and transplanting of BM-derived cells during the same surgical procedure. Though fraction of MSCs out of the nucleated cells is very small (0.001%) [12], BM aspirate concentrate (BMAC) for cartilage pathologies, such as cartilage degeneration, defect, and OA, has showed noteworthy effects. However, further research with well-designed, randomized, controlled clinical trials is still needed to elucidate the exact BMA molecular mechanism of action [13–15]. BMAC is frequently obtained through density gradient centrifugation of BMA, usually collected from the posterior or anterior iliac crest, and rarely from the distal femoral or proximal tibia metaphysis, the latter option being proposed in particular for knee pathology treatment [16]. Moreover, although there are several works which characterized and compared BMA collected using different methods [17] and collected from different anatomical sites, no one has defined yet the precise concentration and characteristics of MSCs that may provide clinical benefits. The better clarification of this aspect would also be useful in order to consider other withdrawal sites than iliac crest, such as the tibia bone that could be a more feasible surgical practice with a better morbility.

Objective of the study

Primary objective of this study was:

To assess the superior efficacy of BM aspirate concentrate treatment than autologous platelet-rich plasma treatment (Arm Crest and Arm Tibia vs. Arm PRP) in terms of WOMAC index change at 12 months (it will be presented in a subsequent paper).

Secondary objective(s) of this study were:

To assess the superior efficacy of BM aspirate concentrate treatment than autologous platelet-rich plasma treatment (Arm Crest and Arm Tibia vs. Arm PRP) in terms of WOMAC index change at 6 months.

To assess the superior efficacy of BM aspirate concentrate from iliac crest treatment than autologous platelet-rich plasma treatment (Arm Crest vs. Arm PRP) in terms of WOMAC index change at 12 months.

To assess the superior efficacy of BM aspirate concentrate from tibia treatment than autologous platelet-rich plasma treatment (Arm Tibia vs. Arm PRP) in terms of WOMAC index change at 12 months.
To assess safety and collect adverse events in each of the three arms at each visit.
To compare pain, as measured by VAS score, between BM aspirate concentrate from iliac crest arm and from tibia arm at 12 months.
Cellular characterization of BM aspirate concentrate from iliac crest and tibia. To compare the number of MSCs between BM aspirate concentrate from iliac crest and from tibia.

Methods

Study design

In order to evaluate the beneficial yield and concentration of MSCs derived from BM harvested from the posterior iliac crest and proximal tibia, and to investigate any differences in terms of cell characterization between the two withdrawal sites, we carried out a parallel-randomized controlled study. Patients with knee OA grade I-IV according to Kellgren-Lawrance [18], were distributed into 3 groups: 30 patients in Group Crest received BMA from the iliac crest, 30 patients in group Tibia received BMA from the tibia bone, and 30 in group PRP (as a control) received PRP. PRP has been using in our hospital since a long time in the treatment of osteoarthritis, however, there is no consensus in the literature on the formulation of PRP, and most society guidelines provide inconclusive recommendations for its use [19]. Furthermore, in order to avoid the centrifuge step that could compromise the quality of the harvested product [20], BM was harvested by Marrow Cellution™ Aspiration System (MC System) [20, 21]. This method employs small draws from a single puncture that promotes only lateral flow from multiple sites (SSLM method) without the need to centrifuge the product, allowing also to saving procedural time. In this way, MC System maximizes stem and progenitor cells recovery and minimizes excess blood within the BMA final product. The first patient considered in this intermediate analysis was treated on February 2023, and the last one on February 2024, therefore the study will conclude 12 months after the treatment of the last patient. Here, we present midpoint 6-month follow-up results for 15 of the 30 patients of each arm.

Study population and main criteria for inclusion/exclusion:

All Subjects enrolled were responding to the following inclusion criteria:

1. 35 years ≤ Age ≤ 65 years
2. Uni-compartmental knee osteoarthritis grade I-IV KL
3. Failure of conservative treatment with corticosteroids
4. Patients agree to take part in the study and sign an informed consent
5. Ability to provide written, personally signed, and dated informed consent to participate in the study, in accordance with the ICH GCP Guideline E6 and applicable regulations, before completing any study related procedures
6. An understanding, ability, and willingness to fully comply with study procedures and restrictions

Subjects who met any of the following exclusion criteria were not included in the study and were treated with other standard treatments:

1. Knee or leg or pelvic trauma within previous six month
2. Neoplasia
3. Rheumatic diseases
4. Constitutional deformity of the lower limb > 10°
5. BMI < 18, BMI > 35
6. Pregnancy
7. Positive inflammation index (ESR, PCR)
8. Subject is a participating investigator, sub-investigator, study coordinator, or employee of a participating investigator, or is an immediate family member of the aforementioned
9. Any factor, which in the opinion of the investigator would jeopardize the evaluation or safety or be associated with poor adherence to the protocol.

Evaluation methods for clinical outcomes

In order to control the clinical outcomes of the treatments, we had thoroughly examined the patients and for each step of our follow-up (0, 6 months) we have collected information on the evolution of pain and knee function through dedicated scores (VAS and WOMAC).

Platelet-Rich plasma preparation

PRP production at the Casa Sollievo della Sofferenza Transfusion Center is done with a manual, closed system, using a TERUMO TSCD connector [Old Belfast Road, Millbrook, Larne BT 402SH, United Kingdom] and a FRESENIUS KABI CompoSeal Universal welder (61352 Bad Homburg- Germany), to ensure product sterility. The method involves collecting 150 ml of autologous whole blood in a bag containing 100 ml of CPDA-1 (calcium phosphate-dextrose-adenine). The blood component after a short stabilization period from collection is

subjected to an initial centrifugation for 15' at 900 rpm at 22 °C [Sorwall RC 12BP]. Buffy coats, containing platelets and leukocyte cells, and supernatant (PPP), obtained by gradient separation, are collected by manual separator in a sterile satellite bag. In order to assess platelet yield and the absence of erythrocyte cells, a cell count is performed on an aliquot of the product using the ABX Micros ES analyzer (Horiba). The BUFFY COATS/PPP mix is further centrifuged to compact the cell contents at 3338 rpm for 12'. After removal of the supernatant, the pellet is resuspended in a volume of platelets-poor plasma (PPP) chosen according to platelet yield, number of sessions and sites to be treated, respecting the final PLTs concentration of 0.8–1 x10^6/ul, aliquoted sterilely into a multiple bag and stored at −30 °C.

Randomly selected patients received three doses of autologous PRP, the first dose one week after the apheresis and the subsequent doses at seven-day intervals.

Bone marrow aspiration technique

In order to aspire the BM we used the Marrow Cellution™ Bone Marrow Aspiration System, a multi-level, multi-directional harvesting system as shown in Fig. 1. Due to its patented design, this system can obtain pure BM from numerous locations within the marrow space from just one single insertion. One of its most attractive features is that there is no need to perform time-consuming manipulation outside of the sterile field (e.g. centrifugation). Unlike traditional Jamshidi needles, which contain only one opening (at the distal tip), newer needle designs feature multiple lateral holes to help aspirate BM in multiple, simultaneous directions. Some newer, improved Jamshidi needles contain lateral holes as well; however, the distal hole at the end of the improved Jamshidi remains the main aspiration path and can still pull in peripheral blood that dilutes the BMA. To solve this problem, the Marrow Cellution™ next-generation BMA device, includes an aspiration cannula that blocks the distal tip, forcing aspiration to occur through the lateral holes only. Additionally, the Marrow Cellution™ was specially designed with a screw mechanism that allows the user to adjust easily the depth of the device within the marrow space, enabling precise relocation of the aspiration holes to a fresh harvest site. This design ensures that proper harvesting technique is maintained during the complete aspiration process. Simply rotating the handle after every 1–2 mL aspiration allows marrow to be harvested from multiple depths while minimizing infiltration with peripheral blood. With this technique no mesh has been adopted to concentrate and purify the final product. The final aspirate contained a high proportion of high-quality stem and progenitor cells [22]. The procedure was carried out under mild sedation and no

Fig. 1 Marrow Cellution™ Bone Marrow Aspiration System, a multi-level, multi-directional harvesting system

patients required additional, post-procedure analgesia for pain, both in the posterior iliac crest and proximal tibia groups, which patients reported to be either mild or non-existent. A total of 10 mL of BM was harvested from the posterior iliac crests of 15 patients and 10 mL of BM from the proximal tibia of other 15 patients. Five mL of aspirate was injected into the knee of the same patient, and the remaining 5 mL were collected in tubes containing 1000 U/mL of heparin (Sigma-Aldrich, St. Louis, MO, USA), for further analysis as described below.

BM purity and MNC count

BM purity was calculated according to Holdrinet et al [23]. The day before the BM harvesting, a sample of venous blood was taken for complete blood count. On the day of the injection, a small amount of BM withdrawn was used for the complete blood count. Cells were counted using an automatic Hematology Analyzer ABX Micros ES 60 (HORIBA Medical). Some of the samples aspirated from proximal tibia were diluted before counting in Phosphate Saline Buffer (PBS), pH 7.4 (Thermo Fisher Scientific, US) to avoid the clogging of the analyzer because the presence of lipid droplets that clogged the analyzer. The number of leukocytes and erythrocytes of BM and peripheral blood (PB) was used for calculating the BM purity using the following

formula [23]: BM purity = [1 − (erythrocytes$_{BM}$/erythrocytes$_{PB}$) × (leukocytes$_{PB}$/leukocytes$_{BM}$)] × 100%. Additionally, the number of mononucleated cells (MNCs) in the BM aspirated was derived by the summary of lymphocytes and monocytes count.

Flow cytometric analysis

Erythrocyte-lysed whole bone marrow (BM) samples were immunophenotyped using an eight-color direct immunofluorescence panel technique. The following combination was used to identify MSCs: CD45-V500/CD19-V450/CD71-APC-H7/CD105-PerCP-Cy5.5/CD34-PE-Cy7/CD271-PE/CD73-FITC/HLADR-APC. Monoclonal antibodies were purchased from BD Biosciences. Gating strategy to identify MSCs was performed as follows (Fig.2): (1) the CD271-positive population was selected; (2) the CD271-positive events which expressed both CD73 and CD105 markers were gated; (3) finally, a back-gate on CD45- negative / low expressed events was provided to confirm the population previously defined as MSCs. MSCs were quantified as percentage respect to BM total cells. An intra –assay quality check of the whole cell sample was provided by the identification of B-cell precursors (CD19+, HLADR+, CD45+lo), hematopoietic stem cells (CD34+, HLADR+, CD45+int), and nucleated red blood cells (CD71+, HLADR-, CD45-). In all samples, an isotype matched negative control with no BM reactivity was used. At least 100.000 events were acquired by using a FACS Canto flow cytometer and a FACS Diva software.

Isolation of bone marrow nucleated cells and MSCs expansion

2–3 mL of undiluted BMA were centrifuged at 300 g for 5 min. Plasma was removed and 1 × 10^5 cells/cm^2 MNCs were plated on 25-cm^2 culture flask with α-Modified Minimum Essential medium (α-MEM; Gibco^{TM}, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS; Gibco^{TM}, Thermo Fisher Scientific, USA) and 5% L-Glutamine 200 mM (Gibco^{TM}, Thermo Fisher Scientific, USA). The flasks were incubated at 37 °C in a humidified atmosphere of 5% CO_2 with medium change every 3–4 days. When the cells reached ~70–80% confluence, they were detached by mild trypsinization (Trypsin-EDTA (0.05%), Thermo Fisher Scientific, USA) for 3 min at 37 °C and counted. Adherent cultured cells were reseeded into a new 75 cm^2 flask at a density of 5000 cells/cm^2 and expanded up to 5–8 passages.

Immunohistochemistry

A calcium chloride and plasma solution was added to BM aspiration samples allowing a clot formation. A fixation in 10% buffered formalin was performed. The solid

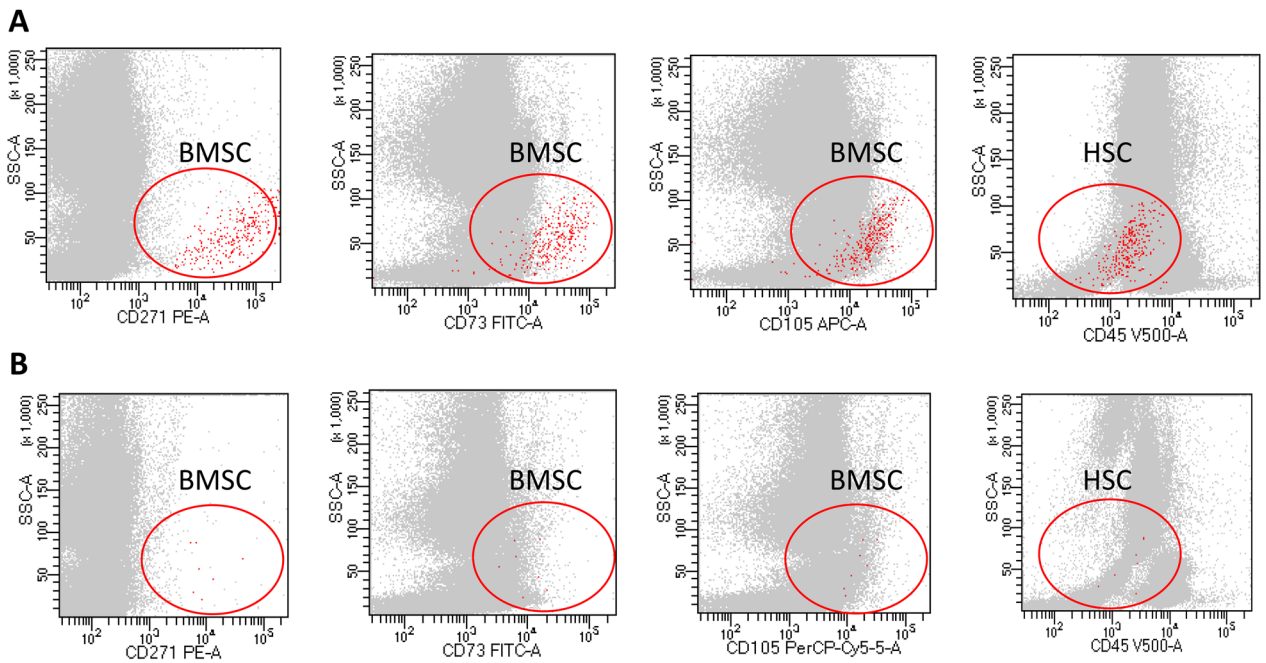

Fig. 2 Exemplary representation of a gating strategy to identify MSCs from posterior iliac crest (**A**) and proximal tibia (**B**). CD271-positive events, which expressed both CD73 and CD105 markers were gated; a back-gate on CD45- negative / low expressed events was provided to confirm the population previously defined as MSCc

clot was automatically processed and embedded in paraffin and subsequently four micron-thick sections were stained with hematoxylin and eosin (H&E) to evaluate adequacy and percentage of cells. Serial sections from each cell-block underwent immunohistochemical (IHC) analysis on the Dako Autostainer Link 48 platform (Agilent Dako, Santa Clara, CA, USA) following appropriate staining protocols and manufacturer's instructions. Briefly, formalin fixed paraffin-embedded sections (3 µm) were selected for IHC analysis and collected on polarized slides. The sections were deparaffinised in xylene, hydrated in gradient alcohol, and warmed in Tris –EDTA buffer (0.01 M, pH = 9.0) for antigen retrieval at 98°C. The sections were then incubated with hydrogen peroxide (0.3% v/v) in methanol for 5 min to quench the endogenous peroxidase activity. Thereafter, the slides were incubated with primary antibodies represented by CD73 (polyclonal antibody, Proteintech, USA), CD90 (clone 2D7D11, Proteintech, USA) and CD105 (polyclonal antibody, Proteintech, USA) for 45 min at RT. The primary antibody was detected by using commercially available detection kit (EnVisionTMFLEX+, Dako, Glostrup, Denmark) following the manufacturer's protocol and diaminobenzidine as chromogen. Slides were washed with Tris-buffered saline (TBS, 0.1 M, pH = 7.4), 3–5 times after each step. Finally, the sections were counterstained with Mayer's hematoxylin and mounted with Biomount (BIO-OPTICA, Milan, Italy). The sections were then evaluated by light microscopic examination using Olympus BX51 microscope.

PRP and BMA injection

With the patient seated or in a supine position, a superolateral injection approach was preferred for intra-articular knee injections, especially when an effusion was present. The physician was standing on the injection side of the affected knee and injected 5 mL of PRP or BMA through intra-articular knee injection (Fig. 3), using a needle with 27–22 gauge and 1.5–2.0 inch. If swollen knee was present, a needle with a gauge of 22–18 and a length of 1.5–2.0 inches was used for preliminary arthrocentesis, before the biological injection. PRP treatment was repeated three times every 7 days, while BMA treatment only once.

Statistical analysis

Continuous variables were summarized using medians and interquartile ranges (IQR). Categorical variables were summarized using frequencies and percentages. The non-parametric Kruskal-Wallis test was used to compare the distributions of continuous variables across the three study arms. For categorical variables, Fisher's exact test was applied. The significance of pre/post changes in VAS and WOMAC scores within each arm was evaluated using the Wilcoxon matched-pairs signed-rank test. The relationship between pairs of continuous variables was assessed using Spearman's rank correlation, while the association between a continuous variable and a categorical variable was measured using the coefficient of determination (R^2, proportion of explained variance). The associations between variables were jointly analyzed within a single model, which also considered some potential confounders, using multivariable linear regression models. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using Stata 18 software (StataCorp. 2023. College Station, TX).

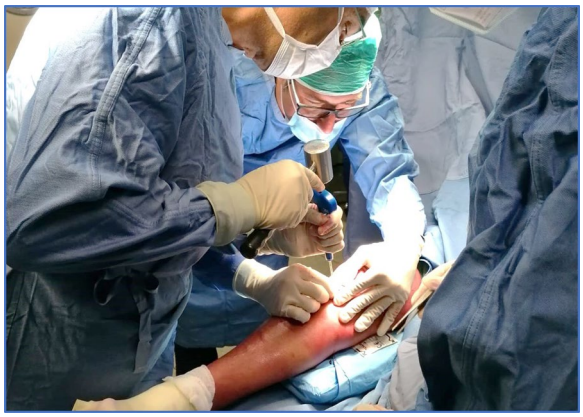

Fig. 3 Anterolateral injection of BMA in the knee

Results

PRP treatment

For the preparation of PRP, a protocol as described in the Materials and Methods section was followed. There were no complications during the procedure, such as edema or hematoma.

BMA treatment

The technique used to obtain BM samples from the proximal tibia and from the posterior iliac crest was straightforward and reproducible in all patients. For the proximal tibia the anatomical landmarks were easily identified by palpation, while for the posterior iliac crest in overweight patients because was slightly challenging to identify the landmarks, the harvesting was ultrasound guided. There were no complications (such as fractures or neurovascular damage) during the procedure in the posterior iliac crest group; we observed only two edema and one hematoma. Conversely, in the proximal Tibia Arm there were two fractures on 15 (13.3%) patients treated ($p < 0.001$). Macroscopically, most of the BM samples from the

proximal tibia used for the analysis presented, after centrifugation at 300 g for 5 min, an appreciable supernatant of fat that was rarely present in samples from the iliac crest (Fig. 4).

Bone marrow purity and MNCs amount

A total of 30 BMA samples were analyzed, with 15 samples from Crest Arm and 15 from Tibia Arm. Calculation of the BM Purity using Holdrinet [23] formula showed that BM collected from proximal tibia was significantly less pure compared to that from posterior iliac crest. The mean BM purity percentage were 71% in the posterior iliac crest and 30% in proximal tibia ($p=0.002$) (Table.1). This difference also reflected the difference observed in the concentration of MNCs at the 2 sites, in fact the mean MNCs concentrations (in millions of cells per mL) were 7.10 in posterior iliac crest and 3.58 in proximal tibia ($p=0.005$) (Table. 1), values consistent with those observed in other studies [24, 25]. However, we found out that improving the BM harvesting technique following the Snap-Back method [26], the number of MNCs increased significantly in the samples collected by the other 15 patients included in the 2 BMA arms (data not shown).

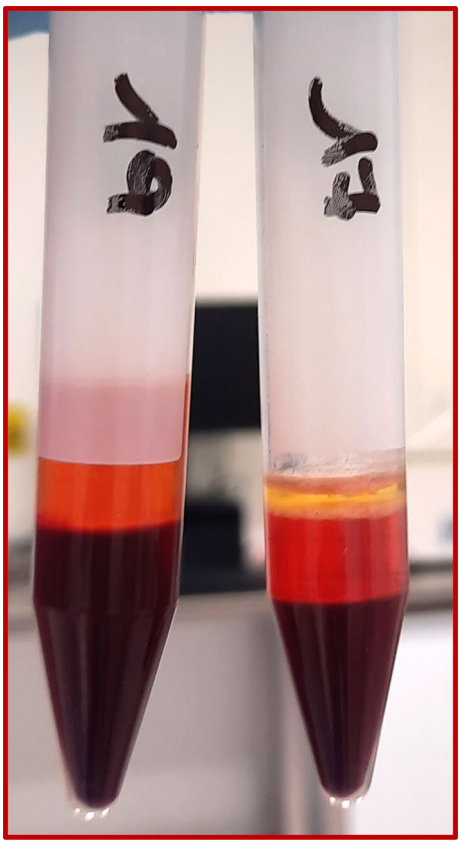

Fig. 4 BM sample from the tibia (on the right), after centrifugation, presents an appreciable supernatant of fat that was rarely present in samples from the iliac crest (on the left)

Quantification and phenotype characterization of BMA from the posterior iliac crest and from proximal tibia

The quantification of MSCs population at the two sites and the characterization of the two BMA were among the secondary objectives of this study. BM derived-cells from both posterior iliac crest and from proximal tibia, showed the same phenotypic pattern as analyzed by flow cytometry for specific surface antigen expression [27]. BMA harvested from the 2 different sites revealed comparable percentage of expression for all the markers considered, with a positive expression for the MSCs markers CD90, CD105 and CD271 in the region were cell population was negative for CD19, CD71, HLADR, CD45 and CD34 (Fig. 2). In vitro culture of MSCs isolated from BM and expanded in vitro for 5–8 passages confirmed the phenotyping and morphological similarity, with MSCs that grew up in the typical spindle-like shape and plastic adherent, from both anatomical sites, if they were present (Fig. 5). Conversely, cellular quantification by flow cytometer showed that the number of MSCs in the BM was significantly higher in posterior iliac crest ($p<0.001$), with $\leq 50\%$ of samples containing a percentage of MSCs equal to or less than 0.058%, compared to proximal tibia, where $>50\%$ of samples lacked MSCs (Table 1). In other words, the majority of BM samples derived from tibia do not have MSCs, instead those that have showed a very small number of MSCs compared to superior iliac crest.

MSCs characterization by morphological and immunohistochemical analysis

BMA cell blocks derived from eight proximal tibiae and 13 posterior anterior crests were stained with hematoxylin and eosin (H&E), and evaluated by an expert pathologist to assess cellular adequacy. An adequate cellularity ($>1\%$) was obtained only in BMA samples derived from posterior iliac crests. IHC analysis highlighted and confirmed the presence of mononuclear cells immune-reactive for CD73, CD90 and CD105, consistent with MSCs in ex vivo samples (Fig. 6).

Primary outcome parameters: BMA vs PRP treatment

Table 1 provides a statistical description of the demographic and clinical variables characterizing the sample at baseline and after 6 months, separately for the 3 study groups.

The average age at the time of treatment was 57 y.o. for Crest Arm, 53 y.o. for PRP arm and 59 y.o. for Tibia Arm ($p=0.1$). No significance differences was observed in the gender distribution ($p=0.9$) and K-L

Table 1 Clinical and demographic variables across the three study groups; medians with interquartile ranges summarized continuous variables, while frequencies and percentages described categorical variables

	Crest	PRP	Tibia	P
N	15	15	15	
Sex (male)	8 (53.3%)	8 (53.3%)	9 (60.0%)	0.9
Age (years)	57.00 (48.00–58.00)	53.00 (45.00–58.00)	59.00 (51.00–62.00)	0.1
BMI (Kg/m^2)	26.42 (24.78–29.38)	27.28 (26.12–27.69)	27.36 (24.31–28.74)	0.9
Side (right)	10 (66.7%)	9 (60.0%)	7 (46.7%)	0.5
K-L				
I	4 (26.7%)	3 (20.0%)	0 (0.0%)	0.034
II	6 (40.0%)	10 (66.7%)	7 (46.7%)	
III	3 (20.0%)	2 (13.3%)	8 (53.3%)	
IV	2 (13.3%)	0 (0.0%)	0 (0.0%)	
HKA	179.80 (175.20–183.80)		182.60 (177.40–185.30)	0.4
%BM	71.00 (53.00–80.00)		30.00 (17.00–40.00)	0.002
MSCs (10^3%)	58.00 (18.00–275.00)		0.00 (0.00–5.00)	< 0.001
MNC (10^6/ml)	7.10 (5.34–8.95)		3.58 (3.18–4.72)	0.005
Monocytes (10^6/ml)	1.55 (1.19–2.66)		1.53 (0.89–1.97)	0.6
PLTs (10^6/ml)	189.50 (157.00–239.00)		64.50 (42.00–87.00)	< 0.001
HCT	41.10 (39.15–42.90)		41.00 (36.80–44.00)	0.9
VAS at T0	10.00 (10.00–10.00)	8.00 (6.00–9.00)	8.00 (7.00–10.00)	< 0.001
VAS at 6 mo	5.00 (1.00–8.00)	4.00 (1.00–6.00)	3.00 (1.00–7.00)	0.5
ΔVAS	−5.00 (−9.00–−2.00)	−4.00 (−5.00–−2.00)	−4.00 (−7.00–−2.00)	0.8
−ΔVAS%	−50 (−90–−20)	−55.5 (−83.3—−25.0)	−62.5 (−80–−30.00)	0.9
WOMAC at T0	37.00 (28.00–71.00)	30.00 (22.00–39.00)	42.00 (35.00–64.00)	0.067
WOMAC at 6 mo	23.00 (9.00–49.00)	16.00 (7.00–21.00)	25.00 (16.00–34.00)	0.4
ΔWOMAC	−14.00 (−20.00–0.00)	−11.00 (−20.00–−7.00)	−16.00 (−35.00–−6.00)	0.6
WOMAC Pain at T0	8.00 (6.00–13.00)	6.00 (3.00–8.00)	9.00 (6.00–12.00)	0.1
WOMAC Pain at 6 mo	5.00 (3.00–7.00)	3.00 (1.00–6.00)	4.00 (3.00–6.00)	0.3
ΔWOMAC Pain	−2.00 (−5.00–0.00)	−2.00 (−4.00–−1.00)	−3.00 (−7.00–−1.00)	0.5
WOMAC Stiffness at T0	3.00 (2.00–6.00)	3.00 (2.00–4.00)	4.00 (2.00–6.00)	0.2
WOMAC Stiffness at 6 mo	2.00 (1.00–4.00)	1.00 (0.00–2.00)	2.00 (1.00–4.00)	0.066
ΔWOMAC Stiffness	−1.00 (−3.00–0.00)	−2.00 (−3.00–−1.00)	−2.00 (−3.00–0.00)	0.6
WOMAC ADL at T0	24.00 (19.00–49.00)	21.00 (17.00–27.00)	31.00 (24.00–45.00)	0.069
WOMAC ADL at 6 mo	17.00 (3.00–37.00)	12.00 (7.00–15.00)	17.00 (12.00–23.00)	0.4
ΔWOMAC ADL	−13.00 (−17.00–0.00)	−7.00 (−15.00–−4.00)	−11.00 (−25.00–−3.00)	0.6

BMI body mass index, *KL* Kellgren Lawrance, *HKA* Hip-knee-ankle, *BM* bone marrow, *MSCs* mesenchymal stem cells, *MNCs* mono-nucleated cells, *PLTs* platelets, *HCT* hematocrit, *VAS* Visual Analogue Scale, *WOMAC* Western Ontario and McMaster Universities Arthritis Index, *ADL* Activities of Daily Living, *P* p-value from the Kruskal–Wallis test assessing the significance of differences across the three arms

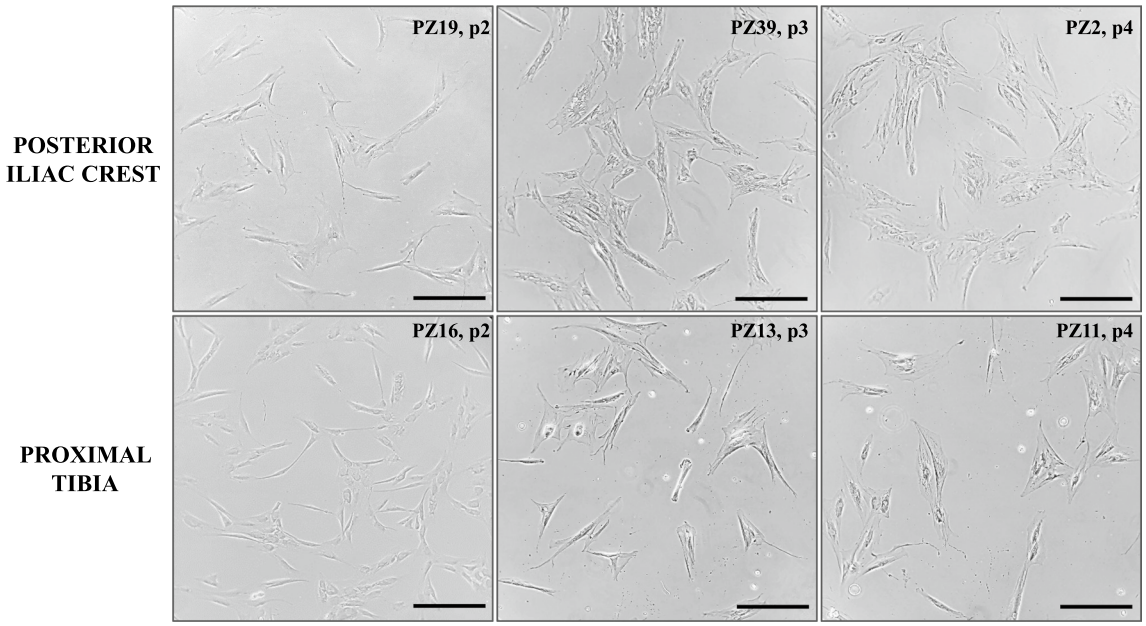

Fig. 5 On the upper panel representative photomicrographs (patients: 19, 39, 2) of undifferentiated MSCs from posterior iliac crest, on the bottom panel representative photomicrographs (patients: 16, 13, 11) of undifferentiated MSCs from proximal tibia, cultured for 2, 3 and 4 passages. The images were captured at 10×magnification with scale bar of ~100 µm

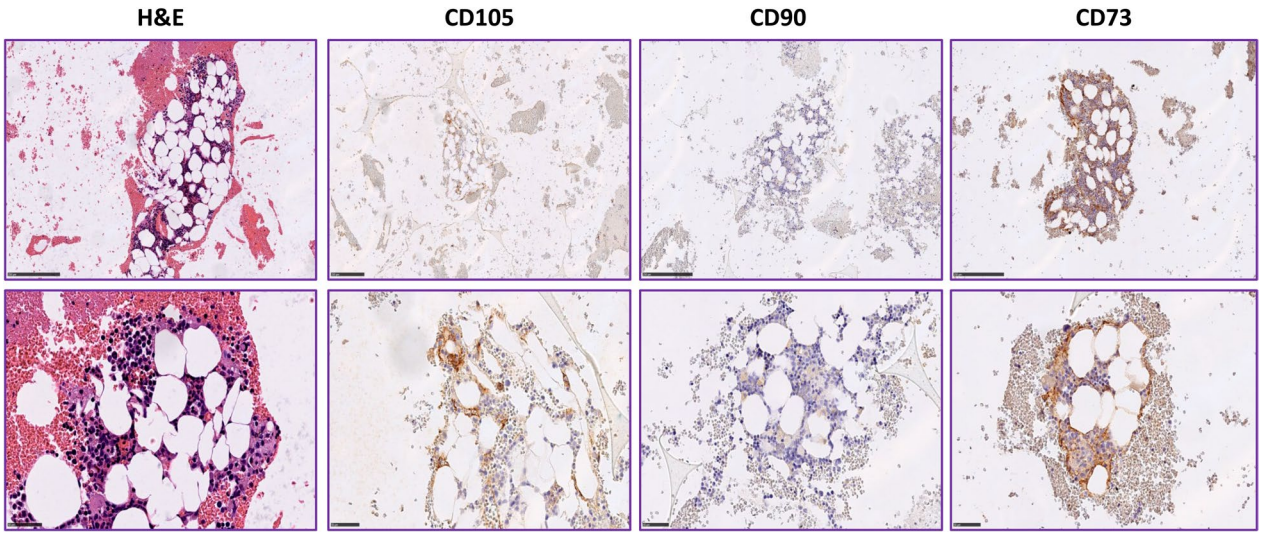

Fig. 6 Representative Images (higher magnification on the bottom) show the positive cells for all 3 MSCs markers (CD73, CD90 and CD105) derived from crest, confirming the presence of these cells in ex vivo samples

grade ($p = 0.034$). VAS and WOMAC score were used for the clinical outcome evaluation. Figure 7A indicates that, out of 15 patients, 2 in the Crest Arm and 1 in the PRP Arm showed no improvement in VAS, while in the Tibia Arm, one patient worsened slightly and one showed no improvement. Figure 7B indicates that in the Crest Arm, one patient worsened and three showed no improvement in WOMAC; in the PRP Arm, 1 patient worsened and 1 showed no improvement; and in the Tibia Arm, 2 patients worsened. Statistical analysis evidenced significant clinical efficacy for both BMA and PRP treatments, as indicated by changes in VAS and WOMAC scores before and after treatment. Median VAS decreases were −4 for the PRP Group

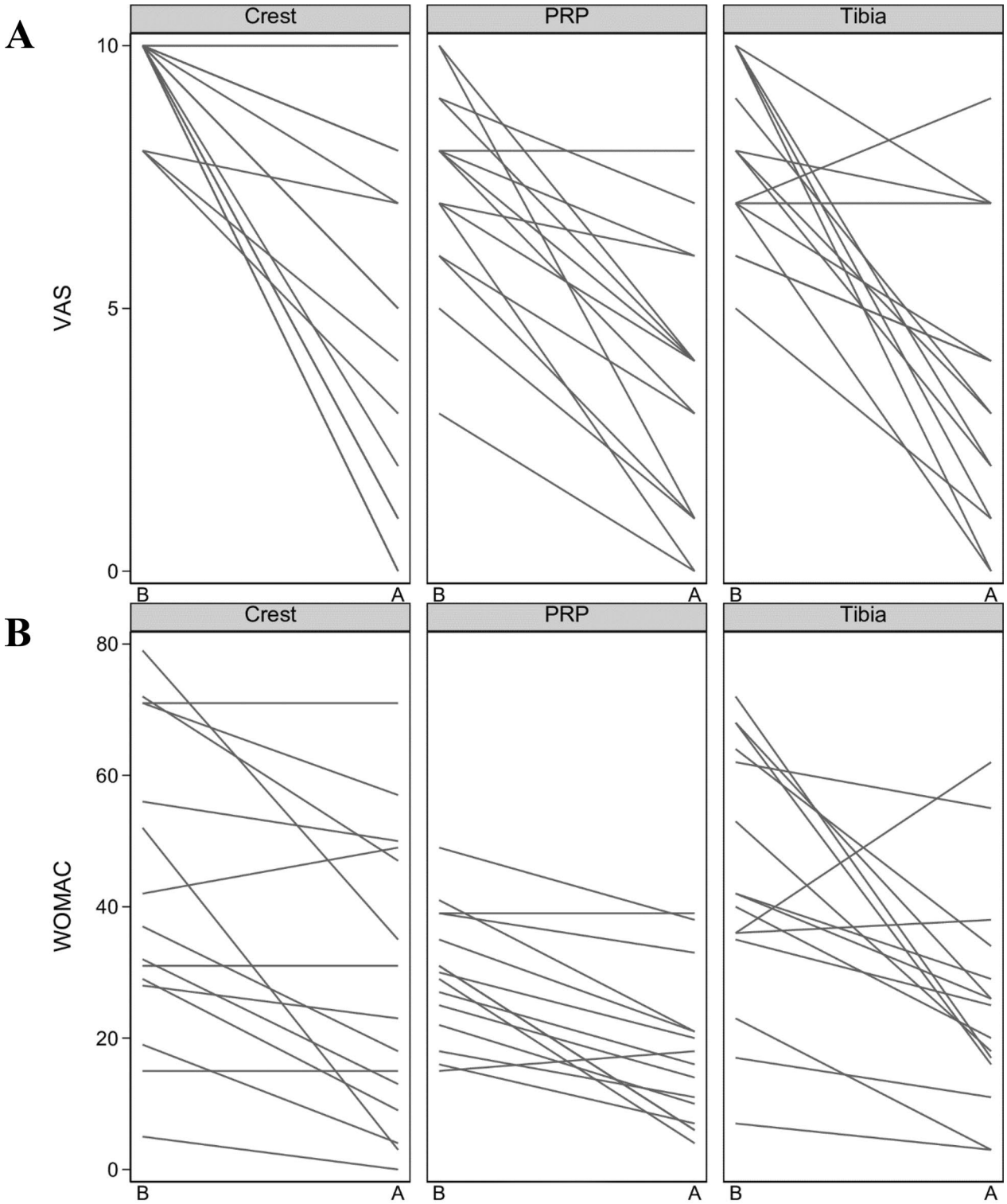

Fig. 7 VAS (**A**) and WOMAC (**B**) values for each patient in the 3 study groups before and after treatment

($p < 0.001$), −5 for the Crest Group ($p < 0.001$) and −4 for the Tibia Group ($p = 0.001$). Median WOMAC decreases were −11 for the PRP Group ($p < 0.001$), −14 for the Crest Group ($p = 0.002$) and −16 for the Tibia Group ($p = 0.003$). More details are provided in Table 2. The reductions in VAS and WOMAC scores across the three study arms did not differ significantly, indicating that BMA and PRP are equally effective 6 months post-treatment (Table 1).

Table 2 Median values (with IQRs) of pre/post changes in VAS and WOMAC in the three study arms

Variable	Crest	P	PRP	P	Tibia	P
ΔVAS	−5.00 (−9.00–−2.00)	< 0.001	−4.00 (−5.00–−2.00)	< 0.001	−4.00 (−7.00–−2.00)	0.001
ΔWOMAC	−14.00 (−20.00–0.00)	0.002	−11.00 (−20.00–−7.00)	< 0.001	−16.00 (−35.00–−6.00)	0.003
ΔWOMAC PAIN	−2.00 (−5.00–0.00)	0.005	−2.00 (−4.00–−1.00)	< 0.001	−3.00 (−7.00–−1.00)	0.005
ΔWOMAC STIF	−1.00 (−3.00–0.00)	0.020	−2.00 (−3.00–−1.00)	< 0.001	−2.00 (−3.00–0.00)	0.020
ΔWOMAC ADL	−13.00 (−17.00–0.00)	0.007	−7.00 (−15.00–−4.00)	< 0.001	−11.00 (−25.00 –−3.00)	0.003

VAS Visual Analogue Scale, *WOMAC* Western Ontario and McMaster Universities Arthritis Index, *ADL* Activities of Daily Living, *P* p-value from the Wilcoxon matched-pairs test assessing the significance of differences between pre- and post-treatment within each arm

Table 3 Association between pre/post changes in VAS and WOMAC and clinical variables within the three arms: Spearman's rank correlation for pairs of continuous variables, and the coefficient of determination (R^2) for associations between a continuous and a categorical variable

Variable	Crest	P	PRP	P	Tibia	P
ΔVAS						
Age	−0.14	0.6	0.07	0.8	−0.19	0.5
BMI	0.07	0.8	0.16	0.6	−0.60	0.019
HKA	−0.20	0.5	–	–	0.14	0.6
HCT	−0.26	0.4	–	–	0.03	0.9
%BM	−0.42	0.2	–	–	0.09	0.8
MSCs%	0.26	0.3	–	–	−0.59	0.021
MNC	−0.02	0.9	–	–	−0.02	0.9
Monocytes	−0.06	0.9	–	–	0.55	0.1
PLT	0.12	0.7	–	–	−0.27	0.4
Sex	0.07	0.3	0.07	0.3	0.05	0.4
Side	0.19	0.076	0.07	0.3	0.37	0.008
K-L (I + II) vs (III + IV)	0.56	< 0.001	0.26	0.004	0.06	0.4
ΔWOMAC						
Age	−0.12	0.7	−0.10	0.7	−0.35	0.2
BMI	−0.06	0.8	−0.15	0.6	−0.41	0.1
HKA	0.12	0.7	–	–	−0.02	0.9
HCT	−0.30	0.3	–	–	0.09	0.8
%BM	−0.62	0.045	–	–	0.25	0.5
MSCs%	0.18	0.5	–	–	−0.22	0.4
MNC	−0.14	0.7	–	–	0.35	0.3
Monocytes	0.10	0.8	–	–	0.75	0.016
PLTs	−0.27	0.4	–	–	−0.21	0.6
Sex	0.08	0.3	0.01	0.7	0.08	0.3
Side	0.00	1	0.09	0.2	0.40	0.005
K-L (I + II) vs (III + IV)	0.19	0.076	0.10	0.1	0.01	0.7

BMI body mass index, *HKA* Hip-knee-ankle, *HCT* hematocrit, *BM* bone marrow, *MSCs* mesenchymal stem cells, *MNCs* mono-nucleated cells, *PLTs* platelets, *KL* Kellgren Lawrance, *VAS* Visual Analogue Scale, *WOMAC* Western Ontario and McMaster Universities Arthritis Index, *ADL* Activities of Daily Living, *Δ* pre/post change, *P* p-value of the test for the significance of the association

Association between VAS change and secondary parameters

Data in Table 3 evidenced a significant negative correlation between the pre/post change in VAS and BMI only in the Tibia Arm ($r=-0.60$, $p=0.019$). This indicates that patients with a higher BMI have a better outcome with BMA treatment but only if BM is withdrawn from the tibia. Additionally, when we analyzed the correlation between the change in VAS and MSCs % in the same arm, we observed that an increase in MSCs % is associated with an increased effect of the BMA treatment ($r=-0.59$, $p=0.021$) (Table 3 and Fig. 8). In the Crest and PRP Arms, data also showed that the VAS change was significantly different between KL grades: patients with KL I-II grade responded better than patients with KL III-IV grade. Table 4 shows that the median value decreases from −6.5 to −1 in the Crest Arm, and from −4 to −1 in the PRP Arm. Furthermore, multivariable regression analysis confirms that, when considering variables together within the same model (including potential confounding variables such as sex and age), the pre/post VAS change continues to show an association with KL grade and MSCs %, the latter only in the Tibia Arm (Fig. 8). Conversely, the correlation with BMI was not confirmed.

Association between WOMAC change and secondary parameters

A significant correlation between the pre/post change in WOMAC score and BM % was found, but only in the Crest Arm ($r=-0.62$, $p=0.045$) (Table 3). This indicates that increasing the purity of BM withdrawal, enhances the effect of the BMA treatment, when BM is derived from the crest. Additionally, we observed that increasing the number of monocytes, significantly decreases the effect of the BMA treatment, but only in the Tibia Arm ($r=0.75$, $p=0.016$) (Table 3 and Fig. 9). Multivariable regression analysis confirms the association between WOMAC score change and number of monocytes when BMA is derived from the proximal tibia. However, the correlation with BM % in the Crest Arm was not confirmed.

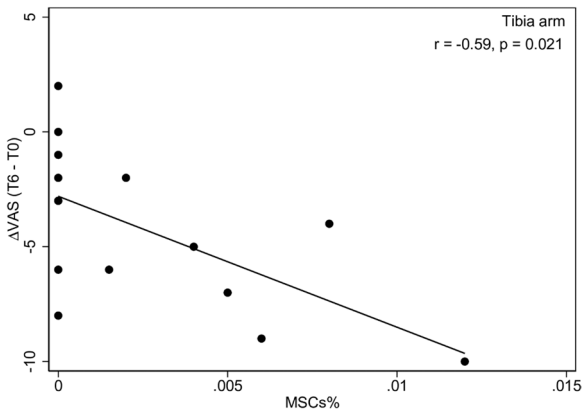

Fig. 8 Association between pre/post VAS change and MSC % in the Tibia arm

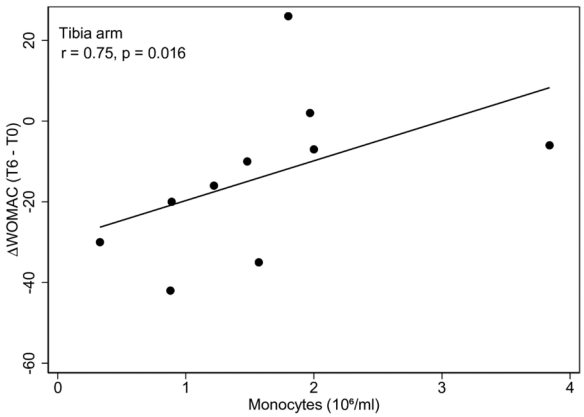

Fig. 9 Association between monocyte count and pre/post change of WOMAC score in the Tibia arm

Table 4 Median values (with IQRs) of pre/post changes in VAS and WOMAC in the three study arms and in the KL subgroups (I + II vs. III + IV)

	Crest	P	PRP	P	Tibia	P
ΔVAS						
KL I + II	−6.5 (−9–−5)	< 0.001	−4 (−5–−3)	0.004	−5 (−8–−2)	0.4
KL III + IV	−1 (−2–0)		−1 (−2–0)		−3 (−6.5–−0.5)	
ΔWOMAC						
KL I + II	−17 (−20–−5)	0.076	−11 (−20–−9)	0.1	−13 (−42–−6)	0.7
KL III + IV	0 (−6–0)		−5.5 (−11–0)		−20 (−32–−2.5)	

VAS Visual Analogue Scale, *WOMAC* Western Ontario and McMaster Universities Arthritis Index, *KL* Kellgren Lawrance, *Δ* pre/post change, *P* p-value of the test for the significance of differences between KL groups I + II vs. III + IV

Complications and adverse effects

The safety and the adverse effects were also included among the secondary objectives of this study.

Patients treated with PRP did not show any complication after the administration; they reported only mild soreness in the respective knee for 1 day to 1 week. Conversely, in Tibia Arm, we recorded two non-displaced fractures that were resolved in about 20 days without surgical or orthopedic treatment. In Crest Arm, we observed two cases of edema and one hematoma in the 7 days after the procedure that were resolved with rest, ice and anti-inflammatory therapy.

Discussion

The employment of uncultured BM-derived stem cells, progenitor cells and other cells to improve tissue repair and tissue regeneration, may avoid the risks associated with *in vitro* expansion of stem cells, as well as the long manufacturing times. Recently, BMAC, which contains concentrated MNCs and PLTs, was proposed as a new "platinum standard" for bone reconstruction [28]. In fact, the PLTs in BMAC may provide a more rapid and effective bone regeneration by MSCs [29]. MSCs yield from anatomically different harvest sites has already been studied, showing as the concentration varies among different sites [25] and using different harvesting devices and harvesting techniques [17, 26, 30]. Narbona-Carceles et al. [24] showed that MSCs concentration from both distal femur and proximal tibia was lower than iliac crest, although phenotype and differentiation potential were similar. Hyer et al. also confirmed that iliac crest showed the greatest yield of BMSCs compared to distal tibial metaphysis and calcaneal body, two alternative harvest sites often used for ankle surgery [31]. Pierini et al. [32] showed that posterior crest was better than anterior crest in term of yield and concentration of connective-tissue progenitors. Nevertheless, to our knowledge, there are not previously published studies comparing the clinical effects and the *ex vivo* cellular characterization of BMA, using BM harvested (BMA) with a Marrow Cellution device, from posterior iliac crest and proximal tibia, in comparison to PRP treatment of OA. Therefore, the present study evaluated the clinical effects in terms of function improvement, referred to as ΔWOMAC score, and pain decrease, referred to as ΔVAS score, of BMA obtained from the proximal tibia and posterior iliac crest, in relationship to their *ex vivo* MSCs composition, versus PRP. We choose PRP as autologous source of healing factors, as the control because the increasing number of clinical studies showing better outcomes compared to other conventional injectable treatments for symptomatic knee osteoarthritis [33, 34]. As already described in the introduction, BMAC contains an enriched population of MNCs and high concentration of PLTs and growth factors which are reported to have anabolic and anti-inflammatory effects [11, 35], as well as BMA, that was shown to have a comparable or even higher concentration of MNCs [20, 21].

Cellular characterization

Our result confirmed, as shown by others, that posterior iliac crest was significantly more densely populated with MNCs than the proximal tibia [25], however the number of monocytes was comparable between the 2 sites ($1.55X10^6$/mL and $1.53X10^6$/mL). Moreover, we found a significantly greater percentage of *ex vivo* MSCs in BM derived from the posterior iliac crest compared to that derived from proximal tibia, within the rage calculated for MSCs in the whole bone marrow (0.01%−0.1%) [36, 37]. This last result was expected also, although the MSCs percentage calculated from us, was directly derived from flow cytometry analysis on *ex vivo* BMA, rather than derived from the fibroblast-CFU [25, 30], as showed in most of the published studies. The calculated difference in MSCs % between the two sites could be relevant, as it has been demonstrated that a greater number of connective-tissue progenitors results in better outcome in treating bone defects. Moreover, we did not find differences among the donors between the sites with respect to the phenotype, as demonstrated by the flow cytometric analysis, and the morphology, as demonstrated by MSCs expended in vitro for up 8 weeks. Our data showed also that BMA from the posterior iliac crest had a significant higher number of PLTs compared to the proximal tibia ($189.50X10^6$/mL and 64.50 $X10^6$/mL, $p<0.001$) (Table 1). Moreover, the PLTs count in the posterior iliac crest was even higher compared to that calculated by others using the same device and harvesting the same BM volume [26].

Clinical outcomes

However, although BMA derived from the posterior iliac crest showed better purity, higher MSCs % and a greater number of PLTs there were no significant differences in terms of ΔWOMAC and ΔVAS across all the three arms, after 6 months from the treatment. This result is in agreement with other clinical studies that did not prove BMAC to be superior to PRP [38, 39] in the treatment of OA, although there are other studies showing the opposite [40]. We found that the improvement in early pain and function scores after treatment was significant in all three arms, with the greatest ΔWOMAC observed when BM derived from the proximal tibia and highest ΔVAS when BM derived from the posterior iliac crest. Our intermediate analysis also showed interesting correlations, giving us more information about the features of BMA derived

from the two different anatomical sites. The effect in term of ΔVAS is dependent on KL grade when considering BMA from crest and PRP; specifically, there is a better outcome for patients classified as KL I-II grade. Recently, also others have proved the importance in the selection of patients, based on KL grade, in the treatment of OA with BMAC [41] and PRP [42], although they got satisfactory results also with patients classified as KL III-IV. Moreover, the effect of BMA treatment depends on MSCs % in term of ΔVAS only in Tibia Arm, where the number of PLTs is 3 times lower than the number found in posterior iliac crest. Furthermore, when we consider the WOMAC at 6 months, we see an inverse correlation with monocytes when the BMA derived from proximal tibia. Multivariable regression analysis confirmed these results. This would confirm the previous correlation between MSCs % and ΔVAS, suggesting that the smallest amount of both PLTs and MSCs, although the product is clinically comparable to BMA from the crest, is more dependent and susceptible to the effect of other cells and factors present in the BM. As showed by others there is a great variability not only in MSCs yield and concentration but also in protein concentration, and cytokine profile between BMA and BMAC and between patients [17].

Synergism between Stem Cells and PLTs

Therefore, we can hypothesize that in the proximal tibia where there is a lower cellularity and BM %, is the synergistic effect of different healing cells like PLTs and MSCs to have a bigger influence on clinical outcome, compared to the posterior iliac crest. In fact, in posterior iliac crest the greater number of PLTs alone could provide, at 6 months, the same clinical effects as observed in PRP Arm. This in agreement with the ESSKA consensus group that recommends the use of PRP as a 1st line orthobiologic injectable treatment option in the knee OA [42]. Although the importance of evaluating the results at 6-month post-treatment results, this intermediate analysis was limited by including only half of patients enrolled in the study, leading to a preliminary data interpretation. This may reduce statistical power and, consequently, the generalizability of the findings. Additionally, variability was introduced due to procedural improvements over the course of the study.

Conclusion

In conclusion, this preliminary 6-month analysis, suggests that BM harvested from the posterior iliac crest, using a needle equipped with multiple lateral holes, has the same relevant clinical effect, as the BM harvested with the same needle from the tibia, when used in patients with OA. Moreover, the clinical results were independent from the concentration of connective-tissue progenitors and PLTs. This would suggest that the quality of MSCs is as important to consider as the quantity of MSCs injected and would indicate the importance of the synergic effect of MSCs and PLTs, in the anatomical sites where these cells are less concentrated. Moreover, BMA from both harvesting sites and PRP possess similar clinical outcome, as showed also by others [43, 44], suggesting that are the factors released by MSCs and PLTs likely responsible for the clinical effects in terms of pain relief. Although the bioactive factors may have differing effects on musculoskeletal tissue [45] in longer time. However, in a regular clinical setting, the transplantation of PRP may be a more feasible method for enhancing pain relieve. We expect that at 12 months, data from all 30 patients in each arm will not only confirm the 6-month results but also will provide more information about any differences between BMA and PRP on long-term treatment. Furthermore, other studies are being carried out to evaluate the clinical performance of these two BMA fractions in terms of cartilage regeneration as well.

Abbreviations

OA	Osteoarthritis
IA	Intra-articular injections
PRP	Platelet rich plasma
MSC	Mesenchymal stem cell(s)
BM	Bone marrow
BMAC	Bone marrow aspirate concentrate or concentration
BMA	Bone marrow aspirate
KL	Kellgren-Lawrance
MC	Marrow Cellution
WOMAC	Western Ontario and McMaster Universities Arthritis Index
VAS	Visual analogue score
BMI	Body mass index
PLTs	Platelets
MNCs	Mononuclear cells

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Author contributions

EM, made substantial contributions to the study design, analysis, interpretation of data, in vitro cells culture and construction of the manuscript. LS and FM made substantial contributions to the design, collection of data and analysis and interpretation of results. GDM contributed to collection of data. GR and NPS contributed substantially to the FACS analysis. GB and PG contributed to immunohistochemical analysis. MS contributed to the statistical analysis, interpretation of data and manuscript reviewing. MC designed the studio. EDS, VG and EC contributed to interpretation of results and construction of manuscript. CC set up the PRP protocol. All authors agree to be accountable for all aspects of this manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study was approved by the local ethics committee (protocol number 263/01/DG). All subjects gave their written informed consent before procedure.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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