ADVANCED THERAPEUTIC STRATEGIES FOR ISCHEMIC HEART FAILURE

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Advanced Therapeutic Strategies for Ischemic Heart Failure

Vooruitstrevende Therapeutische Strategieën voor de Behandeling van Ischemisch Hartfalen

(met een samenvatting in het Nederlands)

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Wouter Adriaan Gathier

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Promotoren:

Prof. dr. S.A.J. Chamuleau
Prof. dr. P.A.F.M. Doevendans

Copromotoren:

Dr. F.J. van Slochteren
Dr. M.J. Cramer

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voor papa

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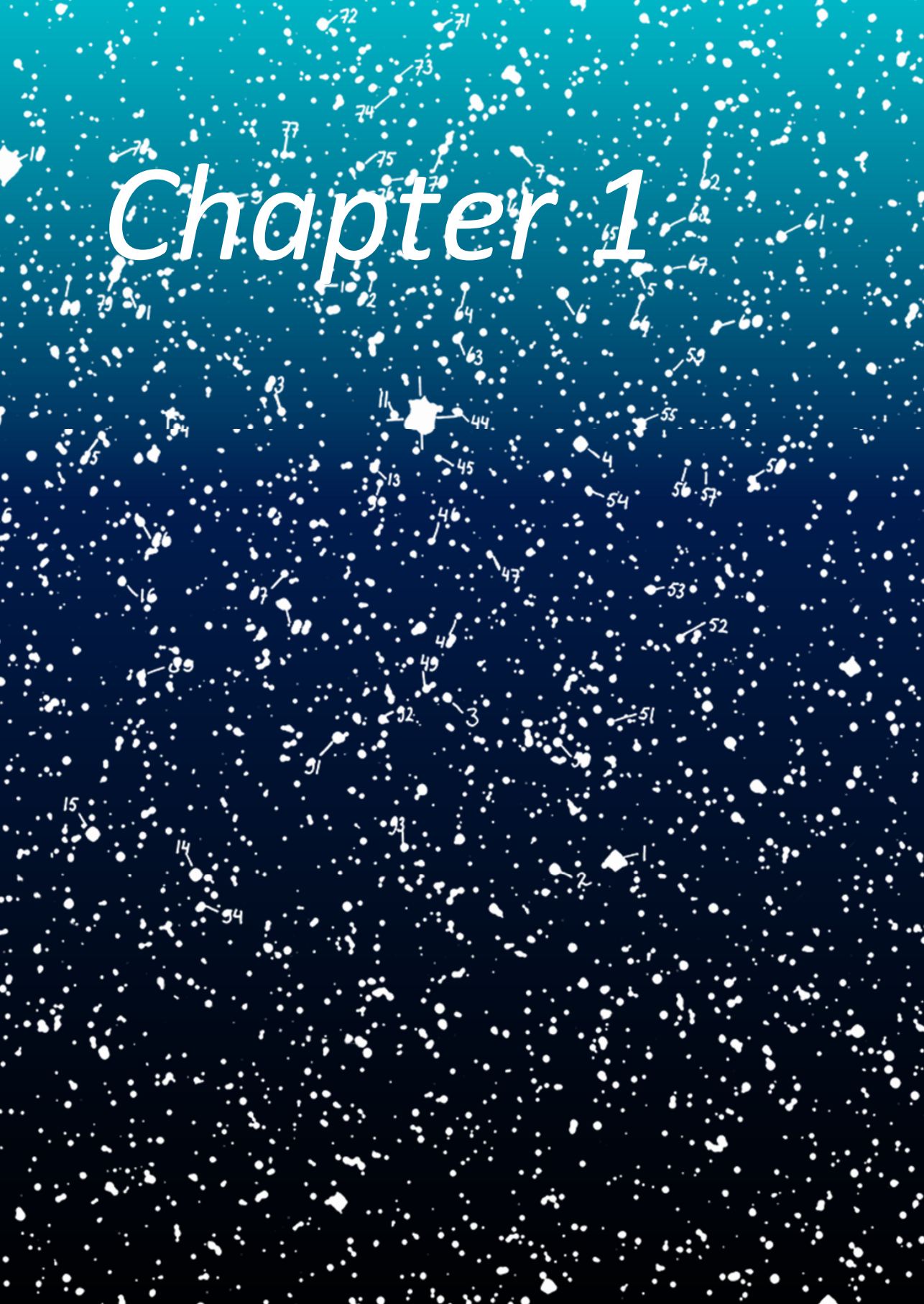
Chapter 1

General Introduction & Outline of Thesis

Ischemic Heart failure

Heart failure is a clinical syndrome characterized by typical symptoms and signs. These include shortness of breath, ankle swelling, fatigue, elevated jugular venous pressure, pulmonary crackles and peripheral edema and are caused by a structural and/or functional cardiac abnormality. The result is a reduced cardiac output and/or elevated intracardiac pressures at rest or during stress [1]. Heart failure can be caused by a multitude of conditions such as myocardial scarring due to ischemic conditions, genetic predisposition, toxic damage (e.g. radiation, medication, recreational substances), myocarditis, metabolic conditions, hypertension, valve disease, arrhythmias, and malignancy [1]. Etiology of heart failure is often specified as non-ischemic versus ischemic with ischemic cardiomyopathy making up approximately 60% of all heart failure cases [2]. Data from the Dutch Heart Foundation show that general prevalence estimates of heart failure in the Dutch population range from approximately 1.2% in men to 1.4% in women. These numbers increase greatly with age, resulting in a prevalence of 2-3% in the population aged 65-74 years, 7-8% for those aged 75-84 years, and 22% for those ≥85 years [3]. Based on calculations made by the Dutch National Institute for Public Health and the Environment (RIVM), the prevalence of heart failure is expected to increase by approximately 111% between 2011 and 2040 due to an increase in life expectancy [4].

Survival after heart failure diagnosis is low, with survival at 1, 2, and 5 years of 90%, 80%, and 60% respectively [5], making heart failure survival rates comparable to these of some common cancers [6]. Besides cardiac transplantation [7], there is currently no cure for heart failure and preventing development of heart failure and treating patients as effectively as possible are key to improve quality of life and survival. Depending on the cause of heart failure, different treatment options are available. Medication is the mainstay of treatment for heart failure, and usually consists of a combination of medications such as angiotensin converting enzyme inhibiters, diuretics, beta blockers, aldosterone antagonists, and digoxin. Other treatment options include surgical treatment, for instance coronary artery bypass grafting in case of severe coronary artery disease, or valve repair or replacement in case of abnormal valve function.

Device therapy has become an important treatment option during the last decades and involves implantation of cardiac defibrillators (ICDs) to prevent sudden cardiac death caused by abnormal heart rhythms. More advanced, biventricular pacemakers were developed later for cardiac resynchronization therapy (CRT) to resynchronize the dyssynchronized heart and improve pump function.

In search for a cure for heart failure, research on regenerative strategies for heart failure took flight in the early 2000s. Both cellular and non-cellular regenerative approaches have been pursued to

repair the damaged heart [8–10]. Cellular approaches encompass administration of either non-cardiac cells such as skeletal myoblasts and bone marrow-derived cells, cardiac-derived stem cells, and pluripotent stem cells. Non-cellular therapies include administration of secretory factors such as growth factors, extracellular vesicles, and micro RNA's.

Although major advances in therapeutic strategies for ischemic heart failure have been achieved in the past decades, better treatment for ischemic heart failure is needed to improve patient well-being and ensure optimal allocation of resources in a healthcare system that is becoming increasingly expensive. This thesis focusses on advanced therapeutic strategies for ischemic heart failure in the form of regenerative therapies and improved CRT using advanced imaging techniques.

Regenerative Therapy

Cell therapy

Cell therapy is focused on improvement of cardiac function both in acute and chronic heart disease. Two mechanisms to achieve this goal have been proposed, namely transdifferentiation of administered cells into functioning cardiomyocytes and the paracrine effect of cells on the heart. With little to no cells transdifferentiating into functioning cardiomyocytes and functional improvement already seen within 72 hours, it became apparent that the effect seen after cell administration was caused by the paracrine effect, rather than cardiomyogenesis [11–13]. Treatment of injured hearts with mesenchymal stromal cells (MSCs) and bone marrow-derived mononuclear cells (BMMNCs) resulted in increased tissue concentrations of cytokines such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF) and insulin-like growth factor I (IGF-I) [14,15]. The effect of cell therapy is governed by a multitude of factors, including the cell type to be administered and the chosen delivery strategy [8,16]. Part of the research incorporated in this thesis is focused on improving effects of cell therapy by addressing certain factors that influence efficacy of cell therapy.

Translational axis

One of the challenges of cardiac cell therapy is the diminishing effect along the translational axis. The effect of cell therapy decreases from Δ LVEF of ~12% in small animals [17], to ΔLVEF of ~8% in large animals [18], to ~4% in humans [19]. The reason for this decline can be found in part in the increasing complexity of the models [20], but also in the qualitatively suboptimal way that preclinical research is performed, leading to an overestimation of the effect size. Strategies to improve the translational axis of preclinical research have been proposed and include, among others, the reduction of publication bias, improved internal and external viability, sound methodologies, improved reporting, and prospective registration of preclinical trials in registries such as http://preclinicaltrials.eu [21]. Either way, the effect of cell therapy on improvement in cardiac function in humans remains low, emphasizing the need for improved techniques to improve outcome.

Two important factors influencing outcome of cardiac cell therapy include the cell type used, and the delivery method used to ensure that the cells remain in the targeted area for as long as possible.

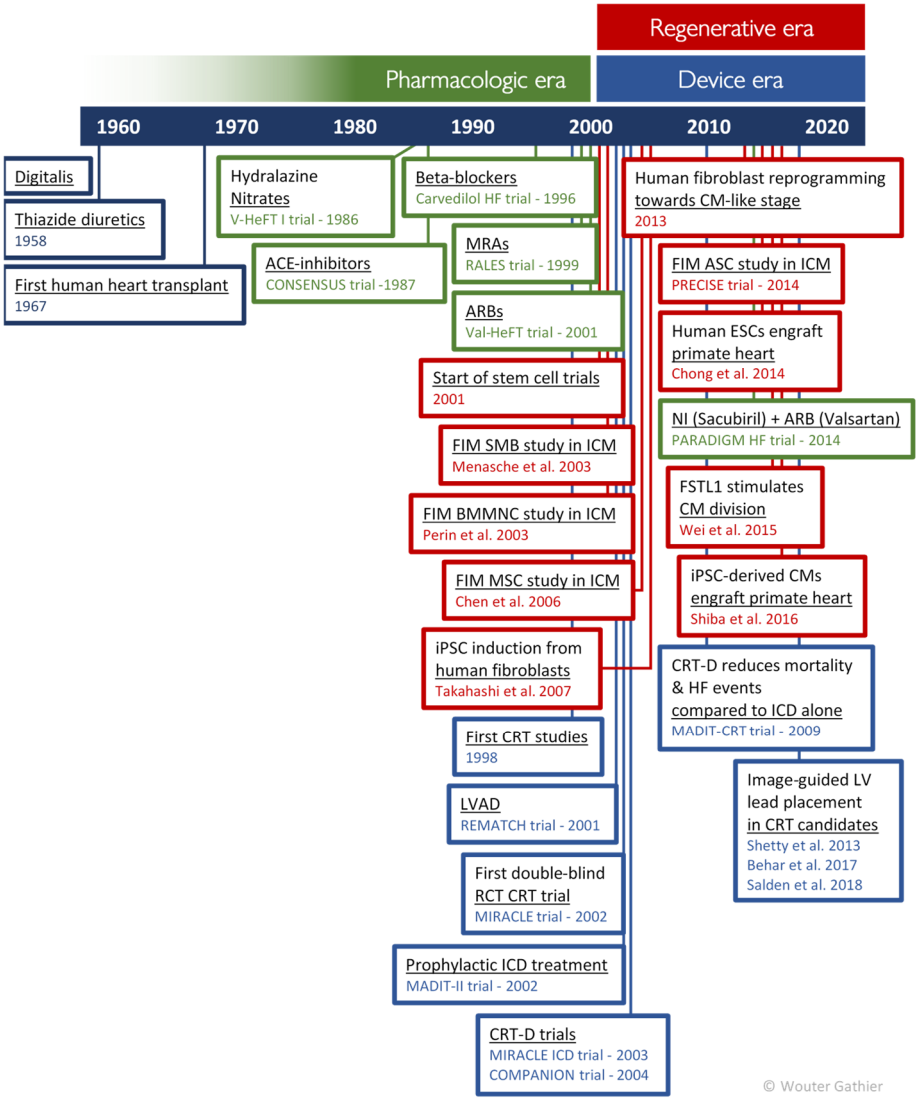

Fig. 1 A (not nearly complete) overview of research and development that contributed to improved treatment options for patients with ischemic heart failure.
ACE = angiotensin converting enzyme, *ARB* = angiotensin receptor blocker, *ASC* = adipose-derived stem cell, *BMMNC* = bone marrow mononuclear cell, *CM* = cardiomyocyte, *CRT* = cardiac resynchronization therapy, *CRT-D* = CRT including ICD, *ESC* = embryonic stem cell, *FIM* = first-in-man, *FSTL1* = Follistatin-like 1, *ICD* = implantable cardioverter-defibrillator, *ICM* = ischemic cardiomyopathy, *iPSC* = induced pluripotent stem cell, *LV* = left ventricle, *LVAD* = left ventricular assist device, *MSC* = mesenchymal stem cell, *MRA* = mineralocorticoid receptor antagonist, *NI*= neprilysin inhibitor, *RCT* = randomized controlled trial, *SMB* = skeletal myoblast.

Cell type

One of the first cell types administered to the heart was the skeletal myoblast [22]. Treatment with skeletal myoblasts has been associated with ventricular arrhythmias after which this cell type was largely abandoned [23–26]. The field eventually shifted to other types of cells, such as BMMNCs and MSCs, which appeared to possess excellent paracrine abilities. A cell type closely resembling BMMNCs in multipotency and immunophenotype is the adipose tissue-derived stem cell or ASC. The main benefit from this cell type is the ease with which these cells can be harvested and isolated from adipose tissue depots [27]. Other cell candidates include cardiac stem and progenitor cells which are natural resident cells in the heart and induced pluripotent stem cells which are generated from, for instance, dermal fibroblast through introduction of reprogramming factors.

A meta-analysis on BMMNCs as a treatment option for ischemic heart disease including 2500+ patients showed that BMMNCs are able to improve LVEF by approximately 3%, reduce infarct size by approximately 2%, and ameliorate remodeling [28]. The multinational, multicenter BAMI trial (ClinicalTrials.gov, Identifier: NCT01569178) recruited patients with reduced LVEF after reperfusion for myocardial infarction to investigate if BMMNC therapy effects all-cause mortality in this patient population. Inclusion stopped in November 2017 with approximately 400 patients included. Follow-up duration for the BAMI trial is set at two years with the first results are expected in the first quarter of 2020. A negative result of the BAMI trial might herald the end of BMMNC therapy for AMI patients, while positive results are needed to reinstate confidence in cell-based therapies.

Just like BMMNCs, MSCs have proven to be effective in patients with acute myocardial infarction [29–32] and ischemic cardiomyopathy [33–36]. MSCs can be easily obtained from bone marrow and cultured, making them ideal candidates for off the shelf, allogeneic cell therapy.

Lately, the field of cell research has been discredited by marginal therapeutic results and the publication of unreliable data by certain scientists. This led to negative attention in the media and scientific field. As a result of poor scientific conduct, including publication of falsified and fabricated data, 16 papers published by the group of Piero Anversa have been retracted from peer reviewed journals. An editorial from 2017 published in Nature Biotechnology addresses these points and states that it is necessary to ‘reassess the trajectory of the field as a whole and develop rational priorities [37]. Cell therapy has been shown to be safe, although the efficacy of cell therapy in patients remains uncertain by inconsistent results of clinical trials [19,38]. Discrepancies in trials that investigated the effect of autologous bone marrow-derived stem cell therapy on ejection fraction are described in the DAMASCENE paper. The authors show that discrepancies in trials and reports on effects of cell therapy on ejection fraction are common and numerous and that the discrepancy count is related to the effect size [39]. These results show the importance of rigorous trial design and open access reports, with open access to study data and insight in methodologies. However, encouraging results make that it is too early to abandon cell therapy as a whole.

Regenerative factors

Partly due to the negative attention and disappointing results, the focus of regenerative strategies shifted away from cell therapy towards other cardiovascular regenerative, cell-free products such as

biological or synthetic factors that possess the ability to influence endogenous regenerative processes. These products include secretory factors such as glycoproteins, growth factors, and extracellular vesicles. A multitude of these factors mimic the secretome of cells, and can be used as a cell-free treatment option.
Extracellular vesicles (EVs) are released into extracellular fluids and one of a number of ways of cells to communicate with each other. These vesicles can transport lipids, proteins and RNA's and can be used to stimulate angiogenesis or protect the heart against ischemic conditions. EVs can be purified from specific cardiac cells and have been suggested as treatment options for, among others, ischemic heart disease [40,41]. The secreted glycoprotein Follistatin-like 1 (FTSL1), has been reported to possess cardioprotective capabilities in the setting of myocardial infarction. Furthermore, FSTL1 might be capable of eliciting a regenerative response by stimulating cell cycle entry and division of cardiomyocytes after epicardial application [42].

These cell-free regenerative strategies could present as a promising therapeutic alternative for cell therapy for patients with ischemic heart failure. However, these therapeutic strategies each have their own limitations, ranging from cumbersome production processes or largely unknown pharmacokinetics to the need of advanced and tailor-made delivery strategies.

Delivery Strategies

Cell delivery strategies have been evolving over the last decades in order to find a more effective way to deliver cells to the heart and make sure that they are retained. Intracoronary infusion has been extensively used to deliver cells to the heart [43,44], Approximately only 5-10% of cell delivered to the heart are still present in the myocardium hours after administration [16,45]. With intracoronary infusion, cells are disseminated throughout the myocardium along the trajectory of the coronary artery used for infusion and the venous system. Intramyocardial injection, a technique in which cells are directly injected into the myocardium, is another viable option for cell delivery to the heart. This technique allows very precise delivery of cells to specific areas of the heart, such as the borderzone of the infarcted area in case of scarred myocardium [16,46]. Cardiac retention of cells after intramyocardial injection only reach approximately 10-15% due to effective venous drainage of the heart, with substantial numbers of administered cells flushed out within minutes [16,47]. To improve results of cell therapy, it is important to improve delivery of cells to the heart.

Device Therapy

Cardiac resynchronization therapy

Device therapy is one of the major advances in heart failure treatment of the past two decades. Cardiac resynchronization therapy (CRT) involves the implantation of a biventricular pacemaker to target ventricular dyssynchrony in the form of a left bundle branch block or interventricular conduction delay. Ventricular dyssynchrony is present in approximately one third of all patients with highly symptomatic systolic heart failure, and causes the heart to pump in an inefficient way [48]. CRT aims at restoring normal contraction patterns and has proven to be beneficial in patients with a LVEF

≤35%, prolonged QRS duration of ≥130ms, and New York Heart Association (NYHA) class II-IV symptoms [1,49–51]. Unfortunately, approximately 30-40% of patients undergoing CRT do not benefit substantially [52–54]. Especially patients with an ischemic cause of heart failure are less often CRT responders [53–56]. Prediction and improvement of response to CRT is important for patient well being and optimal allocation of resources in a healthcare system that is becoming increasingly expensive. Therefore, it is important to expand our knowledge of mechanisms that determine outcome to CRT and improve CRT implantation by, for instance, targeting specific areas of the left ventricle (LV) for LV lead implantation [57–60].

Larger scar burden and LV pacing in or near an area with myocardial scar are associated with diminished CRT outcome [61–63]. For this reason, it is important to delineate areas of myocardial scar and scar transmurality of these areas. Cardiac magnetic resonance imaging (CMR) has proven to be a valuable tool to assess the location and severity of scarred myocardial tissue, but can also be used to perform contraction timing analysis using postprocessing techniques such as CMR-tagging and feature tracking (FT) [59]. Information on the location and extent of scarred myocardium and the areas of delayed mechanical contraction can be used to determine the optimal LV pacing site in ICM patients undergoing CRT.

Research focused on the use of advanced imaging and post-processing techniques is needed to allow for tailor-made delivery of the LV pacing electrode in ICM patients in order to improve CRT response rates in this patient population.

Outline of Thesis

The aim of this thesis is to find solutions to existing challenges in the field of cell therapy, myocardial regeneration, and device therapy for ischemic heart failure in order to improve these therapeutic solutions and increase their potential in patients with ischemic heart failure.

Different cell types and regenerative compounds will be the topic of **Chapters 2, 3 and 4**.
Cell delivery strategies will be discussed in **Chapter 5 and 6**.
Advanced imaging strategies for optimal CRT delivery will be discussed in **Chapter 7**.

In **Chapter 2** the comparison between BMMNCs and MSCs is made in order to determine if either one of these cell types has a higher potential to improve cardiac function in a porcine model of chronic ischemia. Furthermore, the effect of repetitive cell injections is addressed to determine whether a second delivery of cells could augment the effect of the first delivery. In **Chapter 3** the potential of adipose tissue-derived stem cells is reviewed. This cell type is easy to obtain in large numbers from adipose depots in patients, circumventing the process of cultivation of cells to obtain sufficient numbers for transplantation. The process of cell harvesting, isolation and culturing, differentiation potential, working mechanisms, and results in preclinical and clinical research are covered. In **Chapter**

4 the potential role of the secreted glycoprotein Follistatin-like 1 (FSTL1) in cardiomyocyte survival, cardiomyocyte proliferation and angiogenesis is reviewed. In **Chapter 5** an overview is provided of preclinical and clinical data concerning retrograde coronary venous infusion as a cell delivery strategy in regenerative cardiac therapy. Because retention of cells in the heart is still far from optimal with the established ways of delivery, insight in possible alternatives is needed. The search for a more effective cell delivery method is continued in **Chapter 6** where retrograde coronary venous infusion is compared with the more commonly used intracoronary infusion in a chronic ischemia model in pigs. Cell delivery is an important aspect of cell therapy, and if cell delivery is not effective, results of cell therapy will suffer. Furthermore, cell delivery should be safe and, preferably, easy to perform. In **Chapter 7** cardiac magnetic resonance imaging and post-processing techniques are used to assess the optimal LV lead position in patients with ischemic heart failure that received cardiac resynchronization therapy at the University Medical Center Utrecht during the last decade. The aim of this study is to determine the optimal LV lead position relative to the infarcted area and area of latest contraction. Determining the optimal target for the LV lead in advance of the implant procedure will allow for better LV lead placement. The insights from this study will aid future research on targeted LV lead placement and can attribute to real-time image guided LV lead placement. **Chapter 8** provides an in-depth summary of the research incorporated in this thesis.

In summary, this thesis will cover advanced therapeutic strategies for ischemic heart failure and provide insight in advances in cell therapy, novel regenerative strategies, cell delivery strategies, and optimization of cardiac resynchronization therapy.

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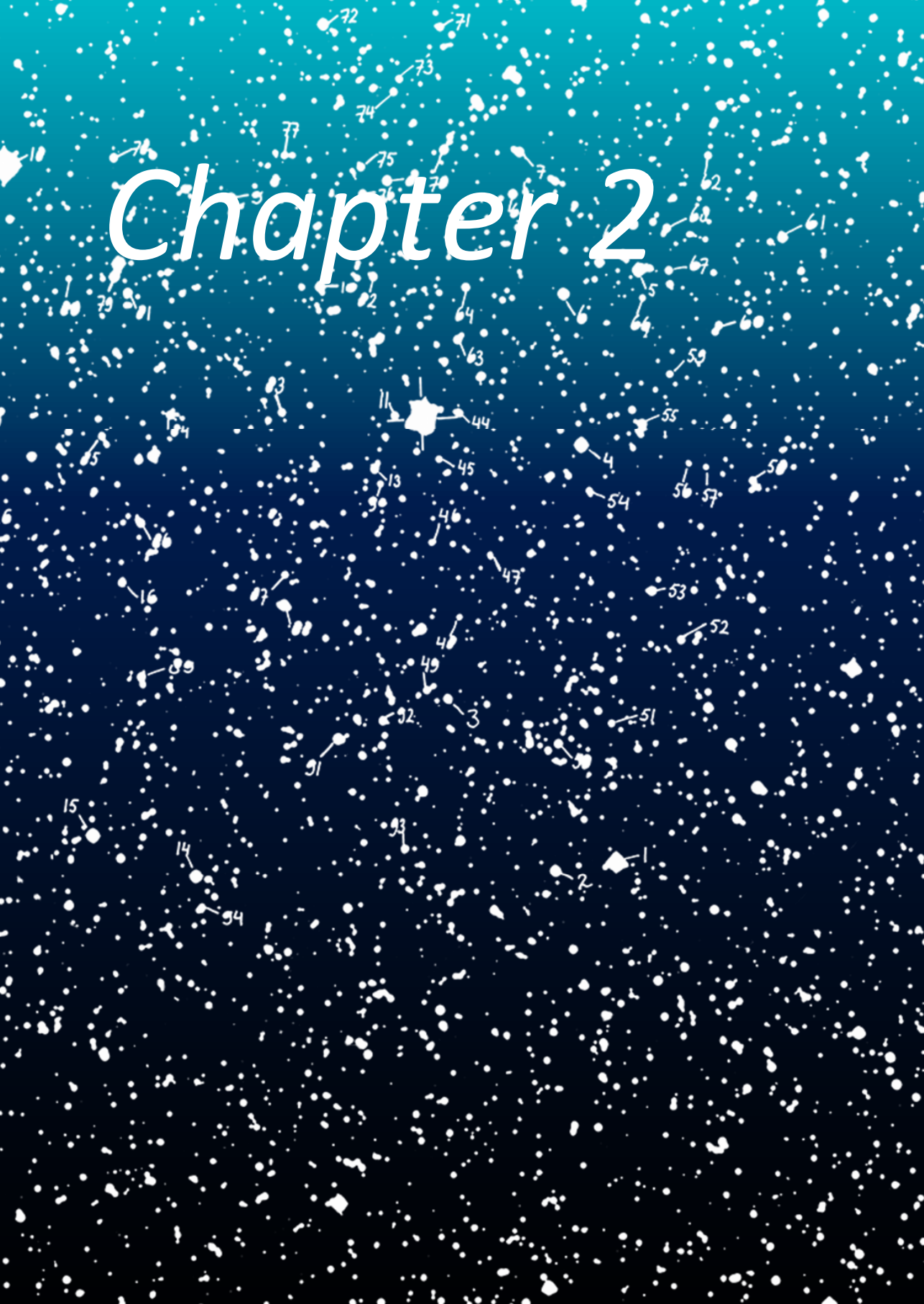
Chapter 2

Autologous Mesenchymal Stem Cells Show More Benefit on Systolic Function Compared to Bone Marrow Mononuclear Cells in a Porcine Model of Chronic Myocardial Infarction

Tycho I.G. van der Spoel[1], Wouter A. Gathier[1], Stefan Koudstaal[1], Frebus J. van Slochteren[1], Sanne Jansen of Lorkeers[1], Joost P.G. Sluijter[1,2], Imo E. Hoefer[3], Paul Steendijk[4], Maarten-Jan Cramer[1], Pieter A. Doevendans[1,2], Eric van Belle[1], Steven A.J. Chamuleau[1]

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[1] Department of Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands

[2] Interuniversity Cardiology Institute of The Netherlands (ICIN), Utrecht, The Netherlands

[3] Department of Experimental Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands

[4] Department of Cardiology, Leiden University Medical Center, Leiden, The Netherlands

Abstract

Background

Cardiac cell therapy is a strategy to treat patients with chronic myocardial infarction (MI). No consensus exists regarding the optimal cell type.

Methods

First, a comparison between autologous bone marrow-derived mononuclear cells (BMMNC) and mesenchymal stem cells (MSC) on therapeutic efficacy after MI was performed. Next, the effect of repetitive, NOGA-guided transendocardial injection was determined via a crossover design. Nineteen pigs were allocated in three groups: (1) placebo (at 4 and 8 weeks), (2) MSC (followed by placebo at 8 weeks), or (3) BMMNC (followed by MSC at 8 weeks) delivery including a priming strategy to enhance MSC effect.

Results

At 4 weeks, ejection fraction (EF) was significantly improved after MSC injection and not by BMMNC injection. After 8 weeks, no difference was observed in EF between cell-treated groups demonstrating the positive systolic effect of MSC.

Conclusions

This study showed that MSC rather than BMMNC injection improves systolic function in chronic MI.

Introduction

Ischemic heart failure remains a major cause of morbidity and mortality [1]. Stem cell therapy emerged as an innovative and attractive therapeutic approach for patients with chronic myocardial infarction (MI). The ultimate goal of this treatment is to support and enhance the endogenous repair mechanisms by replacing dysfunctional cardiomyocytes and inducing angiogenesis.

In clinical and preclinical studies, a modest improvement in left ventricular ejection fraction (LVEF) was observed using a single injection of bone marrow cells after MI [2, 3]. Our preclinical meta-analysis showed that the choice of cell type is an important significant predictor of improvement in LVEF [3] suggesting a trend towards more pronounced effects of mesenchymal stem cells (MSC). Till now, bone marrow mononuclear cells (BMMNC) and MSC have been well studied in patients with ischemic heart disease [4]. However, it is known that functional differences between MSC and BMMNC exist [3]. A direct comparison on functional endpoints between these cell types has not been performed so far. We hypothesized that pretreatment of the area of interest could be helpful to further enhance the effects of MSC. Thus, we incorporated a repetitive cell injection strategy in the study design.

Percutaneous transendocardial (TE) delivery, guided by electromechanical mapping (NOGA), was shown to be safe in patients with chronic ischemic cardiomyopathy [5] and has the advantage of detecting hibernating myocardium which is the area that will probably profit most from cell delivery [6].

Our objective was to determine the most potent regenerative strategy using autologous bone marrow cell types, i.e., BMMNC and MSC, in a large animal model of ischemia/reperfusion injury. First, a direct comparison between BMMNC and MSC was performed 4 weeks after transplantation. Second, the effect of repetitive injection after initial priming was determined by including a crossover with MSC in the BMMNC group with an additional follow-up period of 4 weeks.

Methods

Animals

Nineteen female Dutch Landrace pigs received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals," published by the National Institutes of Health (National Institutes of Health publication 85-23, revised 1985). The study protocol was approved by the Animal Experimentation Committee of the University of Utrecht.

Study Design

Animals were allocated to one of three groups: (group 1) placebo (phosphate buffered saline (PBS), Invitrogen, Carlsbad, CA, USA), (group 2) 10^7 autologous MSC, or (group 3) 10^7 autologous BMMNC injection at 4 weeks. Eight weeks after MI (thus, 4 weeks after initial injection), the animals in group 3 received an additional injection of MSC to determine whether priming could rescue the damaged myocardium, while the other groups received an injection with PBS for control purposes. Twelve weeks after the initial MI, the animals were euthanized and tissue was prepared for histology. Cardiac

function was assessed by pressure-volume (PV) loops and echocardiography. The study design is shown in Fig. 1.

Premedication and Anesthesia

After an overnight fast, animals were sedated with an intramuscular injection of ketamin (10 mg/kg), midazolam (0.5 mg/kg), and atropin (0.04 mg/kg). Next, thiopental (4 mg/kg) was administered intravenously before intubation. They were intubated with an endotracheal tube and anesthetized in the supine position. The animals were mechanically ventilated with the use of a positive pressure ventilator with a mix of oxygen and air (FiO2 0.5). General anesthesia/analgesia was maintained with midazolam (0.5 mg/kg/h, Roche, Woerden, the Netherlands), sufentanyl citrate (2 µg/kg/h, Janssen-Cilag, Tilburg, the Netherlands), and pancuronium bromid (0.1 mg/kg/h, Organon, Oss, the Netherlands). Metoprolol (Centrafarm, Etten-Leur, the Netherlands) was administered intravenously (5 mg) to reduce the mechanical irritation of the heart. During surgery, animals were anticoagulated with heparin (ACT > 250 s). At the end of the experiment, the animals were euthanized by pentobarbital overdose.

Myocardial Ischemia/Reperfusion Model

During the entire procedure, electrocardiogram, arterial pressure, and capnogram were continuously monitored. Prior to MI, all animals received an oral dose of amiodarone (400 mg/day; starting 10 days prior to MI) and clopidogrel (75 mg/day; starting 3 days prior to MI; Sanofi Aventis, Gouda, the Netherlands) [7]. A bolus of 500 mg acetylic salicylic acid (Centrafarm, Etten-Leur, the Netherlands) was given the day before the occlusion. Myocardial infarction was created by a percutaneous balloon of equivalent size to the proximal left circumflex artery (LCX). The balloon was inflated for 75 min at 5–8 atm [8]. Complete occlusion of the LCX was confirmed by angiography. To prevent ventricular arrhythmias, 300 mg amiodarone (Centrafarm, Etten-Leur, the Netherlands) intravenously was given. External defibrillation (150–200 J) was used when ventricular fibrillation occurred. After the procedure, coronary angiography was performed to confirm vessel patency. After recovery, the animals received daily an oral dose of 50 mg metoprolol, 400 mg amiodarone, 75 mg clopidogrel, and 160 mg acetylic salicylic acid until termination to prevent thrombosis and arrhythmias [7].

MSC Culture and Labeling

Bone marrow was aspirated (35–40 mL) from the sternum by a heparinized syringe. BMMNC were isolated by Ficoll density gradient centrifugation and frozen in 10 % DMSO and 90 % culture medium.

MSC were isolated and characterized as previously described [9]. Autologous MSC were cultured at 37 °C in Alpha MEM (Invitrogen, Carlsbad, CA, USA), supplemented with 10 % FBS, heparin, and 1 % penicillin/streptomyocin. Cells were cultured, replacing medium every 3 days and used between passage 5 and 7. Before injection, cells were resuspended in 2 mL PBS and viability was assessed via trypan-blue (Sigma-Aldrich, St. Louis, MO, USA) counting.

Transendocardial Delivery

To enable TE injection, an 8-F sheath was placed in a carotid artery. Next, a mapping catheter (Biosense Webster, Cordis, Johnson & Johnson, USA) was placed retrogradely through the aortic valve into the left ventricle (LV). First, a 3-dimensional electromechanical map of the LV was obtained using the NOGA system (Biosense Webster, Cordis, Johnson & Johnson, USA), as described before [10, 11]. Hereafter, 10 injections of 0.2 mL were slowly placed using the MYOSTAR® injection catheter (Biosense Webster, Cordis, Johnson & Johnson, Diamond Bar, USA). Two injections were placed in the infarct zone and eight in the border zone. Four weeks after the first injection, this procedure was repeated and the second injections were given at the same location. Injections were only given in areas with a unipolar voltage greater than 6 mV [10, 11].

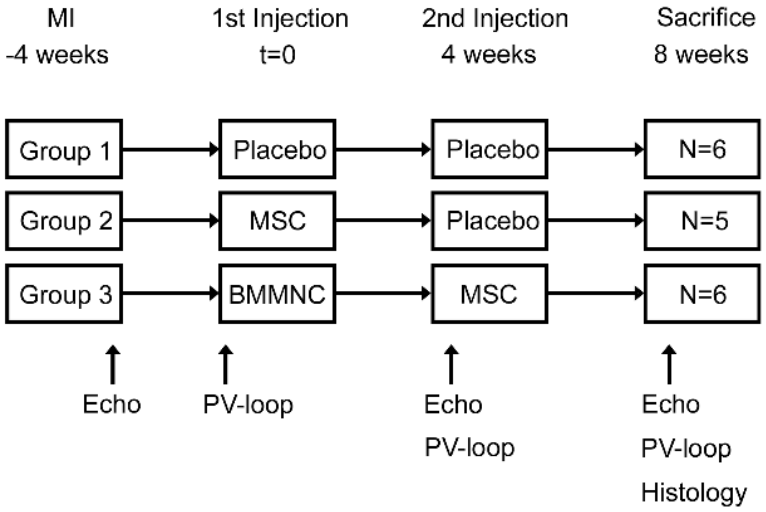

Fig. 1 Study design. *BMMNC* = bone marrow mononuclear cells, *Echo* = echocardiography, *MI* = myocardial infarction, *MSC* = mesenchymal stem cells, *PV loop* = pressure-volume loop

Echocardiography

A transthoracic echocardiogram (5-MHz probe, IE-33, Philips, Best, the Netherlands) was performed directly after MI, 8 weeks after MI, and at sacrifice as described before [7]. Short axis images were obtained at the papillary level, and three consecutive cardiac cycles were acquired. Wall thickness (WT) of the posterolateral wall was assessed in end-systole and end-diastole. The left ventricular internal area (LVIA) was obtained without including the papillary muscles in end-systole and end-diastole. The fractional area shortening was calculated as ((LVIAenddiastole − LVIAendsystole)/LVIAenddiastole) × 100.

Pressure-Volume Loop protocol

Pressure-volume loops were obtained using a 7-F conductance catheter that was inserted via a carotid artery and placed along the long axis of the LV. The catheter was connected with a signal processor (Leycom CFL, CD Leycom, Zoetermeer, the Netherlands). The correct position of the conductance

catheter was verified by angiography and by inspection of the segmental conductance signals. The conductance signals were calibrated by thermodilution and hypertonic saline dilution via a 7-F Swan-Ganz catheter that was placed into the right or left pulmonary artery [12, 13]. Data were collected during steady-state conditions with the respirator systems turned off at end-expiration. From these signals, hemodynamic indices were derived. Data analysis and calculations were performed using custom-made software (CD Leycom, Zoetermeer, the Netherlands), as previously described [14]. Parameters of global systolic and diastolic function were calculated during steady-state conditions at 4, 8, and 12 weeks after MI. Cardiac output (CO) measured by Swan-Ganz was corrected by multiplying each measurement with 0.62. This number was based on the following equation (CO Swan-Ganz at sacrifice/CO transonic aorta flow probe at sacrifice). The isovolumic relaxation time constant (Tau) was calculated by phase-plot analysis. The end-systolic pressure-volume relationship was measured by its slope end-systolic elastance (Ees). Diastolic stiffness (Eed) was determined as the linear slope of the end-diastolic pressure-volume relationship. Both were calculated by single-beat analysis as described earlier [15].

Histology

After euthanasia, the LV was weighed and tissue samples from the infarct, borderzone, and remote region of the heart were obtained. Samples were fixed in 4 % formalin at room temperature. Before cutting 5-µm sections, samples were embedded in paraffin for analysis. For quantification of collagen content, picrosirius red staining and detection with circularly polarized light and digital image microscopy was used [16]. Five random images at ×20 magnification of the infarcted, borderzone, and remote area were obtained per animal. After conversion into gray value images, the average number of grey values was expressed as a mean grey value per square micrometer. Capillary density was assessed by Lectin staining (Sigma-Aldrich) and counterstained with Hematoxylin and Eosin to identify nuclei. In total, five fields per section at ×20 magnification were counted per animal per zone.

Statistical Analysis

Values derived from echocardiography were analyzed in a blinded fashion. For statistical analysis, we used a linear mixed effects model to account for repeated measurements on each animal. In this model, we included a generalized estimation equations-type matrix to account for the association between residual covariance, e.g., time point of measurement (8 and 12 weeks after MI). Statistical comparison of data between groups was done using a one-way ANOVA with a post hoc Tukey or Kruskal-Wallis test. Data are presented as mean ± SE or median with interquartile ranges in case of non-normal distributed data. All statistical analyses were performed using SPSS 18.1.1, and *P* values <0.05 were considered statistically significant.

Results

Procedural Data

In total, 19 animals underwent the MI procedure. One animal in the placebo group died due to severe heart failure evidenced by obduction (group 1; day 71 after MI), and one animal had to be terminated for reaching a human-defined endpoint due to an abscess at the right foot not related to the study (group 2). MSC viability (group 2, 92 ± 4 % vs. group 3, 93 ± 1 %; $P = 0.10$) and number of MSC (group 2, $1.0 \pm 0.1 \times 10^7$ vs. group 3, $0.9 \pm 0.2 \times 10^7$; $P = 0.10$) did not differ between the cell-treated groups. BMMNC viability was 92 ± 4 % and the injected number $1.7 \pm 0.2 \times 10^7$. No cardiac tamponade or sustained ventricular arrhythmias were observed after any cell or placebo injection.

Comparison Between MSC and BMMNC on Cardiac Function at 4 weeks after Cell Transplantation

Four weeks after MI (baseline), no difference in LVEF between groups was observed ($P = 0.30$; Table 1). When comparing LVEF differences between baseline and 4 weeks after injection (Fig. 2), placebo-treated animals showed a reduction in LVEF whereas in MSC-treated animals, LVEF was significantly improved (group 2, 11.9 ± 3 % vs. group 1, −7.8 ± 8 %; $P = 0.002$). Animals treated with MSC showed a tendency for having a decrease in ΔESV (group 2, −6.0 ± 7 mL vs. group 1, 10 ± 10 mL; $P = 0.10$). No significant difference in ΔLVEF between BMMNC and placebo treatment was observed (group 3, −1.6 ± 6 % vs. group 1, −7.8 ± 8 %; $P = 0.748$). Consequently, MSC injection led to a significant increase in ΔLVEF compared to BMMNC injection (group 2, 11.9 ± 3 % vs. group 3, −1.6 ± 6 %; $P = 0.028$) but also significantly improved ΔCO (group 2, 0.7 ± 0.3 L/min vs. group 3, −0.4 ± 0.4 L/min; $P = 0.037$) and thereby reflects an increased systolic cardiac performance. After BMMNC injection, a trend for impaired $\Delta dP/dt_{MAX}$ was observed compared to MSC-treated animals (group 2, −38 ± 154 mmHg vs. group 3, −277 ± 57 mmHg; $P = 0.08$).

Table 1 Hemodynamics derived from pressure-volume loops at baseline, before the second injection, and at sacrifice

Hemodynamics	Baseline (4 weeks after MI)			Injection (8 weeks after MI)			Sacrifice (12 weeks after MI)		
Parameter	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
General									
Weight (kg)	73±3	71±2	73±1	80±3	76±2	76±1	83±3	82±3	82±1
LV weight (g)							168±7	159±5	175±9
FAS (%)				50±5	55±3	47±2	50±2	51±2	43±3
HR (beats/min)	52±2	59±4	52±6	63±8	56±1	57±7	51±7	55±1	53±4
CO (L/min)	3.5±0.3	2.8±0.2	3.0±0.4	3.2±0.2	3.5±0.3§	2.7±0.2	2.8±0.5	3.5±0.3	3.1±0.1
Systole									
ESV (mL)	41±3	37±4	49±7	50±12	31±8	44±7	51±11	23±5#	30±6*
ESP (mmHg)	96±7	87±11	100±5	86±7	90±8	85±4	91±7	81±5	74±4*
EF (%)	62±2	57±2	55±5	54±7	69±3#§	54±5	52±3	74±3#	69±4*
dP/dt_{MAX} (mmHg/s)	1586±131	1390±208	1374±46	1372±152	1351±134	1096±64	1460±102	1402±40§	1033±71*
Ees (mmHg/ml)	3.9±0.5	4.2±0.6	3.7±0.1	3.7±0.7	3.7±0.4	3.2±0.2	4.1±0.9	3.7±0.5	2.5±0.3*
Diastole									
EDV (mL)	107±8	85±5	109±6	106±13	92±13	95±9	106±16	86±9	91±10
EDP (mmHg)	16±1	13±1	16±1	14±2	16±1	13±2	15±1	14±2	11±1*§
dP/dt_{MIN} (mmHg/s)	-1428±131	-1345±165	-1393±91	-1350±224	-1447±119	-1275±99	-1239±224	-1328±99	-1148±93
PHT (ms)	34±2	31±2	39±3	36±7	31±1	34±3	44±7	28±1#	31±1*
Tau (ms)	58±4	52±4	67±6	62±14	51±2	57±5	72±16	48±2	49±2*
Eed (mmHg/mL)	0.38±0.04	0.42±0.02	0.37±0.06	0.46±0.8*	0.54±0.06§	0.29±0.02	0.30±0.04	0.38±0.07	0.24±0.03

Data are presented as mean ± SE

CO = cardiac output, *HR* = heart rate, *EDP* = end-diastolic pressure, *ESP* = end-systolic pressure, dP/dt_{MAX} = maximal rate of LV pressure increase, dP/dt_{MIN} = maximal rate of LV pressure decrease, *EDV* = end-diastolic volume, *Eed* = myocardial stiffness, *Ees* = end-systolic elastance, *ESV* = end-systolic volume, *EF* = ejection fraction, *FAS* = fractional area shortening, *LV* = left ventricle, *PHT* = pressure halftime, *tau* = isovolumetric relaxation time constant

*P<0.05 BMMNC vs. placebo, #P<0.01 MSC vs. placebo §P<0.05 MSC vs. BMMNC

Regarding global diastolic function, no significant difference in Δend-diastolic volume between groups could be observed (group 1, −0.2 ± 4 mL; group 2, 7.7 ± 13 mL; group 3, −14 ± 8 mL; all $P > 0.1$). In addition, dP/dt_{MIN}, Tau, end-diastolic pressure (EDP), and pressure halftime (PHT) were similar in the different treatment groups (Table 1). However, passive diastolic function was improved in the BMMNC group compared to the other groups, indicated by ΔEed (group 1, 0.08 ± 0.05 mmHg/mL; group 2, 0.12 ± 0.08 mmHg/mL; group 3, −0.08 ± 0.05 mmHg/mL; BMMNC vs. placebo, $P = 0.04$; MSC vs. BMMNC, $P = 0.004$; MSC vs. placebo, $P = 0.349$).

Directly after MI, echocardiographic recordings showed that end-systolic WT was similar between groups (group 1, 1.25 ± 0.2 cm; group 2, 1.38 ± 0.1 cm; group 3, 1.01 ± 0.2 cm; $P = 0.78$). Also, no difference in end-diastolic WT was observed (group 1, 1.21 ± 0.3 cm; group 2, 1.18 ± 0.3 cm; group 3, 1.01 ± 0.2 cm; $P = 0.48$). Four weeks after treatment, no significant effect on Δend-diastolic WT (group 1, 0.03 ± 0.06 cm; group 2, 0.01 ± 0.03 cm; group 3 0.10 ± 0.05 cm) and Δend-systolic WT (group 1, 0.28 ± 0.07 cm; group 2, 0.06 ± 0.06 cm; group 3, 0.16 ± 0.09 cm) was found.

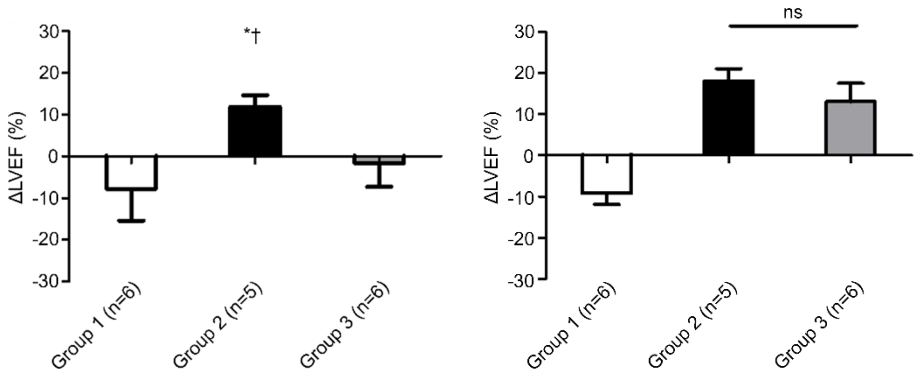

Fig. 2 (left panel) Effects at 4 weeks after cell therapy: group 2 (MSC injection) improves systolic function compared to group 3 (BMMNC) and group 1 (placebo). Percentage of change in LVEF between baseline and 4 weeks after injection in each treatment group. *$P < 0.01$ compared to group 1. †$P = 0.028$ compared to group 3. *LVEF* = left ventricular ejection fraction

Fig. 3 (right panel) Effect at 8 weeks after (repeated) cell therapy: no difference on EF between single (group 2) versus pretreated injections (group 3) with MSC. No significant effect on ΔLVEF (baseline and 8 weeks after injection) between single and repeated cell injection was observed. *LVEF* = left ventricular ejection fraction

Effect of Repeated Cell Injection on Cardiac Function at 8 weeks after Cell Transplantation

Since no effect of BMMNC on ΔLVEF was observed, we now did not expect a synergistic effect of repetitive BMMNC injection in group 3. However, we now were able to study whether a second injection of MSC could rescue the damaged myocardium.

When comparing ΔLVEF between baseline and at sacrifice (Fig. 3), placebo-treated animals showed a reduction in ΔLVEF, whereas in cell-treated animals, ΔEF was significantly improved (group 2, 18 ± 3 %; group 3, 13 ± 4 % vs. group 1, −9 ± 3 %; all $P < 0.01$) caused by a significant reduction in ΔESV (group 2, −14 ± 4 mL; group 3, −20 ± 4 mL vs. group 1, 11 ± 10 mL; all $P < 0.01$). However, no difference in ΔEF or ΔESV between single MSC injection and repeated cell delivery could be observed

($P = 0.28$ and $P = 0.79$). Contractility measured by $\Delta dP/dt_{MAX}$ was significantly increased after single MSC injection, compared to BMMNC and MSC injection (group 2, 105 ± 193 mmHg; group 3, −340 ± 63 mL; $P = 0.003$). In fact, the second MSC injection on top of the first BMMNC injection (without significant difference compared to placebo) once more revealed the magnitude of effect on systolic function by MSC.

Overall, both cell groups showed an improvement in diastolic active relaxation parameters compared to placebo-treated animals. This was reflected by a shortened ΔTau and decreased ΔPHT. Myocardial stiffness (Eed) was unaffected by cell therapy. No statistical difference in active and passive diastolic function between the cell-treated groups could be observed, except for EDP.

No significant difference in echocardiographic parameters between single cell injection and repeated cell injection was observed.

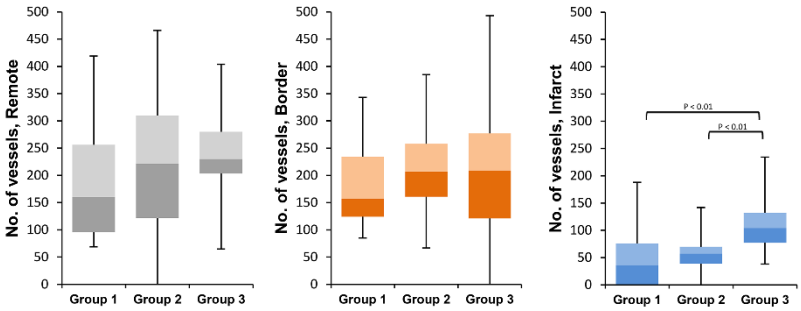

Fig. 4 Microcirculatory remodeling in the damaged myocardium after cell delivery 12 weeks post-MI. Microvascular formation determined by Lectin staining at sacrifice was significantly higher in the infarcted zone in group 3 (BMMNC + MSC injection) compared to the other groups ($P < 0.01$). Furthermore, a nonsignificant increase in vessel density was observed in both cell-treated groups in both the remote and border zones (both $P > 0.1$). Group 1: 4 animals, 13 images used for analysis of remote zone, 14 for border zone, 13 for infarct zone. Group 2: 5 animals, 14 images used for analysis of border zone, 23 images for border zone, 18 for infarct zone. Group 3: 6 animals, 23 images used for analysis of remote zone, 27 for border zone, 30 for infarct zone

Capillary Density and Collagen

Histological samples were not available for two animals (both group 1). Due to technical issues, in 175 of the 225 samples (78 %), representative Lectin stainings were obtained and used for analysis. Both collagen and vascular density data showed a non-normal distribution.

A significantly higher number of capillaries in the infarcted area was seen in group 3 compared to both group 1 and group 2 (median value 104 vs. 36 vs. 57, respectively; $P < 0.01$, Fig. 4). Between groups, no significant difference between the number of vessels in the border zones was found (median value for group 1, 157; group 2, 207; group 3, 209). Also, no difference was found for the remote areas. Within groups, the number of vessels was lower in the infarcted area as compared to border zone and remote area as expected. Furthermore, no significant difference was found in the number of vessels in the remote and border zone. Figure 5 shows representative Lectin-stained images from each group.

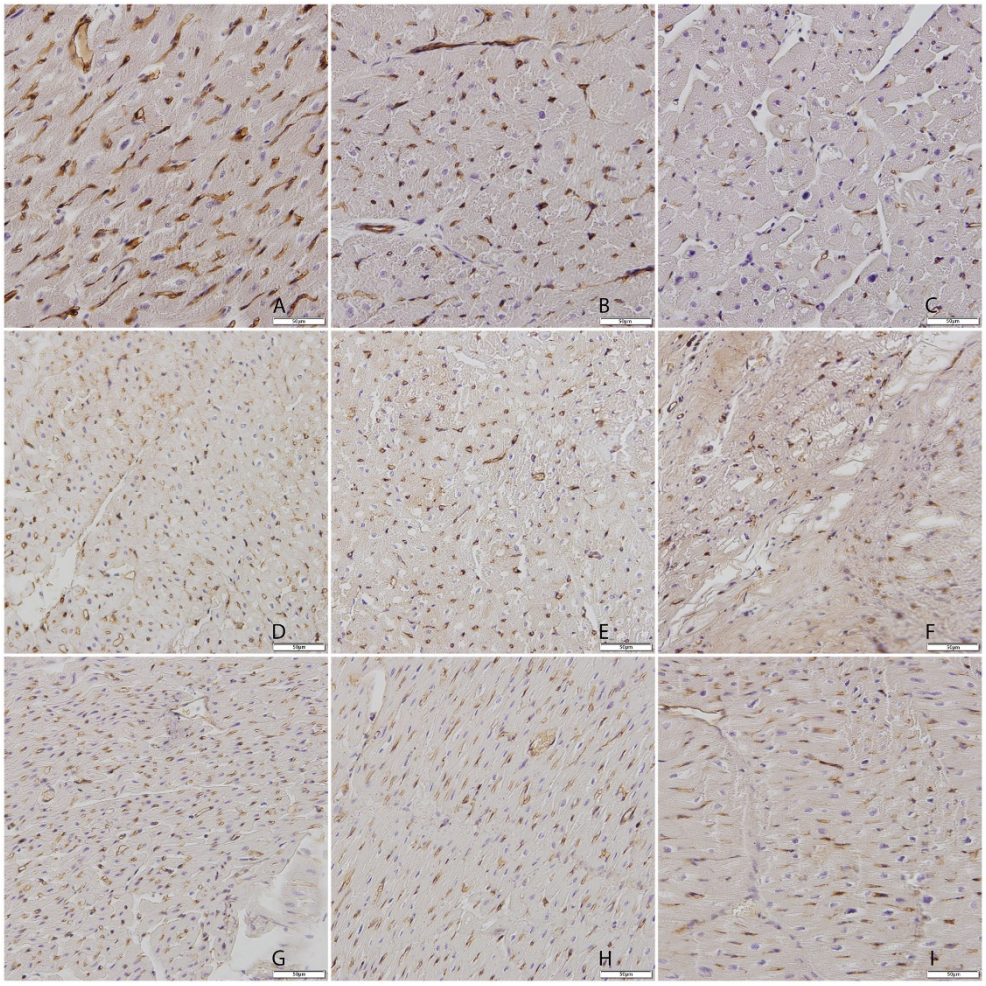

Fig. 5 Representative images of Lectin staining at sacrifice from remote area, border zone, and infarcted tissue. **A**: Group 1 (placebo + placebo) remote, **B**: group 1 border, **C**: group 1 infarct. **D**: Group 2 (MSC + placebo) remote, **E**: group 2 border, **F**: group 2 infarct. **G**: Group 3 (BMMNC + MSC) remote, **H**: group 3 border, **I**: group 3 infarct. Magnification ×20

Collagen density assessment could be performed on 100 % (225 samples) of the picrosirius red stainings. As expected, infarcted tissue from all groups showed a substantial increase in collagen density compared to tissue from remote areas and border zones.

Group 3 showed a significantly lower collagen density in the infarcted area compared to group 1 (median 64×10^{-7} vs. 318×10^{-7}, respectively; $P < 0.01$, Fig. 6), but not group 2 (median 288×10^{-7}). However, a significantly higher collagen density was observed in the border zone of group 3 compared to group 2 (median 29×10^{-7} vs. 3×10^{-7}, respectively; $P < 0.05$, Fig. 6). Figure 7 shows representative picrosirius red-stained images from each group.

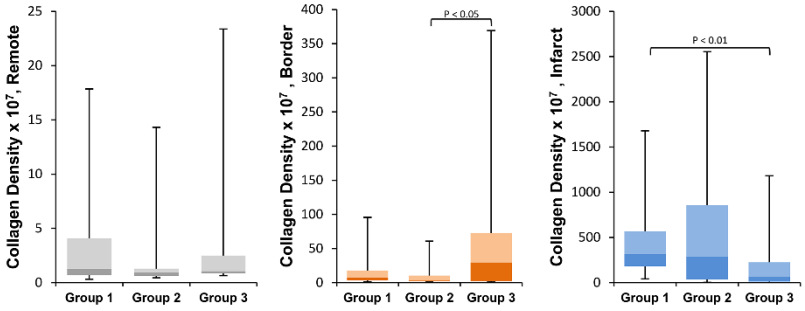

Fig. 6 Collagen density after cell therapy. Collagen quantification 12 weeks post infarction of remote areas, border areas, and infarcted areas. A significantly decreased collagen density was observed in the infarcted zone in group 3 (BMMNC + MSC) compared to group 1 (placebo + placebo) ($P < 0.01$). However, in the border zone of group 3, an increase in interstitial fibrosis was measured compared to group 2 (MSC + placebo) ($P < 0.05$). Group 1: 4 animals, 20 images used for analysis per section. Group 2: 5 animals, 25 images used for analysis per section. Group 3: 6 animals, 30 images used for analysis per section. Note differences in *Y*-axis

Discussion

In this study, we performed a comparison between MSC and BMMNC via TE cell delivery in a porcine model of chronic ischemic heart disease. The main novel findings of our study are the following: (1) MSC are superior to BMMNC in improving systolic function, and (2) the delivery strategy of repeated cell injection was safe and feasible. Interestingly, MSC on top of BMMNC led to normalization of LV function, supporting the notion that MSC rather than BMMNC improve systolic function.

MSC Treatment Improves Systolic Function in Contrast to BMMNC

We performed a head-to-head comparison of treatment with autologous BMMNC and MSC and demonstrated a beneficial effect for MSC on systolic function (EF 11.9 ± 3 %), whereas no effect of BMMNC on LVEF was found compared to placebo (EF −1.6 ± 6 % and −7.8 ± 8 %, respectively).

This was despite the fact that even a slightly higher number of cells were used in the BMMNC group (1.7×10^7 BMMNC vs. 1.0×10^7 MSC). This observation is in line with the results of our large preclinical meta-analysis, showing more benefit of MSC in ischemic heart disease compared to BMMNC [3]. On the contrary, Li et al. did not find significant difference between MSC and BMMNC. However, they infused far more BMMNC than MSC (BMMNC $4.7 \pm 1.7 \times 10^7$ vs. MSC $6.2 \pm 1.6 \times 10^5$) [17]. It is known that the number of cells is related to the magnitude of effect [3, 4]. In our study, no statistical difference between injected cell number was observed. Our results may appear to be in contrast with the data from previous clinical studies that did show modest but significant improvements of LVEF after treatment with BMMNC (approximately 3–5 %) [18–20]. However, such studies were mainly performed in the setting of *acute* MI and these effects were predominantly found in subgroups of large infarctions (baseline EF < 48 %) [18]. In fact, several trials with BMMNC in *chronic* patients did not show improvement of systolic function [5, 21, 22]. On the contrary, in a comparable patient cohort, it was demonstrated that indeed MSC were able to improve cardiac function [23].

Recently, a clinical study (TAC-HFT trail) directly compared these cells and demonstrated the safety of both cell types [24]. However, after MSC injection, a reduction in infarct size was observed which was not the case after BMMNC. Unfortunately, this study was not powered to provide a definitive statement on therapeutic efficacy. Taken together, these results provide a robust rationale for a larger trial comparing both cell types to determine whether or not bone marrow stem cells have a clinical future.

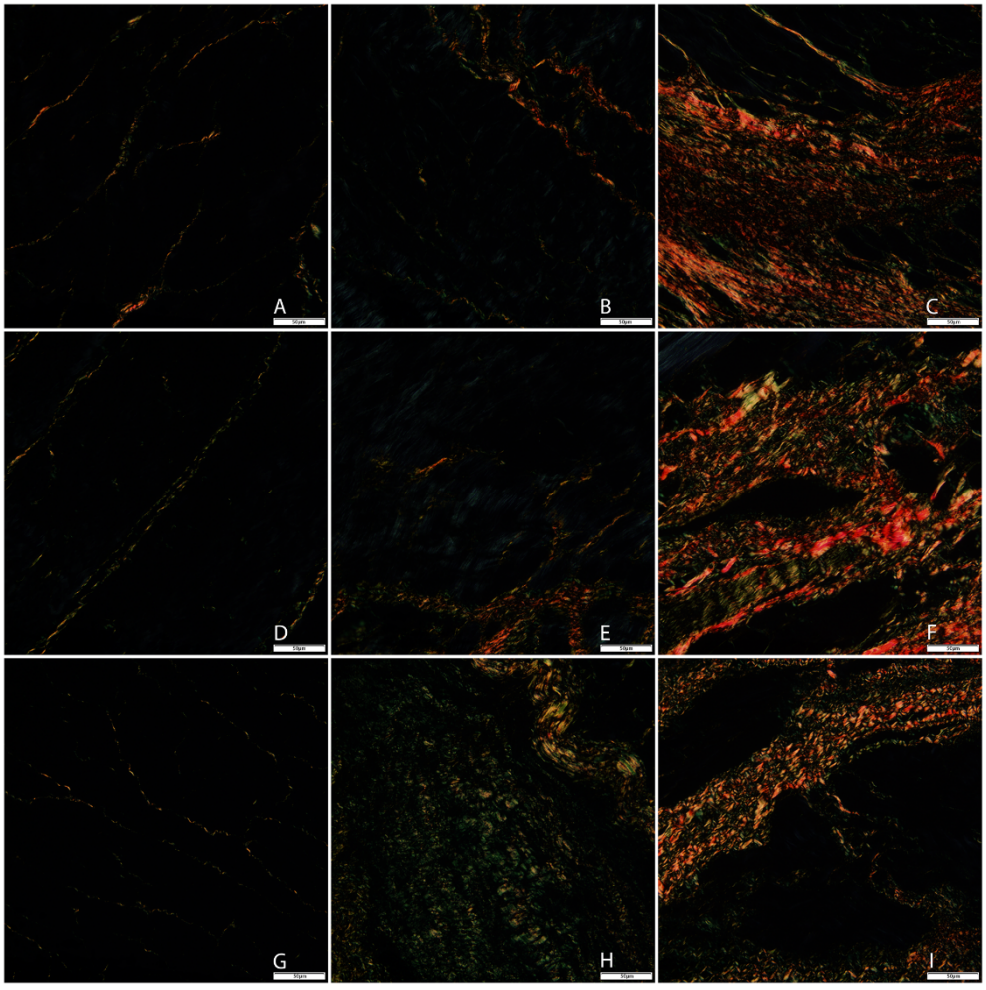

Fig. 7 Representative images of picrosirius red staining at sacrifice from remote area, border zone, and infarcted tissue. **A**: Group 1 (placebo + placebo) remote, **B**: group 1 border, **C**: group 1 infarct, **D**: group 2 (MSC + placebo) remote, **E**: group 2 border, **F**: group 2 infarct, **G**: group 3 (BMMNC + MSC) remote, **H**: group 3 border, **I**: group 3 infarct. Magnification ×20

Repeated Cell Injection was Safe but Does Not Further Improve Cardiac Function

Repetitive cell injections led to no serious adverse events (e.g., death, persistent ventricular arrhythmias) but did not further improve systolic function compared to single MSC injection. This is largely due to the lack of an effect of BMMNC; this was surprising and not anticipated. Several studies investigated in particular the effect of repetitive cell transplantations [25]. Our observations are in line with a clinical trial investigating the effect of repeated BMMNC injections in patients with chronic heart failure showing no additional benefit of repeated BMMNC treatment on LVEF [26]. However, Yao et al. demonstrated that repeated BMMNC injection in patients with large acute MI resulted in a significant improvement in ΔLVEF compared to single cell injection [27]. This effect may be explained by the low baseline LVEF values (20–39 %) which were higher in our study. Our results are in line with an observation [28], in which skeletal myoblasts were sequential injected in a chronic infarcted porcine myocardium. Although a different cell type was used, repeated cell injections showed no difference in ΔLVEF (repeated 15.1 % vs. single 11.1 %).

Histological Effects of MSC Injection

It is postulated that a decreased collagen density and an increase in vascular density in border and/or infarct zone could be the explanation for the observed effects on systolic function in groups 2 and 3. Indeed, collagen density was significantly reduced in the infarcted area in group 3 (BMMNC + MSC) compared to group 1 (placebo + placebo). However, this was not the case for group 2. Surprisingly, the opposite was found for the border zone of group 3. Therefore, the histological analysis of infarcted tissue only partly explains the found effects in the present study.

Second, measurement of capillary density showed a significant increase in the number of microvessels in the infarcted area after BMMNC + MSC injection compared to placebo treatment and MSC + placebo treatment. These data do not directly explain the functional changes although there seems to be a slight trend of increased capillary density in both the border zone and infarcted area of both cell groups; however, a significant increase in the number of vessels was only found in the infarcted area of the BMMNC-primed group as seen in Fig. 4. This suggests a more pronounced angiogenic response after priming with BMMNC before injecting MSC (group 3).

Finally, some MSC were observed in the infarcted tissue by fluorescent microscopy (data not shown), but it is unlikely that the observed effect was caused by differentiation of MSC into cardiac lineages as also suggested by others [29]. However, MSC may lead to prolonged secretion of paracrine factors activating capillary angiogenesis.

Study Limitations

Our ischemia/reperfusion model resulted in a limited decrease in LVEF (appr. 50 %), but not severe heart failure. This is related to the chosen model (temporary occlusion of LCX for 75 min) and maybe by the fact that animals were treated with similar medication protocols (e.g., beta blockers, which may be cardioprotective) compared to the patients suffering from MI. Nevertheless, significant effects on LVEF were observed.

The porcine model is considered the best possible model to resemble the clinical situation, although major differences exist (e.g., risk factors, cell isolation protocols [30], comorbidity, follow-up duration), which prevent direct extrapolation to patient management. Nevertheless, our group demonstrated that large animal models can accurately predict human clinical outcome and these models are frequently used for translational purposes [3].

Care was taken to perform adequate analysis of collagen and vascular density; however, both sampling error and staining issues may have had impact on this histological assessment. By analyzing five sections per sample, we attempted to minimize this bias.

Nowadays, cardiac MRI is considered the gold standard to measure LVEF and volumes. However, due to practical reasons, we performed echocardiographic and pressure-volume loop analysis. These techniques are still considered reliable, reproducible, and a valid measure of LV function and are therefore most often used in preclinical research models.

Finally, our results show that the anticipated synergistic effect by pretreatment with BMMNC before MSC treatment in group 3 was suggested by the increased capillary density, but was not represented in functional endpoints. Knowing this, a fourth group comparing repetitive MSC injections would have been helpful to elucidate this effect for repetitive MSC treatment. Nevertheless, we feel that the take-home message from this particular group is now reassuring that indeed MSC outperform BMMNC in this setting.

Conclusions

We demonstrated that MSC are more potent in terms of improvement of LVEF than BMMNC in a chronic model for ischemic heart disease. Our data do not support strategies using repetitive injections, although using different combinations of cells may be of value in more severe heart failure. These data should encourage researchers and clinicians to focus future studies on other cell types (i.e., MSC) than BMMNC.

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Conflict of interest

The authors declare they have no competing interests.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This study was performed in compliance with the "Guide for the Care and Use of Laboratory Animals," published by the National Institutes of Health (National Institutes of Health publication 85-23, revised 1985). The study protocol was approved by the Animal Experimentation Committee of the University of Utrecht.

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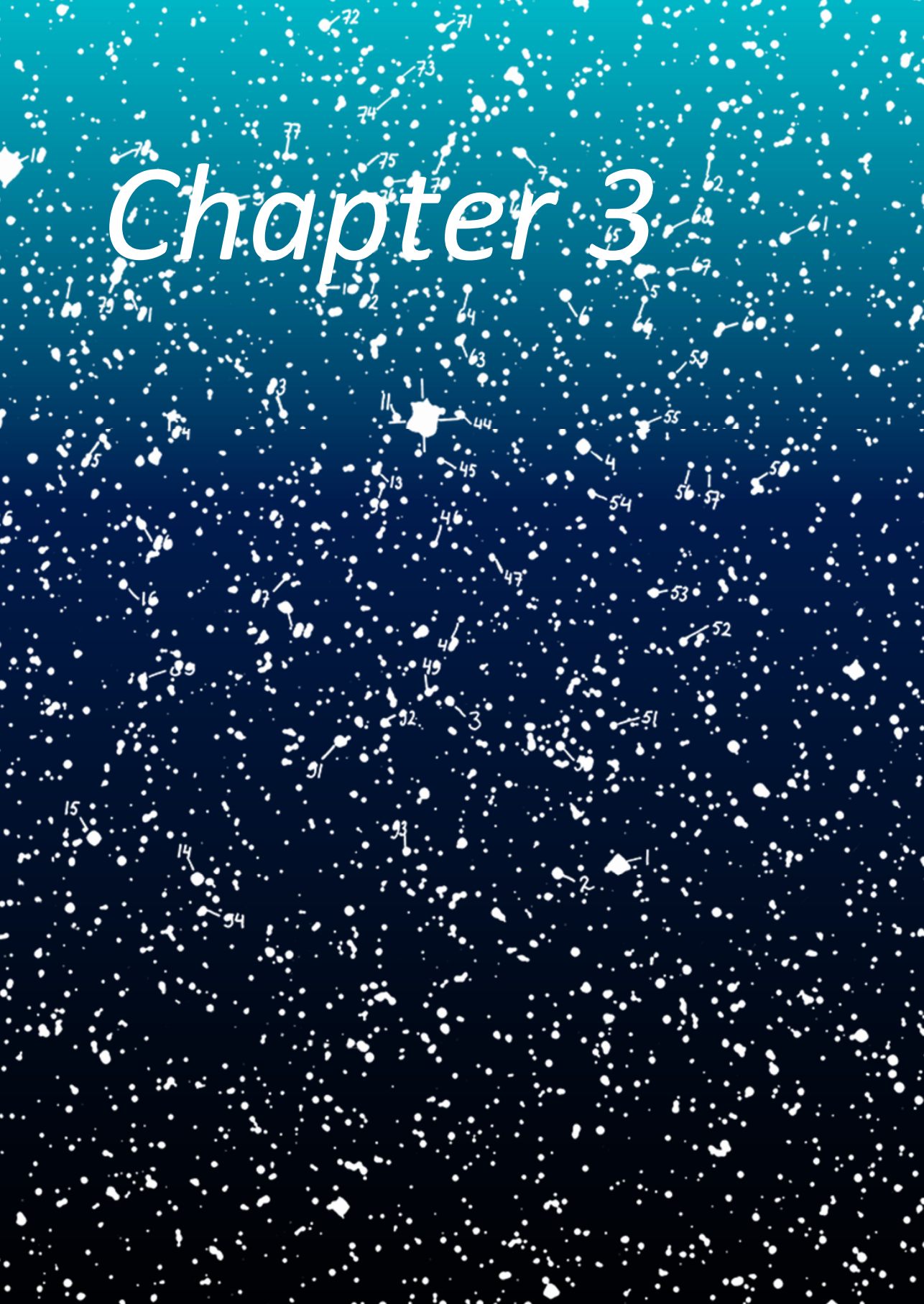
Chapter 3

Adipose-Derived Stem Cells

Wouter A. Gathier[1], Zeliha Türktaş[1], Henricus J. Duckers[1]

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[1] *Department of Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands*

Introduction

Despite current advances in drug therapy, the number of people afflicted with some form of cardiovascular disease (CVD) worldwide continues to rise, due in large part to the advancing age of populations in both developed and undeveloped countries. This major health problem mandates the development of new cost-effective strategies. Many acute forms of CVD, including acute myocardial infarction (AMI) and stroke, progress to chronic phases, which cause significant impairment in functional capacity and quality of life. Native repair mechanisms are inadequate to effect meaningful tissue repair. Delivery of large numbers of stem cells promises to be a strategy that can potentially drive native repair to achieve clinical recovery of cardiac function. This chapter will review the progress with the use of adipose-derived stem cells (ASCs) as a promising new cell type for all forms of CVD.

Criteria for Cells for Cardiovascular Therapy

The ideal stem cell for cardiovascular cell therapy should meet the following criteria for regenerative medicinal applications [1]:

1. Stem cell can be harvested in large quantities
2. Can be harvested in a minimally invasive fashion
3. Has the potential to differentiate along multiple cell lineages
4. Can be transplanted in an autologous or allogeneic fashion in a safe and effective manner
5. Can be produced in accordance with current Good Manufacturing Practice guidelines

ASCs or adipose-derived regenerative cells (ADRCs) meet all of the above criteria.

Adipose Tissue Depots

Subcutaneous fat harbors a wealth of cells with regenerative capacities, which are readily available. The regenerative cells that can be isolated from subcutaneous fat have been designated adipose-derived stem or stromal cells, ADRCs, adipose-derived adult stem cells, adipose-derived adult stromal cells, adipose-derived stromal cells, adipose mesenchymal stem cells, lipoblasts, pericytes, preadipocytes, vascular stromal fraction, and processed lipoaspirate cells. The International Fat Applied Technology Society (iFATS) has chosen the nomenclature “adipose-derived stem cell (ASC)” to designate these cells to provide uniformity, and this designation will be used throughout the current chapter.

Five different types of adipose tissues can be distinguished on a macroscopic level, each with a distinct function, including white adipose tissue (WAT), brown adipose tissue (BAT), bone marrow-associated fat tissue, mechanical, and mammary adipose tissue [2].

White adipose tissue can be found in subcutaneous and surrounding organs (visceral adipose tissue). The location of WAT determines the specific function and growth properties of the adipose tissue. For instance, adipose tissue surrounding the reproductive organs is able to secrete sex hormones, whereas subcutaneous fat tissue primarily functions as energy storage and structural fat depots, for instance in fat pads on the feet do not secrete any growth factors or cytokines at all. Remarkably, this visceral (white) fat, including omental, retroperitoneal, and mesenteric fat tissue, has been shown to be a risk factor for the development of diabetes and CVD [3].

Brown adipose tissue can be found primarily in supraclavicular and cervical tissue and has the unique ability to generate heat through expression of uncoupling protein-1 (UCP1), which mediates uncoupling of the respiratory chain in brown adipocyte mitochondria. Oxidation of metabolic substrates in these uncoupled mitochondria converts chemical energy into body heat. Activation of the sympathetic nervous system, through for instance exposure to cold or overfeeding, induces the thermogenic properties of BAT. This is accomplished by expression and activation of UCP1, as well as of the individual components involved in the oxidation of metabolic substrates, and components involved in the glucose and lipid uptake from the circulation [4]. BAT is found in children but regresses to the point of only small deposits along the sternum and thymus in adults and thus is not a realistic source of ASCs in adults.

A variant of brown adipocytes is referred to as paucilocular, brite (brown in white), or inducible brown adipocytes [5]. These brite adipocytes are able to differentiate into cells capable of thermogenesis through the expression of UCP1, however do not express the conventional brown-fat specific or myogenic markers [6]. Although white and brown adipocytes originate from the embryonic mesodermal germ layer [4], brown adipocytes develop from the Myf5+ progenitor cell, while Myf5- progenitor cells develop into both white and brite adipocytes [7].

Bone marrow-associated adipose tissue is generally found in long osseal cavities that originally harbored functional bone marrow and functions as energy storage, as well as a source of cytokines involved in hematopoiesis and osteogenesis [2].

Mechanical adipose tissue functions mainly as mechanical support for certain areas of the body, including the retro-orbital cavity of the eye, in palmar fat pads of the hand, and a number of other critical structures [2]. Finally, mammary adipose tissue functions as a source of energy and nutrients specifically during lactation [2].

ASCs harvested from different adipose depots may differ in their capacity for proliferation, apoptosis, and Peroxisome proliferator-activated receptor-γ (PPAR-γ) expression (in direct correlation with adipogenesis). For instance, ASCs isolated from the abdominal subcutaneous space were significantly less susceptible to apoptosis when compared with the other depots, whereas ASCs isolated from subcutaneous fat from the arm displayed higher PPAR-γ expression. In addition, ASCs harvested from younger patients showed a trend toward higher cell proliferation, suggesting that younger patients would be more suitable for ASC harvest and cell therapy than older patients. Since ASCs harvested from the superficial abdominal area appear to be less susceptible to apoptosis, and sufficient amounts of lipoaspirate can be harvested from the periumbilical area, this area is recommended for liposuction and ASC isolation for regenerative therapy [8].

Harvest of Lipoaspirate in Coronary Artery Disease (CAD) Patients

Subcutaneous adipose tissue consists predominantly of adipocytes, but also contains other cell populations generally referred to as the stromal vascular fraction (SVF). This SVF consists of a pleiotropic ASCs, comprised of mesenchymal(-like) stem cells, immune-competent cells, and endothelial progenitor cells, as well as mast cell precursors and hematopoietic precursor cells, as well as differentiated endothelial cells and smooth muscle cells [9-11]. The various subpopulations in the SVF can be characterized and separated by their specific CD cell surface expression: for instance, the mesenchymal-like stem cells express CD13, CD29, CD44, CD73, and CD90, whereas hematopoietic cell subpopulations express CD11, CD14, and CD45. The differentiated endothelial cells express CD31 (Platelet Endothelial Cell Adhesion Molecule-1 (PECAM1)) and CD144, whereas adult smooth muscle cells typically express smooth muscle actin [11-13].

In nearly every patient, sufficient subcutaneous adipose tissue is readily available (for up to 3 L) and accessible by a simple percutaneous liposuction procedure even in cardiovascular-challenged patients [14], rendering it an attractive source of stem cells. One milliliter of lipoaspirate can yield up to 400,000 ASCs, which can be separated from the irrelevant adipocytes by a simple centrifugation within hours, rendering the ad hoc acquisition of a therapeutically relevant number of ASCs feasible [15]. A retrospective survey amongst dermatologic surgeons, conducted between 1994 and 2000, concluded that liposuction is a safe procedure with 66,570 operations performed by 261 dermatologic surgeons with no mortality and a serious adverse event (SAE) rate of only 0.68 per 1000 cases [16]. An additional benefit of ASCs, as compared to bone marrow-derived (mesenchymal) stem cells, is the high density of relevant stem cells per gram of tissue in lipoaspirate, which is approximately 500-fold higher than in freshly aspirated bone marrow [17]. The high yield of stem cells per gram of adipose tissue and the ability to harvest larger volumes of lipoaspirate by a simple intervention eliminates the need for culture expansion in order to generate therapeutic dosages of mesenchymal stem cells. This convenient harvesting and direct isolation of larger numbers of mesenchymal cells also enables the treatment of patients within the first few hours of an acute coronary syndrome at a time point when cardiomyocyte cell death is predominant [13]. Although there is no consensus among key opinion leaders whether (sub)acute or late administration of stem cells yields the best results in ST-elevation myocardial infarction (STEMI) patients, we postulate that stem cells are most needed (and therefore most effective) directly after a myocardial infarction to salvage as much compromised myocardium as possible and to protect the remaining myocardium against inflammation, oxidative stress, and ongoing necrosis. On the other hand, administration of stem cells directly following a myocardial infarction exposes them to a hostile environment of ongoing inflammation, with oxidative stress, hypoxia, and necrosis, which limit the life span of the donor stem cells, thereby limiting the therapeutic effect of these cell transplants. Preclinical studies have indicated that the administration of ASCs directly after an AMI consistently resulted in improved left ventricular contractile function and myocardial perfusion [18-20], suggesting an effect on cardiomyocyte survival, induced neoangiogenesis with improved myocardial perfusion, resulting in improved or preserved cardiac performance [19,21-23]. Clinical studies including the TOPCARE trial [24] (bone marrow-derived or

circulating blood-derived, unfractionated mononuclear cells) and BOOST trial [25] (bone marrow-derived mononuclear cells) administered stem cells only after 4-8 days [18], but also showed significant improvements of global left ventricular ejection fraction (LVEF), by cardiac magnetic resonance imaging (CMR) and LV angiography respectively, despite that the stem cells were administered a few days after the acute phase of myocardial infarction [24,25].

Cell Isolation

The major advantage of ASCs over bone marrow-derived mesenchymal stem cells (BMMSCs) is that ASCs can be isolated from fat tissue in a safe, simple, and expedited way, by simple cell centrifugation into a buoyant fraction (containing the irrelevant adipocytes and lipids) and a nonbuoyant cell pellet (containing the ASCs) within a matter of hours. This circumvents the necessity of the costly and time-consuming cultivation of stem cells in order to obtain a sufficient number of mesenchymal stem cells. ASCs can be isolated from adipose tissue with the help of specialized automated devices that are able to process the lipoaspirate into an injectable cell suspension (Cytori Celutiont system, TGI-1200, Icellator Cell Isolation System). These devices enable the direct isolation of autologous ASCs in a conventional coronary catheterization laboratory without specific logistic or infrastructural requirements and within 1 h after tissue harvesting, paving the way for administration of ASCs within the first hours of myocardial infarction.

During automated or manual processing, the lipoaspirate is enzymatically digested into a single cell suspension using collagenases, after which debris and lipid-laden adipocytes are separated by centrifugation into a nonrelevant buoyant fraction and nonbuoyant fraction, which contains the adipose tissue stromal cells [26-28] (Figure 1).

Culturing and Differentiation of ASCs

By culturing ASCs in Dulbecco's modified Eagle's Medium (DMEM) and supplementing with specific agents, ASCs can be coerced to develop into specific lineages, including adipocytes, osteocytes, chondrocytes, and myocytes as discussed below [26,29].

Myogenesis into Striated and Smooth Myocytes

Myogenic medium, consisting of conventional medium supplemented with hydrocortisone and dexamethasone, can be used to culture ASCs and coerce them to develop into a myocyte phenotype, as suggested by the expression of myosin heavy chain and MyoD1, a muscle-specific transcription factor. Multinucleation can also be found in ASCs that have developed into myocyte morphology [26].

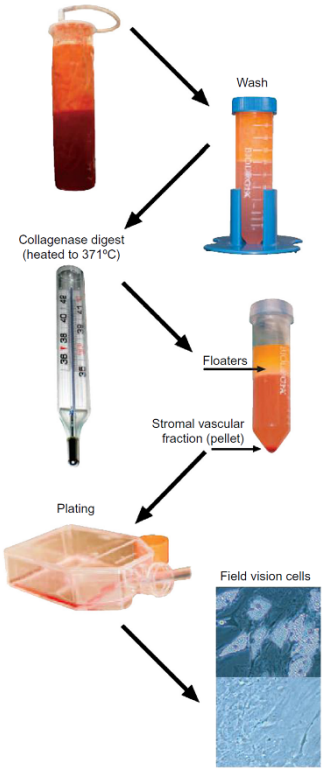

Fig. 1 Processing of lipoaspirate and isolation of adipose derived stem cells. Source: Gimble et al. [2].

Chondrogenesis

ASCs are capable of differentiating into chondrocytes *in vitro* under specific culturing techniques (duplication of cellular condensation via micromass culturing technique) [30], for instance by adding ascorbate-2-phosphate, transforming growth factor (TGF)-β1, and insulin to the medium of adipose-derived cells. Under these culture conditions, chondrogenesis is observed, with the expression of sulfated proteoglycans and expression of collagen type II, a cartilage-specific collagen isoform [26].

Osteogenesis

ASCs cultured in medium supplemented with dexamethasone, ascorbate-2-phosphate, and β-glycerophosphate differentiate into osteocytes and show the osteogenic lineage-specific determinants, including alkaline phosphatase activity and calcification of the extracellular matrix [26].

Adipogenesis

Development of ASCs into adipocytes can be controlled by supplementing the DMEM with a combination of isobutyl-methylxanthine, dexamethasone, indomethacin, and insulin. Lipid accumulation in adipocytes is a lineage-specific determinant for adipocytes and can be visualized by Oil Red O [26].

Immunophenotype of ASCs

ASCs closely resemble BMMSCs in their ability for plastic adherence, multipotency, and immunophenotype [13]. Both ASCs and BMMSCs express CD13, CD29, CD44, CD71, CD90, CD105/SH2, and SH3 surface markers. In addition, neither ASCs nor BMMSCs express the hematopoietic markers CD31 and CD45 [31], whereas the expression of the hematopoietic marker CD34 by ASCs has been questioned [31,32]. Likewise, unequivocal data have been reported regarding the expression on ASCs of Stro-1, a classic BMMSC marker [31,33,34]. ASCs and BMMSCs differ in their specific expression of CD49d by ASCs and CD106 by BMMSCs [31] (Table 1).

To date, no unique marker for ASCs has been identified, but ASCs do express the characteristic markers that comprise the minimal phenotypic criteria for the definition of MSCs as determined by the International Society for Cellular Therapy [35]. Variability in CD expression profile has been noted between donors for CD200, CD201, CD34, and CD36 [36]. The identification of a unique immunophenotype of ASCs may enable the development of more specific mechanisms to capture ASCs from the heterogeneous SVF [37-39].

For now, CD34-coupled magnetic microbeads can be used to (immune)isolate CD34+ cells from the SVF [38]. This immune isolated population consists of both CD34+/CD31+ cells and CD34+/CD31- cells [38]. The CD34+/CD31+ cell population, characteristic for endothelial cells [39], can be depleted again from the CD34+ selected population by a supplemental CD31 immune selection column [38], resulting in a CD34+/CD31- enriched population, primarily containing ASCs, without contamination with endothelial cells [39].

Table 1 Surface marker expression comparing ASCs and BMMSCs

	ASC	BMMSC
CD13	+	+
CD29	+	+
CD44	+	+
CD71	+	+
CD90	+	+
CD105/SH2	+	+
SH3	+	+
CD31	-	-
CD45	-	-
CD34	+/-	-
Stro-1	+/-	+
CD49d	+	-
CD106	-	+

ASC = adipose-derived stem cell, *BMMSC* = bone marrow-derived mesenchymal stem cells

Bone Marrow-derived versus Adipose-derived Mesenchymal (-like) Stem Cells

Several studies suggested that ASCs have a higher tendency to differentiate into adipocytes compared to cultured BMMSCs [40,41], whereas other studies failed to show any difference in adipogenic differentiation capacity [28,42-44].

Also *in vitro* studies regarding capacity to differentiate into osteocytes did not find clear differences between BMMSCs and ASCs [43,44]. The osteogenic capacity of ASCs appeared to be influenced by gender differences. *In vitro* studies suggested that males tend to differentiate ASCs more rapidly and effectively than females. Moreover, the osteogenic differentiation potential of female ASCs decreases with age, whereas the adipogenic potential remains unchanged [45,46]. A comparative study of the chondrogenic differentiation capacity of BMMSCs versus ASCs showed conflicting results: whereas some studies show a lower capacity of ASCs to differentiate into chondrocytes *in vitro* [47-49], other studies report no differences in chondrogenic potential [42]. The potential lower capacity to differentiate into chondrocytes could be corrected by coculture with particular growth factors, including insulin-like-growth factor 1, TGF-β2, and bone morphogenetic protein 6 [50,51].

Taken together, there appears to be no clear differences between the differentiation capacity of MSCs derived from bone marrow or adipose tissue. However, the suboptimal culture conditions may have affected the differentiation potential of BMMSCs and ASCs in these studies, whereas potential heterogeneity of MSC populations or heterogeneity in the MSC preparations may also have resulted in differences in differentiation capacity, due to the presence of distinct subpopulations [52-55].

Working Mechanism of ASCs in CVD

Modulation of Cardiomyocyte Survival and Local Immune Response by the Paracrine Action of ASCs

Myocardial infarction generally results in a chronically scarred myocardium since the current standard of care therapy, reperfusion by primary percutaneous coronary intervention (PCI), can only rarely completely prevent myocardial damage [13]. A number of mechanisms have been proposed through which ASCs can mediate myocardial “regeneration” and induce neoangiogenesis of the ischemic heart muscle [2].

ASCs have been shown to secrete multitude of growth factors and cytokines *in vitro* and *in vivo* that stimulate recovery of injured tissue and to protect the heart muscle from additional damage much alike (bone marrow-derived) mesenchymal stem cells, including hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), fibroblast growth factor 2, TGF-β [56], but also adipokines [57,58]. HGF is able to promote angiogenesis much alike VEGF. When HGF and VEGF are combined however, their angiogenic potential is amplified [59].

In addition, HGF has been shown to reduce cardiomyocyte apoptosis and to preserve LV geometry and contractile function of the heart after myocardial infarction [60]. In addition, the release of not only anti-inflammatory cytokines (TGF-β, interleukin 10), antioxidative proteins, free radical

scavengers, but also the expression of survival factors and heat shock proteins by ASCs may dampen the local immune response and render the local cardiomyocytes more resistant to loss by hypoxia and oxidative stress, thereby promoting myocardial salvage in the acute phase of myocardial infarction [61].

TGF-β expression is significantly upregulated in pressure-overloaded hearts [62] and in the postmyocardial heart [63,64]. Sustained upregulation of TGF-β in these settings eventually leads to a negative effect on LV dilatation and dysfunction, but expression of TGF-β in the early phase of myocardial infarction protects the heart against myocardial dysfunction [65].

ASC Differentiation along Specific Lineage

ASCs have shown the potential to differentiate into cardiomyocytes in cell culture. Planat-Bénard et al. demonstrated that ASCs spontaneously differentiated into cells with morphological, molecular, and functional properties of cardiomyocytes. The isolated ASC is a heterogeneous cell population which consists of differentiated vascular cells (including endothelial cells, vascular smooth muscle cells, circulating blood cells) and a fibroblast-like cell population, described as a multipotential stem cell population for various mesodermal lineages. After 6 days of cell culture, clusters of preadipocytes and adipocytes, large spreading cells with a highly branched shape and fibroblast-like appearance, and clones of rounded cells together with small tube cells appeared. Some of the cultured cells independently started to show contractile activity at days 11-14 of culture reminiscent of cardiomyocytes. Furthermore, myotube-like structures appeared, grew in size and proliferated, and were still surrounded by some of the rounded cells. After 20-30 days of culture, the areas gave rise to a cohesive group of cells, with the presence of branching fibers and sharing tight connections, which were beating at a single rate, suggestive of clusters of cardiomyocytes.

These differentiated adipose-derived cells displayed molecular and functional characteristics of cardiomyocytes and expressed cardiomyocyte-specific transcripts including GATA-4 and Nkx2.5, the ventricular and atrial myosin light chain (MLC)-2v and MLC-2a, respectively, and atrial natriuretic peptide. The adipose-derived cells additionally express myosin-enhancing factor 2C, sarcomeric-actinin, and connexins as determined by immunocytochemistry. Finally, using patch clamp analysis showed that these adipose-derived cardiomyocytes displayed spontaneous, as well as triggered, action potentials [66].

In addition, the ASCs were shown to have a significant proangiogenic potential by secretion of a multitude of proangiogenic growth factors and cytokines, as well by direct differentiation into endothelial cells, which incorporated into functional vessels in a hind limb ischemia model in nude mice, but also develop into vessel-like structures in subcutaneous implanted Matrigel plugs. *In vitro*, these ASCs represent a homogeneous population of CD34+/CD13+ cells, which can spontaneously express the endothelial cell markers CD31 (PECAM1) and von Willebrand factor, when cultured in semisolid medium. Interestingly, mature human adipocytes, as well, have the potential to dedifferentiate and rapidly acquire the endothelial phenotype *in vitro*. *In vivo*, these cells are able to promote neovascularization in ischemic tissue, and form vessel-like structure in subcutaneous Matrigel plug model, suggesting that cells of endothelial and adipocyte phenotypes may have a common precursor [67].

Further evidence of differentiation of ASCs toward cardiac cells include the study by Traktuev et al. which described the spatial relationship between endothelial cells and ASCs in adipose tissue sections. Double-labeling studies showed that CD31+ endothelial cells formed the microvasculature, whereas CD140b+ ASCs were mainly positioned in the perivascular space
(secondary stem cell niche) [68]. Traktuev et al. also studied the relation between ASC-endothelial cell double cultures in Matrigel in more detail. Monocultures of human microvascular endothelial cells (HMVECs) under serum-free conditions resulted in transient neocapillary network that deteriorated after 24 h. Cocultures of HMVECs with ASCs resulted in a stabilized neocapillary network for up to 5 days of cell culture. Fluorescently labeled HMVECs formed the lumen of the capillary structures whereas the ASCs were located perivascular in the Matrigel 2D cultures. In nude mice, subcutaneous implantation of collagen/fibronectin plugs containing human ASCs (hASCs) and endothelial cells resulted in vascular structures with human endothelial cells and perivascular layers of ASCs. Taken together, these studies suggest a possible tertiary stem cell niche of ASC [68].

In addition to the paracrine function of ASCs, administered ASCs may recruit resident stem cells to the site of injury and promote their (trans)differentiation into relevant cardiovascular lineages in the recipient. Alternatively, ASCs are able to differentiate into specific cardiovascular lineages including endothelial cells and vascular pericytes, as well as into cardiomyocytes in cell culture and *in vivo* animal models. However, the frequency of transdifferentiation of ASCs into contractile cardiomyocytes appears to be an infrequent phenomenon in the animal models [38,68-73]. Alternatively, cell culture studies by March and colleagues suggested that ASC-EC 2D Matrigel double cultures were stabilized, suggesting a role for lipoaspirate ASCs as a secondary vascular niche to promote vascular stabilization rather than differentiation into endothelial cells [68].

In this way, ASCs contribute to the regeneration of the heart by preventing cardiomyocyte loss and promoting local neoangiogenesis and myocardial perfusion in the process of myocardial infarction or congestive ischemic heart failure and postmyocardial infarction adverse remodeling.

In vitro, ASCs are also able to differentiate into contractile cardiomyocytes by coincubation with 5-azacytidine, a methylation-inhibiting agent [74]. For instance, Rangappa and coworkers showed that after treatment with 5-azacytidine, cultured rabbit ASCs changed their morphology from fibroblast-like into a cardiomyocyte morphology, as suggested by multinucleation, extended cytoplasmic processes, spontaneous beating of cells after 3 weeks of culture and expression of α-actinin, myosin heavy chain, and troponin-I, specific for cardiomyocytes [70]. In contrast, in studies of Choi and coworkers, coincubation of hASCs with 5-azacytidine failed to induce expression of the cardiac markers, actin and troponin-T, with only expression of both Nkx2.5 and α-actin or Nkx2.5 and troponin-I in a minority of the cultured cells, whereas no contractile cells were observed [71].

Trichostatin A (TSA), a histone deacetylase inhibitor [71], has also successfully been used to drive the differentiation of cultured embryonic stem cells and cardiac side population cells into the cardiomyocyte lineage [75-77]. Choi and coworkers found that culturing ASCs in special cardiomyocyte culture medium, supplemented with TSA, resulted in cardiac myosin heavy chain, α-actin, and troponin-I protein expression [71]. Moreover, TGF-β1 was shown to promote cardiomyocyte-specific expression of troponin-I, cardiac myosin heavy chain, and α-sarcomeric actin in cultured rat ASCs [72].

Alternatively, coculture and direct contact with rat cardiomyocytes and hASCs induced spontaneous beating in synchronism with the rat cardiomyocytes after a week, concomitant to expression of Nkx2.5, GATA-4, troponin-I, α-actin, and cardiac myosin heavy chain in these cocultured ASCs [71]. Contact, but also direct exposure, of ASCs to cardiac extracellular matrix, that is upregulated after myocardial infarction, has also been shown to promote differentiation into the cardiomyocyte phenotype [78], including laminin and fibronectin. Laminin is an extracellular matrix protein that is upregulated in the early phase of myocardial infarction [79], whereas fibronectin is expressed after myocardial infarction [80] in both the infarcted area and the border zone, but not in the noninfarcted area [81]. *In vitro* exposure of hASCs to laminin-coated wells resulted in a high percentage of ASCs differentiating into cardiomyocytes, whereas ASCs seeded in fibronectin-coated wells failed to express cardiomyocyte protein markers [78].

In vivo, attachment and proliferation of ASCs in the infarcted heart was improved by fibronectin expression. Since fibronectin levels are significantly upregulated 12 h after myocardial infarction, ASC therapy may be most effective after this point in time of myocardial infarction [81].

Remarkably, hASCs cultured in VEGF-conditioned medium, without supplement of other cytokines or agents, were able to spontaneously differentiate and express cardiomyocyte markers, whereas anti-VEGF antibodies abrogated the cardiac troponin T expression in the ASCs, suggestive that increased VEGF levels were likely to be responsible for the differentiation of ASCs into the cardiomyogenic phenotype [82].

Planat-Bénard and coworkers also described the spontaneous differentiation of ASCs into the cardiomyocyte phenotype in culture without addition of cytokines or 5-azacytidine to the culture medium; however, beating cells were infrequently observed after 3 weeks of culturing (0.02-0.07%, depending on the source of the adipose tissue). Although the studies of Song et al. and Planat-Bénard show the inherent capacity of ASCs to cardiomyocyte differentiation, the *in vivo* relevance for the therapeutic effect appears to be limited since *in vivo* differentiation into functional contractile cardiomyocytes is rarely observed [66,82].

Other Sources of Stem Cells

In the past, skeletal myoblasts have been used to repair damaged myocardium. These cells have been shown to be able to improve New York Heart Association (NYHA) class, walking distance, and LV function [83-85]. Unfortunately, the use of skeletal myoblasts as a therapeutic agent has been associated with ventricular tachyarrhythmias [85-88], which makes them less suitable as donor cells.

Embryonic stem cells have also been advocated as a potential candidate for cardiovascular cell therapy, although the use of human embryonic stem cells is controversial because of ethical issues and the possibility of teratoma formation [89].

BMMSCs have also been extensively assessed as a potential therapeutic agent for patients with CVD. BMMSCs are similar to ASCs in terms of immunophenotyped [31] and gene expression profiles [90]. In contrast, harvesting of MSCs from bone marrow yields markedly lower number of mesenchymal stem cells as compared to harvesting of ASCs from adipose tissue. This renders these autologous cells less suitable for administration in the acute setting of CVD.

Potential Use of ASCs in the Treatment of Degenerative Disease

ASCs are under evaluation for the treatment of a wide variety of diseases including not only systemic sclerosis, osteoarthritis, partial mastectomy defects after breast cancer, facial lipoatrophy, and urinary incontinence, but also for the use in craniofacial reconstruction, autoimmune disease, and nerve repair.

The safety and feasibility of ASC therapy for myocardial infarction and congestive heart failure will be reviewed here. The advantage of ASCs over other stem cells like BMMSCs is that lipoaspirate can be easily and safely harvested in large quantities and can be isolated from fat tissue by a simple digestion and centrifugation step within hours, rather than by subculture in a Good Manufacturing Practice (GMP)-level clean room facility (which requires months of labor-intensive subculturing). Therefore, ASCs are an ideal source for autologous stem cells for regenerative therapy in the acute phase of a myocardial infarction. The working mechanisms of ASCs will be reviewed here based on the preclinical and clinical trials to date.

Preclinical Studies in Animal Models of AMI

Multiple studies have assessed the beneficial effects of autologous ASCs in the treatment of AMI in various rodent and large animal models. To date, approximately 80 papers have been published describing the effects of ASCs on cardiac disease in animal models, the majority of which involved ASC therapy in myocardial infarction models. A significantly smaller amount of papers was published on the subject of autologous ASC therapy in congestive heart failure animal models, and will be discussed below.

ASC Therapy in AMI Animal Models

Cardiovascular therapies, such as PCI, implantable defibrillators, and pharmacotherapeutics, do not restore or regenerate damaged myocardial tissue actively, post-AMI. To retain cardiac function and prevent heart failure development, stem cell therapy was hypothesized to be a solution. In 2002, Zuk and coworkers demonstrated that ASCs are another interesting source for post-AMI stem cell therapy [31].

Different studies using ASC as therapy in different animal models post-AMI were performed during the last 13 years. The focus in these studies is on the important features of ASC therapy post-AMI and could be important for successful clinical application.

The effect of ASC therapy post-AMI has been studied extensively in immune-deficient and immune-competent animals. Intramuscular injection of allogeneic or xenogeneic ASCs was the preferred method of administration in immune-deficient rodents with cell dose ranging from 0.1×10^6 to 3.0×10^6 ASCs and delivery time point ranging from ,1 h to 7 days post-AMI. Treatment of AMI with hASCs in immune-deficient models showed a significant reduction in infarct size and a significant improvement in LVEF as determined by echocardiography.

Histopathological analysis indeed confirmed that the number of neocapillaries in the infarcted area was significantly enhanced after ASC treatment [21,22,91-97].

The microenvironment of the infarcted myocardium in immune-deficient mice is however

different from immune-competent animals (and humans). In the "severe combined immunodeficient" mice, T- and B-cells are absent, whereas in athymic rats T-cells are depleted [98,99]. These T-cells have multiple functions. They play a crucial role in the healing process post-AMI, by removing damaged and necrotic cells, but they also play a role in removing foreign cells, such as the administered ASC. In immune-deficient animals, ASCs have a greater ability to survive, which could influence the results and cause an overestimation of their therapeutic benefit.

Immune-competent AMI models were overall less positive in outcome after ASC treatment. Almost all immune-competent rodent AMI models used intramuscular injection as delivery method of ASC, with only a small fraction using intravenous administration. Time point of administration ranged from ,1 h after infarct induction to 8 days afterward with cell dose ranging from 0.4 x 10^6 to 100 x 10^6 ASCs, with most studies using a dose between 1.0 x 10^6 and 5.0 x 10^6 ASCs.

Although a number of immune-competent small animal studies demonstrated a significant reduction in infarct size [100-106], this was not a consistent finding in other studies [23,105,107-114] Furthermore, LVEF was found to be significantly improved in some immune-competent small animal studies [23,100-104,106-108,115-118], but not in others [105,109,111-113,119,120], which may be related to the variability in the composition of the pleiotropic ASC. Finally, capillary density was investigated in the infarcted zone after administration of ASC in immune-competent rodents. These results were more ambivalent in immune-competent animals than in studies using immune-deficient animals, which mainly showed an increase in the number of blood vessels [23,101-107,110,111,115-118,120].

For instance, intramyocardial injection of cultured, hASCs in a rodent model of AMI in athymic nude rats using permanent Left Anterior Descending (LAD) coronary artery ligation showed at 4-weeks follow-up (FU), a significant improvement of global contractile function (LVEF and LV fractional shortening (LVFS)) and both left ventricular end-diastolic volume (LVEDV) and left ventricular end-systolic volume (LVESV) by echocardiographic analysis, suggesting a persistent, long-term benefit on LV remodeling and the development of post-AMI heart failure.

Histomorphological analysis showed that cultured hASCs resulted in an attenuation of infarct size (26 ± 6% (hASC) vs 34 ± 6% (saline control)) and a higher density of (neo)arterioles in the infarct border zone (60 ± 9 (hASC) and 37 ± 2 (saline control) SMA+ cells per mm^2) compared with control animals. Viable hASCs were detected in the infarct border zone in 3 out of 10 rats at 4-weeks FU suggesting long-term cell engraftment; however, differentiation of hASCs into cardiomyocytes was not observed, as determined by α-sarcomeric actin immunohistological analysis. This study shows that cultured hASCs in the acute phase of a myocardial infarction rat model bring about a persistent improvement of cardiac function and reduced adverse remodeling on long term. Since cardiac differentiation of hASCs could not be detected, it was postulated that the ASC paracrine functions are most likely responsible for these beneficial effects [22].

Beside the small animal studies, large animal studies were performed to study the effect of ASC therapy in AMI. The physiology and morphology of ovine and porcine hearts are more comparable to the human condition in terms of heart rate, oxygen consumption, and contractility. The animal studies were all performed in immune-competent animals, with some studies comparing multiple delivery methods [121]. Both intramyocardial injection and intracoronary delivery of ASCs were used and the

time point of administration ranged from ,1 h after infarct induction to 14 days post-AMI, with cell dose ranging from 2.0 x 10^6 to 213 x 10^6 ASCs.

After ASC therapy, a significant decrease in infarct size was observed in large animal studies [19,122-124], while only in a few studies no statistically significant reduction was observed [18,121]. In addition, global LVEF was increased in most large animal AMI models [18,19,123,124], whereas some failed to show a statistically significant improvement [121,122]. Capillary density in the border and infarct zone was stimulated in most large animal AMI studies [18,19,121-124].

Although several studies have shown that hASCs are able to differentiate into cardiomyocytes and endothelial cells *in vitro* [21,22,97], no apparent transdifferentiation of ASCs into new cardiomyocytes were observed in the large animal models. The results of immune-competent large animal studies are consistent with the results of the immune-competent rodent AMI models.

For instance, Alt and coworkers assessed the effect of freshly isolated, autologous, adipose-derived stromal cells on AMI outcome in 16 pigs using a transient LAD balloon inflation for 3 h. ASCs were transferred via intracoronary infusion through the angioplasty balloon during LAD occlusion. At 8-weeks FU, LVEF was significantly improved in the ASC-treated group (43 ± 7% (treated) vs 35 ± 3% (control)), whereas myocardial perfusion by quantitative Single Photon Emission Computed Tomography (SPECT) analysis was enhanced in the LAD perfusion myocardial segments (perfusion defect 35 ± 9% (treated) vs 44 ± 5% (control)). Histomorphological analysis corroborated these findings with increased wall thickness of the scar area and enhanced capillary density in the border zone of cell-treated animals [19].

Delayed ASC therapy in an AMI reperfusion model was assessed by Mazo and coworkers in the Goettingen minipig (n = 20). At 9 days post-AMI, animals were treated with intramyocardial injected ASC or culture medium. ASC-treated animals showed improvement in LVEF from 46.69 ± 5.62% at day 9 post-AMI to 64.73 ± 5.14% at 3-months FU, with improvement of both LVESV (46.44 ± 8.03 mL at day 9 post-AMI to 25.58 ± 4.76 mL at 3-months FU) and LVEDV (87.06 ± 10.34 mL at day 9 post-AMI to 64.67 ± 4.64 mL at 3-months FU) indicative for prevention of progression into heart failure, as opposed to placebo treatment. In parallel, myocardial fibrosis formation was attenuated with improved myocardial capillary density in the infarct area in the ASC group, which may explain the improved cardiac performance and reduced late cardiac remodeling [124].

ASC Therapy in Animal Models of Congestive Heart Failure

Initially, studies have focused on the use of ASC in the treatment of AMI. These studies already established the long-term benefit of acute and subacute therapy of ASC on long-term cardiac contractile function and adverse remodeling with prevention of post-AMI heart failure with reduced infarct size, reduced fibrosis formation, attenuation of ongoing cardiomyocyte apoptosis, and improved myocardial perfusion due to neoangiogenesis.

More recently, studies assessed the effect of autologous and allogeneic ASC in both ischemic [73,125,126] and nonischemic [127-129] heart failure models. The majority of these studies have been conducted using rodent models [73,125,127,128] and only a few in large animals [126,129]. In ischemic heart failure models, ASCs were transferred by intramyocardial injections or as an epicardial scaffold applied at 7 days to 4 weeks post-AMI in order to prevent progression of heart failure, with

dose ranging from 1 x 10^6 to 1 x 10^7 ASCs [73,125,126]. In nonischemic heart failure models, ASC therapy was applied by epicardial or intravenous injection, or retrograde coronary sinus injection with dose ranging from 3 to 10 x 10^6 ASCs [127-129]. LVEF improved significantly after ASC administration in the ischemic heart failure models [73,125-129]. Significant attenuation of left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) dimensions, indicative of adverse remodeling and progression of heart failure, has been reported [127,128], whereas local LV wall thickening in the infarcted area was observed in one study [73,125]. Effects of ASC therapy on the extent of myocardial fibrosis were observed, as well as a reduction on brain natriuretic peptide (BNP) levels [125,127,128]. Histologically, a significant increase in capillary density was observed all ischemic models, while this was not investigated in nonischemic models [73,125,126]. Remarkably, ASC transdifferentiation into cardiomyogenic lineage have been reported [73,128], while other studies failed to detect significant transdifferentiation [125-127]. These findings suggest that ASC therapy could prove to be an effective treatment for patients with both ischemic and non-ischemic heart failure. While effects of ASC therapy in ischemic heart failure could possibly be linked to neovascularization, the effects of ASCs on cardiac dimensions and hypertrophy in nonischemic heart failure remain unclear.

For instance, in a murine dilating (nonischemic) cardiomyopathy model in conditional serum response factor knockout mice, Hamdi et al. assessed the effect of cultured allogeneic mouse ASC monolayers, applied as epicardial scaffolds. At 3 weeks after ASC scaffold therapy, histological analysis showed migration of some donor ASCs into the myocardium. Expression of cardiomyocyte-specific markers however could not be detected. Although LVFS was significantly declined in the sham-operated group due to developing heart failure, LV function was preserved in the ASC-treated group correlating with attenuated LV remodeling, as suggested by preserved LVESD and LVEDD at 3-months FU. These results suggest that ASCs may also play a role in the prevention of nonischemic dilating cardiomyopathy (irrespective of the proangiogenic benefit of ASC). Only a limited number of heart failure animal studies have been conducted and more studies should be performed to determine if there is a place for ASC therapy in the treatment of patients with heart failure [127].

The Use of Autologous ASCs in the Treatment of Patients with CVD

To date, only a limited number of clinical trials have assessed the effect of ASCs on CVD.

The APOLLO trial was the first-in-man experience with the use of ASCs in the treatment of 14 patients with a STEMI by intracoronary infusion of these autologous ASCs. Patients were initially treated with a primary PCI and drug-eluting stent placement, after which they were treated within 24 h with an intracoronary infusion of incremental doses of autologous ASCs or placebo control (3:1 randomized) to assess the safety and feasibility of ASCs in STEMI AMI. Within hours after the AMI and primary PCI, patients were subjected to a liposuction procedure to harvest 200 cc of periumbilical fat tissue for the isolation of 20 million ASCs. Remarkably, the liposuction procedure was very well tolerated in the acute phase of AMI with no apparent hemodynamic consequences. Subsequent intracoronary infusion in the culprit coronary artery was performed within 24 h after the primary PCI. During the intracoronary infusion of the adipose-derived mesenchymal stem cells, no apparent microvascular obstruction was observed as assessed by Thrombolysis in Myocardial Infarction (TIMI)

flow analysis pre-, during, and postinfusion, and by analysis of coronary flow reserve under adenosine hyperemia. Therefore, liposuction and intracoronary infusion of adipose-derived mesenchymal-like stem cells appeared to be safe and feasible during the acute phase of a myocardial infarction (Figure 2).

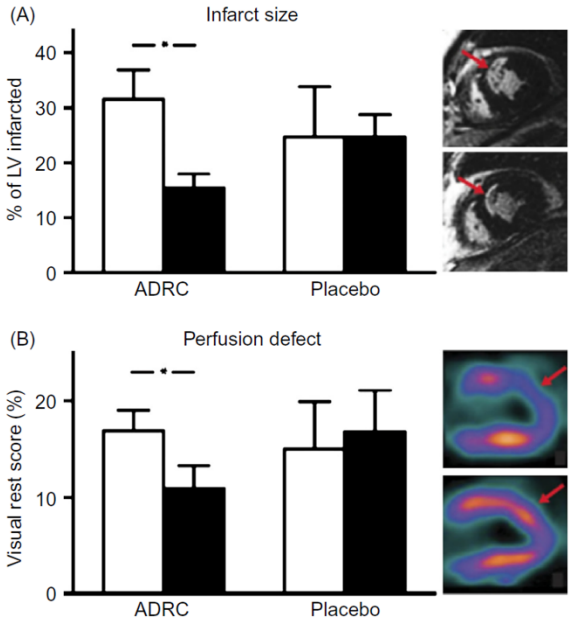

Fig. 2 Infarct size and perfusion defect in APOLLO patients. **A**: The percentage of the left ventricle (LV) infarcted improved significantly in adipose-derived regenerative cell (ADRC)-treated patients from baseline (open column) to the 6-months follow up (FU) time point (solid column; example to the right, red arrows depict infarct at baseline (upper right panel) and 6-months FU (lower right panel)) improvement in placebo patients. **B**: A significant improvement of the perfusion defect was seen in ADRC-treated patients (example to the right, red arrows) from baseline (open column) to the 6-months FU time point (solid column) compared with a deterioration in placebo patients. Source: Houtgraaf et al. [130].

Consistent to the preclinical animal studies, ASC treatment significantly reduced infarct size by 51.3% at 6-months FU, and 38.2% at 18-months FU. In addition, autologous ASC treatment markedly reduced the perfusion defect by 269% and 188% at respectively 6- and 18-months FU, as analyzed by MIBI SPECT. More specifically the perfusion defect in the LAD territory was reduced by 867% and 800% at 6- and 18-months FU. Reduced infarct size and improved myocardial perfusion resulted in improved global myocardial contractile function by 5.7%, and reduced adverse cardiac remodeling at long-term FU. Both LVESD and LVEDD were improved at 6-, 18-, and 36-months FU, suggestive of prevention into post-AMI heart failure as seen in their placebo-treated counterparts. Placebo-treated patients showed progression into heart failure with a 43% increase of LVEDV at 6-months FU as compared to 25% in the treatment group. LVESV increased 35% in placebo patients versus 15% in the ASC-treated patients. In this small first-in-man study, no significant differences were observed in major adverse cardiac and cerebrovascular events (MACCE; defined as incidence of cardiac death, target site

revascularization, reinfarction, and/or stroke), SAEs, or changes in BNP between the ASC-treated group and the placebo group. Remarkably, reduced infarct size, improved myocardial perfusion, and reduced long-term adverse remodeling and developing heart failure resulted in a marked reduction of ventricular (nonsustained) ventricular arrhythmias as well as ventricular ectopy (as a manifestation of post-AMI cardiomyopathy) in the ASC-treated patients. Control patients experienced an average of 2.8 episodes of nonsustained ventricular tachycardia, compared to only 0.6 episodes on average in ASC-treated patients [130,131].

Given the encouraging clinical and functional effects, a phase IIb trail was initiated. The ADVANCE was initiated as a multicentered, randomized placebo-controlled trial to study the efficacy and safety of 20 million ASCs in the treatment of 275 patients with an anterior STEMI by intracoronary infusion within 24 h after the primary PCI. The study was terminated prematurely, due to logistical and economic reasons after inclusion of 27 patients. The FU and analysis of these patients are currently pending, but initial analysis suggested that intracoronary infusion of these mesenchymal-like stem cells was safe, feasible, and did not lead to any adverse events, including no reduction of coronary flow in the epicardial vessels reminiscent of microvascular flow obstruction.

The potential benefit of autologous ASCs was then examined for the treatment of heart failure in the PRECISE trial, the effect of intramyocardial injections of autologous ASC was assessed in a prospective, randomized, placebo-controlled, double-blinded phase 1 study, in 27 patients with advanced ischemic cardiomyopathy. Patients were randomly assigned in a 3:1 fashion to several doses of ASCs or placebo treatment (doses 0.4 x 10^6 ADRC/kg (n = 9), 0.8 x 10^6 ADRC/kg (n = 9), and 1.2 x 106 ADRC/kg (n = 3) vs placebo-controlled patients (n = 6)). The baseline LVEF in this patient population was 34.2 ± 9.5% in the control group and 36.7 ± 7.5% in the ASC-treatment group (average age 63.6 ± 7.5 years, 78% male patients). A total of 400-500 cc of autologous lipoaspirate was harvested from the periumbilical subcutaneous area, without any apparent complications in this fragile end-stage heart failure population. Autologous ASCs were delivered by targeted percutaneous endoventricular injections using electromechanical mapping (J&J MyoStar catheter delivery system). No sustained ventricular
tachyarrhythmias were detected during the procedure and clinical FU using extensive telemetric and Holter screening.

Echocardiographic and CMR analysis did not show any changes in global or regional LV contractility, nor any change in cardiac dimensions at 6-months FU or long-term FU. However, at 6-months FU, CMR analysis did show a significant increase in LV mass in the ASC treatment patients (however, this could not be confirmed at 18-months FU), whereas wall motion score index (WMSI) [132] was improved in patients treated with ASCs, but not in the placebo group at 6-months FU. Interestingly, myocardial oxygen consumption (mVO_2) level was also preserved in the ASC treatment group, whereas in the placebo group mVO_2 progressively regressed at 6 and 18-months FU. Both the treatment group and the placebo group showed a modest improvement in NYHA heart failure classification, as well as the Canadian Cardiovascular Society (CCS) angina pectoris classification. It has to be noted that patients in the control group were significantly younger than patients in the treatment group (55.7 ± 6.1 years vs 65.8 ± 6.3 years, respectively) (Figures 3 and 4).

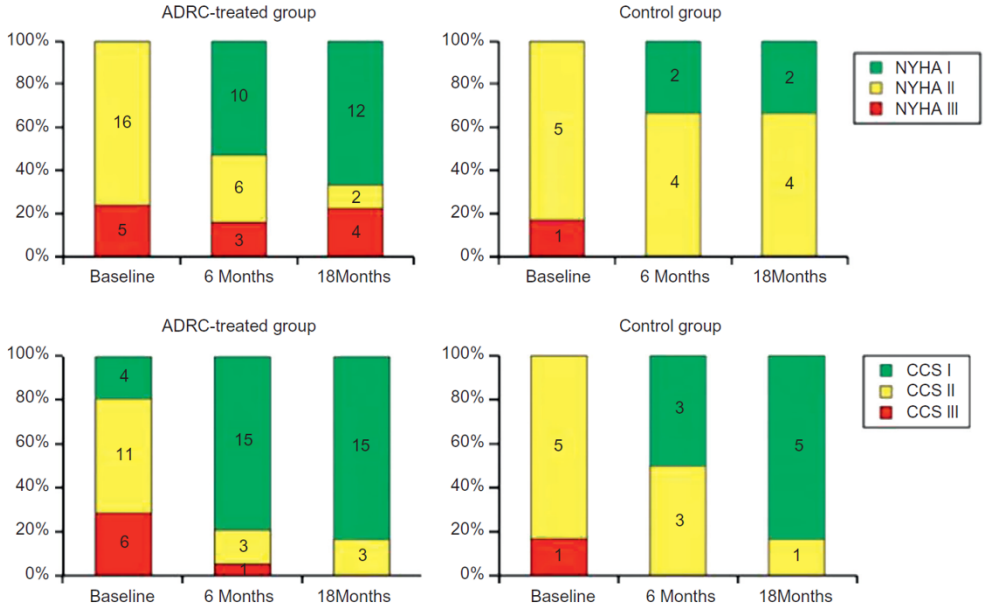

Fig. 3 NYHA functional and CCS angina classification in control and ADRC-treated patients at baseline and 6 and 18 months after transendocardial injections. P = not significant. *ADRC* = adipose-derived regenerative cell, *CCS* = Canadian Cardiovascular Society angina classification, *NYHA* = New York Heart Association functional class. Source: Perin et al. [133].

The PRECISE trial provided the first evidence that the harvesting and transendocardial injection of ASCs is safe and feasible in patients with advanced ischemic cardiomyopathy, and result in preserved maximal oxygen consumption, with improved WMSI [133].

In the phase 1 ANGEL trial, the safety and efficacy of intramyocardial injections of autologous ASCs were also assessed in 5 patients with advanced congestive heart failure (using the BioHeart MyoCath catheter). Preliminary results at 3-months FU showed an average improvement in 6-min walk test of 47 m, whereas the majority of patients walked 65 m further as compared to their baseline. Furthermore, an average improvement of 13 points in the Minnesota Living with Heart Failure questionnaire was reported by patients, with 80% of the patients reporting a clinically significant improvement of more than 5 points [134].

The ATHENA I-II trials are set up as prospective, randomized, placebo-controlled, double-blind phase 2 trials to test the safety and feasibility of two doses of ASCs in the treatment of chronic myocardial ischemia not amenable to conventional percutaneous or surgical revascularization (0.4 x 10^6 cells/kg body weight (ATHENA I) and 0.8 x 10^6 cells/kg body weight (ATHENA II, not to exceed respectively 40 and 80 x 10^6 cells). At the moment of writing, this study is still ongoing and no results have been published as of yet. Feasibility endpoints include the change in mVO_2, cardiac dimensions, LVEF, and perfusion defect at 6-months FU. At 12-months FU, the change in heart failure symptoms, angina symptoms, and quality of life will be assessed. Safety endpoints include the frequency of ventricular arrhythmia, SAEs, and MACCE (defined as cardiac death and hospitalization due to heart failure). The expected completion date of the studies is estimated at May 2019 [135,136].

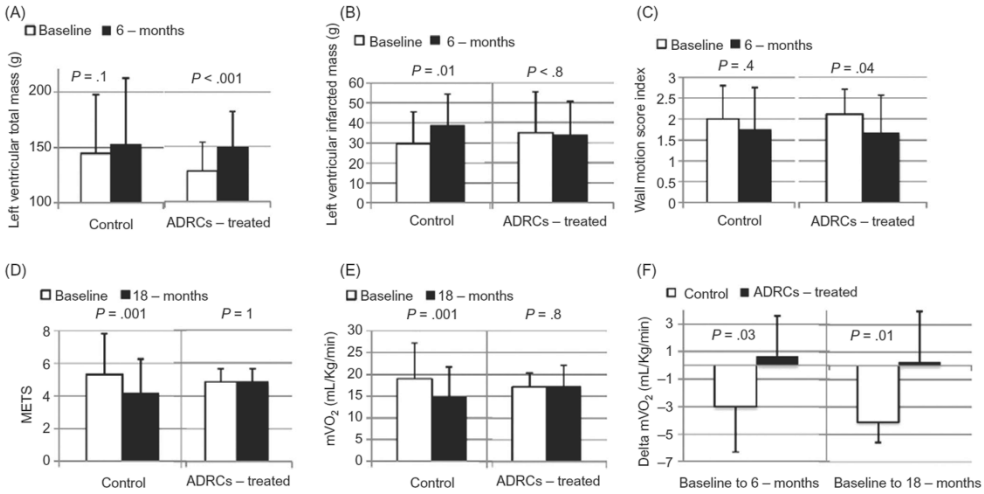

Fig. 4 A-C: Evolution of CMR parameters from baseline to 6-months FU in ADRC-treated and control patients: **A**: left ventricular total mass, **B**: left ventricular necrotic mass, and **C**: WMSI. **D-F**: Treadmill exercise results in control and ADRC-treated patients at baseline, 6, and 18 months: **D**: METS, **E**: mVO_2, and **F**: Δ values of mVO_2. *ADRC* = adipose-derived regenerative cell, *METS* = metabolic equivalents, mVO_2 = myocardial volume oxygen (consumption). Source: Perin et al. [133].

The initial positive results of the APOLLO and PRECISE trials show that ASC harvesting is safe and feasible, and is well tolerated even in patient with AMI and end-stage heart failure patients. No apparent ventricular tachyarrhythmias were linked to ASC treatment in the arrhythmia prone heart failure patients (NYHA class III-IV), whereas in the APOLLO trial even a marked reduction was observed in arrhythmic activity in patients treated with ASCs. Both the PRECISE and APOLLO trials showed beneficial effects of ASC treatment on patients with either an AMI or ischemic cardiomyopathy. Larger clinical trials are however warranted in order to verify the therapeutic value of ASCs in the field of CVD. As liposuction appears to be well tolerated in both the PRECISE and the APOLLO trials, fat harvesting and ASC isolation appears to be ideal to obtain therapeutically relevant number of autologous mesenchymal stem cells within matter of hours. This could prove to be very useful in the acute setting of myocardial infarction. More important, autologous stem cell treatment rules out the possibility of any adverse (allo)immune response toward the administered cells [20].

Conclusions and Future Perspectives

A large body of evidence in small and large animal models for cardiovascular ischemic and nonischemic heart disease has established the effectiveness of ASCs as a therapeutic option, and here we have reviewed part of these findings. Consistent results from both preclinical studies and clinical trials point toward the beneficial effects of treatment with ASCs, including the reduction of infarct (scar) formation, modulation of local inflammatory response and oxidative stress, and promotion of local neoangiogenesis, manifested in improved myocardial perfusion (by SPECT and Coronary Flow

Reserve (CFR)). Protection against ischemic damage and improved myocardial perfusion result in preserved myocardial contractile function, and inhibition of long-term adverse cardiac remodeling and progression into heart failure, with markedly less concomitant ventricular tachyarrhythmias and long-term improvement of overall cardiac performance (icc mVO_2 levels). Perhaps the most important advantage of ASC therapy is that these ASCs can be generated safely, and within a matter of hours and in sufficiently large numbers, which renders ASCs attractive candidates for autologous stem cell transplantation in the acute setting of disease (here, myocardial infarction) or in the setting in which GMP clean room facilities for subculture and preparation of MSC suspensions are not immediately available or warranted. If a decision toward autologous mesenchymal stem cell therapy is made, ASCs would be the first choice of cell.

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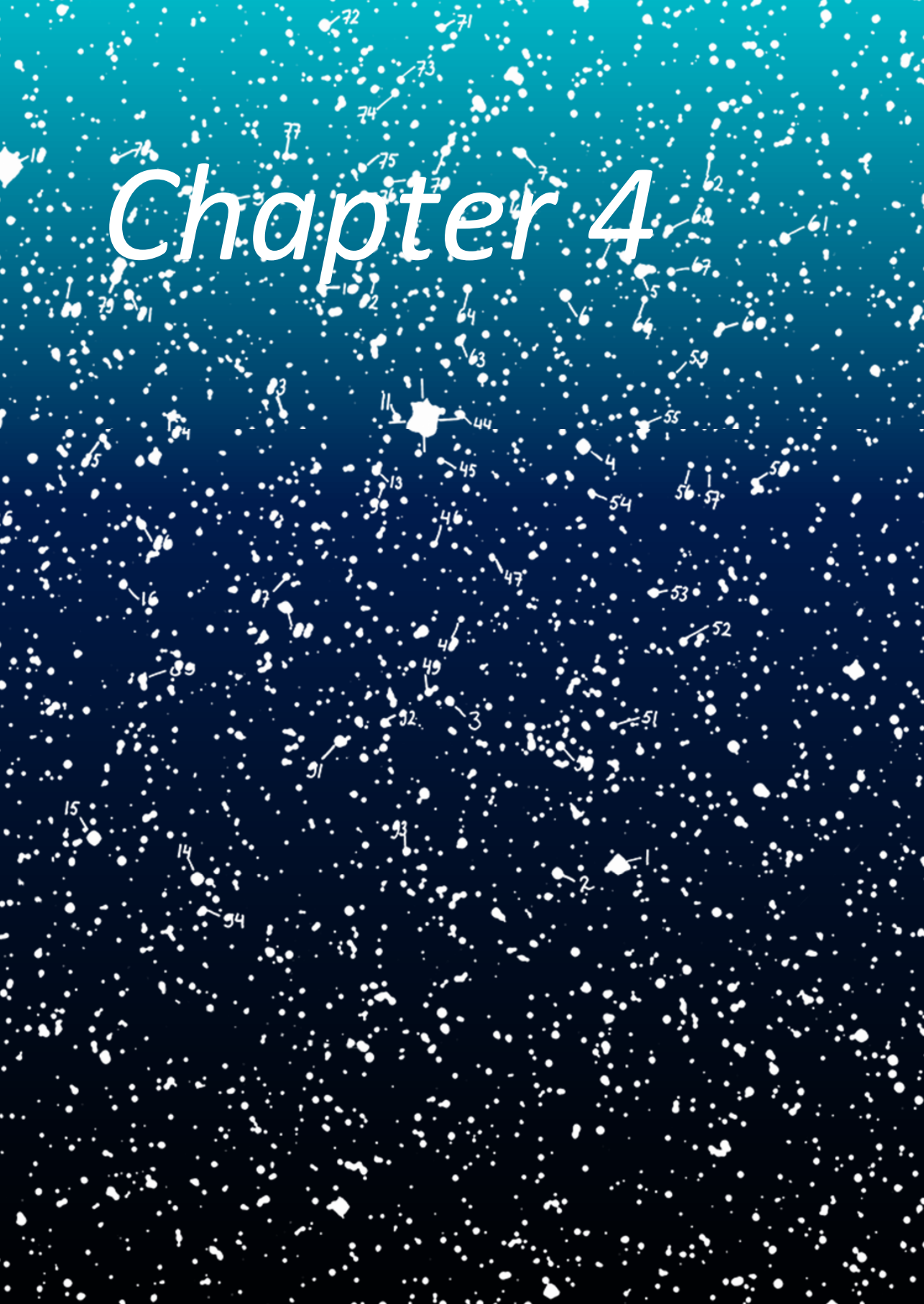
Chapter 4

Follistatin-like 1 in Cardiovascular Disease and Inflammation

Marijn M.C. Peters[1*], Timion A. Meijs[1*], Wouter A. Gathier[1], Pieter A. Doevendans[1], Joost P.G. Sluijter[1], Steven A.J. Chamuleau[1], Klaus Neef[1]

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[1] Department of Cardiology, Experimental Cardiology Laboratory, UMC Utrecht Regenerative Medicine Centre, University Medical Centre Utrecht, University Utrecht, Utrecht, The Netherlands

** These authors contributed equally*

Abstract

Follistatin-like 1 (FSTL1), a secreted glycoprotein, has been shown to participate in regulating developmental processes and to be involved in states of disease and injury. Spatiotemporal regulation and posttranslational modifications contribute to its specific functions and make it an intriguing candidate to study disease mechanisms and potentially develop new therapies. With cardiovascular diseases as the primary cause of death worldwide, clarification of mechanisms underlying cardiac regeneration and revascularization remains essential. Recent findings on FSTL1 in both acute coronary syndrome and heart failure emphasize its potential as a target for cardiac regenerative therapy. With this review, we aim to shed light on the role of FSTL1 specifically in cardiovascular disease and inflammation.

Introduction

Follistatin-like 1 (FSTL1), also known as TSC-36 and follistatin-related protein (FRP), was first discovered in an osteoblastic cell line as a gene induced by transforming growth factor (TGF) β1 expression [1]. This 308 amino acid extracellular glycoprotein is a member of the SPARC (secreted protein acidic rich in cysteine) family of proteins [2,3] and has a molecular mass ranging from 40 to 55 kDa [4,5]. Analysis in mice indicated it to be most abundantly expressed in the lungs [6], the heart [7], skeletal muscle [8], smooth muscle [9], and vascular endothelium [9]. Multiple effects of FSTL1 have been reported, including a regulatory role in embryogenesis [10], inhibition of proliferation in cancer cells [11], and modulation of inflammatory responses [6]. Mechanistically, these various roles of FSTL1 are mediated through different pathways targeting BMP for developmental processes, AKT/AMPK in the context of cardiac disease and CD14/TLR4 for immunological processes [12].

Cardiokines as potential therapeutic targets for heart failure

Heart failure represents a significant health burden within the spectrum of cardiovascular diseases affecting approximately 26 million people worldwide [13]. The treatment options are limited which leads to a poor prognosis with a five-year survival rate of 51.5% [14]. Over the last years, cardiovascular research on heart failure has focused on methods to repair the heart. The use of stem cells in the context of cardiac regeneration has been studied extensively. However, the results of (pre-)clinical trials using stem cell therapy are inconsistent [15]. The main obstacles that are encountered are the complex molecular mechanisms underlying cardiac repair, but also the delivery method [15], low cellular retention [16] and functional integration [17]. Furthermore, the focus on repair of the injured myocardium is accompanied by undervalued importance of the restoration of the microvasculature of the heart and the functional integration of the injected cells within the host myocardium and the extracellular matrix [18]. The use of extracellular vesicles has also received interest as the vesicles can mediate paracrine effects and multiple reparative functions, including stimulating angiogenesis and cardiomyocyte proliferation [19]. There is increasing evidence suggesting that endogenous factors secreted from cardiac tissue, referred to as cardiokines, play an essential role in regulating cellular mechanisms in the heart [20]. They can function through autocrine, paracrine, and endocrine signaling and are involved in responses to injury, cardiac remodeling, and inter-cellular and inter-organ communication [21-25].

FSTL1 in cardiac disease

A protective effect of FSTL1 has been reported in various cardiac diseases (Figure 1) and development [26]. In transaortic constricted hearts, FSTL1 reduces cardiomyocyte apoptosis via phosphorylated AMPK signaling [20]. Furthermore, the loss of FSTL1 in transaortic constricted hearts is associated with more severe myocardial hypertrophy and loss of ventricular function, emphasizing a role for FSTL1 in maintaining cardiac function [27]. Similarly, in infarcted hearts FSTL1 protects from apoptosis and induces angiogenesis via phosphorylated AKT signaling [7,28,29]. For the stimulation of angiogenesis, phosphorylated AKT signaling is dependent on the interaction between FSTL1 and transmembrane

protein DIP2A, which functions as a receptor for FSTL1. A 22% increase in the secretion of FSTL1 by skeletal muscle fibers during exercise could also be the underlying mechanism of cardioprotection acquired through exercise [8]. The capacity to modulate cardiovascular diseases via both cardiac secreted FSTL1 and skeletal muscle secreted FSTL1 indicates the potential role of FSTL1 in myocardial repair after injury.

FSTL1 may enhance cardiac function following I/R injury

Cardioprotection

FSTL1 has been described as an active modulator of cellular responses in various cardiac conditions and may have the capacity to generate both cardioprotective and regenerative responses. Previous studies showed that FSTL1 acts as a cardioprotective factor after ischemia/reperfusion (I/R) injury. FSTL1 overexpression attenuated apoptosis in neonatal rat cardiomyocytes after exposure to hypoxia and reoxygenation. Additionally, systemic administration of recombinant FSTL1 after myocardial infarction in mice significantly reduced the rate of cardiomyocyte apoptosis and the size of the infarct [30]. Similarly, anti-apoptotic effects of FSTL1 have been shown in vitro and in murine and porcine I/R models [30]. Upregulation of endogenous FSTL1, as a response to exercise, attenuated cardiac fibrosis, while intraperitoneal administration of FSTL1 was associated with increased systolic function in a rat model of myocardial infarction [31]. There is increasing evidence suggesting that these cardioprotective properties may be mediated by activation of AMP-activated protein kinase signaling [30,32]. Furthermore, a recent study shows FSTL1 expression is suppressed by miRNA-9-5p, a non-coding RNA which is upregulated after ischemic injury [33]. Antagomir mediated inhibition of miRNA-9-5p in a murine acute MI model attenuated fibrosis and inflammation and preserved cardiac function.

Proliferative responses

Recently, FSTL1 was found to act as a regenerative factor by promoting proliferation of mouse embryonic stem cell-derived cardiomyocytes in vitro [34] raising hopes to unleash the limited endogenous regenerative capacity of the adult human heart [35] beyond the reported 0.8% of cardiomyocytes dividing annually to maintain homeostasis [36]. This baseline proliferation rate can be increased to some extent, for instance by signaling from cellular damage induced by the increased concentration of reactive oxygen species (ROS) in response to ischemia/reperfusion injury [37], via the Nrg1/ErbB2 pathway [38] and via the Hippo-YAP pathway [39], but stimulation of cardiomyocyte proliferation to allow for substantial restoration of the myocardium was considered illusive until recently. However, analysis in a mouse model of MI revealed a switch in FSTL1 expression from epicardial to myocardial cells going along with a switch in posttranslational modification from the hypoglycosylated to the hyperglycosylated isoform [34]. Since this isoform has been shown in vitro to be cardioprotective (anti-apoptotic) but not regenerative (proliferative), reconstitution of hypoglycosylated FSTL1 expression was mediated by surgical application of a FSTL1-loaded collagen-based patch to the epicardium immediately after the induction of myocardial infarction. Restoring epicardial FSTL1 expression significantly reduced scarring, stimulated cardiomyocyte proliferation, and enhanced cardiac function following ischemic injury in murine and porcine models [34].

FSTL1 induced increase of GDF-15 expression and activation of TGFβ-Smad2/3 signaling seems to be a potential pathway for induction of cardiomyocyte proliferation [31,40].

These results indicate that the decrease of epicardial FSTL1 is an adverse reaction to ischemic stress and that reconstitution comprises a promising method to preserve cardiac function and attenuate cardiac remodeling. However, a recent study found no evidence of epicardial FSTL1 expression in a *Fstl1*-eGFP reporter mouse, but could confirm FSTL1 dependent activation of cardiac fibroblasts preventing post-myocardial-infarction ventricular wall rupture [41].

Stimulatory effects on revascularization

Besides inhibiting apoptosis and stimulating proliferation of cardiomyocytes, FSTL1 may also attenuate ischemia/reperfusion injury by inducing revascularization. Recent studies have demonstrated that FSTL1 promotes angiogenesis during ischemic stress through activation of Akt-eNOS-dependent signaling [42], and TGFβ-Smad2/3 after myocardial infarction [31]. Epicardial injection of FSTL1 following myocardial infarction resulted in increased vascularization of the myocardium, particularly in the border zone of the infarcted area [34]. Furthermore, there is evidence suggesting that FSTL1 enhances endothelial cell function and survival [42] and plays a role in vascular remodeling following arterial injury by preventing neointimal hyperplasia [43].

FSTL1 as biomarker: Elevated expression in acute coronary syndrome and heart failure

FSTL1 is strongly expressed under cardiac hypoxic conditions and has been proposed to be a potential diagnostic biomarker in acute coronary syndrome (ACS) and heart failure.

Patients with ACS showed serum concentrations of FSTL1 increased by 88% compared to healthy controls [40]. Upregulation of FSTL1 in ACS patients was independently correlated with an increased incidence of diabetes mellitus, increased N-terminal pro-B-type natriuretic peptide (NT-proBNP) and c-reactive protein (CRP) levels. Serum FSTL1 levels above the median were associated with reduced survival one year after ACS compared to those below the median ($P < 0.019$). In a cohort of 106 ACS patients, patients were 3.7 times more likely to die from a cardiovascular cause when their serum FSTL1 concentration was within the top quartile compared to patients with levels in the three lower quartiles ($P < 0.001$). The prognostic value of FSTL1 in ACS showed similar discriminatory potential as traditional markers CRP and NT-proBNP [40]. Furthermore, GDF-15, which is induced by FSTL1, has strong prognostic value for patients with non-ST-elevation ACS [44].

FSTL1 concentrations are increased in patients with ischemic and dilating cardiomyopathy with a left ventricular ejection fraction of less than 40%. In these systolic heart failure patients, serum FSTL1 levels were elevated by 56% compared to matched controls and associated with increased left ventricular mass, left ventricular posterior wall thickness, and increased brain natriuretic peptide levels [45]. Moreover, significantly elevated serum FSTL1 levels have been found in patients with end-stage heart failure with a left ventricular assist device implantation [9] and in patients with heart failure with preserved ejection fraction (HFpEF) [46]. A recent study showed that the upregulation of FSTL1 expression in heart failure could be accurately detected by using gold nanoparticles. According to the authors, advantages of this technique are the low costs and rapid execution compared to current methods to diagnose heart failure, e.g. NT-proBNP and echocardiography [47].

The efficacy of FSTL1 as a biomarker, especially in relation to established clinical biomarkers and the association with cardiovascular events and mortality, shows promise, but still requires additional research. The increased circulation of FSTL1 in heart failure might suggest further increasing circulating FSTL1 would not improve cardiac function. Contrary to this hypothesis, a recent study showed acute or chronic FSTL1 infusion normalized cardiac metabolism and improved diastolic and contractile function [48].

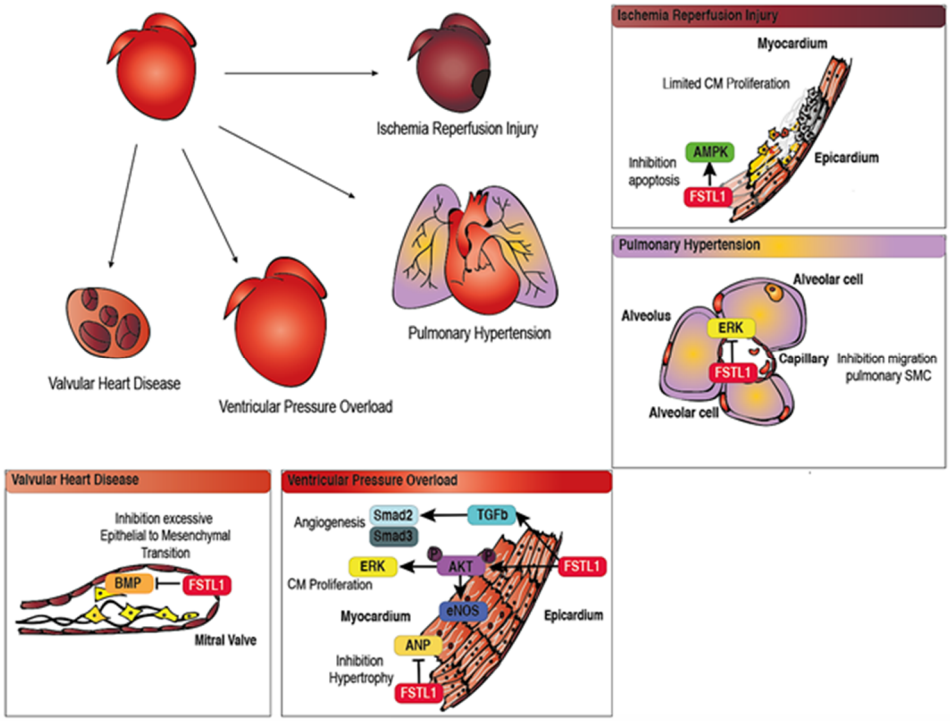

Fig. 1 The role of FSTL1 in cardiovascular diseases. In the injured heart, FSTL1 can influence multiple processes through the interplay with distinct molecular pathways in multiple cardiac cell types. In the healthy and the hypertrophied heart, the epicardial cells secrete FSTL1 inhibiting hypertrophy, stimulating cardiomyocyte proliferation and inducing angiogenesis. Epicardial FSTL1 expression is abolished during ischemia reperfusion injury. Myocardial FSTL1 can inhibit cardiomyocyte apoptosis. In valvular heart disease FSTL1 regulates epithelial to mesenchymal transition. In pulmonary hypertension, FSTL1 inhibits the migration of pulmonary smooth muscle cells (SMC).

Attenuation of cardiac hypertrophy

FSTL1 has been identified as an active modulator of cardiac ventricular hypertrophy. In a mouse model of ventricular pressure overload induced by transverse aortic constriction (TAC), cardiomyocyte-specific knock-out of FSTL1 resulted in excessive hypertrophy and deterioration of systolic ventricular function [27]. In contrast, transgenic mice overexpressing FSTL1, both conditionally and constitutively, were refractory to developing cardiac hypertrophy in this model. Moreover, systemic administration of FSTL1 decreased the hypertrophic response, enhanced systolic function, and attenuated left

ventricular dimensions in both wildtype and FSTL1-knockout (FSTL1-KO) mice. TAC resulted in a 2.2-fold increase of FSTL1 serum levels and this increase was attenuated in the myocyte-specific FSTL1-KO mice, suggesting that cardiomyocytes are the main source of FSTL1 in mice subjected to TAC [27].

The ability of FSTL1 to prevent hypertrophic responses has also been demonstrated in vitro using adult rat ventricular cardiomyocytes [46]. Supplementation of FSTL1 to cell culture attenuated cardiomyocyte hypertrophy induced in vitro by d-aldosterone and reduced expression of atrial natriuretic peptide (ANP), a marker of cardiomyocyte hypertrophy. This was confirmed in vivo showing that increased systemic levels of FSTL1 from transgenic overexpression were associated with reduced cardiac hypertrophy and improved diastolic function in a mouse model of HFpEF [46].

Protection from pulmonary hypertension

Recent evidence suggests that FSTL1 also plays a role in attenuating pulmonary hypertension [49]. During pulmonary hypertension, high blood pressure in the arteries of the lungs increases the workload of the heart and can lead to right ventricular heart failure. In patients with pulmonary hypertension due to chronic obstructive pulmonary disease FSTL1 serum levels are elevated. When comparing heterozygous FSTL1+/– knock-out mice with wild type FSTL1+/+ mice, in a state of hypoxia induced pulmonary hypertension, the heterozygous mice with decreased FSTL1 expression showed higher right systolic ventricular pressures and increased right ventricular hypertrophy. Systemic administration of FSTL1 improved right-sided systolic pressures [49]. Additionally, endothelium-derived FSTL1 appears to regulate the development and remodeling of the pulmonary vasculature and loss of this type of FSTL1 may result in a reduction of right ventricular function [50].

FSTL1 and valvular heart disease

Limited evidence is available regarding the potential association of FSTL1 and valvular heart disease. Recently, it has been described that endocardial and endothelial deletion of FSTL1 resulted in severe deformation of the mitral valve in a mouse model [51]. FSTL1 is normally expressed in both mitral valve leaflets and inhibits bone morphogenetic protein (BMP) signaling to prevent excessive proliferation of valve cells. Echocardiography of a conditional FSTL1 knockout mouse showed mitral regurgitation and progressive left ventricular dysfunction, eventually leading to cardiovascular death [51].

FSTL1 is associated with cardiomyocyte and endothelial cell function, and its pleiotropic function renders its potential to modulate specific processes, including cardiomyocyte survival, cardiomyocyte proliferation and angiogenesis. Additionally, modulation of FSTL1 expression can improve or worsen cardiac function in multiple cardiac diseases. Therefore, FSTL1 has the potential to serve as a therapeutic target to treat I/R injury, pulmonary hypertension, cardiomyocyte hypertrophy, and valvular heart disease.

Inflammation in cardiac diseases

An increasing body of evidence supports the essential role of inflammation in both the development and the progression of cardiovascular diseases [52]. Acute myocardial infarction is often caused by the formation of a thrombus on atherosclerotic plaques in coronary arteries, a process involving the

activation of platelets, the accumulation of immune cells and systemic and local inflammatory events [53]. In the ischemic myocardium, injured myocytes release their intracellular content resulting in a well-orchestrated signaling cascade of neutrophil and monocyte infiltration. Reperfusion of the infarcted area leads to I/R injury mediated by the release of ROS, inducing leukocyte chemokine upregulation. The infiltration of immune cells mediates the secretion of pro-inflammatory cytokines tumor necrosis factor (TNF), IL-1β, and IL-6. The knockdown of TNF ameliorated myocardial I/R injury indicating the role of inflammation in the pathogenesis of I/R injury [54]. But the inflammatory response in the heart after ischemia is not only detrimental for cardiac function: extensive evidence suggests the involvement of inflammatory mechanisms in post-infarction cardiac repair through the clearance of dead cells [55] and also mediating regeneration by macrophages in the neonatal heart [56]. Furthermore, in a mouse model of cardiac pressure overload due to TAC, hypertrophy and myocardial inflammation preceding fibrosis was observed [57]. Additionally, Inflammation plays a role in the pathogenesis of pulmonary hypertension where endothelial dysfunction is accompanied by the upregulation of pro-inflammatory cytokines IL-1, MCP-1 and IL-6 [58].

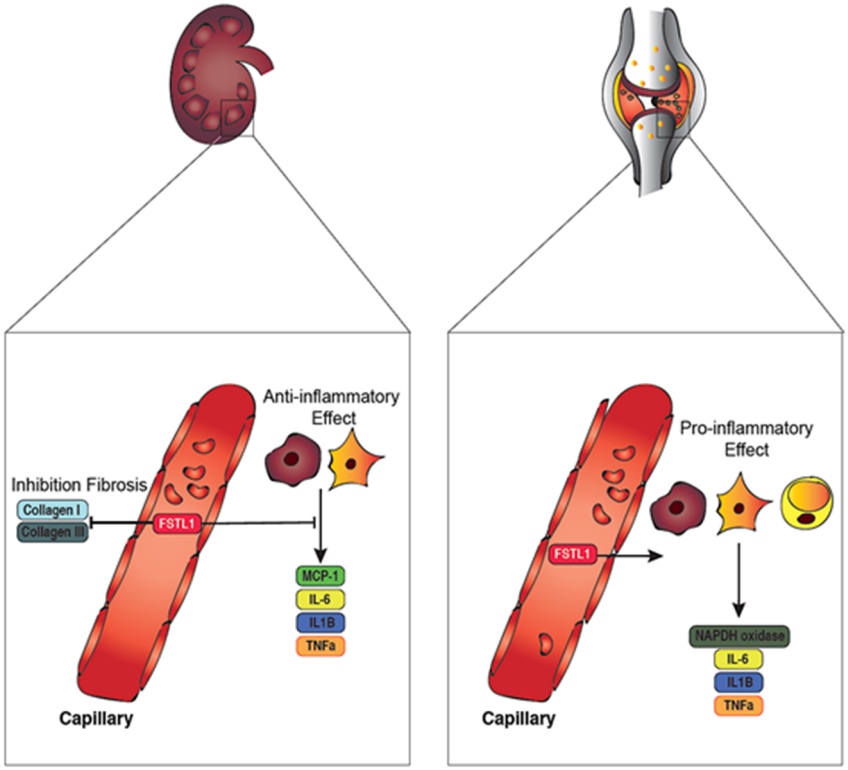

Fig. 2 The bifunctional role of FSTL1 in inflammation. In the kidney, FSTL1 has an anti-inflammatory effect by inhibiting fibrosis and macrophage and fibroblast cytokine secretion. In joints, FSTL1 can mediate the secretion of pro-inflammatory cytokines by macrophages, fibroblasts and adipocytes.

FSTL1 in Inflammation

The identification of FSTL1 as an autoantigen in the synovium of patients with rheumatoid arthritis (RA) [4] led to increased interest to study FSTL1 in inflammatory diseases. Follow-up studies identified both pro-inflammatory and anti-inflammatory effects of FSTL1 (Figure 2).

In multiple inflammatory diseases (e.g. RA [59,60], Sjögrens syndrome, ulcerative colitis, systemic lupus erythematosus, systemic sclerosis, and dermatomyositis / polymyositis, asthma [61]) FSTL1 levels were found to be increased and associated with disease progression.

Expression of FSTL1 was found to be specifically increased in mesenchymal lineage cells and not in the hematopoietic lineage in patients with RA. The plasma levels of FSTL1 were increased in acute Kawasaki disease compared to healthy controls, and a relation was found between increased FSTL1 levels and the likelihood to develop coronary aneurysms [62]. Also in patients with obesity (BMI ≥ 25 kg/m2), serum FSTL1 levels were significantly elevated compared to control patients. Furthermore, elevated FSTL1 mRNA levels were seen in subcutaneous and epididymal adipose tissue in a mouse model of obesity [63]. The pro-inflammatory capacity of FSTL1 is mediated by the expression of pro-inflammatory cytokines IL-6 [6], IL-1β, TNFα, IFNγ-related genes [64], MCP-1 [65] and NF-κB signaling [66]. FSTL1 promotes IL-1β secretion by regulating the activity of the NLRP3 inflammasome both in vitro and in vivo [67]. The injection of FSTL1 in the paws of wild type mice caused swelling only in the presence of IFN-γ [64]. Not only exogenously administered FSTL1, but also endogenously expressed FSTL1 has a pro-inflammatory effect as shown in a mouse model of CIA treated with anti-FSTL1 antibodies. Here, amelioration of arthritis and reduced mRNA levels for IL-1β, IFN-γ, and CXCL10, which is a mediator in bone erosion in CIA, were observed [64,68]. It appears most of the pro-inflammatory effects of FSTL1 are associated with its effect on arthritis. It is unclear whether this will also have implications for the therapeutic application of FSTL1 as a regenerative factor.

Other studies report the anti-inflammatory capacity of FSTL1. An ameliorating effect of recombinant human FSTL1 on arthritis in a mouse model of CIA has been described [69]. Treatment with FSTL1 prevented swelling of footpads and reduced the clinical score used to assess arthritis severity. Furthermore, FSTL1 was able to prevent cartilage breakdown and bone erosion by down regulating expression of c-fos, ets-2, Il-6, MMP-3, and MMP-9, genes that are involved in destructive joint inflammation [69-73]. In an in vitro model of neural inflammation in mouse astrocytes, FSTL1 attenuated the upregulation of pro-inflammatory cytokines after LPS treatment by suppressing the MAPK/p-ERK1/2 pathway [74]. Intravenous administration of FSTL1 in mice 4 weeks after subtotal nephrectomy ameliorated fibrosis and mice that received FSTL1 showed smaller glomerular area and fewer intraglomerular cells [75]. Lower mRNA levels of TNF-α, IL-6, IL-1β, MCP-1, NADPH oxidase components, connective tissue growth factor, TGF-β1, collagen I, and collagen III were found in the remaining kidney tissue of FSTL1 treated mice.

Mechanistically, the dual role of FSTL1 has been linked to pro-inflammatory processes via CD14 and TLR4 and to inhibition of tissue destruction via the downregulation of matrix metallopeptidase (MMPs) regulated via DIP2A, pAKT and up-regulation of FOS [76]. Thus, the capacity of FSTL1 to play either a pro- or anti-inflammatory role might stem from the various pathways through which FSTL1 is able to act.

As the inflammatory response is also important in multiple cardiac diseases and FSTL1 has been reported to have cardioprotective and regenerative effects, it is important to study whether FSTL1 treatment might have a pro-inflammatory effect in the heart. Still, the role of FSTL1 in cardiac inflammation remains largely unknown. Analysis of the expression of pro-inflammatory cytokines after FSTL1 in a mouse and pig model of I/R injury showed decreased levels of TNF-α and IL-6 [30]. Also in cultured neonatal rat cardiomyocytes, lipopolysaccharide (LPS) stimulated expression of pro-inflammatory cytokines was decreased after FSTL1 treatment. FSTL1 supplementation to macrophages, which are abundantly present in the myocardium after cardiac I/R injury led to an AMPK-dependent decrease in TNF-α and IL-6 expression after LPS or BMP-4 stimulation [30].

Furthermore, the effect of cardiac myocyte-derived FSTL1 on chronic kidney disease (CKD) was studied in a mouse model of subtotal nephrectomy comparing healthy mice to cardiac-specific FSTL1 knockout mice [75]. Significantly higher levels of pro-inflammatory cytokines TNF-α, IL-6, IL-1β, MCP-1, and NADPH oxidase components were expressed in FSTL1 knockout mice compared to control mice. It has been suggested that FSTL1 exerts anti-inflammatory effects via the inhibition of BMP-4 dependent inflammatory pathways [30] and pro-inflammatory effects via the activation of TLR4/MyD88/NF-kB and MAPK signaling pathways [77]. Thus, the capacity of FSTL1 to play either a pro- or anti-inflammatory role might stem from the various pathways through which FSTL1 is able to act. Another intriguing explanation of the ambiguous role of FSTL1 in inflammation and inflammatory diseases could be post-translational modification of FSTL1 [2].

Influence of post-translational modification on FSTL1 function

Healthy epicardium expresses FSTL1, which is ceased after myocardial injury. As mentioned, FSTL1 can attenuate detrimental effects from myocardial injury by inducing cardiomyocyte proliferation, reducing apoptosis and inflammation, and promotion of revascularization [34,42]. However, increasing FSTL1 circulating levels after myocardial infarction with FSTL1 from myocardial origin did not induce cardiomyocyte proliferation. Only FSTL1 from epicardial origin was found to be capable to induce a regenerative response [34]. Analyzing and comparing biochemical properties of epicardial and myocardial FSTL1 revealed slower migration of myocardial FSTL1 in SDS polyacrylamide gel electrophoresis, representing increased molecular weight, potentially from post-translational modifications. Application of tunicamycin, an inhibitor and catalyst of reversion of N-linked glycosylation, abolished this difference in migration, suggesting hyperglycosylation as a cause of the observed high molecular weight FSTL1 myocardial isoform. Thus, it seems plausible that glycosylation and potentially other post-translational modifications play a role in modulating the abilities of FSTL1 to generate cardioprotective and regenerative responses.

In previous studies multiple glycosylated isoforms of FSTL1 with varying molecular weights have been detected [2,4,5]. Bacterially expressed recombinant FSTL1, primarily produced in Escherichia coli, is a hypoglycosylated isoform, whereas FSTL1 expressed in mammalian cells is extensively glycosylated. Glycosylated FSTL1 protected mouse embryonic stem cell-derived cardiomyocytes (mESC-CMs) from apoptosis following H2O2 application, although no effect on proliferation, indicating potential regenerative properties, was observed. In contrast, bacterially produced FSTL1 did stimulate proliferation of mESC-CMs but failed to attenuate H2O2-induced apoptosis [34]. FSTL1

produced in insect cells also inhibited apoptosis and inflammatory responses following ischemia/reperfusion injury, as shown in mouse and pig models [30]. However, when comparing FSTL1 expressed in mammalian cells, insect cells, and bacterial cells, no differences were found in the stimulation of fibroblast mobility, despite significant variations in the extent of glycosylation [78]. A recent study analyzed whether ablation of the N glycosylation of the FSTL1 expressed in mammalian cells could increase the regenerative capacity of the human FSTL1 [79]. This study showed that a mutation in a single N glycosylation site (N180Q) of FSTL1 could trigger cardiomyocyte proliferation and cardiac regeneration in a mouse MI model. Based on these results, it is plausible that the upregulation of myocardial and downregulation of epicardial FSTL1 expression following MI alters distribution and ratios of hyper- and hypoglycosylated FSTL1 in the damaged heart.

Since several different isoforms and sources of FSTL1 have been used in previous studies and therefore results are difficult to compare across studies, Table 1 provides an overview of the specifications of previously used recombinant FSTL1 and the main results that have been found with each of these forms. However, precise mechanistic cues of post-translational modifications leading to either cardioprotective or regenerative capacities of FSTL1 still remain to be elucidated.

Method of delivery to the injured heart

Previously described cardioprotective properties of FSTL1 in vitro and in small animal models are considered promising and raise the question which application method would be most suitable for FSTL1 to exhibit its effects in large animals and in humans. Systemic administration of FSTL1 is a conceivable option, however it is unlikely that it will have considerable beneficial effect as FSTL1 is already upregulated in response to various cardiac conditions, including ACS, ischemic cardiomyopathy, end-stage heart failure, and HFpEF. Intramyocardial injections with regenerative factors at the site of injury may potentially induce a cardiac regenerative response [80,81]. However, FSTL1 was shown to exert pronounced cardioprotective and regenerative effects through signaling from the epicardium [34]. It remains to be seen whether the localization or solely the glycosylation status of epicardial (as compared to myocardial) FSTL1 is responsible for the cardioprotective and regenerative effect of FSTL1. Since epicardial FSTL1 expression diminishes in the affected area upon myocardial infarction, reconstitution of epicardial FSTL1 as a cardioprotective intervention has been demonstrated following myocardial infarction in murine and porcine models [34]. It is important to note that application of the patch is an invasive procedure involving surgical open chest access (thoracotomy), hence raising the question whether the potential beneficial effects of FSTL1 application outweigh possible complications of this intervention, including arrhythmias, bleeding, and wound infection. This difficult consideration underlines the importance of developing less invasive strategies in regenerative cardiac surgery. Minimally invasive approaches for epicardial delivery are being developed, e.g. by using a miniature robotic device that can navigate on the epicardial surface of the beating heart [82]. Next to epicardial delivery, it might still be possible to deliver FSTL1 intramyocardially, if solely the hypoglycosylation status is responsible for the regenerative capacity of FSTL1. Here, improved treatment planning and guided injections [83] will enable efficient and localized delivery. In order to ensure localization, stability and sustained release of FSTL1 into the myocardium, a collagen matrix has been used [34] but also injectable biomaterials allowing minimally

invasive access [84] should be tested and optimized. Furthermore, the use of liposomal or polymeric delivery vehicles might enhance the uptake of FSTL1 and increase the efficiency of the therapy, as has been shown for miRNA-based therapeutics [85].
In summary, FSTL1 expression and function has been shown to be closely linked to cardiac disease, both as a marker, and increasingly as a potential therapeutic compound or target. It is important to determine the mechanism and extent of the specific pro- and anti-inflammatory effects when FSTL1 is considered as a potential therapeutic agent or target to treat cardiovascular diseases. Finally, the method of application of FSTL1 in the context of cardiovascular disease will also very likely play a role on how inflammatory responses turn out. Characterization of the effect of FSTL1 on inflammatory cell infiltration and activation in the heart is essential before therapeutic application can be considered.

Conclusion

FSTL1 is a cardiokine with multiple implications in cellular processes in the heart, particularly in response to cardiac injury. An increasing body of evidence indicates that FSTL1 may attenuate I/R injury by inhibiting apoptosis and stimulating proliferation and revascularization, suggesting a potential role in regenerative therapy for heart failure patients. Furthermore, a protective role of FSTL1 has been described in cardiac hypertrophy and pulmonary hypertension. Effects and function of FSTL1 in the context of inflammation remain ambiguous and require further research, especially in acute and chronic cardiovascular disease.
The spatio-temporal organization of FSTL1 expression, its localization and onset after induction of damage, as well as post-translational modifications of FSTL1, mostly in terms of glycosylation, have been shown to be critical parameters of activity. The hypoglycosylated epicardial FSTL1, diminished upon myocardial injury, holds most potential in exerting cardioprotective and regenerative effects. To achieve this, epicardial reconstitution of FSTL1 following I/R injury may be preferred, despite the risks associated with a potentially invasive procedure.
In conclusion, FSTL1 exhibits regenerative and tissue-protective features making it a promising candidate for novel approaches to treat cardiovascular disease, while mechanistic details need further research before advancing to therapeutic applications.

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Table 1 Specifications of recombinant FSTL1 used in research on cardiac disease

Parameter	Study	Origin FSTL1	Expression system	Tag	*in vitro/vivo*	Main findings
Cardioprotection	Gorgens, 2013	Human	*E. coli*	6His at N-terminus	*In vitro*	No alteration of Akt phosphorylation following insulin stimulation.
		Human	*E. coli*	His at N-terminus		
	Maruyama, 2016	Human	HEK293	-	*In vitro*	Promotion of cardiac fibroblast mobility, however no significant differences between the isoforms.
		Human	*E. coli*	6His at N-terminus		
		Mouse	Mouse cell line	6His at C-terminus		
		Mouse	Insect Sf9 cell	FLAG at C-terminus		
	Ogura, 2012	Human	Insect Sf9 cells	FLAG at C-terminus	*In vitro/vivo*	Inhibition of apoptosis and inflammatory responses following cardiac ischemia/reperfusion injury both *in vitro* and *in vivo*.
	Oshima, 2008	Mouse	HEK293	V5 epitope at C-terminus	*In vitro/vivo*	Reduction of apoptosis of neonatal rat cardiomyocytes after ischemia/reperfusion injury by upregulation of Akt and ERK signaling pathways; reduction of myocardial infarct size and apoptosis following myocardial infarction in mice.
	Ouchi, 2010	Mouse	Insect Sf9 cells	FLAG at C-terminus	*In vitro*	Enhanced survival, mobility, and differentiation into network structures in endothelial cells; reduced ischemia/reperfusion-induced apoptosis and stimulation of Akt phosphorylation in cardiomyocytes; effects attenuated by DIP2A.
	Zhang, 2017	Human	Mouse myeloma cell line, NS0-derived		*In vivo*	Decreased right ventricular systolic pressure and right ventricular hypertrophy index in mice with hypoxia-induced pulmonary hypertension.
Modulating cardiac hypertrophy	Tanaka, 2016	Human	Sf9 cells	FLAG at N-terminus	*In vivo*	Decrease of aldosterone-induced cardiomyocyte hypertrophy and diastolic dysfunction in mice with heart failure with preserved ejection fraction (HFpEF).
Cardiomyocyte proliferation	Wei, 2015	Human	*E. coli*	6His at N-terminus	*In vitro/in vivo*	Stimulated proliferation of mouse embryonic stem cell-derived cardiomyocytes but did not protect from H_2O_2-induced apoptosis; faster migration through SDS polyacrylamide gels compared to mammalian-expressed FSTL1; stimulated cardiomyocyte proliferation, cardiac regeneration and survival following ischemia/reperfusion injury in mice and swine models.
		Human	Mouse myeloma cell line, NS0-derived	Asp and 10His at C-terminus	*In vitro*	Inhibited H_2O_2-induced apoptosis in mouse embryonic stem cell-derived cardiomyocytes but did not have a proliferative effect; slower migration through SDS polyacrylamide gels compared to bacterial-expressed FSTL1.

Cardiorenal communication	Hayakawa, 2015	Human	Insect Sf9 cells	FLAG at C-terminus	*In vitro*	Decreased expression of pro-inflammatory cytokines and increased phosphorylation of AMP-activated protein kinase in human mesangial cells.
Endothelial cells and revascularization	Miyabe, 2014	Human	Insect Sf9 cells	FLAG at C-terminus	*In vitro*	Decreased proliferation and migration of human aortic smooth muscle cells by activation of AMPK.
	Wu, 2015	Human	Mouse myeloma cell line, NS0-derived	Asp and 10His at C-terminus	*In vitro*	Attenuated hemin-induced differentiation and survival of erythroid cells; regulated TGF-β and BMP expression.
	Xi, 2016	Human	*E. coli*	21His at N-terminus	*In vivo*	Stimulated angiogenesis and improved systolic cardiac function after myocardial infarction in rats.
Biomarker	Widera, 2012	Mouse	Sf9 insect cells	-	*In vitro*	Increased production of GDF 15 mRNA in adult mouse cardiomyocytes.
	Widera, 2009	Human	NSO mouse myeloma cells	-	*In vitro*	Used for calibration to calculate serum FSTL1 concentrations.
Chemical properties	Hambrock, 2004	Mouse	HEK293	His-Myc at N-terminus	*In vitro*	No structural changes following calcium addition or depletion.
	Li, 2013	Murine	*Drosophila* S2 cells	6His at C-terminus	*In vitro*	Inhibition of BMP4-induced phosphorylation of Smad1/5/8 in Mv1Lu cells.
	Tanaka, 2010	Human	COS-7 cells	FLAG	*In vitro*	DIP2A may be a receptor for FSTL1; FSTL1 binds to DIP2A, CD14, and other proteins of the TGF-β superfamily.

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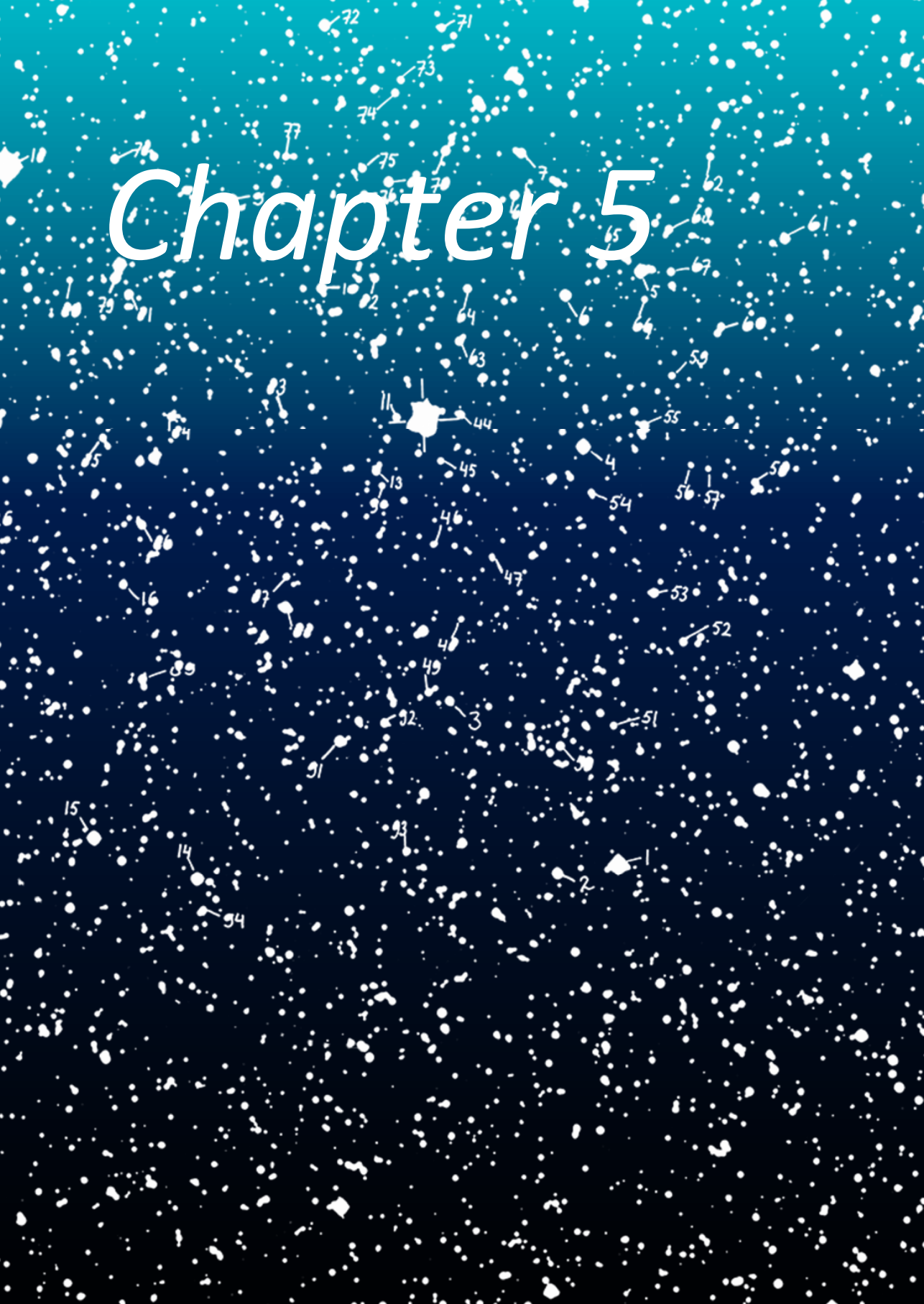
Chapter 5

Retrograde Coronary Venous Infusion as a Delivery Strategy in Regenerative Cardiac Therapy: an Overview of Preclinical and Clinical Data

Wouter A. Gathier[1], Dirk-Jan van Ginkel[1], Mira van der Naald[1], Frebus J. van Slochteren[1], Pieter A. Doevendans[1,2], Steven A.J. Chamuleau[1,2]

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[1] Department of Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands

[2] Regenerative Medicine Center Utrecht, Utrecht, The Netherlands

Abstract

Background

An important aspect of cell therapy in the field of cardiac disease is safe and effective delivery of cells. Commonly used delivery strategies such as intramyocardial injection and intracoronary infusion both present with advantages and disadvantages. Therefore, alternative delivery routes are explored, such as retrograde coronary venous infusion (RCVI).

Aim

Our aim is to evaluate safety and efficiency of RCVI by providing a complete overview of preclinical and clinical studies applying RCVI in a broad range of disease types and experimental models.

Conclusion

Available data on technical and safety aspects of RCVI are incomplete and insufficient. Improvement of cardiac function is seen after cell delivery via RCVI. However, cell retention in the heart after RCVI appears inferior compared to intracoronary infusion and intramyocardial injection. Adequately powered confirmatory studies on retention rates and safety are needed to proceed with RCVI in the future.

Introduction

Cell therapy has proven to be safe and feasible for treatment of cardiac disease. Yet, the clinical relevance of cell therapy is uncertain. Recent meta-analyses show a marginal (2-5%) increase of cardiac function measured by left ventricular ejection fraction (LVEF) [1,2]. Taking into account the dynamic nature and the high perfusion characteristics of the cardiac tissue [3] an important aspect of cell therapy is the location and mode of delivery. Two commonly used administration techniques are intramyocardial (IM) injection and intracoronary (IC) infusion [1,2]. IM injection has the benefit of targeted delivery of cells in a target region, e.g. the border zone of the infarct [4] but this procedure is time-consuming, suffers from rapid wash-out of cells via venous drainage after injection [3], and needs specific systems in the catheterization laboratory. IC infusion is quick and easy to perform but the coronary system is often diseased in the target population, leading to inaccessibility of coronary arteries. Manipulation inside the coronary artery can potentially induce embolisms leading to decreased coronary blood flow [5-7]. Therefore, alternative delivery routes are explored. The coronary venous system is easily accessible and typically free of atherosclerotic disease. Retrograde coronary venous infusion (RCVI) is considered to be a good alternative to IM and IC administration. RCVI is performed by placing a balloon-catheter in the coronary sinus (CS) or into one of the coronary veins. In order to maximize the therapeutic potential, the balloon is kept inflated temporarily to prevent the loss of infused cells due to antegrade, venous flow and to allow the cells to disseminate in the heart. For optimal effect, this occlusion is often prolonged for a certain period after cell infusion. Our aim is to provide a complete overview of preclinical and clinical studies applying RCVI as cell delivery strategy and focus on safety aspects and efficiency measures.

Methods

Search Strategy and Eligibility

The full search strategy is available as Supplemental Material 1. In brief, we have performed a search using the PubMed and Embase databases on May 15th, 2017. Trials were eligible for inclusion if they met the following criteria 1) original (preclinical or clinical) study, 2) full text available in English, 3) covering cell therapy, 4) investigating safety or efficacy of retrograde CS/venous administration. An additional cross-reference screening was performed of included articles. The flowchart of the search is presented in figure 1.

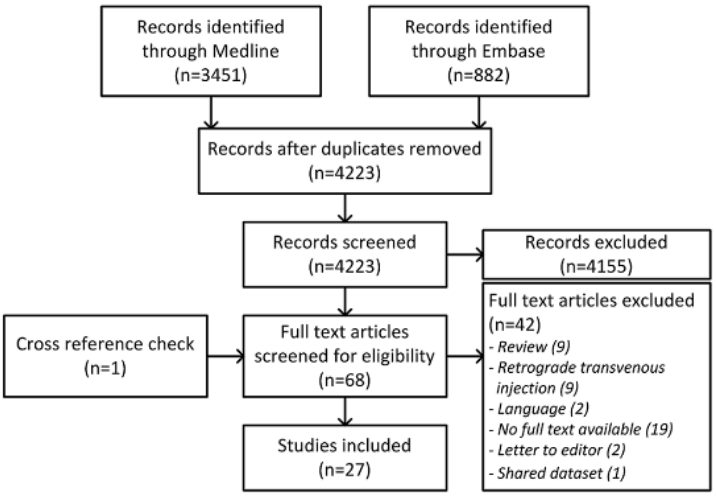

Fig. 1 Flowchart of the systematic search

Results

Search Results

The entire search yielded a total of 4333 (3451 Medline and 882 Embase) hits, of which 110 reports were removed after duplicate screening. Another 4155 reports were excluded after title/abstract screening because they did not fulfill the inclusion criteria. The remaining 68 articles were screened on the availability of full text, leading to another 41 exclusions. One additional article was excluded due to a shared dataset [8]. The cross-reference screening led to one additional inclusion that did not come up in the original search due to the absence of one part of the search string in the title and abstract [9]. The total number of articles included in this review is 27 (figure 1). All articles were published between 2003 and 2016.

Preclinical and Clinical Experience

Retrograde coronary venous infusion has been performed in a number of different studies. In total, 21 preclinical studies are included in this review; 8 rat studies [10-17], 3 dog studies [18-20] and 10 pig studies [21-24,9,25-29]. Patients were treated in 6 studies [30-35].

Preclinical Experience

Treatment was given in acute (acute myocardial infarction; AMI) [9,13-15,19,20,22-25,29], and chronic setting (chronic myocardial infarction (CMI) [10-12,17,21,26-29], and in chronic heart failure (CHF) [18]. One study treated healthy subjects (n=1) [16]. Cell products administered included skeletal myoblasts (n=6) [10,12,15,16,21,26], bone marrow mononuclear cells (n=2) [11,29], peripheral blood mononuclear cells (n=2) [22,24], adipose-derived stem cells (n=3) [18,23,24], mesenchymal stem cells (n=6) [13,14,19,20,25,27], embryonic endothelial progenitor cell (n=1) [9], autologous unfractionated

bone marrow (n=1) [28], and cardiac explant-derived c-Kit+ cells (n=1) [17]. One study administered both adipose derived stem cells and peripheral blood mononuclear cells [24].

Clinical Experience

In the clinical setting, treatment was given in AMI [31], CHF [30,32], and chronic refractory angina (CRA) [33-35]. Infused cell products included bone marrow mononuclear cells (n=3) [30,31,33], umbilical cord sub-epithelial cells (n=1) [32], and autologous unfractionated bone marrow (n=2) [34,35].

Table 1. shows study characteristics on disease model, recipients, and used cell type and number. In summary, there is broad experience with RCVI across species, disease models, and used cells.

Practical Aspects of RCVI

There is high degree of heterogeneity in the way that RCVI is performed. Important differences between models are 1) the infusion duration, 2) the volume of infused cell suspension, 3) the time that the CS or coronary vein is occluded to prevent cells from draining directly into the right atrium, 4) the number of cells infused, and 5) the location of infusion (table 1 and 2).

Preclinical Experience

Cells are predominantly infused via the coronary veins in preclinical trials. The infused cell number ranged from approximately 1x10^6 to 3x10^9. Infusion duration, infused cell volume and the time that the CS or coronary vein was occluded differed both within and between animal species (table 1 and 2).

Clinical Experience

In clinical trials, cells were mainly infused via the CS. The amount of cells infused was generally higher, ranging from approximately 1x10^8 to 4x10^9 cells. Notable differences between preclinical and clinical trials are that infused cell volume were many times greater in clinical trials compared to preclinical trials and that the CS or coronary vein was occluded longer in clinical trials (table 1 and 2).

We found a striking reporting difference regarding practical aspects of RCVI, with roughly 20% of studies not adequately describing procedural characteristics. This hampers the possibility to repeat certain experiments if desired.

Safety Issues

Here safety is described as occurrence of arrhythmias related to RCVI, elevation of heart enzymes, cardiac tamponade, presence of pericardial fluid, microvascular obstruction (MVO), damage to the CS, and mortality. It should be noted that some studies did not report safety aspects due to the purpose and set up of these studies.

Safety Aspects Other than Mortality

Preclinical Experience

Thirteen preclinical studies reported safety aspects of RCVI. One study only described that RCVI is safe without providing data on safety [29]. Seven studies only reported absence of arrhythmias without providing in depth data [10,14-16,22,26,28]. Five articles provided more in-depth data on safety aspects of RCVI [11,12,18,20,23]. These five studies will be discussed in more detail below.

In two studies, IM injection was associated with an increased chance of both spontaneous ventricular tachycardia's and ventricular premature contractions after cell administration compared to RCVI, suggesting that RCVI is safer in these experimental models [11,12]. Another study closely monitored dogs for occurrence of arrhythmias and reported transient atrial fibrillation during CS catheterization in 6 out of 15 dogs and a pre-existent ventricular arrhythmia in one dog [18]. In another dog study no occurrence of arrhythmias or cardiac tamponade associated with RCVI was seen [20]. RCVI did not lead to MVO after cell administration in one pig study [23].

Clinical Experience

All six clinical studies reported safety aspects of RCVI. Two studies only reported absence of arrhythmias without providing in depth data [34,35]. The other four studies provided more in-depth information on safety. In one clinical trial absence of arrhythmias associated with RCVI was reported, but a rise in cardiac enzymes was seen in some patients after RCVI [30]. Rise in cardiac enzymes after RCVI was also reported in some patients in another clinical trial [31]. In a population of patients with heart failure, a transient increase in Troponin-I levels was seen in all patients that resolved within 24 hours after catheterization. No arrhythmias were seen in this patient population and there was no evidence of damage to the CS after infusion [32]. No occurrence of arrhythmias, no rise in cardiac enzymes and no pericardial effusion after retrograde delivery of cells was seen in patients with chronic refractory angina [33].

Mortality

Preclinical Experience

Mortality rates were reported in 16 articles, with no RCVI related deaths occurring in 11 of these 16 studies. The available mortality data are difficult to interpret because it is likely that other factors besides RCVI, such as surgical procedure, have had influence on mortality rates. Loss of subjects that could possibly be attributed to RCVI was seen in 5 studies, described below.

A loss of 11/66 rats (16.7%) after RCVI was seen in one study. This loss could be attributed to the fact that a thoracotomy was performed to access the coronary vein and might not be related to the RCVI procedure itself. Since all animals received cells through RCVI there is no control group for mortality [10]. A comparison was made between mortality rates after IM injection and RCVI in two rat studies. Mortality rates were comparable between IM injection and RCVI with the first study showing mortality rates of 2/34 rats (5.9%) after IM injection and 2/35 rats (5.7%) after RCVI [11]. Similar results were seen in the second study with a mortality of 4/48 rats (8.3%) in the IM injection group compared to 4/49 rats (8.2%) in the RCVI group [12]. Surgical stress and bleeding were suggested to be the cause of mortality.

Table 1 Practical aspects of RCVI regarding disease type, location of infusion, and infused cell type and number

	Study	Species	Number of subjects	Model	Administration	Celltype	Number of cells
Small Animals	Di Lascio [10]	Rat	66	CMI	RCV	SMB	2x10^6 /100g
	Fukushima [11]	Rat	35	CMI	RCV	BMMNC	10^7
	Fukushima [12]	Rat	85	CMI	RCV	SMB	5x10^6
	Huang [13]	Rat	90	AMI	RCV	MSC	10^6
	Huang [14]	Rat	38	AMI	RCV	MSC	10^6
	Suzuki [15]	Rat	62	AMI	RCV	SMB	10^6
	Suzuki [16]	Rat	20	NP	RCV	SMB	10^6
	Zakharova [17]	Rat	32	CMI	RCV	CEDC	10^6
Large Animals	Pogue [18]	Dog	15	CHF	RCV	ASC	10^7
	Sun [19]	Dog	28	AMI	RCV	MSC	10^7
	Wang [20]	Dog	18	AMI	RCV	MSC	10^8
	Formigli [21]	Pig	15	CMI	RCV	SMB	8x10^7
	Hagikura [22]	Pig	15	AMI	RCV	PBMNC	5x10^6
	Hong [23]	Pig	7	AMI	RCV	ASC	10^7
	Hou [24]	Pig	5	AMI	RCV	PBMNC/ASC	10^7
	Kupatt [9]	Pig	ns	AMI	RCV	EEPC	5x10^6
	Lu [25]	Pig	36	AMI	RCV	MSC	10^8
	Prifti [26]	Pig	15	CMI	RCV	SMB	Ns
	Sato [27]	Pig	13	CMI	RCV	MSC	10^7
	Vicario [28]	Pig	16	CMI	RCS	AUBM	Ns
	Yokoyama [29]	Pig	21	AMI & CMI	RCV	BMMNC	3.2±1.2x 10^9
Clinical Trials	Patel [30]	Human	46	CHF	RCS	BMMNC	3.7x10^9
	Silva [31]	Human	9	AMI	RCV	BMMNC	10^8
	Tuma [33]	Human	14	CRA	RCS	BMMNC	8.2x10^8
	Tuma [32]	Human	18	CHF	RCS	UCSEC	1x, 2x, 4x10^8
	Vicario [34]	Human	14	CRA	RCS	AUBM	0,04 or 0,08x10^8/kg
	Vicario [35]	Human	15	CRA	RCS	AUBM	>0,04x10^8 /kg

CMI = chronic myocardial infarction (administration of cells > 1 week post MI), *AMI* = acute myocardial infarction (administration of cells up to 7 days post MI), *CHF* = chronic heart failure, *NP* = no pathology, *CRA* = chronic refractory angina, *MI* = myocardial infarction, *SMB* = skeletal myoblasts, *BMMNC* = bone marrow mononuclear cells, *PBMNC* = peripheral blood mononuclear cells, *ASC* = adipose-derived stem cells, *MSC* = mesenchymal stem cells, *EEPC* = embryonic endothelial progenitor cells, *UCSEC* = umbilical cord sub epithelial cells, *AUBM* = autologous unfractionated bone marrow, *CEDC* = cardiac explant-derived c-kit+ cells.
RCV = retrograde coronary venous infusion, *RCS* = retrograde coronary sinus infusion, ns = not specified

Table 2 Heterogeneity regarding practical aspects of RCI both within and between species

Study type	Infusion duration (min)	Infused volume (ml)	Occlusion time (min)
Rat studies (n=8)	1.0 [0.5 – 1.0] (n=3)	1.0 [0.5 – 1.0] (n=8)	5.0 [1.0 – 5.0] (n=8)
Dog studies (n=3)	No data (n=0)	10.0 [10.0 – 20.0] (n=3)	Insufficient data (n=2)
Pig studies (n=10)	10.0 [0.25 – 40.0] (n=9)	15.0 [10.0 – 25.0] (n=10)	10.0 [5.0 – 20.0] (n=7)
Human studies (n=6)	5.0 [4.0 – 6.0] (n=6)	60.0 [40.25 – 120.0] (n=6)	15.0 [11.0 – 17.0] (n=5)
Overall (n=27)	5.0 [0.88 – 11.25 (n=18)	10 ml [1.0 – 40.0] (n=27)	10.0 [5.0 – 12.75] (n=22)

Data are presented as median with interquartile ranges calculated using IBM SPSS statistics 21. *min* = minute(s), *ml* = milliliter(s), *n* = number of studies that statistics are based on

A common complication with RCVI in small animals is sustained bleeding from the catheter insertion site because the catheter has to be inserted into the fragile left cardiac vein via the left superior vena cava or CS. A comparison was made between conventional RCVI with a modified method of RCVI to see if bleeding could be limited in small animals. Conventional RCVI was described as delivery of cells by direct insertion of a catheter in the left cardiac vein via the CS. Modified RCVI was described as cardiac vein catheterization via the left internal jugular vein. A mortality of 3/7 rats (42.9%) was seen in the group that received cells via conventional RCVI versus 0/20 rats (0%) in the group with modified RCVI [14]. One small animal study reported a loss of 18/62 rats (29%) within 24 hours after RCVI, which the authors linked to development of acute heart failure rather than the RCVI [15].

Clinical Experience

In all 6 clinical trials mortality rates were reported but mortality related to RCVI did not occur.

In conclusion: there seems to be no relation between the way RCVI is performed and the occurrence of adverse events, arrhythmias and mortality. Especially large animal studies and clinical trials do not report mortality or arrhythmias related to RCVI. Although RCVI is reported to be safe in the majority of studies presented here, safety data on RCVI are underreported with the majority of studies providing no or insufficient safety data to conclude that RCVI is a safe method for cell delivery in the heart. Safety and mortality data are provided in table 3.

Efficiency Measures

Retention Rate

Preclinical Experience

The therapeutic benefit of cell therapy is in part based on the retention of cells in the heart. In total, 8 preclinical studies provide data on the percentage of administered cells that retain in the heart after RCVI (table 4). Different methods are used to determine cardiac retention of cells. One method is the use of real-time polymerase chain reaction for the Y-chromosome specific Sry gene to detect the amount of transplanted male cells in female subjects. Other methods include administration of β-

galactosidase-expressing cells, or to label cells radioactively with ^{111}Indium or Tc^{99m}-hexamethylpropylenamineoxime for quantitative analysis using scintigraphy. The retention rates show a high degree of heterogeneity that can partially be explained by differences in animal model, disease model, cell type, infusion time point, follow up time point, and quantification technique. Most studies report a retention ≤ 10% and two studies report a remarkably higher retention of respectively 31.4±4.8 and 29.8±6.9 percent [15,16]. The latter studies applied an indirect measurement of retention by using β-galactosidase-expressing cells, and comparing the level of β-galactosidase activity to the standard curve. One study used a method to optimize retention (magnetic targeting) that resulted in an increase of retention from approximately 2% after routine RCVI to 8.5% with magnetic targeting [13]. It should be noted that the three large animal experiments [9,23,24] consist of very small sample sizes. RCVI appeared to be either inferior to [23,24] or equal to [11,12] IM injection or IC infusion regarding cell retention. Retention rates in table 4 are presented as the percentage of total administered cells that is retained in the heart. In one study [23], retention of cells in the heart was reported as a percentage of cells retained in five major thoracoabdominal organs. We converted the data to a percentage of total administered cells that are retained in the heart in order to achieve comparability between studies. If retention of cells was measured at multiple time points, we reported retention at the first time point, because retention decreased in time in the majority of these studies. A decrease was not seen in 3 studies [23,15,16]. This can be explained by the fact that 2 of these studies used expression of β-galactosidase as a measure of cardiac cell retention [15,16]. Increased expression of β-galactosidase over time was attributed to proliferation of administered cells. The third article [23] presented the retention of cells in the heart as a percentage of the total retention in five major organs. A possible explanation for the increase in retention at a later time point could be that the decrease in number of cells in the heart was relatively less than the decrease in the number of cells in the five major organs, making this decrease in the heart look like an increase [23].

Clinical Experience

Retention of cells in the heart was determined in one clinical trial, showing inferiority of RCVI versus IC infusion [31]. Cells labeled with Tc^{99m}-hexamethylpropylenamineoxime were used to assess retention in the heart. Just like the three pig studies, sample size was small and retention rates were comparable [9,23,24].

Functional Outcomes

The goal of cardiac reparative therapy is improvement of cardiac function or decrease of disease characteristics such as angina complaints in order to improve quality of life and decrease mortality. Here, we focused on the effect of cell administration on 1) LVEF (AMI, CMI, CHF), 2) improvement on the Canadian Cardiovascular Society scale (CSS) (CRA), and 3) myocardial perfusion (CRA).

Table 3 Safety and mortality data

	Study	Species	Reported safety aspects	Mortality related to retrograde infusion procedure
Small Animals	Di Lascio [10]	Rat	no arrhythmias, described as safe	16,7% (11/66) probably related to thoracotomy)
	Fukushima [11]	Rat	more VPC and VT in IM group vs RCVI group, described as safe	RCVI: 5.7% (2/35) vs IM: 5.9% (2/34)
	Fukushima [12]	Rat	more VPC and VT in IM group vs RCVI group, described as safe	RCVI: 8.2% (4/49) vs IM: 8.3% (4/48)
	Huang [13]	Rat	ns	ns
	Huang[14]	Rat	no arrhythmias	conventional technique: 42.9% (3/7) modified technique: 0
	Suzuki [15]	Rat	no arrhythmias, described as safe	29% (18/62) within 24 hours, probably due to acute heart failure
	Suzuki [16]	Rat	no arrhythmias	0%
	Zakharova [17]	Rat	ns	0%
Large Animals	Pogue [18]	Dog	Transient AF during procedure in 6/15 dogs, described as safe	0%
	Sun [19]	Dog	ns	0%
	Wang [20]	Dog	no arrhythmias, no cardiac tamponade, described as safe	0%
	Formigli [21]	Pig	ns	0%
	Hagikura [22]	Pig	no arrhythmias, described as safe	0%
	Hong [23]	Pig	No MVO, described as safe	0%
	Hou [24]	Pig	ns	0%
	Kupatt [9]	Pig	ns	ns
	Lu [25]	Pig	ns	ns
	Prifti [26]	Pig	no arrhythmias, described as safe	0%
	Sato [27]	Pig	ns	0%
	Vicario [28]	Pig	no arrhythmias	ns
	Yokoyama [29]	Pig	described as safe	ns
Clinical Trials	Patel [30]	Human	Rise in cardiac enzymes in some patients, no arrhythmias associated with RCVI, described as safe	0%
	Silva [31]	Human	Rise in cardiac enzymes in some patients	0%
	Tuma [33]	Human	No rise in cardiac enzymes, no arrhythmias, no pericardial effusion, described as safe	0%
	Tuma [32]	Human	No arrhythmias, rise in cardiac enzymes in all patients, no evidence of CS leak or damage, described as safe	0%
	Vicario [34]	Human	no arrhythmias, described as safe	0%
	Vicario [35]	Human	no arrhythmias, described as safe	0%

VPC = ventricular premature contraction, *VT* = ventricular tachycardia, *IM* = intramyocardial injection, *RCVI* = retrograde coronary venous infusion, *ns* = not specified, *AF* = atrial fibrillation, *MVO* = microvascular obstruction, *CS* = coronary sinus

Preclinical Experience

Most of the preclinical studies that reported changes in LVEF (12/15) showed a significant increase in LVEF versus baseline and/or controls. Three studies only showed improvement of LVEF when cells were combined with growth factors [22] or no effects on LVEF at all [18,19].

Clinical Experience

Three out of four clinical studies reported significant improvement of LVEF. The study that did not show improvement of LVEF after RCVI compared IC infusion with RCVI and reported that patients receiving cells through IC infusion did show improvement in LVEF [31]. The difference in cell retention between IC infusion and RCVI in these patients might be the explanation for this difference in functional outcome. Two other studies show comparable retention rates between IM injection and

RCVI and both groups show comparable functional gains [11,12]. In case of CRA, changes in CCS scale and improvement in myocardial perfusion were reported [33-35].

In the majority of cases, cells administered with RCVI are able to effectuate improvement of cardiac function in a range of different experimental models. An overview of functional outcomes is presented in Supplemental Material 2.

Table 4 Retention of cells in the heart

						Application method				
	Study	**Species**	**#**	**Retention method**	**Retention timepoint**	**RCVI retention**	**IC retention**	**IM retention**	**Peripheral IV retention**	**Sign comparison**
Small Animals	Fukushima [11]	Rat	35	Sry	3 d	1.84±0.27 %	-	1.45±0.27 %	-	ns
	Fukushima [12]	Rat	85	Sry	3 d	10±5%	-	14±5 %	-	ns
	Huang [13]	Rat	90	Sry	24 h	2 % / 8.5 %[a]	-	-	-	P < 0.001[d]
	Suzuki [16]	Rat	20	β-galactosidase	10 min	31.4±4.8 %	-	-	-	na
	Suzuki [15]	Rat	62	β-galactosidase	10 min	29.8 ± 6.9 %	-	-	-	na
Large Animal	Hong [23]	Pig	7	Radiolabel	1 h	±8 % [c]	±25 % [c]	-	-	P = 0.037
	Hou [24]	Pig	5	Radiolabel	1 h	3.2±1 %	2.6±0.3 %	11.3±3 %	-	Not sign[b]
	Kupatt [9]	Pig	6	Radiolabel	1 h	2.7 %	-	-	0.5 %	ns
Clinical trials	Silva [31]	Human	9	Radiolabel	4 h	4.62%	16.14%	-	-	P = 0.01

In case retention was not measured as % of total administered dose (e.g. as a % of uptake in major organs), we calculated the retention % of total administered dose. This was the case in one study [23]

Sry = polymerase chain reaction for the Y-chromosome specific Sry gene, *β-galactosidase* = presence of β-galactosidase expressing cells, *radiolabel* = retention measured by scintigraphy after radiolabeled cell infusion. *RCVI* = retrograde coronary sinus/venous infusion, *IC* = intracoronary infusion, *IM* = intramyocardial injection, *IV* = intravenous, *ns* = not specified, *na* = not applicable, #: number of subjects, [a]: 2% in case of normal delivery, 8.5% in case of magnetic targeting, [b]: Comparison between RCVI infusion and IM retention, [c]: corrected for total injected dose, [d]: normal delivery versus magnetic targeting

Discussion

Cell delivery strategies should meet two important demands. First and foremost, the technique should be safe. Second, it should be effective in delivering cells to the heart. In this paper, we provided an overview of RCVI.

There is a high degree of heterogeneity regarding technical aspects of RCVI both between and within species. Furthermore, roughly 20% of studies do not adequately describe procedural characteristics, which hampers the possibility to repeat these experiments technically.

The main finding is that relevant data regarding technique and safety are poorly reported. For instance, 30% of included studies do not report on safety aspects of RCVI at all, while 33% only report absence of arrhythmias without mentioning other safety parameters. Only a limited number of studies provide more in-depth safety information regarding RCVI. The six clinical trials included in this overview report cardiac enzyme rise as the only safety issue associated with RCVI and show no arrhythmias associated with RCVI, no development of pericardial fluid, and no sustained damage to

the CS after RCVI. It is understandable that the first priority of research focused on cell therapy lies with validating the effectiveness of cell therapy in itself. From this perspective, it is logical that some studies do not report on safety of delivery because this was not the purpose of the study. Nevertheless, due to the poor reporting of safety aspects we cannot make an accurate assessment of the safety profile of RCVI.

However, retrograde accessing of the coronary venous system has been performed for a long time in the field of cardiac surgery in a great number of patients. With retrograde cardioplegia (RC), the myocardium is retrogradely perfused during cardiac surgery to induce cardiac arrest and protect the myocardium. With RC, a balloon-catheter is used to occlude the opening of the CS, in a way comparable to RCVI. RC is reported to be safe, with injury to the CS occurring in 0.06% to 0.6% of patients [36,37], resulting in formation of hematoma on the atrioventricular groove, perforation of the CS wall, pericardial effusion, or laceration of the right ventricle or CS [37-40]. These data would suggest that the technical part of RCVI, namely the insertion of a balloon-tipped catheter in the CS followed by infusion of fluid, should be safe.

Cells delivered through RCVI are able to improve cardiac function and alleviate angina symptoms. However, in terms of cell retention, the data suggest that RCVI is a limitedly effective delivery strategy for cell therapy. In fact, IC infusion and IM injection show either higher or equal retention rates. It is likely that inferior retention rates decrease the efficacy of RCVI.

Due to the limited number of studies included in this review, we cannot conclude that RCVI is favorable in certain disease types or that certain cell types performed better than others in the included studies.

In conclusion, the available data on technical and safety aspects of RCVI are insufficient and incomplete. Furthermore, retention data show inferior results compared to IC infusion and IM injection. We conclude that at present, there are not enough arguments to proceed with this technique in the clinical arena. Well-designed confirmatory studies on retention rates and safety data are required to proceed with RCVI in the future.

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Conflict of Interest

All authors declare to have no conflict of interest.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Supplemental Material

Supplemental Material 1: Search Identification

PubMed and EMBASE libraries were searched for relevant articles on May 15th 2017.
Duplicates were removed using RefWorks. Any duplicates that were not properly removed with RefWorks were excluded by hand. After duplicate removal, title/abstract screening was performed followed by full text screening. One article was later added that was found through cross-reference check. Only full text original papers published in English were included in this review. Studies that used transvenous injection were not included in this review. Two researchers were involved in the search and screening phase: W.A. Gathier and D.J. van Ginkel. The search string is presented in the appendix of this article. Figure 1 shows the flowchart depicting the search outcome.

PubMed:
Retrograde intravenous[Title/Abstract] OR Retrograde venous[Title/Abstract] OR Coronary sinus[Title/Abstract] OR Coronary vein[Title/Abstract] OR Coronary veins[Title/Abstract] OR Coronary venous[Title/Abstract] OR Coronary vessel[Title/Abstract] OR Coronary vessels[Title/Abstract] OR Retrograde intracoronary[Title/Abstract]

AND

Retroinfusion[Title/Abstract] OR Retro-infusion[Title/Abstract] OR Infusion[Title/Abstract] OR Infusions[Title/Abstract] OR Infused[Title/Abstract] OR Infusing[Title/Abstract] OR Delivery[Title/Abstract] OR Delivered[Title/Abstract] OR Delivering[Title/Abstract] OR Deliver[Title/Abstract] OR Administer[Title/Abstract] OR Administered[Title/Abstract] OR Administering[Title/Abstract] OR Administration[Title/Abstract] OR Route[Title/Abstract] OR Routes[Title/Abstract]

Embase:
'Retrograde intravenous':ti,ab OR 'Retrograde venous':ti,ab OR 'coronary sinus':ti,ab OR 'coronary vein':ti,ab OR 'coronary veins':ti,ab OR 'coronary venous':ti,ab OR 'coronary vessel':ti,ab OR 'coronary vessels':ti,ab OR 'retrograde intracoronary':ti,ab AND [embase]/lim NOT [medline]/lim

AND

'retroinfusion':ti,ab OR 'retroinfusions':ti,ab OR 'retro-infusion':ti,ab OR 'retro-infusions':ti,ab OR 'infusion':ti,ab OR 'infusions':ti,ab OR 'infusing':ti,ab OR 'infused':ti,ab OR 'deliver':ti,ab OR 'delivered':ti,ab OR 'delivery':ti,ab OR 'delivering':ti,ab OR 'administer':ti,ab OR 'administered':ti,ab OR 'administering':ti,ab OR 'administration':ti,ab OR 'route':ti,ab OR 'routes':ti,ab AND [embase]/lim NOT [medline]/lim

Two articles described the same dataset (Moreira et al. [8] and Silva et al. [31] of which only data from Silva et al. is included in this review to prevent double reporting of data. Two other articles describe the same patient dataset [34,35]. We decided to include both studies in this review because one of these articles presents one-year follow up data [35]. We ended up with a total of 28 articles, of which 27 were included in this review after exclusion of Moreira et al. All articles were published between 2003 and 2016.

Supplemental Material 2: Functional outcomes after RCVI

Supplemental table 2 Functional outcomes presented as improvement in LVEF and/or myocardial perfusion and/or CCS score

	Study	Change in LVEF (%) myocardial perfusion or CCS	Follow up timepoint	Versus	P- value
Small Animals	Di Lascio [10]	*LVEF: Echocardiopgraphy*			
		12 ± 11.2 % (cells)	1 month	Baseline	P=0.0016
		16 ± 8.9 % (cells + RLX)		Baseline	P<0.0001
	Fukushima [11]	*LVEF: Echocardiopgraphy*	7 days		Described as
		15.5 ± 1.7		Baseline	significant
	Fukushima [12]	*LVEF: Echocardiopgraphy*	7 days		
		9.4 ± 0.9 %		Baseline	P<0.05
	Huang [13]	*LVEF: Echocardiopgraphy*			
		~5 %	3 weeks	PBS controls	P<0.05
		~12 % (magnetic targeting)		PBS controls	P<0.05
	Suzuki [15]	*LVEF: Echocardiopgraphy*			
		10.7 ± 4.0 %	4 weeks	PBS controls	P<0.05
		11.1 ± 3.7 %		Sham	P<0.05
	Zakharova [17]	*LVEF: Pressure volume derived*	3 weeks		
		11.4 ± 6.7 %		DMEM controls	P<0.05
Large Animals	Formigli [21]	*LVEF: Echocardiography*			
		~17 % (cells)	1 month	DMEM controls	P<0.001
		~20 % (cells + RLX)		DMEM controls	P<0.001
	Hagikura [22]	*LVEF: Pressure volume*			
		0.5 ± 3.8 % (cells)	4 weeks	Saline controls	Described as not significant
		9.7 ± 3.1 % (cells + VEGF)		Saline controls	P<0.05
	Lu [25]	*LVEF: Echocardiopgraphy*			
		~5 % (cells + Adnull)		Baseline	P<0.05
		~11 % (cells + VEGF)		Baseline	P<0.05
		~27 % (cells + HGF)		Baseline	P<0.05
		LVEF: SPECT	4 weeks		
		~8 % (cells + Adnull)		Baseline	P<0.05
		~10 % (cells + VEGF)		Baseline	P<0.05
		~22 % (cells + HGF)		Baseline	P<0.05
	Pogue [18]	*LVEF: Echocardiopgraphy*			
		-28.5 %	2 years[d]	Baseline	P<0.001

Clinical Trials	Prifti [26]	*LVEF: Echocardiopgraphy* 12 ± 9.8 % *LVEF: SPECT* 13 ± 8.6 %	1 month	Controls (no infusion procedure) Controls (no infusion procedure)	P=0.001 P=0.001
	Sato [27]	*LVEF: Left ventriculography* Preserved, no data ~12 %	4 weeks	Baseline DMEM controls	No p-value P<0.01
	Sun [19]	*LVEF: Echocardiopgraphy* -0.1 ± 12.2 % (cells) -3.1 ± 12.9 % (cells + bFGF)	40 days	Baseline Baseline	Both described as not significant
	Wang [20]	*LVEF: Echocardiopgraphy* ~ 7 % (cells) 11 % (cells + bFGF) 14.9 ± 3.8 % (cells) 17.5 ± 3.3 % (cells + bFGF)	4 weeks	Baseline Baseline Saline controls Saline controls	P=0.124 P<0.01 P<0.01 P<0.001
	Yokoyama [29]	*LVEF: Pressure volume derived* ~5 % (AMI) ~6 % (OMI)	4 weeks	Baseline Baseline	P<0.05 P<0.05
	Patel [30]	*LVEF: Left ventriculography* 6.0 % (niCMP) 8.1 % (iCMP) not specified (niCMP) not specified (iCMP)	12 months	Baseline Baseline Controls, no infusion procedure Controls, no infusion procedure	P=0.007 P=0.006 P=0.954 P=0.814
	Silva [31]	*LVEF: Radionuclide ventriculography* 0.4 ± 14.3 %	6 months	Baseline	P=0.88
	Tuma [32]	*LVEF: Echocardiopgraphy* 4.1 ± 7.4 % (100M cells) 11.3 ± 6.9 % (200M cells) 13 ± 6.5 % (400M cells)	12 months[a]	Baseline Baseline Baseline	P<0.05 P<0.05 P<0.05
	Tuma [33]	*CCS class* 1.4 ± 0.7 *Myocardial perfusion: SPECT*[c] 14.7 % *LVEF: SPECT* 4.3 %	2 years[b]	Baseline Baseline Baseline	P<0.001 P=0.001 P=0.019

Baseline = timepoint before cell infusion, *follow up timepoint* = time after cell infusion, *LVEF* = left ventricular ejection fraction, *RLX* = relaxin, *PBS* = phosphate buffered saline, *DMEM* = Dulbecco's modified Eagle medium, *VEGF* = vascular endothelial growth factor, *Adnull* = null adenovirus, *HGF* = hepatocyte growth factor, *SPECT* = single-photon emission computed tomography, *bFGF* = basic fibroblast growth factor, *AMI* = acute myocardial infarction, *OMI* = old myocardial infarction, *niCMP* = non-ischemic cardiomyopathy, *iCMP* = ischemic cardiomyopathy, *M* = million, *CCS* = Canadian Cardiovascular Society, ~: approximate, in case of figure only in source text

[a]: follow up also occurred at 1 and 4 months post cell infusion. The 100M cell group only showed significant increase in LVEF at 12 months post infusion

[b] follow up also occurred at 1 year

[c]: absolute reduction in ischemic myocardium

[d]: only 4/15 dogs were alive at this point. LVEF never significantly improved during 2 years

If changes in cardiac function had to be determined by calculating the difference between cardiac function at baseline and follow up or between cases and controls, the standard deviation of the change in cardiac function was calculated by taking the square root of the sum of the squares of the standard deviations belonging to cardiac function at baseline and at follow up, or cases and controls.

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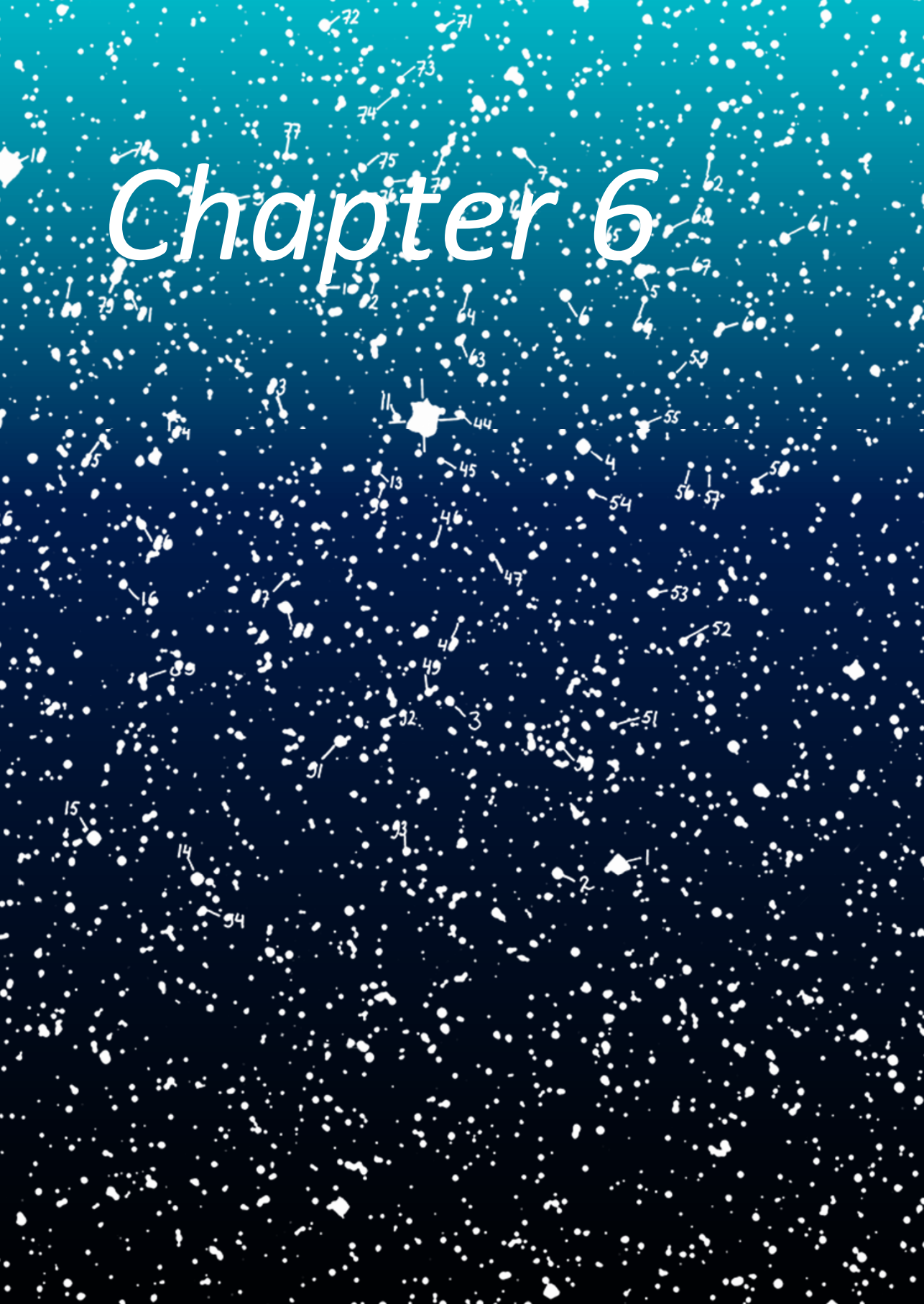
Chapter 6

Lower Retention after Retrograde Coronary Venous Infusion Compared with Intracoronary Infusion of Mesenchymal Stromal Cells in the Infarcted Porcine Myocardium

Wouter A. Gathier[1], Mira van der Naald[1], Bas R. van Klarenbosch[1], Anton E. Tuinenburg[1], John L.M. Bemelmans[2], Klaus Neef[1,4], Joost P.G. Sluijter[3,4,6], Frebus J. van Slochteren[1], Pieter A. Doevendans[1,4,5,6], Steven A.J. Chamuleau[1,4]

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[1] Department of Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands
[2] Department of Nuclear Medicine, University Medical Center Utrecht, Utrecht, The Netherlands
[3] Department of Experimental Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands
[4] Regenerative Medicine Center Utrecht, Utrecht, The Netherlands
[5] Central Military Hospital, Utrecht, The Netherlands
[6] NL-HI (Dutch Heart Institute), Utrecht, The Netherlands

Abstract

Background

Commonly used cell delivery strategies to the heart are intramyocardial injection and intracoronary (IC) infusion, both having their advantages and disadvantages. Therefore, alternative strategies are explored, such as retrograde coronary venous infusion (RCVI). The aim of this confirmatory study was to compare cardiac cell retention between RCVI and IC infusion. As secondary endpoint, the procedural safety of RCVI is assessed.

Methods

Four weeks after myocardial infarction, twelve pigs were randomized to receive mesenchymal stromal cells, labeled with Indium-111, via RCVI (n=6) or IC infusion (n=6). Four hours after cell administration, nuclear imaging was performed to determine the number of cells retained in the heart both *in vivo* and *ex vivo.* Procedural related safety measures were reported.

Results

Cardiac cell retention is significantly lower after RCVI compared to IC infusion (*in vivo:* RCVI: median 2.89% vs IC: median 13.74%, p=0.002, *ex vivo:* RCVI: median 2.55% vs IC: median 39.40%, p=0.002). RCVI led to development of pericardial fluid and hematomas on the frontal wall of the heart in 3 cases. Coronary venous dissection after RCVI was seen in 3 pigs, of which one also developed pericardial fluid and a hematoma. IC infusion led to no-flow in one pig.

Conclusion

RCVI is significantly less efficient in delivering cells to the heart compared to IC infusion. RCVI led to more procedural related safety issues than IC infusion, with multiple cases of venous dissection and development of hematomas and pericardial fluid collections.

Introduction

Cell therapy is a suggested as a potential treatment option for ischemic heart disease, yet only moderate improvement in cardiac function is achieved [1, 2]. The delivery of cells to the myocardium is an important limitation of current cell injection methodologies [3]. The ideal strategy is safe, easy to perform and efficient in cell delivery. Intracoronary (IC) infusion and intramyocardial (IM) injection have been thoroughly tested [4-7]. Both techniques present with disadvantages such as the need for patent coronary arteries and the risk of embolization leading to decreased blood flow in case of intracoronary infusion [8-10]. The IM injection procedure is time consuming and requires specialized equipment in the catheterization laboratory. Furthermore, rapid loss of cells via venous drainage is seen after IM injection [11]. Alternative delivery strategies could possibly overcome these drawbacks. Retrograde coronary venous infusion (RCVI) is less commonly applied, but could be a good alternative to IC infusion and IM injection. However, the available data on technical and safety aspects of RCVI are insufficient and incomplete. At present, there are not enough arguments to proceed with this technique in the clinical arena because well-designed confirmatory studies on retention rates and safety data are required to prove its value [12].

With RCVI, cells are retrogradely infused in the coronary venous system, which is typically free of atherosclerotic disease, and therefore could potentially improve delivery to the target area compared to IC infusion. An important limitation of cardiac cell therapy is the retention of cells in the heart after delivery. IM injection and IC infusion show comparable retention rates of 10-15% [4, 13, 14]. However, there is only limited data available on safety and the retention of cells in the heart after RCVI in large animal models and in the clinical setting. Currently, no direct comparison is available on cardiac cell retention after RCVI versus IC infusion in the setting of chronic myocardial ischemia. In view of future clinical trials, it is important to determine whether RCVI is a good alternative to IC infusion. Therefore, the aim of this confirmatory study is to compare the retention rates of radiolabeled mesenchymal stromal cells (MSCs) in the heart after RCVI and IC infusion and provide an estimate of safety of RCVI in a porcine model of chronic myocardial infarction. We did not aim to provide data on cardiac repair because animals were terminated four hours after cell infusion to enable ex-vivo scintigraphy of different organs.

Methods

Ethical statement

All animals received care in compliance with the “Guide for the Care and Use of Laboratory Animals”, published by the National Institutes of Health (National Institutes of Health publication 85-23, revised 1985). The study protocol was approved by the Animal Experiment Committee of the University of Utrecht and the governing national Central Animal Experiment Committee (AVD115002015257, 105119-2). It was not possible to perform this experiment without animals due to the fact that the hemodynamics and biologic nature of the heart and the whole body cannot be replicated in such a way that the results of this study would be translatable to the real situation. We minimized the

number of animals used by performing a sample size calculation beforehand. Refinement was done by using proven techniques, performed by trained personnel. Furthermore, maximum effort was put into ensuring the best conditions for the animals in terms of housing, enrichment, and analgesia.

Study design

Myocardial infarction (MI) was induced in sixteen female Dutch Topigs pigs (Van Beek SPF varkensfokkerij B.V., Lelystad, The Netherlands). Pigs were selected as the preferred animal for this experiment because of the resemblance of the pig and human heart in terms of anatomy and hemodynamics. Animals that survived four weeks after MI (n=12) were randomized (1:1) to receive MSCs labeled with Indium-111 (In^{111}) via RCVI (n=6) or IC infusion (n=6). Randomization was performed using a closed envelope system. Nuclear imaging was carried out four hours after MSC delivery, after which the anesthetized animals were euthanized by potassium chloride overdose. Nuclear imaging data was analyzed by lab technicians blinded to the infusion procedure.
The protocol of this study was registered on https://www.preclinicaltrials.eu/ (PCTE0000104) and the ARRIVE guidelines were followed for reporting. Heart rate, mean arterial pressure, left ventricular internal diameter at diastole and systole (LVIDd, LVIDs) were determined prior to MI (baseline) and directly prior to cell infusion.

Experimental outcomes

The primary endpoint of this study is retention of radiolabeled cells in the heart four hours after delivery. Cell retention was determined *in vivo* and *ex vivo*. *In vivo* analysis was performed by nuclear total body imaging of the live pig after which the percentage of total radioactive signal (counts) coming from the heart was divided by the total radioactive counts coming from the total body of the pig, including the bladder catheter. Because the heart is partially superimposed over the lungs during total body scanning, termination of the pigs and *ex vivo* scanning of individual organs (heart, lungs, kidneys, liver, spleen) was performed directly after *in vivo* scanning to check whether this superposition would influence the results of the total body imaging. The total radioactive signal (counts) coming from the heart was then divided by the sum of all radioactive counts coming from all aforementioned organs. The secondary endpoint is safety in terms of procedural related complications such as occurrence of vessel dissections, flow obstruction during or after cell administration, development of pericardial effusion, and development of hematomas on the left ventricular wall. Experimental setup is shown in figure 1.

Experimental procedures

Anesthesia and Analgesia

Prior to MI induction, all animals received a Butrans patch 5 μg/h. Animals were pretreated with Amiodarone (1200 mg/day, 7 days), Clopidogrel (75 mg/day, 3 days) and Carbasalate Calcium (loaded with 320 mg, 1 day), which was continued until the end of the experiment (daily dose 80 mg). Premedication (ketamine 10-15 mg/kg, midazolam 0.7 mg/kg, and atropine 0.5 mg) was delivered intramuscularly. Anesthesia was induced with thiopental sodium 4 mg/kg delivered through the ear vein. General anesthesia and analgesia were maintained with a bolus of midazolam 10 mg and

sufentanil 0.25 mg followed by intravenous delivery of midazolam 1 mg/kg/h, sufentanil 10 µg/kg/h, and pancuronium bromide 0.1 mg/kg/h. Animals received 300 mg amiadarone in 500 ml venofundin 6% infused in 30 minutes. Mechanical ventilation was performed using a mixture of O2 and air (1:2) with a tidal volume of 10 ml/kg with 12 breaths per minute. Animals received 5000 IU of heparin every two hours during the procedure.

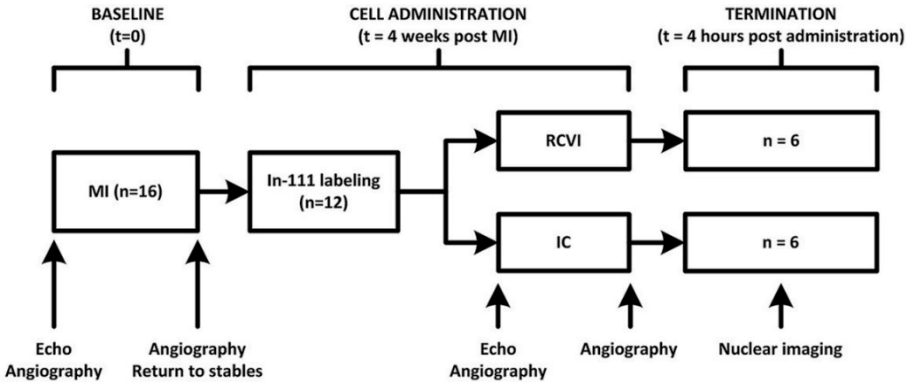

Fig. 1 Experimental setup. *MI* = myocardial infarction, *IC* = intracoronary infusion, *RCVI* = retrograde coronary venous infusion, *t* = timepoint, *n* = number of animals

Myocardial infarction

Myocardial infarction was induced percutaneously by a temporal (90 minute) occlusion of the left anterior descending artery (LAD) using an angioplasty balloon. The preferred occlusion site was after diagonal branch two, but the infarct site was determined per pig based on the anatomy of the coronary arteries (thickness and tract). In case of ventricular fibrillation or ventricular tachycardia without output, 200-joule shocks were delivered using an external defibrillator in order to restore sinus rhythm. Chest compressions were given between shocks to maintain circulation. In addition, amiodarone (maximum of 3 times 150 mg), adrenalin (0.1 mg) and/or atropine (0.5 mg) were administered. Arterial blood pressure, ECG and capnogram were monitored during the entire procedure.

MSC culture and In^{111}-labeling

Allogeneic MSCs were isolated and cultured in αMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 0.2 ng/ml vitamin C (Sigma-Aldrich, St. Louis, MO, USA), 1 ng/ml basic fibroblast growth factor (Sigma-Aldrich, St. Louis, MO, USA) and 1% Penicillin/Streptomycin. The cells were incubated at 37°C and medium was changed every 3 days. Cells were cultured in 75cm^2 flask and passaged when they reached confluence, until passage 2–3. MSCs were frozen in 10% dimethylsulfoxide and 90% culture medium. Characterization of MSCs was performed as previously described [15, 16]. Seven days prior to transplantation, MSCs were thawed, plated in flasks, and grown to confluence, until passage 5–7. At the day of cell delivery, 10^7 MSCs were labeled with Carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA, USA) dissolved in DMSO

(Sigma-Aldrich, St. Louis, MO, USA) to a concentration of 5 mM after which cells were trypsinized and subsequently labeled with a median of 36.3 [interquartile range (IQR) 33.5 – 40.5] megabecquerel (MBq) of In^{111} at 37°C for 20 minutes. After incubation, cells were washed up to three times with Hank's Balanced Salt Solution $CaCl_2$+ $MgCl_2$+ (Life Technologies Corp, Grand Island, NY, USA) to remove unbound label. Radiolabel uptake efficiency was measured with a dose calibrator. After labeling, cell viability was assessed via trypan-blue (Sigma-Aldrich, St. Louis, MO, USA) counting. Before injection, MSCs were resuspended in 10 ml phosphate buffered saline pH 7.4 (Life Technologies Corp, Grand Island, NY, USA).
The protocol on labeling of MSCs with In^{111} can be found at: *https://www.protocols.io/view/labeling-of-porcine-mesenchymal-stromal-cells-mscs-mr9c596*

Histochemistry
Directly after termination, representative myocardial tissue samples were collected from areas of the heart that showed activity during nuclear imaging and were snap frozen in liquid nitrogen. Tissue samples were cut with the Cryostar NX70 (ThermoFisher, Waltham, MA, USA) at 10 µM. The EVOS FL (ThermoFisher, Waltham, MA, USA) cell imaging system was used to check for CFSE positivity. Histological samples were subsequently fixed with acetone, permeabilized with 0,1% Triton X100 (Sigma-Aldrich, St. Louis, MO, USA), and blocked with 10% normal goat serum S-1000 (Vector Laboratories, Burlingame, CA, USA). Monoclonal Anti-α-Actinin (Sarcomeric) Mouse anti-Human (Sigma-Aldrich, St. Louis, MO, USA) (1:350) was used as the primary antibody followed by the secondary antibody Goat anti-Mouse-568 (1:350) (Invitrogen, Carlsbad, CA, USA), and 1 µg/ml Hoechst 33342 (Invitrogen, Carlsbad, CA, USA). Samples were mounted with fluormount-G (ThermoFisher, Waltham, MA, USA). Imaging was performed with a confocal Leica SP8X microscope (Leica, Amsterdam, Netherlands).

Retrograde coronary venous infusion
Two different infusion catheters were used for RCVI. In case the coronary sinus (CS) was ≥ 5 mm in diameter a dedicated CS infusion catheter was used (Advance® CS Coronary Sinus Infusion Catheter, Cook Medical, Bloomington, IN, USA). In case the diameter of the CS was < 5 mm, an over the wire balloon catheter (Advance® 35LP Low-Profile PTA Balloon Dilatation Catheter, Cook Medical, Bloomington, IN, USA) was used. Balloons were inflated at low pressure (maximum of 2 atmosphere) in the CS after which a venogram was made to ensure total occlusion of the CS. When total occlusion was observed, 2 ml of cell suspension followed by 8 ml of sodium chloride 0.9% was infused during 60 seconds. This procedure was performed a total of five times in order to infuse a total of 10 ml of cell suspension flushed with 40 ml of sodium chloride 0.9% in five minutes. Occlusion of the CS was maintained for ten minutes after infusion to prevent washout of cells.

Intracoronary infusion
Intracoronary infusion was performed by placing an over-the-wire balloon (Emerge™ over-the-wire PTCA dilatation catheter, Boston Scientific Corp, Natrick, MA, USA) of equivalent size to the LAD at the same site where occlusion was created during MI induction. After inflation of the balloon at low

pressure, 3.3 ml of cell suspension was infused in 30 – 45 seconds. The balloon was deflated after three minutes to reinstate flow. After three minutes of flow, the procedure was repeated another two times to infuse a total of 10 ml of cell suspension.

Nuclear imaging and analysis

In vivo and *ex vivo* scintigraphy was performed four hours after MSC administration using a dual head gamma camera (Phillips Skylight). A whole-body scan was acquired at both 174 and 247 kiloelectronvolt energy windows using the following imaging parameters: medium-energy general-purpose collimator and 512 x 1024 projection matrix. The retained activity in syringes was measured with a dose calibrator (Azbil Telstar Benelux). Both anterior and posterior images were captured for each total body scan (*in vivo*) and each individual organ (*ex vivo*). The number of counts used for analysis was based on the geometric mean of the anterior and posterior counts. After *in vivo* scanning, regions of interest were placed over the major visceral organs and total body of the pig (figure 2), using manufacturer's software (JETStream workspace; Philips, Best, The Netherlands). The retention of In^{111}-labeled cells in the heart was calculated as a ratio of the total radioactive signal (counts) coming from the heart divided by the total counts coming from the total body of the pig (including bladder catheter), after correction for anatomy. After *ex vivo* scanning of individual organs, the retention of In^{111}-labeled cells in the heart was calculated as a ratio of the total radioactive signal (counts) coming from the heart divided by the total counts coming from all individual organs combined. Data analysis was performed by two to three laboratory analysts per animal coming from a pool of four analysts, supervised by an expert analyst, all blinded for treatment allocation.

Echocardiography

Transthoracic echocardiography (X5-1 probe, IE-33, Philips, Best, The Netherlands) was performed directly before MI induction and four weeks later, directly before MSC infusion. Chamber dimensions (LVIDd and LVIDs) were obtained in short-axis view at mid-papillary level. Analysis was performed in a blinded fashion by a trained physician.

Experimental animals

Sample size

A total number of twelve animals (median age and weight at time of MI: 20 weeks [IQR: 18 - 22], and 72 kilograms [IQR: 68 - 76] respectively) was allocated to receive MSCs via either RCVI (n=6) or IC (n=6) infusion. This sample size was predefined, and calculated for an α of 0.05, power of 80%, maximum standard deviation of 4%, and an expected maximum absolute difference in cell retention of 7.5%. Because four animals died during or after MI induction, a total of sixteen animals had to be used to include twelve animals in the analysis.

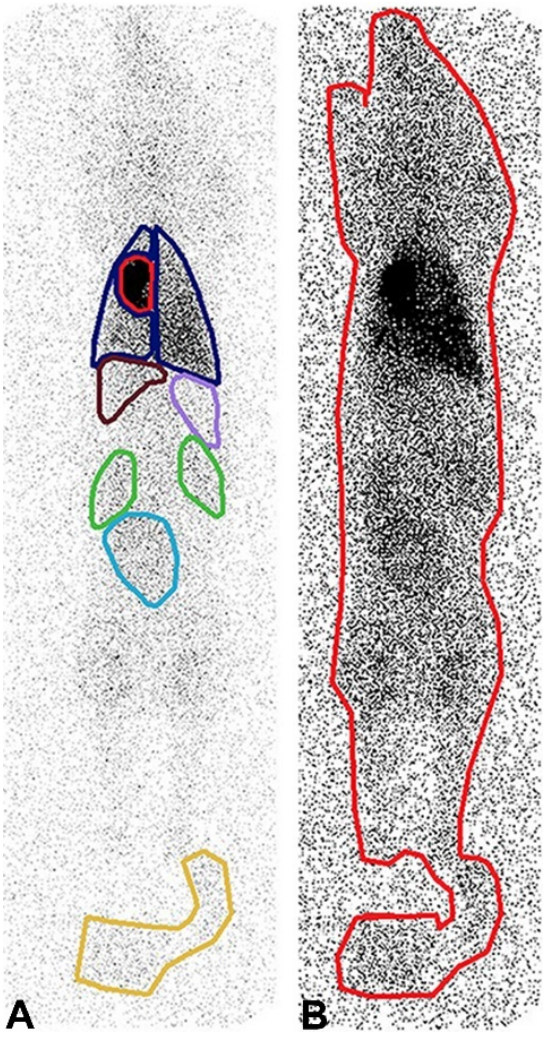

Fig. 2 Total body scintigraphy with regions of interest. **A**: regions of interest placed over visceral organs (heart in red, lungs in blue, kidneys in green, liver in brown, spleen in pink, bladder in light blue), and catheter bag in yellow. **B**: region of interest placed over total body of pig including catheter bag. Of note: both image **A** and image **B** are anterior captures. Both anterior and posterior images were captured for each animal and the number of counts used for analysis was based on the geometric mean of the anterior and posterior counts.

Housing

Animals were housed in stables with up to two pigs in the same stable before MI. After MI, animals were housed in separate stables to minimize stress. Animals were still able to see, smell, and hear each other through the grates that divide the stables. Straw was used for bedding and environmental enrichment was provided in the form of special rods that the animals could nibble on and play with. Welfare was assessed daily by animal caretakers.

Statistical analysis

Statistical analysis was performed using IBM SPSS statistics 25 (IBM, Armonk, New York, USA). Baseline characteristics and cell retention are presented as median with interquartile ranges. Comparison of data between two groups was performed using Mann-Whitney U test. A p-value of <0.05 was considered significant.

Results

Procedural data

Ventricular fibrillation (VF) during MI induction occurred in thirteen out of sixteen pigs, of which two died due to refractory VF. Another two pigs died in the stables due to acute heart failure or a heart rhythm disorder (day four and day nineteen) as a result of the MI. The remaining twelve pigs were randomized to RCVI (n=6) or IC infusion (n=6). No significant differences in heart rate, mean arterial pressure, LVIDd, and LVIDs were seen between groups as seen in table 1a, although a trend was seen towards a larger LVIDs in pigs that were allocated to IC infusion both at baseline and at follow up.

Table 1a Heart rate (HR), mean arterial pressure (MAP), left ventricular internal diameter at diastole (LVIDd) and systole (LVIDs) before myocardial infarction (baseline) and directly prior to cell infusion.

Table 1b Cell viability, total administered cells, and total administered live cells. Values are depicted as median with interquartile ranges.

1a	**Baseline**			**Prior to cell infusion**		
Parameter	**IC (n=6)**	**RCVI (n=6)**	**P-value**	**IC (n=6)**	**RCVI (n=6)**	**P-value**
HR (beats/min)	71 [63 – 81]	72 [66 – 73]	0.937	73 [64 – 82]	69 [63 – 76]	0.589
MAP (mmHg)	74 [67 – 89]	70 [64 – 88]	0.699	79 [72 – 87]	75 [72 – 82]	0.699
LVIDd (mm)	49 [48 – 50]	47 [46 – 49]	0.342	58 [47 – 59]	58 [47 – 62]	0.985
LVIDs (mm)	35 [34 – 37]	32 [29 – 34]	0.063	45 [40 -51]	38 [37 – 38]	0.115

1b			
Parameter	**IC (n=6)**	**RCVI (n=6)**	**P-value**
Cell viability after labeling (%)	66.8 [62.1 – 72.4]	53.6 [49.8 – 73.8]	0.418
Total administered cells (x 10^6)	3.2 [3.2 – 3.7]	2.8 [2.1 – 3.1]	0.180
Total administered live cells (x 10^6)	2.4 [1.6 – 2.4]	1.6 [1.3 – 1.7]	0.167

Cell viability and numbers

The median viability of MSCs after labeling with In^{111} was 66.8% [IQR: 62.1 – 72.4] in the IC group versus 53.6% [IQR: 49.8 – 73.8] in the RCVI group (p=0.418). The median total administered cells was 3.2M [IQR: 3.2 – 3.7] in the IC group versus 2.8M [IQR: 2.1 – 3.1] in the RCVI group (p=0.180) The median number of administered live cells was 2.4M [IQR: 1.6 – 2.4] in the IC group versus 1.6M [IQR: 1.3 – 1.7] in the RCVI group (p=0.167). Results are shown in table 1b.

Cell retention

In vivo analysis

A significant difference in MSC retention in the heart was seen between the RCVI and IC infusion group with a median retention of 2.89% [IQR: 2.14 – 3.86] in the RCVI group versus 13.74% [IQR: 10.20 – 15.41] in the IC infusion group (p=0.002).

No significant differences in cell retention were seen in lungs, kidneys, liver, spleen, and bladder between RCVI and IC infusion, although a trend was seen towards higher retention of cells in the lungs after RCVI. Data are presented in table 2 and figure 3a and 3b.

Table 2 *In vivo* analysis of activity in heart, lungs, kidneys, liver, spleen, and bladder as a percentage of total body activity. Values are depicted as median with interquartile ranges.

	Median activity (%) [interquartile range]	**Median activity (%) [interquartile range]**	**P-value**
Organ	**RCVI (n=6)**	**IC (n=6)**	
Heart	2.89 [2.14 – 3.86]	13.74 [10.20 – 15.41]	0.002
Lungs	35.45 [26.53 – 45.22]	22.07 [20.36 – 29.22]	0.132
Kidneys	1.39 [0.97 – 2.12]	2.32 [1.14 – 3.24]	0.240
Liver	2.76 [2.20 – 3.27]	2.95 [2.56 – 3.44]	0.310
Spleen	0.89 [0.61 – 1.08]	0.81 [0.77 – 1.05]	0.818
Bladder	0.96 [0.38 – 2.74]	0.88 [0.64 – 1.22]	0.937

Ex vivo analysis

In accordance with the *in vivo* results, a significant difference was seen in MSC retention in the heart between the RCVI and IC infusion group after *ex vivo* analysis. The median retention was 2.55% [IQR: 1.86 – 3.16] in the RCVI group versus 39.40% [38.54 – 44.64] in the IC group (p=0.002). Significant differences between RCVI and IC infusion were also seen for lung and liver retention (p=0.002 and p=0.04 respectively), with a significantly higher number of cells retained in the lungs after RCVI and a significantly higher number of cells retained in the liver after IC infusion. Data are represented in table 3 and figure 4.

Table 3 *Ex vivo* analysis of activity in heart, lungs, kidneys, liver and spleen as a percentage of the total counts coming from all individually scanned organs combined. Values are depicted as median with interquartile ranges.

	Median activity (%) [interquartile range]	**Median activity (%) [interquartile range]**	**P-value**
Organ	**RCVI (n=6)**	**IC (n=6)**	
Heart	2.55 [1.86 – 3.16]	39.40 [38.54 – 44.64]	0.002
Lungs	87.10 [76.95 – 90.13]	35.32 [30.22 – 46.53]	0.002
Kidneys	2.53 [1.77 – 4.03]	3.95 [1.95 – 5.28]	0.394
Liver	7.17 [5.28 – 14.83]	17.71 [13.74 – 21.01]	0.041
Spleen	0.78 [0.64 – 0.84]	0.99 [0.89 – 1.08]	0.065

Histological analysis

Histology shows CFSE labelled cells in the heart in the areas that are active on the scintigraphy. As expected, very few CFSE positive cells were found in the myocardial tissue samples from pigs belonging to the RCVI group because myocardial cell retention was low in these animals. In line with

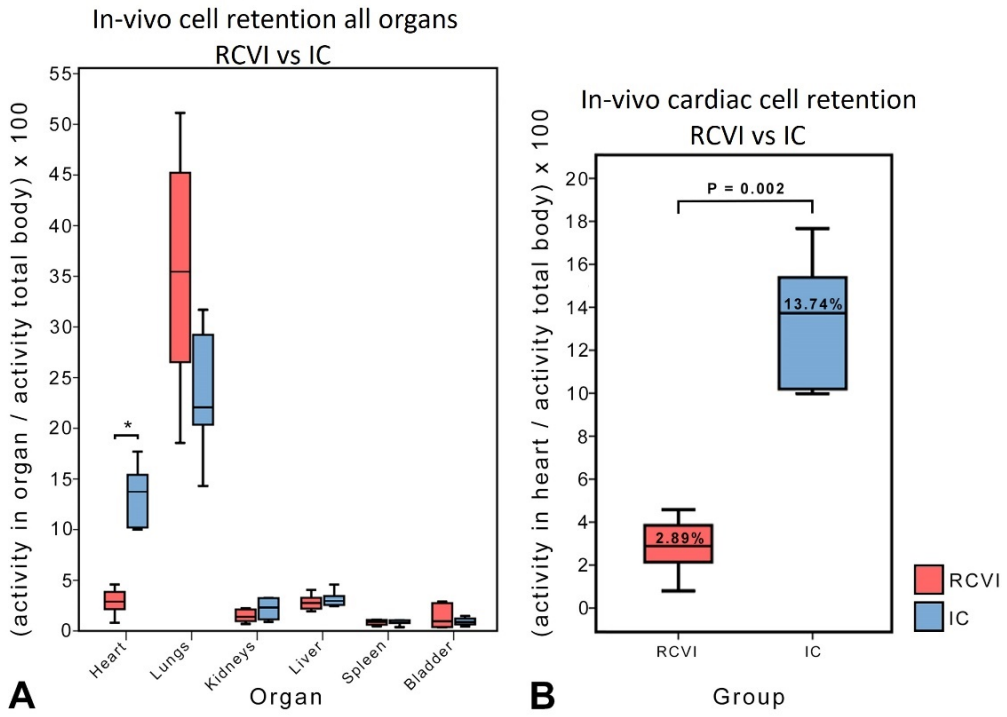

Fig. 3 In vivo retention of cells in major organs presented as a percentage of total body activity. **A**: In vivo analysis of activity in heart, lungs, kidneys, liver, spleen, and bladder presented as a percentage of total body activity: RCVI versus IC infusion. Only activity in the heart differed significantly between RCVI and IC infusion (* = p=0.002). **B**: Magnification of fig 3.A. Retention of MSCs in the heart is significantly worse after RCVI compared to IC infusion. *RCVI* = retrograde coronary venous infusion, *IC* = intracoronary infusion.

our expectations, CFSE positive cells were more abundant in tissue samples from the IC infusion group. Representative histological images are presented in figure 5.

Safety aspects

RCVI group

Dissection of the CS occurred in three out of six pigs at the site of the balloon catheter tip. Two animals with the largest dissection later showed a radioactive hotspot in the heart instead of a more disseminated activity pattern as would be expected in case of cell infusion. Cardiac cell retention in these two pigs was the highest of all RCVI pigs and well above the median of 2.89% with 3.86% and 4.59% (*in vivo* data*)*, respectively.

Three animals presented with a small to moderate, clear pericardial effusion and a hematoma of approximately four cm^2 on the atrioventricular groove of the left ventricle (LV) at termination. Only one animal was free of dissection and development of hematoma and pericardial fluid. In this one animal, the occlusion of the CS was found to be compromised after the infusion was completed,

possibly leading to direct drainage of cells into the right atrium. Nevertheless, the retention in this pig was 2.97% (*in vivo* data).

IC group

One animal in the IC group showed no-flow directly after cell infusion, probably due to thrombus formation. Flow was restored after five minutes of angioplasty.

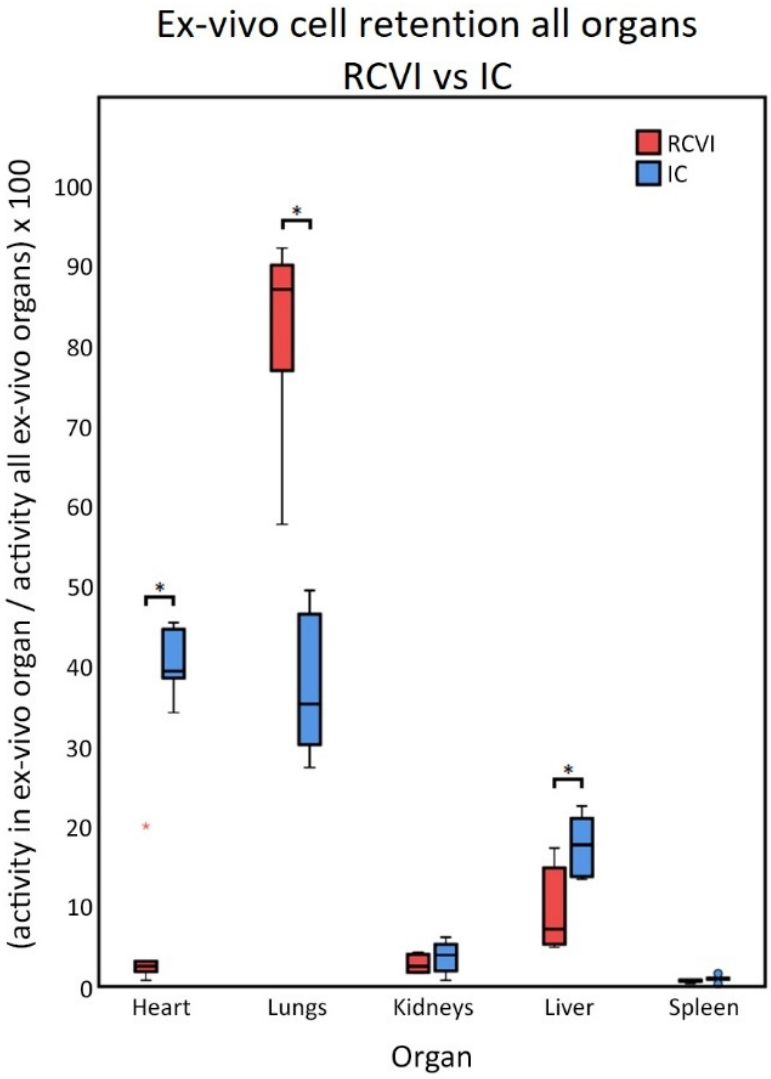

Fig. 4 Ex vivo retention of cells in individual organs. Ex vivo analysis of activity in heart, lungs, kidneys, liver, and spleen as a percentage of total activity from all individual organs combined. Activity in the heart, lungs, and liver differed significantly between RCVI and IC infusion ($p<0.05$) *RCVI* = retrograde coronary venous infusion, *IC* = intracoronary infusion.

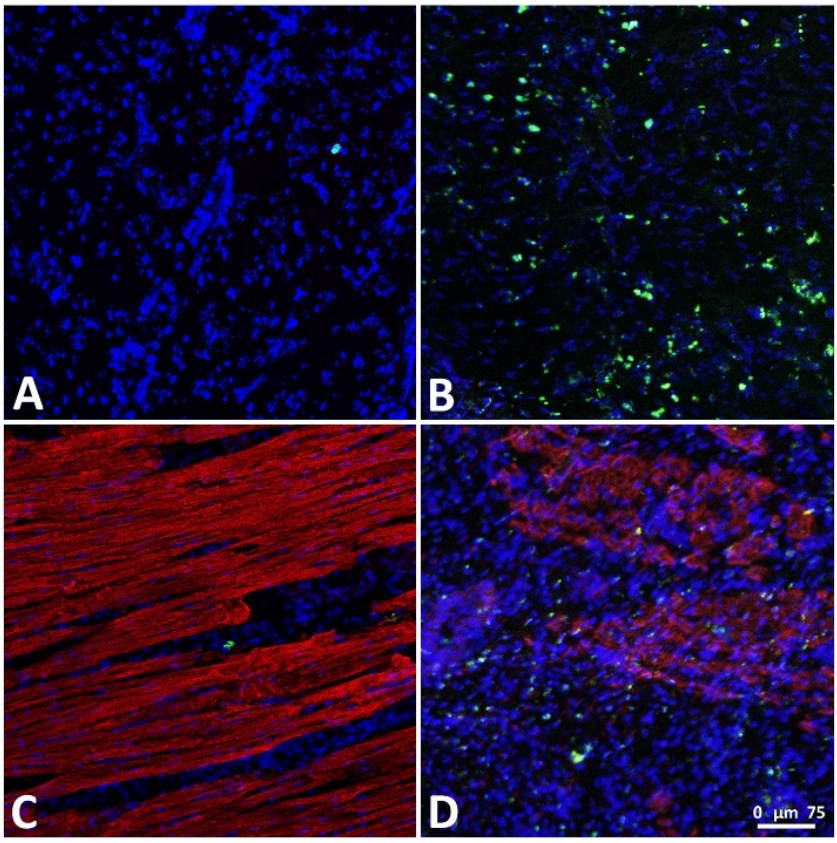

Fig. 5 CFSE positive cells in myocardial tissue samples. **A**: Myocardial tissue sample after RCVI, Hoechst staining and CFSE signal. **B**: Myocardial tissue sample after IC infusion, Hoechst staining and CFSE signal. **C**: Myocardial tissue sample after RCVI, Hoechst and α-actinin staining and CFSE signal. **D**: Myocardial tissue sample after IC infusion, Hoechst and α-actinin staining and CFSE signal. Blue = Hoechst signal, Red = α-actinin signal, Green = CFSE signal.

Discussion

Cell retention

The purpose of this study was to compare cardiac cell retention after RCVI and IC infusion and assess safety of RCVI. It was not possible to generate results on cardiac repair because the study design required termination of animals four hours after cell administration. To our knowledge, this is the first confirmatory study that directly compared retention between RCVI and IC infusion in a chronic MI pig model. We showed that RCVI of MSCs is inferior to IC infusion in terms of cardiac cell retention with RCVI showing a mean retention of 2.89% versus 13.74% with IC infusion (*in vivo* data). One can imagine that higher cell retention in the heart equals a greater effect of cell therapy, making IC infusion preferable over RCVI. We chose to infuse the same number of cells in both the IC infusion group and

the RCVI group in order to make sure that the results are comparable. Because retention of cells in the heart is calculated as a percentage of total administered cell dose in case of *in vivo* analysis, it is probable that a higher cell dose would not result in a higher retention rate. However, it is known that infusion of larger volumes of cells ($30 \times 10^6 - 50 \times 10^6$) via the IC route can result in a higher index of microcirculatory resistance [17, 18].
Currently, there is no evidence that larger cell volumes infused via the retrograde route would impair venous flow. This could implicate that more cells can be infused with RCVI, making up for the lower retention. The retention rates that we observed for IC infusion are comparable to results of other studies [4, 13, 14]. Three pig studies with small sample sizes (n=5, n=6 and n=7) and only 1 clinical trial (n=9) reported retention rates after RCVI in a model of acute myocardial infarction. However, no data on cell retention in a chronic ischemia model in large animals are available. Retention of cells, measured as radioactive positive signals coming from the heart, was low in these four trials, ranging from 3% - 8% of total injected activity, corresponding with our results [13, 14, 17, 19]. A possible explanation for the low retention of cells with RCVI is that cells are maintained in the CS after infusion but are directly flushed into the right atrium after abrogation of the CS occlusion and reinstitution of flow. This could explain the higher retention of cells in the lungs of RCVI treated animals. It is also possible that the cells are not adequately pushed though the microvascular bed as is the case with IC infusion, possibly effecting cell retention. Additionally, a low retention with RCVI could occur due to the existence of aberrant (and/or collateral) veins draining directly in the right atrium, effectively negating the blockade of the CS. An experienced cardiologist analyzed the fluoroscopy images made during cell infusion in this study and found anatomical variations of the coronary veins strongly suggesting the presence of aberrant venous drainage in three out of six RCVI treated animals. We could not find a relation between possible aberrant venous drainage and cell retention in these pigs, possibly due to the small number of pigs and other factors present such as coronary sinus dissection and pericardial effusion.

Differences between in vivo and ex vivo data

With *in vivo* analysis we only found a significant difference in cell retention in the heart between RCVI and IC infusion, while we find a significant difference in cardiac, pulmonary and hepatic retention after *ex vivo* analysis. Also, a different magnitude of retention is seen between *in vivo* and *ex vivo* measurements.
To explain these differences, it is important to understand that cell retention in organs is calculated differently for the *in vivo* analysis and *ex vivo* analysis. In case of *in vivo* analysis, cell retention in a certain organ (numerator) is calculated as a fraction of total body activity (denominator). In case of *ex vivo* analysis, cell retention in a certain organ (numerator) is calculated as a fraction of total *ex vivo* organ activity (denominator). The difference in denominator between *in vivo* and *ex vivo* analysis means that *in vivo* and *ex vivo* data cannot be directly compared to each other. However, both analyses are relevant. *In vivo* data show the percentage of total administered cell dose that is retained in the heart. However, *in vivo* analysis has a few shortcomings. With *in vivo* analysis, regions of interest (ROI's) are drawn around individual organs to determine the amount of radioactive counts coming from these organs. In this study, the regions of interest were determined by experienced technicians

and were accurately defined. However, there is always a margin of error with ROI definition for total body scans. When few cells are retained in an organ, the radioactive signal coming from this organ is low, making the contours of this organ difficult to discern from the surrounding tissue. This makes ROI definition more difficult in organs such as liver, spleen and kidneys, as seen in figure 2. Cardiac and pulmonary borders are usually easier to define, because of the higher retention in lungs and heart. In case of low cardiac retention, as is the case after RCVI, the cardiac border is still easy to define because of the large difference of signal between the heart and surrounding lung tissue. A second drawback of *in vivo* total body imaging is overprojection of organs such as heart and lungs. This could lead to overestimation of signal coming from the heart. Excision of organs followed by *ex vivo* scanning ensures that only counts coming from the individual organ are identified, overcoming errors caused by ROI definition and superposition of organs such as heart and lungs. Thus, differences in significance in pulmonary and hepatic retention between *in vivo* and *ex vivo* imaging can be explained by the difference in the way that retention is calculated, ROI definition and superposition of organs. However, total body scanning is the best option to determine the number of cells retained in the heart as a percentage of total administered cell dose. With *ex vivo* analysis, the retention of cells in the heart cannot be expressed as a percentage of total administered cells, because part of the activity and thus the cells are distributed outside of the organs, for instance in muscles and bloodpool. For this reason, we decided to incorporate both *in vivo* and *ex vivo* data in this study. Both methods show that cell retention is significantly lower in the RCVI group compared to the IC infusion group.

Safety aspects of RCVI

RCVI was associated with multiple safety issues in this study. We found pericardial fluid and hematoma development on the atrioventricular groove of the LV in three pigs and occurrence of CS dissection in three pigs, of which one also showed a hematoma and pericardial fluid at termination. Only one animal in the RCVI group was free of adverse events. It is striking that in this specific animal, the occlusion of the CS appeared to be incomplete after infusion. We do not know at which time point during the infusion procedure the occlusion was compromised.

In one animal, overinflation of balloon of the Advance® CS Coronary Sinus Infusion Catheter (>2 atmosphere) could have been the cause of development of a CS dissection, hematoma on the atrioventricular groove, and pericardial fluid collection.

The most likely explanation for the development of pericardial fluid and hematoma is a sudden rise in pressure in the coronary venous system during infusion even though we infused cells slowly at 10 milliliters per minute. Significant contrast blushing was seen on the fluoroscopy images made after infusion, supporting this hypothesis. We identified ten studies that used RCVI for cell delivery in pigs [14, 17, 19-26]. The median infused volume in these studies was 15 ml [IQR: 10 – 25 ml], with two studies infusing a higher volume of 40 ml [26], and 250 ml [19]. The study that infused 40 ml did so during 4 hours, making it likely that no pressure or volume overload could develop [26]. However, the study that infused 250 ml did so during 10 minutes, making both the infused volume and infusion rate higher than in our study [19]. Unfortunately, it is unclear if the CS was occluded during infusion in these two trials, so it is not possible to make a statement on pressure or volume overload in these cases. Three other trials infused cells at a much higher rate and did not report development of

pericardial fluid and hematomas [14, 17, 22]. However, the infused volume was only 10 ml in these three trials.

It is unfortunate that the majority of the RCVI pig studies reported did not state anything on procedural safeties. The studies that do, mention absence of arrhythmias and microvascular obstruction, but nothing on occurrence of dissection of the CS or development of hematomas or pericardial fluid. It is also possible that pericardial fluid collection and hematoma formation were related to CS injury in some of the cases. Contrary to RCVI studies, development of hematomas on the atrioventricular groove, pericardial fluid collections, and damage to the CS have been reported in the field of cardiac surgery and have been related to traumatic catheter insertion, overinflation of the balloon in the CS, and elevated CS infusion pressure during retrograde cardioplegia [27-30]. With retrograde cardioplegia, the CS is accessed with a balloon-catheter to occlude the CS and subsequently infuse fluid to arrest the heart and protect the myocardium. This procedure is in a way comparable to RCVI. Injury to the CS was reported to occur in 0.6 to 0.06% of the patients that underwent retrograde cardioplegia, essentially proving safety of this technique [27, 31]. A possible explanation for the high number of adverse events in the RCVI group in this study compared to an event rate of only 0.6 to 0.06% in human cases could be the difference in anatomy of the coronary sinus between humans and pigs. Contrary to humans, the hemiazygos vein drains in the coronary sinus in pigs. This leaves less room for balloon positioning in pigs, increasing the chance to perforate the CS with the catheter tip due to the small operating area. Clinical trials that have used RCVI did not report safety issues beside a rise in cardiac enzymes in some cases [13, 32-36].

The occurrence of CS dissections did not appear to have a negative effect on cell retention in the heart. On the contrary, the two pigs with the largest dissection showed the highest retention rates of all six RCVI pigs. It is likely that the infused cells collected between the wall layers of the dissected area, effectively trapping the cells and preventing them from washing out. IC infusion was associated with less safety issues with one animal showing no-reflow directly after cell infusion, which could be restored within 5 minutes. Decreased blood flow after IC infusion is a known drawback and has been attributed to coronary embolisms leading to microvascular plugging in the past [8-10].

Future implications

Here, we found that retention rates with both RCVI and IC infusion are low (<14%), which may hamper the effectiveness of cell therapy. Therefore, alternative approaches to increase cell retention and survival are being investigated. These include the use of carrier materials such as nanomatrix gels, microspheres and cell sheets or patches [37-39], but also pretreatment of grafted cells or target tissues, for instance by overexpressing pro-survival genes to increase survival of grafted cells in a hostile environment [40, 41].

Conclusion

Cardiac cell retention after RCVI is significantly lower compared to IC infusion. Our results confirm previous research comparing retention of cells after RCVI with IC infusion in the setting of acute MI. Furthermore, RCVI presented with more safety issues than IC infusion. Taking both efficiency and safety into account, IC infusion is the preferred method of cell delivery between the two.

Strengths and limitations of the study

- To our knowledge, this is the first confirmatory study performed on cell retention after RCVI versus IC infusion in a porcine model of chronic MI.
- Adequate steps were taken to limit the risk of bias: the primary endpoint was prespecified, sample size was calculated beforehand to ensure adequate power of the study and prevent unnecessary use of animals.
- The study was performed in a randomized matter and outcome assessment was performed by blinded investigators.
- Radiolabeling with In^{111} made it possible to quantify cell retention in a very precise way.
- Precise determination of cell retention in the heart on total body images of pigs is challenging due to over projection of lungs and heart. This means counts coming from areas of the lungs that are positioned over the heart are attributed to the heart, leading to a slightly higher cell retention in the heart than was actually the case. *Ex vivo* measurements of cell retention were performed to overcome this drawback of *in vivo* imaging.

Data availability

The dataset generated and/or analyzed during the current study is available in the Open Science Framework (http://osf.io) repository:
https://osf.io/n8wg4/?view_only=55de4fda913d450899e95c3052dcf79b [42]

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Conflict of interest

WAG, MvdN, KN, JPGS, PAD, and SAJC report grants from the Netherlands CardioVascular Research Initiative (CVON): the Dutch Heart Foundation, Dutch Federations of University Medical Centers, the Netherlands Organization for Health Research and Development, and the Royal Netherlands Academy of Sciences, during the conduct of the study.

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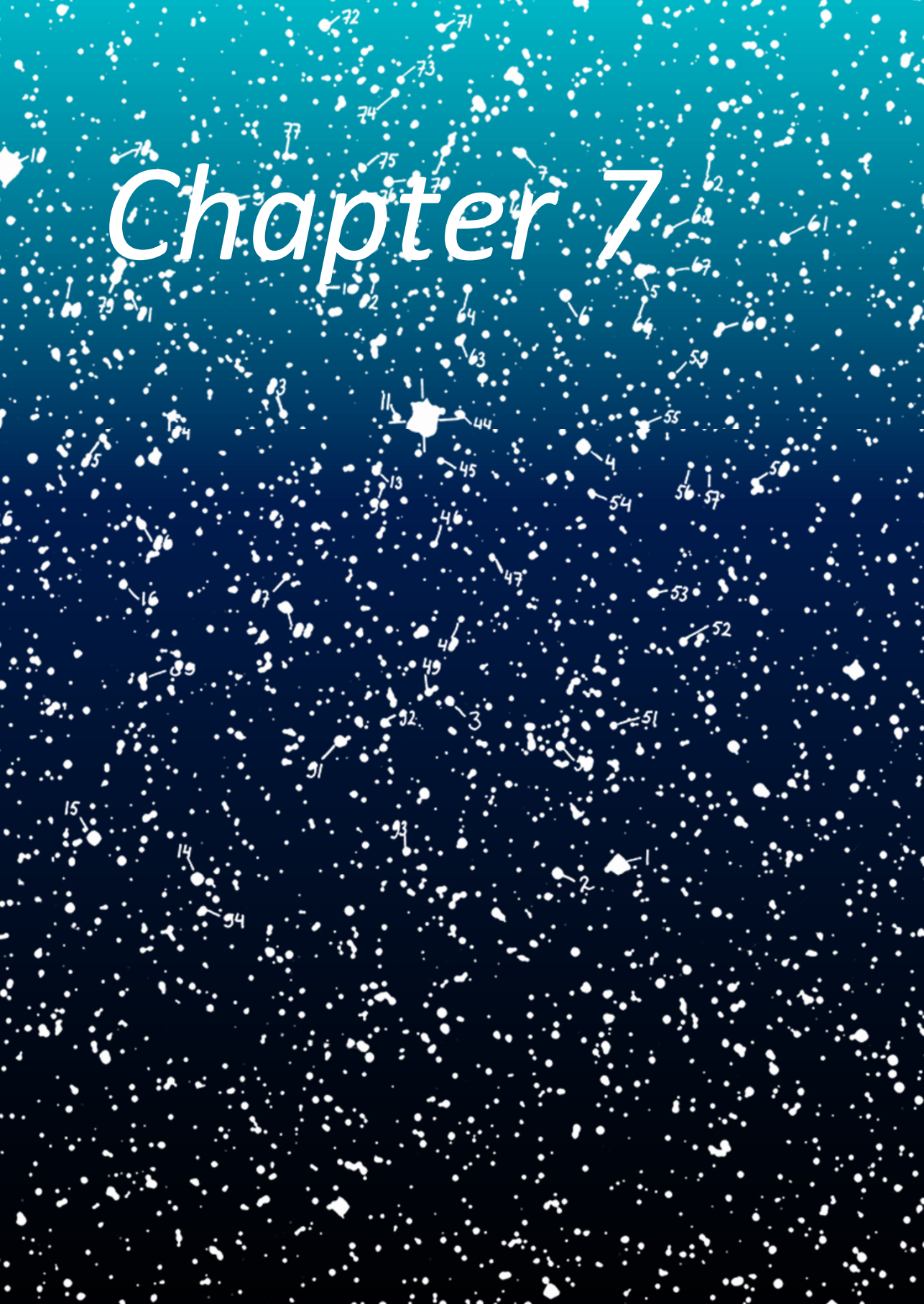
Chapter 7

Feasibility and Potential Benefit of Pre-procedural Cardiac Magnetic Resonance Imaging in Patients with Ischemic Cardiomyopathy Undergoing Cardiac Resynchronization Therapy

Wouter A. Gathier[1*], Odette A.E. Salden[1*], Dirk-Jan van Ginkel[1], Wouter M. van Everdingen[1], Firdaus A.A. Mohamed Hoesein[2], Pieter A. Doevendans[1,4,5,6], Mathias Meine[1], Steven A.J. Chamuleau[1,3,4], Frebus J. van Slochteren[1,3]

Submitted to the Netherlands Heart Journal

[1] Department of Cardiology, University Medical Center Utrecht, Utrecht, the Netherlands
[2] Department of Radiology, University Medical Center Utrecht, Utrecht, the Netherlands
[3] CART-Tech B.V., Utrecht, The Netherlands
[4] Regenerative Medicine Center Utrecht, Utrecht, The Netherlands
[5] Central Military Hospital, Utrecht, The Netherlands
[6] NL-HI (Dutch Heart Institute), Utrecht, The Netherlands
** These authors contributed equally*

Abstract

Aim

To determine the feasibility and potential benefit of a full cardiac magnetic resonance (CMR) work-up for assessing the location of scarred myocardium and the latest contracting region (LCR) in patients with ischemic cardiomyopathy (ICM) undergoing cardiac resynchronization therapy (CRT).

Methods

In thirty patients, scar identification and contraction timing analysis was retrospectively performed on CMR late gadolinium enhancement and CMR-CINE images. Fluoroscopic left ventricular (LV) lead positions were scored with respect to the location of scar, and when placed outside scar, with respect to the LCR. The association between the LV lead position with respect to scar, the LCR and echocardiographic LV end-systolic volume (LVESV) reduction was subsequently evaluated.

Results

The CMR work-up was feasible in all but one patient due to poor CMR image quality. Scar and contraction timing data were succesfully displayed on 36-segment cardiac bullseye plots. Patients with LV leads placed out of scar had superior LVESV reduction (-21±21%, n=19) compared to patients with leads in scar (1±25%, n=11). There was a trend towards superior LVESV reduction in patients with leads in the scar free LCR, compared to leads situated in scar free segments but not in the LCR (-34±14% versus -15±21%, p=0.06).

Conclusions

Reverse remodeling was superior in patients with LV leads situated in a scar free LCR, and neutral in patients with leads situated in scar. These findings demonstrate the feasibility of a CMR work-up and add support for preprocedural treatment planning in ICM patients undergoing CRT.

Introduction

Cardiac resynchronization therapy (CRT) is an effective therapy for patients with chronic heart failure, impaired left ventricular (LV) ejection fraction and prolonged QRS duration [1]. Yet, 30-40% of patients do not benefit from the treatment [2]. Patients with ischemic cardiomyopathy (ICM) derive less benefit from CRT with 50% of patients displaying volumetric or clinical non-response [2–5]. Both a larger scar burden and pacing in or near an area with myocardial scar are associated with a suboptimal response to CRT [6–10]. On the other hand, echocardiographic studies suggested that pacing in the latest contracting region (LCR) is associated with improved CRT response [11,12]. In current clinical practice, the LV lead is implanted empirically at the basal lateral segment, where statistically the most response is obtained. For individual ICM patients, the location of myocardial scar sets an additional requirement for the LV lead location. Given the wide variation in scar distribution and scar burden, as well as a heterogeneity of electrical activation patterns, pre-procedural determination of the location of myocardial scar and mechanical delay may be of key importance in these patients. With advancements in implanting techniques, such as real-time image-guided LV lead delivery, the snare-technique, and multipoint pacing, tailor-made, individualized therapy has become available to patients undergoing CRT [13-16]. These advances call for image postprocessing techiques that can determine the location of myocardial scar and delayed contraction, so that these areas can either be avoided or targeted. Cardiac magnetic resonance imaging (CMR) has been suggested as a promising tool for this purpose. Late gadolinium enhancement (LGE) CMR is the gold standard for determining the location and transmurality of scar tissue. Furthermore, tissue tracking software packages, such as CMR feature tracking (FT), can be used to perform LV contraction timing analysis on standard CMR-CINE images [17,18]. In the present study, we therefore investigated the feasibility and potential benefit of a CMR-based approach to identify scar location, scar transmurality, and LV contraction timing. Furthermore, we assessed the effect of tissue characteristics (e.g. scar and delayed contraction) at the LV pacing electrode on LV reverse remodeling after CRT.

Materials and Methods

Patient selection

Patients with ICM that underwent CRT implantation and had a pre-implantation CMR scan and pre- and postimplant echocardiography acquisition were retrospectively included in the study. Patients received a CRT device according to the ESC guidelines between 2006-2016 in the University Medical Center Utrecht [19]. Standard CRT device implantation was performed with the LV lead placed empirically in a coronary vein overlying the LV free wall, the right atrial lead in the right atrial appendage and the right ventricular lead in the right apicoseptal segment. Patient medical records were screened for an ischemic origin of heart failure based on the presence of ischemic delayed enhancement on CMR-LGE sequences, prior coronary artery bypass grafting, or percutaneous coronary intervention. The study was approved by the local medical ethical committee (METC), by whom the need for informed consent was waived.

Study design

In all patients, the location of myocardial scar, the LCR and the location of the LV pacing electrode were determined and scored on cardiac bullseye plot models. This was done by deviding the LV myocardium in a custom-made 36-segment bullseye plot representation (**Figure 1, 2**). Subsequently, the association of the LV lead position with respect to the CMR-defined location of scar, LCR and its relation with LV reverse remodeling was assessed. LV reverse remodeling was evaluated in terms of LV end systolic volume (LVESV) reduction.

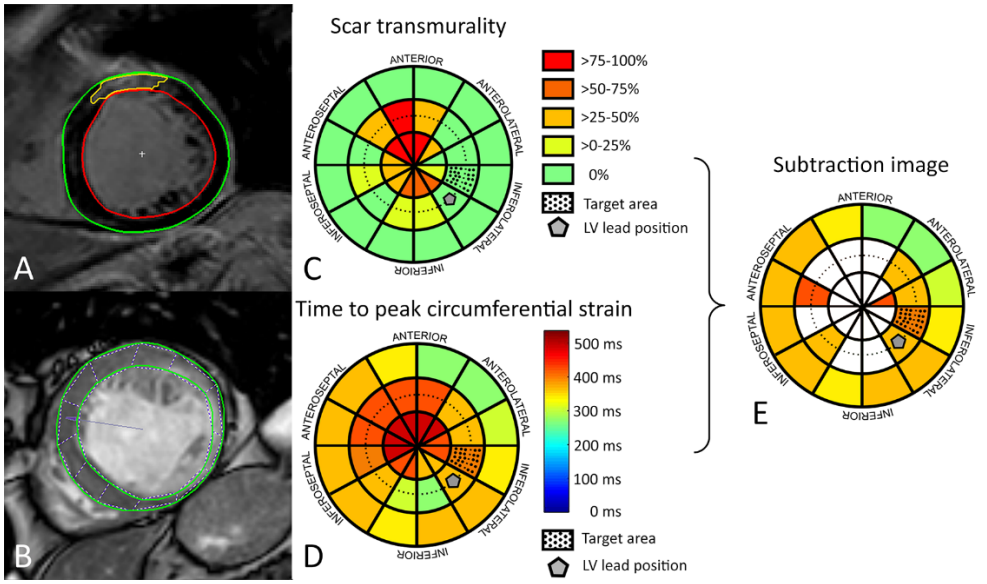

Fig. 1 CMR processing.

A: Segmentation of CMR-LGE sequences used to determine myocardial scar transmurality and position. **B**: Feature tracking of CMR-CINE sequences used for strain analysis and determination of the area of latest time-to-peak circumferential strain. **C&D**: 36-segment cardiac bullseye plots depiciting segmental scar transmurality (**C**) and time-to-peak circumferential strain (**D**). The subtraction image (**E**) shows the contraction timing as shown in (**D**) with subtraction of scarred segments from (**C**). The left ventricular target area is depicted as a dotted segment and the left ventricular lead position is marked by a pentagon. *LV* = left ventricular.

LV lead position

Assessment of the position of the programmed LV pacing electrode on fluoroscopic projections made during CRT implantation was performed by two investigators blinded to all other study data (interobserver variability κ=0.92). The 30º right anterior oblique (RAO) view was used to determine the long axis position of the LV lead (basal, mid or apical), and the 40º left anterior oblique (LAO) view was used to determine the circumferential position of the LV lead on the free wall (anterior (A), anterolateral (AL), lateral 1 (L1), lateral 2 (L2), inferolateral (IL), and inferior (I)) (**Figure 2**).

CMR analysis

CMR scans were performed on a 1.5T MRI scanner (Achieva, Philips Medical Systems, Best, The Netherlands) using a standardized protocol as described in detail before [14]. Scar segmentations were processed using Segment CMR software (Medviso, Lund, Sweden). With this approach, scar transmurality per myocardial segment was evaluated in each patient, as well as total LV scar burden (**Figure 1**). Scar free segments were defined as segments with scar transmurality of 0-5%. This was done to correct for artefacts and noise from, for instance, bloodpool or epicardial fat. For detection of the latest mechanical contracting segments, time to peak (TTP) analysis was performed on short axis CMR-CINE images using CMR-FT software (TomTec Arena, 2D Cardiac Performance Analysis MR, Unterschleissheim, Germany) as described before [14]. In short: endo- and epicardial borders of the short axis CMR-CINE sequences were drawn manually in the end-diastolic frame for all slices. CMR-FT software then automatically followed the myocardial borders throughout the remainder of the cardiac cycle. This resulted in automatically generated circumferential strain data, which were manually checked and corrected when necessary to ensure optimal strain data. Scar transmurality and TTP-strain data were expressed on cardiac bullseye plots using an in-house developed software program running in MATLAB and Statistics Toolbox (The MathWorks, Inc., Natick, Massachusetts, United States) (**Figure 1**). The location of the fluoroscopic LV pacing electrode was scored in a blinded fashion as *in scar* or *out scar*. In patients with an LV lead in a scar free segment, the LV lead location was subsequently scored with respect to the segment with highest TTP strain (latest contracting region) and defined as *within the LCR* or *outside of the LCR*.

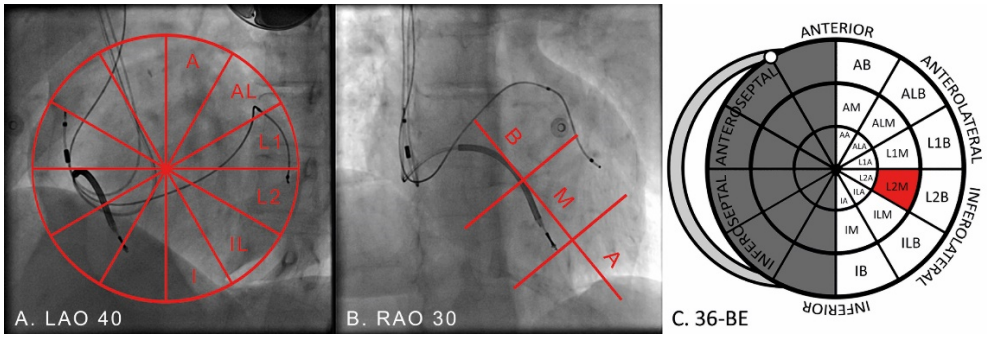

Fig. 2 Identification of the left ventricular lead position on fluoroscopy images.

A: LAO 40º view of the heart with the lateral part of the left ventricle divided in six segments (A, AL, L1, L2, IL, I) with a grid placed over the fluoroscopy image to determine the segment of pacing. **B**: RAO 30º view of the heart divided in three levels (basal, mid, apical). **C**: 36-segment cardiac bullseye plot representing the fusion of the LAO 40º view and RAO 30º view. The distal pacing electrode, which was configurated for biventricular pacing, is located in the red segment. *A* = anterior, *AL* = anterolateral, *BE* = bullseye, *LAO* = left anterior oblique, *L1* = lateral 1, *L2* = lateral 2, *IL* = inferolateral, *I* = inferior, *RAO* = right anterior oblique.

Statistics

Statistical analysis was performed using IBM SPSS Statistics 25 software (IBM, Armonk, New York, USA). Continuous variables were tested for normality with a Shapiro-Wilk test, and were described using mean±standard deviation or, in case of non-normal distribution, with the median [interquartile

range]. Categorical data were described by an absolute number of occurrences and associated frequency (%). Between-group comparisons were performed with Mann-Whitney U tests (continuous data with non-normal distribution), unpaired Student t-test (normally distributed data) and Pearson Chi-Square test or, in case there was an expected cell count of <5, the Fishers exact test (dichotomous variables). A *p*-value of <0.05 was considered to be significant and all tests were two-tailed.

Results

Baseline characteristics

A total of 35 patients met all inclusion criteria. Four patients had insufficient echocardiography quality. CMR processing was feasible in all but one patient due to insufficient CMR quality to perform FT-analysis. Therefore, 30 patients were included in the analysis of whom baseline characteristics are described in **Table 1**.

Table 1 Baseline characteristics

Patient characteristics	All patients (n= 30)	LV lead in scar free region and LCR (n=6)	LV lead in scar free region, not in LCR (n=13)	LV lead in scar (n=11)
Age at implantation (y)	69.9±5.8	68±7	72±5	68±6
Male gender n (%)	24 (80)	4 (66.7)	10 (76.9)	10 (90.9)
LBBB conduction n (%)	23 (76.7)	5 (83.3)	10 (76.9)	8 (72.7)
QRS duration (ms)	150±19	151±29	146±18	154±14
NYHA n (%)				
I/II	13 (43.3)	2 (33.3)	6 (46.2)	5 (45)
III/IV	15 (50)	3 (50)	7 (53.8)	5 (45)
Scar burden (%)	19 [14-24]	13 [5-31]	14 [12-22]*	21 [18-75]*
LV end systolic volume (ml)	151±56	174±79	143±39 (p=0.06)	148±62
LV end diastolic volume (ml)	198±63	215±91	193±41	196±72
LV ejection fraction (%)	24.8±7.0	20±5	26±7	26±7*
Comorbidities n (%)				
Atrial fibrillation	9 (30)	1 (16.7)	5 (38.5)	3 (27.3)
Hypertension	16 (53.3)	5 (83.3)	8 (61.5)*	3 (27.3)*
Smoking	19 (63.3)	5 (83.3)	7 (53.8)	7 (63.6)
Medication n (%)				
Betablocker	21 (70)	4 (66.7)	11 (84.6)	6 (54.5)
ACE-i/ARB	29 (96.7)	5 (100)	14 (100)	10 (90.9)
Diuretics	25 (83.3)	5 (83.3)	12 (92.3)	8 (72.7)

Data presented as mean with standard deviation, median with interquartile range. * significant difference (p<0.05) LBBB = left bundle branch block according to ESC 2013 criteria[2]. *NYHA class* = New York Heart Association functional classification, *LV* = left ventricular, *ACE-i* = angiotensin converting enzyme inhibitor, *ARB* = angiotensin receptor blocker.

LV lead position in relation to myocardial scar, delayed contraction and LVESV reduction

Eleven patients (37%), had LV leads situated in scarred myocardium. Of these, three patients had the LV lead placed in a segment with >75% scar transmurality, two in an area with >50-75% scar transmurality, four in >25-50% scar transmurality and two in >0-25% scar transmurality. Patients in

whom the LV lead was placed in a scar free segment (n=19) had significantly more LVESV reduction at follow-up compared to patients in whom the LV lead was placed in scar (-21±21% versus 1±25% respectively, *p*=0.02). There was a trend towards superior LVESV reduction in patients with LV leads placed in a scar free segment *and* in the LCR compared to patients with leads in a scar free segment but *not* in the LCR (-34±14% versus -15±21%, *p*=0.06) **(Figure 3**).

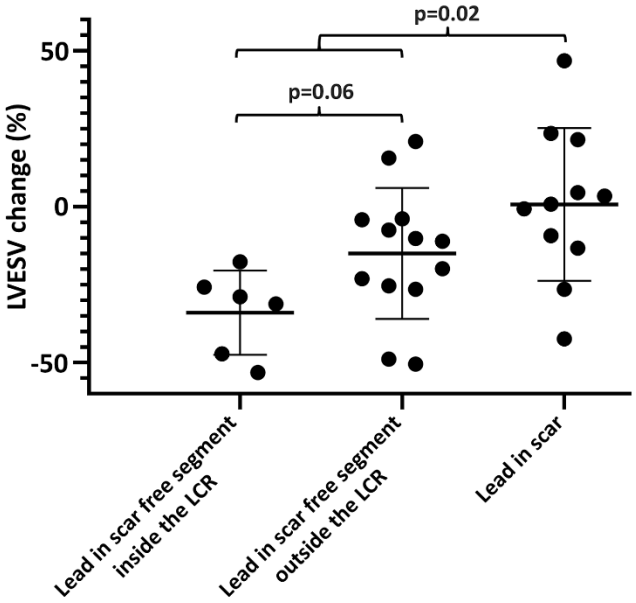

Fig. 3 Echocardiographic response versus lead location in relation to the myocardial scar and the latest contracting region.
Relation between scar location, the location of the latest contracting region, the LV pacing electrode and its relation to left ventricular end systolic volume change. *LCR* = latest contracting region, *LVESV* = Left ventricular end systolic volume

Myocardial scar burden

The amount of reverse remodeling after CRT is not only affected by the presence or absence of myocardial scar or significant mechanical delay at the LV pacing electrode, but is also subject to total LV scar burden. Median LV scar burden was 19% [14-24]. Scar burden was significantly higher in patients with leads in scar compared to patients with leads in scar free myocardial segments (21% [18-75] versus 15% [10-22], *p*=0.02). Scar burden did not vary between patients with leads in a scar free segment and in the LCR, and patients with leads in a scar free segment and out of the LCR (15% [5-35] versus 15% [11-22], *p*=0.677).

Discussion

The present study demonstrates the feasibility and potential benefit of a CMR-workup for the assessment of the optimal site for LV pacing based on myocardial scar transmurality and the area of

latest contraction. For the visualization of scar and delayed contraction, 36-segment cardiac bullseye plots were used. LVESV reduction was most evident in patients with LV leads situated in scar free segments. In addition, there was a trend towards improved LVESV reduction when the LV lead was placed out of scar and in the LCR compared to out of scar and outside the LCR. Because scar burden did not differ significantly between these groups, it is possible that this trend is based on a favorable lead position in these patients. In patients with LV leads placed in scar there was an overall neutral effect on reverse remodeling. This can both be caused by higher scar burden or the LV lead position. These results are in line with previous publications that show that pacing in scar is associated with CRT non-response, while pacing out of scar and in the area with most delayed contraction leads to superior results [9-12,20].

Pre-procedural identification of target sites for LV lead delivery

Despite the promising results from echocardiographic studies showing benefit of targeted LV lead placement, such as the TARGET and STARTER trial, no prospective, randomized clinical studies currently have been published using CMR to guide LV lead delivery in CRT [11,12]. An important limitation of echocardiography is the inability to visualize scar. Yet, in ICM patients undergoing CRT, scar identification is of key importance given the association between scar and poor outcomes [6–10]. CMR, in contrast to echocardiography, is a useful imaging modality to identify and quantify both scar and dyssynchrony in a 3-dimensional fashion and, therefore, may represent the optimal imaging modality for treatment planning [20-21]. In line with our results, Taylor et al. demonstrated in a retrospective study that an LV lead position over nonscarred, late contracting segments, assessed with CMR, was associated with improved echocardiographic response and superior outcomes in CRT patients [20]. In contrast to the present study, only 50% of patients had ICM. Importantly, Taylor et al. did not assess total LV scar burden and its impact on the relation between the LV lead location and CRT response. In addition, a novelty of the present study is that we used smaller LV segments to visualise scar and mechanical delay. When displaying these data on American Heart Association 17-segment cardiac bullseye plots, we believe that the accuracy of CMR is not fully exploited, hence we created smaller LV segments.

Clinical implications

This study shows that CMR is a potentially useful tool that could be used to identify target sites for LV stimulation and, hence, prospectively plan and guide LV lead delivery in patients undergoing CRT implantation. This is especially valuable in patients with scarred myocardium, in whom it is unlikely that a single, empirical location for LV lead placement will adequately resynchronize all patients. Eleven patients (37%) in our study had LV leads positioned in scarred myocardium while a pre-implantation CMR-LGE scan was at the implanting cardiologist's disposal. These data suggests that pre-procedural visual inspection of the plain MRI dataset can not always prevent lead implantation in scarred segments. More advanced, image-guided LV lead implantation, in which scarred myocarium or target sites for LV lead delivery are projected on top of the live fluoroscopy during implantation, might overcome this problem [13,14,21]. When retrospectively assessing the availability of a suitable

coronary branch on fluoroscopy in the current study, a suitable alternative target vein was available in a *scar-free* segment in nine out of these eleven patients. This is an interesting finding because it further fuels the concept of careful assessment of CMR-images before performing CRT implantion. Still, we recognize that we do not know whether acceptable capture thresholds without phrenic nerve stimulation would have been available at non-scarred segments in these nine patients.

Limitations and challenges

The two main limitations of this study are the retrospective design and small sample size. This is caused by the fact that we included only patients with myocardial scar on CMR-LGE scans that were performed before CRT implantion. Differences in baseline characteristics between patients could have influenced our results, for example, patients with LV leads placed in scar had more unfavorable chracteristics at baseline (e.g. higher scar burden, lower frequence of LBBB and more males). The advantage of CMR-FT is that it is a relatively easy technique for contraction timing analysis since it can be performed on CMR-CINE images, which are obtained during standard cardiac imaging protocols. Still, there are some limitations when assessing TTP-strain data. Both electrical substrates (which are generally responsive to CRT) and non-electrical substrates, such as hypocontractility and myocardial scar (which do not respond to CRT) may cause TTP-strain delay [22]. To avoid noise from scarred segments causing TTP-strain delay, we only determined contracting timing in segments outside scarred myocardium (**Figure 1E**). Due to between-group differences and the restrospective study design we cannot draw firm conclusions regarding the superior effect of placing the LV lead in a CMR defined LV target segment. Yet, the present study is a feasibility study of a full-CMR workup. Larger trials are needed to further determine whether this approach leads to improved CRT response.

Conclusion

This study demonstrates the feasibility and potential benefit of a CMR work-up to determine optimal LV pacing sites in ICM patients undergoing CRT implantation. Patients in whom the LV lead was placed in a scar free region with most delayed contraction showed marked LV reverse remodeling, while patients with leads in scarred segments showed an overall neutral effect on LVESV change.

Sources of funding

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Conflict of interest

W.A. Gathier, P.A. Doevendans and S.A.J. Chamuleau report grants from CVON: The Dutch Heart Foundation, Dutch Federations of University Medical Centers, the Netherlands Organization for Health Research and Development, and the Royal Netherlands Academy of Sciences. F.J. van Slochteren is Chief Technology Officer and co-founder of CART-Tech, a spin-out of the division heart and lungs of the University Medical Center in Utrecht focusing on imaging support for CRT placement. S.A.J. Chamuleau is co-founder of CART-Tech. All other authors declare that they have no conflict of interest.

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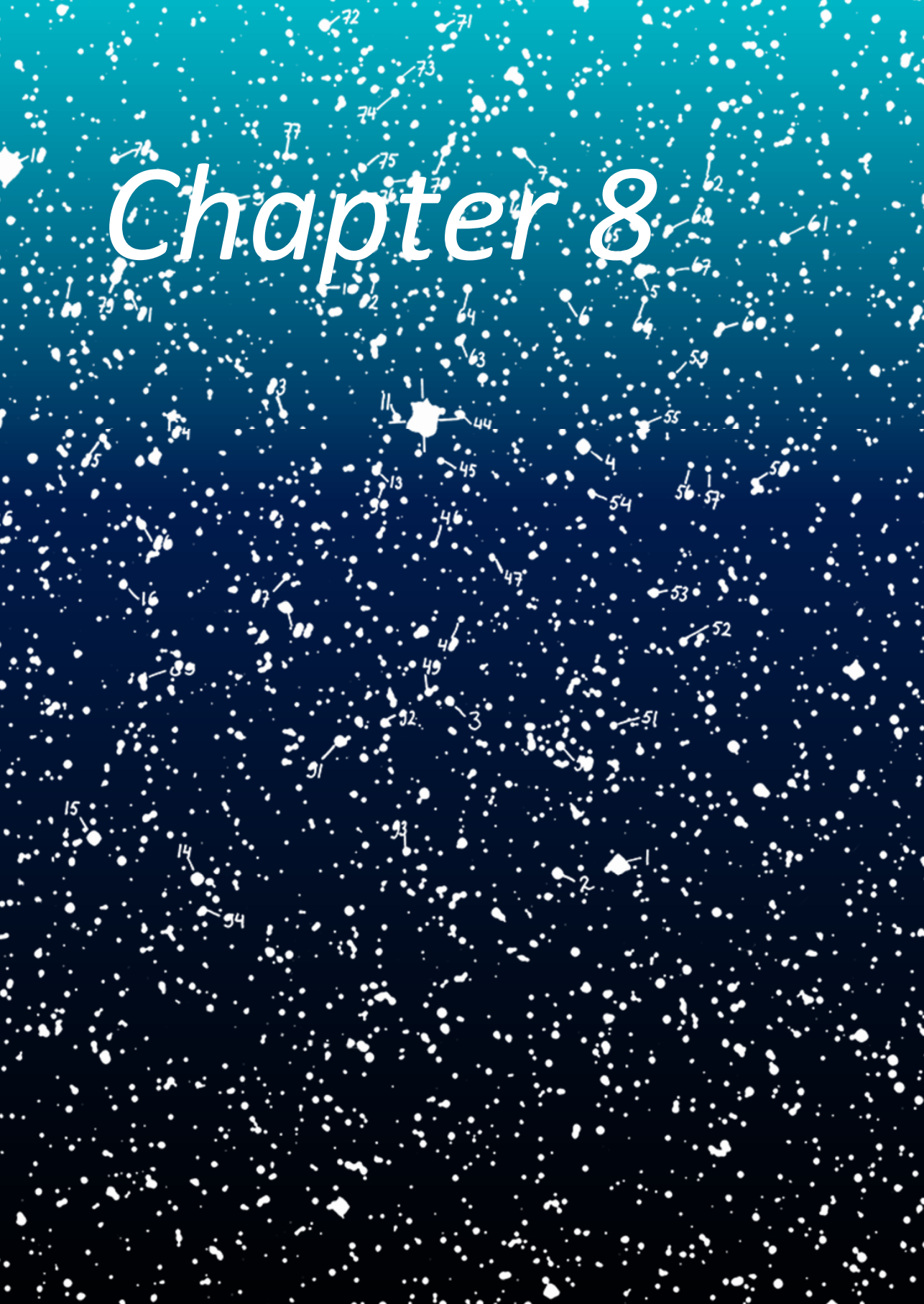
Chapter 8

English Summary

The need for improved and novel therapeutic solutions for heart failure is greater than ever, with the number of patients suffering from heart failure expected to increase dramatically in the coming years [1,2]. The research described in this thesis was performed with improvement of advanced therapeutic strategies for ischemic heart failure in mind. This summary will cover cell therapy, regenerative strategies, and the use of advanced imaging strategies to improve outcome after cardiac resynchronization therapy (CRT).

In **Chapter 2** we compared autologous mesenchymal stem cells (MSCs) and bone marrow-derived mononuclear cells (BMMNCs) in a porcine model of chronic myocardial infarction (CMI) in order to determine if one of these cell types is superior to the other in terms of therapeutic efficacy. We found a beneficial effect for MSCs on systolic function, whereas no effect of BMMNCs on left ventricular ejection fraction (LVEF) was found compared to placebo. This difference in therapeutic efficacy between BMMNCs and MSCs has also been described in a large preclinical meta-analysis by our group [3] and in other publications [4–6]. A study by Li et al. showed comparable therapeutic benefits of BMMNCs and MSCs. However, the number of BMMNCs infused was roughly a factor 100 higher than the number of MSCs infused [7]. It is known that the number of administered cells affect the degree of cardiac recovery [3,8]. Other studies did show a significant effect of BMMNCs on cardiac function, but these studies were mainly performed in the setting of acute myocardial infarction (AMI) and in subgroups of large infarctions [9–12]. The multicenter randomized controlled BAMI trial included approximately 400 patients with reduced LVEF after reperfusion for AMI to investigate if BMMNC therapy effects all-cause mortality in this patient population. First results from this trial are expected early in 2020 and will determine whether BMMNCs will play a future role in the treatment of ischemic heart disease [13].

Furthermore, we studied the effect of repeated cell therapy on systolic function improvement in **Chapter 2**. Despite the fact that transplanted cells are rapidly flushed out of the heart after administration, effects of cell therapy on LV function are seen. We hypothesized that repeated cell therapy might improve the effect of cell therapy, just as effectiveness of most pharmacologic therapies is based on repeated administration of agents. We found that repetitive cell injections led to no serious adverse events, but no further improvement of systolic function was seen compared to single administration of MSCs. The lack of added effect can be attributed to the fact that a single BMMNC injection failed to improve cardiac function. In retrospect, it would have been interesting to add a fourth group in which two injections with MSCs were given to assess if a second MSC injection could increase cardiac function even further compared to single MSC injection. In line with our results, a study by Diederichsen et al. failed to show added effects of repeated BMMNC treatment on LVEF in patients with chronic heart failure. Furthermore, no significant effect of a second injection of skeletal myoblasts (SMBs) in pigs with chronic infarcted myocardium on ΔLVEF was seen in a study Gavira et al. However, a third injection of SMBs was able to further increase LVEF, leading to a significant difference in ΔLVEF between animals that received a single injection and animals that received three

injections [14,15]. More evidence of the benefit of repeated cell treatment was provided by Yao et al. who found a significant improvement in LVEF after repeated infusion compared to single infusion. The reason why Yao et al. did show improvement after repeated administration while we failed to show benefit could be the low baseline LVEF values of the treated patients [16]. Another study in rats with AMI showed added therapeutic effects of repeated MSC injections compared to rats that received a single MSC injection [17]. Other recent studies in rats and mice with ischemic cardiomyopathy showed that multiple administrations of cardiac progenitor cells or cardiac mesenchymal cells significantly improved cardiac function compared to single dose [18,19]. This could mean that a lot of studies on cell therapy that used single administration might have underestimated the effect of cell therapy in the field of cardiology [20]. Multiple cell administrations will only be appealing for patients if the delivery strategy is minimally invasive, effective, and safe. The issue of cell delivery to the heart is also discussed in **Chapter 5** and **Chapter 6**.

The ideal cell for cardiac cell therapy should be easy to harvest, preferably in large quantities and in a minimally invasive way. Furthermore, the cell should possess good therapeutic properties, and should be easily and safely transplantable. A cell type that potentially checks all these boxes is the adipose tissue-derived stem cell (ASC), and an overview is described in **Chapter 3**. ASCs can be harvested in great numbers with relative ease from subcutaneous fat depots, making it possible to deliver autologous cells to the patient within one hour after harvesting [21]. ASCs possess excellent paracrine abilities and secrete a wide array of growth factors and cytokines, including hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), transforming growth factor (TGF)-β, and certain adipokines [22–24]. ASCs have been shown to be able to increase left ventricular ejection LVEF, attenuate both systolic and end systolic diameters of the left ventricle, and reduce brain natriuretic peptide levels and myocardial fibrosis in preclinical research [25–30]. Results from the PRECISE trial, a clinical trial in which our institution participated, showed that ASCs are able to preserve maximal oxygen consumption and improve wall motion score index in patients with heart failure [31]. Furthermore, our group is participating in the SCIENCE trial (clinicaltrials.gov identifier NCT02673164), an international multicenter double-blind placebo-controlled trial, currently recruiting patients to investigate efficacy of ASCs in patients with an LVEF ≤45 and heart failure. First results are expected in 2020 [32]. ASC therapy in a limited number of patients with symptoms of either heart failure (NYHA II-III) or angina (CSS II-IV) showed a favorable change in maximal oxygen consumption, the number of heart failure hospitalizations, and differences in NYHA and CSS classes compared to placebo. However, no differences were seen in LVEF and left ventricular (LV) volumes [33]. Intracoronary infusion of freshly isolated ASCs in nine patients with AMI was shown to be feasibly and safe in the APOLLO phase I/IIa trial. Also, a trend was seen towards reduction of myocardial scar, improvement of the perfusion defect, and improved cardiac function [34].

During the last decades it became clear that administered cells do not effectively reside in cardiac tissue and are not able to regenerate lost tissue after injury. With ambiguous results of cell therapy and negative attention in the media due to publication of unreliable data by certain scientists, the focus of regenerative strategies gradually shifts from cell therapy towards regenerative factors and

secretory factors such as glycoproteins, growth factors, extracellular vesicles, and micro RNA's. In **Chapter 4** the potential of the secreted glycoprotein Follistatin-like 1 (FSTL1) is investigated and its cardioprotective and regenerative properties are discussed. FSTL1 plays a regulatory role in embryogenesis [35], inhibition of cancer cell proliferation [36], and modulates inflammatory processes [37]. FSTL1 is a cardiokine, an endogenous factor secreted from cardiac tissue, and plays a cardioprotective role in case of ischemia/reperfusion injury, but is also capable of promoting proliferative responses in cardiomyocytes. These different effects of FSTL1 can be explained by the occurrence of different isoforms of FSTL1. The hypoglycosylated isoform possesses regenerative (proliferative) properties and is secreted by the epicardium, while the hyperglycosylated isoform is secreted by the myocardium and is able to mitigate ischemia/reperfusion damage. A study by Wei et al. showed that expression of FSTL1 in mice with myocardial infarction shifted from the hypoglycosylated (regenerative) isoform to the hyperglycosylated (cardioprotective) isoform. Reconstitution of hypoglycosylated FSTL1 by application of this isoform with epicardial patches was performed in mouse and swine models of ischemic injury and led to reduced scarring, stimulated cardiomyocyte proliferation, and improvement in cardiac function [38]. These data suggest that the decrease of hypoglycosylated FSTL1 is an adverse response after myocardial infarction and that reconstitution is a promising therapeutic strategy to enhance cardiac function and induce cardiomyocyte proliferation. A recent study by Kretzschmar et al. found no evidence of epicardial FSLT1 expression in mice, but did show that FSTL1-activated fibroblasts contribute to myocardial remodeling after cardiac injury and prevent ventricular wall rupture [39]. Studies by Xi et al. and Magadum et al. have also shown beneficial effects of FSTL1 in the form of cardioprotection and cardiomyocyte proliferation in small and large animals [40,41]. Although FSTL1 shows promising results as a potential therapeutic agent for ischemic heart disease, additional research is required before a first-in-man trial can be realized. Recently, our group has received a Life Sciences & Health grant from Health Holland to fund the DELICATE (A novel DELIvery platform for the regeneration of damaged CArdiac TissuE) project. More on this project in the 'future research' section.

A major factor that determines the therapeutic effect of cell therapy is the delivery method used to administer cells to the heart. Commonly used cell delivery techniques are intramyocardial (IM) injection and intracoronary (IC) infusion. Cardiac cell retention after IM injection only reaches approximately 10-15% because effective venous drainage causes cells to be rapidly flushed out of the heart [42,43]. IC infusion is easy to perform and does not need specialized equipment in the catheterization laboratory, contrary to IM injection. Cardiac cell retention after IC infusion only reaches approximately 10% [42,44]. IC infusion can be complicated by the fact that the coronary arteries are often diseased in the target population leading to inaccessibility of the coronary system. However, the coronary venous system is easily accessible and typically free of atherosclerosis and could be a good route for cell delivery. **Chapter 5** provides an overview of current literature on preclinical and clinical studies that used retrograde coronary venous infusion (RCVI) as a cell delivery strategy to the damaged heart. Research has been performed in small animals, large animals and patients after AMI, CMI, congestive heart failure, and chronic refractory angina with a multitude of different cells, including BMMNCs, MSCs, ASCs, and SMBs. We found that data regarding technical

and safety aspects of RCVI are generally poorly reported. Safety aspects are especially poorly reported in preclinical research, but no severe safety issues were described. RCVI did not lead to severe safety issues in six clinical trials, with three trials reporting (transient) elevation of cardiac enzymes after RCVI [45–47]. RCVI was not associated with arrhythmias or damage to the coronary sinus (CS) in clinical research. Cardiac retention of cells delivered through RCVI was either equal or lower than retention rates after IC infusion or IM injection. It is likely that these inferior retention rates decrease the efficacy of RCVI. Because no well-designed confirmatory studies on retention rates in large animal CMI models were available, we performed a randomized controlled large animal experiment comparing cardiac retention after IC infusion versus RCVI in a model CMI. The results of this study are discussed in **Chapter 6**. MSCs were administered in a randomized fashion through either RCVI or IC infusion. In order to obtain accurate data on cardiac cell retention, MSCs were labeled with the radioactive isotope Indium-111 and cell retention was determined four hours after cell administration through total body scintigraphy. We found that cell retention was significantly higher after IC infusion compared to RCVI (median 13.74% vs 2.89% respectively, p=0.002). The retention rates we observed after IC infusion and after RCVI are comparable to the results of other studies [42,45,48–50]. We infused the same number of cells in RCVI and IC treated pigs. It is known that larger volumes of cells ($30 \times 10^6 - 50 \times 10^6$) though IC infusion can lead to an increase in microvascular resistance [50,51]. There is currently no evidence that higher cell volumes administered via RCVI would lead to venous flow impairment, meaning that it might be possible to deliver more cells through RCVI and make up for the lower retention rate. We encountered several safety issues with RCVI. Three out of six pigs treated with RCVI showed dissection of the CS at the site of the balloon catheter tip. Furthermore, three pigs developed pericardial effusion and a hematoma at the atrioventricular groove of the left ventricle following RCVI. In pigs, there is little space to inflate a balloon catheter in the CS because, contrary to humans, the hemiazygos vein drains directly in the CS. This smaller area for balloon placement increases the chance of CS injury during RCVI in pigs. As reported in **Chapter 5**, RCVI was not associated with injury to the CS in the clinical setting [45–47,52–54]. In **Chapter 5** and **6** we show that cardiac retention after RCVI and IC infusion is low (<14%). Although fundamental differences in anatomy exist between pigs and humans, results from **Chapter 6** strongly suggest inferiority of RCVI compared to IC infusion. There are currently no indications that RCVI is superior to, or even equally as effective as, IC infusion or IM injection. As mentioned before, low retention rates might hamper effectiveness of cell therapy, showing the need for better delivery strategies of cells or compounds to the heart. Alternative approaches to increase cell retention include the use of carrier materials such as microspheres, nanomatrix gels, and cell sheets or patches [55–57]. Increasing the survival of grafted cells in a hostile environment might also further boost effectiveness of cell therapy [58,59].

CRT is an effective therapy for patients with chronic heart failure, impaired LVEF and prolonged QRS duration [60]. Unfortunately, approximately 30-40% of patients undergoing CRT with current implantation techniques do not significantly benefit from the therapy. Especially patients with an ischemic cause of heart failure show less benefit from CRT, with only approximately 50% of patients showing volumetric of clinical response [61–64]. LV pacing in or near an area with myocardial scar is associated with decreased CRT response [65–69], while pacing in the area of latest mechanical

activation is associated with improved response to CRT [70,71]. Advancements in imaging, implantation, and pacing techniques, such as real-time image guided LV lead placement [72,73], the snare technique [74] and multipoint pacing [75], enable tailor-made, patient specific CRT. In current clinical practice, the LV lead is implanted empirically at the basal lateral segment, where statistically the most response is obtained. For individual ICM patients, the location of myocardial scar sets an additional requirement for the LV lead location. Given the wide variation in scar distribution and scar burden, as well as a heterogeneity of electrical activation patterns, pre-procedural determination of the location of myocardial scar and mechanical delay may be of key importance in these patients.

In **Chapter 7** we describe the tissue characteristics *myocardial scar* and *delayed contraction* at the site of the LV pacing lead and their effect on LV reverse remodeling in a retrospective feasibility study in thirty CRT patients with ICM. We investigated the feasibility and potential benefit of a cardiac magnetic resonance imaging (CMR)-based approach to identify scar location, scar transmurality, scar burden, and mechanical delay. We determined the position of the actual LV lead position based on fluoroscopic projections made during CRT implantation. The extent and location of scarred segments could be accurately determined on late gadolinium enhanced (LGE) short axis CMR images. For detection of the latest mechanical contracting segments, time to peak (TTP) circumferential strain analysis was performed on short axis CMR-CINE images using CMR feature tracking software. Subsequently, the location of the LV pacing electrode was scored as *inside scar* or *outside scar*. In patients with an LV lead outside scar, the LV lead location was subsequently scored with respect to the segment with highest TTP strain (latest contracting region) and defined as *within the latest contracting region (LCR)* or *outside of the LCR*. Then, we assessed the effect of scar and delayed contraction at the site of the LV pacing electrode on LV reverse remodeling after CRT. LV reverse remodeling was based on echocardiographic data at baseline and six months follow up and was evaluated in terms of LV end systolic volume (LVESV) reduction. Eleven patients (37%), had LV leads situated in scarred myocardium. Of these, three patients had the LV lead placed in a segment with >75% scar transmurality, two in an area with >50-75% scar transmurality, four in >25-50% scar transmurality and two in >0-25% scar transmurality. Patients in whom the LV lead was placed in a scar free segment (n=19) had significantly more LVESV reduction at follow-up compared to patients in whom the LV lead was placed in scar (-21±21% versus 1±25% respectively, p=0.02). There was a trend towards superior LVESV reduction in patients with LV leads placed in a scar free segment *and* in the LCR compared to patients with leads in a scar free segment but *not* in the LCR (-34±14% versus -15±21%, p=0.06). These results are in line with previous publications that show that pacing in scar is associated with CRT non-response, while pacing out of scar and in the area with most delayed contraction leads to superior results [66,67,70,71,76]. Furthermore, a prospective, single center study by Shetty et al. showed improved CRT response when patients were paced from a CMR defined target segment [77]. This study demonstrates the feasibility and potential benefit of a CMR work-up to determine optimal LV pacing sites in ICM patients undergoing CRT implantation. Combining this technique with real-time fluoroscopy images during CRT implantation will allow electrophysiologists to guide the LV lead to an optimal, tailor-made and patient specific area.

Future research

Ischemic heart failure is a severe clinical condition with a high mortality rate. Although many therapeutic advances have been made in the past decades, improved therapeutic options are still very much needed. The research described in this thesis was aimed to help find better therapeutic strategies, and improve existing treatment options in order to expand treatment options for patients suffering from heart failure. Our group will continue research that builds upon the results described in this thesis. Consequently, in the University Medical Center Utrecht, two relevant projects have been started recently namely the ADVISE-2 study (ZonMW grant), and the DELICATE project (Health Holland grant). These projects are described in more detail below.

Insights generated by the research on optimal LV lead positions in ICM patients undergoing CRT as described in **Chapter 7** have recently been applied in a pilot study in 15 patients, conducted at the University Medical Center Utrecht. The platform was tested in nine patients, and real-time image-guided CRT implantation was succesfully performed in six patients. All LV leads could be placed close to the predetermined target area and away from scar and the left phrenic nerve. Patients showed marked reverse remodeling at six months follow up [73].
The ADVISE-2 study will start in the near future in the form of amulticenter, randomized trial including 120 patients eligible for guided CRT implantation.

As described in **Chapter 4**, FSTL1 shows promising results as a mediator of cardioprotective and proliferative pathways. In the DELICATE project, research on safety and therapeutic efficacy of FSTL1 will continue with several studies planned. The cellular mechanism underlying the regenerative effects of FSLT1 will be studied by our group in a human *in vitro* model of cardiac damage. Collagen patches have proven to be an effective depot for FSTL1 and can be used to ensure sustained delivery. A current drawback of the clinical use of epicardial patches is that they can only be applied on the epicardium surgically after performing a thoracotomy. For this reason, a minimally invasive, epicardial delivery route for sustained FSTL1 delivery will be developed in the DELICATE project. Finally, feasibility, safety and efficacy of the combined approach will be tested by our group in a large animal model, preparing the way for a first-in-human trial.

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Appendix

Nederlandse Samenvatting

Hartfalen is een ernstige aandoening waarbij het hart niet kan voldoen aan de zuurstofbehoefte van het lichaam in rust of tijdens inspanning. Er zijn meerdere oorzaken van hartfalen bekend zoals onder andere hoge bloeddruk, niet goed werkende hartkleppen en het doormaken van een hartinfarct. Bij een hartinfarct krijg het hart gedeeltelijk geen zuurstof omdat de bloedtoevoer verstoord is. Vaak komt dit door een verstopte kransslagader. Het doormaken van een hartinfarct kan leiden tot verlies van gezond hartweefsel en littekenvorming in het hart. Hartfalen dat ontstaat door verminderde bloedtoevoer wordt *ischemisch* hartfalen genoemd. In ongeveer 60% van hartfalen is er sprake van ischemisch hartfalen. In de Nederlandse populatie heeft circa 1.2% van de mannen en 1.4% van de vrouwen hartfalen. Dit percentage neemt toe met de leeftijd waarbij 2-3% van de bevolking tussen de 65-74 jaar hartfalen heeft, oplopend tot 7-8% van de bevolking tussen de 75-84 jaar en 22% van de bevolking ≥85 jaar. Omdat mensen tegenwoordig steeds ouder worden, wordt verwacht dat het aantal mensen met hartfalen met ongeveer 111% zal stijgen tussen 2011 en 2040. De levensverwachting na het stellen van de diagnose hartfalen is slecht, met overlevingspercentages na 1, 2 en 5 jaar van respectievelijk 90%, 80% en 60%. Dit maakt dat de prognose van hartfalen te vergelijken is met enkele veel voorkomende kankers.

De behandeling van hartfalen is afhankelijk van de oorzaak. De basis van de behandeling bestaat uit medicamenteuze therapie. Daarnaast kan chirurgische behandeling worden overwogen in het geval er sprake is van verstopte kransslagaderen (bypassoperatie). Ook kunnen slecht werkende kleppen vervangen worden. Sinds 20 jaar is ook de behandeling met medische apparaten (*devices*) in de vorm van pacemakers en inwendige defibrillatoren (ICDs) niet meer weg te denken.

Vanaf circa 2000 is er veel onderzoek gedaan naar het behandelen van hartfalen met zogenaamde stamcellen. Een stamcel is een cel die kan uitgroeien tot elk type cel, dus ook hartcellen. Men dacht dat het inbrengen van stamcellen in het hart ervoor zou kunnen zorgen dat deze cellen zich in het hart zouden nestelen om daar vervolgens uit te groeien tot functionerend hartweefsel. Zo zou hartschade gerepareerd kunnen worden en zou het hart *geregenereerd* kunnen worden. Al vrij snel bleek dat de toegediende stamcellen niet goed in het hart konden nestelen omdat ze snel uit het hart werden weggepompt. Toch werd er een positief effect op het hart waargenomen na de toediening van stamcellen. Dit bleek te komen doordat de cellen stofjes uitscheiden die een positief effect op het hart hebben. Dit wordt het *paracriene effect* genoemd. Dit paracriene effect zorgt niet voor de aanmaak van nieuw hartweefsel, maar maakt het bestaande hartweefsel gezonder.

Naast stamceltherapie wordt er tegenwoordig ook veel onderzoek gedaan naar ‘echte’ regeneratieve therapie, behandelingen die het hart daadwerkelijk kunnen repareren. Zo wordt er bijvoorbeeld onderzoek gedaan naar het aanzetten van de bestaande hartcellen tot delen, iets wat hartcellen van mensen na de geboorte vrijwel niet meer doen. Ook probeert men hartweefsel te laten groeien in het laboratorium om dit vervolgens aan te brengen op het beschadigde hart.

In dit proefschrift presenteren wij onderzoek dat gericht is op het verbeteren van behandelingsopties voor ischemisch hartfalen. Wij hebben onderzoek gedaan naar het verbeteren van stamcel

behandelingen, het aanzetten van het hart tot vernieuwde celdeling en het verbeteren van de behandeling met bepaalde pacemakers.

In **hoofdstuk 2** beschrijven we de resultaten van een studie waarbij we de effectiviteit van twee typen stamcellen hebben vergeleken om te kijken welk celtype de beste behandelingsresultaten laat zien in varkens met ischemisch hartfalen. De varkens werden behandeld met zogenaamde *mesenchymal stem cells* (mesenchymale stamcellen; MSCs) of *bone marrow-derived mononuclear cells* (uit beenmerg afkomstige mononucleaire cellen; BMMNCs). Daarnaast werd een groep varkens behandeld met een placebo. We vonden dat behandeling met MSCs een positief effect op de hartfunctie had, terwijl behandeling met BMMNCs geen effect liet zien. Waarschijnlijk komt dit doordat MSCs een sterker paracrien effect kunnen uitoefenen op het hart. Daarnaast hebben we gekeken of herhaalde toediening van stamcellen zou kunnen zorgen voor een beter behandeleffect. Dieren die eerst BMMNCs hadden ontvangen kregen een tweede injectie met MSCs. We vonden geen extra effect na de tweede celtoediening vergeleken met toediening van alleen MSCs. Dit zou kunnen komen doordat de BMMNCs in eerste instantie geen effect lieten zien waardoor een extra injectie van MSCs geen verbetering liet zien ten opzichte van een enkele injectie met MSCs.

In **hoofdstuk 3** gaan we in op de uit vetweefsel verkregen stamcel, de *adipose derived stem cell* of ASC. Dit is een celtype dat sterk overeenkomt met de MSC en over uitstekende paracriene eigenschappen beschikt. Stamceltherapie kan onderverdeeld worden in behandeling met lichaamseigen stamcellen (autologe behandeling) of behandeling met cellen die afkomstig zijn van een donor (allogene behandeling). Aangezien ASCs makkelijk en in grote hoeveelheden te verkrijgen zijn uit eigen lichaamsvet is dit celtype bij uitstek geschikt voor autologe celtherapy. Onderzoek met ASCs in patiënten heeft een positief effect laten zien op de hartfunctie en het aantal ziekenhuisopnames per patiënt. Momenteel werkt onze onderzoeksgroep mee aan de SCIENCE-studie. Dit is een studie waarbij patiënten met ischemisch hartfalen worden behandeld met ASCs.

In **hoofdstuk 4** geven we een overzicht van de literatuur op het gebied van het eiwit Follistatin-like 1 (FSTL1). FSTL1 is een eiwit dat onder andere in het hart voorkomt en in staat is het hart te beschermen tegen schade in het geval van een periode van ischemie of verminderde bloedtoevoer. Daarnaast is uit onderzoek gebleken dat FSTL1 er onder de juiste omstandigheden voor kan zorgen dat bestaande hartcellen zich gaan delen. Dit lijkt alleen te gebeuren als FSTL1 zich aan de buitenzijde van het hart, het epicardium, bevindt. Uit onderzoek is echter gebleken dat bij het optreden van ischemie de hoeveelheid FSTL1 aan de epicardiale zijde van het hart juist afneemt. Dit is een belangrijke ontdekking aangezien dit zou kunnen betekenen dat het aanvullen van FSTL1 aan de epicardiale zijde ten tijde van ischemie juist zou kunnen leiden tot reparatie van de ontstane schade. Toekomstig onderzoek door onze groep zal moeten uitwijzen of FSTL1 inderdaad het hart kan aanzetten zichzelf te repareren bij ischemische schade. Ook zal er door onze groep een methode worden ontwikkeld om FSTL1 op een minimaal invasieve manier aan te brengen op het hart.

Een belangrijke beperking van stamcel therapie is de manier van toediening van cellen aan het hart. Veel gebruikte toedieningsmethoden zijn intracoronaire (IC) infusie en intramyocardiale (IM) injectie. Bij IC-infusie wordt een katheter via de slagaders (dus met de bloedstroom mee, ook wel antegraad genoemd) in de arm of de lies opgevoerd tot in de kransslagaderen (coronairarteriën) waar de cellen worden geïnfundeerd. Kort na IC-infusie is nog maar circa 10% van de geïnfundeerde cellen terug te vinden in het hart, de rest spoelt vrijwel direct weg. Bij IM-injectie wordt een katheter met een naald via een slagader opgevoerd tot in de linkerkamer van het hart. Hier kan dan gericht op vooraf bepaalde plekken in de hartspier (myocard) worden geprikt om cellen in te spuiten. Kort na IM-injectie is maar ongeveer 10-15% van de geïnjecteerde cellen in het hart terug te vinden. Beide technieken hebben plus en minpunten. Zo zijn de kransslagaderen vaak verkalkt bij patiënten met ischemisch hartfalen. Als dit het geval is kan er niet doeltreffend worden geïnfundeerd. Een nadeel van IM-injectie is dat er gespecialiseerde uitrusting nodig is om deze techniek uit te voeren. Dit is duur en vereist speciaal getraind personeel. Een alternatieve toedieningsmethode is retrograde sinus coronarius infusie (RCVI). In tegenstelling tot IC-infusie en IM-injectie wordt bij RCVI een katheter via de aders (dus tegen de bloedstroom in, ook wel retrograad genoemd) opgevoerd in het hart in plaats van via de slagaders. Het voordeel is dat de aders in tegenstelling tot de slagaders vrijwel nooit verkalkt zijn. In tegenstelling tot IC-infusie en IM-injectie is er relatief weinig onderzoek gedaan naar de effectiviteit van RCVI.

In **hoofdstuk 5** geven we een overzicht van dierstudies en studies in mensen waarbij gebruik is gemaakt van RCVI om stamcellen toe te dienen. Uit dit overzicht blijkt dat er relatief weinig wordt gerapporteerd over veiligheidsaspecten van RCVI, met name in de dierstudies. Aan de hand van de beschikbare gegevens lijkt RCVI veilig te zijn. Echter is er geen overtuigend bewijs dat RCVI kan zorgen voor een betere beschikbaarheid (retentie) van stamcellen in het hart.

Omdat er geen goed ontworpen studies bestonden waarin de retentie van stamcellen na IC-infusie en RCVI werd vergeleken in een groot proefdier model (varkens) in de setting van ischemisch hartfalen hebben we zelf een dergelijk experiment opgezet. De resultaten hiervan worden beschreven in **hoofdstuk 6**. Om te kunnen zien welk percentage van de toegediende cellen vier uur na toediening nog in het hart aanwezig is hebben we aan de stamcellen (MSCs) een radioactief label gehangen. Hierdoor konden we met een gammacamera (een detector die radioactieve straling meet en daar een beeld van maakt) het radioactief signaal uitlezen dat uit het hart komt. We konden op een zeer precieze manier de retentie van cellen in het hart meten door het radioactieve signaal uit het hart te delen op het totale radioactieve signaal dat afkomstig was uit het varken. We zagen een duidelijk verschil in celretentie tussen RCVI en IC-infusie ten nadele van RCVI. Vier uur na IC-infusie was gemiddeld circa 14% van de cellen nog aanwezig in het hart versus circa 3% na RCVI. Daarnaast traden er bij RCVI vaker complicaties op, maar dit kan te maken hebben gehad met het feit dat de anatomie van het varken verschilt van die van de mens. We zijn tot de conclusie gekomen dat RCVI geen voordeel heeft over IC-infusie als stamcel toedieningsmethode.

In **hoofdstuk 7** richten we ons op *cardiac resynchronization therapy* (CRT), een therapie waarbij een speciale pacemaker wordt geïmplanteerd om het zieke hart beter te maken. In een gezond hart

ontstaat de elektrische prikkel in de sinusknoop die gelegen is in de rechterboezem (atrium) waarna het signaal via de atrioventriculaire (AV) knoop wordt doorgegeven aan de linker en rechter kamer (ventrikels). Bij sommige patiënten met hartfalen knijpt het hart niet meer symmetrisch samen. Dit komt doordat, bijvoorbeeld na een hartinfarct, de elektrische geleiding van het hart verstoord is geraakt. Dit is meestal het geval in het linkerventrikel. Doordat de elektrische activatie ervoor zorgt dat het hart kan samenknijpen, knijpt het linkerventrikel later samen dan het rechterventrikel wanneer de elektrische geleiding door het linkerventrikel verstoord is. Het gevolg hiervan is dat het hart minder goed het bloed kan rondpompen. Uiteindelijk is dit slecht voor het hart en kan de hartfunctie verder achteruitgaan. Om dit te voorkomen is het belangrijk om ervoor te zorgen dat het rechter en linker ventrikel weer tegelijk knijpen. CRT is een behandeling waarbij patiënten een pacemaker geïmplanteerd krijgen met drie draden (*leads*) die ervoor moet zorgen dat de elektrische stimulatie van het hart weer zo goed mogelijk hersteld wordt, zodat het hart weer synchroon kan samenknijpen. Eén CRT-lead ligt in het rechter atrium en kan zo nodig de taak van de sinusknoop overnemen. Daarnaast ligt één lead in het rechterventrikel en één lead in een ader die aan de buitenkant van het linkerventrikel loopt. Het is gebleken dat de positie van de linkerventrikel lead een belangrijke factor is die mede bepaalt of een patiënt baat heeft bij CRT. Wij hebben in een retrospectieve studie in dertig patiënten met ischemisch hartfalen bepaalde eigenschappen van het hartweefsel rondom de linkerventrikel lead bepaald, namelijk de mate van verlittekening na een infarct en hoeveel het gebied rondom de linkerventrikel lead later samenknijpt ten opzichte van de rest van het linkerventrikel. We hebben gebruik gemaakt van MRI-beelden van het hart om deze eigenschappen te bepalen. We vonden dat met name de hoeveelheid littekenweefsel ter plaatse van de linkerventrikel lead bepalend is voor het effect van CRT. Dit komt overeen met wat er uit de literatuur bekend is. Daarnaast lijkt het ook belangrijk te zijn dat de lead in een laat samenknijpend gebied ligt, omdat hier dan de meeste winst te behalen is wanneer dit eerder samenknijpt. Het uiteindelijke doel is om voorafgaand aan de implantatie van een CRT-pacemaker per patiënt het optimale gebied te bepalen waar de linkerventrikel lead moet komen te liggen. Met de huidige technieken kan de cardioloog die de CRT-pacemaker implanteert dan doelgericht de linkerventrikel lead op deze plek proberen te krijgen om een optimaal effect van CRT te bereiken. Binnenkort start een studie in 120 patiënten waarbij deze techniek wordt toegepast.

Ischemisch hartfalen is een ernstig ziektebeeld met een hoog sterftecijfer. Hoewel de afgelopen jaren veel vooruitgang is geboekt op het gebied van therapeutische behandelingen voor ischemisch hartfalen, zijn verbeterde, geavanceerde behandelingsopties hard nodig. Zeker aangezien verwacht wordt dat het aantal mensen met hartfalen de komende jaren zal stijgen. Het onderzoek beschreven in dit proefschrift heeft als doel om bij te dragen aan de ontwikkeling van verbeterde behandelingsopties van hartfalen.
Zoals hierboven beschreven zal door onze onderzoeksgroep verder onderzoek worden gedaan naar de effectiviteit en toepasbaarheid van het eiwit FSTL1. Daarnaast zal onze groep verder gaan met onderzoek naar het verbeteren van CRT-implantatie om zo hopelijk de effectiviteit van CRT te verbeteren.

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List of Publications

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*authors contributed equally

Curriculum Vitae

Wouter Adriaan Gathier was born on December 6th, 1986 in Leiderdorp, The Netherlands to Roel Gathier and Wilma Gathier-Leijten and grew up in the town of Voorschoten with his two sisters Celine and Anouk. After graduation from secondary school (Stedelijk Gymnasium Leiden) in 2005 he moved from Voorschoten to Utrecht where he studied Biomedical Sciences from 2005 to 2007 at the University of Utrecht. In 2007 he started his medical school, also at the University of Utrecht. During the master years of his study he did a senior internship at the department of cardiology at the University Medical Center Utrecht and a senior internship at the department of radiology at the Meander Medical Center in Amersfoort. After graduating from medical school in April 2014, Wouter started as a PhD student at the department of cardiology at the University Medical Center Utrecht, first under supervision of prof. dr. P.A. Doevendans and dr. H.J. Duckers, and later under supervision of prof. dr. P.A. Doevendans, prof dr. S.A.J. Chamuleau, dr. M.J.M. Cramer, and dr. ir. F.J. van Slochteren. Wouter was part of the CVON-HUSTCARE (Cardiovasculair Onderzoek Nederland - HUman STem cells for CArdiac REpair) consortium during his work as a PhD student. In March 2018 he started his radiology residency at the Gelre ziekenhuis Apeldoorn under supervision of K.E. Droogh-de Greve. In 2020 Wouter will continue his residency at the University Medical Center Utrecht under supervision of dr. R.A.J. Nievelstein. Wouter currently lives in Utrecht with his wife Anjuli Gathier-Groenewegen.