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ORIGINAL PAPER

Subchondral bone or intra-articular injection of bone marrow concentrate mesenchymal stem cells in bilateral knee osteoarthritis: what better postpone knee arthroplasty at fifteen years? A randomized study

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Abstract

Purpose There is an increasing number of reports on the treatment of knee osteoarthritis (OA) using mesenchymal stem cells (MSCs). However, it is not known what would better drive osteoarthritis stabilization to postpone total knee arthroplasty (TKA): targeting the synovial fluid by injection or targeting on the subchondral bone with MSCs implantation.

Methods A prospective randomized controlled clinical trial was carried out between 2000 and 2005 in 120 knees of 60 patients with painful bilateral knee osteoarthritis with a similar osteoarthritis grade. During the same anaesthesia, a bone marrow concentrate of 40 mL containing an average 5727 MSCs/mL (range 2740 to 7540) was divided in two equal parts: after randomization, one part (20 mL) was delivered to the subchondral bone of femur and tibia of one knee (subchondral group) and the other part was injected in the joint for the contralateral knee (intra-articular group). MSCs were counted as CFU-F (colony fibroblastic unit forming). Clinical outcomes of the patient (Knee Society score) were obtained along with radiological imaging outcomes (including MRIs) at two year follow-up. Subsequent revision surgeries were identified until the most recent follow-up (average of 15 years, range 13 to 18 years).

Results At two year follow-up, clinical and imaging (MRI) improvement was higher on the side that received cells in the subchondral bone. At the most recent follow-up (15 years), among the 60 knees treated with subchondral cell therapy, the yearly arthroplasty incidence was 1.3% per knee-year; for the 60 knees with intra-articular cell therapy, the yearly arthroplasty incidence was higher ($p = 0.01$) with an incidence of 4.6% per knee-year. For the side with subchondral cell therapy, 12 (20%) of 60 knees underwent TKA, while 42 (70%) of 60 knees underwent TKA on the side with intra-articular cell therapy. Among the 18 patients who had no subsequent surgery on both sides, all preferred the knee with subchondral cell therapy.

Conclusions Implantation of MSCs in the subchondral bone of an osteoarthritic knee is more effective to postpone TKA than injection of the same intra-articular dose in the contralateral knee with the same grade of osteoarthritis.

Keywords Mesenchymal stem cells · Synovial fluid · Cartilage degeneration · Knee osteoarthritis · Synovium · Subchondral bone · Bone marrow

Osteoarthritis (OA) is not only a pure cartilage disorder; it is rather a whole-joint disease affecting various parts of the joint inside and outside the capsule envelope. These structures include the subchondral bone and the synovial tissue [1]. The advent of MRI during the two last decades has underlined the role of both the synovitis and subchondral bone in OA pathology. Bone marrow lesions are now used as a marker for osteoarthritis, as they occur early in the subchondral bone and reverse earlier than the cartilage degradation (as do synovitis). Therefore, new systemic drugs [2] for OA treatment are targeting enzymes, cytokines, and growth factors.

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Local treatments as administration of bone marrow concentrates [3, 4] in the joint have also been proposed. However, for a local treatment with injection or implantation of mesenchymal stem cells (MSCs), the best target (synovitis or BMLs) to postpone total knee arthroplasty (TKA) has not been determined. Despite absence of MSCs in the subchondral bone of the osteonecrosis, there is a high number of MSCs in the synovial fluid (SF) of osteoarthritis hips or knees [5, 6]. If MSCs are increased in SF, one can wonder what is the rational to increase "more" by injecting MSCs in the SF. At contrary, as the number of MSCs is low in the subchondral bone, there could be some advantage to inject MSCs in the subchondral bone of OA patients. The safety, feasibility, and efficacy of subchondral injection versus intra-articular injection have never been compared in the same patients.

For these reasons, with a prospective randomized trial, the purpose of this study was to assess in bilateral knee osteoarthritis what would better drive osteoarthritis stabilization to postpone total knee arthroplasty: targeting synovitis by injection of MSCs in joint fluid or targeting subchondral lesions with percutaneous implantation of MSCs in subchondral bone marrow.

Material and methods

Study patients and data collection

This study was approved by the Institutional Review Board. A total of 60 patients seen for painful bilateral knee osteoarthritis between 2000 and 2005 were included in this study. Patients were considered for inclusion if they had bilateral similar knee pain (Table 1) related to bilateral medial knee osteoarthritis (OA) with a Kellgren-Lawrence grading from 1 to 4 [7] despite conventional treatments such as activity modification, weight loss, physical therapy, analgesics, nonsteroidal anti-inflammatory drugs, or injection therapy for at least 6 months. Inclusion needed that the two knees of the same patient had no more difference than 1 grade with Kellgren-Lawrence grading and absence of prior surgery in the two knees. Exclusion criteria were severe rheumatological or other systemic disease, diabetes, malignancy, or infections. All patients were required to wait three months from any prior intra-articular injection before participating.

Same patient's knees were randomized into intra-articular (IA) injection group or subchondral (SC) group. Each patient was asked (Fig. 1) to choose two identical envelopes for the right knee and the left knee with the subchondral or intra-articular group indicated inside. In this prospective randomized controlled blinded trial, the patients were under general anaesthesiology and had no knowledge of which knee was injected intra-articular or subchondral since the skin entry points were the same; of course, the operating physician did.

Outcomes were measured by an orthopaedic research fellow independent of the operating physician at three months, six months after surgery, and each year after surgery until the most recent follow-up (end of 2019). Five patients had died after a mean follow-up of eight years (range 8 to 13 years); death was not related to knee surgery; none these five patients had another surgery. Two other patients did not return for full clinical evaluation, but local radiographic evaluation was arranged and obtained, and clinical examination performed locally by their medical doctor. The follow-up after surgery for the 55 living patients (mean age 76 years at follow-up; range 62 to 87 years) was an average of 15 years (range 13 to 18 years).

Table 1 Characteristics of the 60 patients and 120 knees

Characteristic	60 patients		
Gender (male/female)	25/35		
Age at inclusion (mean, range)	61 (48–72) years		
Body mass index (kg/m^2)	28.1 (20.4–32.5)		
Follow-up (mean, range)	15 (13–18) years		
Age at follow-up	76 (62–87) years		
	Knees	Knees	
Pre-operative scores	Intra-articular MSCs	Subchondral MSCs	*p* value
(VAS) pain score	3.8 (2 to 5)	4.1 (2 to 6)	0.36
Knee society scores	52 ± 15	55 ± 18	0.32
Range of motion	116° ± 15	118° ± 17	0.22
Kellgren and Lawrence grading			
Grade 1	10	12	
Grade 2	22	18	
Grade 3	16	22	0.47
Grade 4	12	8	

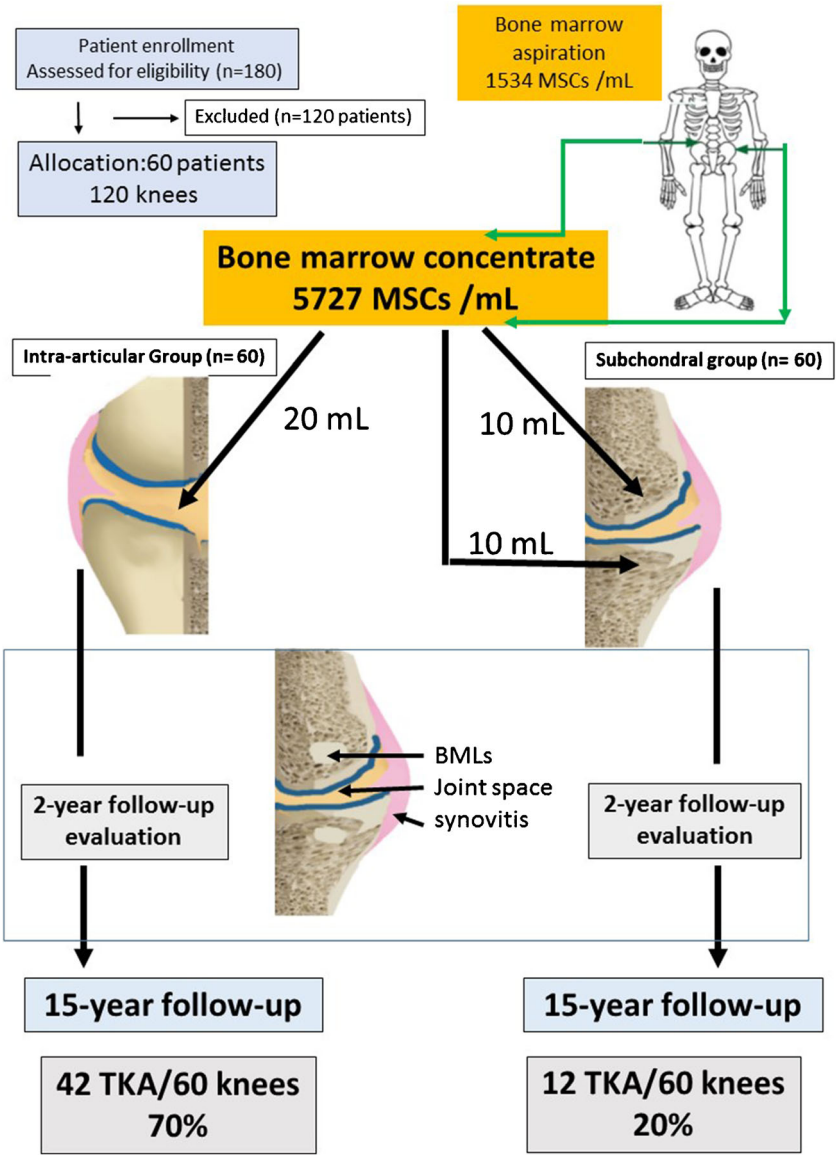

Fig. 1 Flow diagram of the study

Surgical technique

Surgery was performed under general anaesthesia. Patients were placed on supine position. Tourniquet was not needed for both knees.

Mesenchymal stem cell aspiration in the iliac crest was first performed: marrow was aspirated on the both iliac crests with 10-cc syringes as previously reported [8]. The quantity of bone marrow aspirate extracted from the two patient's anterior iliac crests was mixed and averaged 155 mL (range 145 to 160), which was then concentrated by centrifugation to obtain a bone marrow concentrate (BMC) graft (Fig. 1).

Each knee received a BMC graft containing the same number of MSCs, average 5727 MSCs/mL (range 2740 to 7540). After aspiration of the synovial fluid, intra-articular injection of 20 mL was performed into the randomized joint (Fig. 1). For the other side, a 20 mL sterile saline was first injected in the joint so that the two knees had the same post-operative aspects. Then this contralateral knee received a percutaneous implantation of 20 mL BM concentrate in the subchondral medial tibiofemoral compartments, i.e., 10 mL in the medial tibial plateau and 10 mL in the medial condyle. For bone marrow concentrate injection in the femoral condyle, we used fluoroscopy as previously reported [9] to perform a retrograde "outside-in" trocar implantation; for the tibia, the trocar was directed with fluoroscopy in direction of the bone marrow lesions, remaining 5 mm below the tide-mark to avoid perforation of the cartilage. Patients were discharged with instructions for immediate full weight-bearing. Physical therapy was not necessary.

Analysis of bone marrow of iliac crest

Four parameters were measured or calculated from the cell analysis of bone marrow aspirate samples as from the concentrate cell suspension. The technique has been previously reported in details [8, 10] by the authors and is briefly summarized here. Roughly, the first parameter was to quantify the dilution of nucleated cells coming from the bone marrow by cells of the peripheral blood; for that, the haematocrit of the bone marrow was compared with the haematocrit of the peripheral blood. The second parameter was the bone marrow mono-nuclear nucleated cell count (the number of bone marrow mono-nucleated cells per 1.0 mL of marrow aspirate). The third parameter was the CFU-F (colony fibroblastic unit forming) per mL of aspirate in the bone marrow. The CFU-F assay was performed by plating two million mono-nucleated cells from the bone marrow aspirate into 25 cm^2 tissue culture flasks. Following ten days under standard growth conditions, the number of colonies containing at least 50 cells was evaluated. The frequency of progenitor cells was assessed by determining the number of colony-forming units per million mono-nucleated cells present in the cultured sample. The fourth parameter was the MSC concentration calculated as the product of the mono-nucleated cell count and the prevalence of colonies per mL.

Clinical outcome

All inflammatory drugs were stopped before the entry to the study. Glucosamine was allowed, when the patient was using it before the study. During surgery, no steroid was injected. Analgesics were given for 15 days; Patients were asked to avoid anti-inflammatory drugs for three months and to avoid any intra-articular injection in both knees. The clinical results were graded [11] according to the Knee Society knee score and according to the visual analog scale (VAS) pain score. Patients were proposed to compare their knees.

Clinical outcomes were measured by an orthopaedic research fellow independent of the operating physician at three months, six months after surgery, and each year after surgery until the most recent follow-up (end of 2019). The follow-up was average 15 years (range 13 to 18 years).

Radiological and imaging outcome of the patients (synovitis and BMLs)

Bilateral knee radiographs with standard posteroanterior lateral views were obtained. Osteoarthritis bone marrow lesions (BMLs) were defined as previously described [12] as ill-defined areas of decreased signal intensity on T2 MRI sequences. MRI was performed at baseline and at 24 months on a 2.5 T MRI (Siemens) using a standard knee coil. The cartilage volume was measured by using a computer program. The change in the volume of the knee's cartilage was calculated by subtracting the volume at follow-up from the baseline volume (software program Osiris).

Synovitis was scored on MRI based on post-gadolinium T1-weighted fat-suppressed images as per the methodology of Guermazi et al. [13]. Synovitis was scored semi-quantitatively from grades 0 to 2 based on the maximal synovial thickness at predefined locations. The sum of these different locations "synovitis scores" yielded a total synovitis score.

Statistical analysis

Demographic and medical variables were determined by the mean, standard deviation, range, and percent. For this study, a pair protocol analysis was used. Comparisons were performed by Student's test for paired sample parametric data; Wilcoxon signed-rank test was used for paired sample nonparametric data, and Kendall or Spearman test evaluated correlation. In consideration of a three time point evaluation (baseline, 2-year follow-up, most recent follow-up), a repeated measured ANOVA was used. Differences in variables by surgery status (arthroplasty compared with site of stem cell injection) were assessed for categorical variables by chi-square tests. Kaplan-Meier survival curves were generated to assess the yearly incidence of total knee arthroplasty after cell therapy. Finally, to determine the independent (adjusted) association of demographic data and imaging of OA with the risk of total knee arthroplasty, a multivariate Cox proportional hazards model was created. All risks factors as demographic and radiographic factors presented in this study were included and particularly age, sex, weight, and osteoarthritis severity.

Results

Descriptive statistics and cellular product at baseline

Descriptive statistics

One hundred-eighty patients with OA (Fig. 1) were screened for including sixty patients (120 knees) in this study. Demographic data of patients is summarized in Table 1. Patients (35 female) were average 61 years old (range, 48–72 years) with a median body mass index of 28.3 kg/m^2 (range, 20.4–32.5 kg/m^2). Of the 120 knees, 22 were radiographically graded Kellgren-Lawrence 1 (twelve subchondral injections, ten intra-articular injections), 40 knees were radiographically graded Kellgren-Lawrence 2 (18 SC, 22 IA), 38 were graded Kellgren-Lawrence 3 (22 SC, 16 IA), and 20 knees were graded Kellgren- Lawrence 4

(8 SC, 12 IA). Five patients had died after a mean follow-up of eight years (range 8 to 13 years); death was not related to knee surgery; none these five patients had another surgery. Two other patients did not return for full clinical evaluation, but local radiographic evaluation was arranged and obtained, and clinical examination performed locally by their medical doctor. The follow-up after surgery for the 55 living patients (mean age 76 years at follow-up; range 62 to 87 years) was 15 years (range 13 to 18 years).

Cellular product

In the aspiration product before concentration, the number of MSCs was decreasing with age (Fig. 2) with a mean value of 1534 MSCs/mL (range 637 to 2545) and a decrease of average 15% for each decade between 50 and 70 years. For an aspiration of 155 mL, the concentration product (Fig. 1) of 40 mL was divided in two parts (one for each knee) of bone marrow concentrate (BMC) of 20 mL containing average 5727 MSCs/mL (range 2740 to 7540). Compared with the usual concentration in the subchondral bone [14], this BMC graft represents for the randomly assigned knee subchondral knee between a 60-fold increase to a 200-fold increase of MSCs. For the contra-lateral knee where cells were injected in the synovial fluid, the increase was less as regards to the concentration usually present in the SF [15, 16], with an increase ranging from five fold to 20-fold. There were no serious adverse events. Effusions were often seen for several (< 15) days after the procedure in both knees; these were anticipated findings, and probably these initial effusions were likely to be residual to the 20-mL infiltration performed in each knee.

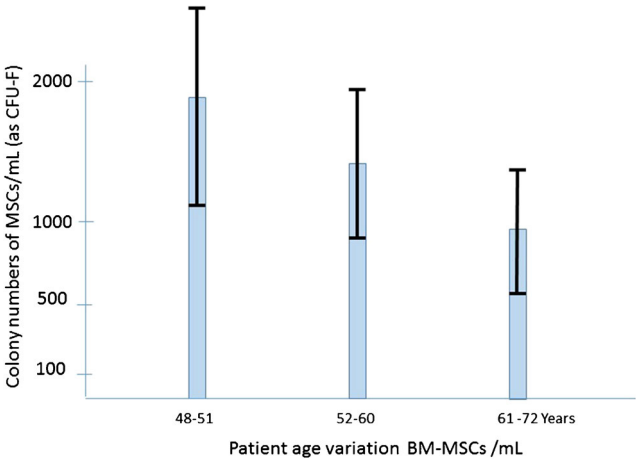

Fig. 2 MSCs obtained by aspiration in bone marrow of the iliac crest (BM-MSCs) from osteoarthritis patients. Data are displayed as the average and range for each age group

Injecting cells in synovitis or in bone marrow lesions: different results at two year follow-up

Clinical improvement was higher on the side that received cells in the subchondral bone

Clinical pre-operative (Table 1) knee scores (55 points ± 18) of subchondral (SC) cell therapy knees were similar ($p = 0.08$) to those (52 points ± 15) of intra-articular (IA) knee's cell therapy. The mean overall changes in knee scores improved on both sides but, at two year follow up, were higher ($p = 0.03$) for the SC group (79.3 points ± 12) when compared with the IA pre-operative group (64 points ± 21).

This also was evident for pain score when VAS pain scores are displayed separately. Of note, improvement in VAS pain scores from baseline was observed for Kellgren-Lawrence grades 1 to 4 knees within each treatment group and for each follow-up time point. For the intra-articular BM-MSC group, pain improvement (Fig. 3a) was maintained at 12 months, at which time point the highest effect was observed; after 12 months, there was still improvement as compared with baseline, but this difference was no more significant ($p = 0.08$) at 24 months. With subchondral injection, improvement remained the same at two year follow-up and highly significantly ($p = 0.001$) increased as compared with baseline.

Imaging improvement was present on the side with the subchondral bone injection

Joint space The evaluation of the K-L score at two year follow-up demonstrated some joint space narrowing at one year in the IA group MSCs ($p = 0.05$ at 12 months), which was not observed in patients treated with subchondral dose MSCs. According to the K-L classification (Fig. 3b), 11 knees with IA cell therapy had progressed to higher grades at two year follow-up. During the same period, there were only two knees with progression for K-L grading in the SC cell therapy group (Fig. 3b). These results suggest that BM-MSCs injected in the subchondral bone may halt the progressive loss of cartilage observed in patients with OA. This was confirmed when cartilage volume changes over time were measured with MRI; the percentage cartilage volume measured with MRI (excluding osteophytes) on the medial compartment increased compared with baseline (2.1% ± 1.4% at 2 years) in the SC group while decreasing on the IA group. No ectopic ossification was observed in the joints or at the sites of BMC injections.

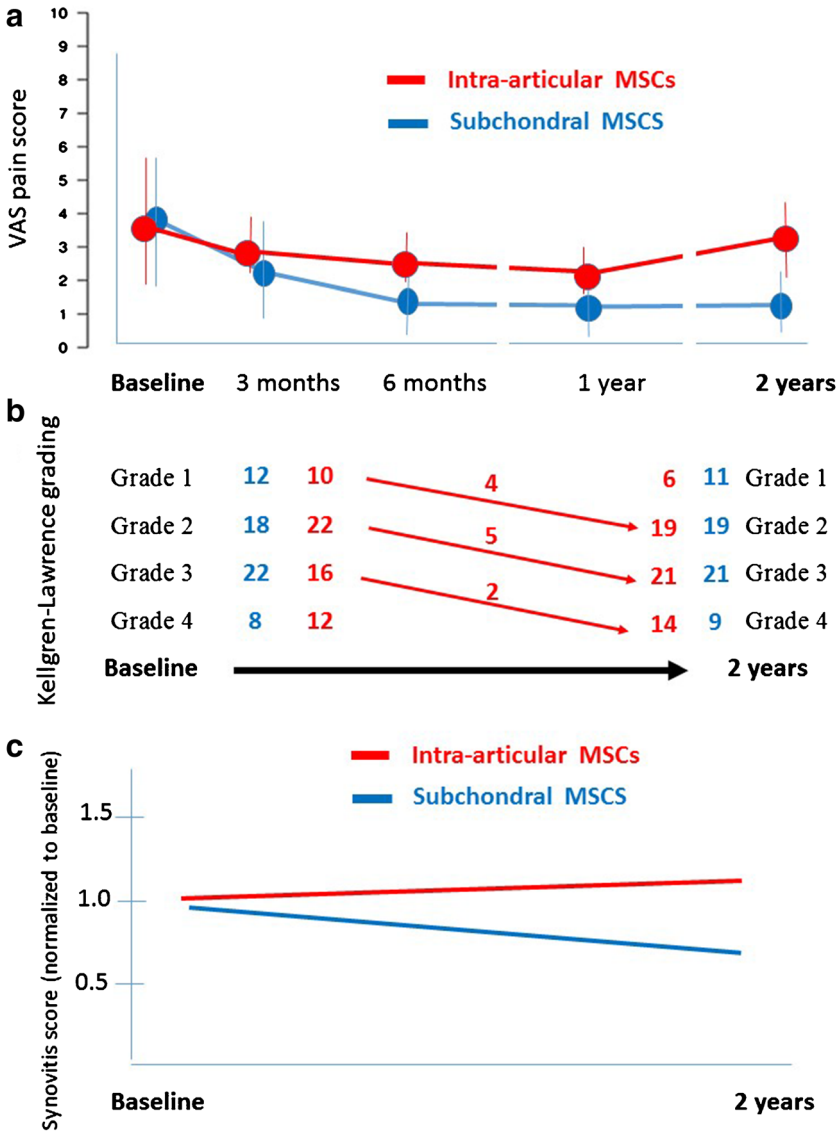

Fig. 3 Results at 2-year follow-up. **a** Visual analog scale (VAS) pain score. **b** Osteoarthritis progression between baseline and 2-year follow-up. **c** Synovitis score (shown as fold change relative to baseline)

Bone marrow lesions The average baseline BML volume of the medial femorotibial compartments (both femur and tibia) was 2.9 cm^3 (range 0.4 to 5.2 cm^3) in the SC group and 2.6 cm^3 (range 0.6 to 6.1 cm^3) in the IA group ($p = 0.34$). After treatment with MSCs injection in the subchondral bone, medial femorotibial compartment BML volume experienced regression over 24 months (mean 2.1 cm^3, range 1.4 to 2.9 cm^3) in all the knees, while no regression was observed for the IA group ($p = 0.01$).

Synovitis Synovitis scores also were not significantly ($p = 0.14$) different at baseline (Fig. 3c) between the two groups. At 24 months, the knees with subchondral MSC implantation demonstrated a better ($p = 0.02$) outcome with a 25% regression of the synovitis scores as compared with baseline, while knees with MSCs intra-articular injection had increased ($p = 0.03$) in worse the synovitis scores (Fig. 4c).

Subchondral bone MSC implantation was more efficient to postpone TKA at 15 years

Incidence of subsequent surgery after cell therapy

At the most recent follow-up of 15 years (range 13 to 18 years), a total of 54 (45%) of the 120 knees treated with cell therapy underwent total knee arthroplasty (TKA) at average seven years (range, 3 to 14 years). The overall incidence of TKA was 3% per knee-year. Among the 60 knees treated with subchondral cell therapy, a total of 12 knees (20%) underwent TKA at a mean of 14 years (range, 9 to 16 years); the yearly arthroplasty incidence was 1.3% per knee-year for this group (Fig. 4), which conforms to another series [12] with subchondral injections for osteoarthritis; for the 60 knees with intra-articular cell therapy, 42 knees (70%)

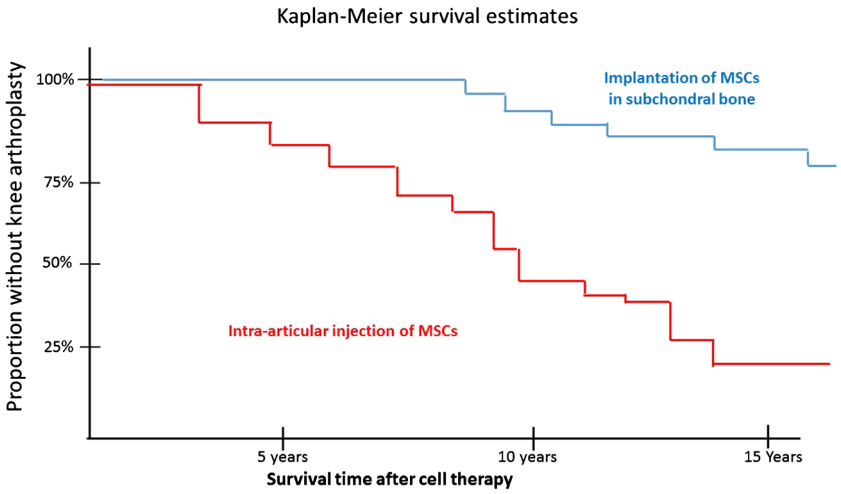

Fig. 4 Arthroplasty-free survival knees (red line) with intra-articular injection of MSCs compared with arthroplasty-free survival knees (blue line) with MSC implantation in the subchondral bone

underwent TKA at a mean of seven years (range, 3 to 14 years) with a higher ($p = 0.01$) yearly arthroplasty incidence of 4.6% per knee-year.

Among the 60 knees treated with subchondral injections, after adjusting for confounders, the decrease of BMLs observed after cell therapy at two year follow-up in the subchondral bone was an independent factor for postponing total knee arthroplasty (hazard ratio HR = 3.12 (95% CI = 2.04 to 6.15); $p < 0.01$). We analyzed the effect of the number of progenitor cells on the decrease risk of TKA, or on the duration of time before TKA; a decrease risk of TKA was associated with a higher number of MSCs ($p = 0.04$); and for those patients who had TKA, a longer time before the arthroplasty was associated ($p = 0.02$) with a higher number of progenitor cells injected in the subchondral bone.

Clinical data at the most recent follow-up

At the most recent follow-up, 18 patients had no subsequent surgery on both sides, 12 patients had a subsequent surgery (TKA) on both knees, and 30 patients had subsequent TKA on the side with intra-articular cell therapy; For the 66 knees without TKA revision at the most recent follow-up (average of 15 years, range 13 to 18 years), the Knee Scores remained higher (76.2 points ± 18) in the group with SC cell therapy as compared with the group with IA cell therapy (58.4 ± 25). Among the 18 patients who had no subsequent surgery on both sides, all preferred the knee with SC cell therapy (odds ratio (OR), 0.0007; 95% CI, 0.0000 to 0.0388; $p = 0.0004$).

A KL grade-4 OA increased the risk of a total knee arthroplasty compared with grade 1 to 3 OA (HR = 2.03 (95% CI = 1.12 to 3.18); $p = 0.02$). Increased age (per year, HR = 1.12 (95% CI = 1.03 to 1.25); $p = 0.03$) and weight (per kg, HR = 1.21 (95% CI = 1.09 to 1.32); $p = 0.01$) at the time of study enrollment were independent risk factors for future total knee arthroplasty; also, male participants were less likely to undergo total knee arthroplasty, as compared with women, but this was not significant ($p = 0.08$).

Discussion

The main finding of this study was that *both subchondral and intra-articular injections of BM-MSC injection resulted in a significant relief of pain symptoms able to postpone TKA; moreover, time conversion to TKA was longer in patients who underwent subchondral BM-MSCs injection*.

In recent years, the role of synovitis and subchondral bone on cartilage degradation has been confirmed. Cartilage integrity is dependent on the underlying subchondral bone and vice versa, as well as on a healthy synovium and the synovial fluid [17]. We have a limited understanding of the factors that predict success of a cell therapy to postpone total knee arthroplasty. It is now admitted that the joint cavity may be considered as a reservoir of MSCs and these MSCs might play for cartilage a role for joint homeostasis and for repair. The presence of MSCs in the synovial fluid of knees with primary osteoarthritis was first reported by Jones [15] and is numerically increased even in early osteoarthritis [16]. These MSCs are probably mobilized from the synovium (through the synovial fluid) towards the degenerative cartilage. Therefore, a treatment increasing their number in the joint [1, 18] may induce pain relief and functional improvement during a short time. Although this intra-articular injection was able to reduce pain during the first 12 months, it was not able to reduce synovitis and to decrease BMLs in the subchondral bone; and the pain improvement was not sustained in the long term with as results conversion to TKA for many knees; we can only speculate on these failures: joint MSCs are naturally shed from the synovium and are elevated with early OA, but their repair capacity might be limited due to interactions with SF hyaluronan that limit their adhesion to cartilage; additionally, the synovium, cartilage, and other joint structures share a

common progenitor that is distinct from that of bone tissue [16] suggesting that the synovium harbors a joint tissue–specific stem cell population, which could explain less effective results with bone marrow MScs injected in the joint.

The current study highlights the importance of subchondral bone marrow lesions as a determinant target for success. In adults with tibiofemoral osteoarthritis, the radiographic severity is not the only predictor of symptom's evolution; the MRI-based findings demonstrate that regression of subchondral bone marrow lesions after cell therapy had greater likelihood of postponing total knee arthroplasty than synovitis changes. The number of MSCs present in the subchondral bone decreases with age. So, there could be some advantage to inject MSCs or other substances [19–22] in the subchondral bone of OA patients where they can provide many bioactive mediators which have been shown to exert positive effects on joint tissues. With assumption that in OA the abnormality of the subchondral bone is related to a dysfunction of mesenchymal stem cells (low number and/or abnormal function), this deficiency might be corrected by transplantation of MSCs from the iliac crest. This new population of cells may have effect on pain relief, resolve bone marrow lesions, and decrease cartilage wear (by multiplication of cells or cytokine effect). In a bolus manner, MSCs could stick to the subchondral bone, when they are washed by the synovial fluid when injected in the joint.

The most important finding of this study is that, based on our results, with KL grade 1 to 3 knee osteoarthritis, subchondral cell therapy treatment could be utilized first instead of TKA treatment as primary treatment, since the treatment is able to postpone TKA during 15 years in some patients. Patients and surgeons 25 years ago embarked on total knee arthroplasty procedures in hope and expectation [23]: hope that loosening would not occur, infection would not appear, and hope that the TKA would provide pain relief for some period. Fortunately, the surgical problems now have been solved, and TKA has been changed from high-risk procedure for OA treatment to a successful surgical practice. However, expectations from patients have also changed over the past 25 years, and may be sometimes, the procedure has reached a stage where patient's expectations exceed what a TKA can offer. If success of TKA is patient's satisfaction with pain-relief, it falls short 20% of the time. Furthermore, incidence of primary TKA in young patients is increasing, and there is evidence that young age increases the risk of revision and dissatisfaction. Furthermore, due to a high prevalence of comorbidities, patients older than 85 years are 41% less likely to receive TKA than younger patients [24]. Due to all these reasons, there is a growing need for less invasive therapies in patients. Such therapy could be the use of autologous, bone marrow concentrate injected into the subchondral bone marrow of the affected joint(s).

Several limitations to our study exist; we do not know the factors that run the duration of the transplanted cells [25], and we do not know how long time they can survive in bone. However, the effects appear long-lasting since some knees are improved during 15 years. Some demographic factors as weight are unfavorable factor for treatment, but this factor may be partially corrected. The recipient knee is a "diseased" joint where several alterations have already occurred in the subchondral bone. Indeed, we can suspect that only a very small proportion of the administered cells are engrafted by the osteoarthritis bone. As the number of MSCs injected in the subchondral bone is a factor of improvement and as the number present in the bone marrow of the iliac crest decreased with age [26], this could a limit of the technique. However, we can remark that, whatever the age, we were able to get MSCs in the iliac crest.

In conclusion, implantation of MSCs in the subchondral bone of an osteoarthritic knee was more effective to postpone TKA than *intra-articular injection* of the same dose in the contralateral knee with the same grade of osteoarthritis.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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