

Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study

Marjolijn Duijvestein,¹ Anne Christine W Vos,¹ Helene Roelofs,² Manon E Wildenberg,¹ Barbara B Wendrich,¹ Henricus W Verspaget,¹ Engelina M C Kooy-Winkelaar,² Frits Koning,² Jaap Jan Zwaginga,² Herma H Fidder,¹ Auke P Verhaar,¹ Willem E Fibbe,² Gijs R van den Brink,¹ Daniel W Hommes¹

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¹Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, The Netherlands

²Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands

Correspondence to

Dr M Duijvestein, Department of Gastroenterology and Hepatology, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, the Netherlands; m.duijvestein@lumc.nl

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ABSTRACT

Background and aim Mesenchymal stromal cells (MSCs) are pluripotent cells that have immunosuppressive effects both in vitro and in experimental colitis. Promising results of MSC therapy have been obtained in patients with severe graft versus host disease of the gut. Our objective was to determine the safety and feasibility of autologous bone marrow derived MSC therapy in patients with refractory Crohn's disease.

Patients and intervention 10 adult patients with refractory Crohn's disease (eight females and two males) underwent bone marrow aspiration under local anaesthesia. Bone marrow MSCs were isolated and expanded ex vivo. MSCs were tested for phenotype and functionality in vitro. 9 patients received two doses of $1-2 \times 10^6$ cells/kg body weight, intravenously, 7 days apart. During follow-up, possible side effects and changes in patients' Crohn's disease activity index (CDAI) scores were monitored. Colonoscopies were performed at weeks 0 and 6, and mucosal inflammation was assessed by using the Crohn's disease endoscopic index of severity.

Results MSCs isolated from patients with Crohn's disease showed similar morphology, phenotype and growth potential compared to MSCs from healthy donors. Importantly, immunomodulatory capacity was intact, as Crohn's disease MSCs significantly reduced peripheral blood mononuclear cell proliferation in vitro. MSC infusion was without side effects, besides a mild allergic reaction probably due to the cryopreservant DMSO in one patient. Baseline median CDAI was 326 (224-378). Three patients showed clinical response (CDAI decrease ≥ 70 from baseline) 6 weeks post-treatment; conversely three patients required surgery due to disease worsening.

Conclusions Administration of autologous bone marrow derived MSCs appears safe and feasible in the treatment of refractory Crohn's disease. No serious adverse events were detected during bone marrow harvesting and administration.

INTRODUCTION

Inflammatory bowel diseases are chronic inflammatory disorders of the gastrointestinal tract, including Crohn's disease and ulcerative colitis. Despite the improvements in the management of inflammatory bowel diseases with the introduction of anti-tumour necrosis factor (TNF) compounds,

Significance of this study

What is already known about this subject?

- MSCs have immunosuppressive effects on T and B cells.
- MSCs ameliorate experimental colitis.
- MSCs are effective in the treatment of acute and severe GvHD of the gut.

What are the new findings?

- MSCs derived from the bone marrow of refractory Crohn's disease patients have identical characteristics compared to MSCs from healthy donors and have intact immunomodulatory capacities in vitro.
- Administration of autologous bone marrow-derived MSCs is safe and feasible in the treatment of refractory Crohn's disease.

How might it impact on clinical practice in the foreseeable future?

- In this manuscript we describe the results of a phase I trial on the application of MSCs in luminal Crohn's disease. Our observations can be the basis for further clinical trials on autologous bmMSCs as a potential treatment for Crohn's disease.

remission often remains difficult to maintain. Many patients suffer from a poor quality of life due to disease relapse, repeated surgeries, extra-intestinal manifestations and drug side-effects. Therefore, novel therapeutic approaches need to be explored.

Mesenchymal stromal cells (MSCs) are non-haematopoietic stromal cells exhibiting multilineage differentiation capacity and the ability to mediate immunosuppressive and anti-inflammatory effects.¹⁻³ MSCs are easily isolated from various tissues,⁴⁻⁶ including the bone marrow, and are capable of ex vivo expansion. Moreover, MSCs can be cryopreserved without loss of phenotype or differentiation potential.⁷ Systemic infusion of MSCs ameliorated the clinical and histopathological severity of experimental colitis, abrogating body weight loss, diarrhoea, and inflammation and

increasing survival.^{8–9} Furthermore, in humans, transplantation of bone marrow (bm) derived MSCs has led to improvement of corticoid refractory graft versus host disease (GvHD), including GvHD of the gut^{10–11} and MSCs obtained from adipose tissue induced healing in complex perianal fistulas in patients with Crohn's disease.¹² Although the mechanisms underlying these effects are not fully elucidated, it has been shown that both cell–cell contact and the secretion of growth factors and cytokines are involved.^{13–14} The potential role of MSCs in the modulation of immune responses and tissue regeneration aroused interest to use MSCs as a novel cellular therapy to treat Crohn's disease.¹⁵

In this clinical phase I study, we evaluated the safety and feasibility of intravenous infusion of autologous bmMSC in refractory Crohn's disease patients. Additionally, the functionality of these bmMSCs was studied focussing on culture potential, morphology, cell surface marker profiling, differentiation potential and immunosuppressive properties. Finally, the effect of bmMSCs on various drugs used to treat Crohn's disease was assessed.

METHODS

Patient selection

On 14 January 2008, the Medical Ethical Committee of the Leiden University Medical Center (LUMC) and the Central Committee on Research involving Human Subject (CCMO, The Hague, the Netherlands) approved this phase I study on autologous bmMSCs in the treatment of refractory Crohn's disease (registered in the Netherlands National Trial Register under study number NTR1360 <http://www.trialregister.nl/>). All patients gave written informed consent. Criteria for patient inclusion were that patients were at least 18 years of age and had moderate to severe Crohn's disease, as defined by a baseline Crohn's Disease Activity Index (CDAI) score between 220 and 450. Furthermore, patients had to be refractory to the standard treatment options for Crohn's disease. We defined refractory patients as patients that, at some time during the course of the disease, must have received steroids, immunosuppressive agents (eg, azathioprine, 6-mercaptopurine or methotrexate) or anti-TNF therapy which did not result in an adequate response to treatment. The following medications were allowed: 5-aminosalicylates and corticosteroids (at a stable dosage regimen for at least 4 weeks) and methotrexate, azathioprine or 6-mercaptopurine (at least 12 weeks, with stable dosage regimen for at least 8 weeks). Infliximab was discontinued at least 8 weeks prior to enrolment. All patients continued current treatment at the time of infusion. Before bone marrow harvest for MSC isolation and expansion, patients were thoroughly screened including medical history, physical examination, standard laboratory investigations and chest x-ray to rule out tuberculosis. Each patient was also screened for HIV, syphilis, hepatitis B and C virus. Patients were excluded if they had a history of lymphoproliferative disease or malignancy within the past 5 years, when they exhibited serious infections or when in need of immediate surgery. Colonoscopy was performed at baseline to confirm disease activity.

Laboratory methods for clinical expansion of MSCs

MSC isolation and expansion

MSCs were expanded according to a common protocol devised by the European Group for Blood and Bone Marrow Transplantation developmental committee, as previously described.¹⁰ Bone marrow was harvested by aspiration from the iliac crest from patients under local anaesthesia in the outpatient clinic.

Bone marrow mononuclear cells (MNCs) were isolated by Ficoll density gradient (density 1.077 g/cm³) centrifugation. Washed cells were resuspended in Dulbecco's modified Eagle's-low glucose medium (Invitrogen, Paisley, UK) supplemented with penicillin and streptomycin (Lonza, Verviers, Belgium) and 10% fetal bovine serum (FBS; HyClone, Logan, Utah, USA), without any additional growth factors. MNCs were plated at a density of 160 000 cells/cm². Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂ in 175 cm² flasks (Greiner Bio-One, Frickenhausen, Germany). When the cultures reached near confluence (>80%), the cells were detached by treatment with trypsin/EDTA (Lonza, Verviers, Belgium) and replated at a density of 4000 cells/cm². MSCs were passaged up to a maximum of three times. When sufficient MSCs were expanded, cells were harvested and cryopreserved in isotonic buffered salt solution supplemented with 10% dimethyl sulphoxide (LUMC Pharmacy, Leiden, The Netherlands). Data on MSCs obtained from healthy donors matched for age and gender, was obtained from previous studies.^{10–16–17} MSCs for these studies were sourced either from a family or non-related (third party) donor. Donors were informed about and consented to the possibility of the use of their MSCs for preclinical studies/analysis. All donors underwent routine donor control examination and screening tests, according to the standard procedures required for bone marrow donors. Following eligibility, donors donated 50–100cc of bone marrow under local anaesthesia as described above.

Characterisation of MSC products

Morphology was monitored twice a week throughout the culture period by light microscopy. *Cell viability* was determined at each passage and harvest procedure by Trypan blue staining in a Bürker chamber. *Immunophenotyping* of cultured MSCs was performed using flow cytometry. The following markers were analysed: HLA II (DR), CD73, CD90, CD31, CD34, CD45, CD80 (Becton Dickinson, Franklin Lakes, New Jersey, USA) and CD105 (Ancell, Bayport, Minnesota, USA). The samples were analysed on a FACSCalibur using CellQuest Pro software (Becton Dickinson). *Absence of contamination by pathogens* was tested at culture initiation and harvest of the MSC product by aerobic and anaerobic cultures (Bactec plus aerobe/F and Bactec plus anaerobe/F; Becton Dickinson). *Genetic stability* of the expanded MSCs was tested by karyotype analysis using a standard G-banding procedure.

Clinical application of MSC products

Release criteria for clinical use of MSCs included product sterility, absence of visible cell clumps, spindle-shape morphology, expression of CD73, CD90 and CD105 surface molecules (>90%) and a normal karyotype in at least 20 observed metaphases.

Laboratory methods for supportive research

In vitro differentiation

MSCs were plated at 5 × 10⁴ cells/cm² in 24-well culture plates and kept in complete medium until 80–90% confluency was reached. For osteogenic differentiation cells were stimulated for 21 days in standard medium supplemented with 50 µg/ml ascorbic acid, 10 mM β-glycerolphosphate and 10⁻⁸ M dexamethasone and were stained with fast blue for alkaline phosphatase. For adipogenic differentiation, cultures were stimulated for 21 days with complete medium supplemented with 0.5 mM 3-isobutyl-1-methylxanthine, 100 µM indomethacin, 5 µg/ml insulin and 10⁻⁶ M dexamethasone. Lipid droplets were revealed by staining with Oil Red O. Control MSCs were grown in

non-conditioned medium. All chemicals were from Sigma-Aldrich (St Louis, Missouri, USA).

MSC/peripheral blood mononuclear cell proliferation assay

Cultured MSCs from patients with Crohn's disease were plated in flat-bottom 96-well plates (Costar, Zwijndrecht, the Netherlands) and allowed to attach overnight. PBMCs were isolated from whole blood of patients with Crohn's disease before MSC infusion. PBMCs were stimulated anti-CD28/anti-CD3 coated Dynabeads (1 bead/5 cells; Invitrogen) per 1×10⁶ cells and were seeded in IMDM with 5% human serum (Sanquin, Amsterdam, The Netherlands), 5% FBS and 100 IU/ml IL-2 (LUMC Pharmacy) per well. Proliferation was measured by ³H-thymidine incorporation. In order to test the effect of MSCs in addition to Crohn's disease medication on proliferating PBMCs, the proliferation assay was also performed in the presence of indicated medications: 10 µg/ml infliximab, 10 µg/ml adalimumab, 10 µM dexamethasone (Sigma-Aldrich), 1 µM azathioprine (Sigma-Aldrich), 4 µM 6-mercaptopurine (Sigma-Aldrich), and 100 µg/ml methotrexate (Pharmachemie BV, Haarlem, The Netherlands) performed as described above.

Cell bead array cytometric assay

Production of TNFα, interleukin (IL)-1b, IL-10, and IL-6 in MSC/PBMC supernatants, colon biopsy homogenates and serum was determined using a CBA kit according to the manufacturer's instructions (BD Biosciences, San Jose, CA).

Study design

Patients received two doses of MSCs, 7 days apart, at weeks 0 and 1. Just before clinical application, cryopreserved cells were thawed and cells were infused intravenously at a target dose of 1–2×10⁶ cells/kg body weight. Patients were clinically assessed at weeks 0, 1, 2, 4, 6 and 14. At each visit, adverse events were ascertained, concomitant medications were recorded and samples for clinical laboratory evaluations and the patients' CDAI scores were obtained. Colonoscopies were performed at weeks 0 and 6, and mucosal inflammation was assessed using the Crohn's disease endoscopic index of severity (CDEIS). The study flow chart is depicted in figure 1.

Statistical analysis

Data were analysed using SPSS or GraphPad. Analyses included the Kruskal–Wallis test followed by Dunn's multiple comparisons (multiple comparisons), two-sided t test and the Wilcoxon signed-rank test for paired data. p Values <0.05 were considered significant.

RESULTS

Patients

In total ten patients (eight females/two males, median age 32.5 and range 19–42 years) with moderate to severe Crohn's disease

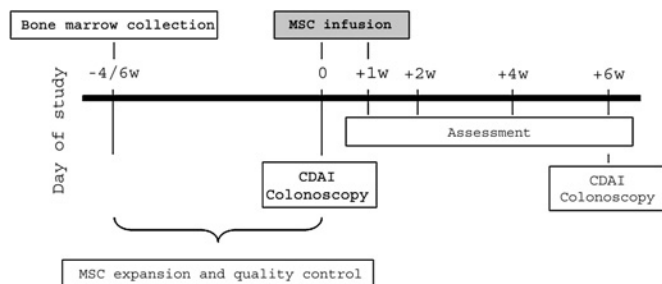


Figure 1 Study flow chart.

Table 1 Baseline characteristics of included patients

	1	2	3	4	5	6	7	8	9	10
Age (years)	24	36	33	34	34	42	29	32	19	30
Sex	F	F	F	F	F	M	M	F	F	F
Disease duration (years)	10	5	15	3	11	4	7	12	3	10
CDAI at screening	442	346	283	316	337	237	316	280	255	277
Baseline CDAI	332	341	254	326	350	266	378	224	NA	304
Disease localisation	Ileocolonic	Colonic, ileum	Colonic, ileum ND	Ileum	Ileum	Colonic	Colonic, ileum	Colonic, ileum ND	Colonic, ileum ND	Colonic
Perianal disease	y	n	y, perianal abscess (inactive)	n	y (inactive)	n	y (inactive)	n	n	y (inactive)
Extra-intestinal manifestations	Arthralgias	Arthralgias	n	Arthralgias	n	n	cheilitis granulomatosa	n	n	n
Current medical therapies	CS 50mg, AZA, MTX, ADA	MTX, ADA	CS 10mg	CS 5mg	CS 40mg	MTX	CS 30mg, 6-MP	CS 5mg, MTX	NA	CS 5mg, MTX
Height (cm)	175	167	174	172	158	160	187.5	173	157	161
Weight (kg)	59.3	68.6	74	99.9	46	78	107.5	113.2	50.5	53.1
Current smoker/smoking history	n/n	n/n	n/n	n/y	n/n	n/y	y/y	n/n	n/y	n/y
Prior medical therapies	CS, AZA, IFX, MTX, CZP, ADA	5-ASA, CS, AZA, IFX, MTX, ADA	5-ASA, CS, ATB, AZA, IFX, MTX, CZP, ADA	5-ASA, CS, 6MP, IFX, MTX, CZP, ADA	5-ASA, CS, AZA, IFX, MTX, CZP, ADA, TAC	CS, AZA, IFX, MTX, ADA	5-ASA, CS, AZA, IFX, MTX, CZP, ADA	5-ASA, CS, AZA, IFX, MTX, CZP, ADA	5-ASA, CS, AZA, IFX, MTX, CZP, ADA	5-ASA, CS, AZA, IFX, MTX, CZP, ADA, HSCT
Prior surgeries	Ileocecal resection	n	Ileocecal resection	Ileocecal resection	Ileocecal resection, colectomy on colon transversum	n	n	n	n	n

ADA, adalimumab; 5-ASA, mesalazine; ATB, antibiotics; AZA, azathioprine; CS, corticosteroids; CZP, certolizumab pegol; F, female; HSCT, mobilisation phase of haematopoietic stem cell transplantation; IFX, infliximab; M, male; 6-MP, 6-mercaptopurine; MTX, methotrexate; n, no; y, yes; NA, not applicable; ND, not determined; TAC, tacrolimus;

Table 2 Adverse events in MSC treated patients (weeks 0–6)

	n	Patient number
<i>Likely related to MSC infusion</i>		
Allergic reaction	1	4
Typical taste and smell	9	All
Headache	3	1, 4, 7
<i>Unlikely related</i>		
Worsening CD*	2	1, 7
Dizziness	1	1
Nausea	2	1, 2
Vomiting	1	1
Bloating	1	3
Abdominal pain	3	3, 5, 8
Haemorrhoid	1	4
Fever	1	4
Lack of appetite	2	1, 4
Fatigue	2	5, 8
Diarrhoea	1	8
Common cold	1	10
Otitis media acuta	1	2

*Two serious adverse events were reported due to worsening of disease requiring hospitalisation.

(median CDAI score at screening of 299.5 and range 255–442) were included in the study and underwent bone marrow aspiration under local anaesthesia. Besides some local pain at the puncture site afterwards, bone marrow aspiration was well tolerated by all patients. Baseline characteristics are presented in table 1. The bone marrow aspiration procedure resulted in sufficient bone marrow to expand MSC up to the required therapeutic doses (supplementary table 1).

One patient with a CDAI score of 255 at screening showed no active disease on colonoscopy and was therefore excluded for further MSC administration (patient 9). The baseline median CDAI score of the nine treated patients was 326 (range 224–378). During MSC infusion, patients were closely monitored. MSC infusion was successful and without relevant side effects. In one patient a transient mild allergic reaction occurred which was probably due to the cryopreservant DMSO.

Moreover, all patients noticed the typical smell and taste due to the DMSO up to 48 h after infusion. Other adverse events in the first 6 weeks of the protocol were ruled unlikely to be associated with MSC treatment and included adverse events such as common cold and headache (table 2).

bmMSC from refractory Crohn’s disease patients are comparable to MSCs from healthy donors

Approximately 100 ml of bone marrow was aspirated from each patient and bmMSCs were isolated and cultured. MSCs from Crohn’s disease patients showed the typical spindle-shaped morphology and similar growth potential and yield compared to MSCs from healthy donors (Supplementary figure 1). Depending on the number of cells needed and the yield of cells, MSCs were harvested in the first, second or third passage (Supplementary table 1). All MSC cultures showed normal karyotyping. Immunophenotypical characterisation was performed by FACS analysis, and showed similar phenotyping as described for healthy controls (table 3).

Furthermore, Crohn’s disease MSCs were able to differentiate along the osteogenic and adipogenic lineages when cultured in appropriate culture medium (figure 2).

bmMSCs from refractory Crohn’s disease patients suppress immune responses in vitro

In the presence of autologous bmMSCs proliferation of PBMCs was reduced in a cell dose-dependent fashion (figure 3A) and decreased TNF α production was observed. An increase of IL-1b and IL-6 was seen, as well as an increase in the regulatory cytokine IL-10 (figure 3B). MSC proliferation was not affected by co-medication (infliximab, adalimumab, dexamethasone, azathioprine, 6-mercaptopurine and methotrexate) in vitro (data not shown). However, MSCs do have an additive effect on inhibition of PBMC proliferation by various Crohn’s disease medication in vitro (figure 4).

Clinical response

Clinical assessment was performed on all patients using CDAI scoring. Two patients were excluded before the primary endpoint was met. The first patient was a chronic severe steroid

Table 3 Flow cytometric analysis of cultured bone marrow mesenchymal stromal cells from patients with Crohn’s disease and healthy donors matched for gender and age (mean/median age of patients with Crohn’s disease patients 31.3/32.5 and healthy donors 32.1/32.5)

	Patient number										Average
	1	2	3	4	5	6	7	8	9	10	
HLA-DR	5.1	5.7	7.9	5.7	1.0	5.4	0.8	0.9	5.0	5.8	4.3
CD31	1.9	2.1	1.7	2.1	1.7	1.1	2.2	1.6	2.0	2.0	1.8
CD73	99.9	99.7	99.9	99.6	99.3	99.7	99.9	99.2	90.4	98.1	98.6
CD45	1.3	1.6	1.9	2.1	3.6	1.3	0.9	1.2	1.4	1.3	1.7
CD105	100.0	99.9	100.0	100.0	100.0	100.0	100.0	99.9	99.9	100.0	100.0
CD80	3.1	6.2	10.3	18.0	4.2	3.3	1.0	0.2	0.3	29.4	7.6
CD90	100.0	100.0	99.9	99.9	99.3	99.6	99.8	100.0	99.8	99.9	99.8
CD34	6.0	4.0	4.6	9.5	2.8	5.6	6.5	0.8	10.1	4.3	5.4
	Healthy donor number										Average
	1	2	3	4	5	6	7	8	9	10	
HLA-DR	10.5	3.7	0.6	4.2	12.8	10.1	1.4	9.1	5.3	3.1	6.1
CD31	2.6	1.7	2.5	1.7	2.8	2.9	1.7	3.1	0.4	1.8	2.1
CD73	99.4	97.4	99.7	99.2	99.7	96.7	94.0	96.4	99.4	99.4	98.1
CD45	2.6	0.7	1.4	1.9	1.2	0.6	1.8	1.2	0.6	1.9	1.4
CD105	99.9	99.9	99.8	100.0	100.0	99.9	99.9	100.0	100.0	99.9	99.9
CD80	1.5	6.6	3.6	6.2	2.2	19.6	19.8	2.2	2.3	3.8	6.8
CD90	99.9	98.9	99.9	99.8	99.9	99.9	99.0	99.8	100.0	99.9	99.7
CD34	4.2	1.7	2.9	4.0	7.1	19.2	6.6	16.5	5.1	4.0	7.1

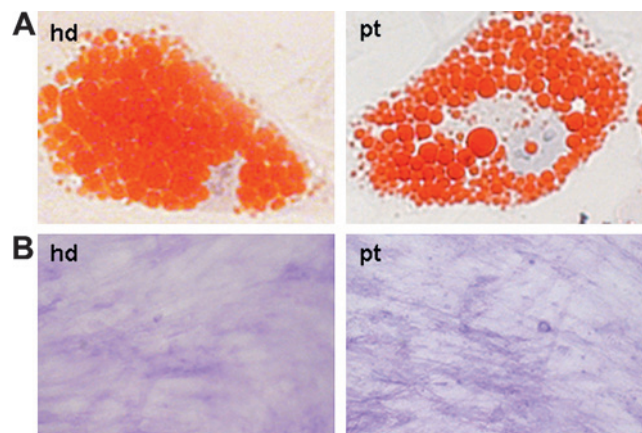
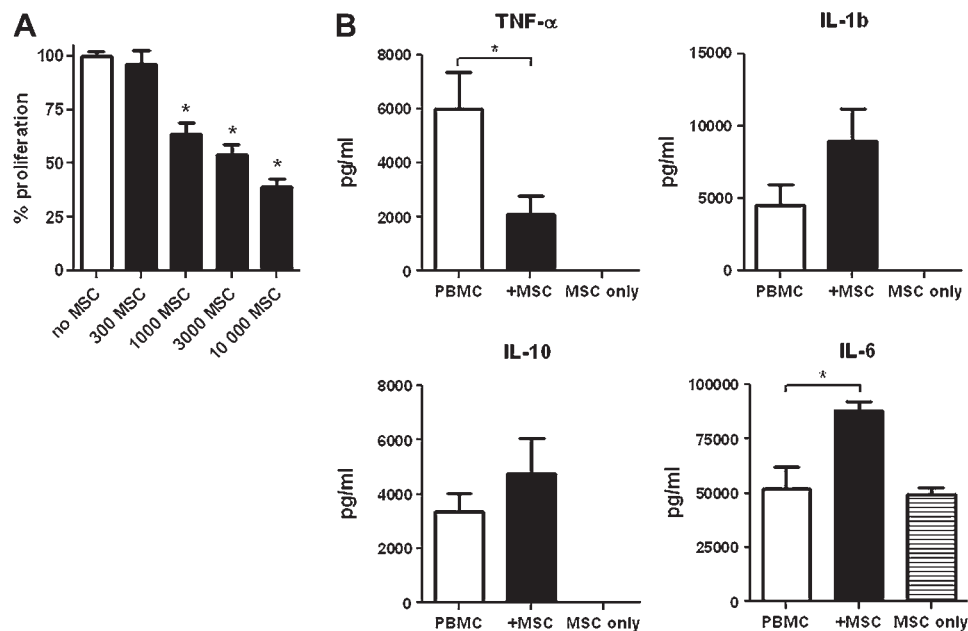


Figure 2 Mesenchymal stromal cells (MSCs) from Crohn's disease patients differentiate into mesenchymal lineages. (A) Adipocyte differentiation was demonstrated in MSCs cultured from a healthy donor (hd) and from a patient with Crohn's disease (pt) after which cells were stained with Oil Red O to show lipid droplets in the cytoplasm of the cell. (B) For osteoblast differentiation MSCs were stained with fast blue to show alkaline phosphatase activity. Non-conditioned MSCs did not stain for Oil Red O, whereas fast blue gave slight background staining (not shown).

refractory patient on the waiting list for surgery. Although an initial decrease of CDAI score was seen this patient was excluded when presented with a CDAI >450 due to poor general condition and persistent rectal blood loss. Patient 7 was withdrawn from the study 4 days after the first MSC infusion because of continuing abdominal aches and bloody diarrhoea. In this case infliximab treatment was resumed. CDAI scores improved in five patients, clinical response (defined as a drop in CDAI >70) was seen in three patients at week 6 (table 4).

Remission (CDAI <150) was not achieved in any of the patients. Three patients had a reduction of 70 points in CDAI score, this decrease could in most cases be ascribed to solid stools and a decrease in soft stool frequency. In a period of 14 weeks, three patients required surgery due to disease worsening (table 4). No significant differences in C-reactive protein (CRP) levels were seen.

Figure 3 Mesenchymal stromal cells (MSCs) from Crohn's disease patients significantly inhibit the proliferation of autologous peripheral blood mononuclear cells (PBMCs) and this inhibition is dose-dependent. (A) 100 000 PBMCs were stimulated with anti-CD3/CD28 beads in the absence (white column) or presence (black columns) of indicated numbers of autologous MSCs. Proliferation measured by ³H-thymidine uptake in counts per minute was expressed as a percentage of PBMC proliferation without MSCs for each individual patient. (B) Cytokine production in the supernatants of PBMC cultures and 10 000 MSC/100 000 PBMC co-cultures. MSCs alone produce interleukin 6 (IL-6) (lower right). In the presence of MSCs, tumour necrosis factor α (TNF- α) production is lowered, whereas IL-1b, IL-6 and IL-10 production is increased. Bars represent the mean and SEM of data from 10 patients in triplicate. * p <0.05 for significant differences.



Endoscopy

Endoscopic improvement, observed by a decrease in CDEIS of 10.0 and 24.7 points, was seen in two patients with extensive Crohn's disease localised in the colon (figure 5). In the other five patients no significant endoscopic improvement was seen between baseline and 6 weeks post-infusion. At weeks 0 and 6, levels of CD4+, CD8+ and CD4+CD127+ populations were determined in biopsies of inflamed mucosa (figure 6A left panels). A trend of lower CD4+ T cells and higher CD4+CD127+ regulatory T cells were observed at week 6 when compared to week 0. Cytokine levels of TNF α , IL-1b, IL-10 and IL-6 were determined in mucosal biopsies and serum at weeks 0 and 6. In general, cytokine levels decreased in the mucosa (figure 6A right panels), whereas an increase of cytokine levels in the serum was seen (figure 6B).

DISCUSSION

This phase I study shows that bone marrow harvesting and expansion of bmMSCs from patients with refractory Crohn's disease is feasible and that MSCs cultured from patients with Crohn's disease are similar to MSCs from healthy donors, in plastic adherence, spindle-shaped morphology, growth potential (supplementary figure 1), surface marker expression, lack of haematopoietic markers (table 3) and differentiation capability (figure 2). In addition, MSCs from refractory Crohn's disease patients are able to inhibit autologous PBMC proliferation and inhibit TNF α production in vitro (figure 3). Furthermore, autologous bmMSC infusion appears to be safe as intravenous MSC infusions were clinically well tolerated. Reported adverse events directly related to MSC infusion were a mild and transient allergic reaction in one patient and the typical taste and smell of the cryopreservant DMSO noticed by all patients (table 2).

Although the design of this study does not allow conclusions on efficacy, after two infusions with autologous bmMSCs, endoscopic improvement was seen in two patients (figure 5), while three patients required surgery due to worsening of disease (table 4). Patients included were chronic active patients refractory to all currently available medical therapeutic options. One could speculate that the immunomodulatory effect of MSCs

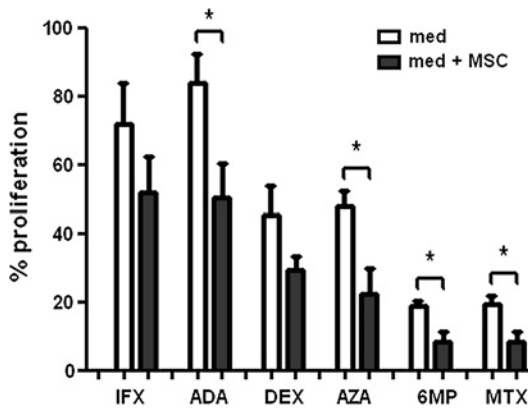


Figure 4 Mesenchymal stromal cells (MSCs) have an additive effect on the inhibition of peripheral blood mononuclear cell (PBMC) proliferation by Crohn's disease medication. In the presence of indicated Crohn's disease medication proliferation of 100 000 stimulated PBMCs added to 1000 MSCs (black bars) compared to PBMC without MSCs (white bars) was measured. Proliferation measured by ³H-thymidine uptake in counts per minute was expressed as a percentage relative to PBMC proliferation in control medium (set to 100%). Bars represent mean and SEM. **p*<0.05 for significant differences between test conditions based (two-sided t test). ADA, adalimumab; AZA, azathioprine; DEX, dexamethasone; IFX, infliximab; 6-MP, 6-mercaptopurine; MTX, methotrexate.

might not be sufficient to induce clinical remission in this category of patients. Further (randomised) trials in also less refractory patients are therefore warranted

In order to study the biological effects of systemic MSC infusion in patients with refractory Crohn's disease we analysed CD4+CD127+ expression on T cells obtained from colonic

Figure 5 Endoscopy at week 0 (upper panels) and at 6 weeks (lower panels) after mesenchymal stromal cell (MSC) treatment (two administrations of 2×10^6 autologous bmMSCs) shows clear mucosal healing. Pictures A and B are from patient 2, pictures C and D from patient 3.

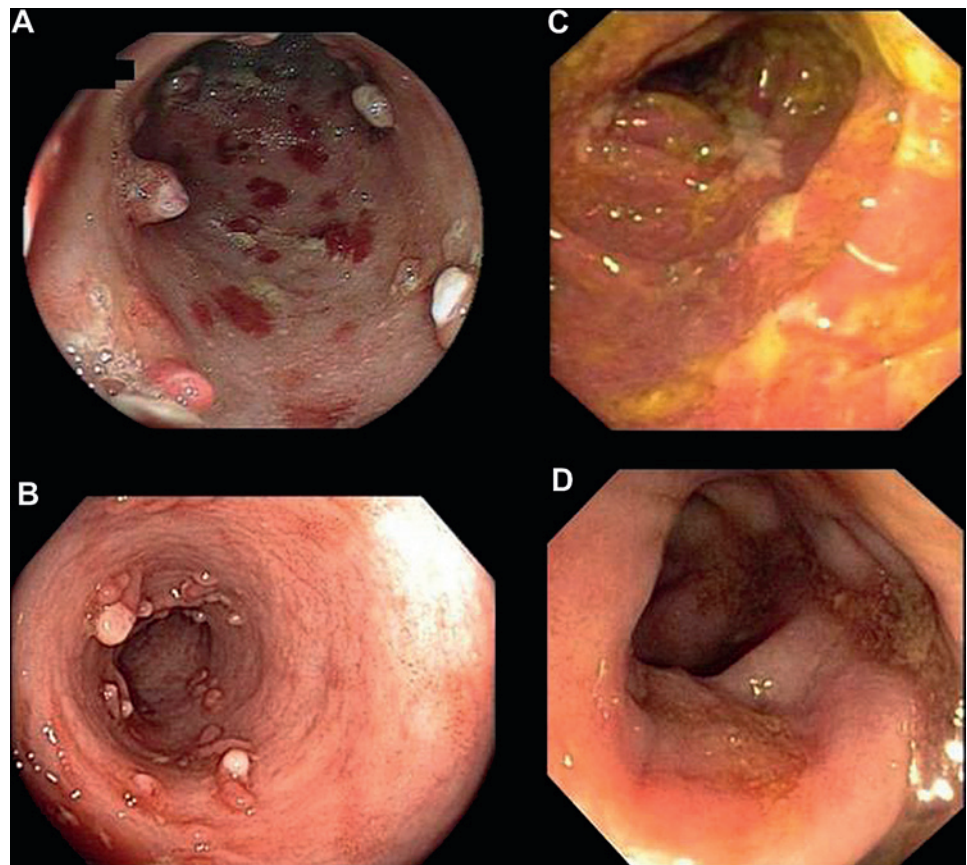


Table 4 Clinical scores of patients at week 0, 1 and 6. In the 6 month follow-up period, three patients underwent surgery in the indicated week after mesenchymal stromal cell infusion

		Patient number									
		1	2	3	4	5	6	7	8	10	
CDAI (0–600)											
Week 0*	First infusion	332	341	254	326	350	266	378	224	304	
Week 1	Second infusion	305	281	182	318	306	247	452†	167	ND	
Week 6	Primary endpoint	473†	185	179	267	314	160	ND	340	354	
Surgical resection											
In week number		7	n	n	n	12	n	n	14	n	

*Baseline.

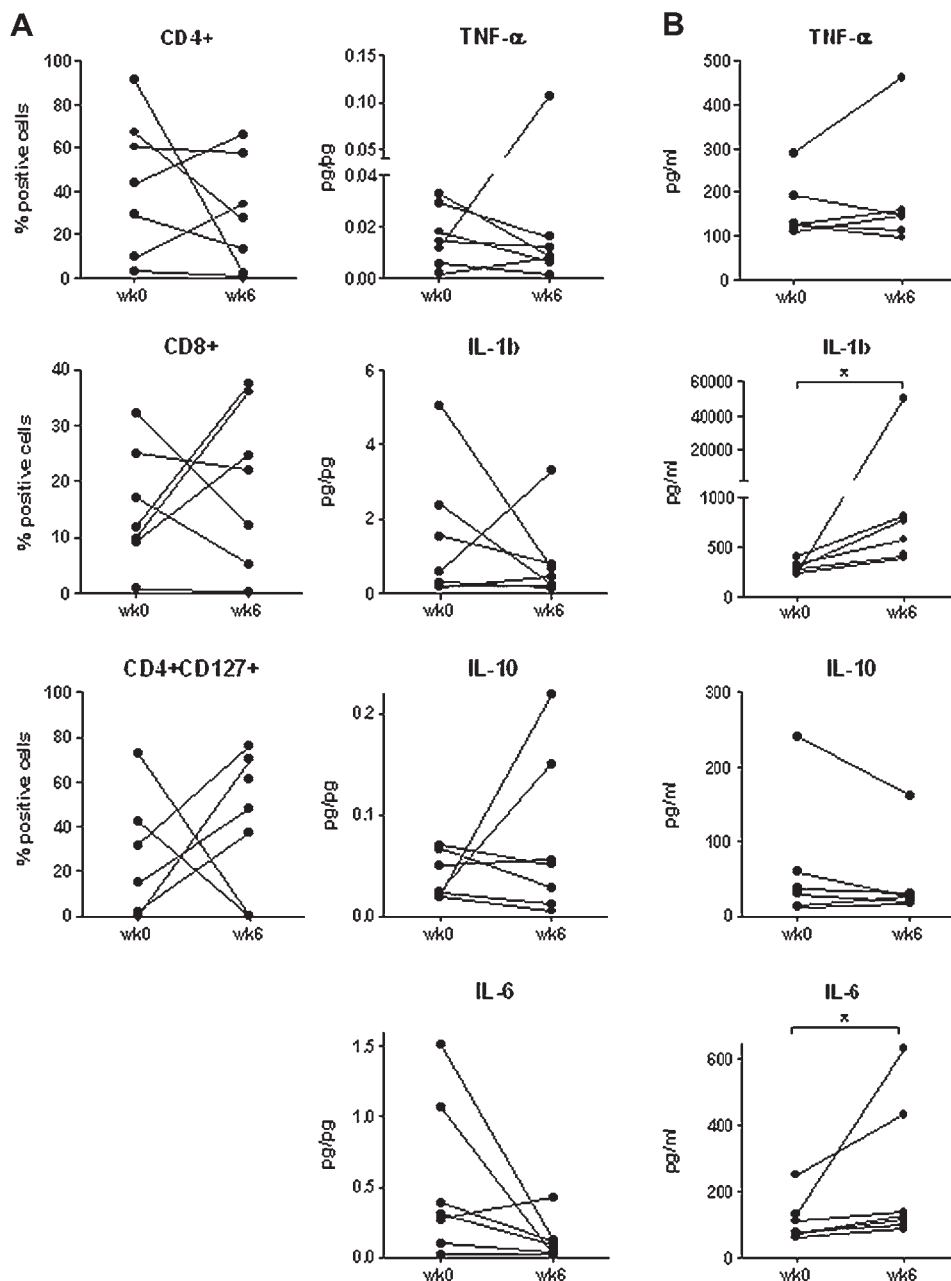
†Withdrawn from study.

ND, not determined; n, no.

biopsies and determined cytokine production in both colon homogenates and serum. We observed a trend of lower CD4+ T cells and higher CD4+CD127+ regulatory T cells at week 6 when compared to week 0, although the number of patients in this study was not enough to reach statistical significance. In addition, cytokine levels went down in mucosal biopsies, indicating a decrease in intestinal inflammation (figure 6A right panels). The apparent reciprocal increase in serum cytokine levels (figure 6B) may be the result of altered distribution of inflammatory cells. Due to the decreased local inflammation, leucocytes are no longer recruited to the intestine, but remain in the circulation, thus increasing the systemic cytokine levels. Similar findings have been observed for regulatory T cells in Crohn's disease¹⁸ and plasmacytoid dendritic cells in dermal inflammation.¹⁹

The intravenous route of administration and target dose of $1-2 \times 10^6$ cells/kg body weight were based on experience with other protocols in GvHD. Intravenous infusion of cells is an

Figure 6 CD4+, CD8+ and CD4+CD127+ populations in biopsies of inflamed mucosa at weeks 0 and 6 (A, left panels). Cytokine levels of tumour necrosis factor α (TNF α), interleukin-1b (IL-1b), IL-10 and IL-6 were determined in mucosal biopsies (A, right panels) and serum (B) at weeks 0 and 6. Bars represent mean and SEM. * $p < 0.05$ for significant differences.



easy, minimal invasive and routinely performed procedure with proven safety so far. Although it has been suggested that MSCs home to sites of inflammation, it is unknown how many cells will eventually reach the intestine in patients with Crohn's disease. In a case report, Dinesen *et al*²⁰ showed that administration of MSCs via selective mesenteric artery cannulation was safe and feasible. This approach may possibly increase the number of cells reaching the affected organ.

In the case of autologous MSCs, an ongoing discussion is whether MSCs are affected by or may contribute to the underlying disease. For instance, MSCs from patients with systemic lupus erythematosus are difficult to expand in culture and yield low cell numbers²¹ and those from patients with multiple myeloma have been shown to be impaired and possibly contribute to the pathogenesis of the disease.²² In this study we demonstrate that MSCs obtained from patients with refractory Crohn's disease show similar growth potential, yield and properties when studied in vitro in comparison to MSCs from healthy donors. Our data support work published recently²³ and

suggest that bmMSCs from patients with refractory Crohn's disease are not affected by the disease. Unfortunately, there is no 'gold standard' test to assess the functionality of MSCs and it has not been demonstrated that in vitro effectiveness of MSCs can be translated to clinical effectiveness, making true extrapolation of this topic difficult.

Another important issue is the effect of MSCs on concomitant medication.²⁴ To address this matter, we studied the effect of MSCs on various routinely used Crohn's disease medications in vitro. We found that adding various Crohn's disease medications to MSC cultures did not affect cell proliferation. Furthermore, adding MSCs to proliferating PBMCs in the presence of tested medications, gave a cumulative effect on the inhibition of PBMC proliferation (figure 4), suggesting that MSCs can be used in addition to concomitant Crohn's disease medication.

A concern in cell-based therapies with ex vivo expanded cells is the formation of tumours. Previous work indicated that, in mice, MSCs stimulate the growth of cancers^{25 26} and promote metastasis.²⁷ Additionally, extensive in vitro expansion of cells may

induce genetic instability. Although an increased risk on tumour formation has never been confirmed in humans, patients with a history of malignancy were excluded from this study. To minimise the risk of transformation of cells we have expanded MSCs in the absence of growth factors, plated MSCs in moderate cell concentrations and used only low passage numbers. Furthermore, we karyotype the MSC product before clinical release to confirm normal karyotyping and did not observe any aberrancies.

In conclusion, our data suggest that intravenous application of autologous bmMSCs is feasible and well tolerated. Furthermore, bmMSC administration may produce clinical benefits in severe refractory Crohn's disease. Therefore, further studies should be designed to examine MSCs as a potential treatment for Crohn's disease.

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REFERENCES

1. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;**105**:1815–22.
2. Di Nicola M, Carlo-Stella C, Magni M, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002;**99**:3838–43.
3. Krampera M, Glennie S, Dyson J, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood* 2003;**101**:3722–9.
4. Zuk PA, Zhu M, Ashjian P, et al. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002;**13**:4279–95.
5. Tondreau T, Meuleman N, Delforge A, et al. Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity. *Stem Cells* 2005;**23**:1105–12.
6. Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol* 2000;**109**:235–42.
7. Bruder SP, Jaiswal N, Haynesworth SE. Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J Cell Biochem* 1997;**64**:278–94.
8. Gonzalez-Rey E, Anderson P, Gonzalez MA, et al. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. *Gut* 2009;**58**:929–39.
9. Gonzalez MA, Gonzalez-Rey E, Rico L, et al. Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. *Gastroenterology* 2009;**136**:978–89.
10. Le BK, Frassoni F, Ball L, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* 2008;**371**:1579–86.
11. Le Blanc K, Rasmusson I, Sundberg B, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 2004;**363**:1439–41.
12. Garcia-Olmo D, Garcia-Arraz M, Herreros D. Expanded adipose-derived stem cells for the treatment of complex perianal fistula including Crohn's disease. *Expert Opin Biol Ther* 2008;**8**:1417–23.
13. Ren G, Zhang L, Zhao X, et al. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell* 2008;**2**:141–50.
14. Parekkadan B, van PD, Suganuma K, et al. Mesenchymal stem cell-derived molecules reverse fulminant hepatic failure. *PLoS One* 2007;**2**:e941.
15. Duijvestein M, van den Brink GR, Hommes DW. Stem cells as potential novel therapeutic strategy for inflammatory bowel disease. *Journal of Crohn's and Colitis* 2010;**4**:99–106.
16. Bernardo ME, Ball LM, Cometa AM, et al. Co-infusion of ex vivo-expanded, parental MSCs prevents life-threatening acute GVHD, but does not reduce the risk of graft failure in pediatric patients undergoing allogeneic umbilical cord blood transplantation. *Bone Marrow Transplant*. Published Online First: 2010 Apr 19.
17. Ball LM, Bernardo ME, Roelofs H, et al. Co-transplantation of ex vivo expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem cell transplantation. *Blood* 2007;**110**(7):2764–2767.
18. Li Z, Arijis I, De HG, et al. Reciprocal changes of Foxp3 expression in blood and intestinal mucosa in IBD patients responding to infliximab. *Inflamm Bowel Dis* 2010;**16**:1299–310.
19. Nestle FO, Conrad C, Tun-Kyi A, et al. Plasmacytoid dendritic cells initiate psoriasis through interferon-alpha production. *J Exp Med* 2005;**202**:135–43.
20. Dinesen L, Wang A, Vianello F, et al. Mesenchymal stem cells administered via novel selective mesenteric artery cannulation for the treatment of severe refractory Crohn's disease. *Journal of Crohn's and Colitis* 2010;**4**:S51–2.
21. Sun LY, Zhang HY, Feng XB, et al. Abnormality of bone marrow-derived mesenchymal stem cells in patients with systemic lupus erythematosus. *Lupus* 2007;**16**:121–8.
22. Arnulf B, Lecourt S, Soulier J, et al. Phenotypic and functional characterization of bone marrow mesenchymal stem cells derived from patients with multiple myeloma. *Leukemia* 2007;**21**:158–63.
23. Bocelli-Tyndall C, Bracci L, Spagnoli G, et al. Bone marrow mesenchymal stromal cells (BM-MSCs) from healthy donors and auto-immune disease patients reduce the proliferation of autologous- and allogeneic-stimulated lymphocytes in vitro. *Rheumatology (Oxford)* 2007;**46**:403–8.
24. Hoogduijn MJ, Crop MJ, Korevaar SS, et al. Susceptibility of human mesenchymal stem cells to tacrolimus, mycophenolic acid, and rapamycin. *Transplantation* 2008;**86**:1283–91.
25. Studeny M, Marini FC, Champlin RE, et al. Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. *Cancer Res* 2002;**62**:3603–8.
26. Djouad F, Plence P, Bony C, et al. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. *Blood* 2003;**102**:3837–44.
27. Karnoub AE, Dash AB, Vo AP, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 2007;**449**:557–63.