Reduction in infarct size, but no functional improvement after bone marrow cell administration in a porcine model of reperfused myocardial infarction

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Received 1 August 2006; revised 19 October 2006; accepted 7 November 2006; online publish-ahead-of-print 29 November 2006

Introduction
Regeneration of infarcted myocardium by transplanting bone marrow (BM)-derived stem cells into the infarct region has been proposed to prevent heart failure by angiogenesis and/or myogenesis.¹,² Early experimental studies in animals with a myocardial infarction (MI) reported improvements in left ventricular (LV) function following cell therapy with BM-derived mononuclear cells (MNC),³ or a cell sub-population selected from MNC.¹ These highly promising initial observations sparked a large number of non-randomized clinical trials, reporting beneficial effects of MNC therapy on global LV function and myocardial viability.⁴⁻⁸ However, out of four recent randomized trials (BOOST-update,⁹ Leuven trial,¹⁰ REPAIR-AMI,¹¹ ASTAMI¹²), three failed to show an improvement in global LV function,⁹,¹⁰,¹² although one reported a reduction in infarct size.¹⁰ In contrast to the discordant results obtained in clinical trials to date, the majority of experimental studies reported positive effects of MNC therapy on global LV function,³,¹³⁻¹⁵ although one study reported no effect.¹⁶ However, the beneficial effects of MNC on infarct size, which have been observed clinically,⁴,⁶,¹⁰ have not been investigated in these studies. Furthermore, all studies used a permanent coronary artery ligation, while all but one¹⁵ (which injected the MNC cells in a coronary venous vessel) injected the MNC directly in the peri-infarct area. Hence, these experimental studies have a markedly different design compared with the clinical trials. Furthermore, most animal studies used more selected, enriched populations, such as the CD34⁺, c-kitpos, or lin-BM-derived cells or BM-derived mesenchymal stem cells.

Consequently, we designed an experimental study that matches the clinical trial protocols more closely, using a porcine reperfused MI model and intracoronary cell injections. MI was induced by PTCA-balloon inflation followed by reperfusion. LV remodelling as well as global and regional LV function was assessed using cine-magnetic resonance imaging (cine-MRI). Contrast-enhanced MR Imaging (Ce-MRI) was used to assess infarct size and infarct remodelling over time.

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Although the clinical trials suggest that intracoronary BM-derived cell delivery appears safe, recently Yoon et al. reported increased calcifications after unselected BM cell injection. Therefore, we compared the effects of MNC to those of unselected BM.

Methods

Experiments were performed in 2-3-month-old Yorkshire-Landrace pigs of either sex (n = 49), in compliance with the ‘Guide for the Care and Use of Laboratory Animals’ (NIH publication 1996) and after approval of the Animal Care Committee of the Erasmus MC.

Myocardial infarction

Animals were sedated (ketamine 20 mg/kg im and midazolam 1 mg/kg im), anesthetized (thiopental, 12 mg/kg iv), intubated and mechanically ventilated with a mixture of oxygen and nitrogen (1:2 vol/vol). Anaesthesia was maintained with fentanyl (12.5 μg/kg/h) and isoflurane (0.6–0.8% started after onset of occlusion). Subsequently, animals received antibiotic prophylaxis (200 mg penicillin and 250 mg dihydrostreptomycinesulfate im) and underwent coronary catheterization through a carotid artery. Subsequently, animals received antibiotic prophylaxis (200 mg procaine benzylpenicillin and 250 mg dihydrostreptomycinesulfate im) and underwent coronary catheterization through a carotid artery, guided by fluoroscopy, followed by balloon occlusion of the proximal left circumflex coronary artery (LCX), for 2 h followed by reperfusion.

One week after MI, all animals were anaesthetized as described above and anaesthesia was maintained with 1–1.5% isoflurane. Approximately 40 mL of BM was aspirated from the iliac crest, using the same BM aspiration/biopsy needles (Kendall monoject, Tyco Healthcare, Gosport, UK) that are routinely used for clinical purposes in our hospital. MNC were isolated by Ficoll Paque-plus (Amersham Biosciences Europe GmbH, Freiburg, Germany) density gradient separation (25 min at 400 g) and suspended in 10 mL Modified Eagle’s Medium (MEM). Crude BM (40 mL) was prepared for injection by filtering through a 100 micron filter. MNC were counted using a cell counter (Sysmex CDA 500, Malvern Instruments Ltd, Malvern, UK). In view of the identical volume of BM that was aspirated, the amount of MNC administered in either group was considered to be similar. BM and MNC were injected within 1 h after aspiration.

Efficacy of cell delivery

To investigate the efficacy of cell delivery, we tested injection with a selective, non-flow-limiting injection catheter (Multifunctional probing, Boston Scientific Co., Boston, MA, USA). One week after MI, BM was aspirated and five swine received an intracoronary injection of ~50 × 10^6 PKH-labelled MNC (PKH 26, Sigma-Aldrich, Schnelldorf, Germany) suspended in 10 mL saline; 5 mL was injected into the LCX (infarct area). The other 5 mL was injected into the left anterior descending coronary artery (LAD; non-infarct area) to investigate if cell injections cause micro infarctions. MNC were injected slowly (1 mL/min) into the coronary artery perfusing the MI area using a probing catheter. The site of injection was identical to the position of the occlusion balloon during MI induction. Four days after cell injection, animals were sacrificed for histological and immunocytochemical analyses.

Histology and immunohistochemistry

The hearts were excised and cut into 6–8 transverse slices similar to the MRI short-axis slices. From the basal plane the first, third, fifth, and seventh slices were fixed in 4% buffered formaldehyde and embedded in paraffin. The second, fourth, sixth, and eight slices were embedded in tissue tec OCT and frozen in liquid nitrogen. Sections (5 μm) were stained with haematoxylin eosin (H&E) and resorcin-fuchsin (collagen). Immunohistochemistry was performed in the infarct sections to determine the expression of the pan-leukocyte marker CD45 (MAC1447, Setotec, Oxford, UK), macrophage surface marker (MAC378, Setotec, Oxford, UK), vimentin (Clone DM-1, DakoCytomation, California, USA) and desmin (Clone V9 DakoCytomation, California, USA). Desmin is expressed by myocytes and smooth muscle cells; vimentin is expressed by fibroblasts and endothelial cells. Sections were semi-quantitatively assessed as negative (0) or the degree (from 1 to 5) of calcium deposition, collagen deposition, and vascularization. The infarct sections were scored for surface area covered with collagen, calcifications, or vascularization. In each animal, one section was scored per LV slice (corresponding with each of the 5–6 MRI LV short-axis slices). For each section, the total infarct area was scored (power field 10×).
Statistical analysis

Data were analysed with SPSS 11.0. All data were analysed using a one-way ANOVA and post hoc analysis using unpaired t-testing with Bonferroni correction to test for significant intergroup differences at corresponding time points. Effect of BM and MNC therapy at the follow-up MRI was tested using analysis of co-variance (ANCOVA) with baseline values as covariate. Statistical significance was accepted when \( P < 0.05 \) (two-tailed). All data are presented as mean \( \pm SD \).

Results

Efficacy of cell delivery

In three additional in vitro experiments, trypan blue exclusion staining showed that 99.7% of the cells were viable after the aspiration procedure. Furthermore, following subsequent injection through the probing catheter, 99.5% of the aspirated cells were viable. These results indicate negligible cell loss due to the BM cell aspiration and handling.

PKH labelled cells could be detected in the infarcted LV lateral wall of five swine, 4 days after injection into the LCX, whereas only a few cells (<1 cell/cm²) could be detected in the healthy non-infarcted LV anterior wall following injection into the LAD (Figure 1). Systematic histological analysis did not reveal any myocardial damage in the normal LAD-perfused myocardium. Quantitative analysis showed that an average of 248 ± 136 PKH positive cells/cm² could be detected in the infarct zone, 4 days after injection with a probing catheter, corresponding to ~6.5% of the injected cells.

Functional assessment of cell therapy

Infarct-related mortality

A total of 44 swine entered the study. Out of 34 MI swine, three animals encountered ventricular fibrillation during the 2 h occlusion, for which they were treated successfully by DC shock. Four animals died prematurely, of which three died within 24 h after induction of MI, while one animal died shortly after the baseline MRI (i.e. prior to any therapeutic intervention). Consequently, 40 surviving animals completed the protocol.

Magnetic resonance imaging

MI swine had a transmural MI of the lateral LV wall encompassing 14.3 ± 5% of the left ventricle. There were no differences in body weight (BW), SV, or CO between controls and MI-animals at baseline (Table 1). Due to normal growth, BW, SV, and CO increased in all four groups over the 4 week follow-up period. Consequently, there were no significant differences in BW or systemic haemodynamics during the endpoint scan (Table 1).

One week after MI, LVW, EDV, and ESV were greater, whereas EF was lower in MI compared with control swine (Figure 2). Changes in LV volumes and mass from baseline to 4 weeks after cell injection did not differ significantly between medium, BM, and MNC-treated MI-animals (Figure 2).

One week after MI, there were no significant differences in diastolic wall thickness between the different groups (Figure 3). SWT in the LCX area was abolished in the MI animals compared with control, which was not affected by BM or MNC treatment.

Infarct size measured with delayed enhancement revealed that MNC treatment resulted in a significant decrease in infarct mass from 10.8 ± 5.5 to 8.7 ± 4.2 g, compared with medium treatment (11.1 ± 5.0 and 12.3 ± 3.9 g; \( P = 0.045 \) change from baseline MNC vs. medium), whereas BM treatment had no significant effect (Table 1). Similarly, there was a greater decrease in infarct size expressed as a percent of the LV in the MNC, but not in the BM-treated animals, compared with the medium-treated swine (Table 1, Figure 4).

Histology

Histology confirmed that all MI animals had a transmural infarct in the LCX region with total loss of viable

### Table 1 Systemic haemodynamics and infarct size measured by delayed enhancement

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Endpoint</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BW (kg)</strong></td>
<td>Control</td>
<td>23 ± 5</td>
<td>40 ± 7</td>
</tr>
<tr>
<td>Mi + medium</td>
<td>26 ± 3</td>
<td>43 ± 6</td>
<td></td>
</tr>
<tr>
<td>Mi + BM</td>
<td>26 ± 1</td>
<td>43 ± 4</td>
<td>0.669</td>
</tr>
<tr>
<td>Mi + MNC</td>
<td>24 ± 1</td>
<td>39 ± 3</td>
<td>0.185</td>
</tr>
<tr>
<td><strong>HR (b.p.m.)</strong></td>
<td>Control</td>
<td>107 ± 15</td>
<td>83 ± 14</td>
</tr>
<tr>
<td>Mi + medium</td>
<td>87 ± 16</td>
<td>84 ± 19</td>
<td></td>
</tr>
<tr>
<td>Mi + BM</td>
<td>103 ± 23</td>
<td>83 ± 15</td>
<td>0.702</td>
</tr>
<tr>
<td>Mi + MNC</td>
<td>85 ± 21</td>
<td>86 ± 17</td>
<td>0.733</td>
</tr>
<tr>
<td><strong>SV (mL)</strong></td>
<td>Control</td>
<td>37 ± 6</td>
<td>51 ± 8</td>
</tr>
<tr>
<td>Mi + medium</td>
<td>38 ± 10</td>
<td>60 ± 11</td>
<td></td>
</tr>
<tr>
<td>Mi + BM</td>
<td>36 ± 7</td>
<td>56 ± 9</td>
<td>0.466</td>
</tr>
<tr>
<td>Mi + MNC</td>
<td>27 ± 9</td>
<td>50 ± 11</td>
<td>0.041</td>
</tr>
<tr>
<td><strong>CO (mL/min)</strong></td>
<td>Control</td>
<td>3.9 ± 0.9</td>
<td>4.2 ± 1.0</td>
</tr>
<tr>
<td>Mi + medium</td>
<td>3.3 ± 1.0</td>
<td>5.0 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Mi + BM</td>
<td>3.8 ± 1.2</td>
<td>4.6 ± 1.0</td>
<td>0.324</td>
</tr>
<tr>
<td>Mi + MNC</td>
<td>3.2 ± 1.2</td>
<td>4.2 ± 1.0</td>
<td>0.099</td>
</tr>
<tr>
<td><strong>Infarct size</strong></td>
<td>Control</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Mi + medium</td>
<td>11.1 ± 5.0</td>
<td>12.3 ± 3.9</td>
<td></td>
</tr>
<tr>
<td>Mi + BM</td>
<td>12.0 ± 3.3</td>
<td>11.6 ± 5.6</td>
<td>0.340</td>
</tr>
<tr>
<td>Mi + MNC</td>
<td>10.8 ± 5.5</td>
<td>8.7 ± 4.2</td>
<td>0.045</td>
</tr>
<tr>
<td>Infarct size (%)</td>
<td>Control</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Mi + medium</td>
<td>14.3 ± 5.6</td>
<td>11.0 ± 4.2</td>
<td></td>
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<tr>
<td>Mi + BM</td>
<td>14.3 ± 3.6</td>
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<td>0.236</td>
</tr>
<tr>
<td>Mi + MNC</td>
<td>14.3 ± 6.5</td>
<td>8.2 ± 4.2</td>
<td>0.024</td>
</tr>
</tbody>
</table>

Baseline, 1 week post-MI; endpoint= 5 week post-MI; P-values, change from baseline in BM or MNC vs. medium-treated MI-animals; NA, not applicable. Data are mean ± SD. Ten animals per group, except for infarct size (medium: \( n = 8 \), BM: \( n = 8 \), and MNC: \( n = 10 \)).

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Figure 1 PKH-labelled cells do not home to healthy myocardium (A), but PKH positive cells (in red) home in infarcted myocardium after injection with a probing catheter (B).
mammals. Conversely, it has been shown that stem cells
or MNC. There were no significant differences (all variables
point (open bars) in control swine and MI swine receiving either medium, BM,
entrate into cardiomyocytes
Although it has been shown that BM-derived cells can differ-
mechanism for the infarct size reduction by MNC treatment.
From the present study, we cannot determine the underlying
study by Janssens
weeks after injection, a finding also reported in the clinical
ract. However , MNC reduced infarct size 4
weeks later. In addition, BM-derived MNC or unselected BM
treatment did not reverse the remodelling of the left ventri-
national benefit of BM-derived cells in this study cannot be
environment for injected cells. Therefore, the lack of func-
idea that the acute inflammatory response is most pro-
choose to inject cells 1 week after MI, with the underlying
 days post-MI is the optimal timing of cell therapy. We
chose to inject cells 1 week after MI, with the underlying

Table 3
  Model  1000–2000  2000–4000  4000–8000  >8000
  BM  4.5%  4.2%  3.3%  3.0%
  MNC  6.5%  6.2%  5.2%  4.8%
  Medium  4.5%  4.2%  3.3%  3.0%

Table 3 suggests that in permanent ligation MI
models, the timing of MNC administration does not appear
to be critically important. Thus, MNC injections either
immediately or up to 2 weeks after MI were reported to
improve global LV function. In contrast, the optimal timing
of stem cell delivery in reperfused MI remains incompletely
understood. However, the REPAIR-AMI trial showed that the
optimal timing of cell injection appears to be at least 5
days after MI.12 Also Bartunek et al.26 suggest that 3 to 10
days post-MI is the optimal timing of cell therapy. We
chose to inject cells 1 week after MI, with the underlying
idea that the acute inflammatory response is most pro-
nounced shortly after MI, whereas the inflammatory
response is diminished 1 week later, thus creating a better
environment for injected cells. Therefore, the lack of func-
tional benefit of BM-derived cells in this study cannot be
explained by the timing of cell administration. It is likely
that a proximal 2 h during LCX occlusion, followed by reper-
fusion creates a transmural infarct that represents a too
hostile environment for injected cells to contribute to LV
function recovery.

Route of administration
In contrast to previous experimental studies, which either
used intramyocardial or retrograde coronary venous injec-
tion (Table 3), clinical trials have invariably used intracor-
ary arterial injections of MNC. Intracoronary injected cells
are thought to disappear from the coronary circulation
into liver, lung, or kidney within a few hours.25 Therefore,
in clinical trials the intracoronary cell delivery typically
involves repeated proximal balloon occlusion during cell
delivery in order to prevent wash-out of cells and facilitate
attachment of the injected cells onto the vascular wall.28
We observed that only ~6.5% of the injected MNC were
present in the infarcted area 4 days after injection, which
could be interpreted to suggest that the injection via the
probing catheter could have resulted in suboptimal cell
engraftment in the myocardium and induce angiogenesis23 that
could result in improved perfusion, particularly in the
border zone. It could be speculated that this might aid in
preventing further ischaemic damage, thereby rescuing
viable tissue in the border zone; alternatively, the
MNC-induced angiogenesis may enhance infarct healing.24

Although there was a reduction in infarct size after MNC
treatment, this was not associated with an improvement
in global or regional LV function. This is in apparent contrast
to the majority of preclinical studies that reported signifi-
cant increases in EF or fractional shortening (Table 3). However, it should be emphasized that the experimental
protocols in these studies differed considerably from the
present study. For example, all these studies performed
intramyocardial or intravenous cell injection in an infarct
model of permanent coronary artery occlusion. In contrast,
we employed a model that more closely mimics the clinical
setting, by using intracoronary cell injection in a reperfused
MI model, making direct comparison to previous preclinical
studies difficult.

Several explanations can be forwarded why cardiac func-
tion failed to improve after intracoronary injection of MNC
or BM in the MI zone.

Timing of cell administration
Inspection of Table 3 suggests that in permanent ligation MI
models, the timing of MNC administration does not appear
to be critically important. Thus, MNC injections either
immediately or up to 2 weeks after MI were reported to
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Discussion
The present study investigated the effect of intracoronary
injection of MNC or BM on LV function and histology at 4
weeks in a porcine model of acute MI followed by reperfu-
sion. Our study shows that in swine an intracoronary injec-
tion of both MNC and unselected BM 1 week after MI does
not improve global or regional indices of LV function 4
weeks later. In addition, BM-derived MNC or unselected BM
treatment did not reverse the remodelling of the left ventri-
cle induced by the MI. However, MNC reduced infarct size 4
weeks after injection, a finding also reported in the clinical
study by Janssens et al.10

Infarct size reduction
From the present study, we cannot determine the underlying
mechanism for the infarct size reduction by MNC treatment.
Although it has been shown that BM-derived cells can differ-
entiate into cardiomyocytes in vitro, it remains unclear
whether BM-derived stem cells are capable of differentiat-
ing into cardiomyocytes in vivo,20–22 especially in large
mammals. Conversely, it has been shown that stem cells

mammal, which matched the delayed enhancement
scans performed at 1 and 5 weeks after MI. All treatments
resulted in extensive collagen deposition in the centre of
the infarct (Figure 5). Semi-quantitative analysis showed that
there were no significant differences in the degree of
calcium or collagen deposition, or of vascularization (Table 2).
Immunohistochemistry showed that most of the
cells in the infarct area were desmin negative, vimentin
positive, CD45 positive, and occasionally positive for macro-
phage surface marker (Figure 6), suggesting that the
majority of cells were fibroblasts (vimentin), inflammatory
cells (CD45 and macrophage surface marker), and endo-
thelial cells (vimentin).

Figure 2: LV EDV, LVW, LV EF, and LV ESV at baseline (solid bars) and at end-
point (open bars) in control swine and MI swine receiving either medium, BM,
or MNC. There were no significant differences (all variables P > 0.20)
between BM, MNC, and medium-treated MI animals in the response over
the 4 week follow-up period.

Figure 6
delivery. Therefore, in 5 additional MI pigs, MNC were injected intracoronary via an over-the-wire balloon catheter during repetitive balloon occlusions and pigs were sacrificed 4 days later. These experiments yielded a similar number of PKH positive cells (252 ± 144 cells/cm²) in the infarct area as with the probing catheter. These findings indicate that injection during balloon occlusion does not result in better cell engraftment, and that the low number of MNC present in the infarct area 4 days after injection is not the result of the probing catheter.

The cell delivery study showed that injection of MNC in healthy myocardium did not induce myocardial damage. In
contrast, intracoronary injection of BM-derived mesenchymal stromal stem cells have been shown to cause microinfarctions in dogs. No PKH positive cells could be detected in the LAD area (in contrast to the MI area). It is important to note that the MNC are smaller in size (5–7 μm measured with the Sysmex Cell Counter) than cultured mesenchymal stem cells (≈20 μm), and therefore MNC are less likely to occlude micro vessels after intracoronary injection.

Follow-up time
It could be argued that the lack of effect of MNC on global LV function, despite the reduction in infarct size, was due to the relatively short follow-up period of 4 weeks. However, inspection of Table 3 shows that previous studies in swine did report improvement in cardiac function 3 or 4 weeks after MNC injections, although these were performed in a model of permanent coronary artery occlusion. Furthermore, recent clinical trials such as the ASTAMI-trial, the BOOST-update, and the trial performed in Leuven do not suggest that a longer follow-up will lead to an improvement in LV function. Thus, the BOOST-update showed that 18 months after MNC administration, the inter-group comparison between MNC-treated group and placebo was no longer significant. The ASTAMI-trial as well as the Leuven trial also did not see a beneficial effect on EF or EDV after MNC injection after 6 and 4 months. The preclinical study by Dai et al. showed a similar trend as the BOOST-update, in that the initial positive effect observed at 4 weeks was lost after 6 months. Taken together, the weight of available evidence from experimental and clinical

Figure 4 Change in infarct size from baseline in percentage of the LV. Regression lines; *P < 0.05 vs. medium-treated MI swine.

Figure 5 Infarct area of medium treated (A and B) compared with crude BM (C and D) and MNC (E and F) treated swine. HE staining (A, C, and E) showed many fibroblasts. RF staining showed a lot of collagen (B, D, and F).

Table 2 Semi-quantitative histology score

<table>
<thead>
<tr>
<th></th>
<th>MI + medium (n = 8)</th>
<th>MI + BM (n = 8)</th>
<th>MI + MNC (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium deposits</td>
<td>0.60 ± 1.31</td>
<td>1.37 ± 1.69</td>
<td>1.04 ± 1.64</td>
</tr>
<tr>
<td>Collagen</td>
<td>3.95 ± 1.10</td>
<td>4.11 ± 0.89</td>
<td>4.36 ± 0.99</td>
</tr>
<tr>
<td>Vascularization</td>
<td>4.05 ± 1.36</td>
<td>3.78 ± 1.22</td>
<td>3.54 ± 1.32</td>
</tr>
</tbody>
</table>

Data are mean ± SD. There are no significant differences between groups.
studies suggests that a longer follow-up period may not yield a significant improvement in LV function.

Infarct composition

There were no significant differences between the MI group receiving medium, and either BM or MNC with respect to the histology. In all MI swine, a transmural infarct was observed with transmural loss of viable myocytes. There were no signs of cardiomyocyte regeneration since immunohistochemistry showed that all cells in the infarct were inflammatory cells, fibroblasts, or capillaries. A transmural infarct seems a hostile environment for the undifferentiated stem cells in the mononuclear fraction. It is not unlikely that MNC will differentiate into fibroblasts in such an environment, and therewith contribute to infarct reduction by infarct remodelling, i.e. scar contracture, but do not contribute to contractility.

Yoon et al.\textsuperscript{17} reported that injection of unselected BM aggravated calcifications within the infarct zone. Similarly, we observed a trend towards increased calcifications following BM administration, but this failed to reach statistical significance ($P = 0.084$). Together with the lack of effect of BM on infarct size in the present study, these observations support the concept that MNC should be favoured over unselected BM.

Conclusions

In a porcine model of MI followed by reperfusion, we could not demonstrate improvements in global or regional LV function by the injection of BM-derived MNC. However, we did observe a reduction in infarct size 4 weeks after MNC-injection, which is in accordance with recent clinical observations.\textsuperscript{10}

Acknowledgements

The authors gratefully acknowledge Marcel de Jong from the Cardiology department of the Erasmus MC for the isolation of the MNC. Financial support by ESA ESTEC (AO-99-LSS-006) is gratefully acknowledged.

Conflict of interest: none declared.
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