Original Article

Patient Demographic Factors Are Not Associated With Mesenchymal Stromal Cell Concentration in Bone Marrow Aspirate Concentrate

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Purpose: To describe the capacity for concentration of a single processing machine for bone marrow aspirate concentrate (BMAC) production and investigate the effects of demographic factors on the number of mesenchymal stromal cells (MSCs) in BMAC. **Methods:** Patients enrolled in our institution's randomized control trials involving BMAC who had complete BMAC flow cytometry data were included. Multipotent MSC phenotype, defined as cell-surface coexpression of specific-identifying antigens ($\geq$95% positive) and the absence of hematopoietic lineage markers ($\leq$2% positive), was determined for both patient bone marrow aspirate (BMA) and BMAC samples. The ratio of cells in BMA:BMAC samples was calculated and Spearman correlations (i.e., body mass index [BMI]) and Kruskall–Wallis (i.e., age: <40, 40-60, >60 years) or Mann–Whitney (i.e., sex) tests were used to determine the relationship of cell concentration to demographic factors. **Results:** Eighty patients were included in analysis (49% male, mean age: 49.9 $\pm$ 12.2 years). Mean concentration of BMA and BMAC was 2,048.13 $\pm$ 2,004.14 MSCs/mL and 5,618.87 $\pm$ 7,568.54 MSC/mL, respectively, with a mean BMAC:BMA ratio of 4.35 $\pm$ 2.09. A significantly greater MSC concentration was observed in the BMAC samples when compared with BMA ($P = .005$). No patient demographic factors (age, sex, height, weight, BMI) were found to predict MSC concentration in the BMAC samples ($P \geq .01$). **Conclusions:** Demographic factors, including age, sex, and BMI do not impact the final concentration of MSCs in BMAC when using a single harvest technique (anterior iliac crest) and a single processing system. **Clinical Relevance:** As the role of BMAC therapy expands in clinical application, it becomes increasingly important to understand the determinants of BMAC composition and how it is affected by different harvesting techniques, concentrating processes, and patient demographics.

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Bone marrow aspirate concentrate (BMAC) is one type of orthobiologic that has gained increased popularity in the field of orthopaedic surgery over the last decade. BMAC has been shown to improve healing through immunomodulating effects preclinically.[1] Numerous clinical studies have suggested a potential role for BMAC in a variety of orthopaedic conditions, including rotator cuff repair (RCR),[2] osteoarthritis (OA),[3] bone and chondral defects,[4] treatment of fracture nonunion,[5] and treating tendinopathies.[6] Despite its increasing utility, the mechanism of BMAC is unclear. Previous studies have suggested that improvement is through multiple pathways, including cytokine alterations and inhibition of interleukin-1 receptor antagonist.[7] However, mesenchymal stromal cells (MSCs) in BMAC likely play a substantial role. MSCs are multipotent stem cells that have been used in orthopaedic applications as the result of their ability to differentiate into all musculoskeletal cell lineages and generate various anti-inflammatory agents, chemokines, cytokines, and growth factors.[8]

Preparation of BMAC can result in a 10-fold increase in MSCs as compared with unprocessed bone marrow aspirate (BMA), although the reliability of this remains unclear.[9,10] Numerous preparation devices are commercially available; however, the literature is limited on the differences between these products.[11]

Multiple factors have been demonstrated to influence the MSCs concentration and chondrogenic potential in BMA and thus BMAC, ranging from aspiration location (iliac crest, proximal humerus, proximal tibia, or calcaneus) to amount extracted to peripheral blood platelet count.[12-22] These studies have suggested that age may affect chondrogenic potential of BMAC derived MSCs but may not affect initial MSC concentration. Although several studies have identified a correlation between increasing age and decreasing MSC concentration, this remains controversial, with several conflicting findings.[15,23-25] Therefore, the role of demographics on the MSC count in BMAC remains unclear. This question becomes relevant especially in the setting of poor tissue quality (e.g., older age), where the use of BMAC may be considered for healing augmentation.

The purposes of this study were to describe the capacity for concentration of a single processing machine for BMAC production and investigate the effects of demographic factors on the number of MSCs in BMAC. We hypothesized that the single processing machine would significantly and reliably increase the concentration of MSC in BMAC compared with BMA. In addition, we hypothesized that donor age and sex would not influence MSC concentration in BMAC.

Methods

Patient Selection

This study comprised data from 4 randomized control trials at the Rush University Medical Center designed to investigate the clinical effect of augmentation of orthopaedic surgical procedures (anterior cruciate ligament [ACL] reconstruction, osteochondral allograft [OCA] transplantation, RCR, and meniscectomy) with BMAC. The 4 randomized control trials received institutional review board approval by the Rush University Medical Center, and all patients consented to one of the studies before enrollment. The trial databases were queried for patients who were part of the BMAC intervention arm of these 4 trials, which was randomized by electronic sequence generation for enrollment order. Each study had its own relevant inclusion and exclusion criteria (Table 1).

After patient selection, chart review was performed to identify the following variables: age, sex, body mass index (BMI), height, and weight. Anthropometric factors (e.g., BMI) which were recorded closest to the date of bone marrow aspiration were chosen for

Table 1. Inclusion and Exclusion Criteria for Component Studies

Demographics	Study 1 (ACL) N = 19	Study 2 (RCR) N = 35	Study 3 (OCA) N = 3	Study 4 (Meniscectomy) N = 23
Inclusion criteria	18-60 years old	At least 18 years old	18-50 years old	18-70 years old
Exclusion criteria	Cancer diagnosis that is not in long-term remission (except BCC), high risk of surgical bleeding or infection, history of HIV, active hepatitis B, active hepatitis C, pregnant or breast-feeding women, or current alcohol/drug abuse	History of diabetes, rheumatoid arthritis, cancer diagnosis that is not in long-term remission (except BCC)	History of rheumatoid arthritis	Cancer diagnosis that is not in long-term remission (except BCC), high risk of surgical bleeding or infection, history of HIV, active hepatitis B, active hepatitis C, pregnant or breast-feeding women, current alcohol/drug abuse)

ACL, anterior cruciate ligament; BCC, basal cell carcinoma; HIV, human immunodeficiency virus; OCA, osteochondral allograft; RCR, rotator cuff repair.

analysis. Of note, the demographics of the 4 included randomized trials were found to be similar in terms of sex ($P = .501$), height ($P = .336$), weight ($P = .581$), and BMI ($P = .645$). As expected, the 4 studies did significantly ($P < .01$) differ in age (ACL: 36.4 ± 9.8 years; RCR: 55.8 ± 8.3, OCA: 35.2 ± 3.6; meniscectomy 54.0 ± 9.1); however, this was expected, given the difference in age group for these orthopaedic conditions and this was found to be advantageous from a study design perspective as it allowed for a great ranger of ages to be correlated to BMA/BMAC concentration.

BMA and Concentration

Bone marrow aspiration was performed in a sterile manner with the patient in supine positioning. Palpation of bony landmarks identified the anterior superior iliac spine and iliac crest, following by sterile prepping and draping around these areas. Local anesthetic was injected, and a 2-mm stab incision was made down to bone. A bone marrow trocar is then placed into bone at approximately 3 cm of depth. Approximately 60 mL total of bone marrow were aspirated for subsequent concentration.[26] At least 1 mL of BMA was removed for flow analysis before concentration.

A single processing machine (Angel System; Arthrex, Naples, FL) was used for concentration of all patient samples according to manufacturer instructions. At least 1 mL of BMAC was removed for flow analysis before its intended clinical use.

BMA and BMAC Staining for Flow Cytometric Analyses

BMA and BMAC samples were collected intraoperatively and held overnight at room temperature before staining for mesenchymal cell content. BMA and BMAC samples were stained in 12 × 77-mm tubes according to the BD Stemflow hMSC Analysis Kit instructions (BD Biosciences, San Jose, CA) per the manufacturers' instruction. After antibody staining, samples were sequentially washed with BD Pharm Lyse lysing buffer (BD), fluorescent-activated cell sorting buffer, and resuspended to a final volume of 0.5 mL in 1% paraformaldehyde (PFA). Samples were refrigerated until preparation for flow cytometry.

Flow Cytometry

Immediately before flow cytometry analyses, the cells (0.5 mL final volume) were transferred into BD Trucount tubes (BD, Franklin Lakes, NJ). BMA samples were run undiluted with 500 µL of sample, whereas BMAC samples were run at a 1:1 dilution with PFA comprising 10% formaldehyde (5 mL of formaldehyde to 45 mL of Hyclone phosphate-buffered saline buffer). Acquisition was performed on a BDFortessa flow cytometer using FACSDiva software (version 6.1.3 or version 8.0.2). Fluorescence parameter PMTs were normalized using Rainbow Calibration Particles, Peak 7 (Spherptech, Lake Forest, IL). Compensation was acquired and applied using BD CompBeads Set Anti-Mouse Ig, K particles, and the calibration data for parameters for fluorescent antibodies including fluorescein isothiocyanate, PerCP-Cy5, allophycocyanin, and phycoerythrin. Forward and side scatter voltages were normalized using BD Trucount beads by analyzing 500 µL of PFA in a BD Trucount tube using parameters acquired during compensation. A 1:2 dilution of BMAC to PFA (250 µL of cells BMC to 250 µL of 1% PFA) was analyzed to confirm cell counts within appropriate ranges, with a goal event count between 1,000,000 and 2,000,000. The same 1:2 dilution was used to analyze BMAC in a BD Trucount tube. Then, 500 µL of BMA was analyzed in a BD Trucount tube with no dilution. After acquisition, the samples were analyzed using FlowJo v9.9.6 (TreeStar Inc., Ashland, OR) software to identify the number of Trucount bead events and the number of events within the multipotent MSC phenotype, defined as cell-surface co-expression of the antigens CD105, CD73, and CD90 (≥95% positive) and the absence of hematopoietic lineage markers CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR (≤2% positive) as seen in Figure 1. Absolute MSC count was determined by dividing the number of BD Trucount beads acquired by the known total number of beads in the tube lot (human MSC even count/ bead event count * Trucount concentration).

Statistical Analysis

Statistical analysis was performed in STATA (version 13; STATAcorp, College Station, TX). Data normality was assessed with the Shapiro–Wilk test. This test

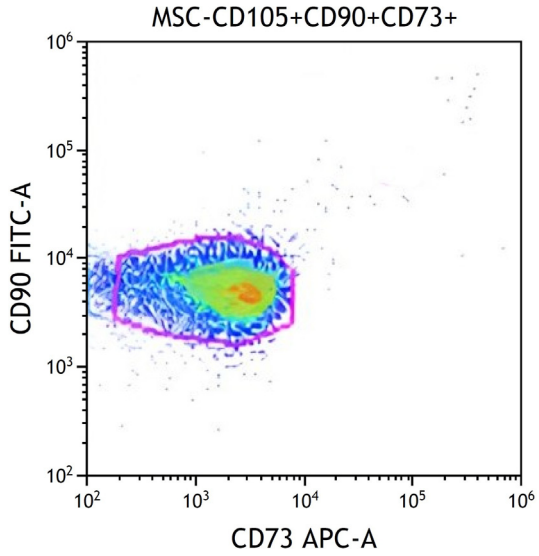

Fig 1. Final gating used to identify the MSC cell population. (MSC, mesenchymal stromal cell.)

demonstrated the presence of non-normally distributed data therefore nonparametric statistical analyses were performed. To compare group differences, a Kruskall–Wallis (comparison of >2 groups) or Mann–Whitney *U* test (2 groups) was used when appropriate. Medians are reported in the results as well as means and standard deviations as indicated. In addition, to investigate correlations between demographic factors and MSCs, Spearman correlations were utilized. Due to the use of multiple statistical tests, significance was set at $P < .01$.

Power analysis was performed in G*Power (Version 3.1.9.7, Heinrich Heine University, Dusseldorf, Germany).[27,28] An a priori power analysis was performed for the study's primary end point to determine the sample size needed to demonstrate a moderate effect size of 0.4 between preprocessing BMA and postprocessing BMAC. Sample size was powered to pre- and postprocessing BMA/BMAC concentration, given that this was the primary outcome of the presented study. Furthermore, a conservative numeric effect size of 0.4 was chosen, given the previously established substantial variability of baseline cell counts and concentration data.[9,10] Power analysis resulted in a minimum expected sample size of 54 patients at a power of 0.8 and alpha of 0.05. For secondary endpoints, such as age, sex, height, weight, and BMI, we determined that an effect size of 0.6 could be achieved with subgroup comparisons of 37 patients per group using the above power and alpha values, requiring at least a total of 74 patients based on 1:1 allocation for powered primary and secondary comparisons.

Results

In total, 85 patients with complete BMA and BMAC flow cytometry data were identified and 5 outliers were removed as a result of an excessively high reported numbers of BMA cells, BMAC cells, or ratio of the 2. These were confirmed through laboratory analysis to be true outliers due to staining or processing error. Of the 80 remaining patients, 19 were from ACL trial, 35 were from the RCR trial, 3 were from the OCA trial, and 23 were from the meniscectomy trial (Table 1). In addition, 49% were male, with a mean age of 49.93 ± 12.17 years and a mean BMI of 29.00 ± 6.34 (Table 2). The distribution of BMA and BMAC cell concentrations is presented in Figure 2. The mean concentration of BMA and BMAC was 2,048.13 ± 2,004.14 (range: 6.80-9,031.47) MSCs/mL and 5618.87 ± 7568.54 (range: 3.65-46,230) MSC/mL, respectively. The mean BMAC:BMA was 4.35 ± 2.09 (range: 0.01-33.94). The mean difference in concentration between BMAC and BMA was 3570.74 ± 7384.69 (range: 8,208.35-44,868.23) MSC/mL (Fig 3). There was a significant increase in concentration of MSCs in the BMAC compared with the BMA samples (medians: 2,912.52 vs 1,363.95, $P = .005$).

Table 2. Cohort Demographics

Variable	Outcome
N	80
M/F	39/41
Age, yr	49.93 ± 12.17
Height, in	67.01 ± 4.05
Weight, lb	186.43 ± 48.20
Smoking Status (never/former/current)	57/15/5*
BMI, m/kg^2	29.00 ± 6.34
BMA MSC/mL	2048.13 ± 2004.14

BMA, bone marrow aspirate; BMI, body mass index; F, female; M, male; MSC, mesenchymal stromal cell.
*Status unknown for n = 3 patients.

Patient demographic factors and their association to BMAC concentration were evaluated with a Kruskall–Wallis (comparison of >2 groups) or Mann–Whitney *U* test (2 groups) when appropriate. No significant association between age was observed ($r = 0.1573$, $P = .1635$) (Fig 4). However, when comparing BMAC concentration in patients younger than 40 years to those between 40 and 60, and older than 60 years, a trend toward significance between groups was observed (medians: <40 years: 889.84, 40-60 years: 1329.57, >60 years: 2685.46, $P = .087$). No significant differences in concentration were observed between sexes (medians: male: 1007.19, female: 1414.59, $P = .3997$) (Fig 5). Lastly, no significant correlations were observed between BMAC concentration and height ($r = -0.1330$, $P = .2424$), weight ($r = 0.0907$, $P = .4268$), or BMI ($r = 0.1512$, $P = .1835$) (Fig 6).

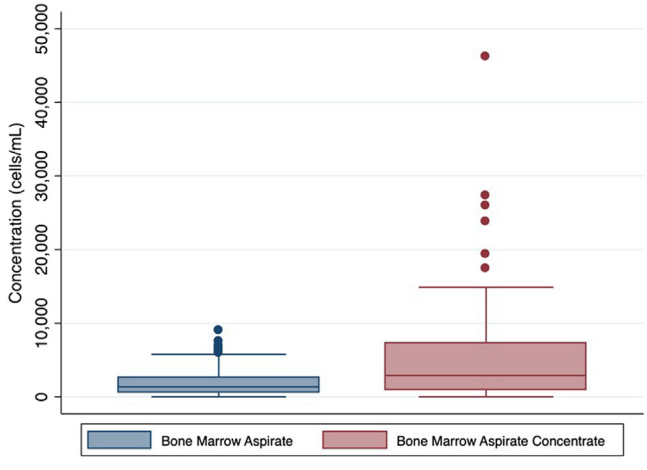

Fig 2. Distribution of concentration of MSC/mL in BMA and BMAC samples. (BMA, bone marrow aspirate; BMAC, bone marrow aspirate concentrate; MSC, mesenchymal stromal cell.)

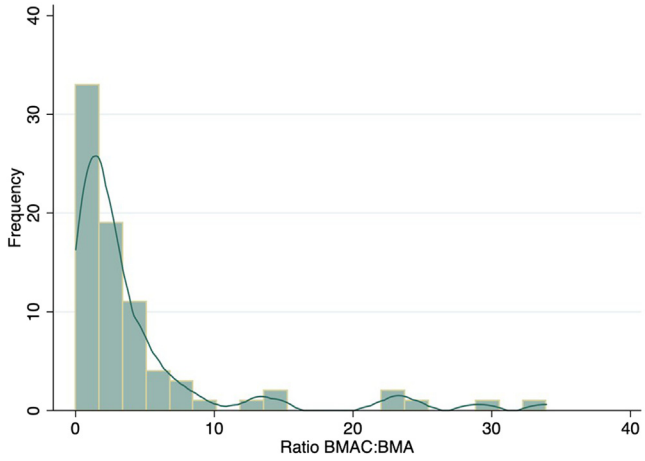

Fig 3. Distribution of the ratio of BMAC to BMA. (BMA, bone marrow aspirate; BMAC, bone marrow aspirate concentrate.)

Discussion

The single BMAC processing machine used in this study was capable of increasing MSC concentration in BMAC compared with BMA samples. The final concentration of MSCs in BMAC was unaffected by baseline patient demographics, such as age, sex, height, weight, and BMI. This confirms our hypotheses. These findings are clinically relevant, given that as the role of BMAC therapy expands in clinical application, it becomes increasingly important to understand the determinants of BMAC composition and how it is affected by different harvesting techniques, concentrating processes, and patient demographics.

In regards to harvest site, the iliac crest has been long considered the preferential source for BMAC.[29] It has been suggested that there is a significantly improved potential for differentiation of cells harvested from the anterior iliac crest as opposed to the proximal tibia or calcaneus.[30] Recent studies have suggested equitable harvests occurring between the iliac crest and other locations such as the proximal humerus, but data is conflicting.[31,32] The current disagreement in the literature compelled us to pursue a single harvest site for our study to further control for this possible confounder in our data.

Further, given the heterogeneity in reporting on qualitative and quantitative characteristics of different BMAC devices, we chose to control for this using a single concentration process and a single harvesting technique at the anterior iliac crest. For our single processing machine for producing BMAC, the Arthrex Angel system, a significant increase MSC concentration was found in BMAC compared with the BMA samples. This finding is consistent with our hypothesis and what has previously been described in the literature for other commercially available BMAC concentrating systems.[9,33,34] This further corroborates the ability of commercial systems to concentrate MSCs from BMA, even though MSCs are estimated to only make up 0.01% to 0.02% of the cellular concentrate in bone marrow compared to other cell types.[9]

Moreover, our study provides evidence that the final concentration of MSCs in BMAC is unaffected by baseline patient demographics such as age, sex, height, weight, and BMI. Previous studies have suggested significant variations in the composition of bone marrow with regards to age, gender, skeletal location, and various pathologic states such as osteoporosis.[35-37] However, there are underlying confounding factors in BMAC production such as harvest site, processing technique, and variable application techniques that make the results of these studies difficult to apply to MSC concentration in BMAC. Thus, controlling for

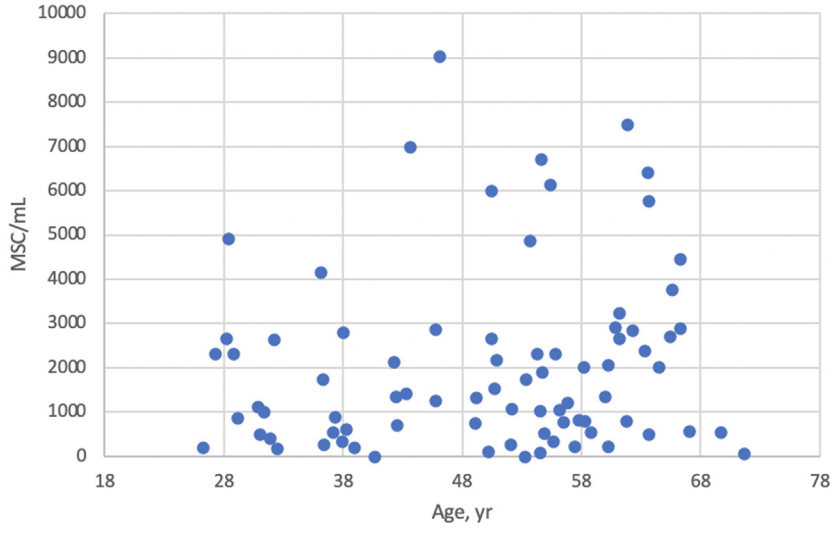

Fig 4. Age versus MSC concentration in BMA. (BMA, bone marrow aspirate; MSC, mesenchymal stromal cell.)

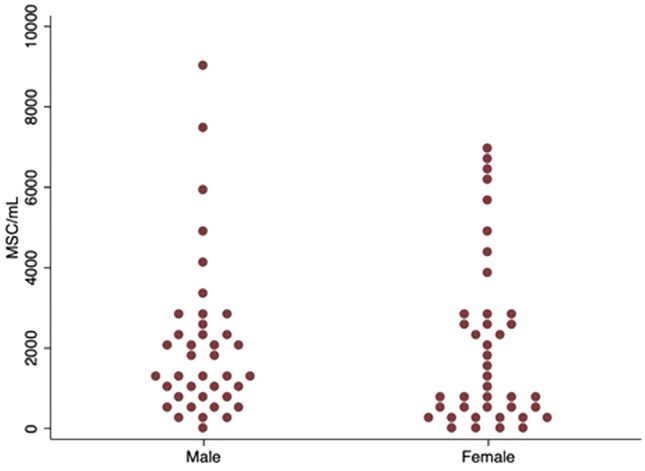

Fig 5. Sex versus MSC concentration in BMA. (BMA, bone marrow aspirate; MSC, mesenchymal stromal cell.)

these factors has allowed us to study these associations more reliably.

Although there are numerous other preparation devices are commercially available, there are limited high quality head-to-head comparisons of the differences between these products in the literature. A review by Gaul et al.[11] compared technical features and quality centrifugation parameters of final BMC products on 8 commercially available devices, including the Arthrex Angel system, and found an inability to compare data between devices due to a lack of standardized reporting in describing biologic potency. Furthermore, they could not recommend any one device over another due to lack of standardized reporting and establishment of device efficacy with clinical outcomes.[11] This was similarly highlighted in a systematic review by Piuzzi et al.[38] studying BMAC used in clinical trials for orthopaedic treatments. They found only 14 of 46 (30%) studies provided quantitative measures of BMAC composition and that not a single study gave thorough enough protocol reporting to allow future investigators to duplicate the study methodology. In addition, Dragoo et al.[39] compared the Arthrex Angel system with 2 other commercially available systems. Although they did not perform flow cytometric analysis for MSCs, they found comparable performance of the Arthrex system in concentration of fibroblastic colony-forming units , CD34+ cells, platelets, and white blood cells, which suggests equitable performance of the system utilized by our study from quantitative metric perspective.

BMAC has been investigated as a promising therapy for full-thickness cartilage lesions, osteochondral lesions, OA, bone healing, and tendon repair, specifically RCR and Achilles tendon repair.[40] In a recent prospective randomized control trial published in 2021 by Cole et al.,[2] BMAC shoulder injections at the time of RCR were found to lead to improved functional outcomes and tendon quality 1-year postoperative magnetic resonance imaging scans. These findings build upon previous 10-year data published by Hernigou et al.,[41] which also demonstrated increased early healing and lower long-term re-tear rates in rotator cuff repairs augmented by MSC-based therapy.

Our study further informs this field of augmentation and suggests that that patient factors should not restrict the use of BMAC based on potential for diminished MSC concentration, as increased MSC concentration has been shown to lead to improved healing in orthopaedic settings such as total hip arthroplasty and atrophic nonunions.[42,43] This becomes relevant when considering the use of BMAC for augmentation in the setting of poor tissue quality and host factors—the very reason augmentation is often considered in this patient population (e.g., older patients). Furthermore, our study sets the stage for future studies aimed at determining potential thresholds of BMAC constituents that correlate with positive clinical outcomes, both from a qualitative and quantitative standpoint in various orthopaedic applications. The upper limits of age when BMAC cells lose concentration also should be examined. Previous studies have shown the equilibrium between red and yellow bone marrow is generally reached at age 25 years.[37] Red marrow and its associated hematopoiesis lies primarily in the axial skeleton in bones such as the pelvis until then and redistributes to the rest of the skeleton in a generally heterogeneous fashion as patients age. Thus, MSC quantity and quality from BMAC harvests from sites like the iliac crest could potentially diminish in the elderly. In addition, our study showed a trend towards significance in the age bracket older than 60 years old. In the general population, over half of people aged 65 years or older show radiographic evidence of OA, which increases to more than 80% of patients older 75 years.[44] Therefore, future studies aimed at examining quantitative and qualitative aspects of MSCs in BMAC for ages brackets for 65-70, 70-75, and 75 years and older could have broad clinical implications. As the production of BMAC continues to standardize, our findings paired with these future studies will help better characterize the biologic potency and reliability of BMAC.

Limitations

The limitations of this study lie primarily in the lack of qualitative examination of our BMAC and MSCs. Future studies should investigate whether morphology or functional phenotype of MSCs relate to demographic characteristics. These studies could include polymerase chain reaction and protein analysis to better understand gene expression as well as investigation of differentiation capabilities of MSCs harvested.[37,44] This study was additionally limited by the potential for selection bias of

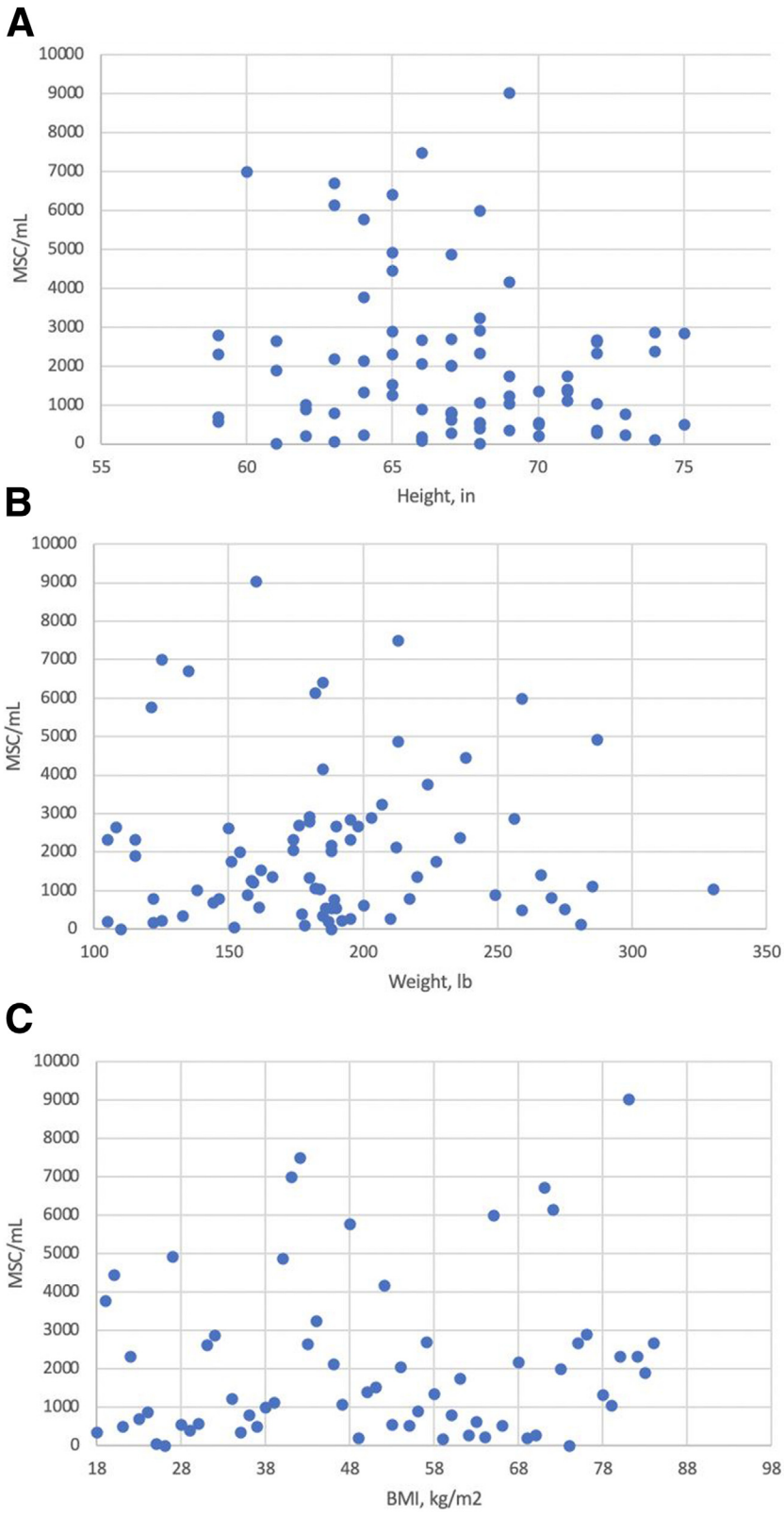

Fig 6. (A) Height, (B) weight, and (C) BMI versus MSC concentration in BMA. (BMI, body mass index; MSC, mesenchymal stromal cell.)

patients included for the 4 randomized control trials that comprised the patient population. However, all 4 trials were included in order to most effectively analyze the effect of demographic variety on BMAC composition.

Conclusions

Demographic factors, including age, sex, and BMI do not impact the final concentration of MSCs in BMAC when utilizing a single harvest technique (anterior iliac crest) and a single processing system.

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