Cell-based Therapies for Cardiovascular Repair

How small things matter

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Cover: These six patients, who suffered from a large acute myocardial infarction, were among the first in the world to receive intracoronary infusion of adipose tissue-derived regenerative cells.
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The greatest glory in living lies not in never falling, but in rising every time we fall.

Nelson Mandela

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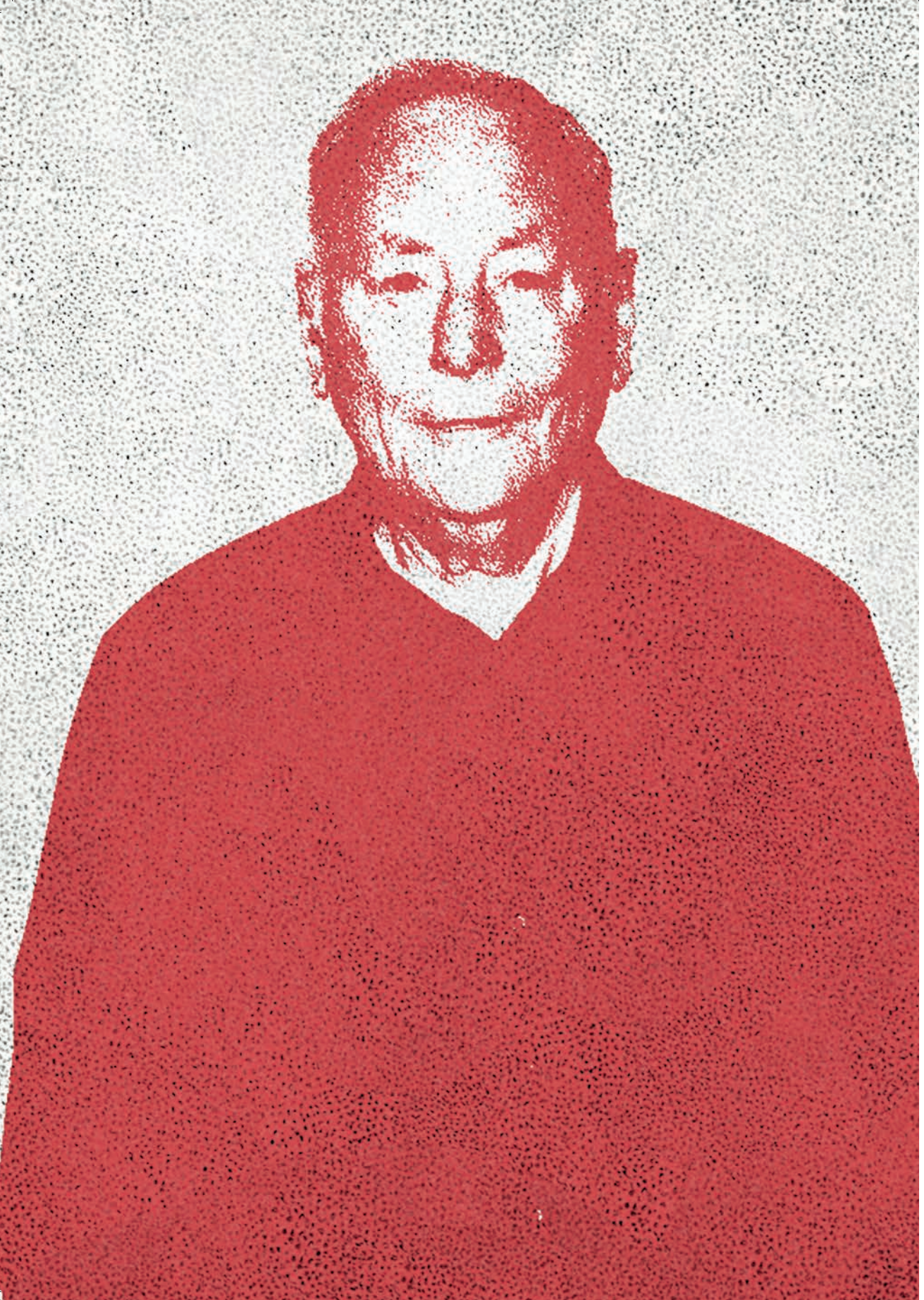

PART I
Introduction

Chapter 1

General introduction and outline of the thesis

General introduction and outline of the thesis

Cardiovascular disease accounts for almost half of the deaths in the Western world and 25% in developing countries, despite significant therapeutic and interventional advances. It is estimated that by the year 2020, cardiovascular disease will surpass infectious disease, to become the world's leading cause of mortality and morbidity. Approximately half of the cardiovascular mortality is due to acute myocardial infarction, whereas subsequent heart failure and stable coronary artery disease account for the majority of morbidity. Stem cell therapy to reduce the burden of cardiovascular disease has been the topic of extensive research over the past decade.

The assumption that multipotent cells can graft to the injured heart and incorporate into the host's myocardium, thereby contributing to contractility and improving cardiac function, is considered to be the holy grail for cardiac cell therapy. Hence, the first attempt of cardiac cell therapy more than a decade ago was with skeletal myoblasts (SkM) in end-stage heart failure patients. These progenitor cells are derived from striated skeletal muscle, and are destined to become contracting myofibers. It was hypothesized that, once injected into myocardial scars, these SkM would differentiate into contractile units *in vivo*, and actually contribute to the contractile apparatus. Unfortunately, clinical reality turned out to be less manipulable, and subsequent research has indicated that the road towards the holy grail is still long and comprises many obstacles on the way.

More specifically, injected SkM did not actually incorporate into scars, but rather formed re-entry circuits for ventricular arrhythmias, whereas cardiac function was not enhanced following SkM injection. In the clinical arena, these disappointing results were rather generalized into the notion that cardiovascular cell therapy did not work. However, as there are several different cardiovascular pathologies and even more different stem cell types, cell therapy is not a single entity. Therefore, the last decade has been devoted to unraveling numerous questions, as 1) what is the ideal stem cell type and dose; 2) what cardiovascular disease types qualify for stem cell therapy; 3) when should stem cell therapy be initiated; 4) how should cells be administered; 5) what is the best surrogate end point to evaluate the effect of cell therapy; etcetera.

This thesis aims to clarify some of these questions, and summarizes our current knowledge about cardiovascular cell therapy. The latter is described and discussed in **part I**, in which a systematic review covers most relevant pre-clinical and clinical experience. Moreover, in two meta-analyses, the clinical efficacy of the first generation of stem cells is assessed in both myocardial infarction and heart failure patients. This thesis also aims to elaborate on various cell types, as well as the cardiovascular diseases these cells aim to heal. More specifically, safety and efficacy of first generation stem cells in both heart failure and acute myocardial infarction (AMI) patients is depicted in **part II**. In **part III**, first-in-man clinical experience with intracoronary infusion of adipose tissue-derived cells (second generation stem cells) is described in AMI patients. These initial results formed the basis for a larger phase II/III study. **Part IV** covers third generation stem cells for the adjunctive treatment of AMI, which comprise allogeneic mesenchymal stem cells (MSC). Safety and efficacy of both single cell suspensions of MSC, as well as encapsulated MSC, were investigated in large animal models of AMI. In contrast to previous reports,

it was found that intracoronary infusion of these cells is both safe and effective when infused briefly following AMI. This finding resulted in the design of a phase I/II clinical study that is currently enrolling AMI patients. **Part V** encompasses studies with the Genous™ stent, which is designed to attract endothelial progenitor cells (second generation stem cells). This stent is hypothesized to promote vascular healing post stent implantation, and theoretically reduces thrombogeneity and in-stent restenosis. **Part VI** summarizes the current findings and touches upon future steps that should be taken to accommodate stem cell therapy as a clinical entity in the future of cardiovascular medicine.

Chapter 2

A concise review of cell-based therapies for cardiovascular repair

What the clinician needs to know

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Introduction

Stem cell therapy to heal scarred myocardium or abrogate adverse remodeling following acute myocardial infarction (AMI) has raised high hopes over the past decades. As stem cells are by definition multipotent cells, in theory they can differentiate into cardiomyocytes and replace scar tissue. Moreover, many stem cell types were found to secrete high levels of cardioprotective proteins and pro-angiogenic factors, thereby representing transplantable micro factories of anti-remodeling and angiotrophic agents. To date, numerous preclinical and clinical efforts have attempted to meet the high expectations with variable outcomes.

More than a decade ago, the first patients with ischemic heart failure were treated with stem cell therapy, using intramyocardial injection of skeletal myoblasts (SkM).[1,2] Briefly afterwards the first pilot study was published, in which AMI patients were treated with intracoronary infusion of bone marrow-derived mononuclear cells (BMMNC).[3,4]

Initial optimism concerning the use of SkM in heart failure patients was toned down by issues of possible pro-arrhythmogenicity of the cells, and disappointing results on efficacy in randomized studies.[5–7] Also, the regenerative capacity of bone marrow (BM)-derived cells in AMI patients has been under debate, since the numerous trials that were performed to date show conflicting results. More specifically, a recent meta-analysis evaluating >2,000 AMI patients, who received BM-derived cellular therapy, showed a modest effect of only +2.14% on left ventricular ejection fraction (LVEF) with no effect on clinical end points (see chapter 3 of this thesis).

Importantly, pre-clinical investigations and phase I clinical studies revealed that newer generations of stem cells might have more regenerative capacities than these first generation stem cells[8–10], whereas the future role of totipotent, embryonic stem cells is still undetermined. Moreover, the age-old dogma that the heart only comprises terminally differentiated and post-mitotic myocytes has recently been abandoned. Studies have shown that the heart contains a resident cardiac stem cell niche, and that cardiomyocytes are replaced several times in a life time.[11,12] This new finding implicates that myocardial tissue has endogenous regenerative capacities, and initiated a new era in cardiovascular regenerative medicine.[13–15]

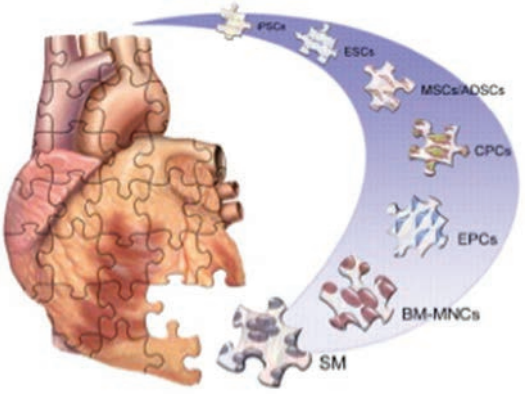

Figure 1. Candidate cell types for cardiovascular regenerative therapy
A variety of cell sources with differing cardiomyogenic potential and developmental origins are under active investigation for cardiac cell therapy after myocardial infarction. BM-MNCs - Bone marrow mononuclear cells. CSCs -Cardiac stem cells. EPCs - Endothelial progenitor cells. ESC - Embryonic stem cells. iPSC - Induced pluripotent stem cells. MSCs - Mesenchymal stem cells. SM - Skeletal myoblasts. *Courtesy of Deutsch et al. Circ Res. 2013;112:884-890*

We therefore pose that there are two potential working mechanisms for cell-based repair of cardiac dysfunction: 1) the enhancement of endogenous regenerative potential, or 2) the delivery of exogenous cells to stimulate or repopulate the heart. This review aims to summarize our current understanding of cardiovascular regenerative medicine by discussing cell types that already have been evaluated in the clinical setting, but also cell types that are currently being evaluated in pre-clinical research. We thereby divide stem cells into first to fifth generation, and will elaborate on the proposed working mechanisms and cardiac disease type they aim to heal.

First generation stem cells for cardiovascular repair – Autologous stem cells

Autologous skeletal myoblasts (table Ia)

Skeletal myoblasts (SkM) are progenitor cells residing in striated skeletal muscle, and are responsible for regeneration of skeletal muscle upon damage. They do not meet the stem cell criteria, as they do not have multi-lineage differentiation potential, but the injection of SkM into scarred myocardium of ischemic heart failure (HF) patients, was considered stem cell therapy in the early days. SkM are easily expandable in cell culture, and because they are destined to become contracting cells, they were a logical candidate for cell replacement therapy. Moreover, in pre-clinical investigations, SkM were able to form functional skeletal myotubes, repopulate the damaged heart, and integrate into host myocardium with formation of electromechanical junctions between host cardiomyocytes and injected skeletal myotubes.[16–18] These data resulted in the first HF patients treated with epicardial SkM injections during bypass surgery, or endomyocardial injections using specialized injection catheters.[1,2] Initial and short-term follow up results were promising with positive effects on global cardiac function, when assessed by stress echocardiography, MRI and/or pressure-volume loop analysis.[2,19,20] However, early enthusiasm subsided, when SkM injection was associated with an increased incidence of sustained ventricular arrhythmias.[21] These ventricular arrhythmias were presumed to be caused by the lack of electromechanical coupling between injected SkM and host myocardium, which is due to the lack of connexin-43 expression. Collections of injected SkM thereby formed electrically isolated islands that functioned as re-entry circuits for ventricular arrhythmias.[22]

The only double-blind, and randomized study performed to date (MAGIC trial; NCT00102128; table Ia), in which patients with depressed cardiac function and scheduled for CABG were randomized between two doses of SkM or placebo, was negative.[6] Also, most other studies that assessed endomyocardial, catheter-based injections failed to show long-term improvement of global cardiac function[5,7], whereas all initiatives for large randomized studies (CAUSMIC: NCT00626314; MARVEL[23]: NCT00526253; table Ia) were halted. Due to the association with ventricular arrhythmias, the lack of obvious long-term efficacy, and more attractive alternatives, SkM therapy currently seems to be abandoned as cellular therapy for cardiac repair.

Table Ia. Skeletal Myoblasts Trials (First Generation)

	Phase	Cell type	Status	Design	No.	Delivery Method	Condition	Transfer day after PCI	Cell number (mill)	Primary Clinical Outcome	Reference
MAGIC	II	Skel MB	terminated	RDBPC	97	IM (Epi)	ICHF	–	400/800	Safety/Efficacy: MACE/ LVEF (Echo) at 6M,	NCT00102128
CAUSMIC II	II	Skel MB	unknown	RDBPC	23	IM (Endo)	ICHF	–	300	Safety/Efficacy: MLHFQ at 1 year	NCT00626314
SEISMIC	II	Skel MB	complete	ROPC	40	IM (Endo)	ICHF	–	150-800	Safety/Efficacy: LVEF (MUGA) at 6M	NCT00375817
MARVEL	II/III	Skel MB	unknown	RDBPC	170	IM (Endo)	ICHF	–	400/800	Safety/Efficacy: QOLQ, 6min walk test at 1 year	NCT00526253

RDBPC: randomized double-blind placebo controlled, ROPC: randomized open-label placebo controlled, SKelMB: skeletal myoblast, IM: intramyocardial, ICHF: ischemic congestive heart failure, LVEF: LV ejection fraction, MACE: major adverse cardiovascular event, MLHFQ:Minnesota living with heart failure questionnaire, QOLQ: quality of life questionnaire, MUGA: multi gated acquisition scan

Bone marrow-derived mononuclear cells (table Ib)

By far the most clinical experience in cardiac cell therapy has been obtained with BM-derived mononuclear cells (BMMNC). The first clinical application was preceded by a pivotal mouse study that showed that BM-derived cells could regenerate infarcted hearts by transdifferentiation into cardiomyocytes.[24] It should be noted however, that only a small percentage of the mononuclear cell fraction of BM consists of stem cells, and that the majority of these stem cells are committed to hematopoietic lineages.[25] It is currently believed that actual transdifferentiation of BMMNC into functioning cardiomyocytes is practically non existing, and that the beneficial effect is primarily evoked by paracrine pro-angiogenic actions, and possibly by incorporation of BMMNC into neo-capillaries.[26]

Although the results of the initial mouse study in 2001 turned out to be controversial[27], reports on the treatment of the first AMI patients followed shortly thereafter.[3,4] Since then, more than 1,300 AMI patients have been treated with BM-derived cells. Even so, results of the various trials have been conflicting. In 2006, the landmark REPAIR-AMI trial was published, which was a double-blind and randomized study to assess the safety and efficacy of BMMNC in over 200 AMI patients (NCT00279175).[28] REPAIR-AMI showed improved LVEF, as assessed by LV angiography at 4 months, and, more importantly, long-term follow up revealed a reduction in clinical end points.[29] Other early studies included the BOOST and ASTAMI randomized trials that showed promising early results, but no sustained benefits after 3 and 5 years of follow up.[30–32] Moreover, recent trials with similar patient numbers, that used cardiac MRI as imaging modality, contradict the beneficial findings on global LV function that were found in the early trials.[33–36] A recent meta-analysis that compiled all clinical evidence to date, revealed a small, but significant, improvement of 2.10% on LVEF (see chapter 3 of this thesis). Intriguingly, this modest beneficial effect disappeared when sub-group analyses were performed for trial design (excluding non-controlled and cohort studies) or imaging modality (only including studies that used MRI-derived measures), thereby reinforcing conclusions from recent negative trial results.[33–36]

The fact remains that long-term follow up of the REPAIR-AMI trial showed sustained effects on clinical end points as recurrent AMI[29], although this effect was not confirmed in the long-term follow up of the BOOST trial (NCT00224536).[32,37] It is hoped that the forthcoming, phase III, BAMI study (NCT01569178), initiated by professor Zeiher and funded by the European Union, which aims to include 3,000 AMI patients, will render definite answers to this unrequited issue.

Although most trials using BMMNC were performed in AMI patients 3-30 days after the index event, some other studies evaluated the effect of BMMNC in ischemic or non-ischemic HF patients. A recent meta analysis analyzed 13 randomized, controlled trials and concluded that BMMNC therapy resulted in a modest increase in global LV function (see chapter 4 of this manuscript).[38] Moreover, intramyocardial delivery was found to be superior to intracoronary delivery. It should be noted however, that also in the treatment of HF patients, the benefit of BMMNC is controversial[39], and several well-designed studies with proper end points rendered only modest or negative results.[40–42]

Also in the field of refractory myocardial ischemia, several exploratory studies have been performed using BMMNC. In most studies, a beneficial effect was found on either cardiac function or angina

rates.[43–45] Patient numbers in these studies were low though, and it seems plausible that enriched cell populations with more pro-angiogenic potential will soon replace BMMNC therapy in this specific patient population.

Second generation stem cells for cardiovascular repair – Autologous enriched stem cells

Endothelial progenitor cells (i.e. CD34+ and CD133+ cells; table Ib)

Endothelial progenitor cells (EPC) were first described in 1997 by Asahara *et al.* as circulating CD34+ cells that can differentiate into endothelial cells.[46] They originate from the BM and were shown to repair and repopulate damaged endothelium, and incorporate into foci of pathological or physiological neovascularization. The ability to promote new blood vessel formation renders these cells ideal candidates for cellular therapy in ischemic diseases. However, fifteen years after their discovery, there is still debate on the exact definition of EPC, and a distinctive cell surface marker is lacking.[47] The majority of EPC is derived from BM, and share a progenitor cell with hematopoietic stem cells (HSC), called the hemangioblast. Both EPC and HSC are positive for CD34, CD133, and KDR, which makes distinction based on cell sorting difficult. Once plated in a culture dish, EPC differentiate into so-called early outgrowth cells (EOC) and late outgrowth cells (LOC). The latter appear only after 2-3 weeks in culture, and are able to form vessel-like structures *in vitro*. EOC are positive for the hematopoietic marker CD45, whereas LOC are not. It is therefore believed that LOC functionally represent true EPC, whereas EOC are more related to the white blood cell line.[48]

Nonetheless, these discriminating features did not enter the clinical arena yet. Thus far, studies in cardiac disease pragmatically used the surface markers CD34 or CD133 to define a pro-angiogenic precursor cell type.

In a few pilot studies in AMI patients, BM-derived cell fractions enriched for CD34+ or CD133+ cells resulted in improved perfusion only, but trends towards increased cardiac function.[49–51] Peri-operative intramyocardial injection during bypass surgery of BM-derived CD133+ cells showed favorable safety results, but failed to improve cardiac function.[52] Nevertheless, a phase III trial was designed to definitively evaluate the efficacy of surgical injection adjunct to bypass surgery in HF patients.[53] EPC have also been successfully applied in patient populations with chronic myocardial ischemia.[47] For instance, Losordo *et al.* performed intramyocardial injections in 167 patients with refractory angina pectoris with a positive response on angina rates[54], and the same group also reported favorable results in patients with critical limb ischemia.[55]

Table Ib. Bone Marrow-derived Cells Trials (First Generation)

	Phase	Cell type	Status	Design	No.	Delivery Method	Condition	Transfer day after PCI	Cell number (mill)	Primary Clinical Outcome	Reference
BOOST	I	MNC	complete	RDBPC	60	IC	AMI	4.8	2460	Safety/Efficacy: Regional systolic wall thickening (MRI)	NCT00224536
LEUVEN-AMI	II	MNC	complete	RDBPC	67	IC	AMI	1	304	Efficacy: LVEF (MRI) at 4M	NCT00264316
REPAIR-AMI	III	MNC	complete	RDBPC	204	IC	AMI	4	236	Efficacy: LVEF (LVG) at 4M	NCT00279175
ASTAMI	II	MNC	complete	RSBPC	100	IC	AMI	6	68	Safety/Efficacy: LVEF, EDV, Infarct size	NCT00199823
TIME	II	MNC	not yet recruiting	RDBPC	120	IC	AMI	3 or 7	150	Efficacy: LVEF (MRI) at 6M	NCT00684021
LateTIME	II	MNC	complete	RDBPC	87	IC	AMI	17.4	150	Efficacy: LVEF (MRI) at 6M	NCT00684060
SWISS-AMI	II	MNC	complete	ROPC	200	IC	AMI	5-7 vs. 21-28	156	Efficacy: LVEF (MRI) at 4M	NCT00355186
HEBE	II	MNC	complete	ROPC	200	IC	AMI	6	296	Efficacy: Regional LVEF (MRI) at 4M	ISRCTN 95796863
BAMI	III	MNC	not yet recruiting	ROPC	3000	IC	AMI	3 to 6	–	Safety/Efficacy: All cause death at 3Y	NCT01569178
REGENT	II	CD34/ CXCL4	complete	ROPC	200	IC	AMI	7	1.9	Safety/Efficacy: LVEF (Echo, LVG) at 6M	NCT00316381
Rostock	I	MNC/CD133	complete	N/A	32	IM (Epi)	ICHF	–	N/A		N/A
PERFECT	III	MNC/CD133	recruiting	RDBPC	142	IM (Epi)	ICHF	–	0.5-5	Efficacy: LVEF (MRI) at 6M	NCT00950274
ESCAPE	III	MNC	complete	RDBPC	250	IM (Epi)	ICHF	–	150	Safety/Efficacy: Survival at 1Y	NCT00841958
FOCUS	II	MNC	recruiting	RDBPC	92	IM (Endo-NOGA®)	ICHF	–	100	Safety/Efficacy: MVO2, LVESV, Reversible defect size	NCT00824005
STAR heart	–	MNC/ CD133/34	complete	Cohort	391	IC	ICHF	–	66	(LVEF (LVG), ETT at 1 and 5Y)	–
RENEW	III	CD34/G-CSF	recruiting	ROPC	444	IM (Endo-NOGA®)	CMI	–	0.1	Safety/Efficacy: ETT at 1Y	NCT01508910
Cell-Wave	I/II	MNC/ shock wave	complete	RDBPC	100	IC	CMI	–	N/A	Safety/Efficacy: LVEF (LVG) at 4M	NCT00326989
Chagas	III	MNC	terminated	RDBPC	183	IC	Chagas CM	–	250	Safety/Efficacy: LVEF at 1Y	NCT00349271

RDBPC: randomized double-blind placebo controlled, RSBPC: randomized single-blind placebo controlled, ROPC: randomized open-label placebo controlled, MNC: bone marrow-derived mononuclear cell, G-CSF: granulocyte-colony stimulating factor, IC: intracoronary, IM: intramyocardial, AMI: acute myocardial infarction, ICHF: ischemic congestive heart failure, CMI: chronic myocardial ischemia, CM: cardiomyopathy, LVEF: LV ejection fraction, EDV: LV end diastolic volume, ESD: LV end systolic volume LVG: left ventriculogram, ETT: exercise tolerance test, MVO2: maximal oxygen consumption

Autologous bone marrow-derived mesenchymal stem cells (table II)

The adult BM harbors another stem cell type, which is probably the most investigated adult stem cell type for cardiac repair in the current preclinical arena. They have shown significant beneficial effect in almost all experimental models of heart disease, and the first clinical applications exerted promising safety results.[56] The mesenchymal stem cell (MSC) was discovered 40 years ago as a rare population of plastic adherent cells, comprising 0.01-0.001% of BMMNC cells.[57] They represent a heterogeneous cell population, are self-renewing, can be culture expanded innumerably, and are defined as: 1) plastic adherent; 2) expressing CD90, CD73 and CD105, and not expressing CD34, CD45, HLA-DR, CD14 or CD11b, CD79a, or CD19 by FACS analysis; and 3) able to transdifferentiate into adipocytes, osteoblasts and chondrocytes *in vitro*.[58] Aside from this consensus, the exact phenotype is still under debate, and MSC identification prior to culture remains undetermined.

Over the past few years, it has become apparent that MSC can be found in most post-natal organs, including the heart[59], liver, spleen, thymus, tendon, periodontal ligament, lungs, menstrual blood, and adipose tissue.[60] The physiological role for MSC in the BM is to support the hematopoietic microenvironment. In other organs, it is believed that MSC reside in perivascular tissue, might have pericyte features, and have a role in maintaining tissue homeostasis.[60,61] Adipose tissue-derived MSC are discussed separately below.

MSC have several characteristics that make them very suitable for cardiac repair, excellently reviewed by Choi *et al.* and Williams *et al.*[56,62] First, they are able to transdifferentiate into cardiomyocytes *in vitro* and *in vivo*.[63] It is important to note, however, that *in vitro* differentiation of MSC requires epigenetic modulation, including DNA demethylization and/or histone acetylation.[64,65] The effect of chemically induced DNA demethylization or histone acetylation on genetic stability and possible chromosome aberrations is yet unknown, and may ultimately interfere with cell product safety.

Second, and more importantly, MSC are known to secrete a vast amount of paracrine factors, which can affect angiogenesis, cardiomyocyte survival, extracellular matrix remodeling, cardiac stem cell recruitment, and the cardiac immunologic milieu (table A).[56] It is believed that these paracrine factors are packed in nano particles, called exosomes.[66] Infusion of MSC conditioned medium containing these exosomes improves cardiac function, without the actual presence of MSC.[67,68] However, administration of these factors alone is believed to be less effective than infusion or injection of MSC, which is probably caused by the rapid wash out of the medium and its exosomes. Also, MSC are immune privileged, thereby enabling allogeneic cell transfer. The use and clinical application of allogeneic MSC is discussed separately below.

The biggest limitation of MSC is the very low frequency in which they are found in regular BM aspirations. Before sufficient cell numbers can be obtained for clinical applications, MSC need extensive culture expansion in clean room facilities. This implicates extra costs, but also at least 2-3 weeks between BM harvest and initiation of cell therapy. This excludes the application of autologous MSC in, for instance, the acute phase of an AMI. Importantly, in a meta-analysis of all large animal studies performed to date, MSC were found to be superior to BMMNC.[8]

Autologous MSC have been investigated in clinical pilot studies of AMI, as well as ischemic and non-ischemic HF. Chen and colleagues investigated the effect of intracoronary infusion of autologous MSC in 34 patients with sub-acute AMI, showing improved perfusion and LV function in treated patients when compared to placebo controls.[69] In a recent pilot study, autologous MSC were injected intramyocardially into the border zone of ischemic HF patients, resulting in reversed remodeling on MRI in 4 patients.[70] These results, together with the conflicting results with BMMNC in HF patients, formed the basis for the TAC-HFT trial (NCT00768066).[71] In this trial, a total of 65 ischemic HF patients were randomized to receive either BMMNC (n=22), MSC (n=22) or placebo (n=21). Intramyocardial injection using the Helix catheter was safe. Injection of MSC, but not BMMNC, resulted in a significant reduction of infarct size and regional cardiac function, when compared to placebo controls.[72] This study suggests that MSC are superior to BMMNC, although these data need to be judged with caution due to the small sample size of the TAC-HFT trial. Also, Mathiasen and co-workers will assess the safety and efficacy of autologous MSC versus placebo treatment in ischemic HF patients.[73]

Cardiogenic-oriented mesenchymal stem cells (table II)

Very recently, clinical trial results were published from a specific sub category of autologous MSC. The group of Terzic *et al.* found ways to direct MSC towards a cardiogenic phenotype using a mix of growth factors, but without genetic engineering or modification.[74] Autologous, patient-derived MSC are culture expanded following BM harvest, after which these MSC are exposed to a cardiogenic cocktail of growth factors and chemokines to acquire myocyte-like features. When transplanted in an animal model of ischemic HF, these cells were shown to improve cardiac function,[75] which resulted in the design of the phase I C-CURE trial (NCT00810238). In this randomized study, a total of 48 patients with ischemic HF were included, who received NOGA-guided intramyocardial injection of cardiogenically-oriented MSC or placebo. Two-year follow up results showed safety and feasibility of this approach, whereas global LV function was significantly enhanced after 6 months of follow up, when compared to standard of care.[76]

Autologous adipose tissue-derived regenerative cells (table II)

Adipose tissue was first identified as an alternative source of abundant numbers of multipotent mesenchymal-like stem cells in 2002.[77] Like BM-derived MSC, these cells stimulate neo-angiogenesis and cardiomyocyte survival both *in vitro* and *in vivo* by release of various angiotrophic, anti-apoptotic and immunomodulatory factors.[78,79] The frequency of ADRCs in freshly isolated adipose tissue digestates is ~ 2,500 fold greater than that of freshly aspirated BM, which implies that culture expansion is not required to generate sufficient numbers of therapeutic cells.[80] On average, 20-40 million cells can be isolated within two hours after a liposuction from as little as 200 grams of lipo-aspirate. In a large animal model of AMI, administration of freshly isolated adipose tissue-derived regenerative cells (ADRCs) improved LV function and myocardial perfusion by cardiomyocyte salvage and stimulated neo-angiogenesis in the infarct border zone, resulting in reduced infarct scar formation.[81]

Table II. Clinical Autologous Mesenchymal Stem Cell Trials (Second Generation)

	Phase	Cell type	Status	Design	No.	Cell source	Delivery Method	Condition	Primary Clinical Outcome	Reference
STEMMI	II	Autologous	complete	RDBPC	78	BM (mobilized G-CSF)	IC	AMI	Safety/Efficacy : regional systolic wall thickening MRI	NCT00135928
PROMETHEUS	I/II	Autologous	complete	RDBPC	45	BM	IM (Epi)	ICHF	Safety/Efficacy: Serious adverse events, Infarct size (MRI)	NCT00587990
C-CURE	II/III	Autologous	complete	RSBPC	240	Guided BM	IM (Endo) (C-Cath®)	ICHF	Safety/Efficacy: LVEF	NCT00810238
APOLLO	I	Autologous	complete	RDBPC	13	Adipose-tissue	IC	AMI	Safety	NCT00442806
ADVANCE	II/III	Autologous	not yet recruiting	RDBPC	360	Adipose-tissue	IC	AMI	Safety and Efficacy: infarct size (MRI)	NCT01216995
TAC-HFT	I/II	Autologous	complete	RDBPC	60	BM	IM (Endo) (Helix®)	CMI	Safety/Efficacy: MRI	NCT00768066
PRECISE	I	Autologous	recruiting	RDBPC	36	Adipose-tissue	IM (Endo) (NOGA®)	CMI	Safety: MACCE at 3Y	NCT00426868
MyStromalCell	II	Autologous	recruiting	RDBPC	60	VEGF-ADRC	IM (Endo) (NOGA®)	CMI	Safety/Efficacy: EET at 6M	NCT01449032

RDBPC: randomized double-blind placebo controlled, RSBPC: randomized single-blind placebo controlled, BM: bone marrow, G-CSF: granulocyte-colony stimulating factor, IC: intracoronary, IM: intramyocardial, AMI: acute myocardial infarction, ICHF: ischemic congestive heart failure, CMI: chronic myocardial ischemia, LVEF: LV ejection fraction, MACCE: major adverse cardiovascular and cerebrovascular event, EET: exercise tolerance test

The cardioprotective and pro-angiogenic effect in large animal models were the basis for the phase I APOLLO (NCT00442806) and PRECISE (NCT00426868) trials. APOLLO was the first-in-man experience with ADRCs in the treatment of patients with ST-elevation AMI. It showed in 14 patients that performing a liposuction in the acute phase of the AMI, as well as intracoronary infusion of ADRC within 24 hours following the primary PCI, is safe and feasible.[9] Moreover, significant effects were obtained on reduction of infarct size and the perfusion defect, which is concordant to the proposed working mechanism of ADRC therapy. Also, a trend towards improved global cardiac function and decreased LV volumes was found. The currently recruiting phase III ADVANCE trial (NCT01216995), which aims to include a total of 216 AMI patients, will evaluate the true value of ADRC therapy in AMI patients.

In the PRECISE trial, ADRC were injected intramyocardially in patients with depressed cardiac function and proof of refractory ischemia with no other treatment options, using NOGA-XP cardiac mapping. The PRECISE trial demonstrated a statistically significant improvement in VO_2max in patients treated with ADRC, when compared to those treated with placebo, although LVEF did not change (unpublished data). Two other clinical phase I/IIa studies that are aimed to assess safety and feasibility of ADRC in patients with chronic myocardial ischemia, are currently enrolling.[82]

Third generation stem cells for cardiovascular repair – Allogeneic stem cells

Allogeneic mesenchymal stem cells (table III)

As stated above, MSC are immune-privileged cells. This is achieved by several immunological features of MSC: 1) lack of expression of MHC class II antigen, and low levels of MHC class I; 2) lack of co-stimulatory molecules as CD40, CD80, and CD86; 3) secretion of immuno-modulatory factors including nitric oxide, heme-oxygenase I, and interleukin-6; 4) suppress innate immune cells via direct cell-cell contact, but also 5) suppress T-cell proliferation and alter naïve T-cells into an anti-inflammatory state.[56,83] These immunologic properties extend the applicability of MSC as a therapeutic for ischemic heart disease, as the immune system plays a pivotal role in infarct remodeling.[84,85] Moreover, its immuno-modulatory effects enable allogeneic cell transfer without the need for immunosuppressive therapies, which has several important advantages. It avoids a laborious, time-consuming, and potentially dangerous BM puncture, as well as the subsequent culturing steps in clean room facilities. Moreover, it enables the production of "off-the-shelf", and even commercially available, cell preparations derived from young and healthy donors. Such stable stem cell banks ensure adequate quality control with inherent batch-to-batch consistency. Also, a negative correlation was found between the number and functionality of progenitor cells, and age and cardiovascular risk factors.[86,87] This would make the use of allogeneic MSC, derived from young and healthy donors, in the typically elderly, cardiovascular patient population preferable over autologous cells. More importantly, cell therapy can be initiated directly after the revascularization of an AMI, thereby maximally utilizing the anti-apoptotic and immuno-modulatory capacities of the cells. However, the ideal timing of cell therapy following AMI is still a matter of debate (see below).[8]

Table A. Cardioprotective paracrine factors secreted by MSC

Secreted factor		Function
Pro-survival		
Insuline-like growth factor-1	IGF-1	Inhibits apoptosis
Secreted frizzled-related protein-2	SFRP-2	Inhibits apoptosis
Homing/recruitment of stem cells		
Thymosin β-4	Tβ-4	Promotes cell migration
Stromal-derived factor	SDF	Promotos cell homing
Cell proliferation		
Basic fibroblast growth factor	bFGF	Proliferation of smooth muscle cells and endothelial cells
Fibroblast growth factor -2	FGF-2	Proliferation of smooth muscle cells and endothelial cells
Fibroblast growth factor -7	FGF-7	Proliferation of endothelial cells
vascular endothelial growth factor	VEGF	Proliferation of endothelial cells en migration
Platelet-derived growth factor	PDGF	Proliferation of smooth muscle cells
Tumor necrosis factor-α	TNF-α	Cell proliferation
Granulocyte colony stimulating factor	G-CSF	Neutrophil proliferation and differentiation
Insuline-like growth factor-1	IGF-1	Regulates cell growth and proliferation
Macrophage colony stimualting factor	M-CSF	Monocyte proliferation and differentiation
Secreted-frizzled-related protein-1	SFRP-1	Enhances cell development
Secreted frizzled-related protein-2	SFRP-2	Enhances cell development
Vessel fomation		
Vascular endothelial growth factor	VEGF	Tube formation
Placental growth factor	PlGF	Promotes angiogenesis
Transforming growth factor-β	TGF-β	Promotes vessel maturation
Metalloproteinase-1	MMP-1	Tubule formation
Metalloproteinase-2	MMP-2	Tubule formation
Remodeling of extracellular matrix		
Metalloproteinase-1	MMP-1	Loosens extracellular matrix
Metalloproteinase-2	MMP-2	Loosens extracellular matrix
Metalloproteinase-9	MMP-9	Loosens extracellular matrix
Plasminogen activator	PA	Degradation of matrix molecules
Tumor necrosis factor-α	TNF-α	Degradation of matrix molecules
Immunomodulatory		
Heme oxygenase-1	HO1	CD4+ T-cell proliferation inhibitor
Hepatocyte growth factor	HGF	T-cell proliferation inhibitor
Indoleamine 2,3-dioxygenase	IDO	Inhibits innate and adaptive immune cell proliferation
Inflammation		
Interleukin-6	IL-6	Inflammation regulator, VEGF induction
Prostaglandin E2	PGE-2	Decreases inflammation
Inducible nitric oxide synthase	iNOS	Decreases inflammation

Most pre-clinical, but also clinical experience with MSC thus far was obtained using allogeneic MSC.[8] Interestingly, there is some pre-clinical evidence in large animals that small percentages of injected MSC have the capacity to engraft in cardiac tissue, and to transdifferentiate into cardiomyocytes, endothelial cells, and smooth muscle cells.[63,88,89] These results were obtained in pigs, but could not be confirmed in other species. In dogs, MSC seemed to transdifferentiate into vascular cells only[90], whereas in a sheep model of HF no engraftment or transdifferentiation could be detected at all.[91] The fact that there seem to be inter-species differences, as well as the low rate of actual transdifferentiation, make the clinical relevance of this phenomenon questionable. Moreover, the robust functional improvement following MSC transplantation in both AMI and chronic HF models[8], is disproportionate to this low rate of engraftment and transdifferentiation. Hence, other mechanisms must be at play. Most of these mechanisms were already mentioned above, summarized in table A, and are primarily based on the paracrine properties of the cells. More specifically: MSC are known to have anti-apoptotic and pro-survival capacities, secrete pro-angiogenic proteins, and influence the local immune system and extracellular matrix composition. Moreover, recent studies have shown that also the postnatal heart contains resident stem cells.[13] Delivery of MSC to infarcted or hibernating myocardium may regenerate myocardium and improve cardiac function by stimulating these resident cardiac stem cells and cardiomyocytes to (re-)enter the cell cycle, thereby initiating cardiomyocyte generation or proliferation.[13,92–95]

Numerous pre-clinical investigations in small and large animal models preceded the few clinical studies that have been performed in AMI and HF patients using allogeneic MSC to date (see both reviews and the meta-analysis by Van der Spoel *et al.*[8,56,96]). In AMI patients, one clinical study investigated the intravenous administration of allogeneic MSC in 39 AMI patients versus 21 placebo controls, briefly following an AMI.[10] It was found that infusion of a considerable number of allogeneic cells was safe, and did not result in adverse reactions or an immunologic response. Moreover, patients exhibited a reduction of ventricular arrhythmias, increased pulmonary function, and improved LVEF after 3 months. Other clinical experience with AMI patients is lacking, because MSC delivery to recently infarcted hearts has been troublesome. More specifically, endomyocardial injection is prone to perforation so briefly following AMI, and intracoronary delivery resulted in vascular plugging with no-reflow phenomena in several pre-clinical studies.[97–100] This issue was recently overcome in a large pre-clinical AMI study using 88 sheep. In this study, the safety, feasibility and efficacy was assessed of intracoronary infusion of a specific Stro3+, immune-selected, immature sub type of MSC directly following an AMI. It was found that intracoronary infusion of these so-called mesenchymal precursor cells (MPC) is safe, does not hamper coronary flow, and has marked beneficial effects on global and regional cardiac function.[95,101] These effects are evoked by myocardial salvage, neo-vascularization, and stimulation of endogenous cardiac regeneration. The observations in this study resulted in the design of a multicenter, phase IIa/b, double blind, randomized and placebo-controlled clinical trial. The Allogeneic-Mesenchymal-precursor-cell-Infusion-in-myoCardial-Infarction (AMICI) trial (NCT01781390), in which European, Australian and US sites will participate, is aimed to prove safety, feasibility and efficacy of MPC therapy in a minimum of 225 patients with ST-elevation AMI, and recently the first patient was treated successfully.

Table IV. Clinical Allogeneic MSC/Cardiac Progenitor Cell Trials (Third/Fourth Generation)

	Phase	Cell type	Status	Design	No.	Cell source	Delivery Method	Condition	Primary Clinical Outcome	Reference
PROCHYMAL	II	Allogeneic	recruiting	RDBPC	220	BM	IV	AMI	Safety: (LVESV)	NCT00877903
Mesoblast AMI	I/II	Allogeneic	recruiting	RSBPC	25	BM	IM(Endo) (NOGA®)	AMI	Feasibility/Safety	NCT00555828
MultiStem	I	Allogeneic	complete	ONPC	25	BM	IM(adventitia of CA) (Cricket®)	AMI	Safety: Adverse Event at 1M	NCT00677222
AMICI	II	Allogeneic	not yet recruiting	RDBPC	225	BM	IC	AMI	Safety/Efficacy: Infarct size (MRI) at 6M	EUCTR2010-020497-41-NL
POSEIDON	I/II	Auto/Allo	complete	RONPC	30	BM	IM(Endo) (Helix®)	ICHF	Safety/Efficacy: TE-SAE at 1M	NCT01087996
Mesoblast CHF	II	Allogeneic	unknown	RSBPC	60	BM	IM(Endo) (NOGA®)	CHF (ischemic/ idiopathic)	Feasibility and Safety	NCT00721045
SCIPIO	I	Autologous	recruiting	RONPC	40	CPC (c-kit)	IC	ICHF	Short term Safety	NCT00474461
CADUCEUS	I	Autologous	complete	RONPC	31	CPC (Cardiospheres)	IC	ICHF	Safety	NCT00893360

RDBPC: randomized double-blind placebo controlled, RSBPC: randomized single-blind placebo controlled, ONPC: open label, non-placebo-controlled, RONPC: randomized, open label, non-placebo-controlled, BM: bone marrow, CPC: cardiac progenitor cells, IV: intravenous, IC: intracoronary, IM: intramyocardial, CA: coronary artery, AMI: acute myocardial infarction, CHF: congestive heart failure, ICHF: ischemic congestive heart failure, LVESV: LV end systolic volume

These Stro3+ MSC are an interesting cell type for cardiac repair, as they were shown to exert extensive cardioprotective effects that exceed the cardioprotective effects of regular MSC.[102,103] It is believed that this difference is evoked by more potent paracrine activity, as well as more extensive multilineage differentiation potential.[102,104] In several pre-clinical investigations, intramyocardial injection of these cells resulted in marked improvement of cardiac function and LV remodeling in models of AMI, ischemic, and non-ischemic heart failure.[91,102,105,106] Recently, the results from a clinical, phase I/II study, assessing the effect of percutaneous endomyocardial injections of allogeneic MPC in 60 HF patients, were presented (Mesoblast-CHF; NCT00721045). Allogeneic MPC injections up to a dose of 150 million cells were shown to be safe and feasible without a clinically significant anti-allogeneic immune response. More importantly, MACCE rate, cardiac mortality and composite end points for heart failure were markedly decreased at 12 month clinical follow up (unpublished data). This study resulted in the preparations of a phase III study analyzing the therapeutic effect of MPC therapy via intramyocardial injections in 120 congestive HF patients.

In the POSEIDON trial (NCT01087996) the difference between autologous and allogeneic MSC in the treatment of ischemic HF was evaluated in 30 patients.[107] It confirmed the safety data of the Mesoblast-CHF study, as no significant anti-allogeneic immune response was found in patients treated with allogeneic MSC. Moreover, cardiac function improved equally in both autologous and allogeneic groups. The future POSEIDON-DCM (NCT01392625) will evaluate the effect of MSC in patients with non-ischemic dilated cardiomyopathy, whereas larger phase III initiatives are much anticipated.

CellBeads

One of the biggest challenges in the cell therapy field today is the poor retention rate of therapeutic cells upon local delivery in the heart, with retention rates as low as 1% after intracoronary delivery.[108,109] Even though permanent engraftment of stem cells is not required to elicit the cardio-protective effect, it seems logical that the greater the number of cells that are retained in the injured myocardium and the longer they reside there, the more pronounced the potential beneficial effect will be. A new concept of stem cell delivery has recently become available owing to advances in the field of biotechnology, as it is currently possible to encapsulate MSC in a biocompatible alginate shell.[110] Alginate encapsulation of varying numbers of MSC results in so-called CellBeads™. These MSCs have been genetically modified to secrete a proprietary recombinant GLP-1 fusion protein, which consists of two GLP-1 molecules bound by an intervening peptide. This form of recombinant GLP-1 is more stable than endogenous GLP-1, rendering a longer half-life and thus prolonged therapeutic potential. The alginate coating of the CellBeads is permeable to the GLP-1 fusion protein and MSC-derived paracrine factors, allowing for continuous delivery, while protecting the MSC from the patient's immune system. Also, oxygen and nutrients can freely pass through the alginate shell, which renders the MSC viable for a long period of time. Thus, Cellbeads are potentially a unique, biological, long-term, local drug delivery platform that is capable of delivering GLP-1, or other therapeutic proteins, in addition to MSC-derived factors (VEGF, MCP-1, IL-6, IL-8, GDNF and NT-3) to any target tissue. These CellBeads can be delivered safely to infarcted myocardium by intracoronary infusion, resulting in engraftment

and production of the recombinant protein for at least 7 days post infusion.[111] The potential efficacy of CellBeads in a large animal model of AMI is currently being analyzed, after which the clinical potential of this promising new therapy will become evident.

Fourth generation stem cells for cardiovascular repair – Cardiac-derived stem cells

Cardiac stem cells (table III)

For decades, the heart has been considered a post-mitotic organ, without the capacity to self-renew or regenerate upon inflicted damage. This dogma has recently been abandoned by the discovery by several groups that considerable cardiomyocyte turnover occurs throughout life in healthy, aged, and damaged hearts.[12,112,113] This self-renewing capacity was found to be based on both the intrinsic capacity of senescent cardiomyocytes to re-enter the cell cycle, as well as the presence of endogenous cardiac stem cells (CSC).[13,114–116] Although the rate of myocyte turnover varied between 40% in a lifetime[112] to over 40% per year[12] depending on the way it was measured, there is now concensus that the cardiomyocyte compartment is substituted approximately 8 times during the adult life time of a healthy indiviual.[113,117] The fact that the heart contains endogenous regenerative potential caused a paradigm shift with regard to cellular therapies to mend broken hearts. It initiated a quest to find ways to direct resident cardiomyocytes to re-enter the cell cycle and start proliferation. Moreover, the isolation of CSC was deemed a potential holy grail for regenerative therapies for CV disease, as these cells might be very effective in regenerating damaged myocardium.

To date, several types of CSC have been identified.[13] The most extensively studied stem cell type is the cKit+ CSC, first described in 2003.[118] They reside in niches in the post-natal heart, which are primarily localized in the atria and in the apex of the heart.[13] This CSC is multipotent, and has the ability to transdifferentiate into endothelial cells, smooth muscle cells and cardiomyocytes.[115] They were found to ameliorate cardiac function when transplanted in pre-clinical models of HF[118,119], which laid the basis for the SCIPIO trial (NCT00474461). In this trial, 16 ischemic HF patients were treated with intracoronary infusion of 1 million autologous CSC. The main finding of SCIPIO was that intracoronary infusion of these cells is feasible and safe, whereas it also resulted in improved cardiac function and decreased infarct size at 1 year follow up.[15] These results warrant further investigation of these cells in larger phase II studies.

Cardiosphere derived cells

Cardiospheres were first described in 2004, and are defined as spherical clusters of undifferentiated cells that evolve when adult cardiac tissue specimens are placed in suspension culture.[120] These cardiospheres contain a heterogeneous cell population with proliferating cKit+ cells in its core, which are

surrounded by differentiating cells that express endothelial and cardiac markers. When cardiospheres are plated and culture expanded, cardiosphere-derived cells (CDC) can be obtained reproducibly.[121] These CDC express stem cell markers, as well as markers vital for contractile and electrical function. It is believed that they possess greater regenerative potential than CSC, as they mimic the stem cell niches that are also found *in vivo*.[122] Also, they have more paracrine activity than pure cKit+ CSC populations.[123] When transplanted in both small and large animal models of ischemic HF, these cells form new cardiac tissue, improve cardiac function, and attenuate adverse remodeling.[121,124] These promising pre-clinical findings initiated the CADUCEUS trial (NCT00893360), in which a total of 17 patients with ischemic HF with baseline LVEF of 25-40% were treated with intracoronary infusion of CDC, as opposed to 8 patients who received standard of care. After 6 months, CDC infusion proved to be safe, whereas MRI analysis showed reduction of infarct size, and improved regional cardiac function. However, LV dimensions and LVEF were not significantly enhanced.[14] It seems that the high hopes that were raised in pre-clinical studies could not be confirmed, although only larger studies as the forthcoming ALLSTAR trial (NCT01458405) can provide definitive answers.

It should be noted that, to obtain cells for both therapies, a cardiac muscle biopsy, but also clean-room facilities, are required. Cardiac biopsy is an invasive procedure with considerable risk of perforation of the right ventricle, and poses a big disadvantage of both CSC and CDC therapy, whereas resident CSC can also be stimulated by MSC transplantation (table A).[92,94,95]

Fifth generation stem cells for cardiovascular repair – Pluripotent stem cells

Embryonic stem cells

Embryonic stem cells (ESC) are derived from the inner cell mass of the developing embryo, and are able to transdifferentiate in all cell and tissue types. This is why the ESC is the prototypical stem cell, capable of unlimited expansion and self-renewal. Theoretically, given their versatility and the possibility of generating beating cardiomyocytes, ESC are the ultimate candidate for cell-based regenerative therapies for cardiovascular disease.[125,126] Moreover, when transplanted into rodent and large animal models of HF, ESC-derived cardiomyocytes engraft into host tissue and improve cardiac function.[127,128] However, cell therapy using ESC raises several concerns that have not been addressed sufficiently yet to proceed to a clinical application: 1) as ESC are derived from embryos, there are ample ethical and societal issues; 2) ESC are prone to teratoma formation, when cells remain in their undifferentiated state; 3) ESC are by definition allogeneic and not immune-privileged, which may lead to immune rejection; 4) the yield of cardiomyocytes from ESC cultures is still too low; 5) competency of ESC-derived cardiomyocytes to electrically and/or mechanically integrate into host myocardium. Extensive research is currently ongoing in order to solve these issues.

Induced pluripotent cells

Recently, a newer cell type was discovered that might circumvent several of the concerns that were raised in the previous paragraph. Induced pluripotent stem cells (iPS) are pluripotent cells that are derived from mature, differentiated cells, such as skin fibroblasts. By overexpressing some reprogramming factors (*i.e.* Sox-2, c-Myc, Oct 3/4, and Kfl4) that are also expressed by ESC, such specialized somatic cells can be reprogrammed to reverse to an embryonic state.[129] iPS have almost identical pluripotent and proliferative potentials as ESC, and can be differentiated into any desired cell type, including cells from the cardiovascular lineage.[130,131] Thus far, human iPS have been successfully transplanted into the murine heart, resulting in regeneration of the myocardium, and improving cardiac function.[132] Because iPS can be patient-derived cells, the ethical issues that were raised around ESC are circumvented, whereas immune rejection is likely to be absent. However, also with iPS, several issues remain to be solved before the field can advance to a clinical application in cardiovascular patients. Most importantly, the disruption of the genome by inserting genes can cause gene mutations ranging from mild aberrations to tumorigenesis.[133,134] Although several groups have reported the possibility to use vectors that do not permanently change the host's DNA, these new techniques are highly inefficient and need extensive fine tuning. The issues that need to be addressed can be summarized as: 1) effectuate more efficient induction of cardiomyocyte lineages; 2) selective expansion of cells of the cardiomyocyte lineage; 3) purification of differentiated cardiomyocytes, as undifferentiated iPS might develop teratomas; 4) address issues with possible acquired immunogenicity; 5) ensure electromechanical coupling of implanted iPS-derived cardiomyocytes.[133]

Induced cardiomyocytes

The fact that somatic cells can be re-programmed into pluripotent cells raises the question if these somatic cells can not be re-programmed towards a cardiomyogenic fate, without first becoming a progenitor cell. This would circumvent the issue of teratogenesis. Indeed, recently Ieda and coworkers were able to reprogramme murine dermal fibroblasts into functional cardiomyocytes by inserting three developmental transcription factors (Gata4, Mef2c, and Tbx5).[135] Although such reprogrammed fibroblasts might be a source of cardiomyocytes for regenerative purposes, the *in vitro* reprogramming efficiency should be improved significantly. Nonetheless, other reports suggest that cardiac-derived fibroblasts can undergo the same reprogramming into functional cardiomyocytes, even in *in vivo* situations. Resident cardiac fibroblasts, reprogrammed into cardiomyocytes, were shown to improve cardiac function and reduce ventricular remodeling.[136,137] This new approach might signify the next paradigm shift in cellular/gene therapy, as it would enable direct reprogramming of scar tissue into functional myocardium without the need for actual cell transplantation.

What cell for what cardiovascular disease type?

This review describes the use of cells for the treatment of cardiovascular diseases. Although it describes many cell types, we do not claim completeness, and several cells have not been mentioned. Moreover, the cardiovascular patient as such does not exist, and there are many disease types with all different pathogeneses. We will only briefly discuss AMI and HF below, and elaborate on the most logical candidate cell for these two specific disease types, whereas many other cardiovascular diseases will not be addressed.

Most clinical studies to date were performed in AMI patients, and used BMMNC. To determine the optimal cell for AMI patients, however, one should ask what we aim to treat by using cell therapy. Obviously, we should strive to minimize damage inflicted by ischemia and reperfusion, thereby reducing infarct size and thus minimizing LV remodeling.

We believe that the ideal cell for AMI patients has the following charateristics: 1) pronounced paracrine anti-apoptotic, pro-angiogenic, and immuno-modulatory capacities; 2) mobilize or stimulate resident CSC and/or cardiomyocytes to proliferate; 3) available during the (hyper)acute phase of the AMI; 4) non-embryonic; 5) multipotent; 6) autologous or non-immunogenic. BMMNC contain some of those characteristics, but MSC harbor all, whereas its paracrine capacities exceed those of BMMNC.[56] Moreover, the immune-privileged state of MSC renders the possibility of an "off-the-shelf" allogeneic cell product, which enables delivery directly following reperfusion of the AMI. It should be noted that also autologous adipose tissue-derived MSC can be available in the acute phase of the AMI, given their high frequency in, and easy accessibility of, adipose tissue.[9] All other cell types necessitate cell culture expansion, which makes the application within hours following the AMI impossible. Thus, to date, MSC seem the most logical candidate for cellular therapy in AMI patients. The AMICI trial, as well as several other forthcoming phase II studies, will render more insight in the safety and efficacy of allogeneic MSC in AMI patients.

In contrast to AMI, HF is a chronic condition with a much broader time window of possible stem cell transplantation, thereby enabling the use of other autologous cell types. Ideally, in patients with heart failure due to systolic dysfunction, cells should be applied that can 1) contribute to the contractile apparatus; 2) influence the remodeling process; and/or 3) enhance blood and nutrients supply. It is still far from feasible to transplant pluripotent cells that have differentiated into therapeutic amounts of cardiomyocyte-like cells, and that engraft and electromechanically couple with the host myocardium. However, in recent years, several cell types that are already committed towards a cardiomyogenic lineage have entered the (pre-)clinical arena, whereas the role for BMMNC seems to subside.[38,40] Interestingly, the proof of principle of cardiac stem cells and cardiosphere-derived cells in HF has been established, and the near future will probably prove whether these cells have a definite place in this disease type.[14,15,124] Currently the most feasible cell type in HF, however, is the MSC. They are readily available, and both autologous and allogeneic MSC have been shown to reverse cardiac remodeling following intramyocardial injection, which might be associated with their stimulatory effect on resident cardiac stem cells and cardiomyocytes, as well as their pro-survival and pro-angiogenic potential.[8,63,92,106,138]

Moreover, MSC driven towards a cardiogenic phenotype were recently shown to have beneficial effects in HF patients.[76] In conclusion, in our opinion, MSC are still the cornerstone of contemporary cardiac cellular therapy until potential new players take over.

Timing of stem cell delivery

One of the unsolved issues in cardiac cell therapy is the ideal timing of cell transplantation following the AMI. Almost all clinical studies that assessed the effect of BMMNC infused the between 2-30 days following the AMI. This was primarily based on results in pilot studies, the landmark REPAIR-AMI trial, as well as logistical considerations.[139–141] The recent SWISS-AMI, TIME and late-TIME trials, but also several meta-analyses showed no difference in benefit, if there was any benefit at all, between the early and late time points within these limits.[34,36,142,143] It should be noted, however, that in AMI patients one of the predominant working mechanism of cell therapy is believed to be through cardiomyocyte salvage, which is evoked by the anti-apoptotic and pro-survival properties of the cells. This suggests that cell therapy should be initiated soon after reperfusion, as in that period most cardiomyocytes are at risk for necrosis or apoptosis.[144,145] We believe that the anti-apoptotic and pro-survival characteristics of, for instance, MSC are best utilized when these cells are infused briefly following the primary PCI. This implicates that the cells are infused in a hostile environment, and that many transplanted cells may not survive. However, it is this hostile environment that the cells need to ameliorate, not only by their anti-apoptotic and pro-survival capacities, but also by influencing the local immunologic milieu[83] and reducing oxidative stress.[68] It was recently posed that the ideal time window for cell transplantation is within 6 hours after the primary PCI, or 5 days later.[84] Indeed, our group found that intracoronary infusion of Stro3+ MSC directly during reperfusion in an ovine model of AMI, resulted in extremely high cell retentions, a marked reduction of infarct size, and improved global and regional cardiac function.[95] This seems to confirm that, although many MSC may subside directly following AMI, the surviving MSC can exert pronounced beneficial effects.

However, injection of stem cells into sub-acute AMIs of 2-4 weeks old can still preserve myocardium and reverse cardiac remodeling.[8,14] Moreover, as discussed above, stem cell therapy in remodeled and already failing ventricles can still result in reduction of LV volumes.[6,70,71,107] The most beneficial effect in post-AMI patients though, is likely to be achieved in the yet non-dilated ventricle.

Delivery methods

Although the field of cell therapy has advanced considerably, and many cell types are currently in phase II clinical testing, several issues still remain. Finding the most appropriate cell delivery method is one of those issues, and still a matter of debate. Several techniques have been described, including intravenous, intracoronary, percutaneous endomyocardial, surgical epicardial, and retrograde

transvenous into the coronary sinus, but percutaneous intracoronary and endomyocardial delivery are the most widely used techniques.[146,147] The best delivery technique largely depends on the disease type. Intracoronary delivery necessitates homing to the site of injury, which includes passage through the endothelial barrier. We currently know that homing signals (*i.e.* SDF-1 expression) are highest within the first few days following an AMI, whereas they subside to sub-clinical levels in chronic HF.[148] This implicates that briefly following an AMI, intracoronary infusion can be applied, and stem cells are attracted to the site of injury.[149–151] This was confirmed in several pre-clinical studies that revealed that intracoronary infusion efficiently targets post-AMI myocardium.[95,99,108] In most clinical studies thus far, a stop-flow technique was adopted by using an over-the-wire balloon that is briefly inflated during stem cell delivery. However, recent studies suggest that continuous infusion without balloon occlusion might result in comparable efficacy results, and even better stem cell homing.[9,95,152]

Because homing signals are mostly absent in failing hearts, intracoronary infusion results in sub-optimal stem cell homing in HF patients.[153] Hence, in this patient population, direct intramyocardial injection has been the preferred mode of stem cell delivery since the beginning of cellular therapy[1,2], and was applied in numerous clinical studies thus far. Intramyocardial injection ensures delivery of the stem cells directly into the interstitial space of the myocardium. However, defining and reaching the target area remains a challenge. Endomyocardial injection using an endovascular approach, combined with electromechanical mapping (NOGA-XP in combination with MyoStar[154] injection catheter), is currently most widely used[2,40,70,107,155–157], and ensures accurate localization of ischemic, hibernating, or scarred areas.[158] Also, several other injection catheters were developed, all with their specific advantages or disadvantages (Helix[159], MyoCath[160], Stiletto[161], SilverPoint). Direct epicardial injection during (bypass) surgery is yet another option for intramyocardial stem cell delivery, and enables direct visualization of the scar and border zone.[1,6,52,162] However, this approach necessitates sternotomy or thoracotomy, which might be unwanted in this frail patient population.

Considerations and future directions

Cellular therapy for both AMI and HF patients has progressed substantially, since the first patients were treated more than a decade ago. Safety and feasibility has been shown for numerous cell types in phase I/IIa studies, and the field is slowly progressing towards phase IIb and III clinical trials. However, many questions still remain unanswered. It is yet unknown what cell type will prevail, what delivery method is safest and most efficient, and the optimal timing of cell delivery is still controversial.

We believe that the forthcoming, EU-sponsored, phase III BAMI trial will definitively show if there is a place for BMMNC in AMI patients, as it is supposed to be powered to find differences on hard clinical endpoints. Moreover, phase II studies using intracoronary or intravenous delivery of allogeneic MSC will assess their presumed superiority to BMMNC, whereas the role for CDC in AMI patients is yet to be determined. Also the field of HF treatment is progressing, recent promising results of the TAC-HFT trial

favoring MSC over BMMNC. Moreover, a phase III study was initiated to assess the efficacy of Stro3+ MSC in 225 ischemic HF patients, and the sequel of the C-CURE trial is much anticipated.

The pre-clinical field is currently progressing rapidly in the ongoing search for new cells, or optimizing existing therapies. Of note are the numerous investigations that are ongoing to enhance stem cell homing and engraftment by gene therapy, or the application of micro-RNA.[163–165] Moreover, numerous biomaterials are currently assessed to improve cell retention, including hydrogels, alginate, and extra cellular matrix surrogates.[166,167] Genetic engineering or preconditioning of stem cells with pro-survival or anti-apoptotic factors were shown to have beneficial effects on stem cell survival, whereas preconditioning of the receiving environment were also beneficial.[168–173]

In conclusion, our knowledge about cellular therapy for cardiovascular repair has progressed significantly over the past two decades. Although it seems that the role for first generation cells is subsiding, cells from the third and fourth generation show pronounced effects in preclinical investigations and favorable results in phase I clinical studies. It seems obvious that cell therapy for cardiovascular disease is here to stay, although several questions remain to be answered in future investigations.

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PART II

First generation stem cells in ischemic heart disease patients

Bone marrow-derived mononuclear cells and skeletal myoblasts

Chapter 3

Intracoronary Stem Cell Infusion Following Acute Myocardial Infarction:

A Meta-analysis and Update on Clinical Trials

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Abstract

Background Several cell-based therapies for the adjunctive treatment of acute myocardial infarction (AMI) have been investigated in multiple clinical trials, but the benefits still remain controversial. This meta-analysis aims to evaluate the efficacy of BMMNC therapy in AMI patients, but also explores the effect of newer generations of stem cells.

Methods and Results A random-effects meta-analysis was performed on randomized controlled trials (RCT) investigating the effects of stem cell therapy in patients with AMI that were published between January 2002 and September 2013. The defined endpoints were left ventricular ejection fraction (LVEF), left ventricular end-systolic and end-diastolic volumes (LVESV/LVEDV), infarct size and major adverse cardiac and cerebral event (MACCE) rates. Also several subgroup analyses were performed on BMMNC trials. Overall, combining results of 22 RCTs, LVEF increased by +2.10% (95% CI, 0.68- 3.52 , P=0.004) in the BMMNC group as compared to controls, evoked by a preservation of LVESV (-4.05 ml ; 95% CI, -6.91- -1.18, P=0.006), and a reduction in infarct size (IS; -2.69%; 95% CI, -4.83- -0.56, P=0.01). However, there is no effect on cardiac function, volumes, nor infarct size, when only randomized controlled trials (n=9) were analyzed that used MRI-derived endpoints. Moreover, no beneficial effect could be detected on MACCE rates following BMMNC infusion after median follow-up duration of 6 months.

Conclusions Intracoronary infusion of BMMNC is safe, but does not enhance cardiac function on MRI-derived parameters, nor does it improve clinical outcome. New and possibly more potent stem cells are emerging in the field, but their clinical efficacy still needs to be defined in future trials.

Introduction

Despite advancements in treatment options, ischemic heart failure (IHF) remains the leading cause of morbidity and mortality in the Western world.[1] Therefore, the search for new therapeutic strategies to prevent adverse ventricular remodeling following acute myocardial infarction (AMI) and subsequent development of IHF is ongoing. More than a decade after the first patient with an AMI was treated with intracoronary infusion of unfractioned bone marrow-derived mononuclear cells (BMMNC)[2], numerous clinical studies have investigated cell-based therapy as an adjuvant treatment in AMI patients. These studies have repeatedly shown that stem cell therapy is safe and feasible. However, although initial results were promising with significant improvement in left ventricular (LV) function and volumes [3–5], other studies showed ambiguous or even negative results.[6–9] This controversy resulted in an continued search for new cell types, and methods to improve outcomes, but still many questions remain.

Thus far, it has been difficult to make solid statements on efficacy and long-term effects on clinical outcomes of cellular therapy due to the limited number of treated patients, and the relatively short follow up (FU) period. However, recently, some larger studies reported their primary results, whereas other studies presented long-term FU data.[5,10–12]

Several meta-analysis regarding BMMNC for the treatment of AMI and ischemic heart failure have been published to date.[13–15] These analyses showed an improvement of only 2-3% on left ventricular ejection fraction (LVEF), and a significant reduction of major adverse cardiac and cerebrovascular events (MACCE; for example all-cause mortality; OR 0.39; 95% CI 0.27-0.55).[14] However, these manuscripts only evaluated the use of autologous BMMNC on cardiac repair, whereas several other cell types have now been investigated to date. Moreover, in the most cited meta-analysis of Jeevanantham, data of AMI and IHF patients were pooled for the evaluation of clinical outcome parameters and subgroup analyses, which might have clouded the outcome in AMI patients.[14]

The current meta-analysis focuses solely on AMI patients, who have been treated with an infusion of BMMNC, but also autologous or allogeneic mesenchymal stem cells (MSC), adipose tissue-derived regenerative cells (ADRC) or cardiosphere-derived cells (CDC). It thereby provides a side-by-side comparison of BMMNC and other cell populations in AMI patients.

Methods

This meta-analysis was executed according to the Quorum statements.[16] Briefly, a random effect meta-analysis was performed that included all randomized controlled trials regarding stem cell therapy for the treatment of AMI, published on Medline (Pubmed) between July 2002 and September 2013. BMMNC were the main focus in this study, as the majority of studies to date assessed this specific cell type. Moreover, the effects of BMMNC therapy were compared with newer generations of stem

cells, including MSC, bone marrow progenitors (CD133+/CD34+ cells), ADRC and CDC. The following search strategy was applied: "stem cells", "progenitor cells", "mononuclear cells", "adipose tissue-derived regenerative cells", "mesenchymal stem cells', "cardiac-derived stem cells", "bone marrow", "vascular stromal fraction", "adipose stem cells", "mesenchymal-like stem cells", "coronary artery disease", "myocardial infarction", "cardiac repair", and "myocardial regeneration". Only articles published in English were included (Supplement Table I). Studies were included that met the following criteria: (1) randomized controlled trials with an appropriate control group who received standard therapy, (2) conducted in patients with an AMI that occurred less than 3 months before, (3) using stem cells that were administered by intracoronary or intravenous injection, (4) total of number of patients enrolled should exceed 10, (5) stem cells were derived from adipose tissue, bone marrow or heart, (6) given in an allogeneic or autologous setting.

Data abstraction and analysis was performed by three different researchers (RdJ, JH, SS) and reported on standardized forms. LVEF, left ventricular end-systolic volume (LVESV), left ventricular end-diastolic volume (LVEDV) and infarct size were assessed as outcome measures as well as clinical outcome. Additional subgroup analyses were performed within the RCTs that investigated BMMNC therapy, in an attempt to gain more insight into possible discriminating parameters or conditions that might improve outcome in future trials.

Subgroup analyses that were conducted are: (1) follow up (FU) duration of 6 months, 6-18 months, and 18-60 months; (2) the different imaging modalities that were used to assess LVEF, LV volumes and infarct size; (3) LVEF at baseline (<40%, <45%, <50%, >50%); (4) the amount of infused cells (<50 million, <100 million, >100 million), (4) timing of delivery (< 2 days, 2-7 days (7 days was the median in this analysis), > 8 days after MI); (5) delivery method (intracoronary 'stop-flow' technique, continuous intracoronary infusion); (6) location of AMI (anterior wall versus all other AMI locations); (7) the used cell preparation method and the use of heparin in the final cell suspension; and (8) Lymphoprep versus Ficoll-based isolation.

Data analysis

Left ventricular function was the primary endpoint of our analysis. In particular, we studied the difference in mean LV ejection fraction change (LVEF, from baseline to follow-up) between patients receiving stem cells and control treatment. We have applied inverse-variance weighting to combine the results from independent studies. Most studies reported mean LVEF ± one standard deviation (SD) at baseline and follow-up. The mean $LVEF_{change}$ was then determined as $LVEF_{follow\text{-}up}$ - $LVEF_{baseline}$, whereas the SD_{change} was estimated according to the method that is described by Hristov *et al*.[17] For studies that report standard errors of the mean (SEM), SDs were determined as SEM*√(sample size). In case interquartile ranges are reported, SDs are estimated as range/4. We applied a random effects model to obtain an overall estimate of the treatment effect, which we report as point estimate and 95% confidence interval (CI). Heterogeneity was analyzed with the I^2 statistic, and was defined as low (25%-50%), intermediate (50%-75%) or high (>75%).

We have applied similar methodology to study several secondary endpoints, including (mean changes in) left ventricular end systolic (LVESV) and end diastolic (LVEDV) volumes, infarct size as measured by cardiac MRI, and perfusion defect as measured by SPECT. We applied the Mantel-Haenszel odds ratio to obtain an overall estimate of the odds ratio for MACCE, again assuming random effects.

All analyses were performed using Review Manager 5.2 analysis software (Rev Man, Version 5.2, Copenhagen, The Nordic Cochrane Centre, The Cochrane collaboration, 2012). We considered p-values <0.05 (two-sided) as statistical significant. Funnel plots were constructed to explore publication bias.

A detailed description of the methods can be found in the material and methods section in the data supplement.

Results

Search results

The final search on September 1st 2013 resulted in a total of 386 articles. The majority of articles were excluded, due to study subject (chronic heart failure or G-CSF treatment), duplicate reports or reviews, resulting in a total of 47 studies. When cohort studies were omitted, 42 articles were used in final analysis.[3–7,10,11,18–52] Finally, a total of 30 RCTs were used in this meta-analysis, comprising a total of 2037 patients, 1218 of whom were treated with cells (Supplemental Figure A). Twenty-two RCTs investigated BMMNC for cardiac repair, whereas 3 trials investigated MSC or MSC-like cells, 4 trials subjected bone marrow progenitors and 1 trial investigated the effects of CDC.

Study Quality

The quality of the RCT was assessed by the Jüni criteria (Supplemental Table II).[53] In 60% of the RCTs, patients and or investigators were not blinded for the cell intervention. Control patients did not undergo a sham biopsy and infusion of cells in most of these studies. Patient follow-up was completed in all studies.

Study Characteristics

The average of participating patients per study was 68 ± 51 patients, whereas the median was 45 patients (range 14-200). Most studies used a 1:1 randomization scheme. The median follow-up duration in all studies was 6 months (range 3-60 Months). The median amount of infused viable cells was 100 million (range from 5 x 10^6 to 60 x 10^9) and the cells were infused after a median of 7 days (range <24 hours to 3 months). MRI was the imaging modality of choice for FU of LV function in 40% of the RCTs (Supplemental Table III).

BMMNC: Cardiac parameters

Overall, BMMNC infusion increased LVEF by +2.10% (95% CI, 0.68-3.52, P=0.004; Figure 1A; 21 trials). LVEDV decreased by -2.80 ml (95% CI, -6.03-0.44, P=0.09; Figure 1C), whereas LVESV decreased by -4.05 mL (95% CI, -6.91- -1.18, P=0.006; Figure 1D) in the cell therapy group. Infarct size was reduced by -2.69% (95% CI, -4.83- -0.56, P=0.01; Figure 1B).

BMMNC: Subgroup analyses

1. Effects of BMMNC transplantation over time

Subgroup analysis revealed that at 6 months FU (n= 21 RCT) the LVEF increased by +2.08% (95% CI, 0.55- 3.60, P=0.008; Table 1). At 12 months (n= 8) this beneficial effect was sustained and increased to more pronounced effect of +3.04% (95% CI, 1.27-4.81, P= 0.0008) when compared to control. At 36-60 months FU (n=3), this treatment effect disappeared to +1.19% (95% CI, -2.74- 5.12, P=0.55) (Table 1). This increase in LVEF up to 18 months FU, was mainly due to a preservation of LVESV in the BMMNC group (as opposed to the control group). In the treatment group, LVESV progressively decreased by -4.84 mL (95% CI, -7.69- -2.00, P=0.0008) at 6 months FU and -3.56 ml at 18 month FU (95% CI, -6.87- -0.25, P=0.03). Infarct size was significantly reduced at 6 months FU (-2.69%, 95% CI,-4.83 - -0.56, P=0.01) and 18 month FU (-3.71%, 95% CI, -6.99- -0.43, P=0.03). This significant effect on infarct size diminished at long term FU (>18 month FU; -0.82%, 95% CI, -3.78-2.15, P=0.59).

Table 1. Effects of BMMNC transplantation over time

	Difference in mean, (95% CI)	P-value
LVEF		
≤ 6months	2.08 [0.55, 3.60]	0.009
6-18 months	3.04 [1.27, 4.81]	0.0008
> 18-60 months	1.19 [-2.74, 5.12]	0.55
LVEDV		
≤ 6months	-3.18 [-6.59, 0.24]	0.07
6-18 months	-1.75 [-6.28, 2.79]	0.45
> 18-60 months	-1.75 [-6.57, 3.07]	0.48
LVESV		
≤ 6months	-4.84 [-7.69, -2.00]	0.0008
6-18 months	-3.56 [-6.87, -0.25]	0.03
> 18-60 months	-0.44 [-9.94, 9.07]	0.06
IS		
≤ 6months	-2.69 [-4.83, -0.56]	0.01
6-18 months	-3.71 [-6.99, -0.43]	0.03
> 18-60 months	-0.82 [-3.78, 2.15]	0.59

The beneficial effect of BMMNC therapy on left ventricular ejection fraction (LVEF) was maintained during 18 year follow-up. This effect on LVEF was mainly due to a preservation of left ventricular end-systolic volume (LVESV). The effects diminished after the 18 months FU. LVEDV: left ventricular end-diastolic volume; IS: infarct size.

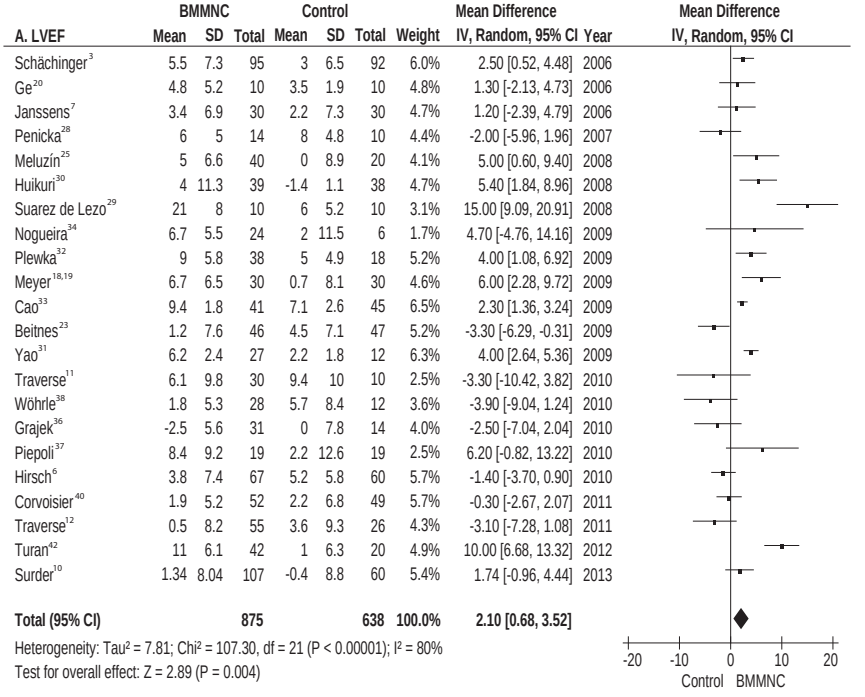
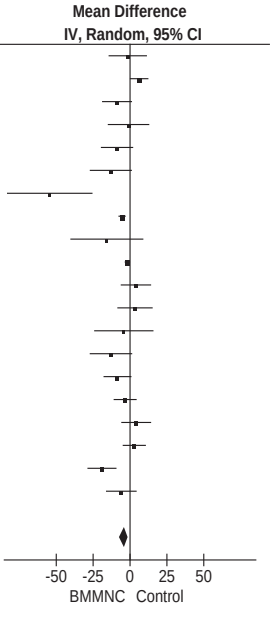


Figure 1A. Forest plot of change in left ventricular ejection fraction of BMMNC transplantation (unadjusted difference in mean, 95% CI). Overall LVEF is increased by +2.10 % (95% CI, 0.68-3.52, P=0.004).
Figure 1B. Forest plot of unadjusted difference in mean change in infarct size. Infarct size was reduced by -2.69 (95% CI, -4.83- -0.56 P=0.01).

	BMMNC			Control				Mean Difference	
C. LVESV	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI	Year
Meyer[18,19]	-0.85	28.1	30	0.68	21.3	30	3.6%	-1.53 [-14.15, 11.09]	2009
Schächinger[3]	5.6	22	95	-0.6	19	92	8.0%	6.20 [0.31, 12.09]	2006
Janssens[7]	-7.8	19	30	1	19.7	30	5.0%	-8.80 [-18.59, 0.99]	2006
Penicka[28]	-3	14.5	14	-2	18.4	10	3.2%	-1.00 [-14.70, 12.70]	2007
Huikuri[30]	-10	30.3	36	-1.2	11.5	36	4.5%	-8.80 [-19.39, 1.79]	2008
Meluzín[25]	-6	21.1	40	7	28	20	3.1%	-13.00 [-26.90, 0.90]	2008
Suarez de Lezo[29]	-47.6	24.8	10	6.8	38.9	10	0.9%	-54.40 [-82.99, -25.81]	2008
Cao[33]	-13.2	4	41	-7.8	5.7	45	11.3%	-5.40 [-7.47, -3.33]	2009
Nogueira[34]	-7.6	12.6	24	8.1	29.8	6	1.2%	-15.70 [-40.07, 8.67]	2009
Yao[31]	-4.3	1.3	27	-2.5	2.4	12	11.6%	-1.80 [-3.24, -0.36]	2009
Beitnes[23]	-5	22.9	46	-9	25.87	47	4.9%	4.00 [-5.92, 13.92]	2009
Wöhrle[38]	5.1	16.3	28	1.7	17.5	12	4.0%	3.40 [-8.20, 15.00]	2010
Piepoli[37]	-2.4	26.6	19	1.8	34.9	19	1.8%	-4.20 [-23.93, 15.53]	2010
Grajek[36]	3.2	12.1	31	16.1	25.5	14	3.1%	-12.90 [-26.92, 1.12]	2010
Traverse[11]	-11.9	5.6	30	-3.4	14.3	10	5.4%	-8.50 [-17.59, 0.59]	2010
Hirsch[6]	-0.9	22.8	67	2.4	19.9	60	6.6%	-3.30 [-10.73, 4.13]	2010
Traverse[12]	0.3	24.3	55	-3.9	19.1	26	5.0%	4.20 [-5.55, 13.95]	2011
Corvoisier[40]	12.1	19.7	52	9.2	18.7	49	6.6%	2.90 [-4.59, 10.39]	2011
Turan[42]	-21	13.5	42	-2	19.5	20	5.2%	-19.00 [-28.47, -9.53]	2012
Surder[10]	12.6	30.4	107	18.5	32.4	60	4.9%	-5.90 [-15.92, 4.12]	2013
Total (95% CI)			**824**			**608**	**100.0%**	**-4.05 [-6.91, -1.18]**	

Heterogeneity: Tau² = 17.81; Chi² = 57.48, df = 19 (P < 0.00001); I² = 67%
Test for overall effect: Z = 2.77 (P = 0.006)



	BMMNC			Control				Mean Difference	
D. LVEDV	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI	Year
Schächinger[3]	14	33	95	12	31	92	6.8%	2.00 [-7.17, 11.17]	2006
Meyer[18,19]	12.9	34	30	5.8	18.7	30	4.0%	7.10 [-6.79, 20.99]	2006
Janssens[7]	4.8	25.8	30	4.8	25.5	30	4.4%	0.00 [-12.98, 12.98]	2006
Penicka[28]	9	17.6	14	12	20.6	13	3.8%	-3.00 [-17.50, 11.50]	2007
Huikuri[30]	5.4	37.1	36	8.2	34.3	36	3.1%	-2.80 [-19.30, 13.70]	2008
Meluzín[25]	2.5	30.5	40	16	32.8	20	2.9%	-13.50 [-30.70, 3.70]	2008
Suarez de Lezo[29]	-13.6	30.8	10	32.3	47.6	10	0.8%	-45.90 [-81.04, -10.76]	2008
Beitnes[23]	-9	27.9	46	-3	32.12	47	4.8%	-6.00 [-18.22, 6.22]	2009
Cao[33]	-3.2	6.2	41	1.5	7.8	45	13.5%	-4.70 [-7.67, -1.73]	2009
Yao[31]	-2.4	2.9	27	-1.5	2.02	12	14.7%	-0.90 [-2.48, 0.68]	2009
Nogueira[34]	8	16	24	2.2	27.8	6	1.7%	5.80 [-17.35, 28.95]	2009
Hirsch[6]	9.2	22.8	67	13.94	23	60	7.9%	-4.74 [-12.72, 3.24]	2010
Wöhrle[38]	16.3	26	28	18	24.3	12	3.0%	-1.70 [-18.49, 15.09]	2010
Grajek[36]	2.6	17.9	31	13.7	30.6	14	2.9%	-11.10 [-28.32, 6.12]	2010
Traverse[11]	-6.8	37.4	30	28.9	18.7	10	2.7%	-35.70 [-53.40, -18.00]	2010
Piepoli[37]	-10	29.6	19	2.2	36.2	19	2.1%	-12.20 [-33.23, 8.83]	2010
Corvoisier[40]	12.1	19.7	52	9.2	18.7	49	8.4%	2.90 [-4.59, 10.39]	2011
Traverse[12]	5.6	39.8	55	4.6	30.8	26	3.3%	1.00 [-14.84, 16.84]	2011
Turan[42]	19	33.9	42	2	19.5	20	4.2%	17.00 [3.65, 30.35]	2012
Surder[10]	20.6	33.9	107	27.1	38.5	60	5.1%	-6.50 [-18.17, 5.17]	2013
Total (95% CI)			**824**			**611**	**100.0%**	**-2.80 [-6.03, 0.44]**	

Heterogeneity: Tau² = 17.97; Chi² = 42.51, df = 19 (P = 0.002); I² = 55%
Test for overall effect: Z = 1.69 (P = 0.09)

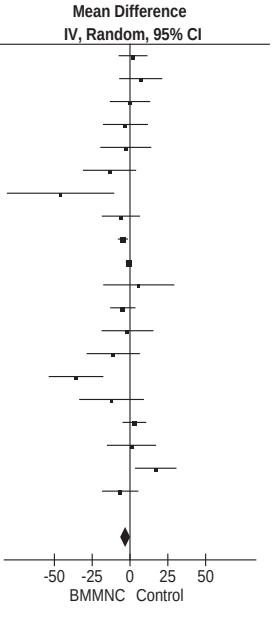


Figure 1C. Forest plot of unadjusted difference in mean change in LVESV. LVESV decreased by -4.05 ml (95% CI, -6.91- -1.18, P= 0.006) in the treatment group.

Figure 1D. Forest plot of unadjusted difference in mean LVEDV(with 95% CI). LVEDV decreased by -2.80 ml (95% CI, -6.03-0.44, P=0.09).

2. Imaging modality

Interestingly, when subgroup analysis is performed based on MRI (n=9), which is currently considered as the golden standard to assess cardiac function and volumes, the significant effect of BMMNC therapy on LVEF diminished (0.13%, 95% CI -2.67 -2.93, P=0.93). Also, the beneficial effect on LV volumes and infarct size disappeared (Table 2). This finding could indicate that BMMNC therapy is not beneficial in AMI patients.

Table 2. BMMNC therapy per imaging modality

Imaging modality	Difference in mean (95% CI)	P for Z	P for subgroup differences
LVEF			
MRI	0.13 [-2.67, 2.93]	0.93	0.03
Echo	3.05 [1.45, 4.64]	0.0002	
SPECT	5.63 [1.81, 9.44]	0.004	
LVG	7.69 [2.43, 12.95]	0.004	
LVEDV			
MRI	-0.86 [-4.66, 2.94]	0.66	0.13
Echo	-3.21 [-5.99, -0.43]	0.02	
SPECT	-15.24 [-30.88, 0.40]	0.06	
LVG	1.85 [-4.44, 8.13]	0.56	
LVESV			
MRI	-2.65 [-5.28, 0.02]	0.06	0.01
Echo	-6.17 [-8.31, -4.03]	<0.00001	
SPECT	-12.71 [-24.41, -1.01]	0.03	
LVG	-14.88 [-26.85, -2.90]	0.01	
Infarct size			
MRI	-1.11 [-3.74, -1.53]	0.82	0.44
SPECT	-2.40 [-2.85, -1.95]	0.00001	

Parameters are represented as unadjusted difference in mean, with 95% CI. Subgroup analysis of MRI revealed that BMMNC therapy is not beneficial for improvement of left ventricular ejection fraction (LVEF). LVEDV: left ventricular end-diastolic volume; LVESV: left ventricular end-systolic volume; LVG: left ventricular angiography.

3.BMMNC versus other cell types

IC infusion of bone marrow progenitor cells (*i.e.* CD34/CD133+ cells) resulted in a significant increase in LVEF of +2.67% (95% CI: -0.05- 5.40, P=0.05; Figure 2), whereas MSC transplantation resulted in an increase of +6.28% (95% CI: -1.07 - 13.64, P=0.09). LVEF increased with +0.80% after transplantation of CDC (95% CI: -1.21-2.82, P=ns). When data of all stem cells are combined, LVEF increases by +2.51% (95% CI 1.24 - 3.78, P=0.0001; Figure 2). Remarkably, we found a significant increase of LVEDV in the CD133/CD34+ patient group by +14.06 mL (95% CI 9.63-18.48) as opposed to a decrease by -2.80 ml in the BMMNC patient group (-6.03 -0.44, P for subgroup differences<0.00001). Table 3 summarizes functional parameters of other cell types opposed to BMMNC and the other subgroup analysis. CDC were omitted from further comparison, because only one trial investigated this new cell type.

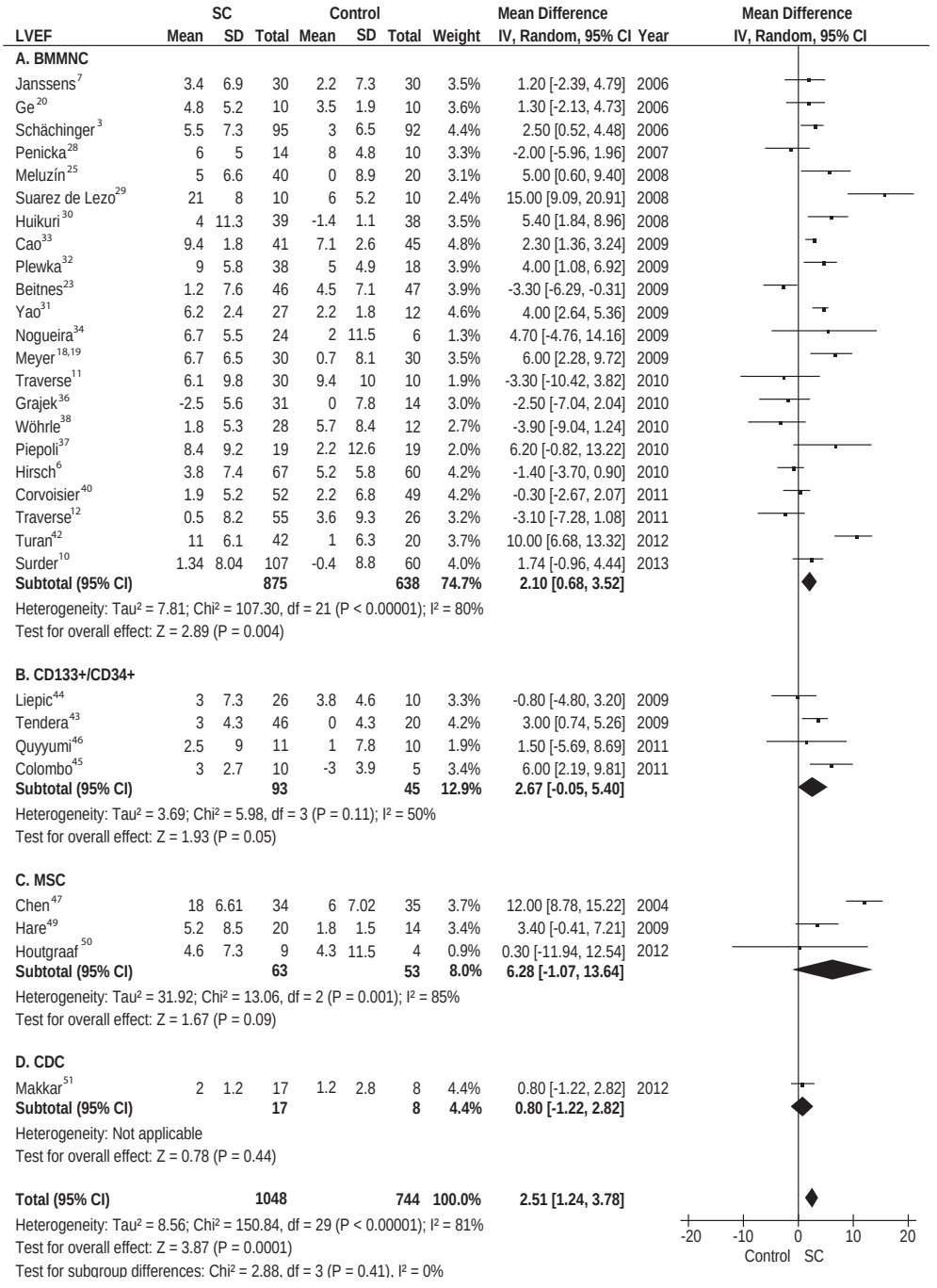


Figure 2. Forest plot of unadjusted difference in mean LVEF (with 95% CI) of all trials and stem cells to date. The overall effect on LVEF on all stem cells combined is +2.51% (95% CI: 1.24-3.78; P=0.0001). BMMNC: Bone marrow Mononuclear cell, MSC: mesenchymal stem cell: CDC: cardiosphere derived cells.

4. Infarct location and LV function at baseline

Patients with an anterior wall AMI due to occlusion of the LAD did not benefit more from stem cell therapy than patients with an AMI located elsewhere (Table 3). According to our data, patients with a lower LVEF (*LVEF* <40% or *LVEF* <45%) at baseline did not benefit more from cell therapy, than patients with a higher LVEF. Only, the beneficial effect of BMMNC therapy on infarct size was significantly greater in patients with a LVEF below 45% (-2.39%; 95% CI, -2.75- -2.02), as opposed to almost no reduction (-0.50%; 95% CI, -2.36- 0.27) in patients with a LVEF above 45% (P for subgroup differences 0.05).

5. Parameters related to cell infusion

The median cell number infused was 100 million cells. Intriguingly, total cell number did not predict outcome. More specifically, patients treated with infusion of <100 million cells did not benefit more or less from cell infusion than patients with higher cell doses. BMMNC transplantation before day 8 resulted in an improved LVEF (+3.10%, 95% CI: 1.49 – 4.70, P=0.009) as opposed to late infusion (-0.37%, 95% CI -2.40 – 1.66).

6. Intracoronary delivery technique

We found that performing a subgroup analysis on intracoronary delivery technique was not feasible, as in all, except 2 studies, the 'stop-flow' technique was adopted.

7. Cell preparation

Subgroup analysis based on the methods of BMMNC isolation revealed that all trials, except for 2[6,33], used Ficoll-based isolation, therefore subgroup analysis was not useful. Final suspension of the cell preparation in heparin-containing saline or in non-heparinized solutions did not have a significant effect on treatment outcome.

Safety of IC cell infusion and MACCE-rates

In the majority of trials, IC infusion of stem cells did not result in procedure-related adverse events, or adverse events were not reported in the manuscript. Only 1 study described a case of thrombosis in the infarct related artery after AMI and 3 cases of intima dissection following balloon inflation during transplantation.[26]

The median follow-up duration for MACCE rates was 6 months. IC infusion of BMMNC did not result in reduction of any MACCE event. More specifically, and in contrast to previous reported meta-analysis, no differences on all-cause mortality, cardiac mortality, hospitalizations for heart failure, restenosis rate, thrombosis, target vessel revascularization (TVR), stroke, recurrent AMI and ICD implantations were detected between BMMNC patients and controls (Table 4). Bone marrow progenitor cell infusion resulted in a reduction in re-hospitalizations for heart failure (OR 0.14, 95% CI: 0.04-0.52; P=0.003) and MSC transplantation resulted in a reduction in VF/VT (OR 0.08, 95% CI: 0.01-0.79; P=0.03) and ICD implantations (OR 0.08; 95% CI: 0.01-0.79, P=0.03)

Table 3. Subgroup analysis of BMMNC and comparison between BMMNC and other stem cells

Subanalyses	No of RCT	EF Difference in mean	EF P-value (subgroup differences)	EDV Difference in mean	EDV P-value (subgroup differences)	ESV Difference in mean	ESV P-value (subgroup differences)	IS Difference in mean	IS P-value (subgroup differences)
EF prior to infusion									
EF < 40%	8	3.46 [1.12, 5.81]	0.53	-0.69 [-3.49, 2.12]	0.84	-4.51 [-8.51, -0.52]	0.85	-2.46 [-4.72, -0.21]	0.06
EF ≥ 40%	15	2.52 [0.69, 4.34]		-1.41 [-7.58, 4.77]		-4.31 [-9.36, 0.73]		0.08 [-1.38, 1.55]	
EF < 45%	10	3.19 [1.07, 5.30]	0.46	-1.51 [-4.41, 1.39]	0.70	-4.76 [-8.23, -1.29]	0.78	-2.39 [-2.75, -2.02]	0.05
EF ≥ 45%	12	1.95 [-0.54, 4.45]		0.17 [-7.85, 8.19]		-3.80 [-9.72, 2.31]		-0.50 [-2.36, 1.36]	
EF < 50%	17	2.80 [1.07, 4.53]	0.67	-1.20 [-4.82, 2.42]	0.97	-5.62 [-8.84, -2.41]	0.57	-1.81 [-3.64, 0.02]	0.34
EF ≥ 50%	5	1.85 [-2.10, 5.80]		-1.01 [-9.69, 7.67]		-3.09 [-11.33, 5.14]		-1.32 [-2.90, 0.27]	
Target Vessel									
LAD/LCx/RCA	13	1.42 [-0.06, 2.79]	0.25	-0.73 [-4.38, 2.92]	0.36	-2.04 [-7.03, 2.94]	0.28	-0.26 [-1.57, 1.04]	0.19
LAD infarct	9	3.11 [0.61, 5.61]		-3.78 [-9.14, 1.57]		-5.50 [-9.40, -1.60]		-2.47 [-5.51, 0.56]	
Timing of infusion									
Infusion <8 d	19	3.10 [1.49, 4.70]	0.009	-1.27 [-4.74, 2.20]	0.03	-5.16 [-8.32, -2.00]	0.30	-1.41 [-3.13, 0.32]	0.46
Infusion ≥ 8 d	3	-0.37 [-2.40, 1.66]		8.38 [0.36, 16.39]		-0.44 [-8.79, 7.91]		-0.40 [-2.46, 1.66]	
Amount of infused cells									
< 50 M cells	3	4.06 [2.81, 5.31]	0.10	-4.70 [-12.27, 2.88]	0.38	-6.49 [-8.84, -4.14]	0.26	-2.40 [-2.78, -2.02]	0.19
≥ 50 M cells	19	2.18 [0.32, 4.04]		-0.94 [-4.72, 2.83]		-4.00 [-7.60, -0.39]		-0.97 [-3.07, 1.13]	
< 100 M Cells	6	3.80 [0.66, 6.94]	0.39	4.01 [-8.40, 16.42]	0.35	-7.05 [-11.89, -2.20]	0.64	-2.05 [-3.71, 0.38]	0.57
≥ 100 M cells	16	2.07 [0.15, 3.99]		-2.27 [-6.45, 1.90]		-3.88 [-7.63, -0.13]		-3.16 [-6.64, 0.32]	
Heparine use									
Heparine	15	3.56 [1.60, 5.51]	0.07	-2.81 [-6.76, 1.13]	0.34	-3.69 [-4.82, -2.56]	0.28	-1.57 [-3.62, 0.48]	0.55
No Heparine	5	0.91 [-1.16, 2.99]		0.49 [-5.08, 6.07]		-1.12 [-5.65, 3.41]		-0.72 [-2.93, 0.10]	
BMMNC vs other SC			versus BMMNC						
BMMNC	22	2.10 [0.68, 3.52]		-2.80 [-6.03, 0.44]		-4.05 [-6.91, -1.18]		-2.69 [-4.83, -0.56]	
CD133+/CD34+	4	2.67 [-0.05, 5.40]	0.74	14.06 [9.63, 18.48]	<0.00001	-3.00 [-8.07, 2.07]	0.72	-2.84 [-6.38, 0.70]	0.96
MSC	3	6.28 [-1.07, 13.64]	0.27	-9.76 [-33.27, 13.74]	0.07	-6.68 [-23.86, 10.50]	0.77	-14.02 [-17.06, -10.79]	<0.00001

RCT indicates randomized controlled trials; LVEF: left ventricular ejection fraction; LVEDV: left ventricular end-diastolic volume: LVESV; left ventricular end-systolic volume; LAD: left descending artery; LCx: left Circumflex artery; RCA: right coronary artery; BMMNC: bone marrow mononuclear cells; MSC: mesenchymal stem cells.

Table 4. MACCE events represented as Mantel-Haenszel odds ratio (OR)

	BMMNC			BM progenitor cells			MSC		
Outcome	**OR**	**95% CI**	**P**	**OR**	**95% CI**	**P**	**OR**	**95% CI**	**P**
All cause mortality	0.68	0.36-1.31	0.25	0.50	0.09-2.67	0.41	3.18	0.13-81.01	0.48
Cardiac mortality	0.73	0.32-1.65	0.45		no events			no events	
Recurrent MI	0.5	0.24-1.06	0.07	2.25	0.04-1.42	0.70		no events	
TVR	0.86	0.58-1.27	0.44	0.96	0.1-2.29	0.93	1.59	0.05-7.52	0.79
In-stent restenosis	0.95	0.51-1.79	0.88		no events			no events	
In-stent thrombosis	0.75	0.08-7.45	0.81	0.61	0.10-3.84	0.60		no events	
Heart failure	0.84	0.44-1.60	0.60	0.14	0.04-0.52	0.003		no events	
CVA	0.62	0.13-2.84	0.53	0.29	0.01-7.76	0.46		no events	
VT/VF	0.60	0.30-1.21	0.16		no events		0.08	0.01-0.79	0.03
ICD	0.98	0.37-2.64	0.97	0.93	0.05-16.39	0.96	0.08	0.01-0.79	0.03

MACCE rates were not reduced in BMMNC treated patients. In patients treated with Bone marrow progenitor cells, the rehospitalizations for heart failure were lower opposed to the control group. Intracoronary infusion of mesenchymal stem cells (MSC) resulted in lower number of VT/VF and ICD implantations. It has to be kept in mind that the trials to date were not sufficiently powered to detect differences in clinical outcome. OR; odds Ratio; CI; confidence interval; MI: myocardial infarction; TVR; target vessel revascularization; VT; ventricular tachycardia; VF: ventricular fibrillation; ICD: Implantable Cardioverter Defibrillator.

Publication Bias

A funnel plot for LVEF showed that studies were equally distributed around the overall estimate, suggesting that there was no sign for publication bias (Supplemental Figure B).

Discussion

In this meta-analysis, which comprises a total of 2037 AMI patients, cell therapy proved to be safe. BMMNC therapy modestly improves LVEF at short and long term FU when all imaging modalities are combined for analysis. The modest improvement in LVEF was mainly due to a sustained LVESV, accompanied by a reduction in infarct size. Interestingly, when only studies are analyzed that used cardiac MRI for measuring volumes and LVEF, this beneficial effect of BMMNC therapy on cardiac function disappeared. Furthermore, the occurrence MACCE events is not reduced in patients treated with BMMNC when compared to controls.

MACCE rates

One of the most salient findings of the current meta-analysis comprises the fact that, despite over 1,500 patients analyzed to date, BMMNC therapy did not affect clinical outcome measures in AMI patients. Our findings seem contradictory to findings in recent meta-analyses by Jeevanantham and coworkers who described a reduction in all-cause mortality, cardiac mortality, recurrent AMI, hospitalizations for heart failure and in-stent thrombosis after BMMNC transplantation. However, in this meta-analysis, both AMI and IHF patients were combined for the assessment of clinical outcome. We hypothesize that ischemic heart failure patients may benefit more from cell therapy, which was corroborated in a recent meta-analysis that solely focused on IHF patients.[15] The current analysis includes, for the first time, recent negative publications as the SWISS-AMI trial and LATE-TIME trial which has modified the results. It should be noted that the median follow-up duration for the assessment of MACCE rates is only 6 months, which might be too short to draw conclusions regarding clinical outcome. We performed a power analysis to calculate the number of patients needed to discriminate a possible beneficial effect of cell therapy in AMI patients. Based on our data, we found that a study of 2,994 patients would be needed to demonstrate a possible effect on MACCE when the incidence of an event is 20% (Supplemental Table IV), whereas >30,000 patients would be needed to demonstrate an effect if the incidence of an event was 2%. In our meta-analysis, the incidence of all-cause mortality was only 2% at a median follow-up duration of 6 months. The forthcoming phase III BAMI trial (NCT01569178) is designed shed more light on the value of BMMNC therapy in improving clinical outcome. It is designed to compare BMMNC transplantation in AMI patients with baseline LVEF <45% to a control group that receives optimal medical care. The primary endpoint in this study is time from randomization to all-cause mortality during 3 year FU. The secondary outcome measures consist of the occurrence of other MACCE events from randomization up until 3 years FU. LV function, volumes and infarct size, are no outcome measures in this trial.

Our power analysis is based on multiple small studies. All these individual studies were primarily designed as safety and feasibility studies, and thus inadequately powered to detect an effect on clinical end points, whereas the BAMI trial was powered based on long-term FU data of the landmark REPAIR-AMI trial.[24] More importantly, the median FU in our meta-analysis is only 6 months, which is rather short to notice effects on clinical outcome measures in this era of aggressive primary interventions and pharmacotherapy. However, it remains questionable if the BAMI trial, is sufficiently powered to establish definitive answers. Nonetheless, we believe that the BAMI trial will shed more light on several questions concerning BMMNC therapy as adjunctive treatment for AMI patients, and the final results are much anticipated. Supplemental Table V summarizes all upcoming clinical trials on stem cell therapy for AMI. Currently, newer generations of more potent stem cells are emerging in the field of cardiology. Our meta-analysis revealed a reduction in rehospitalizations for heart failure or reduction in ventricular arrhythmias and ICD implantations in patients treated with these newer generation cells. However, the number of clinical trials to date is limited, which is why no statement could be made about the superiority of these cells yet.

Cell therapy-related parameters possibly influencing efficacy outcomes

Timing of cell delivery

The optimal timing of cell therapy with respect to AMI remains unclear to date. Thus far, it was believed that cell therapy should be initiated 3-10 days after the AMI, based on findings in phase I studies, logistical issues, and the assumption that in the first 72 hours, the infarct territory encompasses a too hostile environment for the infused cells. Others argued that stem cells should be infused as soon as possible to prevent cardiomyocyte loss by secreted anti-inflammatory, pro-survival and anti-apoptotic paracrine factors.[54] This hypothesis was recently supported by preclinical and clinical evidence.[50,55] Nevertheless, to date almost all (>90%) other clinical studies infused stem cells >72 hours after the AMI. In this meta-analysis, we found that timing of BMMNC infusion later than 8 days did not appear to be effective. This was confirmed by recent trials aimed to address the question of cell therapy timing.[10–12,39] The TIME and LATE-TIME and SWISS-AMI failed to show any beneficial effect of late infusion opposed to early infusion. Of note, the forthcoming phase II AMICI (NCT01781390) and phase III ADVANCE (NCT01216995) trials will render important information on early infusion of MSC-like cells, whereas the BAMI (BMMNC; NCT01569178), REVITALIZE (BMMNC; NCT00874354), REGEN-AMI (BMC; NCT00765453), AMIRST (BMC; NCT01536106) and the phase II study with Prochymal (allogeneic MSC; NCT00877903) will possibly provide new insights in timing of stem cell administration between 2 and 7 days.

Cell type

It is currently hypothesized that culture-expanded sub populations of BMMNC or other specialized cell types, might exhibit more cardio-protective effects than BMMNC.[56] Indeed, a recent meta-analysis that compared all pre-clinical, large animal studies that were performed to date concluded that MSC appear to have more pronounced beneficial effects on LV function than BMMNC [57], whereas newer generation cells might be even more effective. In this meta-analysis we found a trend towards an

improvement in cardiac function in patients treated with MSC. However, only limited numbers of patients are treated with this stem cell type to date, rendering a high heterogeneity between trials. A power calculation revealed that a study of 106 patients per group is needed to detect a possible significant benefit of MSC over BMC. Forthcoming AMICI (NCT01781390) and ADVANCE (NCT01216995) clinical trials are both phase IIa/IIb trials designed to investigate the effects of mesenchymal-like cells on cardiac repair in over 200 patients. Therefore, they might provide evidence of superiority of mesenchymal stem cells. It should be noted, however, that both studies do not perform head-to-head comparisons of MSC and BMMNC.

Cell preparation and infusion

Recently, it was suggested that the use of heparin in the final stem cell suspension might interfere with the SDF-1/CXCR4 axis , thereby resulting in decreased homing of BMC.[58] However, we found that the use of heparin during cell preparation did not appear to influence therapy outcome. Contrarily, there seems to be a trend towards a beneficial effect on LVEF in heparin-treated cells, which was also found by Jeevanantham et al.[14] We also confirm their finding that the BMMNC isolation protocol did not appear to influence therapy outcome. Noteworthy, only 2 trials prepared BMMNC via a different method than Ficoll isolation.

Cardiac MRI and study design as effect modifier?

Although the effect that we found on LVEF in this meta-analysis is limited, it can have significant clinical implications. For instance, in the studies that assessed the effect of primary PCI following AMI, a similar modest 4% improvement in LVEF was found that eventually mounted up to pronounced effects on mortality.[59,60] However, despite early enthusiasm and several previous positive meta-analyses on cellular therapy [9,14], it seems that some consideration is justified.

First, and most importantly, in most of the earlier cell therapy trials that drove initial enthusiasm, LVEF and volumes were assessed by LV-angiography or echocardiography, whereas cardiac MRI is currently considered as the golden standard.[61] Most recent stem cell trials however, used MRI-based analysis for primary endpoint measures of efficacy and volumes, and 40% of all trials in the current meta-analysis used MRI.[6,7,11,12,23,31,38,49] Intriguingly, when our data are corrected for the use of MRI as imaging tool, the positive effect of cell therapy on LVEF, volumes, and infarct size diminishes. This finding corroborates the exploratory findings of Traverse et al[62], and puts the initial enthusiasm concerning BMMNC-based therapies for AMI patients in a different perspective.

Noteworthy, about 50% of the RCTs in this meta-analysis was not executed according to the Jüni criteria (Supplemental Table II), as they do not perform a bone marrow biopsy and sham injection procedure in placebo patients. However, in most studies an unbiased outcome was ascertained by blinded core lab analysis.

Considerations

We believe that the current meta-analysis shows strong indications that BMMNC therapy in AMI patients is not effective in improving clinical outcome. Although the number of patients treated with next generation cell therapies is still too low, and studies performed to date were primarily designed to prove safety and feasibility, these new therapies might prove to be more effective. It is believed that mesenchymal cell populations, or cardiac derived stem cells, exhibit more cardio-protective and regenerative potential. Importantly, preclinical and preliminary clinical evidence indeed show promising benefits of these cell types.[49–51,55,57] Moreover, MSC are immune-privileged cells, and can be administered in an allogeneic setting. This renders the possibility of an allogeneic "off-the-shelf" cell product, which is readily available directly following the primary PCI. This has several logistical advantages, but might also enhance outcome, as it was shown that stem cells derived from young and healthy donors perform better, and have more regenerative potential, than autologous stem cells from the typically elderly cardiovascular patient.[63]

Novelty / Significance

The current meta-analysis, consisting of 30 published, randomized controlled trials, and comprising a total of 2037 patients, is the largest meta-analysis on stem cell therapy for the treatment of AMI patients to date. It includes recently published, relatively large RCTs that used MRI-derived parameters as surrogate end point, and were not included in any meta-analysis yet.[6,10] Also, for the first time, studies that investigated other cell types than BMMNC were included in a subgroup analysis.[50,51] In contrast to a recently published meta-analysis that combined AMI and heart failure patients in most of its subgroup analyses, the current manuscript focuses solely on AMI patients, rendering different and sometimes opposing conclusions.[14]

Conclusion

Intracoronary infusion of BMMNC improves LVEF by +2.10%, mostly by reduction of LVESV and infarct size. However, there is no beneficial effect on global LVEF when restricted to cardiac MRI analysis. The improvement in LVEF did not lead to a reduction in clinical outcome. Newer generations of stem cells with a better profile for cardiac repair are emerging, but their future role still needs to be defined in phase II and III studies.

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SUPPLEMENTAL MATERIAL

Content

Materials and Methods

Search strategy

Medline (July 2002-September 2013) and the Cochrane Central Register of Controlled trials (CENTRAL) and the website of US Food and drug administration www.fda.gov were searched for relevant articles. The search included all studies reported up to September 1st 2013. We also searched for relevant abstracts and presentations on this topic reported in major cardiology meetings. References in other articles were also investigated and included in the analysis whenever deemed appropriate. Websites, including *www.clinicaltrials.gov* and *www.clinicaltrialregister.eu,* were searched for ongoing trials and future trials. The following search strategy was applied: "stem cells", "progenitor cells", "mononuclear cells", "adipose tissue-derived regenerative cells", "mesenchymal stem cells', "cardiac-derived stem cells", "bone marrow", "vascular stromal fraction", "adipose stem cells", "mesenchymal-like stem cells", "coronary artery disease", "myocardial infarction", "cardiac repair", and "myocardial regeneration". Only articles published in English were included. Limitations used in the search were the publication of the study within the last 10 years, limited to clinical trials and randomized controlled clinical trials (Supplemental Table I).

Inclusion and exclusion of studies

Studies were included that met the following criteria: (1) randomized controlled trials with an appropriate control group who received standard therapy, (2) conducted in patients with an AMI that occurred less than 3 months before, (3) using stem cells that were administered by intracoronary or intravenous injection, (4) total of number of patients enrolled should exceed 10, (5) stem cells were derived from adipose tissue, bone marrow or heart, (6) given in an allogeneic or autologous setting.

Only studies with a complete dataset and specified data on the amount of infused cells were included in this meta-analysis. Studies that described the combination of circulating progenitor cells (CPC) or CPC with granulocyte-colony stimulating factor (G-CSF) were excluded from this analysis to circumvent the potential confounding effect of G-CSF therapy on LV function and dimensions, although G-CSF was previously proven ineffective as a mono-therapy for cardiac repair in AMI. When studies compared C-GSF and stem cells, only the patients in the control and stem cell arm were used in this analysis. Cohort studies were excluded from further analysis due to a limited number of studies.

Data abstraction

Three reviewers (RdJ, JH, SS) independently screened abstracts and reported their results in a standardized form. Data extracted from the articles were categorized in trial characteristics, functional outcome, scar size and safety. The following parameters were extracted from the articles: Left ventricular ejection fraction (LVEF), LV end-systolic volume (LVESV), LV end-diastolic volume (LVEDV), infarct size (MRI), perfusion defect (SPECT) and major adverse cardiac and cerebral events (MACCE) rates. MACCE

was specified as: all-cause mortality, cardiac mortality, hospitalization for heart failure, in-stent thrombosis and restenosis, target vessel revascularization, ventricular arrhythmia, ICD implantation and stroke. Infarct size was expressed as the percentage of left ventricle infarcted (in %volume or mass). In the various studies, different imaging modalities have been used to determine left ventricular ejection fraction. Cardiac magnetic resonance imaging (MRI) was considered the golden standard. If more than one imaging modality was included, all data was extracted for subgroup analysis. For studies with more than 1 intervention arm (e.g. multiple doses) the weighted mean was calculated and applied for the main analysis.[1] In trials with multiple follow-up time points, the last published follow-up was used in the main analysis.

Quality

The methodological quality of randomized controlled trials was tested by the Jüni criteria.[2]

Supplemental Table I. Search strategy

Database:
PubMed
User query:
((((((acute myocardial infarction)) OR (coronary artery disease)) OR (myocardial regeneration)) OR (myocardial infarction))) AND ((((((((stem cells)) OR (bone marrow stem cells)) OR (progenitor cells)) OR (mononuclear cells)) OR ((mesenchymal stem cells) OR mesenchymal-like stem cells)) OR (adipose tissue derived regenerative cells)) OR (vascular stromal cells)) OR (cardiac derived stem cells)) AND ((Clinical Trial[ptyp] OR Letter[ptyp] OR Controlled Clinical Trial[ptyp] OR Randomized Controlled Trial[ptyp]) AND "last 10 years"[PDat] AND Humans[Mesh])
Translations:
myocardial infarction "myocardial infarction"[MeSH Terms] OR ("myocardial"[All Fields] AND "infarction"[All Fields]) OR "myocardial infarction"[All Fields]
coronary artery disease "coronary disease"[MeSH Terms] OR ("coronary"[All Fields] AND "disease"[All Fields]) OR "coronary disease"[All Fields] OR ("coronary"[All Fields] AND "artery"[All Fields] AND "disease"[All Fields]) OR "coronary artery disease"[All Fields] OR "coronary artery disease"[MeSH Terms] OR ("coronary"[All Fields] AND "artery"[All Fields] AND "disease"[All Fields])
myocardial "myocardium"[MeSH Terms] OR "myocardium"[All Fields] OR "myocardial"[All Fields]
regeneration "regeneration"[MeSH Terms] OR "regeneration"[All Fields]
stem cells "stem cells"[MeSH Terms] OR ("stem"[All Fields] AND "cells"[All Fields]) OR "stem cells"[All Fields]

bone marrow
"bone marrow"[MeSH Terms] OR ("bone"[All Fields] AND "marrow"[All Fields]) OR "bone marrow"[All Fields]

progenitor cells
"stem cells"[MeSH Terms] OR ("stem"[All Fields] AND "cells"[All Fields]) OR "stem cells"[All Fields] OR ("progenitor"[All Fields] AND "cells"[All Fields]) OR "progenitor cells"[All Fields]

cells
"cells"[MeSH Terms] OR "cells"[All Fields]

mesenchymal stem cells
"mesenchymal stromal cells"[MeSH Terms] OR ("mesenchymal"[All Fields] AND "stromal"[All Fields] AND "cells"[All Fields]) OR "mesenchymal stromal cells"[All Fields] OR ("mesenchymal"[All Fields] AND "stem"[All Fields] AND "cells"[All Fields]) OR "mesenchymal stem cells"[All Fields]

adipose tissue
"adipose tissue"[MeSH Terms] OR ("adipose"[All Fields] AND "tissue"[All Fields]) OR "adipose tissue"[All Fields]

stromal cells
"stromal cells"[MeSH Terms] OR ("stromal"[All Fields] AND "cells"[All Fields]) OR "stromal cells"[All Fields]

vascular
"blood vessels"[MeSH Terms] OR ("blood"[All Fields] AND "vessels"[All Fields]) OR "blood vessels"[All Fields] OR "vascular"[All Fields]

cardiac
"heart"[MeSH Terms] OR "heart"[All Fields] OR "cardiac"[All Fields]

Humans[Mesh]
"humans"[MeSH Terms]

Result:386

Supplemental Table II. Quality assesment score for RCT included in the meta analysis According to Juni Criteria[2]

Author	Year	Was allocation adequate?	Adequate method of randomization described?	Were groups similar at the start of the study?	Were patients/ caregivers blinded for intervention?	Was outcome ascertained blinded?	What percentage was lost in follow up?	Were all patients analyzed in the group to which they were assigned?
BMMNC								
Meyer[3–6]	2004	Y	Y	Y	Y	Y	0	Y
Ge[7]	2006	Y	Y	Y	Y	Y	0	Y
Janssens[40,41]	2006	Y	Y	Y	Y	Y	0	Y
Lunde/ Beitnes[8–10]	2006	Y	Y	Y	N	Y	0	Y
Schachinger[11–13]	2006	Y	Y	Y	Y	Y	0	Y
Meluzin/Panovsky[14–16]	2006	Y	N	Y	N	Y	10	Y
Penicka[17]	2007	Y	N	Y	NR	NR	0	Y
Suarez de Lezo[18]	2007	Y	N	Y	NR	Y	0	Y
Huikuri[19]	2008	Y	Y	Y	Y	Y	0	Y
Yao[20]	2009	Y	N	Y	N	Y	0	Y
Plewka[21]	2009	Y	N	Y	N	Y	0	Y
Cao[22]	2009	Y	Y	Y	NR	Y	0	Y
Nogueira (Silva)[23,24]	2009	Y	Y	Y	N	Y	0	Y
Grajek[25]	2010	Y	Y	Y	N	Y	0	Y
Piepoli[26]	2010	Y	Y	Y	N	Y	0	Y
Wohrle[27]	2010	Y	Y	Y	Y	Y	0	Y
Traverse TIME[28,29]	2010	Y	Y	Y	Y	Y	0	Y
Hirsch[30]	2010	Y	N	Y	Y	Y	0	Y
Corvoisier[31]	2011	Y	Y	Y	N	Y	0	Y
Travers LATE-TIME[32]	2011	Y	N	Y	N	Y	1.1	Y
Turan[33]	2012	Y	N	Y	N	Y	0	Y
Surder[34]	2012	Y	Y	Y	N	Y	?	Y

CD133+/CD34+								
Tendera[35]	2009	Y	N	Y	N	Y	0	Y
Liepic[36]	2009	Y	N	Y	N	Y	0	Y
Colombo[37]	2011	Y	Y	Y	Y	Y	0	Y
Quyumi[38]	2011	Y	N	Y	N	Y	0	Y
MSC/MSC-like								
Chen[39]	2004	Y	N	Y	Y	Y	0	Y
Hare[42]	2009	Y	Y	Y	Y	Y	0	Y
Houtgraaf[43]	2012	Y	Y	Y	Y	Y	0	Y
CDC								
Makkar[44]	2012	Y	Y	Y	N	Y	0	Y

Y indicate yes; N: no; NR: Not reported

Supplemental Table III. Study characteristics of all RCT included in this analysis

Author	Year	Total no	CT pt	Randomization	Cell type	Number of cells	Time to application	Infusion method	Location of MI	FU (mo)	Imaging modality
BMMNC											
Meyer[3–6]	2004	60	30	1:1	BMC	24.6 ± 9.4 x 10^8	4.8 ± 1.3 d	stop flow	all	60 (6,18)	MRI
Ge[7]	2006	20	10	1:1	BMMNC	40 x 10^6	1 d	Stopflow	all	6	Echo, SPECT
Janssens[40,41]	2006	67	33	1:1	BMMNC	172 ± 72 x 10^6	1 d	stopflow	all	4	MRI, echo
Lunde[8] and Beitnes[9,10]	2006	100	47	1:1	BMMNC	87 ± 47.7 x 10^6	5 -8 d	stopflow	anterior	36 (6,12)	MRI, SPECT, echo
Schachinger[11–13]	2006	204	101	1:1	BMMNC	236 ± 174 x 10^6	3-7 d	stopflow	all	12	LVG
Meluzin[14,15] and Panovsky[16]	2006	66	44	1:1:1	BMMNC	Low 1 x 10^7 High 1x 10^8	7 ± 0.3 d	stopflow	all	12 (3,6)	SPECT
Penicka[17]	2007	27	17	2:1	BMMNC	26.4 x 10^8	4 ± 11 d	stopflow	anterior	4	Echo, SPECT
S. de Lezo[18]	2007	20	10	1:1	BMMNC	9 x 10^8	7 ± 2 d	stopflow	anterior	3	LVG
Huikuri[19]	2008	80	40	1:1	BMMNC	402 ± 196 x 10^6	2-6 d	stopflow	all	6	LVG, Echo
Yao[20]	2009	39	12	1:1:1	BMC	1,9 ± 1,3 *10^8	3-7 d	stopflow	anterior	12 (6)	MRI
			15		BMC	2,1 ± 1,7 *10^8	3-7 d and 3 mo				
Plewka[21]	2009	56	38	2:1	BMC	1.44 ± 0.49 x 10^8	7 ± 2 d	stopflow	anterior	6	Echo
Cao[22]	2009	86	41	1:1	BMMNC	5 ± 1.2 x 10^7	7 d	stopflow	anterior	48 (6,12)	Echo
Nogueira[23,24]	2009	30	14	2:1	BMMNC	100 x 10^6	5.5 ± 1.2 d	stopflow ICA	all	6	Echo, SPECT
			10					stopflow ICV			
Grajek[25]	2010	45	31	2:1	BMMNC	2.34 ± 1.2 x 10^9	5-6 d	Stopflow	anterior	12 (3,6)	Echo
Piepoli[26]	2010	38	19	1:1	BMMNC	418.8 x 10^6	4-7 d	stopflow	anterior	12 (6)	SPECT/echo
Wohrle[27]	2010	42	29	2:1	BMMNC	381 ± 130 x 10^6	5-7 d	stopflow	all	6	MRI
Traverse TIME[28,29]	2010	40	30	3:1	BMMNC	100 x 10^6	3-10 d	infusion catheter 1ml/min	anterior	6	MRI
Hirsch[30]	2010	134	65	1:1	BMC	BMC 296 ± 164 x 10^6	3-8 d	stopflow	all	4	MRI
Corvoisier[31]	2011	101	52	1:1	BMC	98.3 ± 8.7 x 10^6	7-10 d	stopflow	all	3	SPECT

Traverse LATE-TIME[32]	2011	87	58	2:1	BMC	150 x 10^6	14-21 d	stopflow	all	6	MRI
Turan[33]	2012	62	42	2:2	BMC	9.6 ± 3,2 x 10^7	7 d	stopflow	all	6	LVG
Sürder[34]	2013	192	64	1:1:1	BMMNC	160 x 10^6	5-7 d	stopflow	all	12(4)	MRI
			64		BMMNC	140 x 10^6	21-28 d				
Bone marrow progenitors											
Tendera[35]	2009	200	80	2:2:1	CD34+CXCR4+ BMC	1.90 x 10^6	3-12 d	stopflow	anterior	6	MRI
			80		BMMNC	1,78 x 10^6					
Lipiec[36]	2009	39	26	2:1	CD 133 + BMC CD34+	0.33 ± 0.17 x 10^6 3.36 ± 1.87 x 10^6	4-11 d	stopflow	anterior	6	SPECT, echo
Colombo[37]	2011	10	5	1:1	CD 133 + BMC	5.9 x 10^6	10-14 d	stopflow	anterior	12(6)	Echo, PET
Quyumi[38]	2011	31	16	1:1:1:1	CD 34+ BMC	5-15 x 10^6	8.3 d	stopflow	all	6	MRI, SPECT
		280	207								
MSC											
Chen[39]	2004	69	34	1:1	bone marrow MSC	48-60 x 10^9	18.4 ± 0.5 d	stopflow	all	6	LVG, PET
Hare[42]	2009	53	34	2:1	allogneic MSC	dose escalating 0.5, 1.6 or 5.0 x10^6 MSC/body weight	1-10 d	intravenous	all	12 (3,6)	MRI
Houtgraaf[43]	2012	14	10	3:1	ADRC	17.4 ± 4.1 x 10^6	<24 hours	infusioncatheter	anterior	6	MRI, SPECT
CDC											
Makkar[44]	2012	25	17	2:1	cardiosphere derived cells	low 12.5 x 10^6 High: 25 x 10^6 intermediate 17,3 x 10^6	1,5 - 3 mo	stopflow	all	12 (6)	MRI

Overview of RCT characteristics. No: number; pt: patients; d: days; m: months; CT: cell therapy; BM-MSC: Bone Marrow Mesenchymal stem cells; BMMNC: Bone-marrow mononuclear cells, MSC; mesenchymal stem cells; ICA: intracoronary arterial; ICV: intracoronary venous; ADRC: adipose tissue derived regenerative cells; CDC: Cardiosphere-derived cells; LVG: left ventricular-angiography; all: LCx, LAD and RCA; Mo: months

Supplemental Table IV. Sample size calculations MACCE rates

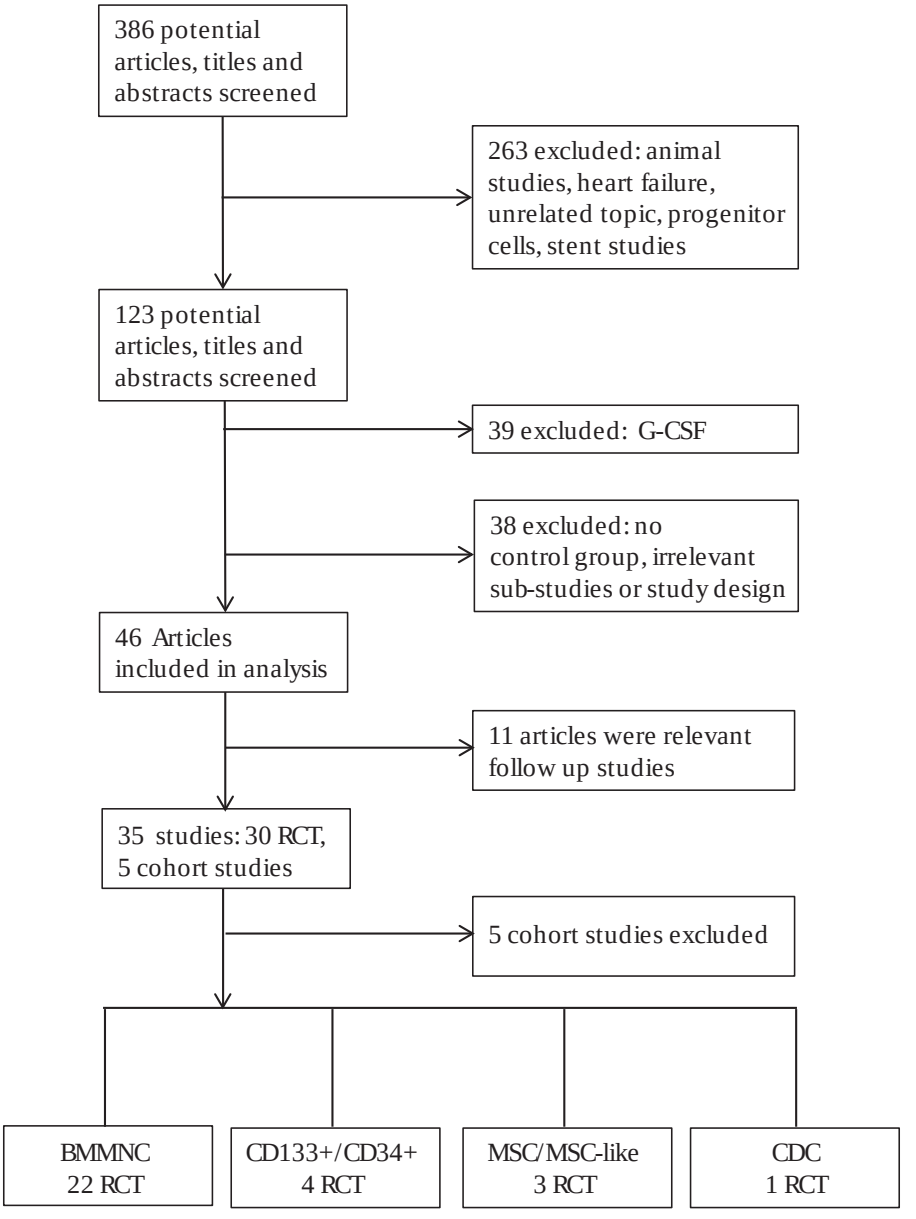
% of MACCE in control	effect	% of MACCE in treatment group	N per group	Total number of patients	effect	% of MACCE in treatment group	N per group	effect	% of MACCE in treatment group	N per group	total number of patients
2	0,60	1,2	4107	8214	0,70	1,4	7615	0,80	1,6	17837	35674
3	0,60	1,8	2716	5432	0,70	2,1	5034	0,80	2,4	11785	23570
4	0,60	2,4	2021	4042	0,70	2,8	3744	0,80	3,2	8759	17518
5	0,60	3,0	1604	3208	0,70	3,5	2970	0,80	4	6943	13886
6	0,60	3,6	1326	2652	0,70	4,2	2454	0,80	4,8	5733	11466
7	0,60	4,2	1128	2256	0,70	4,9	2085	0,80	5,6	4868	9736
8	0,60	4,8	979	1958	0,70	5,6	1808	0,80	6,4	4220	8440
9	0,60	5,4	863	1726	0,70	6,3	1593	0,80	7,2	3716	7432
10	0,60	6,0	770	1540	0,70	7,0	1421	0,80	8	3312	6624
15	0,60	9,0	492	984	0,70	10,5	905	0,80	12	2102	4204
20	0,60	12,0	353	706	0,70	14,0	647	0,80	16	1497	2994

Table V. Upcoming clinical trials on stem cell therapy for the treatment of an AMI

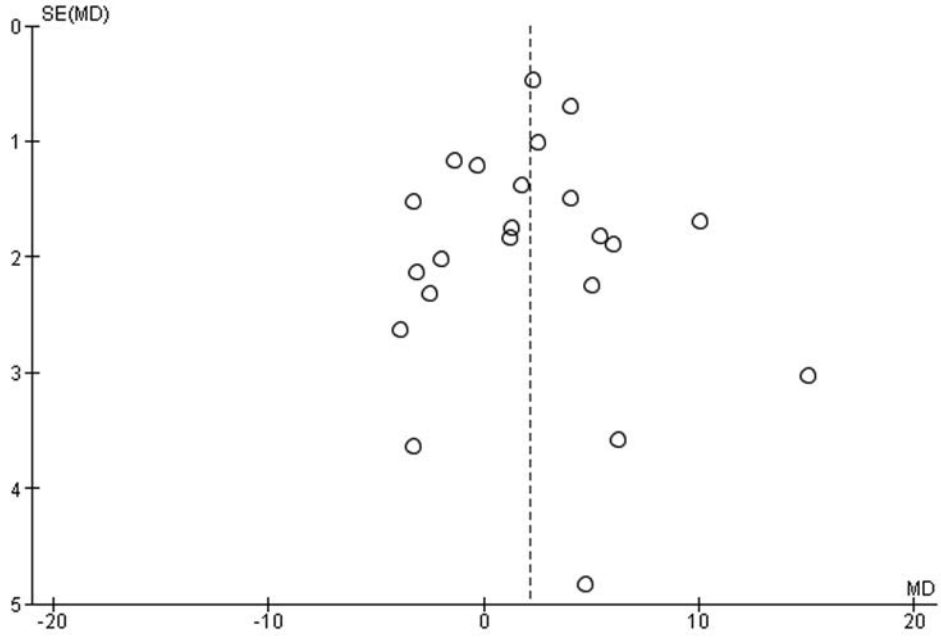
Study name	Trial number	Phase	Route of administration	No. of patients	Cell type	Primary endpoint
BAMI	NCT01569178	III	IC	3000	BMMNC	all-cause mortality during 3 year FU
AMICI	NCT01781390	I/II	IC	225	allogeneic MSCs	reduction of infarcts size between baseline and 6 months FU
ADVANCE	NCT01216995	II/III	IC	216	ADRC	reduction of infarcts size between baseline and 6 months FU
AMIRST	NCT01536106	I/II	IC	30	BMC	adverse events
RELIEF	NCT01652209	III	IC	135	autologous MSC	change in LVEF on MRI
Prochymal-2	NCT 00877903	II	IV	220	allogeneic MSCs	change in ESV on cardiac MRI
REVITALIZE	NCT00874354	I	IC	30	BMMNC	safety and change in LVEF on echo and MRI
ALLSTAR	NCT01458405	II	IC	274	allogeneic CDC	safety and change in infarct size on MRI
REGEN-AMI	NCT00765453	II/III	IC	102	BMC	change in LVEF on MRI at 1 year FU

When the occurrence of a MACCE event in the control group is 2% and the odd ratio (OR) of this event is 0.60, 1.2% of the treated patients will have an event during the study. To show significance between the groups 4107 patients are needed per group. If the occurrence of events rise to 10% only 492 patients are needed in the study. If the odds ratio is 0.80, the differences between the treatment and control group are smaller and more patients will be needed to reach significance. When the occurrence of an MACCE event is 2% in the control group, expected 35.674 patients in total are needed to show a beneficial effect on that event. The longer the follow-up time in the study, the higher the incidence of a MACCE event. Less patients are needed to show a beneficial effect. In our meta-analysis, the all-cause mortality in the control group was 3.4% opposed to 2.1% in the treatment group. The OR of all-cause mortality is therefore 0,60. This would mean that based on this meta-analysis and our calculations, 35.674 patients are needed to show a beneficial effect of cell therapy on all-cause mortality. These calculations are based on a median follow-up duration of 6 months.

Trial number as found on *www.clinicaltrials.gov*. IC indicates intracoronary; IV: intravenous; BMMNC: bone marrow derived mononuclear cells; MSC: mesenchymal stem cell; ADRC: adipose tissue derived regenerative cells; BMC: Bone marrow stem cells; CDC: cardiosphere derived cells; LVEF: left ventricular ejection fraction; LVESV: left ventricular end-systolic volume



Supplemental Figure A. Flowchart of search: "stem cell therapy for acute myocardial infarction"
G-CSF: Granulocyte-Colony Forming Units; RCT: randomized controlled trial; BMMNC: Bone marrow derived mononuclear cells; MSC: mesenchymal stem cells; CDC: cardiosphere derived cells.



Supplemental Figure B. Funnel plot of left ventricular ejection fraction of BMMNC trials.
The dotted line indicates the treatment effect of stem cell therapy. SE (MD): standard error of mean difference; RCT indicates randomized controlled trials; MD: mean difference.

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Chapter 4

First generation cell-based therapy for ischemic heart disease:

A meta-analysis and overview of future perspectives

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Abstract

Background Several cell-based therapies for the adjunctive treatment of acute myocardial infarction (AMI) and heart failure (HF) have been investigated in multiple clinical trials, but the benefits still remain controversial. The first generation of cells, defined as bone marrow-derived mononuclear cells (BMMNC) and skeletal myoblasts (SkM), have been investigated in such extent to merit a meta-analysis. This meta-analysis aims to evaluate the efficacy of this first generation stem in AMI (BMMNC only) and HF (BMMNC and SkM) patients, and gives an overview of newer generations stem cells for cardiovascular repair.

Methods and Results A random-effects meta-analysis was performed on randomized controlled trials (RCT) investigating the effects of stem cell therapy in patients with AMI or HF that were published between January 2002 and December 2013. The defined endpoints were left ventricular ejection fraction (LVEF), left ventricular end-systolic and end-diastolic volumes (LVESV/LVEDV), infarct size and major adverse cardiac and cerebral event (MACCE) rates. Overall, in AMI, LVEF increased by +2.10% (95% CI, 0.68- 3.52 , P=0.004) in the BMMNC group and +3.97% (95% CI: 2.33 – 5.62; P<0.00001), both related to an effect on LVESV. However, there is no effect on cardiac function, volumes, nor infarct size, when only randomized controlled trials were included that used MRI-derived endpoints in AMI. No significant effect on MACCE rates was detected in AMI patients following BMMNC therapy. Interestingly, in HF patients, the significant improvement on LVEF remained, whereas also an effect on all-cause mortality was shown.

Conclusion BMMNC therapy does not improve cardiac function in AMI patients, but mildly improves cardiac function in HF. New generations of stem cells are emerging in the field that might be more potent to mend broken hearts, but they still have to prove their superiority in larger clinical trials.

Introduction

Cardiovascular disease accounts for the highest mortality worldwide, despite improvements in treatment options.[1] Approximately half of the cardiovascular deaths is related to acute myocardial infarction (AMI), whereas subsequent heart failure (HF) and stable coronary artery disease account for the majority of morbidity. To reduce the burden of cardiovascular disease and health care costs, new therapeutic strategies are continuously developed and investigated. In the past decade, stem cell therapy emerged as a potent candidate for cardiac repair following an AMI and in ischemic HF.

More than a decade ago, the first patients were treated with the first generation of stem cell therapy, using intramyocardial injection of skeletal myoblasts (SkM) in patients with HF,[2,3] and intracoronary infusion of bone marrow-derived mononuclear cells (BMMNC) in AMI patients.[4,5] Initial optimism concerning the use of SkM in HF patients were toned down by issues of possible pro-arrhythmogenicity of the cells, and disappointing results on efficacy in randomized studies.[6–8] Also, the regenerative capacity of bone marrow (BM)-derived cells in AMI and HF patients has been under debate, since the numerous trials that were performed to date, show conflicting results. Several meta-analyses suggested beneficial effects of BMMNC. However, in most of these meta-analyses, both non-randomized and randomized studies were included, whereas AMI and HF patients were pooled for analysis of clinical end points,[9,10] or clinical end points were included in the analysis.[11]

In the current paper, we aim to give an overview of the safety and efficacy of the first generation of stem cell therapy for the treatment of ischemic heart disease by including some relevant sub group analyses. Also, we aim to give a concise overview regarding newer generations of stem cells for cell-based cardiac repair.

Methods

A detailed description of the search for the meta-analysis can be found in the online supplement. Briefly, the meta-analysis was executed according to the Quorum statements.[12] A random effect meta-analysis was performed that included all clinical trials regarding stem cell therapy for the treatment of AMI, chronic heart failure or ischemic cardiomyopathy, published on Medline between July 2002 and December 2013. BMMNC and SkM (first generation of stem cells) were the main topic in this analysis. Other stem cells included in this analysis were cardiac-derived stem cells (CDC), mesenchymal stem cells (MSC) and adipose tissue-derived regenerative cells (ADRC), although a meta-analysis on these cell types is not feasible due to the small number of clinical trials that were executed to date. However, these clinical trials are briefly described in the review section below.. The following search strategy was applied: "stem cells", "progenitor cells", "mononuclear cells", "adipose tissue-derived regenerative cells", "mesenchymal stem cells', "cardiac-derived stem cells", "bone marrow", "vascular stromal fraction", "adipose stem cells", "mesenchymal-like stem cells", "skeletal myoblasts", "coronary artery disease", "myocardial infarction", "heart failure" "cardiac repair", and "myocardial regeneration".

Studies were included that met the following inclusion criteria: (1) randomized controlled trials with an appropriate control group that received standard therapy; (2) conducted in patients with an AMI that occurred less than 3 months before or patients diagnosed with heart failure or ischemic cardiomyopathy; (3) using stem cells that were administered by intracoronary, intravenous or intramyocardial injection; (4) total of number of patients enrolled should exceed 10; (5) stem cells were derived from bone marrow, skeletal muscle, adipose tissue, or the heart; (6) given in an autologous or allogeneic setting.

A pre-specified sub-group analysis was performed on the use of the current golden standard to measure cardiac volumes and function: cardiac MRI.

Data abstraction and analysis was performed by two different researchers (RdJ, JH) and reported on standardized forms. Left ventricular ejection fraction (LVEF), left ventricular end-systolic volume (LVESV), left ventricular end-diastolic volume (LVEDV) and infarct size were assessed as outcome measures, as well as the occurrence of major adverse cardiac or cerebral events (MACCE rates). The data of the primary endpoint was used in this analysis.

Results

Autologous skeletal myoblasts in ischemic heart failure patients

Skeletal myoblasts (SkM) are progenitor cells residing in striated skeletal muscle, and are responsible for regeneration of skeletal muscle upon damage. SkM can be easily expanded in cell culture. Because they are destined to become contracting cells, they were a logical candidate for cell-based heart repair. In pre-clinical investigations, SkM were able to form functional skeletal myotubes, repopulate the damaged heart, and integrate into host myocardium with formation of electromechanical junctions between host cardiomyocytes and injected skeletal myotubes.[13]

These promising data resulted in the first HF patients treated with epicardial SkM injections during bypass surgery, or endomyocardial injections using specialized injection catheters.[2,3] Our search revealed 4 clinical trials using SkM,[8,14–16] of which only 2 trials were performed in a double-blind and randomized fashion (MAGIC; NCT00102128; table 1a, and MARVEL; NCT00526253; table 1a[15,16]). Only the MAGIC trial was completed, whereas MARVEL was terminated early with inclusion of only limited patient numbers.[16] In both the CAUSMIC and SEISMIC trials, patients and caregivers were not blinded for the treatment, resulting in exclusion of these studies.[8,14] Because only 1 RCT reached the pre-specified end point, a meta-analysis on cardiac function and volumes was not deemed useful. It should be noted, that in this trial, intramyocardial injection of SkM during bypass surgery failed to improve cardiac function after 6 months of follow up.[15]

Moreover, SkM injection has been associated with increased incidence of ventricular tachyarrhythmias. Indeed, according to our analysis, SkM injection is associated with an increased incidence of VT/VF (OR 2.52; 95% CI 0.99-6.40; P=0.05; Figure 1). These ventricular arrhythmias are hypothesized to be

Table 1a. Skeletal Myoblasts Trials

	Phase	Cell type	Status	Design	No.	Delivery Method	Condition	Transfer day after PCI	Cell number (mill)	Primary Clinical Outcome	Reference
MAGIC	II	SkM	terminated	RDBPC	97	IM (Epi)	ICHF	–	400/800	Safety/Efficacy: MACE/ LVEF (Echo) at 6M,	NCT00102128
CAUSMIC II	II	SkM	unknown	RDBPC	23	IM (Endo)	ICHF	–	300	Safety/Efficacy: MLHFQ at 1 year	NCT00626314
SEISMIC	II	SkM	complete	ROPC	40	IM (Endo)	ICHF	–	150-800	Safety/Efficacy: LVEF (MUGA) at 6M	NCT00375817
MARVEL	II/III	SkM	unknown	RDBPC	170	IM (Endo)	ICHF	–	400/800	Safety/Efficacy: QOLQ, 6min walk test at 1 year	NCT00526253

RDBPC: randomized double-blind placebo controlled, ROPC: randomized open-label placebo controlled, SkM: skeletal myoblast, IM: intramyocardial, ICHF: ischemic congestive heart failure, LVEF: LV ejection fraction, MACE: major adverse cardiovascular event, MLHFQ: Minnesota living with heart failure questionnaire, QOLQ: quality of life questionnaire, MUGA: multi gated acquisition scan

Table 1b. Bone Marrow-derived Mononuclear Cell Trials

	Phase	Cell type	Status	Design	No.	Delivery Method	Condition	Transfer day after PCI	Cell number (mill)	Primary Clinical Outcome	Reference
BOOST	I	MNC	complete	RDBPC	60	IC	AMI	4.8	2460	Safety/Efficacy: Regional systolic wall thickening (MRI)	NCT00224536
TCT-STAMI	I	MNC	complete	RDBPC	20	IC	AMI	1	40	Efficacy: LVEF, volumes(echo) at 6M	-
REPAIR-AMI	III	MNC	complete	RDBPC	204	IC	AMI	4	236	Efficacy: LVEF (LVG) at 4M	NCT00279175
Janssen	II	MNC	Complete	RDBPC	67	IC	AMI	1	172	Efficacy: LVEF (MRI) at 4M	NCT00264316
Meluzin	II	MNC	Complete	RSBPC	66	IC	AMI	7	LD 10	Efficacy; perfusion defect at 3,6,12M	

									HD 100	LVEF (SPECT)	
Penicka	II	MNC	Early terminated	RSBPC	27	IC	AMI	4	2640	Efficacy: LVEF and volumes(echo), Infarct size (SPECT) at 4M	NCT00389545
S. de Lezo	II	MNC	Complete	RSBPC	20	IC	AMI	7	900	Safety/efficacy LVEF (LVG) 3M	-
FINCELL	II	MNC	Complete	RDBPC	80	IC	AMI	2-6	402	Safety : IVUS/arrhythmias, Efficacy: LVEF(LVG/echo) at 6M	-
ASTAMI	II	MNC	complete	RSBPC	100	IC	AMI	6	68	Safety/Efficacy: LVEF, EDV, Infarct size	NCT00199823
Yao	II	MNC	Complete	RSBPC	39	IC	AMI	3-7 d or 3-7d and 3 M	190 210	Efficacy: LVEF and volumes (MRI), perfusion defect (SPECT) at 6 and 12M	-
Plewka	II	MNC	Complete	RSBPC	60	IC	AMI	7	144	Efficacy: LV diastolic and systolic function (echo at 6M)	-
Cao	II	MNC	Complete	RSBPC	86	IC	AMI	7	50	Efficacy: LVEF and volumes (echo), perfusion defect (SPECT) at 6,12 and 48M	-
Nogueira	I/II	MNC	Complete	RSBPC	30	IC	AMI	5.5	100	Safety, feasibility	
Grajek	II	MNC	Complete	RSBPC	45	IC	AMI	5-6	2340	Efficacy: perfusion defect (SPECT) and LVEF radionucleotide ventriculography at 3,6, 12M	-
Piepoli	I/II	MNC	Complete	RSBPC	38	IC	AMI	4-7	418	Mortality, efficacy: LVEF (Echo) at 6 and 12M	NCT00437710
SCAMI	II	MNC	Complete	RSBPC	42	IC	AMI	5-7	381	Efficacy: LVEF and volumes (MRI) 1,3,12M MACCE 1,3,6,12	NCT00669227
TIME	II	MNC	complete	RDBPC	120	IC	AMI	3 or 7	150	Efficacy: LVEF (MRI) at 6M	NCT00684021
HEBE	II	MNC	complete	ROPC	200	IC	AMI	6	296	Efficacy: Regional LVEF (MRI) at 4M	ISRCTN 95796863

BONAMI	II	MNC	Complete	ROPC	101	IC	AMI	3-8	98.3	Efficacy:LVEF and myocardial viability Thalium scintigraphy 3 and 12M, LVEF echo 1,3,6,12M	NCT00200707
LateTIME	II	MNC	complete	RDBPC	87	IC	AMI	17.4	150	Efficacy: LVEF (MRI) at 6M	NCT00684060
Turan	II	MNC	Complete	RSBPC	62	IC	Ami	96	7	Efficacy: LVEF, infarctsize(LVG) 6M	-
SWISS-AMI	II	MNC	complete	ROPC	200	IC	AMI	5-7 vs. 21-28	156	Efficacy: LVEF (MRI) at 4M	NCT00355186
Assmus	II	MNC	Complete	ROPC	51	IC	Healed AMI	3 months	205	Efficacy: LVEF (LVG) at 3M	NCT00289822
Hendrikx	II	MNC	Complete	RSBPC	23	IM(Epi)	Post AMI HF	214	60.3	Efficacy: LVEF (MRI), perfusion defect (SPECT) at 4M	-
PROTECT-CAD	II	MNC	Complete	RDBPC	28	IM (Endo-NOGA®)	ICHF	-	16.7	Exercise capacity, CCS. NYHA, LVEF(MRI), perfusion defect 6M	-
Ang	II	MNC	Complete	RSBPC	62	IC or IM (Epi)	ICHF	-	IC 115 IM 84	Efficacy: LVEF and volumes (MRI and echo),regional wall motion 6M	-
Yao	II	MNC	Complete	RDBPC	47	IC	ICHF	-	12	Efficacy: LVEF and infarct size (MRI), LV volumes Echo at 6M	-
Zhao	II	MNC	Complete	RDBPC	36	IM(Epi)	ICHF	-	659	MACE, Efficacy: LVEF(echo), perfusion defect SPECT at 6M	-
Hu	II	MNC	Complete	RSBPC	60	IM(Epi)	ICHF	-	132	Efficacy: LVEF and volumes (MRI), NYHA, exercise test	-
ESCAPE	III	MNC	complete	RDBPC	250	IM (Epi)	ICHF	–	150	Safety/Efficacy: Survival at 1Y	NCT00841958
Turan	II	MNC	Complete	RSBPC	56	IC	ICHF	-	1	Efficacy: LVEF and volumes(LVG)	-
FOCUS-HF	I	MNC	Complete	RSBPC	30	IM (Endo-NOGA®)	ICHF	-	30	Safety/efficacy: MVO2, perfusion defect, LVEF and volumes (echo) at 3 and 6M. electromechanical mapping 6M	NCT00203203

FOCUS-CCTRN	II	MNC	complete	RDBPC	92	IM (Endo-NOGA®)	ICHF	–	100	Safety/Efficacy: MVO2, LVESV, Reversible defect size	NCT00824005
TAC-HFT*	I/II	MNC	Complete	RDBPC	29	IM (helix)	ICHF	-	?	Safety 30d, SAE 1Y, Infarct size and regional myocardial function (MRI) 1Y	NCT00768066

RDBPC: randomized double-blind placebo controlled, RSBPC: randomized single-blind placebo controlled, ROPC: randomized open-label placebo controlled, MNC: bone marrow-derived mononuclear cell, IC: intracoronary, IM: intramyocardial, AMI: acute myocardial infarction, ICHF: ischemic congestive heart failure, CMI: chronic myocardial ischemia, CM: cardiomyopathy, LVEF: LV ejection fraction, LVEDV: LV end diastolic volume, LVESD: LV end systolic volume LVG: left ventriculogram, ETT: exercise tolerance test, MVO2: maximal oxygen consumption, SAE: serious adverse event. *TAC-HFT compares both MSC and BMNNC to a control group.

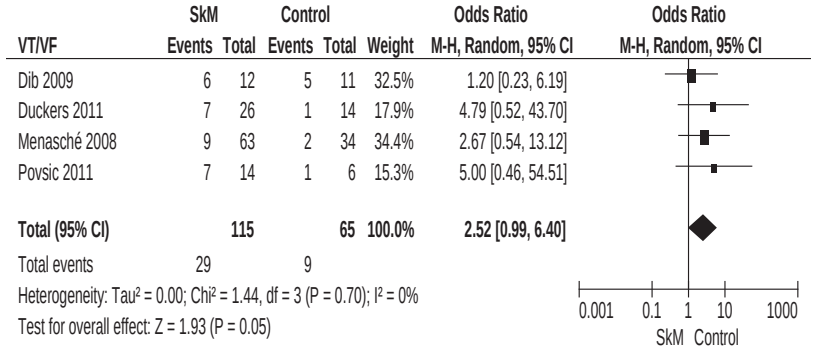

Figure 1. Forest plot of ventricular tachycardia (VT) and ventricular fibrillation (VF) in patients treated with Skeletal myoblasts. Intramyocardial injection of SkM was associated with a significant increase in VF/VT

caused by the lack of electromechanical coupling between injected SkM and host myocardium, due to the lack of connexin-43 expression. Collections of injected SkM thereby form electrically isolated islands that can function as re-entry circuits for ventricular arrhythmias.[17] This finding, in combination with the lack of obvious long-term efficacy, and the development of new and better alternatives, SkM therapy currently is abandoned as cellular therapy for cardiac repair.

Bone marrow-derived mononuclear cells in AMI patients

The search resulted in 21 trials that evaluated the effect of BMMNC transplantation in AMI patients (table 1b, supplemental table 3a). A total of 845 patients were treated with BMMNC opposed to 608 control patients. The average of participating patients per study was 68 ± 51 patients, whereas the median was 45 patients (range 14-200). Most studies used a 1:1 randomization scheme. The median follow-up duration

Table 2. Treatment effect and imaging modality

	all imaging modalities		MRI only	
AMI	**difference in mean (95% CI)**	**p**	**difference in mean (95% CI)**	**p**
LVEF	2.10 [0.68, 3.22]	0.004	-0.13 [-2.67, 2.93]	0.93
LVEDV	-2.69 [-4.83, -0.56]	0.09	-0.86 [-4.66, 2.94]	0.66
LVESV	-4.05 [-6.91, -1.18]	0.006	-2.65 [-5.28, 0.02]	0.06
Infarct Size	-2.80 [-6.03, -0.44]	0.09	-1.11 [-3.74, 1.53]	0.82
HF				
LVEF	3.97 [2.33, 5.62]	<0.00001	3.06 [1.12, 5.01]	0.002
LVEDV	-9.96 [-23.05, 3.13]	0.14	-14.57 [-33.48, 4.34]	0.13
LVESV	-16.80 [-29.62, -2.47]	0.02	-14.41 [-35.03, -6.20]	0.17
Infarct Size	-2.57 [-5.48, 0.35]	0.08	-0.73 [-1.72, 0.27]	0.15

The treatment effect of BMMNC therapy diminished in AMI patients when data is pooled for studies that used MRI derived endpoints. However, in HF patients, the effect on LVEF remains. AMI: acute myocardial infarction; HF: heart Failure; LVEF: left ventricular ejection fraction; LVEDV: left ventricular end-diastolic volume; LVESV: left ventricular end-systolic volume:

	Control			BMMNC				Mean Difference	Mean Difference
LVEF	**Mean**	**SD**	**Total**	**Mean**	**SD**	**Total**	**Weight**	**IV, Random, 95% CI**	**IV, Random, 95% CI**
A. AMI									
Beitnes 2009	1.2	7.6	46	4.5	7.1	47	3.4%	-3.30 [-6.29, -0.31]	
Cao 2009	9.4	1.8	41	7.1	2.6	45	4.4%	2.30 [1.36, 3.24]	
Corvoisier 2011	1.9	5.2	52	2.2	6.8	49	3.7%	-0.30 [-2.67, 2.07]	
Ge 2006	4.8	5.2	10	3.5	1.9	10	3.2%	1.30 [-2.13, 4.73]	
Grajek 2010	-2.5	5.6	31	0	7.8	14	2.6%	-2.50 [-7.04, 2.04]	
Hirsch 2010	3.8	7.4	67	5.2	5.8	60	3.8%	-1.40 [-3.70, 0.90]	
Huikuri 2008	4	11.3	39	-1.4	1.1	38	3.1%	5.40 [1.84, 8.96]	
Janssens 2006	3.4	6.9	30	2.2	7.3	30	3.1%	1.20 [-2.39, 4.79]	
Meluzín 2008	5	6.6	40	0	8.9	20	2.6%	5.00 [0.60, 9.40]	
Meyer 2009	6.7	6.5	30	0.7	8.1	30	3.0%	6.00 [2.28, 9.72]	
Nogueira 2009	6.7	5.5	24	2	11.5	6	1.1%	4.70 [-4.76, 14.16]	
Penicka 2007	6	5	14	8	4.8	10	2.9%	-2.00 [-5.96, 1.96]	
Piepoli 2010	8.4	9.2	19	2.2	12.6	19	1.6%	6.20 [-0.82, 13.22]	
Plewka 2009	9	5.8	38	5	4.9	18	3.4%	4.00 [1.08, 6.92]	
Schächinger 2006	5.5	7.3	95	3	6.5	92	3.9%	2.50 [0.52, 4.48]	
Suarez de Lezo 2008	21	8	10	6	5.2	10	2.0%	15.00 [9.09, 20.91]	
Surder 2013	1.34	8.04	107	-0.4	8.8	60	3.6%	1.74 [-0.96, 4.44]	
Traverse 2012	6.1	9.8	30	9.4	10	10	1.6%	-3.30 [-10.42, 3.82]	
Traverse 2011	0.5	8.2	55	3.6	9.3	26	2.8%	-3.10 [-7.28, 1.08]	
Turan 2011	11	6.1	42	1	6.3	20	3.2%	10.00 [6.68, 13.32]	
Wöhrle 2010	1.8	5.3	28	5.7	8.4	12	2.3%	-3.90 [-9.04, 1.24]	
Yao 2009	6.2	2.4	27	2.2	1.8	12	4.2%	4.00 [2.64, 5.36]	
Subtotal (95% CI)			**875**			**638**	**65.3%**	**2.10 [0.68, 3.52]**	
Heterogeneity: Tau² = 7.81; Chi² = 107.30, df = 21 (P < 0.00001); I² = 80%									
Test for overall effect: Z = 2.89 (P = 0.004)									
B. HF									
Ang 2008	2.16	6.36	18	0.7	4.8	7	2.5%	1.46 [-3.15, 6.07]	
Assmus 2006	2.9	3.6	28	-1.2	3	18	4.0%	4.10 [2.18, 6.02]	
Hendrikx 2006	6.1	8.6	10	3.6	9.1	10	1.4%	2.50 [-5.26, 10.26]	
Hu 2011	10.6	4	31	5.7	2.3	28	4.1%	4.90 [3.25, 6.55]	
Perin 2012	1.4	5.2	54	-1.3	5.1	28	3.8%	2.70 [0.36, 5.04]	
Perin 2011	3	6.6	20	3	6.1	10	2.5%	0.00 [-4.76, 4.76]	
Pokushalov 2010	4.5	2.88	49	-1.6	2.03	33	4.3%	6.10 [5.04, 7.16]	
Tse 2007	3.7	5.1	18	-0.4	7.5	8	2.1%	4.10 [-1.61, 9.81]	
Turan 2011	6	5.2	33	0	6.7	16	3.0%	6.00 [2.27, 9.73]	
Yao 2008	2.4	3.1	24	1.6	2.1	23	4.2%	0.80 [-0.71, 2.31]	
Zhao 2008	13.3	6.7	16	3.9	4.5	18	2.9%	9.40 [5.51, 13.29]	
Sub1total (95% CI)			**301**			**199**	**34.7%**	**3.97 [2.33, 5.62]**	
Heterogeneity: Tau² = 4.93; Chi² = 46.26, df = 10 (P < 0.00001); I² = 78%									
Test for overall effect: Z = 4.73 (P < 0.00001)									
Total (95% CI)			**1176**			**837**	**100.0%**	**2.74 [1.63, 3.85]**	
Heterogeneity: Tau² = 7.11; Chi² = 176.97, df = 32 (P < 0.00001); I² = 82%									
Test for overall effect: Z = 4.83 (P < 0.00001)									
Test for subgroup differences: Chi² = 2.86, df = 1 (P = 0.09), I² = 65.0%									

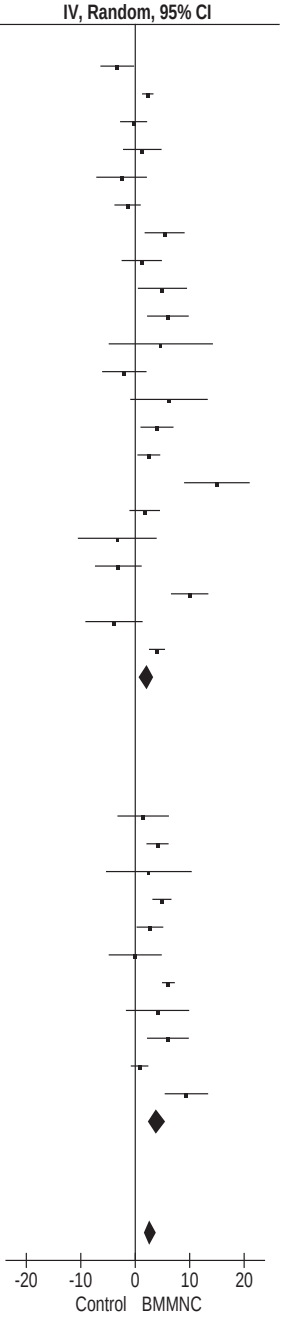

Figure 2. Forest plot of LVEF in patients treated with BMMNC. Figure A represents intracoronary infusion of Bone marrow mononuclear cells (BMMNC) in patients with an acute myocardial infarction (AMI). Figure B represents intramyocardial injection of BMMNC in patients with HF.

in all studies was 6 months (range 3-60 Months). The median amount of infused viable cells was 100 million (range from 5 x 10^6 to 60 x 10^9) and the cells were infused after a median of 7 days (range <24 hours to 3 months). MRI was the imaging modality of choice for follow up of LV function in 40% of the RCTs.

Intracoronary infusion of BMMNC resulted in a minimal increase in LVEF by +2.10% (95% CI: 0.68-3.52; P=0.004; figure 2a; table 2), mostly by a preservation in LVESV which decreased by -4.05 mL (95% CI: -6.91- -1.18 mL; P=0.006). Intriguingly, this modest beneficial effect disappeared (0.13, 95% CI -2.67-2.93; p=0.93) when a sub-group analysis was performed based on imaging modality, and only including studies that used MRI-derived measures (figure 3a; table 2). LVEDV and infarct size did not improve in patients treated with BMMNC, irrespective of imaging modality.

Intracoronary infusion of stem cells did not result in procedure-related adverse events, or adverse events were not reported in the manuscript. No effect on the incidence of MACCE events was detected in this meta-analysis (table 3).

Table 3. MACCE rates Bone Marrow-derived Cells in AMI

		BMMNC	
Outcome AMI	**OR**	**95% CI**	**P**
All cause mortality	0.68	0.36-1.31	0.25
Cardiac mortality	0.73	0.32-1.65	0.45
Recurrent MI	0.50	0.24-1.06	0.07
TVR	0.86	0.58-1.27	0.44
In-stent restenosis	0.95	0.51-1.79	0.88
In-stent thrombosis	0.75	0.08-7.45	0.81
Heart failure	0.84	0.44-1.60	0.60
CVA	0.62	0.13-2.84	0.53
VT/VF	0.66	0.32-1.35	0.25
ICD	0.98	0.37-2.64	0.97
Outcome HF*			
All-cause mortality	0.34	0.15-0.75	0.008
Cardiac mortality	0.70	0.14-3.58	0.66
VT/VF	1.64	0.03-83.48	0.8

MACCE rates were not reduced in BMMNC treated patients. It has to be kept in mind that the trials to date were not sufficiently powered to detect differences in clinical outcome. OR; odds Ratio; CI; confidence interval; MI: myocardial infarction; TVR; target vessel revascularization; VT; ventricular tachycardia; VF: ventricular fibrillation; ICD: Implantable Cardioverter Defibrillator; HF: heart failure; AMI: acute myocardial infarction
*In HF patients, only all-cause mortality, cardiac mortality and VT/VF were included in the analyses. Other MACCE parameters were omitted due to a limited number (2-3) trials that presented these results in their paper.

Bone marrow-derived mononuclear cells in ischemic heart failure patients

Although most trials using BMMNC were performed 3-30 days following the AMI, some other studies evaluated the effect of BMMNC in ischemic or non-ischemic HF patients (table 1b, supplemental table 3b). The search resulted in 12 trials, including a total of 500 patients (301 were treated with BMMNC)

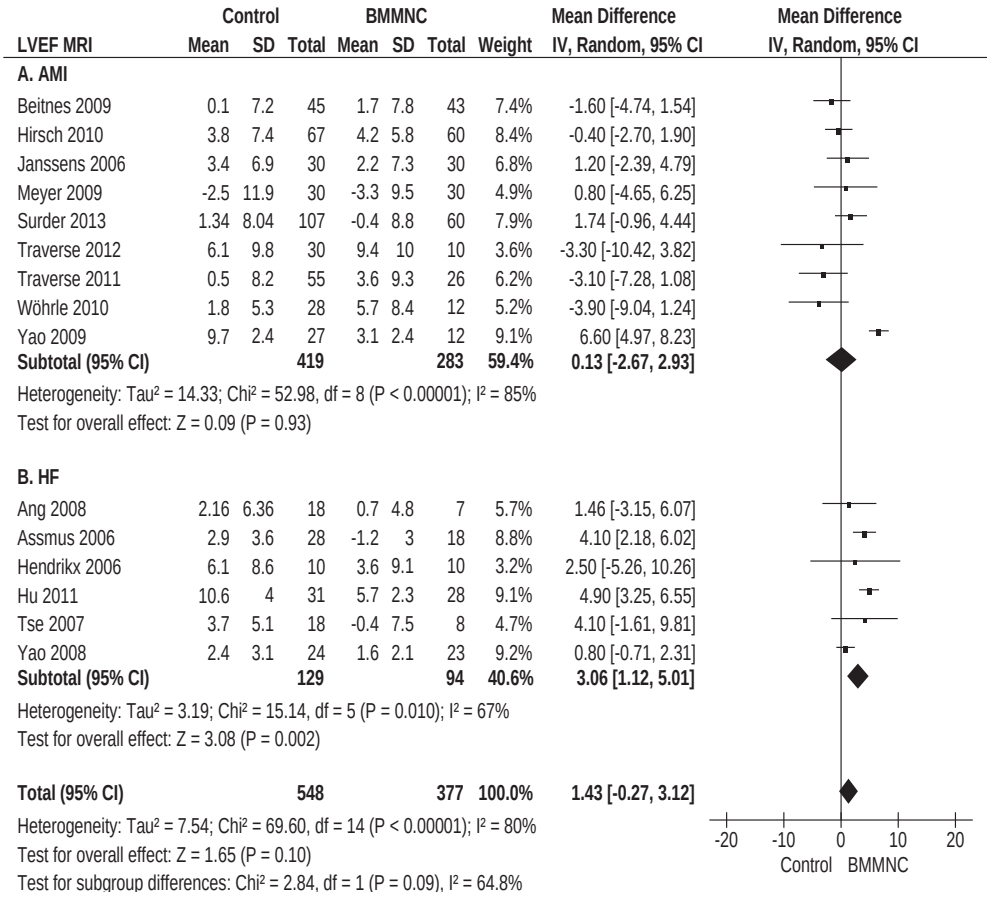

Figure 3. Forest plot of MRI-derived LVEF in patients treated with BMMNC. Figure A represents intracoronary infusion of Bone marrow mononuclear cells (BMMNC) in patients with an acute myocardial infarction (AMI). Figure B represents intramyocardial injection of BMMNC in patients with HF.

[s1-11] The average number of BMMNC injected was 128.6 ± 186.6 x 10^6. The median follow-up duration was 6 months and most trials assessed cardiac function by 2D-echocardiography or LV angiography. Endpoints were mainly specified as the effect on LVEF and LV volumes, or exercise capacity in combination with improvement in NYHA classification. Three studies used intracoronary infusion as delivery route; nine studies investigated the effects of intramyocardial injection.

Overall, LVEF improved by +3.97% (95% CI: 2.33 – 5.62; P<0.00001; figure 2b; table 2) mainly evoked by a reduction in LVESV of -16.05 mL (95% CI: -29.62 - -2.47, p=0.02). LVEDV remained unchanged. Moreover, most trials revealed an improvement in NYHA classification. Unfortunately, the recent study by Heldman *et al.*, which compared BMMNC with autologous mesenchymal stem cells (MSC), did not report the absolute effects on LVEF and volumes.[18] Therefore, this study is not used in our analysis of functional data.

When corrected for studies that used MRI derived parameters, LVEF remains significantly enhanced in patients treated with BMMNC (+3.06%, 95% CI 1.12- 5.01; P=0.002; Figure 3b, Table 2). However, the significant effect on LVESV disappeared (-14.45 ml, 95% CI -35.03 - 6.20; p=0.17).

No serious adverse events were reported in the trials regarding BMMNC transplantation, despite the vulnerable patient population. BMMNC treatment resulted in a reduction of all-cause mortality in ischemic HF patients (OR 0.34, 95% CI 0.15 – 0.75, p=0.008; table 3) but not in other MACCE events.

Discussion

The current meta-analysis comprises 1453 AMI patients and 501 HF treated with BMMNC, as well as 107 HF patients treated with SkM. We defined these cells as first generation of cardiac stem cell therapy, because initial and most experience has been obtained with these cells. We found that BMMNC therapy was safe in both patient groups. However, SkM injection resulted in a significant increase in ventricular arrhythmias in treated HF patients. Our meta-analysis revealed that there is a modest increase of 2% in LVEF in AMI patients treated with BMMNC, but this effect diminishes when only trials are included that use MRI as imaging modality. In HF patients, we found a significant increase in LVEF, irrespective of the imaging modality that was used in the trials. This beneficial effect resulted in a decrease in all-cause mortality in treated patients with HF, which was absent in AMI patients.

Clinical outcome

Several previous meta-analyses have reported beneficial effects of BMMNC therapy on clinical end points in AMI patients as all-cause mortality, cardiac mortality, and the incidence of recurrent myocardial infarction and stent thrombosis.[9,10,19] These results do not correspond with our data. Indeed, we found some discrepancies between these meta-analyses and the current manuscript. First, and most importantly, in these analyses, data of both AMI and HF patients were pooled to increase power. Also, some important negative studies as the HEBE trial were not included in their analysis, whereas other studies were mentioned twice. Moreover, in the current study the odds ratio was calculated to assess the effect on clinical end points, whereas another meta-analysis displayed clinical outcome measures using the relative risk.[11]

The current paper for the first time separates both patient groups, and describes functional outcome, as well as clinical outcome, in one manuscript. We found no clear effect on any pre-specified clinical end point in AMI patients treated with BMMNC, whereas in HF patients the beneficial effect was reduced to all-cause mortality alone. We believe that combining the results of these two distinct patient populations in earlier meta-analyses has clouded outcome, and overestimated the effect of BMMNC therapy. We should also keep in mind that all studies to date regarding BMMNC therapy were designed as safety and feasibility studies. Hence, they were not powered to detect differences on

Table 4. Clinical Autologous and Allogeneic Mesenchymal Stem Cell Trials

	Phase	Cell type	Status	Design	No.	Cell source	Delivery Method	Condition	Primary Clinical Outcome	Reference
STEMMI	II	Autologous	complete	RDBPC	78	BM (mobilized G-CSF)	IC	AMI	Safety/Efficacy : regional systolic wall thickening MRI	NCT00135928
APOLLO	I	Autologous	complete	RDBPC	13	Adipose-tissue	IC	AMI	Safety	NCT00442806
ADVANCE	II/III	Autologous	not yet recruiting	RDBPC	360	Adipose-tissue	IC	AMI	Safety and Efficacy: infarct size (MRI)	NCT01216995
PROMETHEUS	I/II	Autologous	complete	RDBPC	45	BM	IM (Epi)	IHF	Safety/Efficacy: Serious adverse events, Infarct size (MRI)	NCT00587990
C-CURE	II/III	Autologous	complete	RSBPC	240	Guided BM	IM (Endo) (C-Cath®)	IHF	Safety/Efficacy: LVEF	NCT00810238
TAC-HFT	I/II	Autologous	complete	RDBPC	30	BM	IM (Endo) (Helix®)	IHF	Safety/Efficacy: MRI	NCT00768066
PRECISE	I	Autologous	recruiting	RDBPC	36	Adipose-tissue	IM (Endo) (NOGA®)	IHF	Safety: MACCE at 3Y	NCT00426868
PROCHYMAL	I	Allogeneic	complete	RDPBC	53	BM	IV	AMI	safety	-
PROCHYMAL	II	Allogeneic	recruiting	RDBPC	220	BM	IV	AMI	Safety: (LVESV)	NCT00877903
Mesoblast AMI	I/II	Allogeneic	recruiting	RSBPC	25	BM	IM(Endo) (NOGA®)	AMI	Feasibility/Safety	NCT00555828
MultiStem	I	Allogeneic	complete	ONPC	25	BM	IM(adventitia of CA) (Cricket®)	AMI	Safety: Adverse Event at 1M	NCT00677222
AMICI	II	Allogeneic	not yet recruiting	RDBPC	225	BM	IC	AMI	Safety/Efficacy: Infarct size (MRI) at 6M	EUCTR2010-020497-41-NL

POSEIDON	I/II	Auto/Allo	complete	RONPC	30	BM	IM(Endo) (Helix®)	ICHF	Safety/Efficacy: TE-SAE at 1M	NCT01087996
Mesoblast CHF	II	Allogeneic	unknown	RSBPC	60	BM	IM(Endo) (NOGA®)	CHF (ischemic/ idiopathic)	Feasibility and Safety	NCT00721045

RDBPC: randomized double-blind placebo controlled, RSBPC: randomized single-blind placebo controlled, BM: bone marrow, IC: intracoronary, IM: intramyocardial, AMI: acute myocardial infarction, ICHF: ischemic congestive heart failure, CMI: chronic myocardial ischemia, LVEF: LV ejection fraction, MACCE: major adverse cardiovascular and cerebrovascular event, EET: exercise tolerance test

Table 5. Cardiac Progenitor Cell Trials

	Phase	Cell type	Status	Design	No.	Cell source	Delivery Method	Condition	Primary Clinical Outcome	Reference
SCIPIO	I	Autologous	complete	RONPC	40	CSC (c-kit)	IC	ICHF	Short term Safety	NCT00474461
CADUCEUS	I	Autologous	complete	RONPC	31	CDC (Cardiospheres)	IC	ICHF	Safety	NCT00893360
ALLSTAR	I-II	Allogeneic	recruiting	RDBPC	274	Allogeneic CDC	IC	ICHF	Safety/ Infarct size	NCT01458405

RDBPC: randomized double-blind placebo controlled, RSBPC: randomized single-blind placebo controlled, ONPC: open label, non-placebo-controlled, RONPC: randomized, open label, non-placebo-controlled, BM: bone marrow, CSC: cardiac stem cells, CDC: cardiosphere derived cells; IC: intracoronary, ICHF: ischemic congestive heart failure, LVESV: LV end systolic volume

clinical endpoints. We hope that the forthcoming BAMI study (NCT01569178), with all-cause mortality during 3-year follow up as primary end point, will render a definitive answer regarding the role of BMMNC therapy in AMI patients. In this trial, 3000 AMI patients with EF below 45% will be included and randomized to BMMNC therapy or optimal medical care. However, although this study has been announced several years ago, thus far no patient has been included.

Cardiac MRI as reference imaging modality

The studies that drove initial enthusiasm of BMMNC-based heart repair following AMI used either left ventriculography or echocardiography as imaging tool to assess cardiac function.[20,21] Both imaging modalities are known to be less accurate in determining cardiac volumes than the current golden standard, which is cardiac MRI.[22] We believe that this, in combination with relatively low patient numbers, could have overestimated the effects of BMMNC in AMI patients. In the current meta-analysis, 40% (8 out 21) of all trials used cardiac MRI as imaging modality, and correction for this parameter resulted in a reduction of the treatment effect to non-significant values. This finding corroborates the exploratory findings of Traverse et al.[23], and should put the initial enthusiasm concerning BMMNC-based therapies for AMI patients in a different perspective.

In patients with HF, however, improvement of LVEF remained within significant values, despite correction for MRI as imaging modality. Although this effect of only ~3% seems modest, it could have significant clinical implications. For instance, in the studies that assessed the effect of primary PCI following AMI, a similar modest improvement in LVEF was found that eventually resulted in pronounced effects on mortality.[22,24] The community of HF specialists has embraced these positive findings by incorporating BMMNC therapy as a possible adjunctive treatment of HF patients in the guidelines of the European Society of Cardiology for the treatment of HF.[25]

Are BMMNC the optimal cells for ischemic heart disease?

The mononuclear cell fraction of BM consists of a heterogeneous population of cells, predominantly comprising white blood cells and its precursors. Less than 1% of all BMMNC are actual stem cells, the majority of which are hematopoietic stem cells, but this fraction also includes endothelial progenitor cells and mesenchymal stem cells (~0.01%).[26,27] Following BM harvest, BMMNC are separated from red blood cells and plasma, for instance using a Ficoll gradient, after which this whole heterogeneous fraction is infused or injected.

This meta-analysis shows that even *if* there is an effect of BMMNC therapy in ischemic heart disease patients, it is only modest. In the meanwhile, the field has progressed, and several newer generations of stem cells have emerged. Some of these cells have already been tested is phase I and II clinical studies, whereas others are still in the preclinical testing phase. The next paragraphs summarize our knowledge about some of these next generations of stem cells for cell-based cardiac repair.

What cell for what disease type?

As AMI and HF are two distinct conditions within different stages of the disease process, it seems logical that the cell that fits one condition might not be the ideal cell for the other. Therefore, the purpose of cell therapy should be defined for both diseases separately.

In AMI patients, we should strive to minimize damage inflicted by ischemia and reperfusion, thereby reducing infarct size and thus delaying or abrogating LV remodeling. We believe that the ideal cell for AMI patients has the following characteristics: 1) availability during the (hyper)acute phase of the AMI, and; 2) have pronounced paracrine anti-apoptotic, pro-angiogenic, immuno-modulatory, and anti-remodeling capacities, and/or; 3) mobilize or stimulate resident cardiac stem cell niches or cardiomyocytes, and; 4) be autologous or non-immunogenic.

In contrast to AMI, HF is a chronic condition with a much broader time window for possible stem cell transplantation. Ideally, in patients with heart failure due to systolic dysfunction, cells should be applied that can 1) influence the remodeling process, and have anti-apoptotic capacities and/or; 2) mobilize or stimulate resident cardiac stem cell niches or cardiomyocytes, and/or 3) enhance blood and nutrients supply by inducing angiogenesis; and/or 4) be multipotent, transdifferentiate into functional cardiomyocytes, and contribute to the contractile apparatus.

Although BMMNC contain some of the characteristics mentioned above, we believe that there are more potent candidates. For a complete overview of all cell types we refer to chapter 2 of this thesis.

Conclusion

BMMNC are not effective for the treatment of AMI-patients, whereas their benefit in HF patients is modest at most. We believe that, to date, MSC are the promising candidate for cardiac repair in both AMI and HF patients. Autologous and allogeneic MSC have been shown to reverse cardiac remodeling following intracoronary and intramyocardial injection, in AMI and HF patients. This is probably associated with their potent paracrine capacities, resulting in a stimulatory effect on resident cardiac stem cells and cardiomyocytes, as well as reduction of apoptosis and extracellular matrix remodeling, and a stimulation of neo-angiogenesis. Next to MSC, CDC and CSC are emerging, and the proof of principle of these cells in HF has been established. The near future will probably prove whether these cells have a definite place in this disease type.

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SUPPLEMENTAL MATERIAL

Content

Material and Methods

Search strategy

Medline (July 2002-December 2013) and the Cochrane Central Register of Controlled trials (CENTRAL) and the website of US Food and drug administration www.fda.gov were searched for relevant articles. The search included all randomized controlled trials regarding stem cell therapy for the treatment of and AMI, chronic heart failure or ischemic cardiomyopathy reported up to January 1st 2014. We also searched for relevant abstracts and presentations on this topic reported in major cardiology meetings. References in other articles were also investigated and included in the analysis whenever deemed appropriate. Websites, including *www.clinicaltrials.gov* and *www.clinicaltrialregister.eu*, were searched for ongoing trials and future trials. The following search strategy was applied: "stem cells", "progenitor cells", "mononuclear cells", "adipose tissue-derived regenerative cells", "mesenchymal stem cells', "cardiac-derived stem cells", "bone marrow", "vascular stromal fraction", "adipose stem cells", "mesenchymal-like stem cells", "skeletal myoblasts", "coronary artery disease", "myocardial infarction", "heart failure""cardiac repair", and "myocardial regeneration". Only articles published in English were included. Limitations used in the search were the publication of the study within the last 10 years, limited to clinical trials and randomized controlled clinical trials.

Inclusion and exclusion of studies

Studies were included that met the following criteria: (1) randomized controlled trials with an appropriate control group who received standard therapy, (2) conducted in patients with an AMI that occurred less than 3 months before or patients with diagnosed heart failure or ischemic cardiomyopathie (3) using stem cells that were administered by intracoronary, intravenous injection or intramyocardial injection, (4) total of number of patients enrolled should exceed 10, (5) stem cells were derived from adipose tissue, heart, skeletal muscle, bone marrow (6) given in an allogeneic or autologous setting.

Only studies with a complete dataset and specified data on the amount of infused cells were included in this meta-analysis. Studies that described the combination of circulating progenitor cells (CPC) or CPC with granulocyte-colony stimulating factor (G-CSF) were excluded from this analysis to circumvent the potential confounding effect of G-CSF therapy on LV function and dimensions, although G-CSF was previously proven ineffective as a mono-therapy for cardiac repair in AMI. When studies compared C-GSF and stem cells, only the patients in the control and stem cell arm were used in this analysis.

Data abstraction

Two reviewers (RdJ, JH) independently screened abstracts and reported their results in a standardized form. Data extracted from the articles were categorized in trial characteristics, functional outcome,

scar size and safety. The following parameters were extracted from the articles: Left ventricular ejection fraction (LVEF), LV end-systolic volume (LVESV), LV end-diastolic volume (LVEDV), infarct size (MRI), perfusion defect (SPECT) and major adverse cardiac and cerebral events (MACCE) rates. MACCE was specified as: all-cause mortality, cardiac mortality, hospitalization for heart failure, in-stent thrombosis and restenosis, target vessel revascularization, ventricular arrhythmia, ICD implantation and stroke. Infarct size was expressed as the percentage of left ventricle infarcted (in %volume or mass). In the various studies, different imaging modalities have been used to determine left ventricular ejection fraction. Cardiac magnetic resonance imaging (MRI) was considered the golden standard. If more than one imaging modality was included, all data was extracted for subgroup analysis. For studies with more than 1 intervention arm (e.g. multiple doses) the weighted mean was calculated and applied for the main analysis.(SI-3) In trials with multiple follow-up time points, the primary endpoint was used in the main analysis.

Quality

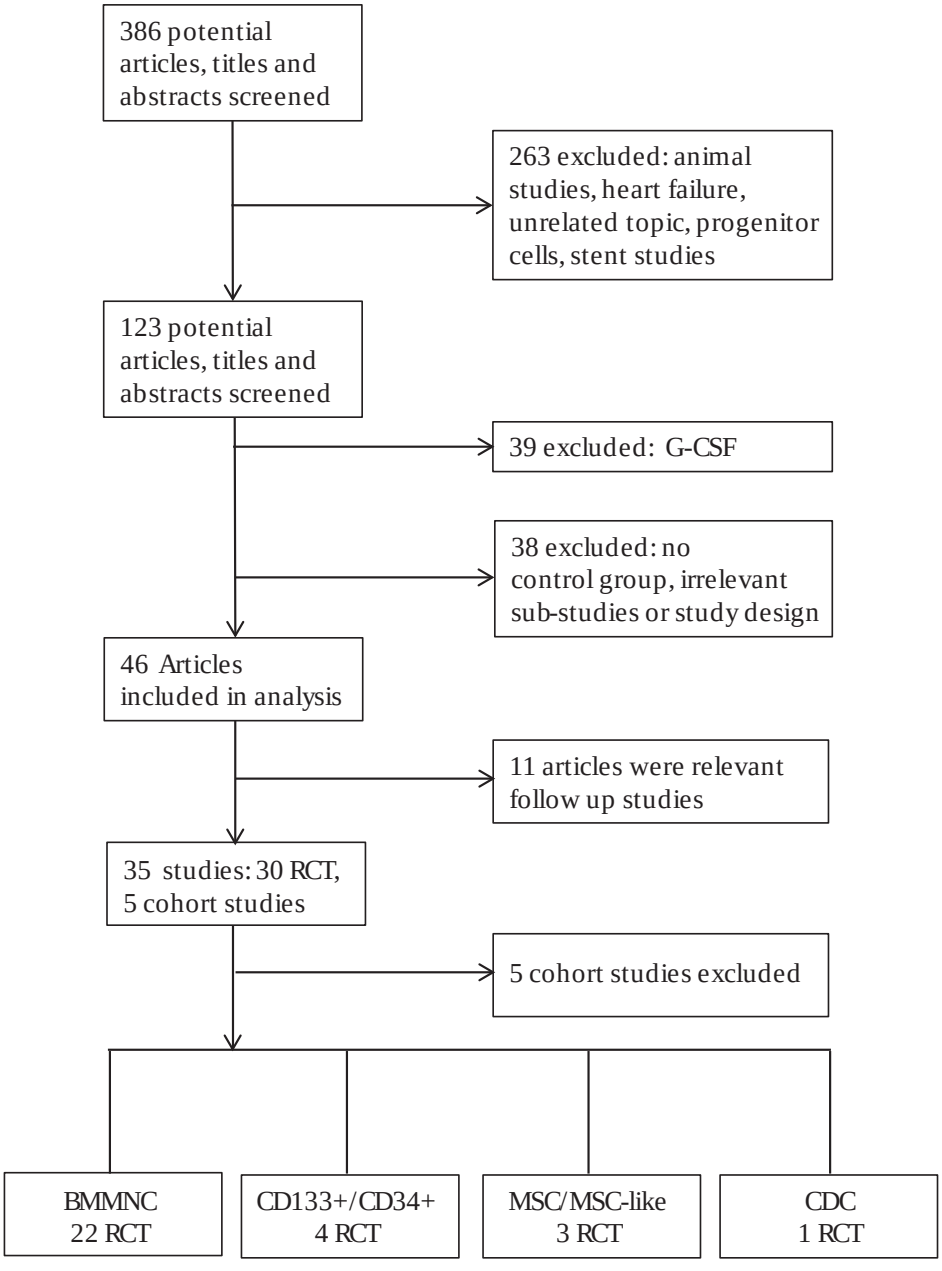
The methodological quality of randomized controlled trials was tested by the Jüni criteria.[1]

Data analysis

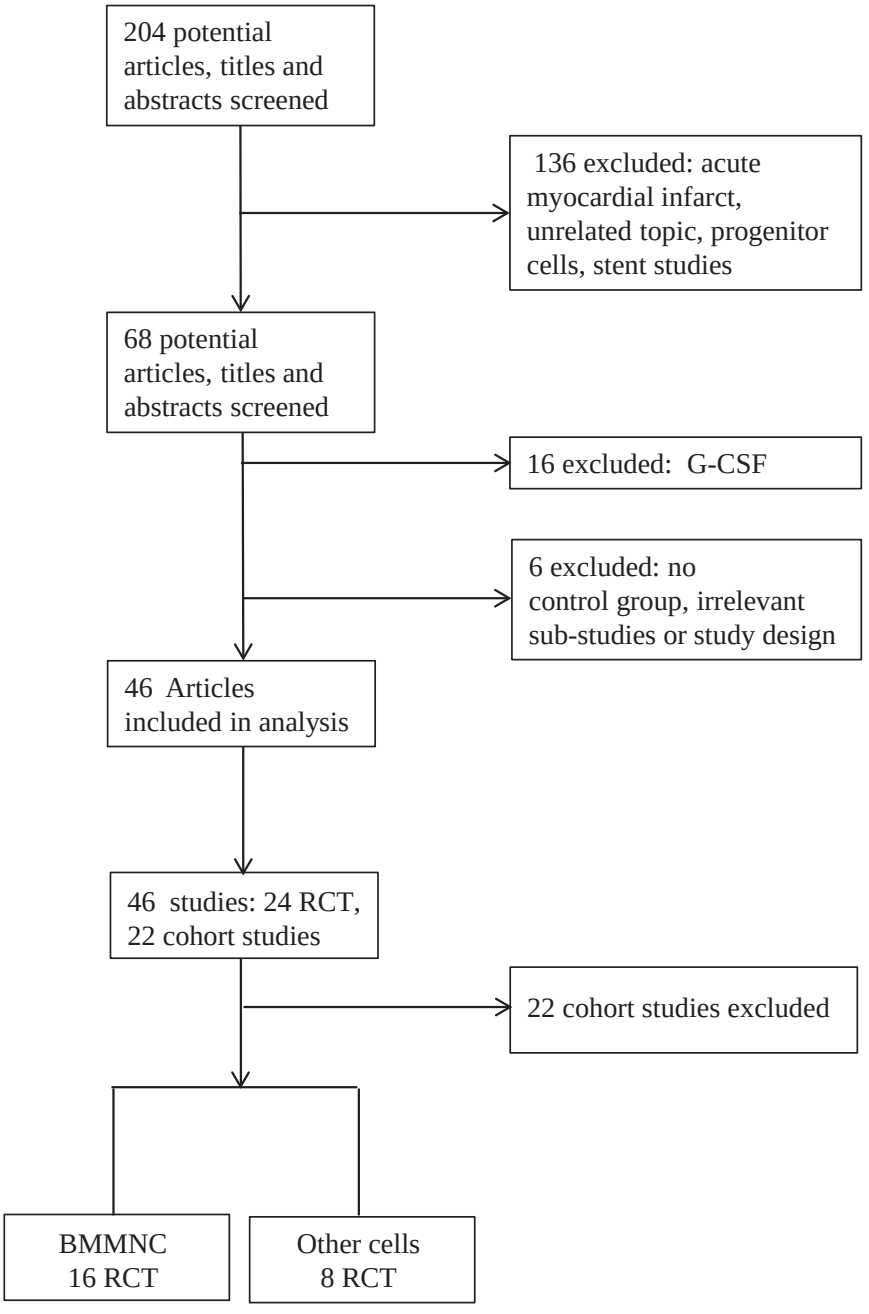
Left ventricular function was the primary endpoint of our analysis. In particular, we studied the difference in mean LV ejection fraction change (LVEF, from baseline to follow-up) between patients receiving stem cells and control treatment. We have applied inverse-variance weighting to combine the results from independent studies. Most studies reported mean LVEF ± one standard deviation (SD) at baseline and follow-up. The mean $LVEF_{change}$ was then determined as $LVEF_{follow\text{-}up}$ - $LVEF_{baseline}$, whereas the SD_{change} was estimated according to the method that is described by Hristov *et al.*[2] For studies that report standard errors of the mean (SEM), SDs were determined as SEM*√(sample size). In case interquartile ranges are reported, SDs are estimated as range/4. We applied a random effects model to obtain an overall estimate of the treatment effect, which we report as point estimate and 95% confidence interval (CI). Heterogeneity was analyzed with the I^2 statistic, and was defined as low (25%-50%), intermediate (50%-75%) or high (>75%).

We have applied similar methodology to study several secondary endpoints, including (mean changes in) left ventricular end systolic (LVESV) and end diastolic (LVEDV) volumes, infarct size as measured by cardiac MRI, and perfusion defect as measured by SPECT. We applied the Mantel-Haenszel odds ratio to obtain an overall estimate of the odds ratio for MACCE, again assuming random effects.

All analyses were performed using Review Manager 5.2 analysis software (Rev Man, Version 5.2, Copenhagen, The Nordic Cochrane Centre, The Cochrane collaboration, 2012). We considered p-values <0.05 (two-sided) as statistical significant. Funnel plots were constructed to explore publication bias.



Supplemental Figure 1. Flow chart of search stem cell therapy in acute myocardial infarction
Supplemental figure S1: flowchart of search meta-analyses on stem cell therapy for the treatment of an acute myocardial infarction. G-CSF indicates granulocyte- colony stimulating factor; RCT: randomized controlled trial; BMMNC: bone marrow mononuclear cell.



Supplemental Figure 2. Flow chart stem cell therapy in heart failure patients

Chapter 5

Final results of a phase IIa, randomised, open-label trial to evaluate the percutaneous intramyocardial transplantation of autologous skeletal myoblasts in congestive heart failure patients: *the SEISMIC trial*

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Abstract

Objectives The SEISMIC study was an open-label, prospective, randomized study to assess the safety and feasibility of percutaneous myoblast implantation in heart failure patients with implanted cardioverter-defibrillators (ICD).

Methods Patients were randomized 2:1 to autologous skeletal myoblast therapy vs. optimal medical treatment. The primary safety end-point was defined as the incidence of procedural and device related serious adverse events, whereas the efficacy endpoints were defined as the change in global LVEF by MUGA scan, change in NYHA classification of heart failure and in the distance achieved during a six-minute walk test (6MW) at 6-month follow-up.

Results 40 Subjects were randomized to the treatment arm (n=26), or to the control arm (n=14). There were 12 sustained arrhythmic events and one death after episodes of ventricular tachycardia in the treatment group and 14 events in the control group (P=ns). At 6-month follow-up, 6MW distance improved by 60.3±54.1 meters in the treated group as compared to no improvement in the control group (0.4±185.7 meters; P=ns). In the control group, 28.6% experienced worsening of heart failure status (4/14), while 14.3% experienced an improvement in NYHA classification (2/14). In the myoblast-treatment arm, one patient experienced a deterioration in NYHA classification (8.0%), whereas five patients improved one or two classes (20.0%; P=0.06). However, therapy did not improve global LVEF measured by MUGA at 6-month follow-up.

Conclusions These data indicate that implantation of myoblasts in patients with HF is feasible, appears to be safe and may provide symptomatic relief, though no significant effect was detected on global LVEF.

Introduction

Heart failure (HF) constitutes a growing patient population, in large part due to advances in percutaneous revascularization procedures, optimized pharmacotherapeutic guidelines and HF care, which contribute to an aging population. An estimated 5 million patients in the US suffer from clinically manifested HF and account for the approximately 1 million hospital admissions per year.(1) This profound socioeconomic burden merits the exploration of novel therapeutic strategies for end-stage HF patients. Cell therapy may provide a new option in the treatment of chronic left ventricular dysfunction.

Skeletal myoblasts (SkMs) are an easily accessible source of autologous precursor cells committed to a myogenic, functionally contractile phenotype. Moreover, SkMs can be expanded extensively in vitro to therapeutic relevant dosages from limited muscle biopsies. SkMs are resistant to ischemia, inflammatory conditions and oxidative stress, and are able to form new myotubes in scarred myocardium, which has been shown to contribute to the function of damaged myocardium in large animal infarction models.(2)

In 2003, Menasche et al. extended these observations to the treatment of CAD patients with congestive HF undergoing surgical revascularization.(3) We previously demonstrated the feasibility of percutaneous autologous SkM transplantation following endoventricular NOGA mapping and targeted intramyocardial injection into the scar areas of chronically infarcted myocardium.(4)

We have now taken these studies further with the SEISMIC study, which was designed as a multicenter, prospective, controlled, randomized study to assess the safety and feasibility of percutaneous intramyocardial transplantation of autologous SkMs and to explore the efficacy in the treatment of congestive heart failure patients. Study patients were stringently monitored for arrhythmias possibly associated with cell therapy, and further analyzed using exercise testing, NYHA classification of HF, Minnesota Living With Heart Failure Questionnaire (MLWHFQ) and MUGA scan analysis of global LVEF at 6 months.

Methods

Study Design

SEISMIC is a phase IIa, prospective, open-label, randomized study performed at 13 sites in 6 European countries to assess the safety, feasibility and cardiovascular effects of endoventricular autologous SkM implantation by a catheter delivery system (MyoCell® and MyoCath™, Bioheart Inc.) in congestive HF patients. Patients were randomized 2:1 to SkM cell therapy versus optimal HF pharmacotherapy. All patients received prophylactic treatment with amiodarone, 4 weeks prior to cell transplantation for up to 4 weeks after SkM transplantation to minimize the risk of ventricular arrhythmias (200mg qd). Subjects assigned to the cell treatment group received a skeletal muscle biopsy for SkM isolation and

culture. Myoblasts were intramyocardially injected into akinetic segments under fluoroscopic guidance. Clinical evaluations were scheduled at baseline, 1, 3, and 6 months.

The primary safety end point was defined as the proportion of patients experiencing serious adverse events (SAEs) at 3 and 6 months follow-up. Serious adverse events were defined as a) any adverse event that was fatal or life-threatening, b) led to prolonged or required hospitalization, c) resulted in any major medical intervention or d) any sustained arrhythmia (>30 seconds). SAEs were adjudicated by an independent DSMB committee. The secondary safety end points included deviations of routine clinical laboratory tests, 12-lead ECG recording, and the occurrence of ventricular arrhythmias on 24hr Holter monitoring. An additional secondary safety objective was to investigate the safety of the MyoCath™ catheter to percutaneously deliver the cellular graft.

The primary efficacy end point comprised the effect on global left ventricular ejection fraction (LVEF) at 3 and 6 months as assessed by a MUGA scan. Secondary efficacy endpoints included the change in six-minute walk (6MW) distance. The overall condition of the patients was assessed by NYHA classification of HF, and the Minnesota Living With Heart Failure Questionnaire (MLWHFQ), frequency and duration of hospitalizations, need for medical treatment, and patient survival. Clinical parameters and analyses of functional outcome were collected and analyzed by an independent clinical research organization and independent core laboratories respectively, all blinded to the treatment of the individual patients.

Patient Eligibility: In/Exclusion Criteria

The study intended to enroll 46 patients, randomized 2:1 to the treatment arm and the control arm. Patients were eligible for study enrollment if they had a diagnosis of ischemic cardiomyopathy with clinically manifest HF, NYHA classification II-III, and a global left ventricular ejection fraction between 20 and 45%, as measured by MUGA. Qualified patients had a defined akinetic region confirmed by either left ventricular angiography or echocardiography. Optimal pharmacological therapy was initiated at least 2 months prior to screening, whereas an ICD was required to be fitted at least 6 months prior to randomization.

Exclusion criteria included CABG or PCI within 3 months prior to cell implantation, HF secondary to valvular disease, ventricular thrombus or significant aortic stenosis.

Quadriceps Muscle Biopsy and Cell Culture Expansion and Transplantation

Autologous SkMs were isolated and cultured from approximately 10 grams of a skeletal muscle biopsy derived from the quadriceps or gastronemicus muscle as previously described.(4) The SkM grafts were required to contain at least 50% SkMs, as determined by flow cytometric analysis of CD56 and desmin immunostaining. The myoblast cellular graft was intramyocardially injected into the targeted treat-

ment region utilizing the MyoCath® catheter delivery system under fluoroscopic guidance as previously described.(5)

The treatment group received a dose in the range of 150-800 million cells, dependent on the size of ventricular scar tissue to be treated. Since the cell concentration was maintained at 50 million cells/mL, the final number of injections varied per patient. The volume per injection was 0.5 mL, whereas a maximum number of 32 injections was allowed per implantation procedure.

Holter Monitoring of Arrhythmia

To distinguish a possible pro-arrhythmic effect of intramyocardial cell therapy, patients were assessed for pre-existing arrhythmias using weekly 24-hour Holter monitoring, initiated at least 6 weeks prior to MyoCell® implantation. Following intramyocardial SkM transplantation, the patient was monitored by continuous telemetry monitoring for a minimum of 72 hours after the procedure, followed by 48 hour Holter monitoring. From the one-week follow-up period onward, patients were subjected to 48 hour Holter monitoring every week, for up to the 4 weeks post-transplantation, and at 3 and 6 months. Any abnormal finding(s) or firing identified by the ICD were assessed immediately by the investigative team at the clinical site.

Statistical Analysis

As SEISMIC was designed to be an exploratory randomized phase IIa study to primarily assess the safety and feasibility of the percutaneous MyoCell® transplantation procedure, and secondarily to provide preliminary data regarding the efficacy of intramyocardial SkM transplantation, no formal power calculations were performed. Two-tailed t-tests were performed for all analyses and confidence intervals are presented with 95% degree of confidence. A two-way ANOVA with repeated measures was used for the comparison of NYHA classification of HF and MUGA left ventricular ejection fraction. All statistical tests used a significance level of á≤0.05.

The parametric assumption of homogeneity of variance for the two treatment groups was checked using the F-test and normality of the two samples was explored using the Wilk-Shapiro test for normality. To support a non-parametric analysis, a 95% confidence for the difference in medians is presented. For analysis of SAEs, a Chi-square test (with continuity correction) was used, presented with a 95% confidence interval for the difference in proportions. If expected cell frequencies were small (≤5), then Fisher's exact test was used instead.

Results

Patient Cohort and Baseline Evaluation

Between October 2005 and May 2007, a total of 62 patients were screened for enrollment in the study at 13 European sites. Of the 62 subjects, 12 failed screening, and were excluded. Three subjects voluntarily withdrew during the screening process. The remaining 47 patients were enrolled and randomized in a 2:1 fashion. 31 Patients were randomized to receive SkM transplantation and 16 were randomized to the control group. Of the 31 patients randomized to the MyoCell® therapy group, 5 patients voluntarily withdrew from the study following changes in the German regulations concerning biopsies. Two patients withdrew from the control group upon knowledge of their randomization allocation. Thus, a total of 40 patients entered the study, 26 of whom entered the SkM cell therapy

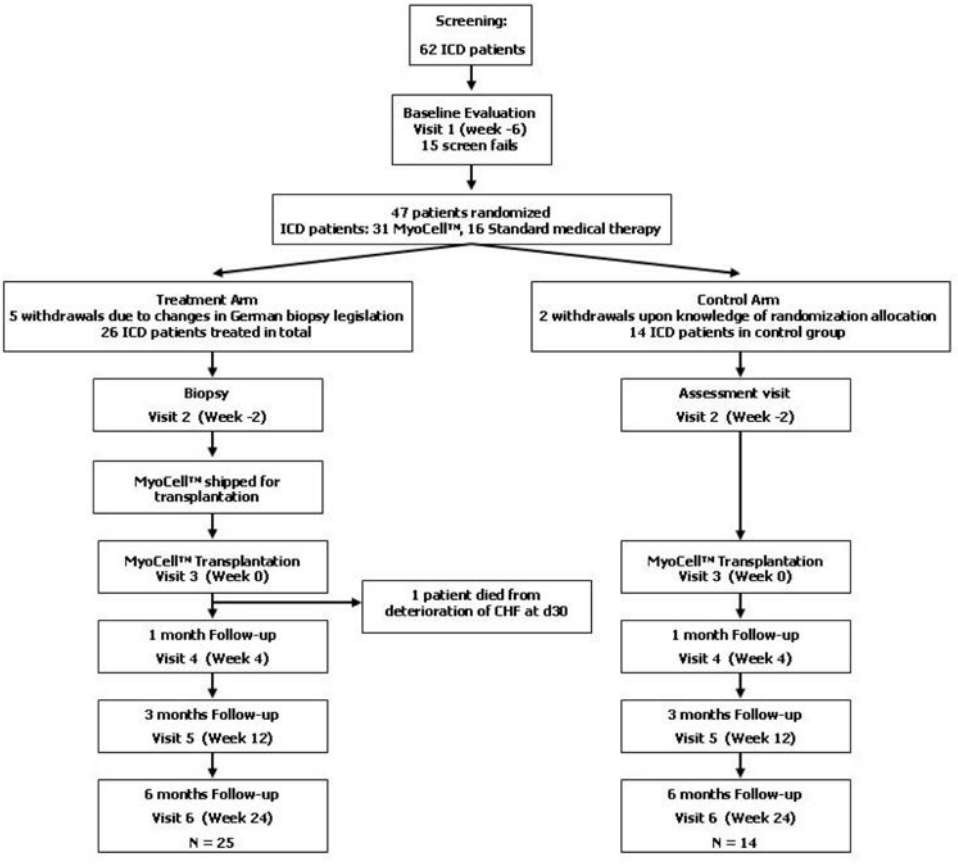

Figure 1. Flow chart of the SEISMIC trial. From the 62 patients that were initially screened, 47 were randomized due to screen failure of 15 patients. Five German patients that were allocated in the treatment arm were withdrawn due to changed biopsy legislation. In the control arm, two patients withdrew upon knowledge of their randomization allocation. 26 patients entered the treatment arm of the study and 14 patients entered the control arm.

Table 1. Baseline Patient Demographics and Clinical Characteristics

Patient Parameter Measured	MyoCell® (n=26)	Control (n=14)	p-value[1]
Sex			
Male	92 % (24/26)	71 % (10/14)	0.16
Female	8 % (2/26)	29 % (4/14)	
Age (years)			
mean ± SD	59.2 ± 8.64	61.9 ± 8.38	0.35
(min - max)	(32 - 72)	(44 - 75)	
Pharmacotherapy			
Beta blockade	89 % (23/26)	79 % (11/14)	0.64
ACE inhibition or ARB	96 % (25/26)	100 % (14/14)	1.0
Diuretics	69 % (18/26)	71 % (10/14)	1.0
Anti arrhythmic drugs	100 % (26/26)	100 % (14/14)	1.0
Coumarin derivative	35 % (9/26)	36 % (5/14)	1.0
Previous Cardiovascular History			
Myocardial infarction	100% (26/26)	100% (14/14)	1.0
Ventricular arrhythmia	73% (19/26)	71% (10/14)	1.0
Aborted sudden cardiac death/ OOHA	4% (1/26)	0% (0/14)	1.0
ICD implanted prior to study requirement	96% (25/26)	100% (14/14)	1.0
Previous PTCA	27% (7/26)	14% (2/14)	0.45
Previous CABG	23% (6/26)	21% (3/14)	0.44
Diabetes Mellitus	31% (8/26)	14% (2/14)	0.45
CVA/TIA	0% (0/26)	14% (2/14)	0.14
LVEF (MUGA)	35 ± 9 %	33 ± 11%	0.54
NYHA			0.32
-NYHA I	0 % (0/26)	0 % (0/14)	
-NYHA II	62 % (16/26)	79 % (11/14)	
-NYHA III	38 % (10/26)	21 % (3/14)	
-NYHA IV	0 % (0/26)	0 % (0/14)	
MLWHFQ (QOL)	40.4 ± 21.9	45.1 ± 29.1	0.32

Numbers are % (counts/available field sample size) or mean ± 1 Standard Deviation.

[1] P-value is from a Fisher's Exact test.

SD Standard Deviation

ICD Implantable Cardioverter Defribillator

MI Myocardial Infarction

CABG Coronary Artery Bypass Graft

PTCA Percutaneous Transluminal Coronary Angioplasty

MLWHFQ Minnesota Living With Heart Failure Questionnaire

arm, and 14 entering the control arm (Figure 1). Baseline clinical characteristics and demographics of the two patient groups are listed in table 1.

Muscle Biopsy and Cell culturing

All procedures were well tolerated and uneventful, resulting in a biopsy of 8.6 ±4.2g. Two patients developed a post procedural hematoma that resolved without sequelae. On average 920 ±302x10^6 myoblasts were generated with a viability of 96.7 ±2.1%. In all cultured cellular grafts, the CD56+ cell content was at least 50% at the time point of cell harvest (89.0 ±13.2% desmin+; 57.2% ±23.9% CD56+).

Transplantation Procedure

The transplantation procedure was successful in 26/27 patients (96.3%). Patients scheduled for myoblast transplantation received an average dose of 596 ±194x10^6 cells in 26 ±7 injections (range 12-35; in 18.8 ±6.8 cc (range 9-30 cc)).

In one patient, the implantation procedure was complicated by a dissection of the left main coronary artery, the ascending and descending aorta, upon dislocation of the MyoCath™ injection catheter during intramyocardial injection. The transplant procedure was ceased after seven injections, with the coronary dissection successfully treated by direct stent implantation, resulting in recovery of coronary flow and stabilization of the patient. The aortic dissection was treated conservatively, and the patient recovered without persistent myocardial or neurological sequelae (CK_{max} < 300). This patient was excluded from the study resulting in 26 patients in total in the treatment arm.

All MACCE

An overview of the adjudicated SAEs is summarized in table 2. No unanticipated adverse events attributable to the SkM therapy were reported, whereas no difference in the incidence of SAEs was observed in the cell therapy and control arm. A total of 21 SAEs occurred in 11 out of 26 patients subjected to SkM transplantation (42%), as opposed to 23 SAEs occurring in 5 out of the 14 control patients (36%; P=0.68). Specifically, 20 cardiovascular SAEs occurred in 11 patients treated with SkM (11/26;42%), whereas 18 cardiovascular events occurred in 4 patients in the control arm (4/14;29%; P=0.39).

One patient in the myoblast therapy group died during the study period (3.8%), whereas no deaths occurred in the control patient group during study follow-up. This particular patient had a previous medical history of ischemic cardiomyopathy, impaired renal and hepatic function, ventricular arrhythmias and ICD implantation four years prior to enrollment. The MyoCell implantation of 625 million cells was uneventfull. From day 5 to 10 post implantation, the patient experienced several episodes of monomorphic, sustained VT, which were successfully terminated via anti-tachycardia pacing. In

Table 2. Serious Adverse Events & Adverse Events at 6 months.

Serious Adverse Event	MyoCell® (n=26)	Episodes	Control (n=14)	Episodes	p-value[1]
Cardiovascular					
Bradycardia	3.8 % (1/26)	1	14.3 % (2/14)	2	0.27
Ventricular Fibrillation	3.8 % (1/26)	1	0.0 % (0/14)	0	1.0
Ventricular Tachycardia	23.1 % (6/26)	11	21.4 % (3/14)	12	1.0
Idioventricular rhythm	0.0 % (0/26)	0	14.3 % (2/14)	2	0.12
Non-sustained VT with hospitalization	3.8 % (1/26)	1	0.0 % (0/14)	0	1.0
Worsening of CHF	3.8 % (1/26)	1	7.1 % (1/14)	1	1.0
Hypotension	0.0 % (0/26)	0	7.1 % (1/14)	1	0.35
Pulmonary Edema	3.8 % (1/26)	1	0.0 % (0/14)	0	1.0
Aorta dissection	3.8 % (1/26)	1	0.0 % (0/14)	0	1.0
Pericarditis	3.8 % (1/26)	1	0.0 % (0/14)	0	1.0
Gastrointestinal					
Diverticulitis	0.0 % (0/26)	0	7.1 % (1/14)	1	0.35
Gastroenteritis	3.8 % (1/26)	1	0.0 % (0/14)	0	
Renal dysfunction	0.0 % (0/26)	0	14.3 % (2/14)	2	0.12
Pulmonary					
Exacerbation COPD	3.8 % (1/26)	1	0.0 % (0/14)	0	1.0
Infection					
Urinary tract	0.0 % (0/26)	0	7.1 % (1/14)	1	0.35
MRSA	0.0 % (0/26)	0	7.1 % (1/14)	1	0.35
Death	3.8 % (1/26)	1	0.0 % (0/14)	0	1.0
Total number of SAEs		21		23	
Number of subjects	42.0 % (11/26)		36.0 % (5/14)		0.95
Subjects with cell therapy-related SAEs[2]	30.8% (8/26)				
Unanticipated cell therapy-related SAEs[3]	0.0% (0/26)				

Subjects reporting a particular event more than once are only counted once using the most severe intensity for that event

'*' denotes statistical significance at the 0.05 level.

[1] P-value is from a Fisher's Exact test.

[2] Cell-Therapy-Related events are those events where relationship is possible/probable/definite to MyoCell, Related to MyoCath Device or Related to Implant Procedure.

[3] Unanticipated events have been identified through a medical review of all serious adverse event information by a medically qualified individual.

addition to beta-blockers and Amiodarone, flecainide was initiated, rendering the patient bradycardic and ICD/pacemaker dependent. At 27 days, the patient deteriorated with the clinical manifestation of progressive HF, which progressed into multi organ failure and death at 30 days post cell transplantation. The local investigator and two independent cardiologists considered the cause of death to be progression of terminal HF, with secondary multiple organ failure, possibly provoked by flecainide with acute renal failure. Alternatively, multiple episodes of VT not manageable with anti-arrhythmic therapy may have initiated the progressive deterioration of cardiac and renal function. Upon autopsy, no evidence of thrombus formation or other signs of inflammation could be detected at the site of intracardiac cell injection by macroscopic or microscopic analysis. In the examined samples, the pathologist did not find clusters of injected SkMs or newly formed myotubes. A relation of this SAE with the MyoCell implantation, however, cannot be excluded.

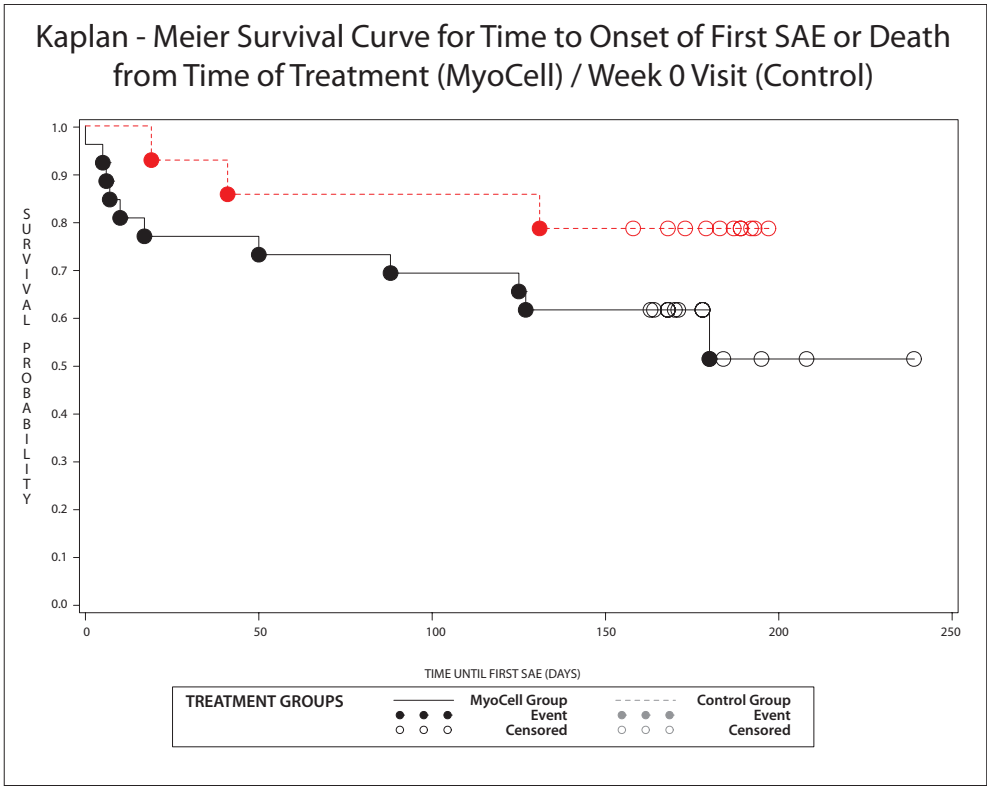

Figure 2. Kaplan Meyer curve of time to first serious adverse event (SAE).

Ventricular Arrhythmias

All patients in the myoblast and control arm received oral Amiodarone therapy at the time of cell transplantation. In the control patient group, 3 patients experienced one or more episodes of sustained VT (3/14 pts; 21%), as opposed to 7 patients in the myoblast treatment group (6/26; 23%). Further, the 14 control patients experienced a total 12 periods of sustained VT, as opposed to 11 events in the 26 cell therapy patients. One of these patients in the treatment group experienced 2 episodes of sustained VT that required ablation of the ventricular focus localized at the myocardial scar area where SkM were injected.

Previously, ventricular tachyarrhythmias were mainly reported in the first 4 weeks following myoblast transfer. However, in the control and the SkM group at hand, the number of patients and the frequency of arrhythmic events were equally distributed among the 6 month observation period and did not appear to correlate with the dose of SkMs administered (data not shown). Thus, there was neither a time, nor a dose relationship between tachyarrhythmic events and the index procedure, as compared to the untreated control group.

Progression of congestive heart failure

Other cardiovascular SAEs included the worsening of congestive HF of subjects requiring hospitalization during the six month observation period. This occurred on 3 occasions in the 26 patients of the SkM therapy group (3/26 patients; 11.5%) as compared to one admission in the matched control group (7.1%). A Kaplan-Meier survival curve for the time to onset of the first SAE or death is shown in figure 2.

Global ventricular ejection fraction by MUGA-scan

The average baseline LVEF in the myoblast treatment arm was 32.3 ±9.1% (n=24) as compared to 32.6 ±11.1% in the control arm (n=14; figure 3). One patient in the cell therapy group died prior to the 3 month MUGA evaluation and was omitted from the analysis. Sequential data were obtained in 24 of the 26 patients in the cell therapy arm and in all 14 patients in the control arm. At six months follow-up, global LVEF was 32.5 ±8.0% in the control patient group, and 31.5 ±11.1% in the SkM-treated group (figure 3; P=0.46).

NYHA Heart Failure Classification and Minnesota Living With Heart Failure Questionnaire

During the 6-month study follow-up, the NYHA HF classification deteriorated in 28.6% in the control arm (4/14), whereas 57.1% remained stable. In the active cell therapy arm, only 8% showed a deterioration of NYHA classification, whereas the majority of patients even improved in 20.0% or remained

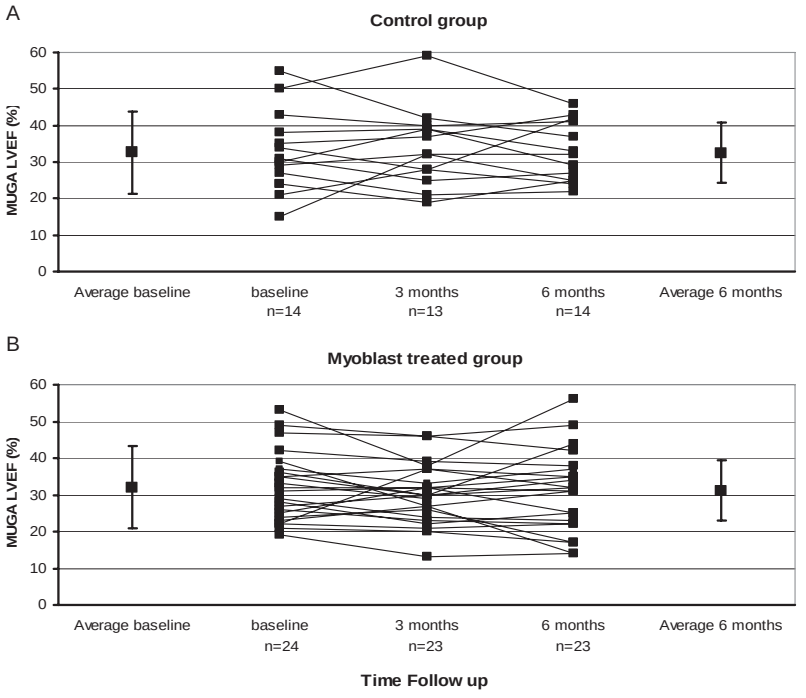

Figure 3. Individual six month change in ejection fraction from baseline to six month follow up in the control group (A) and the skeletal myoblast treated group (B) as measured by MUGA scan. The mean data and standard deviation are denoted separately. No significant change in LVEF was found between the myoblast treated group and control.

unchanged (72.0%) as compared to baseline (figure 4A; P=0.06). Thus, a trend towards improvement of NYHA classification of heart failure was noted in the SkM-treated patients.

The number of patients that experienced improvement of MLWHFQ over the 6 months follow-up increased from 33.3% to 52.2% with cell therapy, whereas the patients who did not notice a change in their quality of life was reduced from 41.7% to 8.7%. However, the mean MLWHFQ score was not significantly different between both arms (figure 4B; P=0.99).

6-Minute Walking Test

At baseline, the average distance walked in the six-minute walking (6MW) test for patients randomized to the cell therapy arm was lower than in the control arm (404.6±94.8 meters (n=23) vs. 442.5±154.3 meters (n=13)), although this difference was not statistically significant.

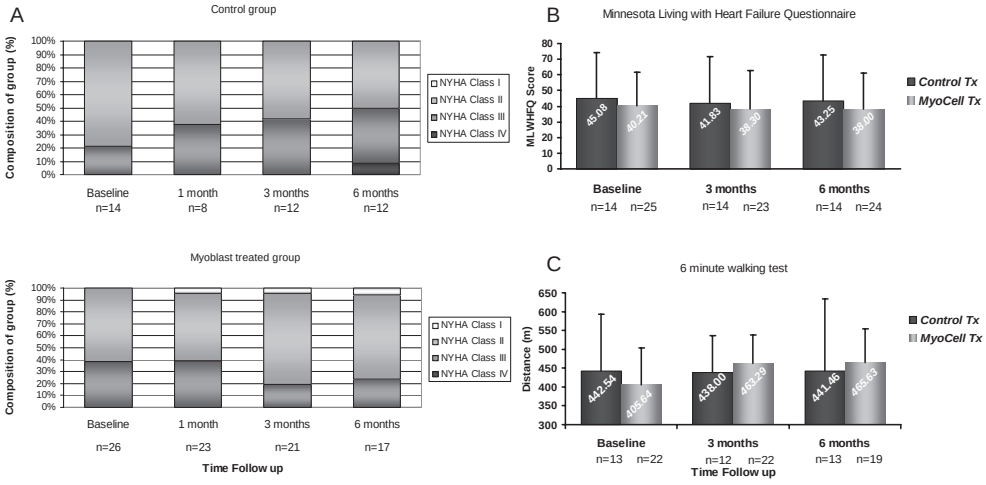

Figure 4. Six month change from baseline in NYHA Class (A), Minnesota Living With Heart Failure Questionnaire (B) and exercise tolerance (C). NYHA Class deteriorated in 28.6% of the cases in the control arm, whereas 57.1% remained unchanged. In the active cell therapy arm NYHA classification either improved or remained unchanged (20.0% and 72.0% respectively, P=0.06). Also in the Minnesota Living With Heart Failure Questionnaire a trend to improvement was seen in treated patients (grey) vs. control patients (black), but this difference was not statistically significant (p=0.99). Exercise tolerance was measured by six minute walking test in treated (black) and control (grey) patients. Although a trend to an increased exercise tolerance was seen, the difference between the treated and control group was not significant (p=0.49).

The distance walked in the control group remained virtually unchanged at 6 month FU (+0.4±185 meters). In contrast, the distance walked in the treated group improved by +60.3 meters to 465.6±89.5 meters (figure 4C: P=0.49). The walking distance improved or remained unchanged in 87% of the myoblast-treated patients, as opposed to 30.8% in the control arm. In contrast, exercise tolerance deteriorated in 69.2% in the control arm as opposed to 13.0% in the cell therapy arm.

Discussion

The rationale for the investigation of cell therapy for HF is based on the assumption that development of HF is linked to irreversible loss of contractile myocardium to below a certain threshold and that myocardial function can be improved by replacing lost muscle with cells that graft to, and function as native myocardium. In experimental models, SkMs have been transplanted into infarcted cardiac muscle and non-ischemic, globally dilated myocardium, and have been shown to subsequently develop into elongated, striated muscle cells which retain characteristics of both skeletal muscle and cardiac cells.(6-9) In these models, intramyocardial myoblast engraftment has been associated with a dose-dependent improvement in LV function. Additionally, SkMs are able to release trophic growth factors that exert an anti-fibrotic effect and enhance cardiac performance and myocardial perfusion through the stimulation of local angiogenesis.(2,10)

The safety concerns regarding endomyocardial SkM therapy predominantly relate to a possible pro-arrhythmogenic effect of the intramyocardial injection technique and the SkM graft. Irrespective of whether cells were transferred as an adjunct to revascularization surgery, or by percutaneous endomyocardial injection, SkM therapy was previously associated in a small number of patients with recurrent premature ventricular systoles and (non)sustained ventricular arrhythmias. This was seen particularly during the first 30 days after transplantation.(3)

In the SEISMIC trial, no significant difference was detected between treatment groups, frequency of ventricular arrhythmic events or the percentage of patients with documented ventricular tachyarrhythmia via ICD interrogation (Table 2). SkM transplantation was neither associated with an increased incidence of ventricular tachyarrhythmias, nor in the absolute number of episodes in the treatment group. In the control patient group, 21% of the patients experienced one or more episodes of serious ventricular tachyarrhythmias (a total of 12 episodes of VT/VF), as opposed to 27% in the myoblast treatment group (total of 12 episodes of VT/VF).

Ablation of a ventriuclar focus was indicated in one patient due to refractory tachyarrhtymias at 10 and 16 weeks following SkM transplantation. Although a causal relationship between these tachyarrhytmic events and the SkM transplantation can not be excluded, the time interval between the cell injection procedure and the onset of VTs renders a relationship less probable. The SkMs were injected in scar tissue that by itself could give rise to ventricular tachyarrhythmias. In another patient, tachyarrhythmias at 5 days following SkM therapy may have provoked deterioration and progression into terminal HF. Even though this particular patient was known with multiple episodes of VT before cell injection and an ICD was implanted four years earlier, causality between the intra myocardial injections and the VTs can not be ruled out.

Although the current study did not suggest a time and dose correlation between tachyarrhythmic events and the index procedure, a pro-arrhythmic effect associated with the myoblast transplantation procedure in these two patients cannot be excluded. However, the current data provide a basis to further explore the therapeutic value of SkM transplantation in larger randomized and placebo-controlled trials with prophylactic Amiodarone and ICD implantation.

In various pre-clinical animal models, SkM therapy has been shown to reduce myocardial remodeling, impede ECM deposition and cardiomyocyte apoptosis and replenish lost cardiomyocytes in the chronic scar by introducing new contractile myocytes into the region of dysfunction.(2,6,11) Immunohistological and patch clamp studies of myoblasts have shown effective repopulation of the fibrotic tissue and transition from a 'fast' to a 'slow twitch' phenotype upon cardiac transplantation eventually leading to restoration of contractile function. Skeletal myoblast therapy is to date the only regenerative cell therapy approach primarily aimed at neomyogenesis, rather than neoangiogenesis in the myocardial scar, in post AMI heart failure patients.

In the MAGIC trial, intramyocardial SkM transplantation as an adjunctive to CABG was studied in patients with ischemic HF with an ejection fraction under 35%.(12) Although SkM transfer failed to aug-

ment the restoration of regional and global LV function as determined by echocardiography in these patients undergoing surgical revascularization, an improvement in LV dimensions was observed in the high dose cell group. In contrast, in the open-label, dose-escalating, randomized MYOHEART and CAUSMIC trials, trends towards beneficial clinical and functional effects of myoblast therapy were reported in patients with ischemic heart failure following endoventricular injection in respectively 20 and 12 patients (13).

In the current study, SkM therapy appeared to attenuate further deterioration of NYHA classification of HF, with an improvement in exercise tolerance in a 6MWT, as opposed to a lack of improvement in the control arm. However, this could not be substantiated by an improvement in global LVEF, quantified by nuclear ventriculography.

The lack of a clear effect on global LVEF can be partly explained by our previous studies in preclinical and clinical myoblast transplantation, which suggested that on short term, akinesia in scarred or hibernating myocardium might be compensated by hyper-contractility in remote myocardial segments to preserve global LV function. A possible beneficial effect of stem cell therapy leading to improved local wall motion in the afflicted wall segment may be concealed by normalization of the compensatory hyper contractility in remote wall segments, resulting in a modest overall effect on global cardiac function. Moreover, the long term follow-up of patients with LV dysfunction by conventional echocardiographic or scintigraphic LVEF quantification is based on the presumption of a comparable hemodynamic state of patients at baseline and follow-up and cannot be corrected for change in LV loading. As myocardial cell therapy aims to improve the contractile function and hemodynamic state, comparing LVEF with the assumption of static LV loading may underestimate thebeneficial effect on LV function. Invasive hemodynamic analysis of systolic and diastolic LV function by the analysis of pressure volume-loop relations under dynamic LV loading (LV unloading by use of gradual preload reduction), can however provide load-independent indices of global and intrinsic systolic and diastolic function. Indeed, in a long term follow-up of patients that received endoventricular myoblast injection, pressure-volume relation analysis provided proof of improved systolic and diastolic function at 6 and 12-month follow-up, whereas nuclear radiography and magnetic resonance imaging failed to reflect this improvement.

Although SEISMIC suggests that the use of SkM therapy is safe, the mixed results on functional efficacy end points, coupled with the general underpowering of the study, render any interpretation inconclusive. Further, since the current trial was an open-label study, a potential effect of patient bias on the positive effects on clinical end points cannot be ruled out, and underscores the necessity to conduct appropriately powered clinical studies to evaluate cell therapy, blinded to both clinical staff and patients, with outsourcing of study end point analyses to independent core laboratories. Hence, the recently initiated MARVEL study seeks to explore the therapeutic efficacy of NOGA guided endomyocardial myoblast therapy in chronic LV dysfunction in a prospective, randomized, double-blinded, placebo-controlled study enrolling 390 patients in up to 35 US and EU clinical centers.

Conclusion

The current study sought to define the safety and feasibility of percutaneous intramyocardial transplantation of autologous SkM in 40 patients with chronic HF with an ischemic origin. One could argue whether this would be the most appropriate target population to provide proof of principle of a neomyogenesis therapy concept, since end-stage heart failure patients may lack the plasticity to respond to reverse remodeling by stem cell therapy. The current study failed to detect a benefit on global LV function, possibly because it was outweighed by the advanced LV dysfunction in end-stage HF or fell beyond the detection limits of radionuclide ventriculography. In addition, the small sample size in a heterogeneous population could have further concealed a possible beneficial effect on global LVEF. Since the studies in the chronic HF stem cell therapy field are still at a very preliminary stage, they are exploratory in nature, and thus by virtue, underpowered. Therefore a true evaluation of efficacy and safety cannot be guaranteed by the study at hand, and should be validated in forthcoming phase II/III studies. However, the current study does provide encouraging feasibility data regarding the endomyocardial stem cell treatment, and suggests some clinical benefit of SkM therapy in HF patients.

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PART III

Second generation stem cells in acute myocardial infarction patients

Adipose tissue-derived regenerative cells

Chapter 6

First-in-Man Experience using Adipose Tissue-Derived Regenerative Cells in the Treatment of Patients with ST-Elevation Myocardial Infarction:

The APOLLO Trial

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In pre-clinical animal models of AMI, administration of freshly isolated adipose tissue-derived regenerative cells (ADRCs) directly following the AMI improved LV function and myocardial perfusion (1,2). The predominant working of ADRC therapy in AMI is believed to be through paracrine release of anti-apoptotic, immunomodulatory and pro-angiogenic factors. These factors evoke cardiomyocyte salvage and stimulate neo-angiogenesis in the infarct border zone, and eventually result in reduced infarct scar formation and remodeling (1-3). ADRCs comprise among others immune-competent cells, endothelial progenitor cells, and mesenchymal stem cells (MSCs). The amount of these MSCs in freshly isolated adipose tissue digestates is ~2,500 fold greater than that of freshly aspirated BM, which makes cell culture unnecessary to generate therapeutically sufficient cells (4). On average, 20-40 million cells can be isolated and prepared within two hours after a liposuction from as little as 200 grams of lipo-aspirate, enabling the treatment of AMI patients within hours following the PPCI.

Table 1. Demographics and Baseline Characteristics.*

	Placebo (n=4)	ADRC (n=9)
Age (years)	55 ± 7.5	61 ± 2.1
Male (%)	100	78
Caucasian (%)	100	100
Body Mass Index	27.6 ± 3.3	27.5 ± 3.0
Smoking (%)	50.0	66.7
Hypertension (%)	50.0	66.7
Creatine Kinase MB (µmol/L)	92.0 ± 5.7	78.0 ± 3.9
NT-proBNP† (pmol/l)	225 ± 116	250 ± 86
Left Ventricular Ejection Fraction (%)		
2D Echocardiography (at screening)	43.5 ± 3.3	46.1 ± 2.5
Cardiac Magnetic Resonance Imaging	47.9 ± 5.4	52.4 ± 4.8
MIBI-SPECT‡	52.0 ± 10.0	52.1 ± 2.5
Infarct Size (% of Left Ventricle)	24.7 ± 9.2	31.6 ± 5.3
Perfusion Defect (%, VRS§)	15.0 ± 4.9	16.9 ± 2.1

*Only includes per treatment evaluable (PTE) patients
†NT-proBNP: n-type brain-natriuric peptide; ‡MIBI-SPECT : sestamibi single photon-emission computed tomography; §VRS: visual rest score

The APOLLO trial was a randomized, double-blind, placebo-controlled, phase I/IIa study, designed to assess the safety and feasibility of intracoronary infusion of ADRCs in the treatment of patients in the acute phase of a large ST-elevation AMI. Patients were eligible for enrolment after successful interventional treatment for their first AMI, had no history of heart disease, and the area of LV hypo- or akinesia had to correspond to the culprit lesion. The residual LVEF needed to be between 30% and 50% as measured by TTE after the PPCI. Following informed consent, the patients underwent a liposuction procedure of the peri-umbilical region, after which ADRCs were isolated using a Cytori Celution® device. Within 24 hours following the PPCI, the ADRCs were infused intracoronarily, while carefully monitoring for coronary flow related side-effects. The main safety end-points were defined as the change in coronary flow pre- versus post-infusion, occurrence of MACCE or other SAE, or hospitalization due

to congestive heart failure during the six-month follow-up. Feasibility end-points were defined as the change in LVEF, infarct size as determined by DE-CMR, and perfusion defect as assessed by MIBI-SPECT (visual rest score; VRS).

A total of 14 patients presenting with an anterior wall AMI were enrolled in the trial, and 3:1 randomized to receive intracoronary infusion of either 20 million ADRCs (n=10) or placebo solution (n=4) in the culprit artery (see table 1 for baseline demographics). One patient in the treatment group was omitted from analysis due to the inadvertent use of an inappropriate cell strainer, resulting in nine analyzable patients. The liposuction procedure was well tolerated in all patients, although in two patients a significant bleeding event occurred. Following these two bleeding events, a protocol amendment that regulated stricter control of heparin use following the PPCI and excluding the use of glycoprotein IIb/IIIa inhibitors, resulted in no more serious bleeds in the next ten patients. Intracoronary infusion of on average 17.4 ± 4.1 million ADRCs was successful and well tolerated in all patients, and did not result in any coronary flow impediment as measured by coronary angiography and coronary flow reserve. One patient in the treatment group experienced a MACCE event (target lesion revascularization) as opposed to none in the control group (p= ns). SAE occurred in 2 out of 4 patients (50%) in the placebo group and 3 out of 9 (33%) patients in the ADRC group (p= ns). Importantly, no unanticipated adverse effects related to the ADRC therapy were reported.

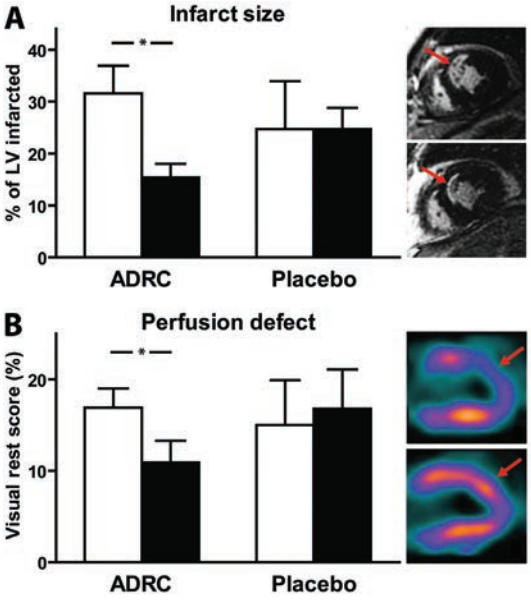

Figure 1. Infarct size and perfusion defect in APOLLO patients.
1A. The percentage of LV infarcted improved significantly in ADRC-treated patients (left panel, red arrows) from baseline (grey bar) to the six-month follow-up time-point (black bar), with no improvement in placebo patients (right).
1B. A significant improvement of the perfusion defect was seen in ADRC-treated patients (left panel, red arrows) from baseline (grey bar) to the six-month follow-up time-point (black bar), compared to a deterioration in placebo patients (right panel).

MIBI-SPECT analysis demonstrated a +4% improvement in global LVEF in ADRC-treated patients from 52.1% to 56.1%, whereas the placebo group deteriorated by -1.7% (52.0% to 50.3%), rendering an absolute difference between the treatment groups of +5.7% (p= 0.114). A similar positive trend of improved cardiac function was found by CMR analysis, which demonstrated a +4.6% improvement of

global LVEF in the ADRC-treated group from baseline to six-month follow-up (p=0.091). The percentage of LV infarcted was reduced by -52% (31.6 ± 5.3 % to 15.3 ± 2.6% at six-month follow-up, p=0.002; figure 1A) in the ADRC-treated patients, as opposed to no change in the placebo-treated AMI patients (24.7 ± 9.2 % vs. 24.7 ± 4.1%; p= 0.48 for difference between groups). Also, in the placebo group, the perfusion defect deteriorated by +1.8% (15.0 ± 4.9% to 16.8 ± 4.3%), as compared to a significant improvement of the perfusion defect in ADRC-treated patients from 16.9 ± 2.1% to 10.9 ± 2.4% at six-month follow-up (change of -6.0%, p=0.004; figure 1B; p= 0.23 for difference between groups).

The main findings of the APOLLO trial at the six-months clinical and angiographic follow-up timepoint are: 1) a liposuction to harvest ADRCs in the acute phase of an AMI is safe and feasible; 2) intracoronary infusion of freshly isolated ADRCs was safe and did not result in an alteration of coronary flow or any indication of micro-vascular obstruction; 3) no serious adverse events were related to the ADRC therapy and 4) ADRC infusion resulted in a trend to improved cardiac function, accompanied by a significant improvement of the perfusion defect and a 50% reduction of myocardial scar formation. The latter is consistent with findings in pre-clinical studies and concordant with the presumed pro-angiogenic, anti-apoptotic, and immunomodulatory working mechanism of ADRC therapy.

The forthcoming ADVANCE study is a multi-center, prospective, 4:1 randomized, placebo-controlled phase IIb/III trial in up to 375 patients, which will assess the safety and efficacy of intracoronary infusion of ADRCs in patients with an ST-elevation AMI. This study was initiated in Q2 of 2011.

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Chapter 7

Long-term patient follow up after intracoronary infusion of adipose tissue-derived regenerative cells in patients with ST-segment elevation myocardial infarction:

final results of the APOLLO trial

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Abstract

Objectives APOLLO is a first-in-human, randomized, placebo-controlled trial to assess safety and feasibility of intracoronary infusion of adipose tissue-derived regenerative cells (ADRCs) after percutaneous coronary intervention (PCI) for acute myocardial infarction (AMI).

Background ADRC treatment following AMI showed favorable safety results and hints of efficacy at 6 months follow-up in a previous concise report. This is the first complete report of the APOLLO trial, and extends functional findings to 18 months, and clinical follow-up to 36 months.

Methods A total of 14 patients were randomized to receive intracoronary infusion of ADRCs (n=10) or placebo (n=4) within 24 hours following the primary PCI. The patients were monitored for procedural and postoperative safety, and the occurrence of adverse events with follow-up to 36 months. Functional analysis was performed by echocardiography, cardiac MRI, and nuclear imaging at baseline, 6 and 18 months.

Results Liposuction, but also intracoronary infusion of ADRC, was well tolerated in all patients. Through 36 months of follow-up, 2 ADRC and 1 placebo patient had experienced major adverse cardiac events requiring target lesion revascularization. No adverse events were associated with ADRC therapy. At 6 and 18 months, intracoronary ADRC infusion, but not placebo infusion, significantly improved perfusion defect, and also coronary flow reserve, while significantly decreasing the percentage of left ventricle infarcted.

Conclusions ADRC infusion proved to be safe during 36 months of clinical follow-up. Moreover, ADRC infusion was associated with improved perfusion and decreased infarct size through 18-month functional follow-up.

Introduction

Although regenerative cell therapy holds great promise for the adjunctive treatment of acute myocardial infarction (AMI) patients, the ideal progenitor cell still needs to be defined. Autologous adipose tissue-derived regenerative cells (ADRCs) can be readily obtained from subcutaneous adipose tissue in amounts that are sufficient for therapy.[1-3] ADRCs are similar to bone marrow-derived mesenchymal stem cells (MSCs) in their potential for differentiation and secretion of relevant growth factors and cytokines.[4-6] In contrast with the challenging and lengthy process required for deriving MSCs from bone marrow, the abundance of adipose tissue in patients and the higher frequency of regenerative cells per unit mass in that tissue can allow for isolation of a relevant number of cells in fewer than 2 hours from the time of donor-tissue acquisition. These factors of processing time and quantity with ADRCs eliminate the need for extensive *ex vivo* cell culturing and allow for treatment of AMI patients within hours after the primary PCI.

In vitro studies have shown that ADRCs secrete significant amounts of pro-angiogenic, anti-apoptotic and immunomodulatory factors, and that they can differentiate into spontaneously beating cells with cardiomyocyte features.[7-11] Also, several studies have demonstrated therapeutic efficacy of ADRCs in animal models of experimentally induced AMI and of chronic myocardial injury.[12-16]

The goal of the first-in-human "AdiPOse-derived stem ceLLs in the treatment of patients with ST-elevation myOcardial infarction" (APOLLO) trial was to extend the findings in pre-clinical investigations into the clinic and determine the long-term safety and feasibility of intracoronary infusion of ADRCs in patients within hours after successful PCI and stenting for acute ST-segment elevation myocardial infarction (STEMI). The earlier published concise report on the six-month follow up of the APOLLO trial showed a favorable safety profile and, among others, decreased infarct size and increased myocardial perfusion in ADRC-treated patients.[17] Here, we fully describe the trial, and extend the clinical follow up to 36 months. Also, several indices of cardiac function, remodeling, infarct size and myocardial perfusion have been included, thereby adding important, yet exploratory, parameters of efficacy.

Methods

Study population and design

The APOLLO trial is a prospective, double-blind, randomized, placebo-controlled, phase I/IIa, first-in-man trial of the safety and feasibility of ADRC therapy via intracoronary infusion in the treatment of acute STEMI patients, successfully treated by PCI and stenting. The trial was conducted between November 2007 and May 2009. The trial was approved by the institutional and national review boards at the participating sites, and was conducted in accordance with the Declaration of Helsinki and ICH E6 Good Clinical Practice Guidelines. Written informed consent was obtained from all patients before trial enrollment.

Patients were eligible for enrollment in the trial, if they were 20 to 80 years of age and had been successfully treated with standard care (PCI and drug-eluting stent placement) within 2 to 12 hours of the acute onset of STEMI symptoms. All other inclusion and exclusion criteria are detailed in Table 1.

Table 1. Selected inclusion and exclusion criteria in the APOLLO study

Inclusion criteria
Clinical symptoms consistent with acute myocardial infarction (AMI) (pain, etc.) for a minimum of 2 and a maximum of 12 hours from onset of symptoms to percutaneous coronary intervention (PCI), and unresponsive to nitroglycerine
Successful revascularization of the culprit lesion in the major epicardial vessel within 2 to 12 hours of the onset of AMI symptoms
Area of hypo- or akinesia corresponding to the culprit lesion, as determined by left ventriculogram at the time of primary PCI
Left ventricular ejection fraction (LVEF) ≥30% and ≤50% at the time of successful revascularization.
Ability to undergo liposuction
Exclusion criteria
Prior MI, prior known cardiomyopathy, or prior hospital admission for congestive heart failure (CHF)
Significant valvular disease, need for mechanical intervention, or cardiogenic shock
Staged treatment of coronary artery disease, or other interventional or surgical procedures to treat heart disease (eg, valve replacement, PCI, or CABG) planned or scheduled within 6 months after the study procedure
Hemodynamic instability within 24 hours prior to randomization, defined as the presence of any of the following: · Systolic blood pressure <90 mmHg · Heart rate >100 bpm for more than 1 hour · Prior ventricular fibrillation or sustained ventricular tachycardia
Patients with increased bleeding risk including but not limited to: (a) those who have received any glycoprotein inhibitor within 7 days preceding the liposuction; or (b) those who have received any anticoagulant within 1 hour of liposuction or who have an aPTT result of ≥1.8 times the control value
Persistent atrial fibrillation
Neoplasia
Pacemaker, ICD, or any other contraindication for MRI
LVEF <30% or >50%
Moderate or severe COPD

Pre-enrollment screening of the post-PCI patients included, among others, 2D-TTE to confirm that post-AMI LVEF was between 30 and 50%, after which informed consent was obtained. Following enrollment, patients underwent a standard liposuction procedure to harvest approximately 200cc of lipoaspirate as described below. At this point, the patients were randomized 3:1 (by interactive voice-response system) to receive an injection of either autologous ADRC or placebo solution (lactated Ringer's solution with autologous peripheral blood making it indistinguishable from the study substance) via intracoronary infusion into the stented culprit coronary artery within 24 hours of the PCI.

In the immediate postoperative period, patients were monitored for arrhythmias using telemetry in the first 72 hours and Holter monitoring at week 1 through 3, and month 1 through 4, 6, 12, 18, 24, and 36. Per protocol, 2D-TTE, cardiac magnetic resonance imaging (CMR), and gated cardiac single photon emission computed tomography (SPECT) were scheduled 2 to 4 days post procedure and at 6 and 18 months. Coronary angiography and Doppler coronary flow measurements were performed before ADRC infusion, directly following ADRC infusion and at 6 month follow up. The imaging studies and Holter recordings were analyzed by blinded core laboratories: Medstar Research Institute, Washington, DC, USA, for 2D echocardiography; Cardiovascular Core Laboratories, Boston, MA, USA, for CMR; Tufts New England Medical Center, Boston, MA, USA, for SPECT; and Agility Centralized Research Services, Bannockburn, IL, USA, for Holter monitoring.

Cell collection, preparation, and infusion

Adipose tissue was harvested by syringe-based lipoaspiration in a standard protocol-detailed procedure.[18] ADRCs were isolated from the lipoaspirate by use of the Celution® system (Cytori Therapeutics Inc., San Diego, CA), as previously described.[19] The Celution® system enzymatically digests the adipose tissue into a single cell suspension and uses differences in buoyancy to separate ADRCs from fat cells. The ADRCs are then further enriched and concentrated in suspension via series of washing and centrifugation steps within a procedure of approximately 120 minutes. Following randomization and blinding by the hospital pharmacist, the ADRC or placebo suspension was subsequently infused using a micro catheter (Twin Pass, Vascular Solutions, USA) placed in the culprit vessel at an infusion rate of 2 mL/min (~2.5 million ADRCs/min).

In order to ensure patient safety in this first-in-man application of ADRCs into coronary arteries, the current trial was originally conceived with a dose-escalation design, progressively employing doses of 20×10^6, 40×10^6, 60×10^6, and 80×10^6 ADRCs. The initial dose in the trial, up to 20×10^6, is a quarter of the maximum dose that was deemed safe and efficacious in preclinical porcine AMI studies.[14] When proof of concept was established with 20 million cells, and trial enrollment reached 14 patients, the trial steering committee and data safety monitoring board recommended that the original plan of dose escalation be discontinued in favor of a larger prospective, phase 2A/2B trial.

Endpoints

Standard safety assessments were performed at 1, 3, 6, 12, 18, 24, and 36 months. Safety endpoints included: the rate of major adverse cardiac and cerebrovascular events (MACCE; defined as the incidence of cardiac death, re-AMI, target site revascularization and/or stroke), the rate of serious adverse events (SAEs) and adverse events (AEs); and the occurrence of arrhythmic events as documented by the scheduled Holter recordings. Additional safety follow-up assessments included coronary flow reserve in the treated vessel before and after cell infusion, and differences and change from baseline in pro-brain natriuretic peptide type B levels (pro-BNP).[20] An independent, international Data Safety

Monitoring Board (DSMB) and critical event committee (CEC) reviewed and adjudicated all MACCE and SAE events.

Efficacy endpoints included: measurement of the LV volumes and LVEF and of the change in LV volumes and LVEF from baseline, to 2 to 4 days post treatment, and 6 and 18 month follow up (2D-TTE), and from 2 to 4 days post treatment to 6 and 18 month follow up (CMR); determination of the change in percentage of LV infarcted and infarct mass (as measured by delayed-enhancement CMR (DE-CMR)); and determination of the change in perfusion defect from 2 to 4 days post treatment to 6 and 18 month follow up (SPECT).

Perfusion defects were expressed as the Visual Rest Score (VRS) and Total Severity Score (TSS), and are determined by the uptake of sestamibi into non-ischemic myocardium using a semi-quantitative, 17-segment scoring system in three short-axis slices. The VRS is defined as the weighted mean of the reader's visual perfusion scores over the target segments, whereas TSS represents an integration of the extent and the severity of the perfusion abnormality at rest by quantitative analysis. The latter represents the extent and severity of infarct or hibernating viable myocardium at rest.

Statistical analysis

APOLLO was primarily a safety and feasibility study, whereas first signs of possible efficacy were explored. All statistical tests were therefore considered exploratory, in the absence of prespecified hypotheses. All statistical tests are two-sided, and statistical significance is assessed with respect to a nominal p value ≤0.05. No prior sample size calculations were performed. The sample size was based on clinical judgment, with the goal of obtaining meaningful safety and feasibility information, while minimizing unnecessary patient exposure. Continuous variables are summarized by means and standard deviations. Categorical variables are summarized by counts and percentages of patients in the respective categories. All analyses and tabulations were performed using SAS software, version 8.2 or higher (SAS Institute Inc., Cary, NC, USA).

Results

Patient population and baseline revascularization procedure

Between November 2007 and May 2009, 14 patients (12 men, 2 women, all Caucasian) were enrolled at the 2 study sites (Figure 1). Of the patients enrolled, 10 patients (61.0 ± 6.4 years [49 to 72 years]) were randomized to treatment with ADRCs; and 4 patients (55.0 ± 14.9 years [42 to 72 years]) were randomized to treatment with placebo solution. Table 2 summarizes the demographic and angiographic characteristics of both groups at baseline. All patients were admitted with an acute anterior myocardial infarction based on a proximal occlusion of the LAD.

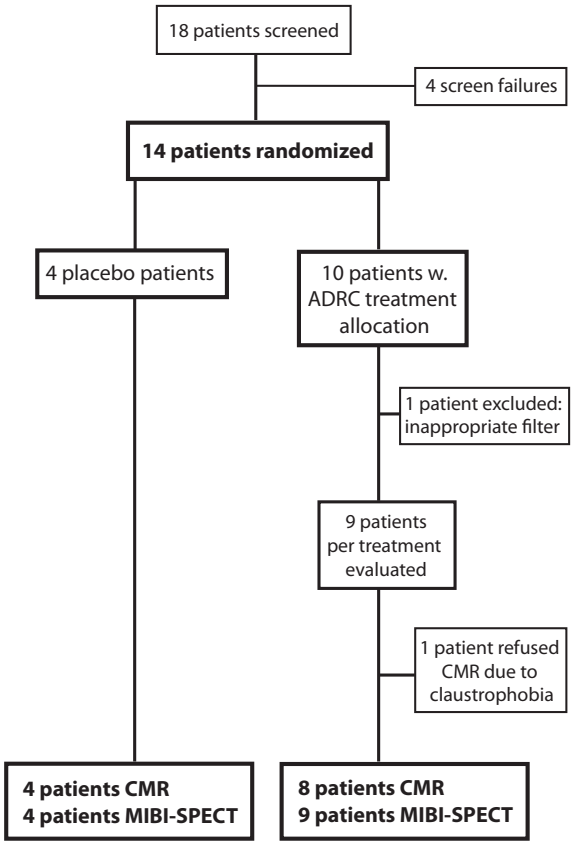

Figure 1. Flow chart of the APOLLO trial. ADRC: adipose tissue-derived regenerative cells; CMR: cardiac magnetic resonance imaging; MIBI-SPECT: sestamibi single photon-emission computed tomography

Before enrollment and randomization, all patients were successfully revascularized. One patient presented with an acute myocardial infarction based on an in-stent thrombosis of a previously treated mid-LAD lesion 10 years before, and was treated by balloon angioplasty only. After enrollment, this patient was randomized to placebo and was included in safety, feasibility and efficacy analyses, although the balloon angioplasty of the previously implanted stent technically represented an inclusion criterion violation.

Adipose tissue harvest and intracoronary injection results

The syringe-based lipoaspiration procedure was well tolerated in all patients, although the first two enrolled patients experienced significant subcutaneous bleeding in the peri-umbilical area following lipoaspiration. After these 2 bleeding events, protocol amendments were made to exclude the use of glycoprotein IIb/IIIa inhibitors within 7 days preceding liposuction and to strengthen strict control of anticoagulation by heparin after the primary PCI, permitting lipoaspiration only in patients with an aPTT-ratio ≤1.8. After these protocol amendments, there were no more serious bleeding events re-

Table 2. Patient demographics and baseline characteristics (n = 13).

	ADRC (n = 9)	Placebo (n = 4)
Age (yrs)	61 ± 2.1	55.0 ± 7.5
Male sex (%)	78	100.0
Weight (kg)	86.2 ± 9.1	82.3 ± 12.5
Body mass index (kg/m^2)	27.5 ± 3.0	27.6 ± 3.3
Hypertension (%)	66.7	50
Smoking (%)	66.7	50
Diabetes (%)	20	25
CK-MB (µmol/l)	78.0 ± 3.9	92.0 ± 5.7
NT-proBNP (pmol/l)	250 ± 86	225 ± 116
Residual TIMI 3 coronary flow	100	100
LAD lesion	100	100

Data are reported as mean ± standard deviation
CK-MB = Creatine kinase-myocardial band
NT-proBNP = N-terminal pro-B-type natiuretic peptide

ported in the remaining patients. The mean amount of adipose tissue harvested was 210 ± 43.3 mL for the treatment group and 181.8 ± 41.5 mL for the placebo group. The intracoronary infusion required between 6 to 5 minutes to complete**.**

The 10 patients in the active treatment group received a mean dose of 17.3 x 10^6 ADRCs. For one of these patients, the use of an inappropriately sized filter (0.2 µm instead of 43 µm) during the intracoronary injection may have reduced the number of infused ADRCs. Therefore, data for this patient were therefore excluded from all feasibility outcome analyses.

Safety results

Bleeding events

In the first four patients enrolled, there were 2 TIMI grade major bleeding events, which were considered SAEs related to the liposuction procedure. In one of these patients, glycoprotein IIb/IIIa inhibition was initiated in the ambulance. In the other patient, a similar but less severe hematoma occurred at the liposuction site, probably associated with a prolonged aPTT (>240 seconds). After the protocol amendments related to the anticoagulation status of these post-PCI patients, the only bleeding events that were reported were regarded as TIMI grade minor or minimal, and required no intervention.

Intracoronary infusion, TIMI flow and coronary flow reserve

Intracoronary infusion of ADRCs was successful and well tolerated in all patients, and did not result in any coronary flow impediment, as assessed by TIMI flow rate or quantified by coronary flow reserve (CFR). In both ADRC-treated patients, as well as controls, CFR remained unchanged before and after cell infusion (Figure 2A).

Interestingly, at six-month follow-up, the CFR had increased significantly by 60% in ADRC-treated patients from 1.57 ± 0.39 to 2.51 ± 0.74 (p= 0.031), as opposed to a non-significant increase of only 4% (from 2.37 ± 0.85 to 2.47 ± 0.72; p=0.93; Figure 2B) in the placebo group.

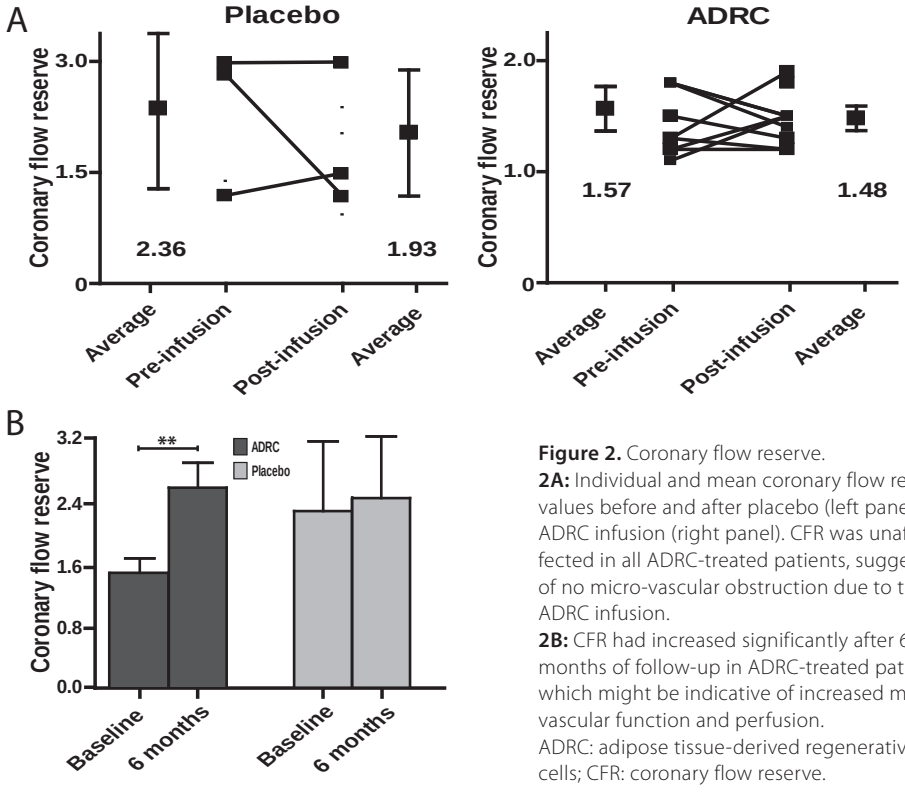

Figure 2. Coronary flow reserve.
2A: Individual and mean coronary flow reserve values before and after placebo (left panel) or ADRC infusion (right panel). CFR was unaffected in all ADRC-treated patients, suggestive of no micro-vascular obstruction due to the ADRC infusion.
2B: CFR had increased significantly after 6 months of follow-up in ADRC-treated patients, which might be indicative of increased micro-vascular function and perfusion.
ADRC: adipose tissue-derived regenerative cells; CFR: coronary flow reserve.

Incidence of MACCE / SAE up to 36 months clinical follow-up

There were 3 MACCE events divided over two treated patients (2/9; 22%), and 1 placebo patient (1/4; 25%; p= NS). All MACCE were target vessel revascularizations (TVR). One patient in the treatment group presented with unstable angina pectoris 2 months following the index procedure, due to a thrombus proximal to the stent in the culprit vessel. In this patient, an anti-phospholipids syndrome was diagnosed as the probable cause of the repeated arterial thrombotic events. The other patient had an asymptomatic *de novo* lesion proximal to the stent in the target vessel at 6 months routine angiographic follow-up. Although asymptomatic, the fractional flow reserve was 0.67, and the patient was subsequently treated by direct stenting of the lesion. Also, one patient in the placebo group underwent TVR, after presentation with unstable angina pectoris more than two years following the index event. Ten SAEs were reported in 7 of the 14 patients over the 36 months follow-up – 6 events in 5 ADRC-treated patients, and 4 events in 4 of the placebo-treated patients. Two of these 10 SAEs were the TIMI grade major bleeding events associated with the liposuction procedure in 2 patients as

described above. Six of the SAEs were considered to be related to the underlying disease. One SAE, a surgical excision of a benign pituitary tumor, was considered to be unrelated to either the treatment, or the underlying disease (Table 3). There were no deaths, strokes or repeat AMIs through the 36 month follow-up. There were no significant differences in MACCE-rate or SAEs between both groups.

Table 3. Summary of MACCE*, Serious Adverse Events and Arrhytmias.

	Placebo (n=4)	ADRC† (n=9)	p-value
MACCE*			
Patients with at least 1 MACCE event	1 (25%)	2 (22%)	NS
Target lesion revascularization	1 (25%)	2 (22%)	NS
Serious Adverse Events (SAE)			
Patients with at least 1 SAE	2/4 (50%)‡	5/9 (56%)‡	NS
Cardiac	***1/4 (25%)***	***3/9 (33%)***	NS
Unstable angina pectoris	1 (25%)	1 (11%)	NS
Atrial fibrillation	0	1 (11%)	NS
Coronary artery stenosis	0	1 (11%)	NS
Non cardiac	***2 (50%)***	***2 (22%)***	NS
Non-cardiac chest pain	1 (25%)	0	NS
Neoplasms (pituitary tumour)	1 (25%)	0	NS
Peripheral artery disease	0	1 (11%)	NS
Pain in extremity	0	1 (11%)	NS
Post-procedural	***1 (25%)***	***1 (11%)***	NS
Bleeding post-liposuction	1 (25%)	1 (11%)	NS
Ventricular arrhythmia			
At least 1 NSVT§	2/4 (50%)	4/9 (44%)	NS
NSVT episodes/patient	2.8 ± 1.0	0.6 ± 0.3	0.048

Values are presented as n. *MACCE: major adverse cardiac or cerebral event; †ADRC: adipose tissue-derived regenerative cells; ‡some patients experienced more than one SAE; §NSVT: non-sustained ventricular tachycardia

BNP levels

At 6 months and 18 months follow-up, there was a continued decrease in pro-BNP. In the ADRC group, pro-BNP values decreased from 235.0 ± 240.9 pmol/L at baseline to 108.6 ± 158.2 pmol/L at 6 months (-32,3; p = 0.19) and to 39.4 ± 45.4 pmol/L at 18 months (- 195,6; p = 0.03). In the placebo group, pro-BNP values decreased from 224.8 ± 232.3 pmol/L, to 115.2 ± 175.4 at 6 months (-109,60; p = 0.30), and to 39.8 ± 43.0 pmol/L at 18 months (-185,0; p = 0.18). There was no significant difference between the groups at the different time points.

Holter monitoring for arrhythmia

No ventricular arrhythmias were reported in either treatment group between 1 and 4 days post-treatment by the continuous 72-hour telemetry and Holter monitoring. Following discharge, ambulatory 24 hour holter recordings were performed weekly for the first four weeks after the index procedure,

and subsequently every month afterwards until 6 months of follow-up, and also at 12, 18, 24 and 36 months of follow up.

ADRC therapy was associated with a significant reduction of ventricular ectopy and the number of ventricular arrhythmias. The number of premature ventricular contractions (PVC) per 24 hour holter recording was markedly reduced in ADRC-treated patients as compared to control patients (48 ± 10 PVC vs. 616 ± 151 PVC, p= 0.014). Moreover, a total of 7 episodes of non-sustained ventricular tachycardia (NSVT) were detected in 4 out of 9 ADRC-treated patients (44%), as compared to 11 episodes in 2 out of 4 placebo patients (50%). On average, each patient in the control group experienced 2.8 episodes of NSVT as opposed to only 0.6 episode of NSVT per ADRC-treated patient (p= 0.048). Holter data are summarized in table 4.

Table 4. Ventricular arrhythmia and ventricular ectopy.

	Placebo (n=4)	**ADRCs (n=9)**	**p-value**
Ventricular tachycardia (VT)			
At least 1 episode of VT	2/4 (50%)	4/9 (44%)	
Total episodes	11	7	0.44
Episodes/patient	2.8 ± 1.0	0.6 ± 0.3	**0.048**
PVC/patient/24 hour	616 ± 151	48.2 ± 10	**0.014**

Values are presented as n.; P-values are determined using two-tailed Chi-square tests when applicable

Efficacy results

Left ventricular Infarct size

Treatment with ADRCs resulted in a substantial and significant reduction of the percentage of LV infarcted at 6 months and 18 months follow-up, as opposed to a non-significant change in placebo controls. In the ADRC-treated patients, the percentage of LV infarcted decreased by -51.3% from baseline to 6 months (from 31.6 ± 15.1% to 15.4 ± 7.4%; p = 0.002), and by -38.2% from baseline to 18 months (19.5 ± 10.7%; p = 0.01). In the placebo-treated patients, the percentage of LV infarcted was -25.1% at 6 months (from 24.7 ± 18.3% to 18.5 ± 14.0%; p = 0.11), and -18.2% at 18 months (20.2 ± 15.9; p = 0.16; figure 3A).

Myocardial Perfusion

In the placebo group, the visual rest score (VRS) first deteriorated by +11.6% (15.0 ± 9.9 to 16.8 ± 8.7, P=0.73) from baseline to six months, but eventually remained unchanged at the 18 month time point (14.8 ± 10.2; p= 0.54). In contrast, treatment with ADRC resulted in a significant -36% reduction of the perfusion defect from 16.9 ± 5.9 to 10.9 ± 6.7 after six months (p=0.004), which sustained after 18 months (-33% to 11.3 ± 5.9; p= 0.007; Figure 3D).

The total summed score (TSS) showed similar results, with a minimal improvement from 11.3 ± 9.3 at baseline to 10.5 ± 5.7 after 6 months (p= 0.55), and to 10.8 ± 7.2 after 18 months (p= 0.30) in placebo patients. ADRC-treated patients however, exhibited a 35% improvement from 13.8 ± 4.9 to 9.0 ± 5.2

after 6 months (p= 0.004) and a sustained 36% improvement to 8.9 ± 3.6 (p< 0.001; **Figure 3E**) after 18 months. This represents a 3-fold reduction of the rest perfusion defect of the left ventricle in cell-treated patients at 18 months follow-up, as compared to control patients (p=0.238), suggestive of improved myocardial perfusion with sustained myocardial viability.

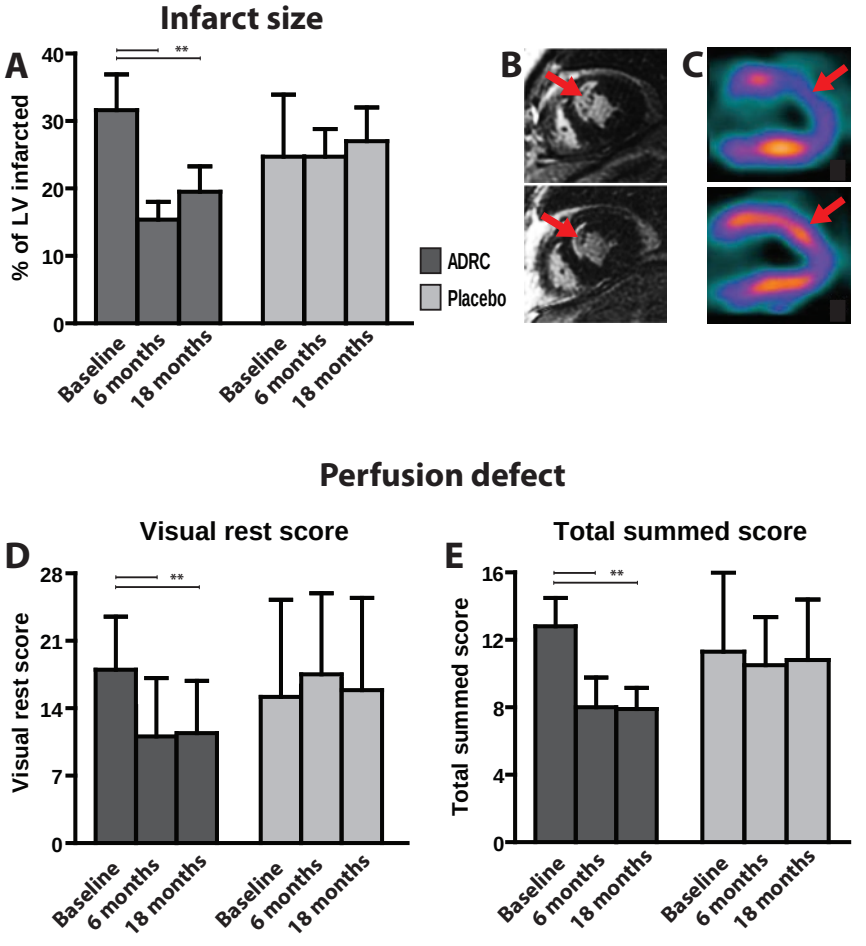

Figure 3. Infarct size and perfusion defect.
3A: The percentage of LV infarcted decreased by 50% after 6 months of follow-up. This effect sustained even after 18 months, whereas there was no change in the control group. **3B.** Representative example of the change in infarct size in ADRC-treated patients as assessed by DE-CMR. The top panel shows an infarct (red arrow) at baseline, the bottom panel shows the infarct in the same patient after 6 months. **3C:** Representative example of enhanced perfusion as seen in ADRC-treated patients as assessed by MIBI-SPECT imaging. The top panel clearly shows reduced perfusion in the anterior wall (red arrow) at baseline, which improved after 6 months (bottom panel). **3D:** Quantification of the enhanced perfusion by the visual rest score (VRS) and **3E:** total summed score (TSS). Both VRS and TSS improved significantly in ADRC-treated patients after 6 and 18 months, as opposed to no change in placebo patients.
ADRC: adipose tissue-derived regenerative cells; DE-CMR: delayed enhancement cardiac magnetic resonance imaging; MIBI-SPECT: sestamibi single photon-emission computed tomography. ** P< 0.01

Table 5. Left ventricular ejection fraction and volumes

Echocardiography								
Treatment		**Baseline**	**2-4 days**	**6 months**	**p-value**	**18 months**	**p-value**	**p-value***
ADRC-treated	LVEF (%)	46.1 ± 7.4	52.2 ± 10.1	50.7 ± 11.3	0.13	51.4 ± 9.6	**0.014**	0.23
	LVEDV (mL)	117 ± 25	120 ± 25	133 ± 39	0.16	146 ± 20	**0.013**	0.76
	LVESV (mL)	63.0 ± 17.9	58.3 ± 22.3	69.4 ± 35.3	0.42	72.4 ± 23.6	0.20	0.32
Placebo	LVEF (%)	43.5 ± 5.8	51.2 ± 8.5	49.5 ± 13.7	0.36	48.8 ± 8.2	0.25	
	LVEDV (mL)	108 ± 19	119 ± 14	133 ± 34	0.10	154 ± 61	0.22	
	LVESV (mL)	60.4 ± 7.6	57.2 ± 6.3	76 ± 25.1	0.46	81.5 ± 45.4	0.37	
CMR								
Treatment		**Baseline**	**2-4 days**	**6 months**	**p-value**	**18 months**	**p-value**	
ADRC-treated	LVEF (%)		52.4 ± 13.6	57.0 ± 10.8	0.18	50.7 ± 15.7	0.47	0.87
	LVEDV (mL)		166 ± 42	193 ± 43	**0.010**	196 ± 45	**0.013**	0.76
	LVESV (mL)		80.1 ± 33	83.2 ± 30.8	**0.028**	100.2 ± 49	**0.021**	0.72
Placebo	LVEF (%)		48.0 ± 10.8	50.3 ± 5.6	0.69	50.1 ± 13.2	0.76	
	LVEDV (mL)		172 ± 13	201 ± 42	0.31	203 ± 61	0.43	
	LVESV (mL)		88.5 ± 15.4	100.9 ± 30.6	0.59	104.2 ± 55.9	0.65	

Left ventricular volumes and ejection faction

The parameters of left ventricular volumes and global LVEF by 2D-TTE and CMR are summarized in Table 5. When assessed by CMR, LV volumes increased equally in both groups. EDV in placebo patients increased by 18% from 171.5 ± 12.8 mL to 202 ± 60.5 mL (p= 0.76) and by 17% in ADRC-treated patients (from 166.5 ± 41.8 mL to 195.6 ± 45.1 mL; p= 0.01), whereas ESV increased from 88.5 ± 15.4 mL to 104.2 ± 55.9 mL (p= 0.65) and from 80.1 ± 33.0 mL to 100.0 ± 49.0 mL respectively (p= 0.021). LVEF slightly increased in placebo patients, as compared to a small non-significant decline in the treatment group (p= 0.47). Importantly, the differences between groups were not statistically significant.

When assessed by echocardiography, LV volumes progressively increased over the 18 months follow up period in both groups, but more in the placebo than in the ADRC-treated group. More specifically, LV end-diastolic volume (EDV) increased by 43% in placebo controls (from 107.5 ± 19.3 mL at baseline to 153.7 ± 61.0 mL after 18 months; p= 0.22), whereas in the treatment group, EDV increased by 25% from 116.8 ± 25.3 mL to 146.4 ± 20.4 mL (p= 0.013). End-systolic volume (ESV) in placebo patients increased by 35% in placebo patients from 60.4 ± 7.6 mL to 81.5 ± 45.4 mL (p= 0.37), and by 15% in ADRC-treated patients (from 63.0 ± 17.9 to 72.4 ± 23.6 mL; p 0.20). LVEF significantly improved in ADRC-treated patients from baseline to 18 months follow up (46.1 ± 7.4% to 51.4 ± 9.6%; p= 0.014) as opposed to a non-significant change in placebo patients (43.5 ± 5.8% to 48.8 ± 8.2%; p= 0.25). However, the difference between groups was not statistically significant (p= 0.09).

Discussion

The APOLLO trial is the first-in-man experience of intracoronary infusion of adipose tissue-derived regenerative cells (ADRCs) in the treatment of patients with ST-elevation AMI. The most important findings over 36 month follow-up are that the liposuction can be performed safely briefly following an AMI, whereas no MACCE or serious adverse events were related to the ADRC therapy. Also, ADRC therapy had no apparent pro-arrhythmogenic effects, but rather appeared to reduce the occurrence of ventricular arrhythmias and ectopy. Although exploratory, ADRC infusion seemed to result in a sustained improvement of the perfusion defect and a reduction of myocardial scar formation, whereas coronary flow reserve in the culprit vessel was significantly enhanced.

In the APOLLO study, limited liposuction in the acute phase of a myocardial infarction appeared to be well tolerated. Although two patients experienced significant bleeding, this was likely to be associated with anti-coagulation therapy in these particular cases. After modification of the protocol to monitor for normalization of the aPTT prior to the liposuction procedure, and to exclude patients with prior treatment with glycoprotein-IIbIIIa-inhibitors, the remaining patients were uneventful. However, post-liposuction bleeding may still represent a concern in the following studies and needs to be carefully monitored.

During cell infusion, coronary flow was monitored carefully by regular assessment of TIMI flow and by CFR analysis. Although intracoronary infusion of BM-derived and cell culture-expanded MSC has raised concerns in pre-clinical studies with respect to micro-vascular obstruction and myocardial infarction [21,22], the intracoronary infusion of freshly isolated ADRCs did not result in any detectable effect on coronary flow. The observed lack of vascular obstruction in the APOLLO patients corroborates the concept that direct isolation and infusion of ADRCs may circumvent the issues of microvascular plugging, although in the APOLLO trial a relatively low dose was applied.

No significant difference was observed between the occurrence of MACCE, SAE or AE in the ADRC-treated and placebo control group. Importantly, the independent DSMB considered no causal relationship between the MACCE and SAE events and ADRC therapy. Furthermore, ADRC therapy did not have any pro-arrhythmogenic effect. On the contrary, ADRC therapy was associated with a reduction of ventricular arrhythmias and ventricular ectopy. This is in line with other clinical and pre-clinical observations in cell therapy studies, and might be correlated with the reduction of scar size and improved myocardial perfusion in ADRC-treated subjects.[23-25]

In the APOLLO trial, ADRC therapy was initiated within 24 hours following the primary PCI. This is in contrast to many previous clinical studies in which cell therapy was initiated in the sub-acute phase of the AMI.[26] However, one of the presumed working mechanisms underlying the beneficial effect of cell therapy is the prevention of cardiomyocyte loss by paracrine release of anti-apoptotic, pro-survival and immunomodulatory factors.[7,8,27] In line with this concept, cell therapy should be initiated as early as possible, when cardiomyocytes are at the highest risk of ischemia/reperfusion-induced apoptosis or necrosis, and the inflammatory response in the infarct area is most pronounced. The feasibility of ADRC therapy directly following reperfusion was demonstrated in various large animal models of AMI.[12,28] Although only 8 patients were analyzed with CMR in the treatment group, the significant and sustained reduction in infarct size may indeed suggest cardiomyocyte salvage evoked by the infused ADRC. A future phase III study will have to confirm these promising data in a larger patient cohort.

In addition to paracrine anti-apoptotic and immunomodulatory factors, ADRCs are known to secrete multiple pro-angiogenic factors [29-31]. As a result, ADRC transplantation in pre-clinical AMI studies consistently resulted in increased capillary density and improved perfusion in the infarct border zone, resulting in preserved cardiac function.[12,28,30] In our clinical study, significant and sustained improvement in both coronary flow reserve (+60%) and perfusion defect (-36%) were found at 6, and 18 months follow-up, as opposed to no change in the control patient group. Interestingly, in the landmark REPAIR-AMI trial, CFR was also profoundly enhanced [32], which seems to confirm the pro-angiogenic and reparative potential of infused regenerative cells in AMI patients. The observations in the current study indicate that ADRCs may promote (neo-)angiogenesis in the peri-infarct region resulting in improved myocardial perfusion, thereby possibly limiting ischemic damage and ultimately improving function.

In conclusion, ADRC therapy appears to be safe and feasible in the acute phase of an AMI. Although in these small patient numbers no statistically significant effect on global LV function was found, significant improvements through 18 months follow up in infarct size, perfusion defect, coronary flow

reserve, and arrhythmia suggest a possible beneficial effect. The obvious major limitation of this phase I/IIa trial is the small sample size, although the study has been performed in a randomized, double-blind fashion with analysis of imaging and holter end-points by independent core laboratories.

Importantly, the possible beneficial effects are consistent with the findings in pre-clinical AMI studies, and concordant with the presumed pro-angiogenic, anti-apoptotic, and immunomodulatory working mechanism of ADRC therapy. ADRCs may thus represent an attractive adjunctive therapy to primary intervention of patients with a large AMI. However, further randomized, controlled trials are needed to confirm these promising results. The ADVANCE trial (ClinicalTrials.gov identifier: NCT01216995) is a prospective, randomized, double-blind, placebo-controlled, phase IIb/III clinical trial that will enroll up to 375 patients with STEMI in up to 35 centers in Europe. The primary endpoint of ADVANCE will be the reduction in infarct size at 6 months by DE-CMR expressed as a percentage of left ventricle infarcted. Completion of ADVANCE is expected in 2014.

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PART IV

Third generation stem cells in acute myocardial infarction

Allogeneic mesenchymal stem cells

Chapter 8

Intracoronary Infusion of Allogeneic Mesenchymal Precursor Cells Directly Following Experimental Acute Myocardial Infarction Reduces Infarct Size, Abrogates Adverse Remodeling and Improves Cardiac Function

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Abstract

Rationale Mesenchymal precursor cells (MPC) are a specific stro3+ sub population of mesenchymal stem cells (MSC) isolated from bone marrow. MPC exert extensive cardioprotective effects, and are considered to be immune-privileged.

Objective This study assessed the safety, feasibility and efficacy of intracoronary delivery of allogeneic MPC directly following acute myocardial infarction (AMI) in sheep.

Methods and results Initially, intracoronary delivery conditions were optimized in 20 sheep. These conditions were applied in a randomized study of 68 sheep with an anterior AMI. Coronary flow was monitored during MPC infusion and cardiac function was assessed using invasive hemodynamics and echocardiography at baseline and during 8 week follow up.

Coronary flow remained within TIMI III definitions in all sheep during MPC infusion. Global LVEF as measured by PV-loop analysis deteriorated in controls to 40.7±2.6% after eight weeks. In contrast, MPC treatment improved cardiac function to 52.8±0.7%. Echocardiography revealed significant improvement of both global and regional cardiac function. Infarct size decreased by 40% in treated sheep, whereas infarct and border zone thickness were enhanced. LV adverse remodeling was abrogated by MPC therapy, resulting in a marked reduction of LV volumes. Blood vessel density increased by >50% in the infarct and border areas. Compensatory cardiomyocyte hypertrophy was reduced in border and remote segments, accompanied by reduced collagen deposition and apoptosis. No micro-infarctions in remote myocardial segments or histological abnormalities in unrelated organs were found.

Conclusion Intracoronary infusion of allogeneic MPC is safe, feasible and markedly effective in a large animal model of AMI.

Introduction

Post myocardial infarction (AMI) left ventricular (LV) remodeling can lead to the clinical syndrome of heart failure, which is an increasing public health issue in the Western world. Although interventional and pharmacological therapy has improved over the past decades, the mortality and morbidity of heart failure is still considerable.[1, 2] Regenerative cell therapy to prevent adverse remodeling is one of the potential adjunctive therapies that has been under extensive investigation over the past few years, with promising results in phase I and II clinical trials.[3]

Although promising, the effects of unfractionated bone marrow (BM) mononuclear cells in these trials have been modest, and it has been suggested that mesenchymal stem cells (MSC) or their sub populations might be more effective.[4-6] Mesenchymal precursor cells (MPC) comprise a Stro-3 immune-selected, immature sub fraction of BM-derived MSC.[7] These MPC are multipotent cells with extensive proliferative potential, and secrete numerous anti-apoptotic, angiogenic factors, and growth factors. It was found that MPC display greater cardioprotective effects than conventional MSC that are selected by plastic adherence alone, which may be evoked by their potent paracrine activity, as well as more extensive multilineage differentiation potential.[8, 9] Interestingly, MPC are immune-privileged and can be transplanted to unrelated recipients, thereby creating the possibility of an allogeneic, "off-the-shelf" cell product, readily available during the acute phase of an AMI. Intramyocardial injection of MPC has been shown to improve cardiac function in small and large animal models of AMI.[9-12]

In contrast to intramyocardial delivery, intracoronary stem cell infusion is a simple, quick, cost-effective and reproducible delivery technique.[13, 14] It does not require specific infrastructure and is available in all interventional coronary cathlabs, while intracoronary infusion of an "off-the-shelf" allogeneic stem cell product can be applied directly following revascularization of the AMI. More importantly, intracoronary infusion omits the risk of myocardial perforation and ventricular arrhythmia that has been associated with intramyocardial injection in infarcted tissue. However, intracoronary infusion of unselected and culture-expanded MSC has previously been associated with impeded coronary flow and micro infarctions in remote myocardial segments in large animal experiments.[15-18]

The primary aim of the current large animal study was to assess the feasibility of intracoronary MPC infusion, and to determine the optimal infusion conditions, while carefully monitoring coronary flow, arrhythmias, and other possible cell therapy related adverse effects. In addition, we investigated the potential efficacy of three different doses of MPC using functional (pressure-volume (PV-loop) analysis, echocardiography) and morphological (morphometry, histology) indices over an 8 week follow-up period.

Materials and Methods

Experimental design

A total of 88 adult sheep were used in this study. All procedures were approved by the institutional animal welfare committee (University of Utrecht, Utrecht, the Netherlands). The study was divided into three distinct phases. In the first phase, the maximum tolerated dose and optimal MPC infusion rate were determined in non-infarcted sheep. In the second phase, we assessed the maximum tolerated MPC dose and optimal infusion rate in animals subjected to an anterior wall AMI. The targeting potential, and cell retention in the infarcted myocardial segment, as well as shedding of MPC to remote myocardial segments and organs, were analyzed in a cell tracking sub study. Finally, in the 3rd phase, the safety and efficacy of intracoronary infusion of three incremental doses of MPC directly following AMI over an 8 week follow-up period was assessed using optimized infusion conditions (see figure 1 for the study flow chart).

Mesenchymal Precursor Cells

MPC used in this study were ovine Stro-3 positive bone-marrow derived cells as previously described.[7, 19] The cells were frozen in cryoprotectant containing 7.5% DMSO and stored at -180ºC in vials at a final concentration of 25 million cells per vial. Before cell infusion, MPC were rapidly thawed, filtered through a 40 micron cell strainer, and suspended in 100 mL of lactated ringers' solution (LR) at a final concentration of 0.5 Mill MPC/mL (see online supplement for cell size measurements).

Phase 1 – Intracoronary MPC infusion in non-infarcted myocardium

A total of 12 sheep (45.2 ± 1.5 kg) were used in phase 1. To assess the optimal infusion rate and maximum tolerated dose, naïve sheep received an intracoronary infusion of incremental doses of MPC (25, 37.5 and 50 million) using an infusion rate of 1.25 or 2.5 million MPC/min (Figure 1A).

A Twin Pass® micro-catheter (Vascular Solutions, Minneapolis, USA) was placed in the proximal LAD and MPC were infused using an infusion pump (Alaris, San Diego, USA). Coronary flow was assessed regularly and troponin I (TnI) was determined at baseline, and 6 hours post cell injection (AccuTnI, Beckman Coulter, Brea, USA). After cell infusion, all animals received a subcutaneously implanted REVEAL DX® event recorder (Medtronic, Minneapolis, USA) to continuously monitor for potential arrhythmias. Two days following infusion, the animals were sacrificed and the heart, lung, liver, kidney and spleen examined by independent pathologists.

Phase 2 – Intracoronary MPC infusion and bio-distribution following AMI

To assess the optimal infusion rate and maximum tolerated dose in AMI, intracoronary MPC infusion was performed in an anterior AMI model in 8 sheep (62.8 ± 1.4 kg). Anterior wall AMI was induced by balloon inflation (Voyager Rx 3.0-3.5x12 mm, Abbott, Illinois, USA) in the mid LAD for 90 minutes.

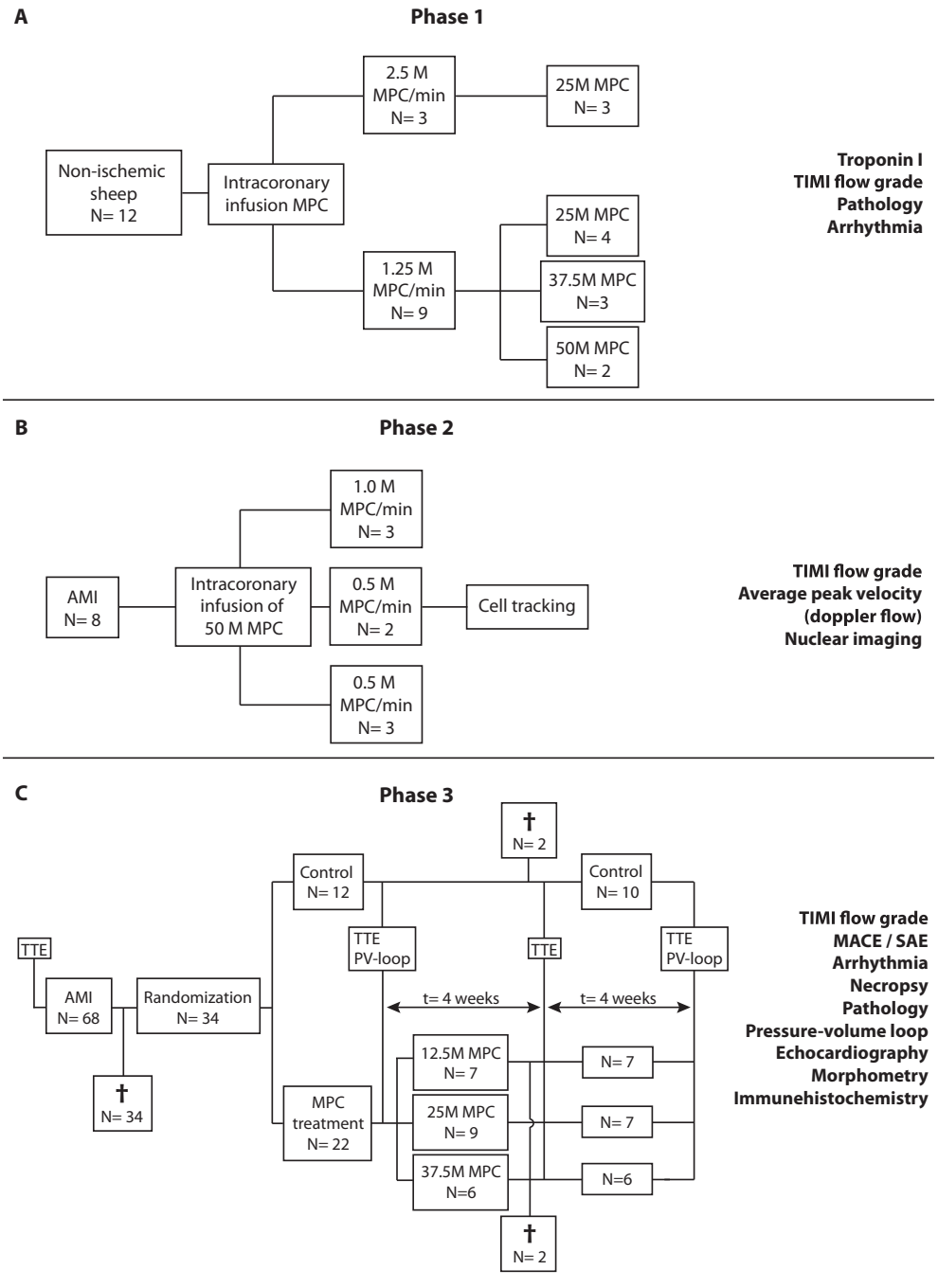


Figure 1. Study flow chart. AMI: acute myocardial infarction; M: million; MPC: mesenchymal precursor cells.

After 15 minutes of reperfusion, a Twin Pass® delivery catheter was positioned in the LAD at the location of prior balloon inflation. Subsequently, 50 million MPC were infused at a rate of 1 million MPC/min (n=3) or 0.5 million MPC/min (n=3). Bio-distribution and myocardial retention was quantified using Indium^{111} labelling in two separate animals (see online supplement). The optimized and safe intracoronary infusion conditions that were found in phase 1 and 2 were subsequently applied in phase 3 of this study.

Phase 3 – Long-term safety effects and dose finding of intracoronary MPC infusion directly following AMI

Induction of myocardial infarction and infusion of MPC

A total of 68 sheep (60.8 ± 1.7 kg) were used in phase 3 of the study (figure 1C). An anterior myocardial infarction was induced by LAD occlusion as described above. After reperfusion, the sheep were randomized by a blinded draw to receive an intracoronary infusion of 12.5, 25, or 37.5 million MPC or LR (control). The cells were infused at an infusion rate of 0.5 million MPC/min. After cell infusion, coronary flow was assessed and a subcutaneous event recorder was implanted. Also, blood was sampled for TnI measurement before AMI and 6 hours post cell or placebo injection.

Pressure–Volume loop analysis

In all animals, baseline PV-loop recordings were acquired directly following MPC or placebo infusion and at 8 weeks follow-up. Also, in a random subset of animals (n=12), pre-AMI PV-loop recordings were performed to obtain reference values of PV-loop parameters of non-infarcted sheep hearts (supplemental online table II). Off-line data analysis was performed by an investigator blinded for the treatment allocation of the individual animals (described in detail in the online supplement).

Echocardiography

In all animals, a transthoracic echocardiogram (TTE) was performed at baseline and directly following the AMI, but also at four and eight weeks follow up. LV volumes, LVEF, regional fractional area change (FAC) and regional systolic wall thickening were analysed off-line by an operator blinded for the treatment allocation of the individual sheep (see online supplement for a detailed description).

Necropsy and pathohistology (long-term safety)

At 8 weeks follow up, the animals were euthanized and routine necropsy was performed to screen for any gross anatomical abnormalities in lung, kidney, spleen, liver and gut, whereas biopsies of these organs were collected for further histological analysis. The heart was excised and prepared as described before, and stained using TTC (supplemental online figure I). Subsequently, the slices were carefully screened for micro-infarctions in remote myocardial segments. Biopsies of the infarct area and infarct border zone were randomly taken and processed for further histological analysis.

Histology samples of the liver, lung, spleen, and kidney were analyzed by an independent pathology core-lab (Druquest International, Leeds, USA) to screen for shedding of MPC and any remote adverse

effects. A section of infarct and border zone tissue of each animal was analyzed by an independent and blinded pathologist, specialized in cardiac pathology (Erasmus University Medical Center, Rotterdam, the Netherlands) to screen for potential local adverse effects of MPC infusion.

Morphometry and histology parameters (efficacy)

Photographs of TTC-stained slices were taken. A blinded technician calculated the percentage of total LV infarcted, and measured infarct and border zone thickness using automated image analysis software.

Collagen content, myocardial salvage index, cardiomyocyte size and cardiomyocyte density

Collagen content and cardiomyocyte size, and cardiomyocyte density were determined using Gomorri trichrome staining.

Blood vessel density

Blood vessel density was determined in the border zone, remote myocardial segments, and in the infarct area. Capillary density in the border zone and remote area was assessed by isolectin-B_4 staining. Arteriolar density in the infarct area was determined using smooth muscle actin staining.

Cardiomyocyte proliferation, apoptosis and cardiac stem cells

The amount of proliferating cardiomyocytes was quantified using Ki-67 staining, whereas the amount of apoptotic cardiomyocytes was assessed using a TUNEL assay. Resident cardiac stem cells were detected by cKit staining.

Statistical Analysis

Efficacy data are depicted as placebo (n=10) versus all MPC-treated (n=20) animals, unless otherwise stated. Continuous data are presented as mean ± standard error of the means. Comparisons of means (morphometry and histology) between groups were performed using a one-way ANOVA with Bonferroni correction for multiple comparisons when applicable. Differences of PV-loop and echocardiography derived parameters between treated animals and controls were analyzed by two-way ANOVA with repeated measures. The MPC-treated group was considered as a homogenous cohort, since ANOVA demonstrated no significant difference in relation to the MPC dose applied. A p-value of £ 0.05 was considered statistically significant. The final data set and statistical analysis were audited and approved by Medical Device Consultants, Inc (MDCI, Reston, Virginia, USA).

Results

Phase 1 – MPC infusion in healthy animals

Coronary flow remained within TIMI 3 range in all animals. However, an infusion rate of 2.5 million MPC/min or infusion of 50 million cells resulted in an increase of TnI levels 6 hours following infusion, and micro infarcts in the LAD territory at 2 days follow-up. In contrast, an infusion rate of 1.25 million MPC/min permitted infusion of doses up to 37.5 million MPC without inducing myocardial necrosis (figure 2A+B). None of the animals experienced ventricular arrhythmias during the two-day follow-up.

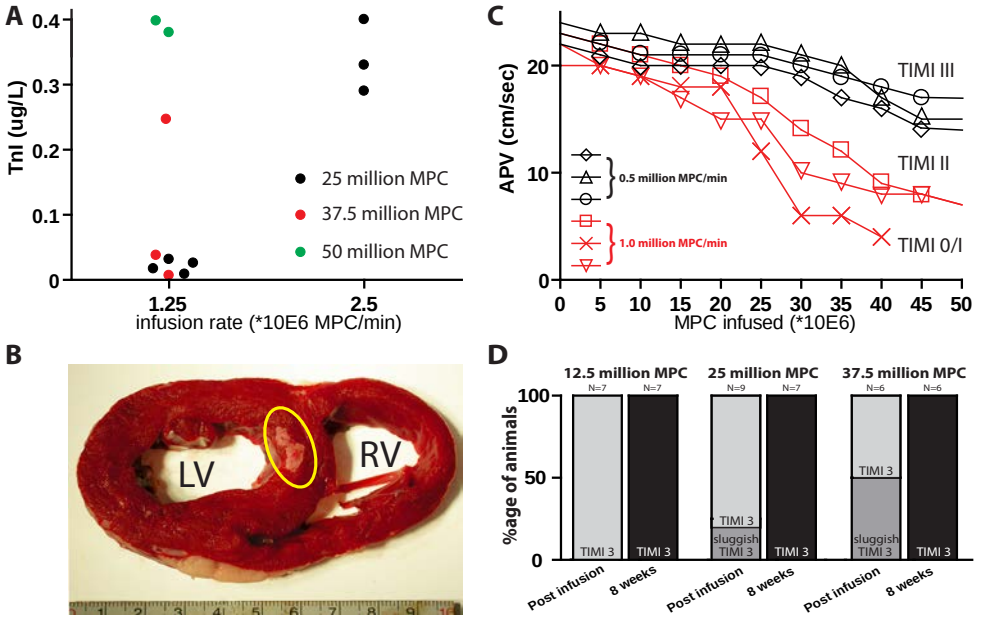


Figure 2. Effects of intracoronary infusion of MPC; **2A.** TnI release six hours after intracoronary infusion of MPC in non-ischemic myocardium. A high infusion rate (right) resulted in significant TnI release in 3/3 animals, irrespective of the low dose infused. When a low infusion rate was adopted (left), infusion of 25 and 37.5 million MPC seemed safe, whereas infusion of 50 million MPC always evoked substantial myocardial necrosis. **2B.** Example of a septal myocardial infarct two days after infusion of 50 million MPC. **2C.** Effect of two different infusion rates on coronary flow in individual sheep directly after an acute myocardial infarction. A high infusion rate (red lines) results in an earlier and more abrupt flow impediment when compared to a low infusion rate (black lines). Coronary flow is depicted as APV and TIMI flow grade; **2D.** The effect on coronary flow of different doses of MPC, when infused directly following the AMI at 0.5 million MPC/min in phase 3 of this study and depicted by TIMI flow grade. Directly following infusion (grey bars), coronary flow was sluggish, but still within TIMI III definition, in 2/9 (22%) animals in the 25 million MPC group and in 3/6 (50%) in the 37.5 million MPC group. At sacrifice (black bars), coronary flow had always returned to normal. APV: average peak velocity; MPC: mesenchymal precursor cells; TIMI: thrombolysis in myocardial infarction; TnI: troponin I.

Analysis of H&E stained sections of all major organs rendered no differences between the sheep that received intracoronary infusion of MPC and healthy controls. More specifically, no shedding of the allogeneic MPC, or any MPC-related acute adverse effects were detected. No acute foreign body or

anti-allogeneic response, defined as extensive or eosinophilic infiltrates, were found in any of the myocardial tissue specimens.

Phase 2- MPC infusion in infarcted myocardium

Infusion of MPC in the culprit artery directly following AMI at a rate of 1 million MPC/min resulted in sluggish flow after infusion of approximately 25 million MPC (n=3, figure 2C). After 25 million MPC, the coronary flow rapidly declined to TIMI grade 1/0 and flow velocities below 10 cm/sec. When applying a reduced infusion rate of 0.5 million MPC/min., sluggish coronary flow was only observed when the absolute dose exceeded 40 million MPC. As a result of these findings, an infusion rate of 0.5 million MPC/min and a maximal dose of 37.5 million MPC were adopted in phase 3 of this study.

Phase 3 – Long-term safety effects and dose finding of intracoronary MPC infusion following AMI

Animal experiments

A total of 68 sheep were subjected to an anterior wall AMI by balloon occlusion in the mid LAD for 90 minutes. Due to ventricular fibrillation refractory to defibrillation, 34 sheep died during infarct induction. The surviving animals were randomized to placebo treatment (n= 12) or treatment with 12.5 (n= 7), 25 (n= 9), or 37.5 (n= 6) million MPC (Figure 1). Two animals in the control group and two animals in the MPC-treated group died during the 8 week follow-up (see below), resulting in 10 analyzable sheep in the control group and 20 in the MPC-treated group (divided in three dose cohorts treated with 12.5 (n=7), 25 (n=7), or 37.5 (n=6) million MPC).

In 6 control animals and 14 MPC-treated animals, serial TnI measurements were available. Both baseline (0.07 ± 0.02 vs. 0.05 ± 0.01; P= 0.89) and post-AMI (272.4 ± 36.6 vs. 297.1 ± 29.2, P= 0.66) measurements did not differ between placebo and cell-treated groups, suggesting a similar degree of injury in both groups.

Coronary flow during and after MPC infusion

MPC infusion was successful in all animals with TIMI grade 3 flow in all dose groups following MPC infusion (Figure 2D). However, infusion of 25 million MPC led to a transient sluggish flow in 2/9 animals (22%), whereas infusion of 37.5 million MPC resulted in sluggish flow in 3/6 (50%) animals. 'Sluggish flow' was defined as a visual difference in the rate of opacification between the culprit artery and reference vessel (circumflex artery), while antegrade flow remained within TIMI grade 3 definitions.[20] At 8 week follow up, coronary flow had normalized in all treated animals.

Death and ventricular arrhythmia analysis

Two sheep in the control group (2/12, 16%), and two sheep in the MPC-treated group (25 million MPC; 2/22, 9%; P= ns) died during the 8 week follow up period (supplemental online table I). Thorough analysis of the implanted event recorders demonstrated that the sheep in the control group died

due to ventricular fibrillation within 12 hours after infarct induction. In the animals in the MPC group, fatal ventricular arrhythmia was excluded as the cause of death after analysis of Reveal® DX data. Subsequent necropsy and histo-pathological examination of the heart, lung, spleen, liver, and kidney by an independent pathologist remained inconclusive about the cause of death in these MPC-treated animals. Specifically, no signs of manifest heart failure were found in lungs, liver and spleen.

In addition to the two animals with a lethal arrhythmia in the control group, another control animal experienced a non-sustained ventricular tachycardia of 20 beats, three weeks following the index procedure.

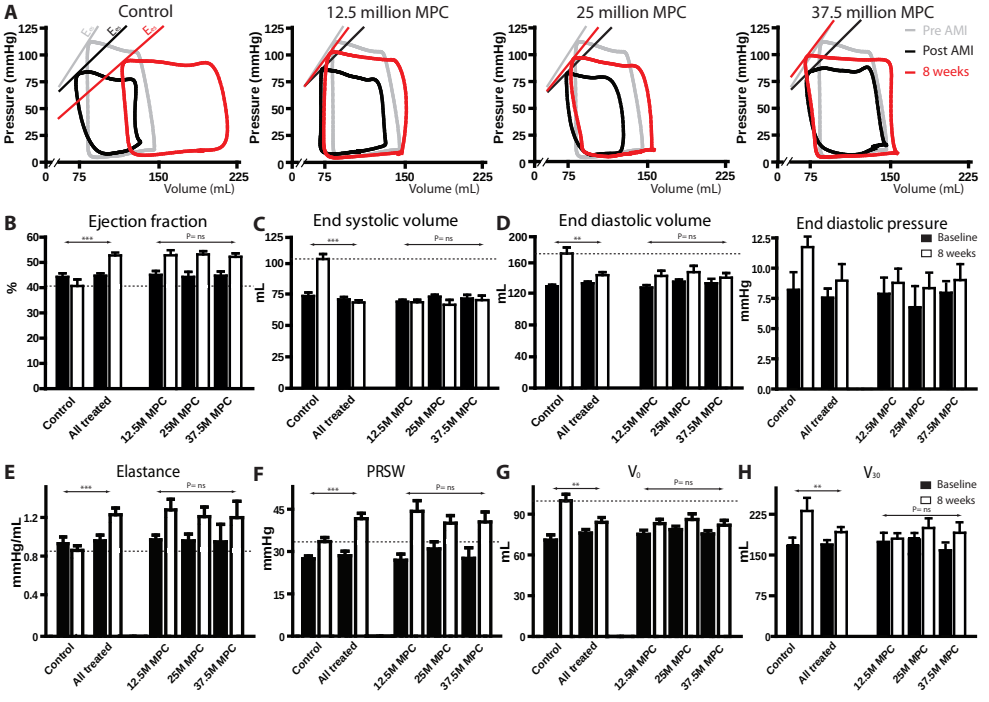


Figure 3. Pressure–volume loop analysis. **3A:** Typical examples of PV-loops of individual animals in the four evaluated groups. The grey loop represents a normal PV-loop of a non-infarcted sheep heart, whereas the black loop represents the PV-relation briefly after an acute myocardial infarction. After eight weeks (red loop), the PV-loop in the control animal shows a rightward shift, indicating increased volumes, further decline of the end-systolic elastance (E_{es}), and increased end-diastolic pressure (filling pressure). In MPC-treated animals, left ventricular dimensions were preserved, whereas E_{es} returned to near baseline levels. **3B:** Left ventricular (LV) ejection fraction further deteriorated in control animals, but was enhanced by over 30% following MPC therapy. **3C/D:** LV volumes increased in the control group, indicative of LV remodeling. This remodeling process was abrogated by MPC therapy. **3E/F:** Pre- and afterload independent parameters of myocardial contractility, E_{es} and PRSW, were enhanced in MPC-treated sheep, as compared to controls. **3G/H:** V_0 and V_{30} are both points on the end-diastolic pressure–volume relation and represent diastolic function and capacitance.
MPC: mesenchymal precursor cells; ns: non significant ; PRSW: pre-load recruitable stroke-work; *P≤ 0.05 ; **P≤ 0.01 ; ***P≤ 0.001

Pressure–Volume loop analysis

All PV-loop derived data can be found in table II of the online supplement.

PV-loop derived LV ejection fraction and volumes

In control sheep, the global LVEF deteriorated from 44.2 ± 1.5% directly following AMI, to 40.7 ± 2.6% at eight weeks. MPC treatment markedly improved cardiac contractile function from 44.7 ± 1.0% to 52.8 ± 0.7% (difference between groups: +12.1%; P<0.001; Figure 3B). No clear dose–effect relationship was observed between the different dose groups.

Following AMI, left ventricular (LV) volumes were comparable between all groups. However, MPC-treatment prevented cardiac remodeling at eight weeks follow-up. End-systolic volume (LVESV) in the MPC treatment group was 68.3 ± 1.8 mL as opposed to 102.8 ± 4.0 mL in the control animals (-34%, P<0.001; figure 3C). Likewise, end-diastolic volume (LVEDV) in the treatment group ameliorated by 16% (149.3 ± 4.1 mL vs. 178.0 ± 8.0 mL, P<0.001) as compared to controls (figure 3D). No significant dose–effect relationship was found between the different treatment groups.

PV-loop derived, load-independent indices of systolic function

LV contractile function is best reflected in the PV-loop derived pre- and afterload independent indices: end-systolic elastance (E_{es}) and pre-load recruitable stroke-work (PRSW). E_{es} and PRSW markedly improved over eight-week follow-up in MPC-treated animals, as opposed to no improvement in controls. In control animals, the baseline E_{es} was reduced to 0.96 ± 0.07 mmHg.mL and remained stable at 0.89 ± 0.05 mmHg.mL at 8 weeks. However, in the treatment group, E_{es} improved from 0.99 ± 0.06 post AMI to 1.26 ± 0.1 mmHg.mL (P= 0.003; Figure 3A/E). In line with these results, PRSW ameliorated to 41.6 ± 1.9 in MPC-treated animals eight weeks, as opposed to 33.5 ± 1.4 mmHg in controls (P= 0.008; Figure 3F).

PV-loop derived indices of diastolic function

Parameters that reflect LV stiffness, including E_{ed}, dP/dT-, and tau, were not significantly different between control and MPC-treated groups (table 1 of the online supplement). Nevertheless, there are indications that diastolic function had improved in MPC-treated animals. First, when corrected for end-diastolic volume, dP/dT- improved significantly, whereas there is a clear trend towards higher end-diastolic pressures in control animals (P= 0.08). Also, V_0 and V_{30} that reflect LV end-diastolic capacitance significantly improved in MPC-treated sheep. V_0 was reduced from 99.8 ± 4.7 mL in the control group to 83.9 ± 2.1 mL in the MPC-treated animals (P= 0.001, Figure 3G), whereas the V_{30} was enhanced from 241 ± 23.5 mL in the control group to 213 ± 8.2 mL in the MPC-treated group (P= 0.047; Figure 3H).

Echocardiography

All echocardiography measures can be found in table III of the online supplement. Global LV function, as depicted by LVEF, was comparable between groups before and directly following AMI (Figure 4A/B/C). Following AMI, LVEF decreased by 20% in both treatment and placebo groups. In the control

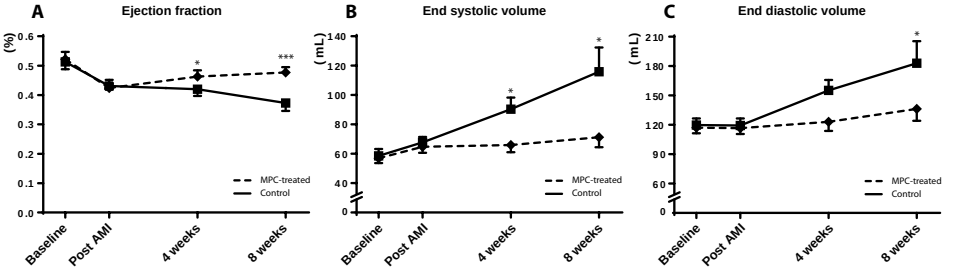


Figure 4. Global LV function and volumes measured by echocardiography. Intracoronary MPC infusion improves global LV function and volumes when compared to controls. **4A.** Global LVEF deteriorated equally in treated and placebo animals after infarct induction, but was significantly enhanced by MPC therapy. **4B/C.** LV end systolic and diastolic volume in MPC-treated animals more or less stabilized after the ischemic insult of the infarct, whereas volumes in control animals further deteriorated. These data corroborate PV-loop derived data on cardiac function and volumes. No significant dose–effect was found. (LV)EF: (left ventricular) ejection fraction. * P≤ 0.05; ** P≤ 0.01; *** P≤ 0.001.

group, LVEF gradually deteriorated further from 43.1 ± 1.2% following AMI to 37.3 ± 1.9% at 8 week follow-up. In contrast, LVEF improved by +21% to 47.7 ± 1.2% in sheep treated with MPC (P= 0.001; figure 4A), when compared to placebo animals, thereby corroborating the PV-loop data.

Echocardiography demonstrated that both LVEDV and LVESV were comparable at baseline and directly following AMI. Importantly, it confirmed PV-loop derived volumes at baseline and the improvement at 8 weeks follow up, as shown in figure 3. LVEDV and LVESV deteriorated in both groups over the 8 week follow-up period, but the increase in the placebo group was significantly greater than in the MPC-treated group. LVEDV increased to 182.9 ± 22.5 mL in control animals, and was reduced by 25% to 136.2 ± 12.0 mL in the treatment group (P= 0.037). LVESV improved by almost 40% from 115.8 ± 16.5 mL in placebo-treated animals to 71.3 ± 6.9 mL in MPC-treated animals (P= 0.042).

Regional function improved by MPC therapy, as FAC in the apex was 39% higher in cell-treated animals as compared to controls (41.4 ± 2.7% vs. 29.8 ± 2.0%; P= 0.027; figure 5A), and FAC was 30% higher in the mid-ventricle (46.7 ± 1.7 vs. 35.8 ± 2.6%; P= 0.007; figure 5B). FAC in the basal segments of the heart did not differ significantly between control and treated animals (figure 5C; P= 0.57). No clear dose–effect was found, and all three doses appeared to be equally effective.

Also, regional contractility improved by MPC therapy. Systolic wall thickening was severely impaired in the apical and mid-ventricular anterior and antero-septal wall segments directly post AMI, whereas compensatory hypercontractility was present in a contra-lateral remote myocardial segment (Figure 5). Systolic wall thickening in the antero-septal segment of the apex improved from 0.5 ± 0.6% in control animals to 23.7 ± 4.2% in treated animals (P= 0.003; figure 5D) and in the anterior wall from 2.7 ± 4.3% to 24.9 ± 2.4% (P< 0.001; figure 5E). Both infarcted segments also markedly improved at the mid-ventricular level (anteroseptal segment: co 9.6 ± 5.5% vs. MPC-treated 39.1 ± 1.8%, P< 0.001; anterior wall: co 13.8 ± 3.6% vs MPC-treated 34.7 ± 1.9%; P< 0.001; figure 5F/G). No significant difference between both groups was found in the contralateral myocardial segment (P= 0.32 and 0.22 respectively; figure 5H/I).

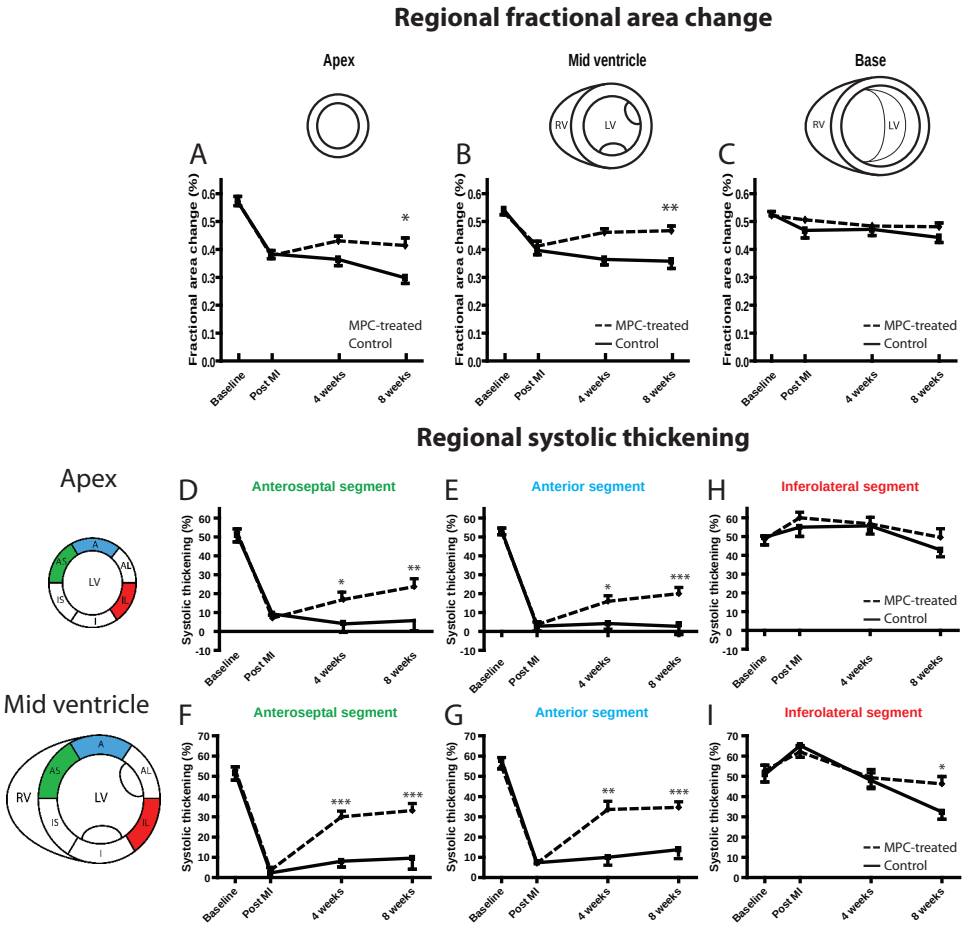


Figure 5. Regional cardiac function as assessed by echocardiography. Intracoronary MPC infusion improves regional function and contractility when compared to controls. **5A-C:** regional cardiac function decreased comparably in both groups directly following the AMI, suggesting similar levels of injury. However, in MPC-treated animals, regional FAC was enhanced in the affected apical and mid-ventricular levels after 8 weeks, whereas the basal level did not show an improvement when compared to controls. **5D-G:** Anteroseptal and anterior systolic wall thickening decreased similarly at apical and mid ventricular levels after the AMI. Systolic wall thickening improved in MPC-treated animals as opposed to no improvement in placebo control animals. **5H/I:** Directly after the AMI, compensatory hypercontractility was seen in the contralateral myocardial segment in all animals, whereas it only improved significantly in MPC-treated animals at the mid-ventricular level after 8 weeks. AMI: acute myocardial infarction; MPC: mesenchymal precursor cells. *P≤ 0.05; **P≤ 0.01; ***P≤ 0.001.

Necropsy and histopathology analysis of tissue samples

During autopsy and macroscopic analysis, no signs of gross anatomical malformations, neoplasms or angiomas were detected in the heart, gut, liver, lungs, kidneys and spleen. This was confirmed by histological analysis by independent pathologists. In the TTC-stained slices of the heart, no signs of micro-infarctions in remote myocardial segments were found.

Infarct size and morphometry

The percentage of LV infarcted in control animals measured 18.4 ± 1.5% in placebo controls, and improved by 33% to 12.0 ± 0.7% in MPC-treated animals (figure 6A; P= 0.001). Also, the average infarct wall thickness in the mid ventricle was enhanced by 25% in treated animals, as compared to control animals (6.4 ± 0.2 vs. 8.0 ± 0.3 mm, P< 0.001; figure 6C). In control animals, the average border zone thickness was 8.5 ± 0.48 mm in the mid ventricle, whereas it improved to 10.5 ± 0.5 mm (P= 0.011) in MPC-treated animals respectively (figure 4D).

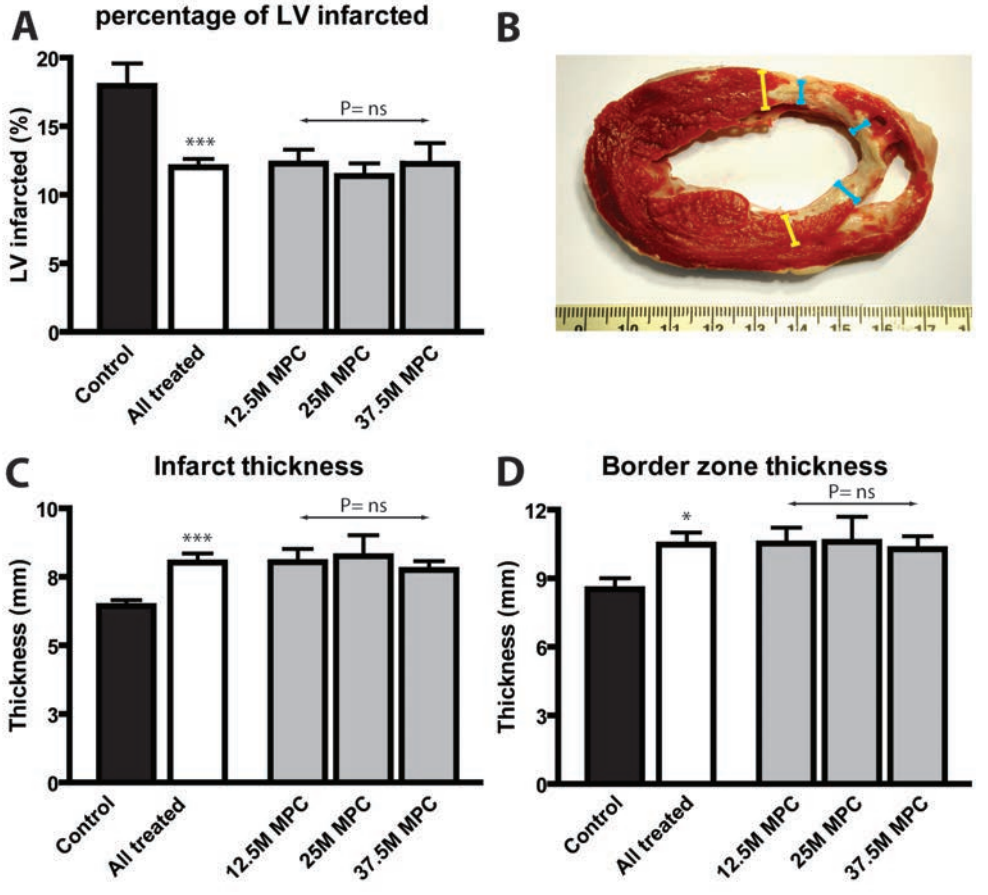


Figure 6. Infarct volume and morphometric analysis. **6A:** Infarct size, calculated as the percentage of the total LV infarcted, significantly improved following MPC therapy. **6B:** Infarct thickness was measured in mid-ventricular slices at three sites in the infarct (blue lines) per slice, whereas the thickness of the border zone was assessed at both sides directly adjacent to the infarct (yellow lines). **6C:** Infarct wall thickness was enhanced by MPC therapy as compared to controls. **6D:** Border zone thickness increased in MPC-treated sheep.
LV: left ventricle; M: million; MPC: mesenchymal precursor cells; ns: non significant ; *P≤ 0.05 ; **P≤ 0.01 ; ***P≤ 0.001

Histology

Collagen content

MPC significantly reduced extracellular matrix deposition in all myocardial areas. Collagen content in the border zone decreased from 16.5 ± 2.1% in the control group to 7.4 ± 0.7% (P<0.001; figure 7A) in the treatment group, whereas collagen content in remote myocardial segments decreased from 2.1 ± 0.4% to 1.0 ± 0.2% (P=0.001; figure 7B).

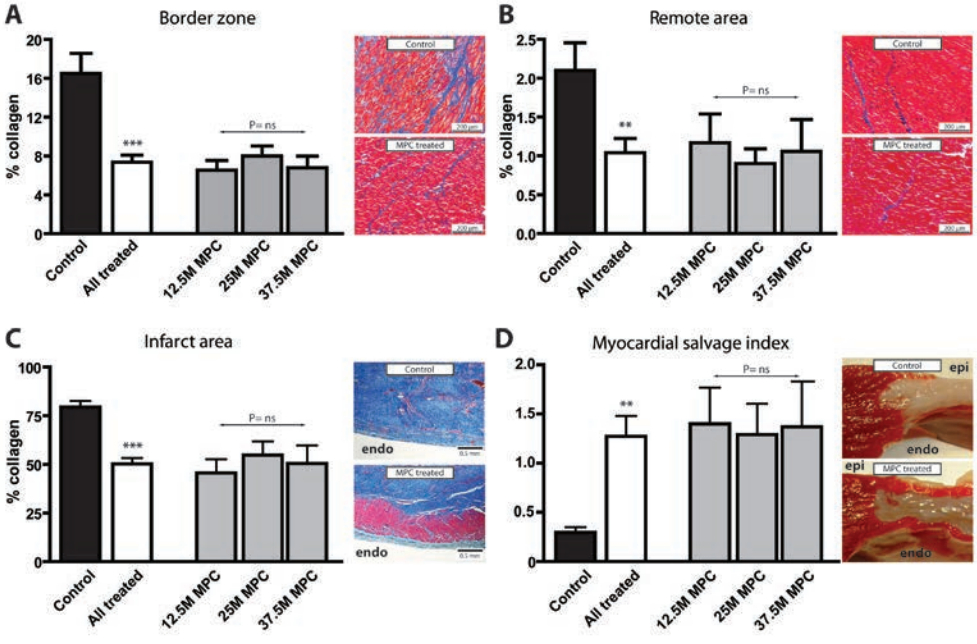
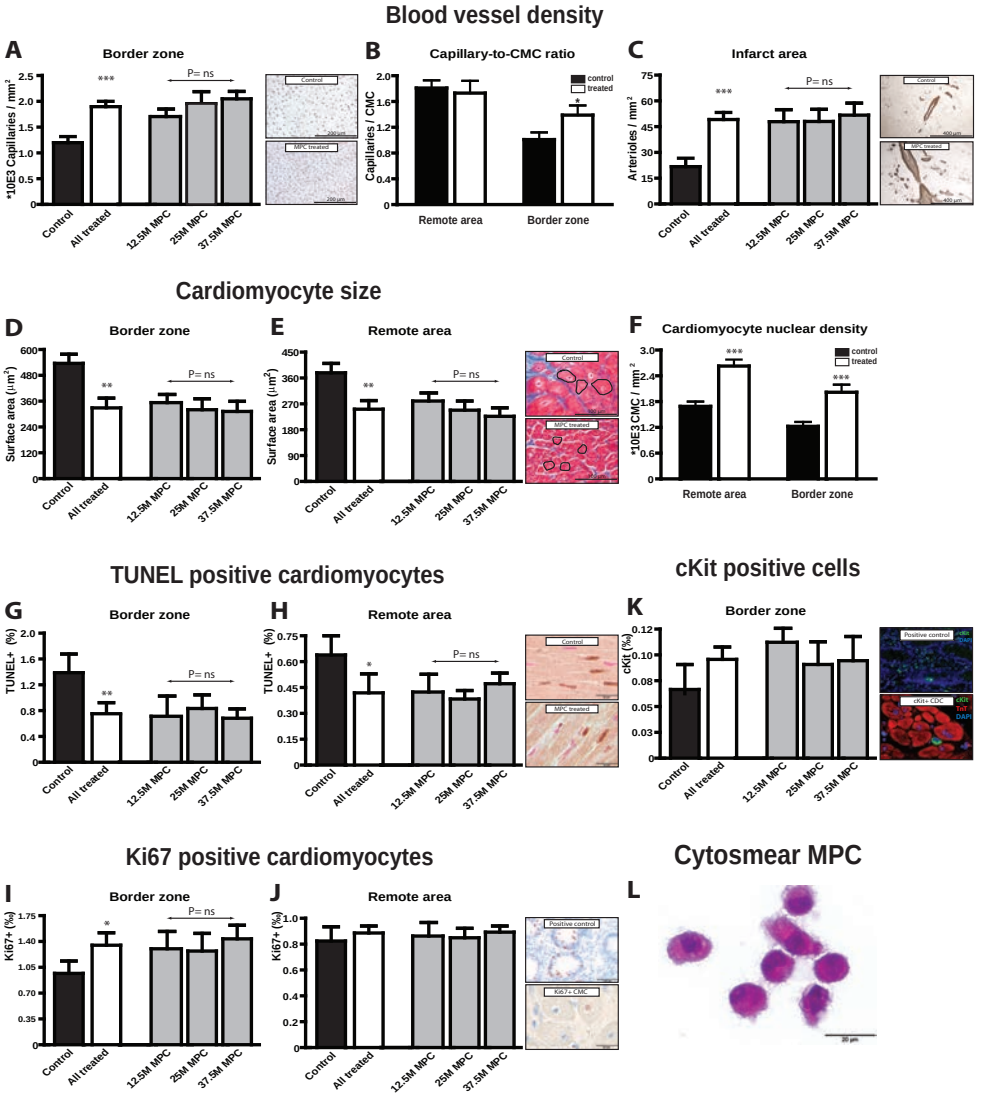


Figure 7. Collagen content and myocardial salvage index. **7A-C:** Collagen content significantly decreased in the infarct, border zone and remote myocardial segments of MPC-treated animals as compared to placebo controls. **7D.** The myocardial salvage index represents the ratio of scar versus viable tissue in the infarct area. AMI: acute myocardial infarction; M: million; MPC: mesenchymal precursor cells; ns: non significant; endo: endocardial side; epi: epicardial side of the left ventricle; *P≤ 0.05 ; **P≤ 0.01 ; ***P≤ 0.001

Interestingly, also in the infarct area, the collagen content was significantly reduced in MPC-treated animals as compared to controls (figure 7C). This decrease in collagen content favored the amount of viable myocardium in infarct specimens, suggesting myocardial salvage. This was represented by a marked improvement in the myocardial salvage index from 0.29 ± 0.06 in controls to 1.30 ± 0.20 in MPC-treated sheep (P=0.002; figure 7D). No clear dose–effect relationship was present in the collagen deposition in all segments.

Blood vessel density, cardiomyocyte size and cardiomyocyte density

The number of capillaries in the border zone was enhanced by 58% from 1196 ± 87 capillaries/mm^2 in the control group to 1894 ± 105 in MPC-treated sheep (P<0.001; Figure 8A). Although not statistically



significant, there appeared to be an incremental dose–effect relation in capillary density between the dose groups (12.5M: 1704 ± 144; 25M: 1953 ± 232; 37.5M: 2046 ± 144 capillaries/mm^2). The higher capillary density in the border zone resulted in a 35% increase of the capillary-to-cardiomyocyte ratio. In MPC-treated animals, each cardiomyocyte was supported by 1.39 ± 0.15 capillaries on average, whereas this was reduced to only 1.01 ± 0.11 in control animals (Figure 8B, P=0.012). In contrast, no difference between groups was found in the capillary-to-cardiomyocyte ratio in remote myocardial segments (figure 8B: controls: 1.81 ± 0.12 capillaries/cardiomyocyte vs. MPC-treated: 1.73 ± 0.19 capillaries/cardiomyocyte; P=0.856).

Figure 8. Blood vessel density, cardiomyocyte size, apoptosis, proliferation and cardiac stem cells. **8A:** Capillary density was assessed in the border zone, revealing increased capillary densities in MPC-treated sheep. **8B:** The capillary-to-cardiomyocyte ratio was only enhanced in the perfusion territory of the culprit artery of MPC-treated sheep as compared to no change in placebo controls, or in remote myocardial segments. **8C:** In the infarct area, a doubling of arteriolar density of MPC-treated animals was observed. **8D/E:** Cardiomyocyte hypertrophy was markedly reduced in the border zones, as well as in remote myocardial segments of MPC-treated animals, when compared to controls. **8F:** This was confirmed by an increase in cardiomyocyte nuclear density in both border zone and remote areas, and is suggestive of delayed or abrogated adverse remodeling that typically precedes clinical heart failure. Together with the profound effect on cardiac function, it also strongly suggests cardiomyocyte regeneration. **8G/H:** MPC therapy reduced cardiomyocyte apoptosis in both border and remote myocardial segments, corroborating reduced adverse remodeling in MPC-treated sheep hearts. **8I/J:** MPC therapy stimulated cardiomyocytes in the infarct border zone to reenter the cell cycle, thereby increasing the number of proliferating cells and inducing endogenous repair. The top picture bordering the graph shows Ki67-positive cells in the gut that served as positive control. The bottom picture shows a Ki67-positive nucleus of a cardiomyocyte. **8K:** cKit staining revealed that the amount of resident cardiac stem cells did not increase in MPC-treated animals in both border and remote areas. The top picture bordering the graph shows cKit-positive cells in the gut that served as positive control. The bottom picture shows a cKit-positive cell (green) in a peri-vascular area of the myocardium (cardiomyocytes are red). **8L:** Microphotograph of MPC in suspension. MPC are considerably smaller than non immune-selected, cultured mesenchymal stem cells that reach sizes of well over 30 micron. M: million; MPC: mesenchymal precursor cells; ns: non significant; CMC: cardiomyocyte; *P≤ 0.05 ; **P≤ 0.01 ; ***P≤ 0.001

The arteriolar density in the infarct area was remarkably enhanced by MPC therapy. In the treatment group arteriolar density doubled, as compared to the control group (49.2 ± 4.1 vs. 21.7 ± 4.0 arterioles/mm^2, P<0.001; Figure 8C).

Post-AMI compensatory cardiomyocyte hypertrophy in border zone and remote areas was more pronounced in the control group as compared to the MPC-treated group. In the control group, cardiomyocyte size was 536 ± 42 µm^2 in the border zone, which was reduced by MPC treatment to 329 ± 45 µm^2 (P< 0.001; figure 8D). In the remote myocardial segment, cardiomyocyte size was 378 ± 32 µm^2 in the control group, and 252 ± 30 µm^2 in the MPC-treated group (P= 0.002; figure 8E). This effect on cardiomyocyte size was confirmed by a significant increase in cardiomyocyte nuclear density in both border en remote myocardial segments. In the border zone, the amount of cardiomyocytes was 1243 ± 102/mm^2 in control animals and increased by 62% to 2026 ± 185/mm^2 in MPC-treated animals (figure 8F; P<0.001). Interestingly, this effect was also found in remote segments, as cardiomyocyte nuclear density in MPC-treated animals was significantly higher than in controls (2645 ± 242/mm^2 vs. 1763 ± 122/mm^2, P<0.001).

Apoptosis, cardiomyocyte proliferation, and resident cardiac stem cells

Apoptotic, TUNEL-positive cardiomyocytes comprised 1.31 ± 0.15% of total cardiomyocytes in the border zone of control animals, and were reduced by 40% in MPC-treated animals to 0.77 ± 0.12% (figure 8G; P= 0.008). MPC therapy also had a favorable effect in remote myocardial segments by lowering the percentage of apoptotic cardiomyocytes from 0.63 ± 0.12% in controls to 0.43 ± 0.03% in MPC-treated animals (figure 8H; P= 0.037).

This effect on programmed cell death was accompanied by a small, but significant, increase in proliferating cardiomyocytes in the infarct border zone, but not in remote segments (figure 8 I/J). In MPC-treated sheep 1.38 ± 0.08‰ of cardiomyocytes were positive for Ki-67 as opposed to 0.97 ± 0.14‰ in placebo controls (P= 0.02).

Resident cardiac stem cells, defined as cKit+ cells, were rarely found in both remote segments and in the infarct border zone (0.071 ± 0.012‰ in controls vs. 0.098 ± 0.022‰ in treated animals; P= 0.30), and were not significantly increased by MPC therapy (figure 8K).

Discussion

In the current study, we investigated a primitive sub population of bone marrow derived MSC. These Stro3+ cells were previously shown to possess potent cardioprotective and immunomodulatory properties *in vitro* and *in vivo*, and can be given to patients in an allogeneic setting. We found that, when certain conditions are adopted, intracoronary infusion of these MPC can be performed safely following AMI without adverse effects, impeding coronary flow, or micro infarctions in remote myocardial segments. Moreover, we demonstrate that intracoronary delivery of MPC prevents LV remodeling and improves residual cardiac function. The results of this study suggest that these effects are evoked by myocardial salvage and subsequent reduction of infarct size, accompanied by induced angiogenesis and reduced myocardial fibrosis.

Previous experience with intracoronary infusion of MSC

Previous studies showed that intracoronary infusion of non-selected MSC was associated with micro-vascular obstruction, coronary flow reduction and myocardial infarctions due to capillary plugging.[15-18] The prominent micro-vascular obstruction that was found in these previous studies might be explained by several factors. First, the size of non-selected MSC progressively increases during cell culturing and higher passages to well over 30 to 50 micrometer.[21] In contrast, MPC comprise an immature sub population of MSC with a median diameter of only 13 micrometer, even when expanded in cell culture (figure 8L and online supplement). As the diameter of capillaries does not exceed 6-10 micrometer, we believe that this small cell size facilitates intracoronary infusion.

Second, in previous studies, higher absolute doses of MSC were used, whereas relatively higher infusion rates were adopted than in the current study. For example, the study of Perin *et al.* infused 100 million MSC at a rate of 1 million cells per minute[15], whereas Freyman *et al.* infused 50 million cells at a rate of 1.5 million cells per minute.[17] In these studies, micro-vascular obstruction and no-flow phenomena are described. On the contrary, infusion of lower cell numbers did not hamper coronary flow in previous experiments. Valina and co-workers infused only 2 million MSC directly following AMI, and Suzuki *et al.* infused 15 million MSC per coronary artery in hibernating myocardium, both without any

flow-related side effects.[22, 23] Also, in a study by Johnston *et al.*, infusion of 10 million cardiosphere-derived cells (20 micrometer in diameter) following AMI was deemed safe, whereas 25 million cells or more caused significant infarctions.[24]

Safety and targeting efficiency of intracoronary MPC infusion

After several pilot experiments divided into two separate phases, we found that, following AMI, a low infusion rate of only 0.5 million MPC/minute permitted intracoronary infusion of 50 million cells without permanently compromising coronary flow, whereas higher infusion rates decreased the maximum tolerated dose. Interestingly, a marked difference between the maximally tolerated dose and infusion rates in animals with or without AMI was noted. This might be associated with increased vascular adhesion of the cells caused by increased expression of chemokines and cell adhesion factors by the activated endothelium following the ischemic insult.[25]

We hypothesize that a low infusion rate might enable the MPC to either pass through the capillary bed or to transmigrate into the peri-vascular tissue without aggregation or capillary occlusion. Indeed, the nuclear imaging retention sub study in two animals revealed that a significant number of MPC still resided in the heart two hours following intracoronary infusion, whereas epicardial coronary flow remained normal (see online supplement). Importantly, no micro-infarctions in, or shedding of MPC to, remote myocardial segments were detected by macroscopic inspection, microscopic analysis and nuclear imaging techniques. This demonstrates that intracoronary infusion of culture-expanded MPC is feasible and can be performed safely. Furthermore, MPC therapy did not have any pro-arrhythmogenic effect. On the contrary, the MPC-treated group showed a trend towards a reduction of ventricular arrhythmias. This might be correlated with the reduction of scar size and improved myocardial perfusion in MPC-treated animals.[26]

Also, no signs of tumorous growth or other focal abnormalities were detected in tissue samples of all major organs or sections of the infarct area by independent and blinded core lab histological analyses. These findings provide additional safety data, as shedding of the cells did not result in significant side-effects, engraftment or aberrant growth in the infused area or remote organs.

Proposed working mechanism of MPC therapy in AMI

The predominant working mechanism of MPC therapy in cardiovascular disease is generally considered to be through paracrine actions of the cells, as long-term engraftment and transdifferentiation into cardiomyocytes of MPC were found to be unlikely in previous studies, and can not account for the profound beneficial effect that has been found in numerous studies.[8-12, 27] Indeed, MPC are known to secrete significant amounts of relevant growth and angiogenic factors as stromal cell-derived factor (SDF)-1, hepatocyte growth factor (HGF)-1, insulin-like growth factor (IGF)-1, VEGF and IL-6. Importantly, the release of these factors exceeds the paracrine abilities of non-selected MSC, resulting in better

cardioprotective properties of MPC when compared to MSC.[8, 9] Although actual cell engraftment and possible transdifferentiation of MPC into cardiomyocytes was not determined in the current study, we believe that it provides insightful data on the regenerative potential and working mechanism of post-AMI cell therapy using allogeneic MPC.

Cardiomyocyte salvage and reduced adverse remodeling

As the cells were administered directly following reperfusion of the AMI, we hypothesize that the therapeutic effect of the MPC is mainly exerted through the release of anti-apoptotic and pro-survival factors, thereby ascertaining cardiomyocyte salvage.[9, 27] In addition, the profound immunomodulatory actions of MPCs may preserve myocardial tissue and contribute to effective tissue healing with limiting scar tissue formation by ameliorating reperfusion injury or attenuating oxidative stress.[28, 29] The presumed efficacy of stem cell therapy within the first hours or days following an AMI was also suggested in two studies that used intracoronary delivery of MSC-like stem cells isolated from adipose tissue.[6, 22]

The reduction of infarct size in MPC-treated animals might have resulted in alleviated LV wall stress and reduced neurohumoral activation. This may then ultimately prevent interstitial fibrosis and compensatory cardiomyocyte hypertrophy in the non-infarcted myocardium and, on the long term, LV dilation.[1, 2] Moreover, in control animals, more apoptotic cardiomyocytes were found in both infarct-related and remote segments, which is a strong indication of ongoing adverse remodeling.[30] Indeed, the placebo-treated animals exhibited increased filling pressures and impaired filling rates, a rightward shift of the PV-relation (*i.e.* increased volumes), and more myocardial fibrosis and cardiomyocyte hypertrophy when compared to MPC-treated animals. These parameters are all part of the structural remodeling process that is generally progressive and precedes the clinical syndrome of congestive heart failure with poor prognosis.[1, 2]

Cardiomyocyte proliferation and resident cardiac stem cells

Recent studies have shown that also the postnatal heart contains resident stem cells.[31] Delivery of MSC to infarcted or hibernating myocardium can regenerate myocardium and improve cardiac function by stimulating these resident cardiac stem cells and cardiomyocytes to (re-)enter the cell cycle, thereby initiating cardiomyocyte generation or proliferation.[23, 31-33] In our study we found a marked difference in cardiomyocyte number and cardiomyocyte size in infarct border, as well as in remote myocardial segments of MPC-treated animals. We believe that this difference in part can be explained by initial myocardial salvage, resulting in subsequent reduction of compensatory cardiomyocyte hypertrophy and apoptosis, and eventually in abrogated adverse remodeling. However, the mere size of the effect on contractile function and cardiomyocyte number, as well as the fact that also remote areas participate, may suggest syngeneic therapeutic working mechanisms induced by the infused MPCs. For instance, the current results indicate that in MPC-treated hearts, increased numbers of cardiomyocytes are in a proliferative state. These proliferating cardiomyocytes might comprise mature proliferating cardiomyocytes that have re-entered the cell cycle, but can also represent the end stage of differentiating cardiac stem cells. Although we found no significant effect on cardiomyocyte proliferation in remote segments after 8 weeks, based on evidence in previous pre-clinical studies[23, 32],

we hypothesize that this is primarily due to the fact that this effect on cardiomyocyte proliferation may have been transient, and the current time point was too late to capture it. We pose that cardiac stem cell niches, which are primarily located in the apex and around the atria of the adult heart[31], might have been activated by the infused MPC, as was also suggested by Suzuki *et al*.[23] As the apical stem cell niche was probably depleted by ischemic damage, we hypothesize that cardiac stem cells originating from peri-atrial tissue might have migrated from the base of the heart to the apical, damaged area, thereby eventually not only regenerating the peri-infarct region, but also repopulating remote segments. It is plausible that increased cardiomyocyte number in basal remote segments, and enhanced proliferation in the apical peri-infarct region, are both late effects of this time-dependent cardiac stem cell activation and migration from base to apex. It should be noted however, that in contrast to previous studies[23, 33], we found no clear difference in the amount of cardiac cKit+ stem cells between treated and control animals, which again might be explained by the longer follow-up period of the current study. Also, the amount of resident cardiac stem cells in sheep myocardium was rather low, possibly caused by the fact that in previous studies[23, 33] mice, and juvenile pigs were used, whereas the current study was performed in adult sheep. We hypothesize that rodents and juvenile pigs may have more resident cardiac stem cells than adult animals, although direct comparative study data are still lacking. Importantly, the fact that MPC therapy also beneficially affects remote regions, which comprise >80% of the injured heart, might explain the profound effect on remodeling, and both global and regional cardiac contractile function that was found in MPC-treated animals.

Induced neo-capillary and arteriole formation

Beside the effect on infarct size, remodeling, and cardiomyocyte proliferation, we also found a marked increase in neo-capillary and arteriole densities in the infarct border zone and infarct area of MPC-treated animals. This increase in blood vessel density in the perfusion territory of the culprit artery suggests a pro-angiogenic potential of MPC therapy and is consistent with previous studies.[9-12, 28] Although we have not directly assessed myocardial perfusion using functional testing, these histologic data suggest improved myocardial perfusion and therefore oxygen and nutrient delivery in the (peri-) infarct region. This might in part explain the enhanced regional cardiac function and contractility that were found in this study.

Previous experience with MPC in acute myocardial infarction

In previous large animal studies that assessed the effect of MPC transplantation following AMI, MPC were injected intramyocardially. MPC transplantation was shown to attenuate LV remodeling and improve cardiac function by enhancing vascular densities, and altering collagen dynamics.[11, 12] These studies also revealed that the low-dose groups (up to 75 million MPC) performed better than the groups that received higher doses (>200 million MPC), suggesting an inverse dose-response relation and a therapeutic threshold of MPC therapy. Accordingly, we found a marked therapeutic effect on both regional and global cardiac function at a relatively low dose range, although no clear correlation was found between efficacy and the cell dose applied. We speculate that higher doses of MPC may be effective, yet also lead to more microvascular obstruction that may counteract the therapeutic

effect, whereas even lower doses might still be effective. In another study, See *et al.* for the first time compared MPC with conventional MSC, thereby showing the extensive cardioprotective and pro-angiogenic paracrine capacities of MPC that exceed the paracrine capacities of non-selected MSC.[9] They showed that paracrine actions were likely the predominant working mechanism of MPC therapy. The current study elaborates on these findings, but also adds to our understanding of the working mechanism of MPC therapy. We confirm that MPC exert cardioprotective effects, reduce fibrosis and increase blood vessel densities in infarct and infarct border zone, but also, for the first time, show effects on CMC proliferation with hints of stem cell activation. By determining optimal intracoronary delivery conditions in a relevant large animal model, we paved the way for clinical studies in the near future that use a protocol based on the results of the current study.

Advantages of allogeneic cell therapy

An allogeneic,"off-the-shelf", cell therapy product, originally derived from a young and healthy donor, has important advantages. It renders a laborious and potentially dangerous BM puncture, as well as the culturing steps in clean room facilities, unnecessary. In addition, the stem cell line ensures adequate quality control with inherent batch-to-batch consistency. Also, a negative correlation was found between the amount and functionality of progenitor cells, and age and cardiovascular risk factors.[34] This would make the use of allogeneic MPC in the typically elderly, cardiovascular patient population preferable over autologous cells. More importantly, the cell therapy can be initiated directly after the revascularization of the AMI, thereby maximally utilizing the anti-apoptotic and immunomodulatory capacities of the cells. Finally, intracoronary delivery of an "off-the-shelf" cell product can be easily performed in any interventional catheterization laboratory in the world, without the need for specific infrastructure or cell delivery techniques.

Clinical experience and prospects

Recently, the results from a clinical, phase IIa study, assessing the effect of intramyocardial injections of allogeneic MPC in 60 heart failure patients, were presented. Allogeneic MPC injections up to a dose of 150 million cells were shown to be safe and feasible without a clinically significant anti-allogeneic immune response. More importantly, MACCE rate, cardiac mortality and composite end points for heart failure were markedly decreased at 12 month clinical follow up. This study resulted in the preparations of a phase III study analyzing the therapeutic effect of MPC therapy via intramyocardial injections in 1,700 congestive heart failure patients.

Likewise, the robust effects of MPC therapy in the current large animal AMI study have led to the design of a multi-center, phase IIa/b, double blind, randomized and placebo-controlled clinical trial. The Allogeneic-Mesenchymal-precursor-cell-Infusion-in-myoCardial-Infarction (AMICI) trial, in which European, Australian and US sites will participate, is aimed to prove safety, feasibility and efficacy of MPC therapy in a minimum of 225 patients with ST-elevation AMI and will start enrollment in Q1 of 2013.

Limitations

Although the current randomized study was performed by blinded operators, and histopathology, PV loop, echocardiography and histology data were analyzed by blinded pathologists or technicians, it also has some limitations. First, the use of cardiac MRI would have supplied additional data on baseline infarct size, and might have rendered slightly more reliable analysis of LV volume. Due to logistical reasons these data are lacking, but we are confident that the combined echocardiography and PV-loop analysis provide adequate, and corroborating functional data on both regional and global LV function. Also, we used a non-atherosclerotic animal model without significant thrombus burden causing the AMI. In the real world, the dynamics of MPC following intracoronary infusion in patients with atherosclerotic and micro-vascular disease might be different and result in earlier flow-related effects. Hence, in the forthcoming AMICI trial, the highest dose tested in our pre-clinical study was omitted. Also, to prevent further loss of animals due to ventricular arrhythmias, all sheep were premedicated with amiodarone. Amiodarone treatment was continued throughout the 8 week follow up, which is different from the real life AMI treatment and might have clouded arrhythmia analysis.

Ideally, a control group of cultured, non-immune-selected MSC should have been part of this study. However, previous studies have shown that intracoronary infusion of comparable amounts of non-selected MSC would have resulted in microvascular obstruction and no-flow phenomena, which would have resulted in a substantial loss of animals. Also, the efficacy of MSC has been established before[35], which makes the addition of an extra group to this already large study obsolete.

Conclusion

Intracoronary infusion of allogeneic primitive mesenchymal precursor cells directly following an AMI is feasible and safe when certain conditions are adopted. It reduces infarct size and prevents subsequent adverse cardiac remodeling by cardiomyocyte salvage and stimulated cardiomyocyte proliferation and angiogenesis, thereby preserving cardiac function and dimensions. The findings of this study might extend the possible application of these cells from specialized cell therapy centers to virtually any interventional cath lab in the world. As MPC can be applied as an "off-the-shelf" product to all AMI patients, the target patient population is considerable, with over 800 primary percutaneous coronary interventions per million inhabitants in Europe alone.[36]

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SUPPLEMENTAL DATA

Materials and Methods

Medication

All sheep were pre-treated with dual anti-platelet therapy (acetylsalicylic acid (Centrafarm, Etten-Leur, the Netherlands) 80 mg qd, clopidogrel (Sanofi-Aventis, Paris, France) 75 mg qd) and amiodarone (Centrafarm, Etten-Leur, the Netherlands) 400 mg qd for ten days prior to the index procedure. Before infarct induction, an intravenous bolus of 10 mg of metoprolol (AstraZeneca, London, United Kingdom) and 10,000 IU heparin (Leo pharma, Ballerup, Denmark) were administered. All sheep received eptifibatide (Merck, Whitehouse Station, USA; bolus of 180 µg/kg and 2 µg/kg/min) during the entire procedure.

Measurement of cell diameter of mesenchymal precursor cells

A vial of 25 million MPC was rapidly thawed, resuspended in a total volume of 25 mL of lactated Ringer's solution and washed twice. Upon the final resuspension step, cells were filtered through a 40 micron cell strainer to obtain a single cell suspension. Subsequently, several cytosmears were made, which were left to air dry. The cytosmears were fixed by submersion in methanol for 15 seconds, after which a common H&E staining was performed. The cytosmears were examined at 1000x magnification and pictures were taken. The diameter of a total of 500 MPC was measured using a routine in automated quantification software as mentioned elsewhere, and the mean and median size were calculated.

Phase 1 – Intracoronary MPC infusion in non-infarcted myocardium

A total of 12 sheep were used in phase 1. To assess the optimal infusion rate and maximum tolerated dose, naïve sheep received an intracoronary infusion of incremental doses of MPC (25, 37.5 and 50 million) using an infusion rate of 1.25 or 2.5 million MPC/min (Figure 1A).

A Twin Pass® micro-catheter (Vascular Solutions, Minneapolis, USA) was placed in the proximal LAD and MPC were infused using an infusion pump (Alaris, San Diego, USA). Coronary flow was assessed by visual estimation of TIMI coronary flow[1] at baseline, every five minutes during MPC infusion, and directly following MPC infusion, to evaluate microvascular obstruction as suggested by reduced antegrade coronary flow. Troponin I (TnI) was determined at baseline, 6 and 24 hours post cell injection (AccuTnI, Beckman Coulter, Brea, USA). TnI levels above 0.1 microgram/L were considered to be an indication of significant cardiomyocyte necrosis due to microvascular obstruction by the infused MPC.

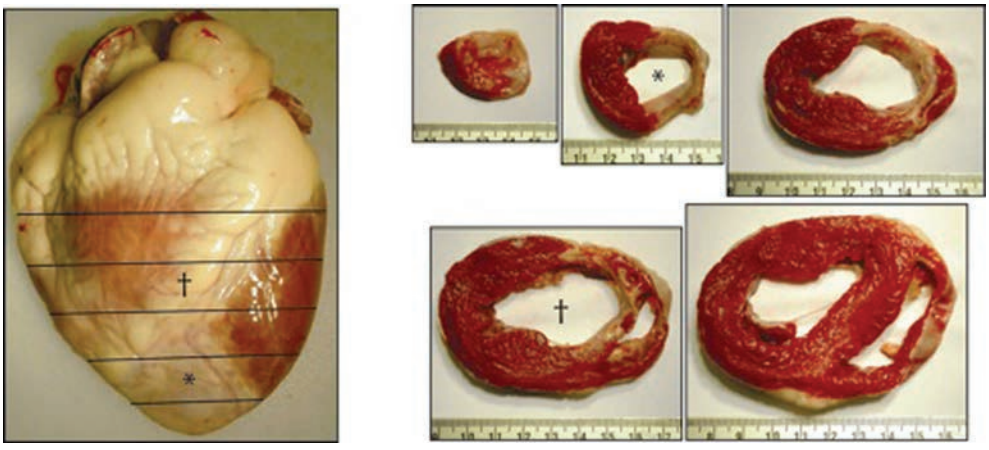

Figure 1. Post-mortem preparation of the heart and TTC staining. After excision, the hearts were cut into five slices in a bread-loaf manner, after which the slices were stained by TTC. TTC staining turns viable myocardium red, whereas infarcted tissue remains white. This facilitates distinction between infarct area, border zones and remote myocardial segments. The slice marked with * represents the apical segment that was used for analysis, the slice marked with † represents the mid ventricular slice.

After cell infusion, all animals received a subcutaneously implanted REVEAL DX® event recorder (Medtronic, Minneapolis, USA) to continuously monitor for potential arrhythmias. After 48 hours, the animals were sacrificed, the hearts excised, and sectioned into five bread-loafed slices of 8-10 mm from apex to base. The sections were stained with 2,3,5-Triphenyltetrazolium chloride (TTC) to visualize (micro-)infarctions. Samples were taken from the inferolateral wall (remote myocardial segment) and anteroseptal wall (target area), as well as from lung, liver, kidney and spleen for histological analysis by an independent pathologist (Erasmus University Medical Center, Rotterdam, The Netherlands) blinded to the individual treatment of the animals.

Table I. Coronary flow, ventricular arrhythmias and death

	Control group	Treatment group	p-value
Coronary flow			
Reduction of TIMI* flow	0/12	0/22	1.000
Death			
Ventricular fibrillation	2/12	0/22	0.144
Unknown	0/12	2/22	0.543
All	2/12	2/22	0.612
Ventricular arrhythmia			
Ventricular fibrillation	2/12	0/22	0.144
Ventricular tachycardia	1/12	0/22	0.371
All	3/12	0/22	0.059

P-values were determined using a two-sided Fischer's exact test.
*TIMI: thrombolysis in myocardial infarction

Phase 2 – Intracoronary MPC infusion and bio-distribution following AMI

To assess the optimal infusion rate and maximum tolerated dose in AMI, intracoronary MPC infusion was performed in an anterior AMI model in 8 sheep. Coronary flow was assessed by visual estimation of TIMI coronary flow, and quantified by intracoronary Doppler flow analysis using a Doppler flow wire (Combowire®, Volcano, San Diego, USA) positioned between the first and second diagonal branch of the LAD, and expressed as the average peak velocity (APV; cm/sec; figure 1B)**.** Bio-distribution and myocardial retention was quantified using Indium^{111} labeling in two separate animals (see below).

Anterior wall AMI was induced by balloon inflation (Voyager Rx 3.0-3.5x12 mm, Abbott, Illinois, USA) in the mid LAD for 90 minutes. After 15 minutes of reperfusion, a Twin Pass® delivery catheter was positioned in the LAD at the location of prior balloon inflation. Subsequently, 50 million MPC were infused at a rate of 1 million MPC/min (n=3) or 0.5 million MPC/min (n=3). The maximum tolerated dose of MPC was assessed by repeated Doppler flow measurements after infusion of every 5 million cells. In addition, TIMI flow was determined after infusion of every 10 million MPC. The optimized and safe intracoronary infusion conditions were subsequently applied in phase 3 of this study.

Phase 3 – Long-term safety effects and dose finding of intracoronary MPC infusion after AMI

Induction of myocardial infarction and infusion of MPC

A total of 68 sheep were used in phase 3 of the study (figure 1C). An anterior myocardial infarction was induced by LAD occlusion as described before. After reperfusion, the sheep were randomized by a blinded draw to receive an intracoronary infusion of 12.5, 25, or 37.5 million MPC or LR (control). The cells were infused via a Twin Pass® delivery catheter at an infusion rate of 0.5 million MPC/min. Coronary flow was assessed by coronary angiography before cell infusion, and every 15 minutes during cell infusion. Coronary angiographies were scored by a blinded interventional cardiologist at Thoraxcenter of the Erasmus University Medical Center in Rotterdam, to prevent bias. After cell infusion, a subcutaneous event recorder was implanted to monitor for ventricular arrhythmias during the 8 week follow-up. Eight weeks following AMI and MPC infusion, coronary angiography and TIMI flow grade assessments were performed and analyzed by independent and blinded investigators.

Nuclear labeling and imaging

MPC were labeled with Indium^{111} at 37°C for 20 minutes (20MBq; GE Healthcare, Pittsburgh, USA). After incubation, cells were washed three times with HANKS buffer (Invitrogen, Carlsbad, USA) and Indium^{111} uptake efficiency was measured with a dose calibrator (Veenstra, Joure, the Netherlands), whereas cell viability was assessed by trypan-blue counting. A total of 37.5 million MPC were infused following reperfusion of the culprit vessel at a rate of 0.5 million MPC/min. Animals were sacrificed two hours after MPC infusion and heart, lungs, liver, spleen and kidneys were excised. Subsequently, the organs, urinary catheter system and the infusion system (syringes, tubing, catheters) were scanned

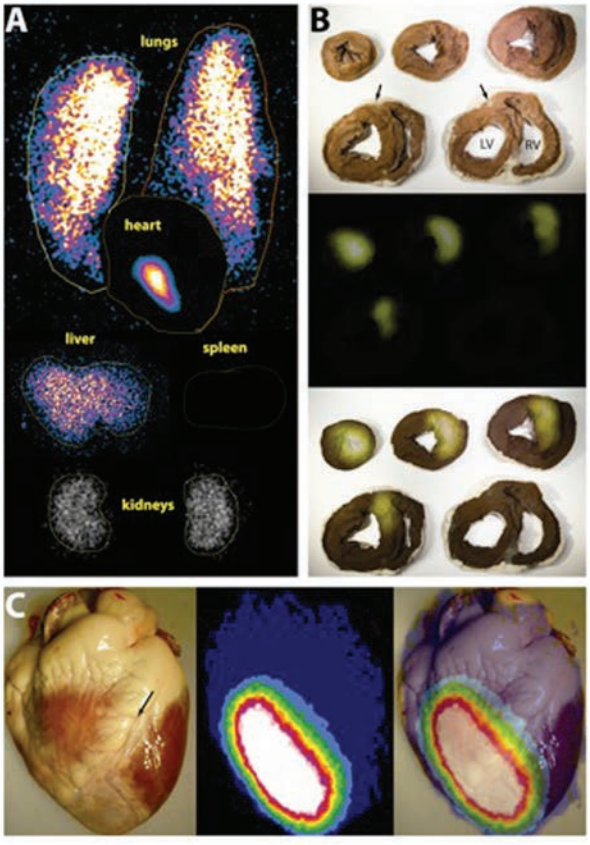

Figure 2. Biodistribution of MPC as assessed by Indium[111] labelling. 3A. Ex vivo scanning of all major organs two hours following intracoronary infusion of 37.5 million MPC. 3B. Top picture: bread-loafed slices from apex to base; middle picture: MPC distribution in these slices; bottom picture: overlay. MPC were only targeted to the perfusion territory of the culprit artery (arrows), whereas no signs of activity were found in remote myocardial segments. 3C. Left picture: anterior view of the intact heart; middle picture: MPC distribution in the anterior wall and apex; right picture: overlay. Arrows: left anterior descending artery; MPC: mesenchymal precursor cells.

using a dual-head gamma camera (Philips, Best, the Netherlands) to quantify MPC bio distribution (acquisition of 5 minutes, 256x256 projection matrix). Bio distribution and retention were determinded using dedicated software (Pegasys, Philips, Best, the Netherlands) and expressed as percentage of the injected dose per organ.

Pressure–volume loop calibration, parameters and analysis

The volume was calibrated by thermodilution and hypertonic saline dilution as previously described.[2,3] PV-loop measurements were performed at baseline and at eight-week follow-up and analyzed using customized software (Conduct NT 2.18, CD Leycom, Zoetermeer, The Netherlands). All values are based on the analysis of ten consecutive beats of sinus rhythm.

End-systolic elastance (E_{es}) was defined as the slope of the end-systolic pressure–volume relation (ESPVR). The E_{es} was calculated using a single-beat estimation method as previously described.[4,5] The V_0 of the end-diastolic pressure–volume relation (EDPVR), which represents the unstretched volume of the LV, was calculated using the following formula: (0.6-0.006*end diastolic pressure (EDP))*end-diastolic

volume (EDV)).[6,7] The V_{30} is the theoretic point on the EDPVR where the pressure is 30 mmHg and was calculated using the following formula: $V_0 + (EDV - V_0)/(EDP/27.8)^{(1/27.6)}$.[6,7] End-diastolic stiffness (E_{ed}) was calculated as EDV/EDP and preload-recruitable stroke-work (PRSW) as stroke-work/EDV.[8]

Echocardiography

In all animals, a transthoracic echocardiogram (TTE) was made before the AMI and directly following the AMI, and at four and eight weeks follow up. Two-dimensional grey scale images at a frame rate of 60-90 frames/s were obtained from a parasternal position with a Philips iE33, equipped with a broadband S5-1 transducer (Philips Healthcare, Eindhoven, The Netherlands). Short axis views were

Table 2. Pressure–volume loop derived parameters.

	reference	control	MPC*	control	MPC*	P value
	Pre AMI†	Post AMI†	Post AMI†	8 weeks	8 weeks	
	n=12	n=10	n=20	n=10	n=20	
HF (beats/min)	70.1 ± 3.4	84.8 ± 4.0	76.5 ± 5.2	92.6 ± 10.0	69.9 ± 4.4	0.11
Volumes						
End systolic volume (mL)	56.1 ± 3.3	73.5 ± 2.8	71.1 ± 1.2	102.8 ± 4.0	68.3 ± 1.8	<0.001
End diastolic volume (mL)	138 ± 7.8	135 ± 2.0	137 ± 1.8	178 ± 8.0	149 ± 4.1	<0.001
Systolic function						
Ejection fraction (%)	63.5 ± 1.7	44.2 ± 1.5	44.7 ± 1.0	40.7 ± 2.6	52.8 ± 0.7	<0.001
Elastance (E_{es})	1.59 ± 0.12	0.96 ± 0.07	0.99 ± 0.06	0.89 ± 0.05	1.26 ± 0.1	0.003
PRSW‡ (mmHg)	47.7 ± 2.8	27.4 ± 1.0	28.5 ± 1.6	33.5 ± 1.44	41.6 ± 1.9	0.008
End systolic pressure (mmHg)	86.8 ± 4.5	70.5 ± 6.0	69.8 ± 3.8	90.5 ± 6.6	84.4 ± 3.7	0.51
Stroke volume (mL)	80.2 ± 5.7	65.9 ± 2.4	72.0 ± 3.0	71.4 ± 6.2	78.1 ± 1.9	0.41
Stroke work (mL.mmHg)	6511 ± 484	3707 ± 179	3954 ± 232	5970 ± 375	6331 ± 325	0.47
Cardiac output (L/min)	5.5 ± 0.3	4.8 ± 0.24	4.5 ± 0.3	6.2 ± 0.5	5.4 ± 0.3	0.22
dP/dtmax	1136 ± 90	980 ± 38	1027 ± 71	1091 ± 82	1155 ± 75	0.15
dP/dTmax / EDV	9.1 ± 0.7	7.3 ± 0.2	7.6 ± 0.6	7.5 ± 0.7	8.5 ± 0.4	0.042
tPER§ (msec)	165 ± 11	165 ± 12	147 ± 9	163 ± 22	165 ± 9	0.30
Diastolic function						
Stiffness	0.05 ± 0.01	0.09 ± 0.03	0.06 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0.22
V_0 (mL)	75.4 ± 4.1	71.0 ± 3.8	76.1 ± 1.1	99.8 ± 4.7	83.9 ± 2.1	0.001
V_{30} (mL)	167 ± 16	185 ± 15	185 ± 7	241 ± 24	213 ± 8	0.047
Tau	29.5 ± 1.5	37.7 ± 7.3	32.9 ± 2.1	27.0 ± 1.8	28.2 ± 0.9	0.27
End diastolic pressure (mmHg)	8.2 ± 1.5	11.0 ± 4.7	7.6 ± 0.9	9.7 ± 1.7	6.0 ± 1.0	0.08
dP/dtmin	-1102 ± 77	-818 ± 56	-823 ± 67	-1042 ± 114	-1126 ± 51	0.29
dP/dtmin / ESV	-12.5 ± 0.9	-11.1 ± 0.6	-11.2 ± 0.7	11.1 ± 1.0	15.3 ± 0.9	0.008
tPFR‖ (msec)	623 ± 42	525 ± 30	604 ± 36	505 ± 30	648 ± 52	0.023

*mesenchymal precursor cells; †acute myocardial infarction; ‡preload recruitable stroke work; §top peak ejection rate;
‖top peak filling rate

recorded at three different levels (basal, mid ventricular and apical) and three consecutive cardiac cycles were acquired. These images were transferred to an Image Arena 4.1 (Tomtec Imaging Systems, Unterschleissheim, Germany) work station for offline analysis. The analysis of echocardiography data was performed by an independent operator, who was blinded for the treatment allocation of the sheep.

The endocardial border was traced at end-diastole and end-systole at each level. LV volumes were calculated using modified Simpson's rule: LV end diastolic volume (LVEDV)= $(A_{bED}) * L/3 + (A_{mED}+A_{pED})/2) * L/3 + 1/3(A_{pED}) * L/3$; LV end systolic volume (LVESV)= $(A_{bES}) * L/3 + (A_{mES}+A_{pES})/2) * L/3 + 1/3(A_{pES}) * L/3$, in which A_b is the area at basal level, whereas A_m and A_p are the areas at mid and apical level respectively. L is defined as the length of the ventricle from apex to base, and was set at 10 cm at baseline, based on cadaver measurements. L at 8 week follow up was measured by counting up the thicknesses of all post-mortem slices (see supplemental figure 1), and L at 4 week follow up was estimated by calculating the mean between baseline and 8 week follow up per animal. LVEF was calculated as follows: [(LVEDV-LVESV)]/LVEDV * 100. These estimations of LV volumes and EF were shown to correspond very well with radionuclide measurement techniques.[9]

Regional fractional area change (FAC) was calculated using the following formula: [(end-diastolic area) - (end- systolic area)]/(end-diastolic area). Also, regional systolic wall thickening was assessed in apical and mid-ventricular recordings. The local wall thickness was measured at end-diastole and systole in the infarct segments (anteroseptal and anterior wall) and one remote segment (inferolateral wall). Systolic wall thickening was subsequently determined by the following formula: [(end-systolic wall thickness) - (end-diastolic wall thickness)]/(end-diastolic wall thickness)].

Infarct volume and morphometry

After excision of the heart, the LV was isolated and cut into 5 slices from apex to base. To discriminate infarct tissue from viable myocardium, the slices were incubated in 1% triphenyltetrazolium chloride (TTC, Sigma-Aldrich Chemicals,Zwijndrecht, Netherlands) in 37 °C Sörensen buffer (13.6 g/L KH2PO4+17.8 g/L Na2H PO4·2H2O, pH 7.4) for 15 min. All slices were scanned from both sides and in each slide the infarct area was compared to total area using digital planimetry software. After correction for the weight of the slices, infarct size was calculated as a percentage of the LV. Infarct thickness was depicted as the average of three measurements from endocardial to epicardial border per slice, whereas the border zone thickness was the average thickness of viable myocardium measured directly adjacent to both sides of the infarct.

Immunohistochemical staining

Collagen content, myocardial salvage index and cardiomyocyte size

Collagen content was assessed using Gomorri trichrome staining. In short, sections of the infarct and border zone, as well as sections of the remote area were deparaffinized and submerged in Bouin's fixation solution (Sigma Aldrich, St. Louis, USA) at 56ºC for 15 minutes. Nuclei were stained with haematoxylin, after which the slides were submerged in Trichrome-LG solution (Sigma Aldrich, St. Louis, USA). After treatment with 0.5% acetic acid solution for one minute, slides were mounted in Entallan (Merck, Darmstadt, Germany).

Three random pictures were taken of each slide at 10x magnification, and collagen content was quantified using a customized software routine as mentioned before and depicted as percentage of the total surface area. The myocardial salvage index was calculated by dividing the area of viable myocardium in the infarct by the area that was composed of collagen.

Also, cardiomyocyte size was measured in trichrome-stained sections of border zone and remote myocardial segments. Three random pictures were taken at 40x magnification and the average surface area of at least 10 cardiomyocytes per field of view was determined. Surface area was only assessed of transversely cut cardiomyocytes in which a nucleus was visible to assure measuring the surface area at the mid level of the cardiomyocyte.

Capillary and arteriolar density

The blood vessel density was determined in border zone, remote area and the infarct area. In the border zone and remote area, blood vessel density was quantified by counting the amount of capillaries per mm^2. Blood vessel density in the infarct area however, was determined by quantifying arterioles, which was necessitated by the disarray of capillaries and pronounced aspecific staining. In brief, sections of the infarct border zone and remote myocardial segment were deparaffinized, rehydrated, pre-treated with trypsin EDTA (Lonza, Verviers, Belgium) and stained for isolectin-B_4 (Bandeiraea simplicifolia Isolectin-B_4 peroxidase, Sigma Aldrich, St. Louis, USA; (20 mg/ml)). Sections of the infarct area were stained for smooth muscle actin (SMA; clone 1A4, Sigma Aldrich, St. Louis, USA; 1:100). All sections were blocked in methanol/H_2O_2 solution for 30 minutes and incubated overnight at 4ºC with isolectin-B_4 or SMA antibody solution. The slides for SMA staining were then washed and immersed in a secondary antibody (HRP-conjugated goat anti-mouse antibody, DAKO, Glostrup, Denmark) for 90 minutes. Subsequently, all slides were immersed in DAB solution (DAKO) for six minutes and finally mounted in Entellan. A technician blinded for the treatment allocation of the individual sheep took three random pictures of the border zone and remote myocardial segment or infarct area at 20x magnification after which capillaries and arterioles were quantified. Capillary density and arteriolar density were expressed as number per mm^2.

The micro-perfusion in the border zone and remote area was quantified as the number of capillaries per cardiomyocyte (capillary-to-cardiomyocyte ratio) and corrected for the collagen deposition in the extra-cellular matrix, and calculated using the following formula: [(capillaries/mm^2)/(cardiomyocytes/mm^2)*(1-collagen content)].

Table 3. Echocardiographic volumes and ejection fraction

	Control	MPC-treated	p-value
LVEF BL (%)	51.3 ± 2.5	52.2 ± 2.5	0.77
LVEF post (%)	43.1 ± 1.2	42.4 ± 1.4	0.58
LVEF 4WFU (%)	42.0 ± 1.7	46.3 ± 0.9	***0.009***
LVEF 8WFU (%)	37.3 ± 1.9	47.7 ±1.2	***0.001***
ESV BL (mL)	58.5 ± 4.4	54.9 ± 3.3	0.51
ESV post (mL)	67.8 ± 3.7	62.8 ± 4.2	0.33
ESV 4WFU (mL)	90.4 ± 7.8	65.9 ± 4.8	***0.025***
ESV 8WFU (mL)	115.8 ± 16.5	71.3 ± 6.9	***0.042***
EDV BL (mL)	119.7 ± 6.7	115.1 ± 5.6	0.58
EDV post (mL)	119.3 ± 7.2	112.6 ± 5.9	0.39
EDV 4WFU (mL)	155.2 ± 10.6	123.0 ± 9.2	***0.018***
EDV 8WFU (mL)	182.9 ± 22.5	136.2 ± 12.0	***0.037***

BL: baseline; post: directly post myocardial infarction; LVEF: left ventricular ejection fraction; 4WFU/8WFU: 4 and 8 week follow up; ESV: end systolic volume; EDV: end diastolic function.

TUNEL, Ki67 and cKit staining

Paraformaldehyde-fixed, paraffin-embedded heart sections of 5 µm thick were used for TUNEL, Ki-67, and c-Kit staining. The amount of apoptosis was quantified using a "In situ cell death detection kit" (Roche, Basel, Swiss) per the manufacturer's instructions. Most antibodies have been used successfully in swine, but not sheep, by other laboratories.[10,11] Antigen retrieval for Ki67 and cKit was done by boiling the slices for 30 minutes in 10mM citrate (pH 6). For Ki67 staining, sections were blocked in methanol/H_2O_2 solution for 30 minutes and incubated overnight at 4ºC with Ki67 antibody (clone MIB-1, DAKO; 1:100) solution**.** The slides were then washed and immersed in a secondary antibody (HRP-conjugated goat anti-mouse antibody, DAKO) for 90 minutes, after which they were immersed in DAB solution (DAKO). Pictures of multiple fields (400x) were used to quantify the frequency of Ki67 staining. cKit staining was performed by immersing the slides in cKit antibody solution (ab5506, Abcam, Cambridge, UK; 1:100) together with anti-cTnI (mouse monoclonal antibody clone 8I-7, Spectral diagnosis, 1:100) to detect myocyte filaments. Samples were posttreated with fluoroisothiocyanate (FITC) conjugated anti-rabbit and TRITC conjugated anti-mouse antibody (Dako). Nuclei were stained with DAPI (Vectashield). Multiple fields were photographed using an Olympus IX55 fluorescence microscope, after which cKit+ cells were quantified.

Results

Measurement of cell diameter of mesenchymal precursor cells

Ovine MPC have a median diameter of 13 micron and a mean diameter of 13.2 ± 2.2 micron (supplemental figure 3)

Nuclear cell tracking experiments

In two separate sheep, bio-distribution was assessed following intracoronary infusion of 37.5 million Indium^{111} labeled MPC. The Indium^{111} labeling efficiency was 79.5 ± 7.5%, and cell viability exceeded 85% after the labelling procedure. *Ex vivo* quantification of Indium^{111} uptake in all major organs estimated a cell uptake in the heart of 40.8% of the total cell dose in sheep 1 and 53.5% in sheep 2, whereas lungs (13.7% and 6.9%), kidneys (2.3% and 1.7%), liver (5.6% and 2.6%), spleen (0.8% and 0.3%), and pericardium (0.3% and 0.1%) had limited uptake (figure 3A). The residual activity was predominantly detected in the infusion and urinary catheter systems. In the heart, the MPC were retained in the perfusion territory of the LAD (figure 3B/C), *i.e.* the anterior and anteroseptal wall. No activity was detected in remote myocardial segments (figure 3B).

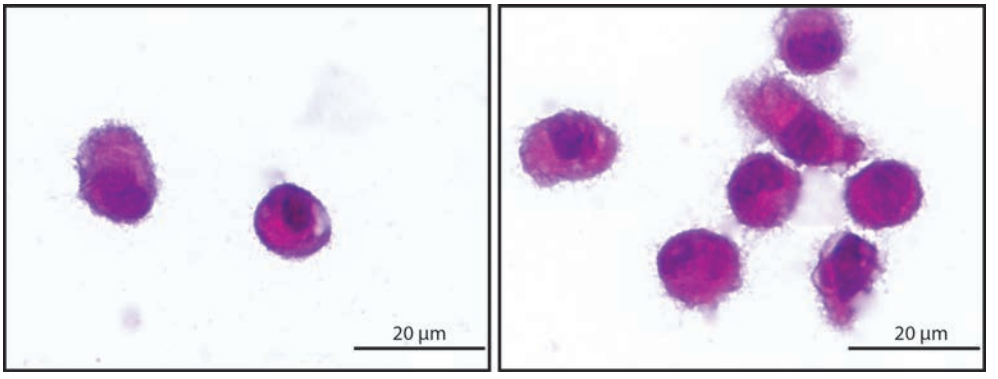

Figure III. Light photomicrographs of mesenchymal precursor cells at 1000x magnification.

Discussion

Analysis of cardiac function

In this study, cardiac function and volumes were assessed using both PV-loop analysis and echocardiography. Invasive hemodynamics by analysis of the pressure-volume relation analysis renders reliable and reproducible quantification of the LV volumes, and thus LVEF, throughout the cardiac cycle.[12,13] Also, indices of intrinsic myocardial contractile function can be determined that are independent of pre- and afterload conditions, which are known to differ substantially between the acute phase of the AMI and at eight weeks follow up.[8,14] Echocardiography analysis showed comparable pre-AMI conditions between groups, provided supportive data on global and regional cardiac function, and corroborated PV-loop data. Importantly, PV-loop and echocardiography data were acquired and analyzed by separate technicians, who were blinded for the treatment allocation of the individual sheep.

Supplemental References

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Chapter 9

Feasibility of intracoronary GLP-1 eluting CellBead™ infusion in acute myocardial infarction

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Abstract

Background Cell therapy is a field of growing interest in the prevention of post acute myocardial infarction (AMI) heart failure. Stem cell retention upon local delivery to the heart, however, is still unsatisfactory. CellBeads were recently developed as a potential solution to this problem. CellBeads are 170 µm alginate microspheres that contain mesenchymal stem cells (MSC) genetically modified to express glucagon-like peptide-1 (GLP-1) supplementary to inherent paracrine factors. GLP-1 is an incretin hormone that has both anti-apoptotic and cardio-protective effects. Transplanting CellBeads in the post-AMI heart might induce cardiomyocyte salvage and ultimately abrogate adverse cardiac remodeling. We aimed to investigate the feasibility of intracoronary infusion of CellBeads in a large animal model of AMI.

Methods and results Four pigs were used in a pilot study to assess the maximal safe dose of CellBeads. In the remaining 21 animals, an AMI was induced by balloon occlusion of the left circumflex coronary artery for 90 minutes. During reperfusion, 60,000 CellBeads (n=11), control beads (n=4) or lactated Ringers' (n=6) were infused. Animals were sacrificed after two or seven days and the hearts excised for histological analyses.

Intracoronary infusion did not permanently affect coronary flow in any of the groups. Histological analysis revealed Cellbeads containing viable MSCs up to seven days. Viability and activity of the MSCs was confirmed by qPCR analysis that showed expression of recombinant GLP-1 and human genes after two and seven days.

CellBeads reduced inflammatory infiltration by 29% (P=0.001). In addition, they decreased the extent of apoptosis by 25% (P=0.001) after two days.

Conclusion We show that intracoronary infusion of 5 million encapsulated MSCs is safe and feasible. Also, several parameters indicate that the cells have paracrine effects, suggesting a potential therapeutic benefit of this new approach.

Introduction

Despite advances in pharmacotherapy and interventional cardiology, heart failure constitutes a growing patient population in the Western world (12). Acute myocardial infarction (AMI) is the major underlying etiology of congestive heart failure (14,28). Because an AMI leads to irreversible loss of cardiomyocytes, and cardiomyocytes have limited regenerative or compensatory capacity, loss of cardiomyocytes irrevocably leads to fibrosis and scar tissue formation (5). Subsequent adverse left ventricular remodeling can cause the heart to fail and lead to the clinical symptoms of heart failure with poor prognosis (6,7).

The cornerstone of the treatment of AMI patients, which is reperfusion in combination with medical treatment, only partly prevents scar tissue formation and remodeling of the damaged heart (8). The prevention of cardiomyocyte loss and remodeling has therefore been the topic of extensive research. The field of regenerative cellular therapy is an area of growing interest, in particular in AMI patients. In fact, small scale clinical cell therapy trials show promising results, using bone-marrow derived mononuclear cells (1,15).

The predominant working mechanism of post-AMI cell therapy is believed to be through the paracrine action of the grafted cells, resulting in myocardial salvage and neo-angiogenesis (13,20,25). The mesenchymal stem cell (MSC) is currently hypothesized to be the most potent, non-embryonic cell with respect to secretion of relevant paracrine growth factors, anti-apoptotic and pro-survival factors, as well as immunomodulatory cytokines, and have been shown to exert cardio-protective effects *in vivo* (3,16,19,30).

Also, a number of recent studies has shown that glucagon-like peptide-1 (GLP-1), one of the most potent incretin hormones, has potential beneficial action on the ischemic and failing heart (2,17,22,24). GLP-1 is a naturally occurring incretin with both insulinotropic and insulinomimetic properties, and has been shown to exert anti-apoptic actions. Interestingly, when added to standard therapy, GLP-1 infusion improved regional and global left ventricular function in a clinical study with AMI patients with severe systolic dysfunction after successful primary coronary intervention (PCI) (18). However, due to its very short half-life, a prolonged infusion of 72 hours with substantial side-effects for the patients was needed to achieve this beneficial effect.

One of the biggest challenges in the cell therapy field today is the poor retention of therapeutic cells upon local delivery in the heart, with retention rates as low as 1% after intracoronary delivery (10,11,27). Even though permanent engraftment of stem cells is not required to illicit the cardioprotective effect, it seems logical that the greater the number of cells that are retained in the injured myocardium and the longer they reside there, the more pronounced the potential beneficial effect may be. Despite wide-ranging efforts to increase cell retention using various delivery techniques, results are still unsatisfactory. A new concept of stem cell delivery has recently become available owing to advances in the field of biotechnology, as it is currently possible to encapsulate MSC in a biocompatible alginate shell (26,29). Alginate encapsulation of varying numbers of MSC results in so-called CellBeads™, available in discrete sizes between 150 and 600 µm.

CellBeads are made from a highly purified alginate material, which is used to encapsulate clusters of adult human MSCs. These MSCs have been genetically modified to secrete a proprietary recombinant GLP-1 fusion protein, which consists of two GLP-1 molecules bound by an intervening peptide. This form of GLP-1 is more stable than endogenous GLP-1, rendering a longer half-life and thus prolonged therapeutic potential. The alginate coating of the CellBeads is permeable to the GLP-1 fusion protein, allowing for continuous delivery, while protecting the MSCs from the patient's immune system. Also, oxygen and nutrients can freely pass through the alginate shell, which renders the MSCs viable for a long period of time. Thus, Cellbeads are potentially a unique, biological, long-term, local drug delivery platform that is capable of delivering GLP-1, or other therapeutic proteins, in addition to MSC-derived factors (VEGF, MCP-1, IL-6, IL-8, GDNF and NT-3) to any target tissue.

We hypothesize that when transplanted in the post-AMI heart, the synergystic effect of paracrine MSC-derived factors together with the cardio-protective GLP-1 peptide, might evoke myocardial salvage, reduce apoptosis and influence the inflammatory response in the acute phase of the AMI. In the long term, CellBead therapy may induce angiogenesis, and decrease post-AMI adverse cardiac remodeling. The aim of the current study was to evaluate the feasibility and safety of intracoronary delivery of 170 µm CellBeads in the acute phase of an AMI in a relevant large animal model.

Methods

Experimental animals

All procedures were approved by the local animal welfare committee. A total of 25 female landrace pigs (Van Beek SPF pigs, Putten, The Netherlands, 69.2 ± 1.0 kg) were used in this study.

Experimental design.

This study was subdivided in two phases. In the first phase (phase 1), the feasibility of intracoronary injection of CellBeads was assessed in naïve, non-infarcted myocardium in four pigs. The goal of this phase was to determine the maximum dose of CellBeads to be delivered safely by continuously assessing coronary flow, or the occurrence of fatal arrhythmias upon CellBead infusion.

In phase 2, 21 pigs underwent an AMI by balloon occlusion, followed by intracoronary infusion of CellBeads to assess the feasibility and safety of CellBead infusion in AMI. Animals received intracoronary infusion of lactated Ringers' (LR) solution (n=6), alginate-only control beads (n=4) or GLP-1 expressing CellBeads (n=11). Coronary flow was assessed prior to, during and after CellBead infusion using coronary angiography, Doppler-aided coronary flow (phase 1) and coronary flow reserve (CFR; phase 2) measurements. Animals were sacrificed after two or seven days and the heart was excised for histological analysis (see figure 1 for study flow chart). In addition, the pigs received an event recorder to monitor for arrhythmic events.

Medication and anesthesia

The animals were pre-medicated for ten days with dual anti-platelet therapy (acetylsalicylic acid 80 mg qd and clopidogrel 75 mg qd) and anti-arrhythmic therapy (only in phase 2; amiodarone 400 mg qd). General anesthesia was induced and maintained with intravenous infusion of midazolam, sufentanil and pancuronium. Upon infarct induction and Cellbead infusion, all animals were therapeutically heparinized and received intravenous infusion of eptifibatide (bolus of 180 µg/kg and 2 µg/kg/min). A fentanyl plaster was applied after the procedure for analgesia. Dual antiplatelet and anti-arrhythmic therapy was continued until the sacrifice procedure.

Induction of the acute myocardial infarction

A myocardial infarction was only induced in phase 2 of the current study. Although in phase 1 the LAD was used to infuse CellBeads, we chose to switch to occlusion of the proximal left circumflex artery (LCX) for induction of the AMI due to an expected higher survival rate. Catheterisation of the left coronary system was performed via the right carotid artery. A left coronary angiogram was made to determine the optimal position for balloon occlusion, followed by inflation of an angioplasty balloon (Voyager Rx 3.5-4.0x12 mm, Abbott, Illinois, USA) for 90 minutes in the proximal LCX to induce an acute posterolateral myocardial infarction.

CellBead infusion and coronary flow assessment

CellBeads were thawed rapidly and diluted in a large volume of LR (150,000 Cellbeads in 250 mL of LR, rendering a concentration of 600 CellBeads per mL).

In naïve animals (phase 1), a micro-catheter (TwinPass, Vascular Solutions, Illinois, USA) was placed in the left anterior descending (LAD) coronary artery, distal to the first diagonal branch. The infusion rate in the first two animals was set at 4 mL/min or 2,400 CellBeads/min. In animals 3 and 4, the infusion rate was reduced to 2 mL/min or 1,200 CellBeads/min. During CellBead infusion, coronary flow was assessed regularly by coronary angiography, and depicted using the conventional TIMI flow grade nomenclature (23). Also, coronary flow was continuously measured during infusion using a Doppler flow wire (Combowire, Volcano, San Diego, USA) and expressed as average peak velocity (APV).

In phase 1, the maximum amount of CellBeads to be safely delivered without compromising coronary flow was found to be 60,000. Thus, in phase 2 (AMI animals), either 60,000 CellBeads (n=11), 60,000 control alginate beads (n=4), or LR alone (n=6) were infused intracoronarily. All animals received two 50 mL syringes, containing 30,000 beads or LR only each. These syringes were infused using a syringe pump (IVAC, Humberside, United Kingdom) at a constant flow rate of 2 ml/min (1,200 CellBeads/min). During infusion, setting of the beads was prevented by regular rocking of the syringe pump.

Beads were delivered after approximately 15 minutes of reperfusion, a TwinPass infusion catheter was placed in the proximal LCX. Coronary angiography was performed before infusion, at 25%, 50%, and 75 % of infusion and ten minutes after infusion of the beads, to determine TIMI flow grade. Also, coronary flow reserve (CFR) was measured before and after bead or LR infusion. CFR was determined by dividing the APV at maximal vasodilatation during adenosine infusion by the baseline APV. Six animals were sacrificed two days after the AMI and bead infusion (four animals that received CellBeads, two control beads), and 15 animals were sacrificed at day seven (seven Cellbeads, two control beads, six LR control; see figure 1). At sacrifice, TIMI flow grade was measured, after which the animals were terminated and the hearts excised for histological analysis. TIMI flow grade was determined by an independent cathlab technician, who was blinded to the treatment of the individual animals.

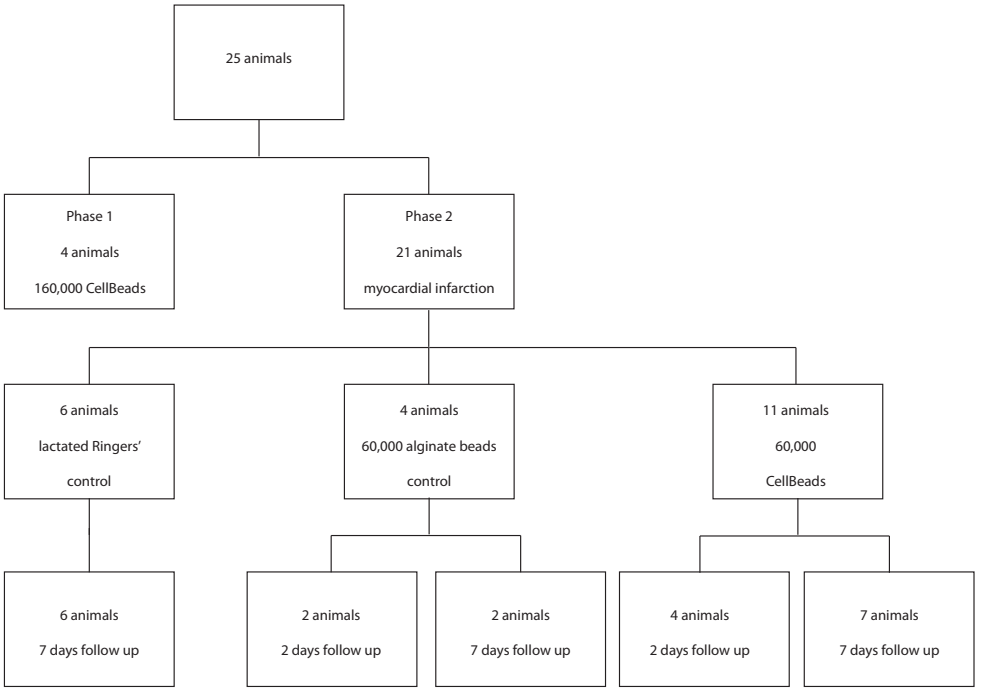


Figure 1. Study flow chart.

Event recorder

After the infusion procedure, all pigs were fitted with a REVEAL DX event recorder (Medtronic, Minneapolis, USA). The recorders were placed subcutaneously in the left thorax. The occurrence of arrhythmias was assessed prior to termination using a CareLink™ programmer (Medtronic, Minneapolis, USA).

Macroscopic and microscopic analysis

In phase two of this study, animals were euthanized at day two or seven and the hearts were excised and sectioned in five to six bread-loafed slices from the apex to the base. The slices were stained in 2,3,5-triphenyltetrazoliumchloride (TTC) to delineate the infarct area from non-infarcted myocardium. The non-infarcted myocardium was thoroughly screened for the presence of micro-infarctions. The entire infarct area and biopsies of remote myocardial segments (anterior and anteroseptal wall) were taken and fixed in formalin, embedded in paraffin and sliced into 5 µm sections. All sections were stained by hematoxylin and eosin staining (H&E-staining) using a standard protocol. Sections were examined by a pathologist specialized in cardiac histopathology.

H&E stained sections of remote myocardial segments were investigated for signs of micro-infarctions or the presence of shedded CellBeads. Sections of infarct tissue of each study animal were investigated for the presence of CellBeads or control beads, inflammatory reactions and/or foreign body response. More specifically, the composition and surface area of the inflammatory infiltrate surrounding each CellBead or control bead was assessed and measured using an Olympus BX45 microscope with a reticle. We only investigated totally transverse cut beads and measured the distance from the bead to the periphery of the surrounding infiltrate.

TUNEL staining for apoptosis

Apoptosis was assessed using an 'In situ cell death detection kit' (Roche, Mannheim, Germany). Samples were counterstained with hematoxylin. At least 30 random pictures were taken from slides of animals that received CellBeads or control beads and were sacrificed at two or seven days. TUNEL and hematoxylin double positive nuclei were counted and expressed as positive cells/100 μm^2.

Quantitative PCR analysis of infarct specimens

For determination of recombinant GLP-1 expression in the target area, small samples of the infarct were taken of animals that survived two or seven days. RNA was extracted using RNA-Bee (Tel-test Inc., Friendswood, Texas, USA) according to the manufacturer's protocol. Quality and quantity of the RNA was verified on an Agilent 2100 Bioanalyzer (Agilent Technologies, UK), and reverse transcribed. Quantitative PCR (qPCR) analysis was performed using an iCycler iQ Detection System (Bio-Rad, the Netherlands). Primers were designed selectively for the recombinant GLP-1 dimer and not endog-

enous GLP-1, a porcine household gene (hypoxanthine-guanine phosphoribosyl transferase 1; HPRT) and a human household genes (HPRT). mRNA levels detected by qPCR were expressed relative to the porcine household genes. The primer sequences are provided in table 1.

Table 1. Primer sequences used for qPCR

Recombinant GLP-1	
CM-1 forward	GTGAGCTCTTATCTGGAAGGCC
CM-1 reverse	AGATAAGAGCTCACATCGCTGG
Human household genes	
HPRT forward	AATGACCAGTCAACAGGGGAC
HPRT reverse	CCTGACCAAGGAAAGCAAAGTC
Porcine household gene	
HPRT forward	AATGACCAGTCAACGGGCGAT
HPRT reverse	CTTGACCAAGGAAAGCAAGGTT

Abbreviations: qPCR: quantitative polymerase chain reaction; HPRT: hypoxanthine-guanine phosphoribosyl transferase 1.

Statistical analysis

The current study was designed as a pilot feasibility study, so no formal power calculations were performed. All statistical analyses were performed post hoc using SPSS 16 statistical software (IBM, Chicago, USA). An analysis of variance (ANOVA) was performed and a Bonferroni correction was used for pair wise comparison between means. Data are presented as mean ± SEM. P values ≤ 0.05 were considered significant.

Results

Results of phase 1 (naïve animals)

All four experiments were a procedural success and it proved to be possible to infuse CellBeads selectively via intracoronary infusion. In the first pig, 75,000 CellBeads were administered without any sign of ischemia or reduction of flow as depicted by APV (Figure 2; delta-APV 5/23) However, after infusion of approximately 80,000 CellBeads several premature ventricular complexes occurred that ultimately progressed into ventricular fibrillation and death of the animal.

In pig 2, APV gradually decreased until the infusion of 60,000 CellBeads, after which a steep decline in APV occurred accompanied by a decrease in TIMI flow from TIMI grade III to I. Hence, we decided to decrease infusion rate in the remainder of the animals from 2,400 CellBeads/min. to 1,200 CellBeads/min. As a result, we found that in the remaining two animals the amount of CellBeads that could be delivered before the decline in APV (defined as >50% of baseline value) and TIMI flow was significant was higher (90,000 Cellbeads). Blood pressure remained stable in all four animals, showing no significant acute effect of CellBead infusion on cardiac performance. After reviewing the results of phase 1,

we concluded that the maximal safe dose that can be infused in naïve/non-AMI pigs is 60,000 CellBeads. Therefore, in phase 2 the feasibility of intracoronary delivery of 60,000 CellBeads was evaluated in a pig LCX AMI model.

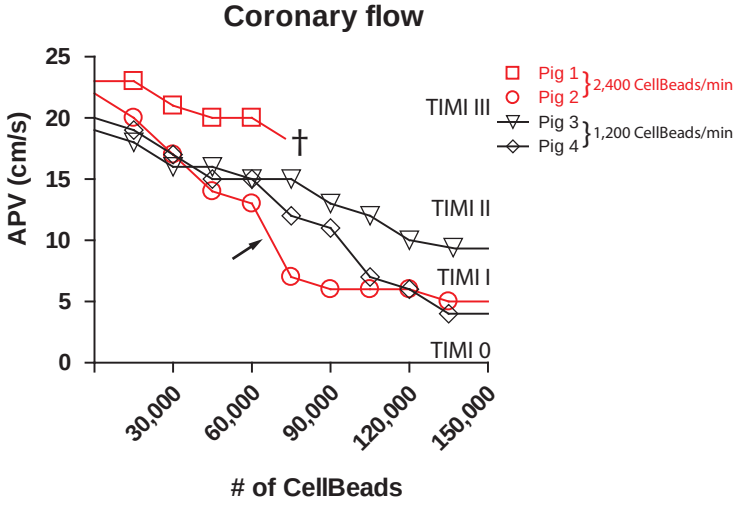


Figure 2. Change of coronary flow upon CellBead infusion in naïve, non-ischemic myocardium. The individual points represent the APV that was measured every 15,000 CellBeads, whereas the corresponding TIMI flow is depicted on the right side of the graph. Intracoronary infusion of CellBeads reduces APV in a dose-dependent manner. An infusion rate of 2,400 CellBeads/min. caused fatal arrhythmia in one animal and a steep decline of coronary flow (both APV and TIMI flow) briefly after the infusion of 60,000 CellBeads (arrow). Reducing the infusion rate to 1,200 CellBeads/min. increased the amount of CellBeads that could be delivered before the APV dropped >50% from baseline. APV: average peak velocity.

Results in porcine AMI model (phase 2)

Animal experiments

A total of 21 pigs were included in this phase of the study. Three animals experienced ventricular fibrillation during infarct induction, but all animals were successfully resuscitated. Animals received 60,000 CellBeads (n=11), 60,000 control beads (n=4) or LR (n=6). No lethal ventricular arrhythmias occurred during CellBead or control bead infusion, and all animals survived the dedicated follow-up time.

Coronary flow reserve

In control animals, average CFR before LR infusion was 1.67 ± 0.09 and 1.73 ± 0.12 after LR infusion (Figure 3A). In the pigs that received intracoronary infusion of CellBeads or control beads CFR remained unchanged with an average CFR of 1.63 ± 0.04 before infusion, and 1.70 ± 0.09 afterwards (*p*=ns; Figure 3B).

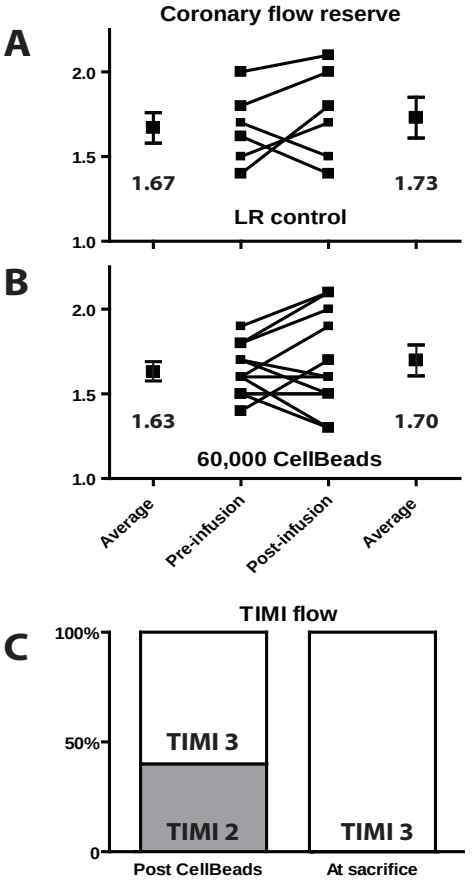


Figure 3. Coronary flow infusion in AMI pigs. **A.** CFR remained unchanged after infusion of LR. **B.** Intracoronary infusion of CellBeads did not alter CFR. **C.** TIMI flow was slightly reduced in 40% of the animals that received intracoronary infusion of 170 µm beads, but had recovered completely at sacrifice.

TIMI flow

All pigs had normal coronary flow after the induction of the AMI, defined as TIMI grade 3 flow. Infusion of 100 mL LR in control animals did not impede TIMI flow. Also, no effect on antegrade coronary flow was found in 60% (9/15) of the animals that received CellBeads or control beads. However, 40% (6/15) of the animals that received CellBeads or control beads experienced a slight reduction of coronary flow, resulting in sluggish flow (TIMI 2) in the culprit artery after the infusion of 60,000 beads (Figure 3C). The occurrence of sluggish flow was predominant during the infusion of the final 15,000 beads, as coronary flow remained unchanged until 45,000 of all 60,000 beads were infused.

Coronary flow returned to normal TIMI 3 flow at the day of sacrifice (day two or seven). There was no difference in coronary flow between animals that survived two days or seven days, or between animals that received CellBeads or control beads.

Analysis of arrhythmias

None of the animals, either control or CellBead-treated, experienced ventricular arrhythmias during the two or seven day follow-up period.

Histology

H&E staining

H&E stained sections of all myocardial segments were examined from all animals included in phase 2. No beads or micro-infarctions could be detected in remote myocardial segments. On the contrary, in infarcted tissue specimens, multiple beads were detected per section, as the majority of 100-200 µm arterioles contained one or more beads. As far as we could determine with conventional histological analysis of sections of the whole infarct area, the CellBeads were distributed equally throughout the infarct. CellBeads in pigs that survived two days as well as in pigs that survived seven days all contained viable MSCs, as was determined by the presence of basophilic nuclei (Figure 4B/C).

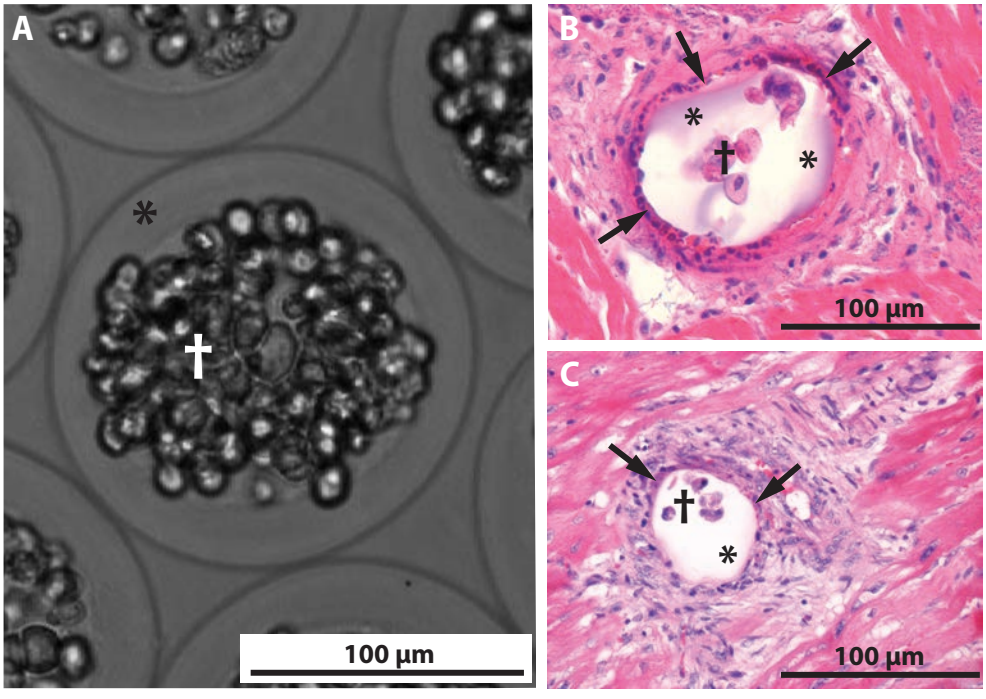


Figure 4. Photomicrograph of micro Cellbeads. **A.** Light photomicrograph of a CellBead prior to infusion. The alginate shell (*) and MSC-containing core (†) can be appreciated. **B.** H&E stained section of a coronary arteriole completely comprised of one CellBead, two days after infusion. Only a thin rim of fibrin and granulocytes surrounds the CellBead (arrows). **C.** H&E stained section of a coronary arteriole containing one CellBead, seven days after infusion. Only a thin rim of fibrin and granulocytes surrounds the CellBead (arrows).
* Intact alginate; †.Mesenchymal stem cells.

Two days after infarction, the cardiac tissue showed infiltration of predominantly neutrofilic granulocytes and loss of cardiomyocyte viability. After seven days, a clear fibrotic response was present with fibroblasts infiltrating the infarcted area. Also, there was a marked increase of the inflammatory infiltrate as compared to the two day animals, which is normal seven days post-AMI.

Inflammatory infiltration surrounding CellBeads

All the beads showed a thin rim of fibrin with neutrophilic granulocytes (Figure 5A). In addition, the beads were variably surrounded by a rim of lymphocytes and plasma cells. We noted that the control alginate beads showed significantly more inflammatory infiltrate directly surrounding the bead when compared to CellBeads (0.038 ± 0.004 mm^2 vs. 0.027 ± 0.004 mm^2, p= 0.003; Figure 5B/C) two days following the AMI.

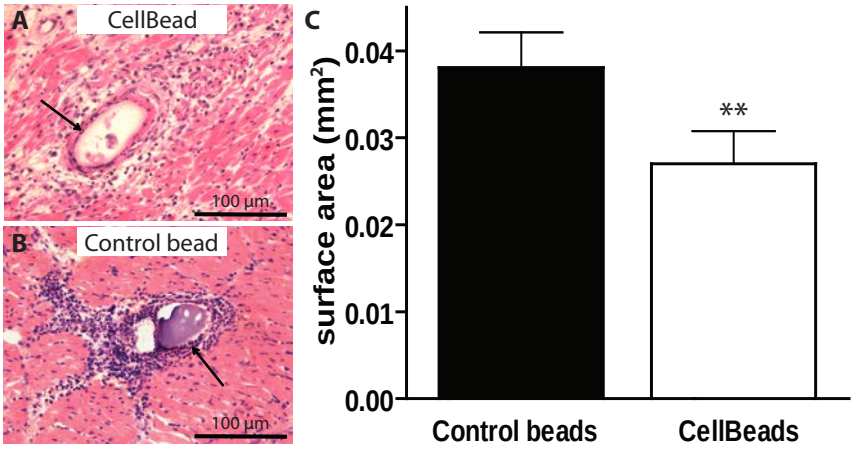


Figure 5. Peri-bead inflammatory infiltration after two days. **A.** A thin rim of neutrophilic granulocytes is evident surrounding a single CellBead (arrow). **B.** A more pronounced inflammatory reaction around a control alginate bead (arrow). **C.** Comparison of inflammatory reaction by measuring the surface area of the inflammatory infiltrate reveals a significant decrease in inflammatory response to the CellBeads as compared to the control alginate beads (** p= 0.003).

At the seven day time-point, the natural healing process of the AMI caused such a background inflammatory infiltrate that measurements of the infiltrate surrounding the beads were not reliable. We therefore performed the analysis at the two day follow-up time-point after AMI only. Since this inflammatory infiltrate after seven days was equally intense in the LR control animals, we considered this as a normal phase in the healing process.

Apoptosis

Apoptosis was significantly reduced in infarct areas of animals that received CellBeads as opposed to animals that received control beads. More specifically, in infarct tissue specimens of animals that received control beads, on average 7.5 ± 0.44 cells were apoptotic per 100 μm^2 after two days. This number was significantly reduced in animals that received CellBeads to 5.6 ± 0.36 per 100 μm^2 (p= 0.003; Figure 6). After seven days, the number of apoptotic cells had decreased to 3.3 ± 0.41 per 100 μm^2 in animals that received control beads as opposed to 2.3 ± 0.29 per 100 μm^2 in animals that received CellBeads (p= 0.34).

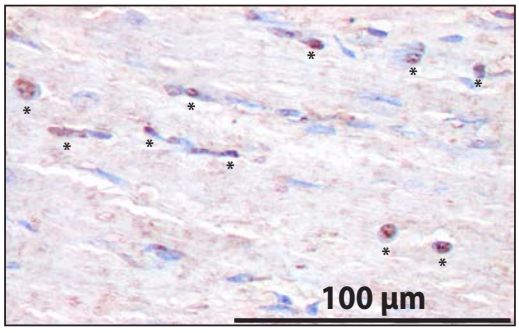
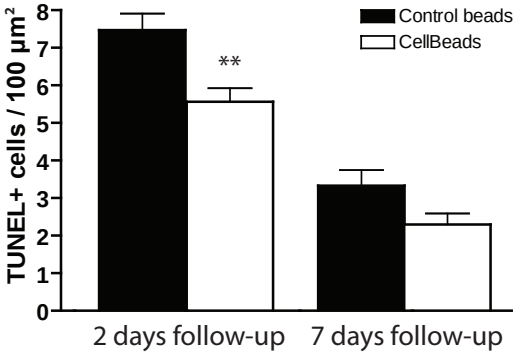


Figure 6. TUNEL staining for apoptosis. (Above) Histological section of infarct tissue two days after the AMI, demonstrating apoptotic cardiomyocytes (*) throughout the tissue. (Below) Quantification of TUNEL positive cells revealed a significant difference in the amount of apoptosis in CellBead-treated tissue compared to controls after two days (** p= 0.003), but not after seven days (p=0.34).



Quantitative PCR analysis of infarct specimens

No expression of recombinant GLP-1 or human genes was found in the hearts of the control pigs. However, expression of recombinant GLP-1, together with the human household gene HPRT was found in infarct segments of animals that received GLP-1 expressing CellBeads, confirming the presence of CellBeads in the infarcted heart that contain viable and actively transcribing human MSC. Expression levels of recombinant human GLP-1 was comparable between specimens from animals that survived two and seven days (Figure 7).

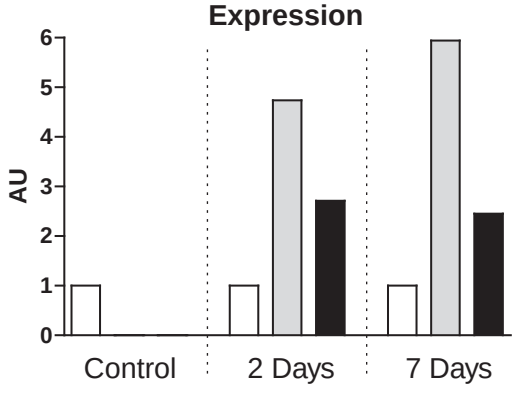


Figure 7. Quantitative PCR analysis of infarct samples. qPCR revealed expression of both the human household gene (grey bar) and recombinant GLP-1 (black bar) as compared to porcine household gene (white bar) in animals that received CellBeads after two and seven days compared to no expression in control animals. The values of the porcine household gene was arbitrarily set to one.

Discussion

The current study demonstrates for the first time that it is feasible to intracoronarily infuse alginate, MSC-containing micro-spheres in infarcted myocardium. Infusion did not permanently affect coronary flow, resulted in precise targeting of the infarct area, and MSCs remained viable for at least seven days. Interestingly, the inflammatory response to CellBeads was negligible in this xenogeneic transplantation model.

We found that intracoronary infusion of significant numbers of 170 µm particles did not severely hamper coronary flow. On the contrary, CFR remained unchanged in all animals that received 60,000 CellBeads or control beads, suggesting little or no micro-vascular obstruction. And although coronary flow as assessed by angiography was slightly decreased to sluggish flow in 40% of the animals, it always returned to normal flow within two or seven days follow-up. This suggests that, even though the coronary vasculature might have been partly obstructed by the beads, this obstruction was temporary, and that alternative collateral routes were found to perfuse the inflicted myocardium. Also, we hypothesize that infusion of limited amounts of obstructive beads into already infarcted myocardium will not increase ischemic damage. Importantly, we did not see any pro-arrhythmic effects of the infused beads. Although longer term follow-up is warranted to draw firm conclusions, these data add to the safety profile.

The current approach is the first that guarantees high stem cell retention. In fact, as 170 µm CellBeads are trapped in the arteriolar tree of the myocardium and can not pass the capillary bed, retention will approximate 100%. Because CellBeads contain ~80 MSCs per bead, infusion of 60,000 beads translates to almost five million delivered MSCs that remain in the targeted area for a substantial period of time. For reference, in cell therapy studies performed thus far, maximally 200 million bone-marrow derived mononuclear cells were transplanted (1,21). Of these cells, 0.001-0.01% comprises MSCs (4), rendering only 20,000 MSCs infused at the best. Retention rates of 1-10% results in negligible cell numbers as opposed to the approach investigated in the current study.

In addition, we show that a substantial amount of the MSCs in the CellBeads remain viable and transcriptively active for at least seven days. In this period of time, the MSCs secrete MSC-derived soluble factors that have been shown to exert cardio-protective and pro-angiogenic effects (3,13,16,20,25). Also, our cells produce therapeutic amounts of GLP-1 that has beneficial, anti-apoptotic effects on the post-AMI heart (18,24). CellBeads are thus small factories of GLP-1 and MSC-derived factors that might have synergistic, favorable effects on cardiac scar tissue formation and adverse remodeling.

Indeed, in our experiments, infusion of CellBeads significantly reduced apoptosis in the infarct area after two and seven days when compared to control alginate beads. This is in line with one of the presumed working mechanisms of GLP-1, and suggests that GLP-1 is factually secreted into the area at risk of cardiomyocyte death following ischemia/reperfusion. The additive effect of GLP-1 remains hypothetical though, as in this pilot study the appropriate control group (MSC that do not express GLP-1) is lacking. However, qPCR analysis confirmed expression of recombinant GLP-1 and human genes in porcine tissue even after seven days.

The fact that there is a significant difference in inflammatory infiltration between CellBeads and control alginate beads is another direct validation of our paracrine hypothesis. Immunomodulatory cytokines like IL-6,IL-8 and IL-10 are secreted by the MSC and prevent the local acute immune reaction and rejection against these human-derived cells. In fact, we observed significantly less immune reaction against the CellBeads than against the biocompatible alginate control beads (9) that evoked some foreign body response in the highly inflammatory post-AMI environment.

There are two major limitations to this study. First, the short follow-up time of at most seven days does not rule out long-term safety issues. Second, the current study did not thoroughly investigate the biodistribution of the CellBeads. Although we hypothesize that retention of the CellBeads in the target area will approximate 100%, additional studies in the near future using nuclear imaging techniques will render more definite answers to this question. This study was designed as a feasibility study and was not aimed at investigating long-term effects and biodistribution. Nonetheless, the results of this pilot study are promising and future studies will focus on long-term safety effects and signs of efficacy. We are currently enrolling over 70 pigs in a large dose-finding and efficacy trial, in which we investigate three different doses of CellBeads versus three control groups. This study will answer questions concerning long-term safety, but also concerning the effect of CellBeads containing MSC that do not express GLP-1 and possible adverse effects caused by the CellBeads, by increased ischemic damage and vascular obstruction.

Conclusion

We show that intracoronary infusion of CellBeads is feasible and appears to be safe in a large animal model of AMI. CellBeads were successfully targeted to the infarct area and MSC remained viable and active for at least seven days. Also, several parameters indicate that the cells sort a paracrine effect, highlighting the potential for cardiovascular repair of this new therapy.

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Chapter 10

Intracoronary infusion of encapsulated GLP-1 eluting mesenchymal stem cells preserves left ventricular function in a porcine model of acute myocardial infarction

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Abstract

Rationale Engraftment and survival of stem cells in the infarcted myocardium remain problematic in cell-based therapy for cardiovascular disease. To overcome these issues, encapsulated mesenchymal stem cells (eMSC) were developed that were transfected to produce glucagon-like peptide-1, an incretin hormone with known cardioprotective effects, alongside MSC endogenous paracrine factors.

Objective This study was designed to investigate the efficacy of multiple doses of intracoronary infusion of eMSC in a porcine model of acute myocardial infarction (AMI).

Methods and Results One-hundred pig were subjected to a moderate AMI (posterolateral AMI; n=50) or a severe AMI (anterior AMI; n=50), whereupon surviving animals (n=36 moderate, n=33 severe) were randomized to receive either intracoronary infusion of 3 incremental doses of eMSC or Ringers' Lactate control. Cardiac function was assessed using invasive hemodynamics, echocardiography and histological analysis.

A trend was observed in the moderate AMI model, whereas in the severe AMI model, left ventricular ejection fraction improved by +9.3% ($p<0.001$) in the best responding eMSC group, due to a preservation of left ventricular end-systolic volume. Arteriolar density increased 3-fold in the infarct area (8.4 ± 0.9/mm^2 in controls, versus 22.2±2.6/mm^2 in eMSC group; $p<0.0001$), capillary density increased by 30% in the border zone (908.1±99.7/mm^2 in control versus 1209.0±64.6/mm^2 in eMSC group; $p=0.02$). Simultaneously, cardiomyocyte apoptosis was reduced in the border area (7.5 ± 2.0% in control versus 2.4 ± 0.7% in eMSC group; $p<0.05$).

Conclusion Encapsulated MSC enable long-term local delivery of cardio-protective proteins to the heart , thereby enhancing angiogenesis and preserving contractile function in an animal AMI model.

Introduction

Regenerative stem cell therapy to promote cardiac repair has been a target of interest in the last 10 years to prevent heart failure after an acute myocardial infarction (AMI).[1,2] Various different stem cells have been investigated for their ability to repair the heart. Mesenchymal stem cells (MSC) seem to be a potent candidate to date. The mechanism of action of MSC is primarily based on the release of paracrine factors to the myocardium.[3] However, retention and survival of stem cells in the myocardium after intracoronary infusion (IC) remains an issue in cell-based therapy, since only a limited number of surviving stem cells remain in the myocardium, thereby limiting the potential benefit of the therapy.[4–6]

CellBeads™, that consist of alginate-encapsulated MSC (BTG International Germany GmbH, Alzenau, Germany), were developed to improve survival of cells in the myocardium, thereby elongating the release of cardio-protective proteins into the infarcted myocardium. Encapsulated MSC (eMSC) are alginate microspheres that contain clusters of human MSC which are genetically modified to produce glucagon-like peptide-1 (GLP-1) alongside MSC endogenous paracrine factors that include vascular endothelial

Table 1. Outcome measures

Study 1: Moderate Posterolateral infarct model.
Primary outcome
LVEF and volumes over time as measured by 2D-echocardiography
Secondary outcomes
Mortality
Ventricular arrhythmias
Change in CFR after eMSC or placebo infusion
Infarct size
Collagen density in infarct, border and remote areas
Arteriole density in infarct, border and remote areas
Capillary density in border and remote areas
Cardiomyocyte apoptosis in border and remote areas
Study 2: Severe Anterior infarct model
Primary outcome
Change in LVEF and volumes over time as measured by 3D-echocardiography
Secondary outcomes
Mortality
Ventricular arrhythmias
Pressure volume loop analysis at 8 week FU
Infarct size
Collagen density in infarct, border and remote areas
Arteriole density in infarct, border and remote areas
Capillary density in border and remote areas
Cardiomyocyte apoptosis in border and remote areas
Cardiomyocyte size in border and remote areas

Overview of primary and secondary outcome measures in both studies. LVEF indicates left ventricular ejection fraction; CFR: coronary flow reserve

growth factor (VEGF), monocyte chemotactic protein-1 (MCP-1), interleukin (IL)-6, IL-8, glial-derived neurotrophic factor (GDNF) and neurotrophin-3 (NT-3).[7–11] Amongst its beneficial effects in type 2 diabetes, GLP-1 has anti-apoptotic and cardio-protective properties.[12–18] Infusion of a GLP-1 analogue, exenatide, after AMI resulted in a reduction of infarct size, thereby improving cardiac function in a preclinical and clinical setting.[12–18] However, GLP-1 has a short half-life in vivo, therefore infusion directly at target site or, in case of the GLP-1 eluting eMSC, production directly on-site could render a long-term release of GLP-1. Encapsulated MSC have a diameter of 170 micron what will result in entrapment in the coronary system following IC infusion where they will locally elute cardio-protective GLP-1 alongside anti-inflammatory and pro-angiogenic paracrine factors.[9,11] The alginate shell surrounding the cells allows diffusion of oxygen and nutrients through the pores of the alginate shell into the MSC as well as diffusion of paracrine factors out of the bead. Moreover, the alginate shell protects the MSC against a host immune respons.[11]

Previously, IC infusion of up to 60,000 eMSC in naïve and infarcted porcine myocardium was well tolerated without any sign of microvascular obstruction.[9] The alginate-encapsulated MSC remain viable for at least 7 days, still secreting the recombinant GLP-1 and MSC paracrine factors.[9]

In this study, we aimed to explore the long term safety, feasibility, and efficacy of incremental doses of intracoronary delivered encapsulated MSC in a porcine AMI model. The primary endpoint in this study was cardiac function as assessed by echocardiography. The secondary endpoints were cardiac contractile function as measured by PV-loop analysis, infarct size, collagen density, capillary density, arteriole density, apoptosis and cardiomyocyte size (table 1).

Materials and Methods

A total of 100 female Landrace pigs (Van Beek, Lelystad, The Netherlands; 70 ± 5 kg) were randomized in this study. All animal experiments were performed according to the "Guide for care and the use of laboratory animals" and all experiments were previously approved by the institutional animal welfare committee of the University of Utrecht, Utrecht, The Netherlands. The efficacy of IC administered eMSC was investigated in a moderate size infarct (posterolateral AMI (LCx-model; study 1) and in the second phase in a severe anterior AMI model (LAD-model; study 2).

Experimental design

The design of this study is summarized in figure 1. Briefly, 100 female pigs underwent an AMI (50 in each group. Pigs that survived infarct induction (n=73; n=36 in the moderate infarct study and n=33 in the severe AMI study), were divided into 4 groups in each study to receive either IC infusion of eMSC or Ringers' Lactate control solution. Cardiac function was assessed by echocardiography and pressure-volume-loop (PV-loop) analysis (in the severe anterior model only). Eight weeks after infarct induction animals were terminated and the hearts were excised for infarct size calculations and histological analysis.

Encapsulated Mesenchymal stem cells

The eMSC that were used in this study had an outer diameter of 170 µm and contained 75 human MSC stably lentivirally transfected to release GLP-1(CellBeads™, BTG Germany, Alzenau, Germany) .[7,9,19] Encapsulated MSC were rapidly thawed and dissolved in 100 ml Ringers' Lactate (RL) just prior to IC infusion. A final concentration of 200 eMSC/mL to 600 eMSC/mL, depending on the dose, or 100 ml of RL were IC infused in 50 minutes at an infusion rate of 2 mL/min via a micro-catheter located in the target vessel (Twin Pass catheter, Vascular Solutions, Minneapolis, USA). All solutions were color coded and administered in a blinded fashion.

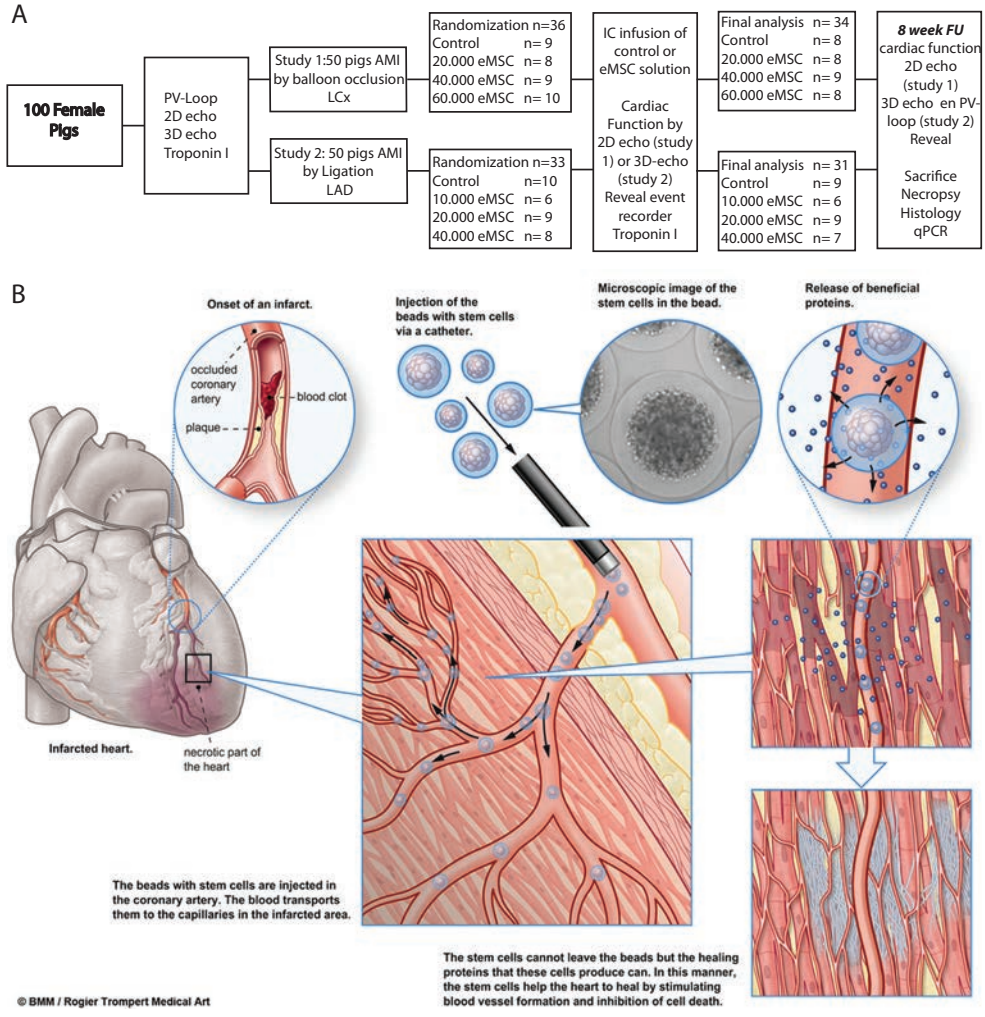

Figure 1. Study design. A: Flowchart of both studies. **B:** image of intracoronary infusion of encapsulated mesenchymal stem cells (eMSC) via a micro-catheter into the target vessel. Following infusion, eMSC will be retained in the vascular bed, behaving like micro-factories that release paracrine factors for cardiac repair. IC indicates intracoronary; LAD: left anterior descending artery; LCX; Left circumflex artery; PV-loop: pressure-volume loop; qPCR: quantitative polymerase chain reaction.

Study 1. Moderate posterolateral infarct model

Infarct induction

All pigs were prepared, anesthetized, intubated and ventilated according to a standardized protocol described in the supplemental data section. An 8F sheath (Cordis, Miami, USA) was introduced into the carotid artery and an angiogram of the left coronary tree was acquired using an 8F JL4 guiding catheter (Boston scientific, Natick, USA). A posterolateral myocardial infarction was induced by inflation of an angioplasty balloon in the proximal LCx for 90 minutes (Trek, 3.5-4.0x12, Abbott, Illinois, USA). Animals were randomized into 4 groups: 20,000 eMSC (n= 8), 40,000 eMSC (n=9), 60,000 eMSC (n=10) or RL control solution (n=9). Thrombolysis in myocardial infarction (TIMI) flow in epicardial coronary arteries was registered pre-, during and post-infusion of eMSC to rule out potential microvascular obstruction (MVO). To further quantify MVO, coronary flow reserve (CFR) was measured before and after eMSC infusion (ComboWire, Vulcano, Zaventem, Belgium, see supplement). All animals received a Reveal™ event recorder for the detection of arrhythmic events (Medtronic, Tilburg, The Netherlands). Cardiac function was assessed using 2D-echocardiography at baseline, after infarct induction and at 8 week FU. Left ventricular ejection fraction (LVEF) and LV volumes derived from 2D-echocardiography were calculated by the modified Simpson rule (see supplement).[20] All analyses were performed by an investigator blinded for the therapy allocation.

Study 2: Severe anterior AMI model

A severe anterior AMI was induced by a sternotomy and ligation of the mid LAD distal to the first diagonal for 90 minutes using a prolene ligature. Due to the open chest procedure, epicardial 3D-echocardiography was performed after reperfusion and animals with an LVEF higher than 45% were excluded from the study (n=4). The remaining animals were randomized into 4 groups: 10,000 eMSC (n=6); 20,000 eMSC (n=9); 40,000 eMSC (n=8) or RL buffer as control solution (n=10). Based on the results of study 1, we decided to infuse lower doses of eMSC in this study. A Twin pass catheter was then positioned in the target vessel and placebo or cell solutions were administered at a fixed infusion rate of 2 mL/min. TIMI flow was assessed before during and after infusion of eMSC or placebo solution. Three-D-echocardiography was performed in this study at baseline, after infarct induction and at 8 week FU. Due to the anatomical position of the porcine ribs, it is not possible to obtain clear 3D-echocardiography images in a close chest model nor is it possible to obtain 2D-echocardiographic images directly after open chest surgery. We therefore decided to perform 3D-echocardiography in this study opposed to 2D-echocardiography in the moderate infarction model. The protocol is described in the online supplement. A Reveal™ recorder was implanted for the detection of arrhythmic events. At 8 week follow-up, PV-loop analysis was performed to obtain data regarding cardiac contractility. The exact protocol has been described elsewhere.[21] Briefly, an admittance catheter (SciSence, London, Canada) was inserted into the left ventricle via the aortic valve.[21] For each measurement, 10 consecutive cardiac cycles were recorded under breath hold. To assess myocardial contractility, caval vein occlusions were performed by ligation of the inferior vena cava until the pressure drop was >50% (see supplement).

Study 1 and 2: Eight weeks post intervention (t= 56 ± 3d)

At 8 weeks post MI, animals were anesthetized, intubated and ventilated according to institutional protocol (see supplement). The Reveal™ event recorder was interrogated. After assessment of cardiac function as described above, animals were terminated and the heart was excised. The left ventricle was separated from the right ventricle and sliced into 5-6 slices of approximately 1 cm thickness as described before.[22] Slices were inspected for the presence of potential ectopic micro-infarctions in non-infarcted segments. Slices were weighed and photographed for infarct size calculations. Biopsies of infarct area, border zone and remote areas were obtained and embedded in paraffin for further histological analysis. Additional biopsies were snap frozen for RNA retrieval and qPCR analysis. Biopsies of the lung, liver, spleen and kidney were taken to exclude shedding of eMSC to remote organs.

Infarct size calculations

Infarct size as percentage of ventricle area was calculated as described before [16,22].

Histological analysis

Paraffin embedded biopsies were sectioned into 5 µm slices (see supplement). To analyze collagen deposition, a Trichrome stain was performed whereupon photographs were made at a 10X magnification that were analyzed using automated image analysis software (Clemex Technologies Inc. Quebec, Canada). Capillary density was determined using an Isolectin B4 staining, and arteriole density was assessed using smooth muscle actin stain (see supplement). Arterioles and capillaries were expressed as number per mm^2. A Terminal deoxynucleotidyl transferase dUTP nick-end labeling assay (TUNEL stain) was performed to quantify for analysis of cardiomyocyte apoptosis and was expressed as percentage apoptotic cardiomyocytes per view.

Quantitative PCR analysis

Quantitative PCR analysis was performed to quantify expression of human GLP-1 and BNP (online supplement for detailed description).

Statistical analysis

Continuous data are presented as mean ± standard error of the mean (SEM). Comparison of means between groups was performed using a one-way-ANOVA, followed by student t-tests to detect differences between groups and dose groups individually. A Mann-Whitney-U test was applied for non-parametric data. P-values < 0.05 were considered significant. All analyses were performed using IBM SPPS Statistics 20 (Chicago, USA).

Results

Study 1: Moderate Posterolateral AMI model

One animal in the control group died of VF one day after the infarct procedure. Two animals in the 60.000 group died of VF, 1 and 5 days following the infarct procedures respectively. None of the

animals needed to be treated for heart failure during follow-up and ventricular arrhythmias were not detected by the Reveal™ event recorder during the 8 week follow-up period in both groups (Figure 1).

Coronary flow

Infusion of the 170 µm eMSC visually did not impede antegrade coronary flow up to infusion of 60.000 eMSC as suggested by TIMI flow analysis. However outwash of contrast was slower in 2/8 pigs in the 60.000 group and 1/9 animals in the 40.000 group. CFR did not change following eMSC infusion confirming that MVO did not occur to a significant level directly following infusion (Figure 2). Based on these results, it was decided to omit CFR measurements in study 2. At 8 weeks follow-up, antegrade flow remained within TIMI 3 range in all groups. The outwash of contrast was impeded (TIMI 2) in 5/8 animals in the 60.000 eMSC group, 3/9 animals in the 40.000 eMSC group and none of the animals in the 20.000 group.

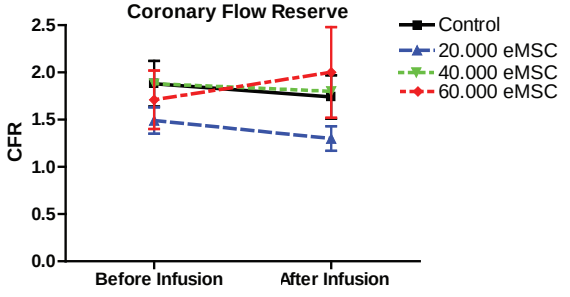

Figure 2: Coronary flow Reserve. Coronary flow reserve after AMI before and after placebo or encapsulated MSC (eMSC) infusion.

2D-Echocardiography

After infarct induction, no differences were observed on LVEF between groups, indicating comparable infarct size. At 8 weeks follow up, LVEF was not different between the control and treatment groups, but a trend was observed towards an improvement in the 20.000 eMSC group opposed to control (+6%; p=0.09; Figure 3; supplemental table 1).

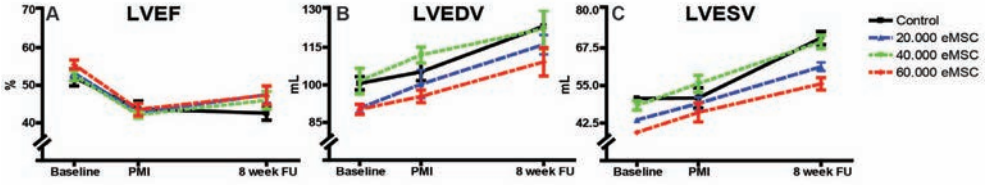

Figure 3. 2D-echocardiography of posterolateral infarct model. **A-C.** Left ventricular ejection fraction (LVEF) and volumes in posterolateral AMI model over time. eMSC: encapsulated mesenchymal stem cells; LVEDV: left ventricular end-diastolic volume; LVESV: left ventricular end-systolic volume; PMI: post myocardial infarct; FU: follow-up.

Infarct size

Infarct size was only 9.6 ± 1.3% in the control group opposed to 7.6 ± 1.2% in the 20,000 group (p=NS). The 40,000 and 60,000 group however, showed similar infarcts sizes as the ringer lactate control (9.1 ± 1.2%; 9.3 ± 1.8% respectively; figure 4A)

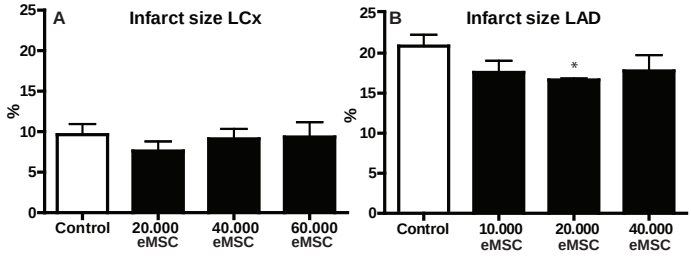

Figure 4. Infarct size. A: infarct size in posterolateral infarct model in different groups . **B:** Infarct size in study 2 was approximately 20% in control and was reduced by 25% in the optimal dose group. eMSC: encapsulated mesenchymal stem cell. *P<0.05

Histology

Capillary density in border and remote areas was not enhanced in all groups (Supplemental table III, supplemental figure I). However arteriolar density increased by almost 200% in the infarct area in the 20,000 group (22.7 ± 2.9 arterioles/mm^2 vs. 12.8 ± 1.1 arterioles/mm^2, p=0.01) compared to the control group (supplemental figure I C and D, Supplemental table III), whereas eMSC did not enhance arteriole formation in the border and remote segments. Moreover collagen deposition and cardiomyocyte apoptosis were not affected (supplemental figure IE-F).

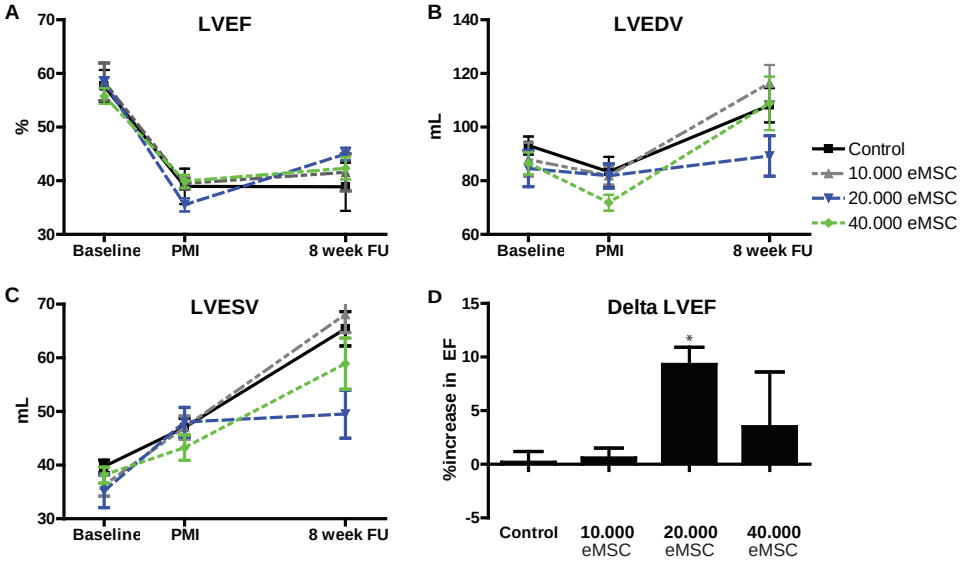

Figure 5. 3D-echocardiography anterior infarct model. **A:** Left ventricular ejection fraction (LVEF) measured by 3D-echo over time. **B:** Left ventricular end-diastolic volume (LVEDV) over time. **C:** Left ventricular end-systolic volume (LVESV) over time. **D:** delta LVEF represents the absolute difference between LVEF at 8 weeks and directly following AMI. There is a remarkable 9.3% increase in LVEF in the 20.000 group. eMSC indicates encapsulated mesenchymal stem cells. *p<0.05

Study 2: Severe Anterior AMI model

One animal in the control group in the died of VF one day after the infarct procedure. None of the animals needed to be treated for heart failure during follow-up and ventricular arrhythmias were not detected by the Reveal™ event recorder (Figure 1). Outwash of contrast was decreased (TIMI 2) in 43% of the animals in the 40.000 group and none of the animals in the other groups directly post infusion and at 8 weeks FU.

3D-echocardiography

LVEF at baseline was comparable between the groups (supplemental table I, figure 5). After ligation of the LAD for 90 minutes, LVEF was comparable between the groups. At 8 weeks follow up, LVEF increased by +0.2 ± 1.0% in the control group to 38.9 ± 1.5%. 20,000 eMSC improved LVEF by +9.3% to 44.7 ± 1.2 % (p=0.003; supplemental table I). The increase in LVEF was mainly due to a preservation of LVESV, which decreased by -2.9 ± 4.8 ml (20,000 eMSC) compared to an increase of +18.1 ± 4.5 ml in control animals (figure 5).

Pressure-Volume loop analysis

End-systolic pressure volume relationship (ESPVR), which reflects systolic contractile function, was dramatically increased by 230% in all treatment groups as opposed to control animals (6.5 ± 1.3 mmHg/ml in 20,000 eMSC group vs. 1.9 ± 0.5 mmHg/ml in control; p=0.008). No differences were observed in other PV-loop derived parameters (figure 6, supplemental table II).

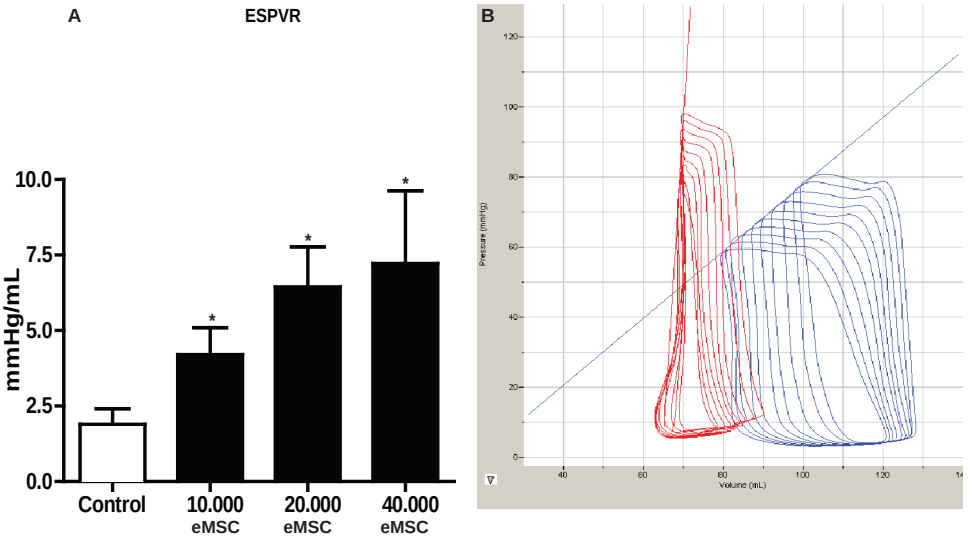

Figure 6. Pressure volume loop analysis of ESPVR. **A:** End-systolic pressure volume relation (ESPVR) is enhanced in all encapsulated MSC (eMSC) groups. **B:** Actual vena cava occlusion of a control animal (blue) and 20.000 eMSC animal. *P<0.05

Infarct size

Infarct size decreased by -20% in the 20,000 eMSC group (16.7± 0.2% vs. 20.5 ± 1.4% in the control group; p=0.05). No significant reduction in infarct size was observed in the other treatment groups (figure 4B, supplemental table III).

Histological analysis

Capillary density was higher in the best responding dose group (1209.0 ± 64.6/mm^2 in 20,000 eMSC group compared to 908.1 ± 99.6/mm^2 in the control group; p=0.02; 20,000 eMSC;figure 7). The other dose groups did not enhance capillary formation, nor increased the capillary density in remote areas.

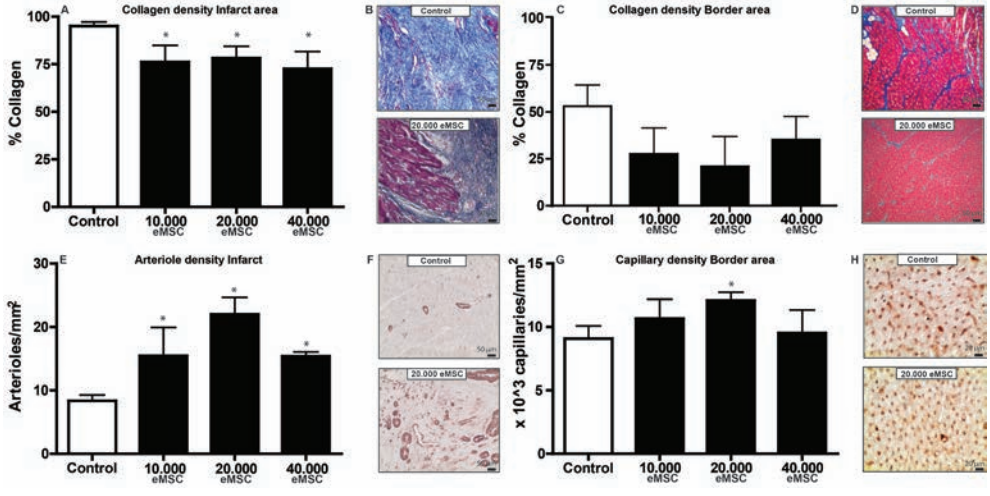

Figure 7. Histological analysis. A-D: Trichrome stain for detection of collagen. **A:** Collagen density in infarct area is decreased in all animals treated with encapsulated mesenchymal stem cells (eMSC). **B:** Example of Trichrome stain infarct area. Pictures are taken at a 10 times magnification. Blue represents collagen, pink viable myocardium. Collagen deposition is more dens in the animals in the control group opposed to animals in the 20,000 eMSC group. **C:** Collagen density border area was not enhanced in treated animals s. **D:** Example of Trichrome stain in border area. **E-F:** Arterioles were stained by smooth muscle actin stain. **E:** In the infarct area, arteriole density was enhanced in treated animals. **F:** Representative images of smooth muscle actin stain in infarct area in a control animal and in a 20,000 eMSC animal at 10 times magnification. G-H: Isolectin stain detect capillaries. **G:** Capillary density was improved in 20.000 eMSC group as opposed to control. **H:** Representative images of Isolectin stain in the border area of a control animal and a 20,000 eMSC animal respectively at 20 times magnification. *P<0.05

Arteriolar density improved in the infarct area in all doses by +200-300% (22.2 ± 2.6/mm^2 versus 8.4 ± 0.9/mm^2; p<0.05; figure 7; supplemental table III).

Treatment with 20,000 eMSC reduced cardiomyocyte apoptosis by -66% (2.4 ± 0.7% vs. 7.5 ± 2.0%; p=0.002; Supplemental table III, figure 8), whereas apoptosis was not reduced in any other group.

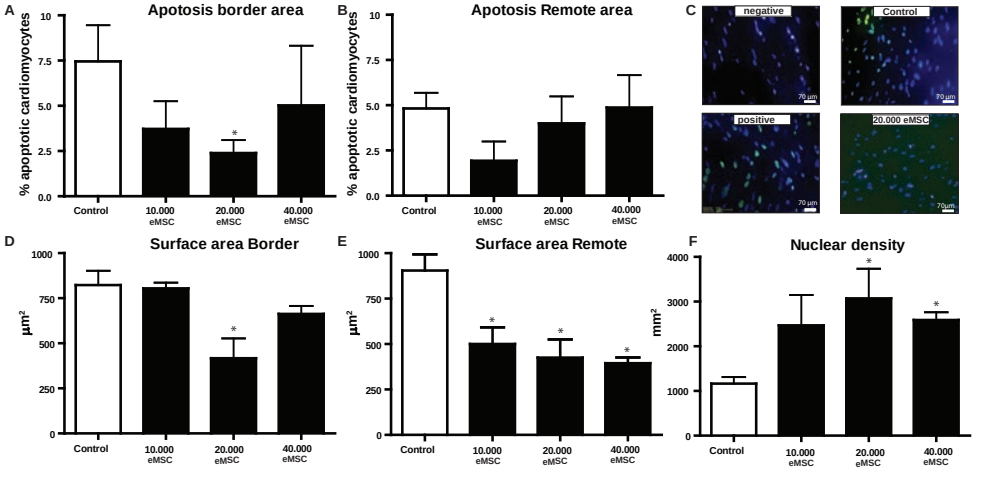

Figure 8. A-C: Cardiomyocyte apoptosis via TUNEL stain in the border area and remote areas. Apoptosis was slightly reduced in the border area in the 20.000 group but not in the other groups. **C:** Example of Fluorescent TUNEL stain. Blue cells are alive, green cells are apoptotic.
D-F: Cardiomyocyte size in the border area was significantly lower in the 20.000 group (**D**). In the remote myocardial segments, the surface area was lower in all animals treated with eMSC (**E**). This finding corresponds with an increased nuclear density in the highest dose groups in remote areas (**F**). *P<0.05 eMSC indicates encapsulated mesenchymal stem cells

Collagen content in the infarct area was reduced in all treatment groups (supplemental table III). Collagen density in the border zone did not show significant differences, whereas a trend was observed in remote myocardial segments (figure 7).

The surface area of the cardiomyocytes in the infarct border zone of animals treated with 20.000 eMSC was almost 50% lower than in control and the other dose groups (417.1 ± 110.2 μm^2 in 20.000 group versus 823.1 ± 74.0 μm^2 in control ; p=0.009; figure 9). The same was observed in the remote myocardial segments.

Survival of encapsulated MSC

At 8 weeks FU, neither human housekeeping gene GAPDH nor human GLP-1 expression could be detected by qPCR, indicating that the MSC inside the beads do not elute paracrine factors at 8 week FU.

Discussion

In the current study, we investigated the efficacy and the optimal dose of encapsulated immunoprotected MSC in a porcine model of AMI. This is the first time that the efficacy of microencapsulated MSC was investigated for treatment of AMI. In the moderate infarct study, only a trend towards an improvent of cardiac function was observed. Histological analysis revealed only an increase in arteriole density in the infarct area. In the severe infarct study, LVEF was remarkably improved following eMSC infusion. Moreover, infarct size was reduced, contractility was enhanced and neovascularization occurred.

Safety

In line with our previous results, eMSC infusion did not show side-effects in a preclinical setting.[9] Previous studies suggested a reduction in TIMI flow and CFR following intracoronary infusion of mesenchymal stem cells.[5,23,24] In this study, only outwash of contrast was slower in animals in the high dose group, what indicates that some MVO occurs. It seems that this can be attributed to the absolute number of eMSC infused. Studies that report MVO after infusion of MSC, usually apply high doses what could eventually result in obstruction. Several other preclinical and clinical studies have shown that under carefully controlled and monitored condition however, e.g. slow infusion rate and low cell number, infusion of MSC or MSC like stem cells or cardiospheres is well tolerated.[22,25,26] In the case of eMSC the same assumption could be made.

Efficacy

When given IC, alginate encapsulated MSC significantly improve LVEF after an AMI. In the moderate infarction model we detected a trend, which was further explored in a more severe AMI model. As expected, the treatment effect turned out to be more pronounced in animals with more severe AMI, presumably due to a larger pharmacological window in the anterior model. This corresponds with a large meta-analysis by Jeevanantham *et al* who concluded that patients with a LVEF blow 43% benefit more from cell-based cardiac repair.[1] However, another meta-analysis failed to show a more prominent treatment effect of BMMNC therapy in patients with lower LVEF. If a treatment effect is already noticeable in a small infarct, the benefit will be expanded in a large infarct.

In absolute terms, IC infusion of eMSC improved LVEF by +9.3% in severe anterior AMI model, which represents a significant improvement compared to the previously reported 2.14 - 3.48% increase after infusion of BMMNC.[1] The most effective dose in our study was 20,000 eMSC what equals 1.6 million MSC, which is several orders of magnitude less than used in most preclinical trials using cultured MSC. However, eMSC are better retained into the myocardium than unprotected stem cells and rendering in prolonged protein release. This complicates direct comparison. Infusing less than 20,000 eMSC was not effective, suggesting that this dose is the lower limit. On the other hand, 40,000 eMSC were not

superior to lower doses, which does not preclude a dose dependent effect but more likely reflects unfavorable effects of incremental MVO. This was confirmed by the finding that outwash of contrast was impeded in animals in higher dose groups. MVO could have resulted in an increase infarct size beyond the reparative mechanism of the eMSC.

Working mechanism

Several preclinical studies showed that MSC therapy after AMI enhanced the formation of arterioles and capillaries by a release of paracrine factors.[22,27] MSC inside the alginate beads have a comparable pro-angiogenic effect.[28] Moreover, eMSC also improved angiogenesis in a hind-limb ischemia model and in porcine interposition grafts.[10,28,29] This pro-angiogenic effect might explain the observed preservation of cardiac function.[28,29]

Moreover, eMSC treatment reduced fibrosis and improved myocardial viability in the infarct zone, effectively reducing total infarct size.[3,22,30] Secretion of immunomodulatory cytokines by MSC, like IL-6 has been shown to increase the lifespan of neutrophils in the hostile post-AMI environment and improves healing of the infarct wound.[31] In addition, MSC trigger the transition of classical M1 to anti-inflammatory M2 macrophages, thereby enhancing infarct healing via increased angiogenesis.[31]

Alongside pro-angiogenic factors and immunomodulatory cytokines, MSCs secrete anti-apoptotic factors that improve cell survival.[22,30] Additionally, eMSC in this study were transfected to secrete recombinant GLP-1. Exenatide, a GLP-1 analogue, has shown to reduce infarct size and improve cardiac function in a pig AMI model.[16] Moreover, in 2 recent clinical trials in which exenatide was injected in AMI patients (TIMI 0-1 flow) before PCI, infarct size was reduced by 50% what indicates that Exenatide prevents reperfusion mediated cell death.[17,18] As eMSC are infused within 30 minutes after reperfusion, the effect on infarct size could be related to limitation of reperfusion damage.

Next to the anti-apoptotic effects of GLP-1, which extended to the 8 weeks follow up, GLP-1 has been shown to directly improve cardiac contractility by increasing intracellular cyclic-AMP concentrations.[32] This latter effect could be responsible for the significant ESPVR improvement in all eMSC groups. The observed preservation in contractility could also be explained by strengthening of the heart's matrix by the infusion of alginate thereby preventing LV remodeling as previously was shown.[33,34] However, in these studies liquid alginate was used that diffuses through the vessel wall.[33] Although eMSC cannot leave the vessel lumen, they seem to provide structural support to the myocardium. There was no dose response relationship, what would be expected. The functional benefit in higher doses were actually less pronounced, probably due to incremental MVO as discussed before

The heart contains a population of resident cardiac stem cells.[35] It is hypothesized that MSC can activate these stem cells in order to stimulate infarct repair following an ischemic event. Suzuki *et al* concluded from a porcine study, in which MSC were IC injected following AMI that MSC stimulate

endogenous cardiac stem cells to home to the site of injury and differentiate into cardiomyocytes.[27] In addition to this, the MSC in their study differentiated into cardiac stem cells. Moreover, cardiomyocytes were stimulated to proliferate in animals that were treated with MSC. All these effects combined resulted in an increased cardiomyocyte nuclear density. In our study, cardiomyocyte nuclear density is increased in border an remote areas what indicates that eMSC stimulate myocardial salvage what results in a reduction of compensatory hypertrophy. Myocardial salvage in this study is based on a reduction of apoptotis by GLP-1 and most likely proliferation of adult cardiomyocytes. It was recently shown, that IL-6 secreted by MSC-like stem cells, stimulated cardiomyocyte proliferation.[36] As the eMSC produce IL-6, this would be the proposed working mechanism of cardiomyocyte proliferation in this study. Differentiation of MSC inside the beads could not have contributed to the increased number of cardiomyocytes, because MSC do not leave their shell. Activation of endogenous stem cells also remains a proposed mechanism of action. As was shown by Suzuki *et al* and Houtgraaf *et al* activation of resident cardiac stem cells is not detectable after 6 weeks following transplantation, the c-kit stain was omitted from current protocol.[22,27] Moreover, cardiomyocyte proliferation could be not detected at 8 week FU in a previous study that used MSC.[22] Therefore ki-67 stain was not executed.

Study limitations

Despite our best efforts, this study has some limitations. First, cardiac function was not assessed by golden-standard cardiac MRI. However, echocardiographic measurements have shown excellent correlations with MRI. We are therefore confident that our findings would be corroborated by MRI.[37]

In the two substudies we used slightly different protocols. In the more severe anterolateral model we used an open chest procedure to minimize mortality. This open chest procedure enabled us to use epicardial 3D-echocardiography instead of the transthoracic 2D-echocardiography used in the moderate posterolateral infarction model. Although the two methods have shown to be in good concordance with one another, values should not be directly compared.[38,39]

Second, MVO was only investigated by CFR measurements and TIMI flow not directly by measuring hyperemic microvascular resistance. CFR and antegrade flow were however not affected, making severe obstruction unlikely.

Third, we did not include all possible controls including eMSC not transduced to produce GLP-1, empty beads or MSC only. This decision was based on our previous pilot data where we found beneficial effects on apoptosis and inflammatory response of eMSC opposed to empty alginate beads without MSC. Moreover, in a proof of concept study by Wright *et al* IC infusion of macro-eMSC resulted in preserved LVEF and reduced infarct size when compared to empty alginate beads without cells and encapsulated human MSC that were not transduced to express GLP-1, suggesting that these beads do not contribute to a beneficial functional effect.[10]

As the GLP-1 analogue produced by the eMSC consists of a GLP-1 fusion protein with a short half-life, a direct comparison with native GLP-1 or GLP-1 analogues with prolonged half-life would be difficult to interpret.

Here, the MSC are derived from humans. This makes sense as we aimed to test the efficacy of the clinical product. Further research is needed to test whether allogeneic encapsulated mesenchymal stem cells are equal or even a superior effect.

Conclusions

IC infusion of encapsulated GLP-1 eluting MSC resulted in a preserved LVEF in a porcine AMI model and is well tolerated up to 8 weeks FU. Infusion of eMSC improves cardiac function by preservation of end-systolic volume and cardiac contractility. Importantly, encapsulated MSC are an interesting platform that enables long term paracrine delivery of MSC proteins and recombinant proteins to the damaged myocardium. Encapsulated MSC are able to prevent cardiomyocyte loss, enhance adaptive neovascularization, thereby limiting left ventricular remodeling.

Acknowledgements

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SUPPLEMENTAL MATERIAL

Content

Material and Methods

Medication and Anesthesia

All animals were pre-medicated starting ten days prior to infarct induction with dual anti-platelet therapy ((acetylsalicylic Acid: 300 mg loading followed by 80 mg qd (Centrafarm, Etten-Leur, The Netherlands) and clopidogrel 75 mg qd(Sanofi-Aventis, Paris, France)) and anti-arrhythmic therapy (amiodarone, loading dose of 1200 mg followed by 800 mg qd; Sanofi-Aventis, Paris, France). Dual antiplatelet and anti-arrhythmic therapy was continued during follow up.

General anesthesia was induced with 0.4 mg/ kg midazolam (Actavis, Zug, Switzerland), 10 mg/kg ketamine (Narketan,Vétoquinol, Lure Cedex, France) and 1 mg of Atropine (Pharmachemie BV., The Netherlands) and maintained with intravenous infusion of midazolam 0.5 mg/kg/h, sufentanil 2.5 µg/kg/h (Janssen-Cilag B.V., Tilburg, The Netherlands) and pancuronium 0.1 mg/kg/h (Inresa, Battenheim, Germany). Upon infarct induction and Cellbead infusion, all animals were therapeutically heparinized with 2 doses of 5000 IE (Leo pharma, Ballerup, Denmark) IV and received intravenous infusion of eptifibatide (bolus of 180 µg/kg and 2 µg/kg/min (GlaxoSmithKline BV, Zeist, The Netherlands).

A fentanyl plaster 25 µg/h (Janssen-Cilag B.V., Tilburg, The Netherlands) was applied before and after the first procedure for analgesia. The animals were treated with one doses of Augmentin intravenously (1000/100 mg, Sandoz, Holzkirchen, Denmark) before the infarct procedure.

All animals in received meloxacam 2 times 0.5 mg/kg daily (Produlab-Pharma B.V. Raamsdonksveer, The Netherlands) for two days after index procedure for additional analgesia.

Coronary flow

In the first study, coronary flow was assessed after myocardial infarct and after the complete infusion of eMSC by coronary flow reserve measurements (CFR). Every 25cc infusion was stopped and TIMI flow was assessed according to the TIMI criteria.[1] During CFR measurements, APV was assessed in normal conditions after AMI and during maximum vasodilation following injection of 140 mcg/kg/min nitroglycerine IV. Peak APV was assessed and CFR was calculated.

Echocardiography

Two-dimensional grey scale images at a frame rate of 60-90 frames/s were obtained in parasternal position using a Philips iE33 (Phillips, Einhoven, The Netherlands), equipped with a broadband S5-1 transducer. Transthoracic echocardiographs were acquired at baseline, post myocardial infarct and eight weeks follow-up in the posterolateral infarct model. A long axis view and three levels of short axis view (basal, mid ventricular and apical levels) were obtained by acquiring three successive cardiac cycles.

Echocardiographic data was transferred to and analyzed using Image Arena 4.1 (Tomtec Imaging Systems, Uterschleissheim, Germany). This analysis was performed by an investigator blinded for the treatment groups. Regional left ventricular function was assessed by measuring the change in LV cavity area (fractional area change) during systole and diastole at basal (mitral valve level), mid ventricular (papillary muscle level) and apical level. Left ventricular volumes were then calculated by the modified Simpson rule: LV end diastolic volume (LVEDV)= (A_{bED}) * L/3 + (A_{mED}+A_{pED})/2) * L/3 + 1/3(A_{pED}) * L/3; LV end systolic volume (LVESV)= (A_{bES}) * L/3 + (A_{mES}+A_{pES})/2) * L/3 + 1/3(A_{pES}) * L/3, in which A_b is the area at basal level, whereas A_m and A_p are the areas at mid and apical level respectively.[2] L is defined as the length of the ventricle from apex to base. The length was obtained of 4 chamber view 3D echo in LAD study at baseline and follow-up. In the LCx study, the average length at baseline in the LAD study was used, whereas 3D-echocardiography was not possible to obtain in the LCx study. The length at follow up in the LCx study was obtained by counting up the thickness of the post-mortem heart slices. LVEF was calculated following standardized formula: ((LVEDV-LVESV)/LVEDV)*100.

3D-echocardiography

In study 2, epicardial 3D-echocardiographs were acquired with X-3 transducer using the iE33 ultrasound machine (Philips, Eindhoven, The Netherlands). The 3D-transducer (X-3, Philips, Eindhoven, The Netherlands) was wrapped in a sterile sleeve. A pocket of gel was positioned under the transducer, to bring the complete apex a vu. The transducer was positioned directly epicardially on the apex of the heart. We positioned the transducer in all animals in the same direction so we obtained a 4 chamber view. The depth and sector size were adjusted to fit the complete ventricle. In each pig, the data sets were acquired in real time using 7 consecutive cardiac cycles (full volume analysis).

The images were offline analyzed using QLab 10.1 (3DQ advanced) analysis software. The tracing of the ventricle was perfomed by semi-automatic border detection as described before by Soliman and coworkers. [3] Briefly, LV quantification starts by proper 4-chamber view and orthogonal views. The end-diastolic volume and end-systolic frames are identified and on both frames and the apex, anterior, lateral, inferior and septal mitral annulus is identified. Qlab 10.1 automatically traces the endocardial border. Traces that are unsatisfactory can be manually adjusted. The ejection fraction is calculated by the Qlab software as (LVEDV-LVESV)/LVEDV*100.

PV-loop measurement and analysis

In the severe infarct model, a 7F tetrapolar admittance catheter (7.0 VSL Pigtail, no lumen, Transonic, Scisence, London, Canada) was introduced in the left ventricle via the aortic valve, and the pigtail tip of the catheter was positioned in the apex. This catheter measures admittance magnitude and phase in combination with pressure. The catheter exists of 7 electrodes, that divide it into 4 segments. The largest segment that was positioned into the LV was used for the measurements. The catheter was connected to the ADVantage sytem (Transonic, Scisence, London, Canada) for real-time data assess-

ment.[4–6] PV loop measurements were performed at eight weeks follow up. In this study, contractility parameters and relaxation parameters were used in the analysis. LVEF and LV-volumes were assessed by 2D-echocardiography and 3D-echocardiography. Systolic PV-loop-derived parameters that were assessed and analysed in current study were: dP/dt max (maximum increase in pressure/s), end-systolic-pressure-volume-relationship (ESPVR), pre-recruitable stroke work (PRSW). Diastolic parameters that were determined were: Tau (an isovolumic relaxance constance), dP/dt min (maximum relaxation over time), end-diastolic pressure volume relationship (EDPVR).

Immuno-histochemical stainings

Collagen content, cardiomyocyte size and myocardial salvage index

Collagen content in infarct, border and remote myocardial segments was assessed via trichrome stain. Briefly, all sections were deparaffinised, fixated in Buoin's fixative (Sigma-Aldreich, St. Louis, USA) at 56º for 15 minutes, Nuclei were stained with haematoxylin for 3 minutes. The slides were submerged in Trichrome-AB solution for 5 minutes after which they were treated with 0.5% acetic acid for 1 minute. Slides were mounted with Entallan (Merck, Darmstadt, Germany). Three random pictures were made at a 10X magnification and collagen content was calculated as percentage collagen of total surface area using automated analysis software (Clemex, Quebec, Canada).

To calculate cardiomyocyte size, 3 random pictures of all slides of the border area and remote area were obtained at 40X magnification. The surface area was determined by the automated analysis software. Only transversely cut cardiomyocytes containing a nucleus were analysed.

Capillary and arteriole density

Arteriole density was obtained in infarct, border and remote myocardial segments, using alpha smooth muscle actin (SMA, clone 1a4, Sigma-Aldreich, St Louis, USA). Endogenous peroxidase activity was blocked by 3% Methanol/H_2O_2 solution for 30% and incubated with SMA 1:1000 overnight. Subsequently, the slides were incubated with secondary HRP-conjugated goat-anti-mouse dilution 1:200 (DAKO, Glostrup, Denmark) for 90%. All slides were immersed in DAB solution for 2 minutes (DAKO, Glostrup, Denmark) and mounted with Entallan. A technician that was blinded for the dose groups took 3 random pictures at 10 times magnification. Arterioles per pictures were counted and expressed as number per mm^2.

Capillary density was only assessed in border en remote areas, because almost all capillaries are destroyed after AMI. All sections were dewaxed and pre-treated with trypsin EDTA (Lonza, Verviers, Belgium). Endogenous peroxidase activity was blocked as described above. All slides were incubated with Isolectin B_4 (Bandeiraea simplicifolia Isolectin B_4, Dako, Glostrup, Germany) diluted 1/50 overnight. After that the slides were immersed in DAB solution at mounted with Entallan. Photographs were taken at 20X magnification and number per mm^2 was calculated.

Apoptosis

Apoptosis was quantified in all regions using In-Situ cell detection kit-FITC labelled(Roche, Basel, Swiss). The manufacturer's instructions were followed. After mounting with Vectashield with DAPI, 3 random photographs at 40X magnification were made, using un Olympus IX55 fluorescence microscope and number of apoptotic cells were counted. Apoptosis is depicted as percentage of apoptotic cells.

Quantitative PCR

The expression of human household genes, GLP-1 and BNP as markers for hypertrophy were investigated using qPCR analysis. RNA was isolated from snap frozen biopsies of infarct area, border zone and remote myocardial using RNA-Bee™ RNA Isolation Solvent (Tel-Test Inc., Friendswood, USA) according to the manufacturer's protocol. CDNA was created using Bio-Rad iScript™ cDNA Synthesis Kit (Bio-Rad, Veenendaal, The Netherlands), according to the manufacturer's instructions. The primers that were investigated can be found in the table below. qPCR was performed using SensiMix™ SYBR & Fluorescein Kit (Bioline, Boston, USA) and expression was detected by Bio-Rad MyiQ System Software of MyiQ™ Optical Module. The threshold cycle (Ct) values from interested genes were normalized to porcine Beta Actin expression(housekeeping gene) or human GAPDH (GLP-1).

Primers for qPCR	
Porcine housekeeping gene	
B actine forward	AAGAGCTACGAGCTGCCCGAC
B actine reverse	GTGTTGGCGTAGAGGTCCTTC
Human housekeeping gene	
GAPDH forward	GCTCATTTCCTGGTATGACAAC
GAPDH reverse	GAGGGTCTCTCTCTTCCTCTT
Recombinant GLP-1	
CM-2 forward	GTGAGCTCTTATCTGGAAGGCC
CM-2 reverse	AGATAAGAGCTCACATCGCTGG
BNP	
BNP forward	GCAGCAGCCTCTATCCTCTC
BNP reverse	TCCTGTATCCCTGGCAGTTC

Results

Brain Natriuretic Peptide (BNP) analysis

Blood was sampled at baseline and at 8 week follow up. The clinical chemical laboratory of University medical center in Utrecht and in Rotterdam, both tried to measure BNP levels using an ELISA. Unfortunately, this was not successful, therefore BNP levels were detected using qPCR analysis. There were no differences in qPCR levels of BNP between groups.

Necropsy

There were no signs of anatomical malformations by macroscopic analysis of the heart, liver, spleen, lungs and kidneys. There were no signs of macro-infarcts in remote areas of the heart.

Supplemental figure I: Histology study I: moderate infarct model

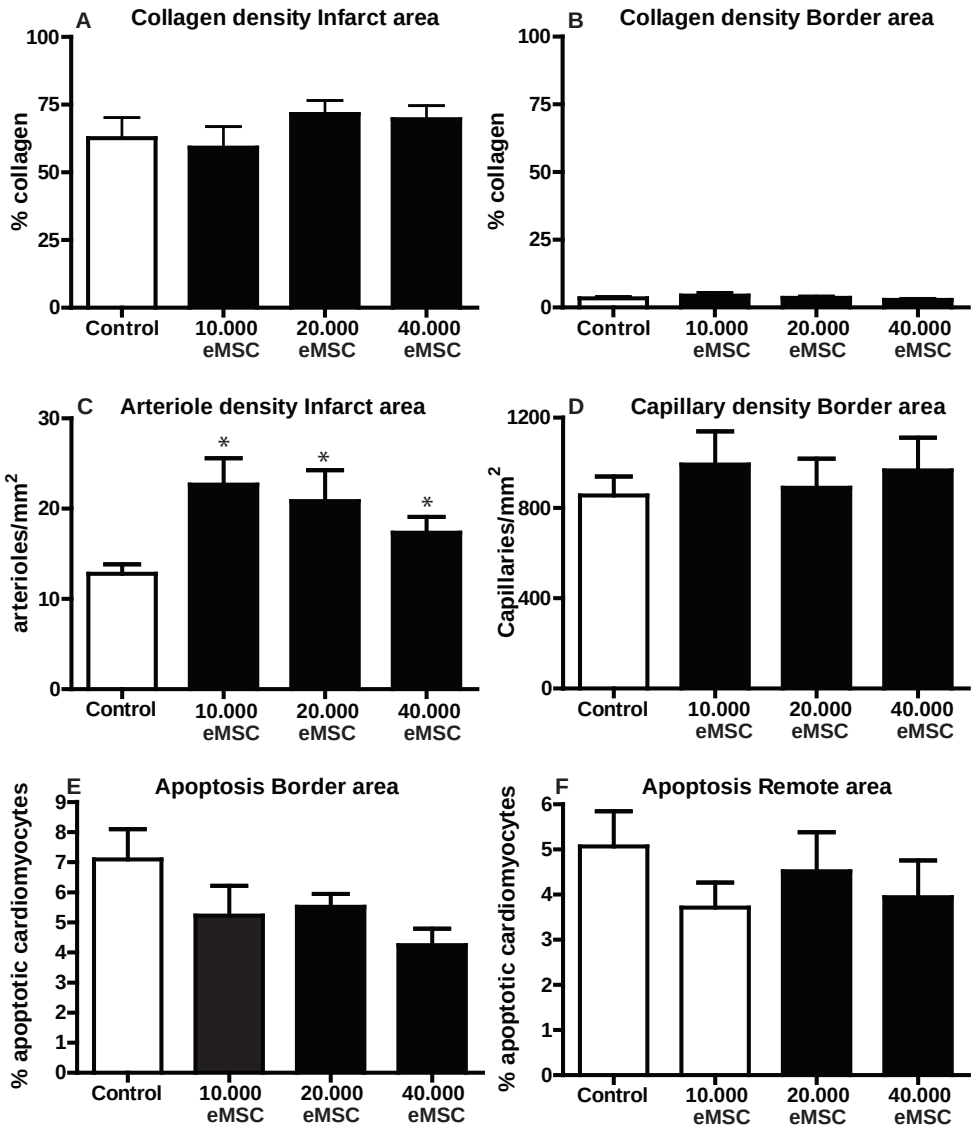

Figure I: Fibrosis, vascular density and apoptosis. **A-B:** Collagen density in infarct and border area in the first phase of the study. **C:** Arteriole density increased in infarct area in all dose groups opposed to control. **D:** Capillary density. **E-F:** Cardiomyocyte apoptosis in border and remote segments respectively. eMSC indicates encapsulated mesenchymal stem cells

Left ventricular weight

No differences were observed regarding left ventricular weight at 8 week follow-up in all groups in both studies.

Supplemental table I: Volumes and ejection fraction assessed by 2D and 3D echocardiography.

Study 1: Moderate infarct model					
2D-echo	**Control**	**20.000 eMSC**	**40.000 eMSC**	**60.000 eMSC**	**P -value ANOVA**
LVEF Baseline	51.2 ± 0.6	52.0 ± 0.2	52.7 ± 0.6	56.3 ± 0.5	NS
LVEDV Baseline	102.2 ± 1.0	90.7 ± 0.7	101.5 ± 2.2	90.2 ± 0.8	NS
LVESV Baseline	50.0 ± 1.0	43.5 ± 0.4	48.4 ± 1.5	39.4 ± 0.5	NS
LVEF PMI	43.7 ± 0.8	42.6 ± 0.4	42.1 ± 0.3	43.6 ± 0.7	NS
LVEDV PMI	105.2 ± 1.4	100.3 ± 2.0	111.9 ± 1.1	95.4 ± 1.0	NS
LVESV PMI	59.3 ± 1.2	59.0 ± 1.0	55.7 ± 1.0	55.7 ± 1.0	NS
LVEF 8W FU	42.6 ± 0.8	47.5 ± 0.7	46.0 ± 0.8	47.3 ± 1.0	NS
LVEDV 8W FU	123.4 ± 3.5	116.1 ± 1.4	122.0 ± 2.7	109.2 ± 2.3	NS
LVESV 8W FU	70.8 ± 2.2	61.2 ± 1.4	69.5 ± 2.2	55.5 ± 2.1	NS

Study 2: Severe infarct					
3D-echo	**Control**	**10.000 eMSC**	**20.000 eMSC**	**40.000 eMSC**	**P-value ANOVA**
LVEF Baseline	57.6 ± 1.0	58.5 ± 1.8	58.4 ± 1.0	55.8 ± 1.4	NS
LVEDV Baseline	93.1 ± 3.4	87.7 ± 7.9	84.6 ± 6.7	86.5 ± 4.1	NS
LVESV Baseline	39.7 ± 1.2	36.3 ± 2.5	35.1 ± 3.0	38.1 ± 1.5	NS
LVEF PMI	38.9 ± 1.1	41.9 ± 1.5	35.3 ± 1.2	39.9 ± 1.3	NS
LVEDV PMI	83.4 ± 5.5	81.9 ± 4.1	81.8 ± 4.5	71.8 ± 2.9	NS
LVESV PMI	51.0 ± 3.6	47.0 ± 2.6	52.0 ± 2.7	43.2 ± 2.4	NS
LVEF 8W FU	38.9 ± 1.5	41.6 ± 1.7	44.7 ± 1.2	42.3 ± 2.1	0.032
LVEDV 8W FU	108.1 ± 6.4	116.4 ± 5.8	89.2 ± 7.1	99.2 ± 13.1	NS
LVESV 8W FU	65.4 ± 3.2	68.0 ± 4.1	49.5 ± 4.2	58.9 ± 8.9	0.032
Delta LVEF	0.2 ± 1.0	0.6 ± 0.9	9.3 ± 1.6	3,5 ± 5,1	0.003
Delta LVEDV	30.9 ± 8.2	38.9 ± 8.1	6.4 ± 7.5	21.9 ± 11.3	NS
Delta LVESV	18.1 ± 4.5	20.8 ± 3.9	2.9 ± 4.8	12.8 ± 7.7	NS

Supplemental table I represents echocardiographic derived volumes and ejection fraction. 2D-echocardiographic volumes are calculated by the modified Simpson method in study one and 3D-echocardiography in study 2. LCx indicates left circumflex artery;; LVEF: left ventricular ejection fraction; LVEDV: left ventricular end-diastolic volume; LVESV: left ventricular end-systolic volume; PMI: post myocardial infarct; 8W FU: eight week follow-up; LAD: left anterior descending artery; delta: indicates the absolute difference between post myocardial infarct measurement and 8 week FU. eMSC indicated encapsulated MSC

Supplemental table II: PV-loop parameters

Parameter						
Study 2: severe Infarct						
Systolic Parameters	**measure**	**Control**	**10.000 eMSC**	**20.000 eMSC**	**40.000 eMSC**	**P-value**
dP/dt + 8 week FU	mmHg/s	974.6 ± 161.7	1417.1 ± 113.2	1071.4 ± 135.3	1226.4 ± 136.2	NS
ESPVR 8 week FU	mmHg/mL	1.9 ± 0.5	5.2 ± 1.0	6.5 ± 1.3	5.2 ± 1.7	<0.05
PRSW 8 week FU	mmHg	59.7 ± 10.2	72.4 ± 13.2	56.7 ± 7.8	51.2± 17.7	NS
Diastolic Parameters						
Tau 8 week FU	ms	68.8 ± 5.8	55.0 ± 4.0	67.9 ± 6.8	47.7 ± 2.1	NS
dP/dt - 8 week FU	mmHg/s	-624.2 ± 129.7	-1051 ± 82.8	-634.5 ± 94.3	-851.0 ± 66.8	NS
EDPVR Baseline	mmHg/ml	0.184 ± 0.07	0.027 ± 0.015	0.0168 ± 0.028	0.02 ± 0.003	NS

Supplemental table SIII represents some admittance derived parameters. End-systolic pressure volume relationship (ESPVR) is significantly better in all animals indicating a preserved contractility. PRSW indicates; pre-recruitable stroke work. EDPVR: end-diastolic pressure volume relationship.

Supplemental table III. Histological analysis

Study 1: Moderate infarct						
parameter	**measure**	**Control**	**20,000 eMSC**	**40,000 eMSC**	**60,000 eMSC**	**P- value**
Infarct size	%	9.6 ± 1.3	7.6 ± 1.2	9.1 ± 1.2	9.3 ± 1.8	NS
Collagen density						
Infarct	% collagen	62.6 ± 7.6	59.1 ± 7.8	71.5 ± 5.0	69.6 ± 5.0	NS
Border	% collagen	3.4 ± 0.6	4.4 ± 1.1	3.5 ± 0.7	2.8 ± 0.6	NS
Remote	% collagen	2.0 ± 0.3	1.4 ± 0.2	1.8 ± 0.3	1.7 ± 0.5	NS
Arteriole density						
Infarct	arterioles/mm^2	12.8 ± 1.1	22.7 ± 2.9	20.8 ± 3.4	17.3 ± 1.8	0.04
Border	arterioles/mm^2	14.7 ± 1.5	18.3 ± 3.3	16.7 ± 1.5	14.2 ± 1.3	NS
Remote	arterioles/mm^2	10.1 ± 0.9	10.4 ± 2.5	11.2 ± 1.7	9.6 ± 2.3	NS
Capillary density						
Border	capillaries/mm^2	855.5 ± 83.7	992.0 ± 147.4	889.3 ± 128.9	966.7 ± 145.2	NS
Remote	capillaries/mm^2	1131 ± 196.2	890.7 ± 142.6	1140.0 ± 1160.4	1157.0 ± 196.5	NS
Cardiomyocyte apoptosis						
Border	% apoptotis/view	7.0 ± 0.7	5.2 ± 0.9	5.5 ± 0.4	4.2 ± 0.5	NS
Remote	% apoptotis/view	5.4 ± 0.6	3.7 ± 0.5	4.5 ± 0.9	3.9 ± 0.7	NS

Study 2: Severe infarct						
		Control	**10.000 eMSC**	**20.000 eMSC**	**40.000 eMSC**	**P-value**
Infarct size	%	20.5 ± 1.4		16.7± 0.2		0.05
Collagen density						

Infarct	% collagen	95.1 ± 2.1	76.1 ± 8.7	78.2 ± 6.2	72.6 ± 9.0	0.04
Border	% collagen	9.2 ± 3.1	4.7 ± 1.3	3.2 ± 1.3	5.2 ± 1.6	NS
Remote	% collagen	7.6 ± 2.6	2.5 ± 0.7	3.2 ± 0.6	1.8 ± 0.9	0.05
Arteriole density						
Infarct	arterioles/mm^2	8.4 ± 0.9	15.5± 4.4	22.2 ± 2.6	15.4 ± 0.6	0.0007
Border	arterioles/mm^2	6.6 ± 0.4	6.0 ± 0.4	9.7 ± 1.4	7.2 ± 2.1	NS
Remote	arterioles/mm^2	3.8 ± 0.5	4.2 ± 0.3	5.7 ± 1.1	3.7 ± 0.8	NS
Capillary density						
Border	capillaries/mm^2	908.1 ± 99.6	1066.0 ± 152.0	1209 ± 64.6	951.3 ± 181.5	0.02
Remote	capillaries/mm^2	1131.0 ± 119.9	899.8 ± 76.8	1232.0 ± 130.9	837.3 ± 137.5	NS
Cardiomyocyte apoptosis						
Border	% apoptotis/view	7.5 ± 2.0	3.7 ± 1.5	2.4 ± 0.7	5.0 ± 3.3	0.02
Remote	% apoptotis/view	4.8 ± 0.9	1.9 ± 1.0	4.0 ± 1.5	4.8 ± 1.8	NS

Supplemental table III. Overview of histological analysis. NS indicates not significant. eMSC: encapsulated mesenchymal stem cells

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PART V

Second generation stem cells in stent design

Endothelial progenitor cell capturing stent

Chapter 11

Endothelial progenitor cell capture to aid vascular repair following coronary stenting:

A new frontier in stent technology?

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Introduction

Drug eluting stents (DES), that locally release antiproliferative, immunosuppressive or anti-inflammatory drugs to retard intimal hyperplasia and restenosis, have significantly improved the outcome of percutaneous coronary interventions. In-stent restenosis was reduced by approximately 70% by the introduction of DES, both in paclitaxel or sirolimus eluting stents[1-3]. However, the local toxicity of the drug on the vessel wall and endothelium have also been shown to concomitantly impair endothelial regeneration and induce local hypersensitivity reactions leading to long-term endothelial dysfunction[4-10]. As endothelial stent coverage was found to be the most powerful histological predictor of late stent thrombosis (LST), delayed re-endothelizalisation remains a big concern[5]. Even though the incidence of LST is low, the sheer volume of coronary interventions nowadays leads to significant numbers with high morbidity and mortality. LST is associated with non-fatal MI and cardiac death in as many as 75 % and 45 % of the cases respectively[11-13]. An accelerated re-endothelizalisation response of the bare stent struts and facilitated arterial repair therefore is desirable. This may not only prevent stent thrombosis by inhibition of platelet adhesion, but it also might impede smooth muscle cell proliferation and migration[14]. This eventually might result in reduced neo-intimal hyperplasia with preserved arterial integrity and endothelial vasomotor function.

Endothelial progenitor cells

The arterial repair response after stent implantation is a multi-step process that involves dedifferentiation, migration and proliferation of neighbouring endothelial cells[14]. Equally important is the incorporation of systemically circulating endothelial progenitor cells (EPCs) originating from the bone marrow. These cells are estimated to contribute to re-endothelizalisation of the neo-intima for up to 25%[15, 16]. The beneficial role of these endothelial progenitor cells in neoangiogenesis and arterial repair was initially suggested in 1997 by Asahara *et al*. [17] EPCs are immature cells that are capable of migrating, proliferating and differentiating into endothelial cells under influence of angiotrophic growth factors, including vascular endothelial growth factor, cell-cell interactions and interactions with the extra cellular matrix. Several studies have suggested, that these EPCs play an essential role in postnatal vasculogenesis as well as in the vascular repair response[4, 15, 18, 19].

Ever since their discovery, there has been controversy about the actual flow cytometric phenotype of EPCs. Whereas the classical phenotype comprises CD45+/ CD34+/ VEGFR2+(KDR) cells, several alternate populations have been identified as potential endothelial cell (EC) progenitors[20]. Moreover, EPCs can be characterized by functional analysis in cell culture (migratory and colony forming potential, expression of EC specific proteins, uptake of acLDL), rather than by flow cytometry[20, 21]. Recently, two functionally distinct subpopulations of EPCs were described: 'early outgrowth EPCs' and 'late outgrowth EPCs'. Early outgrowth EPCs proliferate slowly, express monocytic (CD14), leukocyte (CD45) and EC (VEGFR2, CD31) surface markers and die after a few weeks in cell culture[22, 23]. These cells stimulate angiogenesis in a paracrine fashion by secreting angiotrophic, anti-apoptotic and chemotactic factors and cytokines. Late outgrowth EPCs arise from adherent colonies amongst

early outgrowth EPCs and have been shown to proliferate more rapidly and they can be expanded almost innumerably. These late outgrowth EPCs do not express CD14 and CD45 and are thought to promote neoangiogenesis by transdifferentiation into functional ECs of newly formed structural blood vessels[22-25]. Considering the expansion potential, these late outgrowth cells are speculated to be in fact stem cells. A recent paper by Sieveking *et al*. confirms this hypothesis[26]. Despite the clear in vitro difference in phenotype and function of both early and late outgrowth EPCs, the in vivo (clinical) relevance remains uncertain. Since most of the clinical research and trials to date have been focussed on early outgrowth EPCs, the majority of the data that we will discuss, refer to these specific early outgrowth EPCs.

Clinical implication of endothelial progenitor cells

The titer of EPC is known to increase upon ischemic insults and induction of vascular damage, including myocardial infarction and stent implantation, indicative of ongoing arterial repair and compensatory vasculogenesis[27-30]. EPC number, function and migratory capacities however were found to be decreased in patients with stable coronary artery disease (CAD) [31] and in patients with several cardiovascular risk factors. [32] For instance, EPC function was impaired with age [33] or with smoking [34], as well as in patients with type II diabetes mellitus [35], dyslipidaemia [36] and hypertension[31]. Conversely, low EPC count or impaired EPC function in vitro was shown to be a strong predictor of in-stent restenosis, (progression of) atherosclerotic disease, cardiovascular events and death from cardiovascular causes[37-40].

Several factors are associated with improved function and recruitment of EPCs from the bone-marrow including VEGF, estrogen, exercise and erythropoietin[41-45]. Also, statin therapy is known to augment EPC mobilization from bone marrow and improvement of EPC function in both mice and patients[46, 47]. It is believed that the increase of bio-availability of eNOS by statins and the activation of the PI3-kinase/Akt-pathway underlies these beneficial and pleiotropic effects. [41, 48, 49]

Vascular healing and re-endothelialisation

To accelerate re-endothelialisation after coronary intervention several approaches have been explored to date. Direct seeding of mature endothelial cells on bare metal stents proved to be laborious, whereas the cultured endothelial monolayers covering the stent struts is severely damaged by balloon expansion of the stent. Blood flow along the stent surface after implantation washed away most of the remaining cells. Although this approach is feasable, it has not been pursued in a clinical setting.[50-52]

Alternatively, angiotrophic growth factors such as VEGF have been explored to stimulate the endogenous ability to promote regrowth of the endothelial layer. The mode of delivery of VEGF, that has been described, varied from direct intracoronary infusion of plasmids encoding VEGF prior to PCI [53,

54] to recombinant VEGF protein-coated stents [55] and stents coated with VEGF plasmids (gene eluting stent)[56]. Although these techniques proved to be safe in animals and small scale clinical trials, they failed to show a reduction in neo-intima formation.

The CD34 antibody-coated bioengineered coronary stent (Genous R stent)

The key role of EPCs in the arterial repair response after stent implantation prompted the concept that recruitment of the patient's own EPCs to the site of vascular injury could aid stent re-endothelialization and initiate the endogenous arterial repair response. A few years ago, this 'pro-healing' concept led to the development of the first bioengineered stent by the Thoraxcenter Rotterdam in conjunction with OrbusNeich (Genous R-stent). This stent was specifically designed to promote the arterial healing response by a coating of immobilized murine antibodies raised against human CD34. As a result, the Genous R-stent captures and sequesters circulating CD34-positive progenitor cells to the luminal stent surface and so theoretically initiates re-endothelialisation.

Several pre-clinical studies were conducted to prove safety and feasibility. In pigs, anti-CD34-coated stents exhibited accelerated coverage with an endothelial cell population and, equally important, showed no sign of mural thrombus formation in contrast to bare metal stent control pigs, 48 hours post implantation. Complete coverage of the Genous-stent by a functional endothelial monolayer was achieved after 7 to 14 days, whereas DES controls showed eminent delayed vascular healing.

Re-establishment of a functional endothelial cell layer restored the cellular vascular integrity and homeostasis. The recovery of vascular function resulted in prevention of platelet aggregation and in-stent thrombus formation, inhibition of smooth muscle cell migration and proliferation, therby prevention of neonintimal hyperplasia, preserved vasomotor response, and angiogenesis. [14]

The EPC capturing Genous stent in clinical practise

The encouraging pre-clinical studies in rabbits, pigs and primates led to the HEALING-FIM study in which safety and feasibility of the anti-CD34-coated stent was demonstrated in 16 patients with stable single vessel CAD. In this study the procedural success rate was 100%, whereas the composite major adverse cardiac and cerebrovascular event (MACCE) rate was 6.3% due to a target vessel revascularization in one patient. Despite only one month of dual anti-platelet therapy, there was no acute stent thrombosis observed in the treated patient group. Mean late luminal loss after six months was 0.63 ± 0.52 mm and stent volume obstruction was 27.2% ±20.9%[57].

Where HEALING-FIM demonstrated the safety of the anti-CD34-coated stent, the HEALING II registry was designed to provide additional safety data and initial efficacy data as well by use of QCA and IVUS follow up. HEALING II was a prospective, non-randomized, multi-centered study, enrolling 63 patients with single vessel CAD. The coronary intervention was successful in 98.4% of the cases. No acute or

sub-acute stent thrombosis was found during the 18 month follow up, despite only one month of dual anti-platelet therapy. An analysis of the six and eighteen month data disclosed a clinically-driven target lesion revascularization of 17.4% and a target vessel revascularization of 7.9 %. The overall MACCE rate was 7.9 %. Angiographic follow up revealed a late luminal loss of 0.78±0.39 mm at 6 months and a regression of late luminal loss to 0.59±0.31 mm at 18 months[58, 59]. IVUS confirmed this neo-intimal regression, although less pronounced as compared to the QCA data. Interestingly, it was found that EPC titer correlated closely with angiographic outcome at 6 months, but also with the regression in late luminal loss after 18 months follow up. This would suggest an ongoing vascular repair and remodelling process even after eighteen months of follow up.

Since the anti-CD34-coated stent is capturing circulating EPCs that initiate and govern the vascular healing, the number and function of EPCs appear to be of paramount importance to the response to this bioengineered stent. Indeed, in the HEALING II registry a clear correlation was found between the EPC titer and restenosis rates. All patients with significant restenosis or MACCE/revascularization events had low EPC titers at 6 month follow up, whereas patients with normal EPC titer did not show TLR or MACCE.

Recently, the use of the EPC capturing stent in the treatment of acute myocardial infarction was described by Co *et al*[60]. In this study 120 patients with acute ST elevation MI were treated with a primary PCI using a Genous R stent. Procedural success was achieved in 95% of the cases. At one year follow up, MACE rate was 9.2% and TLR/TVR 5.8%. One patient suffered from acute stent thrombosis and one patient from a sub-acute stent thrombosis. No LST was reported, despite only one month of dual anti-platelet therapy.

Upcoming results and trials

Data analysis of the HEALING II study showed that the majority of patients treated with concomitant statin therapy had normal EPC titers, while patients that were not on statin therapy had low EPC counts [59]. The latter observation led to the design of the HEALING IIb registry, in which all patients receiving the anti-CD34-coated stent also were initiated with statin pharmacotherapy (Atorvastatin 80 mg qd) at least two weeks prior to stent placement. HEALING IIb is a multi-center, prospective, non-randomized trial with angiographic follow-up at six and eighteen months, in which the effectiveness of the anti-CD34-coated stent in combination with optimal statin therapy will be assessed. By the beginning of 2008, inclusion of all 100 patients was finished and final outcome is expected in 2009. This study will render more insight into the correlation between EPC titer, accelerated vascular healing and angiographic outcome after placement of the OrbusNeich Genous R-stent.

Forthcoming studies with the anti-CD34-coated stent comprise the HEALING-AMI as a single center study in which 60 patients with ST elevation myocardial infarction (AMI) receive a Genous stent in the culprit lesion and Atorvastatin 80 mg qd for six months. Enrolment of all 60 patients has recently been completed and the final study report will be due in September 2008.

The e-HEALING registry recently reached complete enrolment of their 5000 patients. The e-HEALING registry is a multi-center (100-120 centers), worldwide, prospective registry of an all-comer patients population treated with the Genous Bio-engineered R stent. The primary objective of this registry is to collect post-marketing surveillance data on patients that have received a Genous R-stent. The study protocol recommends that patients receive standard statin therapy for at least two weeks prior to the intervention and only one month of clopidogrel treatment after the procedure. The primary endpoint of the registry is target vessel failure at 12 months.

Only recently, enrolment has started of the HEALING-Vasomotion study. This is a multi center study of 36 patients, in which the vasomotor response of a stented coronary artery will be assessed 6 months after the stenting procedure. Patients will be randomized to receive either a Genous R stent or a Xience V stent after at least two weeks of optimal statin therapy. At baseline and six months after stent implantation, IVUS will be performed as well as an acetylcholine provocation test. Vasomotor response after acetylcholine provocation of the stented vessel will render more insight into the difference in vascular healing and endothelial function after placement of the bio-engineered stent or DES.

Finally, the TRIAS-HR and TRIAS-LR studies are multi-center, prospective, randomized trials of approximately 1200 patients that only recently have been initiated. In the TRIAS-HR study, non-inferiority of the Genous R-stent versus TAXUS Liberté Paclitaxel eluting stents in *de-novo* lesions with high-risk for restenosis will be investigated. The TRIAS-LR study was designed to show non-inferiority of the Genous R stent versus bare metal stents in *de-novo* lesions with a low risk of restenosis. Primary endpoints of both studies are target lesion failure within one year.

Conclusion

In conclusion, acceleration of the endogenous vascular repair response by EPC capturing is a interesting novel approach to impede in-stent restenosis formation in percutaneous interventions. Safety and proof of principle have been established in several (pre-)clinical studies, although the outcome of larger randomized clinical trials has to be awaited.

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Chapter 12

Capture of circulatory endothelial progenitor cells and accelerated re-endothelialization of a bio-engineered stent in human ex vivo shunt and rabbit denudation model.

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Abstract

Aims The Genous™ Bio-engineered R™ stent (GS) aims to promote vascular healing by capture of circulatory endothelial progenitor cells (EPCs) to the surface of the stent struts, resulting in accelerated re-endothelialization. Here, we assessed the function of the GS in comparison to bare metal stent (BMS), when exposed to the human and animal circulation.

Methods and Results First, Fifteen patients undergoing coronary angiography received an extracorporeal femoral AV shunt containing BMS and GS. Macroscopical mural thrombi were observed in BMS, whereas GS remained visibly clean. Confocal and scanning electron microscope (SEM) analysis of GS showed increase in strut coverage. qPCR analysis of captured cells on the GS demonstrated increased expression of endothelial markers KDR/VEGFR2 and E-Selectin, and decrease of pro-thrombogenic markers TFPI and PAI-1 compared to BMS.

Secondly, a similar primate AV shunt-model was used to validate these findings, and occlusion of BMS was observed, while GS remained patent, as demonstrated by live-imaging of Indium-labelled platelets.

Thirdly, in an *in vitro* cell-capture assay, GS struts showed increased coverage by EPCs whereas monocyte-coverage remained similar to BMS.

Finally, assessment of re-endothelialization was studied in a rabbit denudation model. 20 animals received BMS and GS in the aorta and iliac arteries for 7 days. SEM analysis showed a trend towards increased strut coverage, confirmed by qPCR analysis revealing increased levels of endothelial markers (Tie2, CD34, PCD31 and P-selectin) in GS.

Conclusions In this proof of concept study, we have demonstrated that the bio-engineered EPC capture stent, Genous™ R™ stent, is effective in EPC-capture, resulting in accelerated re-endothelialization and reduced thrombogenicity.

Introduction

Vascular homeostasis is maintained by the endothelial cell (EC) layer that is involved in the regulation of platelet adhesion, vasomotor function and cell cycle quiescence of the cellular constituents of the vascular wall(1). Bone-marrow derived circulating endothelial progenitor cells (EPC) aid in the regeneration of damaged and dysfunctional endothelium and therefore play a central role in the vascular repair response(2-4). Recruitment of EPC to the site of vascular injury has been proposed to promote vascular healing, and has been shown to inhibit neointimal proliferation and restenosis associated with percutaneous coronary intervention (PCI)(5). The GENOUS™ Bio-engineered R™ stent (Genous stent) (Orbus-Neich Medical BV, Hoevelaken, the Netherlands) has been developed to enhance the capture of circulating EPCs to the stent surface using an immobilized antihuman-CD34 monoclonal antibody. CD34 was previously shown to be expressed in circulating haematopoietic cells in humans(4, 6). The sequestered EPC are thought to enhance endothelial healing and thus protect the stented vascular segment against acute thrombosis with minimized neointimal hyperplasia.

The safety, feasibility and efficacy of the Genous stent in human coronary artery disease (CAD) have been the subject of multiple clinical studies(7-11). Although the long-term effect of the Genous stent on clinical outcome has been investigated, the efficacy of the bio-engineered stent to promote initial endothelial recovery has never been shown in humans before. Here, we studied early cellular interactions of the Genous stent within the circulation of CAD patients.

In the first part of the study, a temporary *ex vivo* arteriovenous (AV) shunt was established by canulation of the femoral artery and vein and connection of the two via a synthetic tube comprising the BMS and the Genous stent. The stents were exposed to the human circulation under continuous flow. EPC capture and subsequent EPC differentiation was analyzed using conventional ultrastructeral analysis as well as by quantifying surrogate endothelial markers on the captured stent by qPCR analysis. In the second part of the study, the validation of accelerated endothelialization was further conducted in a well-established primate model for stent-related thrombogenecity. In the third part, CD34+ cells capture specificity was evaluated in an in vitro capture model. In the final part of the study, long term effects of the Genous stent on the vascular endothelium was evaluated in a rabbit model of arterial balloon injury and vascular repair.

Material and Methods

Study population

The study was performed in 15 patients undergoing elective heart catheterization, followed in 11 cases by PCI. Informed written consent was obtained prior to the procedure for all patients. The study was reviewed and approved by the institution's ethics review committee. The baseline characteristics of included patients are shown in the Table 1.

Patients characteristics	N	%
Male	10	66,67
Age	69,4	
PCI	11	73,33
Hypertension	8	53,33
Diabetes Mellitus	0	0
Dyslipidemia	5	33,33
Smoking	6	40
Clinical pattern		
Stable angina pectoris	10	66,67
Unstable angina pectoris	4	26,67
Syncope	1	6,667
Periferal vascular disease	3	20
Stroke	2	13,33
Heart Failure	3	20
Previous myocardial infarction	4	26,67
Previous PCI	7	46,67
Previous CABG	2	13,33
Use of:		
Statin	14	93,33
Aspirin	13	86,67
Clopidogrel	13	86,67
Warfarin	2	13,33
Beta Blockers	3	20

PCI = Percutaneous coronary intervention, CABG = Coronary artery bypass grafting

Ex vivo human AV shunt

The Genous stent (OrbusNeich, Hoevelaken, The Netherlands) is coated with an immobilized murine monoclonal antibodies directed against human CD34, a known antigen expressed on EPCs. It is designed to capture circulating EPCs to promote vascular healing. Patients received an extracorporeal A-V shunt containing one Genous stent and one BMS stent (non-coated, stainless steel R-stent). (Supplemental data: Figure 2). For a detailed description of the protocol, see supplemental dada.

mRNA processing and qPCR analysis

The captured cells were harvested from the stents by directly retrieving the RNA using a lysis buffer provided by a commercial RNAeasy isolation kit, followed by cleanup using RNA isolation columns (Qiagen, The Netherlands) according to the manufacture's protocol as conducted previously (12-14). Briefly; 20 ng carrier RNA was added to the cell lysate. 1 volume of 70% ethanol was added to the homogenized lysate and the sample was mixed and isolated by a commercial RNAeasy column. RNA was reverse transcribed using iScript reagents according to the manufacturer's protocol (Bio-Rad, The Netherlands). *Gene expression* was quantified using qPCR by the SensiMix™ SYBR & Fluorescein Kit (Bioline, The Netherlands), followed by signal detection on a MyiQ Single-Color Real-Time PCR Detection System (BioRad, the Netherlands). Negative were included, whereas GAPDH and beta actin served as housekeeping genes. Primers for the analysis were designed using the sequences available on GenBank (*http://www.ncbi.nlm.nih.gov/*) and Primer3 software to generate up to 3 primer pairs for each target sequence (Supplemental data table 1). All primers were tested and the optimal primer pair was ultimately used for sample analysis.

Rabbit model of arterial balloon injury

The early effects of Genous in accelerated reendothelialization were further assessed in a rabbit endothelial denudation model(15). Stents were implanted in 20 New Zealand white adult male rabbits. One BMS and one Genous stent were implanted per rabbit in the aorta (for qPCR analysis, N=11) and iliac artery (for SEM analysis, N=9). For the aorta, the stents were alternated in order. All stents were deployed at nominal pressure (9 atm) for 30 seconds. Angiography was performed to confirm appropriate stent placement and vessel diameter post-deployment. At 7 days post stenting, follow-up angiography was performed. To obtain stent samples for SEM analysis, the rabbits were perfusion fixed in 10% formalin and the stented arteries were harvested. For qPCR analysis, the vessels were isolated without *in situ* fixation, the stents were removed and incubated in RNA isolation buffer (RLT buffer, Qiagen, The Netherlands) and stored at -80°C until qPCR analysis.

For the scanning EM protocol, baboon AV shunt study and in vitro CD34+ capture stent validation, please see supplemental material and methods.

Results

Part 1: Human AV shunt study reveals evidence of accelerated capture of endothelial progenitor cells and protection against in stent thrombosis and inflammation in Genous versus BM stent.

The Genous stent has been tested extensively in animal models(16), but direct evidence of the *in vivo* EPC capture capability of this stent in the human circulation has not been presented. In the first part of the study, we investigated the acute effect of the Genous stent in patients undergoing

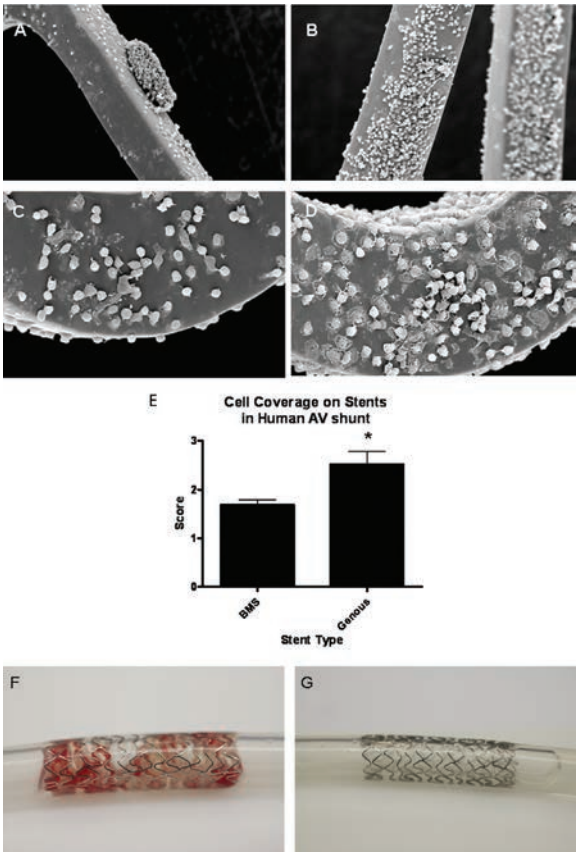

Figure 1. SEM analysis of the EPC capturing stent and BMS in the human AV shunt model revealed marked increase in cell strut coverage compared to BMS. SEM inspection of the study stents showed less strut coverage, and the presence of thrombus-like structures on BMS (A) as compared to Genous stent (B). High magnification SEM revealed more adhesion of cells with a flattened polygonal morphology on the struts of the stent (D) versus BMS (C). Average stent strut coverage was visually rated by blinded (core lab) technicians (CV-Path Institute, USA) on a 0-3 virtual scale (corresponding to 0-25, 25-50, 50-75 and 75-100% stent coverage). Bar graph indicates the level of strut coverage as assessed by SEM in the two stent study groups. * $p<0.05$, N=9 (E). Macroscopic appearance of BMS (F) and Genous (G) stents in the human *ex vivo* shunt model shows mural thrombi in the BMS, whereas the Genous stent remained free of thrombogenic material.

coronary angiography. In addition, assessment of the Genous stent in a CAD patient setting provides relevant evaluation of bioactivity of the CD34 capture antibody that was raised specifically against human CD34 antigen (Supplemental data: figure 1). In this study, the EPC capture capacity of the Genous stent was compared to that of uncoated, stainless steel R-stents (BM stents).

The Genous and the BMS that were tested in the human AV shunt model were exposed to the circulation for up to 120 minutes. Stents selected for SEM analysis revealed marked increase in strut cell coverage in the Genous stent as compared to BMS (Figure 1A and C: BMS, Figure 1B and D: Genous stent). Average stent-strut coverage was visually rated by a blinded (core lab) technician on a 0-4 scale (corresponding to 0-25, 25-50, 50-75 and 75-100% stent coverage). Cell deposition on the struts was enhanced by 32.5% ($p=0.006$, N=9) in Genous stents as compared to BMS in paired analysis (Figure 1E). High resolution assessment of the SEM data revealed cellular deposits present on both stents that could be distinguished into a population of cells with a rounded and flattened morphology, cells with a more monocyte-like appearance, and blood platelets. To further elucidate the identity of this mixed population, we performed by qPCR of the cellular substrate. Macroscopical comparison of Genous and BM stents also revealed the substantial presence of mural thrombi in the 2 BMS stents, whereas all of the Genous stents remained free of thrombogenic material (Figure 1F and G). This observation led us to further examine markers of thrombogenicity and coagulation by qPCR.

For qPCR analysis, cells were directly lysed from the stents and subsequently analyzed by qPCR using the housekeeping genes GAPDH and beta actin to normalize for cell content and quality of total RNA. qPCR analysis of the attached cells showed no significant difference in CD34 expression between the Genous and BMS groups (data not shown). However, evaluation of endothelial markers revealed a marked increase of KDR/VEGFR2 ($p<0.001$), and E-Selectin RNA expression ($p<0.045$) with the Genous stents, as compared to BMS (Figure 2A). Expression of another endothelial specific marker Plvap (Figure 2A) showed a tendency to increase expression in Genous stent, although it was not statistically significant ($p=0.21$). An equivalent CD34 transcript content, with enhanced expression of endothelial markers would suggest accelerated ongoing differentiation into the endothelial lineage concomitant with downregulation of CD34 expression.

qPCR evaluation of markers of thrombosis and coagulation (Figure 2B) revealed a significant decrease in expression of tissue factor pathway inhibitor (TFPI) and plasminogen activator inhibitor-1 (PAI-1) in Genous compared to BMS ($p=0.04$, and $p=0.02$, for TFPI and TF respectively), suggestive of a less pro-thrombotic state of the cells attached to the Genous stent, and thus a reduced risk for stent thrombogenicity.

To assess the potency of the Genous stent to protect the vascular wall against inflammation, inflammatory markers were also included in the study. CD16 is an established neutrophil expression marker. Cells sequestered to the Genous stent, presented lower expression levels of CD16 over time ($p<0.002$), whereas the BMS showed persistent CD16+ expression in attached cells ($p<0.932$), suggesting that adhesion of CD16+ inflammatory cells was prevented by accelerated capture of CD34+ endothelial progenitors and subsequent coverage of the stent struts (Figure 2C). Moreover, the inflammatory markers for immune cell subpopulations were not significantly different between the stents, including CD68, CD14, monocyte chemoattractant protein-1, CXCR-1 and VCAM1 (data not shown).

Combined, these qPCR analysis corroborate and extend the ultrastructural analysis by SEM, and confirm enhanced attachment of circulating EPCs to the Genous stent surface. In addition, these data suggest that the attached cells could undergo rapid endothelial commitment and differentiation (loss of CD34, increased expression of KDR1/ E-selectin), with reduced thrombogenecity and inflammatory response of the injured vascular wall.

Part 2: Genous inhibits in-stent thrombosis in a baboon AV shunt model.

Based on the differences on thrombogenicity by qPCR, and the lack of mural thrombi in the Genous stent as observed in the clinical study, we further assessed thrombogenicity of the Genous stent in an established primate model using a similar AV shunt setup with exclusion of anti-platelet treatment in the protocol. Live deposition of platelets and fibrinogen was studied in the AV shunt set up by measuring the accumulation of Indium labeled platelets with a gamma camera for up to 2 hours. In line with the human data, the Genous stent had a lower thrombogenic potential than BMS in the baboon shunt model. Within 65 minutes after initiation of the experiment, the BMS were occluded with a flow-limiting thrombus, while the Genous stents remained patent for at least 2 hours. (Figure

3A). Further SEM analysis revealed increased platelet deposition and in stent thrombus formation in BMS versus Genous stents (Figure 3B). Platelet deposition was significantly higher in BMS compared to Genous stents after flow exposure as quantified by gamma camera ($1.13 \pm 0.57 \times 10^9$ as compared to $0.50 \pm 0.22 \times 10^9$ platelets, in BMS versus Genous respectively, p=0.04; Figure 3C). Although fibrinogen accumulation seemed less prominent on the Genous as compared to BM stents, no statistical difference was found between the groups. The values were 0.05 ± 0.02 mg/stent and 0.18 ±0.10 mg/stent for the Genous and BM stents respectively.

Part 3: In vitro cell capture assay demonstrates specific adhesion of human peripheral blood derived CD34+ cells on Genous CD34 capture stents.

To examine whether the CD34 antibody on the Genous stent is also able to bind the more abundant circulatory inflammatory cells such as monocytes, an *in vitro* assay was performed to test the CD34+ cell capture specificity of the Genous stent. Genous and BM stents were deployed in silicon tubing and were exposed to a cell mixture of PKH26 red fluorescent labeled human monocytes ($1x10^6$ cells/ml) and PKH2 green fluorescent labeled human CD34+ cells ($2x10^5$ cells/ml), under a constant rotation speed of 0,3 RPM for 2 hours. Confocal assessment of the stent struts showed a greater number of CD34+ cells to adhere to the Genous stent strut surface as compared to BMS; cell density for CD34+ cells was 500±158 cells/cm^2 strut area on the Genous versus 17±8 cells/cm^2 on the BM stent (p=0.0009; Figure 3D,E). In contrast, monocyte adherence was not significantly different between the two stent types (79±44 cells/cm^2 as compared to 58±39 cells/cm^2, Genous versus BMS respectively, p=0.07; Figure 3D,E). Therefore, the specificity of the Genous stent to capture CD34+ cells was significantly higher as compared to BMS, as 86% of the attached cells were CD34+ as compared to only 26% on the BMS.

Part 4: Genous stent promotes reendothelialization at 7 days in rabbit endothelial denudation model.

20 New Zealand white rabbits received stent placement after endothelial denudation in the aorta N=11 and iliac artery n=9 (left Genous, right BMS). From 11 animals, the aortic stents were harvested after 7 days and the cell lysates were evaluated for EC markers. qPCR analysis showed increased levels of endothelial markers in the Genous versus BM stent treated artery, including Tie2 (p=0.02) , CD34 (p=0.08), CD31 (p=0.07), and P-selectin (p=0.05), indicating that the Genous stent promoted long term endothelialization (Figure 4A). These data support our finding of the AV shunt study in CAD patients where we propose earlier endothelialization in Genous stent compare to BMS. In line with these findings, SEM analysis of 9 rabbits that received the Genous (left) or BM (right) in bilateral denudated iliac arteries showed a trend of increased strut coverage at 7 days post implantation (Figure 4B-D). Together, these data indicate that the Genous stent efficiently promotes reendothelialization in a denudated vessel wall environment as shortly as 7 days after placement.

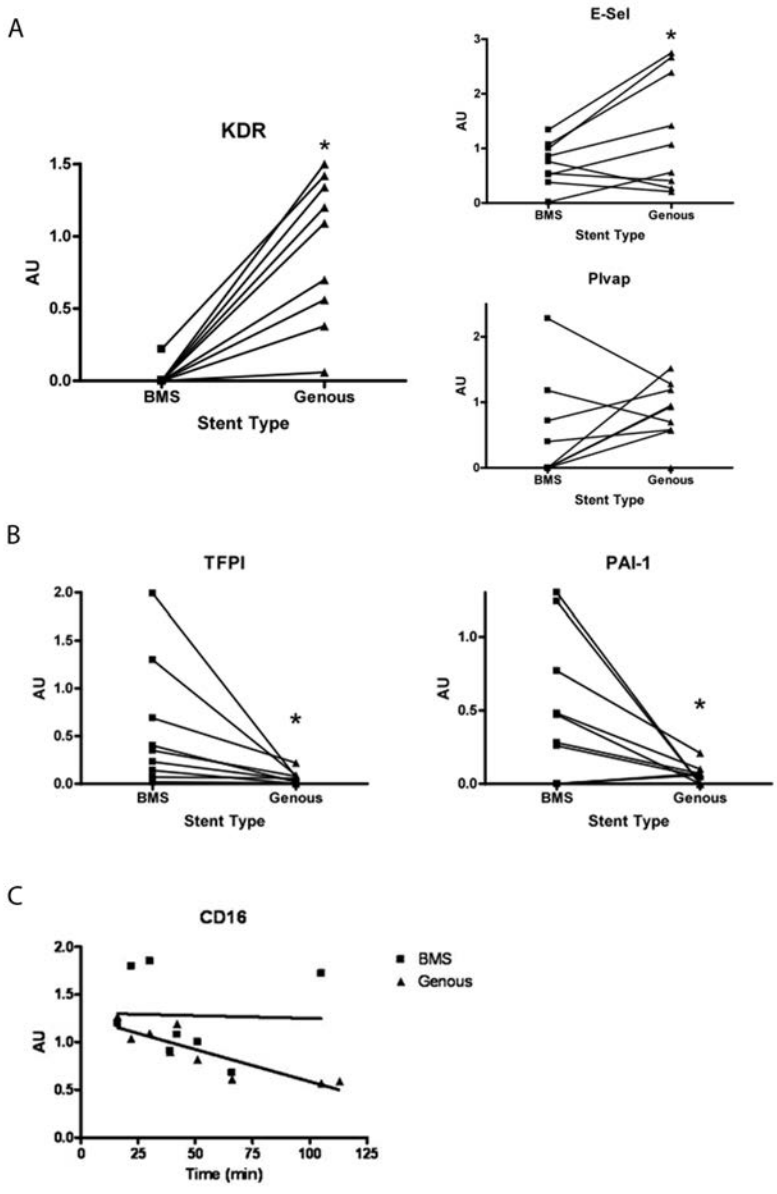

Figure 2. qPCR evaluation of cellular markers in cell lysates of captured cells in the human AV shunt stent model. Paired comparison of the expression levels of the individual genes revealed a marked increase in endothelial markers, including KDR/VEGFR2 ($p<0.001$) and E-Selectin ($p<0.045$) mRNA expression in the stents compared to BMS (A). Expression of another endothelial specific marker PLVAP (A) showed no significant increased expression in the stent ($p=0.21$). qPCR analysis of markers of thrombosis, coagulation, and inflammation: Paired comparison of the expression levels of the individual genes revealed a marked decrease in tissue factor pathway inhibitor (TFPI) and plasminogen activator inhibitor-1 (PAI-1) in the Genous compared to the BM stent (B) ($p=0.04$ and $p=0.02$). qPCR showed a significant decrease in CD16 marker expression in the cells captured by the Genous stent over time, whereas the CD16 mRNA levels on BMS were maintained (C) (*$p<0.05$, $^{\Phi}p<0.1$, N=9).

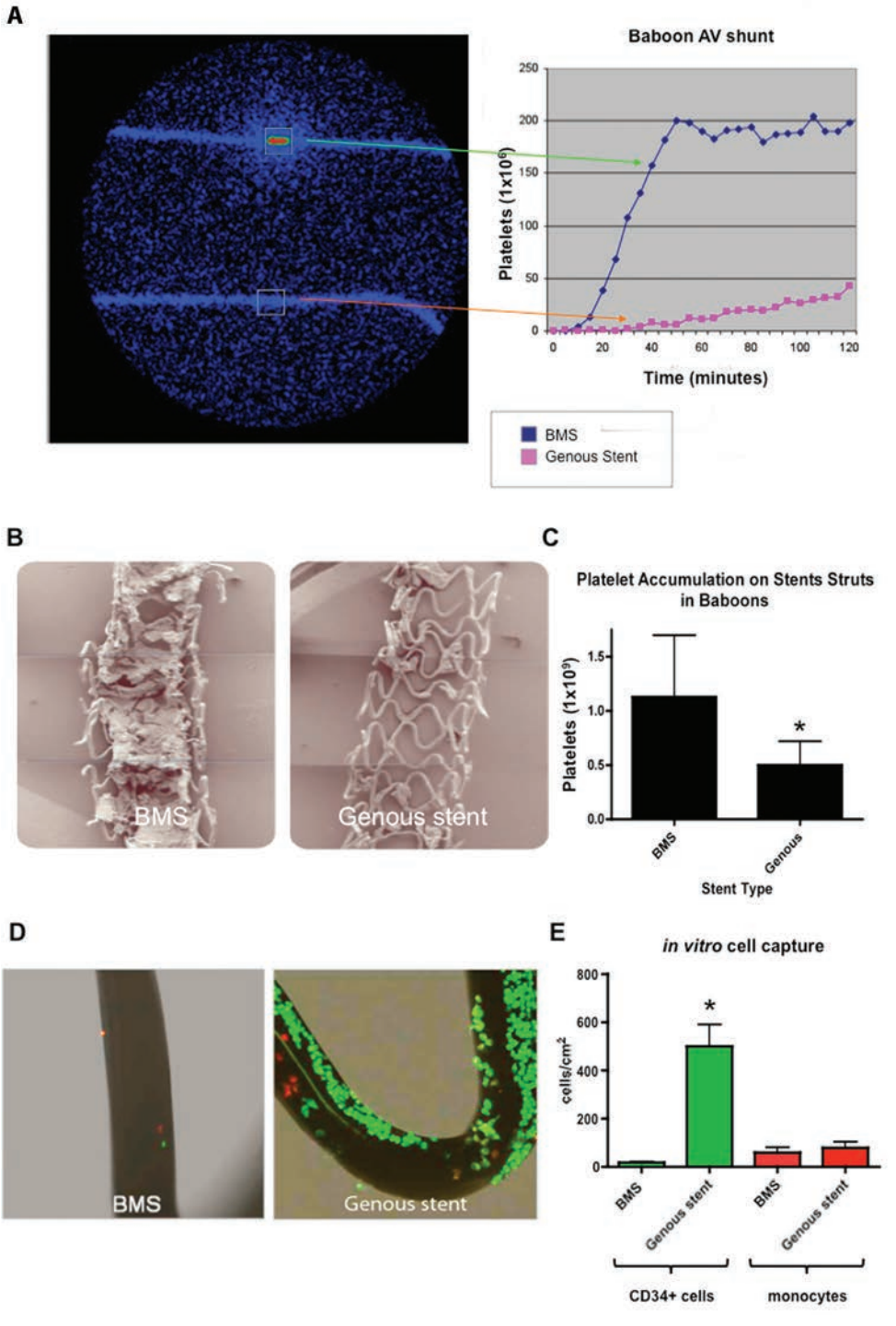
A
Baboon AV shunt
Platelets (1x10⁹)
250
200
150
100
50
0
0
20
40
60
80
100
120
Time (minutes)
BMS
Genous Stent
B
BMS
Genous stent
C
Platelet Accumulation on Stents Struts in Baboons
Platelets (1x10⁹)
1.5
1.0
0.5
0.0
*
BMS
Genous
Stent Type
D
BMS
Genous stent
E
in vitro cell capture
cells/cm²
800
600
400
200
0
*
BMS
Genous stent
BMS
Genous stent
CD34+ cells
monocytes

Figure 3. Live imaging of AV shunt set up using a gamma camera to measure deposition of Indium labeled platelets on the study stents (A). Line graph shows a typical example of accumulating platelet signal over time. Low magnification SEM images of the BMS and the EPC capture stent in the baboon AV shunt model revealed a decrease in mural thrombus in the Genous versus BMS (B). Bar graph showing the quantified number of platelets accumulated on the BMS and Genous stent after 2 hours of flow exposure (C). Data was acquired from the live imaging AV shunt set up *p<0.05, N=3.
In vitro assay to test the CD34+ cell capture specificity of the Genous stent: Genous and BM stents were deployed in silicon tubing and were exposed to a cell mixture of PKH26 red fluorescent labeled human monocytes ($1x10^6$ cells/ml) and PKH2 green fluorescent labeled human CD34+ cells ($2x10^5$ cells/ml), under a constant rotation speed of 0,3 RPM for 2 hours. Micrographs show confocal images of strut coverage of BMS and Genous stent (D). Bargraph shows the quantified number of CD34+ cells and monocytes per cm^2 strut area. *p<0.05, N=3 (E).

Discussion

Endothelialization is a critical step in the initiation of vascular repair following stent implantation. Re-endothelialization of the damaged area involves activation and migration of resident EC adjacent to the stent area or by recruitment of blood-derived EPC. Early presence of a functional endothelial lining after vascular injury could improves the process of vascular healing and reduces the risk of restenosis an acute thrombosis.

The stent struts of the bio-engineered R stent (GENOUS®) incorporate an immuno-affinity surface, consisting of covalently bound monoclonal antibodies directed against the human CD34 antigen, a cell surface marker found on circulating EPC. EPC capture by the GENOUS® R stent is shown schematically in Figure 1 of supplemental data. The efficacy of EPC capture and re-endothelialization of the Genous stent has been extensively evaluated in porcine models(16) relying on the cross-reactivity of the monoclonal CD34 antibody against porcine CD34. Although these studies gave a clear indication of stent performance, optimal capture efficacy by the human CD34-directed antibody can only be truly tested under circumstances when the stent is exposed to the human circulation. Here, the performance of the BMS and the bio-engineered Genous stent was studied in an *ex vivo* AV shunt construction in which stents were exposed to the human circulation for up to 2 hours. The data of this study provide for the first time, direct evidence of the capture efficiency of a bio-engineered capture stent in the human circulation.

The efficacy of EPC capture was first assessed by conventional scanning electron microscope (SEM) analysis, which revealed the presence of blood-derived cells with a rounded or flat polygonal morphology indicative of EPC, and platelet aggregation on the stent struts. Semi-quantitative analysis of the Genous stent revealed increase in cellular density (platelets were excluded) on the stent surface as compared to BMS. To elucidate the identity of the captured cells, surrogate biomarkers for EPC, EC, and immunocompetent cells and thrombogenicity were assessed by qPCR analysis. Endothelial cell surface markers KDR, E-selectin, and PLVAP mRNA were expressed in the attached cells in the Genous samples, as compared to the BMS samples. However, only low expression levels of the CD34 marker were observed in the lysates obtained from both stents. Limited CD34 expression in the EPC capture stent sample could be due to active downregulation of CD34 in response to shear stress after

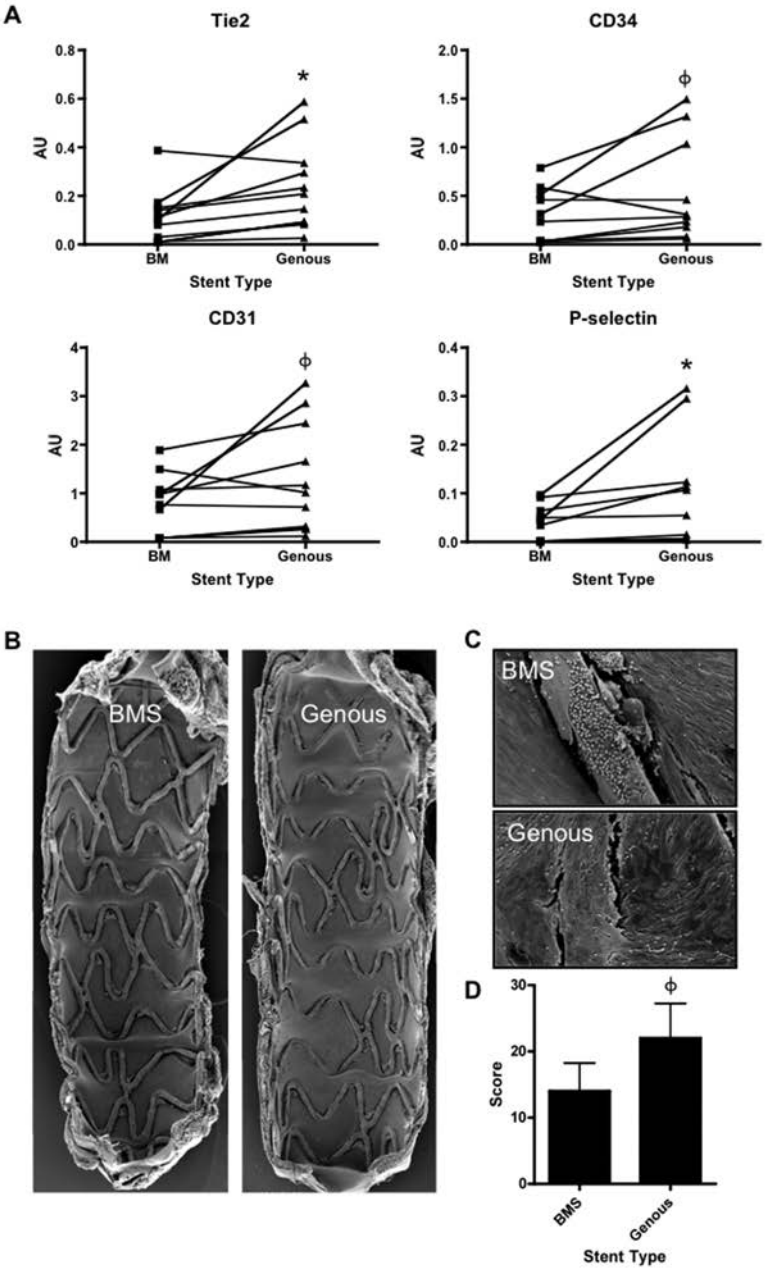

Figure 4. qPCR analysis of the study stents of 11 New Zealand white rabbits was performed to evaluate capture of cells and subsequent expression of EC markers. Paired qPCR analysis showed increased levels of endothelial markers by the cells captured on the Genous versus BMS stent treated arteries, including Tie2 ($p=0.02$), CD34 ($p=0.07$), CD31 ($p=0.08$) and P-Selectin ($p=0.05$). *$p<0.05$, ϕ$p<0.01$, N=11 (A).
SEM analysis of the stents implanted iliac vessels of 9 New Zealand white rabbits: Low (B) and high (C) magnification assessment revealed improved cell coverage between and above struts in the Genous stent versus BMS. Bargraph show the level of strut coverage as analyzed by SEM in the two stent groups. ϕ$p<0.01$, N=9 (D).

cell immobilization on the struts and exposure to blood flow leading into EC differentiation(17). It is known that upregulation of EC markers and adaptation of the cultured EC morphology into a more *in vivo*-like phenotype could be induced within minutes after exposure to mechanical flow(18). In addition, loss of CD34 cell surface expression on circulating EPC is associated with commitment to the endothelial linage. Furthermore, de Boer et al. has demonstrated that *in vivo* platelet activation could rapidly induce differentiation of CD34+ cells into more maturated KDR+ ECs(19). Therefore, the low level of CD34 expression on the captured cells could be explained by rapid differentiation of the progenitor cells towards a mature EC type. In line with this hypothesis, expression of more mature EC markers, including E-selectin were indeed upregulated in the Genous stent samples as compared to BMS. For an extended discussion of the qPCR data, see supplemented data.

Previous studies have shown that, at least in the porcine models, the efficacy of EPC capture stent coverage was similar to that of BMS, with a optimal endothelial coverage of total stented area of 99% in both groups(16). However, it has to be taken into account that the lack of difference in response could be due to a low cross-reactivity of the human CD34 antibody on the Genous stent against porcine CD34 antigen, resulting in a suboptimal EPC capture. The *ex vivo* AV shunt data in the patients and primates in the current study, showed that the Genous stent was capable of rapid capture of circulating progenitor cells within the first hours of exposure. SEM and qPCR analysis have subsequently validated the endothelial phenotype of the adherent population. Taken together, our data indicate that the EPC capture stent is capable of accelerating the re-endothelialization process in exposure to the human circulation and therefore aid the vascular healing after vascular injury as compared to the BMS stent.

The combination of the EPC-capture and drug-elution technology has shown thus far to be a promising strategy in the pre-clinical setting(16). In contrast to that type of study, which focuses on late stent outcome, this study was predominantly designed to provide first-time proof of efficient EPC capture in human patients. Therefore, we have chosen to focuss on capture efficiency alone and compare the Genous to the BM stent. Although we have provided adequate proof for this cell-capture technology, drug-elution may compromise the cell capture efficiency and should be investigated for each of the new generation of combo-devices that are currently being developed.

A second important finding reported in this study is the effect of the Genous stent on thrombogenesis. The rapid coverage of the Genous stent by a protective endothelial lining was hypothesized to protect the stented area from thombogenesis and inflammation, thereby promoting a more efficient healing of the vascular wall. Indeed, macroscopic comparison of the Genous and BMS revealed the clear presence of mural thrombi in the BMS stent of one of the patients, whereas all the EPC capture stent remained free of visible thrombi. It should be mentioned that this particular patient only received ASA (no Clopidigrel therapy) before PCI. Although the patient's blood was exposed to both Genous and BM stents and the Genous stents showed no signs of thrombi, the striking difference in thombogenic response between the two stent types could be related to the absence of anti-platelet aggregation surface markers. To further elucidate this, biomarkers of coagulation and thrombosis were further examined in the human shunt material. We observed significant downregulation in PAI and TFPI expression in the lysates of EPC capture stent as compared to BMS, pointing to platelet ag-

gregation on the stent struts(20). Similarly, TFPI mRNA enrichment in the attached cells indicates in the BMS a rich platelet environment promoted by recruited inflammatory cells(21)·(22). Summarized, these data point to active EPC recruitment as playing a putative role in vascular protection against stent thrombosis. These findings were further validated in the baboon shunt experiments. For an extended discussion on this subject, see supplemental data.

The early EC lining on the stent could protect against active accumulation of inflammatory cells involved in the innate immune response. However, in line with the in vitro findings, there was no difference in other inflammatory markers or innate immunity markers such as CD68, CD14, MCP1 and CXCR-1. This could be due to the short exposure time, but more importantly, paracrine stimulation by the injured vessel wall is lacking in the human AV shunt setup. In the absence of cytokine and chemokine release to trigger inflammatory cell activation, the protective effect of the stent reendothelializing by circulating EPC on the inflammatory response, may only be limited. Previously, Granada et al. performed a comparison study of stents (combining CD34 capture with the Sirolimus-eluting strategy) with conventional drug eluting stents including Xience and Cypher in a porcine experimental model. It was demonstrated that the EPC capture technology further diminished overall intimal inflammation and giant cell accumulation after 28 days of implantation in the coronary arteries as compared to the Cypher and Xience stents(16). This was associated with a decrease in neointimal growth. This suggests that active re-endothelialization of drug eluting stents could indeed protect the injured vascular wall from further inflammatory activation, thereby protecting the stented area from further platelet adhesion and restenosis(16). Based on these findings, the Genous stent should provide vascular protection against thrombosis in the patients in short and long term follow-up. Recently, supporting data was presented by the e-HEALING (Healthy, Endothelial Accellerated Lining inhibits Neointimal Growth) multi-center registry in which the long-term effect of the Genous biogenineered R stent was followed in 5000 patients. Indeed, low levels of in-stent thrombosis and repeat revascularization of 1.1% and 5.7% respectively was observed at 12-month post intervention (26). New clinical trails are currently under evaluation in which the CD34 capture technology will be combined with Sirolimus-elution to assess novel combination strategies (REMEDEE: NCT00967902).

In conclusion, we showed in an AV shunt construction in human CAD patients and baboons that the CD34+ EPC recruitment promotes re-endothelialization and inhibited platelet adhesion. This specific aspect of the biological behavior of the Genous stent is especially promising as it could, combined with a drug-eluting strategy(16), yield safe and efficient therapy against restenosis, while diminishing the need for dual antiplatelet therapy after stent implantation.

Acknowledgement

Dedicated to Katarina Larsen, a hardworking and talented colleague, a loving and caring friend, and a kind and wonderful person who will be greatly missed by us all.

Conflict of Interest

Katarína Larsen *none*, Caroline Cheng *none*, Dennie Tempel *none*, Sherry Parker is employed by Orbusneich medical, Saami Yazdani *none*, Wijnand K den Dekker *none*, Jaco H Houtgraaf *none*, Renate de Jong *none*, Stijn Swager-ten Hoor *none*, Erik Ligtenberg is employed by Orbusneich medical, Steve Rowland is employed by Orbusneich medical, Frank Kolodgie *none*, Patrick W. Serruys *none*, Renu Virmani *none*, and Henricus J. Duckers *none*.

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SUPPLEMENTAL MATERIAL

Table 1. Human primer sets

GENE	SENS(5′ → 3′)	ANTISENS (3′ → 51′)
CD 14	CTTGTGAGCTGGACGATGAA	CCAGTAGCTGAGCAGGAACC
CD 16	GGCCTCGAGCTACTTCATTG	TTCCAGCTGTGACACCTCAG
CD31	ATGATGCCCAGTTTGAGGTC	ACGTCTTCAGTGGGGTTGTC
CD34	GCAAGCCACCAGAGCTATTC	TCCACCGTTTTCCGTGTAAT
CD68	ACCAAGAGCCACAAAACCAC	GGACTGTGAGTGGCAGTTGA
CXCR-1	AATCTGTCCCTGCCCTICTT	AGTGTACGCAGGGTGAATCC
E-SELECTIN	AGCCCAGAGCCTTCAGTGTA	AACTGGGATTTGCTGTGTCC
BETA ACTIN	AGCACTGTGTTGGCGTACAG	TCCCTGGAGAAGAGCTACGA
GAPDH	GGAGTGGGTGTCGCTGTTG	TGCCAAATATGATGACATCAAGAA
KDR	AGCGATGGCCTCTTCTGTAA	ACACGACTCCATGTTGGTCA
MCP-1	TCTGTGCCTGCTGCTCATAG	CAGATCTCCTTGGCCACAAT
PAI-1	CAACTTGCTTGGGAAAGGAG	GGGCGTGGTGAACTCAGTAT
PLVAP	GAGCTGGCCATCAGAAACTC	GGGACTCCAGGATCTTCCTC
P-SELECTIN	CCCAGACCACATCTCTGTGA	ACAGGGATGAGATGCAGACC
TF	GCGCTTCAGGCACTACAAAT	CGTCTGCTTCACATCCTTCA
TFPI	GGCCCATGTAAAGCAATCAT	TGGCTTTTCTTGTTGCAATG
VVVF	TGCTGACACCAGAAAAGTGC	AGTCCCCAATGGACTCACAG

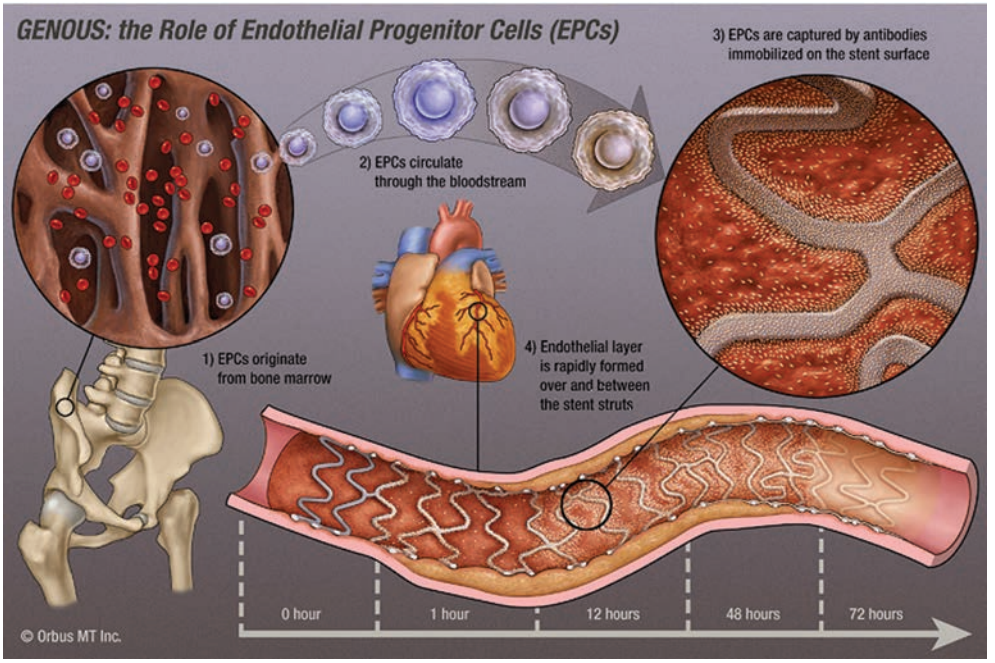

Figure 1. Schematic representation of a stented vascular wall with the Genous stent capturing circulating EPC.

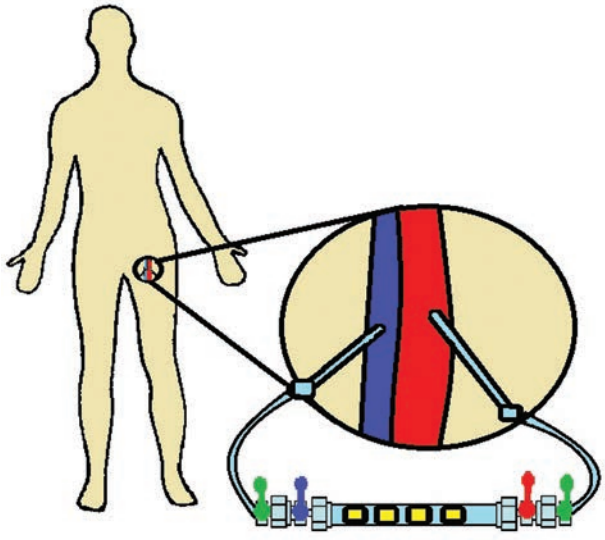

Figure 2. Schematic extracorporeal shunt connection to arterial (red stopcock) and venous (blue) catheter sheath introducer in femoral artery. Yellow squares indicate presence of 4 stents (2 BMS and 2 Genous stents) in the shunt tubing.

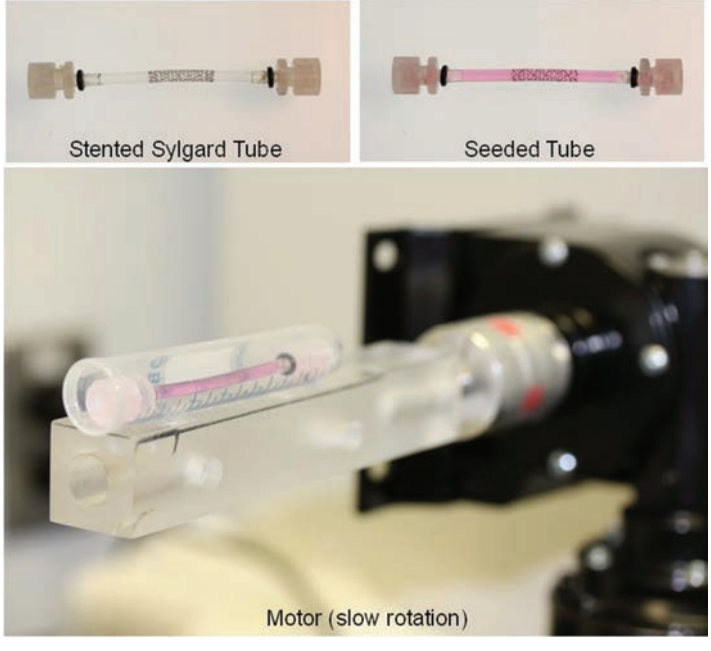

Figure 3. For the cell capture assay, a custom-made setup was created using sylgard 184 silicone tubes (with an inner diameter 3,14 mm, an a thickness of 0,5 mm). BM and Genous stents were deployed to obtain a stent-tube ratio of 1,1 to 1,0. The labeled monocyte and CD34+ cell suspensions were injected in the stented tube, and incubated under slow rotation for 2 hours at a rate of 0,3 RPM at 37°C/5% CO_2.

Material and Methods

Scanning Electron Microscopy (human AV shunt)

The study stents were fixed in situ with 4% formalin/PBS, retrieved from the tubing, and bisected longitudinally to expose the lumen surface. The stents were further processed for scanning electron microscopy (SEM) and were rinsed in sodium phosphate buffer (0.1-mmol/L, pH 7.2) and post-fixed in 1% osmium tetroxide for 30 minutes, followed by dehydration in a graded series of ethanols. After critical point drying, the samples were mounted and sputter-coated with gold. The specimens were visualized using a Hitachi Model 3600N scanning electron microscope and low power photographs of 10x were taken of the lumen surface to estimate the degree of EC coverage of the implant. Regions of interest were photographed at incremental magnifications of 200x to 600x.

Acute thrombogenicity

The thrombogenicity of Genous and BM stents was assessed using the ex vivo AV shunt baboon model of arterial thrombogenicity(1), in which accumulation of Indium-labeled platelets is measured in the stents implanted in a chronic arteriovenous femoral shunt in non-anticoagulated baboons. The baboons were not treated with anti-platelet drugs, but were monitored daily for general health and CBC counts. All the animals included in our experiments had normal clotting times as monitored weekly by aPT and PT.

After rinsing in sterile saline, the test stents were expanded in sterile tubing to 3.2 mm diameter utilizing an inflation device and the sterile tubing was connected to the arteriovenous loop and placed over a gamma camera. Platelet accumulation was measured at five minute intervals by imaging ^{111} Indium labeled platelets with a gamma camera, for up to 2 hours under continuous flow. The stents were subsequently visualized using scanning electron microscopy, after radiation had dissipated(1).

In vitro assessment of human CD34+ cell capture and confocal microscopy

Human adult monocytes (advanced Biotechnologies, USA) were cultured in RPMI1640 medium (Gibco, USA) supplemented with 20% FCS at 37°C/5% CO_2. Human peripheral blood derived CD34+ cells (Allcells, USA) were expanded in expansion medium composed of Stemline II Basal Media (Sigma, USA), supplemented with 2% G-CSF (Granulocyte CSF, Sigma, USA), and 1% Stemspan Cytokine Cocktail (Stemcell Technologies, USA). Human monocytes and CD34+ cells were labeled prior to the cell capture experiment, using a fluorescent labeling kit according to the manufacturer's instructions (PKH26 and PKH2 for monocytes and CD34+ cells respectively, Sigma, USA). For the cell capture assay, a custom-made setup was created using sylgard 184 silicone tubes (inner diameter 3,14 mm, 0,5 mm thickness). BM and Genous stents were deployed to obtain a stent-tube ratio of 1,1 to 1,0 (see supplemental figure 3). Labeled monocyte and CD34+ cell suspensions were injected in the stented tube, and was incubated under slow rotation for 2 hours at a rate of 0,3 RPM at 37°C/5% CO_2. After

the rotation cycle, stents were fixed in 10% formalin for 10 minutes, washed in PBS, cut longitudinally and imaged under confocal microscope (Zeiss, USA), using 490 nm/504 nm and 551 nm/567 nm excitation and emission for green fluorescently labeled CD34+ cells and red fluorescently labeled monocytes respectively. Quantification of captured cells was performed by counting individual cells on the struts, normalized to stent length.

Ex vivo human AV shunt

The study stents were introduced and deployed into a sterilized medical grade Silastic™ tubing (inner diameter 3,14 mm, 0,5 mm thickness, Dow Corning, USA,). BM and Genous stents were deployed to obtain a stent-tube ratio of 1,1 to 1,0 and the shunt was connected between arterial and venous sheaths that were placed in patients for vascular access to form an AV shunt. The relative position of the BMS and the Genous stent in the AV shunt was alternated to eliminate position bias. The patients were anti-coagulated with sodium heparin to achieve an activated clotting time (ACT) greater than 300 seconds during their PCI procedure. Additionally, the blood flow in the AV shunt was assessed continuously throughout the experiments with a coronary flow wire to monitor for changes in blood flow indicative of thrombotic occlusion in order to reduce the risk thrombo-emboli formation during the procedure. The flow was maintained at 47.6 ± 14.6 cc/ml comparative to coronary flow.

Statistical analysis

All data are expressed as means ± sem. Comparisons between the patients groups are performed using a paired or non-paired student's t-test when appropriate for the type of comparison. A P value less than 0.05 is considered statistically significant.

Discussion

KDR is an EPC and EC marker, but is also a vital receptor for EC proliferation, implying that the captured progenitor cells were also proliferative. Re-endothelialization efficiency of the stent at a later time point was further studied in a rabbit model for arterial balloon injury and vascular repair. Ultrastructural analysis demonstrated a modest increase in strut coverage by spindle shaped (endothelial) cells at the proximal and distal ends of the Genous versus BM stent, at 7 days after implantation. qPCR analysis subsequently validated the presence of EC on the stent surface by mature endothelial (progenitor) cell markers including CD34, CD31, Tie2, and P-selectin, as all these markers were upregulated in the EPC capture stent versus the BMS group.

To further substantiate these findings, platelet aggregation was assessed in a baboon AV shunt model. Accelerated re-endothelialization by CD34+ progenitor cells prevents platelet adhesion in AV shunts

in primates, potentially by competing with the adhesion of blood-derived platelets and inflammatory cells. Similarly, the adherent progenitor cell population could contribute to stent surface protection against platelet aggregation(2, 3), rendering the overall effect of the Genous stent to be vascular-protective. Additional *in vitro* experiments using labeled human blood derived monocytes and CD34+ cells, clearly showed specific capture of CD34+ cells on the Genous stent strut surface, whereas monocyte adhesion was similar to that of BMS after a short cell-seeding procedure of 2 hours. This *in vitro* data would suggest that the involved protective mechanism is not physical competition in cell adhesion, but rather a potential paracrine effect of captured EPCs that benefits the prevention of platelet aggregation.

To assess whether the stent coverage provides protection against inflammation in the injured vessel segment, expression levels of CD16 was quantified. CD16 is expressed on neutrophils, monocytes, macrophages and natural killer cells. When interpreting the data it has to be taken into account that leukocyte adhesion during the human immune response is not a static process. It includes cell activation, rolling, and adhesion, followed by either firm adhesion or diapedesis over the basal membrane, or detachment from the vascular surface after which the leukocyte re-enters the blood flow(4). Prolonged retention times on an activated vascular area indicate an inflammatory response. CD16 levels correlated with the exposure of stents to blood flow, showing a decrease in CD16 signal over time.

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Chapter 13

Final results of the HEALING IIB trial to evaluate a bio-engineered CD34 antibody coated stent (Genous™ Stent) designed to promote vascular healing by capture of circulating endothelial progenitor cells in CAD patients.

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Abstract

Objective to assess the safety and efficacy of the Genous™ endothelial progenitor cell (EPC) capturing stent in conjunction with HmG-CoA-reductase inhibitors (statins) to stimulate EPC recruitment, in the treatment of patients with de novo coronary artery lesions.

Methods and results The HEALING IIB study was a multi-center, prospective trial, including 100 patients. The primary efficacy endpoint was late luminal loss by QCA at 6 month follow-up (FU). Although statin therapy increased relative EPC levels by 5.6-fold, the angiographic outcome at 6 month FU was not improved in patients with an overall in-stent late luminal loss of 0.76±0.50 mm. The composite major adverse cardiac events (MACE) rate was 9.4%, whereas 6.3% clinically justified target lesion revascularizations (TLRs) were observed. 2 Patients died within the first 30 days after stent implantation due to angiographically verified in-stent thrombosis. At 12 month FU, MACE and TLR increased to 15.6% and 11.5% respectively and stabilized until 24 month FU. 18 Month angiographic FU showed a significant decrease in late luminal loss (0.67±0.54, 11.8% reduction or 10% by matched serial analysis, P=0.001)

Conclusion the HEALING IIB study suggests that statin therapy in combination with the EPC capture stent does not contribute to a reduction of in-stent restenosis formation for the treatment of de novo coronary artery disease. Although concomitant statin therapy was able to stimulate EPC recruitment, it did not improve the angiographic outcome of the bio-engineered EPC capture stent. Remarkably, angiographic late loss was significantly reduced between 6 and 18 months. (ClinicalTrial.gov number, NCT00349895.)

Introduction

Drug eluting stents (DES) have emerged as an effective means of attenuating stent-related restenosis formation and has enabled the field of percutanuous coronary intervention to move forward to complex coronary angioplasty, with an efficacy equivalent to surgical intervention in the short and long term [1]. The mainstay of drugs eluted from the stent polymer comprise cytostatic or cytotoxic compounds to impede neointimal formation that arise from vascular smooth muscle cell (VSMC) activation and proliferation. Lately, DES have been associated with late in-stent thrombosis, presumably based on a concomitant impeded arterial repair response characterized by incomplete endothelial coverage of the stent struts, persistent fibrin deposition and inflammation beyond 24-month post implantation [2]. Therefore, prolonged (6-12 months) dual anti-platelet therapy has been recommended in conjunction with DES in order to mitigate the risk of stent thrombosis that is associated with incomplete healing. In addition, the polymer coating of the DES, which ensures prolonged release of the anti-proliferative compounds, inherently invokes inflammation and cytotoxicity with delayed stent coverage. Furthermore, non-erodable polymers were associated with granulomatous and hypersensitivity reactions in relevant animal models. Finally, DES have been shown to interfere with proper endothelial function in arterial segments adjacent to the implanted stent, as suggested by an impaired or paradoxical vasomotor response, which could pose the segment at risk for ischemia and coronary occlusion [3].

Alternatively, therapeutic interventions aimed to facilitate the vascular repair response following coronary intervention could reinstate endothelial integrity and maintain vascular senescence to prevent VSMC proliferation, local inflammation and vascular platelet activation, while maintaining local vasomotor function to ensure short and long-term success of the treated arterial segment. The underlying strategy of accelerated endothelialization aims to provide a non-thrombogenic coating of exposed stent surfaces to reduce in-stent thrombosis and potentially decrease neointimal hyperplasia. Reinstatement of the endothelial integrity can be augmented by local delivery of endothelial mitogenic compounds, including VEGF [4], or facilitation of attachment of circulating endothelial progenitor cells (EPCs) to the strut surface.

In 2003, development was begun of a bio-engineered stent with a proprietary coating containing an anti-human CD34 antibody (Genous™ Bio-engineered R stent, OrbusNeich) that sequesters circulating CD34+ haematopoietic cells to the stent strut surface, facilitates strut coverage and initiates the arterial repair response. Moreover, the combination of this CD34 antibody-coating with conventional sirolimus-eluting stents appeared to overcome the delayed endothelial coverage in stented porcine coronary arteries [5].

The first clinical experience with the EPC capturing Genous Stent was obtained in 16 patients in the HEALING-FIM study. The healing technology approach was deemed safe and feasible with an in-stent late luminal loss of 0.63±0.21 mm at the 6 month angiographic follow-up (FU) [6]. The original design of this stent platform was adapted before proceeding to the phase I/II study, including modification of monoclonal antibody with a higher affinity and transfer from a wet to a dry lyophilized prepara-

tion CD34-antibody coated stent premounted on an Evolution2™ PTCA balloon catheter. In the HEALING II study, treatment with the Genous Stent of 63 elective patients with a de-novo lesion resulted in a 6-month angiographic late loss of 0.78±0.39 mm and percent in-stent volume obstruction of 22.9±13.7% [7,8]. A sub-analysis of the individual patients suggested that specifically patients with a lower EPC titer responded relatively poorly to the EPC capture technology compared to patients with a normal EPC titer, with more prominent late loss and higher incidence of revascularization events. Low EPC titer concurred with a lack of HMG-CoA-reductase inhibitors (statins) in the pharmacotherapy of these CAD patients.

As statins have been shown to augment EPC titer, survival, and activity in vitro and in vivo, we hypothesized that concomitant treatment of CAD with statin therapy could stimulate the EPC titer and efficacy in these patients and therefore the overall response to the EPC capture technology. This led to the design of the HEALING IIB study that aimed to assess the safety and efficacy of the Genous Stent in conjunction with optimal statin therapy to stimulate EPC recruitment in the treatment of stent-related restenosis formation in 100 elective patients with de novo native coronary artery lesions.

Methods

For full description of the Methods, an expanded Methods section is available in the On-line Data Supplement.

Results

Baseline characteristics and procedural outcome

One hundred patients were included in the HEALING IIB study. Four patients were per protocol excluded from post-procedural analysis; two patients did not meet the in- and exclusion criteria, one patient was not on Atorvastatin during two weeks pre-procedure as required per protocol and one patient had a post-procedural diameter stenosis of 32% despite post dilatation (see figure 1 for flow chart). The baseline patient demographics, lesion characteristics and clinical outcomes of the current HEALING IIB and earlier HEALING II study are summarized in tables 1-3. The study population had an average age of 64 years, whereas 20% of the patients suffered from diabetes mellitus. Compared to the HEALING II study, patients in the HEALING IIB study had significantly more hypertension, hypercholesterolemia and stable angina and there were more current smokers. On the other hand, there were significantly less previous smokers and patients with unstable angina. At the index procedure, 100% of the patients were initiated on high dose Atorvastatin therapy (80 mg qd) for at least 2 weeks according to the protocol outline. At one month FU, 91% of the patients were still being treated with Atorvastatin 80 mg qd. In 82 patients, one single index lesion was treated, whereas in 14 patients, 2 lesions in 2 independent coronary arteries received a study stent (total 110 lesions treated). The

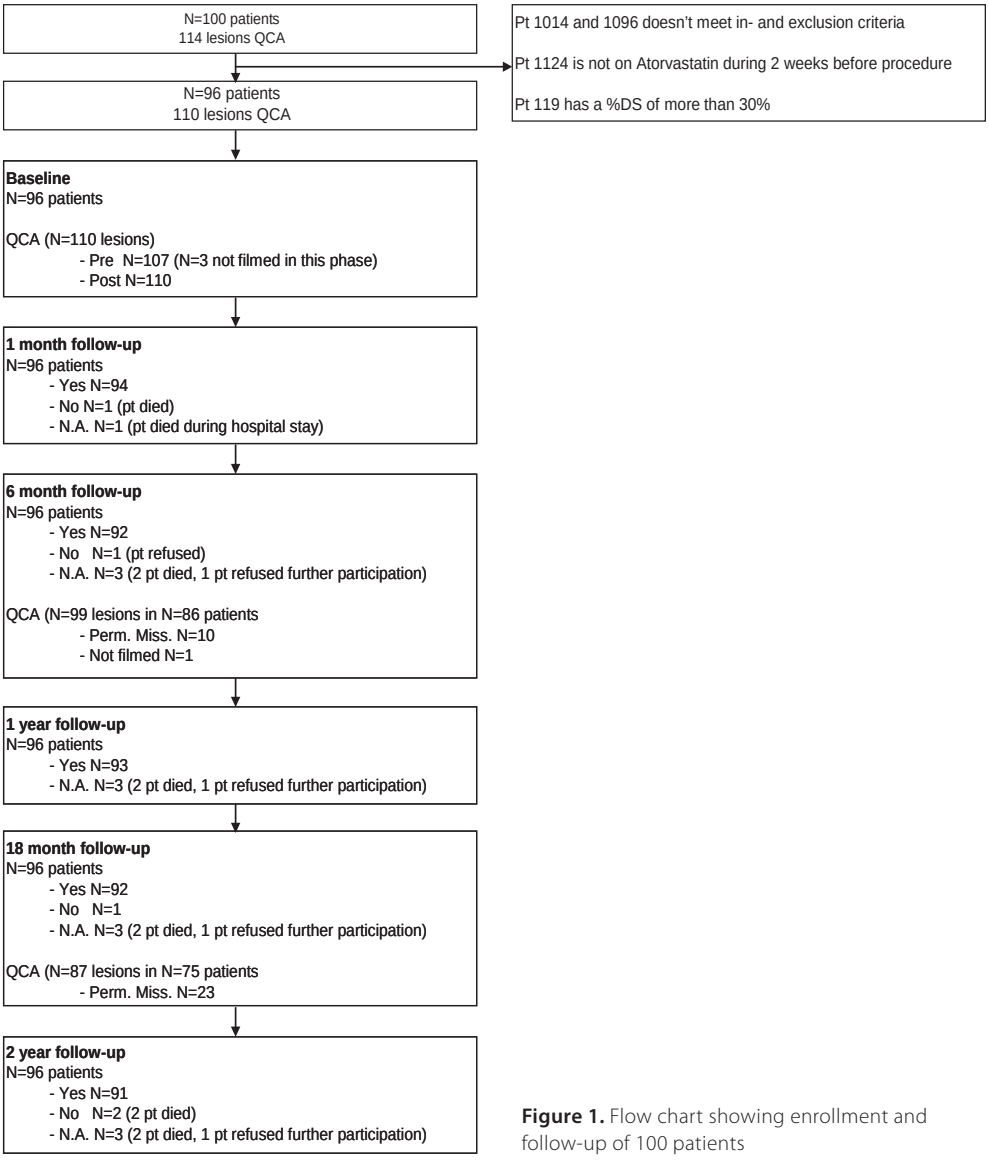

Figure 1. Flow chart showing enrollment and follow-up of 100 patients

average lesion length comprised 12.64±5.69 mm in length, with a diameter stenosis of 57.9%±9.9%, as assessed by QCA. Angiographic and overall procedural success were respectively 97.3% (107 out of 110 lesions treated) and 93% (89/96 patients; table 4). A second overlapping EPC capture stent was implanted in three patients to treat a dissection following implantation of the first stent, in three patients due to a type A/B dissection, and in eight patients due to incomplete coverage of the target lesion by the first stent. Two patients displayed a transient TIMI II coronary flow following stent deployment. None of the patients received GPIIb/IIIa inhibitors during or after the index procedure.

Table 1. Patient demographics and clinical characteristics

Patient parameter	HEALING II (N=63)	HEALING IIB (N=96)
Age (years)		
Mean ± SD	60.8 ± 10.2	63.7 ± 9.8
Min-max	43-78	43-78
Body Mass Index (kg/m^2)		
Mean ± SD	26.8 ± 3.7	27.3 ± 4.5
Min-max	18.7-38.1	17.1-41.9
Male	66.7% (42/63)	73.7% (71/96)
Diabetes mellitus	12.7% (8/63)	19.8% (19/96)
Hypertension	46.0% (29/63	66.7% (64/96)[a]
Hypercholesterolemia	66.7% (42/63)	84.4% (81/96)[a]
Previous stroke	3.2% (2/63)	3.1% (3/96)
Congestive heart failure	0.0% (0/63)	3.0% (3/96)
Family history of MI	52.4% (33/63)	55.2% (53/96)
Peripheral vascular disease	3.2% (2/63)	9.4% (9/96)
Previous MI	23.8% (15/63)	26.% (25/96)
Previous CABG	3.2% (2/63)	2.0% (2/96)
Previous PTCA	9.5% (6/63)	18.8% (18/96)
Smoking history		
Previous	2.2% (14/63)	42.7% (41/96)[b]
Current	41.3% (26/63)	20.8% (20/96)[b]
Unstable angina	27.0% (17/63)	8.3% (8/96)[b]
Braunwald I	9.5% (6/63)	1.0% (1/96)
Braunwald II	1%2. (8/63)	5.2% (5/96)
Braunwald III	4.8% (3/63)	2.1% (2/96)
Stable angina	60.3% (38/63)	80.2% (77/96)[b]
CCS I	7.9% (5/63)	11.5% (11/96)
CCS II	28.6% (18/63)	56.3% (54/96)
CCS III	20.6% (13/63)	10.4% (10/96)
CCS IV	3.2% (2/63)	2.1% (2/96)
Silent ischemia	12.7% (8/63)	11.5% (11/96)

Numbers are % (counts/available field sample size) or mean ± 1 Standard deviation

SD	Standard deviation
MI	Myocardial Infarction
CABG	Coronary Artery Bypass Graft
PTCA	Percutaneous Transluminal Coronary Angioplasty
Braunwald	Braunwald Classification
CCS	Canadian Cardiovascular Society Classification

[a] P<0.05
[b] P<0.01

Table 2. Lesion characteristics

	HEALING II		HEALING IIB	
Parameter	#	SD or %	#	SD or %
Study stents per patient	1.1	0.4	1.3	0.5[b]
Treated segments	63		110	
RCA	24	38.1%	40	36.5%
LM	0	0.0%	0	0.0%
LAD	25	39.7%	43	39.1%
LCX	14	22.2%	27	24.5%
Total stent length (mm)	17.0	2.9	21.4	8.5[b]
Lesion type[a]	63		107	
A	0	0.0%	1	0.9%
B1	27	42.9%	49	45.8%
B2	36	57.2%	53	49.5%
C	0	0.0%	4	3.7%

LAD	Left Anterior Descending
LCX	Left Circumflex Artery
LM	Left Main
RCA	Right Coronary Artery

[a] According to ACC/AHA classification
[b] P<0.0001

Clinical outcomes

Compliance to clinical FU at 6, 12 and 24 months was 96%, 96% and 95% respectively. Table 3 provides an overview of the major adverse cardiac events (MACE) at 1, 6, 12, 18 and 24 months. Clinical outcomes were comparable with the HEALING II study, as there was only a significant difference in TVR at 18 months. Acute in-stent thrombosis was verified in two patients within the 30 days FU. One patient suffered from an angiographically verified in-stent thrombosis at day one post-implantation and died. Another patient suffered from an angiographically verified acute in-stent thrombosis at day 9 post-implantation and the patient died. This patient received stents in a bifurcation with residual dissection following stent implantation, whereas a second stent could not be implanted. The procedure was assumed not to be a procedural success with suboptimal stent implantation. Both of these events have been adjudicated as cardiac deaths due to a definite in-stent thrombosis by the independent CEC. Finally, one patient had a clinically driven target lesion revascularization (TLR) at 181 days following the index procedure, and 4 days after the re-intervention due to a clinically-driven TLR, designated as a secondary, definite in-stent thrombosis. During the re-intervention the interventionist reported a left main dissection and thrombus in situ in LAD and left circumflex with normal coronary flow.

Clinically driven TLR by repeat PCI occurred in 6.3% at 6 month FU and increased to 9.4% at 12 month FU. After 12 month FU, no further TLRs were reported and the TLR rate remained stable at 9.4%. Total MACE rate of the patients treated with the EPC capture device was 9.4%, 15.6% and 16.6% at 6,12 and 24 months respectively (see figure 3 for Kaplan-Meier curve)**.** Target vessel failure (clinically-driven

Table 3. Clinical outcomes in HEALING II and HEALING IIB studies

	At discharge		1 Month FU		6 Month FU		9 Month FU		12 Month FU		18 Month FU		24 Month FU	
	HEALING II	HEALING IIB	HEALING II	HEALING IIB	HEALING II	HEALING IIB	HEALING II	HEALING IIB	HEALING II	HEALING IIB	HEALING II	HEALING IIB	HEALING II	HEALING IIB
N	63	96	63	96	63	96	63	96	63	96	63	96	63	96
MACE	0.0%	5.2%	0.0%	5.2%	NA	9.4%	7.9%	NA	NA	15.6%	7.9%	15.6%	NA	16.6%
Cardiac death	0.0%	1.0%	0.0%	2.1%	NA	2.1%	1.6%	NA	NA	2.1%	1.6%	2.1%	NA	2.1%
MI	0.0%	4.2%	0.0%	5.2%	NA	5.2%	0.0%	NA	NA	5.2%	0.0%	5.2%	NA	5.2%
Q-wave	0.0%	1.0%	0.0%	2.1%	NA	2.1%	0.0%	NA	NA	2.1%	0.0%	2.1%	NA	2.1%
Non Q-wave	0.0%	3.1%	0.0%	3.1%	NA	3.1%	0.0%	NA	NA	3.1%	0.0%	3.1%	NA	3.1%
TLR clinically driven	0.0%	1.0%	0.0%	2.1%	NA	6.3%	6.3%	NA	NA	11.5%	6.3%	11.5%	NA	11.5%
CABG	0.0%	0.0%	0.0%	0.0%	NA	0.0%	0.0%	NA	NA	2.1%	0.0%	2.1%	NA	2.1%
Re-PCI	0.0%	1.0%	0.0%	2.1%	NA	6.3%	6.3%	NA	NA	9.4%	6.3%	9.4%	NA	9.4%
TVR	0.0%	1.0%	0.0%	2.1%	NA	6.3%	20.6%	NA	NA	15.6%	25.4%	15.6%	NA	16.6%
TVF	0.0%	2.1%	0.0%	3.1%	NA	7.3%	11.2%	NA	NA	14.6%	11.2%	14.6%	NA	14.6%
Stent thrombosis	0.0%	1.0%	0.0%	2.1%	NA	2.1%	0.0%	NA	NA	3.1%	0.0%	3.1%	NA	3.1%
Definite/probable	0.0%	1.0%	0.0%	2.1%	NA	2.1%	0.0%	NA	NA	3.1%	0.0%	3.1%	NA	3.1%
Definite	0.0%	1.0%	0.0%	2.1%	NA	2.1%	0.0%	NA	NA	3.1%	0.0%	3.1%	NA	3.1%
Probable	0.0%	1.0%	0.0%	2.1%	NA	2.1%	0.0%	NA	NA	3.1%	0.0%	3.1%	NA	3.1%
Possible	0.0%	1.0%	0.0%	1.0%	NA	1.0%	0.0%	NA	NA	0.0%	0.0%	0.0%	NA	0.0%

CABG Coronary Artery Bypass Graft
MACE Major Adverse Cardiac Events: Cardiac Death, Myocardial Infarction (Q-wave, non Q-wave, emergent CABG or Clinically driven TLR as determined by the CEC
TLR Target Lesion Revascularization
TVR Target Vessel Revascularization, clinically and non-clinically driven
TVF Target Vessel Failure: clinically driven target vessel revascularization, Q-wave or non Q-wave MI, or cardiac death that could be clearly attributed to a vessel other than the target vessel

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[a] $P<0.05$

Table 4. Principal effectiveness and safety (results n=96 patients, n=110 lesions)

Effectiveness measures	Post procedure		6 Months FU		18 Months FU	
Angiographic succes	97.3%	(107/110)				
Procedural succes	93.0%	(89/96)				
In-stent binary restenosis rate			23%	(23/99)		
RVD	2.95±0.50	(n=110)	2.79±0.61	(n=96)	2.72±0.60	(n=84)
MLD	0.57±0.41	(n=110)	1.81±0.68	(n=99)	1.68±0.68	(n=87)
% DS in-stent	12.73±6.20	(n=110)	35.2±18.74	(n=99)	31.75±19.96	(n=87)
Stent thrombosis			3.1%	(3/96)	3.1%	(3/96)
Late loss in-stent (mm)			0.76±0.50	(n=99)	0.67±0.54	(n=86)
Acute gain in-stent (mm)	1.42±0.38	(n=107)				
In-stent malapposition	0.0%	(0/62)				
QCA in-stent volume obstruction			32.68±25.2	(n=96)	22.5±28.33	(n=84)

MACE	Major Adverse Cardiac Events: Cardiac Death, Myocardial Infarction (Q-wave, non Q-wave, emergent CABG or clinically driven TLR as determined by the CEC
RVD	Reference vessel diameter (mm)
MLD	Minimal luminal diameter (mm)
%DS	Percent diameter stenosis

Numbers are % (counts/available field sample size) or mean ± standard deviation

TVR, recurrent infarction or cardiac death that cannot be attributed to a vessel other than the target vessel) was 7.3% at 6 month FU and 14.6% at 12 and 24 month FU (table 3).

Angiographic FU occurred in 89.9% (86/96 patients; 99 lesions) at 6 months and in 78.1% (75/96 patients; 87 lesions) at 18 months. The mean in-stent luminal diameter (MLD) in the Genous Stent at 6 months was 1.81±0.68 mm (post-procedural in-stent MLD 2.57±0.41 mm), whereas late luminal loss was 0.76±0.50 mm by QCA (table 4). At 18 month angiographic FU, mean MLD increased to

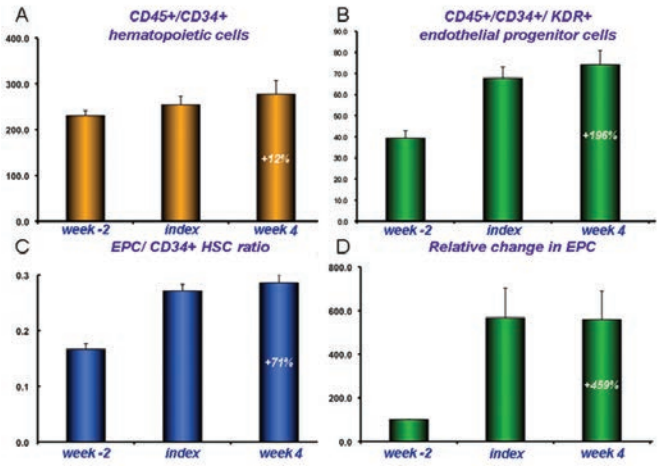

Figure 2. Effect of high dose Atorvastatin on circulating CD34+ haematopoietic cells and EPC levels. High dose Atorvastatin leads to a mild increase in CD34+ haematopoietic cells (A), whereas absolute and relative EPC count weremarkedly increased (B and D). There was a subsequent increase in EPC/CD34+ haematopoietic stem cell (HSC).

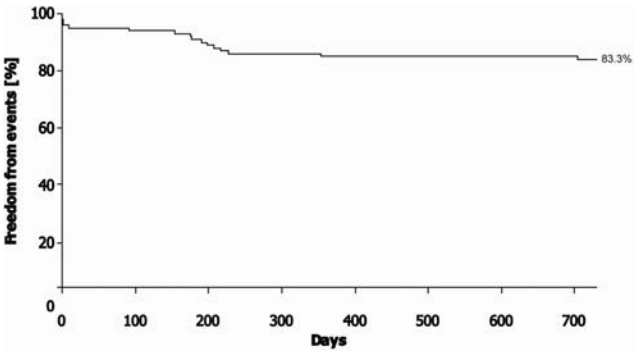

Figure 3. Kaplan-Meier curve showing the event free survival for Major Adverse Cardiac Events (MACE). Mace are defined as the incidence of cardiac death,Q-wave or non Q-wave myocardial infarction, emergency cardiac surgery and clinically justified target lesion revascularization

1.86±0.68, whereas late luminal loss significantly decreased to 0.67±0.54 (12% reduction as compared to 6 month FU; 10% reduction using serial matched analysis, P=0.001). There was no difference in late luminal loss between diabetics and non diabetics (0.76±0.56 mm vs. 0.76±0.50 mm).

Circulating EPC levels in patients receiving the Genous Stent

EPC titer was analyzed before initiation of Atorvastatin 80 qd at screening, at the index procedure (at 2 weeks statin treatment) and at 1 month FU. Blood samples were quantified in a blinded fashion in all 100 patients. At screening, 49% of patients were maintained on some statin therapy at conventional doses. Two weeks following conversion to high dose Atorvastatin, relative EPC levels were increased by 5.6-fold +/- 1.30 to 63.4 EPCs/100µl whole blood, as compared to baseline values (fig. 2D), whereas CD34+ circulating hematopoietic cells remained largely unaffected by statin therapy with an increase of only 12% (fig. 2A). These data suggest that high dose statin therapy facilitates commitment of the CD34/CD45 hematopoietic cell lineage into the committed endothelial (progenitor) cell fate, rather than recruitment of endothelial progenitor cells and hematopoietic stem cells from the primary niche. EPC levels remained elevated until the 30 day FU after stent implantation.

Discussion

The HEALING IIB clinical trial was a multi-centered, open-label, prospective study to evaluate the safety and efficacy of a stent bioengineered to capture circulating EPCs to promote stent re-endothelialization and initiate vascular healing in combination with optimized statin therapy. Previously, post-hoc stratification of study patients in the HEALING II study suggested that in particular patients with low EPC titers responded poorly to the EPC capture stent technology with a late loss and TLR events equivalent to bare metal stents [7]. Moreover, several studies have suggested that cardiovascular risk factors, including hypertension, aging, hypercholesterolemia, and diabetes mellitus, are associated with low EPC titers and EPC dysfunction [9]. Low EPC titer in the HEALING II study was also associated with a lack of statin therapy in these CAD patients (statin use in patients with low vs. normal EPC levels: 41% vs. 92%) [7]. Indeed, statin therapy has been shown to augment EPC levels in-vivo, and improve EPC survival, clonal capacity and adhesion in in-vitro assays by improved eNOS bioavailability, as well

as via activation of the PI3-Akt dependent eNOS pathway [9-11]. Therefore, we hypothesized that combined treatment of high-dose statin therapy with the EPC capture stent technology may improve the response of the patient population with low EPC titers, and the overall response to this pro-healing concept of percutaneous coronary intervention.

At enrollment, 79% of CAD patients were already maintained on statin therapy (34% Atorvastatin, 45% other statin therapy**).** Within two weeks after initiation of high dose Atorvastatin pharmacotherapy, relative EPC levels increased by 5.6-fold at the time of the index procedure and maintained elevated during a 30 day FU. Despite effective EPC recruitment, treatment of elective patients with de-novo coronary artery disease with the EPC capture Genous Stent resulted at 6 month angiographic FU in a late luminal loss of 0.76±0.50, and a QCA-derived percent volume obstruction of 34±26% (table 4). The overall ischemia-driven and non-ischemia-driven revascularization rate at 6 month FU comprised 6.3%, whereas all MACE were 9.4%. These data are not significantly different from the results of the HEALING II study, although MACE seems somewhat increased in the HEALING IIB study. This difference may be explained by the fact that in the Healing II study patients with only one vessel disease were included, whereas in the HEALING IIB study, patients with multivessel disease were also included, making them more complex patients and more susceptible to MACE. Consequently, the number of study stents per patient and total stent length was significantly higher in the HEALING IIB study (see Table 2). Remarkably, comparable with the HEALING II study, angiographic late loss was significantly reduced by 0.10 mm (by matched serial analysis, P=0.001) between 6 and 18 months. This finding contrasts with findings in DES treated patients, where a progression of late loss is generally observed [12]. Furthermore, one could speculate whether this regression in late luminal loss was reflected in clinical outcome, as MACE and TLR stabilized between 12 and 24 month FU. The question can therefore be raised whether the apparent advantage of DES in comparison with the EPC capture stent is only a temporal advantage, or whether longer-term clinical outcomes will prove otherwise. To address this question, randomized studies comparing DES and the Genous Stent, with clinical and angiographic FU after 24 months are required.

Re-endothelialization to inhibit in-stent restenosis formation

The assumption that regrowth of endothelium may limit neointimal accumulation has raised inconsistent results in pre-clinical and clinical studies. In gently denudated rat aortas, smooth muscle cell proliferation is not increased subjacent to the areas of endothelial loss [13]. In rat carotid arteries, neointimal accumulation after arterial injury is more related to medial injury than endothelial loss [14]. In balloon-injured rabbit iliofemoral arteries, early restoration of endothelium by autologous endothelial seeding did not decrease neointimal accumulation [15]. Human atherosclerotic plaques have been shown to develop under morphologically intact endothelium [16], whereas endothelialized human coronary stents also contained substantial neointimal hyperplasia [17].This suggests that the presence of endothelium and a resistance to intimal growth hence is not inextricably linked. In contrast, in a rabbit arterial balloon injury model, local delivery of VEGF accelerated stent endothelialization and reduced neointimal hyperplasia at 28 days FU [18] **.**

Various studies have shown that circulating EPCs are up-regulated following arterial injury and incorporated at the site of balloon or stent injury to form a new endothelial lining and initiate the vascular repair response [19]. Also, low circulating EPC levels or dysfunctional EPCs in CAD patients were associated with an increased risk of restenosis formation [20] . Stents bearing coatings of antibodies that specifically capture EPCs and immobilize these to the strut surface have been shown to accelerate endothelialization of the stent within 24 hours following stent implantation in rabbit aortic and porcine iliofemoral and coronary arteries (RGD coating [21], anti-CD105 coating [Active Endothelial Cell Capture by Stents Coated with Antibody against CD105, Song et al. TCT Asia Pacific 2009], and anti-CD34 coating).

However, the role of circulating EPCs on progression of atherosclerosis and neointimal hyperplasia is poorly understood and various lines of research have yielded conflicting results. Whereas Rausher and co-workers demonstrated that bone marrow-derived mononuclear cells attenuated the atherosclerotic burden in ApoE-deficient mice, others have shown that in the ApoE-deficient mice intravenous infusion of bone marrow derived mononuclear cells promoted atherosclerosis formation and in some studies was also associated with a more vulnerable composition of the atherosclerotic lesions with enhanced vascularization, larger lipid cores, thinner fibrous caps and higher numbers of infiltrating CD3 cells [22]. Also, the effect of CD34+ circulating endothelial and smooth muscle progenitor cells on post-injury neointimal hyperplasia is poorly understood. Whereas some studies indicated a benefit on in-stent neointimal hyperplasia in stented pig arterial segments explanted at different time points [23], others have failed to demonstrate a benefit in in-stent neo-intimal hyperplasia in porcine coronary arteries, despite a benefit on EPC capture and accelerated endothelialization (EPC capture in stented porcine coronary arteries increases endothelialization, but does not affect intimal thickening, HM van Beusekom et al., accepted in CCI, 2011). Also, accelerated coverage of PTFE prosthetic vascular in a porcine AV shunt graft model failed to prevent neointimal hyperplasia at the venous anastomosis, and actually, stimulated cellular proliferation led to prominent neointima formation [24]. The current study suggests that despite stimulation of EPC titer in all elective CAD patients, accelerated coverage of the EPC capture stent did not seem to impede restenosis formation and clinical MACE at 6 month FU and questions the paradigm that early re- endothelialization is a prerequisite for appropriate attenuation of neointimal hyperplasia. Even though we were unable to find an early effect on restenosis, there was a significant long term effect in terms of a reduction in late luminal loss between 6 and 18 months. This regression was accompanied by a stabilization of MACE and TLR rates between 12 and 24 months FU.

Re-endothelialization to inhibit in-stent thrombosis

Alternatively, accelerated re-endothelialization by an EPC capture stent technology may provide a non-thrombogenic coating of the exposed stent struts, thereby reducing the risk of in-stent thrombosis and potentially decreasing or eliminating the need for anti-thrombotic therapy. Recently, we showed in an ex-vivo human and baboon shunt model that in-stent thrombosis is decreased in the Genous Stent when compared to BMS [25]. Together with a decrease in mural thrombi, we showed a

significant decrease in expression of tissue factor pathway inhibitor (TFPI) and plasminogen activator inhibitor-1 (PAI-1), markers of thrombosis and coagulation, in the Genous Stent compared to BMS. On the contrary, some animal studies of arterial injury have previously suggested that thrombogenicity following arterial injury decreases in the absence of endothelialization. For instance, in balloon-injured rabbit aortas platelet deposition occurred within minutes, but did not increase over the subsequent 24 hours [26]. Despite the absence of endothelialization, over the course of a week, the number of vessel-adhered platelets actually decreased, indicating that thrombogenicity decrease preceded endothelial regrowth. Moreover, exposed smooth muscle cells in balloon-injured rat carotid arteries were able to maintain a relatively non-thrombogenic surface [27] . Local delivery of VEGF in stented iliac arteries of non-atherosclerotic rabbits resulted in a near complete re-endothelialization by day 7 following stent implantation versus placebo delivery with an associated reduction of mural thrombus formation. Even though the strength of the inverse correlation between stent endothelialization and the development of organized thrombus was relatively low (r^2=.52), these results suggest that next to endothelialization, other factors play an important role in thrombus suppression [28].

Recent autopsy and angioscopy studies suggested that following implantation of sirolimus eluting stents (SES) and paclitaxel eluting stents (PES), reendothelialization and arterial healing was incomplete for up to 6-12 months, therefore rendering the vessel more prone to late in-stent thrombosis [29]. More extensive endothelial dysfunction and impeded arterial repair following implantation of SES/PES in remote vascular segments have been suggested by a persistent aberrant coronary vasomotor response to exercise and acetylcholine stimulation of the vascular segments adjacent to the implanted stent. The secondary end points of the HEALING IIB study therefore included in-stent thrombosis at 30 days and incidence of MACE at 6-month FU. 3 Patients suffered from a definite in-stent thrombosis at day 1, 9 and 181 days following the index procedure (by ARC definitions)**.** The overall 3% incidence of definite in-stent thrombosis does not suggest an anti-thrombotic capacity of the EPC capture stent technology in the current study; however, all three events had documented significant procedural complications, which might have facilitated the in-stent thrombosis. For example, in the HEALING II study 0% stent thrombosis was reported and in the e-HEALING clinical registry of a real-world population (n=4939) treated with the Genous Stent, the 12 month definite and probable stent thrombosis rate was 1.1% [30]. Moreover, it should be noted that the current study was largely underpowered to obtain a reliable estimate of the stent thrombosis rate with the Genous Stent. The stent thrombosis rate reported in the e-HEALING registry seems to confirm the favorable real world results with the Genous Stent, even in the absence of prolonged dual anti-platelet therapy.

A limitation of the study is the low compliance to 18 month angiographic follow up (78.1%, 75/96), which may have resulted in loss-to-follow up bias and underestimation of the stent thrombosis rate or late luminal loss.

In conclusion, in the HEALING IIB study, 100 elective patients with de novo coronary artery lesions received a Genous Stent in conjunction with HmG-CoA-reductase inhibitors (statins) to stimulate EPC recruitment. Although high dose statin therapy adequately enhanced EPC titers at the index procedure, the EPC capture stent technology did not sufficiently impede clinical restenosis rates and late

luminal loss at 6 month angiographic FU. However, a significant reduction in late luminal loss was observed between 6 and 18 month angiographic FU which was accompanied by a stabilization of MACE and TLR rates between 12 and 24 months FU.

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Chapter 14

Efficiency of statin treatment on EPC recruitment depends on baseline EPC titer, and does not improve angiographic outcome in coronary artery disease patients treated with the Genous™ stent.

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Abstract

Objective To asses the effect of high dose Atorvastatin treatment on endothelial progenitor cell (EPC) recruitment and angiographic and clinical outcome in coronary artery disease (CAD) patients treated with the Genous™ EPC capturing stent.

Methods The HEALING IIB study was a multi-center, open-label, prospective trial that enrolled 100 patients. Patients were started on 80mg Atorvastatin qd, at least two weeks before index procedure and continued for at least four weeks after the index procedure.

Results 87 Patients were included in this analysis. EPC levels significantly increased as early as 2 weeks after start of statin. Remarkably, among this group, 31 patients proved to be non-responder to Atorvastatin treatment (i.e no increase in EPC levels) while 56 patients were responders (i.e. rise in EPC count between week -2 and 0). Compared to responders, non-responders had a significantly higher baseline EPC count (76±10 vs. 41±5, $p<0.01$) with a lower LLL at 6 and 18 month FU (0.61±0.07 vs. 0.88±0.08 $p<0.05$ and 0.50±0.08 vs. 0.82±0.08 $p<0.01$ respectively). Furthermore, baseline EPC count inversely correlated with LLL at 6 month follow-up (FU) ($R=-0.42$, $p<0.001$).

Conclusion Patients with higher EPC count at baseline showed no increase in EPC recruitment in response to statin treatment but had favorable LLL at 6 and 18 month FU, whereas patients with lower EPC count were responsive to statin therapy but EPCs might be less functional as they had higher LLL at 6 and 18 month FU. These data imply that, although statin treatment can enhance EPC titer in these patients with low baseline levels, there is no indication for a possible beneficial clinical effect with EPC capture stents.

Introduction

In recent years there has been a tremendous development in the treatment of coronary artery disease (CAD), from balloon angioplasty to bare metal stent placement to stents with active coatings. The introduction of bare metal improved the outcome of percutaneous coronary intervention (PCI), but on the long-term, restenosis of the stent can occur due to vascular smooth muscle cell (VSMC) proliferation. Nowadays, more advanced stents bearing cytostatic compounds have been developed. These so-called drug eluting stents (DES) have largely overcome the problem of restenosis as they inhibit the proliferation of VSMCs. However, DES have been associated with stent thrombosis as they also inhibit the regrowth of the endothelium. The endothelium plays a pivotal role in maintaining vascular integrity and function and injury or dysfunction can trigger the onset of cardiovascular disease. It is therefore important to maintain vascular integrity by rapid restoration of any damage to the endothelium. Vascular repair can be ensured by proliferation and migration of adjacent mature endothelial cells or incorporation of circulating endothelial progenitor cells (EPCs). EPCs were first described by Asahara et al in 1997 and were defined as CD34+ or KDR+ cells with the ability to differentiate into endothelial cells *in vitro* (2). Furthermore, they showed *in vivo* that those cells contributed to neoangiogenesis in a murine and a rabbit hindlimb ischemia model. Since this initial study, many researchers have reported a beneficial effect of EPCs on neovascularization, reendothelialization and reduction of neo-intima formation in animal studies (11,12,13,21). Observational clinical studies showed that circulating EPC levels were correlated with cardiovascular risk factors and outcome (5,19,22).

As EPCs can enhance the arterial repair, an anti-human antibody coated stent (Genous™ Bio-engineered R stent, OrbusNeich) was designed to bind circulating CD34+ hematopoietic cells and facilitate accelerated vascular repair and decrease neo-intima formation after stent placement (1,15). The HEALING II study showed that use of the Genous stent for the treatment of de novo coronary artery disease is safe and feasible with an in stent late luminal loss (LLL) after 6 months of 0.78±0.39 mm (6,7) . Remarkably, patients with low circulating EPC levels responded relatively poorly to the Genous stent with worse clinical and angiographic outcome. We therefore hypothesized that an increase in the number of circulating EPCs would improve the response to the Genous stent and subsequently improve clinical and angiographic outcome. As statins can increase circulating EPCs (16,20,22), we designed the HEALING IIB study in which we evaluated the efficacy of the Genous stent in conjunction with high dose statin (Atorvastatin 80mg qd) therapy. We previously reported the clinical and angiographic outcome of the HEALING IIB study (4). Here, we present the in depth-analysis of the effect of Atorvastatin treatment on circulating EPC levels and correlate these findings with patient clinical outcome of the HEALING IIB trial.

Material and Methods

Study population and protocol

The design and clinical results of the HEALING IIB trial were recently published[16]. Briefly, 100 patients, from 13 sites in 7 European countries were enrolled, with a diagnosis of de novo stable or unstable angina or silent ischemia with a maximum of two de novo lesions in two independent native coronary arteries eligible for coronary stenting. Patients were started on 80mg atorvastatin qd at two weeks before index procedure and continued for at least four weeks after index procedure. Patients already on statin therapy were switched to 80 mg atorvastatin qd. Aspirin treatment was initiated 12 hours before the procedure (75 mg qd), whereas a loading dose of 300 mg of clopidogrel was administered prior to the procedure, proceeded by 75 mg qd for the period of 4 weeks. Administration of GPIIb/IIIa was left at the investigator's discretion.

Anginal status (according to the Canadian Cardiovascular Society Classification of Angina and the Braunwald Classification for Unstable Angina) and the documentation of Major Adverse Cardiac Events (MACE) were assessed at 1, 6, 12, 18 and 24 month follow-up (FU). Circulating CD34+ EPC titers were quantified by flow cytometric analysis at screening (baseline), 2 weeks after initiation of high dose Atorvastatin and at 30 days FU of the index procedure.

Quantitative angiographic analysis was performed at 6- and 18-month FU. Coronary angiograms were obtained in perpendicular views following an intracoronary injection of nitrates. Off-line quantitative analyses of pre-procedural, post-procedural, 6- and 18-month FU angiographic data were performed at an independent imaging core laboratory (Cardialysis, Rotterdam, the Netherlands).

The trial was reviewed and approved by the local Medical Ethics Review Committees, and written informed consent was obtained from all patients.

Genous™ R stent - Endothelial progenitor cell capture technology

The Genous™ Bio-engineered R stent is based on a 316L stainless steel stent platform with study devices available in diameters of 2.5, 3.0, and 3.5mm and lengths of 9, 18, 23, and 33mm (OrbusNeich Medical, Fort Lauderdale, FL) with a polysaccharide matrix coating covalently coupled with murine anti-human CD34 antibodies which specifically target the CD34-positive circulating endothelial cell progenitor population.

Blood sample preparation

Venous blood was drawn under sterile conditions and collected in EDTA collection tubes. Whole blood was used to measure circulating EPC levels

Quantification of circulating endothelial progenitor cells

EPCs were measured using the Stem Cell Kit (Beckman Coulter Comp, Cedex, F) and a modified ISHAGE protocol. Whole blood was incubated with complete antibody mix, i.e. APC-labeled anti-hCD45, FITC-labeled anti-hCD34 (Pharmingen) and PE-labeled anti-hKDR (R&D systems). As negative control we used PE labeled anti-hIgG (Pharmingen). Blood was incubated for twenty minutes at room temperature. Dead cells were excluded using 7-AAD viability dye. Samples were lysed for ten minutes at room temperature using two ml of lysis buffer. Hundred microliter counting beads were added to correlate cell amounts to blood volume. Duplicate samples and one isotypic control were analyzed on an automated flow cytometer (FACSCanto®, Becton&Dickinson). Final data analysis was done with Flowjo software (Tree Star, Inc., USA, see figure 1). We first excluded dead cells by gating on a 7-AAD vs. side scatter (SSC) dot plot. The remaining viable cells were displayed on a CD45 vs. SSC dot plot. We gated the CD45 positive and dim population to select lymphocytes and exclude red blood cells and debris. On the third plot, showing the CD34 vs. SSC dot plot, we gated the bright CD34 cell population and further excluded irrelevant cells by a lymphogate. EPCs were characterized as 7-AAD-, CD45 dim/+, CD34+, FSC/SSClow and VegfR2+. The EPC gate is set according to the IgG1-PE isotypic control.

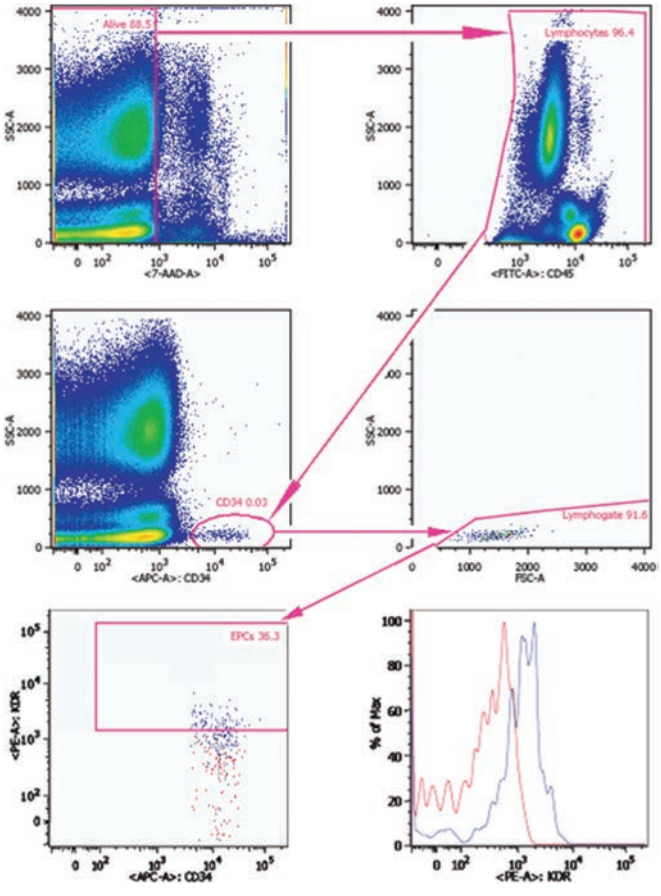


Figure 1. We used a novel method to measure EPCs in whole blood using a modified ISHAGE protocol. Dead cells are excluded with a 7-AAD viability dye. Lymphocytes are selected as CD45 dim and positive cells. The bright CD34 cell population is selected and a lymphogate excludes irrelevant cells. EPCs are characterized as *7-AAD-, CD45dim/+, CD34+, FSC/SSClow and KDR+*. The gate is always set according to the IgG1-PE isotypic control

Statistical analysis

Continuous data are presented as mean ± standard deviation (SD) and categorical variables are expressed as numbers and percentages. The Kolmogorov-Smirnov test was used to analyze whether continuous data were normally distributed.

Differences in baseline characteristics between responders and non-responders were analyzed by student's t-tests, the Mann-Whitney-U test, chi-square tests, and Fisher's Exact test as appropriate. EPC counts at different time points were compared using repeated measurements ANOVA for normally distributed variables, with Newman-Keuls as post-hoc test and Friedman test for non-normally distributed variables, with Dunn's multiple comparison test as post-hoc test. Spearman correlation coefficient was used to correlate circulating EPC counts with clinical outcome.

We applied a univariable logistic regression (LR) analysis to detect clinical characteristics that influenced the risk of being a non-responder to Atorvastatin treatment. To determine independent risk factor of being a non-responder, we applied a multivariable LR analysis. Non-responder was considered the dependant variable and the following independent variables: baseline EPC count, age, sex, hypertension, diabetes mellitus, hypercholesterolemia, previous statin use, BMI and smoking history were considered potential determinants . Because of the relatively small sample size, we performed multivariable LR with backward deletion and all variables with a *P*-value <0.15 were maintained in the model.

We report crude and adjusted odds ratios (ORs) along with their 95% confidence intervals (CIs). For all tests, a two-sided P-value of less than 0.05 was considered significant. All statistical analyses were performed using SPSS 17.0 for Windows (SPSS, Inc., Chicago, IL) and GraphPad Prism 4.

Results

Baseline characteristics

We included one hundred patients in the HEALING IIB study. Four patients were per protocol excluded from post-procedural analysis; two patients did not meet the in- and exclusion criteria, one patient was not on Atorvastatin during two weeks pre-procedure as required per protocol and one patient had a post-procedural diameter stenosis of 32% despite post dilatation. Another 9 patients were excluded from this analysis because of missing values in EPC count on baseline or two weeks FU.

Among the final 87 patients, 31 patients were non-responders to Atorvastatin treatment (i.e. no rise in EPC count between week -2 and week 0) and 56 patients were responders (i.e. rise in EPC count between week -2 and 0). Baseline characteristics for the non-responder and responder groups are summarized in table 1. Baseline characteristics between non-responders and responders were relatively similar, except for baseline EPC count, sex and smoking status. There was no difference in medication use between the two groups (data not shown). Seventy-one percent of the patients in the responders group and 74 percent of the patients in the non-responders group were already on statin therapy before start of the study (p=0.78) and switched to 80 mg of Atorvastatin treatment. At the index procedure, 100% of the patients were initiated on high dose Atorvastatin therapy (80 mg qd) for at least 2 weeks according to the protocol outline. At one month FU, 91% of the patients were still being treated with Atorvastatin 80 mg qd.

Table 1. Patient demographics and clinical characteristics

Patient parameter	Responders (N=56)	Non responders (N=31)	P value
Age (years)			
Mean ± SD	62.110.5	64.9 ± 8.3	>0.05
Min-max	37-80	49-82	
Body Mass Index (kg/m^2)			
Mean ± SD	27.2 ± 3.5	27.9 ± 6.0	>0.05
Min-max	21.5-40.4	17.1-41.9	
Male	82.1% (46/56)	54.8% (17/31)	<0.05
Diabetes mellitus	17.9% (10/56)	25.8% (8/31)	>0.05
Hypertension	62.5% (35/56)	77.4% (24/31)	>0.05
Hypercholesterolemia	87.5% (49/56)	77.4% (24/31)	>0.05
Previous stroke	3.6% (2/56)	0.0% (0/31)	>0.05
Congestive heart failure	5.4% (3/56)	0.0% (0/31)	>0.05
Family history of MI	48.2% (27/56)	58.1% (18/31)	>0.05
Previous MI	32.1% (18/56)	16.1% (5/31)	>0.05
Previous CABG	3.2% (2/56)	0.0% (0/31)	>0.05
Previous PTCA	14.3% (8/56)	19.4% (6/31)	>0.05
Smoking history			<0.05
Never	29,8% (17/56)	51.6% (16/31)	
Previous	50.9% (29/56)	22.6% (7/31)	
Current	17.5% (10/56)	25.8% (10/31)	
Ischemic status			
Unstable angina	3.5% (2/56)	16.1% (5/31)	>0.05
Stable angina	86.0% (49/56)	71.0% (22/31)	>0.05
Silent ischemia	10.5% (6/56)	12.9% (4/31)	>0.05

Numbers are % (counts/available field sample size) or mean ± 1 Standard deviation

SD	Standard deviation
MI	Myocardial Infarction
CABG	Coronary Artery Bypass Graft
PTCA	Percutaneous Transluminal Coronary Angioplasty

Effect of Atorvastatin treatment on serum lipid profile in responders and non-responders

As shown in table 2, Atorvastatin treatment resulted in a significant decrease in total cholesterol, LDL-cholesterol and triglyceride level in the responders group, while there was no effect on HDL-cholesterol. Likewise, the non-responders showed a significant decrease in total cholesterol and LDL-cholesterol and a trend towards a decrease in triglyceride level. HDL cholesterol was not affected by Atorvastin in the non-responders.

Table 2. Serum lipid levels before and after start of 80 mg Atorvastatin treatment

		T=-2 weeks	T=+4 weeks	p value
Non responder	Total cholesterol	178,2±6,8	136,2±5,9	p<0,01
	LDL cholesterol	48,2±2,4	44,7±2,7	p=0,3
	HDL cholesterol	99,5±6,6	69,5±5,2	p<0,01
	Triglycerides	124,9±16,8	90,2±13,3	p=0,13
Responder	Total cholesterol	186,2±6,4	127,9±17,1	p<0,01
	LDL cholesterol	45,0±1,8	42,9±5,7	p=0,39
	HDL cholesterol	112,7±5,8	67,1±9,0	p<0,01
	Triglycerides	125,5±10,2	82,3±11,0	p<0,01

Effect of Atorvastatin on circulating endothelial progenitor cell levels

First we assessed the effect of Atorvastatin treatment on circulating EPC levels for the complete HIIB cohort. As shown in figure 2A, baseline EPC levels were 53.7 ± 5.4 cells/100 µl. Two weeks of Atorvastatin treatment resulted in a significant increase in EPC levels to 74.9 ± 6.3 cells/100 µl ($p<0.05$ by repeated measurements ANOVA) and this was maintained until six weeks after start of treatment (85.2 cells ± 8.5 cells/100 µl, $p<0.05$ by repeated measurements ANOVA and compared to baseline). When we divided the patients into responders to Atorvastatin treatment and non–responders, the responders had a significant increase in EPC levels from 41.1 ± 5.2 cells/100 µl to 89.4 ± 7.9 cells/100 µl at two weeks ($p<0.001$) and 96.4 ± 11.8 cells/100 µl at six weeks ($p<0.001$, see figure 2B), while the non-responders showed a significant decrease upon Atorvastatin treatment from 76.4 ± 10.9 cells/100 µl to 48.6 ± 8.3 cells/100 µl at two weeks ($p<0.001$) and 65.7 ± 9.9 cells/100 µl at six weeks ($p<0.05$, see figure 2C). At baseline, there were significantly higher numbers of circulating EPCs in the non–responder group compared to the responder group (76.4 ± 10.9 cells/100 µl vs. 41.1 ± 5.2 cells/100 µl, $p<0.01$). Two weeks of Atorvastatin treatment reversed this and the non–responders had a significantly lower number of circulating EPCs (48.6 ± 8.3 cells/100 µl vs. to 89.4 ± 7.9 cells/100, for non-responders and responders respectively, $p<0.001$, see figure 2D).

Table 3. Multivariate regression model

Charasteristic	Crude OR, 95% CI	Adjusted for confounders OR, 95% CI
Baseline EPC count	1.015 (1.005-1.025)	1.019 (1.007-1.031)
Age (per year)	1.032 (0.986-1.079)	1.049 (0.985-1.116)
Sex (Male)	3.282 (1.268-8.495)	2.409 (0.735-7.901)
BMI per kg/m2	1.036 (0.944-1.137)	-
DM (yes)	1.600 (0.562-4.556)	-
Never smoked (yes)	0.733 (0.411-1.305)	0.273 (0.082-0.913)
Hypercholesterolemia (yes)	0.571 (0.187-1.748)	-
Hypertension (yes)	1.943 (0.750-5.029)	-
Previous statin use (yes)	1.150 (0.427-3.100)	-

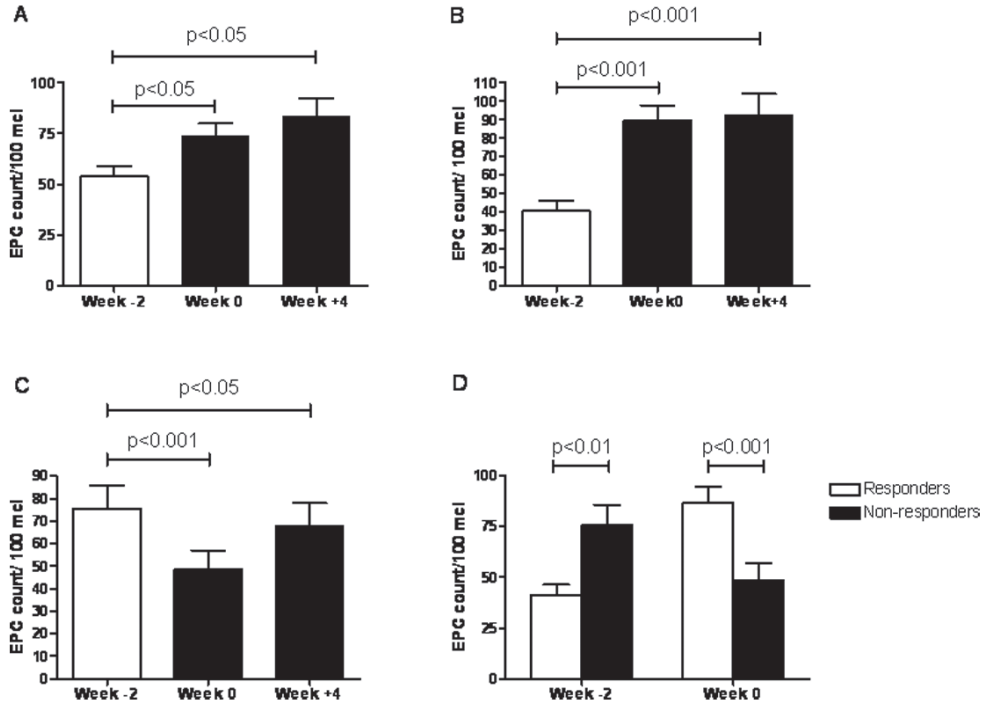


Figure 2. Statin increased circulating EPC levels for the complete HEALING IIB cohort as early as two weeks and was maintained until six weeks B. Responders to statin therapy had a significant increase in circulating EPC levels, two and six weeks after start of treatment C. Non-responders showed even a significant decrease in circulating EPC levels upon statin treatment. D. Non-responders had a significant higher baseline EPC count compared to responders. Two weeks after start of treatment, this was reversed and responders had a significant higher EPC count.

Determinants of response to atorvastatin treatment

Baseline EPC count and the number of patients that never smoked were significantly higher in non-responders, whereas there were less men. When we applied our LR model, only baseline EPC count (aOR: 1.02 and 95% CI: 1.007-1.031) and having never smoked (aOR 0.27 and 95% CI: .082-.913) were independent risk factors for being a non-responder (see table 3).

Circulating endothelial progenitor cell levels and clinical and angiographic outcome

Complete H2B cohort

Baseline EPC count inversely correlated with late luminal loss (LLL) at six months (see figure 3A, Spearman r= -0.41, p<0.001). EPC levels at baseline did not correlate with major adverse cardiac events (MACE) or target lesion revascularization (TLR) at six months. Although statin significantly increased circulating EPC levels for the complete HEALING IIB cohort, this was not accompanied by an improvement in LLL as LLL was comparable with the HEALING II study.

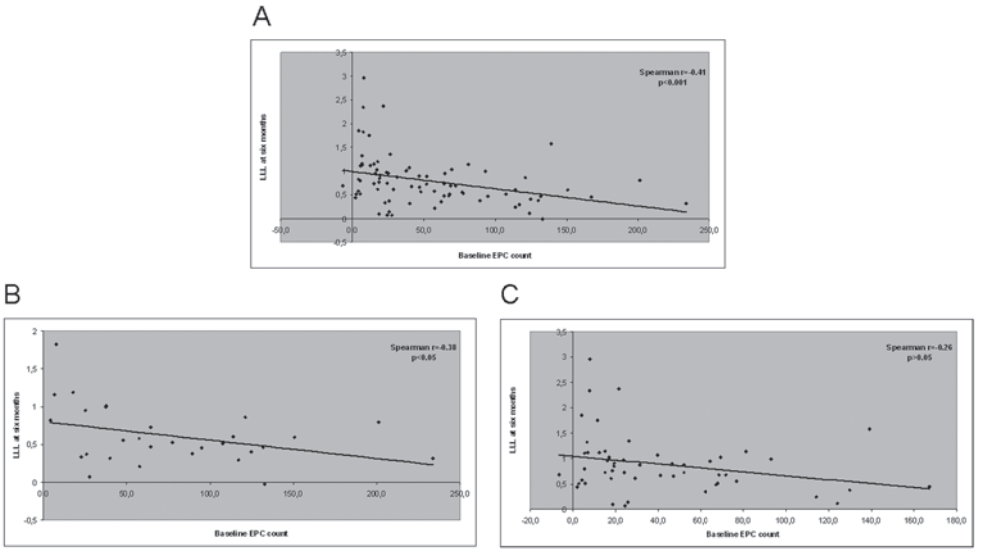
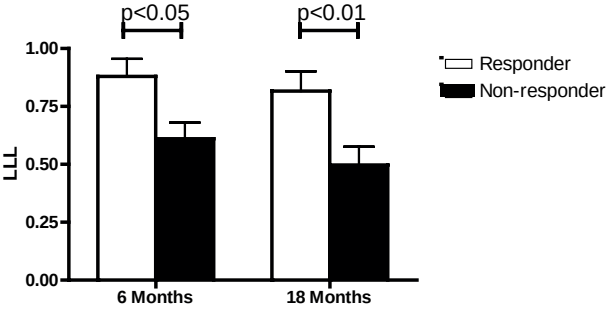


Figure 3. A. Baseline EPC count significantly correlates with LLL in the complete HEALING IIB cohort. B. Baseline EPC also significantly correlates with LLL in the non-responders group but not in the responders group (C).

Non-responders vs. responders

Baseline EPC count of non-responders inversely correlated with LLL (Spearman r=-038, p<0.05 and Spearman r=-0.26, p>0.05, see figure 3B and 3C). Responders did not show such a correlation. LLL at six and eighteen months were significantly lower in non-responders compared to responders (0.61 ± 0.07 vs. 0.88 ± 0.08, p<0.05 and 0.50 ± 0.08 vs. 0.82 ± 0.08, p<0.01, see figure 4). There were no target lesion revascularizations (TLRs) in the non-responders group and six in the responders group (p=0.09).

Figure 4. Non-responders group has a significantly lower LLL at six and eighteen month FU.



Discussion

In this subanalysis of the HEALING IIB study we evaluated the effect of high dose Atorvastatin treatment on circulating EPC levels and correlation with angiographic and clinical follow-up. Prati and coworkers already showed that high dose Atorvastatin treatment could reduce in stent restenosis and late luminal loss (18). However, in contrast to the RESTART study, we specifically looked at the effect of high dose Atorvastatin treatment on EPC levels and its effect on late luminal loss. Our study supplies data on the possible role of EPCs in LLL and demonstrate that statin treatment in patients might contribute to vascular healing via EPC recruitment.

In line with earlier studies (16,20,22), statin treatment significantly increased circulating EPC levels for the complete HEALING IIB cohort, as early as two weeks after start of statin treatment. When we evaluated EPC levels for each individual patient at every time point, we could divide the cohort in patients responding to statin treatment with a rise in EPC level (responder group) and patients who did not respond to statin treatment with an increase in EPC level (non-responder group). Interestingly, the non-responder group had a significantly higher baseline EPC count in comparison to the responders group, suggesting that the response to statin treatment on EPC level depends on baseline EPC count and that patients with a high baseline EPC count are unable to recruit or produce more EPCs despite high dose statin treatment. An alternative, and maybe more logical explanation would be that non-responders react to vascular disease with a more efficient vascular repair response. That would explain the high basal level of EPC count in blood in non-responders versus responders (76.4 ± 10.9 cells/100 µl vs. 41.1 ± 5.2 cells/100 µl, $p<0.01$), which indicates that the non-responders are more capable of recruiting EPCs from the bone marrow into the blood stream. Likewise, the blood circulatory pool of EPCs of non-responders may be more efficient in homing to the affected vascular area, an intrinsic capacity that was further amplified by statin treatment (3,14). For the responders, the data might imply that although recruitment of EPCs from bone marrow by statin treatment is successful, the recruited EPCs in the bloodstream remain inefficient in homing capacity to the target vessel wall. Further research is required to assess this hypothesis on the complex effect of statins on EPC recruitment and homing.

EPC levels and cardiovascular risk factors

Earlier publications showed that several cardiovascular risk factors, for example hypertension, diabetes, age, smoking status or increased body mass index could affect EPC titer. Vasa and coworkers reported that patients with coronary artery disease had lower EPC levels compared to healthy volunteers. Moreover, the number of risk factors was significantly correlated with lower EPC levels and multivariate analysis showed that smoking status was an independent predictor of reduced EPC levels. Later, Hill and coworkers showed in a relative small group of healthy volunteers that the number of EPC colony forming units was significantly reduced in patients with diabetes, hypercholesterolemia or hypertension. When they adjusted for age, only hypercholesterolemia remained significant. Multivariate analysis revealed that only flow mediated brachial reactivity, a composite measure of endo-

thelial integrity, was an independent predictor of reduced EPC colonies. Here we assessed whether cardiovascular risk factors could affect being a non-responder or responder. We found a significant difference between responders and non-responders in baseline EPC count, smoking status and sex. However, when we applied our LR model, only baseline EPC count and smoking status remained significant predictors for being a non-responder, i.e higher baseline EPC count.

EPC levels and cardiovascular outcomes

Baseline EPC count inversely correlated with late luminal loss (LLL) for the complete HEALING IIB cohort. Furthermore, patients from the non-responders group had a lower LLL at six months FU compared to patients from the responders group. This difference in LLL was even more pronounced at eighteen months FU. As Ellis and coworkers showed that LLL can serve as surrogate clinical endpoint (8), we evaluated the occurrence of target lesion revascularization (TLR). Indeed, we found a trend towards more TLRs in the responders group. These results suggest that baseline EPC count can predict the response to the GENOUS stent in terms of LLL and perhaps clinical benefit. There is much debate about the significance of baseline EPC count in the pathophysiology of cardiovascular disease. It was already shown that low circulating EPC levels are associated with less favorable cardiovascular outcomes (5,9,19). As circulating EPCs play an important role in maintaining integrity of the endothelial layer by accelerating re-endothelialization in injured arteries, low levels of EPCs could result in delayed re-endothelialization and increased cardiovascular risk. Our study confirmed these results, but furthermore showed that baseline EPC count is correlated with angiographic outcome, not only at six months FU but also at 18 months FU. Pelliccia and coworkers were the first to prospectively assess circulating EPC levels and the occurrence of in-stent restenosis or progression of coronary artery disease in patients treated with a bare metal stent (17). In contrast to our findings, they showed that high circulating EPC count correlated with the occurrence of in-stent restenosis and high late LLL, possibly due to engraftment of EPCs in the neointima and subsequently differentiation into vascular smooth muscle cells. There was no correlation between EPC count and progression of atherosclerosis, stable coronary artery disease or healthy controls. There are some differences between our work and the work of Pelliccia. First of all, as with many papers on EPCs, there is a difference in characterization of EPCs, as there is no standardized way to measure EPCs. Originally, EPCs were described as CD34+ or KDR+ cells and now different combination of cell surface markers have been advocated to define EPCs, most commonly CD133+/CD34+/KDR+, CD34+KDR+, CD34+/KDR+/CD45- or CD14+/CD34^{low}. We measured EPCs using a modified ISHAGE protocol using whole blood in which the EPC population was defined as CD45+/CD34+/KDR+, whereas Pelliccia and coworkers used mononuclear cells, defining EPCs as CD45-/CD34+/KDR+. Secondly, Pelliccia included only stable angina patients, whereas we also included patients with unstable angina and silent ischemia. Thirdly, our patients were treated with the GENOUS stent and Pelliccia used the bare metal stent. However, it seems unlikely that this influenced baseline EPC count, as blood was drawn at least one day before PCI. More recently, in contrast to our work and the work of Pelliccia, Haine et al. reported that they found no correlation between circulating EPC levels and angiographic and clinical restenosis (*Blood endothelial progenitor cells as predictive markers for coronary in-stent restenosis*, moderated poster session, ESC, Paris 2011),

making it even more complex to understand the role of EPCs in cardiovascular disease and outcome. We believe that, at least for the clinical outcome after GENOUS stent placement, baseline EPC count is a valid biomarker for and reflects the regenerative potency of the vascular bed.

Interestingly, although statin treatment significantly increased circulating EPC count in the responders group, this increase was not reflected in a favorable LLL. One explanation could be that the responders group not only had lower baseline EPC count but also less functional EPCs and that statin therapy can increase EPC count but can not improve the angiogenic capacities of these cells. However, we can only speculate on this as we only performed a quantitative analysis of the EPCs, but no functional analysis. Future studies to elucidate the angiogenic potential of EPCs of this specific subset of non-responder patients will help to understand the complex biology behind these clinical findings.

Conclusion

To our best knowledge, this is the first prospective study to show that there is a significant difference in baseline EPC count between responders and non-responders to Atorvastatin 80 mg treatment for EPC recruitment in coronary artery disease patients treated with the GENOUS stent. Furthermore, higher baseline EPC count correlated with favorable angiographic outcome with less LLL at six and eighteen months FU compared to lower baseline EPC count. Although statin treatment significantly increased circulating EPC levels in the responders group, this was not reflected in improved angiographic outcome, suggesting that low EPC count reflects less functional EPCs. EPCs might be used as predictor for angiographic outcome for patients treated with the GENOUS stent.

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PART VI
Discussion

Chapter 15

General discussion, conclusions, and future directions

Introduction

Despite advancements in prevention and treatment options of ischemic heart disease, it still remains the major cause of morbidity and mortality in the western world.[1–3] Therefore, the search for new treatment strategies is ongoing. About two decades ago, stem cell therapy emerged as a promising new tool in the cardiologist's arsenal. Indeed, numerous small and large animal studies showed favorable effects of stem cell therapy in the treatment of acute myocardial infarction (AMI) and heart failure (HF) patients.[4,5] Nevertheless, clinical data thus far show only moderate beneficial results, which do not meet the high expectations. Thus, it is of paramount importance to increase our understanding of the various working mechanisms of stem cell therapy, and to constantly strive to take the field to the next level.

When considering the current status of stem cell therapy for cardiovascular diseases, it is important to keep a historical perspective. Tremendous progress has been made, in which some previously unthinkable developments changed the way we look at cardiac regeneration. Many promising candidates for stem cell therapy have been identified, and the results of numerous pre-clinical and clinical studies start to show the direction in which the field is heading (see also **chapter 2** of this thesis). Moreover, the age-old dogma that the heart is a post-mitotic organ, has been abandoned, which means that the heart has endogenous regenerative potential.[6,7] Our knowledge how to address this potential and benefit from it, is currently exponentially growing.

Nevertheless, we are only at the beginning of the era of regenerative medicine in heart disease. This thesis represents a small paving stone of the long road that still lies ahead, and aims to depict the

Table 1. Generations of stem cells

Generation	Cell types*
1. Autologous stem cells	Skeletal myoblasts
	Bone marrow-derived mononuclear cells
2. Autologous enriched stem cells	Endothelial progenitor cells
	Autologous mesenchymal stem cells
	Cardiogenic-oriented mesenchymal stem cells
	Adipose tissue-derived regenerative cells
3. Allogeneic stem cells	Allogeneic mesenchymal stem cells
	Encapsulated mesenchymal stem cells (CellBeads)
4. Cardiac-derived stem cells	Cardiac stem cells
	Cardiosphere derived cells
5. Pluripotent stem cells	Embryonic stem cells
	Induced pluripotent cells
	Induced cardiomyocytes

progress in the field of cardiovascular stem cell therapy over the past decade. By categorizing stem cells in five sub categories, from first to fifth generation (table 1), an orderly distinction was made from past to present, and from present to future. This thesis comprises clinical and pre-clinical experience with the first three generations of stem cells, and evaluates clinical efficacy of the first generation.

Overview

This thesis starts with a review of our current understanding of cardiovascular regenerative medicine by discussing cell types that already have been evaluated in the clinical setting, but also cell types that are currently being developed and/or tested in pre-clinical research (**Chapter 2**). In this review, we pose that there are two potential working mechanisms for cell-based repair of cardiac dysfunction: 1) the enhancement of endogenous regenerative potential, or 2) the delivery of exogenous cells to stimulate or repopulate the heart (figure 1). In the following chapters, except for chapter 4, it is either

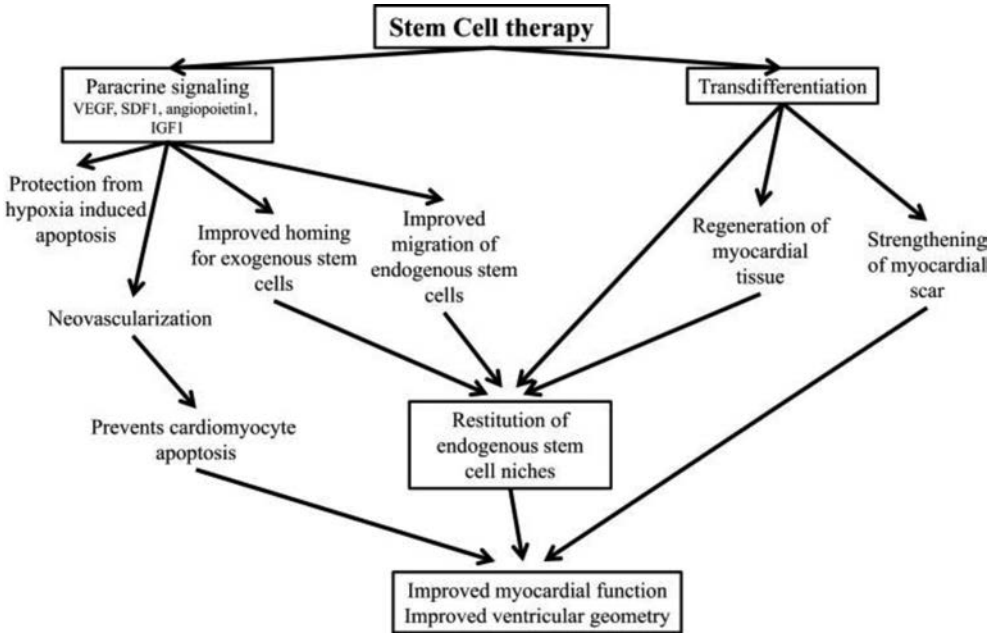

Figure 1. Mechanisms underlying cardiac regeneration due to cell-based therapy. The improvement in myocardial function and ventricular geometry after transplantation of stem results from multiple coordinated actions of cells used as a therapeutic. Successful cell-base therapy, as observed as a result of MSC and CSC therapy, likely arises from the actions of both administered cells and host cellular elements. Notably, MSCs both have the capacity for trilineage differentiation, as well as stimulating the recruitment, survival, and differentiation of host CSCs. This action of MSCs results from both secretion of cytokines and growth factors [vascular endothelial growth factor (VEGF), stromal-derived factor 1 (SDF1), insulin-growth factor 1 (IGF1), angiopoietin 1] and antifibrotic mediators that together promote neovascularization and reduce fibrosis. Together these actions and the recruitment of endogenous CSCs could represent the reconstitution of stem cell niches in the myocardium.

one of these working mechanisms that prevails. The predominant working mechanism in current cell therapy is believed to be through paracrine actions of secreted factors by the transplanted stem cells. Almost all stem cells studied to date secrete growth factors, as well as anti-apoptotic, anti-remodeling, and pro-angiogenic proteins. These factors have been shown to affect survival of resident cardiomyocytes, enhance angiogenesis and reduce or reverse LV remodeling. Although most stem cells have paracrine properties, it is believed that mesenchymal stem cells, and stem cells that are derived from the heart (*i.e.* cardiac stem cells and cardiosphere-derived cells), have the most pronounced paracrine activity.[8–11] Also, it has been shown that for instance mesenchymal stem cell (MSC) transplantation stimulates endogenous cardiac stem cells, as well as increases proliferation of adult cardiomyocytes. Indeed, cardiomyocytes, which were considered to be senescent cells for decades, can be activated to re-enter the cell cycle by cell therapy.[12,13]

Bone-marrow derived mononuclear cells and skeletal myoblasts

Most clinical experience to date has been obtained with autologous, adult stem cells. In **chapter 2**, these cells were classified as first generation stem cells. The first clinical studies with bone-marrow derived mononuclear cells (BMMNC) showed promising and favorable results, which resulted in several large studies that included over 2,000 AMI and HF patients in total.[5] In **chapter 3** and **chapter 4**, the results of these clinical studies are combined, and analyzed in two meta-analyses. In **chapter 3**, we analyzed all clinical stem cell studies in AMI patients. Interestingly, and in conflict with several earlier meta-analyses, it was concluded that when only studies are included that use state of the art measures of LV function, the effect of BMMNC infusion in AMI patients is negligible. Moreover, the assumed beneficial effect of BMMNCs on clinical end points could not be confirmed in AMI patients alone.

In **chapter 4**, a meta-analysis was performed on all randomized controlled studies with first generation stem cells, *i.e.* skeletal myoblasts and BMMNC. We confirmed our previous finding that BMMNC therapy did not yield functional recovery in AMI patients, nor improved clinical outcome measures. Interestingly however, a modest beneficial effect was found in HF patients that received BMMNC injection. This effect sustained, even when corrected for MRI-derived parameters of cardiac function. Moreover, a beneficial effect on all-cause mortality was found. This explain why BMMNC therapy was included as adjunctive treatment in the latest ESC guideline for the treatment of heart failure patients.[14]

It should be noted that in most meta-analyses to date, AMI and HF patients were pooled to increase power. This resulted in the general notion that BMMNC therapy had a beneficial effect on cardiac function and survival in both patient groups. We found that when these groups are separated, and only MRI-derived measures are analyzed, the beneficial effect in AMI patients disappears, whereas the effect in HF patients is sustained. It can therefore be concluded that combination of these two distinct patient groups resulted in provisional and faulty conclusions in these previous meta-analyses.

It is of importance to note that the most-cited meta-analyses regarding cardiovascular cell therapy, and showing beneficial effects of BMMNC therapy, were sometimes published in high-impact journals.[5,15,16]

The results of the SEISMIC study, which was a clinical study with another first-generation cell type, *i.e.* skeletal myoblast (SkM), are reported in **chapter 5**.[17] In contrast to earlier reports[18], but in conjunction with a later large randomized controlled trial (MAGIC trial)[19], NOGA-guided SkM injection into the scar and scar border zone of end-stage HF patients, did not result in improved cardiac function. However, SkM injection did result in increased walking distance during a 6-minute walk test and improved NYHA classification. These beneficial effects should be interpreted with caution, though, because this was an open-label study, and a placebo effect can therefore not be excluded. The most important disadvantage of SkM injection, however, turned out to be the occurrence of ventricular arrhythmias. In most reports, an increase of ventricular arrhythmias was observed in patients who received intramyocardial injection of SkM.[20] In this end-stage HF patient population, which is already prone to ventricular arrhythmias, the cluster of injected SkM formed a macro re-entry circuit that facilitated sustained ventricular arrhythmias. This major side effect is confirmed in **chapter 4**, which is the first meta-analysis to include data on ventricular arrhythmias following SkM injection. In **chapter 5**, however, it is demonstrated that ventricular arrhythmias following SkM injection can be prevented by prophylactic amiodaron use, as in the SEISMIC study no difference was found in ventricular arrhythmias between treated and placebo patients. Nevertheless, due to the association with ventricular arrhythmias, the lack of obvious long-term efficacy, and better alternatives, SkM therapy currently seems to have been abandoned as cellular therapy for cardiac repair.

Adipose tissue-derived regenerative cells

Adipose tissue was first identified as an alternative source of abundant numbers of multipotent mesenchymal-like stem cells in 2002.[21] Like BM-derived MSC, these cells stimulate neo-angiogenesis and cardiomyocyte survival both in vitro and in vivo by release of various angiotrophic, anti-apoptotic and immunomodulatory factors.[22–24] In animal models of AMI, administration of freshly isolated adipose tissue-derived regenerative cells (ADRC) consistently improved left ventricular (LV) function and myocardial perfusion by cardiomyocyte salvage and stimulated neo-angiogenesis in the infarct border zone, resulting in reduced infarct scar formation.[25,26] On average, 20-40 million cells can be isolated within two hours after a liposuction from as little as 200 grams of lipo-aspirate.[27]

The consistent therapeutic effect in small and large animal models was the basis for the APOLLO trial, which was designed as the first-in-man experience with ADRCs in the treatment of patients with an ST-elevation AMI. Primary objectives of this study were to determine the safety and feasibility of harvest and intracoronary infusion of ADRCs in the acute phase of an AMI, as well as to explore the potential efficacy of this cell therapy. In **chapter 6** and **chapter 7**, the first exploratory data, as well as long-term safety and feasibility results are described respectively.[28] The most important findings are

that the liposuction procedure can be performed safely briefly following an AMI, whereas no MACCE or serious adverse events were related to the ADRC therapy. Also, ADRC therapy had no apparent pro-arrhythmogenic effects, but rather appeared to reduce the occurrence of ventricular arrhythmias and ectopy. Thus, ADRC therapy appeared to be safe and feasible in the acute phase of an AMI. Although exploratory, ADRC infusion seemed to result in a sustained improvement of the perfusion defect and a reduction of myocardial scar formation, whereas coronary flow reserve in the culprit vessel was significantly enhanced.

These possible beneficial effects are consistent with the findings in pre-clinical AMI studies, and concordant with the presumed pro-angiogenic, anti-apoptotic, and immunomodulatory working mechanism of ADRC therapy.[26,27] ADRCs may thus represent an attractive adjunctive therapy to primary intervention of patients with a large AMI. However, further randomized, controlled trials are needed to confirm these promising results. The ADVANCE trial (ClinicalTrials.gov identifier: NCT01216995) is a prospective, randomized, double-blind, placebo-controlled, phase IIb/III clinical trial that will enroll up to 375 patients with STEMI in up to 35 centers in Europe. The primary endpoint of ADVANCE will be the reduction in infarct size at 6 months by DE-CMR expressed as a percentage of left ventricle infarcted.

Allogeneic mesenchymal stem cells

As stated previously, the effects of unfractionated bone marrow (BM) mononuclear cells in clinical AMI trials have been modest at most (**chapter 3** and **4** of the current thesis). It has been suggested that mesenchymal stem cells (MSC) or their sub populations might be more effective.[28,43,44] In part III of this thesis, autologous adipose tissue-derived cells (ADRC) were shown to be a safe and feasible alternative to BMMNC. However, to obtain ADRC, a liposuction procedure is necessary in vulnerable patients with dual anti-platelet therapy. Although we found no major bleedings in most APOLLO patients, several patients experienced substantial hematomas of the abdominal wall. Moreover, patients need a reasonable amount of subcutaneous fat. The typical male, cardiovascular patient is overweight, but the fat supply predominantly lays intra-abdominally and is therefore not accessible by standard liposuction.

A different source for MSC is the bone-marrow (BM). However, MSC comprise only 0.001-0.01% of all cells in the BM, which necessitates cell culture expansion in clean room facilities to obtain clinically relevant cell numbers.[8] This excludes MSC from the use in the acute phase of an AMI. Also, the function of stem cells is known to decline with age and cardiovascular comorbidities.[45] It was found that MSC do not express major-histocompatibility-complex class II proteins, and that they exert immunosuppressive effects *in vitro* and *in vivo*.[8,46] As a result, allogeneic MSC do not trigger an acute immune rejection, and can thus be administered without immunosuppressive therapy. This implies that MSC can be used as an 'off-the-shelf' product and that they can be transplanted directly as adjunctive treatment of acute coronary syndromes and other cardiac conditions. The downside of culture-expanded MSC is that they increase in size to well over 30 micron, and that intracoronary delivery can result in micro-vascular obstruction and no-reflow phenomena.[47–49]

Mesenchymal precursor cells (MPC) comprise a Stro-3 immune-selected, immature sub fraction of BM-derived MSC.[50] These MPC are multipotent cells with extensive proliferative potential, and secrete numerous anti-apoptotic, angiogenic factors, and growth factors. MPC display greater cardioprotective effects than conventional MSC that are selected by plastic adherence alone, which may be evoked by their potent paracrine activity, as well as more extensive multilineage differentiation potential.[10,11] As MSC, these MPC are immune-privileged and can be transplanted to unrelated recipients, and are thus readily available during the acute phase of an AMI. The efficacy of intramyocardial injection of MPC was already proven in several studies in small and large animal models of AMI.[10,51–53]

Because MPC are considerably smaller that regular MSC, we hypothesized that it might be possible to infuse these cells via the intracoronary route without vascular obstruction. This hypothesis resulted in the design of the DOMESTICATE study. The primary aim of this large animal study was to assess the feasibility of intracoronary MPC infusion, and to determine the optimal infusion conditions, while carefully monitoring coronary flow, arrhythmias, and other possible cell therapy related adverse effects. In addition, we investigated the potential efficacy of three different doses of MPC using functional (pressure-volume (PV-loop) analysis, echocardiography) and morphological (morphometry, histology) indices over an 8 week follow-up period. In **chapter 8**, the results of this study are described.[13]

The main conclusions of this study were that, when low infusion speeds were adopted, intracoronary infusion of MPC can be performed safely following AMI without adverse effects, impeding coronary flow, or micro infarctions in remote myocardial segments. More specifically, we found that, following AMI, a low infusion rate of only 0.5 million MPC/minute permitted intracoronary infusion of 50 million cells without permanently compromising coronary flow, whereas higher infusion rates decreased the maximum tolerated dose. We hypothesize that a low infusion rate enables the MPC to either pass through the capillary bed or to transmigrate into the peri-vascular tissue without aggregation or capillary occlusion. Indeed, the nuclear imaging retention sub study revealed that a significant number of MPC still resided in the heart two hours following intracoronary infusion. Importantly, no micro-infarctions in, or shedding of MPC to, remote myocardial segments were detected by macroscopic inspection, microscopic analysis and nuclear imaging techniques.

Also, it was demonstrated that intracoronary delivery of MPC prevents LV remodeling and improves residual cardiac function. The results of this study suggest that these effects are evoked by myocardial salvage and subsequent reduction of infarct size, accompanied by induced angiogenesis and reduced myocardial fibrosis. One of the most important differences between the DOMESTICATE study and almost all other pre-clinical and clinical efforts is, apart from the cells, the timing of delivery. We believe that by administering the MPC directly following reperfusion of the AMI, the therapeutic effect of the MPC is mainly exerted through the release of anti-apoptotic and pro-survival factors, thereby promoting cardiomyocyte salvage.[8,10] In addition, the profound immunomodulatory actions of MPCs may preserve myocardial tissue and contribute to effective tissue healing with limiting scar tissue formation by ameliorating reperfusion injury or attenuating oxidative stress.[54,55] Additionally, we found that MPC infusion stimulated endogenous regenrative potential of the heart, as has been previously described for other cells as well.[12] And, beside the effect on infarct size, remodeling, and cardiomyo-

cyte proliferation, we also found a marked increase in neo-capillary and arteriole densities in the infarct border zone and infarct area of MPC-treated animals. This increase in blood vessel density in the perfusion territory of the culprit artery suggests a pro-angiogenic potential of MPC therapy and is consistent with previous studies.[51–53,56]

Because of the safety profile, accompanied by the pronounced effects on cardiac function and remodeling, the results of the DOMESTICATE study led to the design of a first-in-man clinical trial. The AMICI trial is a multi-center, phase IIa/b, double blind, randomized and placebo-controlled clinical trial in which European, Australian and US sites participate. Its aim is to prove safety, feasibility and efficacy of two different doses of MPC therapy in a minimum of 225 patients with ST-elevation AMI and started enrolment in the second half of 2013. It is obvious that the results of this trial are much anticipated.

Allogeneic encapsulated mesenchymal stem cells (CellBeads™)

One of the biggest challenges in the cell therapy field today is the poor retention of therapeutic cells upon local delivery in the heart, with retention rates as low as 1% after intracoronary delivery (10,11,27). Even though permanent engraftment of stem cells is not required to elicit the cardioprotective effect, it seems logical that the greater the number of cells that are retained in the injured myocardium and the longer they reside there, the more pronounced the potential beneficial effect will be. Despite wide-ranging efforts to increase cell retention using various delivery techniques, results are still unsatisfactory. A new concept of stem cell delivery has recently become available owing to advances in the field of biotechnology, as it is currently possible to encapsulate MSC in a biocompatible alginate shell (26,29). Alginate encapsulation of varying numbers of MSC results in so-called CellBeads™, available in discrete sizes between 150 and 600 µm.

CellBeads™ are made from a highly purified alginate material, which is used to encapsulate clusters of adult human MSC. These MSC have been genetically modified to secrete a recombinant GLP-1 fusion protein, which consists of two GLP-1 molecules bound by an intervening peptide. This form of GLP-1 is more stable than endogenous GLP-1, rendering a longer half-life and thus prolonged therapeutic potential. The alginate coating of the CellBeads is permeable to the GLP-1 fusion protein, allowing for continuous delivery, while protecting the MSCs from the patient's immune system. Also, oxygen and nutrients can freely pass through the alginate shell, which renders the MSC viable for a long period of time. Thus, Cellbeads are potentially a unique, biological, long-term, local drug delivery platform that is capable of delivering GLP-1, or other therapeutic proteins, in addition to MSC-derived factors (VEGF, MCP-1, IL-6, IL-8, GDNF and NT-3) to any target tissue.

Although the therapeutic potential of CellBeads™ is obvious, the smallest version is still 170 µm in diameter, which is as large as a small arteriole in the heart. Intracoronary infusion of such CellBeads will therefore result in blockage of arterioles and a reduction of perfusion. However, the potential benefit

of prolonged cell therapy in post-AMI hearts validated an exploratory safety and feasibility study. It was hypothesized that when transplanted in the post-AMI heart, the synergystic effect of paracrine MSC-derived factors together with the cardio-protective GLP-1 peptide, might evoke myocardial salvage, reduce apoptosis and influence the inflammatory response in the acute phase of the AMI. In the long term, CellBead therapy may induce angiogenesis, and decrease post-AMI adverse cardiac remodeling, whereas infusion of the CellBeads in readily infarcted tissue might not be harmful.

In chapter 9, the results of the CELEBRATE study is described, which aimed to evaluate the feasibility and safety of intracoronary delivery of 170 µm CellBeads™ in the acute phase of an AMI in a relevant large animal model. It was found that intracoronary infusion of CellBeads™ is feasible and appears to be safe. Epicardial blood flow remained unchanged in all animals that received up to 60,000 CellBeads or control beads, suggesting little or no vascular obstruction. Moreover, CellBeads were successfully targeted to the infarct area and MSC remained viable and active for at least seven days. Also, several parameters indicate that the cells sort a paracrine effect, highlighting the potential for cardiovascular repair of this new therapy.

These promising pilot results initiated a study sequel, which was designed as a dose-finding study in large animal models of moderate and severe cardiac dysfunction, which is described in **chapter 10**. In the first group, a moderate size myocardial infarction was induced by balloon occlusion of the proximal left circumflex artery. This results in infarct that comprises approximately 10% of the total LV and impairs cardiac function by 15%. The second group underwent surgical ligation of the left anterior descending artery, resulting in larger infarcts (20-25% of the LV) and subsequent more severe LV dysfunction. Following the balloon occlusion, the animals were blindly randomized to 3 different dose groups or placebo. These were then infused in the culprit vessel directly following induction of the AMI using a microcatheter.

It was confirmed that infusion of CellBeads™ can be performed safely without hampering coronary flow. More importantly, CellBeads™ significantly improve cardiac function after an AMI. In the moderate infarction model we detected a trend, which was further explored in a more severe AMI model. As expected, the effect of CellBeads™ turned out to be more pronounced in animals with more severely depressed cardiac function, presumably due to a larger pharmacological window in the anterior model. In absolute terms, intracoronary infusion of CellBeads™ improved LVEF by +9.3% in severe anterior AMI model, which is more or less comparable to the effect of MPC.[13] However, it significantly exceeds the effect of BMMNC that was shown to be 2-3% at most (**chapter 3**). The most effective dose in this study was 20,000 CellBeads containing 1.6 million MSC, which is several orders of magnitude less than used in most preclinical trials using cultured MSC including our MPC study. However, CellBeads™ have a better retention than unprotected stem cells and ensure prolonged protein release, complicating direct comparisons. Infusing less than 20,000 CellBeads was not effective, suggesting that this dose is the lower limit. On the other hand, 40,000 CellBeads™ were not superior to lower doses, which does not preclude a dose dependent effect but more likely reflects unfavorable effects of incremental microvascular obstruction of non-infarcted segments.

Additional histologic analyses showed that the predominant mode of action of these transplanted MSC was through myocardial salvage and reduction of fibrosis, thereby effectively reducing infarct size. Myocardial salvage might have been caused by the pro-survival and anti-apoptotic proteins secreted by the encapsulated MSC, in conjunction with the release of recombinant GLP-1. Indeed, apopotosis was significantly reduced in treated animals, when compared to controls. Moreover, Cell-Bead infusion enhanced angiogenesis in the infarct and infarct border zone, thereby improving oxygen and nutrient supply to the inflicted region. These results confirmed our findings in the MPC study, thereby corroborating the cardioprotective potential of mesenchymal cells.

Genous R stent

Drug eluting stents (DES) that locally release antiproliferative, immunosuppressive or anti-inflammatory drugs to retard intimal hyperplasia and restenosis, have significantly improved the outcome of percutaneous coronary interventions.[29] However, the local toxicity of the drug on the vessel wall and endothelium has also been shown to concomitantly impair endothelial regeneration and induce local hypersensitivity reactions leading to long-term endothelial dysfunction.[29–32] As endothelial stent coverage was found to be the most powerful histological predictor of late stent thrombosis (LST), delayed re-endothelialisation remains a big concern.[31] Even though the incidence of LST is low, the sheer volume of coronary interventions nowadays leads to significant numbers with high morbidity and mortality. LST is associated with non-fatal MI and cardiac death in as many as 75% and 45% of the cases respectively. An accelerated re-endothelialisation response of the bare stent struts and facilitated arterial repair is therefore desirable. This may not only prevent stent thrombosis by inhibition of platelet adhesion, but it also might impede smooth muscle cell proliferation and migration. This eventually might result in reduced neo-intimal hyperplasia with preserved arterial integrity and endothelial vasomotor function.

The key role of endothelial progenitor cells (EPC) in the arterial repair response after stent implantation prompted the concept that recruitment of the patient's own EPC to the site of vascular injury could aid stent re-endothelialisation and initiate the endogenous arterial repair response. Several years ago, this "pro-healing" concept led to the development of the

first bio-engineered stent (Genous R-stent). This stent was specifically designed to promote the arterial healing response by a coating of immobilised murine antibodies raised against human CD34. As a result, the Genous R-stent captures and sequesters circulating CD34-positive progenitor cells to the luminal stent surface and so theoretically initiates re-endothelialisation. This accelerated endothelial healing would reduce the risk of stent thrombosis and shorten the required period of dual antiplatelet therapy.[33,34]

In **chapter 12**, a proof of principle study was performed to show that the Genous stent indeed enhances EPC capturing.[35] We showed in an *ex vivo* human arteriovenous shunt model that the Genous

stent had a higher stent-strut coverage. Further evaluation of these cells by qPCR showed that the Genous stent had a higher percentage of endothelial cell coverage compared to the bare metal stent. This increased number in endothelial cells was accompanied by decreased thrombogenitic, not only macroscopic but also by qPCR analysis. These ex vivo human results were confirmed by subsequent studies in a baboon arteriovenous shunt model and a rabbit endothelial denudation model. Furthermore, we showed *in vitro* that the Genous stent indeed captures more CD34 positive cells, but not monocytes.

Previously conducted studies that assessed safety and feasibility of the Genous™ R stent in patients with coronary artery disease were the HEALING FIM and HEALING II studies.[33,34] A sub-analysis showed that patients with low circulating EPCs had a relatively high late luminal loss and higher incidence of revascularizations at six months follow up. More interestingly, patients with low levels of circulating EPCs did not receive HMG-CoA-reductase inhibitors (statins) therapy. As statins have been shown to increase circulating EPC levels[36,37], we designed the HEALING IIB study in which we evaluated the efficacy of the Genous stent in conjunction with high dose statin (Atorvastatin 80mg once daily) therapy. Patients were started on 80mg atorvastatin once daily two weeks prior to the index procedure and continued for at least four weeks after the index procedure. Patients already on statin therapy were switched to 80 mg atorvastatin once daily.

In **chapter 13** we report the results of the HEALING IIB study.[38] The patient population of the HEALING IIB study was comparable with the HEALING II study. Statin therapy significantly increased circulating CD34+ EPC levels. Despite this increase in circulating EPC levels, late luminal loss at six months FU remained comparable to the HEALING II trial. Major adverse cardiac events at six months follow up seemed somewhat increased in the HEALING IIB trial compared to the HEALING II trial. This could be partially explained by study design, as the HEALING II trial included only single vessel disease whereas the HEALING IIB trial also included multi-vessel disease. Likewise, we also found a significant reduction in late luminal loss at long term follow up. This reduction in late luminal loss was accompanied by a low rate of major adverse cardiac events and target lesion revascularization between twelve and 24 months follow up. Late luminal loss of the Genous™ stent is higher than most drug-eluting stents (DES) to date. However, late luminal loss seems to increase in DES over time, while we have consistently shown that late luminal loss in the Genous™ stent declines. Therefore, one could speculate whether DES and Genous™ stent would yield comparable results beyond 24 months follow up. To address this question, randomized studies comparing DES and the Genous™ stent, with clinical and angiographic follow up beyond 24 months are required. Disappointingly, in the HEALING IIB trial there was a 3% incidence of stent thrombosis. In three patient stent thrombosis occurred at day 1, 9 and 181 post-procedure. However, all three events had documented significant procedural complications, which might have facilitated the in-stent thrombosis. The low stent thrombosis rate of 1.1% reported in the e-HEALING registry seems to confirm the favorable real world experience with the Genous Stent, even in the absence of prolonged dual anti-platelet therapy.[39]

In **chapter 14** we performed a sub-analysis of the HEALING IIB trial to characterize the patients that had an increase in EPC levels upon statin treatment.[40] Furthermore, we evaluated whether baseline

EPC levels could predict angiographic and clinical follow up. We divided the patients into a group that did not respond to Atorvastatin treatment with an increase in EPC levels, and a group that did respond to Atorvastatin treatment. Independent risk factors for being a non-responder were baseline EPC levels and non smoking. In other words, non-responders had a higher baseline EPC level and had never smoked when compared to responders. It is already known that endothelial dysfunction and different cardiovascular risk factors negatively influence the number of circulating EPCs. In this study we were able to show that smoking was associated with a low baseline level of circulating EPCs. However, we were unable to show a correlation between other cardiovascular risk factors or risk factor for endothelial dysfunction, including diabetes, hypertension or hypercholesterolemia. Interestingly, non-responders had a higher baseline EPC count than responders, suggesting that non-responders were unable to recruit more EPCs from the bone marrow despite efficient treatment with Atorvastatin. Alternatively, non-responders might have an increase in EPCs, but may be more efficient in homing of the recruited EPCs to the affected vascular area, an intrinsic capacity that was further amplified by statin treatment.[41] Therefore, no real increase in circulating EPCs may be found. Vice versa, for the responders, the data might imply that although recruitment of EPCs from bone marrow by statin treatment is successful, the recruited EPCs in the bloodstream remain inefficient in homing to the target, stented vessel wall. The fact that the non-responders had a more favourable late luminal loss at six months may hint that the latter mechanism indeed plays a role. This difference was even more pronounced at eighteen months follow up. This observation seemed clinically relevant as there was a trend towards a decrease in target lesion revascularizations in the non-responders group. Consequently, in our study baseline EPC count was negatively correlated with late luminal loss at six and eighteen months FU for patients treated with the Genous™ stent and high dose Atorvastatin.

Although we proved that the Genous™ stent indeed promotes re-endothelialization, this was not accompanied by less late luminal loss in the HEALING IIB trial, questioning the paradigm that early re-endothelialization is necessary to prevent neo-intima hyperplasia. The decreased thrombogenicity was also not observed in the HEALING IIB trial, but we did find less in-stent thrombosis in the HEALING II trial and the e-HEALING registry. Furthermore, one must bear in mind that patients only used one month of dual antiplatelet therapy, making the GENOUS stent suitable for patients that have a (relative) contra-indication for dual antiplatelet therapy.

To combine the two benefits of drug eluting stents and CD34 antibody coated stents, the Combo™ stent (OrbusNeich Medical, Ft. Lauderdale, Florida) was developed. The Combo™ stent is a combined sirolimus-eluting CD34 antibody coated stent. Recently, the results of the first-in-man, randomized, controlled multicenter REMEDEE trial (Randomized study to Evaluate the safety and effectiveness of an abluMinal sirolimus coatED bio-Engineered StEnt) was published.[42] The Combo™ stent was non-inferior to the paclitaxel eluting stent in terms of late luminal loss at nine months or the incidence of MACE at twelve months follow up. Also, no stent thrombosis was observed. This reduction in late luminal loss compared to the Genous™ stent was at the expense of a longer duration of dual antiplatelet therapy compared to the Genous™ stent. It is interesting to see whether long term follow up also shows a reduction in late luminal loss and stabilization in clinical events, as we have shown with the Genous™ stent.

Future directions

The pre-clinical field is currently progressing rapidly in the ongoing search for new cells, or optimizing existing therapies. The studies in part IV of the current thesis contributed considerably to our knowledge, and the fact that one of these studies progressed into a clinical sequel is very exciting. Moreover, investigations with fourth and fifth generation cellular therapies will enhance our knowledge and understanding, and clinical applications will take place rather sooner than later.

Also, our knowledge concerning the clinical application of cellular therapy for both AMI and HF patients has progressed substantially, since the first patients were treated more than a decade ago. Safety and feasibility have been shown for numerous cell types in phase I/IIa studies, and the field is slowly progressing towards phase IIb and III clinical trials. The APOLLO trial, described in part III of this thesis, is a good example of such a trial. However, many questions still remain unanswered. It is yet unknown what cell type will prevail, what delivery method is safest and most efficient, whereas the optimal timing of cell delivery is still controversial.

We believe that the forthcoming, EU-sponsored, phase III BAMI trial will definitively show if there is a place for BMMNC in AMI patients, as it is supposed to be powered to find differences on hard clinical endpoints. Moreover, phase II studies using intracoronary or intravenous delivery of allogeneic MSC will assess their presumed superiority to BMMNC, whereas the role for CDC in AMI patients is yet to be determined. Also the field of HF treatment is progressing with recent promising results of the TAC-HFT trial favoring MSC over BMMNC. Moreover, a phase III study was initiated to assess the efficacy of Stro3+ MSC in 225 ischemic HF patients, and the sequel of the C-CURE trial, with cardiogenically directed MSC, is much anticipated.

Conclusion

The main findings of the current thesis include: 1) First generation stem cells are not beneficial in AMI and heart failure patients; 2) liposuction can be performed safely briefly following AMI, when attention is paid to the anti-platelet and anti-thrombotic regimen; 3) intracoronary infusion of ADRC within 24 hours following the AMI is safe and feasible up to 36 months following the index event, and renders clear hints of efficacy; 4) intracoronary infusion of allogeneic mesenchymal precursor cells is safe when certain conditions are adopted, and results in; 5) pronounced effects on infarct size and cardiac function when compared to placebo-treated animals; 6) Intracoronary infusion of encapsulated mesenchymal stem cells (CellBeads) is safe and feasible, when you go low and slow; 7) Intracoronary infusion of CellBeads results in an increase in cardiac function in large animals with severely depressed cardiac function, when 20,000 CellBeads are infused; 8) The EPC-capturing stent is able to enhance vascular healing and reduce thrombogenicity; 9) but it does not improve outcome, even when; 10) EPC counts are raised artificially by high-dose statin treatment.

In conclusion, one might say that much progress has been made in the field of regenerative cardiovascular medicine over the past 15 years. This thesis contains some of this progress, but has no definitive answers. I feel that we currently are at the end of the beginning of an era, and that there will be a definite place for cell therapy in the future treatment of heart disease.

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Summary

Despite numerous advances in medical and interventional treatment options, ischemic heart disease, caused by atherosclerosis, still is the major cause of mortality and morbidity in the Western World. Acute myocardial infarction (AMI), followed by adverse ventricular remodeling, can evolve into the clinical syndrome of heart failure. Therapies to prevent or reduce adverse remodeling following AMI, have been the topic of extensive research over the past 50 years. Moreover, the search for cures of left ventricular dysfunction causing manifest heart failure is ongoing. Despite these efforts, the only real cure for end-stage heart failure is heart transplantation, which has several disadvantages of its own, even apart from the poignant shortage of donor hearts.

Stem cell therapy to either reduce adverse remodeling, or replace scar tissue by contracting cells, has raised high hopes in the last two decades. It is an appealing idea that transplantation of stem cells results in the replacement of lost cardiomyocytes, thereby restoring cardiac function of the failing heart. However, despite initial enthusiasm, it proved more difficult to obtain such an effect. It turned out that the road towards effective stem cell therapy contained countless hurdles, many of which have not been overcome yet.

The current thesis represents a small paving stone of this long road that still lies ahead, and aims to depict the progress in the field of cardiovascular stem cell therapy over the past decade. **Chapter 1** is a brief introduction to this thesis and summarizes the contents, whereas **chapter 2** represents an actual overview of the field of cardiovascular cell therapy. It is a synopsis of what is known, of what are the current challenges and questions, and aims to give a direction in which the field is heading. In **chapter 2**, we divide stem cells into five generations. The first generation comprises the first stem cells ever tested in humans: autologous skeletal myoblasts (SkM) and bone marrow-derived mononuclear cells (BMMNC). The second generation are autologous cells that are enriched in one way or another (*i.e.* endothelial progenitor cells, adipose tissue-derived regenerative cells, mesenchymal stem cells etc.), whereas the third generation exists of *allogeneic* mesenchymal stem cells. The fourth generation is defined as stem cells derived from the heart, either autologous or allogeneic. The fifth generation is the only generation with no clinical applications yet, and consists of pluripotent cells, either embryonic or induced.

In **chapter 3**, the safety and efficacy of BMMNC in AMI patients is assessed in a meta-analysis. Despite over 2,000 patients evaluated, we found no significant effect on MRI-derived measures, and no benefit on clinical end-points. BMMNC therapy did prove to be safe. **Chapter 4** confirms these findings, and extends the meta-analysis by also evaluating the benefit of first generation stem cells in heart failure patients. In contrast to the lack of effect in AMI patients, in heart failure patients, there seems to be a modest beneficial effect of BMMNC therapy. **Chapter 5** describes the results of the SEISMIC trial, in which end-stage heart failure patients received intramyocardial injections of SkM. In this open-label trial, no benefit on cardiac function was found, which contradicts various pre-clinical studies.

Chapter 6 and **7** focus on adipose tissue-derived regenerative cells (ADRC; second generation stem cells) in AMI patients. In these chapters, the results of the APOLLO trial are depicted. The APOLLO trial was a first-in-man study to evaluate the safety and feasibility of ADRC in the treatment of 14 patients with a large AMI. **Chapter 6** is a concise report of the first 6 months of follow up, whereas **chapter 7** describes the complete trial results. Moreover, **chapter 7** extends the functional follow up to 18 months, and the clinical follow up to 36 months. Briefly, we found that performing a liposuction in these fragile patients is feasible and safe. Moreover, intracoronary infusion of ADRC within 24 hours of the AMI seemed to be safe, whereas it resulted in remarkable reductions of infarct size and perfusion defects. However, these hints of efficacy were not substantiated by improved cardiac function, which necessitates further research in a larger trial. The ADVANCE trial is a phase III trial to prove efficacy of ADRC in >300 AMI patients, and is currently enrolling.

In **chapter 8**, we investigated the safety, feasibility and most effective dose of allogeneic mesenchymal precursor cells (third generation) in a large animal model of anterior AMI. Because intracoronary infusion of mesenchymal stem cells was previously associated with vascular plugging and the occurrence of micro infarctions, in this study we first sought the ideal and safest intracoronary delivery conditions. Following these pilot experiments, we performed a dose-finding study. We found that intracoronary infusion of these allogeneic mesenchymal precursor cells directly following the AMI reduced infarct size and improved cardiac function. The favorable results of this study resulted in the design of a clinical sequel. In this Allogeneic-Mesenchymal-precursor-cell-Infusion-in-myoCardial-Infarction (AMICI) trial, the safety, feasibility ans efficacy of these stem cells will be evaluated.

Chapter 9 describes a novel way to increase stem cell retention following intracoronary infusion. For the first time, it was found that mesenchymal stem cells encapsulated by a shell of alginate (CellBeads™), can be infused safely into coronary arteries of healthy and infarcted pigs. These Cellbeads get trapped in small arterioles in the infarct region, and theoretically serve as micro factories of paracrine factors. Indeed, one week following infusion, a part of the mesenchymal stem cells was still alive and reduced local inflammation and apoptosis. The results of the study that are described in **chapter 9** led to a large sequel, which was designed to test feasibility and efficacy of intracoronary infusion of CellBeads™ directly following AMI. **Chapter 10** covers the effects of different doses of CellBeads™ on cardiac function in large animal models of moderate and severe left ventricular dysfunction. We found that in a large animal model of severe left ventricular dysfunction, infusion of CellBeads™ reduces infarct sizes and preserves cardiac function.

In **chapter 11**, a concise review relates to the Genous R stent™. This stent was designed to attract endothelial progenitor cells in a attempt to enhance vascular healing following stent implantation. It was found in **chapter 12** that this design indeed attracts EPC, accelerates vascular healing and reduces thrombogenicity. Because it is known that statin therapy increases the numbers of circulating endothelial progenitor cells, the HEALING IIb trial was designed. In this trial patients who would receive a Genous R stent™ in the near future, were pre-treated with a high dose regimen of atorvastatin. In **chapter 13** and **14**, the results of the HEALING IIb study are described. In brief, high dose atorvastatin was able to increase EPC numbers in patients with low baseline titers, but it did not have

a beneficial effect on restenosis rates. Moreover, we found that patients with higher EPC count at baseline showed no increase in EPC recruitment, but less restenosis. These data imply that, although statin treatment can enhance EPC titer in patients with low baseline levels, there is no indication for a possible beneficial clinical effect with EPC capture stents.

In **chapter 15**, the results of the studies in this thesis are evaluated, and the following conclusions are drawn: 1) First generation stem cells are not beneficial in AMI and heart failure patients; 2) liposuction can be performed safely briefly following AMI, when attention is paid to the anti-platelet and anti-thrombotic regimen; 3) intracoronary infusion of ADRC within 24 hours following the AMI is safe and feasible up to 36 months following the index event, and renders clear hints of efficacy; 4) intracoronary infusion of allogeneic mesenchymal precursor cells is safe when certain conditions are adopted and leads to; 5) pronounced effects on infarct size and cardiac function when compared to placebo-treated animals; 6) Intracoronary infusion of encapsulated mesenchymal stem cells (CellBeads) is safe and feasible; 7) Intracoronary infusion of CellBeads results in an increase in cardiac function in large animals with severely depressed cardiac function, when 20,000 CellBeads are infused; 8) The EPC-capturing stent is able to enhance vascular healing and reduce thrombogenicity; 9) but it does not improve outcome, even when; 10) EPC counts are raised artificially by high-dose statin treatment.

Samenvatting

Ondanks vooruitgang in medische behandelmogelijkheden en interventies blijven ischemische hartziekten, veroorzaakt door atherosclerose, een belangrijke oorzaak voor mortaliteit en morbiditeit in de Westerse wereld. Het acuut myocardinfarct (AMI), gevolgd door remodellering van de linker ventrikel, kan uiteindelijk het klinisch syndroom van hartfalen veroorzaken. De afgelopen 50 jaar is er zeer uitgebreid onderzoek gedaan om wegen te vinden om dit remodelleren van de linker ventrikel te voorkomen, en zo het optreden van hartfalen uit te stellen. Ook de zoektocht naar middelen om een eenmaal verminderde pompfunctie te herstellen of verdere achteruitgang te vertragen is onverminderd gaande. Ondanks al deze pogingen blijft de enige èchte behandeling voor eindstadium hartfalen een harttransplantatie, waaraan ook vele beperkingen kleven, los nog van het nijpende tekort aan donorharten.

De afgelopen 20 jaar zijn er hooggespannen verwachtingen geweest betreffende stamceltherapie om remodellering van de linker ventrikel te vertragen, dan wel om geïnfarceerd en verlittekend myocard te vervangen. Het is inderdaad een aantrekkelijk idee, dat getransplanteerde stamcellen eenmaal afgestorven cardiomyocyten zouden kunnen vervangen, waardoor de pompfunctie van het falende hart verbetert. Helaas is de waarheid weerbarstiger en bleek het veel moeilijker om dit gewenste doel te bereiken dan aanvankelijk gedacht. De weg naar effectieve stamceltherapie is lang en vol met obstakels, waarvan velen nog steeds niet geslecht zijn.

Dit proefschrift vertegenwoordigt slechts een kleine tegel in de lange weg die nog te gaan is, en heeft tot doel de voortgang van stamceltherapie voor cardiovasculaire ziekten over de afgelopen decennia weer te geven. **Hoofdstuk 1** is een korte introductie en samenvatting van de inhoud van dit proefschrift. **Hoofdstuk 2** betreft een compleet overzicht van stamceltherapie voor cardiovasculaire ziekten. Het kenschetst wat we weten, wat de huidige uitdagingen en hiaten in de kennis zijn, maar probeert ook een richting te wijzen, waarin het vakgebied zich verplaatst. In dit hoofdstuk delen we stamcellen in in vijf generaties. De eerste generatie bestaat uit de eerste cellen die ooit in mensen werden onderzocht, te weten: skeletspiermyoblasten (SkM) en beenmerg mononucleaire cellen (BMMNC). De tweede generatie bestaat uit autologe cellen (van de patiënt zelf) die op één of andere manier geïsoleerd of gekweekt zijn (endotheliale voorlopercellen (EPC), (uit vetweefsel verkregen) mesenchymale stamcellen (ADRC en MSC respectievelijk), etc.), terwijl de derde generatie bestaat uit allogene mesenchymale stamcellen (cellen van een niet-gerelateerde donor). De vierde generatie is gedefinieerd als alle stamcellen die hun origine vinden in het hart, autoloog dan wel allogeen. De vijfde generatie is de enige generatie waarmee nog geen enkele klinische ervaring is opgedaan en bestaat uit pluripotente stamcellen, zoals embryonale stamcellen en geïnduceerde pluripotente stamcellen.

In **hoofdstuk 3** wordt de veiligheid en effectiviteit van BMMNC in AMI patiënten geëvalueerd in een meta-analyse. Ondanks het feit dat er al meer dan 2.000 AMI patiënten behandeld zijn met BMMNC, werd er geen significant behandeleffect gevonden op functionele eindpunten die met cardiale MRI bepaald waren. Ook werd er geen gunstig effect gevonden op harde klinisch eindpunten, alhoewel

wel bleek dat de behandeling veilig is. **Hoofdtuk 4** bevestigt deze bevindingen, maar breidt de meta-analyse ook uit naar patiënten met hartfalen. Hieruit blijkt dat er wel een bescheiden positief effect wordt gevonden bij patiënten met hartfalen, nadat ze met BMMNC zijn behandeld. In **hoofdstuk 5** worden de resultaten van de SEISMIC studie beschreven. In deze studie werden meerdere intramyocardiale injecties met SKM toegediend bij patiënten met eindstadium hartfalen. In deze open-label studie werd geen effect gezien van deze injecties, hetgeen enkele pre-klinische studies tegenspreekt.

Hoofdstukken 6 en **7** concentreren zich op uit vetweefsel verkregen mesenchymale stamcellen (ADRC; tweede generatie) in AMI patiënten. In deze hoofdstukken worden de resultaten van de APOLLO studie besproken. De APOLLO studie was een first-in-man studie, die erop gericht was om aan te tonen dat het mogelijk en veilig is, om in patiënten met een groot AMI deze stamcellen te verkrijgen en te injecteren. **Hoofdstuk 6** beschrijft beknopt de resultaten van de eerste 6 maanden van follow up, terwijl in **hoofdstuk 7** de complete studie wordt beschreven. Bovendien wordt de functionele follow up verlengd tot 18 maanden en de klinische follow up tot 36 maanden. Kort gezegd: het blijkt veilig om zo kort na een hartinfarct een liposuctie uit te voeren in deze kwetsbare patiëntengroep. Ook bleek dat het intracoronair infunderen van deze stamcellen veilig was, zonder dat het een nadelig effect had op de bloedstroom in de betreffende kransslagader. Na 6 en 18 maanden bleek er sprake van een opmerkelijke afname in infarctgrootte en perfusiedefect in behandelde patiënten. Deze effecten waren echter niet significant verschillend van de controle groep en leidden niet tot verbetering van pompfunctie. Omdat de APOLLO studie slechts bestond uit 14 patiënten (10 behandeld versus 4 placebo patiënten), moeten deze hoopvolle resultaten nog bevestigd worden in een grotere vervolgstudie. De ADVANCE studie is een fase III studie die is opgezet om de effectiviteit van deze cellen aan te tonen en includeert momenteel patiënten in >10 landen in Europa.

In **hoofdstuk 8** wordt een studie beschreven in grote proefdieren (schapen). In deze studie werd onderzocht of het mogelijk is om allogene mesenchymale stamcellen (MSC) intracoronair te infunderen kort na een AMI. Tevens onderzochten we welke dosis het meest effectief is. Uit eerdere studies was gebleken dat intracoronaire infusie van MSC de bloedstroom in de kransslagaderen kan vertragen en zelfs aanleiding kan geven tot het optreden van kleine hartinfarcten. Allereerst werd in deze studie dus bepaald of intracoronaire infusie mogelijk is, en wat de ideale infusie-condities zijn. Toen dit inderdaad het geval bleek, werd vervolgens een gerandomiseerde studie uitgevoerd om de optimale behandeldosis te bepalen. Wij ontdekten dat intracoronaire infusie van deze allogene MSC direct na een AMI de grootte van het infarct gunstig beïnvloedt, waardoor de pompfunctie van het hart behouden blijft. De positieve resultaten van deze studie hebben geresulteerd in het opzetten van een klinisch vervolgstudie. In deze studie, genaamd de AMICI trial, zal worden onderzocht of deze cellen in mensen net zo veilig en effectief zijn als in het schapen model van AMI.

Hoofdstuk 9 beschrijft een zeer vernieuwende manier om de retentie van stamcellen na intracoronaire infusie te vergroten. Door MSC te verpakken in kleine kraaltjes van alginaat ontstaan zogenaamde CellBeads™. Deze kraaltjes hebben een diameter van ongeveer 150 µm. Door de cellen te verpakken in alginaat worden ze beschermd tegen het immuunsysteem van de ontvanger, maar kunnen zuurstof en voedingsstoffen de cellen nog steeds bereiken. Wij vonden dat het mogelijk is om

deze CellBeads™ veilig te infunderen in een varkensmodel voor AMI. De CellBeads™ lopen na infusie vast in kleine arteriolen en zouden daar in theorie kunnen fungeren als kleine fabrieken van paracriene factoren. Inderdaad bleek dat 1 week na infusie een deel van de CellBeads™ en MSC nog intact waren en dat er een gunstig effect leek te bestaan op lokale ontsteking en celdood. Deze positieve resultaten zijn aanleiding geweest voor een grote pre-klinische vervolgstudie. In deze vervolgstudie onderzochten we de veiligheid en effectiviteit van verschillende doses CellBeads™ in grote proefdieren direct na een middelgroot of groot AMI. **Hoofdstuk 10** omschrijft de resultaten van dit onderzoek in meer dan 100 varkens. We vonden dat er geen nadelige effecten zijn van intracoronaire infusie van CellBeads™, als het totaal aantal de 60.000 niet overschrijdt. Tevens bleek er een gunstig effect op infarctgrootte en hartspierfunctie in bepaalde dosisgroepen, maar dit alleen in de proefdieren die een groot (voorwand)infarct hadden doogemaakt.

In **hoofdstuk 11** vindt u een beknopt review betreffende de Genous R stent™. Deze stent is ontworpen om endotheliale voorlopercellen (EPC) aan te trekken in een poging om de genezing van de vaatwand direct na stentplaatsing te bevorderen. Uit **hoofdstuk 12** blijkt dat dit ontwerp inderdaad endotheliale voorlopercellen aantrekt, de genezing van de vaatwand bevordert en thrombogeniciteit vermindert. Omdat uit eerdere onderzoeken ook al was gebleken dat therapie met statines de hoeveelheid circulerende EPC verhoogt, werd de HEALING IIb studie ontworpen. In deze studie werden patiënten, die in de nabije toekomst een Genous R stent™ zouden krijgen, minimaal twee weken voorbehandeld met een hoge dosis atorvastatine. In **hoofdstuk 13** en **14** worden de resultaten van deze studie beschreven. Het bleek inderdaad mogelijk om met hoge dosis atorvastatine de titer van EPC te verhogen in patiënten bij wie de titer bij baseline laag was. Deze hogere titer na behandeling had echter geen effect op de mate van in-stent restenose. Tegenovergesteld bleek dat hoge dosis atorvastatine bij patiënten met een hoge baseline titer geen effect had, terwijl deze patiënten significant minder restenose lieten zien. Uit deze data blijkt dat, alhoewel hoge dosis statine de hoeveelheid EPC positief kan beïnvloeden, dit geen effect sorteert op het optreden van restenose in patiënten met een Genous R stent™.

In **hoofdstuk 15** worden de resultaten van de studies uit dit proefschrift besproken, waarbij de volgende conclusies kunnen worden getrokken: 1) Eerste generatie stamcellen sorteren geen gunstig effect in patiënten met een acuut myocardinfarct. 2) Een liposuctie kan kort na een AMI veilig uitgevoerd worden, als er gelet wordt op het gevolgde beleid ten aanzien van bloedplaatjes-aggregatie remmers en antithrombotische middelen. 3) Intracoronaire infusie binnen 24 uur na het AMI van uit vetweefsel verkregen stamcellen is veilig en er zijn duidelijke aanwijzingen dat dit aanleiding kan geven tot gunstige effecten op infarctgrootte en perfusiedefect. 4) Intracoronaire infusie van allogene mesenchymale stamcellen is veilig als er aan strikte voorwaardes wordt voldaan en leidt tot; 5) uitgesproken gunstige effecten op infarctgrootte en pompfunctie. 6) Als infusiesnelheid en absoluut aantal van CellBeads laag gehouden wordt, is het intracoronair infunderen van deze CellBeads™ veilig en kan aanleiding geven tot; 7) een verbetering van pompfunctie in proefdieren met een groot hartinfarct. 8) De Genous R stent™ verbetert genezing van de bloedvatwand na stentplaatsing en vermindert thrombogeniciteit, maar ; 9) in-stent restenose wordt hierdoor niet verminderd, zelfs niet als; 10) de EPC titer kunstmatig wordt verhoogd met hoge doses statine.

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About the author

Hendrik Jacob Houtgraaf was born on December 23rd, 1976 in Rotterdam, the Netherlands. He finished high school in Oud-Beijerland in 1995, and subsequently moved to Rotterdam to start medical school at the Erasmus University Rotterdam. During his study, he was involved in several research projects at the departments of Cell Biology and Genetics (Prof. dr. H.J. Hoeijmakers) and Experimental Cardiology (late Prof. dr. W.J. van der Giessen). In 2000, he started with the Master of Science program Molecular Medicine, which eventually resulted in a Master of Science degree in 2001. He obtained his medical degree, with honors, in 2004.

In 2005 he started as a resident at the department of Internal Medicine at the Ikazia Hospital in Rotterdam, as part of his cardiology training (Prof. dr. M.L. Simoons). In 2007, he continued his training as a resident at the department of Cardiology at the Albert Schweitzer Hospital in Dordrecht. In late 2007, he began his PhD program at the departments of Interventional Cardiology (Prof. dr. P.W.J.C. Serruys) and Molecular Cardiology (supervisor Dr. H.J. Duckers), Erasmus University Medical Center, Rotterdam on the topic of cell-based therapies for cardiovascular repair. In 2011, he continued his clinical cardiology training, which he successfully completed in 2014. He currently works as a cardiologist at the Maasstad Ziekenhuis in Rotterdam.

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toegewijde, consciëntieuze en geordende research nurse van het Erasmus MC. Samen hebben we ervoor gezorgd dat er 13 patiënten geïncludeerd zijn in de APOLLO trial, wat een grote prestatie is geweest. De inclusie was echter nog het makkelijke deel; daarna begon pas de hectiek van het regelen van een echo, plastisch chirurg, cathlab, CCU bed en alle onderzoeken. Ik denk dat we geen enkele keer voor tien uur 's avonds het ziekenhuis hebben verlaten. Toch was het een leuke, bijzondere en leerzame ervaring. Aan ons gezamenlijke werkbezoek aan Cytori in San Diego, waar we onder leiding van Bobby "where are the cells?" Burns de geheimen van het Celution apparaat onder de knie kregen, denk ik met veel plezier terug. Ik wens jou, samen met jouw Piet, een hele gelukkige toekomst!

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Speaking of beer pong: I was introduced to the game by my other good friend, Brian Strem, on a legendary day in New York (after a legendary night). Brian, you and Anya have become good friends, which was illustrated by our trip to Budapest. Although we do not see each other very often anymore, I'm sure that we will keep in touch. The exchange of Gijs and Sasha a couple of years from now is a done deal, and we will definitely get to Napa valley rather sooner than later!

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