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Allogeneic Mesenchymal Stem Cells Stimulate Cartilage Regeneration and Are Safe for Single-Stage Cartilage Repair in Humans upon Mixture with Recycled Autologous Chondrons

TOMMY S. DE WINDT,[a] LUCIENNE A. VONK,[a] INEKE C. M. SLAPER-CORTENBACH,[b] MARCEL P. H. VAN DEN BROEK,[b] RAZMARA NIZAK,[a] MATTIE H. P. VAN RIJEN,[a] ROEL A. DE WEGER,[c] WOUTER J. A. DHERT,[d] DANIEL B. F. SARIS[a,e]

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[a]Department of Orthopaedics, [b]Cell Therapy Facility, Department of Clinical Pharmacy, [c]Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands; [d]Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands; [e]MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, The Netherlands

Correspondence: Daniel B.F. Saris, M.D., Ph.D., Department of Orthopaedics, University Medical Center Utrecht, Heidelberglaan 100, 3584CX Utrecht, POB G05.228, The Netherlands. Telephone: 0031 88 75 569 51; Fax: +31 30 25 106 38; e-mail: d.saris@umcutrecht.nl Received February 18, 2016; accepted for publication June 29, 2016

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ABSTRACT

Traditionally, mesenchymal stem cells (MSCs) isolated from adult bone marrow were described as being capable of differentiating to various lineages including cartilage. Despite increasing interest in these MSCs, concerns regarding their safety, in vivo behavior and clinical effectiveness have restrained their clinical application. We hypothesized that MSCs have trophic effects that stimulate recycled chondrons (chondrocytes with their native pericellular matrix) to regenerate cartilage. Searching for a proof of principle, this phase I (first-in-man) clinical trial applied allogeneic MSCs mixed with either 10% or 20% recycled autologous cartilage-derived cells (chondrons) for treatment of cartilage defects in the knee in symptomatic cartilage defect patients. This unique first in man series demonstrated no treatment-related adverse events up to one year postoperatively. At 12 months, all patients showed statistically significant improvement in clinical outcome compared to baseline. Magnetic resonance imaging and second-look arthroscopies showed completely filled defects with regenerative cartilage tissue. Histological analysis on biopsies of the grafts indicated hyaline-like regeneration with a high concentration of proteoglycans and type II collagen. Short tandem repeat analysis showed the regenerative tissue only contained patient-own DNA. These findings support the novel insight that the use of allogeneic MSCs is safe and opens opportunities for other applications. Stem cell-induced paracrine mechanisms may play an important role in the chondrogenesis and successful tissue regeneration found. STEM CELLS *2016; 00:000–000*

SIGNIFICANCE STATEMENT

This is the first study showing allogeneic MSCs are safe and effective in stimulating cartilage regeneration in the knee when combined with autologous chondrons. The fact that one year after surgery, no stem cell DNA could be traced in the regenerative tissue, may confirm the recent view on MSCs as cellular moderators, that stimulate autologous tissue repair through paracrine mechanisms.

INTRODUCTION

Mesenchymal stem cells (MSCs, also known as multipotent mesenchymal stromal cells) are a nonhematopoietic adult stem cell population that are present in various tissues such as bone marrow, adipose tissue, synovial membrane, and others. Their ability to differentiate into lineages of mesenchymal tissues, including osteogenic, chondrogenic, neurogenic, myogenic and adipogenic, make them promising cells for the use in regenerative medicine [1]. Successful cartilage regeneration using cultured autologous MSCs has been shown in various small and large animal models and pilot studies in humans [2]. This is promising as it would eliminate the need for ex vivo chondrocyte expansion, which is necessary for the widely used autologous chondrocyte implantation (ACI) procedure pioneered by Brittberg et al. in 1994. [3] While successful treatment has been shown by this clinically approved advanced therapy medicinal product (ATMP), the downside is that the procedure requires two separate surgeries; one to harvest patient-own cartilage cells, and one to implant the expanded cells into the defect. Millions of cells are required for the repair of these large defects, which if left untreated, cause symptoms such as pain, limit function and may lead to osteoarthritis [4]. From a patient, treating physician and payers

Table 1. In- and exclusion criteria

Inclusion criteria	Exclusion criteria
• Age $\geq$18 and $\leq$45 years old • Symptomatic isolated cartilage defect (femoral condyle/trochlea) • Defect size $\geq$2cm^2 and $\leq$8 cm^2 • $\geq$50% of functional meniscus remaining • Stable knee ligaments (i.e. anterior and posterior cruciate ligaments)	• (History of) osteoarthritis, (Kellgren-Lawrence grade $\geq$3 x-ray. • Concomitant inflammatory disease (rheumatoid arthritis, metabolic bone disease, psoriasis, gout, symptomatic chondrocalcinosis) • (History of) septic arthritis • Malalignment requiring osteotomy • (History of) total menisectomy in the target knee joint • Any surgery in the knee joint 6 months prior to inclusion • Risk groups for MRI scanning (pacemakers, nerve stimulators, metal particles, stents, clips or implants • (Possible) pregnancy or breast feeding • Patients with severe anxiety for MRI or needles

perspective, a single-stage noncultured cell-based therapy would be a great advance. To ensure sufficient cells, expanded allogeneic MSCs could be used as an off-the-shelf cell product.

It was initially believed that engraftment and differentiation of MSCs would lead to neotissue formation and tissue repair [5]. However, more recently, it has been shown that MSCs can stimulate tissue repair by the secretion of potent paracrine factors and only a limited amount, if any, of MSCs actually engraft and differentiate in vivo [6]. Although several studies support this "paracrine or chondroinductive role" for tissue restoration by MSCs, no consensus has been reached on what the cell fate and mechanism of action of administered MSCs precisely are in vivo. No clinical proof of allogeneic MSCs mixed and used for signaling function has been shown while engraftment rate and paracrine signaling is thought to be affected by the source and dose of MSCs and the timing and route of administration [7].

In addition to their trophic properties, MSCs have also shown to possess anti-inflammatory and immunomodulatory effects [8]. The limited engraftment and immunomodulatory actions of MSCs, together with the fact that they have been shown to have low immunogenicity based on their low expression levels of human leukocyte antigen (HLA) major histocompatibility complex (MHC) class I and II, makes them candidates for allogeneic therapies. Clinical trials that use allogeneic MSCs focus primarily on their immunosuppressive role and include treatment of steroid-resistant graft-versus-host disease, acute respiratory distress syndrome and Crohn's disease in clinical trials [9]. However, differentiation of allogeneic MSCs induces immunogenicity, which might affect their use in tissue regeneration [10]. For local cartilage regeneration it would be desirable to administer cells directly to the defect. So far, no clinical data have been reported on the survival of allogeneic MSCs, or cell combinations, administered directly into a tissue defect. Therefore, it is especially relevant to know what the cell fate of allogeneic MSCs is in a clinical setting.

This study provides the unique initial description of the successful treatment of 10 patients with focal cartilage defects using a combination of 90% allogeneic MSCs and 10% recycled autologous chondrons (standard yield) or 80% allogeneic MSCs with 20% recycled autologous chondrons (high yield) in fibrin glue to focal cartilage defects in a phase I (first-in-man) clinical trial (the Instant MSC Product accompanying Autologous Chondron Transplantation (IMPACT) study, NCT02037204, https://www.youtube.com/watch?v=S3rIBjA03AA). Full ethical approval for trial execution was received (Central Committee on Research Involving Human Subjects (CCMO) and the UMC Utrecht under protocol number NL.40142.000.12). Patient and data safety was monitored by external independent observers and safety and efficacy of this treatment was already tested over a time frame of 6 months in a large animal model [11].

Methods

Study Design and Objectives

This is a phase I/II prospective monocenter study, investigating the feasibility and safety of a new tissue engineered ATMP for isolated articular cartilage defects. It is academically driven without commercial conflict or involvement. The primary objective of this study was to prove clinical safety and feasibility of IMPACT and demonstrate noninferiority in adverse event rate compared to ACI. The secondary objective was to determine the level of clinical improvement while the third objective was to examine parameters of structural repair.

Subject Enrollment Criteria

Patients were enrolled at the specialized knee clinic of the University Medical Center Utrecht. Confirmation of an isolated articular cartilage defect was obtained using a magnetic resonance imaging (MRI) scan and/or a previous arthroscopy performed in another orthopaedic center. If an isolated cartilage defect was present, patients were screened for eligibility to participate based on the inclusion and exclusion criteria. The inclusion criteria were defined as patients having a symptomatic isolated Modified Outerbridge Grade III or IV cartilage defect of 2 to 8 cm^2 on the femoral condyle or trochlea, with at least 50% of functional meniscus and stable knee ligaments. Exclusion criteria were signs of OA as defined by a Kellgren-Lawrence grade $\geq$ 3 on a x-ray, concomitant diseases that may have affected the joint (e.g., rheumatoid arthritis), malalignment of the knee requiring correction osteotomy, previous surgeries in the affected knee 6 months prior to inclusion and anxiety for MRI or needles. The inclusion and exclusion criteria are summarized in Table 1. Patients received the study information and a 3-week reflection period prior to signing informed consent. An independent physician was available for further questions prior to- and during the study period.

Surgical Procedure and ATMP Manufacturing Process

The surgical procedure was performed using a mini-arthrotomy. Cartilage defects were debrided to create stable surgical base and borders. This debrided tissue was transported to the Cell Therapy Facility where it was used for cellular recycling. Briefly,

minced cartilage was subjected to 40 minutes of enzymatic digestion using a mixture of thermolysin/collagenase II (Liberase GMP grade, Roche, Germany). This rapid digestion of cartilage was previously shown to produce chondrocytes with their pericellular matrix (chondrons), which have superior chondrogenic properties compared to chondrocytes [11, 12]. Allogeneic cryopreserved MSCs were thawed for mixture with chondrons. The MSCs used are classified as ATMPs and manufactured in the GMP-licensed Cell Therapy Facility of the UMC Utrecht from healthy donors as approved by the CCMO (Biobanking bone marrow for MSC expansion, NL41015.041.12).

Bone marrow was aspirated under general anesthesia from the iliac crest of 2 third party non-HLA matched healthy donors (age 2 and 5) for the treatment of sib patients. The surplus of the bone marrow was used for MSC expansion. The parent or legal guardian of the donor signed the informed consent as approved by the CCMO. Bone marrow aspirates were density separated and MSCs were isolated by plastic adherence and expanded using the MC3 systems and α-MEM (Minimal Essential medium) with L-glutamine from Macopharma (Tourcoing, France) supplemented with 5% platelet lysate and 3.3 IU/ml Heparin up to passage 3 as previously described [13, 14]. At passage 3 the MSCs were cryopreserved in 0.9% Sodium Chloride (Fresenius Kabi, Bad Homburg, Germany); 10% CryoSure-DMSO (WAK-Chemie Medical GmbH, Steinbach, Germany); 5% Human Serum Albumin (Cealb, Sanquin, Amsterdam, The Netherlands). Cell viability and fulfillment of the release criteria of MSCs was assessed for each vial thawed. The following release criteria were used: immunophenotype of the MSC: $>$70% $CD73^+$ cells, $>$70% $CD105^+$ cells, and $>$70% $CD90^+$ cells, and $<$10% $CD45^+$ cells and $<$1% $CD3^+$ cells [15] (Supporting Information Fig. S1); cell viability $>$ 90%, sterility tests according to the European Pharmacopeia: negative for aerobic and anaerobe bacteria, fungi, and yeast; mycoplasma $<$10 CFU/ml and endotoxin $<$ 1 IU/ml ($<$5 IU/kg/hour). After thawing, the MSCs were washed in 0.9% Sodium Chloride/10% Human Serum (Sanquin, Amsterdam, the Netherlands) Albumin and the concentration of DMSO in the end product is $<$0.001%. Autologous chondrons were run over a 100-μm cell strainer (BD Biosciences, San Diego, CA) to get rid of matrix residues, washed twice to reduce the presence of Liberase, counted using 3% acetic acid with methylene blue and mixed with the allogeneic MSCs at a 10:90 ratio (standard yield) or 20:80 ratio (high yield), depending on the amount of chondrons isolated which was dependent on the available amount of defect rim-derived cartilage. Cells were mixed in the fibrinogen component of fibrin glue (Beriplast, CSL Behring, Global) at 1.5–2 million cells/ml. After approximately 90 minutes, the knee was reopened through the mini-arthrotomy and the fibrin glue injected in the defect. Upon injection, the cell-laden fibrinogen was mixed with the thrombin component causing it to immediately gelate. The knee was flexed several times to guarantee local adherence of the fibrin glue scaffold before the knee was closed in layers. The procedure is illustrated in an animation (https://www.youtube.com/watch?v=S3rIBjA03AA).

Rehabilitation

All patients were dismissed one day after surgery and followed the same standardized phased rehabilitation protocol supervised by their own physiotherapist and adjusted to individual goals [16]. To ensure correct use if this protocol, a specialised physiotherapist contacted each patient and their physiotherapist prior to starting the protocol. Briefly, patients were nonweight bearing for 3 weeks with a gradual increase to full weight bearing at 9 weeks. Patients with a trochlear defect received a brace that was locked in extension for 3 weeks and worn during walking for 6 weeks. Joint circulation exercises such as heel slides and stationary cycling were recommended starting one day after surgery. Strength training started from week 7 onward and consisted of isometric quadriceps exercises followed by progressive closed chain exercises. Propriocepsis, open chain exercises and exercises on a home trainer were initiated from week 13 onwards. After 6 months, more intense walking exercises were allowed with a gradual increase in load. Higher impact activities were adjusted to sports and not allowed until 9 months after surgery. Sports with pivotal movements were not allowed during this study.

Follow-up

Safety Assessment. A standardized assessment performed by a physician (rheumatologist) was performed independent from the surgeon and investigators. All patients were assessed one day, two and four weeks, and 3, 6, and 12 months after surgery according to a standard assessment on general condition i.e. impression, temperature, heart rate and blood pressure and local inspection of the knee i.e. arthralgia, swelling, crepitation and motion. To monitor inflammation and signs for a foreign body response blood analysis (i.e., C-reactive protein [CRP] erythrocyte sedimentation rate [ESR] and leukocyte count) was performed after 1 day, 2 and 4 weeks after surgery. A data safety monitoring board consisting of an orthopaedic surgeon, a professor in rheumatology research and a statistician reviewed all patient data according to set intervals.

Patient Reported Outcome. To evaluate the clinical status of the patients treated with the IMPACT therapy, the included patients were asked to complete the Knee injury and Osteoarthritis Outcome Scoring (KOOS), The visual analog scale (VAS) for pain and the EuroQoL 5-Dimension Health Questionnaire (EQ5D) at baseline (before IMPACT therapy) and at 3, 6, and 12 months follow-up. The KOOS has been shown to be more sensitive and responsive than WOMAC in younger and more active patients and has been validated to assess the clinical improvement after cartilage regeneration [17]. The EQ5D is a widely used health-related quality of life (QoL) measure that contains five domains, namely, mobility, self-care, usual activities, pain/discomfort and anxiety/depression and includes a VAS for overall health [18]. It has been shown to be applicable to, and valid for, a wide range of health conditions and treatments [19–21].

MRI

A baseline and follow-up MRI scan (12 months) was used after surgery to assess structural repair. All MRI scans were performed on a 3-T clinical MR scanner (Achieva, Philips Healthcare, Best, The Netherlands). A standard protocol including proton density-weighted sequences in three planes and T2 multiecho sequence was used.

Second-Look Arthroscopy

One year after surgery, the consent for a second-look arthroscopy given at the start of the trial was re-evaluated and performed if patients gave approval. During the second look arthroscopy the lesion site was re-evaluated for degree of defect repair, integration

Table 2. Summary of the demographics and baseline characteristics ($n = 10$)

Characteristic	
Mean age in years (s.d.)	26 (5)
Males (n)	8
Mean length (m)	1·82 (0·1)
Mean weight (kg)	82·4 (9·4)
Mean symptom duration in months (s.d.)	15·3 (10·8)
Acute symptom onset (*n*)	7
Gradual symptom onset (*n*)	3
Previous knee surgery $n = 0$ (n)	5
Previous knee surgery $n = 1$ (n)	4
Previous knee surgery $n = 2$ (n)	1
Single cartilage defect (*n*)	10
Defect size postdebridement (cm^2) (s.d)	3·6 (0·7)
Defect location	
Medial femoral condyle	5
Lateral femoral condyle	4
Trochlea	1
Standard yield IMPACT treatment (*n*)	5
High yield IMPACT treatment (*n*)	5
Concomitant defect treated during surgery (*n*)	0
Concomitant meniscal damage (*n*)	2

with the native tissue and macroscopic appearance according to the International Cartilage Repair Society (ICRS) macroscopic evaluation system of cartilage repair [29, 30]. In addition, a 2-mm biopsy was taken from the center of the repair tissue to procure tissue for DNA analysis and stained for histological analysis.

Histological Analysis

To evaluate morphology, biopsies were formalin- fixed, embedded in paraffin and stained for proteoglycans, type I and II collagen. To evaluate collagen fiber orientation a Picrosirius red staining and polarized light microscopy was used. Briefly, samples were dehydrated using graded alcohol steps, immersed in xylene and embedded in paraffin. To evaluate morphology and proteoglycan content 0.125% Safranin-O (Merck, Germany counterstained with Weigert's haematoxylin (Klinipath, The Netherlands), 0.4% fast green (Merck) was used. A type I and II collagen immunostaining was used to determine the collagen deposition. Briefly, antigen retrieval was performed by subjecting the sections to 1 mg/mL pronase (Sigma-Aldrich) for 30 minutes at 37°C followed by 10 mg/mL hyaluronidase (Sigma-Aldrich) incubation for 30 minutes at 37°C. Subsequently, the sections were blocked using a 5% BSA in PBS solution for 30 minutes (type I collagen) or 1 hour (type II collagen) followed by an overnight incubation at 4°C with a primary antibody against human collagen type I (mouse-anti human type I collagen, 1/1,000 dilution in PBS = BSA-5%, AB6309, Abcam, Cambridge, UK) or type II collagen (mouse-anti human type II collagen, II-II6B3, 1/100 dilution in PBS-BSA-5%; Developmental Studies, Hybridoma Bank). After washing, the slides were incubated with a horseradish peroxidase-conjugated anti-mouse secondary antibody (1/100 dilution in PBS-BSA-5%) for 60 minutes at ambient temperature. Immunoreactivity was visualized using 3,3′-diaminobenzidine (DAB, Sigma-Aldrich). The sections were counterstained with Mayer's haematoxylin. For the Picro-Sirius-Red staining, sections were deparaffinized, stained using 0.1% sirius red F3B (Klinipath) in saturated aqueous picric acid for 1 hour followed by rapid dehydration in graded steps. Slides were mounted with Vectamount permenant mounting medium and analysed using a polarized light microscope (Olympus BX51) All samples were processed and stained using the exact same procedure (e.g., color baths).

Short Tandem Repeat Analysis

To identify the cellular composition of the repair tissue at 12 months, genomic DNA was isolated from both the chondrons and MSCs prior to implantation as well as from the one-year biopsies. Ten loci were amplified and sequenced and specific alleles for the donors were determined. The loci D2S1360, D7S1517, D8S1132, D9S1118, D10S2325, D11S554, D12S391, MYCL, P450CYP19 and SE33 were amplified and sequenced based on the EuroChimerism STR marker panel. The amount of DNA present for each donor was calculated from the areas of the electropherogram from which the ratio between two cell types could be calculated.

Statistical Analysis

Differences in clinical outcome between baseline and 3, 6, and 12 months after surgery were tested by a repeated-measures analysis of variance (ANOVA). To test the difference in outcome between the standard and high yield an independent samples *t* test was used. Statistics were performed using SPSS version 21.0 (IBM, Chicago, IL). To limit observer bias, clinical monitoring was performed by a rheumatologist independent of the treating surgeon. Similarly, videos of the second look arthroscopies with subsequent ICRS II macroscopic scores and MRI scans were reassessed by an investigator who was not part of the treatment regime (RN).

Results

Baseline Characteristics

The mean age of the 10 patients included in this study was 26 ± 5 and included 8 males. The mean post-debridement defect size was $3{\cdot}6\ cm^2 \pm 0{\cdot}7$. Five patients received the standard and 5 patients the high yield mixture. No difference in demographic data was found between the high and low yield group, respectively. Previous surgeries were performed in 5 patients. These included partial menisectomy ($n = 3$), debridement ($n = 1$) and bone marrow stimulation by microfracture ($n = 1$). The demographics and baseline characteristics are presented in Table 2.

Safety Assessment

All patients showed an increase in serum CRP levels one day after surgery, typical for a post-surgical procedure response. One week postoperatively, the CRP levels were decreased from a mean value of $13{\cdot}8 \pm 15{\cdot}2$ mg/L one day after surgery to $3{\cdot}4 \pm 6{\cdot}6$ mg/L at 6 weeks (Fig. 1A). The serum ESR remained low and stable over the measurement points (Fig. 1B). No patient showed any clinical sign of a foreign body response (fever/warmth of the knee, erythema, effusion or disproportionate swelling) as shown by the standardized assessment completed by the independent rheumatologist. One of the patients had increased CRP and ESR levels at week 6 (21 mg/L and 13 mm/hour, respectively), but showed no signs of rejection to the cell product as the knee was not red or warm, no effusion was observed and the knee could be flexed to 110°. One week later, all serum levels showed normal values. No serious unexpected suspected unexpected serious adverse reactions (SUSARS) were found and no reinterventions were performed. Adverse events included post-surgery events and symptoms during rehabilitation (Table 3). One adverse event included an incidental

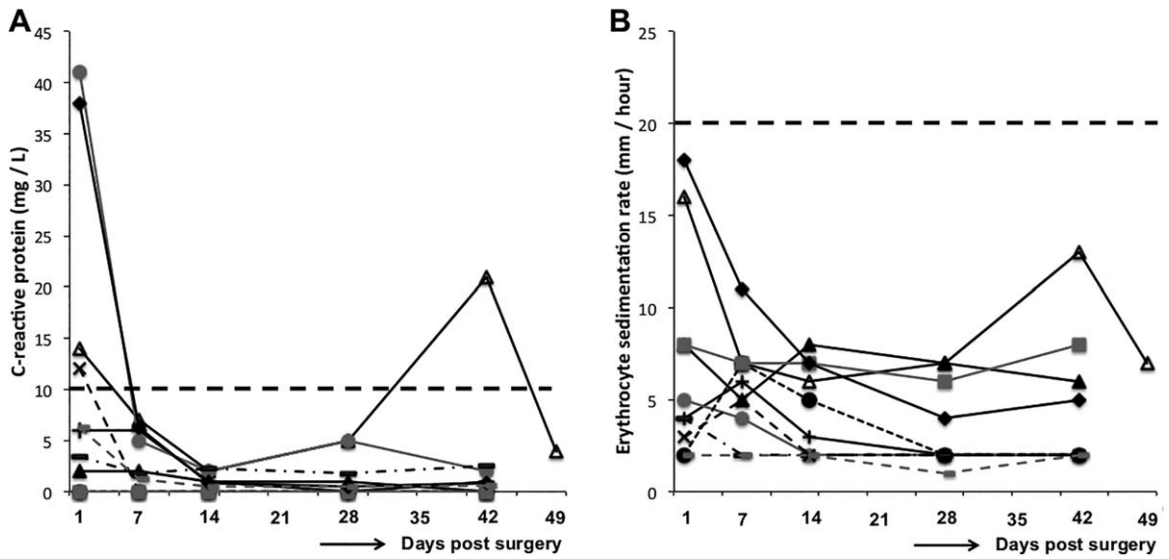

Figure 1. Initial safety assessment outcome. Serum levels of C-reactive protein (CRP, **A**) and erythrocyte sedimentation rate (ESR, B) at 1, 7, 14, 21, 21, 35, 42 and if applicable 49 days after surgery. The data are presented in line graphs for each patient and the maximum of the normal values are indicated by the dotted line.

Table 3. Treatment-related adverse events

Adverse event	*n*
Patients with at least one adverse event	8
Post-surgery (24 hours)	
Nausea and vomiting	2
Urinary retention	2
Headache	1
Vasovagal episode	1
Musculoskeletal (new episode)	
Arthralgia	3
Instability	1
Joint swelling	1
Crepitation	1
Second lesion (incidental finding second-look arthroscopy)	1

(asymptomatic) new defect found in the trochlea in a patient that had been treated with IMPACT for a defect on the femoral condyle (Table 3). In this patient, the index lesion was fully regenerated and the patient had good clinical improvement (improvement in overall KOOS from 60.1 to 89.8 at 12 months).

Short Tandem Repeat Analysis

Ten short tandem repeats (STRs), based on the EuroChimerism STR marker panel, were amplified by PCR from genomic DNA isolated from the cartilage part of seven biopsies. Subsequently, the lengths of the STR amplicons found in the biopsies were compared to the lengths of the amplicons measured from the MSC donors and the recipient patients. For each donor-recipient combination, at least seven loci could be used to define the origin of the genomic DNA from the biopsies (Supporting Information Table S1). For both the standard and high yield ratio's, the biopsies contained only autologous DNA, thus no DNA of the allogeneic MSCs could be detected at the detection limit of the assay (1 in 100,000 cells) (Supporting Information Fig. S2).

Clinical Outcome

The mean improvement in KOOS showed a gradual positive change from baseline to 12 months. The mean overall KOOS showed an improvement from $66{\cdot}1 \pm 19{\cdot}4$ to $87{\cdot}6 \pm 4{\cdot}8$. ($p = .009$) Statistically significant improvement ($p < .05$) in all subscales was seen with the biggest effect in the Sports and Recreation subscale (mean baseline score: $41{\cdot}0 \pm 28{\cdot}4$, mean 12 month score: $81{\cdot}0 \pm 4{\cdot}8$) ($p = .008$). All patients showed a statistically significant reduction in mean VAS pain score from baseline ($40{\cdot}4 \pm 22{\cdot}3$) to 12 months after surgery ($12{\cdot}4 \pm 9{\cdot}5$ ($p = .03$). No significant difference in clinical outcome was found between the standard yield and high yield groups ($p = .40$). The clinical outcome scores are presented in Figure 2.

MRI

Compared to baseline, MRI scans made 12 months after surgery showed complete filling of the defect, integration with both the subchondral bone and host tissue, and reduced subchondral bone reaction. Example figures are provided in Figure 3.

Second-Look Arthroscopy and Histology

Nine patients consented with a second-look arthroscopy at 12 months follow-up, which confirmed effective defect fill and integration in the surrounding tissue in all patients without loosening of the graft upon manipulation with an arthroscopic probe (examples provided in Supporting Information Fig. S3). Macroscopic ICRS evaluation suggested grade I (normal tissue) repair in six patients and grade II (nearly normal tissue) repair in three patients (Supporting Information Table S2). Abundant proteoglycans were present in the repair tissue as shown by Safranin-O staining on paraffin-embedded sections of full-thickness biopsies taken from the core of the repair tissue during the second-look arthroscopies (Fig. 4, immunostaining controls Supporting Information Fig. S4). Similarly the biopsies showed positive staining for both type I and II collagen, with type II collagen showing a more intense staining. The collagen fibers showed green to yellow polarizing colors for the best and mean biopsy. The picrosirius red staining showed early signs of perpendicular collagen fiber orientation in the best and mean biopsy [22]. The picrosirius red staining on the worst biopsy showed a more red polarizing color. (Fig. 4).

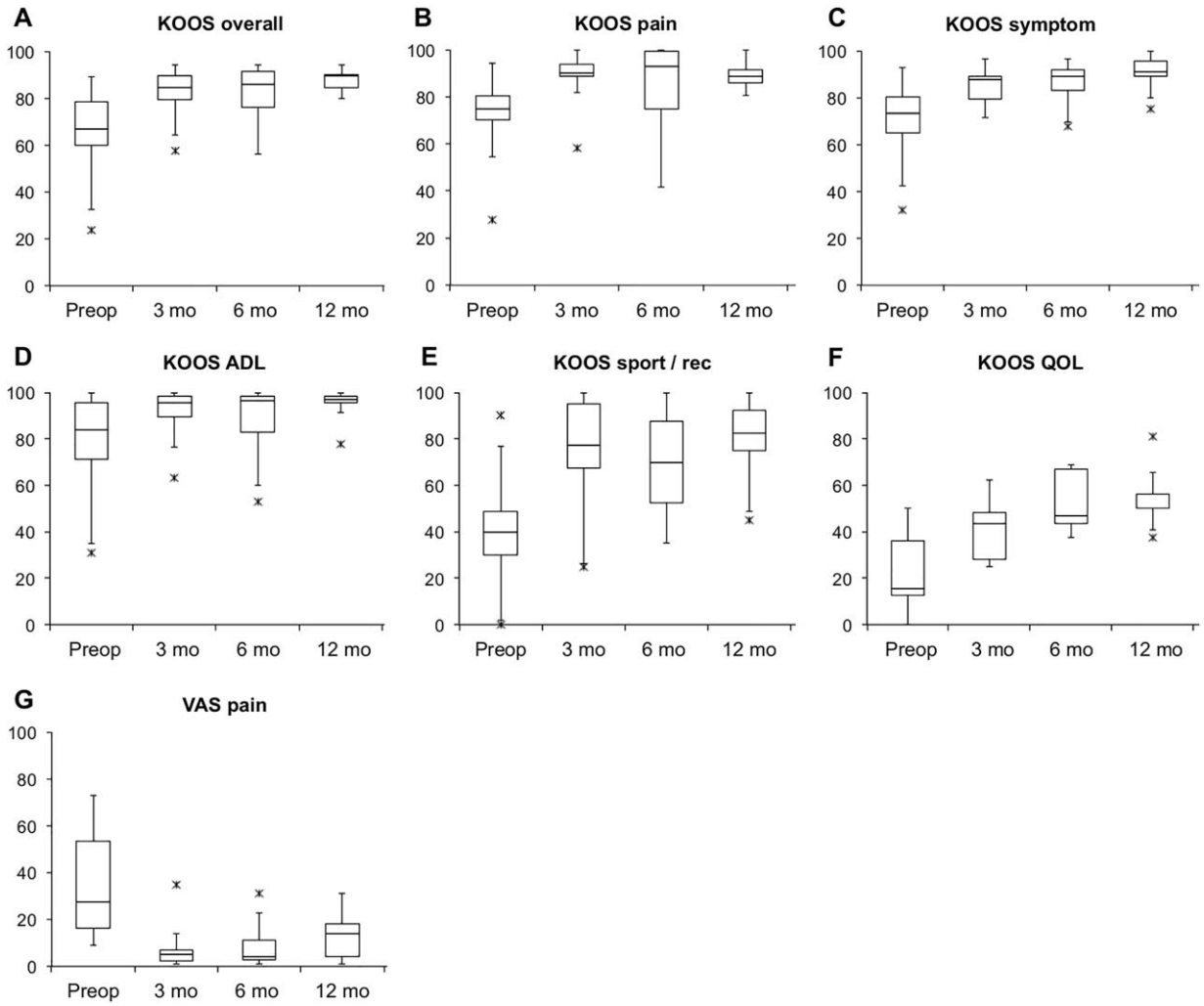

Figure 2. KOOS subgroups and VAS for pain from pre-operative (preop) to 3, 6 and 12 months (mo). All outliers are shown as individual data points. **(A)**: KOOS overall ($p < .0013$); (B): KOOS pain ($p < .0405$); (C): KOOS symptom ($p < .0006$); (D): KOOS activities of daily living (ADL, $p < .0402$); (E): KOOS sport and recreation (sport/rec, $p < .0016$); F, KOOS quality of life (QOL, $p < .0000$); G, VAS pain ($p < .0002$). Abbreviations: ADL, activities of daily living; KOOS, Knee injury and Osteoarthritis Outcome Score; QOL, quality of life; sport/rec, sport and recreation; VAS, visual analog scale.

Discussion

This is the first report to show safety of allogeneic MSC implantation for single-stage cartilage repair. It demonstrates that the proof of concept, in which rapidly isolated chondrons, which were recycled from debrided cartilage instead of harvested from a non-load bearing site of the knee, combined with allogeneic human bone marrow MSCs, is feasible, stimulates reproducible tissue regeneration and provides clinical improvement. No treatment related adverse events were observed up to one-year follow-up. In addition, no inflammation was detected by blood tests in the first 6 weeks after implantation.

These results indicate that the treatment approach is feasible and safe. Such a one-stage approach would have major benefits for patients as they would be able to immediately start with the rehabilitation following surgery, instead of having to wait on a cell expansion period. The early improvement in clinical outcome with fast reduction of joint swelling and normalization of joint function shown from 3 months onward may be explained by an immunomodalatory effect of the MSCs as described earlier [26]. In addition, the rehabilitation protocol was closely monitored and in line with a previous report showing improved outcome after an accelerated weight bearing program after cartilage repair [23]. However, as most reports show clinical outcome from 6 months onward, and this article is focused on safety in a small sample size, future analysis has to show the effect the treatment has on early clinical outcome.

Our findings indicate that the allogeneic MSCs used did not invoke an immune response. In general, cartilage is considered an immune privileged tissue as it has no blood and nerve supply and the resident chondrocytes are embedded in a dense extracellular matrix [24]. However, to allow better attachment and integration of the cell-containing fibrin glue, the subchondral bone was debrided causing bleeding and providing a direct connection to the immune system. It is known that undifferentiated MSCs do not provoke an immune response due to their low expression levels of MHC class I and II. However, for a tissue repair approach as used in this study it was unknown whether (part of) the MSCs would differentiate and induce a secondary (mild) immune response. It can be concluded that no allogeneic cells were present in the repair tissue after one year and that no severe immune responses were observed.

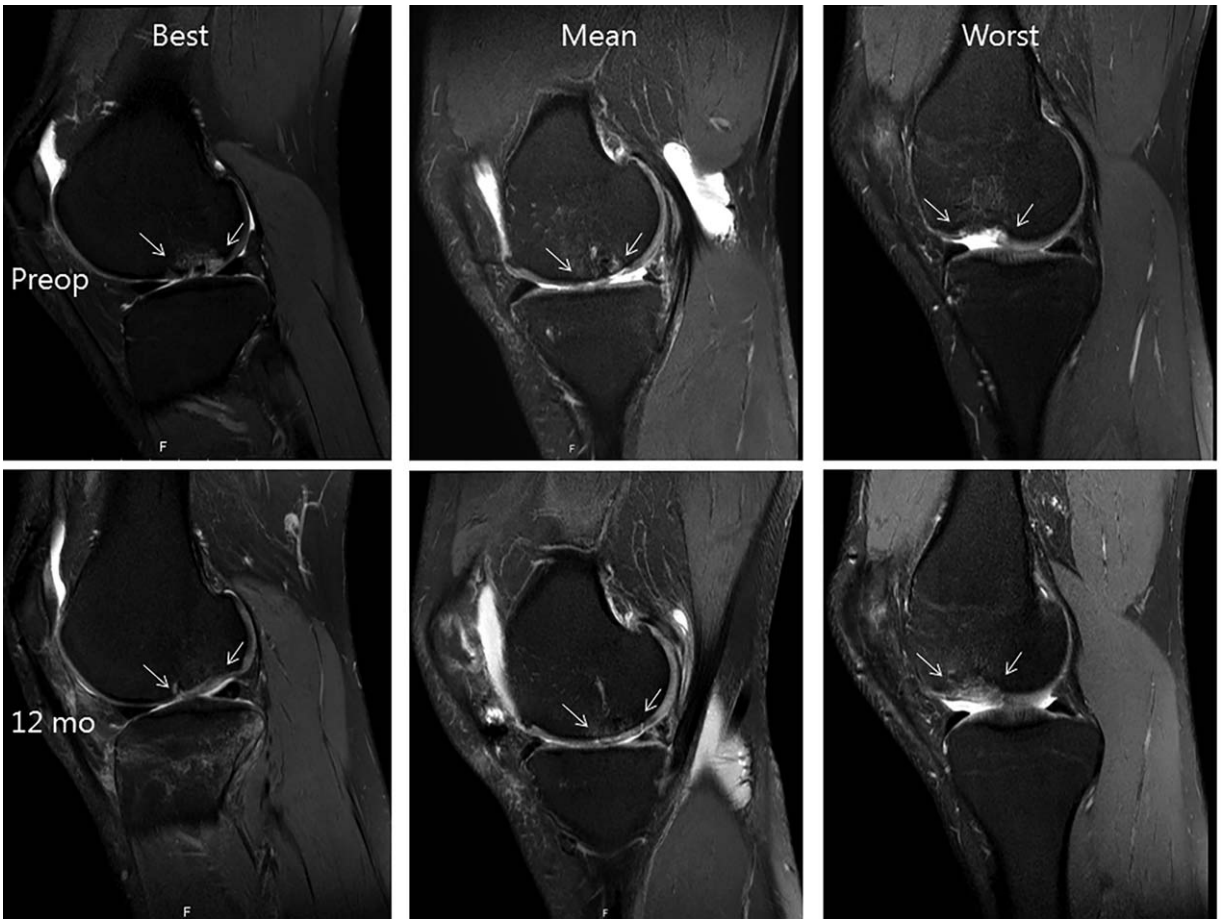

Figure 3. Magnetic Resonance Imaging preoperatively (preop, **A** and C) and 12 months after surgery (12 months, B and D). Representative images of the best, mean and worst results are obtained. Arrows indicate focal defect preoperatively and the repair tissue 12 months postoperatively. Abbreviations: mo, months.

However, the biopsy was taken from the core of the repair tissue, which although seems representable for the whole graft, may have missed (some) allogeneic MSCs in the periphery. It is also still possible that MSCs initially engrafted the repair tissue and differentiated, which might have led to a mild immune response over time with a gradual removal of the allogeneic cells. In rats it has been shown that allogeneic MSCs, which were implanted into infarcted myocardia, were eliminated from the tissue 5 weeks after transplantation due to an immune response invoked by differentiation, but their functional benefits were present for 5 months [25]. Therefore, it remains a question whether reimplantation of allogeneic MSCs for tissue repair strategies will activate a memory T-cell response to differentiating allogeneic cells. On the other hand, in vitro studies on cocultures of chondrocytes and MSCs have also shown that MSCs disappear from the cultures while the chondrocytes differentiate, and these studies were performed in the absence of any immune cells. So, it might very well be that cocultures stimulate the MSCs to disappear, while the MSCs stimulated the structural and functional cartilage restoration by paracrine effects. This is in line with the recent view on stem cells as site-regulated "drug-stores" that by secreting trophic factors, establish a regenerative microenvironment and regulate the local immune response [26]. These "off-the-shelf" cells, allow for a single-stage procedure without having to subject the patient to an additional bone-marrow aspiration. This is in contrast to the more traditional view on MSCs as stem cells with multipotent differentiation capacity [26]. Regardless how long the allogeneic MSCs were present and whether or not they temporarily differentiated, the MSCs must have stimulated the autologous chondrons to produce new cartilage. The number of autologous chondrons implanted in the fibrin glue mixture alone was likely to be insufficient for repairing such large defects which do not show spontaneous healing and are less responsive to bone marrow stimulation alone. Besides, close cell-cell contact is essential for paracrine signalling and cartilage regeneration [8]. Thus, it is most likely that the coimplanted chondrons are stimulated to proliferate and produce (neo)cartilage instead of the resident chondrocytes in the neighbouring cartilage. This would mean that a coimplantation of allogeneic MSCs with chondrons is preferred over using allogeneic MSCs alone. The added value of using chondrons instead of chondrocytes has been shown by in vitro and in vivo studies; chondrons produced more proteoglycans, the type II collagen they produce contains more cross-links and several membrane receptors that stimulate enzymatic cartilage-degradation upon activation are shielded by the pericellular matrix [27, 28]. In our *in vivo* studies, an advantage of using a combination of chondrons and MSCs was also observed when compared to chondrons or MSCs alone [11].

In the present study, MRI scans, second-look arthroscopies, and histology showed hyaline like tissue regeneration

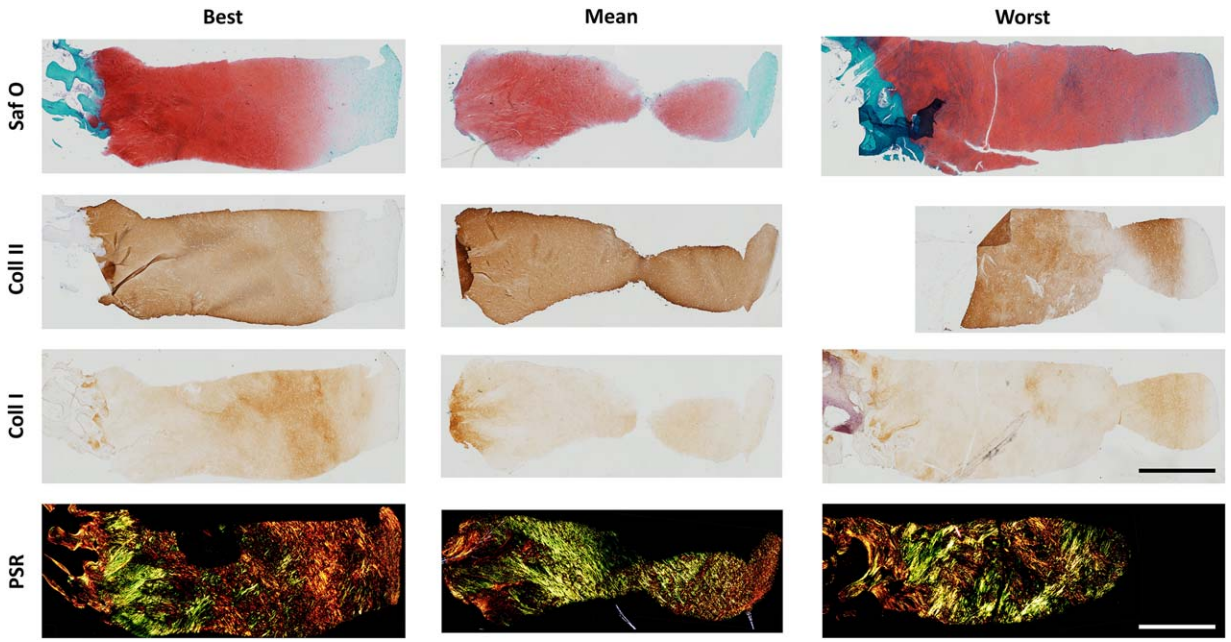

Figure 4. Saf O staining (best, mean, worst), Coll II and I immunostaining (best, mean, worst) and PSR red staining (best, mean, worst) on biopsies from the core of the repair tissue 12 months after surgery. Best, mean and worst samples were selected based on the intensity and distribution of Saf O and Coll II and organization of the collagen fibrils in the PSR stainings. Pictures are taken from the subchondral bone (left) to the cartilage surface (right). Scale bar indicates 1 mm. Positive and negative isotype controls for type I and type II collagen immunostainings can be found in Supporting Information Figure S4. Abbreviations: Coll II and I, type II and I collagen; PSR, picrosirius red staining; Saf O, safranin-O proteoglycan.

with good integration with the native tissue and signs of perpendicular collagen fiber orientation. The quality of the repair tissue was found to be similar or even superior to the histological results shown after ACI, especially considering the collagen type II staining [29, 30]. The picrosirius red staining indicated perpendicular collagen fiber orientation in the best and mean biopsy while showing a more red polarizing color in the worst biopsy. These findings may underline the importance of picrosirius red staining which, to the best of our knowledge, has not been explored for histological evaluation of patient biopsies and warrants future analysis in a larger sample size. In fact, in some of the sections perpendicular collagen fiber orientation is observed on the cartilage surface by picrosirius red staining, without any visible staining for type I or type II collagen. It is yet unclear what the exact composition of this superficial tissue is. For now, the clinical outcome shown in this study is non-inferior to one-year outcomes after ACI, making IMPACT a likely candidate to replace ACI, once long-term safety and efficacy have been shown similar to that of ACI. As a large batch of allogeneic MSCs can be cultured from one donor bone-marrow aspirate and used to treat multiple patients, the cell culture is cheaper, and less susceptible to donor variation compared to ACI. An early health technology assessment predicted a reduction in costs of this single-stage treatment compared to the two-stage ACI, assuming noninferiority, of at least €10.000-per patient treated (data submitted). Shipping frozen allogeneic cells and a closed system to mince the debrided cartilage, isolate and wash chondrons would overcome current logistical challenges and the need for a GMP-licensed cell therapy facility. An increase in patient comfort, by use of a single operation and recycled cartilage tissue rather than iatrogenic harm caused by a biopsy from healthy cartilage is at least as important. Indeed, chondrocytes derived from debrided tissue have shown greater chondrogenic capacity compared to non-weight bearing healthy cartilage which if used for biopsies, is associated with donor-site morbidity [31]. However, a larger cohort of patients as well as long-term follow-up would be necessary to confirm these proposed advantages.

Future analysis (in a larger cohort) will reveal if the clinical outcome achieved will provide durable repair and improve cost-effectiveness. The findings of this unique first-in-man study demonstrate that allogeneic MSCs can be a safe cell source for tissue regeneration in a clinical setting and that instead of engraftment or differentiation, allogeneic MSCs likely stimulate tissue regeneration through paracrine mechanisms with satisfying improvement in clinical outcomes.

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Author Contributions

T.S.W.: Conception and design, provision of study material/patients, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; L.A.V.: Conception and design, provision of study material, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; I.C.S.: Conception and design, provision of study material, collection and assembly of data, manuscript writing, final approval of manuscript; M.H.P.B.: Conception and design, provision of study material, manuscript writing, final approval of manuscript; R.N.: Provision of study material/patients, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; M.H.P. van Rijen: Collection and assembly of data, manuscript writing, final approval of manuscript; R.W.: Conception and design, provision of study material, manuscript writing, final approval of manuscript; W.J.A.D.: Conception and design, manuscript writing, final approval of manuscript; D.B.F.S.: Conception and design, provision of study material/patients, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflict of Interest

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