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Different methods of bone marrow harvesting influence cell characteristics and purity, affecting clinical outcomes

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ABSTRACT

Background: Bone marrow (BM)-derived stem cells were implanted to induce angiogenesis in patients with no-option critical limb-threatening ischemia. Considering the potential for this therapy, conflicting results related to BM harvesting methods have been reported that could affect stem cell concentrations and quality.

Methods: A total of 75 patients with no-option critical limb-threatening ischemia were treated with BM implantation. For 58 patients, BM was harvested using a BM aspirate concentrate system (Harvest Technologies; group HT) with a standard aspiration needle, followed by an automated centrifugation process, to produce BM aspirate concentrate. For 17 patients, BM was harvested using the Marrow Cellution system (Aspire Medical Innovation; group MC). $CD34^+$ cells/mL, $CD117^+$ cells/mL, $CD133^+$ cells/mL, $CD309^+$ cells/mL, hematocrit, and BM purity were compared between the two BM preparations.

Results: The retrospective analysis of a subset group after adjustment for age shows that the quality of BM obtained using the Marrow Cellution system is better, in terms of purity, than the classic harvesting method before centrifugation. Harvested BM before centrifugation is characterized by a higher percentage of $CD133^+$ cells compared with BM after centrifugation. In contrast, the MC aspirate had a larger amount of very small embryonic-like cells, as indicated by the higher percentage of $CD133^+$, $CD34^+$, and $CD45^-$ cells. These differences translated into an increased occurrence of leg amputations in group HT than in group MC and an increase in transcutaneous oxygen pressure in patients treated with BM aspirated using MC.

Conclusions: BM manipulation, such as centrifugation, affects the quality and number of stem cells, with detrimental consequences on clinical outcomes, as reflected by the different amputation rates between the two groups. (JVS—Vascular Science 2023;4:100130.)

Clinical Relevance: Critical limb-threatening ischemia is the most advanced form of peripheral arterial disease with major economic and social effects due to the high amputation rate and mortality. The problem is even greater for diabetic patients, for whom the expected incidence of amputation is ~40% to 50%. Thus, the need for new therapeutic options is urgent. The present report highlights the striking effects of angiogenic therapy by bone marrow-derived stem cells obtained using a novel technology. We found that the choice of bone marrow harvesting method does influence the clinical outcome; however, further studies are needed. The present study presents a meaningful background for future development.

Keywords: Angiogenesis; Bone marrow; Hematopoietic stem cells; Peripheral arterial disease; Very small embryonic-like stem cells

Peripheral arterial disease (PAD) is a growing health emergency. Critical limb-threatening ischemia (CLTI) is the most advanced form of PAD, with major economic and social effects due to the high amputation rates and mortality.[1] Notwithstanding the progress in medical, vascular, and endovascular treatment, the amputation rate has remained constant, accounting for 25% of patients with CLTI.[2] The problem is even greater for

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The editors and reviewers of this article have no relevant financial relationships to disclose per the JVS policy that requires reviewers to decline review of any manuscript for which they may have a conflict of interest.

2666-3503

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https://doi.org/10.1016/j.jvssci.2023.100130

diabetic patients, for whom the expected incidence of amputation is ~40% to 50%; thus, the need for new therapeutic options is urgent.[3] Since the seminal work of Asahara et al,[4] who discovered endothelial progenitor cells in the blood, therapeutic angiogenesis has been introduced clinically, with conflicting results. Bone marrow (BM)-derived stem cells and peripheral circulating stem cells have been used to induce angiogenesis in patients with no-option CLTI.[5,6] The use of BM aspirate (BMA) concentrate (BMAC) to treat no-option CLTI was pioneered at our institution beginning in 2008.[7] Recently, we have changed our approach to obtain BMA without centrifugation to avoid any manipulation steps[8] that could negatively affect the quality of the BM. Moreover, the new method used (Marrow Cellution System; Aspire Medical Innovation) maximizes stem and progenitor cell recovery and mitigates peripheral blood contamination. A recent meta-analysis consolidated the evidence supporting the efficacy of BMAC in reducing amputation risk.[9] This same meta-analysis also highlighted significant variability in amputation outcomes, and a punitive cause of this might be related to the lack of specific guidelines to drive stem cell therapy forward and the use of inappropriate techniques for BM collection and preparation. Thus, the primary goals of the present study were as follows:

1. To assess whether two different approaches to BM aspiration (ie, the Harvest Technologies BMAC system [group HT] vs the Marrow Cellution system [group MC]), influenced the outcomes of leg amputation for patients with chronic obliterative arterial disease
2. To evaluate whether the stem cell number and quality differed between the two methods

To meet our goals, we collected BM using the two different techniques (HT vs MC) from patients with no-option CLTI. The study was conducted from 2008 to 2019. The first part of the study was performed with the BMAC system until 2017. The second part of the study was performed using the MC method to the end of the study period.

ARTICLE HIGHLIGHTS

- **Type of Research:** A single prospective, nonrandomized, single-arm, investigational device exemption study
- **Key Findings:** Bone marrow harvested using the Marrow Cellution system has an higher percentage of very small embryonic-like cells that counteract the occurrence of leg amputations and increase the $TcpO_2$ in patients with CLTI.
- **Take Home Message:** The quality and number of stem cells is affected by bone marrow harvesting methods.

METHODS

Study design. The ethical committee of the Catholic University of the Sacred Heart and the Italian Council of Health approved the study protocol. Overall, 77 consecutive White patients with no-option CLTI underwent autologous fresh BM implantation at our institution (Gemelli Molise, Campobasso, Italy). All patients signed an informed consent form as established by the Catholic University of the Sacred Heart. All 77 patients had presented with critical ischemia of the lower limbs using the Rutheford classification. Previous surgical, vascular, or endovascular revascularization had failed for all patients. No further therapeutic options were deemed possible by the cardiovascular team of our institution, who performed their examinations independently of the investigators involved in the present study.

Patient inclusion criteria. The patient inclusion criteria were as follows: a diagnosis of CLTI not suitable for surgical or endovascular treatment as determined by the anatomic criteria (ie, absence of target vessels, absence of conduits, segmental long occlusions, calcified lesions predictive of poor results) and/or medical conditions of high risk (ie, unstable angina, renal insufficiency); and previous unsuccessful revascularization attempt. Aspirin, oral anticoagulation drugs, and antihypertensive drugs were allowed if deemed necessary to maintain stable values for ≥1 month of the following: hematocrit (HCT) ≥28%, white blood cell count ≤14,000/mm^3, platelet count ≥50,000/mm^3, international normalized ratio, ≤1.6, and partial thromboplastin time <1.5 seconds.

Patient exclusion criteria. The patient exclusion criteria were as follows: life expectancy <6 months due to comorbidities; a history of hematologic disease, contraindicating BM transplantation; chronic renal insufficiency requiring dialysis; malignancy; and a high risk for anesthesia or American Society of Anesthesiologists class V. Additional exclusion criteria included a life-threatening ischemic lesion requiring immediate amputation; extensive necrosis of the limb or other condition requiring amputation; complete occlusion of the iliac axis untreated surgically or endovascularly on the side to treat; and a complete absence of flow in the Dorsalis pedis artery at the color Doppler echocardiography evaluation (ankle brachial index [ABI] 0; the reduced blood flow expressed by an ABI of 0 could impair the homing and survival of the cells). In addition, patients with clinically active infection and antibiotic therapy 7 days before enrollment; patients receiving immunosuppressive therapy; women who were pregnant, lactating, or of fertile age; and patients with a previous cardiovascular accident within 30 days before randomization were excluded.

Preoperative evaluation. Patient screening included arterial color Doppler echocardiography of the epiaortic

vessels and inferior limbs, ABI determination ≤30 days before treatment, measurement of transcutaneous oxygen pressure ($TcPO_2$) on the feet, and microbiologic evaluation of topical lesions. In addition, computed tomography angiography, magnetic resonance angiography, or arteriography of the inferior limbs was performed 3 months before treatment. An assessment of the cardiovascular risk profile was obtained during the 2 weeks before treatment, with evaluation of risk factors such as diabetes mellitus (glycemic value and glycated hemoglobin), smoking, arterial hypertension, hyperlipidemia, and C-reactive protein (hematochemical parameters). Finally, the presence of any malignancy was excluded. The following examinations performed during the 12 months before intervention were considered: prostate-specific antigen testing for male patients aged >45 years, chest radiography for patients aged >50 years, mammography for women aged >40 years, and Papanicolaou testing for women aged >21 years.

For the first 58 patients, the BMAC protocol was used (group HT). For the subsequent 17 patients, we used BMA (group MC). The BM from the 58 patients in group HT was aspirated using a standard Jamshidi needle; 240 mL of BM was collected from both iliac crest wings and subsequently centrifuged. The BM from the remaining 17 patients in group MC was aspirated; 40 mL of BM was collected using the MC system, without subsequent centrifugation.

BM withdrawal. The procedures were performed in the operating room. After sedation (midazolam and propofol), with the patient in a prone position, the BM was harvested bilaterally from the iliac crest.

For group HT, 240 mL of BM was aspirated in accordance with the manufacturer's protocol and processed using an automated, point of care device (Harvest Technologies) to obtain a final concentration volume of 40 mL of BMAC. The BM was harvested from two different points on both iliac crests (120 mL for each site). The BM was centrifuged immediately at room temperature and injected after processing (~15-20 minutes after withdrawal). The 240 mL of BM was automatically centrifuged in one chamber at 1250*g* for 3 minutes and successively at 1050*g* for 9 minutes. During the first phase, the erythrocytes form sediment and pass through the semipermeable septum, which automatically settles at the plasma—erythrocyte interface. During the short interim stop phase of a few seconds, the buffy coat decants by gravity into the anterior chamber, and the semipermeable septum retains the erythrocytes in the posterior chamber. The second phase of the centrifugation process constitutes separation of the cellular components from the plasma. At the end of the second centrifugation, the processing kit is extracted from the centrifuge, excess plasma is removed from the anterior chamber, and the cell fraction is resuspended in residual plasma to form a homogeneous solution (volume of 10 mL for every 60 mL of processed aspirate) of BMAC.

For group MC, 40 mL of BM was harvested using the MC needle, as previously described.[8] In brief, BM was harvested from one site. The device was inserted to reach the distal part of the medullary space, and the sharp point was replaced with a stylet closed at the end and with side port to harvest the BM from a different level, twisting at 360° and raising the device, avoiding excessive blood infiltration from the previous level in the BM. The BM was aspirated using two 20-mL syringes connected to the MC needle starting at the distal part of the medullary space (Fig 1). No further processing of the BMA was performed.

BM injection. Samples of BMAC and BMA were sent to the laboratory for quantitative analysis of the cellular concentration. The ischemic limb was geographically plotted using a dermatologically suitable graphic ink marker to indicate the various injection points according to the preoperative angiographic examinations and angiosomes feeding the ischemic area. The BMAC and BMA were injected deeply intramuscularly, with a distance of 1 to 2 cm between each injection point and 1 cm of distance from the vascular bed for a 40 to 80 cm extension of length along the limb. 1 mL of BMAC or BMA was injected at each point on the leg using a 21-guage needle of 2 to 4 cm and on the foot using a 27-guage needle (Fig 2).

At day 7 after treatment, the patients underwent clinical evaluation of reperfusion and pain therapy. At 1, 3, and 6 months of follow-up, the $TcpO_2$ and ABI measurements were repeated. Arteriography was repeated at 6 months.

Flow cytometric analysis. The cell population was analyzed using flow cytometry. The membrane antigens included CD34, CD45, and CD117 for early hematopoietic cells, CD133 for stem cells, and CD309 (vascular endothelial growth factor receptor 2) for endothelial cells. Cellular phenotyping was performed using both immunostaining (data not shown) and flow cytometry using antibody-fluorochrome conjugates for 30 minutes on ice (Navios; Beckman Coulter Life Sciences). Appropriate isotype controls were also used (Beckman Coulter). The stained cells were washed, suspended in phosphate-buffered saline, and analyzed using a flow cytometer (Navios Ishage; Beckman Coulter Life Sciences). Standard Protocols were used for enumerating the cell populations. The flow cytometry data were analyzed with an appropriate software (Beckman Coulter Life Sciences).

BM Purity. BM purity was calculated according to Holdrinet et al.[10] During BM harvesting, a sample of venous blood was taken. The leukocytes and erythrocytes were counted in the BM and peripheral blood (PB). The purity of BM was derived using the following

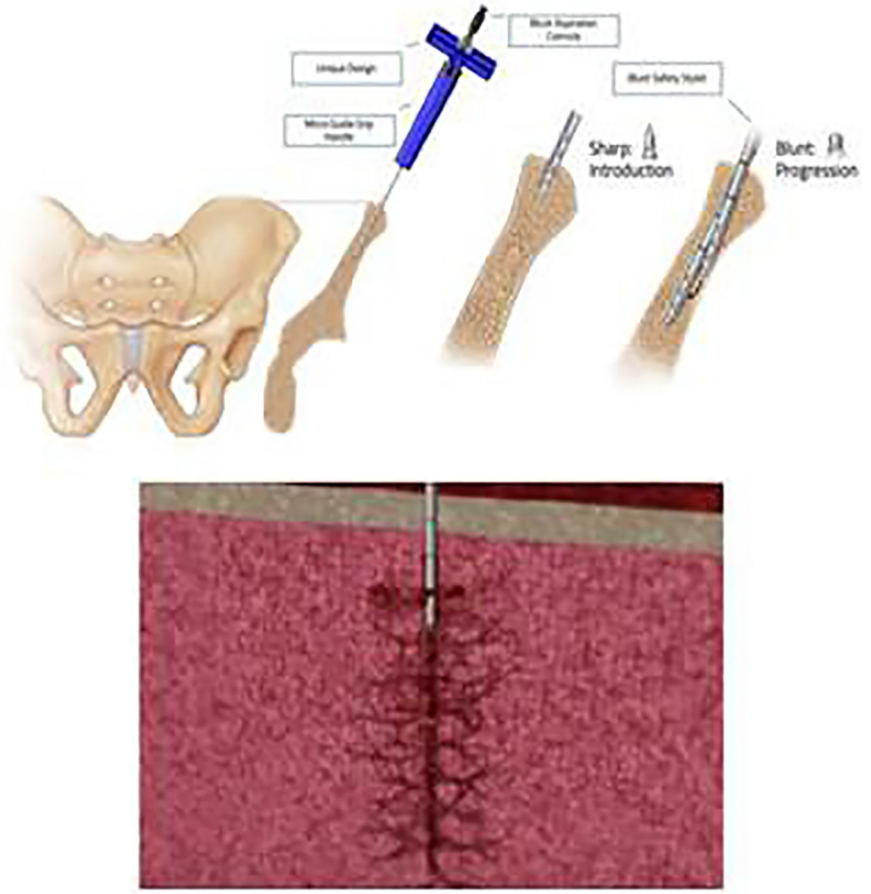

Fig 1. Bone marrow (BM) harvest using the Marrow Cellution (MC) device. Note the sample at the different levels, avoiding contamination of blood from the vacuum left in the inferior level from the BM aspirate (BMA).

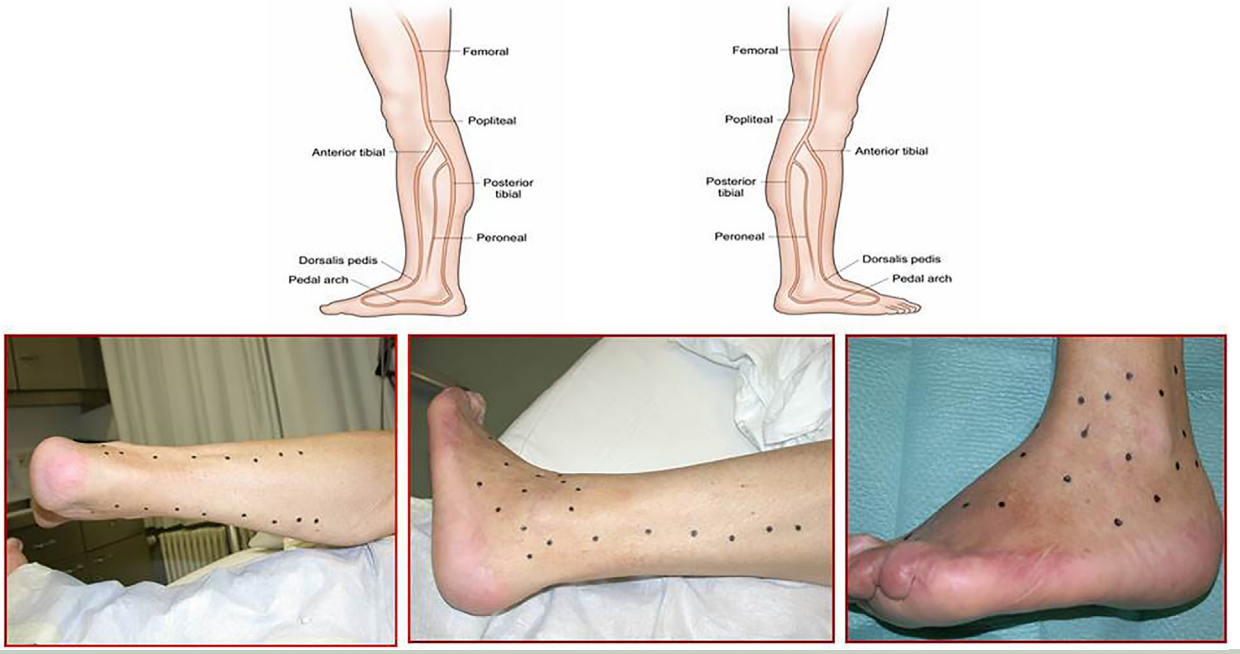

Fig 2. Preoperative mapping of the ischemic territory for the injection sites showing the angiosomes.

formula[10]: BM purity = [1 − (erythrocytes$_{BM}$/erythrocytes$_{PB}$) × (leukocytes$_{PB}$/leukocytes$_{BM}$)] × 100%.

Statistical analysis. The end point of the present study was the occurrence of leg amputation after the injection of stem cells obtained using two different sampling techniques for BM aspiration (HT vs MC). First, we compared the baseline characteristics of the two groups to detect factors other than the technique used for BM aspiration that might influence the occurrence of leg amputation. Continuous variables are reported as the median and interquartile range (IQR) and categorical variables as frequencies and percentages. Continuous variables were compared using the Mann-Whitney *U* test for independent samples and the Wilcoxon signed rank test for paired samples. Categorical variables were compared using the χ^2 test. Statistical significance was set at $P < .05$.

RESULTS

Patient characteristics. Overall, 77 patients were referred to our department for treatment with BM to avoid amputation due to CLTI. Two of these patients received both BM treatments and were excluded from the group analysis but were included in the global assay of BMAC efficacy to treat patients with CLTI with no alternative indication to amputation. Of the remaining 75 patients, 58 were in group HT and 17 were in group MC.

The patients in group HT were significantly older (median age, 74 years; IQR, 63-80 years), by ∼10 years, than the patients in group MC (median age, 64 years; IQR, 53-70 years; $P = .0049$). The major risk factors for arterial disease and comorbidities were balanced in the two groups (Table I). Because of the possible influence of the effects of age on our results, a secondary analysis was conducted of a subgroup of HT patients matched by age with MC patients. For the subset analyses, we included 33 patients from the HT group and 15 patients from the MC group. The patients in the HT group were not significantly older (median age, 65 years; IQR, 54-72 years) than those in the MC group (median age, 63 years; IQR, 53-69 years; $P = .3217$). The incidence of chronic kidney disease was significantly more frequent in the MC group (2 of 33; 6.1%) than in the HT group (4 of 15; 26.7%; $P = .0454$). The major risk factors for arterial disease and comorbidities were balanced between the two groups (Table I).

Circulatory angiogenetic cells. Flow cytometric analysis for expression of the markers CD34$^+$ for hematopoietic stem cells, CD133$^+$ for stem cells, CD117$^+$ for BM stem/progenitor cells, and CD309$^+$ for endothelial cells shows that CD133 expression is significantly higher in the BM harvested before the centrifugation steps compared with the BM after centrifugation (Table II). Moreover, expression of the same marker, CD133, in the BM harvested after centrifugation is significantly higher compared with the aspirated BM using both paired and unpaired nonparametric tests (Table III). No significant results were found for the other markers.

The analysis of HCT and BM purity (Figs 3 and 4) also shows a significant difference in the BM before (HCT, 33.2%; BM purity, 71%) vs after (HCT, 26,6%; BM purity, 93%) centrifugation ($P = .0003$ and $P = .0001$, respectively; Table II), with no significant differences between the BM harvested after centrifugation and the aspirated BM (group MC).

Very small embryonic-like cell quantification. Cells positive for CD34 and CD133 and negative for CD45 were considered to be very small embryonic-like (VSEL) cells (Fig 5). Quantification analysis of this cell population shows a significant difference ($P = .0237$) between BMAC compared with BMA (group MC).

Matched age groups. In the subgroup analysis matched for age, the CD133$^+$ cells of group MC were significantly different than those from group HT using both paired and unpaired nonparametric tests (Tables IV and V). In addition, the BM purity of the patients in the MC group was significantly different than that in HT group only using the paired nonparametric test (Table V).

Amputation. Leg amputation was significantly more frequent among the patients in group HT (20 of 58; 34.5%) than among the patients in group MC (1 of 17; 5.9%; $P = .0209$; Table VI). Furthermore, group MC also showed a significantly higher post-transplant $TcpO_2$ (median, 41 mm Hg; IQR, 38-46 mm Hg) compared with group HT (median, 28 mm Hg; IQR, 21-38 mm Hg; $P = .0004$; Table VI). However, the need for postoperative debridement was more likely for group MC during the folow up (5 of 17; 35.3%) than for group HT (5 of 58; 8.6%; $P = .0063$; Table VI). No statistically significant differences were found in mortality, with 5 of 58 patients (8.6%) dying in group HT and none of 22 patients (0.00%) dying in group MC ($P = .2102$; Table VI). This result was likely influenced by the insufficient power to detect differences and might be improved with longer follow-up.

Multivariable logistic regression analysis confirmed that the MC technique (group MC) is independently associated with an 88% reduced probability of leg amputation compared with the harvested BMAC technique (group HT), even when corrected for patient age (odds ratio, 0.12; 95% confidence interval, 0.006-0.7001; $P = .077$; Table VII).

Matched age groups. Leg amputation was also significantly more frequent in the age-matched groups in the HT group (11 of 33; 33.3%) than in the MC group (1 of 15; 6.7%; $P = .0480$; Table VI). Multivariable logistic regression analysis confirmed that the MC technique is independently associated with a 88% reduced probability of leg amputation compared with the HT technique when corrected for patient age (odds ratio, 0.12; 95% confidence interval, 0.017-1.231; $P = .077$; Table VII).

Table I. Baseline patient characteristics

Parameter	HT	MC	*P* value
Total population	n = 58	n = 17	
Age, years	74 (63-80)	64 (53-70)	.0049
Risk factor			
Smoke	24 (41.4)	6 (35.3)	.6524
Hypertension	51 (87.9)	15 (88.2)	.9729
Comorbidities			
Thromboangiitis obliterans	10 (17.2)	4 (23.5)	.5585
COPD	45 (77.6)	10 (58.8)	.1240
DM	32 (55.2)	11 (64.7)	.4846
CKD	11 (19.0)	6 (35.3)	.1573
CAD	20 (34.5)	5 (29.4)	.6965
Clinical status			
$TcpO_2$ (before transplant), mm Hg	12 (7-15)	12 (9-17)	.2840
Ulcers	17 (29.3)	6 (35.3)	.6380
Necrosis	25 (43.1)	3 (17.6)	.0564
Gangrene	7 (12.1)	1 (5.9)	.4674
Site of ischemia			
Inferior right	17 (29.3)	5 (29.4)	.9936
Inferior left	17 (29.3)	7 (41.2)	.3564
Inferior, nonspecified	24 (41.4)	5 (29.4)	.3729
Subgroups	n = 33	n = 15	
Age, years	65 (54-72)	63 (53-69)	.3217
Risk factors			
Smoke	16 (48.5)	6 (40.0)	.5845
Hypertension	27 (81.8)	13 (86.7)	.6761
Comorbidities			
Thromboangiitis obliterans	10 (30.3)	4 (26.7)	.7972
COPD	26 (78.8)	9 (60.0)	.1746
DM	16 (48.5)	9 (60.0)	.4592
CKD	2 (6.1)	4 (26.7)	.0454
CAD	7 (21.2)	4 (26.7)	.6769
Clinical status			
$TcpO_2$ (before transplant), mm Hg	11 (9-13)	14 (11-18)	.0900
Ulcers	11 (33.3)	5 (33.3)	1.0000
Necrosis	13 (39.4)	3 (20.0)	.1864
Gangrene	5 (15.2)	1 (6.7)	.4100
Site of ischemia			
Inferior right	11 (33.3)	5 (33.3)	1.0000
Inferior left	8 (24.2)	6 (40.0)	.2656
Inferior, nonspecified	14 (42.4)	4 (26.7)	.2959

CAD, Coronary artery disease; *CKD*, chronic kidney disease; *COPD*, chronic obstructive pulmonary disease; *DM*, diabetes mellitus; *HT*, Harvest Technologies bone marrow aspirate concentrate system; *MC*, Marrow Cellution system; *$TcpO_2$*, transcutaneous oxygen pressure.
Data presented as median (interquartile range) or number (%).

Furthermore, the MC group (median, 42 mm Hg; IQR, 39.5-46 mm Hg) also showed a significantly higher post-transplant $TcpO_2$ compared with the HT group (median, 28.5 mm Hg; IQR, 23.5-40 mm Hg; $P = .0021$; Table VI).

No statistically significant differences were found in mortality between the age-matched groups, with no patients in either group dying (0 of 33 in the HT group [0.00%] and 0 of 15 in the MC group [0.00%]; Table VI).

Table II. Laboratory findings using Wilcoxon signed rank test for total population

BM characteristics	Before HT	After HT	*P* value
CD34 (n = 50)	0.96 (0.62-1.40)	0.94 (0.68-1.30)	.3491
CD133 (n = 50)	0.15 (0.62-1.40)	0.10 (0.68-1.30)	.0040
CD117 (n = 50)	0.79 (0.54-1.03)	0.78 (0.53-1.17)	.5559
CD309 (n = 50)	0.01 (0.01-0.03)	0.01 (0.00-0.03)	.1032
CDTOT (n = 51)	2.06 (1.22-2.66)	1.83 (1.45-2.42)	.9216
HCT (n = 31), %	33.2 (31.1-36.9)	26.6 (22.4-36.7)	.0003
BM purity (n = 31), %	71.0 (57.0-83.0)	93.0 (92.0-97.0)	<.0001

BM, Bone marrow; *HCT,* hematocrit; *HT,* Harvest Technologies bone marrow aspirate concentrate system.
Data presented as median (interquartile range).

Table III. Laboratory findings using Mann-Whitney *U* test for total population

BM characteristics	After HT (n = 53)	MC (n = 14)	*P* value
CD34	0.93 (0.62-1.30)	1.02 (0.78-1.15)	.7702
CD133	0.10 (0.06-0.20)	0.02 (0.01-0.02)	<.0001
CD117	0.77 (0.51-1.15)	1.10 (0.63-1.48)	.1660
CD309	0.01 (0.00-0.03)	0.01 (0.00-0.03)	.4323
CDTOT	1.83 (1.45-2.42)	2.25 (1.38-2.94)	.4328
HCT, %	26.6 (22.4-36.7)	34.0 (32.9-38.1)	.0547
BM purity, %	93.0 (92.0-97.0)	95.0 (90.0-97.0)	.5605

BM, Bone marrow; *HCT,* hematocrit; *HT,* Harvest Technologies bone marrow aspirate concentrate system; *MC,* Marrow Cellution system.
Data presented as median (interquartile range).

This result was possibly influenced by the insufficient power to detect differences and might be improved with longer follow-up.

DISCUSSION

The incidence of PAD is increasing due to diabetes and aging of the population.[11] Notwithstanding the excellent progress of vascular and endovascular surgery, 20% of PAD patients develop critical limb ischemia and 4% to 27% require amputation.[12] Only 60% of patients affected by CLTI can be treated using bypass surgery or percutaneous transluminal angioplasty. Patients not eligible for revascularization are treated with vasodilators drugs (ie, prostanoids), with a partial remission of symptoms in 50% of patients. The remaining 50% of patients require amputation. New research efforts are directed toward the following objectives: (1) pain reduction, (2) increased walking function, and (3) a reduced amputation rate. Angiogenesis is a physiologic and pathologic process involving platelets and several tissue and circulating cell populations. Isner et al[13] hypothesized that BM endothelial progenitor cells injected into an ischemic limb could differentiate with appropriate cytokine signals into adult endothelial cells, leading to vascular remodeling and angiogenesis. In the recent years, the paracrine actions of cells, involved in repairing ischemic damage, have assumed particular importance.[14] The pathophysiologic process to repair ischemic damage is profoundly altered in the presence of diabetes and arteriosclerosis.[15-18] In patients with diabetes, a complex derangement of angiogenesis is present, and mobilization of stem cells from the BM is reduced. Gallagher et al[19] demonstrated that endothelial nitric oxide synthase phosphorylation in the BM is impaired in a murine model of diabetes. In the same study, they observed a reduction of the amount of cytokine stromal-derived factor (SDF)-1α in epithelial cells and myofibroblasts. SDF-1α is a key factor for the mobilization and homing of stem cells in the side of ischemic damage.

However, in the absence of flow or the presence of severely reduced flow, as assessed by angiography and expressed by an ABI of 0, the homing and survival of the cells is jeopardized. In a pathophysiologic environment, it is of paramount importance for the modality to obtain and deliver the component necessary to repair the ischemic damage. It has been shown that cells obtained from the blood and BM can lose the homing capacity due to the isolation method used through the loss of chemokine receptors.[20]

Another aspect that must be considered is the presence of human VSEL cells. Human VSEL cells are a resident population of pluripotent stem cells in the BM that express CD133, giving rise to cells from all three germ lineages. They can be mobilized from the BM to the

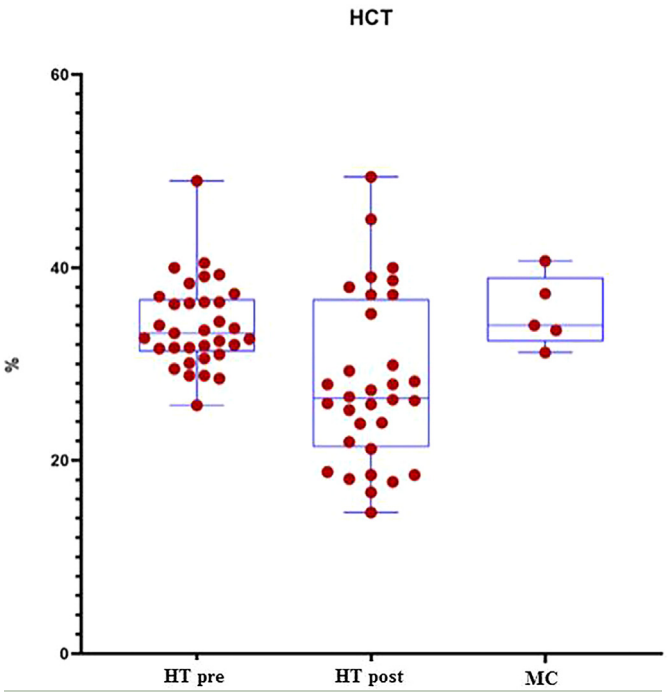

Fig 3. Analysis of hematocrit (HCT) shows a significant difference in the bone marrow (BM) before vs after centrifugation, with no significant differences between the BM harvested after centrifugation and the aspirated BM.

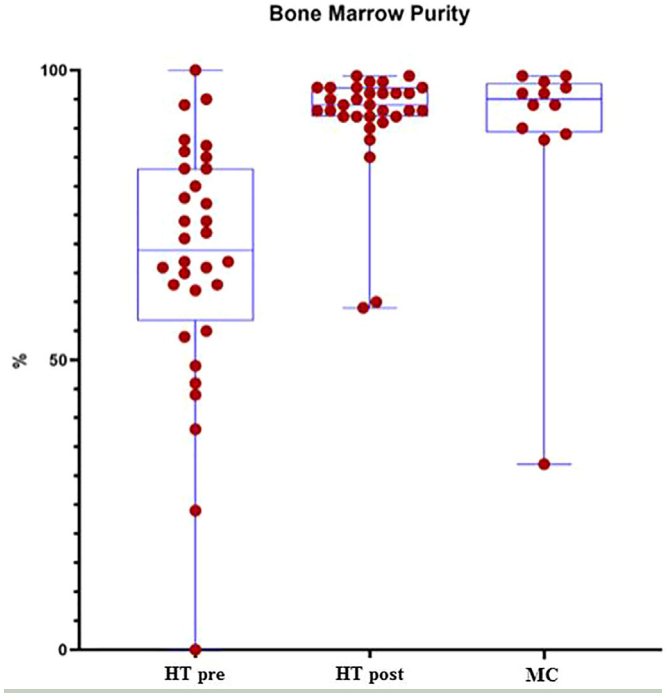

Fig 4. Analysis of bone marrow (BM) purity shows a significant difference in the BM before vs after centrifugation, with no significant differences between the BM harvested after centrifugation and the aspirated BM.

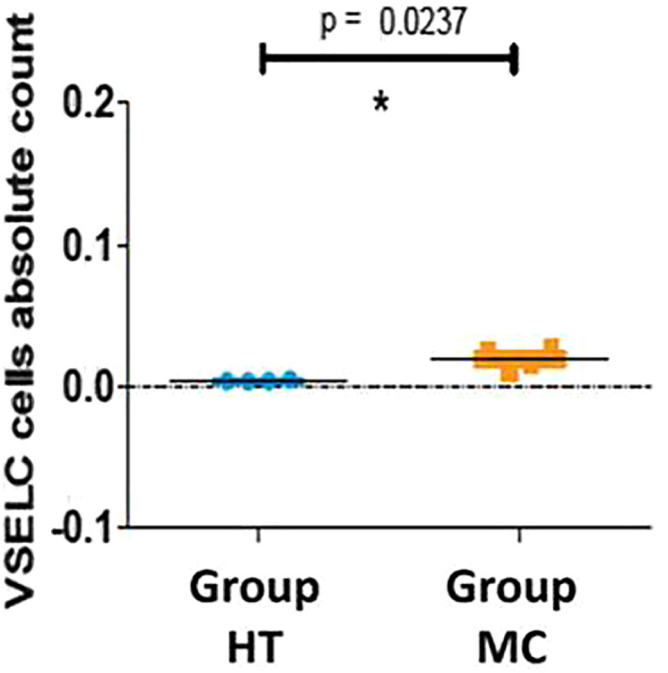

Fig 5. Quantification of very small embryonic-like (VSEL) cells ($CD34^+$, $CD133^+$, $CD45^-$) between group HT (Harvest Technologies) and group MC (Marrow Cellution system).

peripheral blood in CLTI patients and have angiogenic properties in vivo.[21] Human BM VSEL cells isolate and differentiate in vitro under angiogenic conditions allowing for production of so-called VSEL-derived cells able to induce revascularization in an hind limb ischemia model and Matrigel implant.[22] Considering their dimensions,[23] we hypothesize that during the centrifugation process of the harvested method, they are discarded, together with the erythrocytes,[24,25] although the harvested technique resulted in a higher number of $CD133^+$ cells compared with the aspirated technique. We could speculate that although the $CD133^+$ cells observed with the harvested method are the hematopoietic stem cells and endothelial progenitor cells, the $CD133^+$ cells among the aspirated cells could also be VSEL cells (Fig 5), as indicated by the slightly higher HCT in the MC group. In fact, the VSEL cells would be lost during centrifugation, as observed by comparing the harvested BM before and after centrifugation in the HT group.

Moreover, it has been shown that $CD133^+$ cells can secrete SDF-1.[26,27] SDF-1/CXCL12 is a chemokine with attractant effects on cells that harbor the fusin/CXCR4 receptor, notably hematopoietic cells[20] and uncommitted neural cells.[28] The SDF-1/CXCR4 axis induce hematopoietic cell mobilization and migration,[29] adhesion,[30] engraftment,[31] transendothelial migration,[32] retention in the BM,[33] and modulation of hematopoiesis.[34] From our data, we would expect better results in revascularization

Table IV. Laboratory findings using Wilcoxon signed rank test for age-matched subgroups

BM characteristics	Before HT	After HT	*P* value
CD34 (n = 27)	0.95 (0.67-1.42)	0.93 (0.68-1.37)	.5561
CD133 (n = 27)	0.15 (0.09-0.31)	0.10 (0.04-0.20)	.0065
CD117 (n = 27)	0.79 (0.53-1.04)	0.93 (0.51-1.20)	.5560
CD309 (n = 27)	0.01 (0.01-0.02)	0.01 (0.01-0.02)	.6676
CDTOT (n = 28)	2.07 (1.28-2.69)	1.90 (1.46-2.69)	.8554
HCT (n = 17), %	32.6 (30.77-36.90)	27.3 (23.87-38.17)	.0684
BM purity (n = 17), %	0.72 (0.65-0.83)	0.93 (0.92-0.96)	.0004

BM, Bone marrow; *HCT,* hematocrit; *HT,* Harvest Technologies bone marrow aspirate concentrate system; *MC,* Marrow Cellution system.
Data presented as median (interquartile range).

Table V. Laboratory findings using Mann-Whitney *U* test for age-matched subgroups

BM characteristics	After HT (n = 30)	MC (n = 12)	*P* value
CD34	0.93 (0.68-1.37)	1.08 (0.90-1.34)	.5836
CD133	0.10 (0.04-0.20)	0.02 (0.01-0.02)	.0002
CD117	0.93 (0.51-1.20)	1.47 (0.93-1.57)	.0574
CD309	0.01 (0.01-0.02)	0.01 (0.01-0.03)	.7282
CDTOT	1.90 (1.46-2.69)	2.59 (1.79-3.11)	.2499
HCT, %	27.3 (23.87-38.17)	33.5 (31.77-38.90)	.2443
BM purity, %	0.93 (0.92-0.96)	0.96 (0.90-0.98)	.2213

BM, Bone marrow; *HCT,* hematocrit; *HT,* Harvest Technologies bone marrow aspirate concentrate system; *MC,* Marrow Cellution system.
Data presented as median (interquartile range).

Table VI. Outcomes for total population and age-matched subgroups

Outcome	After HT	MC	*P* value
Total population			
Amputation	20 (34.5)	1 (5.9)	.0209
Necrosectomy	5 (8.60)	6 (35.3)	.0063
Death	5 (8.60)	0 (0.00)	.2102
$TcpO_2$ (after transplant)	28.5 (21.5-38.0)	41.0 (38.5-45.7)	.0004
$\Delta TcpO_2$	18.0 (12.0-24.0)	30.0 (22.2-30.0)	.0001
Subgroup			
Amputation	11 (33.3)	1 (6.7)	.0480
Debridement	4 (12.1)	5 (33.3)	.0809
Death	0 (0.0)	0 (0.0)	
$TcpO_2$ (after transplant), mm Hg	28.5 (23.50-40.00)	42.0 (39.50-46.00)	.0021
$\Delta TcpO_2$, mm Hg	20.0 (14.50-25.00)	30.0 (21.75-30.00)	.0039

HT, Harvest Technologies bone marrow aspirate concentrate system; *MC,* Marrow Cellution system; *$TcpO_2$,* transcutaneous oxygen pressure; *$\Delta TcpO_2$,* change in transcutaneous oxygen pressure.
Data presented as number (%) or median (interquartile range).

for group HT because that group had the highest number of $CD133^+$ cells. However, because many leukocytes commonly possess CXCR4, SDF-1 could mobilize cells of the innate immune response to the ischemic region,[35] displaying proinflammatory properties. Therefore, if the harvested BM has a greater number of leukocytes, as shown by the greater number of total nucleated cells/1 mL,[8] the greater content of SDF-1, acting on the leukocytes, might counteract the revascularization process, as shown by the leg amputation frequency.

Several studies have shown that contamination of BM with red blood cells (RBCs) impairs the functionality of isolated BM cells through the secretion of factors such as lipids or proteins.[36] Thus, the purity of the BM is important. We showed that aspirated BM using the MC system has a purity of 96% vs a purity of 72% for the BM before

Table VII. Logistic regression

Risk factors for amputation	β	OR (95% CI)	*P* value
Total population			
Age	0.004876	1.005 (0.9685-1.046)	.800
Extraction technique			.057
HT	Ref	Ref	
MC	−2.091	0.12 (0.006-0.7001)	
Subgroup			
Extraction technique			.077
HT	Ref	Ref	
MC	−1.946	0.143 (0.017-1.231)	

CI, Confidence interval; *HT,* Harvest Technologies bone marrow aspirate concentrate system; *MC,* Marrow Cellution system; *OR,* odds ratio; *Ref,* reference value.

centrifugation using the HT technique. Moreover, the different forces that interact during the centrifugation could damage RBCs.[37,38] Destroyed RBCs release several highly inflammatory hemoglobin split products, such as heme, ferric-hemoglobin, and iron.[39] The presence of damaged RBCs with their toxic effects could impair the functionality and salutary effects of the other component of BM, including stem and progenitor cells.[36,40] Another aspect that should be considered is the pH conditions, which could be altered by the release of toxic products with effects on the quality of factors released by stem and/or progenitor cells and could have a negative therapeutic effect.[41]

Therefore, the superior purity, absence of the damaging effects of RBCs, and increased number of VSEL cells could have resulted in the superior clinical results observed for group MC. The BMA group had a low incidence of amputation, with a more favorable procedure of postoperative debridement performed during follow-up. Altogether, these considerations highlight the necessity of characterizing the precise cell population using rapid and reliable tests for quality control of the cell product obtained using different extraction methods to better use the cell sources available.

Finally, the aspiration of a reduced volume of BM in group MC (40 mL vs 240 mL for group HT) has clinical advantages, in terms of both decreased stress for patients and shorter sedation times for these critically ill patients. These two parameters should be considered when choosing between these two methods.

Study limitations. The present study has some limitations. However, these do not affect the clinical and scientific value of the observed results. The first limitations are the study duration and lack of randomization. The Aspire technology (Aspire Medical Innovation) became available during our study of BMAC to treat PAD at the Gemelli Molise Hospital and, based on the available knowledge, appeared worth using. The present report was conceived as retrospective review, and the results strongly support our decision. Second, patient age between the two overall study groups was significantly different, a factor that could have affected the outcomes for the patients in the MC group, who were younger. However, the subgroup analyses proved the superiority of the Aspire approach, overruling the age factor. In addition, the striking effects observed in the MC groups support its use even for elderly patients whose BM might be deficient, most importantly, who would be at much higher risk should a surgical approach be considered.

CONCLUSIONS

BMA is known to aid in the healing of a variety of orthopedic injuries and to promote angiogenesis in ischemic tissue. However, the technique used for BM harvesting and preparation is important for the different cell compositions. Moreover, manipulation of BM, even minimally, such as with centrifugation, can affect the quality and number of stem cells. Thus, it is fundamental to characterize the cell population in relationship to the harvesting method used to understand properly the effects of BM content for the specific disease treated. In our study, we showed that BM aspirated with the MC method is safe. We did not observe any side effects (eg, infection) due to the procedure. Also, the method mostly resulted in a better product compared with the classic harvesting method. We found the MC method capable of counteracting the occurrence of leg amputations and increasing the $TcpO_2$ in patients with CLTI.

AUTHOR CONTRIBUTIONS

Conception and design: EC, EV
Analysis and interpretation: EC, EM, AM, SP, MF, LP, AP, MF, EF
Data collection: EC, EMC, AM, MF, LP, BA, CD
Writing the article: EC, EM, BA, EV
Critical revision of the article: EC, EM, EMC, AM, SP, MF, LP, AP, MF, EF, BA, EV
Final approval of the article: EC, EM, EMC, AM, SP, MF, LP, AP, MF, EF, BA, CD, EV

Statistical analysis: EC, EMC
Obtained funding: EC
Overall responsibility: EC

DISCLOSURES

None.

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Submitted Jul 14, 2023; accepted Sep 17, 2023.