

# Quantification and Qualification of Stem Cells From Blood After Mobilization With Filgrastim, and Concentration Using a Platelet-Rich Plasma System



Adam W. Anz, M.D., Andrea Matuska, Ph.D., Joseph L. Edison, D.O.,  
Siraj F. Abdullah, D.O., Travis J. Dekker, M.D., Hillary A. Plummer, Ph.D., A.T.C.,  
Kenny V. Brock, Ph.D., D.V.M., and Michael D. Goodlett, M.D.

**Purpose:** To determine the cellular composition of a product created with peripheral blood harvested after systemic mobilization with filgrastim and processed with one point-of-care blood concentrating system, i.e., a platelet-rich plasma (PRP) system. The second purpose was to compare mobilized platelet-rich plasma (M-PRP) with a concentrated bone marrow aspirate (cBMA) and a PRP created from the same subjects with the same PRP system. **Methods:** Ten healthy volunteer subjects were recruited for collection and analysis of 3 tissue sources: non-treated peripheral blood, bone marrow aspirate, and filgrastim-mobilized peripheral blood, involving 4 doses of weight-based filgrastim. One point-of-care blood and bone marrow concentrating system was used to create 3 products: PRP, cBMA, and M-PRP. Automated hematologic analysis was performed on all products to quantify total red blood cells, white blood cells (WBCs), monocyte, platelet, and hematopoietic progenitor cell (HPC) concentrations. Flow cytometry was used to determine hematopoietic and mesenchymal progenitor cell populations. Lastly, concentrates were cultured and fibroblast colony-forming units (CFU-F) and morphology of adherent cells were evaluated. **Results:** M-PRP contained a greater concentration of WBC (mean difference = 53.2 k/ $\mu$ L;  $P < .0001$ ), monocytes (mean difference = 8.3 k/ $\mu$ L;  $P = .002$ ), and a trend toward a greater concentration of HPC (mean difference = 200.5 / $\mu$ L;  $P = .060$ ) when compared with PRP. M-PRP contained a greater concentration of monocytes (mean difference = 5.5 k/ $\mu$ L;  $P = .017$ ) and a trend toward a greater concentration of platelets (mean difference = 348 k/ $\mu$ L;  $P = .051$ ) and HPC (mean difference = 193.4 / $\mu$ L;  $P = .068$ ) when compared with cBMA. M-PRP had a similar concentration of platelets to PRP (mean difference = 110 k/ $\mu$ L;  $P = .051$ ) and PRP had a greater concentration than cBMA (mean difference = 458 k/ $\mu$ L;  $P = .003$ ). cBMA remained the only product capable of producing CFU-Fs ( $446 \pm 247$  /mL) as neither the M-PRP nor PRP produced CFU-Fs. M-PRP produced colonies consistent with WBC. **Conclusions:** M-PRP, produced with filgrastim mobilized blood and a proprietary PRP system, contained more total WBCs, monocytes, platelets, and HPCs than cBMA and more WBCs, monocytes, and HPCs than PRP. **Clinical Relevance:** Filgrastim mobilized PRP may be an alternative to cBMA for use as a point-of-care product for orthopaedic treatments.

See commentary on page 2919

Progenitor cells, including hematopoietic progenitor cells (HPCs) and mesenchymal progenitor cells (MPCs), are found in the blood and bone marrow and are naturally mobilized to the peripheral blood

circulation during episodes of stress, injury, and exercise.<sup>1,2</sup> Progenitor cell therapies have shown great potential for addressing acute, chronic, and traumatic orthopaedic injury. Orthopaedic surgeons have begun

From Andrews Institute for Orthopedics & Sports Medicine (A.W.A.) and Andrews Research & Education Foundation (H.A.P.), Gulf Breeze, Florida (A.W.A.); Arthrex, Naples, Florida (A.M.); Edward Via College of Osteopathic Medicine, Auburn, Alabama (J.L.E., S.F.A., K.V.B., M.D.G.); Eglin Air Force Base, Niceville, Florida (T.J.D.); and Auburn University Sports Medicine, Auburn, Alabama (M.D.G.), U.S.A.

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Address correspondence to Adam W. Anz, M.D., Andrews Institute for Orthopedics & Sports Medicine, 1020 Gulf Breeze Parkway, Gulf Breeze, FL 32561. E-mail: [anz.adam.w@gmail.com](mailto:anz.adam.w@gmail.com)

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to augment surgical procedures and treat degenerative conditions such as osteoarthritis, with injections of bone marrow aspirate and platelet-rich plasma (PRP).<sup>3-7</sup> Although bone marrow aspiration is frequently used for orthopaedic applications, the number of cells harvested is variable and dependent on individual, aspiration technique, and location of harvest.<sup>8-11</sup> Aspiration techniques have been compared with the most efficient method involving rapid, small-volume, and multiple aspirations from multiple locations on the iliac crest.<sup>8-13</sup> Orthopaedic studies have begun to investigate additional sources of cell harvest for orthopaedic purposes to include resident stem cells and autologous peripheral blood monocytes.<sup>14-19</sup>

Pharmaceutical mobilization with a mobilizing drug such as filgrastim, followed by peripheral harvest of cells with apheresis, is now more common than bone marrow aspirate for the hematologic oncologic clinical practice of hematopoietic stem cell transplant.<sup>20,21</sup> Filgrastim is a synthetic form of human granulocyte colony-stimulating factor, a hormone that increases production of progenitor cells in the bone marrow, release into the peripheral circulation, and subsequent increased circulating numbers in the blood. Pharmaceutical mobilization followed by apheresis harvest has improved cell harvest when compared with bone marrow aspirate.<sup>21-23</sup> The long-term safety of filgrastim mobilization has been reported with bone pain as a common adverse effect and no association with neoplastic risks identified.<sup>24-31</sup> Previous work has determined that the optimal dosage of filgrastim for cell mobilization is 10 µg/kg/day for 4 consecutive days.<sup>24</sup> Preclinical studies have determined that mobilized blood contains a cell population that is expandable, pluripotent, and has a genetic profile similar to neural and embryonic stem cells.<sup>32,33</sup>

Although previous studies have evaluated the potential of filgrastim to improve apheresis harvest, the potential of combining filgrastim mobilization with peripheral blood harvest and concentration with a PRP device can be developed further. The primary purpose of this study was to determine the cellular composition of a product created with peripheral blood harvested after systemic mobilization with filgrastim and processed with one point-of-care blood concentrating system, i.e., PRP system. The second purpose was to compare mobilized platelet-rich plasma (M-PRP) product with a concentrated bone marrow aspirate (cBMA) and a PRP created from the same subjects with the same PRP system. It was hypothesized that M-PRP would yield a greater number of multipotent HPCs, colony-forming units, and platelets compared with cBMA and PRP.

## Methods

### Participants

Ten healthy male volunteers were recruited through word of mouth and flyers. Inclusion criteria included male sex, age 19-39 years, and weight 50-100 kg. Additional exclusion criteria included previous allergic reaction to filgrastim or lidocaine, latex allergy, history of diabetes, any autoimmune disorder, blood disorders requiring immunosuppression, cancer, ongoing infectious disease, significant cardiovascular, renal, hepatic, or pulmonary disease, sickle cell or other blood disorders, presence of abdominal tenderness with palpation, signs of splenomegaly, and unclear lung fields. A 5-mL blood sample was collected for a complete blood count (CBC) with white blood cell (WBC) differential. If WBC was greater than 20,000/mL, the participant was also excluded. All participants completed an informed consent, which was approved by Auburn University's institutional review board (approval 16-398 AR 1612). Participants received a stipend of \$500 after completing the study. Funding for this study was provided by Edward Via College of Osteopathic Medicine. Bone marrow aspiration kits and Angel processing kits were donated by Arthrex (Naples, FL).

### Procedures: Peripheral Blood and Bone Marrow Collection

Approximately 1 week after the medical screening, the participants returned to the clinic to have a blood draw and bone marrow aspiration performed. For the blood draw, 8.6 cc of ACDA (anticoagulant citrate dextrose solution) was drawn into a 60-mL syringe and a standard venipuncture on the upper extremity was used to draw blood to fill the syringe to 65 cc. 2.5 cc of blood was put into a vacutainer tube to be analyzed with flow cytometry. The remaining blood in the 60-mL syringe was processed with the Arthrex Angel System (Arthrex) using the 15% hematocrit setting to create a PRP product.

Next, a bone marrow aspirate from the iliac crest was performed by the same orthopaedic surgeon (A.W.A.). The participant was positioned in the lateral decubitus position and ultrasound was used to outline the iliac crest and confirm the location from where the bone marrow aspirate would be obtained. The skin was prepped with ChlorPrep, a 2% CHG/70% IPA solution, (BD, Franklin Lakes, NJ) and the skin was numbed with 20 mL of 1% lidocaine. An 11-gauge bone marrow aspirate needle (Angel cPRP & BMA Tray; Arthrex) was used to collect bone marrow aspirate. A 60-mL syringe pre-filled with 8.6 mL of ACDA was prepared for the sample. A 1-cm incision was made in the skin and the needle was advanced into the bone

**Table 1.** Descriptive Data for Automated Hemocytometer, Flow Cytometry, and Culture

	Platelet-Rich Plasma	Concentrated Bone Marrow Aspirate	Mobilized Platelet-Rich Plasma
Automated hemocytometer			
RBC, M/ $\mu$ L	0.6 $\pm$ 0.4	1.2 $\pm$ 0.8 (PRP: $P = .048$ ; M-PRP: $P = .530$ )	1.0 $\pm$ 0.6 (PRP: $P = .038$ )
WBC, k/ $\mu$ L	11.4 $\pm$ 4.8	46.2 $\pm$ 20.1 (PRP: $P < .0001$ ; M-PRP: $P = .266$ )	58.0 $\pm$ 28.6 (PRP: $P < .0001$ )
MONO, k/ $\mu$ L	3.5 $\pm$ 5.1	6.3 $\pm$ 5.8 (PRP: $P = .001$ ; M-PRP: $P = .017$ )	11.8 $\pm$ 5.5 (PRP: $P = .002$ )
PLT, k/ $\mu$ L	1194 $\pm$ 331	736 $\pm$ 199 (PRP: $P = .003$ ; M-PRP: $P = .051$ )	1084 $\pm$ 516 (PRP: $P = .542$ )
HPC, / $\mu$ L	0.3 $\pm$ 0.5	7.4 $\pm$ 4.8 (PRP: $P = .001$ ; M-PRP: $P = .068$ )	200.8 $\pm$ 295.1 (PRP: $P = .060$ )
Flow cytometry			
HPC, / $\mu$ L	0.2 $\pm$ 0.2	6.0 $\pm$ 6.3 (PRP: $P = .119$ ; M-PRP: $P = .377$ )	9.9 $\pm$ 8.3 (PRP: $P = .008$ )
MPC, / $\mu$ L	3.0 $\pm$ 4.6	23.9 $\pm$ 27.6 (PRP: $P = .01$ ; M-PRP: $P < .001$ )	1.3 $\pm$ 1.4 (PRP: $P = .644$ )
Culture			
CFU-F, /mL	0 $\pm$ 0	446 $\pm$ 247 (PRP: $P < .0001$ ; M-PRP: $P < .0001$ )	0 $\pm$ 0 (PRP: $P = 1.00$ )

NOTE. Hematopoietic progenitor cell markers included a combination of CD45+CD34+CD133+CD38- and mesenchymal progenitor cell markers were CD45-/lowCD271+.

CFU-F, colony-forming unit fibroblast; HPC, hematopoietic progenitor cell; MONO, monocyte; MPC, mesenchymal progenitor cell; PLT, platelet; RBC, red blood cell, WBC, white blood cell.

marrow cavity. The needle was advanced approximately 3 to 4 cm and aspiration of 30 cc was performed while rotating and withdrawing the needle. The needle was advanced a second time in a divergent vector. Aspiration was continued while withdrawing and rotating the needle to fill the syringe to 65 cc. The sample of bone marrow was processed using the same protocols as described above for the peripheral blood sample to create a cBMA product.

### Pharmaceutical Mobilization

Approximately 30 days after the bone marrow aspiration visit (within 7 days of the exact harvest date), participants began a series of 10  $\mu$ g/kg body weight dose filgrastim injections for 4 consecutive days. The 30-day time period to administer filgrastim was to allow for WBC washout as the average lifespan of a WBC is 13-20 days. Dosages were rounded to 300  $\mu$ g, 600  $\mu$ g, 780  $\mu$ g, and 840  $\mu$ g, depending on each participants weight. The injections were administered subcutaneously into the thigh. Each day of injection, the participants also underwent a brief physical examination and 5-mL blood draw to obtain a CBC with WBC differential to monitor potential side effects of the drug. On the fifth day, a 5.0-mL vacutainer tube and a 60-mL syringe, prefilled with 8.6 mL of ACDA, were both filled with peripheral blood. The 60-cc syringe was filled to the 65-cc point as described for the previous harvests. This blood was processed as detailed previously for the initial blood draw and bone marrow aspirate samples to create an M-PRP.

### Analysis

Concentrate products were evaluated on an automated hematology analyzer (XE-5000; Sysmex, Kobe, Japan) to obtain a CBC with differential, including HPC concentrations. BD Accuri C6 flow cytometer (BD Biosciences, Ann Arbor, MI) was used to evaluate HPC

profile, CD45+CD34+CD133+CD38-, and MPC, CD45-/lowCD271+, profile in all samples. The samples were washed and diluted based on the sample concentration and then incubated for 30 minutes with a binding inhibitor. Following initial incubation, the samples were incubated for 30 minutes with antibodies for each lineage panel. Standard isotype control samples were used. Each sample was washed, centrifuged, and then fixed with 10% formaldehyde before the flow cytometry analysis.

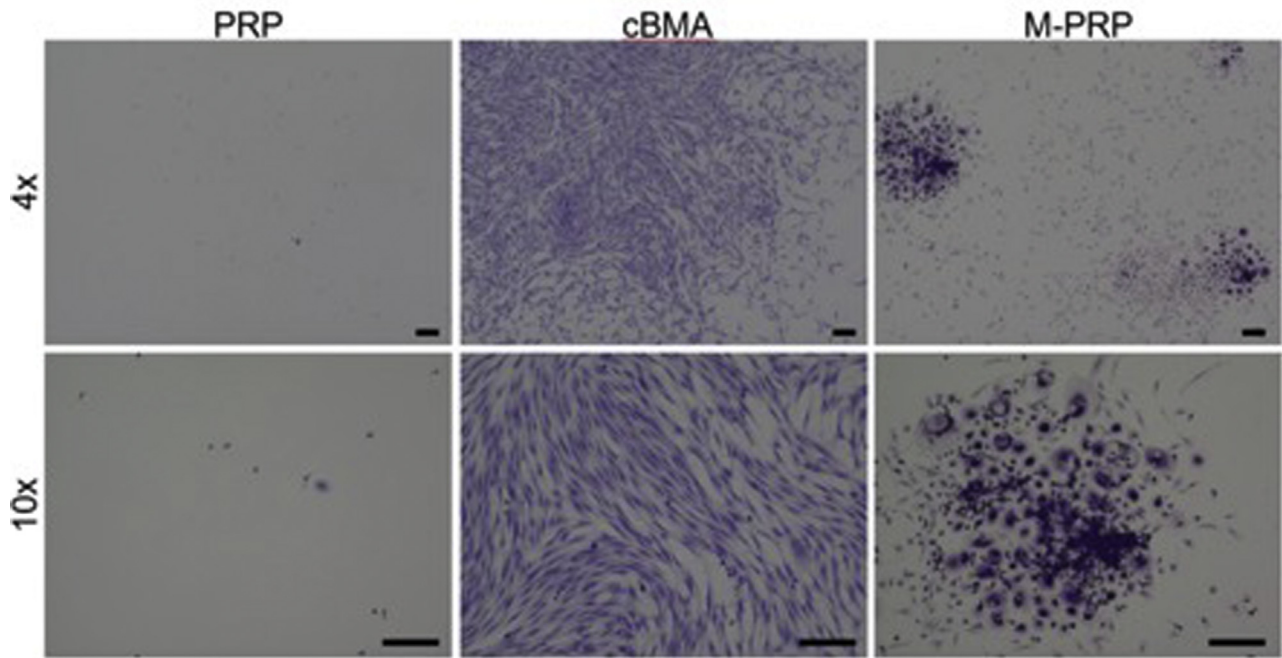
A volume of each sample to plate  $10^6$  total nucleated cells were then cultured in triplicate in complete DMEM/F-12 (Gibco, Gaithersburg, MD) for 10 days at 37°C in humidified conditions. Any remaining samples were also cultured in excess. Plates were washed and stained in 0.5% Crystal Violet solution and air dried before counting. Colonies were macroscopically counted and then confirmed microscopically for the appropriate fibroblastic phenotype and presence of at least 50 cells. Upon observing that colonies in mobilized samples were not fibroblastic, additional samples were stained with a combination of anti CD45-FITC, anti CD14-PE/DAZZLE, and DAPI (BioLegend, San Diego, CA) to further characterize the adherent cell population.

### Statistical Analysis

Statistical analyses were performed using Statistical Package for Social Science (SPSS) software (version 22; IBM Corp., Armonk, NY). Means and standard deviations were calculated for all variables. Paired sample  $t$  tests were performed to examine if the numbers of cellular composition were different between the PRP, cBMA, and M-PRP. An alpha level set a priori at  $P \leq .05$ .

### Results

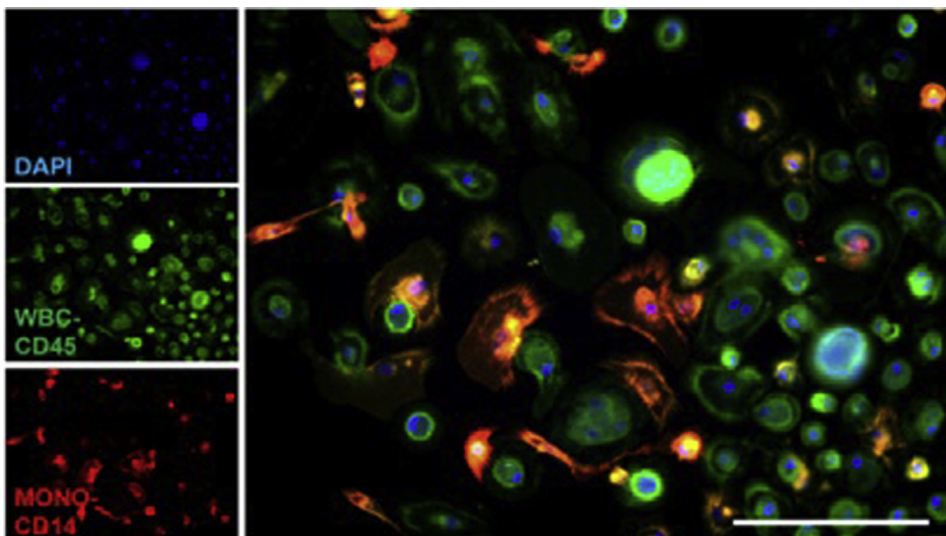
Twelve participants were enrolled in the study; however, 2 participants withdrew following the screening visit due to scheduling conflicts. The average



**Fig 1.** Representative images of adherent cell morphology and size among PRP, cBMA, and M-PRP after crystal violet staining. cBMA had dominant fibroblastic colonies, whereas M-PRP had colonies consisted of multiple cell phenotypes. Scale bars are 200  $\mu\text{m}$ . (cBMA, concentrated bone marrow aspirate; M-PRP, mobilized platelet-rich plasma; PRP, platelet-rich plasma.)

age of the volunteers was  $23.1 \pm 2.8$  years and volunteers had a body mass index of  $25.6 \pm 4.1$   $\text{kg}/\text{m}^2$ . Descriptive data of the cellular components are presented in Table 1. M-PRP contained a similar number of platelets compared with PRP (mean difference = 110  $\text{k}/\mu\text{L}$ ;  $P = .051$ ) and white blood cell concentrations more similar to that of cBMA (mean difference = 11.8  $\text{k}/\mu\text{L}$ ;  $P = .266$ ). The M-PRP had a greater number of monocytes when compared with PRP (mean difference = 8.3  $\text{k}/\mu\text{L}$ ;  $P = .002$ ) and cBMA (mean

difference = 5.5  $\text{k}/\mu\text{L}$ ;  $P = .017$ ). Flow cytometry with a panel evaluating for HPC revealed similar numbers when comparing M-PRP with cBMA (mean difference = 3.9  $/\mu\text{L}$ ;  $P = .377$ ). A panel evaluating for MSP revealed a greater number of MPCs in cBMA than M-PRP (mean difference = 22.6  $/\mu\text{L}$ ;  $P < .001$ ) a PRP (mean difference = 20.9  $/\mu\text{L}$ ;  $P = .01$ ). cBMA produced significantly more CFU-F than M-PRP (mean difference = 446  $/\text{mL}$ ;  $P < .001$ ) or PRP (mean difference = 446  $/\text{mL}$ ;  $P < .001$ ).



**Fig 2.** Immunohistochemical analysis of non-fibroblastic colonies of M-PRP indicated a mixture of hematopoietic surface markers, including CD45 (common leukocyte antigen) and CD14 (monocyte/macrophage marker). Counterstaining with DAPI also demonstrated multinucleated cells. Scale bar is 200  $\mu\text{m}$ . (DAPI, 4',6-diamidino-2-phenylindole; M-PRP, mobilized platelet-rich plasma; WBC, white blood cells.)

After staining with crystal violet, PRP samples had sparse plastic adherent single cells of hematopoietic origin or aggregates with 2-5 cells, with no clusters visible to the naked eye. In cBMA samples, any adherent leukocytes were overshadowed by the dominant presence of CFU-F with fibroblastic morphology (Fig 1). Several M-PRP samples demonstrated small clusters visual to the naked eye. However, upon microscopic examination these aggregates did not display CFU-F morphology, but rather a mixture of leukocyte lineages confirmed with immunohistochemistry (Fig 2).

## Discussion

The most significant finding of this study was that M-PRP, created by systemic mobilization with filgrastim, peripheral blood harvest, and processing with one PRP system, contained a greater concentration of platelets and monocytes when compared with cBMA. M-PRP had significantly greater WBC, monocytes, and HPCs compared with PRP. Although M-PRP did not generate significant CFU-F colonies, it did produce colonies consistent with hematopoietic lineages. These findings are consistent with supporting our hypothesis that M-PRP would create a cellular point-of-care blood product similar in composition to cBMA.

When tissue is damaged, cells are mobilized from the bone marrow through the peripheral circulation to damaged sites for participation in the healing response, and monocytes play a key role in this process.<sup>19,32,34-36</sup> Monocytes isolated from both filgrastim mobilized blood and unmobilized blood have performed as progenitor cells in vitro and similar to cells cultured from bone marrow, i.e., mesenchymal stem cells (MSCs).<sup>17-19,32-34</sup> In vitro studies have confirmed stem capabilities, i.e., proliferative and differentiation potential, and that hypoxia drives the differentiation of peripheral blood monocytes into MSCs.<sup>17-19,37</sup> Two studies have found monocyte-derived progenitor cells to be pluripotent, suggesting that monocytes lineages may have just as much if not more differentiation potential than MSCs.<sup>32,34</sup> Head-to-head cartilage repair models in both small and large animals have found equal potential to improve cartilage repair.<sup>17,19</sup> In a recent randomized controlled clinical trial, a monocyte-rich PRP performed as well clinically as cBMA for the treatment of osteoarthritis.<sup>38</sup> Further research is required to demonstrate the orthopaedic significance of monocytes, monocyte lineage cells, and filgrastim.

Studies for comparison include an evaluation of activated filgrastim mobilized platelet supernatant on the effects of mobilized monocytes and a comparison study of PRP to cBMA in patients undergoing orthopedic procedures. Kang et al.<sup>39</sup> harvested blood from volunteers after 3 days of filgrastim administration. After centrifugation, the platelet-rich plasma was

separated from the red and white cell fraction. The PRP was activated, and the supernatant harvested. Monocytes were fractionated from the cell component. The supernatant contained greater levels of interleukin (IL)-8, IL-7, platelet-derived growth factor (PDGF), and vascular endothelial growth factor when compared with standard platelet supernatant, and the supernatant had a priming effect upon the monocytes toward enhanced angiogenesis. Cassano et al.<sup>40</sup> evaluated the output of 2 concentration systems with BMA and compared BMA to PRP with one of these systems. Considering the cellular cBMA products, the 2 systems yielded different contents. Similarly, when comparing PRP with cBMA, they found a more cellular product in cBMA and greater concentrations of IL-1ra with cBMA. Similarly, we found a more cellular product when comparing cBMA with PRP in this study.

It is becoming clear that differing point-of-care systems yield different byproducts after processing of similar sources with large heterogeneity in concentrations of platelets, leukocytes, and growth factors.<sup>40</sup> In addition, the composition of PRP differs between patients and basic demographics.<sup>41,42</sup> Confusing the situation further, a recent systematic review found that reporting of preparation protocols is highly variable and inconsistent, making it impossible to reliably reproduce testing protocols to allow for direct comparisons of PRP studies.<sup>43</sup> There is a clear need to recognize this variation when considering the use of these point-of-care blood and marrow products and when designing/reporting clinical trials to determine their efficacy.<sup>42</sup> With this in mind, the findings of this study are particular to the point-of-care system used and no extrapolation to other systems should be made.<sup>41,43,44</sup>

Filgrastim has gone through multiple safety and efficacy trials with multiple uses in the field of hematology.<sup>24-28</sup> The most common adverse reactions are bone pain, fever, and headache. In one study involving 126 patients with cancer, 44% reported mild-to-moderate muscle and bone pain and 7% reported headaches.<sup>22</sup> The Spanish National Donor Registry reports that of 736 donors, 90% reported bone pain and 33% had headaches.<sup>26</sup> There are also rare but serious risks associated with the use of filgrastim that include splenic rupture, acute respiratory distress syndrome, serious allergic reactions, sickle cell disorders, glomerulonephritis, capillary leak syndrome, leukocytosis, aortitis, alveolar hemorrhage, and potential for tumor growth stimulatory effects on malignant cells. Despite these risks, the consensus from the hematology oncology profession is that filgrastim is safe in adult and pediatric patients with no long-term risks identified since its usage began in the early 1990s.<sup>21,29-31</sup> Considering the ease of obtaining peripheral blood, it may be a simpler in office procedure when compared with bone marrow aspiration.

One concern regarding the use of filgrastim is the associated cost. The authors have used filgrastim in multiple completed and ongoing clinical research projects and consulted with practicing hematologist oncologists.<sup>7,16</sup> A direct cost available through medical/pharmaceutical supply sources to clinicians in the United States is \$1.1 dollars per microgram. Dosages for one orthopaedic application study involving filgrastim and apheresis involves 3 injections of 300 µg for 3 days, which translates into \$990 for that application.<sup>7,16</sup> This study used 10 µg per kg as a dosage and 4 doses. For the average 70-kg individual, this translates into \$3080 dollars for this potential application. Cost is a hurdle when one considers the translation of biologics as a whole. Human trials focusing on orthopaedic indications evaluating efficacy should proceed before clinical application.

The demographic of this study was thin, healthy male patients. To limit confounding variables, female patients were excluded, age was limited, and weight was controlled. The composition of blood and bone marrow varies dependent on age and sex. The cellularity of bone marrow has been found to decline with age, with approximately 90% of marrow cavities filled with cells at birth, 50% at the age of 30 years, and 30% at the age of 70 years.<sup>45,46</sup> In PRP samples from healthy participants, female participants had greater epidermal growth factor, hepatocyte growth factor, insulin-like growth factor-1, and PDGF-BB than males.<sup>47</sup> In addition, participants 25 years of age or younger had greater epidermal growth factor, insulin-like growth factor-1, PDGF-AB, PDGF-BB, and transforming growth factor-β1 than individuals older than 25 years.<sup>47</sup> Weight was restricted in this study due to the logistics of pre-filled syringes and cost of filgrastim. Further study could determine the variability among different demographics and if smaller dosages of filgrastim would be effective.

### Limitations

There are limitations to this study. Since this is a benchtop study, cell counts and culturing assays may not truly portray the potential of these tissues in vivo. In addition, since samples were from young, thin, male, healthy donors alone, the results do not represent a complete picture. A third limitation is a small sample size, which may subject results to sampling bias. Normality was assumed in data analysis. We believe that with a greater number of subjects that trends seen in the data would further become clear. The study design and cost of the study make it difficult to recruit more subjects. In addition, the findings of this study can only be applied to this unique point-of-care system and thus cannot be applied to all PRP systems.

### Conclusions

M-PRP, produced with filgrastim-mobilized blood and a proprietary PRP system, contained more total white blood cells, monocytes, platelets, and HPCs than cBMA and more white blood cells, monocytes, and HPCs than PRP.

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