Safety and Efficacy of Bone Marrow Derived Progenitor Cells in Patients with Chronic Ischaemic Heart Failure

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Safety and Efficacy of Bone Marrow Derived Progenitor Cells in Patients with Chronic Ischaemic Heart Failure

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ABSTRACT

Bone marrow stem/progenitor cell (BMSC) therapy for cardiac repair in humans is yet to fulfil the exciting potential demonstrated in preclinical experiments. This thesis presents three clinical studies addressing some of the unresolved issues regarding the ideal delivery method, the effect of patient-related factors on progenitor cell concentration/function and the possible biological mechanism(s) of action.

The first study describes the intramyocardial arm of the REGENERATE-IHD trial- a randomised controlled trial assessing the efficacy of mobilised BMSCs in patients with ischaemic heart failure. In summary, 30 patients were randomised 1:1 to receive injection of BMSCs suspended in autologous serum or serum alone (control group). All patients received a 5-day course of G-CSF prior to bone marrow harvest and intramyocardial injection. At 1-year, there was a significant increase in ejection fraction, the primary end-point, in patients treated with BMSCs. There were also significant improvements in the secondary end-points of NT-proBNP and symptoms.

In the second study, progenitor cell concentration and function were assessed in patients with ischaemic heart failure (IHD), dilated cardiomyopathy (DCM) and acute myocardial infarction (AMI). Findings include ageing having an inverse association with circulating CD34+ cell concentration as well as blunting the effects of G-CSF on BMSC mobilisation. DCM patients had

significantly higher baseline circulating progenitor cell concentrations compared to IHD/AMI.

The final study presents preliminary data regarding a novel imaging technique to detect angiogenesis which is recognised as a potential therapeutic effect of BMSCs. Nine patients with heart failure underwent nuclear imaging using a radio-tracer peptide with a high affinity for αvβ3, an angiogenesis-related integrin, before and after intracoronary infusion of BMSCs/serum. Preliminary results showed detectable baseline uptake of the radio-tracer suggesting a novel finding of persistent angiogenesis following remote myocardial infarction and also hint at a tantalising possibility that BMSC infusion may lead to therapeutic angiogenesis.

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CONTRIBUTION TO WORK AND FUNDING

This thesis is based upon work that I carried out whilst working as a Cardiology Research Fellow within the Adult Stem Cell Trials Unit at The London Chest Hospital between August 2010 and August 2012 under the supervision of Professor Anthony Mathur. The REGENERATE-IHD clinical trial had regulatory approval and was recruiting patients when I joined the trial team. I was responsible for completing recruitment by enrolling the final 50 of the total 90 patients. In this thesis I present the safety and efficacy results of the intramyocardial arm of the study. I was involved in designing and successfully obtaining regulatory (ethics and MHRA) approval for the 'angiogenesis' imaging sub-study; methodology and preliminary results of which are also presented in this thesis. I was involved in the treatment phase of the majority of the patients and also reviewed the majority of patients at follow-up assessments. I performed bone marrow aspiration in the majority of patients and also performed the left ventriculography procedure. I was 2nd operator in the intramyocardial injection procedures and follow-up NOGA® mapping. I analysed the contrast echocardiograms, LV angiograms and NOGA® maps. The cardiac CTs and MRIs were analysed by Dr Ceri Davies, Consultant Cardiac Imaging Specialist. I collected and recorded the data on patient safety events, symptoms and quality of life score changes and biochemical (NT-proBNP) and cell concentration data. I have been solely responsible for the data analysis and preparation of this thesis.

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ABBREVIATIONS

AMI	acute myocardial infarction
ADSC	adipose derived stem cells
ASC	adult stem cell
BMMNC	bone marrow mononuclear cells
BMSC	bone marrow stem/progenitor cells
BNP	brain natriuretic peptide
CEPS	circulating endothelial progenitor cells
CSC	cardiac (resident) stem cell
CPC	circulating progenitor cells
CRT	cardiac resynchronisation therapy
DCM	dilated cardiomyopathy
EPC	endothelial progenitor cells
ESC	embryonic stem cell
G-CSF	granulocyte-colony stimulating factor
HRQL	health related quality of life
HSC	haematopoietic stem cells
iPCs	induced pluripotent stem cells
ISR	in-stent restenosis
ICD	implantable cardioverter defibrillator
IVUS	intravascular ultrasound
LV	left ventricular
LVEF	left ventricular ejection fraction
LVSD	left ventricular systolic dysfunction
LVT	left ventricular thrombus
MSC	mesenchymal stem cells
NT-proBNP	N-terminal prohormone of brain natriuretic peptide
PBSC	peripheral blood stem cells
NYHA	New York Heart Association
PCI	percutaneous coronary intervention
QLV	quantitative left ventriculography
RCT	randomised controlled trial
TVIM	trans-(coronary)-venous intramyocardial (injection)

CHAPTER 1: INTRODUCTION

Cardiovascular disease is the leading cause of morbidity and mortality worldwide. Despite the advances in medical and catheter-based therapy for acute myocardial infarction the 1-year mortality remains as high as 11%[1-3] and the long-term prognosis for patients with heart failure and left ventricular systolic dysfunction (LVSD) remains poor[4]. There is currently no treatment option that targets the primary problem of cardiomyocyte loss during an ischaemic insult which results in chronically impaired ventricular function. Hence, a need has been identified for novel therapies that target this issue and the discovery of adult stem plasticity has seen progenitor cell therapy emerge as an exciting therapeutic potential. It is hoped that cell therapy can ameliorate myocyte loss in the acute infarct setting and also improve cardiac function and symptoms in patients with chronically infarcted myocardium. Numerous cell types have been, and continue to be, investigated for their ability to contribute to cardiac repair. Bone marrow derived stem/progenitor cells (BMSCs) have been widely investigated clinically due to the ease of obtaining bone marrow and preclinical experiments demonstrating the potential of this cell type to transdifferentiate to a cardiomyocyte phenotype[5, 6]. The latter remains under considerable debate as this finding has not been consistently reproduced[7, 8] although even in the absence of transdifferentiation improvement in cardiac function has been demonstrated[9]. Thus there are likely to be other mechanisms, such as cell fusion and paracrine effects, which may contribute to potential improvement. Furthermore, it remains unclear which fraction of the bone marrow (i.e. haematopoietic stem cells, CD34+ cells, endothelial progenitor cells, mesenchymal stem cells or even the

monocyte/lymphocyte component) may contribute most to the process of potential repair or whether is requires a combination of two or more of these cell types.

Despite these uncertainties, the past decade has seen a steady increase in the number of clinical trials being performed to assess the safety and efficacy of different cell types and delivery methods for the purposes of cardiac repair in patients with chronic ischaemic heart failure. These small clinical studies have suggested that progenitor cell therapy may improve various functional characteristics of patients with chronic heart failure[10-14]. The need for larger randomised controlled studies to further investigate this potential benefit has been clearly outlined[15]. Additional questions relating to the ideal cell type, use of adjunctive cytokine therapy and the method of cell delivery remain to be answered as the field moves forward and attempts to translate the positive findings of preclinical experiments into clinically meaningful benefits. There is also clearly a need for clinical mechanistic studies to define the biological effects of cell therapy on human cardiac tissue.

The Randomized Controlled Trial to compare the Effects of G-CSF and Autologous Bone Marrow Progenitor Cells Infusion on Quality of Life and Left Ventricular Function in Patients with Heart Failure secondary to Ischaemic Heart Disease (REGENERATE-IHD) is a single centre double-blind, randomised controlled study being conducted at The London Chest Hospital[16]. This is the only trial of its kind in the United Kingdom and addresses some of the unanswered questions mentioned above regarding cell therapy in heart

failure. The trial investigates the potential for autologous BMSCs to improve cardiac function and symptoms in patients with chronic ischaemic heart failure and no further treatment options. As well as testing routes of administration, it uses a novel approach of administering granulocyte-colony stimulating factor (G-CSF) as adjunctive cytokine therapy.

In this thesis, chapter 2 provides background to the relevant pathophysiology of ischaemic heart failure and the current treatment options available. The potential role of adult stem cell therapy and results of previous clinical trials are also described. In chapter 3 the specific hypotheses and aims of this thesis are presented. Chapter 4 then presents the methods and results of the intramyocardial arm of the REGENERATE-IHD trial which examines the safety and efficacy of NOGA® guided intramyocardial injection of G-CSF mobilised BMSCs in patients with chronic ischaemic heart failure. The study methodology and trial design are described and the safety and efficacy results are presented and discussed in detail. The data from this chapter has been submitted for publication in a leading international medical journal.

Chapter 5 describes a study of bone marrow progenitor cell characteristics in patients recruited to the whole REGENERATE-IHD study as well as in patients recruited to two other cell therapy clinical trials being performed at The London Chest Hospital. This study assesses the effects of age, disease state and G-CSF on progenitor cell concentration and function. This analysis is pertinent as there is growing evidence to suggest that efficacy of autologous cell therapy maybe attenuated by patient related factors affecting the quantity and

quality of the cell product. The data from this chapter has been published in the *Stem Cell & Development Journal*[17]- a leading journal in the field of stem cell therapy.

Chapter 6 describes the preliminary results of a novel clinical imaging technique to detect angiogenesis potentially related to BMSC therapy. Despite numerous studies suggesting beneficial cardiac effects of cell therapy there remains considerable conjecture regarding the actual biological mechanism(s) through which this occurs. One of the proposed mechanisms demonstrated in preclinical experiments is neoangiogenesis either directly by differentiation into endothelial cells[18] or indirectly through secretion of paracrine factors[19]. This is yet to be demonstrated clinically and hence this novel imaging technique represents a major step forward in this field and may provide an exciting insight into whether there is clinically relevant neoangiogenesis following BMSC therapy in humans.

Chapter 7 provides an insight into the future directions for the field of cardiac cell therapy and the thesis conclusions are summarised in chapter 8. The appendices include an additional small retrospective study evaluating the incidence of left ventricular thrombus (LVT) in patients with ischaemic heart failure. This analysis was prompted by the withdrawal of 3 patients from the intramyocardial arm of the REGENERATE-IHD study due to LVT being diagnosed on baseline imaging tests. Finally, the appendices also include a list of publications and presentations arising from this period of research.

CHAPTER 2: BACKGROUND

2.1 Heart Failure

2.1.1 Epidemiology

Ischaemic heart disease (IHD) is the commonest cause of death in the world accounting for 7.2 million deaths per year (approximately 12% of all reported deaths)[20]. Acute myocardial infarction (AMI) affects around 250,000 people each year in the United Kingdom (UK)[21] and subsequent heart failure with left ventricular systolic dysfunction (LVSD) is relatively common. In the HORIZONS-AMI trial, a contemporary study of AMI treatment, the incidence of new-onset heart failure was 4.7% at 1-year and this was associated with significantly increased rates of mortality and adverse ischaemic events[22]. The overall prevalence of heart failure is estimated to be 3% of people aged over 45 in the UK[23] and this is likely to increase, partly because of an aging population but also because of improved interventions that prolong survival after AMI[24]. In approximately 30% of patients, heart failure is secondary to causes other than IHD/AMI, such as diastolic dysfunction and dilated cardiomyopathy[25]. Regardless of aetiology heart failure is associated with significant morbidity and mortality reflected by a 5-year mortality varying from 26-75%[26], a prognosis that is worse than most cancers. Patients with heart failure can be significantly limited in term of exercise capacity which can vary from being breathless on moderate exertion to being breathless at rest. Furthermore, once admitted with an episode of decompensated heart failure, the subsequent rate of re-hospitalisation is high- 19% at 30 days in one recent study[27]. This has a major impact on quality of life as well as leading to a major

socioeconomic cost burden. The annual cost of heart failure in the UK is just over £625 million, thus consuming 2–2.5% of the total National Health Service (NHS) budget[28]. It has also been estimated that IHD cost the economy £3.9 billion in lost productivity in 2008[29]. These financial considerations are also important drivers behind the search for novel therapies for heart failure.

2.1.2 Normal resting cardiac function

The principle function of the heart is to generate a cardiac output that sustains an arterial blood pressure necessary to provide adequate perfusion of organs. In a healthy person, cardiac output is matched to the body's metabolic demand and is a product of stroke volume (SV) and heart rate. SV is the volume of blood ejected from the ventricle in one cardiac cycle and is approximately 70ml in an adult human at rest. The ventricular end-diastolic volume (EDV) is the volume of blood in the ventricle just prior to contraction and is typically 120 ml. The end-systolic volume (ESV) is the residual volume after contraction and is therefore usually 50 ml of blood. Left ventricular ejection fraction (LVEF), the most commonly used measure of cardiac function, is calculated as SV/EDV ((EDV-ESV)/EDV)) i.e. it measures the fraction of the EDV that is ejected with each heart beat and is normally 55-60% in an adult human at rest.

There are three major mechanisms that regulate EDV and ESV, and therefore SV: preload, afterload and myocardial contractility (inotropy). The concept of preload regulating myocardial performance was described in the late 19th century by physiologists Frank and Starling. Otto Frank showed that the

strength of ventricular contraction in isolated frog hearts was increased by stretching the ventricle prior to contraction[17]. This work was extended by Ernest Starling and others who found that increasing venous return, and therefore the EDV and filling pressure of the ventricle, led to increased stroke volume in dogs[18]. The ability of the heart to change its force of contraction and therefore stroke volume in response to changes in EDV is termed the Frank-Starling mechanism (figure 1). This important physiological concept is not dependent on neural or humoral activations. The molecular pathway underlying this mechanism involves myofilament length-dependent activation although the exact processes through which sarcomeres detect changes in length and translate this into increased sensitivity to activating calcium remains to be fully defined[30]. The Frank-Starling mechanism is attenuated in states of reduced inotropy, such as following a large myocardial infarction, and stroke volume may not increase significantly in response to increased EDV[31] (figure 1).

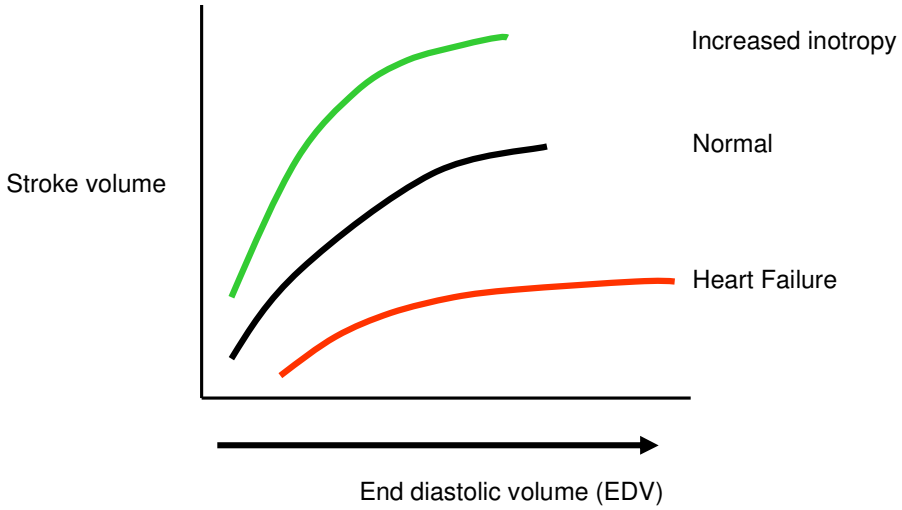


Figure 1. Frank-Starling curves depicting the relationship between end diastolic volume (EDV) and stroke volume. In a normal heart (black line) cardiac output continually increases in response to an increase in pre-load. In states of increased inotropy there is augmented cardiac output at the same EDV (green line). In heart failure (deceased LV contractility) the curve is shifted down and is flattened so that stroke volume and cardiac output do not increase significantly in response to increased EDV leading to further deleterious increases in EDV and end-diastolic pressure.

2.1.3 Pathophysiology of ischaemic heart failure

Ischaemic heart failure is a disease state in which cardiac contractile function is impaired secondary to myocardial necrosis resulting in an insufficient cardiac output to adequately perfuse the body's organs. Several natural compensatory mechanisms are initiated in patients with ischaemic heart failure in an attempt to counteract the fall in cardiac output and maintain sufficient blood pressure to perfuse vital organs. These include: (1) changes to circulatory haemodynamics, (2) neurohormonal alterations and (3) ventricular remodelling.

2.1.3.1 Changes to circulatory haemodynamics

A large myocardial infarction resulting from prolonged occlusion of a major coronary artery leads to myocardial necrosis and loss of upto a billion cardiomyocytes[32]. This leads to impaired contractility of the heart reflected by a reduction in left ventricular ejection fraction (LVEF) and stroke volume leading to increased ESV. This, in addition to normal pulmonary venous return, leads to an increase in EDV. In a normal heart increased EDV would increase contractility (via the Frank-Starling mechanism) but in a failing heart EDV and therefore end diastolic pressure (EDP) remains elevated (figure 1). In the presence of impaired left ventricular systolic function this produces a vicious cycle of further elevations in EDV and EDP and progressive dilatation of the ventricle as seen in patients with chronic heart failure. During diastole the persistently elevated EDP is transmitted to the left atrium and to the pulmonary veins and capillaries causing transudation of fluid into the pulmonary interstitium and symptoms and signs of pulmonary congestion. One

of the major hopes of cellular therapy is potential regeneration of myocardium with subsequent improvement in contractile function (LVEF) and inotropy leading to reduction in EDV/ESV (figure 1). This is partly the reason why improvement in LVEF has been chosen as the surrogate marker to assess the efficacy of cell therapy in the clinical trial described in this thesis (chapter 4).

2.1.3.2 Neurohormonal changes

Neurohormonal activation involves three important compensatory mechanisms in response to reduced cardiac output and hypotension: the stimulation of the sympathetic nervous system, the renin-angiotensin-aldosterone system (RAAS), and the release of natriuretic peptides. In part, these mechanisms serve to increase systemic vascular resistance (SVR) to maintain arterial blood pressure in the setting of reduced cardiac output (blood pressure= cardiac output x SVR).

The fall in cardiac output is sensed as decreased perfusion pressure by baroreceptors in the carotid sinus and aortic arch. This activates the sympathetic adrenergic system which stimulates catecholamine synthesis leading to increased heart rate and SVR in an attempt to maintain blood pressure and perfusion.

The RAAS is activated early in patients with heart failure and this is mediated through an increase in renin release from the juxtaglomerular cells of the kidney in response to decreased renal artery perfusion pressure and also direct stimulation of juxtaglomerular β2 receptors by the activated adrenergic

system. Renin is an enzyme that cleaves circulating angiotensinogen to form angiotensin I, which is then rapidly cleaved by endothelial cell-bound angiotensin-converting enzyme (ACE) to form angiotensin II (Ang II) which is a potent vasoconstrictor[33]. Ang II also stimulates release of aldosterone from the adrenal cortex which expands the intravascular volume by increasing sodium reabsorption by the kidney.

Activation of the adrenergic and RAAS systems is initially beneficial in maintaining a cardiac output but in the long-term continued activation typically proves harmful. The increased intravascular volume adds to the increasing preload/EDV in a failing heart (figure 1) and leads to pulmonary and peripheral oedema. Continuous sympathetic activation results in down-regulation of cardiac β-adrenergic receptors and up-regulation of inhibitory G-proteins, contributing to a decrease in the myocardium's sensitivity to circulating catecholamines and a reduced inotropic response[34]. These neurohormonal mechanisms also play an important role in adverse LV remodelling.

In contrast, the natriuretic peptides are potentially beneficial hormones secreted in heart failure in response to activation of stretch receptors by the increased intra-cardiac pressure. The best studied of these are atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). BNP was originally identified in extracts of porcine brain[35] (hence the name), although in humans it is produced mainly in the cardiac ventricles[36]. The physiological actions of BNP (and ANP) include sodium and water excretion, vasodilation, inhibition of renin secretion and antagonism of the effects of Ang II[37]. These

effects are beneficial to patients with heart failure although are not usually sufficient to fully counteract the vasoconstriction and volume-retaining effects of the other activated neurohormonal systems. BNP is co-secreted along with a 76 amino acid N-terminal fragment (NT-proBNP) which is biologically inactive. The biological half-lives of BNP and NT-proBNP are at least twice as long as that of ANP making these peptides more suitable for diagnostic blood testing[38]. However, the measurement of BNP is not yet routine in clinical practice but has potential uses in the diagnosis of heart failure, monitoring response to treatment and prognostication. Studies have shown that BNP levels correlate with heart failure severity and clinical outcome[37]. Also, greater percentage reduction in BNP with treatment of decompensated heart failure maybe associated with better event-free survival[39]. We have therefore assessed for changes in NT-proBNP level in patients in our REGENERATE-IHD clinical trial (chapter 4) with the hypothesis that if cell therapy improves cardiac function this maybe reflected in a reduction in NT-proBNP level.

In addition to these well recognised neurohormonal mechanisms it is now becoming increasingly realised that there exists a 'cardiac-bone marrow axis' which is affected by myocardial injury[40]. Several experimental and clinical studies in myocardial infarction have shown activation of a bone marrow niche with release of pro-angiogenic cells and various types of haematopoietic stem cells (HSCs)[41, 42]. This effect appears to be beneficial with the extent of initial mobilisation of HSCs shown to be an independent predictor of a more favourable remodelling process following AMI[43].

2.1.3.3 Left ventricular remodelling

Following a large myocardial infarction, there are changes in the structure of the both the infarcted and non-infarcted ventricular muscle and these changes in chamber size and thickness (remodelling) affect long-term ventricular function and prognosis[44]. Figure 2 summarises the recognised pathophysiological processes that are thought to be involved in producing left ventricular remodelling.

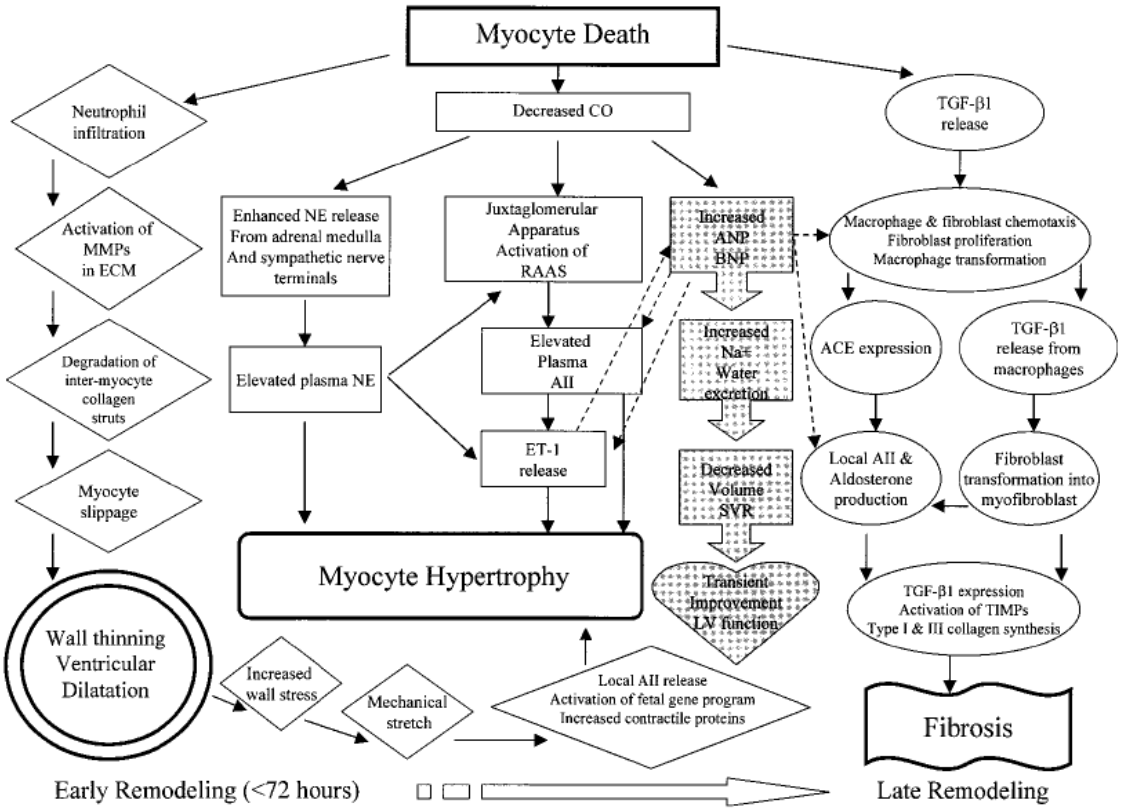


Figure 2. Schematic representing the pathophysiology of left ventricular remodelling following myocardial infarction. The many concomitant processes involved in early and late remodelling are shown in this diagram and discussed in the text. ACE: angiotensin converting enzyme; AII: angiotensin II; ANP: atrial natriuretic peptide; BNP: brain natriuretic peptide; ECM: extracellular matrix; ET: endothelin; MMP: matrix matalloproteinases; SVR: systemic vascular resistance. *Reproduced with permission from Lippincott Publishing*[45].

In the early stages following an MI, the infarct area is infiltrated by macrophages, monocytes, and neutrophils and there is infarct expansion which results from the degradation of the inter-myocyte collagen bridges by serine proteases matrix metalloproteinases (MMPs) released from neutrophils[46]. This inflammatory process is necessary to remove necrotic cells but at the same time is detrimental to surviving cardiomyocytes. Infarct expansion produces wall thinning and ventricular dilatation (figure 3), increased wall stresses and risk of ventricular rupture[47]. Increased wall stress is a potent stimulus for hypertrophy of non-infarcted segments which is mediated partly through intracellular signalling via Ang II release[48]. This increased mass of muscle fibres serves as a compensatory mechanism that helps to maintain contractile function and counteracts the elevated ventricular wall stress[45]. The inflammatory processes involved in early infarct expansion produce a hostile environment and may partly explain why the beneficial effects of stem cell therapy in AMI seem to be limited to when cells are delivered between 4-7 days after the infarct[49].

Late remodelling principally involves myocyte hypertrophy and collagen scar formation as mechanisms to alleviate the increased wall stresses and counteract the distending forces[50]. There is considerable change to the extracellular matrix during this remodelling process. Myocyte hypertrophy is initiated by activation of neurohormonal pathways, mechanoreceptors, local tissue RAAS and paracrine factors[45]. Increased diastolic wall stress leads to the synthesis of new sarcomeres in *series* with the old causing the myocytes to elongate[51]. The radius of the ventricular chamber therefore enlarges, doing

so in proportion to the increase in wall thickness- this is termed mural (or eccentric) hypertrophy[52], as illustrated in figure 3.

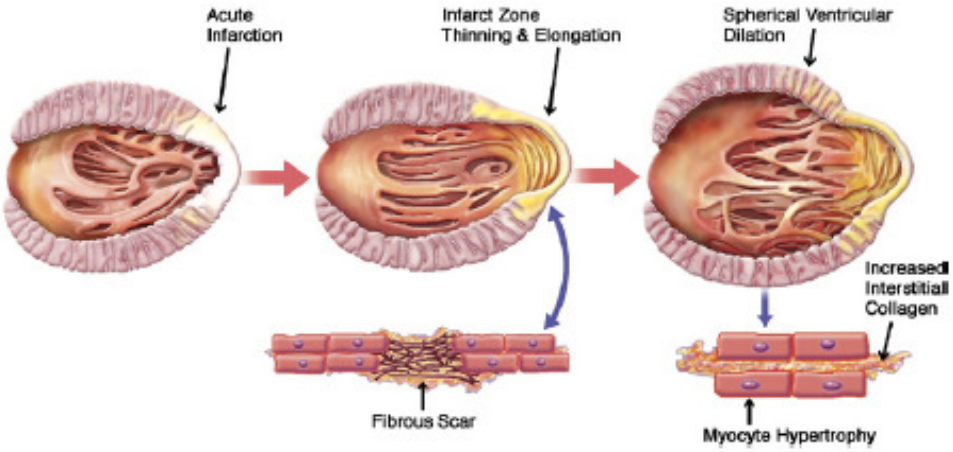


Figure 3. Illustration of the pathological consequences of LV remodelling following an acute myocardial infarction. Early remodelling involves infarct expansion of wall thinning and the initiation of fibrous scar formation. Late remodelling involves increased interstitial collagen and myocyte hypertrophy in non-infarcted segments leading to LV eccentric hypertrophy with spherical ventricular dilation.
Reproduced with permission from Elsevier Science[53].

In humans post infarct healing consists predominantly of myocardial fibrosis and scar formation which is triggered by release of the cytokine transforming growth factor-β1 (TGF-β1) from necrotic myocytes[54]. This cytokine is important in the proliferation of myofibroblasts which are responsible for collagen production[55]. Deposition of collagen occurs predominantly in the infarct zone; however, it also occurs in non-infarcted myocardium (figure 3). Collagen can be detectable within 1 week post infarct and by 4 weeks the necrotic myocytes are entirely replaced by fibrous tissue[46]. There is evidence to suggest that native bone marrow stem cells are involved in this reparative process by homing to the infarct area, transforming into myofibroblasts and contributing to scar tissue remodelling and neoangiogensis[8]. Unlike humans, zebrafish have been show to fully regenerate myocardium, without any scar formation, within 8 weeks of injury[56]. Understanding the processes involved in this 'scar-less'

healing process will hopefully provide insight into the barriers that prevent efficient regeneration in humans.

The early remodelling process is considered an adaptive and necessary process to stabilise the infarct area and augment cardiac output. However, beyond the early stages, remodelling predominantly involves hypertrophy of non-infarcted segments resulting in increased wall mass, chamber enlargement, and a shift from an elliptical to a more spherical chamber configuration[57, 58] (see figure 3). These changes result in a progressive decline in cardiac function and LV dilatation with the eventual development of signs and symptoms of congestive heart failure.

2.1.3.4 Myocyte loss and cellular dysfunction

Acute myocardial infarction leads to the loss of a large number of cardiomyocytes through cellular necrosis. In chronic ischaemic heart failure there is continued cell loss through apoptosis which maybe triggered by elevated catecholamines, Ang II, inflammatory cytokines and mechanical strain on the myocytes[59]. Even viable myocardium in heart failure is abnormal at a cellular and molecular level[59]. Mechanical wall stress, neurohormonal activation, and inflammatory cytokines, such as tumour necrosis factor α (TNF-α), are believed to activate changes in the genetic expression of contractile proteins, ion channels, catalytic enzymes, surface receptors and secondary messengers in the myocyte[60]. These detrimental processes were thought to be irreversible but recently the demonstration of mitotic cells within the human heart following injury[61] and chimerism of transplanted hearts[62]

suggests the potential for myocardial repair/regeneration. It is for these reasons that cell therapy has emerged as a potential treatment option with the hope that it can lead to cardiac repair through preventing apoptosis and improving cellular function in addition to potential myocyte regeneration and reverse remodelling. This is discussed in more detail in section 2.2.

2.1.4 Morbidity and mortality

The predominant symptom in patients with heart failure is dyspnoea on exertion. The mechanism of this is partly due to pulmonary interstitial oedema and also due to reduced perfusion of respiratory muscles. Reduced cardiac output and impaired perfusion of organs is responsible for other multiple symptoms seen in heart failure including lethargy, weakness and dulled mental status. The increase in intravascular volume secondary to maladaptive neurohormonal activation can also lead to peripheral oedema. The New York Heart Association (NYHA) classification can be used to give a simplified assessment of the functional capacity of an individual patient (Table 1). Furthermore, it is now well recognised that NYHA classification is also a strong independent predictor of prognosis with more symptomatic patients having a higher mortality[63, 64].

Table 1. The New York Heart Association Classification of Heart Failure

NYHA Class	Definition	Annual mortality
I	No limitation of physical activity	3-5%
II	Slight limitation of activity. Dyspnoea and fatigue with moderate physical activity (e.g. walking up stairs quickly)	10%
III	Marked limitation of physical activity. Dyspnoea with minimal activity	12-16%
IV	Severe limitation of activity with symptoms even at rest	15-20%

Current therapies which improve clinical outcomes in heart failure also improve NYHA class[65, 66] and hence clinical trials of therapies in heart failure routinely use improvement in NYHA functional class as a surrogate marker of efficacy. We have therefore assessed change in NYHA class at 6-months and 1-year post treatment as a secondary end-point in our study.

Patients with ischaemic heart failure can also suffer from angina and this can be graded according to the Canadian Cardiovascular Society (CCS) classification system[67]: Grade 1: No angina, Grade 2: angina on moderate exertion, Grade 3: angina on mild exertion, Grade 4: angina at rest. The change in CCS angina class has also been assessed in patients in our study, although this is not a pre-defined secondary end-point.

2.1.5 Current treatment options for heart failure

There are three main goals of current medical and device therapy in patients with heart failure:

1. Management of symptoms with vasodilators and diuretics

2. Attenuation of the neurohormonal response to help prevent/retard adverse ventricular remodelling in order to slow progression of LV dysfunction
3. Improvement of long-term prognosis

Standard pharmacologic treatments include ACE inhibitors, angiotensin receptor blockers, β-blockers, aldosterone antagonists, and diuretics. These treatments block key neurohormonal pathways and counteract salt and water retention, thus interrupting the vicious cycle responsible for progressive cardiovascular remodelling, volume overload and decreased exercise tolerance. The European Society of Cardiology heart failure guidelines provide detailed recommendations of how and when to introduce the different medical and device therapies in patients with heart failure[68]. A relatively brief description of the current available therapy is provided below.

Angiotensin-converting enzyme (ACE) Inhibitors

Angiotensin-converting enzyme (ACE) inhibitors are an important class of drugs in the management of patients with heart failure and LVSD. The mechanism of improvement with ACE inhibition is multifactorial and includes peripheral vasodilatation, ventricular unloading, and the attenuation of ventricular dilatation and remodelling. Importantly, ACE inhibition appears to have a direct effect on myocardial tissue preventing the inappropriate growth and hypertrophy stimulated by Ang II and other growth factors[69, 70]. A number of large studies have demonstrated a survival benefit when ACE inhibitors have been used in all patients with myocardial infarction[71, 72] and selectively in

patients with left ventricular dysfunction or heart failure[73]. V-HEFT2 was the first clinical trial of ACE inhibitors in heart failure. In this study, enalapril therapy led to a 28% reduction in mortality as compared to vasodilator therapy with hydralazine/nitrate[74]. The survival benefit of ACE inhibitors compared with other vasodilators further indicates important biological tissue action in addition to vasodilatation[75].

Angiotensin receptor blockers (ARBs)

ACE inhibitors can be associated with a dry cough in a proportion of patients and this is thought be related to accumulation of bradykinin secondary to inhibition of ACE. Angiotensin receptor blockers (ARBs) avoid this problem as they act downstream of ACE inhibitors by blocking the type-1 angiotensin receptor, thereby attenuating the biologic effects of Ang II. The largest clinical trials of ARBs in patients with chronic heart failure are Val-HEFT[76] and CHARM[77]. Both of these studies showed that ARBs were equivalent to ACE inhibitors with regard to heart failure mortality reduction. Therefore, ARBs are generally considered an acceptable alternative for patients who are intolerant of ACE inhibitors[68].

Beta-blockers

In addition to inhibition of the RAAS, antagonism of the sympathetic system with beta-blocker therapy is mandatory for all patients with LVSD. Several randomised trials (including CAPRICORN[78], MERIT-HF[79]) have demonstrated significant reduction in cardiovascular mortality by up to 48%. The mortality benefit from beta-blockade is due to a reduction in both progressive heart

failure and sudden death. Additional heart rate lowering with Ivabradine (a selective I*f* channel blocker acting on the sinus node) has been shown to improve symptoms and outcomes in heart failure[80].

Aldosterone antagonists

Aldosterone is an adrenal hormone and its production is stimulated by RAAS activation amongst other pathways. Aldosterone has been shown to lead to direct adverse effects on the myocardium including fibrosis and remodelling. The effects of inhibiting aldosterone in heart failure were first investigated in the RALES trial[81], where treatment with spironolactone resulted in a 30% reduction in mortality and 36% less hospitalisation in patients with NYHA class III-IV heart failure. More recently, in the EMPHASIS-HF trial[82] eplerenone was shown to reduce death and rehospitalisation in patients with only mild (NYHA class II) heart failure symptoms. Aldosterone antagonists are therefore now recommended in all symptomatic heart failure patients.

Implantable Cardiac Defibrillators (ICDs)

Sudden cardiac death (SCD) from ventricular tachycardia/fibrillation is a major cause of death in patients with heart failure. The ICD represents a significance advance in the primary prevention of SCD. The MADIT-I and MADIT-II studies[83] showed absolute survival benefit of approximately 1-1.5% per year with ICD therapy in patients with ischaemic heart failure and ejection fraction less than 30%. The National Institute of Clinical Excellence (NICE) in the UK recommends ICD therapy should be considered in patients with ischaemic heart failure and LVEF < 35%[84].

Cardiac Resynchronisation Therapy (CRT)

Mechanical ventricular dyssynchrony is present in upto a third of patients with LVSD. CRT with biventricular pacing is designed to resynchronise LV contraction and improve cardiac function and therefore symptoms. In the two largest trials of CRT, COMPANION[85] and CARE-HF[86], biventricular pacing was associated with an improvement in symptoms and a reduction in hospitalisation compared to optimal medical therapy alone. NICE recommends CRT should be considered in patients with symptomatic heart failure with LVSD and QRS duration >150 milliseconds.

Heart transplantation and left ventricular assist device (LVAD) therapy

Currently, heart transplantation is the only definitive therapy for end-stage heart failure but is limited to the sickest patients due to donor-organ shortage and approximately 15 to 30% of potential cardiac recipients die while waiting for a donor heart[87]. Furthermore, patients with ischaemic heart failure tend to be older and have significant co-morbidities which would exclude them from consideration for transplant.

Due to donor organ shortage and prolonged waiting times for a donor heart, there is growing use of mechanical left ventricular assist devices (LVAD) as a means of bridge to transplantation. There is also evidence (such as the REMATCH trial[88]) to suggest that LVADs can be used as 'destination' therapy in selected patients who are not suitable for transplantation. Interestingly, LVAD therapy appears to have biological beneficial effects on the native heart with evidence of reverse remodelling and in some cases significant recovery of

cardiac function[89]. This has led to the possibility of using LVAD therapy as a 'bridge to recovery' in patients with failing hearts and appears to be achievable in some patients[90]. This provides further evidence that the human heart is capable of recovery of contractile function given the right environment such as mechanical unloading of the ventricle. In an attempt to improve the success of 'bridge to recovery' there have been positive case reports of the adjunctive use of cell therapy at the time of LVAD implantation[90]. Clinical trials are underway to investigate this combination further and theoretically a volume unloaded ventricle may provide a better environment for transplanted cells to survive and engraft.

Despite the current available medical and device therapy the morbidity and mortality in patients with heart failure remains considerable. In a recent Canadian study of trends in heart failure outcomes, the 1-year risk-adjusted mortality only decreased from 17.7% in 1997 to 16.2% in 2007[91]. Hence, there are several novel therapeutic interventional strategies being investigated such as vagal nerve stimulation[92], cardiac contractility modulation therapy[93], surgical ventricular reconstruction[94] and percutaneous LV partitioning[95]. Over the past decade stem cell transplantation has emerged as a biological therapy with huge potential which may lead to cardiac repair with initial pilot studies suggesting improvements in cardiac function, symptoms and long-term prognosis in patients with heart failure. The next section provides a review of the field of cardiac stem cell therapy, particularly with regards to ischaemic heart failure.

2.2 Stem Cell Therapy for Heart Disease

2.2.1 Introduction

Myocardial infarction and heart failure are associated with significant loss of cardiomyocytes with subsequent fibrosis and scar formation leading to inevitable remodelling and left ventricular dilatation. Despite the wide variety of contemporary medical and device therapy there is no treatment that targets the primary problem of myocyte loss. The human heart seems unable to repair itself despite evidence of ongoing cell division[61] and renewal[62, 96] and it is possible that these innate self-repair mechanisms are overwhelmed by the magnitude of cell loss following injury. This is in contrast to non-mammalian vertebrates such as the zebrafish which are capable of regenerating up to 20% of myocardium following injury[56]. The aims of cardiac regenerative medicine are to induce repair of the human heart following injury either by up-regulation of its own repair mechanisms or by the addition of biological therapy, such as genes or stem cells. The demonstration of adult stem cell plasticity and in particular the potential for cardiomyocyte differentiation has prompted intense basic and clinical research with the aim of harnessing the regenerative properties of stem cells in order to repair damaged myocardium, improve cardiac function and improve patient morbidity and mortality.

2.2.2 Stem cell potential- an overview

Stem cells are defined by two unique characteristics: they are uncommitted cells capable of unlimited self-renewal and secondly they can differentiate into more specialised cells and organs. The most primitive and ultimate stem cell is the zygote which can develop into all cell types including the embryonic membranes- this potential is termed totipotent. The developing embryo contains embryonic stem cells (ESCs) which are pluripotent as they can develop into cells from all three germinal layers (i.e. mesoderm, endoderm and ectoderm) and produce all the tissue types needed to form a functional organism. There are also multipotent stem cells, such as haematopoietic stem cells (HSCs), which can produce a small range of differentiated cell lineages appropriate to their location

Developed adult organs also contain undifferentiated stem cells but in far fewer numbers, these are called adult (somatic) stem cells (ASCs). These cells were thought to be limited in differentiation potential and only able to replenish and repair injured tissue in the organ that they resided in. However, more recently adult stem cells have been shown to 'transdifferentiate' into cell types from different germ layers, a property referred to as plasticity, and this has been demonstrated in several scientific studies[97, 98]. Regenerative medicine is an emerging interdisciplinary field of research which hopes to utilise the properties of ESCs and/or ASCs in clinical applications focusing on the repair and regeneration of cells, tissues, or organs.

2.2.3 What is the ideal cell type for cardiac repair

The ideal cell type for the purposes of cardiac repair should possess the following characteristics:–

- Ability to repair/regenerate damaged myocardium
- Easy to obtain and expand
- Safe (no tumour formation)
- No immunogenicity issues
- Easy to store
- Easy to deliver
- No ongoing ethical issues
- Cost-effective

A variety of cell types have been explored for the purposes of cardiac repair (see Table 2).

Table 2 Different cell types that have been investigated for use in cardiac repair

Allogeneic	Autologous
Embryonic stem cells (ESCs)	Induced Pluripotent Stem cells (iPSC)
Fetal Cardiomyocytes	Adipose derived stem cells
Human umbilical cord derived cells	Resident cardiac stem cells (cardiospheres)
Allogeneic mesenchymal stem cells	Epicardium derived stem cells
	Skeletal myoblasts
	Mesenchymal stem cells (MSCs)
	Bone marrow derived:
	Endothelial progenitor cells (EPCs)
	Mononuclear cells (MNCs) e.g CD34+
	Mesenchymal stem cells (MSCs)

Embryonic Stem Cells (ESCs)

Given that multiple cell types are generated from a single embryonic stem cell (ESC) origin during embryonic development, the potential of such a cell for cardiac repair is obvious. ESCs have diverse potential and current work has already shown that their differentiation can be controlled to produce specific cell types useful for clinical application. The potential of ESCs is limited by several significant drawbacks: collection of cells from embryos raises ethical concerns; implanted cells will require immunosuppression in recipients to prevent rejection; and lastly; the potential of growth and differentiation that makes ESCs desirable for cellular repair can result in tumour formation. Hence, although ESCs have significant potential for cardiac repair, the inherent drawbacks have prompted researchers to actively investigate the possible use of adult stem cells instead.

Fetal cardiomyocytes

Fetal cardiomyocytes were one of the first cell types to be investigated as potential candidates for cardiac repair. Animal studies have shown fetal cardiomyocyte transplantation improves the function of ischemic and globally failing hearts[99, 100]. However, the use of fetal cardiomyocytes has similar concerns to ESCs including availability, immunogenicity, and ethics thus other cell types have surpassed this as likely candidates for use in cardiac repair.

Human Umbilical Cord Derived Stem Cells

Umbilical cord blood is blood that remains in the placenta and in the attached umbilical cord after childbirth. It has been shown to be a potent source of haematopoietic stem cells. Human umbilical cord blood mononuclear cells (hUCBCs) are currently used clinically for repopulating bone marrow in patients being treated for bone marrow disorders such as acute leukaemia. Cord blood contains a large number of non-haematopoietic stem cells which rarely express HLA class II antigens and appear to be immunologically naive thus reducing the risk of rejection and are therefore an attractive option for regenerative therapy[101]. In animal models of acute myocardial infarction injection of hUCBCs is associated with significant reductions in infarct size particularly when given by the intramyocardial route[102]. Phase I clinical studies are being planned/performed with the use of hUCBCs for the treatment of patients with dilated cardiomyopathy and refractory angina.

Unlike ESCs, autologous adult stem cells are virtually free from the risks of teratoma formation and immune rejection although display more limited differentiation potential. Adult stem cells that have been studied to date include induced pluripotent stem cells, adipose derived stem cells, tissue-resident cardiac stem cells, skeletal myoblasts, mesenchymal stem cells, circulating endothelial progenitor cells and most commonly bone marrow–derived mononuclear stem cells.

Induced Pluripotent Stem Cells (iPSCs)

An exciting alternative to ESCs is emerging in the form of *inducible Pluripotent Stem Cells* (iPSCs)- these are adult stem cells that have been successfully reprogrammed back to an undifferentiated pluripotent state by inserting four genes, Oct3/4, Sox2, KL4 and c-Myc, into differentiated somatic cells[103-105]. These cells have the morphological phenotype of ESC cells and have been demonstrated in vivo and in vitro to have the same differentiation potential as ESCs (i.e. able to form all three germ cell layers). Functioning cardiomyocytes[106] have already been produced from iPSCs demonstrating their potential use in cardiovascular regenerative medicine. Despite the potential inherent ability to produce patient individualised iPSCs, problems remain that must be solved before they can be used in clinical trials. The main concern is tumourigenesis. The majority of iPSC lines have been derived by inserting putative oncogenes using integrating retroviruses, e.g., lentiviruses, into the host genome which have the potential to cause cancer. One study demonstrated up to 20% of off-spring derived using iPSCs developed tumours[107].

Another practical barrier to clinical use of iPSCs will be the ability to produce sufficient cells for therapeutic purposes. Experiments to date have shown low conversion rates in the percentage of cells treated compared to those that are successfully induced into an iPSC phenotype. The percentage of conversion to iPSCs in the literature varies from 1–0.0006%[108]. Once the complete mechanism of reprogramming is understood, and the tumourigenesis problem has been resolved, there are considerable expectations that iPSC production

can be up-scaled to provide a patient specific source of cardiovascular cells for use in regenerative medicine. Until these aims are accomplished, the main cell types in use for cellular therapy remain adult tissue derived stem cells.

Adipose derived stem cells (ADSC)

Adipose tissue has been investigated as a source of adult progenitor/stem cells for the purposes of cardiac repair as this tissue contains a heterogeneous mixture of endothelial, haematopoietic and mesenchymal progenitor cells which can be easily harvested by liposuction[109]. Preclinical studies have shown that adipose derived stem cells (ADSC) are associated with improvement in ejection fraction in animal models of myocardial infarction and neoangiogenesis via paracrine factors has been postulated as a potential mechanism of action of ADSCs[110, 111]. Clinical trials are currently ongoing and include the APOLLO Study (ClinicalTrials.gov Identifier: NCT00442806) and PRECISE Study (ClinicalTrials.gov Identifier: NCT00426868) which are assessing the safety and efficacy of ADSCs in acute myocardial infarction and chronic myocardial ischaemia respectively.

Resident Cardiac Stem Cells

The heart has been considered a terminally differentiated organ lacking self-renewal capabilities but this dogma has been challenged recently, particularly with the demonstration of continued cell division within the adult heart following injury such as myocardial infarction[61]. The discovery of myocardial chimeras further increased the suspicion of the existence of a cardiac stem cell (CSC). Myocardial chimerism was demonstrated by testing for the

presence of Y chromosome in explanted hearts of male patients who had received female donor hearts. Male derived myocytes and endothelial cells were revealed within the female heart[62]. This finding was interpreted as representing cardiac repair affected by circulating progenitor cells. A second similar study, in which the hearts of females who had received male bone marrow were examined for presence of the Y chromosome, concluded that bone marrow progenitor cells were capable of transit to the heart and its subsequent repair[112].

The chimera studies suggest the existence of a population of cardiac progenitors recruited from a non-cardiac source such as bone marrow. However, several independent investigators have now presented strong evidence for a resident population of cardiac progenitor cells within the heart. The isolated cardiac progenitor cells have standard stem cell defining characteristics and are capable of differentiating into multiple cardiac cell lineages such as cardiomyocytes, endothelial and vascular cells[113, 114]. Similar findings have been replicated in mice, rats, dogs and humans. In animal models, the CSCs have been shown to repair and improve cardiac function following myocardial ischaemia[115]. Thus, CSCs are an attractive option for use in clinical trials, as they are intrinsically more aligned to producing all the cells needed to repair the damaged heart. However, they are significantly more difficult to harvest and isolate compared to BMSCs and the way ahead may be activation and stimulation of patients' own intrinsic CSCs rather than transplantation. There is preclinical evidence to suggest that bone marrow

derived stem cell therapy maybe able to stimulate endogenous CSC proliferation to induce cardiac repair[116].

A harvesting technique has been described that involves obtaining human myocardium from surgical or endomyocardial biopsies which were then partially enzymatically digested followed by culture for 14–24 days[114]. Small round cells were seen to bud off from the primary ex-plant and divide in suspension. The isolated cells were then grown in suspension culture, in differentiation media, which induced formation of a ball of cells that has been termed a cardiosphere[114]. When cardiospheres were grown in co-culture with rat neonatal cardiomyocytes, approximately 20% of cardiospheres were seen to contract spontaneously. Moreover, spontaneous calcium transients were seen in a small percentage of these contracting cells.

Human cardiosphere derived cells, when injected into the border zone of mouse model of myocardial infarcts engrafted and migrated into the infarct zone. After 20 days, the percentage of viable myocardium within the infarct zone was greater in the treated group than in the fibroblast treated control group; likewise, echocardiography performed on day 20 post-infarct revealed a higher LVEF in the cardiosphere treated group (42.8±3.3%) than in either the fibroblast-treated (25.0±2.0%, $P<0.01$) or the control (26.0±1.8%, $P<0.01$) group[117]. The CArdiosphere-Derived aUtologous Stem CElls to Reverse ventricUlar dySfunction (CADUCEUS) study[118], which is a Phase 1 Study of 30 patients to receive intracoronary autologous cardiosphere-derived stem cells, recently published 6-month follow-up results. Biopsy samples yielded

prescribed cell doses within 36 days. Compared with controls at 6 months, MRI analysis of patients treated with CSCs showed reductions in scar mass, increases in viable heart mass and regional contractility, and regional systolic wall thickening. However, changes in end-diastolic volume, end-systolic volume, and LVEF did not differ between groups by 6 months.

Epicardium derived progenitor cells

It has recently been demonstrated that the zebrafish can fully regenerate myocardium after cryo-induced injury/infarction of up to 20% of the ventricle[56, 119]. Recent experimental evidence suggests that this regeneration may occur through limited dedifferentiation of existing cardiomyocytes followed by proliferation[120] and through activation and expansion of surrounding epicardial tissue which supports the regenerating myocardium[121]. These studies in zebrafish suggest that the epicardium may play an important role not only in adult heart repair but also during continuous growth of the adult heart[122]. A subset of epicardium derived cells (EPDCs) expressing known markers of stem cells, c-kit and the CD34, have been identified in the subepicardial space of fetal and adult human hearts[123]. Experimental studies have demonstrated that EPDCs have the potential to differentiate into cardiomyocytes[124] and intramyocardial injection of human EPDCs in a mouse model of myocardial infarction has been shown to improve cardiac function supporting the hypothesis that EPDCs may play an important role in cardiac repair. The question arises why human EPDCs remain dormant following myocardial injury and current work is focusing on whether paracrine factors may play a role in activating these cells. A potential stimulus has been identified in the

form of thymosin β4 (Tβ4), which is an actin monomer-binding protein that has been shown to activate EPDCs to a pluripotent state, possibly by an epigenetic effect- a chemical change to DNA which alters gene expression[125]. In a recent landmark experiment, pre treatment of mice with Tβ4 prior to inducing a myocardial infarct led to activation of EPDCs which underwent cardiomyocyte differentiation and infarct size and overall ejection fraction were significantly better in the treated mice compared to controls[126]. This study provides evidence that activation and up-regulation of the hearts own repair mechanism may be possible without the need for additional biological therapy. A clinical study on the safety and efficacy of injecting thymosin β4 for treating acute myocardial infarction has already been planned (ClinicalTrial.gov Identifier NCT01311518).

Skeletal myoblasts

Skeletal myoblasts have been widely studied due to several favourable characteristics: their muscle phenotype with hope for contractile function when transplanted into the heart; autologous source of cells avoiding immune rejection; minimal risk of tumour formation after transplantation; easily harvested; cell numbers can be increased significantly prior to transplantation; and finally, they are relatively resistant to ischaemia which may help long-term survival in the hostile environment within the diseased heart. Early preclinical animal studies have demonstrated the ability for skeletal myoblasts to engraft, form myotubules, and enhance cardiac function after transplantation into infarcted myocardium[127]. However, not all of the pre-clinical studies have provided positive results. For example, consistently, studies have

demonstrated significant cell loss: up to 84% loss in the first 24 hours and death of the transplanted myoblasts within a few days[128, 129]. Furthermore, transplanted cells down-regulate the major adhesion and gap junction proteins, N-cadherin and connexin43, of the intercalated disk[130] and are functionally isolated from the host myocardium with no evidence of electromechanical coupling[131]. This may account for the increased incidence of arrhythmias seen in hearts after myoblast transplantation[132].

Human studies (discussed in more detail later) have shown that epicardial injection of these cells during coronary artery bypass (CABG) surgery is feasible with potential functional benefits[133]. The main limitations regarding the use of skeletal myoblasts is that they remain committed to the skeletal muscle lineage and have been associated with arrhythmias[134], the mechanism of which is yet to be fully defined but may involve the processes described above.

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) can be found in bone marrow, muscle, skin and adipose tissue. They have a fibroblast-like morphology, and can differentiate into bone, cartilage, and fat cells[135]. In addition to this well described tri-lineage potential, MSCs have been shown to transdifferentiate in vitro and in vivo towards cardiomyocyte[136] and vascular cell phenotypes. In vivo pre-clinical studies have demonstrated that MSCs have the capacity to instigate both myocardial repair and neovascularisation in animal models of cardiac injury[137]. Differentiation of MSCs into cardiomyocytes and endothelial

cells in vivo when transplanted into the heart in injury models has also been demonstrated. Such transdifferentiated cells have been strictly characterised by immunohistochemistry and positively stain for cardiac and endothelial specific markers, as well as gap junction proteins[138]. Furthermore, MSC transplantation in acute MI[111] and in ischaemic cardiomyopathy[139] has been reported to induce functional benefits that include reduced scar formation and infarct size, improved regional and global ventricular function, and increased vascular density and myocardial perfusion. There has also been evidence of benefit in studies using non-ischaemic models such as dilated cardiomyopathy[140].

MSCs also appear to be relatively immunoprivileged as they lack various major histocompatibility complex and co-stimulatory cell-surface antigens and therefore may be used as an allogeneic graft[141]. This avoids the need for harvest procedures such as bone marrow aspiration or liposuction and MSCs can potentially be given intravenously as they have been shown to home to injured myocardium following acute myocardial infarction (AMI) in animal models[142]. The safety of intravenous allogeneic MSCs (Prochymal™, Osiris Therapeutics) has been demonstrated in a Phase I randomised controlled trial of patients with AMI. In this trial of 53 patients adverse event rates were similar in the MSC and placebo treated groups and interestingly ejection fraction was significantly higher in the MSC treated group at 6-months. A larger Phase II study is currently underway to investigate these findings further. As mentioned above, clinical trials of adipose tissue derived stem cells (containing MSCs) are also in progress.

MSCs are an attractive option for clinical cardiovascular therapy because of their demonstrated ability to facilitate myocardial and vascular repair. They are easy to collect, manipulate and expand once in tissue culture, and there is potential for their use in allogeneic transplantation. However, many aspects of the biology of their therapeutic use remain to be fully understood and optimised. Continued research into the basic physiology of MSCs, especially with respect to their therapeutic uses, is needed if clinical potential is to be realised.

Endothelial Progenitor Cells

Successful cellular therapy for cardiac regeneration will require transplantation of functioning cardiomyocytes and concomitant generation of an adequate blood supply. Endothelial progenitors cells (EPCs) can contribute to tissue revascularisation and can be isolated from adult bone marrow or from the peripheral circulation (termed circulating endothelial progenitor cells- CEPs). Adult derived EPCs and CEPs can be distinguished from mature endothelial cells by a functional in vitro assay due to their high proliferation rate. Mature endothelial cells, EPCs and CEPs share several endothelial specific markers. However, only EPCs and CEPs express AC133 (CD133)[143]. The current accepted definition of EPCs is co-expression of CD133+VEGFR2+ markers[144]. Human CEPs have shown potential to differentiate to cardiomyocytes. When co-cultured with neonatal rat cardiomyocytes, human CEPs formed cells with a cardiomyocytic phenotype as defined by positive staining for cardiac specific markers, such as troponin, atrial naturetic peptide and MEF-2[145]. EPCs and CEPs play an important role in neovascularisation in vivo [146]. Circulating EPCs

are mobilised in response to organ ischaemia, trauma and acute myocardial infarction[147]. The increase in CEPs post-MI is mirrored by a rise in the growth and migratory cytokine VEGF-A, and suggests a role for this factor in the mobilisation of progenitor[148]. Also, HMG-CoA reductase inhibitors (statins) have been shown to augment mobilisation of EPCs. This important observation may provide an alternative mechanism by which statins decrease morbidity and mortality in patients with ischaemic heart disease [149].

Transplantation of EPCs and CEPS has been shown to promote neovascularisation of the ischaemic heart and improve function. Transdifferentiation to endothelial cells, smooth muscle cells and cardiomyocytes has been characterised by immunohistochemistry[150, 151]. In animal models of MI, transplantation of EPCs or CEPs causes a significant increase in capillary density, regional blood flow and collateral formation in the ischaemic heart. In addition, cardiac function is also significantly improved following transplantation [152, 153]. Encouraging results such as these has led to human clinical studies of EPC transplantation. Two studies have been completed to date; EPCs or CEPs were transplanted either intracoronary following acute MI or transepicardial at the time of coronary bypass surgery. There were no adverse outcomes and both studies were able to show an increase in cardiac function with improved myocardial perfusion [154, 155]. A major limitation of therapeutic use of EPCs in cardiac repair is the current debate regarding their true phenotype[156]. Close consideration of the definition of this cell type is needed to ensure agreement and consistency in the preclinical research and subsequent translation to the clinical arena.

Bone marrow derived stem/progenitor cells (BMSCs)

The most widely studied of the adult stem cells has been bone marrow derived stem cells (BMSCs). These consist of two distinct cell populations: mesenchymal stem cells (MSCs) and mononuclear cells (BM-MNCs). BM-MNCs include haematopoietic stem cells (HSCs) and endothelial progenitor cells (EPCs). All three cell types (MSC, HSC and EPC) have shown the potential to transdifferentiate to a cardiomyocyte phenotype[138, 145, 157]. BMSCs can also be characterised by specific cell surface markers, for example CD34, which is expressed on primitive stem cells and differentiated HSCs cells, and is absent on mature haematopoietic cells. Additionally, cell populations can be further distinguished by the lineage marker (Lin) which if present represents cells committed to a particular lineage, and the stem cell factor receptor c-kit and the stem cell antigen-1 (sca-1) which are both markers of primitive stem cells.

The developmental potential of BMSCs is now recognised to expand beyond the haematopoietic lineages with numerous reports indicating that cells derived from bone marrow were capable of giving rise to multiple unexpected cell types. These include neural cells[158], skeletal muscle[97] and hepatic cells[159]. This plasticity of BMSCs, has led to considerable excitement in using BMSCs in cell-based therapies and as vectors to deliver therapeutic agents. This is particularly attractive clinically because bone marrow can be readily obtained from patients by aspiration from the marrow itself or by harvest from the peripheral circulation following mobilisation with cytokines such as granulocyte colony stimulating factor (G-CSF).

In a controversial landmark animal study[5], $Lin^{-}c\text{-}kit^{+}$ (markers of undifferentiated stem cells) BMSCs were injected directly into the contracting wall bordering a myocardial infarct which had been acutely induced by coronary artery ligation. The transplanted cells appeared to undergo transdifferentiation to cardiomyocytes with newly-formed myocardium occupying a significant proportion of the infarcted area with significant improvement in LV function just 9 days after cell transplantation[5]. This transdifferentiation event has not been universally reproduced and in fact has been challenged by different groups, which have demonstrated that injected BMSCs develop into haematopoietic cell types after transplantation rather than cardiomyocytes[7-9]. However, even in the absence of transdifferentiation there appeared to be a beneficial effect on cardiac function[9]. There have also been studies in large animal (porcine) models of chronic myocardial ischaemia which have shown improvement in cardiac function following BMSC treatment[160, 161].

There have been few experimental studies comparing the efficacy of different cell types in improving cardiac function. In a mouse model of AMI, bioluminescence imaging after intramyocardial cell injection suggested better tissue retention of BM-MNCs which appeared to be present at 6 weeks whilst MSCs and skeletal myoblasts could not be detected after 3 weeks[162]. Furthermore, BM-MNCs had a more favourable effect on cardiac function compared to the other cell types although interestingly there was no evidence of transdifferentiation on histological evaluation. Another study, in a large animal (canine) model of chronic ischaemic heart failure, showed

intramyocardial injection of BM-MNCs to again be superior to MSC injection with regards to improvement in cardiac function, reduction in infarct size and NT-proBNP levels[163]. Interestingly, functional improvement was associated with a neoangiogenesis.

In summary the different cell types described above offer a range of therapeutic options. The BMSC has to date had the most application in clinical studies through ease of use and positive effects on cardiac function. The ESC continues to offer the greatest therapeutic potential whilst the iPSC may well provide the best compromise between these and the BMSC population. Much work is still needed to identify a cell type that meets the ideal criteria and moves the field closer to delivering a viable biological therapy that can be used in routine clinical practice.

2.2.4 Proposed Mechanism(s) of Actions of Stem Cell Therapy

The mechanism(s) by which adult stem cell therapy appears to improve cardiac function and perfusion following pathological injury remains an area of ongoing investigation and debate. The initial theory was that transplanted stem cells formed new cardiomyocytes and endothelial cells directly leading to improved myocardial contraction and perfusion. This was based upon individual publications which appeared to demonstrate transdifferentiation of transplanted BMSCs. However, this finding has not been consistently reproduced by other investigators leading to the ongoing debate regarding the contribution of transdifferentiation to functional benefit and it is now thought that the beneficial effects seen are multi-factorial in origin[164]. Possible

additional mechanisms include neovascularisation by differentiation into an endothelial phenotype, cell fusion, paracrine effects of the cell infusate including promotion of angiogenesis and stimulation of the resident cardiac stem cell population[116, 164, 165] (Figure 4).

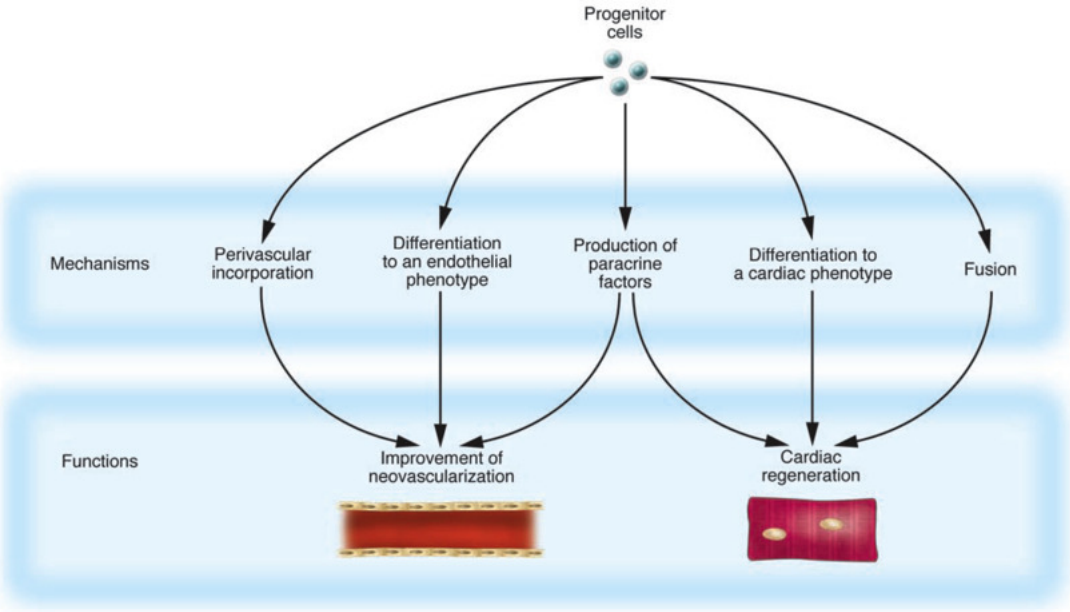


Figure 4. Illustration of the proposed mechanisms of action of stem/progenitor cell therapy. Cell therapy is now thought to exert beneficial effects through one or a combination of the processes shown. An additional recognised mechanism is the stimulation of resident cardiac stem cells.
Reproduced with permission from American Society for Clinical Investigation[164].

The direct transdifferentiation theory was formed from several findings. Initially, the concept of exogenous cell delivery as a possible means of improving cardiac function was demonstrated. For example, when transplanted into the heart, embryonic cardiomyocytes formed stable grafts[166] while foetal cardiomyocytes improved cardiac function after cryoinjury[100]. Following on from these findings, adult stem cells started to be seen as possible donor cells. Such hope was based upon evidence of adult stem cell plasticity, particularly those found within the bone marrow. Animal experiments

demonstrated that circulating endothelial cells of bone marrow origin could contribute to neovascularisation in adult tissues[167]. Evidence for haematopoietic stem cells contributing to cardiac muscle and vasculature were also seen[157]. Furthermore, human mesenchymal stem cells (MSCs) were shown to have multi-lineage potential[168] and were able to form cardiomyocytes in vitro when chemically treated[136]. These findings were given support from the examination of human cardiac sex-mismatch transplants that demonstrated cardiomyocyte and vascular chimerism, i.e. de novo post natal formation of cardiac and vascular tissue- this supported the idea of a native circulating progenitor cell being able to contribute to cell repair[62].

Multiple pre-clinical studies continued to support the transdifferentiation hypothesis, initially suggesting that all three cell lines (MSCs, HSCs and EPCs) from adult bone marrow were able to contribute to cardiac regeneration in small animal models of myocardial ischaemia. MSCs were shown to engraft into ischaemic myocardium, and express muscle proteins in murine models of myocardial infarction[137, 138]. Human EPCs were able to simulate neoangiogenesis within the infarcted vascular bed, reducing apoptosis and improving cardiac function in a mouse model[151]. In one prominent study bone marrow derived cells were shown to regenerate substantial amounts of myocytes, endothelial cells and smooth muscle cells when injected into the hearts of mice following myocardial infarction[5]. The latter study remains controversial and favour for the direct transdifferentiation theory has receded ever since muscle generation and transdifferentiation were not seen in two high profile studies of bone marrow progenitor therapy, although a small

functional benefit was still seen[7, 9]. Other studies observed that the rare transdifferentiation events that were thought to be de novo cardiomyocyte formation could be attributed to donor and host cell fusion[169, 170]. Furthermore, recent studies suggest that BMSCs stimulate proliferation of resident cardiac stem cells and this maybe an important mechanism of cardiac repair[116].

The search for a complete explanation behind the benefits gained with stem cell treatment has led to the proposal that local release of paracrine factors could be the significant key effect of transplanted cells rather than myogenesis. Supporting this, it is clear adult stem cells are capable of secreting a wide range of cytokines[171]. The released paracrine factors have been shown to induce a number of beneficial effects, including induction of angiogenesis in host tissues[151], reduced apoptosis[171] and immunomodulation of injury[172]. A further documented paracrine benefit is the proposed stimulation of the recently described resident cardiac progenitor cells[165 173]. There is a growing body of literature demonstrating the use of stem cells in therapeutic angiogenesis[18, 174, 175]. Furthermore, BMSCs transplanted into injured myocardium express several signalling factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and transforming growth factor (TGF), all key signalling factors involved in angiogenesis, cytoprotection and survival[176].

Two noteworthy studies support the hypothesis for an acute paracrine effect of adult stem cells. Akt (protein kinase B) is a kinase that regulates several cellular processes, including cell cycle progression, transcription, glucose

uptake and apoptosis. In a rat MI model, both MSCs transfected with Akt and Akt-MSC conditioned media significantly limited infarct size when administered into the infarct border zone one hour after ischaemia was established[177]. The genes for VEGF, FGF-2, HGF, IGF-1, and Tβ4 were all found to be up-regulated within the Akt-MSCs, especially in the setting of hypoxia. These data strongly support a paracrine mediated mechanism for myocardial protection in AMI. The second supportive study used cardiac cryoinjury in rats; immediately following injury MSCs were injected into the border zone[178]. Infarct size was assessed 10 weeks later and found to be significantly reduced, but no evidence of myogenesis or angiogenesis could be found- indirect evidence for a paracrine effect over transdifferentiation. Furthermore, bone marrow mononuclear cells have been demonstrated in vitro and in vivo to significantly reduce ischaemia-reperfusion injury. The mechanism of this benefit has been suggested to be due to a paracrine related triggering of mediators of the reperfusion injury salvage kinase (RISK) pathway, the final common beneficial mediator of both pre- and post-conditioning[179-181]. The RISK pathway refers to a group of protein kinases that, when specifically activated during myocardial reperfusion, provide cardioprotection by preventing lethal reperfusion injury. The RISK pathway is thus a mediator of cell survival[182, 183]. Another component of the amelioration of reperfusion injury, aside from activation of the RISK pathway, could be a direct effect on reactive oxygen species (ROS). MSCs in particular can reduce ROS generation. MSCs express the enzymes required to manage ROS, in particular high levels of glutathione peroxidise[184], and have been shown to produce superoxide dismutase[185].

It is possible that different mechanisms are important at different time points as the pathophysiological processes change during the evolution form acute myocardial infarction to chronic heart failure. The mechanism of acute repair could be considered more likely to be related to the release of local factors that act on cells within the myocardium reducing ischaemia-reperfusion injury and thus reducing infarct size. This is unlikely to be the case for chronic heart failure where there is no acute injury and in fact there are no studies that demonstrate benefit of reperfusion injury modulation in this setting[186]. It is more likely that robust reparative processes leading to angiogenesis and myogenesis will be required to improve chronically scarred myocardium. Understanding the complete pathways involved and how they interact at specific time points in the progression from acute myocardial infarction to chronic heart failure will be essential to utilising and optimising adult stem cells in clinical therapy.

A major criticism of clinical trials, using progenitor cell therapy for the treatment of patients with end-stage heart failure, is that they fail to address mechanistic questions, given an overall lack of understanding of how this approach may lead to an improvement in cardiac function and symptoms[187, 188]. It is clear that the field of translational medicine in general is in need of novel new imaging techniques that will help bridge the transition from the bench to bedside. This thesis presents the preliminary results of a 'first in man' feasibility study to detect angiogenesis following BMSC transplantation using nuclear imaging and a novel radio-tracer (Technetium-99m labelled cyclic

RGD peptide: ^{99m}Tc-NC100692; GE Healthcare) targeted at the αvβ3 integrin that is expressed by vessels undergoing angiogenesis[189, 190].

2.2.5 Delivering Stem Cells to the Heart

There are currently five available methods for delivering cell therapy, as illustrated in figure 5: (1) peripheral infusion; (2) cell mobilisation with cytokines such as G-CSF; (3) transvenous via the coronary sinus; (4) intracoronary infusion; (5) intramyocardial injection- which can be either transepicardial, transendocardial or trans-coronary-venous injections.

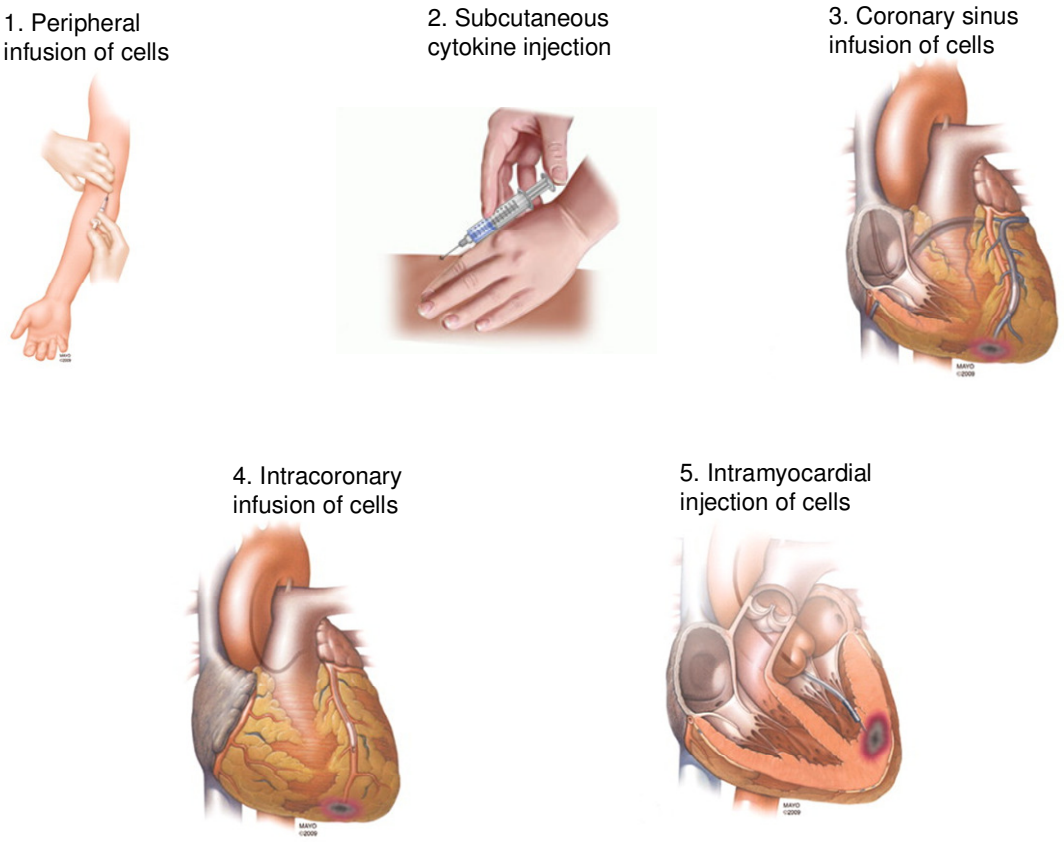


Figure 5. Available methods of delivery for cell therapy. There are currently five different methods for delivering cell therapy: (1) peripheral infusion; (2) cell mobilisation with cytokines such as G-CSF; (3) transvenous via the coronary sinus; (4) intracoronary infusion; (5) intramyocardial injection- which can be either transepicardial, transendocardial or trans-coronary-venous injections.
Adapted from Gersh et al. Mayo Clin Proc. 2009;84(10):876-892.

All the techniques remain in the early stages of clinical investigation and it remains to be determined which of these will prove to be the most efficacious approach. A limitation common to all current techniques is the low target area cell retention achieved which has been highlighted in a preclinical study[191] that demonstrated maximal cell retention rates within the myocardium of 11% with intramyocardial injection, 3% with intracoronary injection and 3% with intravenous infusion. There is ongoing work trying to elucidate the reasons for these losses and finding solutions to improve the efficiency of acute cell retention such as combining the cells with a biological scaffold[192]. Numerous scaffold designs have been created for use in cardiac applications using gelatin, fibrin, collagen, and alginates in the form of gels or 3D scaffolds [193, 194].

2.2.5.1 Peripheral Infusion

Peripheral intravenous infusion of stem cells would be an attractive non-invasive method of stem cell delivery due to its simplicity and applicability. Human bone marrow cells have been shown to 'home' to peri-infarct areas when infused into the peripheral circulation of a mouse model of acute myocardial infarct [151]. However, this technique may not be suitable in the chronic heart failure setting which lacks the temporarily up-regulated biological homing signals present in the AMI setting. Another significant limitation is that only a few cells appear to reach the affected area due to trapping of the cells in the microvasculature of the lungs, liver, and lymphoid tissues [195]. Peripheral infusion has been extensively used in animal models but there has been only one human study to date- a placebo controlled study assessing primarily the safety of peripheral infusion of allogeneic human mesenchymal stem cells

(Prochymal™) after acute myocardial infarction[196]. This was confirmed as a safe delivery method and the treatment group showed significant improvement in symptoms, LV ejection fraction as well as evidence of reverse remodelling on Cardiac MRI. However, given the already small numbers of injected cells that are known to be retained by the heart it is likely that more direct interventional injection will be more efficacious.

2.2.5.2 Cell Mobilisation

Cell mobilisation provides an indirect method of introducing cell therapy to the injured myocardium. In the clinical setting, one of the most studied mobilising factor is granulocyte-colony stimulating factor (G-CSF). Endogenous G-CSF is a potent hematopoietic cytokine that affects progenitor cell proliferation, maturation and functional activation and enhances the mobilisation and recruitment of stem cells from the bone marrow to the blood circulation[197]. G-CSF activates progenitor cell releasing factors such as neutrophil elastases and matrix-metalloproteinase to release the stem cells from the bone marrow[198]. G-CSF is also proposed to have a range of direct effects on the myocardium, including creating a favourable environment for homing and engraftment of stem cells, inhibiting apoptosis and promoting cardiomyocyte survival, including angiogenesis and reducing fibrosis associated with adverse cardiac remodelling [199]. G-CSF is generally well tolerated with most common side effects being bone pain and fevers.

Initial preclinical animal studies[200] and Phase 1 clinical trials in the setting of acute myocardial infarction (AMI) showed that G-CSF treatment appeared to

be safe with possible beneficial effects on left ventricular systolic function[201, 202]. However, a subsequent randomised placebo controlled study of subcutaneous G-CSF after primary PCI for AMI showed no additional improvements in left ventricular function when compared to placebo[203]. This was confirmed in a meta-analysis on the effect of G-CSF in AMI which showed no overall benefit although subset analysis showed there may be benefit limited to patients with LV systolic dysfunction and if the infusion is started early[204]. Concerns have also been raised regarding the potential of G-CSF to cause in-stent restenosis (ISR) as it increases the level of circulating neutrophils [205] which may accelerate the process of neointimal proliferation. One of the initial clinical trials reported an unexpectedly high rate of ISR in 10 patients treated with G-CSF following an AM[206]. However, a more recent trial involving patients with large infarcts and late revascularisation did not show any increased incidence of ISR[207]. Furthermore, an intracoronary intravascular ultrasound (IVUS) based study, 5 months after stent insertion for AMI, showed no increase in in-stent neointimal hyperplasia in the G-CSF treatment group compared to placebo[208].

In stable coronary artery disease patients with angina and normal LVEF, progenitor cell mobilisation with G-CSF was not shown to have any obvious beneficial effect on cardiac function or patient symptoms and with the potential to cause adverse ischaemic events[209]. Two small, non randomised, studies have shown possible beneficial effects of G-CSF in chronic ischaemic heart failure with regards to patient symptoms and improvement in LV function but also raised concerns regarding worsening of angina during the treatment

phase[14, 210]. There has also recently been a third small non-randomised study which again suggested symptomatic improvement following G-CSF treatment in patients with ischaemic heart failure[211].

It remains to be determined if G-CSF treatment could be an effective part of a treatment strategy combining several cytokines and/or local stem cell delivery. The intramyocardial arm of the REGENERATE-IHD trial presented in this thesis combines G-CSF treatment with intramyocardial injection of stem cells and is the first study to do so in patients with ischaemic heart failure.

2.2.5.3 Coronary Sinus Infusion

Retrograde infusion of stem cells through the coronary sinus and coronary venous system has been achieved in experimental models which suggest that this method may provide a more uniform delivery of cells with improved cell retention rates[129]. Theoretically this approach may also be safer than intracoronary or intramyocardial injection. There have been sporadic case reports[212] demonstrating safety of this technique but there have not been any published clinical trials of this technique. In this procedure, the coronary sinus is cannulated using a guidewire and an angioplasty balloon is advanced to the selected cardiac vein related to the coronary artery territory to be treated. The venous blood flow is then halted, by prolonged balloon inflation for approximately 15 min, while the stem cells are infused under pressure. A phase 1 clinical trial of 14 patients with refractory angina suggests this is a safe technique with signs of efficacy[213]. The main limitations to this approach are the technical difficulties related to the more variable coronary venous

anatomy which can at times be difficult to negotiate with guidewires and balloons. This technique may not be possible in patients who already have a coronary sinus device in situ such as a LV lead for resynchronisation therapy or a coronary sinus annuloplasty device although cells could be administered as an adjunct to the implantation of such devices.

2.2.5.4 Intracoronary Infusion

Intracoronary infusion is the method that has been most widely adapted in clinical trials of cell therapy, especially after primary angioplasty for AMI. This method has also been used in patients with heart failure secondary to ischaemic heart disease although in this setting occluded coronary arteries may prevent targeted delivery to the infarcted area. The technique employs the basic skills of balloon angioplasty and therefore can be performed by a interventional cardiologist with minimal additional training. Cells are infused through the central lumen of an over-the-wire (OTW) balloon catheter during low-pressure balloon inflation for 2-4 minutes ('stop-flow' technique). Standard short (6-10mm) balloons are used to minimise endothelial injury and balloons are also undersized by 0.5mm less than the reference vessel diameter. Balloon inflation time is empirically chosen and the purpose is to prolong the contact time between the infused cells and endothelium of the coronary macro/micro circulation as well as to provide an ischaemic stimulus which may improve homing of cells to the target myocardium. Inflation time may need to be curtailed in patients who do not tolerate prolonged occlusion of a clinically important coronary artery. In the AMI setting infusion of cells is limited to the infarct related artery whilst in patients with chronic heart failure cell infusion

may be distributed amongst all patent coronary artery and graft conduits. Some more recent animal work suggests that single bolus infusion may be as effective [214] although stop-flow seems to be the prominent method in trials.

A preclinical study in dogs raised concerns regarding the possibility of mircoinfarcts caused by intracoronary injection of mesenchymal stromal cells[215] and there is likely to be a safety threshold with regards to the size and dose of cells delivered using the intracoronary route. Reassuringly, the safety of this technique has been confirmed in multiple clinical trials and a recent meta-analysis confirmed that the relative risks of mortality and morbidity, measured by incidence of re-infarction, arrhythmias, restenosis, hospital re-admission, and target vessel revascularisation, were not significantly increased in participants who received intracoronary BMSC treatment following AMI compared with controls[49].

2.2.5.5 Intramyocardial injection

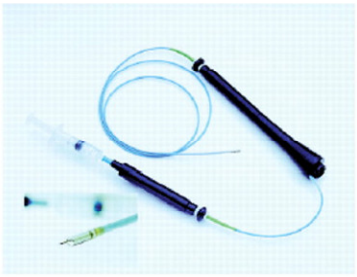
Intramyocardial injection is the most direct method of administering cell therapy and there are several advantages of this technique in patients with heart failure and angina. In contrast to the AMI setting, patients with chronic ischaemic cardiomyopathy are unlikely to release signals from damaged myocardium to induce stem cell homing and theoretically it maybe more effective to use intramyocardial injection to deliver the cells to the target area. Also, in the chronic setting the coronary artery subtending the infarct/ischaemic area maybe occluded without significant collateral formation and intramyocardial delivery may be more effective in these patients.

Experimental data using radionucleotide labelled BM-MNCs suggest that target area cell retention maybe higher with intramyocardial injection when compared to the intracoronary delivery method[216]. Furthermore, a meta-analysis of randomised controlled trials of BMSC therapy in heart failure patients showed that improvement in LVEF was only seen when cells were delivered by the intramyocardial route[217]. Its use has also been mainly limited to chronic conditions due to concerns of myocardial perforation and arrhythmia in the acute setting.

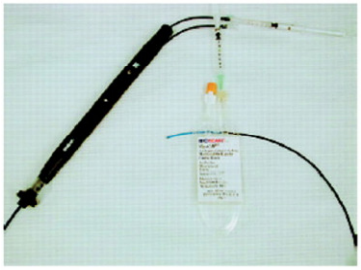
There are currently three methods for intramyocardial injection of cells: open surgical transepicardial injection, percutaneous transendocardial injection and trans-coronary-venous intramyocardial injection of cells. Earlier clinical trials have relied on surgical transepicardial injection of cells where results are biased by simultaneous coronary bypass surgery [134, 218-220]. When bypass is not indicated, percutaneous delivery may provide comparable efficacy with reduced risk. Comparison studies in swine models of chronic ischaemic heart failure have shown that percutaneous and surgical transplantation of skeletal myoblasts both lead to similar improvements in LVEF and reverse remodelling [221].

Percutaneous transendocardial injection is usually performed via 8Fr femoral access although brachial access for transendocardial injection of stem cells has been reported [222]. A percutaneous approach allows this therapy to be delivered to the higher risk population and also provides an option for repeated therapy if required. The main additional risks of this approach are

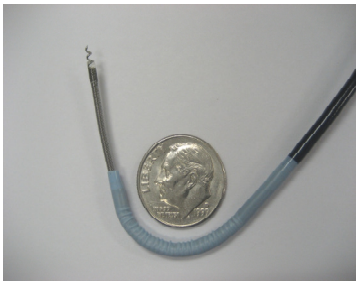
ventricular perforation with pericardial effusion/tamponade and procedure related ventricular arrthymias although the reported incidences of these have been low in the published clinical trials. A deflectable tip injection-needle catheter is advanced retrogradely across the aortic valve and cells can be injected directly into any area of the left ventricular endocardium. There are four commercially available catheter systems currently being used for transendocardial cell delivery: the Myostar™ (Biosense Webster), the MyoCath™ (BioHeart), the Helix™ (BioCardia) and the Stiletto™ (Boston Scientific) catheter delivery systems (Figure 6).



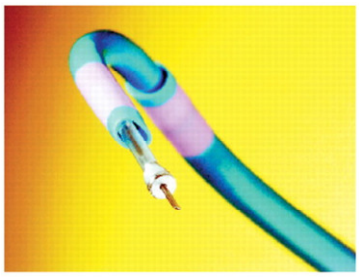
A. MyoStar™ (Biosense-Webstar)



B. MyoCath™ (BioHeart)



C. Helix™ (BioCardia)



D. Stiletto™ (Boston Scientific)

Figure 6. The four commercially available catheters for transendocardial/intramyocardial injection of biologic material which are currently under investigational use in clinical trials.

The Myostar™ catheter system utilises a 3-dimensional (3-D) navigation system to guide injection and the other three catheters are guided fluoroscopically therefore providing 2-dimensional orientation only. The first

two catheter systems are introduced without a guidewire so require manipulation when advanced from the femoral artery and across the aortic valve whilst the second two catheters are introduced over a traditional guidewire system. The relative merits of the different catheter delivery systems are now discussed.

The Myostar™ catheter system utilises nonfluoroscopic guidance using the NOGA® (Biosense Webster) magnetic electromechanical system for intramyocardial navigation and mapping (Figure 7). This technique uses ultra low intensity magnetic fields generated by a triangular magnetic pad positioned beneath the patient and location sensor on the tip of the mapping catheter, which helps determine the real-time location and orientation of the catheter tip inside the left ventricle.

The NOGA® system analyses the movement of the catheter tip at the location of an endocardial point, timed with systole and diastole, and compares it with the movement of neighbouring points- the percentage difference, termed linear local shortening (LLS), represents the degree of mechanical function at that endocardial point. The mapping catheter tip is also capable of measuring endocardial voltage potentials and an electrical map is constructed concurrently with the mechanical map. Normal myocardial cells deliver a resting unipolar potential of 15 mV, whereas voltage values less than 6.9 mV reflect scar tissue; potentials between 7mV and 15 mV suggest viable myocardium [223].

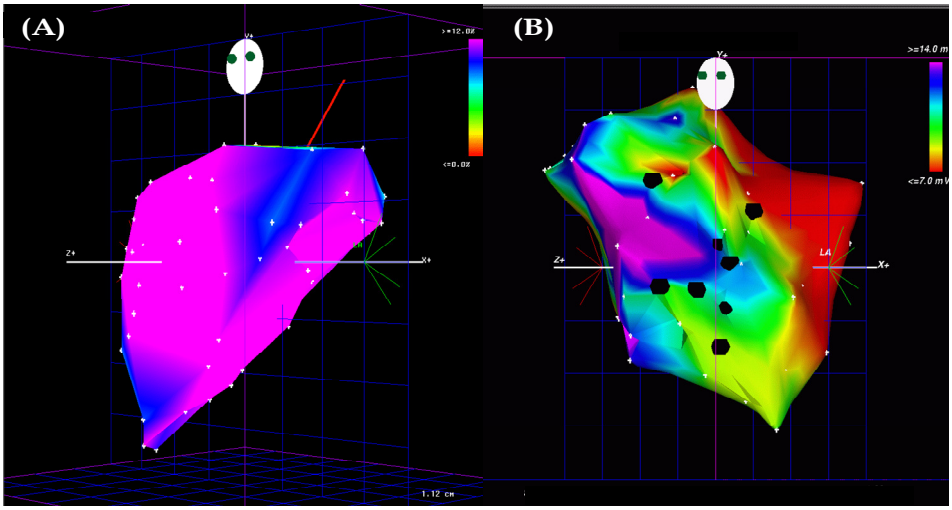


Figure 7. An example of an electromechanical map obtained using the NOGA system. Electromechanical characteristics, including unipolar voltage and local liner shortening, of sampled areas of the endocardial wall are mapped onto a computerised 3D colour-coded construct of the left ventricle. (A) The purple/blue end of the colour spectrum in the 'local linear shortening map' indicates a good wall motion in a healthy viable myocardium as shown on this map taken from a normal subject. (B) By contrast, the red end of the colour spectrum in the so called 'voltage map' represents scarred myocardium devoid of electrical activity as shown in this example taken from a patient with chronic ischaemic heart failure. The green/yellow border zone on this map represents hibernating myocardium which is targeted for intramyocardial injection. Black spots on the map label areas where injections have been delivered.

When the map is complete, all the data points are integrated by the NOGA® workstation and represented in a 3-D colour-coded reconstruction of the endocardial surface which is able to distinguish between normal, ischaemic (but viable) or infarcted myocardium (Figure 7). Furthermore it allows accurate manipulation of the injection catheter in the left ventricle and therefore precise transendocardial injection. This allows targeted delivery of cells into specific areas, for example BMSCs tend to be injected into the peri-infarct zone as it has been shown that BMSCs do not engraft well into scarred myocardium compared to skeletal myoblasts which have been injected into scarred myocardium and seem capable of engrafting in fibrotic tissue [224].

The Myostar™ catheter (Figure 8) incorporates an integrated injection needle and a location sensor at the tip which interfaces with the NOGA® 3-D mapping system.

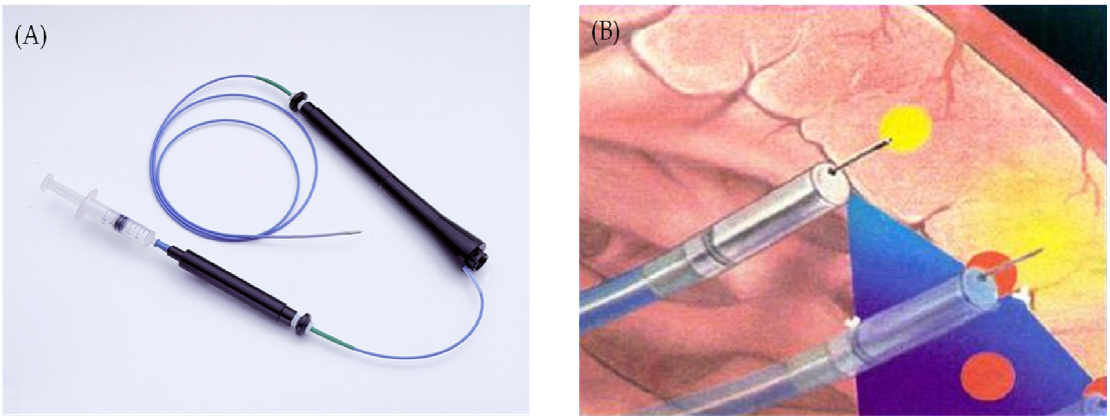


Figure 8. Percutaneous transendocardial injection using the Myostar™ delivery catheter. (A) A flexible, steerable, injection catheter is advanced retrogradely across the aortic valve into the LV cavity and injections are guided by electromechanical mapping. (B) The catheter tip incorporates a fine nitinol 27-gauge needle which is normally retracted. Images courtesy of Biosense Webster.

The 115-cm deflectable-tip catheter consists of an outer shaft and inner core lumen which runs the full length of the catheter and culminates distally in a nitinol 27-gauge needle. The core lumen can be advanced and retracted independently of other catheter movements. The proximal handle contains controls for catheter tip deflection, needle advancement and the injection port for needle lumen injection. The catheter is prepared by adjusting the retractable needle extension length at 0º and 90º flex, setting the needle length to a maximal needle-to-wall ratio of 0.6. A 1ml Luer-Lock syringe containing the injectate is connected to the injection port and the catheter 'dead-space' is filled with the cell suspension prior to introducing the catheter. Multiple injections can be performed at specific sites identified on the 3-D NOGA® electromechanical map- at our institution we perform circumferential 10x0.2mls injections 1-2cms apart into the hibernating myocardium around

scar tissue. Needle injection into the true apex and myocardial segments with wall thickness less than 0.8mm should be avoided to minimise the risk of perforation. This technology appears to be safe from preclinical and clinical studies [223, 225]. The main disadvantages of this system are the additional time and costs required to produce an accurate electromechanical map to guide transendocardial injection. The NOGA-Myostar catheter delivery system has been used to deliver therapy in the intramyocardial arm of the REGENERATE-IHD trial described in this thesis.

The MyoCath™ injection catheter (Figure 9) is similar to the MyoStar™ and consists of a 115-cm long deflectable injection catheter that also contains an integrated core lumen with a 25-gauge stainless steel needle at its distal end.

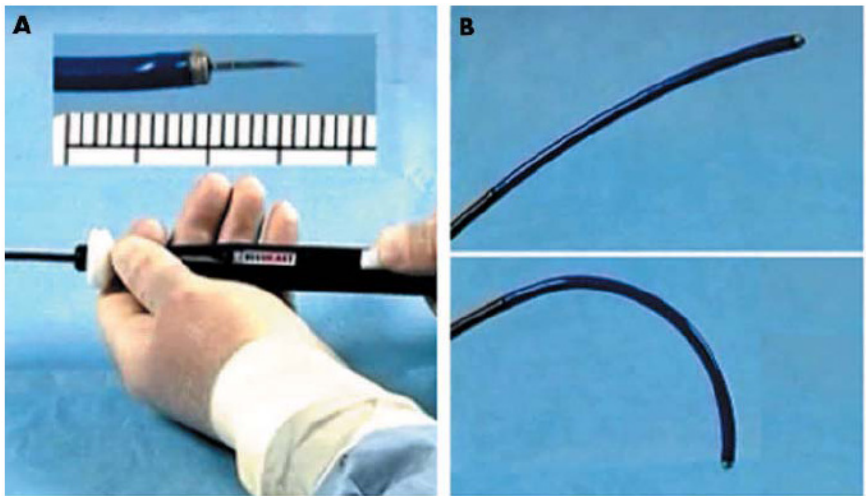


Figure 9. The MyoCath™ (BioHeart) injection catheter. A- integrated catheter system with core lumen containing retractable 25-guage stainless steel needle at its distal end. B- deflectable tip manipulated through a combination of axial rotation and deflection of the distal aspect (the latter under a separate control mechanism capable of inducing up to 180° of flexion). Images courtesy of BioHeart.

The needle can be advanced and retracted from the tip of the catheter and provides for multiple injections to a predetermined needle insertion depth. The

MyoCath™ is however guided by fluoroscopic guidance and additional imaging such as transoesophageal echocardiography is sometimes utilised.

The Stiletto™ catheter (Figure 6D) is also guided fluoroscopically and thus lacks the precision of the NOGA® system although real time MRI guidance has shown promising results[226]. It contains three separate, independently moveable components: two steerable guiding catheters (9Fr and 7Fr), and an inner spring loaded needle component. Catheter tip orientation is accomplished by guiding catheter manipulation, and the injection needle is set to a fixed depth (3.5 mm) and the spring loaded advancement mechanism theoretically allows the device to overcome fibrotic scar tissue resistance to needle penetration. This technology is still under investigation; preclinical studies suggest that this is a safe technique[227] although higher than expected rates of pericardial effusion has led to the use of the Stiletto™ catheter being halted in the GENASIS investigational trial of VEGF-2 biologic for the treatment of severe angina.

Biocardia's Helix™ catheter (Figure 10A) has a small, hollow, distal corkscrew needle which is rotated into the endocardium to provide active fixation (similar to active fixation pacing leads) and to allow local delivery of biological therapeutic products. This catheter is used in combination with the Biocardia Morph® Deflectable (steerable) Guide Catheter (Figure 10B) which helps catheter navigation within the LV under fluoroscopic guidance only. By eliminating the process of electromechanical mapping this technique may have advantages in terms of procedural time and cost. Preclinical studies

have proven safety and efficacy of this delivery method[228] and there is preliminary data to suggest this method may be clinically effective[229].

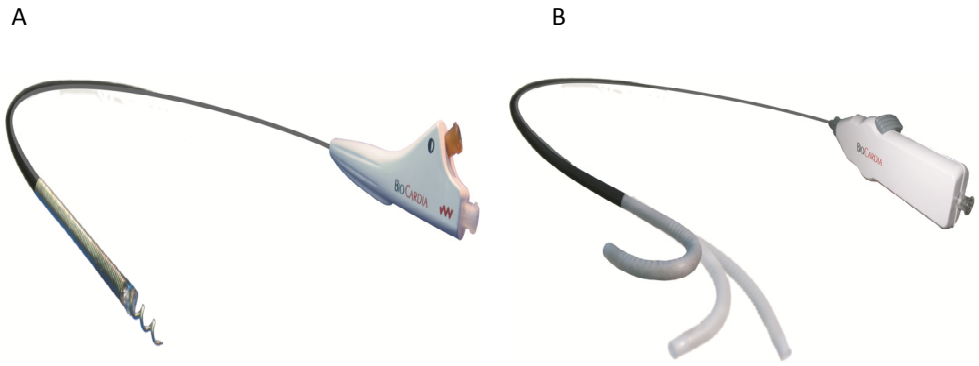


Figure 10. Biocardia Helix® Catheter Delivery System. A- The Helical Infusion Catheter for transendocardial delivery is advanced through the Morph Guide. The therapeutic lumen discharges at the distal tip of the helical needle. A second lumen discharges at the base of the helix for delivering contrast and confirming positioning. B- The Morph Universal Deflectable Guide Catheter is advanced through the aortic valve over a guide wire and provides the ability to guide the Helical Infusion Catheter. Images courtesy of Biocardia.

Trans-coronary-venous intramyocardial (TVIM) injection can be performed using the TransAccess® catheter system (TransVascular Inc) that is placed percutaneously into the coronary sinus and target vein (Figure 11). Then, using intravascular ultrasound (IVUS) guidance, a 24-gauge nitinol needle is extended to perform transvenous myocardial puncture. A 27-guage microinfusion catheter (IntraLume™) is then advanced though the needle to the targeted areas for cell delivery. Initial studies have confirmed the feasibility and safety of this approach in porcine models [230].

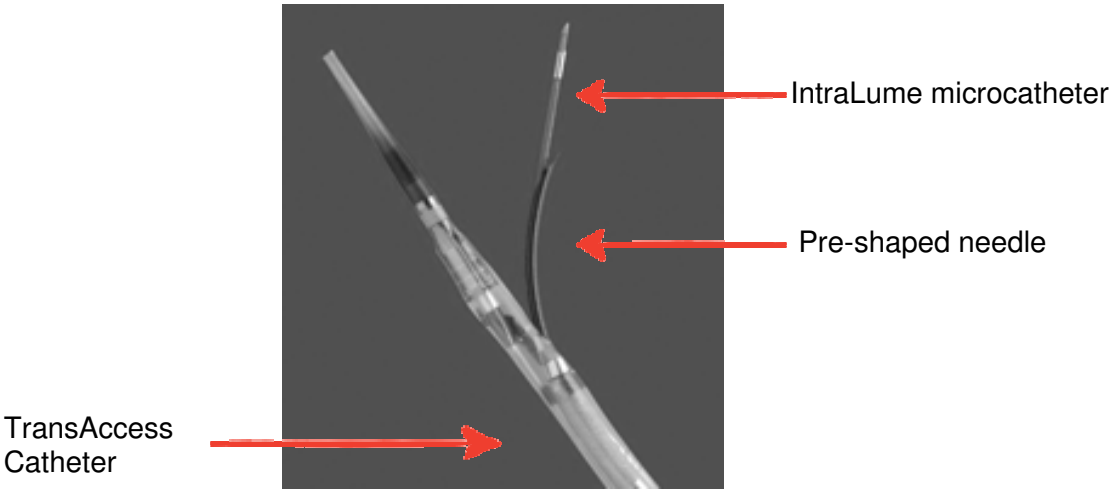


Figure 11. TransAccess® (Medtronic) catheter tip with Intralume micro-catheter advanced through the needle tip. IVUS tranducer is located at tip of TransAccess Catheter. Image courtesy of TransVascular Inc.

A Phase 1 clinical trial has confirmed the safety and efficacy of this delivery method of skeletal myoblasts for the treatment of chronic ischaemic LV systolic dysfunction [231]. Furthermore, an animal study demonstrated that the TVIM injection of BM derived mononuclear cells may lead to a greater retention of the cellular product at the target area as compared to intracoronary infusion [232]. The main limitations to this approach are related to the technical difficulties due to variable coronary venous anatomy which can at time be difficult to access with guidewires and TVIM may be one of the more technically demanding procedures.

Transepicardial delivery of stem cells has been performed in patients already scheduled for CABG in whom cells are injected into infarct border zones and/or areas of infarcted or scarred myocardium under direct visualization[127]. The major disadvantage of this approach is the necessity for a sternotomy and therefore is a highly invasive approach which can be

associated with significant surgical morbidity. However, in planned CABG surgery the concomitant delivery of cell therapy would seem feasible. One important advantage of this technique is that it can provide a high concentration of cells per unit area injected although not all areas of the myocardium (e.g. the septum) can be reached using a this approach.

There have therefore been several techniques utilised for cell delivery in the hope of cardiac regeneration in patients with acute and chronic ischaemic heart disease. In animal models all routes appear to be equally efficacious although the exact mechanisms of action through which cell therapy leads to an improvement in cardiac function remain unanswered. There has been translation of all the delivery methods from animal models to humans and to date the intramyocardial route appears to be the most promising, in particular target area cell retention maybe higher with intramyocardial injection when compared to the intracoronary delivery method [231, 233]. There is yet to be a comparative clinical study of different cell delivery methods and our REGENERATE-IHD trial has been designed to answer this question. This study compares the efficacy and safety of delivering BMSCs using three different routes: indirect cell delivery by mobilisation using peripheral infusion of G-CSF versus direct injection of cells using the intracoronary or intramyocardial route. This thesis presents the results of the intramyocardial arm of the study only.

2.2.6 Trials of Stem Cell Therapy in Chronic Ischaemic Heart Failure

There have been numerous clinical trials in the past decade examining the safety and efficacy of stem cell therapy for patients with ischaemic heart failure. Studies have been heterogonous in design although percutaneous transendocardial injection of cells has been the most widely investigated delivery method and skeletal myoblasts and BMSCs have been the most studied cell types. This section provides a review of recent clinical trials of stem cell therapy in patients with heart failure according to the different delivery methods (see Table 3).

2.2.6.1 Transendocardial Delivery of Cell Therapy

Percutaneous transcatheter intramyocardial injection of skeletal myoblasts has shown promise in early Phase I/II clinical studies. A pilot study of 5 patients, published in 2003, demonstrated that NOGA® guided injection of skeletal myoblasts into the infarcted area of patients with heart failure following previous anterior myocardial infarction was safe with no serious adverse events related to the procedure. There also appeared to be some improvement in regional myocardial thickening as well as global LVEF [234]. The CAuSMIC study also used NOGA® guided transendocardial injection of skeletal myoblasts into areas of viable myocardium in 12 patients with severe ischaemic heart failure. There was improvement in NYHA functional class, quality of life and evidence of reverse ventricular remodelling when compared to controls after 1 year follow-up [235].

Table 3. Clinical trials of percutaneous stem cell therapy for chronic heart failure

Study name (ref)	Study Design	*n*	Cell type	Delivery Method	Primary Outcome
Smits et al[234]	NR	5	SM	Transendocardial *MyoStar™*	LVEF increased from 36 +/- 11% to 41 +/- 9% (3 months, p = 0.009)
CAuSMIC[235] (2009)	NR	23	SM	Transendocardial *MyoStar™*	Improvement in heart failure symptoms, reduction in LV dimensions and evidence of improved viability in cell treated group at 1 year
SEISMIC[236] (2011)	RCT	40	SM	Transendocardial *MyoCath™*	No change in global LVEF but there was a trend towards improvement in patient symptoms
Perin et al[13] (2004)	NRC	20	BMSC	Transendocardial *MyoStar™*	Improvement in myocardial perfusion and exercise tolerance in cell treated group but no significant change in LVEF
Beeres et al[237] (2007)	NR	15	BMSC	Transendocardial *MyoStar™*	Improvement in regional contractility and global LVEF
Williams et al[229] (2011)	NR	8	BMSC	Transendocardial *Helix™*	Improvement in regional contractility and evidence of reverse remodelling
FOCUS-HF[238] (2011)	RCT	30	BMSC	Transendocardial *MyoStar™*	Improvement in symptoms and perfusion but no change in global LVEF
TOPCARE-CHD[10] (2006)	RCT	75	BMSC	Intracoronary	Improvement in global LVEF by 2.9% in BMSC treated group only at 3months follow-up
DanCell-CHF[10] (2008)	NRC	32	BMSC	Intracoronary (two infusions 4 months apart)	No improvement in LVEF even with repeated infusions there was improvement in NYHA Class
STAR-Heart[239] (2010)	NRC	391	BMSC	Intracoronary	Improvement in LVEF and survival in cell treated group over 5 years follow-up
CADUCEUS[118] (2012)	RCT	31	CDC	Intracoronary	Reduction in scar mass, increase in viable heart mass and regional contractility but no improvement in LVEF

Abbreviations: CDC: Cardiosphere-derived cells; ICM: ischaemic cardiomyopathy; DCM: dilated cardiomyopathy; *n*: number of patients; SM: skeletal myoblasts; BMSC: bone marrow stem/progenitor cells; LVEF: left ventricular ejection; NR: non-randomised; NRC: non-randomised with control group; RCT: randomised controlled trial

In the SEISMIC trial, a randomised multi-centre trial in Europe, 40 patients (14 controls) with ischaemic heart failure underwent fluoroscopy only guided transendocardial injection of skeletal myoblasts [236] directly into the area of scar using the MyoCath® catheter. Adverse events during follow-up were similar between cell treated and control group and in particular number of arrhythmic events (all patients were required to have an ICD in-situ) were also similar. However, although there appeared to be some improvement in patient symptoms the study failed to show any significant improvement in LVEF. The results of these small studies have prompted the design of larger RCTS including the MARVEL Trial (ClinicalTrials.gov Identifier: NCT00526253) which was designed as a randomized, double-blind, placebo-controlled, multi-centre Phase II/III Trial. Enrolment in the MARVEL Trial began in October 2007 but unfortunately is currently halted due to limited financial resources.

In view of the underwhelming clinical results with skeletal myoblasts other cell types are now being investigated. In particular, transendocardial injection of BMSCs has been shown to be safe with potentially beneficial effects. An early non-randomised trial in 14 patients with severe chronic ischaemic heart failure showed NOGA® guided intramyocardial injection of BMSCs into viable myocardium was safe and was associated with significantly improved regional and global LV systolic function at 6 months [240]. Another non-randomised study of 20 patients showed NOGA® guided intramyocardial injection of BMSCs was again safe and associated with improvements in exercise tolerance and myocardial perfusion[13]. A further small study also showed improvement in patient symptoms and LV function following NOGA® guided intramyocardial injection of BMSCs[237]. A recent Phase 1 (non-randomised) study, using the

Biocardia Helix™ catheter system, has confirmed the beneficial effects of BMSCs in chronically infarcted myocardium with improvements seen in regional contractility and evidence of reverse remodelling[229].

The most recent RCT, FOCUS-HF, assessing the efficacy of NOGA® guided intramyocardial injection of BMSCs found improvements in symptoms and quality of life but no change in global LVEF[238]. Although the trial was randomised and double-blinded, the control group did not receive a 'sham' injection of placebo but instead underwent a 'mock' procedure.

2.2.6.2 Intracoronary Delivery of Cell Therapy

There have been relatively few studies assessing intracoronary delivery of BMSCs in chronic ischaemic heart failure. In the randomised, cross over design TOPCARE-CHD trial [10], intracoronary delivery of BMSCs showed an improvement in LVEF of 2.9% with no major adverse cardiac events. In contrast the DanCell-CHF Study [239], in which patients received two repeated treatments of intracoronary BMSCs 4 months apart, showed no improvement in LV function at 1 year follow-up although there was significant improvement in patient symptoms. This study adds to the growing belief that using left ventricular ejection fraction (LVEF) as a marker of beneficial effects of cell therapy may be inadequate as patient symptoms do seem to improve without any significant objective change in LVEF. The STAR-Heart Study is the largest study to date of BMSC therapy in chronic ischaemic heart failure[241]. Of the eligible patients screened 191 patients underwent intracoronary BMSC therapy, and the control group consisted of 200 patients who declined to have

the active intervention. Over a 5-year follow-up period intracoronary BMSC therapy was associated with significant improvement in LVEF as well as patients' exercise capacity. Interestingly, there was a significant decrease in long-term mortality in the BMSC treated patients compared with the control group.

There has only been one clinical study of a combined approach of intracoronary and intramyocardial injection of BMSCs. In the MYSTAR study[242], 60 patients were treated with a combined approach either early (3-6 weeks) or late (3-4 months) after myocardial infarction. There appeared to be significant improvement in LVEF (by 3.5%) in both groups as well as reduction in infarct size.

In summary, there have been numerous studies, using different cell types and different delivery methods, assessing whether cell therapy is beneficial in patients with established heart failure secondary to ischaemic heart disease. Cell therapy appears to be safe with potentially beneficial effects in terms of patient symptoms as well as modest improvements in LV function. A recently formed task force of the European Society of Cardiology has identified a need for appropriately sized randomised controlled trials to try and accurately assess the efficacy of cell therapy and to define the ideal cell type, delivery method and patient type which will derive the maximum benefit of this biological therapy[15]. There has also been outlined a need for mechanistic studies to help further the understanding of the biological processes leading to benefit.

The REGENERATE-IHD study[16] has been designed to try and answer some of this questions. This is a unique comparative randomised controlled study of different BMSC delivery methods with adjuvant G-CSF cytokine therapy in patients with ischaemic heart failure (ClinicalTrials.gov Identifier: NCT00747708). This is the only trial of its kind in the UK and is also the first study worldwide, that we are aware of, to combine G-CSF with direct cell delivery in heart failure patients. The study aims to provide important answers regarding the efficacy of cell therapy and the best delivery method. Chapter 4 of this thesis will present the rationale, methodology and results of the intramyocardial arm of the study.

CHAPTER 3: THESIS HYPOTHESES AND AIMS

3.1 REGENERATE-IHD Clinical Trial (Intramyocardial Arm)

3.1.1 Hypotheses

Combined G-CSF therapy and intramyocardial injection of bone marrow derived progenitor/stem cells (BMSCs) may lead to an improvement in cardiac function and symptoms in patients with chronic ischaemic heart failure.

3.1.2 Aims

1. Assess the safety and feasibility of bone marrow mobilisation with subcutaneous injection of G-CSF in patients with chronic ischaemic heart failure.

2. Assess the safety and feasibility of intramyocardial injection of mobilised BMSCs or autologous serum.

3. Measure changes in cardiac function (with cardiac MRI or CT, LV angiography and echocardiography) following intramyocardial injection of BMSCs or autologous serum.

4. Measure changes in NT-ProBNP levels in patients treated with intramyocardial injection of BMSCs or autologous serum

5. Assess changes in patients' symptoms and well-being (using heath related quality of life questionnaires) following intramyocardial injection of BMSCs or autologous serum.

6. Assess changes in unipolar voltage and local linear shortening (as assessed by NOGA® mapping) in patients treated with intramyocardial injection of BMSCs or autologous serum.

7. Assess the impact of progenitor cell concentration and function on the primary and secondary end-points of the study.

3.2 A Study of Progenitor Cell Characteristics

3.2.1 Hypotheses

Bone marrow progenitor cell concentration and function may be affected by age and the disease process itself. G-CSF therapy increases peripheral blood CD34+ cell concentration although this effect may differ between patients with ischaemic heart failure and dilated cardiomyopathy. G-CSF mobilisation may also affect the functional capability of peripheral and bone marrow progenitor cells.

3.2.2 Aims

1. Measure the baseline concentration of circulating CD34+ progenitor cells and endothelial progenitor cells (EPCs) in patients recruited to the

REGENERATE-IHD study and assess for correlation between concentration and clinical parameters including patient age.

2. Compare the concentration and function of progenitor cells in patients recruited to the ischaemic heart failure study with that of patients recruited to two other trials of cell therapy in dilated cardiomyopathy and acute myocardial infarction.

3. Assess the effect of G-CSF on bone marrow and peripheral blood progenitor cell concentration and function in patients with ischaemic heart failure and dilated cardiomyopathy.

3.3 Angiogenesis Imaging Study

3.3.1 Hypotheses

BMSC therapy may partly exert its beneficial effects through de novo angiogenesis and this may be detected using a novel radio-tracer peptide (^{99m}Tc-NC100692: proposed International Non-proprietary Name (INN) ^{99m}Tc-maraciclatide) which binds to αvβ3 integrin, an angiogenesis related integrin.

3.3.2 Aims

Assess the feasibility of using a novel radio-tracer peptide (^{99m}Tc-NC100692) to detect angiogenesis and specifically aim to show that:

1. ^{99m}Tc-labeled peptide (NC100692) injection is well-tolerated in heart failure patients

2. ^{99m}Tc-labeled peptide (NC100692) uptake in heart failure patients can be detected

3. ^{99m}Tc-labeled peptide (NC100692) uptake is affected by intramyocardial injection of BMSCs or serum

CHAPTER 4: THE REGENERATE-IHD STUDY

4.1 Study Design

The Randomized Controlled Trial to compare the Effects of G-CSF and Autologous Bone Marrow Progenitor Cells Infusion on Quality of Life and Left Ventricular Function in Patients with Heart Failure secondary to Ischaemic Heart Disease (REGENERATE-IHD) trial is an ongoing double-blind, randomised controlled study being conducted at a single centre in the UK[16]. The trial investigates the potential for autologous bone marrow-derived stem/progenitor cells (BMSCs) to improve cardiac function and symptoms in patients with chronic ischaemic heart failure and no further treatment options. As well as testing routes of administration, it uses a novel approach of administering G-CSF as adjunctive therapy.

The study design is depicted in figure 12. There are three arms of the study: peripheral mobilization with G-CSF or placebo (saline) injections; intracoronary delivery of BMSC or autologous serum (control) following G-CSF mobilisation; and NOGA® guided intramyocardial delivery of BMSC or autologous serum (control) following G-CSF mobilisation.

The intervention arms of the study are double-blind so that neither the participant nor investigator was aware of treatment assignment. The peripheral arm of the study is single blind so that only the patient was unaware of treatment assignment. The primary outcome measure, change in LVEF, was assessed by an independent cardiologist blinded to all treatment assignments.

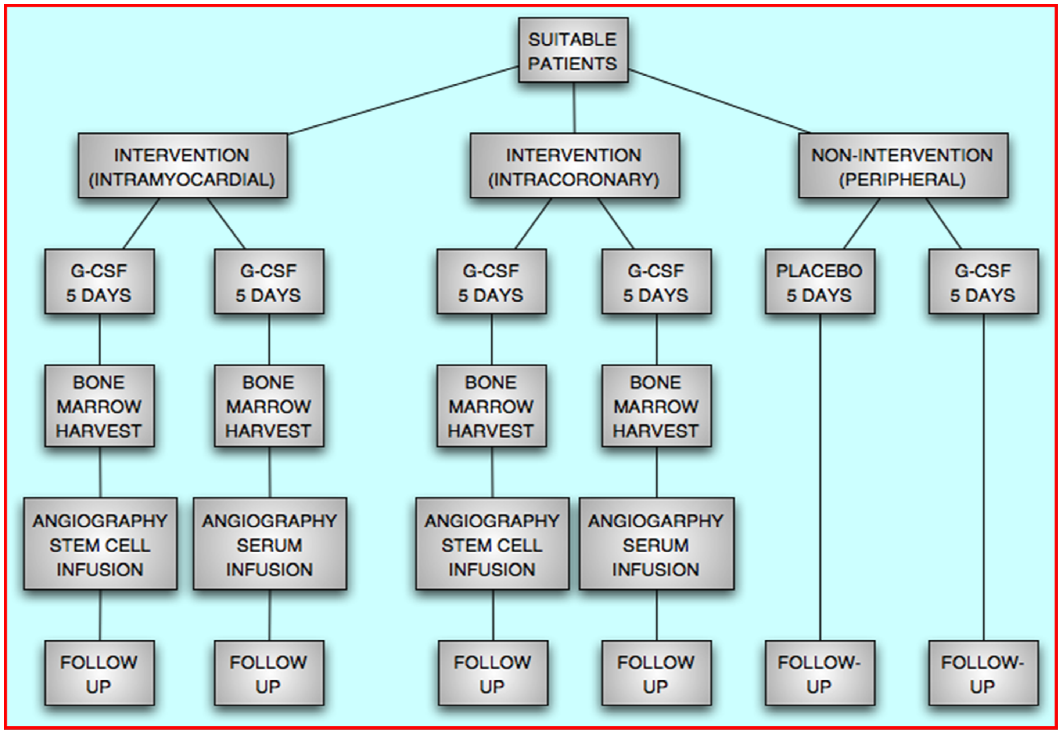


Figure 12. Design of the REGENERATE IHD trial.

We decided to initially complete treatment within the intramyocardial arm (the main focus of this thesis) and patients were then randomly allocated to the other two arms of the trial. The mains reasons for this were to ensure that operators who had undergone training in NOGA® mapping and intramyocardial injection were able to maintain an adequate skill level and to avoid costly equipment expiring beyond recommended date of use. Following allocation to treatment arm patients were further randomised 1:1 to active or control groups. This thesis will primarily present the methodology and results of the intramyocardial arm of the REGENERATE-IHD Trial.

4.2 Study Rationale

The REGENERATE-IHD trial (intramyocardial arm) has several unique features. The rationale behind the choice of cell type, delivery method, control group and primary end-point are described below.

4.2.1 Bone marrow stem/progenitor cells

We chose to study BMSCs, specifically the mononuclear cell fraction, as this cell type has been the most investigated and has shown the most promise so far in preclinical experiments and clinical trials[13, 241, 243]. The mononuclear cell fraction contains a heterogeneous population of cell types including HSCs (CD34+), EPCs (CD133+VEGFR2+), MSCs, monocytes (CD14+) and lymphocyte subsets characterised by CD3+ (T-cells), CD19+ (B cells) and CD16+CD56+ (natural killer cells) cell surface markers. HSCs and EPCs comprise only 1-3% of the mononuclear cell fraction and the contribution of the other cell types to the process of cardiac repair remains unclear. For example, lymphocytes produce TNF-α, which up-regulates vessel-wall expression of VCAM-1[244] which is required for homing of stem cells to injured tissue[245]. In view of the uncertainty regarding which cell type(s) are most influential in the healing process we have taken the pragmatic approach of using the whole mononuclear cell fraction rather than specific sub-sets. Isolation of the bone marrow mononuclear cell fraction requires a density gradient medium, a polysaccharide solution of a specific density which acts as a liquid filter to remove undesired components, such as granulocytes, platelets and red blood cells[246]. We have used the Ficoll-Paque™ density gradient

medium in our clinical trial as this has been the most widely used in clinical trials of BMSC therapy. We have also previously published data from our department showing that the composition and quantity of cell types obtained using this medium is similar to that obtained from Lymphoprep™- the other commercially available density medium[247].

4.2.2 Combination of G-CSF therapy and myocardial delivery

As mentioned previously there have been small non-randomised trials of G-CSF therapy alone in patients with heart failure suggesting possible beneficial effect on symptoms[210, 211]. There has so far only been one study of combined G-CSF mobilisation of cells with subsequent direct delivery of cells to the heart. In the ACT34-CMI study intramyocardial injection of CD34+ cells (obtained by peripheral leukapheresis after 5 days of G-CSF administration at a doe of 5µg/kg/day) improved angina symptoms in patients with refractory angina[248]. Of note, in the latter study patients had relatively preserved LVEF hence our study is the first to examine combined G-CSF and intramyocardial injection in patients with heart failure and severe LV systolic dysfunction. The possible advantages of a combined approach include a) the direct beneficial effects of G-CSF on myocardial tissue, b) increasing the number of progenitor cells that can be harvested and c) potentially improving the quality of progenitor cells in patients with ischaemic heart disease who have been shown to have impaired bone marrow function[249, 250].

G-CSF has been administered at a dose of 10µg/kg/day for 5 days prior to bone marrow harvest in our trial. This dose of G-CSF has been shown to

improve the yield of CD34+ cells compared to a 5µg/kg/day dose[251]. Higher doses, such as 16µg/kg/day, have been associated with increased side effects such as splenic rupture[252].

In our trial progenitor cells have been collected by bone marrow aspiration rather than peripheral leukapheresis. It remains unclear whether peripherally harvested circulating progenitor cells (CPCs) would have the same therapeutic effect as the cells found in the bone marrow (BMSCs). In the TOPCARE-CHD study intracoronary infusion of CPCs was significantly inferior to BMSCs with regards to improvement in LVEF in patients with chronic heart failure[10]. Based on this data and current available evidence we decided to use BMSCs in our clinical trial.

4.2.3 NOGA® guided intramyocardial injection

Intramyocardial injection of cells or serum has been performed using the NOGA® system and Myostar™ catheter. As described previously the main advantage of this system is the ability to delineate the infarcted myocardium so that the cells can be injected accurately around the infarct zone/hibernating area. This technique appears to be safe and has been used to deliver cells in several clinical trials[13, 237, 253] with only one reported case of pericardial effusion[254].

4.2.4 Control group (intramyocardial serum injection)

The control group patients in our clinical trial have also received G-CSF therapy followed by bone marrow aspiration. They also underwent NOGA® mapping and intramyocardial injection of autologous serum only (without cells). This allowed our study to be double-blind thus strengthening the study design[255, 256]. Serum injection (in the control group) also allows us to exclude potential confounding effects such as possible beneficial effect of 'needle puncture' which may elicit a beneficial injury reaction including angiogenesis.

Our study is one of the first trials to perform intramyocardial injection in the control group. Previous studies have either used standard care as the control group or have performed a 'mock' procedure without actually injecting so as to maintain patient blinding[238]. We are only aware of two other studies that have performed 'sham' intramyocardial injection. In the ACT34-AMI study[248] the control group received injection of 0.9% normal saline with 5% autologous serum. In the recently published FOCUS-CCTRN study[257] the control group received 0.9% saline with 5% human serum albumin. The injection of autologous serum in our trial potentially allows us to assess the effects of injection of soluble paracrine factors only on cardiac function and symptoms.

4.2.5 Outcome measures

The primary outcome measure is change in left ventricular ejection fraction (LVEF) measured by sensitive cardiac MRI/CT imaging. This has been chosen as the surrogate marker of efficacy because its relevance can be readily

appreciated by the clinical cardiology community since it is the most commonly used tool to assess cardiac function. Furthermore, the change in LVEF following a therapy has been shown to be closely correlated to subsequent long-term clinical outcome[258]. Secondary outcomes measures include change in NT-proBNP levels as prior studies of various medical therapies in heart failure have demonstrated improved clinical outcomes if reduction in NT-proBNP level is achieved[39]. Subjective improvement in symptoms has been assessed by change in NYHA functional class and health related quality of life (HRQL) questionnaire scores.

4.3 Methods

4.3.1 Declaration of Helsinki

This study is conducted in full accordance with the principles of the "Declaration of Helsinki" (as amended in Tokyo, Venice, Johannesburg, and Edinburgh) and with the laws and regulations of the country.

4.3.2 Ethics and MHRA Approval

Approvals from the local research ethics committee (LREC) and Medicine and Healthcare products Regulatory Agency (MHRA) was granted prior to recruitment of any participants.

4.3.3 Clinical Trial Registration

The REGENERATE-IHD study has been registered with the Clinicaltrials.Gov website. The identifier is NCT00747708. This is in accordance with the International Committee of Medical Journal Editors (ICMJE) initiative requiring prior entry of clinical trials in a public registry as a condition for publication.

4.3.4 Patient Recruitment

4.3.4.1 Inclusion Criteria

All of the following criteria were required for patients to be considered for the study:

- Impaired left ventricular systolic function secondary to ischaemic heart disease
- At least NYHA class II functional class despite optimal medical therapy and no further revascularisation options
- Patient had been considered for implantable cardioverter defibrillator (ICD) and/or cardiac resynchronisation therapy (CRT) as per up-to-date clinical guidelines

4.3.4.2 Exclusion criteria

Patients were excluded if they had any of the following:

- Recent acute coronary syndrome
- The presence of cardiogenic shock
- Decompensated heart failure

- LVEF < 10% prior to randomisation
- Contra-indication for bone marrow aspiration
- Known active infection
- Known infection with human immunodeficiency virus (HIV), hepatitis B virus (HBV), or hepatitis C virus (HCV) syphilis or HTLV
- Lifestyle with high risk for infection with HIV, HBV or HCV syphilis or HTLV
- Serum creatinine >200 umol/L
- Chronic inflammatory disease
- Serious known concomitant disease with a life expectancy of less than one year
- Female subjects of childbearing potential
- Atrial fibrillation (unless pacemaker implanted with regular paced rhythm)
- Patients who had recently responded to the implantation of a biventricular pacemaker
- Weight >140kg

4.3.4.3 Patient selection

Potential candidates for the trial were identified from several sources:

- ICD follow-up clinic at St Bartholomew's Hospital
- Heart failure clinics at The London Chest Hospital, local district general hospitals and also from other hospitals nationally
- Referrals from cardiologists from all regions within the United Kingdom

Potential patients were invited to attend an assessment clinic at The London Chest Hospital to discuss the trial and assess suitability. A patient information sheet (PIS) was sent by post to all patients prior to being seen in clinic. During the clinic visit a full medical history was taken and a full physical examination performed. Patients also had a baseline electrocardiogram (ECG) and screening blood tests including virology testing.

4.3.4.4 Informed Consent

Suitable patients had the trial explained to them in detail with particular reference to the PIS. Patients who agreed to take part then completed the consent form.

4.3.4.5 Randomisation

Randomisation was performed with an online commercial clinical trial randomisation software programme (IHD-CLINICAL- http://www.ihd-clinical.com/).

4.3.5 Study Outline

The outline of the intramyocardial arm of the REGENERATE-IHD is shown in figure 13. The first 30 patients were automatically allocated to this arm of the study and following this patients have then been randomly allocated to the 'intracoronary' or 'peripheral' arm of the study.

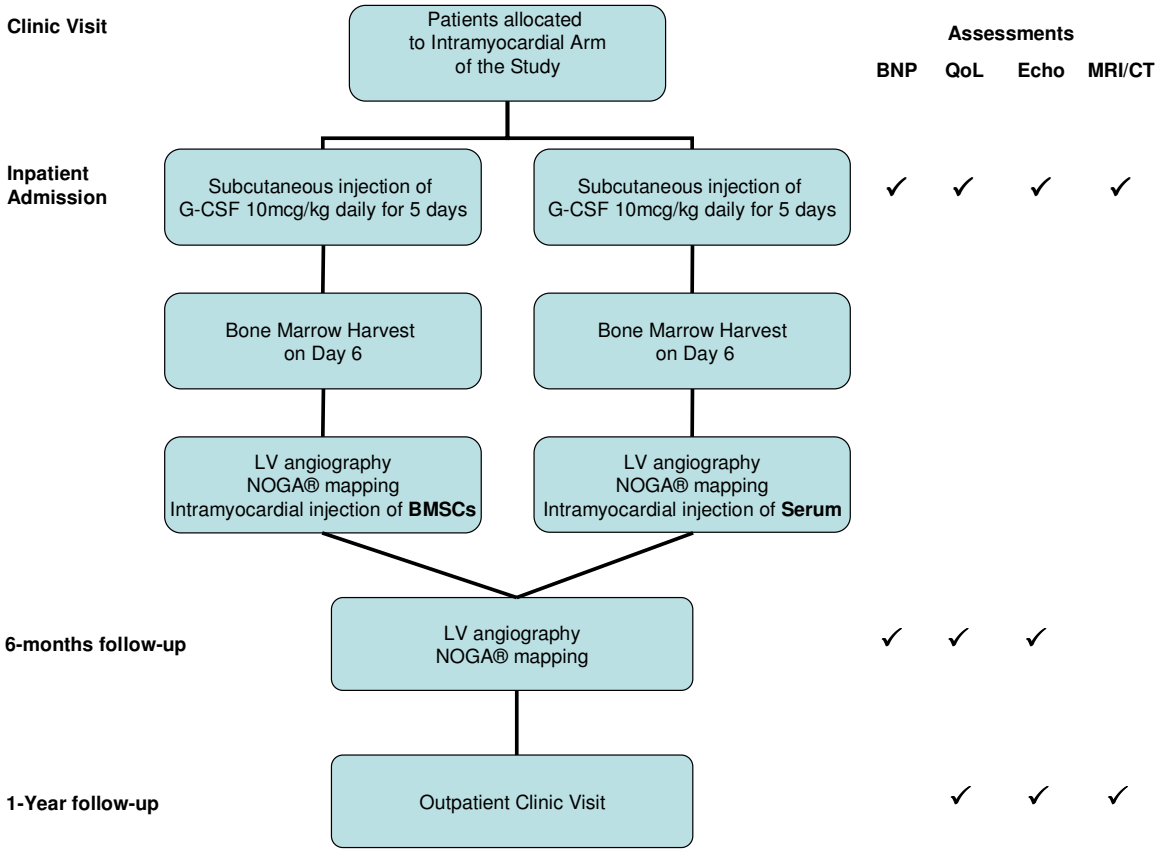


Figure 13. The outline of the intramyocardial arm of the REGENERATE-IHD Study.

4.3.6 Baseline Study Procedures

Patients were typically admitted on a Tuesday afternoon (Day 0) for the study. A baseline contrast echocardiogram was performed on day 0. A Cardiac MRI or CT (if there was a contraindication to MRI scanning) was performed on Day 0 or Day 1 depending on availability. Baseline blood tests were also taken at this stage.

4.3.6.1 Baseline assessment of symptoms and quality of life (QoL)

Patients' heart failure symptoms were graded according to the NYHA classification of functional ability and angina symptoms were graded by the CCS classification, both of which have been described in section 2.1.4. We also assessed quality of life (QoL) using three well validated health related quality of life (HRQL) questionnaires which are discussed below and the full questionnaires are provided in appendix B.

• The MacNew Heart Disease HRQL questionnaire was designed to evaluate how daily activities and physical, emotional, and social functioning are affected by ischaemic heart disease and its treatment[259]. The MacNew (see appendix B) consists of 27 items which fall into three domains (a 13-item physical limitations domain scale, a 14-item emotional function domain scale, and a 13-item social function domain scale)[259]. The maximum possible score in any domain is 7 (high HRQL) and the minimum is 1 (poor HRQL). In our study a global HRQL score was calculated as the average of all scored items. The MacNew instrument can be used to assess change in QoL after an intervention and a change of at least 0.5 is a useful indicator of the minimal important difference in the global HRQL score[260].

• The EQ5D is a HRQL measure developed by the EuroQol Group[261]. The EQ-5D (see appendix B) consists of 2 parts - the EQ-5D descriptive system and the EQ visual analogue scale (EQ VAS). The EQ-5D descriptive system comprises the following 5 domains: mobility, self-care, usual activities, pain/discomfort and anxiety/depression. Each domain has 3 possible

responses: no problems, some problems, extreme problems. The responses from the EQ-5D descriptive system may be converted into a single summary index (as has been done in our study) by applying a formula that essentially attaches values to each of the levels in each domain[262].

The EQ VAS records the respondent's self-rated health on a vertical, visual analogue scale (0-100) where the endpoints are labelled 'worst imaginable health state' and 'best imaginable health state'. This information can be used as a simple quantitative measure of HRQL[263]. The EQ5D has been validated for use in patients with ischaemic heart disease[264].

• The SF-36 Version 2 (see appendix B) is a 36-item questionnaire that measures eight multi-item dimensions of health: physical functioning (10 items) social functioning (2 items), role limitations due to physical problems (4 items), role limitations due to emotional problems (3 items), mental health (5 items), energy/vitality (4 items), pain (2 items), and general health perception (5 items)[265]. For each dimension, item scores are coded, summed, and converted into a scale from 0 (worst possible health state) to 100 (best possible health state)[266]. Two standardised summary scores can also be calculated from the SF-36; the physical component summary (PCS) and the mental health component summary (MCS). The PCS and MCS scores have been standardised using means and standards deviations from general United Kingdom population survey published previously[267]. The use of SF-36 HRQL questionnaire has been validated for the evaluation of a wide variety of medical interventions [268].

4.3.6.2 Baseline assessment of cardiac function

Left ventricular ejection fraction (LVEF) was chosen as the primary surrogate marker to assess change in cardiac function following BMSC therapy. We have also assessed for change in cardiac volumes. LVEF has been assessed with 4 different imaging modalities:

• Transthoracic echocardiography with contrast was performed on Day 0 of the patients' admission. Two-dimensional (2-D) echocardiography with a phased-array electronic ultrasound apparatus was performed in the four standard views: parasternal long and short views and apical four- and two-chamber views (figure 14).

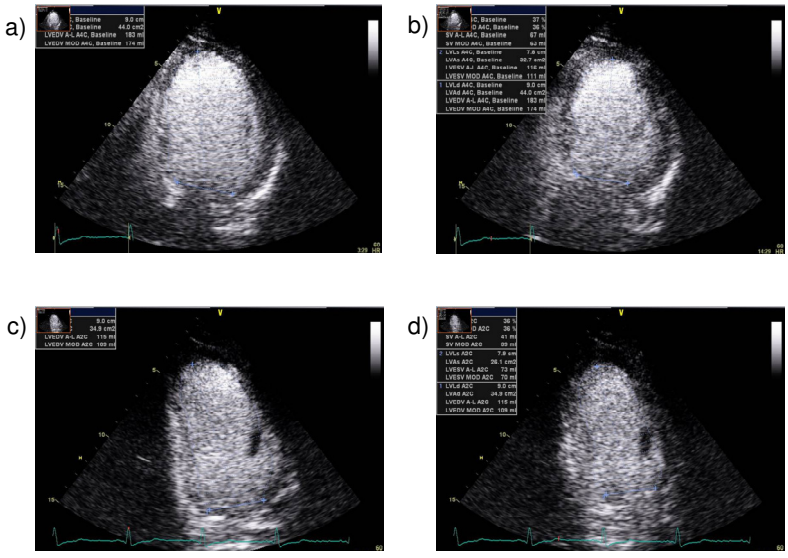


Figure 14. Still frames showing contrast echocardiography for assessment of LVEF and biplane Simpson's method for calculation of LV volumes. Apical 4-chamber views on top row, a) end-diastolic frame and b) end-systolic frame. Apical 2-chamber views on bottom row, c) end-diastolic frame and d) end-systolic frame.

A commercially available contrast agent (Sonovue™) was administered intravenously to opacify the LV and improve endocardial definition. LVEF was

calculated using the biplane Simpson's method[269] (figure 14). Pulsed wave doppler was used to obtain mitral inflow velocity (E) and tissue doppler information (TDI) of the mitral annulus relaxation velocity (E') to obtain the E/E' ratio to assess diastolic function[270].

• Left ventricular angiography was performed on day 6, as part of the NOGA® mapping procedure. This was performed by obtaining a cine image during injection of 30mls of contrast (at a rate of 10mls/second), using a power injector, through a pigtail catheter that was placed retrogradely in the left ventricle from the femoral access site. Cine images were obtained in right anterior oblique (RAO) 30º and left anterior oblique (LAO) 60º views (figure 15). LVEF was obtained by quantitative left ventriculography (QLV) which was performed with dedicated QLV software (QAngio X™).

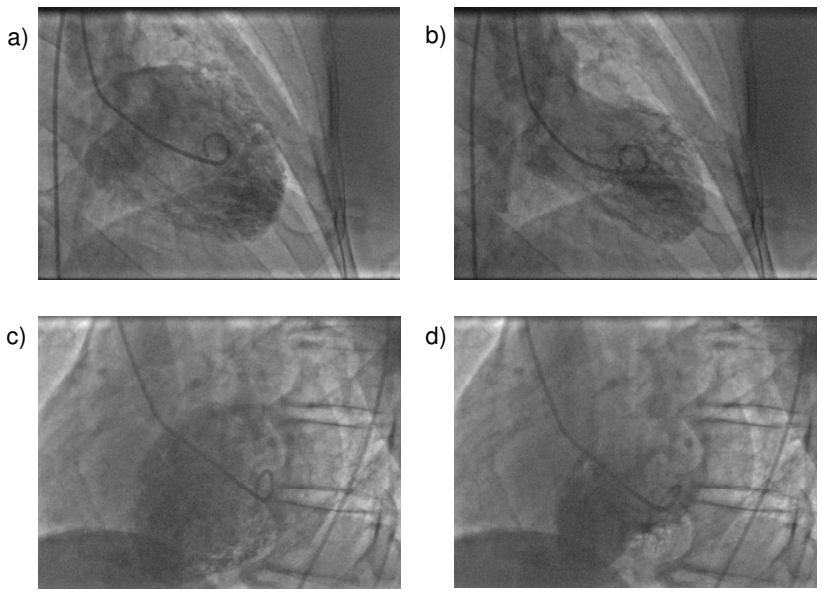


Figure 15. Still frames showing LV angiography for assessment of LVEF. Top row shows RAO 30º a) end diastolic and b) end systolic frames and bottom row shows LAO 60º c) end diastolic and d) end systolic frames.

• CMR was performed in patients without a contraindication to MRI scanning using a Siemens Avanto 1.5T (Siemens Medical Solutions, Erlangen, Germany) scanner, using internationally standardised acquisition protocols [271]. Cardiac magnetic resonance imaging (MRI) is now widely accepted as the gold standard assessment for cardiac function. LV volumes and EF were analysed using a short axis cine-based contour tracing protocol (figure 16).

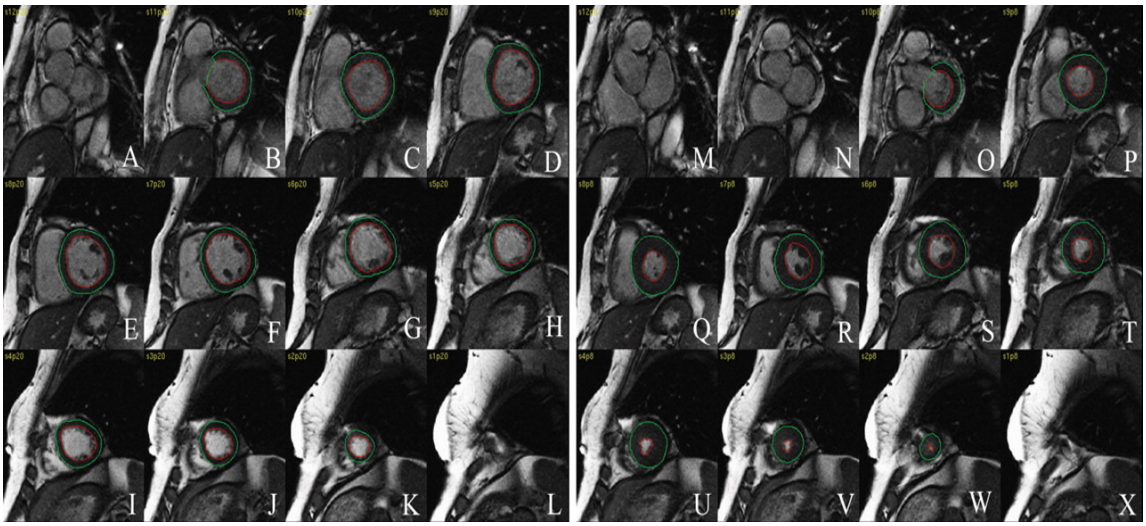


Figure 16. Still images showing the evaluation of LVEF by CMR. Short axis views of the entire left ventricle from base to apex in diastole (A-L) and systole (M-X). Delineation of the epicardial and endocardial contours allows calculation of left ventricular mass, end diastolic volume, end systolic volume, stroke volume and ejection fraction.

• Cardiac CT was performed in patients with a contraindication to MRI scan which was predominantly the presence of an intra-cardiac device. A Siemens 256-slice "FLASH" dual-source scanner was used to obtain retrospectively gated scans with intravenous iodinated contrast for assessment of cardiac structure and function. LV volumes and EF were analysed using a short axis cine-based contour tracing protocol, similar to CMR. Studies comparing MRI and CT have shown good correlation between the two imaging modalities for LVEF assessment [272, 273]. All MRI and CT studies were analysed by an experienced cardiologist blinded to treatment.

4.3.6.3 Baseline blood tests (including NT-ProBNP)

Blood samples were collected from patients on the day of admission (Day 0) for serum NT-proBNP testing. The samples were analysed at an independent laboratory (Doctors Laboratory, London, UK) using a high-sensitivity, quantitative enzyme immunoassay (Modular Analytics E170, Roche Diagnostics). Measurement of cardiac enzymes (creatinine kinase and Troponin-T) was performed on day 6 (pre-procedure) and day 7 (post-procedure) to assess for procedure related myocardial necrosis/infarction. These samples were analysed by the biochemistry laboratory at The Royal London Hospital.

4.3.6.4 Peripheral injection of G-CSF (days 1-5)

Recombinant human G-CSF (Granocyte®, Chugai Pharma, UK) was administered subcutaneously at a dose of 10μg/Kg/day for 5 consecutive days prior to bone marrow harvest on Day 6. Patients had routine blood tests performed daily as well as a sample taken for CD34+ cell count estimation.

4.3.6.5 Bone marrow aspiration (day 6, morning)

Bone marrow was obtained from the posterior iliac crest. 50 ml of bone marrow were aspirated equally into heparin-treated syringes from 3 separate sites over the iliac crest. Thirty-six ml of peripheral venous blood was acquired immediately prior to bone marrow harvest to obtain autologous serum for intramyocardial injections. Blood and bone marrow samples were delivered immediately to the Good Clinical Practice accredited Stem Cell Laboratory for

processing. Isolation and characterisation of BMSCs were performed by a designated lab technician (Natalie Saunders).

4.3.6.6 Isolation of bone marrow mononuclear cells

The bone marrow sample was layered on a Ficoll-Paque™ preparation density gradient medium (Axis shield, Oslo, Norway) and centrifuged at 2500rpm for 30 minutes. The mononuclear cell fraction was extracted and subjected to 3 wash cycles in 0.9% saline (Baxter, Norfolk, UK). Cells were resuspended in 2 ml of autologous serum for intramyocardial injection. Control group injections consisted of 2 ml autologous serum alone. Samples were maintained at room temperature for the entire procedure and the final injectate of stem/progenitor cell suspension or placebo was transported to the cardiac catheter laboratory at London Chest Hospital for the intramyocardial or intracoronary injection procedure. Viability of the cell preparation was checked with 7-AAD (7-amino-actinomycin D) staining immediately prior to infusion and was 98.4 ± 0.7% in the cell treated group.

4.3.6.7 Flow-cytometry of progenitor cells and CFU-GM assays

Bone marrow and peripheral blood circulating progenitor cells (CD34+ cells and endothelial progenitor cells (EPCs)) were characterised using flow cytometry. All flow cytometry analyses were performed using a BD FACSCanto Flow Cytometer with BD FACSDiva v 5.0.3 software (BD Biosciences). For the identification of HSC populations, cells were incubated with fluorescein isothiocyanate (FITC)-labeled antibody against human CD45 (BD Biosciences, Erembodegem-AALST, Belgium) and phycoerythrin (PE)-

labeled antibody against human CD34 (BD Biosciences) for 15 min at room temperature.

EPCs were analysed by initially incubating samples with mouse serum IgG (Sigma, Dorset, UK) for 15 min at 4oC with a cocktail of antibodies comprising allophycocyanin (APC)-labelled antibody to CD133 (Miltenyi Biotec, Surrey, UK) and PE-labelled antibody to VEGFR-2 (R&D Systems, Abingdon, UK) to characterise EPCs and FITC-labelled monoclonal antibodies to CD2, CD13 and CD22 (Beckman Coulter, High Wycombe, UK) to identify and therefore eliminate inclusion of lineage-negative non-progenitor cells. To ensure exclusion of nonviable cells in the final EPC count, cells were also incubated with a PerCP-Cy5-labelled 7AAD stain (BD Biosciences). Cells were then incubated for 15 min at room temperature with 2ml of Pharm Lyse™ buffer (BD Biosciences) to lyse red blood cells. Samples were washed once in phosphate-buffered saline and 20µl of Accucount flow cytometry beads (Saxon Europe, Kelso, UK) were added before analysis.

Functional analysis of CD34+ cells was performed using a colony-forming unit (CFU-GM) assay. BM-MNCs ($2x10^4$ per dish), Day 0 peripheral blood MNCs ($2x10^5$ per dish), and Day 6 peripheral blood MNCs ($2x10^4$ per dish) were seeded, in triplicate preparations, in methylcellulose plates (Methocult H4534, including stem cell factor, granulocyte-macrophage colony–stimulating, and interleukin-3, Stem cell Technologies). Plates were studied under phase-contrast microscopy, and granulocyte-macrophage colony forming units (CFU-

GM; colonies >50 cells) were counted after 14 days of incubation. Results were taken from the mean of the triplicate results.

4.3.6.8 Intramyocardial injection of cells/placebo (day 6, afternoon)

Intramyocardial injection was performed using the NOGA® mapping system and MyoStar™ injection catheter- a detailed description of this delivery system has been provided in section 2.2.5.5. After femoral arterial access (8Fr sheath), a weight adjusted bolus dose of heparin was given as per routine procedure. A LV angiogram was initially performed to assess LVEF and also to guide intramyocardial injection. Patients then underwent LV electromechanical mapping using NOGA® XP Cardiac Navigation System (Biologics Delivery Systems Group, Cordis Corporation, Diamond Bar, CA, USA) to delineate the scar area (unipolar voltage <6.9mV) and surrounding viable but hibernating myocardium. This was correlated with areas of wall dyskinesia on the LV angiogram. Direct intramyocardial injection was performed with the MyoStar™ injection catheter (Biologics Delivery Systems Group, Cordis Corporation, Diamond Bar, CA, USA) as previously described[13, 225]. The total 2 ml volume of injectate was delivered equally over 10 target areas at approximately 1cm intervals. Areas of the myocardium with a wall thickness of <5mm were avoided. The electromechanical mapping data (unipolar voltages and local linear shortening) were recorded so that they could be compared to the follow-up mapping procedure performed at 6-months follow-up. A NOGA® map and injection sites from a patient's procedure are shown in figure 17.

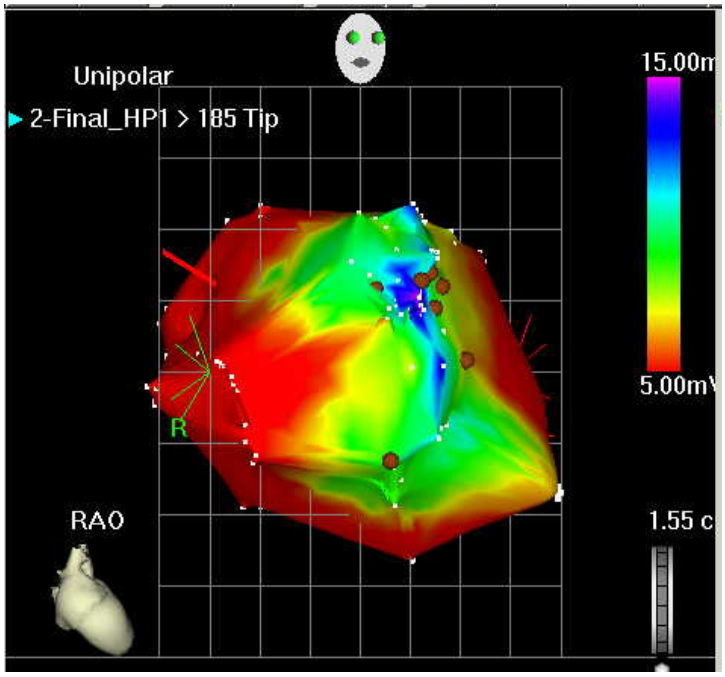


Figure 17. An example of a NOGA® map obtained in a patient in our trial. This map represents endocardial unipolar voltage which is depicted in a colour scale with values <6.9mV (scar tissue) showing as red. As can be appreciated in this map this patient has extensive area of scar tissue. The brown spots represent sites of intramyocardial injection and these have been placed around the area of scar tissue.

4.3.6.9 Patient monitoring and discharge

Following the intramyocardial injection procedure all patients had an immediate transthoracic echocardiogram performed to exclude pericardial effusion. Patients were then admitted to the monitored bed on the cardiology ward and had overnight monitoring. A blood sample was taken the following morning for measurement of CK/Troponin-T levels and patients were discharged home (on day 7) if there were no complications.

4.3.7 Follow-up

Patients were followed up at periods of 6-months at 12-months.

6-month follow-up assessments:

- Clinical history and physical examination
- Contrast echocardiography
- Blood test for NT-proBNP measurement
- LV angiography
- NOGA® electromechanical mapping
- HRQL questionnaires

12-month follow-up assessments:

- Clinical history and physical examination
- Contrast echocardiography
- Cardiac CT or MRI scanning for LV function
- HRQL questionnaires

4.3.8 End-point measures

All outcome measures were assessed blinded to patient treatment allocation.

4.3.8.1 Primary outcome

The primary outcome measure was change in global left ventricular ejection fraction (LVEF) at 12 months relative to baseline as measured by cardiac MRI or CT.

4.3.8.2 Secondary outcome measures

These included:

- the occurrence of a major adverse cardiac event (MACE) including cardiac death or ventricular arrhythmia
- change in global LVEF measured by resting contrast echocardiography
- change in global left LVEF measured by quantitative left ventriculography
- change in serum levels of NT-proBNP
- change in HRQL questionnaire scores
- change in unipolar voltage (UV) and local linear shortening (LLS) maps as assessed by NOGA®

4.3.9 Statistical Analysis

Sample size and power calculations

The sample size for this trial has been determined so there would be a sufficient number of patients to provide a high degree of confidence (power = 90%) for evaluating progenitor cell transplantation therapy. The study has been powered to adequately assess the primary end-point of change in cardiac function as assessed by LVEF on cardiac MRI or CT scanning. An absolute improvement of 3.5% in this LVEF would be considered both a realistic effect and potentially worthwhile. The within patient standard error is estimated to be around 2.8%, we used a pessimistic estimate of this as 4%. This gives a standard error for the difference of the measurements of before and after treatment of $(2x4)^{1/2}$ = 2.8- conservatively estimated as 3. Therefore the standardised effect we aimed to detect is 3.5/3 = 1.17. Using samples size tables (table 4.2) from Machin et. al [274], to detect this targeted difference with a significance level of 5% and power of 90% requires 11 patients per group. Allowing for a (pessimistic) dropout of 4 patients per group we aimed to recruit 15 patients per group i.e. 30 patients in each arm of the study.

Statistical analysis

Analysis is on a per-protocol basis. Continuous variables are presented as means (±SD) or (±SEM) and categorical variables are presented as percentages. Statistical comparisons of continuous variables were made by the parametric 2-sided t-test or Wilcoxon signed-rank test for paired variables or Mann-Whitney U test for independent variables. Categorical variables were

compared by means of Fisher's exact test. A P value of < 0.05 was considered to indicate statistical significance. All reported P values are two-sided. Statistical analyses were performed using Graphpad Prism version 5.0 (GraphPad Software, San Diego, CA) and SPSS 19. Results were reviewed by Professor Mahesh Parmer, Director of the Medical Research Council's Clinical Trials Unit (CTU).

4.4 Results

4.4.1 Patient recruitment and baseline characteristics

The flow diagram below depicts patient recruitment and treatment.

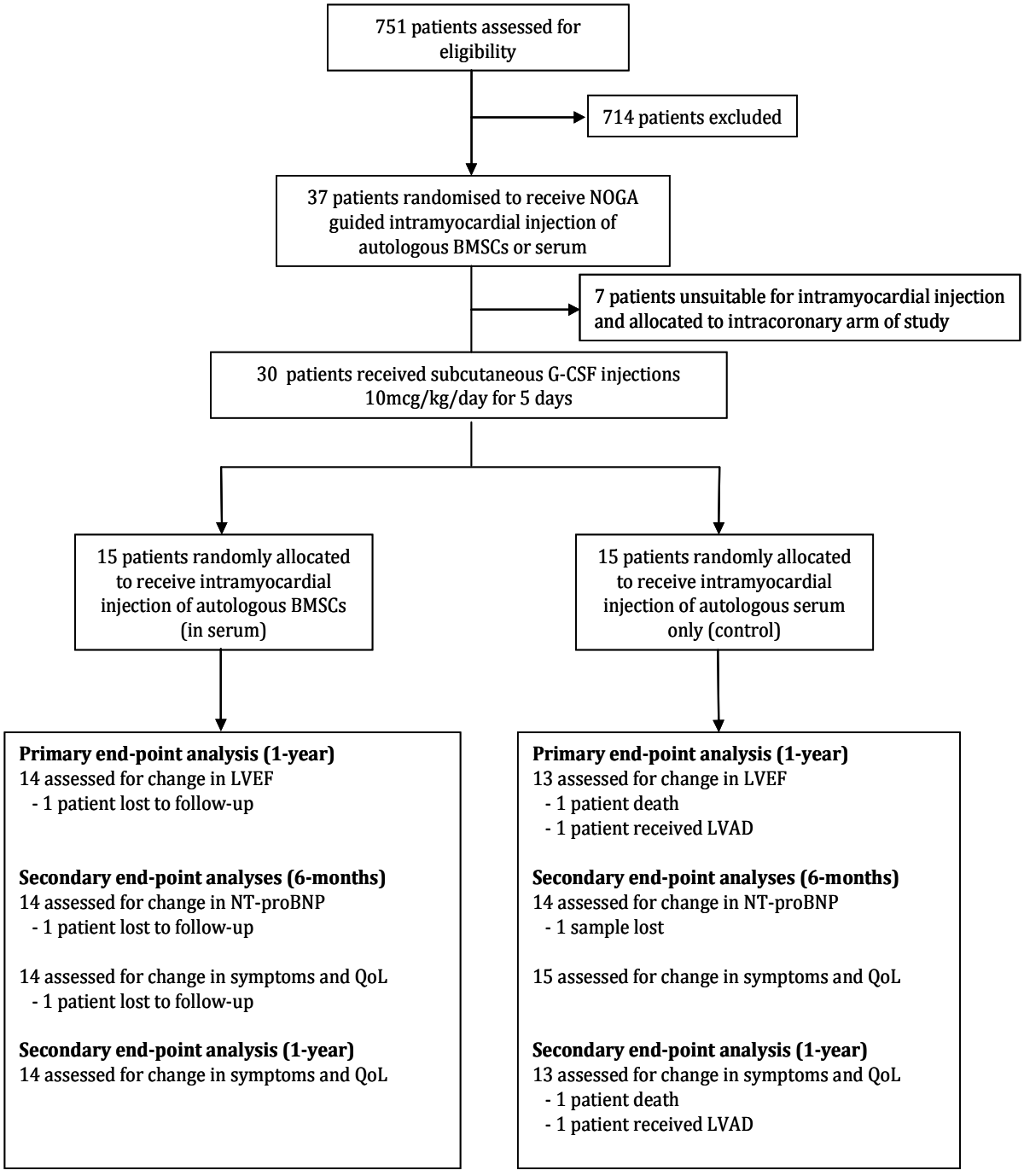


Figure 18. Flow diagram of patient recruitment and treatment

A total of 751 patients were assessed for eligibility between October 2009 and November 2010 (Figure 18). 714 patients were unsuitable based on the predefined exclusion criteria described in the methods (major reasons being atrial fibrillation, NYHA class 1 status, creatinine>200umol/l, ongoing medical/device optimisation, patients declining and randomisation into a separate study). A total of 37 patients were randomised into intramyocardial arm of the study with a final 15 patients receiving intramyocardial injection of bone marrow mononuclear cells suspended in serum and 15 patients receiving intramyocardial serum injections alone. 7 patients (of the initial 37) did not receive treatment as they were not suitable for intramyocardial injection (reasons described below).

The baseline characteristics of the 30 patients who underwent treatment are shown in table 4. Patients in the two different treatment groups were generally well matched although there was a trend towards higher number of implantable cardiac devices in the BMSC treated group. It is important to note that the mean left ventricular ejection fraction (LVEF) was 29% and NT-proBNP was 1048 pg/ml- reflecting a cohort of patients with truly advanced ischaemic heart failure.

Table 4. Baseline characteristics of patients

	All Patients (n=30)	BMSC group (n=15)	Control (serum) group (n=15)	p-value
Age, years	63 ± 10	65 ± 9	60 ± 11	0.202
Male sex, No (%)	30 (100)	15 (100)	15 (100)	-
Medical history				
Diabetes (%)	8 (27)	4 (27)	4 (27)	0.659
Previous MI (%)	26 (87)	13 (87)	13 (87)	0.701
CABG (%)	10 (33)	4 (27)	6 (40)	0.350
Time from last MI (years)	8.5 ± 7	8.0 ± 6.2	8.9 ± 8	0.749
LVEF (%)	29 ± 9	31 ± 8	29 ± 10	0.607
NT-proBNP (pg/ml)	1048 ± 1263	1299 ± 1499	796 ± 959	0.283
Medications at recruitment				
Statins	27 (90)	13 (87)	14 (93)	0.500
ACEI/ARB	30 (100)	15 (100)	15 (100)	-
β-blocker	25 (83)	14 (93)	11 (73)	0.165
Aldos Antagonist	21 (70)	12 (80)	9 (60)	0.213
Diuretics	22 (73)	12 (80)	10 (66)	0.341
Devices				
ICD (%)	21 (70)	13 (87)	8 (53)	0.054
CRT (%)	9 (30)	7 (47)	2 (15)	0.054
Total number of cells isolated				
MNC ($x10^6$)	N/A	138 ± 99	128 ± 141	0.816
CD34+ ($x10^6$)	N/A	3.7 ± 1.5	3.5 ± 2.7	0.857

Data presented as number and (% of patients) or mean ± SD; LVEF: left ventricular ejection fraction; MNC: mononuclear cells; N/A: not applicable; NS: not significant. P-value based on comparison between BMSC and control group.

4.4.2 Feasibility and Safety of G-CSF mobilisation

All patients completed the subcutaneous injection of G-CSF phase of the trial. This was well tolerated in all the patients. The most common complaint was bone pain which was experienced by 5 (17%) patients. Only 1 (3%) patient had clinically evident splenomegaly which resolved by discharge (day 7) and 1 (3%) patient had pyrexia > 38^0C. G-CSF also led to transient rise in liver function tests (LFTs) but these were clinically non-significant and all resolved after completion of G-CSF treatment. There were no episodes of worsening angina and no significant elevations in cardiac enzymes during G-CSF treatment.

There were no complications of the bone marrow aspiration procedure performed on day 6.

4.4.3 Feasibility and safety of intramyocardial injection

4.4.3.1 Feasibility of NOGA® mapping and intramyocardial injection

6 patients who were initially randomised to the intramyocardial arm of the study had to be changed to the intracoronary arm. The reasons were:

- 3 patients were found to have LV thrombus which is a contraindication for NOGA® mapping.
- 1 patient had ventricular tachycardia during NOGA® mapping and it was felt that intramyocardial injection would pose too high a risk.
- 1 patient was felt to have high risk LV anatomy with areas of LV wall thickness <0.5cm.

- 1 patient had to be changed due to a technical issue with Cardiac Catheter Lab being unable to perform NOGA® procedure on that day.

1 patient was withdrawn as no scar tissue was found on NOGA® mapping.

4.4.3.2 Safety of NOGA® intramyocardial injection

The intramyocardial injection was well tolerated in most of the patients. There were 3 (10%) arrhythmia episodes:

- 1 patient had ventricular tachycardia during NOGA® mapping (changed to intracoronary arm)
- 1 patient had self-limiting episodes of paroxysmal atrial fibrillation
- 1 patient had ventricular tachycardia with haemodynamic compromise requiring emergency DC cardioversion during intramyocardial injection. The procedure was then completed without any further complications.

Only 1 patient developed a small pericardial effusion which had resolved on a repeat scan the next day.

There were 5 (17%) access site complications although all of these apart from one were small femoral haematomas which did not delay discharge for the patients. However 1 (3.3%) patient had bleeding from the puncture site which did not seal with manual pressure and he required vascular surgery to repair his femoral artery.

There were no significant arrhythmias during overnight monitoring post procedure. Cardiac enzymes were checked the day after the procedure and

results are shown in Figure 19. Although there was a statistically significant rise in Troponin-T level (mean 0.049 ng/ml), this did not meet the criteria for defining a peri-procedure myocardial infarction[275].

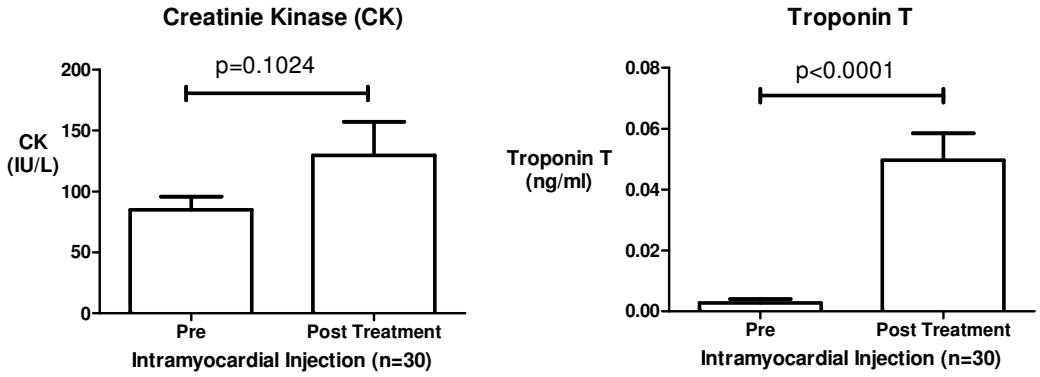


Figure 19. Bar graphs representing changes in cardiac enzymes 1-day post intramyocardial injection. Although there is an overall statistically significant rise in Troponin T level this did not meet the threshold for diagnosis of peri-procedural myocardial infarct.

4.4.4 Safety of BMSC therapy

In general there was a relatively low rate of serious adverse events (SAEs) for the whole cohort of patients during the 1-year follow-up period. In total, there were 7 SAEs which are shown in table 5.

Table 5 List of serious adverse events (mean follow-up 380 days)

Serious adverse event	BMSC treated (n=15)	Control (serum) group (n=15)
Death (all cause)	0	1
Cardiac death	0	0
Myocardial Infarction	0	0
Coronary revascularisation	0	0
Arrhythmia episode	0	1
Hospitalisation for heart failure	1	1
Hospitalisation for chest pain	1	0
Hospitalisation for other cause	0	2

The incidence of serious adverse events during 1-year follow-up is depicted graphically in figure 20 using a Kaplan Meir curve comparison of event free survival. The incidence of events was not significantly different between the two groups (hazard ratio 0.392, 95% confidence interval 0.089-1.730).

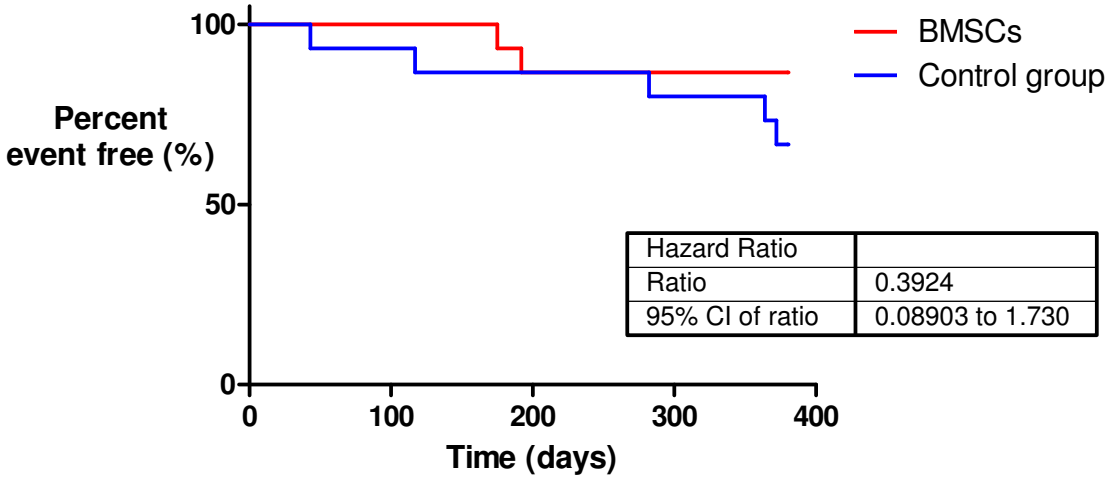


Figure 20. Kaplan Meir curves comparing event free survival (composite of all cause hospitalisation and mortality) between BMSC treated and control (serum) groups.

4.4.5 Effect of BMSC therapy on cardiac function

4.4.5.1 Cardiac CT/MRI

The change in left ventricular ejection fraction (LVEF) and cardiac volumes was assessed in all patients who completed 1-year follow-up: 14 patients in the BMSC treated group (1 patient lost to follow-up) and 13 patients in the control group (1 patient death and 1 patient received LVAD therapy hence was not included).

In the BMSC treated group there was a significant increase in LVEF at 1 year (33.57 ± 11.03% vs 28.59 ± 10.24%, p=0.037). There was also a trend

towards improvement in the control group but this was not statistically significant (33.15 ± 9.91% vs 29 ± 9.21%, p=0.246):

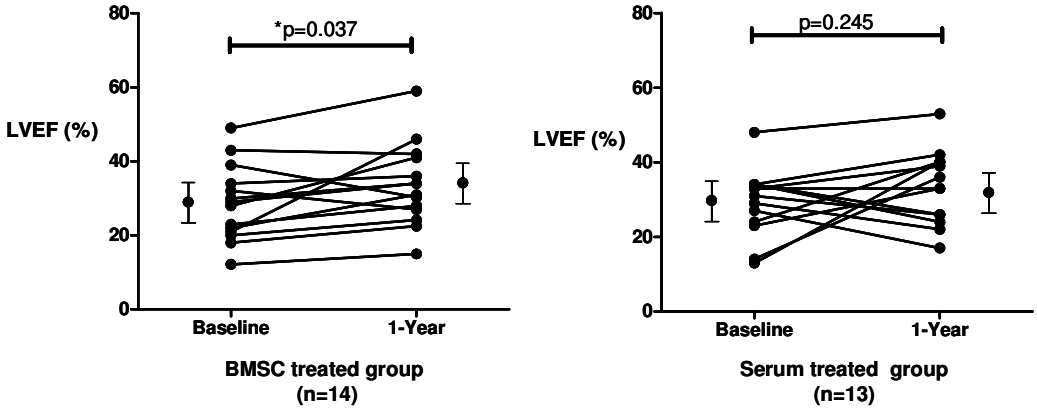


Figure 21. Graphs representing change in LVEF in BMSC treated and control groups at 1-year, measured by CT/MRI. Error bars indicate mean and 95% confidence intervals.

There were no statistically significant changes in the end-diastolic or end-systolic volumes (EDV or ESV respectively) in either group although there was a trend towards reduction in ESV (204±73 vs 196±76 mls, p=0.546) in the BMSC treated group:

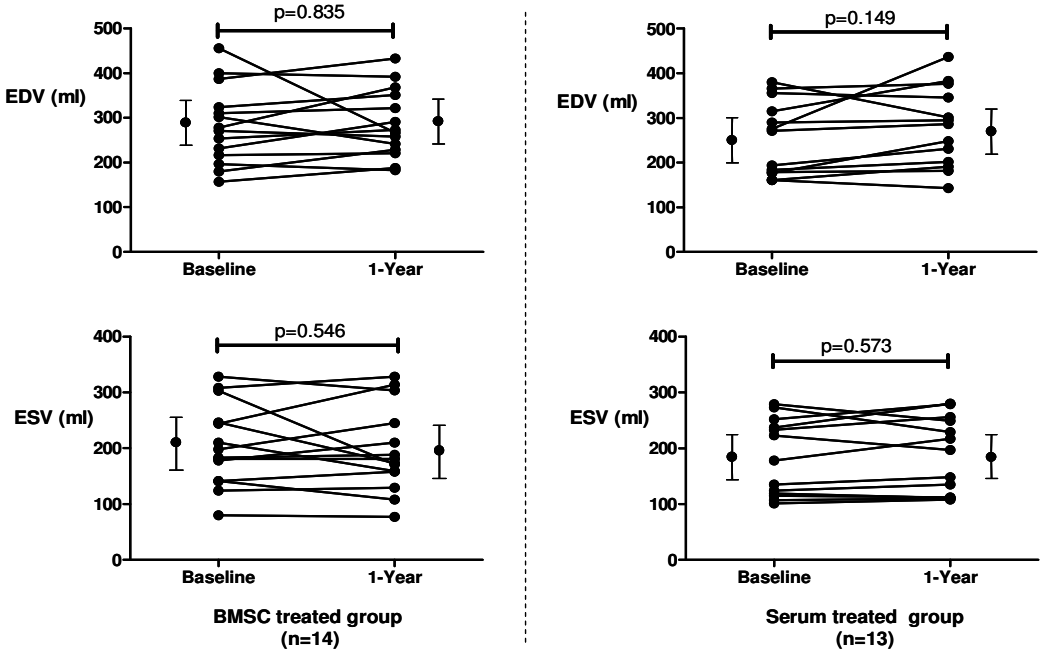


Figure 22. Graphs representing change in cardiac volumes in BMSC treated and control groups at 1-year, measured by CT/MRI. Error bars indicate mean and 95% confidence intervals.

The magnitude of change in ejection fraction in the BMSC treated group appeared to be related to be related to the in vitro functional potential of the cells as measured by a colony forming unit assay (CFU). See section 4.4.8 for more details.

4.4.5.2 Quantitative Left Ventriculography (QLV)

Quantitative left ventriculography (QLV) analysis was performed using dedicated software- QAngio® XA (Medis). An example of QLV analysis for one of the patients is shown in figure 23:

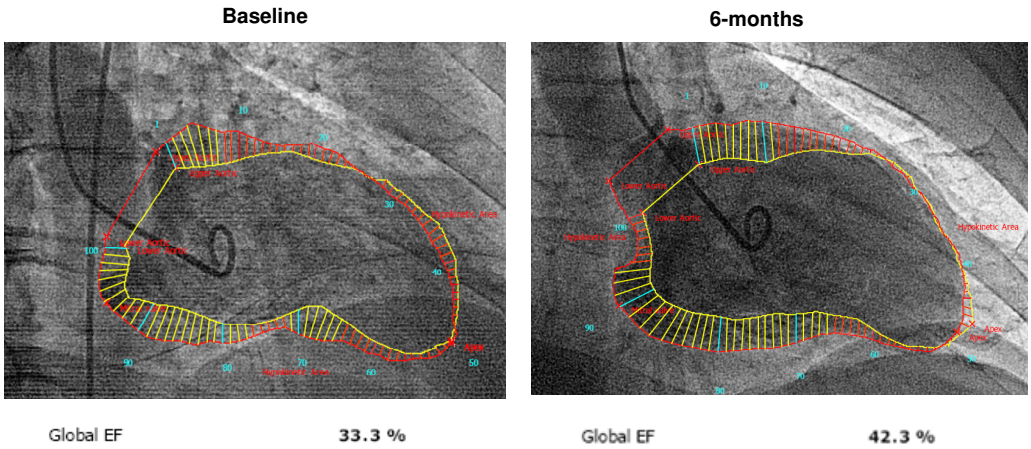


Figure 23. Still images of quantitative left ventriculography (QLV) calculation using the QAngio® XA software. Contour tracing of the LV cavity in end-diastole (red line) and in end-systole (yellow line) was performed to calculate cardiac volumes and ejection fraction.

Overall, there was no significant difference in LVEF, measured by QLV, 6-months after intramyocardial injection of BMSCs or serum alone (control group):

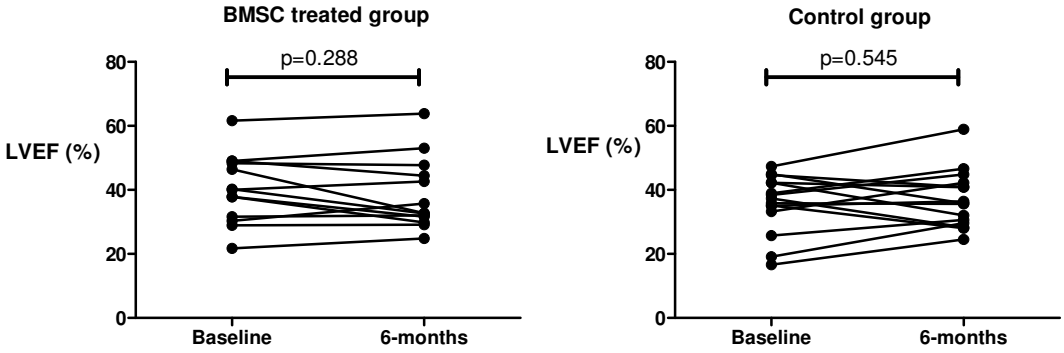


Figure 24. Graphs representing change in LVEF in BMSC treated and control group at 6-months, as measured by quantitative left ventriculography (QLV).

There was also no significant change in LV end-diastolic volume in either the BMSC treated (209±59 vs 203±69, p=0.719) or control (214±56 vs 205±61, p=0.43) groups 6-months post treatment:

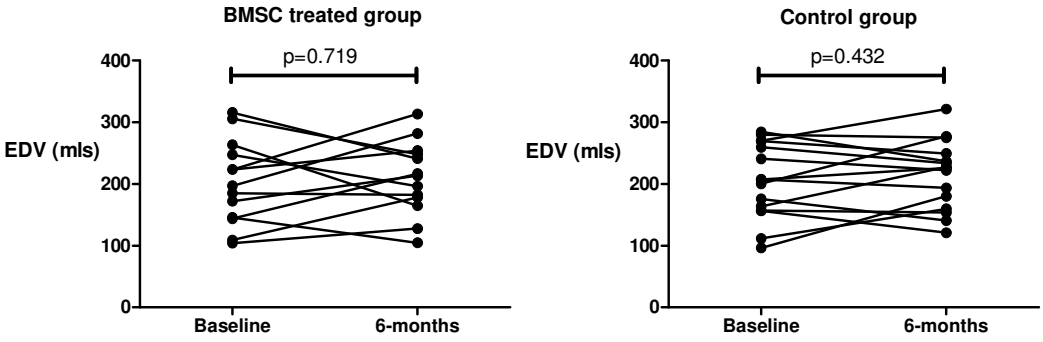


Figure 25. Graphs representing change in end-diastolic volume (EDV) in BMSC treated and control group at 1-year measured by quantitative left ventriculography (QLV).

4.4.5.3 Contrast transthoracic echocardiography

There was no significant overall change in LVEF, measured using the biplane Simpson's methods on contrast echocardiography, in either treatment group at 6-months or 1-year post treatment:

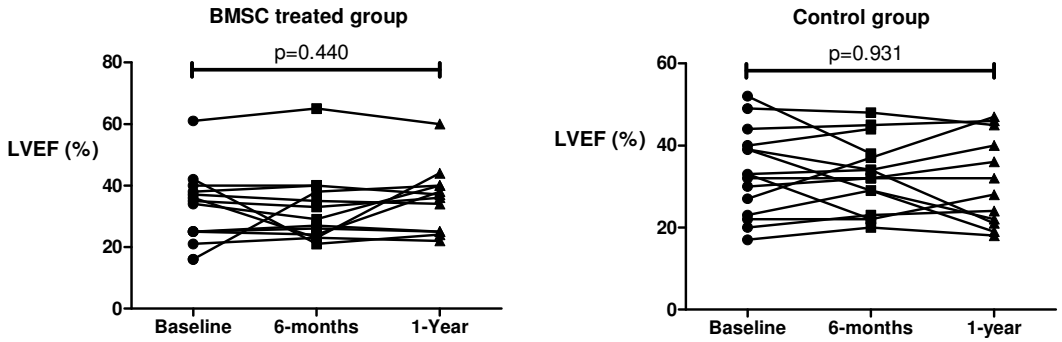


Figure 26. Graphs representing change in LVEF as measured by contrast echocardiography using the biplane Simpson's method.

However, there did appear to be some improvement in diastolic function in patients treated with BMSCs. The E/E' parameter improved significantly from baseline to 1-year in the cell treated group (12.17± 6.1 to 10.46± 4.1, p=0.043) but no improvement was seen in the control group.

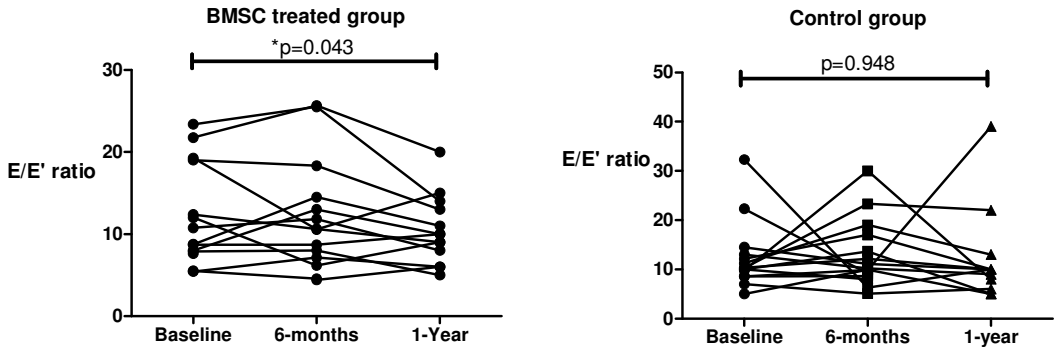


Figure 27. Graphs representing change in diastolic function (E/E') measured using echocardiography, during follow-up.

4.4.6 Effect of BMSC therapy on NT-proBNP levels

Baseline and 6-month follow-up NT-proBNP levels were available in 14 patients in each of the treatment groups.

In the BMSC treated group there was a significant reduction in NT-proBNP levels at 6-months post treatment (769±754 vs 977±867, p=0.045). Interestingly, there was also a significant reduction in NT-proBNP levels in the control (serum only) group (408±302 vs 572±424, p=0.024). To investigate whether this was an effect of G-CSF, the NT-proBNP levels were assessed in patients in the peripheral arm of the REGENERATE-IHD study who received either subcutaneous G-CSF or saline injections only. There was no significant reduction in NT-proBNP levels in either of these patient groups. The data is summarised in figure 28:

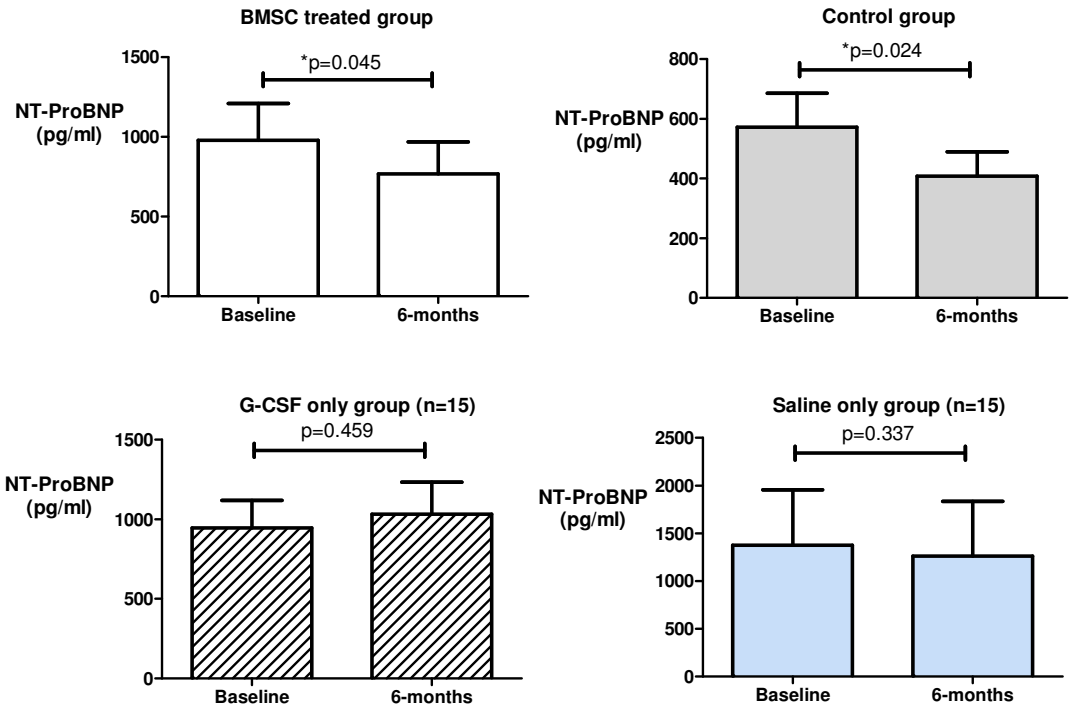


Figure 28. Bar graphs representing change in NT-proBNP levels in the BMSC treated and control groups (top row). Bottom row shows change in NT-proBNP levels in patients receiving only subcutaneous G-CSF or saline injections.

4.4.7 Effect of BMSC therapy on patient symptoms and QoL

4.4.7.1 Change in NYHA and CCS class

There was a significant reduction in NYHA functional class in patients treated with BMSC therapy at 6-months (2.53 vs 2.01, p= 0.02) and 1-year post therapy (2.53 vs 2.07, p=0.04). In the control group there was also a reduction in NYHA class at 6-months (2.26 vs 1.80, p=0.003), however this was not maintained at 1-year follow-up (2.07 vs 2.26, p=0.501):

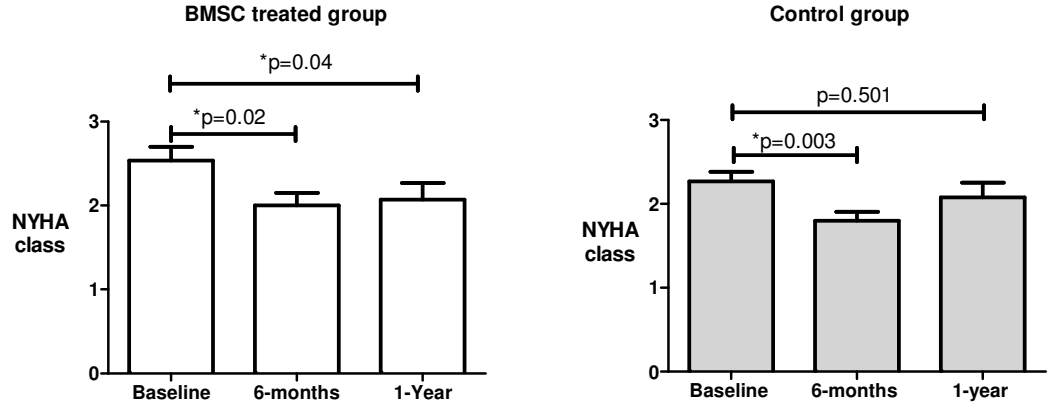


Figure 29. Bar graphs representing change in NYHA class during follow-up

There was no significant change in CCS angina class in the BMSC treated group although there was a trend towards reduction in angina in the control (serum) group:

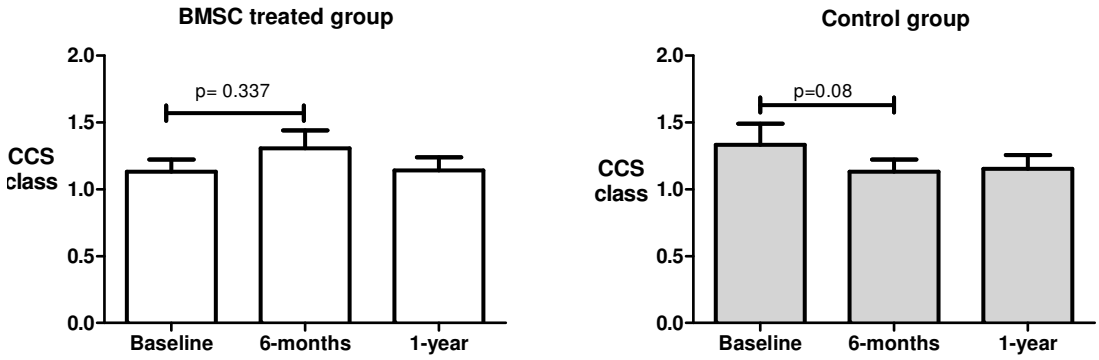


Figure 30. Bar graphs representing change in CCS angina class during follow-up

4.4.7.2 Changes in Health Related Quality of Life (HRQL)

As described in the previous methods section, HRQL was assessed using 3 different questionnaires, each performed at baseline, 6-months and 1-year.

Macnew instrument

The global score was calculated for each patient at the three different time-points and the change in the mean score for each treatment group was assessed. In the BMSC treated group there was a significant increase in the mean MacNew global score from baseline to 6-months (3.8 ± 0.9 vs 4.4 ± 0.9, p=0.02), however there was no significant difference at the 1-year time-point. Interestingly, in the control (serum only) group there was a trend towards improved QoL at 1-year compared to baseline (4.95 ± 1.4 vs 4.2 ± 1, p=0.052). The data is summarised in figure 31.

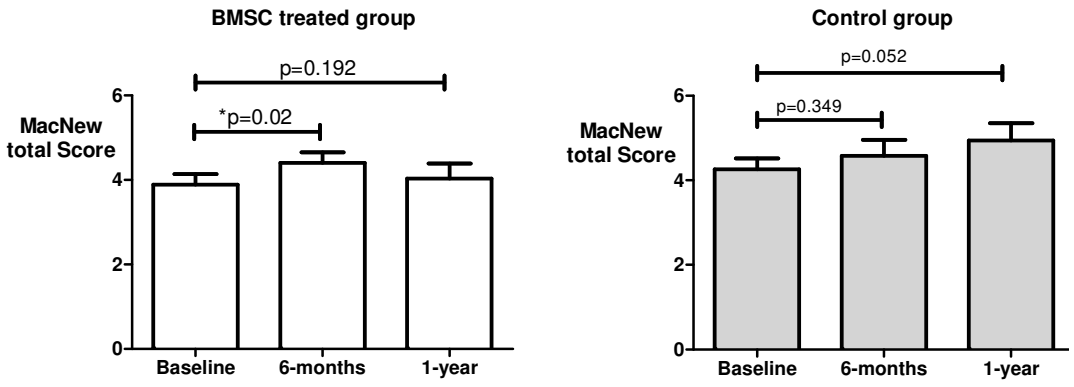


Figure 31. Bar graphs representing change in MacNew questionnaire total score during follow-up

SF-36 instrument

The SF-36 questionnaire responses were scored and summarised according to the SF36™ Version 2 Manual. Two summary scores were obtained, the physical component score (PCS) and mental component score (MCS) and both were normalised using UK normative data [267].

In the BMSC treated group the SF36-PCS improved significantly 1-year post treatment (29.89±15.42 vs 24.91±12.56, p=0.023) but not at the 6-month time-point. In the control group there was no statistically significant improvement although there was a trend towards this. The SF36-MCS did not improve significantly in either group. The data is summarised in figure 32.

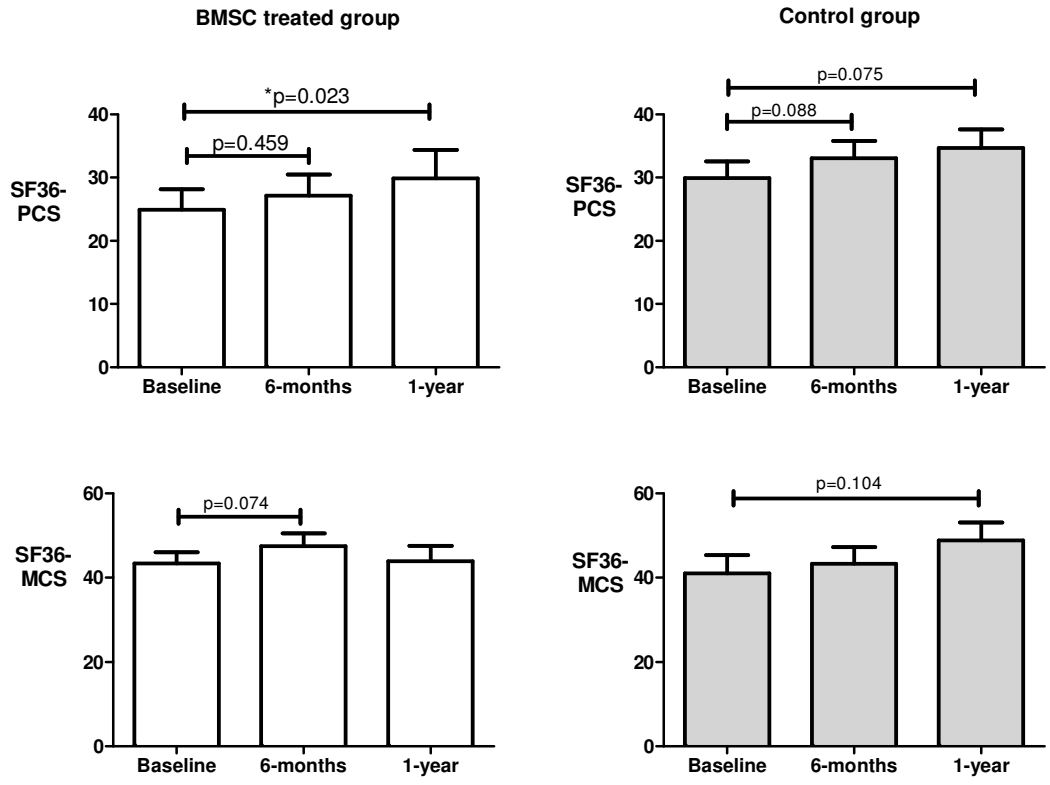


Figure 32. Bar graphs representing change in SF36 questionnaire scores during follow-up

EQ-5D Instrument

The scores from the EQ-5D descriptive system were converted into a single summary index using an online calculator: (www.economicsnetwork.ac.uk/health/EQ_5D_index_calculator.xls).

The visual analogue scale (VAS) scores were tabulated for each patient and the mean summary index score and VAS score were compared in each group over time.

There was no significant change in the EQ-5D summary index score in either the BMSC treated or control (serum only) groups. In the BMSC treated group the EQ-5D VAS score improved significantly at 6-months post treatment (55.21±21.45 vs 45.13±20.34, p=0.035) but had declined to a similar baseline level by 1-year. There was no significant change in the VAS score in the control group.

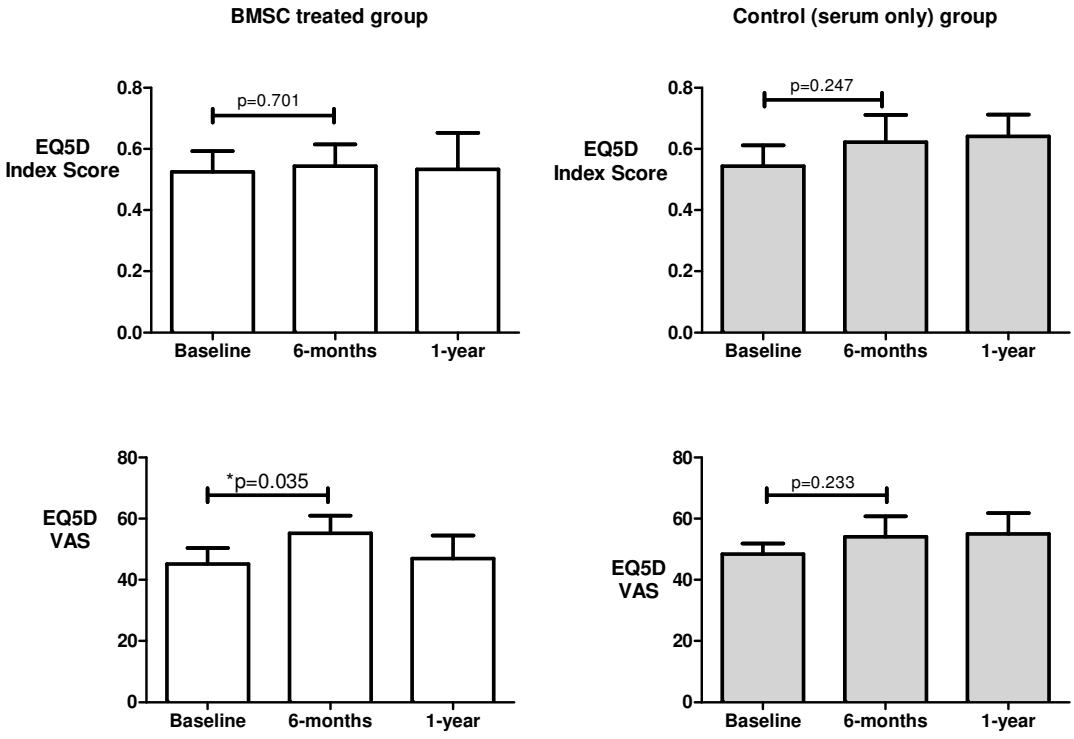


Figure 33. Bar graphs representing change in EQ5D questionnaire scores during follow-up

Summary of HRQL questionnaire scores

A summary of the numerical HRQL questionnaire scores is provided in table 6:

Table 6 Health Related Quality of Life Questionnaire Scores

Questionnaire	Baseline	6-months	1-Year
MacNew			
BMSCs group	3.88 ± 0.97 (n=15)	**4.40 ± 0.91* (n=14)**	4.03 ± 1.29^{NS} (n=13)
Serum alone group	4.26 ± 1.01 (n=15)	4.58 ± 1.46^{NS} (n=15)	4.95 ± 1.47^{NS} (n=13)
SF36 v2 PCS			
BMSCs group	24.91 ± 12.56 (n=15)	27.13 ± 12.59^{NS} (n=14)	**29.89 ± 15.42* (n=12)**
Serum alone group	29.91 ± 10.19 (n=15)	33.05 ± 10.51^{NS} (n=15)	34.68 ± 10.61^{NS} (n=13)
SF36 v2 MCS			
BMSCs group	43.43 ± 10.28 (n=15)	47.50 ± 11.37^{NS} (n=14)	43.95 ± 12.63^{NS} (n=12)
Serum alone group	41.04 ± 16.75 (n=15)	43.31 ± 15.44^{NS} (n=15)	48.80 ± 15.43^{NS} (n=13)
EQ5D Index Score			
BMSCs group	0.52 ± 0.26 (n=15)	0.54 ± 0.27^{NS} (n=14)	0.53 ± 0.41^{NS} (n=12)
Serum alone group	0.54 ± 0.26 (n=15)	0.62 ± 0.33^{NS} (n=14)	0.64 ± 0.25^{NS} (n=13)
EQ5D VAS Score			
BMSCs group	45.13 ± 20.34 (n=15)	**55.21 ± 21.45* (n=14)**	46.92 ± 25.95^{NS} (n=12)
Serum alone group	48.36 ± 13.36 (n=15)	54.00 ± 25.93^{NS} (n=15)	55.00 ± 24.49^{NS} (n=13)

Data presented as mean ± SD
Significance compared to baseline (NS: non-significant, *= P<0.05, significant results in bold)
BMSCs: bone marrow stem cells; MCS: mental component score; PCS: physical component score; VAS: visual analogue score

4.4.8 Effect of progenitor cell concentration and function on primary and secondary end-points of study

Table 7 shows the final concentration of peripheral and bone marrow progenitor cells in the trial patients following G-CSF mobilisation. There were no significant difference between the BMSC treated and control groups with regards to peak peripheral blood CD34+ cell concentration following 5-days of G-CSF therapy. There were also no differences in the final (product) concentration of total mononuclear cells or CD34+ cells obtained from bone marrow aspiration. The in vitro functional potential (colony forming units) of the bone marrow CD34+ cells was also similar between the two groups.

Table 7. Progenitor cell concentration and function data

	BMSC treated group (n=15)	**Control group* (n=15)**	**p-value**
Peak peripheral blood CD34+ concentration (CD34+ cells/µl)	46.22 ± 34.9	66.81 ± 72	0.328
Total MNC/product ($x10^6$)	125 ± 89	136 ± 140	0.798
Total CD34+ cells/product ($x10^6$)	3.29 ± 1.37	3.37 ± 2.41	0.91
Colony Forming Units (CFUs per BM CD34+ cell plated)	0.07 ± 0.08 (n=9)	0.08 ± 0.05 (n=8)	0.799
Cell viability	98.4 ± 0.7	N/A	

Data presented as mean ± SD, *cells stored in the control group
MNC: mononuclear cells

There was no correlation between the total dose of mononuclear or CD34+ cells injected and the magnitude of change in LVEF in the BMSC treated group. However, there did appear to be a positive relationship between the magnitude of LVEF improvement and the in vitro functional potential of the injected CD34+ cells, measured by a colony forming unit (CFU) assay, as shown in figure 34:

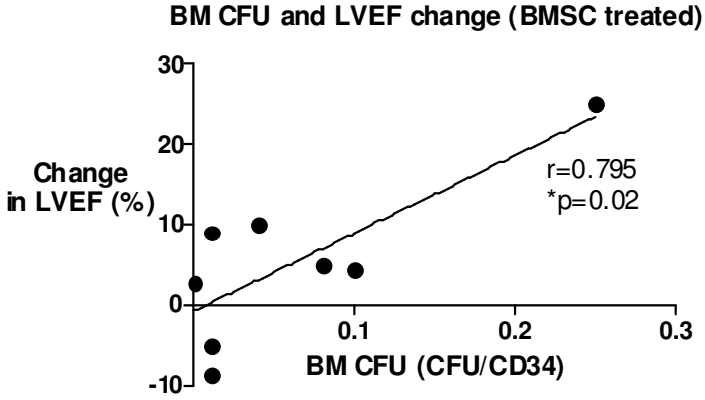


Figure 34. Scatter plot and linear regression diagram demonstrating positive correlation between bone marrow CD34+ cell colony forming CFU potential (BM CFU) and magnitude of change in LVEF in patients treated with BMSC therapy.

There were no other significant correlations between progenitor cell concentration/function and outcome measures.

A more detailed analysis of progenitor cell concentration and function has been performed in the study presented in the next chapter. This includes analysis of all patients recruited to the REGENERATE-IHD trial and compares progenitor cell characteristics between the different study populations.

4.4.9 Effect of BMSC therapy on NOGA® measures of myocardial contractility and voltage potential

4.4.9.1 Measuring endocardial voltages

As has been described in the methods section, endocardial mapping of the left ventricle was performed using the NOGA® XP Cardiac Navigation System. The endocardial map was displayed as a bulls-eye plot (an example is shown in Figure 35) which divides the ventricle into 9 segments: apex, basal anterior, mid-anterior, basal lateral, mid-lateral, basal posterior, mid-posterior, basal septal and mid-septal. For each segment the mean unipolar voltage (mV) and local linear shortening (%) were recorded. NOGA® parameters for comparison between baseline and 6-months were available in 12 patients in each of the two treatment groups.

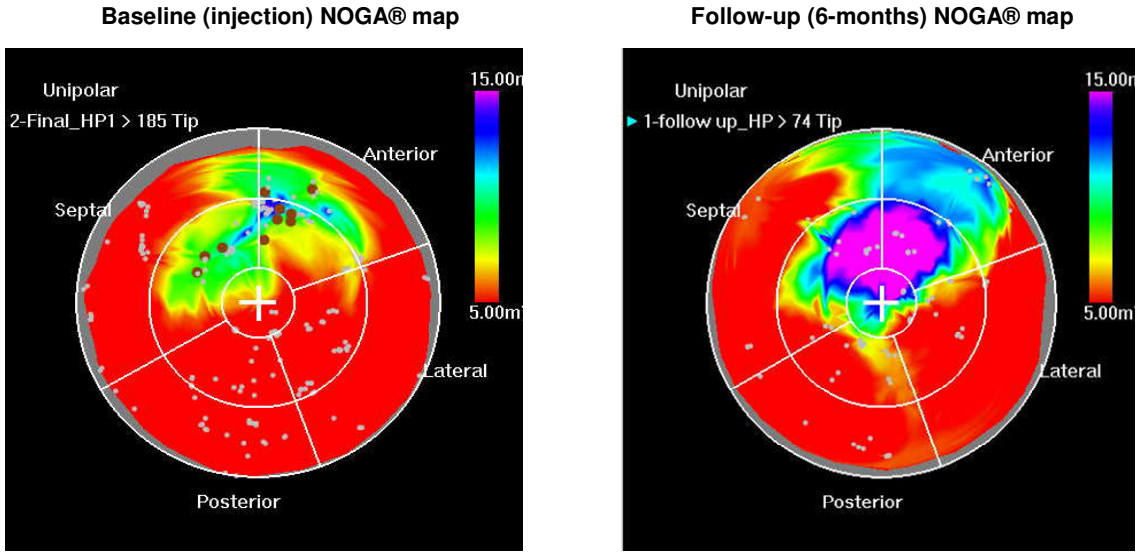


Figure 35. An example of a bulls eye plot of NOGA® derived unipolar voltages in a patient on the trial. On the left is the baseline map and on the right is the follow-up map at 6-months. In this patient there seems to be increased unipolar voltage at the previous injection sites suggesting improved viability.

4.4.9.2 Changes in unipolar voltage (UV) potential

In the BMSC treated group there appeared to be possible improvement in unipolar voltage (UV) from baseline to 6-months (7.71±3.2 vs 7.97±3.2, p=0.637) although this was not statistically significant. Interestingly in the control (serum treated) group there was a statistically significant improvement in UV at 6-months post treatment (7.06±2.8 vs 7.59±3.1, p=0.02). The data is summarised in figure 36:

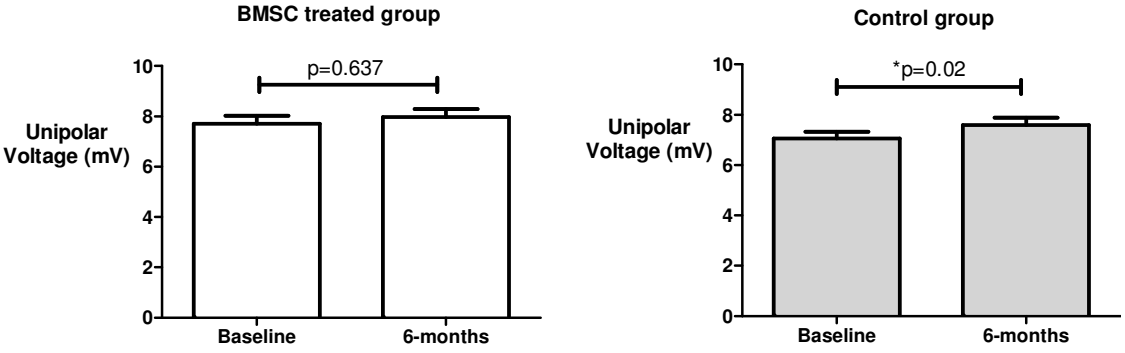


Figure 36. Bar graph representing change in overall unipolar voltage at 6-months follow-up in the BMSC treated and control group

To examine this further, the change in UV was assessed separately for the myocardial areas that had the cells or serum injected. As shown in figure 37, there did not appear to be any significant change in the injected segments.

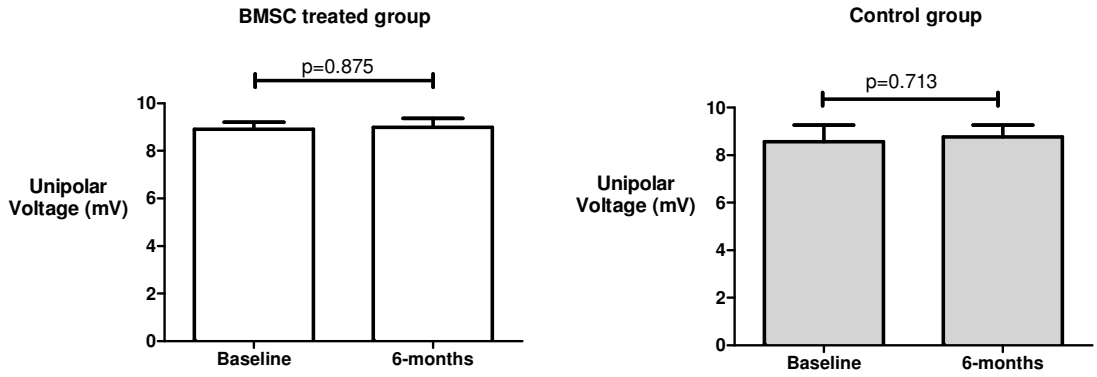


Figure 37. Bar graph representing change in unipolar voltage in the injected myocardial segments

However, in the non-injected areas there did appear to be a trend towards improvement in unipolar voltage in both groups, although this did not reach statistical significance (figure 38).

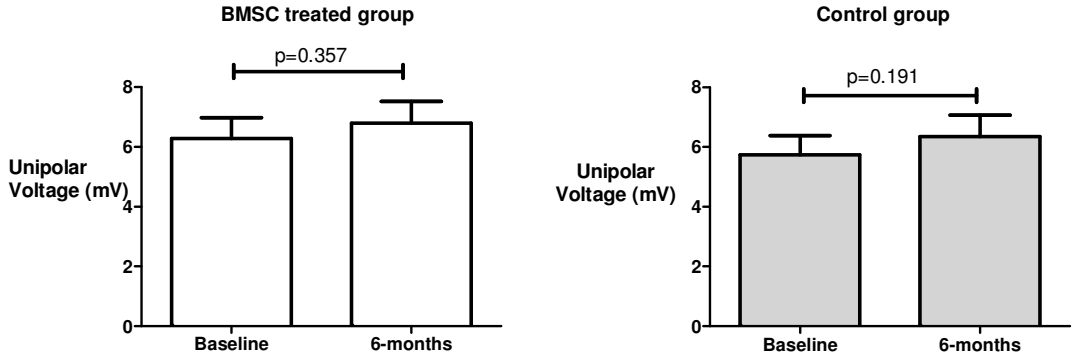


Figure 38. Bar graph representing change in unipolar voltage in the non-injected myocardial segments

4.4.9.3 Changes in local linear shortening

There was no significant change in local linear shortening in the BMSC treated group 6-months post treatment but in the control group there appeared to be a deterioration (statistically non-significant) in this parameter of mechanical function:

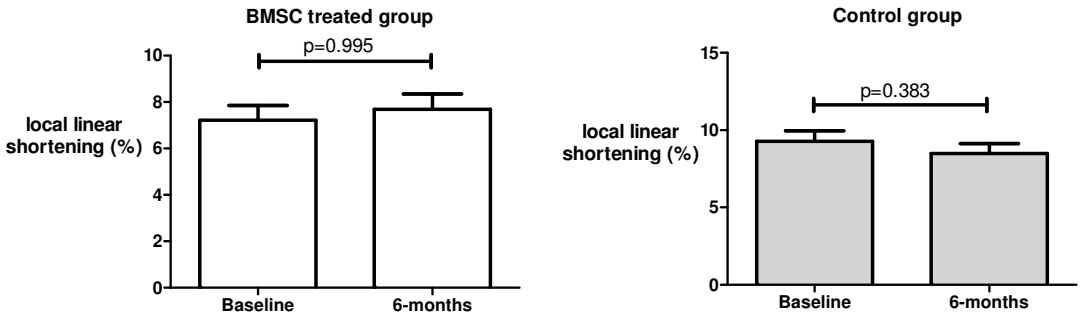


Figure 39. Bar graphs representing change in local linear shortening at 6-months follow-up.

4.4.9.4 Accuracy of NOGA® derived cardiac function

The data derived from the electromechanical mapping was used to calculate cardiac volumes and ejection fraction using an automated computer algorithm on the NOGA® workstation. The baseline NOGA® derived EDV, ESV and LVEF were compared with those obtained from the baseline MRI/CT scans. There appeared to be at least modest correlation between the two methods for assessment of cardiac volumes and function (figure 40):

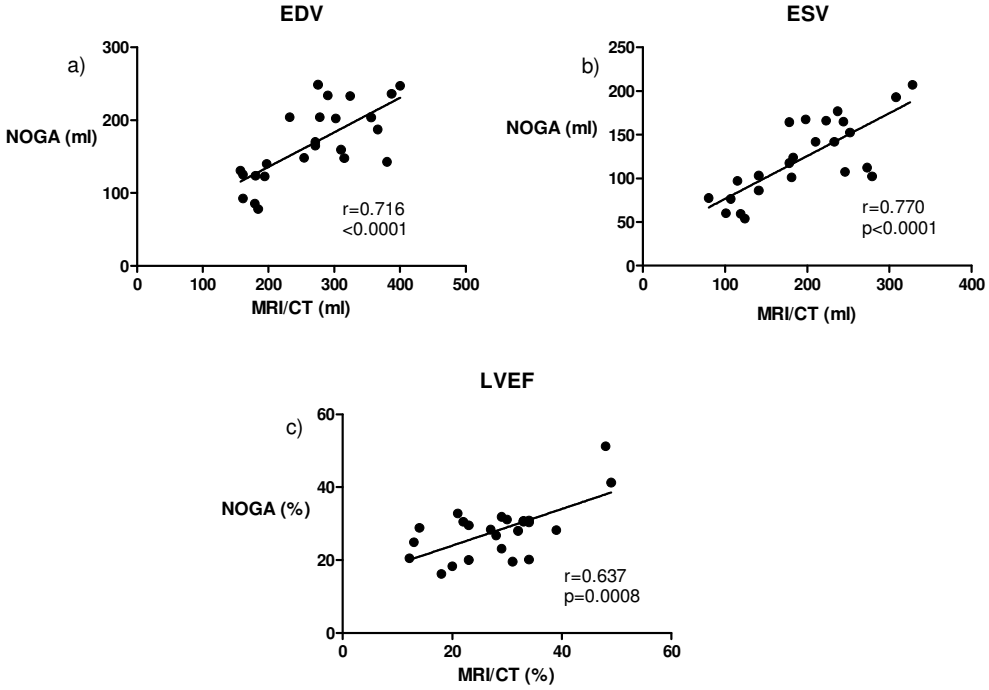


Figure 40. Scatter plots demonstrating correlation between NOGA® and MRI/CT derived cardiac volumes and function: a) end-diastolic volume (EDV); b) end-systolic volume (ESV); c) left ventricular ejection fraction (LVEF).

4.5 Discussion

In this double-blind randomised controlled study, combined therapy with G-CSF and intramyocardial injection of BMSCs led to a statistically significant improvement in LVEF at 1-year (the primary outcome measure). The degree of LVEF improvement appeared to be related to the in vitro functional potential of injected cells. A non-significant increase in LVEF was also seen in the patients treated with intramyocardial injection of serum (control group). There was also improvement in diastolic function seen only in the cell treated group. There was a significant reduction in NT-proBNP levels in both groups and NYHA functional class was improved at 6-months in both groups but this was only maintained at 1-year in the cell treated group. There were significant improvements in HRQL questionnaire scores only in the cell treated group. NOGA® mapping parameters of viability and contractile function suggested improvement in viability after injection of serum with no obvious change in the contractile parameter in either group.

4.5.1 Efficacy of intramyocardial injection of mobilised BMSCs

4.5.1.1 Cardiac function

Intramyocardial injection of mobilised BMSCs was associated with a significant increase in LVEF by 4.99%. The study therefore met its pre-determined primary end-point of an aim to detect a change in LEVF of 3.5%. This is one of the first RCTs of cell therapy in ischaemic heart failure to show this beneficial effect of BMSC therapy. Previous trials have had inconsistent results with earlier small non-randomised trials showing LVEF improvement[237, 253] but

more recent randomised controlled trials (RCTs) not confirming this although still showing symptomatic benefit[238]. The most recent RCT of intramyocardial injection of BMSCs, the FOCUS-CCTRN[257], was reported as negative for its primary end-points of reduction in end-systolic volume (ESV) and improvement in MVO2. One of the key differences of our trial protocol is the combination of G-CSF with direct delivery via intramyocardial injection and it is possible that this maybe the reason why we were able to show a positive result. G-CSF is known to have direct beneficial effects on cardiac tissue[199, 276] in addition to its ability to increase the number of circulating progenitor cells. G-CSF may also improve the functional ability of circulating progenitor cells- a finding we have recently published[17] and demonstrated in chapter 5 of this thesis. This may correct for impaired cell function that has been increasingly recognised in older patients with ischaemic heart disease[238, 249].

Another possible reason why our trial was able to demonstrate an improvement in LVEF is the use of more sensitive imaging techniques, in the form of cardiac MRI or CT, compared to previous trials which used either echocardiography or quantitative left ventriculography (QLV). MRI and CT have higher spatial and temporal resolution thus reducing the variability of function assessment[277]. As can be seen in the results section, echocardiography and QLV failed to demonstrate any significant improvement in LVEF in our study even though we found a positive result using MRI/CT. Cardiac MRI is widely regarded as the gold standard for measuring cardiac volumes and LVEF in patients with heart failure, with the highest level of reproducibility[278]. Unfortunately, cardiac MRI is generally contraindicated in

patients with implanted cardiac devices (unless they have 'MRI safe' devices) and most patients with severely impaired cardiac function will have some form of device therapy (ICD ± CRT). This was the case with our study in which only 8 out of 30 patients were able to have cardiac MRI. However, our second choice imaging modality, cardiac CT, performs similarly to MRI with regards to accuracy of LVEF measurement. For example, comparison studies, using MRI as the reference standard, have shown that cardiac CT is superior to both QLV and echocardiography for accurate and reproducible assessment of global LVEF[272, 279].

The size of our phase II trial required a surrogate marker to assess efficacy rather than a hard clinical end-point such as mortality. LVEF was chosen as the primary surrogate marker of efficacy as it has been widely used in other clinical trials of heart failure therapies and its value is easily appreciated by cardiologists and general physicians. LVEF has also consistently been shown to be a powerful predictor of mortality[280, 281]. Furthermore, a recent meta-analysis of drug/device interventions in heart failure found a significant correlation between longer therapeutic effects on mortality and short-term therapeutic effects of a drug or device on LVEF[258]. Although the magnitude in improvement in LVEF in our trial (4.99%) may seem small it is important to remember that most current medical therapy following myocardial infarction, which have been associated with significant long-term morbidity and mortality benefits, have also only been associated with a similar degree of LVEF improvement[282]. It will require an appropriately powered phase III study to

determine whether improvement in LVEF with BMSC therapy will also translate into significantly improved clinical outcomes such as mortality.

The improvement in LVEF seen in our trial appears to have been mostly driven by an improvement in end-systolic volume (ESV). This hints at a degree of beneficial reverse remodelling of the left ventricle following mobilised BMSC cell therapy. A recent clinical trial of intramyocardial injection of mesenchymal stem cells showed significant improvements in cardiac volumes also suggesting beneficial reverse remodelling with cell therapy[229]. As with LVEF, improvement in ESV with current heart failure therapies has been shown to be associated with significantly better clinical outcomes[283] and larger outcome trials will be required to determine whether this is also the case with cell therapy.

Interestingly, although no improvement in global LVEF was demonstrated on echocardiography, there did appear to be some improvement in diastolic function assessed using the E/E' parameter. The ratio of early trans-mitral flow velocity (E) to early diastolic septal mitral annulus velocity (E') has been shown to be the most accurate non-invasive predictor of elevated LV filling pressure[284, 285]. In our study, BMSC treated patients had a significant improvement in this parameter suggesting an improvement in LV end-diastolic pressure (EDP). This supports the finding of improved LVEF that was demonstrated on MRI/CT imaging, as improved contractile function would lead to an increase in stroke volume and thus reduction in EDV and EDP (via Frank-Starling mechanism, figure 1). Improvement in diastolic function with

BMSC therapy has been previously demonstrated in an animal model of chronic myocardial ischaemia[161]. A recent meta-analysis (including 365 patients from 6 trials) demonstrated a significant improvement in the E/E' parameter of diastolic function in patients treated with BMSC therapy following AMI[286]. Assessment of diastolic function as a marker of efficacy of cell therapy is likely to increase in future trials as it may provide a more subtle and earlier marker of improvement in cardiac function than LVEF alone[287].

In our study there did not appear to be a direct relationship between the dose of mononuclear/CD34+ cells and magnitude of improvement in LVEF in patients who were treated with intramyocardial injection of BMSCs. This supports other recent studies which suggest that dose of cells may not be a critical factor in treating heart failure patients with BMSCs. For example, in a recent study of CD34+ cells in myocardial ischaemia, both low ($5x10^6$) and high ($10x10^6$) dose cell injection led to comparable improvements in symptoms and perfusion[248].

However, the magnitude of improvement in LVEF in the BMSC treated group appeared to be related to the functional (colony forming unit (CFU)) potential of the bone marrow CD34+ cells. This is an interest finding that has not been demonstrated previously in clinical trials. It is being increasingly recognised that the functional capacity of BMSCs in patients with heart failure maybe impaired[288]. In a pre-clinical experiment, BMSCs from patients with ischaemic heart failure were shown to have reduced CFU potential and this was associated with impaired neovascularisation following injection in an animal

model of hind limb ischemia[289]. In the recent FOCUS-HF study, hematopoietic and mesenchymal CFU assays showed decreased progenitor cell activity particularly in patients aged above 60 years[238]. Furthermore, in the TOPCARE-CHD study, sub-study analysis demonstrated infusion of progenitor cells with a high CFU functional capacity was associated with a significantly lower mortality during further follow-up[290]. It appears therefore beneficial effect of cell therapy is related to the quality of the injected product and this may be a limiting factor for the use of autologous cells. There is therefore growing interest in the use of allogeneic cell products such as allogeneic mesenchymal stem cells (MSCs) which has the advantage of a more consistent quality of cell product and may circumvent the issue of patient related cellular dysfunction.

4.5.1.2 NT-proBNP levels

NT-proBNP levels decreased significantly in both the BMSC treated group and the control (serum) group. This does not appear to be solely related to G-CSF treatment received by both groups as NT-proBNP levels did not decrease in those patients who had G-CSF treatment only without intramyocardial injection (the peripheral arm of the REGENERATE-IHD study). Reduction in NT-proBNP provides further evidence to suggest a beneficial biological effect of a combined approach of G-CSF treatment and intramyocardial injection of BMSCs. Current available heart failure therapy that improve ejection fraction also lower NT-proBNP levels[291] so it is encouraging to see this combined therapeutic effect in the cell treated group in our trial. Furthermore, treatments that reduce NT-proBNP have been shown to improve clinical outcomes[39]. In a post hoc analysis of a randomised controlled trial assessing inotropic

intervention in acute heart failure (SURVIVE study), patients with >30% drop in NT-proBNP levels over five days had a 67% reduction in adjusted risk of death from any cause over the next month and a 47% decrease over the next six months [292].

There is also interest in using NT-proBNP levels to guide intensiveness of heart failure therapy and there have been several RCTs evaluating this approach. In the Pro-BNP Outpatient Tailored Chronic Heart Failure Therapy (PROTECT) trial[293], BNP guided adjustment of medications (compared to standard therapy) led to a significant reduction in cardiovascular events (worsening HF, hospitalization for HF, ACS, ventricular arrhythmias, cerebral ischemia, or CV death). This biomarker guided therapy approach has been validated in a meta-analysis of 6 RCTs comprising 1627 patients [294]. Pooled analysis showed a significant mortality advantage for biomarker-guided therapy (hazard ratio was 0.69, 95% CI 0.55-0.86) compared to control.

Hence, the reduction in NT-proBNP levels seen in our study may represent a clinically significant finding and warrants further investigation in future clinical trials, particularly in regards to correlation with clinical outcomes.

4.5.1.3 Symptoms and health related quality of life (HRQL)

It is encouraging to see that in addition to the improvement in cardiac function and NT-proBNP levels there were also significant improvements in symptoms and HRQL in patients treated with BMSC therapy. Although NYHA function class also improved at 6-months in the control group, this effect was only

maintained in the cell treated group at 1-year. Also improvements in the HRQL questionnaire scores were only seen in the cell treated group.

The use of the NYHA functional class provides a simple tool of assessing patients' symptomatic response to a therapy although has limitations of being subjective with only 4 possible outcomes (class I-IV). The use of standardised HRQL questionnaires improves the accuracy of assessing symptomatic response and the three questionnaires used in our study have been well-validated for assessing the response of interventions in patients with cardiac disease. Given the high morbidity and mortality associated with heart failure there is increasing use of HRQL questionnaires as primary and secondary end-points in clinical trials in this setting[295]. Improvements in HRQL have been demonstrated in recent clinical trials of cell therapy in patients with heart failure and in some cases this has been without evidence of significant improvement in cardiac function[238, 257]. The use of quality of life as an outcome measure is also important as it provides healthcare service providers with a tool to assess the cost/benefit of potentially approving this novel therapy for a broad group of heart failure patients.

4.5.2 Efficacy of intramyocardial injection of autologous serum

Interestingly, the trial results appear to suggest signs of efficacy in the control group i.e. patients who received G-CSF followed by intramyocardial injection of autologous serum alone. The serum was obtained by peripheral venesection after 5-days of G-CSF treatment, at the time of bone marrow

aspiration, and was used either as suspension medium for the BMSCs in the active treatment group or was injected alone in the control group. Patients in the control group showed significant improvement in NT-proBNP levels at 6-months and there was also a trend towards improvement in ejection fraction and symptoms (NYHA class and CCS angina class).

The potential reasons for improvement seen in the control group include:

a) chance alone

b) placebo effect

c) confounding effect of G-CSF

d) biological efficacy of autologous serum

e) biological effect of needle puncture of the myocardium

f) mechanical effect of 'material' injection into the myocardium

Placebo effect is unlikely given that there was an objective measure of improvement in the control group with statistically significant reduction in NT-proBNP levels. This is also unlikely to happen by chance in patients who have been clinically stable and on optimal medical therapy with no medication changes during follow-up. It is possible that the improvement maybe related to G-CSF therapy as all patients, including the control group received this. However, as shown in figure 28, there was no improvement in NT-proBNP levels in patients in the peripheral arm of the REGENERATE-IHD study who received G-CSF alone without intramyocardial injection. Therefore, this provides evidence that there maybe a positive biological effect of the

combined approach of G-CSF treatment and intramyocardial injection of serum (which may contain beneficial soluble paracrine factors).

BMSCs produce a wide range of cytokines and chemokines (including VEGF, FGF-2, IL-6, PIGF, and MCP-1) that have shown extensive therapeutic potential[296]. These paracrine mechanisms could be as diverse as stimulating receptor-mediated survival pathways, inducing stem cell homing and differentiation or regulating the anti-inflammatory effects in infarcted areas[177]. There have been preclinical experiments examining the potential benefit of these soluble factors secreted by BMSCs. In a rat model of AMI, intramyocardial injection of conditioned medium alone improved LVEF to a similar extent as bone marrow derived MSC injection although histological evaluation showed that the infarct wall thickness was significantly greater in the MSC treated group[296]. Another preclinical experiment in a rat AMI model also investigated if soluble factors secreted by MSCs could promote cardioprotection[297]. In rats injected with conditioned medium there was a significant reduction in left ventricular end-diastolic pressure and improvement in cardiac contractility. The group also assessed in vitro cardioprotection in neonatal ventricular cardiomyocytes by quantifying apoptosis after 24 hours of serum deprivation associated with hypoxia (1% O_2) in absence or presence of conditioned medium. The in vitro results showed that conditioned medium was able to decrease cardiomyocyte necrosis. These results suggest that soluble factors released in vitro by MSCs are able to promote cardioprotection in vitro and improve cardiac function in vivo[297]. Therefore it is possible that the potential efficacy of intramyocardial injection of autologous serum seen in our

study represents the action of soluble paracrine factors which are likely to be present in the serum. Ideally, we would have measured the concentration of some of these paracrine factors in the serum being injected and this is the subject of ongoing work within the department

An alternative hypothesis is the potential beneficial effect of needle puncture of the myocardium alone. Direct myocardial revascularisation (DMR) procedures using various mechanical devices and energy sources (e.g. laser) have been explored as possible options to produce therapeutic angiogenesis in patients with refractory angina with no targets for conventional percutaneous or surgical revascularisation[298, 299]. It has been suggested that the likely mechanism associated with the initiation of angiogenesis is the induction of local inflammatory processes, and that tissue changes trigger expression of variety of angiogenic cytokines which initiate and maintain microvessel collateral formation[300]. Growing clinical experiences derived from DMR procedures indicate symptomatic benefit despite inconsistent or lack of improvement in myocardial perfusion. In particular, needle insertion has been proposed by several investigators to induce angiogenic responses[301, 302].

Finally, the possibility of a mechanical beneficial effect of 'material' injection into the myocardium has been raised. In an elegant study, utilising a validated finite element model of an ovine left ventricle with an anteroapical infarct, researchers examined the short-term effect of injecting 'material' (volume ranging from 0.5-1.5ml) into the peri-infarct border zone. The results of simulated injections indicated that the addition of non-contractile material to a

damaged left ventricular wall has important effects on cardiac mechanics, with potentially beneficial reduction of elevated wall stresses, as well as changes to clinical parameters of left ventricular function such as LVEF[303]. This suggests both the significant potential for therapeutic application of material implantation to the myocardium as well as potential confounding mechanical effects. Further pre-clinical work is ongoing to validate this hypothesis.

4.5.3 Changes in NOGA® electromechanical parameters

The NOGA® endocardial electromechanical mapping system used in this trial enables measurement of endocardial electrical, unipolar voltage (UV), and mechanical, local linear shortening (LLS), function. Normal myocardial cells deliver a resting UV potential of 15 mV, whereas voltage values less than 6.9 mV reflect scar tissue; potentials between 7mV and 15 mV suggest viable myocardium[223, 304]. The cut-off threshold level for normal LLS ratios has varied between clinical studies, from 9%[304] to 12%[305]. Regions of akinetic/dyskinetic scar are easily delineated by their very low shortening ratio (LLS <2–3%)[223], while intermediate values (between 3-9%) likely represent hibernating myocardium with reversible ischemia[306]. Measurement of UV and LLS enables targeted delivery of BMSCs to hibernating myocardium around an area of scar tissue- this is important as BMSCs do not engraft well into scarred myocardium[224]. Furthermore, follow-up mapping procedures may allow assessment of changes in UV and LLS as surrogate markers of efficacy of cell therapy[235, 307].

The NOGA® mapping data in this thesis shows a statistically significant improvement in overall mean UV (suggesting improved viability) following intramyocardial injection of autologous serum (control group). The BMSC treated group also appeared to show possible improvement but this was not statistically significant. Exploring this further it appears that the UV improvement occurred in the non-injected segments in both groups. Previous studies that have performed follow-up NOGA® mapping after BMSC therapy have actually shown UV improvement only in the cell injected regions[308, 309]. The suggestion of improvement in non-injected segments seen in our study may support the hypothesis of a paracrine effect of the cells/serum on adjacent myocardial segments. As discussed previously, paracrine effects of BMSCs could include neoangiogenesis[151] or stimulation of resident cardiac stem cells[165]. The LLS data did not show any statistically significant changes 6-months post-treatment although there appeared to be a suggestion of deterioration in mechanical function in the control group.

It is difficult to explain why the improvement in UV was statistically significant only in the serum treated group and not the cell (suspended in serum) treated group. The most plausible reason is likely sample size and the small number of patients studied and it is possible that with more patients the improvement in the cell treated group may also have been significant. This is supported by the finding of a trend towards improvement in the non-injected myocardial segments in both groups. The data in this analysis is interesting although must be interpreted cautiously as the trial was not powered to detect any changes in NOGA® parameters. Also the number of patients in our study are fewer than

other studies that have specifically assessed NOGA® parameters. A limitation of the diagnostic performance of NOGA® is potential inaccuracy of the mapping procedure due to cardiac motion which can be further confounded by respiratory variation and subject movement. Also, as NOGA® does not provide visual verification of inter-ventricular septal position, variations of cardiac anatomy and orientation within the thorax may not be identified resulting in possible misrepresentation of LV segments. Furthermore, the accuracy and clinical value of comparing NOGA® derived mapping data between baseline and follow-up procedures as surrogate markers of treatment efficacy remains to be validated. Nevertheless, it is reassuring to see that there was reasonable correlation between NOGA® and MRI/CT for the assessment of cardiac volumes and function (figure 40) which suggests that the mapping procedure was relatively accurate and complete.

4.5.4 Feasibility of intramyocardial injection of mobilised BMSCs

All patients completed the G-CSF mobilisation phase. However, 4 patients who were initially allocated to receive intramyocardial injection of BMSCs had to be re-allocated to the intracoronary arm of the trial as they were found to have LV thrombus (3) or too high risk LV anatomy (1) on baseline imaging. This represents one of the limitations of NOGA® guided intramyocardial injection compared to intracoronary injection as there are certain anatomical considerations which make intramyocardial injection unsafe. A retrospective analysis (see appendix A) of our own REGENERATE-IHD study population revealed an 11.3% incidence of previously undetected LV thrombus. The adherence to randomisation could have been improved by baseline imaging

prior to randomisation to assess for presence of LV thrombus and to accurately delineate the endocardial anatomy. This will be particularly important as larger Phase III trials are designed and it maybe prudent to screen patients for LV thrombus prior to enrolment. The NOGA® mapping and intramyocardial injection was generally well tolerated with only one patient (out of 32 attempted) not being able to be complete treatment due to ventricular arrhythmia. The other patient who did not complete treatment after NOGA® mapping was actually found to have normal myocardial voltages with no area of scar tissue. This patient was withdrawn and this may be an advantage of the NOGA® procedure as it can confirm the presence of previous myocardial infarction and ensure the right patients are being treated in the setting of a trial.

4.5.5 Safety of intramyocardial injection of mobilised BMSCs

The bone marrow mobilisation with G-CSF phase of treatment (days 1-5) was well tolerated in all the patients with no major adverse events. Expected side effects such as bone pain and pyrexia were seen in a small proportion of patients. The NOGA® mapping and intramyocardial injection procedure was generally safe. There was only one incidence of pericardial effusion which resolved with conservative management. This is in keeping with the published incidence from other studies. There was no incidence of acute or short-term cerebrovascular events and it is possible that the exclusion of patients with LV thrombus detected on baseline imaging helped prevent ischaemic embolic events.

Overall mortality at 1 year was low with only 1 death, 3.3% of the whole cohort. This compares favourably with the 5.5% predicted risk of 1-year mortality for this particular group of patients which was calculated using the Seattle Heart Failure Model prediction tool [310]. This is a well validated model for predicting survival in patients with heart failure which was derived by retrospectively investigating predictors of survival among 1,125 heart failure patients. This multivariate risk model identified age, gender, ischemic etiology, NYHA, ejection fraction, systolic blood pressure, K-sparing diuretic use, statin use, allopurinol use, hemoglobin, % lymphocyte count, uric acid, sodium, cholesterol, and diuretic dose/kg as significant predictors of survival.

There is increasing evidence to suggest that BMSC therapy may lead to improved hard clinical outcomes, such as mortality, in addition to improvement in cardiac function and symptoms. For example in the STAR-Heart[241] study of intracoronary infusion of BMSCs in patients with heart failure, there was a 0.75% per year mortality rate in the cell treated group compared to 3.68% per year in the control group ($P<0.01$) during 5-year follow-up. Furthermore, a recent meta-analysis of trials of BMSCs in AMI and IHD, comprising 50 studies and 2,625 patients, has shown significantly lower all-cause and cardiac mortality in those patients treated with BMSCs compared to controls[311]. These signals from Phase II trials and meta-analysis need to be confirmed and a large multi-centre Phase III trial involving 3000 patients receiving BMSC therapy following AMI (BAMI trial) has been designed to answer this question (ClinicalTrial.gov Identifier NCT01569178).

4.6 Study limitations

The results of this study should be cautiously interpreted as it is a phase II study with a relatively small number of patients and uses LVEF as a surrogate marker of efficacy for stem cell therapy. The main strength of the REGENERATE-IHD trial is the double-blind randomised controlled study design. However, the control group in the intramyocardial arm also received G-CSF therapy and injection of autologous serum which may have led to a biological effect and may explain why there appears to be some signs of efficacy in this group. Ideally we would like to have had an additional 'true' placebo arm where patients received intramyocardial injection of saline so that we could explore whether the improvement in NT-proBNP and symptoms in the control arm were due to injection of serum or whether this was due to 'needle' effect. It would also have been interesting to measure the concentration of soluble paracrine factors such as VEGF in the autologous serum of patients undergoing treatment. Finally, although we have demonstrated subjective improvement in symptoms and quality of life we would ideally have assessed patients' functional improvement objectively with tests such as MVO2 exercise testing or the 6-minute walk test.

CHAPTER 5: A STUDY OF PROGENITOR CELL CHARACTERISTICS

5.1 Study Rationale

Although the relative safety of BMSC therapy in cardiac disease has been demonstrated, clinical effectiveness has been less consistently shown [236, 239, 312]. Potential reasons for this include heterogeneity in patient selection and clinical trial design. Studies have varied with regards to use of peripherally harvested progenitor cells (PBSCs) or BMSCs and have also differed as to whether cells have been mobilised with G-CSF or not. Furthermore, the number and functional ability of bone marrow progenitor cells has been shown to be reduced in patients with advanced heart failure and ischaemic heart disease[289]. In particular, aging has been recognised to adversely affect the functional capacity of progenitor cells[313] and the mobilising ability of G-CSF[314]. Hence, as the number of clinical trials using autologous cells continues to increase, a need has been identified for further mechanistic studies that include analysis of cellular composition and function of the BMSC infusate.

The previous chapter presented the results of the intramyocardial arm of the REGENERATE-IHD study. We are currently performing two other randomised, double-blind placebo-controlled trials in patients with dilated cardiomyopathy (REGENERATE-DCM[315], clinicaltrials.gov identifier NCT01302171) and acute myocardial infarction (REGENERATE-AMI, clinicaltrials.gov identifier NCT00765453). The IHD and DCM studies investigate the effects of G-CSF alone or in combination with intracoronary or intramyocardial delivery of

autologous BMSC therapy on cardiac function and quality of life in patients with symptomatic heart failure and no further treatment options. The AMI study assesses the safety and efficacy of early delivery of intracoronary BMSC in patients treated with primary stenting for acute myocardial infarction. Our studies therefore provide an ideal platform to assess the relationship between age and cardiac disease state on the concentration of peripheral and bone marrow progenitor cells as well as the response to G-CSF mediated mobilisation.

5.2 Methods

5.2.1 Objectives

This is an exploratory analysis of progenitor cell characteristics in patients recruited to three randomised controlled clinical trials of BMSC therapy in patients with ischaemic heart failure (IHD), dilated cardiomyopathy (DCM) and acute myocardial infarction (AMI). Specifically, we assess the impact of age, disease state and G-CSF on progenitor cell concentration and function.

5.2.2 Subjects

Peripheral blood and bone marrow samples were obtained from 201 patients recruited to the REGENERATE- IHD, DCM and AMI trials. All patients in the AMI analysis had a bone marrow sample for analysis. In the IHD/DCM trials a proportion of patients were randomised to receive only G-CSF/placebo (saline) so did not have a bone marrow sample for analysis. The Local Research Ethics Committee has approved the protocols of all three trials which are

conducted in accordance with the Declaration of Helsinki. Written informed consent has been obtained from each patient prior to inclusion in the trial, including consent for biochemical and cellular analysis of peripheral blood and bone marrow.

5.2.3 Progenitor cell mobilisation with G-CSF

Recombinant human G-CSF (Granocyte®, Chugai Pharma, UK) was administered subcutaneously at a dose of 10 µg/Kg/day for 5 consecutive days to patients enrolled in the DCM and IHD studies. Patients in the control group received saline injections. A peripheral blood sample was obtained on days 0, 1, 2, 3, 6 and 7 for estimation of peripheral progenitor cell counts. Patients in the AMI trial did not have G-CSF treatment and a single peripheral blood sample was taken for estimation of baseline progenitor cell count.

5.2.4 Bone marrow aspiration

Bone marrow aspiration was performed on day 6 in patients enrolled to the DCM and IHD studies who had been randomised to receive intracoronary injection of cells/placebo. Patients in the DCM/IHD studies who were randomised to receive only G-CSF or placebo did not have bone marrow aspiration performed. In the AMI study bone marrow aspiration was performed within 12 hours of successful primary stenting for acute myocardial infarction. Aspiration was performed from the posterior superior iliac spine. 100mls of bone marrow was collected in 20 10mls syringes- each syringe contained 1ml of heparin into which 5 ml of bone marrow was aspirated. The bone marrow

samples, together with peripheral blood, were then delivered immediately to the Barts Health NHS Trust Stem Cell Laboratory, which is an accredited facility for the production of cellular therapeutic material. Isolation and characterisation of the bone marrow mononuclear cell (BM-MNC) fraction was performed by a designated lab technician (Natalie Saunders).

5.2.5 Isolation of BM-MNCs

Bone marrow aspirates from the heparin-treated syringes were pooled into a single transfusion bag. The entire volume was then passed through a blood component transfusion set with a 200μm filter. Autologous BM-MNCs were isolated by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation and the heparin was washed off at this stage.

5.2.6 Flow cytometry analysis

BM-MNCs were characterised using flow cytometry. All flow cytometry analyses were performed using a BD FACSCanto Flow Cytometer with BD FACSDiva v 5.0.3 software (BD Biosciences). For the identification of HSC populations, cells were incubated with fluorescein isothiocyanate (FITC)-labeled antibody against human CD45 (BD Biosciences, Erembodegem-AALST, Belgium) and phycoerythrin (PE)-labeled antibody against human CD34 (BD Biosciences) for 15 min at room temperature.

EPCs were analysed by initially incubating samples with mouse serum IgG (Sigma, Dorset, UK) for 15 min at 4^{o}C with a cocktail of antibodies comprising

allophycocyanin (APC)-labelled antibody to CD133 (Miltenyi Biotec, Surrey, UK) and PE-labelled antibody to VEGFR-2 (R&D Systems, Abingdon, UK) to characterise EPCs and FITC-labelled monoclonal antibodies to CD2, CD13 and CD22 (Beckman Coulter, High Wycombe, UK) to identify and therefore eliminate inclusion of lineage-negative non-progenitor cells. To ensure exclusion of nonviable cells in the final EPC count, cells were also incubated with a PerCP-Cy5-labelled 7AAD stain (BD Biosciences). Cells were then incubated for 15 min at room temperature with 2ml of Pharm Lyse™ buffer (BD Biosciences) to lyse red blood cells. Samples were washed once in phosphate-buffered saline and 20µl of Accucount flow cytometry beads (Saxon Europe, Kelso, UK) were added before analysis.

5.2.7 Colony-forming unit (CFU-GM) analysis

BM-MNCs ($2x10^4$ per dish), Day 0 peripheral blood MNCs ($2x10^5$ per dish), and Day 6 peripheral blood MNCs ($2x10^4$ per dish) were seeded, in triplicate preparations, in methylcellulose plates (Methocult H4534, including stem cell factor, granulocyte-macrophage colony–stimulating, and interleukin-3, Stem cell Technologies). Plates were studied under phase-contrast microscopy, and granulocyte-macrophage colony forming units (CFU-GM; colonies >50 cells) were counted after 14 days of incubation. Results were taken from the mean of the triplicate results and presented as a ratio of CFU per CD34 cell plated. CFU assays were performed in 25 IHD, 10 DCM and 12 AMI patients due to availability of assay.

5.2.8 Statistical analysis

Data are expressed as mean ± SD unless otherwise stated. Comparison of the distributions of a continuous variable between two independent groups was performed using an unpaired two-sided t-test. Correlation was assessed using the Pearson r method and a p-value calculated using linear regression. Analysis of covariance (ANCOVA) was used to assess for confounders. A p-value of less than 0.05 was assumed to indicate statistical significance. $CD34^+$ stem cell counts are expressed as units per μL (U/μl) and EPC count is expressed as cells per μl (EPC/μl). CFU results have been presented as a ratio of CFUs per CD34 cell plated (CFU/CD34 cell). Statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) and SPSS 19 (IBM).

5.3 Results

5.3.1 Baseline characteristics

We assessed the progenitor cell counts in 201 patients: 110 IHD, 38 DCM and 53 AMI peripheral blood (PB) samples and 78 IHD, 17 DCM and 53 AMI matching bone marrow (BM) samples. Baseline characteristics of all patients are shown in Table 8. Patients in the REGENERATE-IHD study were significantly older, had a higher incidence of diabetes and were more likely to be taking a statin.

Table 8. Baseline characteristics of all patients

	REGENERATE-IHD (n= 110)	REGENERATE-DCM (n=38)	REGENERATE-AMI (n=53)	p-value
Age, years	64 ± 9.7	55 ± 9.5	56 ± 10.5	<0.0001
Male sex, No (%)	105 (95%)	28 (77%)	49 (92%)	0.0003
Medical history				
Diabetes (%)	30 (27%)	3 (8%)	5 (9%)	0.0038
Previous MI (%)	110 (100%)	0	3 (5.6%)	<0.0001
LVEF (%)	33±11	N/A	N/A	
Total cholesterol (mmol/l)	4.2 ± 1	5.2 ± 1.4	5.3 ± 1.2	<0.0001
NT-proBNP (pg/ml)	1286 ± 2151	1177 ± 1489	N/A	NS
Medications at recruitment				
Statins	97 (88%)	15 (40%)	7 (13%)	<0.0001
ACEI/ARB	99 (90%)	38 (100%)	6 (11%)	<0.0001
β-blocker	98 (89%)	30 (79%)	1 (2%)	<0.0001
Aldosterone antagonist	75 (68%)	25 (65%)	0	<0.0001

Data presented as number (% of patients) or mean ± SD. p-value represents ANOVA test of difference between the three groups. LVEF: left ventricular ejection fraction; N/A: not applicable; NS: not significant.

5.3.2 Effect of age on progenitor cell number and function

We found an inverse relationship between age and PB CD34+ cell concentration (r=0.2, p=0.02) (Figure 41a) in the whole study group (n=201). There was a trend towards an inverse relationship between age and EPC cell concentration but this was not statistically significant (Figure 41b).

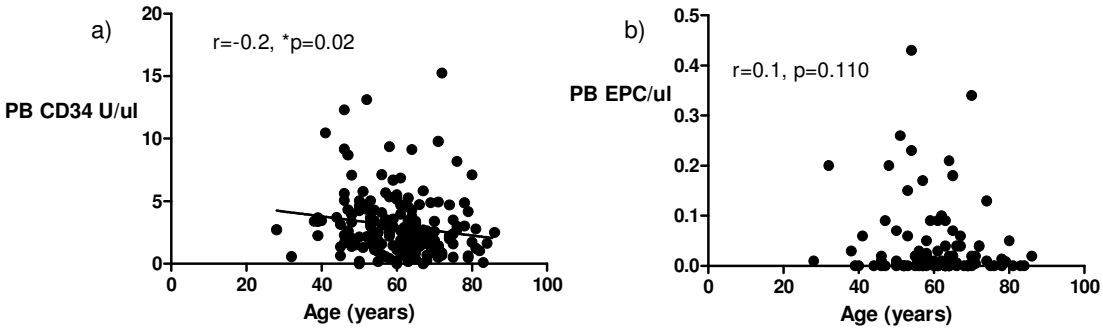


Figure 41. Correlation between age and peripheral blood progenitor cell concentration in the whole study group. a) There was a significant inverse relationship between age and CD34+ cell concentration. b) There was a trend towards an inverse relationship between age and EPC cell concentration but this was not statistically significant.

We also explored the effect of age on the mobilising ability of G-CSF in the chronic ischaemic heart failure (IHD) patient group. The median age of 64 years was used to divide the patients into two groups. We found that the older age group had a lower concentration of CD34+ cells in the bone marrow following mobilisation with G-CSF (75.49±64.49 CD34 U/µl versus 113.5±61.03, p=0.0023) (Figure 42a). There was also a trend (p=0.09) towards lower percentage increase in circulating CD34+ cells (day 6 compared to day 0) with G-CSF in those aged above 64 years (Figure 42b).

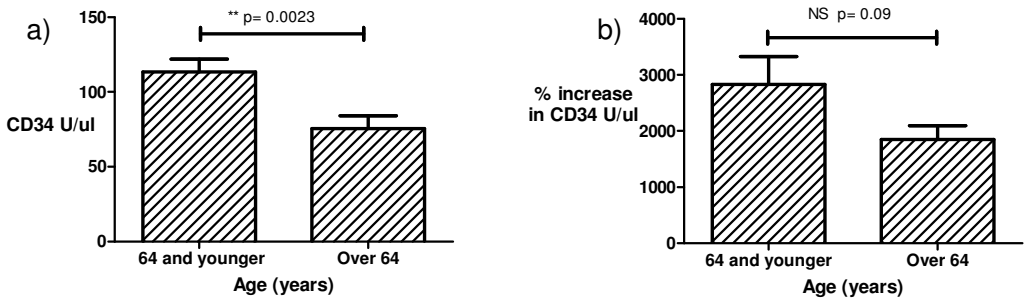


Figure 42. Effect of age on progenitor cell counts following G-CSF. a) There was significantly lower CD34+ cell concentration in the bone marrow of ischaemic heart failure patients aged over 64 years. b) There was a non-significant trend towards impaired peripheral mobilisation of CD34+ cells in patients aged above 64 years as measured by %increase in cells from baseline to day 6.

5.3.3 Correlation between other clinical parameters and progenitor cell concentration

We did not find any statistically significant independent correlations between other clinical parameters and peripheral or bone marrow progenitor cell concentrations in the three cohorts of patients investigated (Figure 43). Renal impairment appeared to have an inverse correlation with bone marrow CD34+ progenitor cell concentration in patients with chronic ischaemic heart failure although this was not independently significant following multiple regression analysis, possible due to confounding between age and renal impairment.

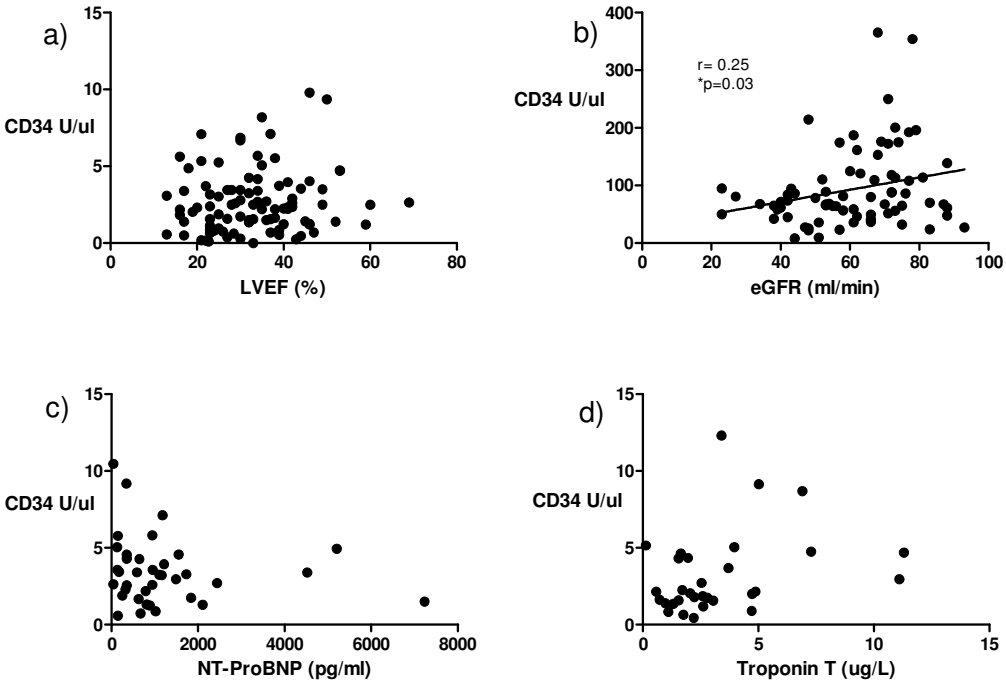


Figure 43. Selected scatter plots demonstrating bivariate relationship between clinical parameters and progenitor cell counts. a) left ventricular ejection fraction (LVEF) and peripheral blood CD34+ cell concentration in ischaemic heart failure; b) eGFR and bone marrow CD34+ cell concentration in ischaemic heart failure; c) NT-ProBNP and peripheral blood CD34+ concentration in dilated cardiomyopathy; d) peak troponin T level and peripheral blood CD34+ concentration in acute myocardial infarction.

5.3.4 Effect of disease state on progenitor cell number and function

The concentration of circulating CD34+ and EPCs was found to be significantly higher in the baseline peripheral blood of DCM patients as compared to IHD patients (3.4±2.1 CD34+ U/µl versus 2.6±1.9, p=0.033, and 0.06±0.1 EPCs/µl versus 0.01±0.03, p<0.0001) (Figure 44a+b). This remained significantly different after adjusting for age, diabetes and total cholesterol. In AMI patients the number of circulating EPCs was also significantly lower compared to DCM patients but similar to IHD patients. Despite there being a higher circulating CD34+ cell count in the DCM patients the CFU potential was not significantly different (Figure 44c).

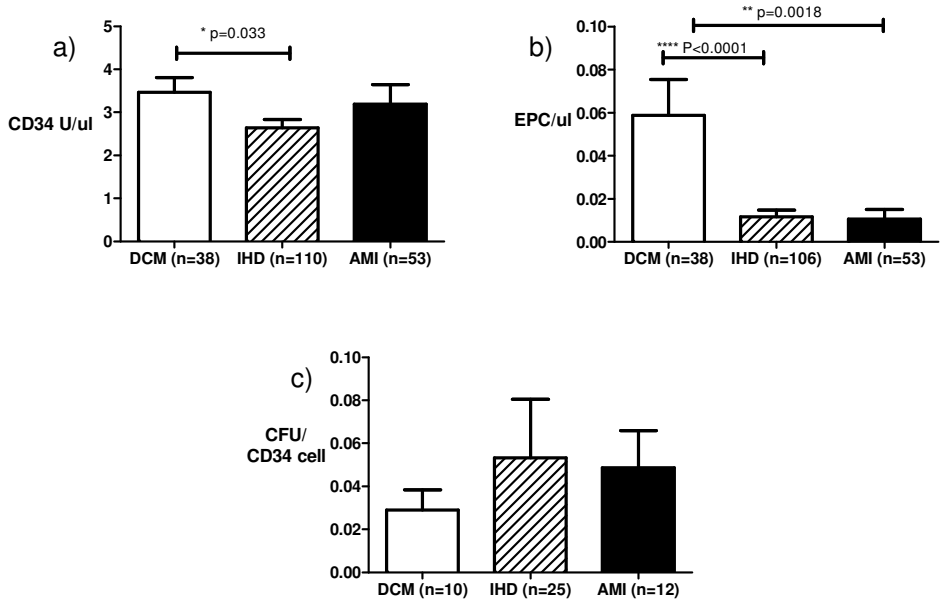


Figure 44. Comparison of baseline concentration of a) circulating CD34+ and b) EPCs in the peripheral blood of dilated cardiomyopathy(DCM), ischaemic heart failure (IHD) and acute myocardial infarction (AMI). c) colony forming units (CFU) in the peripheral blood of the three different patient cohorts.

5.3.5 Effects of G-CSF mobilisation

The G-CSF treatment (10μg/kg/day for 5 days) led to a substantial and comparable increase in the peripheral concentration of CD34+ cells in both IHD and DCM patients, day 6 mean concentration of 52.3 CD34 U/μl compared to 2.7 CD34 U/μl on day 0 (Figure 45). There was no significant increase in cell concentration in patients who received saline injection only (day 6 concentration of 2.7 CD34 U/μl from baseline of 2.2 CD34 U/μl).

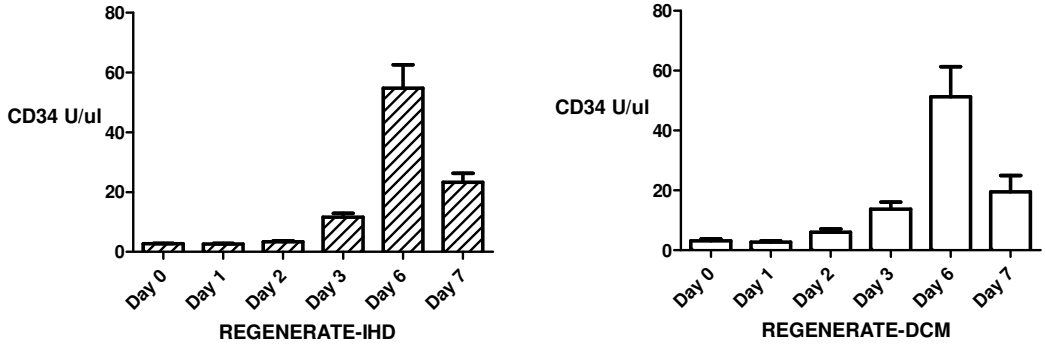


Figure 45. Effects of G-CSF on peripheral blood CD34+ cell counts in ischaemic heart failure (left) and dilated cardiomyopathy (right) showing comparable increase in circulating progenitor cells with a similar peak on day 6.

The progenitor cell count in the bone marrow of IHD and DCM patients was similar following treatment with G-CSF. The increase in bone marrow progenitor cell counts following G-CSF mobilisation can be appreciated by comparing the IHD and DCM bone marrows with that of AMI patients who did not receive G-CSF (Figure 46).

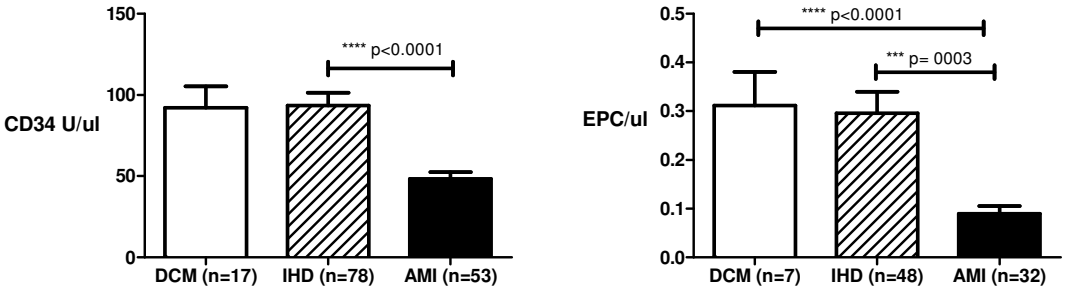


Figure 46. Comparison of bone marrow progenitor cell counts in the three different cohorts showing significantly higher bone marrow progenitor cell concentration with 5-days G-CSF treatment (DCM and IHD patients) compared to no G-CSF (AMI patients).

The concentration of CD34+ cells in the bone marrow aspirated on day 6 was significantly higher than in the peripheral blood on the same day (Figure 7 a+b). However, CFU functional assessment (Figure 47c) suggests that peripheral blood cells appear to be more functionally active than bone marrow cells (0.15±0.14 CFU/CD34 cell versus 0.08±0.05, p=0.0081). Furthermore the CFU/CD34 cell ratio in the peripheral blood increases significantly with G-CSF (Figure 47d) suggesting mobilisation of functionally active progenitor cells.

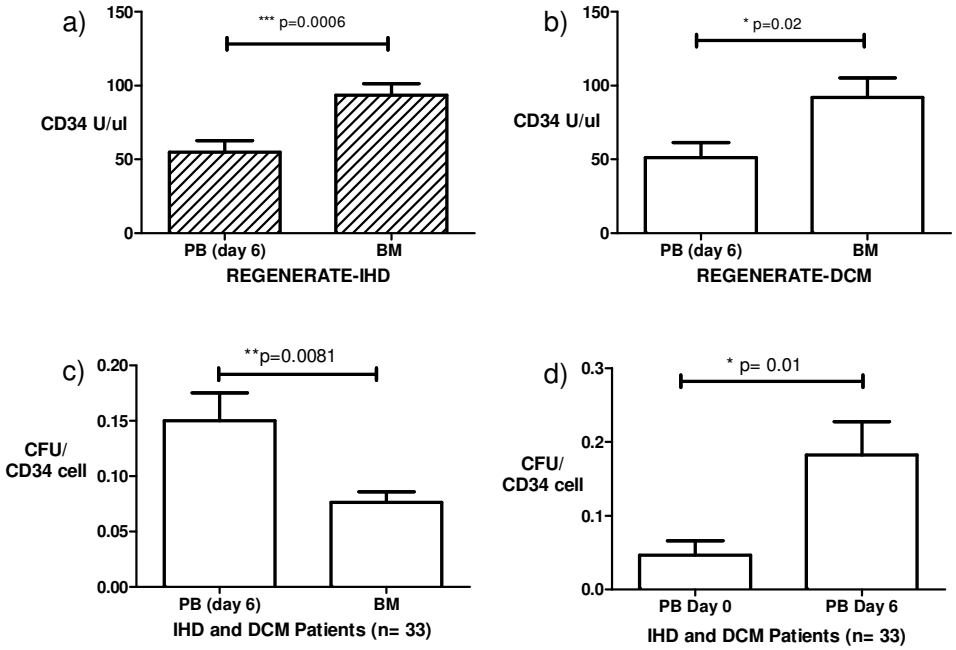


Figure 47. Comparison of peripheral blood and bone marrow CD34+ concentration following G-CSF mobilisation in a) IHD patients and b) DCM patients. The concentration of cells is higher in bone marrow in both ischaemic heart failure and DCM. c) Comparison of functional activity of progenitor cells following G-CSF mobilisation between peripheral blood and bone marrow showing significantly higher CFU/CD34 cell in peripheral blood. d) Comparison of CFU/CD34 cell in peripheral blood at baseline and day 6 showing a significant increase in functional ability of circulating progenitor cells following G-CSF mobilisation.

5.4 Discussion

The past decade has seen a large increase in the number of clinical trials being performed to assess the safety and efficacy of BMSC therapy for cardiac disease. Only a few of these studies have examined progenitor cell

concentration and function in recruited patients [238, 290, 316], an important consideration given the varying degrees of efficacy demonstrated. The data from this present study confirm previous reports of an inverse relationship between age and PB CD34+ cell concentration in patients with cardiac disease (Figure 41). We have also shown that, in ischaemic heart failure patients, the concentration of BM CD34+ progenitor cells, following G-CSF mobilisation, is lower in patients aged above the median age of 64 years (Figure 42a). There was also a trend towards reduced peripheral mobilisation by G-CSF in the older age group (Figure 42b). The adverse effects of age on progenitor cell number and function has been increasingly recognised [313]. In healthy individuals and patients with coronary artery disease, increasing age is associated with reduced number and function of cultured EPCs and CD34+ cells in the bone marrow [313, 317]. Older age is also associated with significantly reduced BM derived progenitor cell mobilisation in patients after AMI [318]. In the recent FOCUS-HF study of intramyocardial injection of BM-MNCs in ischaemic heart failure [238], the number and function of progenitor cells was significantly higher in patients ≤60 years than in patients >60 years. Interestingly, following treatment objective evidence of improvement (by MVO2) was only seen in the patients <60 years. Hence, the impact of age on bone marrow progenitor cell number and function may limit the efficacy of cell therapy in some patients. This may in part explain some of the discrepant clinical trial results and further work is required to identify the age group of patients that will derive most benefit of cell therapy.

We have also shown that concentration of circulating CD34+ cells and EPCs are higher in patients with DCM than in those with ischaemic heart failure (Figure 44a). The number of circulating EPCs is also lower in AMI patients compared to DCM (Figure 44b). This is consistent with previous reports showing circulating EPCs inversely correlate with number of risk factors for coronary artery disease [249]. Although there was a higher concentration of circulating CD34+ cells in DCM patients, the CFU analysis suggested that there was no difference in functional capability. It is possible that this finding maybe due to an adverse effect of heparin on progenitor cell function as has been recently reported by Professor Zeiher's group (unpublished data), although our data is based on a small number of CFU assays and heparin exposure was similar across all samples. The difference in circulating progenitor cells between ischaemic heart failure and DCM has not previously been demonstrated suggesting that the atherosclerotic process rather than chronicity or severity of disease is the important determinant of the progenitor cell concentration.

G-CSF has been used to mobilise bone marrow progenitor cells in patients with cardiac disease either on its own, as adjuvant therapy with peripheral leukapheresis (peripheral blood stem cells, PBSCs) or bone marrow harvest and subsequent direct delivery of progenitor cells to the heart [319-321]. It is still debated whether G-CSF treatment leads to an increase in progenitor cells in the bone marrow in addition to the mobilisation of these cells into the peripheral blood. Studies in patients undergoing myeloablative therapy suggest that transplantation of mobilised allogeneic PBSCs may lead to an

earlier recovery in bone marrow function compared to cells obtained from a marrow harvest[322, 323]. Our data shows that the concentration of CD34+ cells in the bone marrow following 5-days of G-CSF treatment is higher than in the peripheral blood on that day (Figure 47a+b). Conversely, CFU analysis suggests significantly higher functional potential of the CD34+ cells in the peripheral blood compared to bone marrow (Figure 47c). Several studies have suggested that cytokines can shift early repopulating stem cells out of the marrow into the peripheral blood. A pre-clinical study found that after 5 days of G-CSF, mice had only 25% of the baseline long-term repopulating stem cells in the marrow [324] and in another experiment prolonged G-CSF administration to mice led to a depletion of primitive BM stem cells[325].

In contrast to bone marrow transplantation following myeloablative therapy, there is yet to be a published clinical trial comparing the safety and efficacy of mobilised PBSC versus mobilised BMSC for the purposes of cardiac repair. There have however been clinical studies comparing the relative efficacy of ex vivo expanded PBSCs and non-mobilised BMSCs. The recent HEBE trial [312] found no improvement in global or regional LV function with either intracoronary PBSC or BMSC compared to standard therapy. In contrast to this, the earlier TOPCARE-AMI study [154] found significant beneficial effects of both PBSCs and BMSCs on LV function. The same group also compared the efficacy of intracoronary BMSCs or PBSCs in patients with chronic ischaemic heart failure in the TOPCARE-CHD study [10]. In this study, only BMSCs appeared to have a beneficial effect on improving LV function with no significant effect of PBSCs. The potentials reasons given for this include the

smaller number of progenitor cells derived from peripheral blood as well as the fact that circulating progenitor cells are known to be reduced in number and function in patients with heart failure [326]. The PBSCs were not mobilised with G-CSF prior to harvest which could have led to a positive effect, particularly as our study shows that G-CSF can lead to mobilisation of functionally active cells (Figure 47d).

Our findings are clinically important and indicate that further work is required to define whether peripheral or bone marrow harvest of progenitor cells is best for treating patients with heart failure. The possibility that pre-treatment with G-CSF may correct for deceased CFU potential and hence regenerative capacity of cells from patients with ischaemic heart disease is an important observation that needs further investigation.

5.5 Study Limitations

This is an exploratory analysis which limits the conclusions that can be drawn but at the same time generates interesting hypotheses to be examined in future studies. Our study is limited by the lack of a true normal patient control group for comparison although the comparison between disease states provides valuable information. Our analysis is also limited by the lack of CFU-GM assays being performed in all the patients analysed; this was due to an issue with availability.

CHAPTER 6: ANGIOGENESIS IMAGING STUDY

6.1 Study Rationale

6.1.1 Angiogenesis as a mechanism of cell therapy

The mechanism(s) of action of cell therapy are yet to be fully elucidated. There are likely to be several other beneficial effects in addition to potential transdifferentiation of delivered cells into cardiomyocytes. One proposed mechanism is neoangiogenesis either through transdifferentiation to vascular endothelial cells or via expression of several signalling factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and transforming growth factor (TGF), all key signalling factors involved in angiogenesis[18]. Preclinical experiments have demonstrated the ability of BMSCs to induce angiogenesis in infarcted myocardium and have implicated this as the mechanism of cardiac repair[327]. Although there continues to be growth in the number of clinical trials evaluating the potential of cell therapy, there have so far been few mechanistic studies performed in humans. Hence, the recent development of radiolabelled imaging probes, utilising single-photon emission computed tomography (SPECT) imaging, that specifically bind to receptors involved in angiogenesis provides an exciting opportunity to gain insight into the biological effects of cell therapy on human cardiac tissue.

6.1.2 The αvβ3 integrin receptor

Integrins are receptors that mediate the attachment between a cell and the tissues that surround it, such as other cells or the extracellular matrix (ECM). Structurally, integrins are heterodimers containing two distinct chains, called

the α (alpha) and β (beta) subunits and in mammals eighteen α and eight β subunits have been characterized[328]. The αvβ3 integrin receptor (Figure 48a) plays a key role in angiogenesis[329]. It is up-regulated and expressed preferentially on proliferating endothelial cells and limited distribution elsewhere within tissue[188, 330]. By serving as a receptor for a variety of extracellular matrix proteins containing an arginine-glycine-aspartic acid (RGD) sequence, these integrins mediate migration of endothelial cells into the basement membrane and regulate their growth, survival, and differentiation[331].

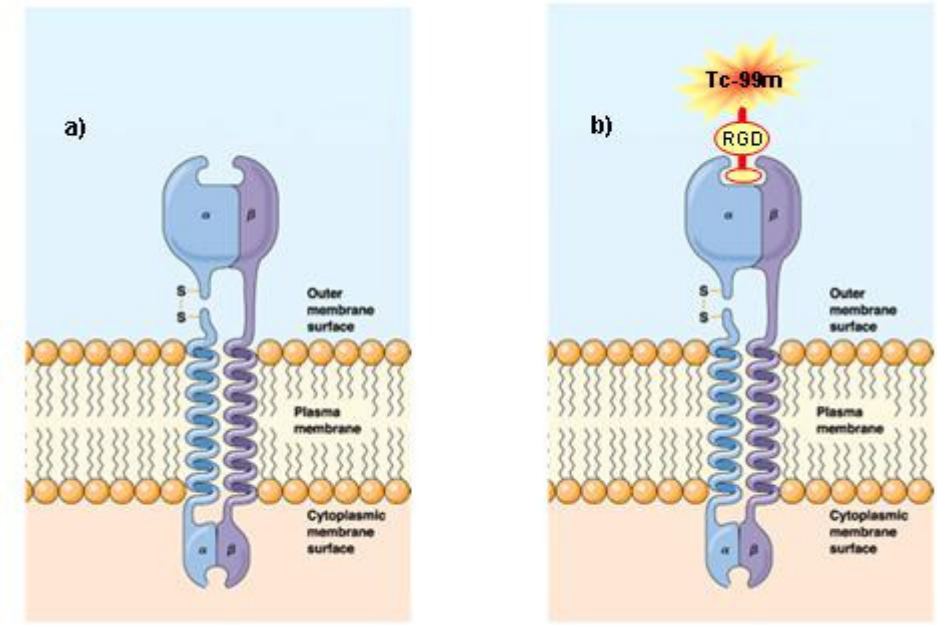


Figure 48. a) The αvβ3 integrin receptor; b) ^{99m}Tc-NC100692 binding site to the αvβ3 integrin receptor

6.1.3 ^{99m}Tc-NC100692 as an imaging agent

^{99m}Tc-NC100692 is a radiopharmaceutical agent manufactured by GE Healthcare Ltd (Amersham, UK) currently being investigated as a diagnostic agent for the detection of angiogenesis. ^{99m}Tc-NC100692 is a technetium labelled small, cyclical peptide that contains the RGD motif in a configuration

that gives the ligand a high affinity for the αvβ3 integrin (Figure 48b). Preclinical experiments in murine models of limb ischaemia have demonstrated increased focal activity of ^{99m}Tc-NC100692 on SPECT imaging 3-7 days following femoral artery ligation which correlated with ex vivo tissue analysis (gamma counting) and immunofluorescence staining[190, 332]. Clinical studies have demonstrated a relationship between tracer uptake, angiogenic status (microvessel density) and integrin expression in malignant tumours and tissue recovering from ischaemia[333].

The high affinity of this agent for the αvβ3 integrin up-regulated in angiogenesis makes it an ideal tool for evaluating angiogenesis in clinical studies. It can be administered as a single bolus injection and uptake detected with SPECT imaging. Excretion is predominantly through the urinary pathway, with about 56% of the injected activity excreted in the urine. About 16% of the injected activity is excreted in the faeces [334]. A phase 1 clinical study in which healthy volunteers were administered ^{99m}Tc-NC100692 intravenously demonstrated only minimal amount of background activity in the thorax but increased uptake of the agent in the liver and intestines[334]. Phase 2a studies have also been conducted in breast and lung cancer patients to detect angiogenesis and ^{99m}Tc-NC100692 was safe and well tolerated[333].

We therefore designed a sub-study to assess the feasibility of using ^{99m}Tc-NC100692 to detect angiogenesis in patients undergoing BMSC therapy in our trial. This sub-study has been carried out in collaboration with the Nuclear Medicine department at University College London Hospital and Department

of Advanced Cardiovascular Imaging at Yale (Professor Sinusas). We had initially planned to perform these imaging studies in patients recruited to the intramyocardial arm but owing to delay in obtaining ethics and MHRA approval these scans have been performed in 10 patients undergoing intracoronary infusion of BMSCs or serum. Patients have undergone baseline angiogenesis imaging with a follow-up scan 4 days post intracoronary infusion to assess for de novo angiogenesis. This time-point is based on the experience of Professor Sinusas in his pre-clinical work that demonstrated a significant angiogenic signal at this time point following a hypoxic stimulus[335]. Patients have also undergone baseline and follow-up perfusion imaging to assess for any change in perfusion related to angiogenesis. Patients receiving intracoronary infusion of serum will provide a 'control' group for the study. The preliminary results from the first three patients undergoing this imaging sub-study are presented in this chapter. Imaging scans from normal patients will be shown for comparison and have been provided courtesy of GE Healthcare.

6.2 Methods

6.2.1 Angiogenesis sub-study protocol

Patients allocated to this sub-study underwent the standard procedures of the REGENERATE-IHD trial protocol as described in chapter 4, except that they had intracoronary infusion of BMSCs/serum instead of intramyocardial injection. They underwent 4 additional scans at The Nuclear Medicine department at University College London Hospital (UCLH)- the timeline of the study protocol is demonstrated in figure 49.

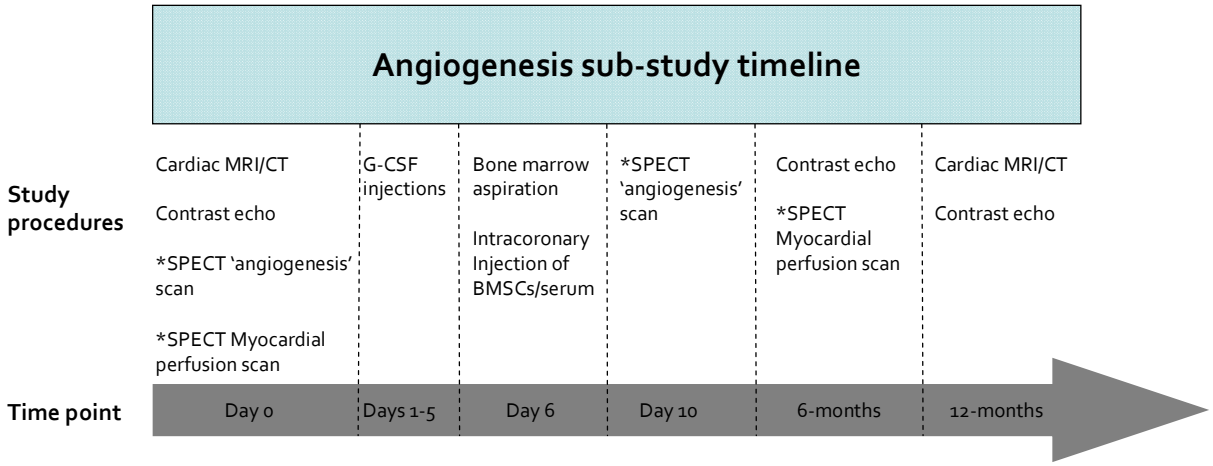


Figure 49. Timeline of angiogenesis sub-study

Details of additional imaging studies:

1. Baseline angiogenesis scan (day 0)- patients received administration of ^{99m}Tc-NC100692 injection 20 minutes before SPECT/CT scan using hospital protocol at baseline. 5.5ml of ^{99m}Tc-NC100692 was injected intravenously at a rate of 2-4mls per second. Each subject received 75µg of NC100692.
2. Baseline perfusion scan- patients also underwent a baseline assessment of myocardial perfusion using a standard thallium-201 protocol.
3. Follow-up angiogenesis scan- a repeat SPECT/CT following administration of ^{99m}Tc-NC100692 was performed 4 days post intracoronary infusion of BMSCs/placebo to assess for therapy induced angiogenesis.
4. Follow-up perfusion scan- a repeat thallium-201 perfusion scan was performed 6-months post therapy to assess for changes in perfusion.

This coincided with the patients' normal 6-months follow-up appointment for the trial.

6.2.1 Acquisition and analysis of data

Image analysis has been performed by the nuclear medicine department at UCLH (Dr Maria Holstensson) and includes ongoing work regarding quantification of uptake of ^{99m}Tc-NC100692 and assessment of relationship to measured changes in functional indices and perfusion.

6.3 Results

6.3.1 Patient Characteristics

Nine patients from the intracoronary arm of the REGENERATE-IHD Study were recruited for this sub-study, the final patient declined consent. The baseline characteristics of these patients are shown in table 9.

Table 9 Baseline characteristics of patients recruited to imaging study

	Patients recruited to 'angiogenesis' imaging sub-study (n=9)
Age (years)	59 ± 8
Male sex, n (%)	8 (88)
Previous MI, n (%)	7 (77)
CABG, n (%)	3 (33)
Time from last MI (years)	7.7 ± 7.1
Diabetes, n (%)	2 (22)
NT-proBNP (pg/ml)	1236 ± 1227
LVEF (%)	30 ± 7.5
ICD/CRT, n (%)	6 (66)

Data presented as number and (% of patients) or mean ± SD

6.3.2 ^{99m}Tc-NC100692 imaging in normal patients

The following figure shows the distribution of uptake of ^{99m}Tc-NC100692 in two 'normal' patients with no known prior history of cardiac disease. There is no appreciable cardiac uptake of the radio-tracer although there is uptake in the liver and intestines.

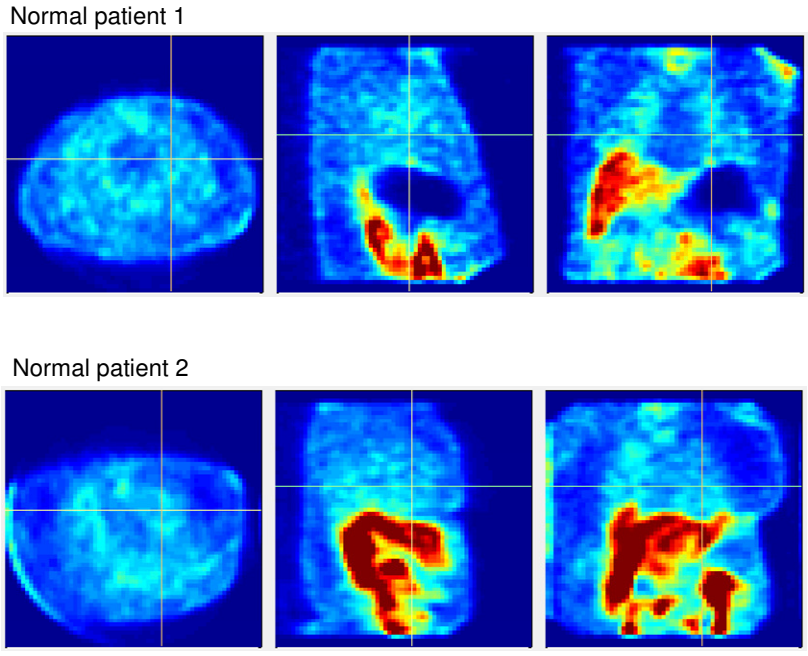


Figure 50. ^{99m}Tc-NC100692 imaging in two normal patients showing no appreciable cardiac uptake. There is however significant uptake within the liver and intestines. Images courtesy of GE Healthcare.

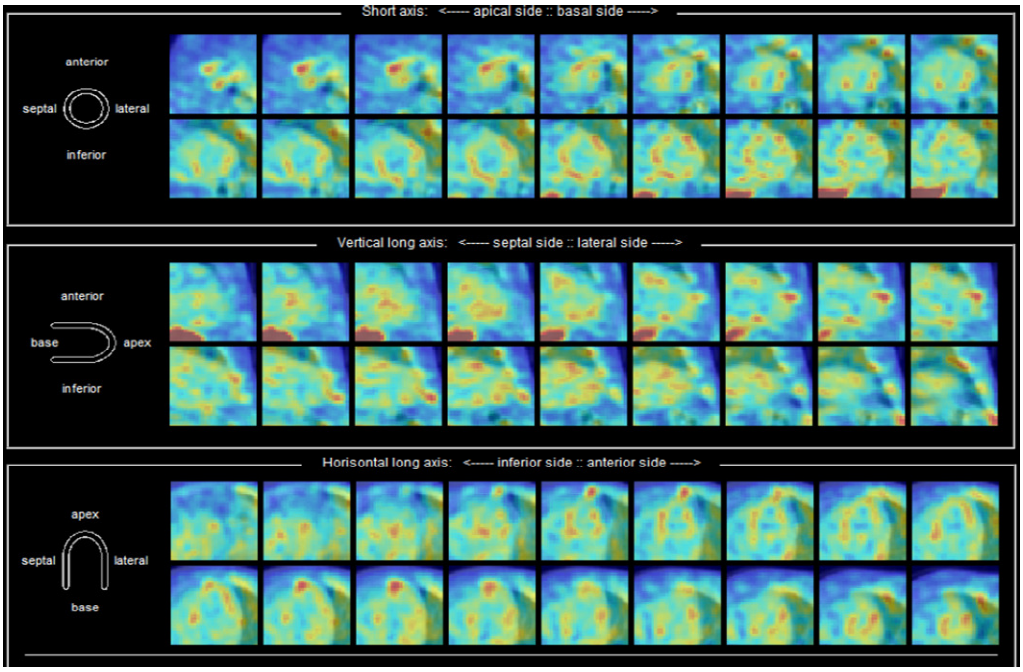
6.3.3 ^{99m}Tc-NC100692 in study patients

The baseline and follow-up ^{99m}Tc-NC100692 imaging in the first 3 patients who underwent this sub-study are shown here. Only the qualitative SPECT imaging data is currently available at the time of writing this thesis and refinement of quantitative analysis is ongoing. We remain blinded to whether these patients received BMSCs or serum alone.

Patient 1

In patient 1 there is detectable myocardial uptake (red/yellow areas) of ^{99m}Tc-NC100692 at baseline and uptake appears to be increased 4 days post intracoronary infusion of BMSCs/serum:

Patient 1. Baseline scan



Patient 1. Follow-up scan, 4 days post intracoronary injection of cells/serum

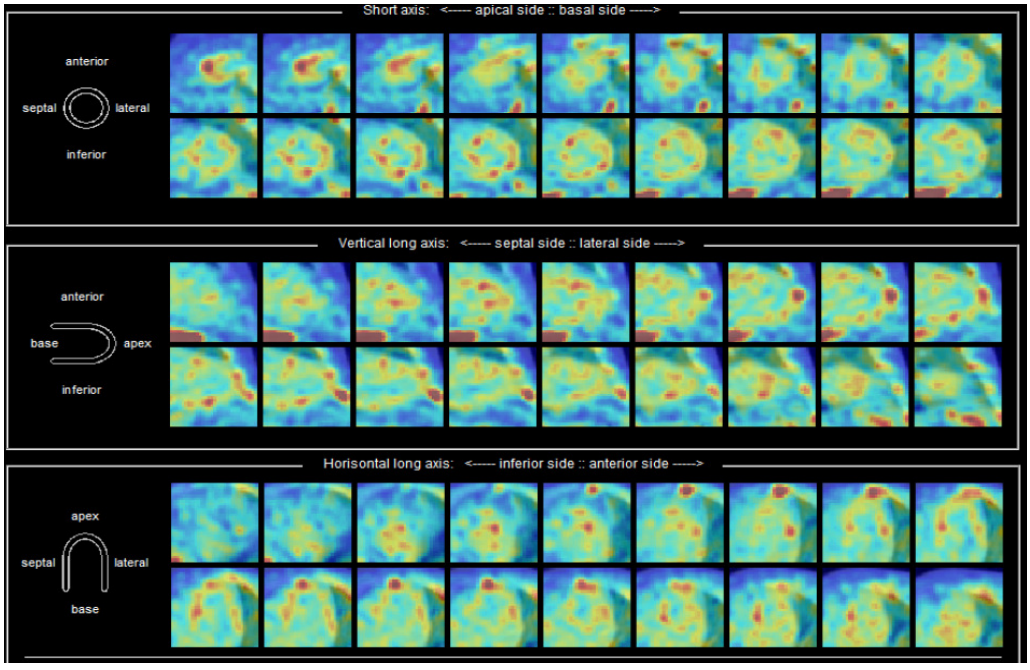
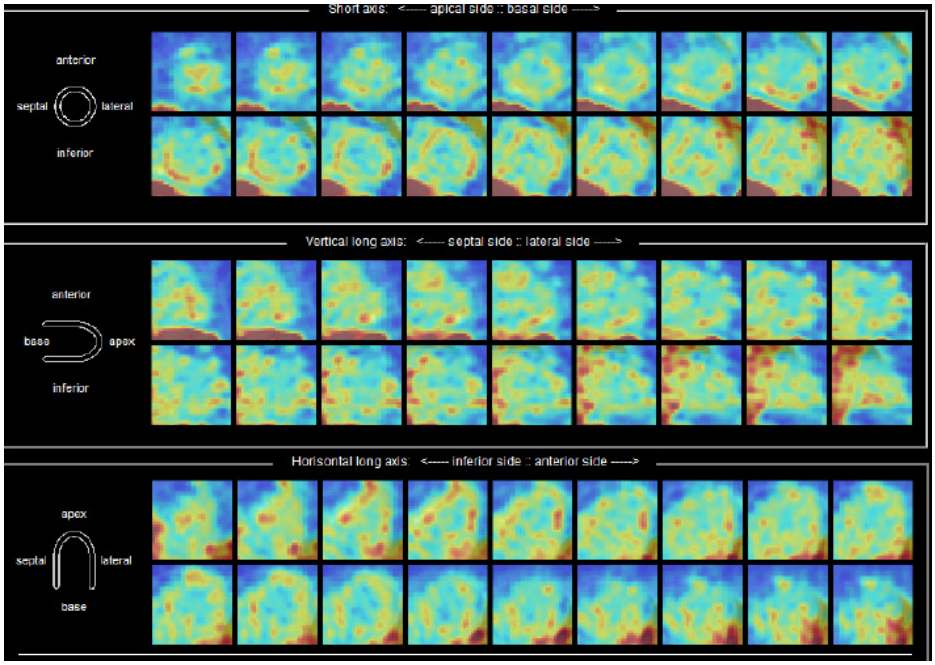


Figure 51. Baseline and follow-up ^{99m}Tc-NC100692 SPECT imaging in patient 1.

Patient 2

In patient 2 there is again detectable uptake of ^{99m}Tc-NC100692 at baseline, however there appears to be relatively similar uptake at the follow-up scan:

Patient 2. Baseline scan



Patient 2. Follow-up scan, 4 days post intracoronary injection of cells/serum

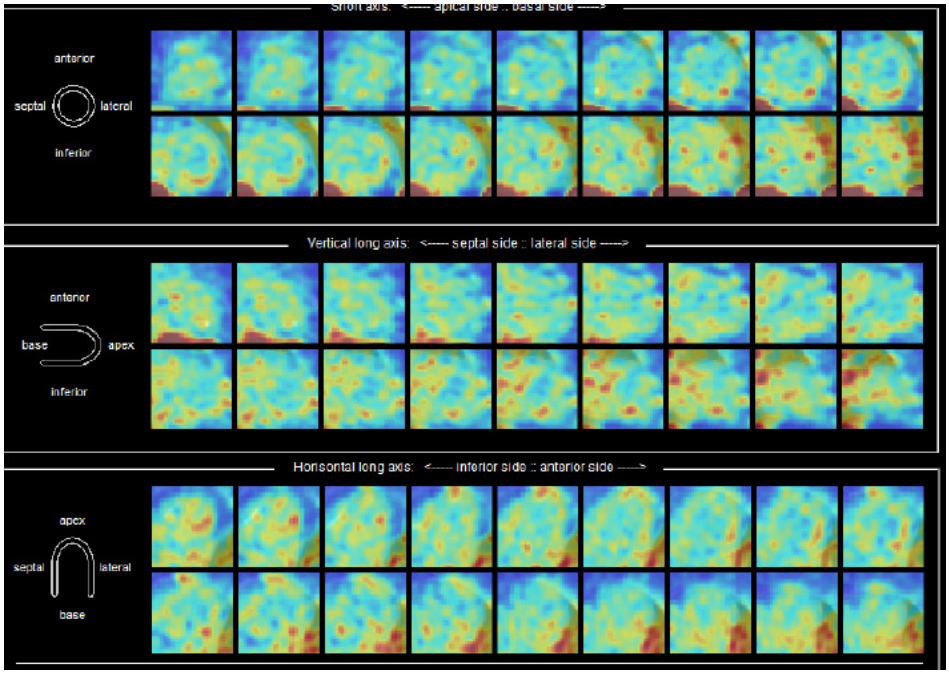
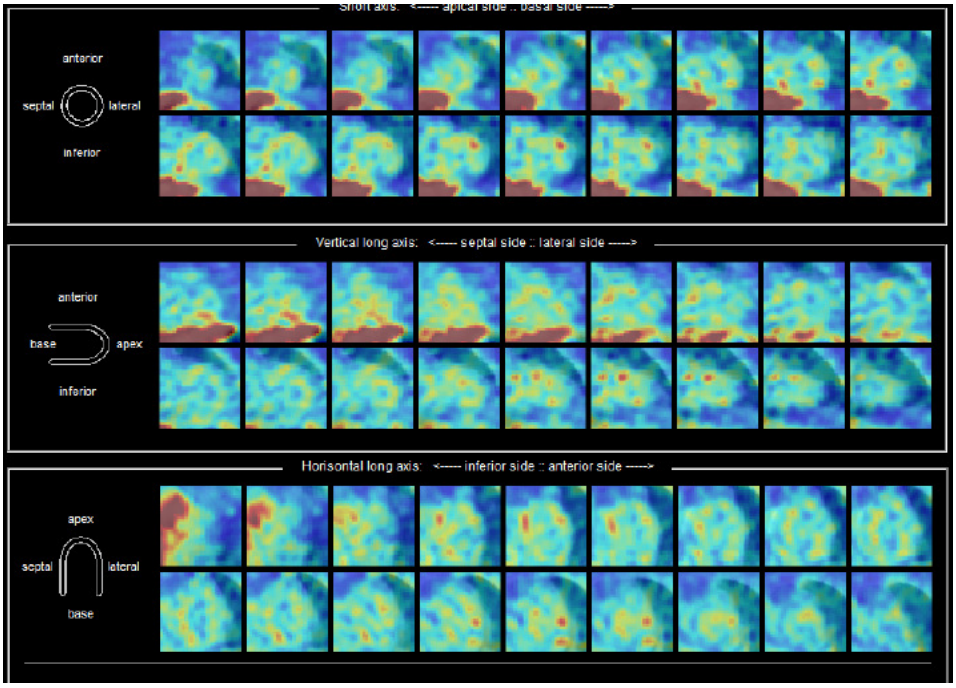


Figure 52. Baseline and follow-up ^{99m}Tc-NC100692 SPECT imaging in patient 2.

Patient 3

In patient 3 there is relatively less uptake of ^{99m}Tc-NC100692 at baseline compared to the previous two patients and there is no significant increase in uptake following intracoronary infusion of BMSCs/serum:

Patient 3. Baseline scan



Patient 3. Follow-up scan, 4 days post intracoronary injection of cells/serum

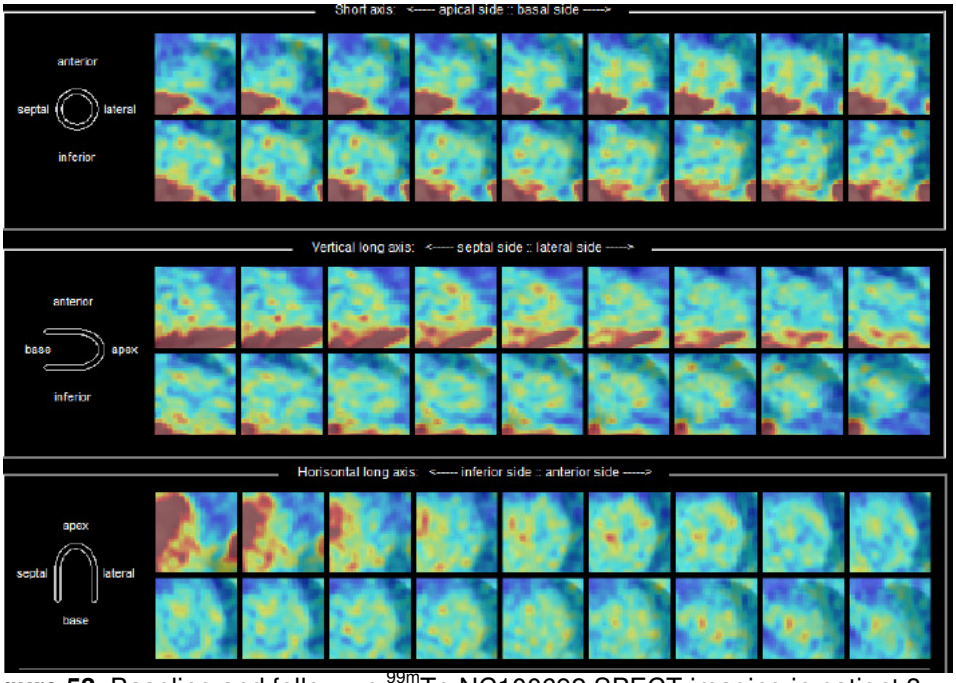


Figure 53. Baseline and follow-up ^{99m}Tc-NC100692 SPECT imaging in patient 3.

6.4 Discussion

This unique study assesses for the first time the uptake of ^{99m}Tc-NC100692, a novel radio-tracer peptide with a high affinity for the αvβ3 integrin, in patients with chronic ischaemic heart failure. The αvβ3 integrin receptor is highly up-regulated in endothelial cells undergoing angiogenesis[336] thus making it an ideal target for assessing possibly neoangiogenesis related to BMSC therapy.

^{99m}Tc-NC100692 administration (at baseline and 4 days post intracoronary infusion of BMSCs or serum alone) was tolerated well by all nine patients who underwent this novel imaging technique. Patients did not experience any adverse symptoms and there were no changes to haemodynamic monitoring during infusion of the radio-tracer.

The baseline and follow-up SPECT images for the first 3 patients are shown in figures 51-53. The imaging results from two 'normal' patients are shown in figure 50. As these figures show, there is no appreciable cardiac uptake of ^{99m}Tc-NC100692 in normal patients. However, in the study patients uptake of ^{99m}Tc-NC100692 was seen in all 3 patients at baseline and in one patient there appears to be increased uptake following the intracoronary infusion of BMSCs/serum. These preliminary results suggest that in patients with chronic ischaemic heart failure and remote myocardial infarction there is evidence of persistent angiogenesis and there is the tantalising possibility that BMSC infusion may lead to an increase in angiogenesis.

The ability of this non-invasive imaging technique to demonstrate baseline levels of angiogenesis in patients with chronic ischaemic heart failure is novel. However, this is not an entirely unexpected finding given that the study patients have previous myocardial infarction and severe coronary artery disease- likely resulting in local tissue hypoxia[337]. Previous animal studies have shown uptake of ^{99m}Tc-NC100692 following surgically induced myocardial infarction[189, 338, 339]. ^{99m}Tc-NC100692 SPECT imaging has also been assessed clinically to detect infarct angiogenesis in patients with recent myocardial infarction[340]. In this study of 10 patients, uptake was demonstrated in the infarct zone, indicating angiogenesis in the healing infarct. Our study suggests that angiogenesis continues in the chronic setting as all patients in our study were at least 1-year post myocardial infarction. Neoangiogenesis is known to play an integral process in the LV remodelling process following myocardial although the degree of new capillary network formation is unable to match the metabolic demands of the hypertrophied myocardium. This mismatch contributes to the loss of viable tissue, infarct extension and fibrous tissue formation[341].

Therapeutic angiogenesis as a concept has been around for some time and aims to improve perfusion via collateral blood vessel formation in ischaemic heart and limbs[342]. Until recently, the focus of this field of research was the use of angiogenic cytokines such as vascular endothelial growth factor (VEGF) or members of the fibroblast growth factor (FGF) family to stimulate angiogenesis[336]. It is now increasingly recognised that angiogenesis is likely to be a significant mechanism through which bone marrow stem cell therapy

exerts a beneficial effect on ischaemic cardiac tissue[18, 151] and hence cell therapy has a major role to play in the field of therapeutic angiogenesis.

Currently, the efficacy of cell therapy in cardiac disease has been assessed using surrogate markers such as LVEF and myocardial perfusion. It remains to be determined whether an improvement in angiogenesis will be associated with a simultaneous improvement in LVEF/perfusion and the final un-blinded analysis of our sub-study will provide valuable information regarding this. The development of similar molecular imaging techniques that target the biological processes implicated in cell therapy may allow direct confirmation of engraftment of cells and the monitoring of the beneficial effects such as neoangiogenesis.

6.5 Study Limitations

We would ideally liked to have performed this sub-study in patients undergoing intramyocardial injection of BMSCs but due to delay in obtaining ethical and regulatory approval this study has been performed in patients in the intracoronary arm of the study. We also remain blinded to whether patients received stem cells or serum (control group) at the time of this thesis submission. The current analysis provides only qualitative information regarding the degree of tracer uptake and further work is ongoing to develop a method of quantification which will allow more objective assessment of the degree of angiogenesis. Nevertheless, there is valuable information that has been gained just in these three patients and a paper will be published in due course detailing the full results of this sub-study.

CHAPTER 7: FUTURE DIRECTIONS

7.1 General

The three studies presented in this thesis add significantly to the growing evidence base regarding the potential for cell therapy to improve cardiac function and symptoms in patients with ischaemic heart disease. The studies have also generated interesting hypotheses that warrant further investigation.

The efficacy of the combined approach of G-CSF and intramyocardial injection of BMSCs demonstrated in the REGENERATE-IHD study (chapter 4) will need confirmation in a larger multi-centre phase III randomised controlled trial. Future trials should also be adequately powered to detect a difference in hard clinical end-points (such as mortality and re-hospitalisation) as there was a hint towards improved clinical outcomes in our study. The results also suggest a possible role for the combination of G-CSF and intramyocardial injection of serum. This warrants further examination and a trial comparing serum injection versus saline injection may help evaluate the efficacy of this combination. It will also be important to perform assays to characterise the various constituent paracrine factors in the serum which maybe responsible for cardiac repair. The final analysis of the full REGENERATE-IHD trial, with the completed intracoronary and peripheral arms of the study, will provide valuable insight into the ideal route of delivery of BMSCs in patients with ischaemic heart failure.

In chapter 5, G-CSF was shown to improve the functional potential of circulating CD34+ cells compared to those in the bone marrow. This finding requires further investigation and a randomised trial of G-CSF followed by intramyocardial injection of peripheral (PBSCs) *or* bone marrow derived stem cells (BMSCs) may be helpful. Previous trials comparing PBSCs versus BMSCs have shown differing results[10, 154] but G-CSF was not administered in those trials and this maybe an important factor.

The novel nuclear imaging technique, presented in chapter 6, is a potentially exciting tool which will allow the assessment of possible therapeutic angiogenesis related to BMSC therapy. The full results of this study are not yet available but are highly anticipated as there did appear to be an increase in tracer uptake in one of the study patients. This technique is very much in the early phase of development and there is ongoing work to refine the qualitative and quantitative assessment of tracer uptake. However, this technique could play a major role in the field of stem cell therapy as it may allow indirect confirmation of cell engraftment as well as measuring the therapeutic angiogenic response. Hence, it may become established as one of the key modalities to assess efficacy in future larger trials in this field. Furthermore, angiogenesis imaging could have a wider application in the field of general/interventional cardiology and in other therapies that aim to improve angiogenesis such as direct myocardial revascularisation.

There are several other ongoing developments in this rapidly developing field of cell therapy for cardiac disease. These can be grouped into: (1) identifying

the ideal cell type; (2) refining the delivery method; (3) improving cell engraftment and retention; (4) performing mechanistic studies and large phase III clinical trials with hard clinical end-points; (5) developing imaging techniques to identify and monitor cell engraftment as well as subsequent biological effects. The key developments are discussed in detail below.

7.2 Cells

In addition to BMSCs, there are several other cell types undergoing clinical evaluation for their effects on cardiac repair. In particular, cardiac resident stem cells (CSCs) have shown promising efficacy results in the setting of AMI (CADUCEUS trial[118]) and ischaemic heart failure (SCIPIO trial[343]). Other developments include allogeneic products and modification of cells prior to delivery.

Allogeneic cell products

It is now increasingly recognised that patient related factors such as age and severity of disease state can adversely affect the function of autologous cells. The advantages of an allogeneic cell product include the ability to provide an 'off the shelf' therapy with uniform quality and consistency and avoid the problem of patient related cell dysfunction. This would also avoid harvesting cells from patients which could reduce time and costs of cell therapy. The main disadvantages relate to potential immune mediated cell rejection. Allogeneic MSCs (Prochymal™) have already been used in the clinical setting and have shown promise in improving cardiac function following myocardial infarction[196]. MultiStem® (Athersys) is another allogeneic product containing

bone marrow derived multi-potent adult progenitor cells (MAPCs). MAPCs demonstrate tri-lineage differentiation potential and have been shown to expand in high numbers in culture[344]. MAPCs also appear to be immunoprivileged making this an ideal allogeneic cell product. A phase 1 clinical trial of MultiStem® injection using a novel adventitial delivery technique in the AMI setting has demonstrated potential efficacy[345].

Cell modification

There is growing interest in guiding the transdifferentiation of cells into a cardiopoietic phenotype (i.e. lineage specified) prior to transplantation into infarcted myocardium in an attempt to improve the efficacy of cell therapy. Cell modification may also overcome the issue of patient related cellular dysfunction. This has been successfully demonstrated in a murine model of myocardial infarction where epicardial injection of lineage specified cardiopoietic MSCs (obtained from human bone marrow) achieved superior cardiac functional and structural improvements compared to injection of unguided MSCs[346]. This is also being examined in the C-Cure trial which is an ongoing phase II/III trial evaluating the safety and efficacy of intramyocardial injection of guided cardiopoietic MSCs in patients with ischaemic heart failure (ClinicalTrial.gov Identifier NCT00810238).

7.3 Delivery systems

New interventional techniques to deliver cell therapy have been developed and an overview of delivery systems has been provided in section 2.2.5 of this thesis. There is ongoing development due to the need for better delivery

systems to improve cell retention. For example, a recent feasibility study has assessed the safety of a novel percutaneous intracoronary micro-needle adventitial delivery technqiue[345]. The development of specialised delivery systems requires that interventional cardiologists acquire the necessary training and proficiency in these techniques if and when cell therapy is established as potentially beneficial therapy in selected patients. Training programmes are already being established around the world following guidelines by the International Society for Cardiovascular Translational Research (ISCTR) providing recommendations for successful training in methods of delivery of biological therapeutic material for cardiac regeneration[347].

7.4 Engraftment

A major limitation of current cell delivery techniques is the low rate of engraftment of transplanted cells. This was highlighted in a preclinical study that demonstrated maximal cell retention rates within the myocardium of only 11% with intramyocardial injection, 3% with intracoronary injection and 3% with intravenous infusion[191]. There are therefore various ongoing developments to try and improve cell engraftment and retention, some of which are described below.

Cell Sheets

Cell sheet-based tissue engineering has been developed by harvesting two-dimensional (2D) cell sheets from culture plates and stacking the sheets to create three-dimensional (3D) tissues without the use of scaffolds[348]. Stacked

cardiomyocyte sheets have been demonstrated to contract simultaneously and have been shown to improve cardiac function following surgical transplantation in rat, canine and pig models[348]. A clinical trial of transplanting cell sheets in heart failure patients has already been instigated and there are reports of promising early results[349].

Cardiac Patch

In this technique stem cells are seeded on 3D porous alginate scaffolds which are then surgically transplanted onto the epicardial surface of infarcted hearts. In a rat infarct, these patches were shown to stimulate intense neovascularisation and attenuate LV dilatation[350]. A clinical study has compared epicardial injection of BMSCs alone versus implanting a collagen matrix seeded with BMSCs in patients with impaired cardiac function undergoing CABG. The matrix group appeared to show better improvements in cardiac structure and function compared to injection of BMSCs suggesting an improvement in the efficiency of cellular cardiomyoplasty[351]. This technique is limited by the need for a surgical procedure.

CellBeads®

CellBeads® (CellMed AG, Germany) are 170 μm alginate microspheres that contain MSCs genetically modified to express glucagon-like peptide-1 (GLP-1) in addition to inherent paracrine factors. GLP-1 is known to have anti-apoptotic and cardioprotective effects[352]. CellBeads® have been shown to have upto 90% myocardial retention following intracoronary infusion in a porcine model of AMI[353]. In this study, there did not appear to be any impairment of coronary

blood flow and there were positive signs of efficacy with reduction in apoptosis. This technology may improve myocardial cell retention as well as providing a means of prolonged secretion of beneficial paracrine factors by the encapsulated cells.

7.5 Clinical trials

The number of clinical trials evaluating cell therapy in cardiac disease continues to increase- at the time of submission of this thesis there were 458 trials registered with ClinicalTrials.gov. The European Society of Cardiology Task Force on cell therapy has provided guidance on the design of future trials[15]. This includes recommendations for large phase III studies assessing clinical end-points and well-designed mechanistic studies.

Phase III trials

So far, there have been many relatively small clinical trials assessing the safety and efficacy of cell therapy in acute and chronic cardiac disease. The relative safety of cell therapy has been uniformly demonstrated but demonstration of efficacy (using surrogate end-points such as LVEF) has been inconsistent. Meta-analyses of cell therapy suggest that there is a modest but significant improvement in cardiac function following cell therapy in AMI and chronic ischaemic heart failure[217, 311]. Interestingly these analyses also suggest improved clinical outcomes such as mortality following cell therapy[49, 311]. Hence, there are now plans to perform large phase III trials to assess the effects of cell therapy on hard clinical end-points. One such study is the proposed BAMI trial which will be a European multi-centre trial recruiting

3000 patients post-AMI to assess the impact of BMSC therapy on mortality (ClinicalTrial.gov Identifier NCT01569178). There is also a US phase III trial evaluating the efficacy and safety of autologous CD34+ stem cells to increase exercise capacity in patients with chronic myocardial ischemia. This study, sponsored by Baxter (USA), follows on from the ACT34-AMI[248] study and aims to recruit 450 patients across 50 sites in the US (ClinicalTrial.gov Identifier NCT01508910).

Head to head comparison of cells

In view of the multiple different cell types undergoing clinical evaluation there is a need for clinical trials comparing the efficacy of different cell types in different disease states. The first pre-clinical comparison of cell types showed superior survival pattern and efficacy of BM-MNCs compared to MSCs and skeletal myoblast in a murine model of AMI[162]. The most recent pre-clinical experiment showed that cardiac derived stem cells were superior to BM-MNCs and MSCs in their ability to improve cardiac function in AMI[354]. Clinical studies have so far only compared PBSCs and BMSCs with mixed results[10, 154, 312]. Our own data presented in this thesis suggests that G-CSF treatment may lead to PBSCs having more functional potential than BMSCs[17] and this warrants further investigation in the design of future trials. In a recent meta-analysis of BMSC trials in AMI and IHD patients, BM-MNC transplantation appeared to have better efficacy results compared to CD133+ or CD34+ cells alone[311]. This also warrants further investigation.

Other mechanistic studies

Proposed mechanistic studies include assessing the roles of stromal cell-derived factor-1 (SDF-1) and microRNAs in cardiac repair. SDF-1 is chemokine that is transiently expressed following tissue injury and is thought to promote stem cell homing to the myocardium[355]. It also limits cardiac injury by initiating a number of protective molecular pathways that are both anti-inflammatory[356] and anti-apoptotic[357]. A recent preclinical study injected naked plasmid DNA encoding SDF-1 into the infarct border zone in a rat model of chronic heart failure[358]. Compared to controls the SDF-1 treated animals showed increased angiogenesis, improved cardiac function and evidence of scar remodelling.

MicroRNAs are small strings of oligonucleotides that regulate molecular switches in cells that control the expression of hundreds of genes (i.e. a class of post-transcriptional regulators)[359]. Specific microRNAs have been shown to be capable of inducing direct cellular reprogramming of fibroblasts to cardiomyocyte-like cells in vitro[360]. Furthermore, administration of microRNAs into ischemic mouse myocardium resulted in evidence of direct conversion of cardiac fibroblasts to cardiomyocytes in situ[360]. This early work is fascinating and if reproduced by others may have huge therapeutic potential.

7.6 Imaging

Currently the role of imaging in cell therapy is limited to the assessment of surrogate end-points of efficacy such as LVEF and cardiac volumes. There are also developments to use real-time MRI guidance to help guide

transendocardial injection of cells[361]. However, there is a lack of clinical imaging techniques targeted at the cellular level and an inability to track the fate of injected cells. Preclinical experiments have utilised direct labelling (with MRI and nuclear imaging) to assess immediate cell distribution[216], reporter genes to assess long-term survival[358] and optical fluorescence imaging to estimate transdifferentiation and engraftment[116]. There are yet to be clinical translation of these methods mainly due to concerns regarding manipulation and disruption of cells prior to delivery. The 'angiogenesis' imaging technique, described in chapter 6 of this thesis, utilising SPECT imaging and a radio-tracer with high affinity for an angiogenesis related integrin is the first clinical study of its kind and once completed may represent a big step forward with regards to clinical imaging of the biological actions of transplanted cells.

CHAPTER 8: CONCLUSIONS

This thesis has presented three clinical studies evaluating the role of bone marrow stem cell (BMSC) therapy in patients with ischaemic heart failure. The aims of these studies were to address some of the unresolved questions regarding this novel therapy, in particular: the delivery method and the role of adjunctive cytokine therapy, the effect of patient-related factors on progenitor cell concentration and function and the possible mechanism(s) of action of cell therapy.

In the first study, complete 1-year follow-up data from the intramyocardial arm of the REGENERATE-IHD were presented. Combined G-CSF treatment and intramyocardial injection of BMSCs in patients with chronic ischaemic heart failure led to a significant improvement in LVEF, NT-proBNP, symptoms and quality of life. There was also improvement in diastolic function in the cell treated group. The improvement in LVEF was related to the in vitro functional potential of injected cells.

Interestingly, there were also signals of efficacy seen in the control group who received G-CSF therapy and intramyocardial injection of autologous serum. There was a significant reduction in NT-proBNP and a statistically non-significant improvement in LVEF. This raises the possibility that there maybe some therapeutic potential of G-CSF therapy combined with direct delivery of soluble paracrine factors.

In a separate study of progenitor cell characteristics, ageing appeared to have an inverse relationship with circulating CD34+ cell concentration. In older patients with ischaemic heart failure, bone marrow concentration of CD34+ cells following G-CSF treatment was lower and peripheral blood (PB) mobilisation was also impaired. The disease process itself also appeared to have an effect on peripheral blood progenitor cell concentration. Non-ischaemic heart failure (DCM) was associated with significantly higher baseline PB CD34+ and EPC concentration compared to IHD. Following G-CSF treatment, CD34+ cell concentration was greater in the BM compared to PB, however the PB CD34+ cells appeared to have a greater and improved (compared to baseline) functional potential. These results suggest treatment with G-CSF improves the functional potential of mobilised circulating progenitor cells compared to those in the bone marrow.

The preliminary results of a sub-study assessing the feasibility of a novel imaging technique to detect angiogenesis related to BMSC therapy has also been presented. In this study the uptake of ^{99m}Tc-NC100692, a radio-tracer peptide with a high affinity for the αvβ3 integrin (which plays a key role in angiogenesis), was assessed in patients undergoing intracoronary infusion of BSMCs or serum. SPECT imaging demonstrated uptake of the radio-tracer in patients prior to any treatment providing evidence for a degree of persistent angiogenesis in patient with chronic ischaemic heart failure. In one patient there appeared to be an increase in tracer uptake following treatment raising the tantalising possibility that BMSC transplantation may lead to an increase in angiogenesis in human cardiac tissue.

The field of cell therapy for the treatment cardiovascular disease continues to gather pace. The results from the intramyocardial arm of our REGENERATE-IHD add valuable insight and suggest a role for combined G-CSF therapy and intramyocardial injection of BMSCs. Signals of efficacy demonstrated in the control group warrants further investigation of intramyocardial injection of soluble paracrine factors. The current aims are to translate the beneficial findings seen in preclinical experiments and small clinical studies into improved clinical outcomes in a large number of patients in the setting of phase III clinical trials. This will require close collaboration between scientists and clinicians to refine cell products, delivery systems and methods to improve cell retention in targeted tissue. Novel imaging techniques which allow detection of cell engraftment and biological action will play a key role in facilitating the refinement of cell therapy. The future holds great promise for cellular therapy which will hopefully provide a realistic treatment option for a large number of patients with heart failure who remain symptomatic and would otherwise continue to suffer a poor outcome.

APPENDIX A: LV THROMBUS STUDY

Study Rationale

In the REGENERATE-IHD Study presented in chapter 4, three patients had to be withdrawn from the intramyocardial arm due to the finding of previously undiagnosed left ventricular thrombus (LVT) on baseline cardiac imaging. Hence, we sought to examine the incidence of LVT in patients recruited to our whole REGENERATE-IHD Study to ascertain whether we should routinely screen patients for LVT as part of the assessment clinic prior to inclusion in future trials. This is particularly important in view of the imminent design of larger Phase III studies recruiting hundreds-to-thousands of patients. The overall feasibility of NOGA® intramyocardial injection is discussed in section 4.5.4.

Patients with heart failure and left ventricular systolic dysfunction (LVSD) are recognised to be at an increased risk of developing arterial and venous thrombo-embolic complications, but reports of the incidence of these events vary widely[362, 363]. In addition to atrial fibrillation, the development of LVT is a possible causal mechanism responsible for thromboembolism in these patients. There are currently no guidelines as to whether patients with LVSD should be screened for the development of LVT and if so at what time interval this should be performed. There is also no firm consensus regarding the period of anticoagulation once LVT has been identified. Hence, current

guidelines and recent clinical trials of anticoagulation in heart failure are also discussed in this study.

Methods

Study objectives

We sought to assess the incidence of previously undiagnosed LVT in consecutive patients with severe chronic ischaemic heart failure recruited to our REGENERATE-IHD study. We also examined the sensitivity of contrast echocardiography imaging compared to cardiac CT/MRI for the diagnosis of LVT.

Patient population

The study population comprised of all the patients who have so far completed follow-up in the REGENERATE-IHD Study.

Cardiac Imaging

The presence of LVT was assessed in all baseline and follow-up imaging studies that were performed in recruited patients. All imaging studies were reviewed by an imaging specialist.

Contrast echocardiogram

Two-dimensional transthoracic echocardiogram (TTE) was performed in all patients at baseline, 6-months and 1-year post treatment. An ultrasound contrast agent, Sonovue™, was used in all patients to improve delineation of the LV endocardial border.

Cardiac MRI (CMR)

CMR was performed in patients without a contraindication to MRI scanning using a Siemens Avanto 1.5T (Siemens Medical Solutions, Erlangen, Germany) scanner, using internationally standardised acquisition protocols[271]. The presence of LVT was assessed following administration of an intravenous contrast agent, gadolinium doterate, (Dotarem, 0.15mmol/kg).

Cardiac CT

Cardiac CT was performed in patients with a contraindication to MRI. A Siemens 256-slice "FLASH" dual-source scanner was used to obtain retrospectively gated scans with intravenous iodinated contrast for assessment of cardiac structure and function.

Results

71 patients have so far completed 1-year follow-up in the whole REGENERATE-IHD trial. All baseline and follow-up imaging studies were evaluated for the presence of previously undiagnosed left ventricular thrombus (LVT). All patients were in sinus rhythm. There were no statistically significant differences between patients with and without LVT in terms of baseline characteristics or severity of LV systolic dysfunction (table 10).

Table 10. Characteristics of patients with and without left ventricular thrombus

	Patients without LVT (n=63)	Patients with LVT (n=8)	p-value
Age, years	63.5 ± 9.6	57.4 ± 7.9	0.09
Medical history			
Previous MI (%)	50 (74)	8 (100)	0.33
Time from prior MI (years)	8 ± 7	11± 6	0.28
LVT at time of prior MI (%)	2 (3.2)	1 (14)	0.30
Previous CABG (%)	21 (33)	3 (38)	0.55
Previous TIA/CVA (%)	4 (6.3)	1 (12.5)	0.46
LVEF (%)	31.9 ± 10.9	27.6 ± 5.1	0.28
LVEDD (cm)	6.2 ± 0.9	6.2 ± 0.8	0.99
NT-proBNP (pg/ml)	1124	1048	0.90
Medications at recruitment			
Aspirin (%)	53 (84)	8 (100)	0.28
DAPT (%)	19 (30)	4 (50)	0.23
Warfarin (%)	12 (19)	0	0.21

Data presented as number (% of patients) or mean ± SD; DAPT: dual anti-platelet therapy; LVEF: left ventricular ejection fraction; LVEDD: left ventricular end-diastolic diameter; LVT: left ventricular thrombus

Incidence of LVT

A total of 8 (11.3%) patients had LVT detected during cardiac imaging performed as part of the trial protocol. 6 patients had LVT detected at baseline and 2 patients had LVT identified during follow-up scans at 6 months and 1 year. Of the 7 patients who had LVT diagnosed with CMR (n=4) or CT (n=3), the corresponding contrast TTE was positive in only 2 of these patients, giving a sensitivity of only 30% compared to CMR/CT. The 2 cases diagnosed accurately on TTE were large protuberant thrombi and in the 5 cases detected

only by CMR/CT the LVT were smaller, laminated or adherent to the antero-septum of the LV cavity (Figure 54).

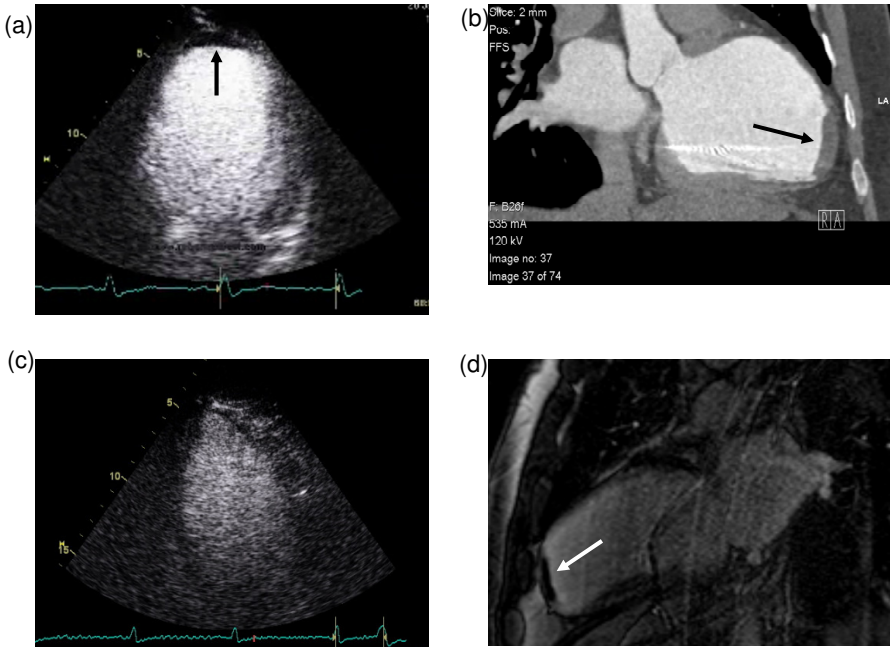


Figure 54. Comparison of contrast echo and cardiac CT/MR for diagnosis of left ventricular thrombus. (a) contrast echo accurately demonstrating a moderate sized apical LVT (black arrow) which was subsequently confirmed on CT which is shown in (b); (c) contrast echo in a patient which does not appear to show LVT, however on subsequent MRI with gadolinium contrast (d) a small, non-mobile laminated apical LVT is clearly demonstrated (white arrow).

Management of LVT

All the patients who had LVT identified were commenced on oral anticoagulation with warfarin following discussion of risk/benefit. There have not been any bleeding complications and we have decided to continue anticoagulation indefinitely in these patients.

Thrombo-embolic episodes during follow-up

Only one patient (1.4%) developed a thrombo-embolic complication during follow-up. This was a pulmonary embolism in a patient without LVT and not on warfarin.

Discussion

We have shown an 11.3% incidence of LV thrombus (LVT) in a population of optimally treated patients with severe LVSD secondary to ischaemic heart disease. In addition we found no statistical difference in the rates of prior MI, CABG or TIA/CVA between patients with and without LVT. There was also no difference between these groups with regards to LVEF and LV end diastolic diameter. There was no difference in the prior use of anti-platelet therapy; however none of the 8 patients with LVT were taking warfarin compared to 19% of patients without LVT. This was not statistically significant which is most likely due to the relatively small study cohort. Finally we established that contrast TTE has a sensitivity of only 30% compared to cardiac CT/MRI for the detection of LV thrombus.

The incidence of LVT in patients with ischaemic LVSD remains considerable despite contemporary therapy. The incidence found in our cohort of patients is similar to other recent published series. In their recent papers Weinsaft et al. quote an incidence of 7-10% in patients with significant LVSD[364, 365]. Their data is based on studies comparing transthoracic echocardiography (TTE) and delayed enhancement-CMR (DE-CMR). The sensitivity and specificity of TTE were 33% and 91% respectively compared to DE-CMR which is similar to the finding in our study. Follow-up in the former study supported DE-CMR as a reference standard, with >5-fold difference in endpoints (embolic events) between patients with versus without LVT by DE-CMR ($p = 0.02$). This is further supported by another study comparing TTE, TOE and DE-CMR for the detection of LV thrombus in patients with surgically/pathologically confirmed

LVT. In this study of 160 patients with all 3 imaging modalities performed within 30 days of surgical or pathological confirmation, contrast-enhanced MRI had the highest sensitivity and specificity (88% +/- 9% and 99% +/- 2%, respectively) compared with TTE (23% +/- 12% and 96% +/- 3.6%, respectively) and TOE (40% +/- 14% and 96% +/- 3.6%, respectively) for thrombus detection[366]. TTE remains the most widely used modality of LV imaging for the detection of LVT due to its ease of use, non invasive nature and low cost. However, with the recent expansion in availability of CMR this is rapidly becoming the imaging modality of choice for assessment of cardiac structure and function. Cardiac CT with contrast also provides a more sensitive approach to LVT detection than TTE; however the associated radiation and contrast exposure make this unattractive as a screening tool.

The incidence of thromboembolism during follow-up was low (1.4%) in our study, although the eight patients with LVT were all commenced on long-term anticoagulation with warfarin. This incidence is in keeping with published data such as the SOLVD study in which the annual risk of stroke was 1.5% in patients with mild to moderate systolic dysfunction and 4% in those with severely reduced EF, as compared with 0.5% in the general population[367]. In another study of 264 ambulatory patients with HF (mean EF 27%), the rate of stroke or transient ischaemic attack was again low (1.7%/year) over a mean of 24±9 years[368]. However, LVT was identified in half the cases, and patients with thrombus had a significantly higher rate of thrombo-embolism (5.3%/year) than those without thrombus (p=0.03). Furthermore, an embolic event in patients with heart failure may increase mortality as was shown in a study in

which patients with an embolic event suffered a significantly higher mortality during follow-up when compared to those without an embolic event ($p<0.001$)[369].

There are limited national and international guidelines regarding anticoagulation in patients with LVT. Currently, the 2008 American College of Chest Physicians do not recommend oral vitamin K antagonists in patients with non-ischemic HF unless another indication above and beyond heart failure itself is present[370]. Similarly, the ACC/AHA 2009 heart failure guidelines state that warfarin is most justified in patients with heart failure who have other indications for anticoagulation (e.g. previous embolic event or AF)[371]. In keeping with US guidelines, the 2008 ESC heart failure guidelines recommend that oral Vitamin K antagonist therapy be instituted for patients with heart failure and concomitant AF (Class I, Level A) or those with a proven intra-cardiac thrombus or evidence of thromboembolic complications (Class I, Level C). However, current guidelines do not provide recommendation regarding the duration of anticoagulant therapy.

The lack of definitive guidance reflects the limited clinical evidence available based on 3 small randomised trials (WASH[372], WATCH[373] and HELAS[374]). Hence the recent presentation of the results of the WARCEF trial[375] was highly anticipated. This was a double-blind, multi-centre study comparing aspirin (325mg) or warfarin in 2,305 patients with LVEF <35% and NYHA classes I-III heart failure who were in sinus rhythm. The study showed no difference in the primary end-point of death, ischaemic stroke and intra-cerebral haemorrhage

between the two groups over a mean follow-up period of 3.5 years (HR 0.93 (0.79-1.10), p = 0.4). However when the primary outcome was separated in its component parts there was a highly significant benefit of warfarin over aspirin for the prevention of ischaemic stroke (HR 0.52 (0.33-0.82) p = 0.005). This came at the cost of higher rates of major haemorrhage with warfarin, although rates of intra-cerebral haemorrhage were similar between the two groups. This study therefore appears to support current guidelines which do not recommend routine anticoagulation in heart failure without another indication such as AF.

However, the recent development of novel oral anticoagulant therapy with lower bleeding risks may re-open the question of anticoagulation in heart failure. Dabigatran, a direct thrombin inhibitor, has recently been approved for the prevention of stroke in patients with non valvular AF. In the RE-LY trial, dabigatran at the higher of 2 doses reduced the risk of stroke and peripheral embolic events by 34% and the risk for hemorrhagic stroke by 74% compared to warfarin[376]. Rivaroxaban is a novel direct factor Xa inhibitor approved in the United States for the prevention of stroke in non-valvular AF patients. In the ROCKET-AF trial[377] rivaroxaban was non-inferior to dose-adjusted warfarin for the prevention of stroke. Major and non-major clinically relevant bleeding were not different between the two groups, but significant reductions in intracranial haemorrhage (0.5% vs. 0.7%, P=0.02) and fatal bleeding (0.2% vs. 0.5%, P=0.003) were seen in the rivaroxaban group. Apixaban is another oral direct factor Xa inhibitor which has been shown to be superior to warfarin in preventing stroke or systemic embolism and caused less bleeding in the

recently published ARISTOTLE study[378]. The improved safety profile of these newer agents warrants their evaluation in patients with ischaemic heart failure and LVSD particularly in the sub-group of patients with LVT who are known to have higher embolic risk which is associated with adverse morbidity and mortality.

Finally, as our analysis shows that the presence of LVT appears to be relatively common in patients with LVSD, it maybe prudent to screen (with MRI/CT rather than echocardiography) for the presence of thrombus prior to randomisation of patients in future trials involving invasive intra-cardiac procedures such as transendocardial injection. This would avoid the issue of withdrawing patients after randomisation which can impact the statistical interpretation of trial results. From a clinical perspective, the relatively common finding of LVT in heart failure patients may limit the number of patients able to receive cell therapy via the transendocardial route. Furthermore, as discussed above there is currently no consensus guideline regarding anticoagulation in patients with heart failure and sinus rhythm. There is also no recommendation regarding the length of anticoagulation once LVT is diagnosed in the chronic setting. Patients with LVT may still be able to receive transendocardial therapy if there is resolution of thrombus following a period of anticoagulation.

Study Limitations

The analysis of the incidence of previously undiagnosed LV thrombus is an exploratory analysis, the findings of which need to be confirmed in a larger prospective database of heart failure patients.

APPENDIX B: HRQL QUESTIONNAIRES

The three Health Related Quality of Life (HRQL) questionnaires (MacNew, SF36 Version 2 and EQ5D) are provided in this appendix.

MACNEW Questionnaire

macnew.org
HEALTH-RELATED QUALITY OF LIFE INSTRUMENT

We would now like to ask you some questions about how you have been feeling **DURING THE LAST 2 WEEKS.**

Please check the box ❑ that matches your answer

1. In general, how much of the time during the last 2 weeks have you felt frustrated, impatient or angry?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

2. How often during the last 2 weeks have you felt worthless or inadequate?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

3. In the last 2 weeks, how much of the time did you feel very confident and sure that you could deal with your heart problem?

1 ❑ NONE OF THE TIME
2 ❑ A LITTLE OF THE TIME
3 ❑ SOME OF THE TIME
4 ❑ A GOOD BIT OF THE TIME
5 ❑ MOST OF THE TIME
6 ❑ ALMOST ALL OF THE TIME
7 ❑ ALL OF THE TIME

4. In general how much of the time did you feel discouraged or down in the dumps during the last 2 weeks?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

5. How much of the time during the past 2 weeks did you feel relaxed and free of tension?

1 ❑ NONE OF THE TIME
2 ❑ A LITTLE OF THE TIME
3 ❑ SOME OF THE TIME
4 ❑ A GOOD BIT OF THE TIME
5 ❑ MOST OF THE TIME
6 ❑ ALMOST ALL OF THE TIME
7 ❑ ALL OF THE TIME

6. How often during the last 2 weeks have you felt worn out or low in energy?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

7. How happy, satisfied, or pleased have you been with your personal life during the last 2 weeks?

1 ❑ VERY DISSATISFIED, UNHAPPY MOST OF THE TIME
2 ❑ GENERALLY DISSATISFIED, UNHAPPY
3 ❑ SOMEWHAT DISSATISFIED, UNHAPPY
4 ❑ GENERALLY SATISFIED, PLEASED
5 ❑ HAPPY MOST OF THE TIME
6 ❑ VERY HAPPY MOST OF THE TIME
7 ❑ EXTREMELY HAPPY, COULD NOT HAVE BEEN MORE SATISFIED OR PLEASED

8. In general, how often during the last 2 weeks have you felt restless, or as if you were having difficulty trying to calm down?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

9. How much shortness of breath have you experienced during the last 2 weeks while doing your day-to-day physical activities?

1 ❑ EXTREME SHORTNESS OF BREATH
2 ❑ VERY SHORT OF BREATH
3 ❑ QUITE A BIT OF SHORTNESS OF BREATH
4 ❑ MODERATE SHORTNESS OF BREATH
5 ❑ SOME SHORTNESS OF BREATH
6 ❑ A LITTLE SHORTNESS OF BREATH
7 ❑ NO SHORTNESS OF BREATH

10. How often during the last 2 weeks have you felt tearful or like crying?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

11. How often during the last 2 weeks have you felt as if you are more dependent than you were before your heart problem?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

12. How often during the last 2 weeks have you felt you were unable to do your usual social activities or social activities with your family?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

13. How often during the last 2 weeks have you felt as if others no longer have the same confidence in you as they did before your heart problem?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

14. How often during the last 2 weeks have you experienced chest pain while doing your day-to-day activities?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

15. How often during the last 2 weeks have you felt unsure of yourself or lacking in self-confidence?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

16. How often during the last 2 weeks have you been bothered by aching or tired legs?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

17. During the last 2 weeks, how much have you been limited in doing sports or exercise as a result of your heart problem?

1 ❑ EXTREMELY LIMITED
2 ❑ VERY LIMITED
3 ❑ LIMITED QUITE A BIT
4 ❑ MODERATELY LIMITED
5 ❑ SOMEWHAT LIMITED
6 ❑ LIMITED A LITTLE
7 ❑ NOT LIMITED AT ALL

18. How often during the last 2 weeks have you felt apprehensive or frightened?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

19. How often during the last 2 weeks have you felt dizzy or lightheaded?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

20. In general, during the last 2 weeks how much have you been restricted or limited as a result of your heart problem?

1 ❑ EXTREMELY LIMITED
2 ❑ VERY LIMITED
3 ❑ LIMITED QUITE A BIT
4 ❑ MODERATELY LIMITED
5 ❑ SOMEWHAT LIMITED
6 ❑ LIMITED A LITTLE
7 ❑ NOT LIMITED AT ALL

21. How often during the last 2 weeks have you felt unsure as to how much exercise or physical activity you should be doing?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

22. How often during the last 2 weeks have you felt as if your family is being over-protective toward you?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

23. How often during the past 2 weeks have you felt as if you were a burden on others?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

24. How often during the past 2 weeks have you felt excluded from doing things with other people because of your heart problem?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

25. How often during the past 2 weeks have you felt unable to socialize because of your heart problem?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME

26. In general, during the last 2 weeks how much have you been physically restricted or limited as a result of your heart problem?

1 ❑ EXTREMELY LIMITED
2 ❑ VERY LIMITED
3 ❑ LIMITED QUITE A BIT
4 ❑ MODERATELY LIMITED
5 ❑ SOMEWHAT LIMITED
6 ❑ LIMITED A LITTLE
7 ❑ NOT LIMITED AT ALL

27. How often during the last 2 weeks have you felt your heart problem limited or interfered with sexual intercourse?

1 ❑ ALL OF THE TIME
2 ❑ MOST OF THE TIME
3 ❑ A GOOD BIT OF THE TIME
4 ❑ SOME OF THE TIME
5 ❑ A LITTLE OF THE TIME
6 ❑ HARDLY ANY OF THE TIME
7 ❑ NONE OF THE TIME
❑ NOT APPLICABLE

That's the end. Thanks very much for answering the questions.
[Version: November 2003]

SF36 Version 2 Questionnaire

Patient Name: _____ Date: _____

1. In general, would you say your health is: (circle one)

   Excellent Very good Good Fair Poor

2. Compared to one year ago, how would you rate your health in general now? (circle one)

   Much better now than one year ago.

   Somewhat better now than one year ago.

   About the same as one year ago.

   Somewhat worse than one year ago.

   Much worse than one year ago.

3. The following items are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much? (Mark each answer with an **X**)

ACTIVITIES	**Yes, Limited A Lot**	**Yes, Limited A Little**	**No, Not Limited At All**
a. **Vigorous activities,** such as running, lifting heavy objects, participating in strenuous sports			
b. **Moderate activities,** such as moving a table, pushing a vacuum cleaner, bowling, or playing golf			
c. Lifting or carrying groceries			
d. Climbing **several** flights of stairs			
e. Climbing **one** flight of stairs			
f. Bending, kneeling or stooping			
g. Walking **more than a mile**			
h. Walking **several blocks**			
i. Walking **one block**			
j. Bathing or dressing yourself			

4. During the past 4 weeks, have you had any of the following problems with your work or other regular daily activities as a result of your physical health? (Mark each answer with an **X**)

	YES	**NO**
a. Cut down on the **amount of time** you spent on work or other activites		
b. **Accomplished less** than you would like		
c. Were limited in the **kind** of work or other activities		
d. Had **difficulty** performing the work or other activities (for example, it took extra effort)		

5. During the past 4 weeks, have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (such as feeling depressed or anxious)? (Mark each answer with an **X**)

	YES	**NO**
a. Cut down the **amount of time** you spent on work or other activities		
b. **Accomplished less** than you would like		
c. Didn't do work or other activities as **carefully** as usual		

6. During the past 4 weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbors or groups? (circle one)

Not at all Slightly Moderately Quite a bit Extremely

7. How much bodily pain have you had during the past 4 weeks? (circle one)

None Very mild Mild Moderate Severe Very severe

8. During the past 4 weeks, how much did pain interfere with your normal work (including both work outside the home and housework)?

Not at all A little bit Moderately Quite a bit Extremely

9. These questions are about how you feel and how things have been with you during the past 4 weeks. For each question, please give the one answer that comes closest to the way you have been feeling. How much of the time during the past 4 weeks – (Mark each answer with an **X**)

	All of the Time	**Most of the Time**	**A Good Bit of the Time**	**Some of the Time**	**A Little of the Time**	**None of the Time**
a. Did you feel full of pep?						
b. Have you been a very nervous person?						
c. Have you felt so down in the dumps that nothing could cheer you up?						
d. Have you felt calm and peaceful?						
e. Did you have a lot of energy?						
f. Have you felt downhearted and blue?						
g. Did you feel worn out?						
h. Have you been a happy person?						
i. Did you feel tired?						

10. During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting with friends, relatives, etc.)? (circle one)

All of the time Most of the time Some of the time A little of the time None of the time

11. How TRUE or FALSE is each of the following statements for you?

	Definitely True	**Mostly True**	**Don't Know**	**Mostly False**	**Definitely False**
a. I seem to get sick a little easier than other people					
b. I am as healthy as anybody I know					
c. I expect my health to get worse					
d. My health is excellent					

EQ5D Descriptive Questionnaire

By placing a tick in one box in each group below, please indicate which statements best describe your own health state today.

Mobility

I have no problems in walking around	❑	PLEASE TICK
I have some problems in walking around	❑	ONE BOX
I am confined to bed	❑	

Personal Care

I have no problems with personal care	❑	PLEASE TICK
I have some problems washing or dressing myself	❑	ONE BOX
I am unable to wash or dress myself	❑	

Usual Activities *(e.g. work, study, housework, family or leisure activities)*

I have no problems with performing my usual activities	❑	PLEASE TICK
I have some problems with performing my usual activities	❑	ONE BOX
I am unable to perform my usual activities	❑	

Pain/Discomfort

I have no pain or discomfort	❑	PLEASE TICK
I have moderate pain or discomfort	❑	ONE BOX
I have extreme pain or discomfort	❑	

Anxiety/Depression

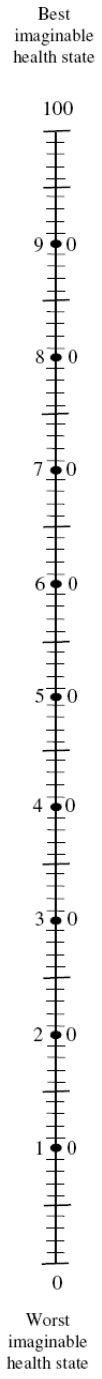
I am not anxious or depressed	❑	PLEASE TICK
I am moderately anxious or depressed	❑	ONE BOX
I am extremely anxious or depressed	❑	

EQ5D Visual Analogue Score

To help people say how good or bad a health state is, we have drawn a scale (rather like a thermometer) on which the best state you can imagine is marked 100 and the worst state you can imagine is marked 0.

We would like you to indicate on this scale how good or bad your own health is today, in your opinion. Please do this by drawing a line from the box below to whichever point on the scale indicates how good or bad your health state is today.

Your own health state today



APPENDIX C: PUBLICATIONS AND PRESENTATIONS

Publications in peer reviewed journals:

- Mathur A, Mozid AM et al. Combined G-CSF and Intramyocardial Injection of Bone Marrow Cells in Heart Failure Improves Cardiac Function and Symptoms at 1-Year. (submitted to Lancet)

- Mozid AM, Jones D, Arnous S, Saunders N, Wragg A, Martin J, Agrawal S, Mathur A. The Effects of Age, Disease State and Granulocyte-Colony Stimulating Factor on Progenitor Cell Count and Function in Patients Undergoing Cell Therapy for Cardiac Disease. Stem Cells Dev. 2012 Jul 26.

- Mozid AM, Arnous S, Sammut E, Mathur A. Stem Cell Therapy for Heart Diseases (review). Br Med Bull 2011; 98(1): 143-159.

- Arnous S, Mozid AM, Martin J, Mathur A. Bone marrow mononuclear cells and acute myocardial infarction. Stem Cell Research & Therapy 2012, 3:2.

- Arnous S, Mozid AM, Mathur A. The Bone Marrow Derived Adult Stem Cells for Dilated Cardiomyopathy (REGENERATE-DCM) Trial– Study design. Regenerative Medicine 2011; 6(4):525-33.

Textbook chapters:

- Mozid AM, Arnous S, Lovell M, Mathur A. Chapter 3.49 Cell Based Regenerative Therapy. Percutaneous Interventional Cardiovascular Medicine- The PCR-EAPCI Textbook. First Edition 2012.

Presentations at Conferences:

- European Society of Cardiology (ESC) Annual Congress (August 2011, Paris):

  Poster presentation of pilot phase of REGENERATE-IHD trial of bone marrow derived cells for patients with heart failure.

- Cell Therapy for Cardiovascular Disease (January 2012, New York): Poster presentation of progenitor cell study results.

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