

# Combination of Filtered Bone Marrow Aspirate and Biomimetic Scaffold for the Treatment of Knee Osteochondral Lesions: Cellular and Early Clinical Results of a Single Centre Case Series

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## Abstract

**BACKGROUND:** Osteochondral injury is a very common orthopaedic pathology, mainly affecting young, active population, with limited current treatment options. Herein we are presenting cellular and early clinical data of a patient series treated for chronic osteochondral lesions in the knee with a filter-based intra-operative bone marrow aspirate (BMA) separation device.

**METHODS:** Fifteen patients with chronic knee osteochondral lesions (60% females, 19–59 years) were included in this prospective case series. Filtered BMA (f-BMA), containing mesenchymal stem/stromal cells (MSCs), was combined with a biomimetic collagen-hydroxyapatite scaffold (CHAS) and implanted into the site of the lesion. Harvested BMA and post-separation f-BMA were analysed for blood cell counts, flow cytometry, and fibroblast colony forming units (CFU-Fs). Patients were followed for serious adverse events and graft failures. Clinical evaluation was assessed using the knee injury and osteoarthritis outcome score (KOOS). In 8 patients a magnetic resonance imaging (MRI)/arthroscopy were performed.

**RESULTS:** Cell suspension contained 0.027% CD271<sup>+</sup> CD45<sup>-</sup> 7-AAD<sup>-</sup> cells, 0.15% CD73<sup>+</sup> CD90<sup>+</sup> CD105<sup>+</sup> cells and 0.0012% CFU-Fs of all nucleated cells with 86% viability. Filtration process resulted in 12.8 (4.0–40.8) fold enrichment in terms of CFU-F content in comparison to initial BMA. No serious adverse events related directly to the osteochondral treatment were reported. After an average follow-up of 20 months (14–25) all KOOS subscales (Symptoms/Pain/Daily activities/Sport and recreation/Quality of life) increased significantly from pre-operative 55/56/67/30/30 to post-operative 73/76/79/51/52 (*p* values < 0.05), respectively. MRI or arthroscopic evaluation revealed nearly normal to normal overall International Cartilage Repair Society assessment in 7/8 patients.

**CONCLUSION:** The filter-based BMA separation procedure significantly increased the frequency of mesenchymal stem/stromal cells (MSCs), however their concentration was not increased. The clinical evaluation revealed high safety profile of the treatment and resulted in improved clinical status of the patients.

**Keywords** Knee joint · Cartilage · Articular · Bone marrow mesenchymal stem cells · Cell separation · Biomimetic materials

## 1 Introduction

Cartilage lesions can be painful and disabling, have poor capacity for repair, and may predispose patients for osteoarthritis [1]. For long-term repair and regeneration of tissue, cells alone or in combination with biomaterials are implanted at the site of injury. Several strategies, including subchondral bone drilling and microfracture techniques are

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employed to supply blood and bone marrow (BM) to the lesion site [2]. The procedure recruits mesenchymal stem/stromal cells (MSCs, sometimes also referred to as connective tissue progenitors—CTPs [3]) from BM, stimulates fibrin clot formation and subsequent fibrocartilage formation [4]. Another technique, autologous chondrocyte implantation (ACI), is regarded as the first application of a tissue-engineering strategy in orthopaedic surgery [5, 6].

Strategy in chondral/osteochondral repair is moving toward intraoperative single-step procedures to reduce the risks associated with two surgical procedures, to reduce costs and the necessity for a good manufacturing practice (GMP) facility for cell cultivation [7]. In this regard, the use of bone marrow aspirate (BMA), which contains both hematopoietic and mesenchymal stem/stromal cells in addition to other cell types that may play a role in tissue regeneration, represents a viable alternative [8]. The mechanisms of action of MSCs in tissue regeneration are related to the secretion of number of cytokines, chemokines and growth factors, which can improve angiogenesis, reduce inflammation, inhibit apoptosis and stimulate endogenous repair [9]. In addition, MSC differentiation into desired phenotype can be achieved as a result of environmental stimuli [7]. However, in bone marrow aspirates, MSC represent only 0.001%–0.01% of the total mononuclear cells [10]. For increasing the density of MSCs, various concentration methods, like bone marrow density gradient centrifugation or recovery of cells from filters and clotting, were developed. These methods are relatively simple, can be performed in the operation theatre and are therefore not associated with major regulatory obstacles [2].

Cartilage and bone need to be restored for a successful outcome after osteochondral lesion treatment. Traditionally, the autologous bone grafting was combined with a superficial cartilage repair. A nano-structured biomimetic collagen-hydroxyapatite scaffold—CHAS (MaioRegen, Fin-Ceramica, Faenza, Italy) was developed to provide simultaneous osteochondral regeneration. Positive clinical results have been shown by many short to mid-term reports when implanted in a stand-alone manner [11]. A recent randomized clinical trial showed superiority of this device toward bone-marrow stimulation for osteochondral lesions [12]. However, some treatments resulted also in incomplete cartilage and poor subchondral bone repair, despite significant clinical improvements [13]. In the treatment of osteochondral lesions in complex situations, such as older patients, early osteoarthritis, and revision cases, success range of stand-alone scaffolds is rather limited [14].

Herein we present first cellular and clinical data results of a cell filter device for collection of nucleated cells from BMA [15, 16]. Filtered BMA (f-BMA), containing MSCs, was used on patients treated for chronic osteochondral

lesion in the knee joints. BM aspiration, cell filtration, their seeding onto CHAS scaffold and surgical implantation were combined intra-operatively in a single step procedure. We hypothesized: (1) the filter-based BMA separation procedure significantly increases concentration and frequency of MSCs; (2) combining f-BMA and CHAS represents a safe therapeutic approach, and (3) improves clinical status of the patient.

## 2 Materials and methods

### 2.1 Patient population and surgical procedures

This prospective case series comprises of 15 patients that were operatively treated for chronic knee osteochondral lesions in a single orthopaedic centre between 2014 and 2016. There were 9 females and 6 male patients enrolled in the study with average age of 33 years (from 19 to 59). The study protocol was approved by the National Medical Ethics Committee (No. 0120-14/2016-2). The patients were suffering from the following: three primary large osteochondritis dissecans (OCD) lesions on medial femoral condyle, two recurrent OCD on medial femoral condyle after failed fragment fixation, three isolated post-traumatic osteochondral lesions, two recurrent post-traumatic osteochondral lesions after failed microfractures, two patellofemoral instability related lesions and three cases of multiple femoral lesions due to osteoarthritis. Surgeries were performed under general or spinal anaesthesia. All knee patients had osteochondral graft implanted through a mini-arthrotomy or classical central arthrotomy, depending on the lesion size, location, and concomitant procedures. There were ten isolated cartilage surgeries, two patients required additional patellofemoral stabilization, one patient received a high tibial osteotomy, and one needed an open synovectomy. The site of the osteochondral lesion was first debrided in quadrangular manner to macroscopically intact articular cartilage on sides and toward a non-sclerotic bone in the depth. One patient with subchondral bone cyst in the medial femoral condyle received an additional cyst curettage and autologous bone grafting. A biomimetic osteochondral scaffold was first cut out to press fit into the lesion. On all four sides, as well as on the articular collagen layer, it was stabilized with a fibrin sealant (Beriplast, CSL Behring, Marburg, Germany). A tri-layer CHAS MaioRegen (Fin-Ceramica, Faenza, Italy) was used in 11 cases. A thinner, bi-layer version of same scaffold, MaioRegen Slim (Fin-Ceramica, Faenza, Italy) was used in 4 cases. MaioRegen is a reabsorbable, implantable, multilayer device indicated for the treatment of osteochondral lesions. This substitute is composed of type I collagen fibers (of equine origin) and Mg enriched hydroxyapatite (HA)

**Table 1** Demographic data and surgery details of patients treated with f-BMA

Patient code	Sex	Age (years)	BMI (kg m <sup>-2</sup> )	Knee location	Main diagnosis	Lesion size (cm <sup>2</sup> )	ICRS grade	Scaffold	Concomitant procedures
PM00814M	F	34	24.4	Patella	Traumatic OC lesion	2.5	4A	MaioRegen	Patella stabilization
TG00914M	M	32	25.2	MFC	OCD	2.5	4B	MaioRegen	None
SR00415M	F	35	23.9	Multiple lesions	Localized OA	9.5	4A	MaioRegen	Knee synovectomy
MG00615M	F	22	27.7	MFC	OCD	4.0	4B	MaioRegen	None
MP00915M	F	36	28.3	MFC	Traumatic OC lesion PF instability	3.0	4A	MaioRegen	Patella stabilization
TP01015M	F	19	25.2	MFC	Recurrent OCD	3.5	4B	MaioRegen	None
MG01315M	F	34	21.5	Trochlea	Recurrent post-traumatic OC lesion	2.5	4A	MaioRegen Slim	None
SK01615M	M	59	25.1	MFC	Recurrent OC lesion, Medial osteoarthritis	7.0	4B	MaioRegen	High tibial osteotomy
DM00116M	M	25	25.0	Trochlea	Traumatic OC lesion after ACL reconstruction	2.0	4A	MaioRegen Slim	None
DD00316M	F	28	25.0	LFC	Traumatic OC lesion	2.4	4A	MaioRegen	None
JS00416M	M	30	20.9	LFC	Recurrent post-traumatic OC lesion	4.5	4B	MaioRegen	None
AK00616M	M	45	25.6	Multiple lesions	Degenerative lesions	3.5	4A	MaioRegen Slim	None
PŠ00716M	F	33	35.6	MFC	OCD	6.5	4B	MaioRegen	None
LL00916MK	F	53	27.3	Trochlea	Degenerative lesion	3.5	4A	MaioRegen Slim	None
16BK017BK	M	34	23.5	MFC	Recurrent OCD lesion	3.5	4B	MaioRegen	Bone grafting of subchondral cyst

*ACL* anterior cruciate ligament, *BMI* body mass index, *ICRS* International Cartilage Repair Society, *LFC* lateral femoral condyle, *MFC* medial femoral condyle, *OA* osteoarthritis, *OC* osteochondral, *OCD* osteochondritis dissecans

crystals, nucleated in the fibers. A biologically inspired approach was applied to nucleate bone-like HA nanocrystals in the collagen fibers. After the nucleation/assembling process the suspensions were freeze-dried; controlling the freezing and heating rate, and a preferential collagen orientation in the first layer was achieved [11]. All details on patient population and surgeries are given in Table 1. Post-operatively, the patients were restrained from weight-bearing for 6 weeks followed by progressive rehabilitation for muscle strength, range of motion, and anti-swelling measures. Three months after index surgery they were allowed to get involved with light activities and moderate activities were allowed after 6 months. Participation in strenuous sport activities (such as ball games, martial arts, athletics training) was allowed after one year post-surgery.

## 2.2 Bone marrow harvesting, cell isolation and application

BM aspiration needle (13-gauge, HS Hospital Service, Aprilia, Italy) was coated with dalteparin (Pfizer, Luxembourg, Luxembourg) prior to BM aspiration. Between 18 and 35 ml of bone marrow was aspirated from iliac crest

into 50-ml or 10-ml syringes, prefilled with acid citrate dextrose solution A (ACD-A, Fresenius Kabi, Bad Homburg, Germany). If 10-ml syringes were used, the needle for BM aspiration was repositioned up to three times. BMA was processed using filter-based cell separation device CelleEffic BM (Kaneka Corp., Osaka, Japan). The procedure was performed according to manufacturer's instructions. Around 50 ml of cell suspension in saline was obtained after the isolation of nuclear cells. Small aliquots (2–4 ml) were sampled for cellular evaluation. The remaining cell suspension was centrifuged for 5 min at 400×g and concentrated to a final volume between 3 and 7 ml. Before the f-BMA administration, the intra-operative tourniquet was released. Osteochondral implant was soaked by fresh blood since this was shown to be crucial for its early stability [18]. Typically, 2/3 of prepared final cell suspension volume was injected diffusely into the upper portion of the scaffold at the very end of surgery. The last 1/3 of the cell suspension volume was injected freely into the joint cavity after the complete wound closure.

## 2.3 Cellular evaluation

To test our hypothesis (1) extensive cellular evaluation was performed.

Samples for cell counting (100  $\mu$ l), CFU-F assay (1–2 ml) and flow cytometry (1–2 ml) were taken before and after BMA processing with filter-based separation device. The samples were stored at 4 °C and analysed for cell count, CFU-F assay and surface marker expression on the same day, except for the first two samples which were analysed on the following day. The viability analysis for all the samples was performed on the same day.

### 2.3.1 Blood cell counts

Concentration of white blood cells (WBCs) and red blood cells (RBCs) were determined with haematology analyser COULTER® Ac-T diff2™ (Beckman Coulter, Fullerton, CA, USA).

### 2.3.2 Fibroblast colony forming units (CFU-F) assay

Cells were seeded at least in duplicates into 6 well culture plates (1.25  $\times$  10<sup>5</sup>, 2.5  $\times$  10<sup>5</sup> or 5  $\times$  10<sup>5</sup> WBCs/well, well area 9.026 cm<sup>2</sup>, TPP, Trasadingen, Switzerland) containing DMEM/Ham's F12 (Life Technologies Corp., Paisley, UK) supplemented with 10% FBS (Life Technologies Corp., Paisley, UK), 1 ng/ml bFGF (Preprotech, London, UK) and antibiotics: penicillin, streptomycin and gentamycin (Life Technologies Corp., Paisley, UK). Plates were incubated at standard conditions (37 °C, 5% CO<sub>2</sub>) with media changes every 3 to 4 days. Samples were fixed with 4% formaldehyde and stained with 0.05% Crystal Violet (Sigma Chemical Co., St. Louis, MO, USA) after 14 days of cell culture. Colonies containing  $\geq$  50 fibroblastic cells were manually counted under stereomicroscope. Frequency and concentration of CFU-Fs was calculated based on average CFU-F plate counts, number of seeded cells and concentration of WBCs in the sample.

### 2.3.3 Flow cytometry

Estimation of MSC content was achieved by determining CD271 positive and CD45 negative cells, concomitantly assessed for viability using 7-aminoactinomycin D (7-AAD), with the similar procedure as described before [19]. Samples were added into the Trucount tubes, labelled with anti-CD45 (FITC, cat. no. 555482), anti-CD271 (PE, cat. no. 557196) antibodies and 7-AAD (all from BD Biosciences, San Jose, CA, USA), incubated 20 min at room temperature in the dark. After red blood cell lysis, the samples were immediately analysed with flow cytometer FACS Calibur (BD Biosciences, San Jose, CA, USA). By

comparing the ratio of counted bead events to CD271 positive and CD45 negative viable cell events, absolute number of MSCs was calculated. The viability of all nucleated cells was assessed by measuring all 7-AAD negative cells.

Additionally, the quantification of MSCs was performed by measuring the cell population coexpressing general mesenchymal markers CD73, CD90 and CD105 [20]. Samples were labeled with anti-CD73 (PE, cat. no. 550257, BD Biosciences or APC, cat. no. 130-097-945, Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD90 (FITC, cat. no. 555595, BD Biosciences), and anti-CD105 (PerCP-Cy5.5, cat. no. 560819, BD Biosciences) antibodies and incubated 20 min at room temperature in the dark. After incubation red blood cells were lysed with a BD FACS lysing buffer for 10 min, centrifuged at 400 $\times$ g for 5 min, cell pellet was resuspended with DPBS and samples were analysed with FACSCalibur or FACSaria (BD Biosciences, San Jose, CA, USA). The cells co-expressing all three markers were gated to determine their percentage. Their absolute numbers were calculated indirectly based on the WBC counts measured with haematology analyser.

### 2.3.4 Calculation of cell separation parameters

To evaluate the proposed hypothesis (1) enrichment and concentration factors were calculated as described below. Additionally, we have also calculated cell recovery rate.

For the measurements of different cell populations, measured with haematology analyser, flow cytometer and CFU-F assay, absolute cell numbers were calculated considering the sample volumes before and after BMA processing with filter-based separation device. For calculation of cell concentrations in the final cell product, potential cell losses during final centrifugation step were neglected. The enrichment factor was calculated in order to compare the frequency of target cells amongst all cells (including RBCs) before and after BMA processing:

$$\text{Enrichment factor} = \frac{\% \text{target cells}_{\text{after separation}}}{\% \text{target cells}_{\text{before separation}}} \quad (1)$$

The concentration factor was calculated to compare the concentration of cells in the final cell product over the baseline in initial BMA:

$$\text{Concentration factor} = \frac{\text{target cell concentration}_{\text{after separation}}}{\text{target cell concentration}_{\text{before separation}}} \quad (2)$$

To compare the absolute cell numbers before and after cell separation process the recovery rate was determined as follows:

$$\text{Recovery rate} = \frac{\text{target cell number}_{\text{after separation}}}{\text{target cell number}_{\text{before separation}}} \times 100\% \quad (3)$$

## 2.4 Clinical evaluation

To evaluate the proposed hypothesis (2) and (3) clinical evaluation was robustly focused only for safety and efficacy.

Patients were prospectively followed at one, three, and then every six months after surgery. Unscheduled visits in cases of any unexpected adverse events were allowed. Since this study group was primarily focused on a filter-based bone marrow separation procedure, we aimed to include all patients in whom this f-BMA was used.

All patients were carefully monitored for any serious adverse events (defined as hospitalisation or repetitive surgical intervention) or documented graft failures (revision cartilage repair or arthroplasty, or low clinical outcome together with radiological or arthroscopic evidence for graft failure). Serious adverse events were classified into one out of four categories in relation to the implanted graft: unrelated, possibly related, probably related, and definitely related.

Knee osteoarthritis outcome score (KOOS) was used to assess efficacy of the treatment [21]. Only the data at the time of surgery and at the last evaluation (patients with minimum of one-year follow-up were included). Biological healing response was evaluated non-systematically by magnetic resonance imaging (MRI) or arthroscopy in situations of surgical re-intervention: four patients had MRI only, two patients had arthroscopy only and another two had both. Simple graft evaluation according to ICRS Cartilage Repair Assessment [22] was performed in all these cases.

## 2.5 Statistical analysis

All data averages, except KOOS scores, are presented as geometric mean with 95% confidence interval (CI) or range, as indicated.

In order to test hypothesis (1), the enrichment and concentration factors of evaluated cell populations were compared to value 1, which indicates equal cell frequencies and cell concentrations before and after BMA processing. To test statistical significance of calculated factors, the data was first transformed using function  $Y = \log(Y)$ . Means of transformed data were then compared to hypothetical value of 0 ( $\log(1) = 0$ ) using one sample  $t$  test. Differences with  $p \leq 0.05$  were considered as statistically significant.

KOOS scores are presented as arithmetic mean with 95% CI. In order to test hypothesis (3) paired two-sided

$t$ -test was used to determine statistical significance between individual pre-OP and post-OP scores. Differences with  $p \leq 0.05$  were considered as statistically significant.

Computer software GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used for all statistical calculations.

## 3 Results

### 3.1 Cellular evaluation

Results were obtained by detailed evaluation of blood cell counts, CFU-F assay and flow cytometry analysis. The measured parameters for each individual patient are presented in Table 2. To evaluate hypothesis (1), enrichment and concentration factors were calculated and are presented in Fig. 1.

#### 3.1.1 Cell suspension volumes before/after separation

The initial BMA (N = 15) with added anticoagulant had an average volume of 29.0 ml (range 19.0–37.0), whereas the final cell suspension had a volume of 5.1 ml (range 3.0–7.0). The volumetric concentration factor was therefore 5.7 (range 3.2–11.0).

#### 3.1.2 Blood cell counts

The BMAs (N = 14) on average contained  $20.9 \times 10^6$  WBC/ml (range  $7.3 \times 10^6$ – $38.7 \times 10^6$ ) and  $3824 \times 10^6$  RBC/ml (range  $3095 \times 10^6$ – $4947 \times 10^6$ ). With the concentration of RBCs being two orders of magnitude higher than the concentration of WBCs, it is evident that RBCs represent the vast majority of cells (99.4%, range 99.0–99.8) in the initial BMAs. Processing of BMA through the separation column removed 98.6% RBCs (range 97.6–99.2), which in the final cell preparation represented 91.7% of all cells (range 88.8–96.0). This resulted in the increase of WBC percentage from initial 0.5% (range 0.2–1.0) to the final 8.0% (range 4.0–11.2). The average absolute number of WBCs before separation was  $600.5 \times 10^6$  (range  $203.0 \times 10^6$ – $1277.1 \times 10^6$ ) and afterwards it decreased to  $132.0 \times 10^6$  WBC (range  $60.0 \times 10^6$ – $208.8 \times 10^6$ ). Therefore, the average WBC recovery rate was 22.0% (range 11.4–35.3). The average enrichment and concentration factors for WBCs were 14.7-fold (range 10.4–23.7,  $p < 0.05$ ) and 1.2-fold (range 0.8–1.9,  $p < 0.05$ ), respectively.

**Table 2** Characterization of cell populations in bone marrow aspirates and in final cell preparations after separation with filter-based intra-operative bone marrow aspirate separation device

Patient code	Before separation					After separation				
	Volume (ml)	WBC ( $10^6 \text{ ml}^{-1}$ )	RBC ( $10^6 \text{ ml}^{-1}$ )	CFU-F ( $\text{ml}^{-1}$ )	CD271 <sup>+</sup> CD45 <sup>-</sup> 7-AAD <sup>-</sup> ( $10^6 \text{ ml}^{-1}$ )	Volume (ml)	WBC ( $10^6 \text{ ml}^{-1}$ )	RBC ( $10^6 \text{ ml}^{-1}$ )	CFU-F ( $\text{ml}^{-1}$ )	CD271 <sup>+</sup> CD45 <sup>-</sup> 7-AAD <sup>-</sup> ( $10^6 \text{ ml}^{-1}$ )
PM00814M	35	19.2	3095	77	0.0040	5	18.5	225	148	0.0023
TG00914M	37	24.5	3930	270	0.0038	5	20.7	225	311	0.0042
SR00415M	37	20.0	3700	40	0.0180	7	26.4	214	106	0.0155
MG00615M	31	34.3	3500	685	0.0130	7	29.2	233	322	0.0103
MP00915M	31	24.6	3705	196	0.0087	5	27.4	240	356	0.0168
TP01015M	32.5	–	–	–	–	5	–	–	–	–
MG01315M	27.5	20.5	3470	150	0.0055	5	27.0	250	54	0.0025
SK01615M	29	13.2	3763	88	0.0010	6	21.2	240	127	0.0017
DM00116M	26	22.8	4947	1216	0.0024	5	28.8	480	1267	0.0041
DD00316M	27	10.1	3807	47	0.0058	5	19.2	288	128	0.0048
JS00416M	29	26.2	3807	454	0.0104	4	37.2	480	248	0.0055
AK00616M	19	23.3	4330	699	0.0069	6	20.2	323	391	0.0076
PŠ00716 M	20	36.6	3836	2584	0.0301	5	36.6	289	2344	0.0303
LL00916MK	28	7.3	3875	203	0.0057	5	12.0	288	288	0.0097
16BK017BK	33	38.7	4065	1754	0.0153	3	69.6	640	1670	0.0096
Geometric mean	29.0	20.9	3824	286	0.0068	5.1	25.8	297	307	0.0065
Lower 95% CI	26.0	15.9	3593	133	0.0041	4.6	20.4	244	164	0.0040
Upper 95% CI	32.3	27.5	4069	616	0.0113	5.7	32.8	361	576	0.0105

CFU-F fibroblast colony forming units, CI confidence interval, RBC red blood cells, WBC white blood cells

### 3.1.3 CFU-F assay

The functional assay for measuring the frequency of MSCs with the colony forming abilities was performed (N = 14). The average dosage of CFU-Fs administered to the patients was 1571 CFU-F (range 270–11,722), which presented 0.0012% (range 0.0002–0.0064) of all nucleated cells. The average CFU-F recovery rate was 19.1% (range 6.5–50.4).

The average CFU-F enrichment and concentration factor were 12.8-fold (range 4.0–40.8,  $p < 0.05$ ) and 1.1-fold (range 0.4–2.7,  $p = 0.677$ ), respectively.

### 3.1.4 Flow cytometric analysis

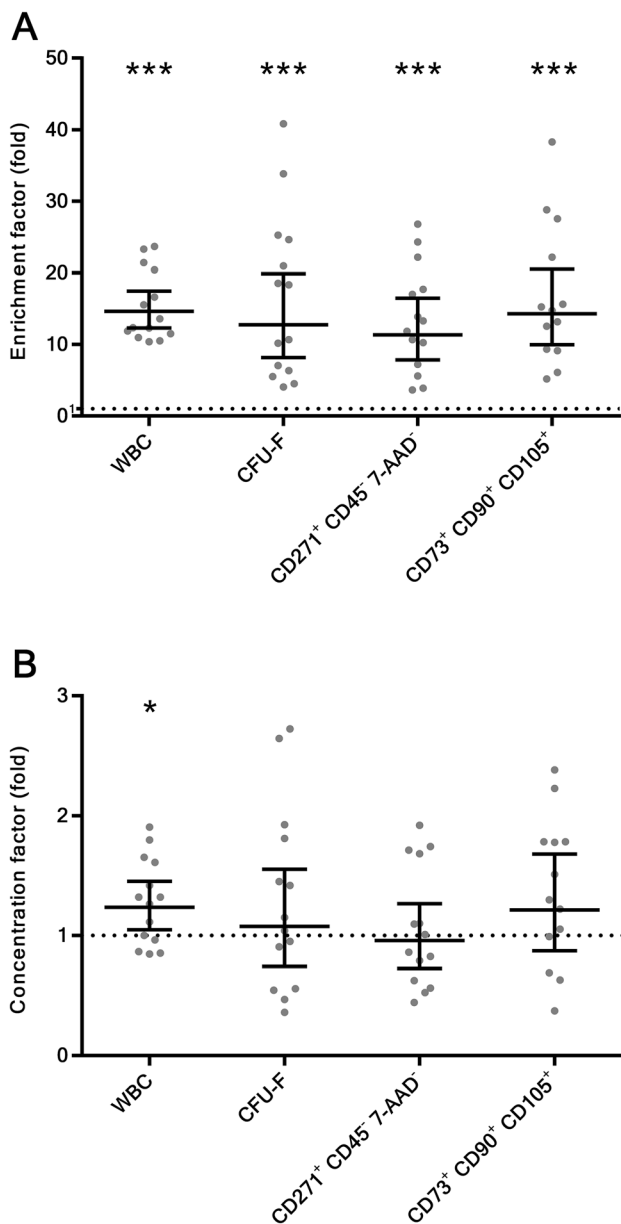
The average viability of all nucleated cells in BMAs was 91% (range 80–98) and has slightly decreased to 86% (range 78–96) after processing through separation column (N = 14).

The estimation of MSC content was performed with flow cytometric analysis for CD271<sup>+</sup> CD45<sup>-</sup> 7-AAD<sup>-</sup> cells (N = 14). The average frequency of CD271<sup>+</sup> CD45<sup>-</sup> 7-AAD<sup>-</sup> cells before separation was 0.037% (range 0.008–0.140) of all nucleated cells and has decreased to 0.027% (range 0.008–0.082) of all nucleated cells after separation. The average CD271<sup>+</sup> CD45<sup>-</sup> 7-AAD<sup>-</sup> cell recovery rate was 15.4% (range 5.7–34.8).

The average enrichment and concentration factors for CD271<sup>+</sup> CD45<sup>-</sup> 7-AAD<sup>-</sup> cells were 11.4-fold (range 3.6–26.8,  $p < 0.05$ ) and 1.0 (range 0.4–1.9,  $p = 0.754$ ), respectively.

Additionally, estimation of MSCs was evaluated by measuring the concentration of CD73<sup>+</sup> CD90<sup>+</sup> CD105<sup>+</sup> cells (N = 13). The average frequency of CD73<sup>+</sup> CD90<sup>+</sup> CD105<sup>+</sup> cells after separation was 0.15% (range 0.01–1.70) of all nucleated cells. The average CD73<sup>+</sup> CD90<sup>+</sup> CD105<sup>+</sup> cell recovery rate was 21.5% (range 6.0–39.8).





**Fig. 1** **A** Enrichment factors and **B** concentration factors of cell populations after separation with filter-based intra-operative bone marrow aspirate separation device. Data are presented as geometric mean with 95% confidence interval ( $13 < N < 14$ ). Statistical significance was determined by one sample t-test comparing means of logarithmically transformed data to hypothetical value of 0 (dotted line represents value 1;  $\log(1) = 0$ ), \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . *CFU-F*, fibroblast colony forming units; *WBC* white blood cells

The average enrichment and concentration factors for CD73<sup>+</sup> CD90<sup>+</sup> CD105<sup>+</sup> cells were: 14.3-fold (range 5.2–38.3,  $p < 0.05$ ) and 1.2-fold (range 0.4–2.4,  $p = 0.223$ ), respectively.

### 3.2 Clinical evaluation

The mean time from surgery to last evaluation was 20 months (range 14–25).

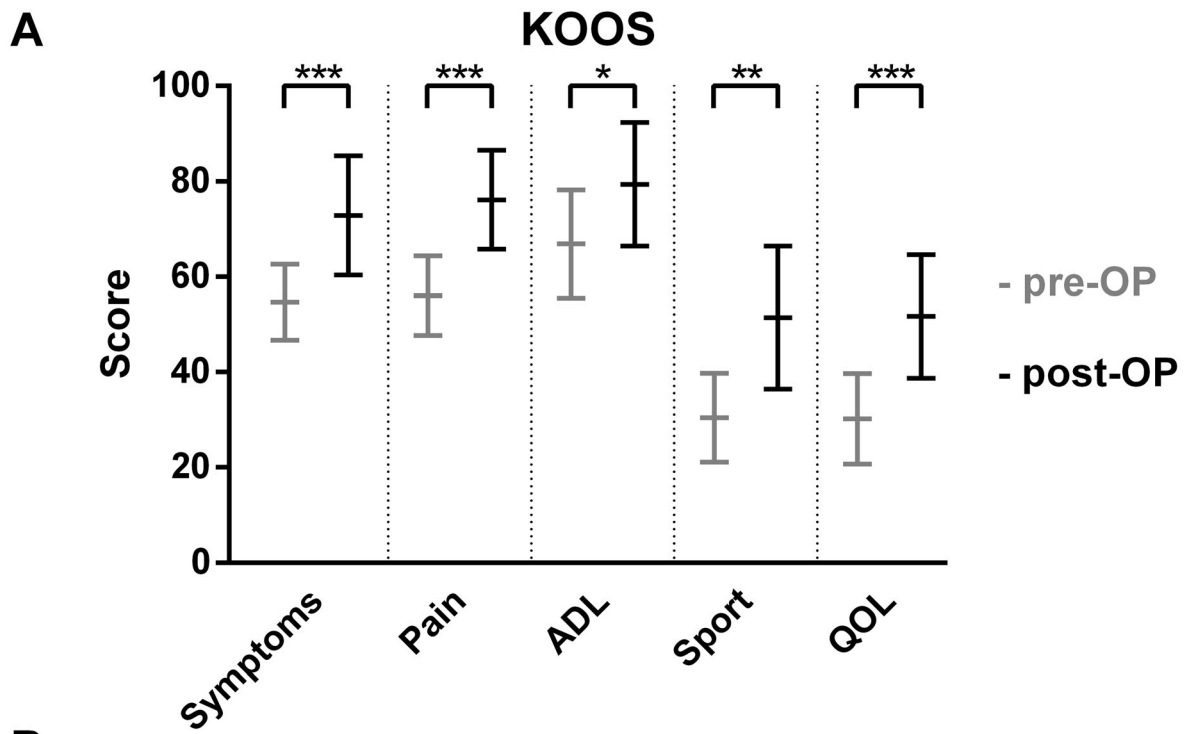
Four patients required surgical re-intervention that was not related to the cartilage repair: removal of an old loosened ACL fixation device, arthroscopy due to a fresh patella lesion, arthroscopic removal of a post-op adhesions in the anterior knee compartment, and HTO plate removal. One patient (SR00415M) encountered partial graft failure due to progression of OA process. Altogether, no serious adverse events related directly to the osteochondral treatment were reported. Therefore, the treatment revealed high safety profile as proposed in hypothesis (2).

All KOOS subscales increased significantly from pre-operative Symptoms 55/Pain 56/ADL 67/Sport 30/QoL 30 to post-operative Symptoms 73/Pain 76/ADL 79/Sport 51/QoL 52, with all  $p$  values  $< 0.05$ , confirming the proposed hypothesis (3). Majority of knee patients (14/15) reported improvements in KOOS pain category comparing to the baseline. Refer to Fig. 2A. The results of safety evaluation and clinical outcome are presented in Table 3.

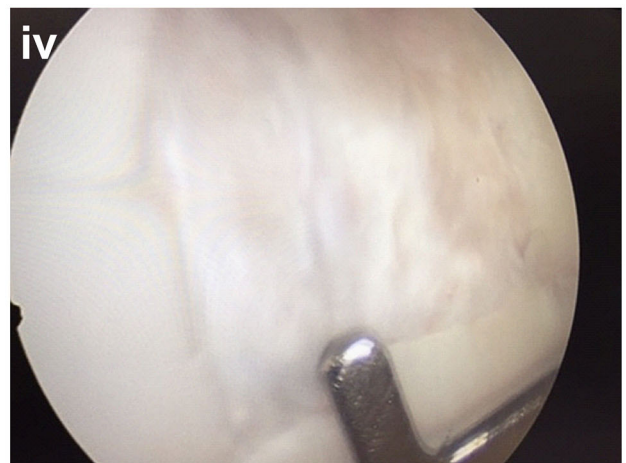
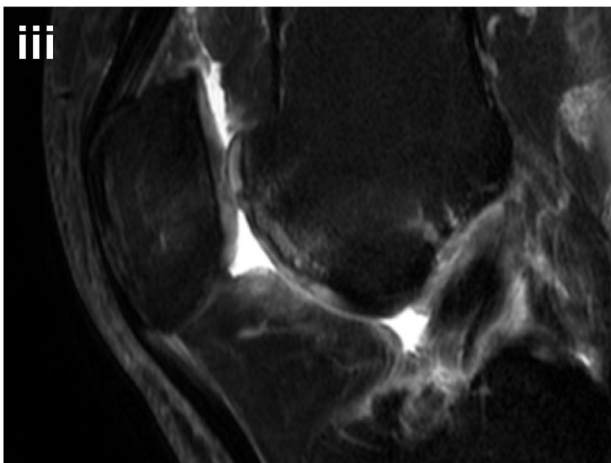
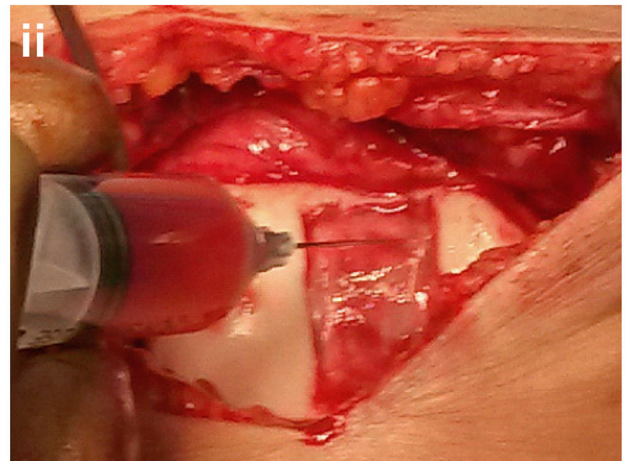
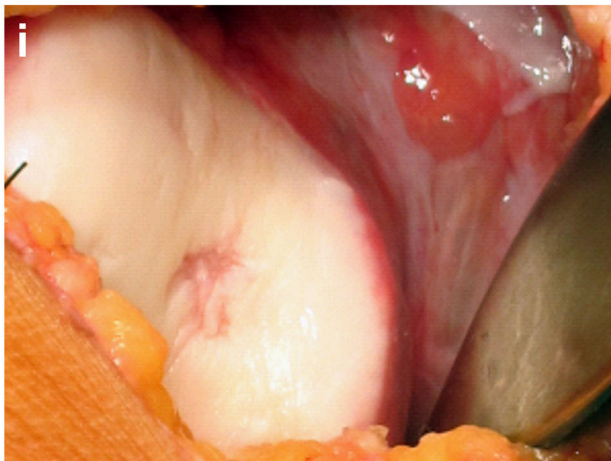
Eight patients in this case series had an MRI (4), arthroscopic (2), or both (2) follow-up evaluations performed. The failed patient SR00415M presented above, revealed overall ICRS graft repair assessment of 6 points. The remaining seven patients revealed normal (3 cases) or nearly normal (4 cases) overall ICRS repair assessment. Figure 2B shows implantation procedure and outcome of a successful cartilage repair for patient MG01315M.

### 4 Discussion

Symptomatic osteochondral lesions require surgical repair to reduce patients' symptoms and to prevent progression toward early osteoarthritis. Combination of biocompatible scaffolds and autologous active cells is prevailing treatment strategy for midsized to large lesions [23]. The concentration of MSCs is considered one of important parameters that influence clinical success of cell therapies utilizing MSCs isolated within one operative procedure [8]. Bone marrow processing through a separation column offers an alternative approach to conventional centrifuge-based bone marrow processing systems enabling concentration of MSCs. The mechanism of cell isolation on the selected filter device is a combination of cell trapping as result of sieving effect and adherence of MSCs to the nonwoven fabrics [15]. According to DOSES cell therapy communication tool [24] the f-BMAs obtained in the present study represent autologous, bone-marrow derived cells, obtained by minimal manipulation through filtration and centrifugation. Cell suspension contained 0.027%



**B**





◀**Fig. 2 A** Knee Osteoarthritis outcome scores before and after treatment with f-BMA. Data are presented as arithmetic mean with 95% confidence interval (N = 15). Statistical significance was determined by paired two-sided t-test, \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . **B** A 34-year old female former handball player suffered from an osteochondral lesion of central trochlea femoris (i) 7 years after anterior cruciate ligament reconstruction (case MG01315M). The site of the lesion was debrided and covered with a bi-layered collagen-hydroxyapatite biomimetic scaffold combined with f-BMA (ii). MRI image taken 18 months after surgical repair shows complete reconstruction of the entire osteochondral unit (iii). A knee arthroscopy (performed at 12 months post-surgery due to a loosened ligament fixation device) demonstrated complete filling of the lesion with a repair tissue that was fully integrated to the surrounding cartilage (iv). *ADL* activities of daily living, *KOOS* knee osteoarthritis outcome score, *QOL* quality of life

CD271<sup>+</sup> CD45<sup>-</sup> 7-AAD<sup>-</sup> cells, 0.15% CD73<sup>+</sup> CD90<sup>+</sup> CD105<sup>+</sup> cells and 0.0012% CFU-Fs of all nucleated cells with 86% viability. f-BMA was delivered intra-articularly and injected onto biomimetic collagen-hydroxyapatite scaffold.

Filtration processing of clinical samples removed 98.6% RBCs from initial BMAs. While the majority of RBCs were washed off the column the greater extent of WBCs (evaluated with haematology analyser) and MSCs (evaluated with CFU-F assay and flow cytometry) were harvested from the column which resulted in 11.4–14.7-fold enrichment of these two cell populations in final cell preparation, depending on the evaluation method. The separation column is equally selective for WBCs and MSCs since the enrichment factors for both cellular populations are in the similar range. The high enrichment factors indicate to high selectivity of the cell isolation method. However the concentrations of CFU-Fs before and after separation were very similar, resulting in concentration factors that were not significantly different to 1 ( $p > 0.05$ ), showing that no concentration of MSCs was achieved with our BMA processing procedure. The average recovery rate for CFU-Fs was 21.5%. Nevertheless, our number of 1571 CFU-F (range 270–11,722) in the final cell preparation falls within

**Table 3** Clinical outcome and serious adverse events or graft failures following treatment with f-BMA

Patient code	Follow-up period (months)	KOOS pre-OP					KOOS post-OP					Serious adverse events or graft failure
		Symptoms	Pain	ADL	Sport	QOL	Symptoms	Pain	ADL	Sport	QOL	
PM00814M	24	50	42	94	50	63	86	83	90	60	63	None
TG00914M	24	64	44	82	40	56	90	83	100	90	80	None
SR00415M	21	25	42	50	5	19	18	36	40	0	6	MUA due arthrofibrosis and joint contracture
MG00615M	21	71	64	91	30	38	96	92	99	90	75	None
MP00915M	17	57	44	66	25	13	64	72	72	30	25	None
TP01015M	16	64	75	82	40	50	82	97	99	75	69	None
MG01315M	14	57	72	94	55	31	80	90	96	75	80	Removal of old loosened ACL fixation device
SK01615M	19	56	64	41	25	12	86	90	90	50	69	HTO plate removal
DM00116M	21	81	57	72	25	19	78	64	68	40	31	Arthroscopic debridement of de novo patella lesion
DD00316M	25	42	50	46	50	25	40	54	40	46	25	None
JS00416M	25	57	61	50	30	38	82	80	96	50	63	None
AK00616M	24	34	32	36	5	32	39	43	38	5	38	Revision arthroscopy due to localized adhesions
PŠ00716 M	24	42	44	46	37	32	80	83	70	46	69	None
LL00916MK	20	56	64	82	40	25	89	82	99	75	44	None
16BK017BK	14	64	86	71	0	0	83	93	94	40	38	None
Arithmetic mean		55	56	67	30	30	73	76	79	51	52	
Lower 95% CI		47	48	56	21	21	60	66	66	36	39	
Upper 95% CI		63	64	78	40	40	85	87	92	66	65	

*ACL* anterior cruciate ligament, *ADL* activities of daily living, *HTO* high tibial osteotomy, *KOOS* knee osteoarthritis outcome score, *MUA* manipulation under anesthesia, *QOL* quality of life

range of post-processing CFU-F numbers measured in other commercial systems: 806–8888 CFU-F [25].

Based on our preliminary investigation of flow-through fractions the majority of MSCs is actually retained on the column (data not shown), however they are not efficiently recovered with the current harvesting procedure. With additional optimization of harvesting technique more MSCs could be recovered from the column which could lead to overall improvement of current cell separation process with selected filter-based separation device.

Our proposed hypothesis (1) was partially confirmed as the population of MSCs was significantly enriched. However, the described procedure did not enable concentration of MSCs.

As a most abundant cell population in BM, RBCs can have a great impact of MSC seeding efficiency on 3D scaffolds simply by preventing their physical contact with the surface. With the removal of majority of RBCs the seeding efficiency of MSCs onto decellularized 3D bone construct was significantly increased in the *in vitro* study [17]. Their BMA filtering device enriched CFU-Fs by 3.7-fold which is lower than 12.8-fold enrichment achieved in our study. Our degree of enrichment is mainly result of 98.6% RBC removal, which is consistent with the previously reported data [16]. In our opinion the most important contribution of filter-based cell separation procedure used in this study is in the removal of RBCs which is expected to be beneficial in terms of MSC seeding efficiency onto biomimetic scaffold. More systematically designed *in vitro* and *in vivo* studies should be performed to better understand the impact of MSC frequency in the final cell product on overall tissue regeneration outcome. With this knowledge we could also make more justified decisions which cell isolation strategy to employ for specific application: ones with higher cell recovery rates or others with higher cell enrichment factors.

During the follow-up no serious adverse events related directly to the osteochondral treatment were reported; primary graft failure was 7%. Clinically, the majority of patients significantly improved their post-operative knee function. MRI or arthroscopic evaluation revealed nearly normal to normal lesion repair in the majority of evaluated cases.

The clinical results, showing high safety profile and positive clinical response, are in favour of hypotheses (2) and (3).

It needs to be stressed out that patient pathology was rather complex (more than half were revision cases, one-third required concomitant procedures, one-fourth of the knees already showed degenerative cartilage changes). This particular biomimetic collagen-hydroxyapatite scaffold has been extensively used in a stand-alone manner for the osteochondral repair in the knee [11]. Albeit our results

are short-termed, they concur with other clinical reports [26]. These early results also correspond to our series of patients, in whom CHAS was combined with cultivated autologous chondrocyte implantation [27]. There are only limited reports on the usage of single step bone-marrow procedures in combination with other scaffolds for the treatment of focal cartilage lesions. Gobbi et al. [28] were using BMAC covered with collagen I/III matrix, and showed this combination to be viable treatment for knee chondral lesions. They reported complete coverage of lesions seen on MRI with hyaline-like cartilage in 80% of patients, and normal to nearly normal tissues (hyaline cartilage-like tissues) on biopsies at second-look arthroscopy. They also compared the techniques above to the classical MACI techniques based on autologous chondrocyte cultivation, and consider both treatments to be effective for large patellofemoral chondral lesions at 3 year follow-up [29]. Enea et al. [30] used collagen-covered microfractures and bone marrow concentrate for focal cartilage defects in the knee. They consider it safe; it improved knee function, and has a potential to regenerate hyaline-like cartilage. Reconstitution of the original cartilage level was achieved, but bone marrow edema and/or subchondral irregularities were observed in all patients. Second-look arthroscopy was performed in 5 patients, resulting in 1 normal, 3 nearly normal, and 1 abnormal result. Histology showed hyaline-like repair tissue. Skowronski et al. [31] applied BMAC to a collagen membrane. They consider this one stage repair of large chondral lesions as an effective treatment modality. Chahla et al. [32] in their recent systematic review conclude that BMAC treatment appears to be a safe procedure that is growing exponentially, most likely because it represents one of the few categories allowed by the FDA to deliver stem cells (minimally manipulated). All the studies included in their systematic review reported good results, but they used different outcome measures and this heterogeneity does not allow for direct comparison. They also stress out a need for well-conducted randomized controlled trials with large sample sizes and defined end points to further evaluate the efficacy of BMAC for the treatment of knee pathologies. Our clinical results concur to the results above, with one very important difference: only deep chronic osteochondral lesions were treated in our case series. Chronic osteochondral lesions namely cannot be treated with thin collagen membranes that were used in combination with BMAC by other authors. Therefore, usage of a biomimetic osteochondral scaffold and well balanced MSC source is recommended.

The clinical results have to be interpreted in light of limited patient series, short-medium follow-up time and a non-randomized study design. On contrary, the entire two-year patient series operated on the knees by a single

surgeon (MD) was included into the study. A single surgeon design avoids possible bias due to different surgical technique. We also cannot delineate what percentage of cartilage repair is attributable to biomaterials versus f-BMA. But we could prove that combination of both offers safe and successful cartilage repair and return to activities, which was a primary target of this publication. f-BMA obtained by the presented procedure may easily be combined with other cartilage repair scaffolds in the future.

Altogether, this patient case series presents clinical application of filter-based separation device, which enabled efficient removal of RBCs, while achieving 12.8-fold enrichment and 19.1% recovery of CFU-Fs. However, the concentration of MSCs was not increased. Combination of f-BMA and biomimetic scaffold prepared for the treatment of osteochondral lesions resulted in no reported serious adverse events directly related to the graft. One patient (6.7%) in the series had documented graft failure. Clinically, all the other fourteen patients improved their post-operative knee function in all five KOOS categories. All average KOOS subscales increased significantly from pre-operative Symptoms 55/Pain 56/ADL 67/Sport 30/QoL 30 to post-operative Symptoms 73/Pain 76/ADL 79/Sport 51/QoL 52, with all  $p$  values  $< 0.05$ . MRI or arthroscopic evaluation revealed nearly normal to normal lesion repair in the majority of evaluated cases.

With respect to proposed hypotheses, we could partially confirm hypothesis (1). The population of MSCs was significantly enriched; however, the concentration of MSCs was not achieved. The clinical results, showing high safety profile and positive clinical response, are in favour of hypotheses (2) and (3).

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**Author contributions** MK and MD conceived and designed the study. MV, JV and MD acquired the data, MV, AB and MD interpreted and analysed the data. MV, JV, AB and MD drafted the article and MK revised it critically for the important intellectual content. All authors approved the final version of the article to be published.

#### Compliance with ethical standards

**Conflict of interest** Educell Ltd., Trzin, Slovenia (co-authors MV, MK, AB) is a commercial cell and tissue institution that is providing separated MSCs and other advanced cell therapies. MD is a clinical consultant to Fin-Ceramica, Faenza, Italy.

**Ethical statement** All procedures performed in studies involving human participants were in accordance with the ethical standards of the national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study protocol was approved by the National Medical Ethics Committee (No. 0120-14/2016-2). Informed consent was confirmed by the National Medical Ethics Committee.

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