THE THERAPEUTIC USE OF ADULT AUTOLOGOUS BONE MARROW DERIVED CELLS IN ISCHAEMIC

CARDIOMYOPATHY

MDRes Thesis

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Abstract

The effect of combined cytokine and cell therapy in ischaemic cardiomyopathy is unclear. Meta-analyses suggest improved cardiac function with cell therapy. The optimal cell delivery route remains unclear. The mechanism of effect on cardiac function with cell therapy remains to be elucidated. This thesis aims to address these unanswered questions. Chapter 1 introduces cell therapy in ischaemic cardiomyopathy. Chapter 2 details the methods. This thesis can be divided into three projects.

The first project investigates whether granulocyte-colony stimulating factor (G-CSF) alone or in combination with intracoronary (IC) or intramyocardial (IM) injection of autologous bone marrow-derived cells (BMC) improves cardiac function as well as functional and biochemical parameters in patients with ischaemic cardiomyopathy. Chapter 3 details the results of the study which suggests an improvement in cardiac function, patient functional characteristics and biochemical parameters in patients who received IM BMC therapy along with G-CSF.

The second and third projects assess the mechanisms by which improvements in cardiac function and/or symptoms may have occurred. The second study looked at the association between various pro- and anti-inflammatory cytokines as well as pro-angiogenic cytokines with G-CSF/cell therapy. Chapter 4 details the results of the cytokine sub-study. Important signals were observed including a reduction in certain pro-inflammatory cytokines (e.g. MCP-1, IL-8 and IL-1b) and an increase in the pro-angiogenic cytokine VEGF in the group with a significant improvement in cardiac function at 1 year i.e. the IM BMC group.

The third project (chapter 5) looked at myocardial scar, diastolic function and cell characteristics in relation to G-CSF/cell therapy and cardiac function. The results highlighted important correlations

including a significant association between colony-forming unit granulocyte-macrophage counts and improvement in cardiac function in the IM BMC group.

Lastly, in chapter 6, I discuss the findings and the implications of the research in everyday practice.

Contribution to work

All contents in this thesis have been written by myself and reviewed by my supervisors Prof Anthony Mathur and Prof Charles Knight, who I wish to thank for their continued support and supervision during my studies. I undertook the work leading to this thesis while I was working as a Clinical Research Fellow in the Adult Stem Cells Unit at the London Chest Hospital between January 2013 and January 2015. My work was based on the REGENERATE-IHD trial, a randomised controlled trial looking at the effect of granulocyte colony stimulating factor (G-CSF) alone (peripheral arm) or in combination with intramyocardial (IM) or intracoronary (IC) bone marrow derived cell (BMC) therapy in patients with ischaemic cardiomyopathy. I followed-up the trial participants in the IC and peripheral (P) arms (60 patients) at 1 and 2 years and the IM arm participants (30 patients) at 2 years. I undertook training to analyse cardiac CT scans for left ventricular ejection fraction. I also undertook training to analyse cardiac MR scans to analyse myocardial scar. Once all follow-ups were completed, I performed analysis of all CT scans for all trial participants in the IC and P arms at baseline, 1 year and 2 years and CT scans for all IM arm participants at 2 year follow-up. Dr Ceri Davies, Consultant Imaging Cardiologist, and Dr Stephen Hamshere, Research Fellow, helped quality control the analyses. I analysed all cardiac MR scans (i.e. IC, IM and P arms) at baseline and 1 year for myocardial scar quantification. I also analysed all transthoracic echocardiograms at baseline and 1 year for diastolic function and all left ventriculograms in the IC and P arms at baseline and 6 months for change in left ventricular function. I collated all the data from the Quality of life questionnaires (3 different questionnaires at 4 different time points for all IC and P arm patients) and calculated the score for each questionnaire using available software. As part of the cytokine sub-study, I analysed the results of all the cytokine measurements at baseline and 1 year. I wish to acknowledge the contribution of the RANDOX technical specialists who analysed the stored

plasma sample for the cytokines mentioned in the study. All data were collated and all statistical analyses were undertaken by myself using standard statistical software packages. The BMC preparation and analysis were undertaken by a stem cell laboratory technician who undertook the cell preparation and analysis in a GMP certified laboratory. The REGENERATE-IHD trial protocol stated that, should the trial show positive results, the trial participants who had not received the active therapy could receive the active therapy on compassionate grounds. I performed bone marrow aspiration on the majority of these patients and assisted the stem cell scientist in cell preparation and analysis work for these patients. To derive a conclusion and to understand the "G-CSF and cell therapy story" from the trial, it was necessary to combine and compare the results in the three arms –P, IC and IM. The IM arm 1 year results (30 patients) had been previously published as the MDRes thesis of Dr A Mozid, my predecessor, who I wish to gratefully acknowledge for providing me with the data from the IM arm that he used for his analysis. I re-analysed all data from the IM arm and performed comparative statistics between all three arms. I also wrote the manuscript for the main paper which is currently under review.

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- Prof Charles Knight, secondary supervisor

- Dr Stephen Hamshere, Clinical Research Fellow, London Chest Hospital

- Dr Ceri Davies, Consultant Imaging Cardiologist

Finally, I would like to most gratefully acknowledge my supervisor Prof Anthony Mathur, who I wish to thank immensely for his continued support, encouragement and supervision during my studies.

CONTENTS

List of tables

Table 5.1. Change in cytokines/chemokines with change in scar

List of figures

Abbreviations

HF	Heart failure
IHD	Ischaemic heart disease
PPCI	Primary percutaneous coronary intervention
AMI	acute myocardial infarction
LVEF	left ventricular ejection fraction
ECM	extracellular matrix
MMP	matrix metalloproteinase
RAS	renin-angiotensin-aldosterone system
BNP	brain natriuretic peptide
NT pro-BNP	NT pro- brain natriuretic peptide
NPR	natriuretic peptide receptors
LVESV	left ventricular end systolic volume
LVEDV	left ventricular end diastolic volume
NYHA	New York Heart Association
CCS	Canadian Cardiovascular Society
IL-1a	Interleukin 1a
IL-1b	Interleukin 1b
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10

TNF-α   Tumour Necrosis Factor- α

MCP-1   Monocyte chemoattractant protein-1

IFN-γ   Interferon- γ

VEGF   Vascular Endothelial Growth Factor

ACEi   Angiotensin Converting Enzyme inhibitor

ARB   Angiotensin receptor blocker

CRT   Cardiac Resynchronisation Therapy

ICD   Implantable Cardioverter Defibrillator

BMC   Bone marrow derived cells

iPSC   induced pluripotent stem cells

CSC   Cardiac stem cells

ASC   Adipose tissue derived stem cells

ESC   Embryonic stem cels

SM   Skeletal muscle

6MWT   6 minute walk test

MvO2   Maximum oxygen consumption

MSC   Mesenchymal stromal cells

UC-MSC   Umbilical cord-mesenchymal stromal cells

CDC   Cardiosphere derived cells

CMR   Cardiac magnetic resonance scans

CT   Computed tomography

Tβ4   Thymosin β4

HSC   Haematopoietic stem cells

EPC   Endothelial progenitor cells

CFU-GM   Colony forming unit- granulocyte macrophage

CD   Cluster of differentiation

G-CSF   Granulocyte colony stimulating factor

SDF-1   Stromal derived factor

IM   Intramyocardial

IC   Intracoronary

P   Peripheral

TTE   Transthoracic echocardiogram

TDI   Tissue Doppler imaging

LA   Left atrium

DT   Deceleration time

QLV   Quantitative left ventriculogram

QoL   Quality of life

Chapter 1 Introduction

1.1 The burden of Heart failure

The prognosis of patients with heart failure (HF) is still dismal despite decades of advances in healthcare. HF {defined as a structural or functional cardiac abnormality that results in a failure to supply oxygen at a rate commensurate with the demands of the metabolising tissues, despite normal filling pressures (or only at the expense of increased filling pressures)[1]} is a common condition associated with significant morbidity, mortality and cost to the healthcare system.

In the UK 900,000 people suffer from HF with more than 5.8 million people affected in the USA.[2] The prevalence of HF is expected to rise in the UK with an increasing ageing population and thus highlights the unmet need for better treatment options. Worldwide an estimated 23 million people suffer from HF [3] with the estimated prevalence expected to increase by 25% in the next 20 years .[4] The prevalence and incidence of HF are known to be related to age with a prevalence of around 1% for those between 45 and 64 years old but 20% in the over 85 age group.[2] According to the Framingham study, the lifetime risk of developing HF in those over 40 years old is 20%.[5]

The impact on healthcare systems is substantial. In the UK, 2% of all hospital bed-days and 5% of all emergency medical admissions are secondary to a primary diagnosis of HF. On average, a GP looks after 30 patients with established HF.[2] In the USA, it is estimated that treatment of chronic HF costs the US economy more than $29 billion per year.[6] The above facts highlight the significant financial burden to the health services of looking after patients with chronic HF.

1.1.1 Aetiology

Heart failure most frequently occurs as a consequence of ischaemic heart disease (IHD), [7] which accounts for more than two-thirds of cases of HF. Hypertension, which had previously been considered the commonest cause of HF has been superseded by IHD and is the second most important contributory factor. The causes of HF are listed in Table 1.1.

Table 1.1. Aetiology of chronic heart failure

Ischaemic heart disease
-Myocardial infarction
-Myocardial ischaemia
Valvular heart disease
-obstructive
-regurgitant
Hypertension
Chronic tachy- or brady-arrhythmias
Non-ischaemic dilated cardiomyopathy
-genetic/familial
-toxic/drug-induced
-infiltrative
-metabolic
-post-viral illness

High-output states
e.g. thyrotoxicosis, chronic anaemia

Ischaemic heart disease is the UK's biggest killer and is responsible for approximately 73,000 deaths per year.[8] The advent of primary percutaneous coronary intervention (PPCI) together with advancements in medical therapy with new anti-platelets and anticoagulation has limited the damage caused by and improved the treatment of acute myocardial infarction (AMI) by reducing mortality and recurrent MI. These improved treatment strategies for AMI have led to the greater survival of patients who in the pre-PPCI era would not have survived. However this appears to be at the expense of developing HF. Data from the Framingham study, reported by Velagaleti et al., shows that the incidence of HF within 30-days post-MI rose from 10% in the period 1970-79 to

23.1% in the period 1990-99. The 5 year incidence of HF was 27.6% in 1970-79 and increased to 31.9% in 1990-99.[9] A few other studies have also supported the Framingham findings including the Worcester Heart Attack Study[10] and a cohort study from Canada.[11] Not all studies demonstrate this trend, however, as in the Olmsted County community study which involved 2596 patients with a first acute MI over a period of 20 years (1990-2010) and showed a decline in post-MI HF incidence in the period 2004-2010 when compared to 1990-1996. [12] However, it is likely that the improved survival of patients undergoing MI is, at least partly, leading to the increasing prevalence of HF.

1.1.2 From myocardial infarction to chronic ischaemic cardiomyopathy – Pathophysiological basis

During an AMI, up to 10^9 cardiomyocytes can be lost as a result of ischaemia.[13] The resulting acute damage activates a chronic, subsequently self-propagating, cascade of biologically active molecules (neurohormones e.g. noradrenaline, angiotensin and cytokines e.g. IL-6, TNF) which have deleterious effects on the heart. These acute (myocardial damage from MI) and chronic (neurohormonal, cytokine activation) insults to the heart set off a progressive, maladaptive change in left ventricular (LV) architecture known as ventricular remodelling (Figure 1.1), ultimately leading to the clinical manifestations of HF. The condition of impairment of left ventricular ejection fraction (LVEF) secondary to IHD is labelled as chronic ischaemic cardiomyopathy.

1.1.2.1 Left ventricular remodelling

Ventricular remodelling refers to the change in ventricular architecture resulting in increased LV volumes and change in the shape of the LV. In the first few days following AMI, the myocytes undergo necrosis followed by thinning and dilatation of the LV wall. This is as a result of expansion of the necrotic area, not by further necrosis but, by "slippage" of cells past one another as a result of degradation of the intercellular collagen support.[14] Cardiac fibroblasts subsequently deposit

collagen over the thinned, infarcted territory, resulting in scar formation. Scar formation in the short-term has a protective effect by restricting further expansion of the necrotic area, however, in the long-term, it serves as a substrate for life threatening arrhythmias. In a significant proportion of patients (approximately 30%) the process of LV remodelling continues but in the non-infarcted, healthy myocardium. [15] The non-infarcted myocardium undergoes hypertrophy. Hypertrophy in HF is usually secondary to a volume overload state and leads to hypertrophy of cardiac myocytes with elongation of myocytes and placement of sarcomeres in series, known as eccentric hypertrophy. (Figure 1.1). This results in further thinning of the LV wall and dilatation of the LV. According to Laplace's law, the dilatation and thinning of the LV results in increased wall stress and thus increased afterload. Although, initially a compensatory mechanism, the increasing afterload and the reducing contractility results in a continuing downward spiral to 'pump failure'. Furthermore, the increased wall stress results in sub-endocardial hypoperfusion and thus worsening ischaemia and LV function, activation of stretch-induced genes {e.g. angiotensin II, tumour necrosis factor (TNF)} and free radical generation resulting in further activation of various cytokines e.g. IL-1β (see section 1.1.2.3).[16] In addition, the stretching of the LV annulus and increased displacement of the papillary muscles, secondary to the annular dilatation, result in functional mitral valve regurgitation, which in turn imposes further volume overload on the heart. It has been noted that the larger the area of infarction, the greater the extent of remodelling[17].

The biological processes underlying remodelling involves alterations in cardiac myocyte biology, the myocardium and left ventricular geometry.[18] Changes in myocyte biology involve 1. Myocyte hypertrophy 2. Alterations in excitation-contraction coupling thus reducing contractility 3. Down-regulation of beta-adrenergic receptors 4. Mitochondrial abnormalities causing abnormal myocardial energy metabolism 5. Progressive destruction of myofilaments and 6. Distortion of

cytoskeletal architecture.[16]   Changes in the myocardium include 1. Increased myocyte loss secondary to increased necrosis and apoptosis and 2. Changes in the extracellular matrix (ECM). The composition and mass of the ECM is altered with changes in the amount and subtypes of collagen, collagen arrangement and cross-linking and interaction of cells and ECM. These changes are driven primarily by the balance between matrix metalloproteinases (MMP), peptidases capable of degrading the ECM proteins, and their inhibitors. [18] Ultimately, the LV geometry changes from an elliptical shape to a more spherical shape with increased LV volumes.

1.1.2.2 Neurohormonal mechanisms

The process of LV remodelling is driven by the activation of a number of biologically active molecules and pathways. The neurohormonal mechanisms include activation of the sympathetic nervous system and the renin-angiotensin-aldosterone system. Activation of the sympathetic nervous system is accompanied by a blunted parasympathetic response. The increased sympathetic activation occurs relatively early in HF and results in elevated noradrenaline levels in the blood. As a result, although an adaptive response initially, the resulting reduction in heart rate variability and increase in contractility and peripheral vascular resistance result, in the long-run, in increased afterload and increased energy demands on a failing heart and thus becomes a maladaptive response.   Relatively late in the course of HF, compared to the sympathetic system, the renin-angiotensin-aldosterone system (RAS) is activated. This occurs secondary to renal hypoperfusion and adrenergic stimulation. Again, similar to the activation of the sympathetic system, activation of the RAS is initially a compensatory response that soon becomes maladaptive. Thus, although angiotensin II and aldosterone, in the short-term, cause vasoconstriction and salt and water retention thus aiding circulatory homeostasis, in the longer term, they result in cardiac fibrosis and hypertrophy, endothelial dysfunction and impaired noradrenaline uptake, amongst other

deleterious effects. Angiotensin II is known to have direct cytotoxic effects on the heart including promoting fibrosis and cell hypertrophy. Despite the already expanded extracellular volume, the stimulation of the RAS leads to further increase in salt and water retention and thus increased afterload. Angiotensin II also stimulates the production of arginine vasopressin (antidiuretic hormone), another vasoconstrictor, which also results in further volume expansion by increasing water reabsorption in the kidneys. The net result is a worsening of haemodynamics and progressive LV remodelling all contributing to a downward spiral of worsening LVEF and symptomatic decline.

To combat this increasing spiral of neurohormonal mediated deleterious effects, proteins with natriuretic and vasodilator properties become activated. These include the natriuretic group of peptides of which brain natriuretic peptide (BNP) is widely used in clinical practice and is now a clinically established marker used in both the diagnosis and prognosis of HF patients. BNP was originally detected in the porcine brain, hence the name. However, in humans, it is synthesised, along with atrial natriuretic peptide (ANP), another natriuretic peptide, in the heart in response to mechanical stress from volume overload. BNP is synthesised as a pre-prohormone that undergoes subsequent cleavage to form the 32 amino acid BNP (the active natriuretic peptide) and the inactive, 76-amino acid by-product, NT pro-BNP. Both, BNP and NT pro-BNP, can be measured using commercially available immunoassays. BNP, along with ANP, binds to natriuretic peptide receptors (NPR-A and NPR-B). This in turn leads to activation of intracellular second messenger systems ultimately leading to vasodilatation, loss of sodium and water, inhibition of the RAS and inhibition of cardiac fibrosis[18].   Although an adaptive response, as HF progresses, the response to natriuretic peptides becomes blunted.
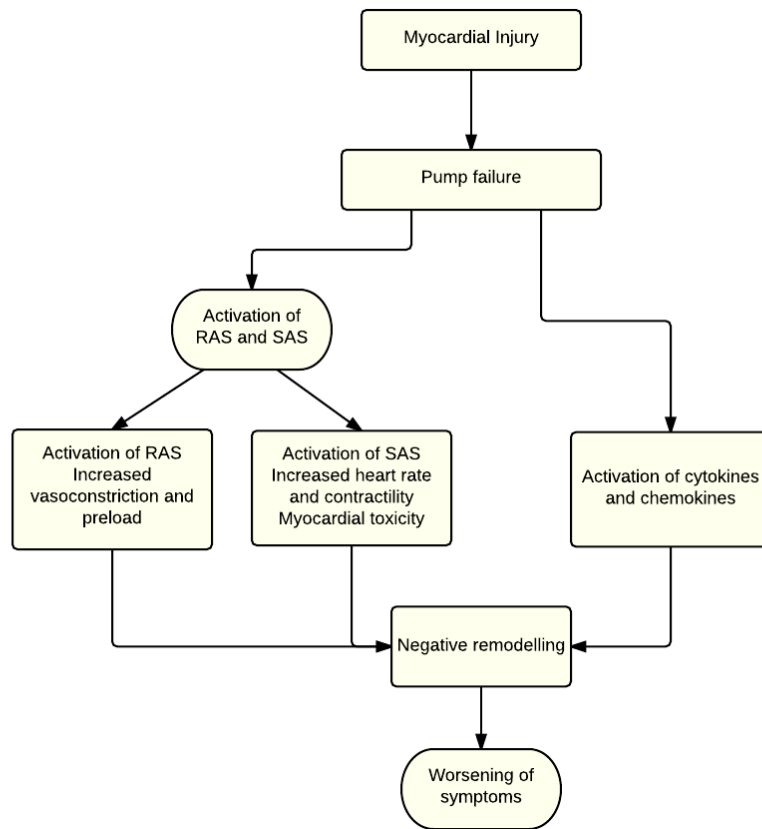
Fig 1.1. Diagram showing the pathophysiology of heart failure. The initial event is usually a myocardial infarction which results in pump failure and subsequent activation of the renin angiotensin system (RAS) and the sympathetic adrenergic system (SAS) as well as cytokines. The eventual result is negative remodelling and symptomatic heart failure

1.1.2.3 Cytokines in heart failure

It has increasingly been recognised that pro- and anti-inflammatory cytokines may play a role in the progression of remodelling in HF in addition to the neurohormonal activation discussed above.[19] In the early 1990's, Levine et al. were the first to show a link between inflammation and HF by showing an elevated level of the pro-inflammatory cytokine, tumour necrosis factor (TNF-α), in patients with HF.[20] TNF-α has been extensively studied in heart failure patients and animal models. Bryant et al, developed transgenic mice expressing TNF-α. These mice subsequently developed severe impairment of biventricular cardiac function, ventricular dilatation and ventricular fibrosis.[21]

TNF-α impairs cardiac function by disrupting calcium homeostasis, production of reactive oxygen species and increased nitric oxide production. Several other cytokines have been studied since. IL-2 is a glycoprotein that is produced by lymphocytes and induces T-cell proliferation. IL-2 has been shown to cause severe but reversible LV dysfunction in isolated rat heats.[22] Furthermore, IL-2 has been studied in patients with renal cell carcinoma and metastatic melanoma. In such patients, IL-2 was found to cause transient, LV dysfunction.[23] Similar LV dysfunction was also observed in another study by Nora et al. who looked at the cardiotoxic effects of IL-2 in patients with renal cell carcinoma, melanoma or colon carcinoma and found similar depressive effects on LV contractility.[24] Another cytokine studied is IL-4, an anti-inflammatory cytokine and pro-fibrotic cytokine that stimulates the production of collagen type I and II. In a study by Gonzalez-Molina et al., they found that increased IL-4 levels was correlated with adverse remodelling and cardiac fibrosis. Patients with chronic heart failure had higher levels of IL-4 than in controls. [25] IL-6, a pro-inflammatory cytokine, has been studied in chronic heart failure patients in several studies and has been found to be elevated in such patients. IL-6 is a multifunctional cytokine that is produced by a variety of cells including lymphocytes and fibroblasts. TNF-α is known to activate IL-6 expression and IL-6 and TNF-α levels have been found to be correlated in heart failure patients.[26] IL-6 has been shown to be raised with deteriorating LV function and is a marker of severity of heart failure and prognosis.[27] Il-6 has been shown to depress LV contractility and potentiate the negative inotropic effect of TNFα and IL-1β and induce myocardial hypertrophy. [28] IL-8, a pro-inflammatory cytokine, is known to be elevated in patients with chronic HF. Damas et al have studied several different cytokines as a sub-study of the statin trial CORONA (Controlled Rosuvastatin Multinational Trial in Heart Failure). They found that IL-8 level was prognostic in predicting mortality and worsening heart failure. [29] IL-1 is a family of cytokines which includes IL-1α and IL-1β, both pro-inflammatory cytokines. Both act

on the same receptor. IL-1β has been shown to depress LV function in animal models. Injection of IL-1β into mice induced systolic dysfunction.[30] In a group of patients with rheumatoid arthritis, treatment with the IL-1 receptor blocker Anakinra showed improvement in LV function.[31]

IL-10 is an anti-inflammatory cytokine as opposed to the pro-inflammatory cytokines discussed above. Although it is raised in heart failure patients, it is not as elevated in comparison to the elevation in levels of the pro-inflammatory cytokine TNF-α, i.e. the increase in this protective cytokine (IL-10) appears to be inadequate.[32] Most recently, Dopheide et al. looked at the IL-10/TNF-α ratio and found that a low ratio was associated with reduced LVEF and a worse prognosis at 10 years.[33] Research has also focused on cytokines involved in neovascularisation, particularly on vascular endothelial growth factor (VEGF). VEGF is a cytokine with multiple functions including neovascularisation and anti-apoptosis. In heart failure, VEGF levels decrease with progression of the disease state, probably by exhaustion of VEGF release.[34] Pathological cardiac hypertrophy occurs in heart failure and this together with reduced VEGF levels results in reduced capillary density, myocardial hypoxia and cardiac dysfunction. Whether VEGF could serve as a novel therapy for heart failure patients has been studied in various studies including gene therapy using VEGF but with mixed results. [35, 36] Other cytokines studied include interferon-γ (IFN-γ), IL-18 and macrophage chemoattractant protein-1 (MCP-1). IFN-γ is a cytokine produced by peripheral blood mononuclear cells i.e. mast cells, macrophages, T-cells. The effect of IFN-γ is as yet unclear. IFN-γ has been shown to induce cardiomyocyte hypertrophy and increased fibroblast proliferation and resultant cardiac fibrosis. However, other data suggest that IFN-γ may have a protective role by actually limiting hypertrophy. [37] IL-18, in synergy with IL-12, activates production of IFN-γ. IL-18, a pro-inflammatory cytokine, has been shown to induce cardiac hypertrophy, contractile dysfunction, myocyte apoptosis and extracellular matrix remodelling. These biological effects of IL-18 are

thought to be mainly due to enhanced IFN-γ production. [27] Macrophage chemoattractant protein-1 (MCP-1) is a chemokine belonging to the C-C chemokine family that has been shown to be elevated in chronic heart failure patients.[38] Furthermore, in mice studies, MCP-1 antagonist gene therapy was shown to attenuate LV dysfunction in post-MI HF models.[39]

In HF, the balance between pro-inflammatory cytokines and anti-inflammatory cytokines appears to be tilted towards the pro-inflammatory state. The complex interactions of the vast array of cytokines in heart failure are still incompletely understood. The cytokines appear to interact with the neurohormonal system. In fact, levels of pro-inflammatory cytokines were lower in patients with HF receiving RAS blockade therapy thus implying significant cross-talk between the systems. [19, 40] Several cytokines have shown correlation with disease severity and prognosis. For e.g. measurement of TNF-α, IL-6 and MCP-1 has been linked to more severe disease and TNF- α levels have been linked with poor prognosis. Table 1.2 shows some of the important cytokines and chemokines associated with the progression and clinical presentation of HF.

1.1.3 Can remodelling be reversed or halted?

In several trials of therapeutic medicines or devices in HF, a reduction in or attenuation of the increase in LV volumes has been noted. The reduction in LV volumes and a change in shape of the LV towards a more elliptical shape is termed reverse remodelling. In the Metoprolol CR/XL Randomised Intervention Trial in Congestive Heart Failure (MERIT-HF) MRI sub-study, administration of metoprolol for 6 months significantly reduced indexed LV end-diastolic volume (LVEDV) by 16% and indexed LV end- systolic volume (LVESV) by 24% and demonstrated a 28% improvement in LVEF.[41] In the Valsartan HF trial (Val-HeFT), a reduction in LVEDV and improvement in LVEF were also demonstrated with treatment with the angiotensin receptor blocker valsartan.[42]

It was noted in the Val-HeFT trial that patients with worse LVEF at baseline had the greatest degree of reverse remodelling. Observation from several trials indicate that clinical improvement in patients and improvements in LVEF have been associated with reverse remodelling.[16] Reverse remodelling has been a prime target of many therapeutic trials in HF. Reduction in myocyte hypertrophy and changes in gene expression including activation of genes involved in sarcomere formation, β-adrenergic signalling and excitation-contraction coupling towards the level found in a normal heart are likely to account for the observed changes of reverse remodelling.

Table 1.2. Effects of selected cytokines/chemokines on cardiac function

CYTOKINE/CHEMOKINE	EFFECT ON MYOCARDIUM / ASSOCIATION WITH CLINICAL OUTCOME
IL-2	Negative inotropic effect[43]
IL-4	Pro-fibrotic effect [44]
IL-6	Negative inotropic effect[43]; elevated in HF patients[45]
IL-8	Predicts outcomes in HF patients[29]; increases with declining functional class
MCP-1	Levels correlates with declining LVEF; recruit monocytes and macrophages which in turn secrete further pro-inflammatory cytokines[46]
TNFα	Elevated in patients with HF; inhibits contractility, extracellular matrix remodelling, LV hypertrophy [47]
IL-1	Adverse remodelling, enhances apoptosis[48]
IL-10	Protective effect in HF; reduced levels in HF; correlates with decline in LVEF[49]
VEGF	Angiogenic factor; clinical benefit of VEGF gene therapy not seen in trials[34]

1.1.4 Clinical presentation

Around 50% of patients with impaired left ventricular systolic function are asymptomatic. [50] However, over time they ultimately present with symptoms. Symptomatic patients with HF usually complain of breathlessness and fatigue. Clinical assessment involves a detailed history including assessment of the severity of symptoms and the functional status. To aid this, the New York Heart Association (NYHA) classification is often used. Originally described in 1928 and subsequently revised several times, the score grades the symptom severity on a scale of I to IV (table 1.3). The score is helpful to assess response to therapy, severity and prognostication in chronic HF patients. It has been shown in HF patients to be associated with mortality, hospitalisation and disease progression. In a study comparing 2441 chronic HF patients with NYHA class III/IV with 1863 patients with NYHA class I/II it was demonstrated that those in NYHA III/IV had a hazard ratio of 1.49 for HF related mortality (95% CI 1.20 to 1.84, p <0.0001) and 1.17 for HF related hospitalisation (95% CI 1.03 to 1.34, p = 0.017).[51]

1.1.5 Current management

The current management of HF consists primarily of pharmacotherapy. Other treatment options include device therapy and surgical therapy for selected patients. Pharmacological therapy consists of drugs to relieve symptoms and drugs to attenuate the progression of the disease and reduce mortality and morbidity. The former group consists primarily of diuretics. The main classes of diuretics used for symptom relief are loop diuretics and thiazide diuretics. Drugs to reduce mortality and attenuate disease progression include angiotensin converting enzyme inhibitors (ACEi) (or angiotensin receptor blockers-ARBs), beta blockers and aldosterone antagonists. ACEi (or ARBs) have been shown to improve LVEF, improve symptoms, reduce ventricular volumes and reduce the risk of mortality in systolic HF patients. [52, 53] In the CONSENSUS trial, enalapril reduced

mortality by 31% at 1 year compared to the control group in patients with HF, as well as a reduction in symptoms.[52]

Table 1.3. New York Heart Association classification of heart failure symptoms

NYHA class	Symptoms
I	Symptoms only on strenuous activity. Asymptomatic with normal activity
II	Symptoms on prolonged or moderate exercise or exertion
III	Symptoms on doing activities of daily living, mild exertion.
IV	Symptoms at rest. Incapacitated.

All patients, unless contraindicated, should be commenced on an ACEi (or ARB) as soon as a diagnosis of systolic HF is confirmed. Beta blockers have also been shown to improve LVEF (by as much as 5-10%), reduce hospitalisations, improve symptoms and reduce mortality. [41, 54, 55] In the MERIT-HF randomised controlled trial, metoprolol significantly reduced all-cause mortality in patients with HF compared to placebo.[41] In patients with very symptomatic HF (NYHA III/IV), addition of an aldosterone antagonist, apart from improving symptoms, has also been shown to reduce hospitalisation and reduce mortality by almost 30%.[56] Important trials of pharmacological agents in HF are presented in table 1.4.

To aid in the diagnosis and assessment of HF and to guide decision making, measurement of biomarkers is often used. BNP (or NT-pro-BNP), as mentioned in section 1.1.2, has been an established biomarker in clinical HF practice for some time. BNP has been shown, not only to aid in the diagnosis of HF, but also to be an independent predictor of prognosis.[57] Higher BNP levels are associated with adverse outcomes. Furthermore, recent trials have shown that serial measurement

of BNP aids in managing patients and that BNP-guided therapy is superior to clinical assessment guided therapy alone.[58] Trials have shown reduction in BNP levels in patients on the RAS antagonist Valsartan in the Val-Heft study [59] and after biventricular pacing. [60] Furthermore, BNP reduction has been noted in association with reverse LV remodelling in HF patients. BNP (or NT pro-BNP) can be measured using commercially available assays. The cut-off for BNP and NT pro-BNP are different for the diagnosis of HF. A serum BNP level <100pg/ml or a NT pro-BNP level of <400pg/ml strongly suggests a diagnosis other than HF. The half- life of BNP is approximately 20 minutes while the half-life of NT pro-BP is around 2 hours. Hence, BNP has to be processed reasonably quickly after collection compared to NT pro-BNP. Serial BNP (or NT pro-BNP) levels measured in trials or clinical practice must be interpreted with caution. As much as 25% variability in measured values from one visit to another can be physiological. [61]

Selected patients with systolic LV impairment can be considered for device therapy. Around 50% of deaths in patients with systolic HF occur secondary to arrhythmias.[62] In patients who have an LVEF ≤35%, are symptomatic (NYHA II or III) despite optimal medical therapy and have a life expectancy of more than a year, implantation of an ICD (implantable cardioverter defibrillator) is recommended as primary prevention. Cardiac resynchronisation therapy (CRT), which involves implantation of a biventricular pacemaker with (CRT-D) or without (CRT-P) a defibrillator function, has been shown to improve symptoms, reduce hospitalisation with HF and prolong survival in patients with advanced HF. In the COMPANION trial, both CRT-P and CRT-D reduced all-cause mortality in patients with advanced HF by 24% and 36% respectively when compared to optimal pharmacotherapy alone. [63] In the MADIT-2 trial, implantation of an ICD in post MI patients with significant LV dysfunction (LVEF<30%) significantly reduced the risk of mortality in patients who received an ICD when compared to optimal medical therapy. [64] The aim of the CRT is to reduce the

interventricular dyssynchrony and thus optimise ventricular stroke volume. In the CARE-HF trial, LVEF increased by 3.4% at 3 months in the CRT group compared to the no CRT group.[65] In the MIRACLE-HF trial, CRT increased LVEF by 3.6% at 6 months compared to 0.4% in the no CRT group. [66] However, almost 20% of patients fail to respond to cardiac resynchronisation. [67]

Surgical treatment options are limited. Cardiac transplant selection criteria are strict and the availability of donors low. This has led to the use of left ventricular assist devices (LVAD's) as both destination and bridge therapy to transplant. However, these devices are still not widely available and are very expensive. Very recently, a new class of drug, known as neprilysin inhibitors, has shown promise in a phase III trial. The drug LCZ696 was compared against standard ACE inhibitor therapy with enalapril. LCZ696 was found to reduce both mortality and hospitalisation from heart failure compared to enalapril. [68] Gene therapy has also shown promise in preliminary trials. A key target for gene therapy has been SERCA2a (Sarcoendoplasmic reticulum calcium ATPase), a protein involved in calcium transport. SERCA2a levels and activity are reduced in advanced heart failure and leads to reduced systolic calcium release. In the phase I and II CUPID trials, (Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease) adeno-associated virus type 1 (AAV-1) vectors were used to deliver SERCA2a gene to patients with advanced heart failure via the intracoronary route with the aim of restoring SERCA2a levels in these patients. The trial demonstrated improvements in patient symptoms (NYHA and QOL questionnaire), LVEF, LVESV, BNP and functional parameters (6-min walk test and MVO2) at 6 months.[69] However, the advent of novel therapies in heart failure are slow in coming and the prognosis remains poor despite the current evidence based treatment of HF.

1.1.6 Prognosis

Both LVEF and LV volumes have been linked closely to mortality and morbidity in patients with HF. The lower the LVEF, the higher is the risk of mortality. The DIG trial was a randomised clinical trial to investigate the effect of digoxin on mortality and hospitalisation in patients with heart failure. In the DIG study, LVEF lower than 45% was associated with an almost linear increase in mortality risk across successive LVEF deciles (figure 1.2).[70, 71] Apart from prognostic information, the assessment of these parameters aids in the management of these patients in terms of monitoring and decision making on device therapy for advanced HF patients (i.e. cardiac resynchronisation therapy). Although LVEF and volumes are both prognostic markers in HF patients, they do not always correlate with HF symptoms or exercise capacity.[72]

Treatment for HF in developed countries is mostly based on existing European Society of Cardiology, American Heart Association or national guidelines. These guidelines are based on extensive evidence from numerous trials, as highlighted in section 1.1.5. Several classes of drugs, most notably ACEi and beta-blockers, as well as cardiac resynchronization therapy and ICD implantation have shown mortality benefit. However, the epidemiological perspective suggests these treatments have only had a modest effect on HF prognosis.
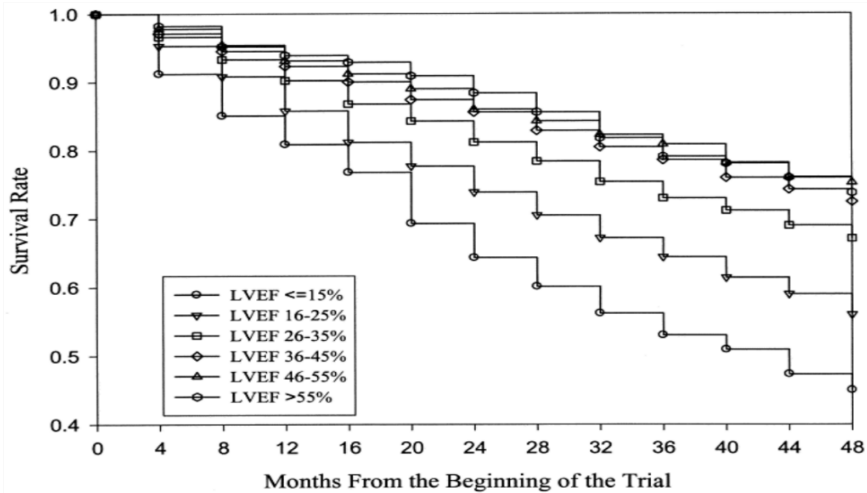
Figure 1.2. Survival and LVEF. Survival curves from the DIG trial showing the relationship between baseline left ventricular ejection fraction (LVEF) and survival. (From Curtis JP, Sokol SI, Wang Y, et al. *Journal of the American College of Cardiology* [71])

Although ACEi have shown significant reductions in the relative risk of mortality, the absolute risk reduction has been small, as highlighted in the paper by V L Roger.[73] In the study by MacIntyre et al. from Scotland, where they looked at event rates in more than 65000 patients with a primary diagnosis of HF, they noted an improvement in median survival from 1.2 to 1.6 years.[74] However, VL Roger notes, that this rise is modest and that, although statistically significant, considering the large sample size, the improvements are not clinically significant. This fact is reiterated by the authors of the study, who in the conclusion of the paper, write "……..there is still much room for improvement". This is reflected by the fact that the current prognosis for patients who are admitted to hospital with HF remains very poor. Almost all other fields in cardiology, apart from HF, have seen dramatic improvements in prognosis. Yet, only half the patients with HF survive at 5 years and the 10 year survival is only 10%. [74, 75] The increasing prevalence of HF poses a significant burden to patients, practitioners, and healthcare systems and highlights the clear need for alternative therapies.

Table 1.4. Landmark trials forming the basis for current medical therapy in heart failure (adapted from

McMurray J, Pfeffer M. *Heart failure* [62]) RR-Relative risk

Trial	N	Severity of HF	Drug	Primary endpoint	Primary outcome
ACE inhibitors					
CONSENSUS	253	End-stage	Enalapril 20mg BD	Death	40% RR reduction
SOLVD-T	2569	Mild-severe	Enalapril 10 BD	Death	16% RR Reduction
Beta-blockers					
CIBIS-2	2647	Mod-severe	Bisoprolol 10mg OD	Death	34% RR reduction
COPERNICUS	2289	Severe	Carvedilol 25mg BD	Death	35% RR reduction
MERIT-HF	3991	Mild-severe	Metoprolol XL/CR 200mg OD	Death	34% RR Reduction
Angiotensin receptor blockers					
Val-HeFT	5001	Mild-severe	Valsartan 160mg BD	Death or morbidity	13% RR Reduction
CHARM-alternative	2028	Mild-severe	Candesartan 32mg OD	Cardiovascular death or HF hospitalisation	23% RR Reduction
CHARM-added	2548	Mod-severe	Candesartan 32mg OD	Cardiovascular death or HF hospitalisation	15% RR Reduction
Aldosterone antagonists					
RALES		Severe	Spironolactone 25-50mg OD	Death	30% RR Reduction
EMPHASIS-HF	2737	Mild-mod	Eplerenone (up to 50mg OD)	Cardiovascular death or HF hospitalisation	HR 0.69 (18.3% in eplerenone group v 25.9% in placebo)
Digitalis					
DIG	6800	Mild-severe	Digoxin	Death	No effect
Cardiac resynchronisation therapy (CRT-P)					
COMPANION	925	Mod-severe	CRT-P	Death or any hospital admission	19% RR Reduction
CARE-HF	813	Mod-severe	CRT-P	Death or any cardiovascular hospital admission	37% RR Reduction
Cardiac resynchronisation therapy plus defibrillator (CRT-D)					
COMPANION	903	Mod-severe	CRT-D	Death or any hospital admission	20% RR Reduction
Implantable cardioverter defibrillator (ICD)					
SCD-HeFT	1676	Mild-severe	ICD	Death	23% RR Reduction

1.2 Regenerative medicine

1.2.1 Introduction

Despite the enormous improvements in cardiovascular therapy, the morbidity and mortality from heart failure remains significant resulting in a need for additional, novel therapeutic approaches. Stem cells as a form of regenerative medicine have emerged as a potential solution to the problem. Cardiac regenerative medicine came to the forefront of medical research more than a decade ago with the goal of regenerating cells in the damaged heart. However the concept of organ regeneration existed long before the term regenerative medicine was coined. Back in the 8th century BC, Greek mythology described the story of Prometheus. Legend has it that the Titan God Prometheus offended Zeus by stealing fire from Mount Olympus for the benefit of mankind.[76] As punishment, he was banished to the Carpathian Mountains and chained to a rock to be tortured by the eagle Ethos. Despite Ethos pecking away part of his liver every night, Prometheus' liver would regenerate everyday only to be pecked on the next night. Hence, the Greeks named the liver from the word "hepanomai" meaning to "repair oneself".

1.2.2 Stem cells

Stem cells are cells that possess the unique ability to divide into multiple different cell types. These cells are present in the human embryo-human embryonic stem cells- and are the cells that give rise to all the cell types and resultant organs that form the complete organism. The adult human also has a reservoir of stem cells- non-embryonic or adult stem cells- in various locations in the body e.g. bone marrow, adipose tissue, umbilical cord, gut and heart. In tissues, such as the gut and liver, these cells repair and replace damaged tissue on a regular basis. In the heart, these cells do exist, and are the focus of much research into their role in cardiac repair. The key feature of stem cells is

that they are undifferentiated cells. The cells resulting from the proliferation of stem cells can either differentiate and commit themselves to a certain cell lineage or form new stem cells. The words "stem" and "progenitor" cells are sometimes, inappropriately, used interchangeably. The definitions of stem and progenitor cells are as follows: stem cells are characterised by their ability to divide indefinitely and into multiple, mature cell types. This ability of stem cells to divide into multiple, mature cell types is defined by varying degrees of potency-multipotence, pluripotence, totipotence- compared to progenitor cells which are characterised by their lack of ability to divide indefinitely and are usually unipotent.[77] Table 1.5 shows the definition of the levels of potency. The adult bone marrow is composed of a mix of stem and progenitor cells i.e. pluripotent and multipotent cells as well as more mature, differentiated cells and has been the most widely used source of stem cells in cardiac research.

Table 1.5. Levels of cell potency and definitions

Totipotent	Able to form all differentiated cells in the organism and trophoblastic cells of the placenta
Pluripotent	Able to form cells from all three germ layers but not the placenta or supporting structures
Multipotent	Able to form multiple but limited cell types
Unipotent	Able to differentiate into only one cell type

1.2.3 The concept of cardiac regeneration

For a long time, the adult human heart has been considered a post-mitotic organ. As mentioned in section 1.1.2, an AMI can result in the loss of a billion cardiomyocytes and the human heart lacks

the ability to regenerate cells to the degree required to replace such a large number of damaged cells. The subsequent replacement of the injured tissue by scar results in impairment of cardiac function and results in HF. The target of regenerative practitioners is to deliver stem and progenitor cells to the affected area, which would proliferate into mature cardiomyocytes and electrically couple with the host cardiomyocytes to replace the scar tissue with functional cardiac tissue. This in turn would improve cardiac function. Studies back in the 1970's by Rumyantsev and others in lower vertebrates, e.g. amphibians, suggested that the heart had regenerative capacity. [78] The zebrafish, a lower vertebrate, has been a popular animal model of cardiac regenerative studies. More than a decade ago, Poss et al. demonstrated that resection of 20% of the adult zebrafish heart resulted in replacement of the excised tissue by new cardiac muscle within a few weeks. [79] To mimic cardiac ischaemic injury rather than direct physical injury through excision, groups have used cryoinjury to create areas of myocardial necrosis in zebrafish models. Again, replacement with new cardiac muscle was evident. [80] However, unlike the zebrafish, the mammalian heart does not have the ability for such large scale cardiac regeneration although the initial concept that the adult human heart is totally post-mitotic has been challenged. In a study by Bergmann et al., they used ^{14}C carbon dating in individuals exposed to nuclear bomb tests during the Cold War to calculate the age of cardiomyocytes and found that cardiomyocytes renew over the course of an individual's lifetime. The turnover decreases gradually with age with a 1% turnover annually at age 25 and 0.45% at age 75.[81] Other evidence for cardiac regeneration comes from chimerism studies where post-mortem examination of cardiac tissue from male patients who had received heart transplants from female donors showed evidence of Y-chromosome positive cardiomyocytes suggesting cardiac regeneration or some form of integration with the recipient tissue. [82] Although the above demonstrate that there is some regenerative capacity in the adult heart, this is by no means on a

scale that would be therapeutically meaningful. However, a key goal of regenerative medicine is to try and revive this innate regenerative property. Furthermore, improvements in cardiac function have been observed in clinical trials using cell therapy but without clear evidence of myocardial regeneration. This has led to the key goal of "regenerative" medicine moving from achieving myocardial regeneration to myocardial repair instead.

1.2.4 Introduction to cell types

Several clinical trials have looked at stem and progenitor cell therapy in both acute MI and chronic ischaemic cardiomyopathy. So what are the attributes of the ideal cell type? The ideal cell type would have to be:

- Safe

- Improve cardiac function and hence symptoms/prognosis

- Able to create new, functional cardiac tissue which integrates well with host tissue

- No immunogenicity

- Easy to deliver

- "Off the shelf" availability of allogeneic products

- No socio-ethical problems

- Ease of availability

Prime among the above attributes are safety, ease of availability and lack of immunogenicity.

To date, several different cell types and sources have been identified and used in pre-clinical and clinical trials. Human stem cell sources can be broadly divided into three different categories: a) embryonic/foetal derived stem cells, b) adult or non-embryonic stem cells and c) adult somatic cells

which have been transformed into stem cells (induced pluripotent stem cells-iPSC). Autologous bone marrow cells (BMC) have been the most widely used in clinical trials due to the extensive experience of using these cells in haematology and the ease of availability and will be discussed in detail in section 1.2.5. Other cell types used in cardiac research are the following:

a) Embryonic stem cell

Theoretically, this is the most attractive cell type in view of its pluripotent nature. Embryonic stem cells (ESC) are obtained from the inner layer of the blastocyst stage of the human embryo. Human ESC lines were first described by Thomson et al.[83] In preclinical studies, ESCs have been found to differentiate into cardiomyocytes which integrate into host tissue in small animal models. In the study by Caspi et al, they injected human ESCs to rat chronic myocardial infarction models and demonstrated an improvement in LV function. [84] However, the use of this cell type has been limited by the risk of tumorigenicity, the immunogenicity due to their allogeneic nature and the ethical concerns in using cells from human embryos. Tumorigenicity was demonstrated at high ESC doses in animal models in a study by Behfar et al.[85] Therefore, clinical trials using ESC in cardiac research are still some way off. Recently, Murry et al. in Nature describe a study where they delivered 1×10^9 cryopreserved, human embryonic derived cardiomyocytes (hESC-CM) intramyocardially to monkeys that had been artificially induced to undergo myocardial infarction and subsequent reperfusion.[86] They report good engraftment of the cells into the native myocardium with good contractile activity and perfusion of the grafts by native vasculature. On the downside, all the hESC-CM monkeys had arrhythmias. This large scale delivery of hESC-CM into a large animal model renews the concept that hESC's might be a realistic prospect in human cardiac regenerative trials in the future although the risk of teratoma formation and arrhythmias remain major obstacles.

b) Adult stem cells

1. Skeletal myoblasts

Skeletal myoblasts (SM) are progenitor cells that regenerate into myotubes and striated muscle fibres. These multipotent cells were attractive candidates for cardiac regeneration in view of the ease of availability from muscle biopsies, their contractile nature, their resistance to hypoxic conditions and their rapid expansion in vitro. Preclinical studies in small and large animal models were quite encouraging with several studies reporting improvements in LVEF and reverse remodelling.[87-89]

However, of note, in experimental studies, skeletal myoblasts implanted into the myocardium differentiated into skeletal i.e. striated muscle fibres rather than cardiomyocytes[90]. It appears that the newly formed myotubes fail to integrate electromechanically with the host cardiomyocytes as a result of failure of expression of key gap junction proteins, like connexion-43 or N-cadherin. This creates "islands of conduction block" which appear to provide a substrate for the arrhythmias. In a study by Roell et al. published in Nature, SM from transgenic mice which were overexpressing connexin-43 showed a reduction in the incidence of ventricular tachycardia when compared to normal SM.[91]

In 2001, SM became the first cell type to be used in stem cell clinical trials in cardiac research by Menasche et al. in a small, non-randomised, uncontrolled study. However, SM have been limited in their use in clinical trials due to the risk of potentially fatal arrhythmias. In the phase II, randomised-controlled MAGIC trial, SM showed no improvement in LVEF compared to controls, with a higher incidence of ventricular arrhythmias in the cell treated group.[92] Similarly, in the SEISMIC phase II, open-labelled, randomised trial, SM injected intramyocardially in patients with HF

showed no improvement in LVEF at 6 months.[93] The prematurely terminated MARVEL-1 trial suggested improvements in the 6 minute walk test (6MWT) at 6 months in cell treated patients. However, ventricular tachycardia was more frequent in this group.[94] Given the adverse effects observed with SM, likely a result of the lack of integration of these cells with the host tissue, in a population already at a higher risk of significant arrhythmias, the future of this cell type in HF patients is questionable.

2. Mesenchymal stem cells

Mesenchymal stem cells (MSC) are available from a wide range of tissues. Their most abundant source is the bone marrow. Other sources of MSC's include adipose tissue, umbilical cord and peripheral blood. These multipotent stem cells have the ability to commit themselves down mesodermal (chondrocytes, adipocytes and osteoblasts) and non-mesodermal lineages. These non-haematopoietic cells are defined in vitro by their ability to adhere to plastic surfaces ("plastic adherence"). There are no specific cell surface markers for MSC's although they are usually accepted to express CD105, CD90 and CD 73 and are negative for haematopoietic markers CD34, CD45 and CD14. In large and small animal models, MSC's have been shown to exert a beneficial effect on cardiac function including improvement in LV ejection fraction, reduction in infarct size, neoangiogenesis and reduction in mortality. [95]   Convincing evidence for transdifferentiation into cardiomyocytes on a scale sufficient to explain these improvements is lacking.[96] MSC's have properties which make them attractive candidates in cell therapy. They lack human leukocyte antigen (HLA) class II molecules and thus, do not induce immunogenic reactions and hence are attractive from a safety perspective. Furthermore, this lack of immunogenicity makes them attractive as allogeneic cell products. The main sources of MSCs used in cardiac regenerative medicine are discussed below.

Bone marrow derived mesenchymal stem cells

In the bone marrow, mesenchymal stem cells (MSC) reside in the bone marrow stroma. They were first described by Friedenstein et al. in the 1970's. [97] MSCs only make up 0.001% to 0.01% of the mononuclear cell fraction. Makino et al. were the first group to show differentiation of BM derived MSC into cardiomyocytes *in vitro* using mice cells[98] followed by the demonstration of differentiation of MSC into cardiomyocytes *in vivo* by Toma et al.[99] However, a number of the experiments used genetic manipulation to induce transdifferentiation into cardiomyocytes-like cells. In Makino's study, epigenetic modulation with DNA methylation was used. Although, experimentally successful, this approach is not clinically practicable. Whether unmodulated MSCs differentiate into functional cardiomyocytes in vitro is controversial but animal studies have shown that MSCs do differentiate into vascular smooth muscle cells and endothelial cells and do improve cardiac function. Thus, it appears that the beneficial effect is likely mediated by mechanisms, other than direct cardiomyocytes differentiation, including proangiogenic differentiation and secretion of paracrine factors, which prolong cell survival, homing and angiogenesis amongst other functions and also possibly by stimulating and mobilising resident cardiac stem cells from their intracardiac niches (see under "Mechanism of action"-section 1.2.9). Clinical trials using bone marrow derived MSCs are discussed together in the section on trials using bone marrow derived cells (section 1.2.5).

Adipose tissue derived mesenchymal stem cells

Adipose tissue is abundant. With increasing liposuction and the low immunogenicity of adipose derived stem cells, these cells are also a potential, attractive allogeneic product. Adipose derived cells have been given various names including adipose derived stromal cells (ADSC), adipose

derived adult stem cells (ADAS) and adipose derived mesenchymal stem cells (AdMSC) to name a few. The current consensus is to call these cells adipose derived stem cells (ASC) to avoid confusion. These cells are multipotent, plastic-adherent stem cells, very similar to bone marrow derived MSC's. Whether these cells are actually MSC's derived from adipose tissue or cells which are just MSC-like cells remains unclear. In one study, ASC and MSC when compared were immunophenotypically >90% identical.[100] However, there were some differences e.g. CD106 expression on MSC but absence on ASC. Although whether or not ASC are the same as MSC's is still unclear, what is clear is that adipose tissue contains a potential, widely available source of stem cells. In fact, adipose tissue is reported to contain more than 2000 times the number of MSC-like cells compared to fresh bone-marrow, thus minimising the time needed to culture expand these cells as is necessary when using BM-derived MSCs. In animal studies, ASC transplantation has been shown to improve cardiac function, reduce infarct size and myocardial fibrosis in post-MI models. [101, 102] In the study by Wang et al, ASC were injected intramyocardially into the peri-infarct zone in female Lewis rat hearts 1 week after an MI. Using cardiac MRI at 1 week and 4 weeks after the cell transplantation and immunofluorescence studies, the group showed a significant improvement in LVEF and as well as an increase in capillary density in the peri-infarct area. However, there was no evidence of differentiation into cardiomyocytes on a scale that would be sufficient to explain the improvements seen.[102] In the preliminary results of the PRECISE (Randomised Clinical Trial of Adipose-Derived Stem Cells in Treatment of Non Revascularisable Ischaemic Myocardium) double-blind, placebo-controlled trial in patients with chronic HF and no revascularisation options, a reduction in infarct size at 6 months and improvement in functional outcomes as demonstrated by an increase in MVO2 and metabolic equivalents at 18 months in the treatment group were seen.[103] However, LVEF deteriorated in the treatment arm at 6 months

compared to control (45 versus 48%). These mixed findings highlight the need for larger trials using ASC to clarify the potential role of ASC's in the treatment of patients with ischaemic cardiomyopathy.   A slightly larger, phase II, randomised, placebo-controlled trial using intramyocardial injection of ASC in patients with chronic ischaemic cardiomyopathy is underway in the USA (ATHENA trial; NCT 01556022)

Umbilical cord derived mesenchymal stem cells

 The umbilical cord (UC) is a rich source of almost all the stem cells found in the bone marrow. These include CD34+ cells as well as mesenchymal stem cells (UC-MSCs). The advantage of using UC is that it is a waste product after baby delivery and so does not pose ethical concerns. Another potential advantage is that these cells could be stored for future use and serve as an autologous storage bank of cells for the individual. However, the disadvantage is that they can only be obtained from delivering mothers which restricts their source. UC-MSCs possess the same qualities as their BM-MSC counterparts in that they are multipotent, have immunomodulatory properties and can be expanded ex vivo. UC-MSCs can be obtained from the whole UC, umbilical vessels, the umbilical lining or the periumbilical vessels.[104] Animal studies with cord blood cells have shown promising results. In mice models of artificially induced MI, there was evidence of increased angiogenesis in the peri-infarct area after receiving UC-MSC's. [105] A few clinical trials are underway or planned using UC-MSC in HF. One of the planned studies is in China where the investigators plan to inject UC-MSC intramyocardially into patients with ischaemic cardiomyopathy (LVEF <45%) (NCT01946048). Their primary endpoint is change in LVEF as measured using echocardiography.

3. Cardiac stem cells

The concept of the heart as a post-mitotic organ has been modified as discussed above. It has increasingly come to the attention of the scientific community that there exists a heterogenous population of resident cardiac stem cells (CSC). These are multipotent and can give rise to cardiomyocytes, smooth muscle and endothelial cells.[106] CSCs include *c-kit+* (c-kit is a receptor tyrosine kinase for the cytokine stem cell factor, which is involved in haematopoiesis) stem cells, cardiosphere-derived cardiac stem cells (CDC), Sca-1 (a murine stem cell marker) cells, side population cells and islet-1+ cells. Beltrami et al. in 2003 identified resident *c-kit+* cells in rat hearts.[107] In 2007, Anversa's group identified *c-kit+* cells from the human heart. [108] CDC's are cardiac cells which when isolated and grown in culture form spherical clusters, which Messina et al. labelled cardiospheres after identifying these cells in 2004. [106] The problem with the original cardiospheres were that there were concerns the cell clusters were too large to safely infuse through the intracoronary route. Marban et al. subsequently refined the process of CDC isolation and expansion so that not only were the cells more compact and thus safely deliverable via the intracoronary route but also could be harvested from percutaneous endomyocardial biopsies, thus obviating the need for surgical procedures. Furthermore, the whole process of isolation to expansion takes 4-6 weeks which is not a considerable length of time considering the chronicity of the patients' condition. The two most relevant cell populations to human studies are the *c-kit+* and CDC populations as there is most clinical data on these cell types. CSC are present in a very low frequency in the adult heart at ~1 per 10000 cardiomyocytes. [107] However, the advantage of CSC is that they can be isolated from cardiac tissue and then expanded ex vivo before being transplanted back into the patient. In several animal studies, using small and large animal models and models of acute and chronic ischaemia, CSC's have been shown to have a range of beneficial effects including

improvement in cardiac function, reduction in fibrosis, reduction in infarct size and reverse remodelling.[109-111] In a chronic ischaemic cardiomyopathy animal model, *c-kit+* cells were infused using the intracoronary route to pigs 90 days post-MI and were found to have improved LVEF, reverse remodelling and evidence of neoangiogenesis. [112] Marban *et al.* also did a head-to-head study using CDC, bone marrow mononuclear cells, BM-MSC and ASC in a post-MI animal model and demonstrated superiority of CDC over the other cell types in terms of improvement in cardiac function and reverse remodelling.[113]

Clinical trials, so far, have been encouraging. The SCIPIO trial, a phase I trial, used CSCs in ischaemic cardiomyopathy patients undergoing coronary artery bypass graft surgery (CABG). [114] Apart from confirming CSC therapy safety and feasibility, the interim CMR results of the trial showed a significant increase in LVEF (27.5±1.6% to 41.2±4.5%; p=0.013) and reduction in infarct size (-9.8±3.5g, p=0.039) at 12 months. The authors also noted improvements in regional contractility. Interestingly, the functional improvement was greatest in segments with the most myocardial dysfunction. Also noted was an increase in viable muscle mass, indirectly suggesting muscle regeneration. However, there has been significant criticism with the way the data was handled and some of the claims could be exaggerated. The publication is under review by the research conduct standards authority at Harvard.[115] In the proof-of-concept, CADUCEUS trial, CSC were administered via intracoronary route to patients with a recent MI. Although no improvement in LVEF was noted, a significant reduction in scar size, increase in tissue viability and regional contractility were observed.[116] This trial has shown safety and feasibility and forms the basis of future trials using this cell type.

4. Epicardial stem cells

The embryonic epicardium contains epithelial cells, which have been shown to contribute to the development of the coronary vasculature and myocardium. For this to happen, the epicardial derived stem cells (EPDC) change from epithelial cells to mesenchymal-like cells, the "epithelial-to-mesenchymal transition", and then migrate to the subepicardial layers to contribute in cardiac development. However, over the course of embryonic development, this tissue generative capacity declines and by the time to adulthood, the "stemness" of the cells becomes dormant. However, some groups have suggested that it might be chemically possible to reactivate the stemness in these cells to regenerate adult cardiac tissue. One such chemical messenger implicated in this process is thymosin β4 (Tβ4). Tβ4 is an actin monomer-binding protein that can "re-awaken" these dormant EPDC's from the adult epicardium[117]. Exactly how it does this is not clear. In experimental animal studies, injection of human EPDCs into mice post-MI hearts showed improvement in cardiac function.[118]

c) Adult somatic cells

Induced pluripotent stem cells

 Takahashi and Yamanaka, in 2006, transduced mouse fibroblasts with four different transcription factors- Oct3/4, Sox2, c-Myc, and Klf4, to yield pluripotent stem cells as a result of reprogramming "stemness" into the cells. These cells were similar to mouse ESCs. A year later, the same group transformed human adult somatic cells into pluripotent stem cells by transducing human dermal fibroblasts with the same four transcription factors using retroviral vectors. These transformed somatic cells, termed induced pluripotent stem cells (iPSC), do not have the same ethical issues as ESCs. Differentiation of iPSCs into functioning and morphological cardiomyocytes, as well as direct

programming of somatic cells into cardiomyocytes bypassing the pluripotent stage, has been demonstrated using animal and human cells.[119-121] Of particular relevance to the HF population, Zwi- Dantsis et al. have derived cardiomyocytes from human iPSCs isolated from HF patients.[122] However, iPSC development has several hurdles to overcome. A potential concern with iPSC is their low and inefficient reprogramming efficiency. The genome stability of iPSCs is low and thus poses the risk of tumorigenicity. Human studies are, therefore, some way from starting. Table 1.6 below shows the advantages and disadvantages of the various autologous cell types.

Table 1.6. Cell types and their advantages and disadvantages. + Advantages - Disadvantages

	ESC	BMC	MSC	EPC	SM	CDC	IPSC
+	Pluripotent	Ease of availability Excellent safety and feasibility profile Autologous (and allogeneic trials ongoing)	Low immunogenicity Widespread availability Multipotent	Available from bone marrow and peripheral blood Pro-angiogenic	Resistant to ischaemic stress Autologous i.e. no immunogenicity Ease of availability	Resident cardiac cells Low tumourigenicity Autologous Transdifferentiation	Pluripotent No ethical issues Autologous
-	Tumorigenicity Ethical hurdles Isolation Immunogenicity	Transdifferentiation debatable Unclear which cell subset beneficial Bone marrow aspiration uncomfortable	Difficult to isolate as low yield Need to expand in vitro Heterogeneity Low transdifferentiation capacity	No myocardial transdifferentiation Phenotypic definition unclear	No transdifferentiation or integration Arrhythmogenic potential Discouraging trial results	Need biopsy to obtain cells Low yield Need lengthy preparation	Low reprogramming rates Poor engraftment Tumourigenicity

1.2.5. Bone marrow derived cells (BMC)

The bone marrow contains a heterogeneous mixture of stem, progenitor and mature cells. The stem and progenitor population make up a very small proportion of the unprocessed cellular component of the bone marrow. The principal stem and progenitor cell components are haematopoietic stem cells (HSC), mesenchymal stem cells (MSC) and endothelial progenitor cells (EPCs)-henceforth, collectively referred to as bone marrow derived cells (BMC). BMC have been by far the most widely used stem cells in the field of cardiac regenerative medicine. The main reasons for this widespread use include ease of availability, high yield of cells thus not necessitating in vitro

expansion and the extensive experience of using these cells in haematology and haemato-oncology. However, a downside of using these cells is the invasive and "uncomfortable" nature of bone marrow aspiration to obtain the cells. The procedure is, however, safe. In a retrospective study by Bain et al. looking at bone marrow biopsy complications over a 7-year period, they found a 0.05% incidence of adverse events out of almost 55000 biopsies[123]. The key side effects reported were haemorrhage, needle breakage and infections. In another study, this time a prospective study, again by Bain et al, they found a 0.07% rate of adverse events over a 1 year period[124]. A common side effect from this procedure is bone pain which is usually transient. Despite these minor side effects, the majority of trials have used BMC. The stem and progenitor cells reside in a heterogeneous mixture of cells in the unfractionated bone marrow mononuclear cell component of the bone marrow.

1.2.5.1 Unfractionated bone marrow mononuclear cells

The bone marrow mononuclear cells are a heterogeneous mixture of BMCs, characterised by the presence of unilobulated, mononuclear cells that lack granules in the cytoplasm. These cells are of similar density and morphology and can thus be separated out by means of density gradient centrifugation. The key stem and progenitor cells of the bone marrow reside in this unfractionated bone marrow mononuclear fraction   However, the stem and progenitor cell population only make up a small fraction of the mononuclear cell population with mature, committed cells making up the remainder of the cells. The commonest way of separating out the mononuclear cell fraction involves, amongst other methods, adding Ficoll medium to the fresh bone marrow sample and centrifuging at room temperature to obtain the "buffy" layer (more details in methods chapter-chapter 2).

The key bone marrow mononuclear stem cell populations includes haematopoietic stem cells, mesenchymal stem cells and endothelial progenitor cells. Other cell types include side population cells [125] and very small embryonic-like stem cells (in mice bone marrow). MSC's have already been discussed in section 1.2.4. HSC and EPC make up only 2-4% of the mononuclear cell population in the bone marrow.

The HSC's give rise to haematopoietic lineages-both myeloid and lymphoid. The identity and isolation of HSC's has been the focus of much research. The HSCs are isolated by means of identifying a certain combination of cell surface proteins or "cell-surface markers" characteristic of HSCs. CD34+ is widely used as a marker of HSC. However, CD34 is not specific to HSCs and HSCs make up a small proportion of CD34+ cells. As the stem and progenitor cells mature into more committed, differentiated lineages, they tend to lose CD34 expression. To increase specificity of purification of HSC's further, some additional cell surface markers have been identified. HSC's do not express the Lin antigen on their cell surface, antigens which are present on mature cells. Furthermore, in humans, HSCs tend to express low levels of or no CD38 and CD45RA and higher levels (compared to differentiated cells) of CD90. HSCs are not exquisitely found in the bone marrow but also in the circulation. Peripheral blood HSC when injected into MI mice models showed transdifferentiation into cardiomyocytes. [126] However, whether human HSCs transdifferentiate into cardiomyocytes in real life, non-laboratory conditions is controversial with some groups showing evidence of transdifferentiation whereas other groups have failed to do so. [126, 127 128]

A subset of the haematopoietic stem cell progeny can differentiate into an endothelial phenotype ex vivo and these progenitor cells are termed endothelial progenitor cells. EPCs derived from bone marrow were first described by Asahara et al. in 1997,[129] although there is still considerable debate

and controversy regarding the correct cellular definition of EPC identity.[130] It is generally accepted that cells bearing the cell surface markers CD34+, CD133+ and the endothelial cell surface marker VEGFR2[131] represent EPC's. EPC in bone marrow are derived from HSC and induce re-endothelialisation and neovascularisation by either directly differentiating into endothelial cells or by secreting paracrine pro-angiogenic factors. EPCs also circulate in the bloodstream (known as circulating endothelial progenitor cells-CEPC). The exact identity and origin of CEPC are also debated.[130, 131] EPCs and HSCs are mobilised from the bone marrow to peripheral blood in response to cytokines such as granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF)[132] as well as hypoxic injury e.g. myocardial infarction or other forms of stress.[133] Low CEPC levels have been correlated with the severity of ischaemic HF in a study by Berezin et al. [134] In pre-clinical studies, human EPCs, injected into mice models of hindlimb ischaemia, showed significant improvement in blood supply and capillary density.[135] Kawamoto et al. culture expanded human CEPC and injected them intravenously into rat MI models and showed the incorporation of these cells into areas of neovascularisation as well as preservation of LV function. [136]

1.2.5.4 The transition from pre-clinical to clinical trials using bone marrow-derived cells

Preclinical studies using BMC have shown mixed results with some showing encouraging results.[137, 138] Tomita et al. showed an improvement in myocardial function and evidence of increased angiogenesis with intramyocardial injection of BMCs in rat models of chronic myocardial infarction. In 2001, Orlic et al., published the results of a "defining but controversial" point in the field of cardiac stem cell research.[139] In their article in Nature, Orlic reported transdifferentiation of BMCs into neo-myocardium when injected into transgenic mice models of myocardial infarction. Within a few months of the publication of Orlic's report, the first human study was underway. Very rarely in

medical science is a shift from bench to bedside seen so quickly and so prematurely. Ironically, a few other groups subsequently failed to reproduce the evidence of transdifferentiation into neo-myocardium as had been demonstrated by Orlic et al.[127] In the study by Murry et al, they injected haematopoietic stem cells into adult normal and injured mouse hearts and then used genetic techniques to track cardiomyocytes specific transgenes and found no evidence of transdifferentiation into cardiomyocytes. In the clinical setting, fewer studies using BMCs or subsets have been undertaken in the chronic HF setting when compared to trials in acute myocardial infarction patients. The results have been mixed. The premature translation of preclinical studies to clinical research is probably partly to blame for the lack of clarification on a number of factors which introduce heterogeneity in trial design including route of delivery, dosing of cells, cell preparation techniques, superiority of cell type and cell functional characteristics. These are likely to have played a part in generating these mixed results. Clinical trials using BMCs are discussed in detail next.

1.2.5.5 Clinical trials of BMC therapy in heart failure

Encouraging results from preclinical trials of cell therapy in HF led to a rapid transition to clinical research. The vast majority of trials to date have been small scale, phase II trials using BMCs with the aim of testing safety and efficacy. The trials have not raised any significant safety concerns. However, the efficacy results have been mixed with some trials suggesting improvement in LVEF whereas others have failed to do demonstrate this.

Perin et al., injected bone marrow mononuclear cells intramyocardially in 14 patients with chronic HF in a prospective, open-labelled, non-randomised study in 2003. They found a 9% improvement in LVEF, measured using echocardiography, in the cell treated group. [140] They also showed significant improvement in myocardial perfusion and functional capacity at 6 and 12 months

follow-up. [141] In 2005, Strauer et al, reported the results of the open-labelled, controlled IACT study, where they infused autologous, bone marrow mononuclear cells via the intracoronary route into 18 patients with ischaemic cardiomyopathy. They reported a 15% improvement in LVEF at 3 months along with a 30% reduction in infarct size. [142] The same author reported the results of the STAR-HEART study in 2010, an open label, non-randomised trial, and showed a significant improvement in LVEF at 5 years in 191 patients with HF who had received intracoronary BMC. [143] However, questions have been raised regarding the validity of this trial. [144] Two of the more recent trials - FOCUS-HF and FOCUS-CCTRN, both from the US, have failed to show any benefit in HF patients treated with intramyocardial, autologous BMC therapy. [145, 146] FOCUS-HF was a small single-blinded, placebo-controlled trial (n=30). The FOCUS-CCTRN trial was a larger (n=92), phase II, randomised, double blinded study. Both trials demonstrated no safety concerns. However, neither trial showed an improvement in the primary efficacy end-points {LVEF, MVO2 (peak oxygen consumption on cardiopulmonary exercise testing) in FOCUS-HF and left ventricular end systolic volume-LVESV index or reversible perfusion defect in FOCUS-CCTRN}. In FOCUS-HF, although there was no improvement in the primary end-point, younger patients (≤60 years) were noted to have a higher BMC proliferative capacity and significant improvement in MVO2. Similarly, in the FOCUS-CCTRN trial, the increase in LVEF was greater in patients with higher BMC counts. Both these trials suggest a mechanistic link between cell numbers and proliferative capacity and trial outcomes and emphasise the need for further studies.

The TAC-HFT [147] study was a phase 1 and 2 randomised, blinded, placebo-controlled study looking at transendocardial injection of MSC or BMC as compared to placebo in a total of 65 patients with ischaemic cardiomyopathy. The study found no safety concerns. In both cell treated groups, the quality of life improved as assessed using Minnesota Living with HF questionnaire. Functional

improvements were noted in the MSC treated groups with significant improvement in the 6 minute walk test (6MWT) distance. However, no significant change in LVEF or LV volumes were detected.

Preclinical and clinical trials with MSCs have been encouraging. The results of the first double-blinded placebo controlled trial with transendocardially injected autologous MSC in HF (MSC HF trial) has been presented at the ACC scientific sessions.[148] The trial showed a significant reduction in LVESV (-8.2±14.2ml) (the primary endpoint) and increase in LVEF (5.5±3.8%) in the cell treated group and there was functional improvement in the cell treated group, although a similar functional improvement was also noted in the placebo group. This raises the question of how important these cardiac functional endpoint improvements are, if they do not translate to well-defined improvements in patients' clinical wellbeing.

Other ongoing clinical trials include the randomised, phase I/II ASSURANCE trial looking at direct intramyocardial injection of BMC in patients undergoing left ventricular assist devices for severe heart failure (NCT00869024). [149] The REPEAT trial, led by Prof Zeiher and team from Frankfurt, is a phase II/III trial investigating whether repeat IC or single IC autologous bone marrow mononuclear cells is more effective in reducing mortality (NCT01693042).[150]

Most trials to date have studied the effect of BMC therapy on systolic function and LV volumes as well as patients' functional status but not on diastolic function. Yao et al. looked at the effect of intracoronary delivery of autologous BMC on systolic and diastolic function and found that BMC improved diastolic function but not systolic function.[151] Further studies are necessary to study the effect of cell therapy on diastolic function.

1.2.6 Use of adjunctive therapy and allogeneic cells

1.2.6.1 Adjunctive therapy

Novel approaches are being used to "manipulate" cells before administration. In the C-CURE trial, autologous bone-marrow derived mesenchymal cells were exposed to a 'cardiopoietic cocktail' (exact ingredients unknown) that enhanced differentiation into cardiac progenitor cells which were then injected intramyocardially. It showed improved LVEF and LVESV as well as increased 6MWT distance.[152] In another approach, extracorporeal cardiac shock wave was administered prior to BMC therapy in the CELLWAVE trial. They demonstrated a significant improvement in LVEF in the shockwave-treated BMC group compared to the group receiving shockwave and placebo infusion only.[153] . Granulocyte colony stimulating factor (G-CSF) is a potent haematopoietic cytokine used as an adjunct as well as on it's own in a few clinical trials to mobilise BMC from the bone marrow and is discussed in more detail in section 1.2.7.

1.2.6.2 Allogeneic cells

Allogeneic products are appealing for at least two reasons: the availability as "off the shelf" products thus obviating the need for invasive bone marrow aspiration in the patient as well as the possibility of using these cells when the autologous cells are found to be dysfunctional. It is known that in HF and with age, stem cell functional characteristics decline. [154] It is not clear how this decline relates to clinical potency for cardiac repair. The key concern regarding the use of allogeneic cells has been the risk of immunogenicity. In the phase 1/2 randomised, but not controlled, POSEIDON trial, which compared autologous and allogeneic mesenchymal stem cells, delivered transendocardially, in patients with ischaemic cardiomyopathy[155], there were no findings suggestive of significant safety concerns. However, although the autologous group showed

improvements in some patient functional outcomes (quality of life questionnaires, 6 minute walk test) and the allogeneic group showed a reduction in LVEDV, no overall improvement in LVEF was seen. The trial demonstrated an inverse relationship between efficacy and number of cells injected as well as a reduction in scar size with cell therapy. However, more definitive phase III studies will be necessary before reaching a conclusion regarding efficacy. The ongoing ALLSTAR trial will look into allogeneic CSC transplantation (NCT01458405 clinicaltrials.gov).

1.2.7 G-CSF in stem cell trials

G-CSF is a potent cytokine known to mobilise haematopoietic cells as well as mesenchymal stem cells.[156, 157] It is commonly used in daily haematological practice and this extensive experience from haematology suggests that it is safe to use in the vast majority of patients. The most commonly reported adverse effects from G-CSF administration include bone pain and headache. In a study by Anderlini et al, of 341 patients receiving G-CSF, 84% complained of bone pain, 54% of headache and 31% of fatigue.[158] In animal studies, G-CSF has been shown to improve cardiac function and survival after myocardial infarction.[159, 160] G-CSF mobilises HSC from the bone marrow by modulating the SDF-1/CXCR-4 axis. SDF-1 or stromal cell derived factor-1, a chemotactic chemokine, and CXCR-4, its receptor, appear to play a critical role in retaining HSC in the bone marrow. The gradient between bone marrow SDF-1 and peripheral blood SDF-1 controls the retention of HSC in the bone marrow. Disruption of this gradient results in mobilisation of stem cells into the peripheral blood. Administration of G-CSF results in the production of a proteolytic environment in the bone marrow with increased levels of metalloproteinase-9, neutrophil elastase and cathepsin G, which appear to degrade and disrupt the SDF-1/CXCR4 axis. [161] In the study by Fukuhara *et al* on mice, administration of G-CSF before and after artificially inducing an infarct, resulted in significant numbers of BMCs in the peri-infarct area compared to those mice that didn't

receive G-CSF. Furthermore, some of these bone marrow derived cells were found to express markers suggesting differentiation into cardiomyocytes[159]. Other studies have suggested that G-CSF might have a direct effect on cardiomyocytes. In the study by Harada et al., published in Nature Medicine, they showed that G-CSF resulted in activation of anti-apoptotic signalling via the JAK-STAT pathway and thus conferred a survival advantage on the cardiomyocytes.[162] Human trials have looked at whether treatment with G-CSF, either by virtue of cell mobilisation or as a paracrine factor, leads to improvement in cardiac function, either alone or in combination with stem cell administration. However, most of these trials have been small and non-randomised, non-controlled and results have been mixed. [156, 157, 163-166] Some have suggested an associated improvement in LVEF [165] although most of the other studies have shown a lack of improvement in LVEF in both ischaemic cardiomyopathy [164] and in subjects with coronary artery disease.[156, 157]A few trials have raised concerns as to whether G-CSF increases the rate of in-stent restenosis in patients with previous PCI [167] although other subsequent trials have not borne this out. [168] Table 1.8 lists cell therapy trials in HF, including trials that have used G-CSF as adjunctive therapy.

1.2.8 Summary of cell therapy trials in HF

The phase II trials of cell therapy have shown mixed signals of effect. The trials have, however, demonstrated that BMC therapy as well as cardiac stem cell therapy appear to be safe. There are several possible reasons for the lack of translation of the beneficial effects seen in preclinical models into clinical research (Table 1.7). The positive and negative signals of effect, bearing in mind the heterogeneity of methods and endpoints in the trials, paves the way, using the experience from these trials, for further phase II trials with modification of method/endpoints as well as, most importantly, phase III trials.

1.2.9 Mechanisms of action

The proposed mechanism of action of stem and progenitor cells is unclear and remains a matter of significant debate. There are several postulated mechanisms and it is likely that they are not mutually exclusive (figure 1.3). They include 1. Transdifferentiation of stem and progenitor cells into cardiomyocytes 2. Transdifferentiation of stem and progenitor cells into blood vessels 3. Cell fusion and 4. Paracrine hypothesis. The original work by Orlic et al. suggested that the primary mechanism of action of cell therapy was transdifferentiation.[139] However since then, numerous studies have demonstrated conflicting results with some animal studies supporting transdifferentiation [137, 169] yet others showing no evidence in favour of transdifferentiation. [127, 170] It is now clear that transdifferentiation does not occur to the extent that would explain the observed improvements in cardiac function seen in clinical and pre-clinical work. Differentiation of stem and progenitor cells into endothelial phenotype cells has been demonstrated in previous studies. In the study by Wang et al., they injected human CD34+ cells in immunodeficient mice models of MI and noted differentiation of the CD34+ cells into human-derived endothelial phenotype cells[171]. In the study by Tillmanns et al, they demonstrated the formation of de novo coronary artery like structures after injection of cardiac progenitor cells into rat models of coronary occlusion.[172] This latter point is particularly relevant, i.e. the presence of total coronary occlusion or flow limiting disease, as this mechanism of action is likely to be beneficial and important to patients with such disease. In patients without occlusive or flow limiting coronary disease, this mechanism is unlikely to explain the majority of benefit in cardiac function, although it is likely to be an important contributory element in the cardiac improvement. The evidence for cell fusion between stem cells and endogenous cardiomyocytes comes from a few studies. [181-183] However, the observed level of

fusion was too low to adequately explain any improvement in cardiac function. This postulated mechanism of action has fallen out of favour.

Table 1.7. Potential reasons why results of preclinical stem cell research fail to translate

(Adapted from [173] Choudhury T, Mathur A. The birth of regenerative pharmacology. 2012)

- **Not enough cells injected in human trials compared to animal studies**

- **Timing of cell injection – different time course of remodelling in animals compared to humans**

- **Cell characteristics- difference in cell characteristics between animals and humans**

- **Delivery method-concentration of cells at target lower in humans than in animals due to low retention**

- **Animal heart failure models not very representative of human heart failure and hence dissimilar results**

- **Low regenerative potential in cells from elderly patients and patients with heart failure**

Table 1.8 Clinical trials using bone marrow derived cells in ischaemic cardiomyopathy. IC-intracoronary; IM-intramyocardial; BMMNC-bone marrow mononuclear cells; CABG-coronary artery bypass graft surgery; MRI-magnetic resonance imaging ; SPECT-single photon emission computed tomography; QLV-quantitative left ventriculography; CPC-circulating endothelial progenitor cells

TRIAL	Cell type?	G-CSF	Randomised	Placebo-controlled	Double blinded	Route(s)	Co-intervention	Imaging modality	Main outcomes
Patel 2005[174]	CD34	No	Yes	No placebo control	No	Subepicardial injection	CABG	Echo, SPECT, angio	Improved LVEF
Hu 2011[175]	BMMNC	No	Yes	Yes	Yes	IM	CABG	MRI	Improved LVEF, LVESVI and WMSI
FOCUS-HF[146]	BMMNC	No	Yes	Yes but placebo was mock injection procedure only	No	IM	No	Echo, SPECT	No change in LVEF CCS and QoL improved
FOCUS-CCTRN[145]	BMMNC	No	Yes	yes	Yes	IM	No	Echo, SPECT	No change in LVESV index, maximal oxygen consumption
Pokushalov 2010[176]-	BMMNC	No	Yes	No-optimal medical therapy	No	IM	No	Echo, SPECT	Improved LVEF, CCS and NYHA
Zhao 2008[177]	BMMNC	No	Yes	Yes	?No	IM	CABG	Echo, SPECT	Improved LVEF, CCS, perfusion
Perin 2003[140]	BMMNC	No	No	Yes	No	IM	No	Echo, SPECT	LVEF up LVESV and LVEDV down
Assmus 2006-TOPCARE-CHD[178]	BMMNC or CPC	No	Yes	Control=no cell infusion; was a crossover study	No	IC	No	Echo, SPECT, MRI	LVEF up and NYHA down (BMMNC only)
Assmus 2012-CELLWAVE[153]	BMMNC	No	Yes	Yes	Yes-in second phase	IC	Shockwave	QLV, MRI	Improved LVEF
Assmus 2012-TOPCARE-G-CSF[164]	CPC	Yes	Yes	No	No	SC (G-CSF) /IC (cells)	No	QLV, MRI	No significant effect on LVEF
Gao 2006[179]	BMMNC	No	Yes	No placebo infusion-control group had standard medical care	No	IC	No	Echo	Improved LVEF; LVESV, BNP decreased
Ang 2008[180]	BMMNC	No	Yes	No-CABG only as control	-	IC (via graft) AND IM-directly into scar not peri-scar	CABG	DSE	No change in LVEF, LV volumes or infarct size
STAR-HEART[143]	BMMNC	No	No	No	No	IC	No	SPECT, QLV	Improved LVEF

The mechanism that has gained increasing support and is currently the most accepted is the paracrine hypothesis. Several studies have shown that administered stem cells secrete numerous cytokines, chemokines and   growth factors that can exert beneficial effects. These 'paracrine' effects include but are not limited to anti-apoptotic cell signalling, stimulation of cell homing and induction of neo-angiogenesis (table 1.9). Gnecchi et al demonstrated a reduction in apoptosis of ischaemic rat cardiomyocytes exposed to conditioned medium from MSC exposed to hypoxic stress. The same group overexpressed Akt, a protein kinase that inhibits cellular apoptosis, in MSC's and then administered conditioned medium from these cells to animal infarct models and demonstrated significant reduction in apoptosis and infarct size. [184, 185] MSCs have also been shown to exert a wide array of proangiogenic cytokines including VEGF (vascular endothelial growth factor) and bFGF (basic fibroblast growth factor).[186] Work on bone marrow mononuclear cells has also suggested a paracrine mechanism. In the study by Kamihata *et al* using rat ischaemic models, they showed significant increase in the levels of proangiogenic cytokines after injection of bone marrow mononuclear cells into ischaemic myocardium.[187] Takahashi *et al.* cultured rat bone marrow mononuclear cells and then analysed the supernatant culture medium. They found various cytokines including VEGF and IGF-1 (insulin like growth factor-1) in the supernatant. In vitro, this supernatant inhibited cardiomyocyte apoptosis and maintained cell contractility. They then injected the supernatant into rat ischaemia models which resulted in increased angiogenesis, reduction in scar size and significant improvement in cardiac function.[188] Other paracrine effects include stimulation of endogenous stem cells by paracrine mediators and modulation of the extracellular matrix. There is evidence that administered stem cells might work by stimulating and recruiting endogenous cardiac stem cells from their 'niches'. The work by Hatzistergos et al. showed that MSC injected in swine models resulted in engrafted and well integrated MSCs,

endogenous CSC's and adult cardiomyocytes on histological examination of the heart tissue.[96] CSC

and SM have been shown to have beneficial effects on the extracellular matrix by modulating levels

of matrix metalloproteinases and their inhibitors, tissue inhibitor of metalloproteinase (TIMP), both

important mediators of extracellular remodelling[189, 190]. To date, several factors have been

identified as paracrine mediators of stem cell action (table 1.9).
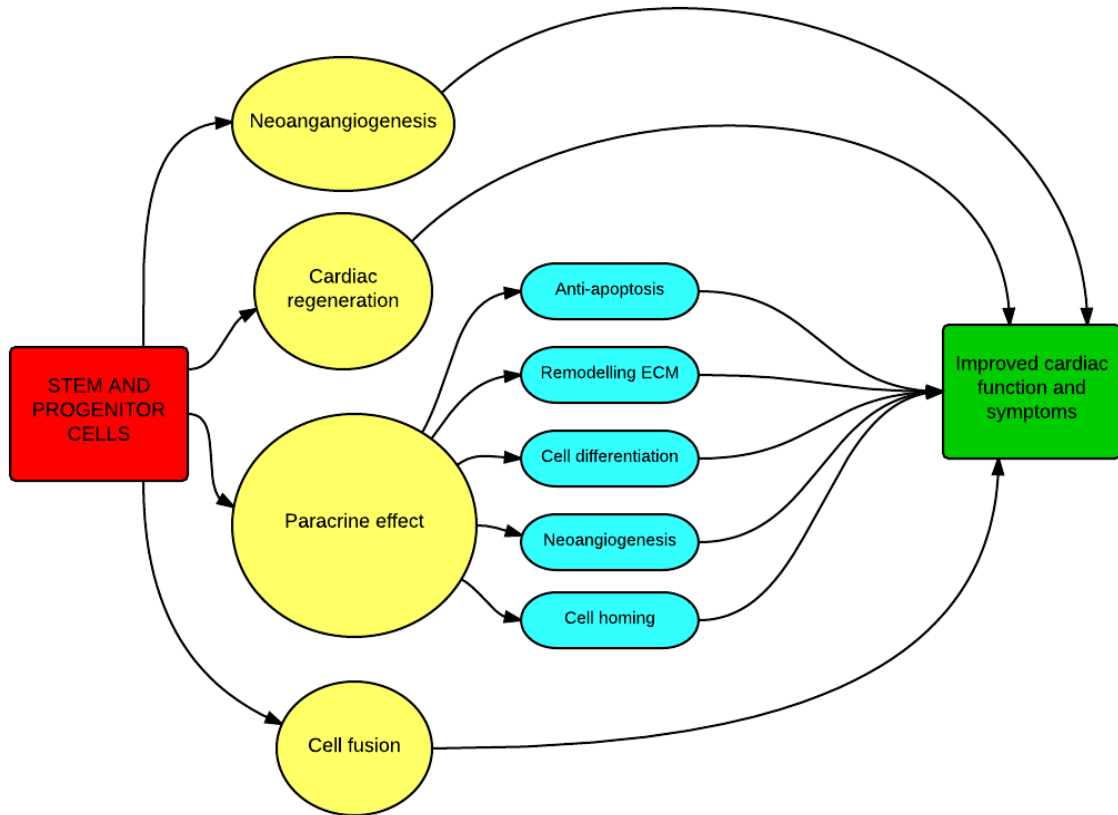
Figure 1.3. Mechanisms of action for cell therapy. Multiple mechanisms of action of stem and progenitor cells in cardiac

repair have been postulated. The paracrine effect has gained most support.

Table 1.9. Paracrine effectors of stem and progenitor cells

PARACRINE EFFECTS	PARACRINE EFFECTORS
Cell survival	SDF-1, bFGF, HGF, VEGF, IGF-1, MCP-1, SFRP-2, IL-10
Induction of neoangiogenesis	VEGF, SDF-1, bFGF, IGF-1, MCP-1, HGF, IL-1β, Angiopoietin 1 and 2
Stimulation of cell homing/migration	SDF-1, TNF-α, TIMP 1 and 2, MIP 1α, IGF-1
Remodelling of extracellular matrix	IL-10, thymosin β4, TIMP 1 and 2, HGF, MCP-1
Cell differentiation	VEGF, IGF-1, HGF, TNF-α

1.2.10 Cell delivery methods

Several cell delivery methods have been used in clinical trials so far. No direct comparison between all the delivery methods has yet been carried out in ischaemic cardiomyopathy patients although meta-analysis[191] suggests that the intramyocardial (IM) route of delivery is superior in HF patients in terms of improvement in LV function. The various routes of cell delivery are discussed in the following section (figure 1.4).

a) Transcatheter intramyocardial injection

The procedure involves administration of the cells via injection directly into the myocardium using a catheter based approach. There are several catheter systems in use, including the Helix, Myostar and Stilletto systems. The operator uses imaging (e.g. MRI guidance) or electromechanical mapping (EMM) guidance to detect areas of infarction and thus helps localise the target areas of injection in the peri-infarct area. The NOGA® electromechanical mapping system is commonly used for the purpose of EMM. It measures transmembrane voltage, a reflection of viability, and linear local shortening, a measure of contractility, to map out areas of infarction and viable tissue. The IM

route has advantages including the option of directly injecting cells into the target area of myocardium. Furthermore, patients with chronic ischaemic heart disease may not have patent coronary arteries supplying the myocardial region of interest and hence, the IM route offers an alternative. The procedure does not require open surgical intervention. However, the procedure is not without its risks. Significant risks include myocardial perforation and significant, ventricular arrhythmias. Furthermore, the retention of cells at the site of interest, although better than the intracoronary route, is still quite poor. Hou et al. looked at cell retention after cell administration via 3 different routes in swine ischaemic models and found that the IM route had the most retention at 11+/-3% when compared to intracoronary delivery where the retention was 2.6+/-0.3%.[192]

b) Intracoronary injection

The intracoronary (IC) route has been the most widely used route in clinical trials for obvious reasons- it can be easily used by interventional cardiologists and does not require any extra training. Furthermore, the procedure can be done at the time of PCI and is safe to do after AMI. Another advantage of this route is that the cells are distributed more evenly over the area of interest. However, as mentioned above, the retention of cells is very low. The most commonly used method of IC infusion of cells involves transient occlusion of the artery. A standard, 0.014" over-the-wire balloon is used and the cell suspension/placebo infused distal to the occluding balloon using a stop-flow technique as previously described by Assmus et al. [193] The reasoning behind this technique is that transient occlusion of the artery proximal to the cells will maximise retention of the cells. The other approach is to infuse the cells while maintaining coronary flow. Most of the

trials using IC delivery show that it is a safe procedure. In a recent retrospective study by Assmus et al., looking at 775 procedures involving IC cell administration using the stop-flow technique, they demonstrated that IC cell delivery posed no additional risk to that of a conventional angiogram.[194] However, the efficacy of this route in terms of improvement in cardiac function is not so clear. In a review article by Sheng et al, they looked at 30 studies since 2002 that used IC cell delivery and questioned the actual effect of IC cell delivery on cardiac function [195]. Furthermore, Dib et al. studied the viability of MSCs before and after passage through a standard, OTW balloon catheter. They noted that the inner lumen of the catheter partially collapses during balloon inflation. Cell viability was reduced at both low and high flow rates and with a range of inflation pressures suggesting that use of standard OTW balloon catheters might affect cell viability and thus efficacy outcome.[196]

c) Surgical intramyocardial injection

In contrast to transcatheter IM injections, this involves a direct, surgical approach, necessitating open-heart surgery or lateral thoracotomies. As a result, this route is more prone to significant complications associated with the invasive nature of the procedure as well as the risk of myocardial perforation and ventricular arrhythmias associated with IM injection. The advantage of this route is the direct visualisation and injection of cells into the peri-infarct area. However, as with other routes, the retention of cells is also a problem as cells have been shown to leak out of the injection site. In a study by Grossman et al., they injected 15 pigs via transcatheter IM approach as well as a direct, open-chest surgical approach and found a lower retention in the direct surgical approach pigs as compared to the transcatheter approach pigs (15% v 43%; p<0.01).[197] The other disadvantage is that this route is usually reserved for patients who are due to undergo cardiothoracic surgery and not an ideal route across the spectrum of cardiac patients.

d) Venous approaches

Intravenous: The intravenous route is obviously the least invasive route and one that can be used across centres as it requires minimal training. However, it has the distinct disadvantage of not delivering cells directly either into the myocardium or into the coronary circulation. As a result, the success of the procedure relies on the ability of the cells to home to the target of interest i.e., the heart. However, in chronic HF patients, the migratory capacity of the cells may be impaired as may the trophic signals from the heart and as a result this route of delivery is unlikely to be very successful in ischaemic cardiomyopathy patients. In a pre-clinical study by Barbash et al, they found that intravenous delivery of bone marrow derived mesenchymal stem cells to post-MI rats had a significant reduction in cell delivery to the heart due to entrapment in the lungs[198].

Coronary sinus and coronary veins: Retrograde cell injection via the coronary sinus has also been used. This allows retrograde delivery of the cells where the coronary arteries of interest are occluded. However, in ischaemic cardiomyopathy patients, a significant number have biventricular pacemakers which involve passing a lead via the coronary sinus. Hence, in these patients, this route cannot be used. Furthermore, the success of the procedure is reliant on the venous anatomy and in patients with complex venous anatomy this can be a problem.

TRANSVENOUS-
intravenous or
coronary sinus

INTRACORONARY
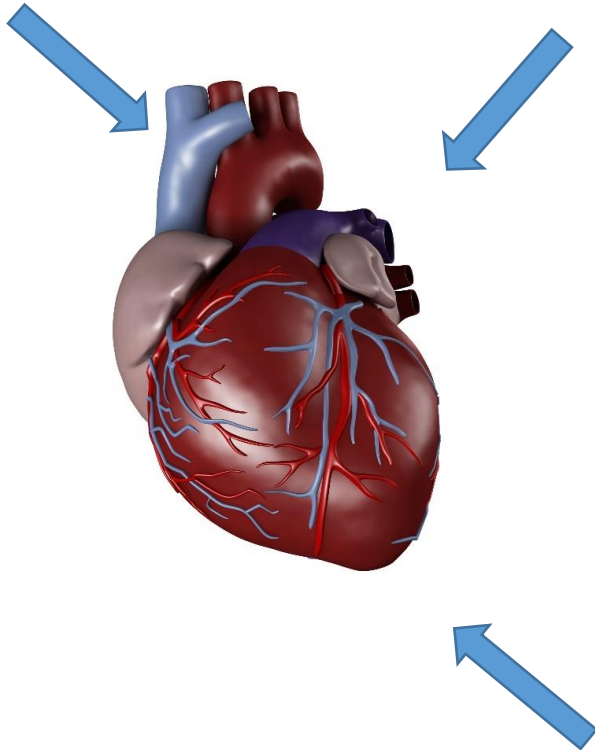
Figure 1.4. Routes of cell delivery.
Figure showing the various routes of
delivering stem and progenitor cells to the
heart.

INTRAMYOCARDIAL
-percutaneous or
surgical

1.2.11 Meta-analyses of BMC therapy trials in HF

To gauge some sort of conclusion regarding the efficacy of BMC therapy and the best route of cell

delivery in heart failure, one has to look at meta-analyses. In a recent meta-analysis, by Fisher et

al., of autologous BMC in chronic heart disease, a +5.95% mean difference in LVEF in the sub-set of

patients with heart failure receiving cell therapy when compared to controls was observed.

Furthermore, a +5.30% mean difference in the intramyocardial (IM) cell therapy group (which was

statistically significant when compared to the 3.19% improvement in the intracoronary (IC) group)

was noted, suggesting superiority of the IM route. The findings are also in agreement with three

other meta-analyses of cell therapy and heart failure: Kandala et al. (5.13% via IM route) [199], Jeevanantham et al. (3.96%; not route specific) [200] and Fisher et al. (3.47%; 8 of 9 trials via IM route).[201] The evidence for using G-CSF as a pharmacological agent in HF comes from small, non-randomised trials and has been mixed with most trials showing no improvement in LVEF as discussed above. Thus the overall conclusion from meta-analyses is that cell therapy does improve cardiac function, albeit modestly, and that the intramyocardial route appears to be superior to the alternative available routes, although no direct trial evidence of this is available in ischaemic cardiomyopathy patients.

1.2.12 Unanswered questions

Firstly, the effects of G-CSF in ischaemic cardiomyopathy have been mixed with a number of studies showing no benefit of G-CSF, with some studies actually suggesting a possible deleterious effect with respect to LVEF and increased rate of restenosis in patients. However, the theoretical attraction of G-CSF, in terms of enhanced cell mobilisation and aid in cell homing, remains. This together with the positive results from pre-clinical studies, means that further research using this cytokine is imperative. Secondly, although G-CSF itself has been used in several trials as a solo "therapeutic" agent, the full benefit may require the delivery of autologous BMC to the heart to be seen. So far, no study has compared the effects of G-CSF alone and in combination with BMC in ischaemic cardiomyopathy patients. Thirdly, the optimal route of cell delivery is still unclear. As discussed in detail in section 1.2.10, the intracoronary and the intramyocardial routes have been the most widely used routes in clinical trials. However, to date, no single trial in ischaemic cardiomyopathy patients using autologous BMC has compared the intracoronary to the intramyocardial route. As discussed in section 1.2.5, to date, several trials have looked at the effect

of BMC on cardiac function but quite a few have lacked true placebo-control arms (table 1.7). There is therefore a need for a study assessing whether G-CSF administration alone or in combination with either the IC or targeted IM injection of autologous bone marrow derived cells (BMC) leads to an additional benefit on cardiac function compared to placebo controls. The vast majority of studies to date have concentrated on systolic cardiac function as the key outcome. However, there are few studies looking at the effect of G-CSF and cell therapy on diastolic function. The prevalence of heart failure with preserved ejection fraction is estimated to be up to 50% of the heart failure population.[18] Thus, studying the effect of G-CSF and BMC on diastolic function would be highly relevant to heart failure therapy. There is also a paucity of studies looking at the effect of G-CSF and cell therapy on the levels of various cytokines involved in heart failure (discussed in section 1.1.2.3) and their association with effects on cardiac function.

1.3 Hypothesis and aims

The main aim of this thesis is to test the hypothesis that subcutaneous G-CSF alone, or in combination with autologous bone marrow derived stem cells, improves cardiac function in patients with ischaemic cardiomyopathy.

Specific aims

1. To determine whether G-CSF administration alone or in combination with bone marrow-derived cells is safe and results in an improvement in left ventricular function in patients with ischaemic cardiomyopathy

2. To determine the optimal route of delivery for therapy: specifically testing whether the intracoronary or the intramyocardial route is superior in terms of efficacy

3. To assess the mechanisms by which improvements in LVEF and/or symptoms may have occurred specifically assessing cytokine levels, myocardial scar size and diastolic function.

4. To assess the relationship between cell characteristics and change in LVEF e.g. numbers of cells delivered and CFU-GM colonies

Chapter 2 Methods

2.1 THE REGENERATE IHD trial

2.1.1 Introduction

The MDRes thesis is based on the results of the REGENERATE-IHD trial primary and secondary outcomes as well as sub-studies designed to gain mechanistic insights into the action of G-CSF and BMC therapy. The REGENERATE-IHD trial was an investigator-initiated, randomised, placebo-controlled, single-centre trial. The trial was designed to fill certain unexplored gaps in knowledge on the effects of G-CSF and BMC therapy in ischaemic HF, as discussed in section 1.2.12. The trial attempted to address this by being a unique clinical trial that 1. investigated the effects of G-CSF alone 2. investigated the effects of G-CSF in combination with autologous BMC 3. administered cell therapy via the intracoronary (IC) and targeted intramyocardial (IM) routes 4. had appropriate comparator groups for each arm of the trial. The trial assessed whether G-CSF administration alone or in combination with either the IC or targeted IM injection of autologous bone marrow derived cells leads to an additional benefit on cardiac function compared to placebo controls (serum). The trial was initiated following a pilot study to address safety and feasibility.[202]

Ethics and trial registration

The Local Research Ethics Committee approved the protocol (REC no. 04/Q0603/13). The trial was registered with www.clinicaltrials.gov (NCT00747708) and the European Clinical Trials register (EudraCT no. 2005-002706-27). Approval was obtained from the MHRA. The trial was conducted in accordance with the Declaration of Helsinki and was in agreement with the revised Declaration of Helsinki (October 2013, Fortaleza, Brazil).

2.1.2 Trial design

The trial was designed to assess the efficacy of G-CSF alone and in combination with autologous BMC via two different routes of cell delivery. Thus, there were three separate arms in the study, namely peripheral, intracoronary and intramyocardial. Each arm had an appropriate comparator or control group. The peripheral arm was designed to test the efficacy of G-CSF alone with the control group being subcutaneous saline. In the intracoronary arm, the participants were designated to either receive G-CSF and intracoronary cells or G-CSF and intracoronary placebo (autologous serum). The third arm-the intramyocardial arm- was designed to test the efficacy of G-CSF with intramyocardial cell therapy (active group) as compared to G-CSF and intramyocardial placebo (autologous serum). The primary end-point was chosen as change in left ventricular ejection fraction (LVEF) as measured using advanced cardiac imaging. The vast majority of cell therapy trials to date have assessed change in LVEF as one of the main outcomes. LVEF is associated with prognosis in heart failure patients. Furthermore, advanced cardiac imaging with cardiac magnetic resonance (CMR) is now recognised as the most accurate way of assessment of LVEF. [203] Hence, it was decided to use LVEF as the primary endpoint in the trial-to enable comparison with other studies and due to availability of advanced cardiac imaging.

Power calculation

Following a pilot study, as requested by the ethics committee, the study was powered to detect a 3·5% within group improvement in the primary endpoint i.e. change in LVEF at twelve months based on changes seen in a contemporary review of cell therapy.[204] Based on a power of 90%, a significance level of 5% and an estimated within observation error of 4%, the calculated required

number of patients in each group was 11. It was estimated that an additional four per group would be needed in order to ensure that 11 patients reached the primary endpoint at 1 year, resulting in a size of 15 patients per treatment group.

Statistical design

A paired t-test was used to detect any statistical significance of within group changes in LVEF. For additional analyses using continuous variables, appropriate parametric (paired-t for paired and independent samples t-test for non-paired data, one-way ANOVA for multiple comparisons) and non-parametric (Wilcoxon signed –rank test for paired and Mann-Whitney for non-paired data) tests were used. Chi-squared or Fisher's exact tests were used for categorical variables. Pearson's linear regression was used for comparison between LVEF and cell function variables as well as cytokines. Values are quoted as mean ± SD unless otherwise stated. All p-values are two sided and $p < 0.05$ considered to indicate statistical significance. Statistical analyses were performed using SPSS® version 21 (IBM Corp., Armonk, NY, USA) and graphs were produced using Graphpad Prism® version 6·0 (GraphPad Software, San Diego, CA).

Study endpoints

Primary endpoint

The primary endpoint was the change in global left ventricular ejection fraction (LVEF) at 12 months relative to baseline, measured by advanced cardiac imaging (CMR or cardiac CT where CMR contraindicated).

Secondary endpoints

Secondary Endpoints at 6 months

Change in global LVEF measured by resting contrast echocardiography

Change in global LVEF measured by quantitative left ventriculography

Change in NYHA class

Change in serum levels of NT-proBNP

Change in quality of life scores-using MacNew, EQ5D and SF-36 questionnaires

Occurrence of major arrhythmias defined as ventricular tachycardia or survived sudden death

Secondary Endpoints at 1 and 2 years

The change in LVEF relative to baseline measured by resting contrast echocardiography

Change in NYHA class compared to baseline

Change in quality of life scores- assessed using MacNew, EQ5D and SF-36 questionnaires

The occurrence of a Major Adverse Cardiac Event (MACE) - MACE defined as cardiac death, percutaneous coronary intervention (PCI), coronary bypass graft surgery (CABG) or myocardial infarction

Patient selection

Patients were referred to the trial with a confirmed diagnosis of HF from local HF clinics. Patients underwent screening by the research nurse or physician prior to inclusion in the study to ensure the inclusion criteria were met and that no exclusion criteria were present. All potential trial participants received detailed information regarding the objectives and methodology of the trial and the post-trial follow-up arrangements. A detailed patient information sheet (PIS) was provided to each patient by the research nurse. The potential participant was given ample time to think about the pros and cons of participating in the trial. Following this period, the research team answered any further questions raised by the patient. Once the patient was happy to go ahead, the

participant was asked to sign a consent form to indicate informed consent. The participant was informed of his/her right to change their mind at any point in the trial. The participant also consented to the use of his/her data gathered during the trial as well as any remaining bone marrow and stored blood sample for future trial related research.

Inclusion and Exclusion criteria

Inclusion criteria
- Confirmed diagnosis of HF secondary to ischaemic heart disease

- Established on optimal and stable medical therapy for at least 6 months

- Documented impaired left ventricular ejection fraction (LVEF)

- NYHA II-IV

- No further treatment options.

Exclusion criteria
- Acute coronary syndrome within the preceding 6 months

- Cardiogenic shock

- Atrial fibrillation

- Impaired renal function (serum creatinine >200μmol/l)

- Serious concomitant illness with a life expectancy of <1 year

- Contraindication to bone marrow aspiration

- Chronic inflammatory disease

- Active infection

- Known infection with human immunodeficiency virus

- Hepatitis B virus, hepatitis C virus, syphilis or Human T-cell lymphotropic virus.

Randomisation

Patients were randomised to 1 of 3 arms: peripheral, intracoronary (IC) or intramyocardial (IM) (figure 2.1). The first 30 patients were allocated to the IM arm as the aim was to complete this arm first. This was done as treatment in this arm required the presence of expensive equipment for electromechanical mapping (NOGA®) as well the presence of senior clinicians and technical staff trained in the use of such equipment. The remainder of the 60 patients were randomised in a 1:1 manner to the other 2 arms (peripheral or IC), using a dedicated trial software system (HD Clinical, Bishops Stortford, Herts, UK).

Once allocated to an arm, the patient was randomized in a 1:1 manner to receive either the active intervention or placebo. In the peripheral arm, patients received either subcutaneous G-CSF (active) or saline (placebo). In the IC arm, patients received either IC BMC (active) or IC autologous serum (placebo). In the IM arm, patients received targeted IM injection (with the aid of electromechanical mapping) of BMC (active) or autologous serum (placebo).
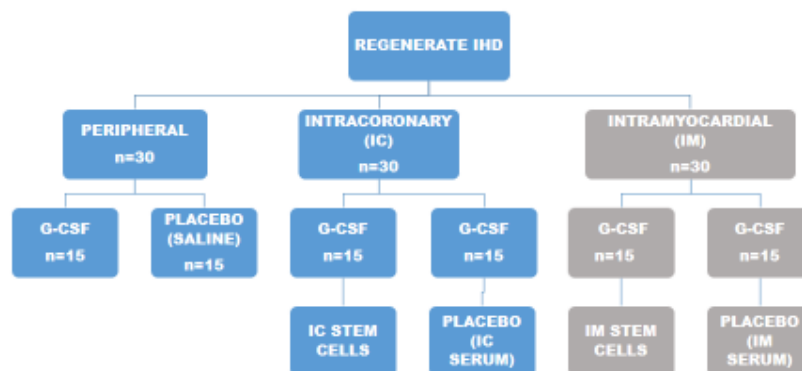
Figure 2.1. Structure of the REGENERATE-IHD trial. Diagram showing the three arms of the trial. Each arm has 30 participants divided into an active intervention and a placebo group. G-CSF-granulocyte colony stimulating factor

Treatment procedures

Peripheral

In the peripheral arm, the participant was admitted to the general cardiology ward for a period of 5 days. All patients, apart from those in the peripheral placebo group, received their first dose of subcutaneous G-CSF (10µg/kg/day), which was continued for 5 days. Patients in the peripheral placebo group received their first dose of subcutaneous saline. At the end of day 5, patients in the peripheral arm were discharged.

Intra-myocardial and intra-coronary

In the IC and IM arms, the participant was admitted to the hospital 6 days prior to the cell/placebo reinfusion. All patients received their first dose of subcutaneous G-CSF (10mcg/kg) on the day of admission and then daily for 5 days.

On the morning of the procedure, all patients had 30mls of blood taken under strict aseptic techniques. This blood was used to obtain serum in which the BMC were suspended. Further blood was obtained for CD34+ count and clotting (if necessary) for patients on warfarin. Following the above, bone marrow aspiration was performed. The blood and bone marrow sample were then sent to the stem cell lab under sterile conditions.

Bone marrow aspiration and cell processing

Bone marrow aspiration

Informed, written consent for the procedure was obtained on the day of the procedure. Under aseptic conditions and using local anaesthetic, 100mls of bone marrow was aspirated from the posterior iliac crest (figure 2.2). [205] 5mls of aspirate was collected in each of 20 syringes. Each syringe was pre-rinsed with heparin and contained 1ml of heparin in it. The syringes were sent by

taxi in ice to the stem cell processing lab at the Royal London hospital, a fully compliant good manufacturing practice laboratory. The cells were suspended in autologous serum to make up a volume of 10mls (for the IC arm) and 2mls (for the IM arm) (see next section). The placebo (serum) was also prepared in identical sterile syringes. These were then transported to the cardiac catheterisation lab for the infusion procedure.
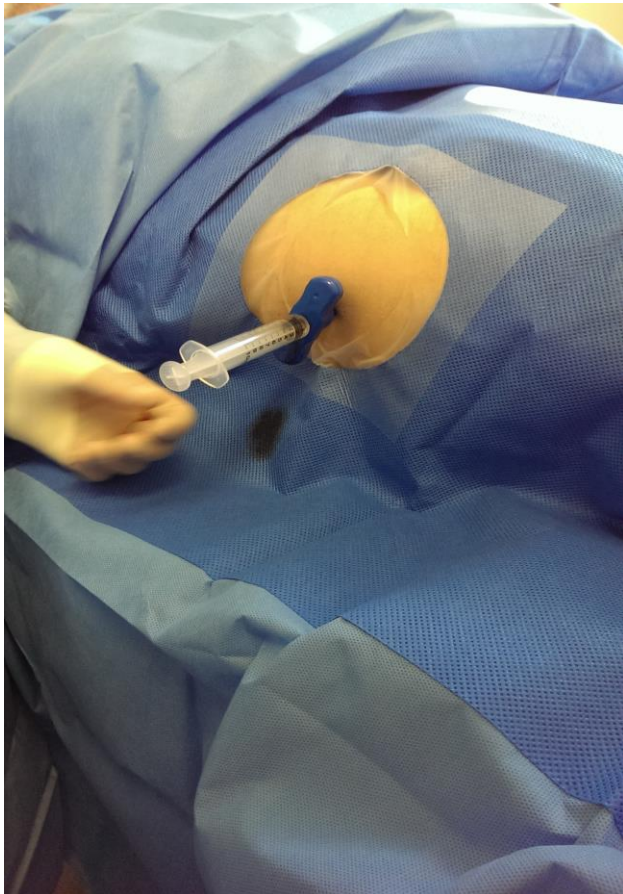
Figure 2.2. Bone marrow aspiration trolley and bone marrow aspiration being performed from posterior iliac crest

Cell processing

The unprocessed bone marrow was transferred to a sterile, glass-shielded, stem cell laboratory. The 50mls of bone marrow harvest was pooled in a transfer bag and diluted with 0.9% saline in a 1:2 ratio. The harvest was then passed through a 200μm filter. In each of four 50ml tubes, 15 ml of Ficoll was added. 30 ml of harvest was then carefully layered on top of the Ficoll in the first three tubes and 10ml in the last, making sure they do not mix. The tubes were centrifuged at 2500rpm for 30 minutes. The resultant "buffy" layer was pipetted out carefully and then resuspended in 10ml of 0.9% saline and re-centrifuged at 2500rpm for 10 minutes (figure 2.3). This was repeated a further two times. The final cell pellet was suspended in 10 ml of autologous sera for the IC BMC group and 2ml of sera for the IM BMC group. For the placebo intervention groups, 10ml and 2ml of autologous sera were prepared for the IC placebo and IM placebo groups respectively.

Plasma layer

Mononuclear "Buffy" layer

Platelets/Erythrocytes

82
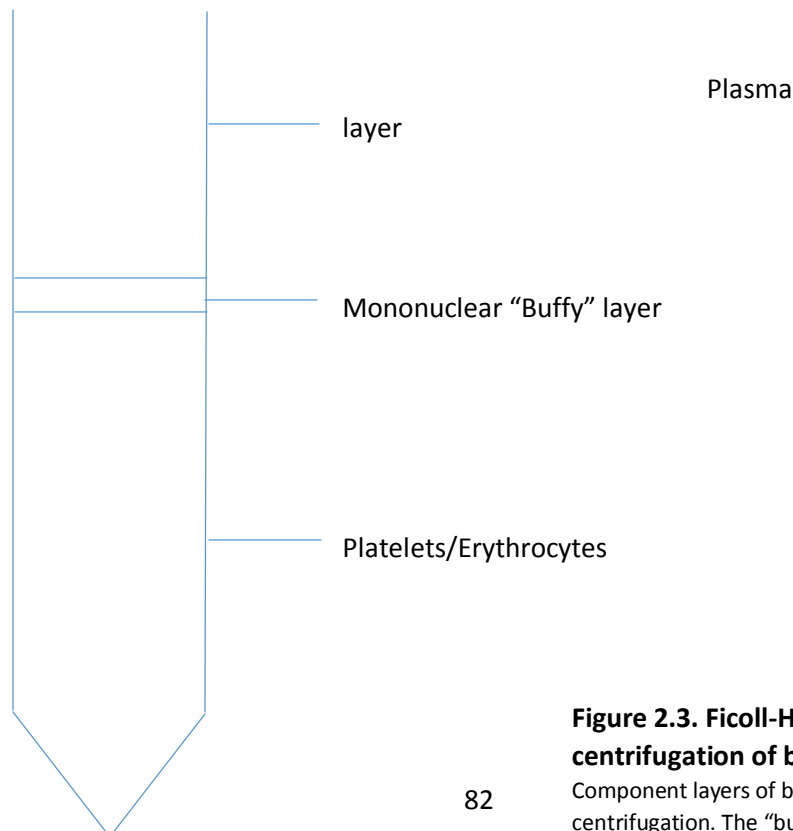
Figure 2.3. Ficoll-Hypaque centrifugation of bone marrow.
Component layers of bone marrow after centrifugation. The "buffy" layer is the mononuclear cell fraction.

Intracoronary injection

Coronary Angiography

Premedication of diamorphine and diazemuls was given prior to the procedure and oxygen therapy at 2 litres per minute was administered throughout. A complete angiogram was recorded before and after the procedure. The angiogram was recorded in a standardised manner suitable for quantitative angiographic analysis. TIMI flow was recorded,[206] as was the Rentrop classification for collaterals.[207] The angiogram was used to confirm the diagnosis of ischaemic heart disease and also to allow decision-making regarding the vessels for injection. The aim was to infuse progenitor cells to as much of the left ventricle as possible utilising the coronary circulation. Ideally 3 suitable conduits (arterial as well as venous in the case of previous coronary bypass surgery) were identified that anatomically supplied the largest amount of left ventricular muscle. The progenitor cell suspension or placebo (serum) was divided equally and injected down all three conduits. A left ventriculogram was also acquired in 2 planes for quantification of left ventricular function.

Weight adjusted bolus dose of heparin (100IU/kg) was given as per routine procedure. All patients were pre-treated with a loading dose of aspirin (300mg) and clopidogrel (600mg) and subsequently received a maintenance dose for 1 month (75mg). A 0.014-inch guide wire was passed into the target coronary artery. An over-the-wire compliant balloon catheter oversized by 0.5 mm, in comparison to the vessel diameter, was advanced into the proximal 1/3 of the target vessel and the wire removed.

The balloon was inflated with low pressure (<4atms) to completely block blood flow for three minutes, while 3.3 ml of the progenitor cells suspension or placebo was infused distal to the

occluding balloon through the central port of the balloon catheter (in patients in whom there are only 2 target arteries, 5mls of progenitor cell suspension or placebo was infused). This procedure was then repeated in the remaining target vessels. After completion of the infusion, coronary angiography was repeated.

Intramyocardial injection

Patients underwent left ventricular electromechanical mapping using NOGA® XP Cardiac Navigation System and IM injection with the MyoStar[TM] catheter (Biologics Delivery Systems Group, Cordis Corporation, Diamond Bar, CA, USA) as previously described [208 209]. The target areas for injection were the border zones around scar tissue based on voltage criteria obtained using the NOGA map as has been demonstrated previously by Perin et al [209]. The total 2 ml volume of injectate was divided and delivered equally to 10 target areas at approximately 1cm intervals.

Following either procedure, the patient was observed overnight and discharged the following morning.

Details of baseline and follow-up visit investigations and other components

Clinical assessment

At admission, a full assessment was undertaken. The following points were specifically recorded:

- NYHA class

- CCS class

- Presence of ICD/CRT (and if ICD, any shocks and date of last check)

- Medications

- Fitness for procedure e.g. ability to lie flat for entire duration of procedure, if on warfarin, whether to be temporarily stopped

- Details of physical examination with particular emphasis on cardiovascular examination

At the follow-up visits, a full assessment was undertaken with special emphasis on the following points:

- Change in cardiac symptoms since last visit i.e. progression, improvement or no change

- NYHA class

- CCS class

- Any other symptoms i.e. non-cardiac

- If ICD-any shocks and date of last check

- Any hospital admission(s) since last visit and if so, details of admission

- Any clinic visits and outcome of visit, if available

- Any change in medications since last visit and if so, details of name of medication, dose, frequency

- Detailed cardiovascular examination with emphasis on any signs of fluid overload

Advanced Cardiac imaging

Cardiac magnetic resonance imaging

Cardiac magnetic resonance (CMR) technology relies on magnetic alignment of the nuclei of hydrogen atoms when placed in a powerful magnetic field. Radiofrequency signals are transmitted to excite the aligned atoms and the signal received from the tissue is received by receiver coils. Two different relaxation times- T1 (longitudinal relaxation time) and T2 (transverse relaxation time)

of the protons are used. The varying relaxation times of the protons as well as the varying proton density in different tissues are exploited to generate the contrast in image between tissues. On T1 weighted images the myocardium appears dark whereas on T2 weighted images the myocardium appears bright.

For this study, imaging was performed on a 1.5 T Philips Achieva scanner with a cardiac 32-channel phased array coil. Each examination used cine-CMR for ventricular volumes and function. Cine CMR is considered the gold standard of the evaluation of cardiac volumes, mass, and systolic function since it does not apply geometric assumptions and has excellent reproducibility and accuracy (standard error for left ventricular ejection fraction, mass and volume is approximately 5%). [210, 211] Thus CMR provides superior endo- and epi-cardial definition and thus allows accurate quantification of LVEF, LVEDV, LVESV and myocardial mass. Not only can it demonstrate abnormalities in myocardial function, volumes and mass but it has a myriad of other applications including myocardial perfusion imaging, detection and quantification of myocardial scar tissue, and detection of non-ischaemic cardiomyopathies, cardiac masses or thrombi. A few trials have used CMR to look at changes in infarct size with cell therapy (Table 1.8). The lack of radiation and the magnitude of information it provides makes it an attractive option over other imaging modalities. However, not all patients are suitable for CMR due to implanted, non-CMR safe, metallic devices e.g. pacemakers, which is a contraindication due to the magnetic field. Hence, a significant number of HF patients have implanted devices which preclude them from undergoing CMR's. In patients who were not suitable for CMR, cardiac computed tomography (CCT) was used as an alternative advanced imaging technique.

For scar imaging, gadolinium-chelated contrast agents are used. Gadolinium based agents tend to be retained for longer in scar tissue-late gadolinium enhancement (LGE)- compared to normal myocardium, secondary to reduced capillary density and increased volume of distribution. By a pulsing sequence known as inversion-recovery using T1 weighted imaging, the normal myocardium is made to look very dark while the LGE myocardium looks bright i.e. hyper enhanced. CMR allows not only the detection but quantification of scar tissue as well. Various methods have been used to quantify scar tissue. These include quantifying as scar any myocardium that is a certain number of standard deviations above the intensity of the normal myocardium. Various SDs including 2, 3, 4, 5 or 6 SD's have been used. Manual quantification is also possible by tracing around the scar tissue of interest. The other commonly used method is the full width half max (FWHM) method which detects as scar any area of the myocardium that has an intensity more than half the maximal signal intensity of the scar. Unfortunately, there is no clear consensus on the best method to use. According to a paper by Bondarenko et al., the 5SD method was the most accurate when compared to visual (i.e. manual) analysis in patients with chronic HF. [212] Hence, the 5SD method was used for scar analysis in this study. T1 weighted inversion-recovery gradient images were acquired 10 min after injection of a dose of 0.2 mmol/kg of gadoterate meglumine (Dotarem) for late gadolinium enhancement. Inversion time was adjusted to null signal from healthy myocardium (voxel size 2.07×2.16 mm, slice thickness 8 mm, FOV 300 mm).

Endocardial and epicardial contours were manually traced for each of the slices in short axis LGE stack (Figure 2.4). The myocardial mass was then calculated. The 5SD semi-automatic method was then used to select areas of late gadolinium hyperenhancement (Figure 2.5). The 5SD method highlights as scar, myocardium that has a signal intensity more than 5 standard deviations above the normal myocardium. Any visible areas of scar not picked up by the software using the 5SD

method or any areas not compatible with scar formation were manually included or excluded

respectively. The software was then used to calculate the actual mass (in grams) of the scar and the

percentage (%) scar (calculated by expressing the scar mass as a % of the myocardial mass). Images

were analysed in blinded fashion by 2 experienced operators. In case of discordance between

operators, blinded review by a level III accredited CMR reader was performed. Analysis was

performed using dedicated software (CVI42, Circle Cardiovascular Imaging Inc, Calgary, Alberta,

Canada).
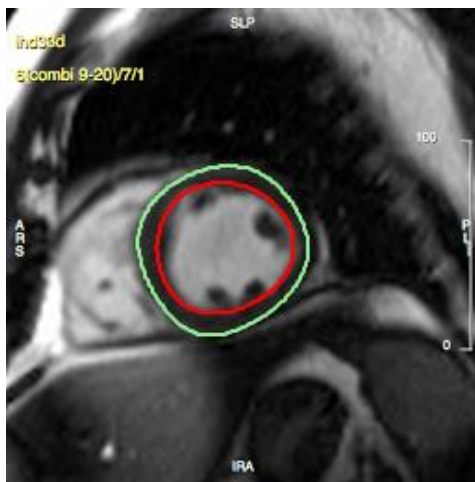
Figure 2.4. Endocardial (red) and epicardial (green) borders traced around short axis slices on CMR
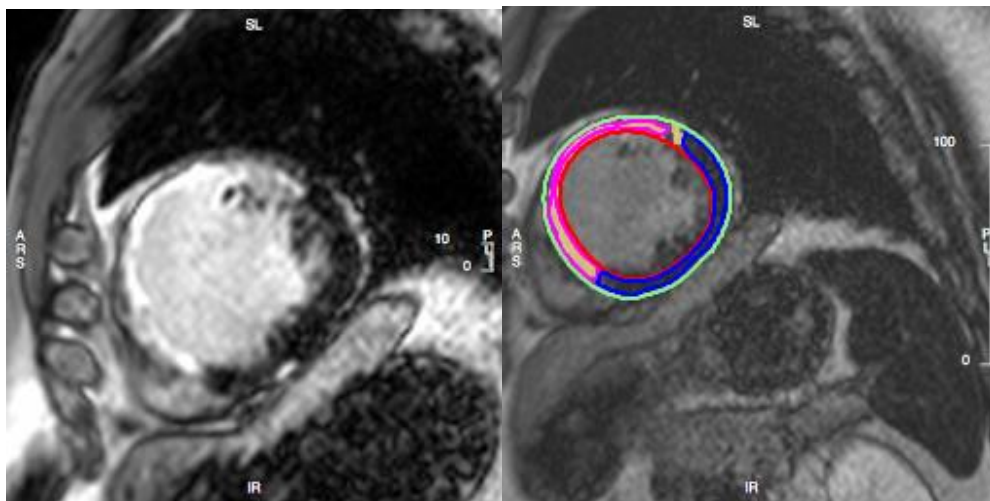
Figure 2.5. Assessment of scar on CMR. Late gadolinium enhancement seen on image in left. 5SD method used to highlight

area of scar (right)

Cardiac computed tomography

Cardiac computed tomography (CCT) is an established imaging modality to quantify cardiac function and volumes. In a head-to-head comparison of 64-row CT, 2D echo, left cine ventriculogram (LCVG) and 3D echo with CMR, CT showed no significant over or underestimation of EF, end-diastolic volume (EDV) or end-systolic volume (ESV) compared to CMR. Furthermore, compared to the other three modalities (i.e. 2D and 3D echo and LCVG), CT was more accurate in assessing EF, EDV and ESV with CMR being the gold-standard. Of note, the study used a semi-automated method of post processing similar to this study.[213] A recent meta-analysis of 27 studies, comparing newer generation (64-row or more) CTs to CMR and 2D echo, lent further support to the previous study and showed no significant difference between LVEF measured using CT and CMR or CT and echocardiography.[214] To negate any effect of differences between imaging modality, a within group analysis was used to assess change in LVEF. Thus, for any single participant in the study, a single imaging modality was used at all follow-up timepoints.

The currently used multislice or multidetector (MDCT) scanners were introduced in the early 1990's.The MDCT consists of an X-ray source (s) and detector rows opposite the X-ray source both of which are loaded on a ring-like structure called a gantry. The patient is positioned within the ring on a table. The gantry rotates at a fixed, pre-specified speed. The X-ray beams emitted are captured by the detector arrays after traversing the patient. The attenuation properties of tissue are detected by the software to reconstruct images of the heart and surrounding tissues. Fast heart rates can reduce the temporal resolution and therefore, heart rate control with beta-blockers has been used to optimise imaging. One of the key factors affecting temporal resolution is the gantry rotation time-the time for the gantry to rotate 360º. To obtain complete cross-sectional views of the heart, a 180º is necessary. This equates to half the gantry rotation time. However, the

newer dual source CT's (DSCT) have two x-ray sources emitting simultaneously. This reduces the time taken to acquire images to $1/4^{th}$ the gantry rotation time. As a result, the temporal resolution has increased significantly to <80ms. This high temporal resolution was one of the key factors favouring CT over other modalities, in patients not suitable for CMR. The other source of motion artefacts is that of breathing movements which necessitates breath-holds when acquiring images. As an example, with a 128 detector DSCT, each detector measures 0.625mm i.e. a total length of 128x0.625=80mm. An average heart is around 120-150mm in length so the scan can be done with virtually one breath-hold thus minimising breathing artefacts.

All patients in the trial were studied on a 64-slice spiral CT (Sensation 64, Siemens, Forchheim, Germany) scanner using retrospective gating to acquire the images. Intravenous B-Blockers were used in patients to achieve optimal pulse rate (<70), where needed, prior to initiation of the scan and all patients were on cardiac monitors during administration. All scans were performed in the cranio-caudal direction. An initial scouting x-ray (topogram) was performed to ensure correct alignment of the patient for the remainder of the scan. The scan was initiated when contrast arrived within the ascending aorta. This was performed manually after test bolus arrived in the area of interest or using an automated CT triggering once the Hounsfield unit crossed a threshold in a set region of the aorta. A total of 50 - 100 mls of contrast was used for the scan with the scan taking 10–15 minutes to complete. Reconstruction algorithms were used to convert the raw data into interpretable images, which were then analyzed on specialist software. The data was reconstructed throughout the cardiac cycle and cine movie images were collected in 10 phases along the cardiac cycle and 10 levels. Analysis of LV volumes and function was performed using dedicated PC-based software Siemens (Forchheim, Germany) by using a semi-automatic method.

The end-systolic and end-diastolic phases were manually selected. Once the end-systolic and end-diastolic phases were selected, the software detected voxels (volumetric pixels) with an attenuation above a set threshold and based on this, delineated the epicardial and endocardial borders. To activate the segmentation required the operator to define the mitral valve plane and mark a point on the anterior interventricular septum. The hinge-to-hinge method was used to define the mitral valve plane to achieve greater consistency.[215] This involves using the hinge points of the mitral and aortic valve leaflets closest to the ventricular wall as the two defining points for the mitral valve plane. A problem with this method, however, is that it excludes the left ventricular outflow tract (LVOT). To compensate for this, manual adjustment to the volumes to include the LVOT was carried out. Manual adjustment to the contours to exclude pacemaker artefacts and to include papillary muscles and trabeculae in the LV cavity was also performed. Further manual adjustments of contours were made where necessary.

Transthoracic echocardiography

A transthoracic echocardiogram (TTE) was performed on each patient at 6 months, 12 months and 24 months. The following views were recorded: parasternal long axis view (PSLAX), parasternal short axis view (PSSAX), 4-chamber (4C) view, 3-chamber view and 2-chamber (2C) view. The scans were performed by accredited, practising echocardiographers. The following information was recorded: left ventricular dimensions, degree of valvular heart disease and forward flow velocities via the aortic, tricuspid and mitral valves. A commercially available contrast (Sonovue®) was injected via a peripheral cannula to enhance the definition of the endocardial borders. The contour of the LV in end-diastole and end-systole were traced in both the 4C and 2C views. The machine then calculated the global LVEF using the Simpson's method whereby the end-systolic volume and end-diastolic volume are calculated by "stacking" together and adding the 2D "slices" or "discs" to

provide the 3D volume (Figure 2.6). The images were then analysed at a later date to assess LV

systolic and diastolic function.

Figure 2.6. Assessment of LVEF on contrast echocardiography. Endocardial definition delineated by used of contrast.

Endocardial border traced in systole and diastole in both 4 chamber and 2 chamber views to yield LVEF.

Assessment of diastolic function on echocardiography

For the assessment of diastolic function, the following parameters were measured: mitral valve

inflow E and A velocities, E:A ratio, Deceleration time (DT). Tissue Doppler imaging (TDI) was used

to measure E' velocity (figure 2.7) and E:E' ratio. Left atrial (LA) size was measured in the parasternal long-axis view.
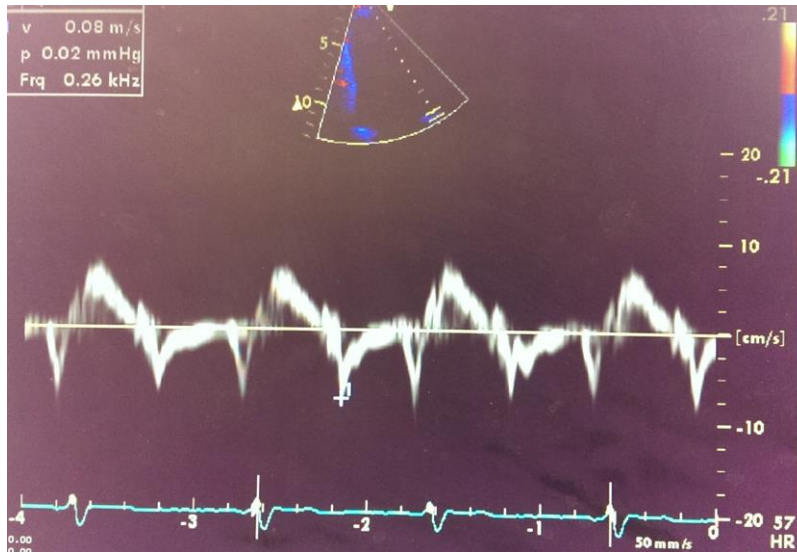
Figure 2.7 Tissue doppler imaging (TDI) on echocardiography. Measurement of E' (+) using TDI imaging.

Quantitative left ventriculography

Quantitative left ventriculography (QLV) was performed at baseline and 6 months in the IC and IM arms. A pigtail catheter was placed into the LV cavity and 30mls contrast injected at a rate of 10ml/second. Image acquisition was carried out in two perpendicular planes- right anterior oblique (RAO) 30° and left anterior oblique (LAO) 60°. Left ventricular ejection fraction and volumes were calculated using the centreline method, using QXAngio 7.1® software (Medis Inc.). The analysis involved manually delineating the LV border in end-systole and end-diastole.

Blood sampling

Venous blood was obtained at admission, 6 months and 1 year. All bloods were processed at the hospital's blood sciences laboratory apart from NT pro-BNP which was sent to a commercial blood sciences laboratory. Peripheral blood was sent to the stem cell lab for CD34+ cell count,

mononuclear cell count (MNC) and circulating endothelial progenitor cell (EPC) count. Bloods were taken for the following: full blood count (FBC), urea and electrolytes (U&E), liver function tests (LFT's), clotting screen and NT pro-BNP. An EDTA tube of additional blood was spun at 1600g for 20mins and the supernatant serum stored at -80°C for further analysis.

Quality of life (QoL) questionnaires

In the trial on which this MDRes is based, the SF36 (short form 36), EQ-5D (EuroQol 5D) and the MacNew questionnaires were used. The three QoL questionnaires were filled in by participants in the trial at baseline, 6 months, 1 year and 2 years. Copies of each of the questionnaires are provided in Appendix 1.

MacNew The questionnaire consists of 27 questions covering three domains-a 13 item physical domain scale, a 14 item emotional domain scale and a 13 item social wellbeing domain scale. A global score is obtained from the sum of the scores to the individual items. The scoring for each answer is from 1 (poor QoL) to 7 (good QoL). On average it takes 10 minutes to fill in. Missing responses do not contribute to the score. [216] A change in score of 0.5 or more in a domain or in the global score is taken as the minimal important difference.[217] The questionnaire is available online and free to use.

SF-36 The SF36 v2 questionnaire consists of 36 questions covering 8 domains- vitality, physical functioning, bodily pain, general health perceptions, physical role functioning, emotional role functioning, social role functioning and mental health.[218] A low score indicates poor perception of health and vice versa. The raw scores are entered into using available SF36 v2 software which then transforms the score into a 0-100 scale (0-worst QoL and 100-best QoL). The software also generates two summary scores-the physical component summary (PCS) score and the mental health component summary (MCS) score. These two aggregate scores were standardised for the

UK population after analysing a large-scale data set of SF-36 responses from a random selection of the UK population. [219] A high score on the PCS or MCS indicates a high level of satisfaction with physical or mental health respectively and vice versa.

EQ-5D The EQ-5D questionnaire consists of two parts- a Visual Analogue Scale (VAS) and the EQ-5D descriptive system.[220] The VAS is a self-rated score using a 0-100 vertical ladder (0 being worst and 100 being best imaginable health state) and taken as an overall indication of the quality of life. The EQ-5D descriptive system consists of 5 dimensions: mobility, self-care, usual activities, pain/discomfort and anxiety/depression. Each dimension has 3 options for the patient to choose from: no problems, some problems, extreme problems. Each of these options are weighted and when the raw data is entered from the EQ-5D descriptive system into EQ-5D software, a summary index score, based on the weighting, is provided which is taken as an overall measure of QoL in the individual.[220]

2.2 Cytokine analysis

Various cytokines including pro-inflammatory, anti-inflammatory and pro-angiogenic cytokines have been implicated in the pathogenesis of ischaemic cardiomyopathy as discussed in section 1.1.2.3. However, no study has yet looked at the association between G-CSF and cell therapy and the levels of these cytokines or their association with the outcomes on cardiac function. As part of the MDRes, a selection of these cytokines was measured at baseline and at 1 year and analysed for any association with cell therapy and primary outcome. These were: IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFN-γ, TNF-α, IL-1a, IL-1b and MCP-1. The reason why these cytokines were chosen was two-fold. Firstly, as detailed in section 1.1.2.3, this selection of cytokines have been linked to the progression of heart failure and prognosis in heart failure patients. Secondly, the availability of a

multiplex assay to measure this collection of cytokines meant it was possible to obtain levels for 11 different cytokines in one sample run.

On the day of the cytokine analyses, the samples were left to thaw at room temperature. The cytokine analyses were performed using the Randox® Evidence Investigator™ by dedicated staff from Randox® Laboratories using their standard assay protocol detailed below. The Evidence Investigator™ Biochip Array was used to simultaneously detect multiple analytes from a single patient sample using ELISA (Enzyme Linked ImmunoSorbent Assay). The technology consists of a solid state device containing multiple regions of immobilised antibodies to the specific cytokines. Binding of horsedradish labelled antibody to the cytokines results in chemiluminescence and higher levels of cytokines results in increased levels of chemiluminescence i.e. using sandwich chemoluminescent immunoassay. The concentration of the analyte was calculated by measuring the light signal emitted from the chemoluminescent antibodies and compared to that of a standard calibration curve.

Materials (all provided by Randox®)

- *Randox® Evidence Investigator™*

- *Cytokine High sensitivity Assay Diluent (1 x 14ml)*

- *Cytokine High sensitivity Conjugate   (1 x 20 ml)*

- *Cytokine High sensitivity Biochip   (54 biochips)*

- *Cytokine High sensitivity Calibrator   (9 x 2ml)*

- *Signal reagent EV805   (2 x 10ml)*

- *Wash buffer concentrate   (1 x 32ml)*

- *Calibrator concentration disc and barcodes*

- *Biochip well cover slips*

- *Cytokine high sensitivity control*

- *Thermoshaker*

Detailed protocol

The RANDOX® investigator machine was calibrated using standard calibration discs. The thermoshaker was equilibrated to +37ºC for 30 minutes before use. The following steps were then carried out (Figure 2.8).

1) 200 µl of assay diluent was pipetted into each biochip well.

2) 100 µl of sample was pipetted per well. See Figure 4.1.

3) The biochips were incubated for 1 hour at +37 ºC and 370rpm. See Figure 4.2.

4) The wells were covered and refrigerated at 2-8 ºC overnight.

5) After overnight incubation, the reagents were discarded from the wells

6) 2 quick wash cycles were carried out using 350µl of diluted wash buffer (32ml concentrate consisting of Tris buffered saline, pH7.4, with 968ml water) into each well

7) A further 4 wash cycles were carried out

8) After the final wash and removal of all residual wash buffer, 300 µl of conjugate (20mM Tris buffered saline, pH7.5, with assay specific antibodies labelled with horse radish peroxidase) and the wells incubated at 37 ºC for 1 hour and 370 rpm

9) Steps 6 and 7 were repeated after discarding conjugate

10) The carriers were filled with wash buffer and left to soak till directly before imaging but for no more than 30 minutes. The carriers were protected from light

11) The wash buffer was removed and 250 µl of the signal reagent immediately added.

12) After 2mins (±10s) the carriers were inserted into the Evidence Investigator™

13) Images were captured and results processed by automated, dedicated software

1. Add 200µl of assay buffer and 100µl of sample (or standard) to each well.

2. Incubate the carrier at 37°C for 60 mins @ 370RPM on the thermoshaker, cover with sealer and further incubate for 16-20hours @ 2-8°C/ 0RPM

3. Decant the liquid and wash each well. Perform 2 quick washes and 4 two-minute soaks. Decant the liquid and tap the carrier onto lint free tissue paper

4. Add 300µl of conjugate to each reaction well.

5. Incubate the carrier at 37°C for 60 mins @ 370RPM on the thermoshaker

6. Decant the liquid and wash each well Perform 2 quick washes and 4 two-minute soaks. Decant the liquid and tap the carrier onto lint free tissue paper.

7. Mix Luminol-EV805 & Peroxide (1:1). Add 250µl of signal reagent-EV805 to each reaction well. Incubate for 2 mins and protect from light.

8. Image each carrier on the Investigator System

Figure 2.8 Randox Evidence Investigator™ Assay Steps (courtesy Mr Allen Huxley, Randox Laboratories)

2.3 Cell Analysis

Bone marrow mononuclear cell count was performed using an inverted, high powered microscope and a manual cell counter. Flow cytometry was used for quantitative analyses of CD34+ cells and EPC's. A FACSCanto flow cytometer with BF FACSDiva v5.0.3 software (BD Biosciences) was used. HSC were identified by incubating the cells 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 minutes at room temperature.

For EPC analysis, the bone marrow mononuclear cell sample was incubated with mouse serum IgG (Sigma, Dorset, UK) for 15 minutes at 4°C and a mix of the following antibodies-allophycocyanin

(APC)-labeled antibody to CD133 (Miltenyi Biotec, Surrey, UK) and PE labeled antibody to VEGFR-2 (R&D Systems, Abingdon, UK) for positive selection of EPC's and FITC-labeled antibodies to CD13, CD2 and CD22 to exclude non-EPCs. The resulting sample was then incubated with a PerCP-Cy5-labeled 7AAD stain (BD biosciences) to check viability. The cells were then incubated with 2ml of PharmLyse™ buffer (BD Biosciences) for 15minutes at room temperature to lyse red blood cells. This was followed by a wash with PBS (Phosphate buffered saline). 20µl of Accucount flow cytometry beads (Saxon Europe, Kelso, UK) were then added prior to analysis using flow cytometry. Colony-forming unit granulocyte-monocyte (CFU GM) assays were performed on selective IC and IM bone marrow samples. The BMCs were plated out in triplicates on methylcellulose plates together with Methocult media, consisting of stem cell factor, granulocyte macrophage colony-stimulating factor and Interleukin-3 (Stem Cell Technologies). The plates were left in the incubator for 14 days. Using phase contrast microscopy, CFU-GM were counted on day 14 (colony>50cells). The mean of the triplicate was taken as the final result.

Chapter 3 The safety and efficacy of G-CSF and autologous bone marrow derived cells in ischaemic cardiomyopathy

3.1 Background

Trials assessing the therapeutic effectiveness of G-CSF in ischaemic cardiomyopathy have demonstrated mixed results as have similar studies assessing the effects of autologous BMC. As detailed in section 1.2.12, no single study has yet looked at the effect of G-CSF alone and in combination with autologous BMC in ischaemic cardiomyopathy patients. Furthermore, the optimal route of delivery of cells is still unclear as no single trial has compared routes of delivery, although meta-analyses suggest the intramyocardial route is superior. The majority of trials that have looked at the effect of G-CSF or autologous BMC have been small in size and have often lacked appropriate placebo-control arms. The REGENERATE-IHD trial was designed with the aim of addressing the above limitations. This phase II, randomised, placebo-controlled trial assessed the safety and efficacy of G-CSF alone and in combination with autologous bone marrow derived cell therapy, delivered either via the intramyocardial or the intracoronary route, in patients with ischaemic cardiomyopathy. As detailed in section 2.1, the trial had three arms-peripheral, intracoronary and intramyocardial. Each arm had an active and a placebo group (Figure 3.1).
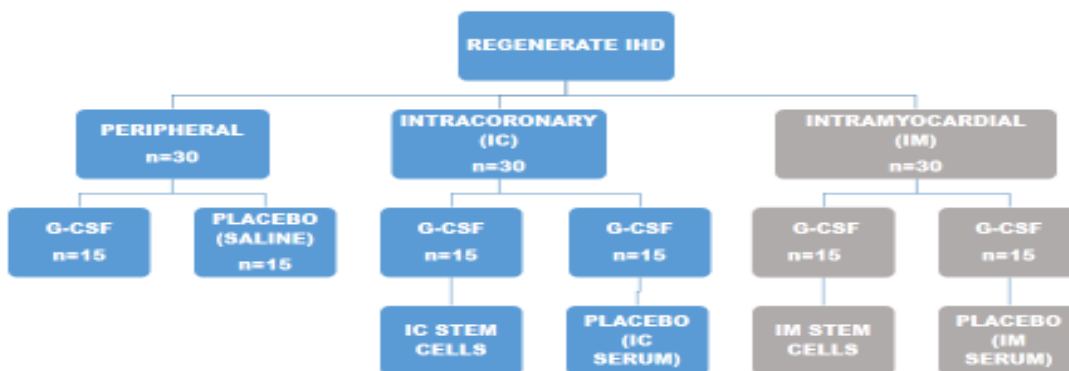
Figure 3.1. Structure of the REGENERATE-IHD trial. Diagram depicting the three arms of the trial and the number of participants in the active and placebo groups in each arm.

3.2 Results

3.2.1 General characteristics of study population

A total of 1133 patients were referred and screened from heart failure clinics. Of these 1028 were ineligible due to the following reasons: non-ischaemic aetiology of heart failure (n=79), NYHA<II/normal LVEF (n=236), atrial fibrillation (n=27), refusal to participate in the trial (n=389), death before formal consent (n=53) and other comorbidities, including valvular heart disease and significantly impaired renal function (n=244). Of the 105 patients who were randomised, 15 patients were withdrawn either because of unsuitability for the intervention or patient withdrawal. 90 received the allocated trial intervention (Figure 3.2).

The mean age of the patient population was 62.8 ±9.6 years while the majority of patients were male (94.4%). The baseline characteristics were similar across the groups with a mean LVEF of 30.6% (95% CI 28.53-32.74), a mean NT-pro-BNP of 1187.0±179.9 pg/ml and 97.8% of patients in NYHA class II/III. Baseline patient demographics for the entire trial are shown in Table 3.1. The majority of patients were on optimal medical therapy (as per ESC guidelines), [221] and there were no significant differences across groups in prescribed medication or implanted device therapy. The mean total number of cells injected in the two stem cell groups was 115.1x10^6 bone marrow mononuclear cells. The mean viability of processed cells was 98.2%.

Table 3.1. Baseline characteristics

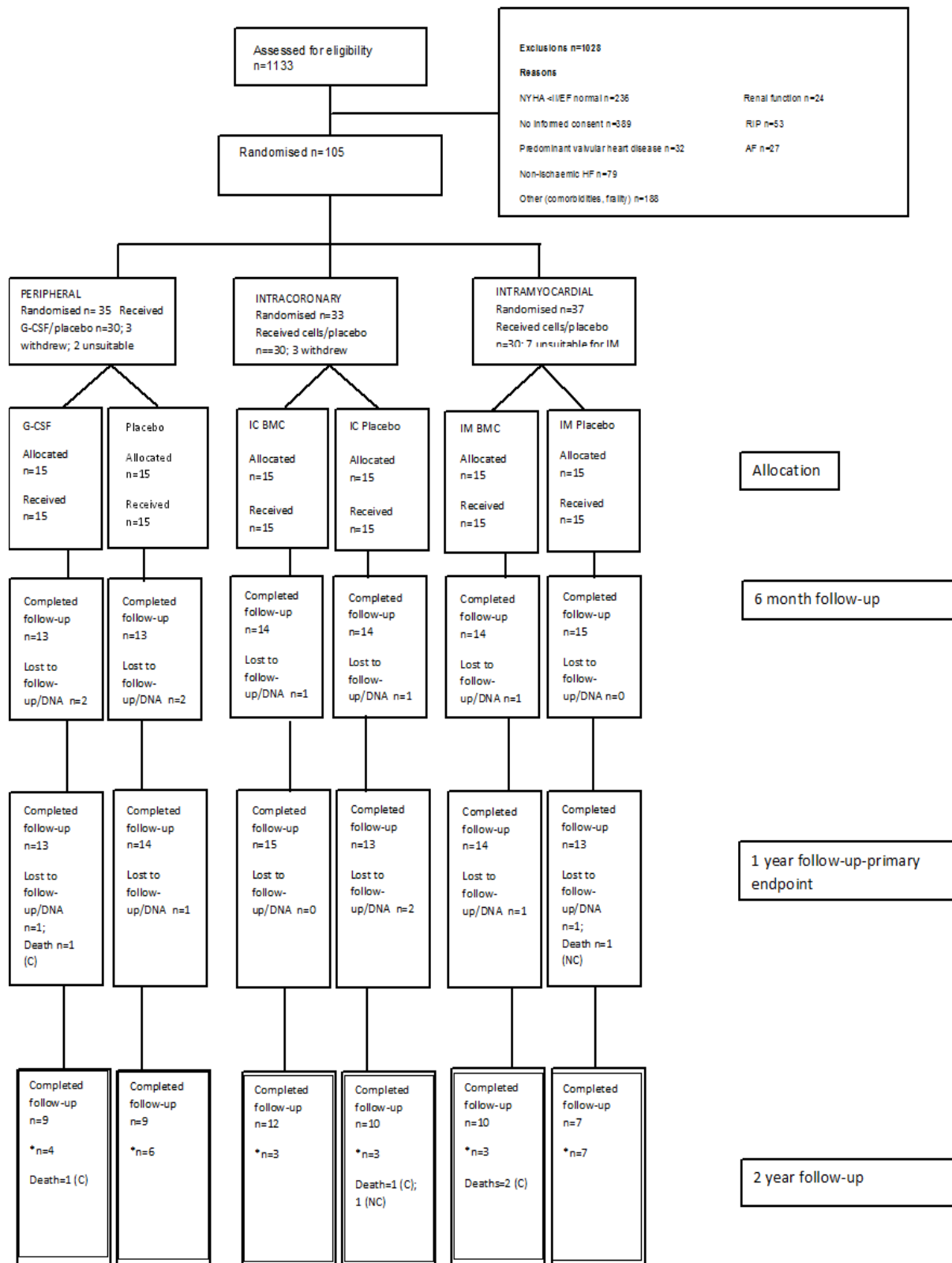|	Saline (n=15)	G-CSF (n=15)	IC serum (n=15)	IC BMC (n=15)	IM serum (n=15)	IM BMC (n=15)	p-value
Age, years (mean ± SD)	63.3±9.3	63.1±8.2	62.8±10.7	62.1±9.7	60.4±11.2	65.3±9.4	0.841
Sex M/F n	14/1	13/2	14/1	14/1	15/0	15/0	0.896
BMI (kg/m^2) (mean±SD)[#]	29.5±4.3	31.4±6.0	31.7±6.5	29.7±4.8	29.6±3.7	30.8±4.0	0.739
Medical History, n (%)							
Hypertension	3 (20.0)	1 (6.7)	2 (13.3)	3 (20.0)	5 (33.3)	5 (33.3)	0.414
Diabetes	4 (26.7)	5 (33.3)	2 (13.3)	2 (13.3)	4 (26.7)	4 (26.7)	0.748
CABG	5 (33.3)	4 (26.7)	3 (20.0)	7 (46.7)	6 (40.0)	4 (26.7)	0.653
MI	13 (86.7)	12 (80.0)	14 (93.3)	13(86.7)	13 (86.7)	13 (86.7)	0.949
Hypercholesterolaemia	4 (26.7)	6 (40.0)	5 (33.3)	5 (33.3)	8 (53.3)	4 (33.3)	0.715
Smoker/ex-smoker,	12 (80.0)	8 (53.3)	13 (86.7)	11(73.3)	14 (93.3)	11 (73.3)	0.150
Time from last MI, days median (IQR)	1307 (1064-5443)	2527 (966-4928)	2856 (1278-6041)	1805 (896-3855)	2406 (706-5402)	2684 (706-5402)	0.964
LVEF (%) (mean ± SD)	34.7±10.1	27.9±12.4	31.6±7.4	31.7±8.8	29.0±9.2	28.6±10.2	0.385
Devices number,n (%)							
CRT-D	4 (26.7)	5 (33.3)	4 (26.7)	4 (26.7)	3 (20.0)	7 (46.7)	0.781
CRT-P	1 (6.7)	1 (6.7)	0 (0)	0 (0)	0 (0)	0 (0)	0.999
ICD only	7 (46.7)	2 (13.3)	4 (26.7)	5 (33.3)	5 (33.3)	6 (40.0)	0.485
Medication history, n (%)							
Statin	12 (80.0)	13 (86.7)	13 (86.7)	13(86.7)	14 (93.3)	13 (86.7)	0.995
ACEi/ARB	14 (93.3)	14 (93.3)	13 (86.7)	14(93.3)	15 (100)	15 (100)	0.896
B-blocker	15(100)	14 (93.3)	12 (80.0)	15(100)	11 (73.3)	14 (93.3)	0.079
Aldosterone antagonist	9 (60.0)	13 (86.7)	9 (60.0)	12(80.0)	9 (60.0)	12 (80.0)	0.351
Diuretics	10 (66.7)	11 (73.3)	13 (86.7)	12(80.0)	8 (53.3)	12 (80.0)	0.363
NYHA at baseline, n (%)							
II	10 (66.7)	9 (60.0)	5 (33.3)	8 (53.3)	11 (73.3)	8 (53.3)	0.376
III/IV	5 (33.3)	6 (40.0)	10 (66.7)	7 (46.7)	4 (26.7)	7 (46.7)	

Figure 3.2. CONSORT diagram

*denotes number of patients who were not seen as they passed the 2 year mark before the decision to undertake 2 year follow-ups

103

At one year, a total of 82 patients were assessed for the primary endpoint (Figure 3.2). In the IM arm, 14 patients were assessed in the BMC group (one referred for LVAD) with 13 patients assessed in the IM placebo group (one death and one patient lost to follow-up). In the IC arm, all 15 patients in the BMC group and 13 patients in the placebo group (one lost to follow-up and one CT scan not analysable) were assessed for the primary endpoint whilst in the peripheral group, 13 patients in the G-CSF group (one death and one CT scan not analysable) and 14 in the placebo group (one patient lost to follow-up) were assessed for the primary endpoint.

At 2 years, a total of 57 patients attended for follow-up. In the IM BMC group 10 patients attended with 7 attended in the IM placebo group. In the IC arm, 12 patients in the BMC group and 10 patients in the placebo group attended for follow-up. In the peripheral arm, 9 patients attended in each of the 2 groups. 55 patients underwent CMR/CT scans at 2 years (2 patients were ineligible due to renal impairment) of which 53 scans were analysable. Mortality data was collected on all 90 participants. Baseline demographics for the 2 year follow-up attendees are shown in table 3.2.

3.2.2 Change in left ventricular ejection fraction using advanced cardiac imaging

At 1 year no change in LVEF was seen in the G-CSF only treated group with a mean decrease of -1.25% (95% CI -5.4-2.9%; p=0.520), compared to a reduction of -0.98% (95% CI -4.4-2.5%; p=0.551) seen in the peripheral placebo group (saline only). No significant change in LVEF was seen in either the IC BMC treated group (0.89% change in LVEF (95% CI -2.2-3.9%; p=0.541)) or the IC placebo group (1.1% (95% CI -1.7-3.9; p=0.413). Only patients treated in the IM BMC group met the primary endpoint of change in LVEF (increase of 4.99%; 95% CI 0.33-9.6%; p=0.038). Although a trend to

improvement in LVEF was seen in the IM serum group {4.15% (95% CI -3.3-11.6%); p=0.246}, this did not reach significance. Figure 3.3 shows the change in LVEF across the 6 groups.

The IM BMC group showed a significant, absolute improvement in LVEF of 5.97% (95% CI 0.4 to 11.5; p=0.035) compared to the peripheral placebo group, with no significant difference between the IM serum group and the peripheral placebo group (5.14%; 95% CI -2.4 to 12.7; p=0.175). There was no difference in LVEF between the IC BMC and IC serum groups (-0.21%; 95% CI -4.2 to 3.8; p=0.916) or between the peripheral G-CSF and placebo groups (0.27%; 95% CI -4.8 to 2.4; p=0.913) (Table 3.3 and figure 3.3).

Left ventricular ejection fraction at 2 years

The IM BMC group showed a 3.18% (95% CI -4.2-10.6; p=0.356) improvement in LVEF at 2 years. The IM placebo group showed a -1.33% (95% CI -14.5-11.8; p=0.804) reduction in LVEF. The IC BMC group showed a 2.01% (95% CI -4.4-8.4; p=0.503) increase in LVEF at 2 years and the IC placebo group a 0.63% (95% CI -4.2-5.5; p=0.775) change in LVEF at 2 years. In the peripheral arm, the G-CSF only group showed a -3.3% decline in LVEF (95% CI -10.8 - 4.3; p=0.333) and the placebo group (saline only) showed a -6.3% decrease in LVEF (95% CI -18.4 – 5.8; p=0.256). Figure 3.4 shows the change in LVEF across the groups.

3.4.2.1 Effect of age and baseline LVEF on primary endpoint in patients receiving BMC therapy

The mean change in LVEF in patients in the IC and IM BMC groups who were <60years old at baseline was 2.5±2.0% and was not significantly different to the change in LVEF at 1 year in those >60 years old (3.0±1.7%; p=0.863). The median LVEF for the entire cohort was 32.0%. An unpaired t-test was performed to compare the change in LVEF at 1 year in patients in the IC and IM BMC groups with baseline LVEF≥32% and those with baseline LVEF<32%. Although not significant, the change in LVEF was greater in patients with a baseline LVEF <32% (3.5±1.5%) compared to those with a baseline LVEF≥32% (0.9±1.9%; p=0.334) (Fig 3.5).

Table 3.2 Baseline characteristics of patients attending for 2 year follow-up

	Saline (n=9)	G-CSF (n=9)	IC serum (n=10)	IC BMC (n=12)	IM serum (n=7)	IM BMC (n=10)	p-value
Age, years (mean ± SD)	65.3±12.2	64.0±9.8	65.4±10.3	58.7±8.9	56.8±9.7	61.8±7.5	0.626
Sex M/F n	8/1	8/1	9/1	11/1	7/0	10/0	0.945
BMI (kg/m^2) (mean±SD)[#]	30.0±4.2	30.3±5.2	30.0±5.7	30.9±4.7	31.4±3.7	30.8±4.6	0.990
Medical History, n (%)							
Hypertension	2 (22.2)	0 (0.0)	1 (10.0)	2 (16.7)	4 (57.1)	2 (20.0)	0.122
Diabetes	1 (11.1)	2 (22.2)	1 (10.0)	2 (16.7)	1 (14.3)	1 (10.0)	0.980
CABG	2 (22.2)	4 (44.4)	2 (20.0)	6 (50.0)	2 (28.6)	2 (20.0)	0.574
MI	8 (88.9)	8 (88.9)	9 (90.0)	11 (91.7)	7 (100.0)	8 (80.0)	0.967
Hypercholesterolaemia	2 (22.2)	3 (33.3)	3 (30.0)	4 (33.3)	5 (71.4)	4 (40.0)	0.504
Smoker/ex-smoker,	6 (66.7)	6 (66.7)	8 (80.0)	10 (83.3)	6(85.7)	6 (60.0)	0.871
Time from last MI, days median (IQR)	3593 (5420)	2788 (4634)	2808 (2288)	1731 (3402)	1894 (5336)	3993 (4957)	0.894
LVEF (%) (mean ± SD)	34.0±10.8	27.4±12.9	31.7±7.3	31.3±8.4	28.1±12.3	29.3±11.7	0.783
Devices number,n (%)							
CRT-D	4 (26.7)	4 (33.3)	3 (26.7)	3 (26.7)	0 (20.0)	4 (46.7)	0.368
CRT-P	1 (6.7)	1 (6.7)	0 (0)	0 (0)	0 (0)	0 (0)	0.373
ICD only	4 (46.7)	2 (13.3)	4 (26.7)	5 (33.3)	3 (33.3)	4 (40.0)	0.946
Medication history, n (%)							
Statin	7 (80.0)	8 (86.7)	9 (86.7)	12 (86.7)	7 (93.3)	10 (86.7)	0.328
ACEi/ARB	8 (93.3)	8 (93.3)	9 (86.7)	12 (93.3)	7 (100)	10 (100)	0.659
B-blocker	9 (100.0)	9 (93.3)	7 (80.0)	12 (100.0)	5 (73.3)	10 (93.3)	0.012
Aldosterone antagonist	4 (60.0)	7 (86.7)	6 (60.0)	11 (80.0)	4 (60.0)	9 (80.0)	0.108
Diuretics	5 (66.7)	5 (73.3)	8 (86.7)	10 (80.0)	4 (53.3)	7 (80.0)	0.612
NYHA at baseline, n (%)							
II	9 (66.7)	6 (60.0)	3 (33.3)	6 (53.3)	6 (73.3)	5 (53.3)	0.017
III/IV	0 (33.3)	3 (40.0)	7 (66.7)	6 (46.7)	1 (26.7)	5 (46.7)	

Figure 3.3 Change in LVEF at 1 year. Dot plots showing change in LVEF at 1 year. LVEF at baseline and 1 year as measured using CMR/CT in each of the treatment groups (A-F). A significant improvement of 4.99% was observed in the IM BMC treated group (group F). A-peripheral placebo; B - peripheral G-CSF only; C – peripheral G-CSF + IC serum; D - peripheral G-CSF + IC BMC; E - peripheral G-CSF + IM serum; F - peripheral G-CSF + IM BMC. IC-intracoronary; IM-intramyocardial; G-CSF- granulocyte-colony stimulating factor; BMC-bone marrow derived cells; LVEF-left ventricular ejection fraction; CMR-cardiac magnetic resonance imaging; CT-computed tomography. * denotes p<0.05. Large solid circles represent the means at baseline and 1 year for each group respectively, and error bars represent 95% CIs.
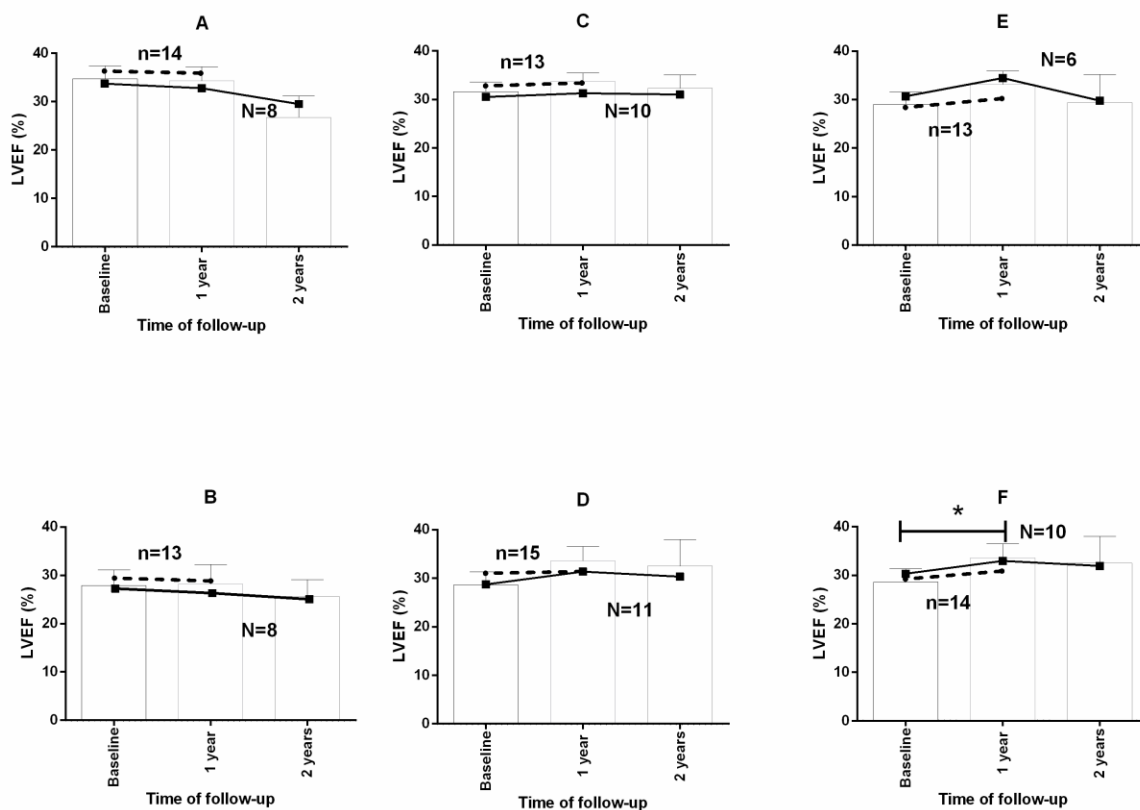
Fig 3.4. Analysis of left ventricular ejection fraction at 2 years. LVEF at baseline, 1 year and 2 years as measured using CMR/CT in each of the treatment groups (A-F). A significant improvement of 4.99% was noted in the IM BMC group at 1 year. An improvement of 3.2% was observed in the IM BMC treated group at 2 years, although not significant compared to baseline (group F). A-peripheral placebo; B - peripheral G-CSF only; C – peripheral G-CSF + IC serum; D - peripheral G-CSF + IC BMC; E - peripheral G-CSF + IM serum; F - peripheral G-CSF + IM BMC. IC-intracoronary; IM-intramyocardial; G-CSF- granulocyte-colony stimulating factor; BMC-bone marrow derived cells; LVEF-left ventricular ejection fraction; CMR-cardiac magnetic resonance imaging; CT-computed tomography. * denotes p<0.05. Dotted line- change in LVEF in patients reaching primary endpoint at 1 year (n); Continuous line-change in LVEF at baseline, 1 year and 2 years in patients attending 2 year follow-up (N)
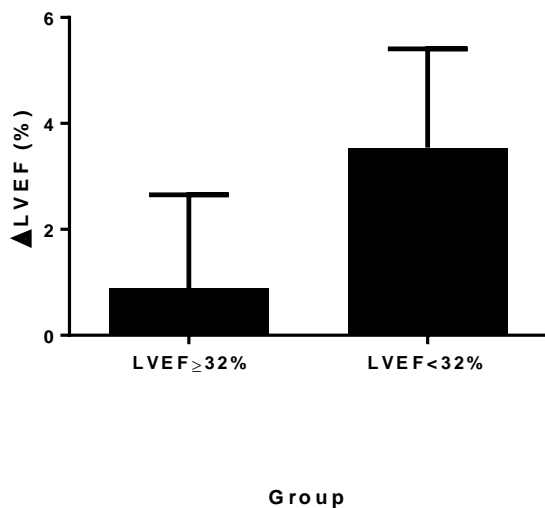
Figure 3.5. Bar charts showing Change in LVEF relative to baseline LVEF. Mean (+SEM) change in LVEF at 1 year according to baseline LVEF in the IC and IM BMC groups. Baseline LVEF was classified as being ≥median baseline LVEF or < median baseline LVEF. Median baseline LVEF was 32.0%. *p<0.05 Independent samples t-test. BMC-bone marrow derived cells; IC-intracoronary; IM- intramyocardial

3.2.3 Left ventricular volumes and mass

Left ventricular end-diastolic volumes

None of the groups showed any significant change in left ventricular end-diastolic volume (LVEDV) at 1 year and all groups showed an increase (or no change) in LVEDV. In keeping with the trend, all groups except for the IM BMC group, showed an increase in LVEDV at 2 years. The IM BMC group showed a -15.1mls reduction in LVEDV, albeit insignificant (95% CI -56.2 to 26.0; p=0.427).

Left ventricular end-systolic volume

Only the IM BMC group showed a reduction in left ventricular end-systolic volume (LVESV) at 1 year (-8.6ml (95% CI -38.5 to 21.3; p=0.546), although not significant. All other groups showed non-significant increases in LVESV. This trend was maintained at 2 years, with the IM BMC group being

the only group to show a reduction in LVESV, albeit non-significant (-14.6mls; 95% CI -48.5-19.3; p=0.355). All other groups showed an increase in LVESV at 2 years.

Myocardial mass

No significant change in myocardial mass (MM) was noted in any of the six groups at 1 year. At 2 years, however, the IM BMC group showed a statistically significant increase in myocardial mass (MM) (35.3g; 95% CI 9.5 to 61.1; p= 0.013). None of the other groups showed any significant change in MM at 2 years. Tables 3.3 and 3.4 show the values for LVEF, LV volumes and MM at 1 year and 2 years respectively in each of the groups. Figure 3.6 shows the change in LVEF, LV volumes and myocardial mass at 2 years.

Table 3.3. Change in LVEF, LV volumes and myocardial mass at 1 year

	Peripheral placebo	Peripheral G-CSF	IC serum	IC BMC	IM serum	IM BMC
	N=14	N=13	N=13	N=15	N=13	N=14
EF baseline	35.3 (29.4-41.2)	29.5 (22.1-36.9)	32.6 (28.5-36.7)	31.7 (26.8-36.6)	29.0 (23.4-34.6)	28.6 (22.7-34.5)
EF 1 year	34.3 (28.0-40.6)	28.3 (19.5-37.0)	33.7 (29.8-37.5)	32.6 (26.6-38.6)	33.2 (27.2-39.1)	33.6 (27.2-39.9)
p-value	0.551	0.520	0.413	0.541	0.246	0.038
ESV baseline	164.0 (119.3-208.6)	205.7 (147.3-264.1)	175.0 (151.5-198.6)	179.2 (135.0-223.4)	182.8 (141.4-224.1)	204.6 (162.0-247.2)
ESV 1 year	173.7 (121.1-226.3)	212.8 (133.5-292.2)	177.7 (151.1-204.2)	180.9 (129.6-232.1)	187.0 (145.6-228.4)	196.1 (151.8-240.3)
p-value	0.179	0.613	0.729	0.870	0.573	0.546
EDV baseline	246.3 (196.9-295.6)	283.3 (226.2-340.3)	258.1 (232.0-284.2)	254.7 (204.7-304.7)	254.5 (204.6-304.5)	283.2 (232.8-333.6)
EDV 1 year	253.9 (200.4-307.4)	291.5 (217.3-365.6)	260.9 (228.3-293.5)	254.7 (204.3-305.1)	278.6 (224.7-332.5)	287.0 (242.8-331.1)
p-value	0.421	0.570	0.696	0.999	0.149	0.836
MM baseline	197.7 (157.3-238.2)	199.0 (150.6-247.3)	178.7 (146.4-211.0)	172.6 (145.5-199.7)	172.3 (143.9-200.7)	182.4 (161.1-203.8)
MM 1 year	204.6 (155.5-253.6)	182.8 (138.0-227.5)	181.2 (140.9-221.6)	175.4 (147.3-203.5)	174.2 (149.7-198.6)	185.3 (167.8-202.8)
p-value	0.553	0.168	0.730	0.582	0.701	0.584

Table 3.4. Change in LVEF, LV volumes and myocardial mass at 2 years

|	Peripheral placebo	Peripheral G-CSF	IC serum	IC BMC	IM serum	IM BMC
	N=8	N=8	N=10	N=11	N=6	N=10
EF baseline	33.1 (23.7-42.4)	28.9 (18.2-39.7)	31.7 (26.5-36.9)	31.4 (25.5-37.3)	30.7 (18.8-42.5)	29.3 (21.0-37.7)
EF 2 years	26.8 (16.4-37.1)	25.6 (17.4-33.8)	32.3 (26.1-38.5)	33.4 (28.2-38.6)	29.3 (14.4-44.3)	32.5 (20.0-45.0)
p-value	0.256	0.333	0.775	0.503	0.804	0.356
Change	-6.3 (-18.4-5.8)	-3.3 (-10.8-4.3)	0.6 (-4.2-5.5)	2.0 (-4.4-8.4)	-1.3 (-14.5-11.8)	3.2 (-4.2-10.6)
ESV baseline	190.6 (117.0-264.3)	191.3 (152.1-230.5)	182.4 (156.7-208.0)	181.2 (126.5-235.9)	170.3 (104.2-236.5)	203.2 (149.6-256.8)
ESV 2 years	224.3 (127.5-321.0)	215.8 (158.0-273.5)	186.1 (158.9-213.3)	188.9 (136.3-241.6)	223.0 (131.6-314.4)	188.6 (134.1-243.1)
p-value	0.061	0.053	0.676	0.493	0.008	0.355
Change	33.6	24.4	3.8	7.7	52.7	-14.6
EDV baseline	276.3 (201.8-350.7)	267.6 (236.2-299.0)	265.8 (240.2-291.3)	256.6 (193.4-319.9)	243.7 (165.4-321.9)	284.3 (218.1-350.5)
EDV 2 years	301.8 (200.2-403.3)	287.1 (231.3-343.0)	275.3 (241.0-309.6)	276.6 (213.8-339.5)	306.3 (240.1-372.6)	269.2 (224.4-314.0)
p-value	0.095	0.200	0.387	0.100	0.007	0.427
Change	25.5	19.6	9.6	20.0	62.7	-15.1
MM baseline	226.4 (186.2-266.6)	222.8 (178.8-266.8)	197.7 (170.1-225.3)	171.3 (136.6-205.9)	159.7 (114.2-205.1)	174.9 (148.4-201.4)
MM 2 years	240.3 (189.2-291.4)	221.4 (161.7-281.1)	187.0 (151.5-222.5)	181.5 (134.9-228.1)	153.2 (89.7-216.7)	210.2 (178.0-242.4)
p-value	0.467	0.928	0.319	0.306	0.634	**0.013**
Change	13.9	-1.4	-10.7	10.3	6.5	35.3
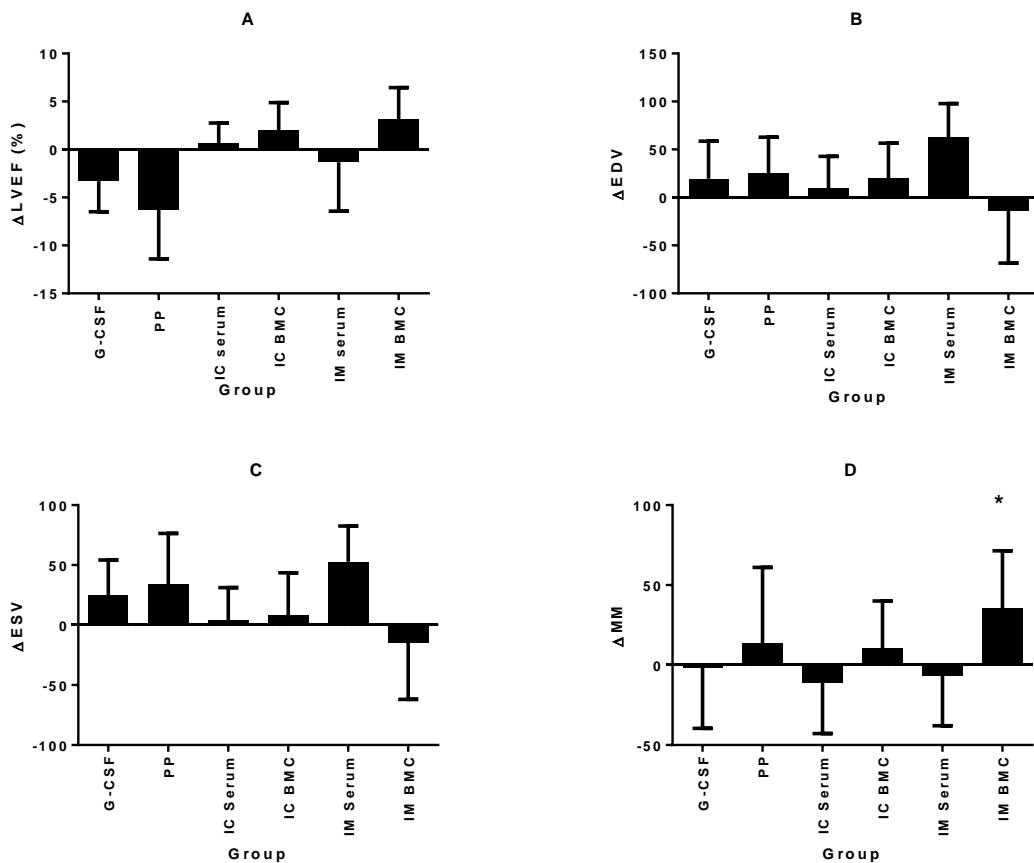
Figure 3.6. Change in LVEF, volumes and myocardial mass by group at 2 years. Change in A) LVEF B) EDV C) ESV D)

MM as measured using CMR/CT in each of the treatment groups (A-F). Only the IM BMC group showed a reduction in EDV and ESV

and a significant increase in myocardial mass at 2 years. A-peripheral placebo; B - peripheral G-CSF only; C – peripheral G-CSF + IC

serum; D - peripheral G-CSF + IC BMC; E - peripheral G-CSF + IM serum; F - peripheral G-CSF + IM BMC. IC-intracoronary; IM-

intramyocardial; G-CSF- granulocyte-colony stimulating factor; BMC-bone marrow derived cells; LVEF-left ventricular ejection

fraction; EDV-end-diastolic volume; ESV- end-systolic volume; MM- myocardial mass; CMR-cardiac magnetic resonance imaging; CT-

computed tomography. * denotes p<0.05 (paired-t-test). Mean + SEM represented

3.2.4 New York Heart Association score

In the peripheral placebo group, there was no significant change in New York Heart Association

(NYHA) score at 6 months (2.29 to 2.25; Δ-0.04; 95% CI -0.45 to 0.38; p=0.862) or at 1 year (2.29 to

2.29; Δ0.00; 95% CI -0.40 to 0.40; p>0.999) at 1 year. In the G-CSF only group, mean NYHA changed

from 2.43 to 2.31 (Δ-0.12; 95% CI -0.57 to 0.33; p= 0.5883) at 6 months and from 2.43 to 2.21, a change of -0.21 (95% CI -0.65 to 0.23; p=0.3302) at 1 year. However, the reduction in NYHA scores at 6 months and 1 year in the G-CSF only group were non-significant.

Neither of the IC groups, showed any significant change in NYHA at 6 months or 1 year. In the IC arm, mean NYHA changed from 2.71 to 2.41 (Δ -0.41; 95% CI -0.83 to 0.014; p=0.058) in the IC placebo group at 6 months and from 2.71 to 2.43 (Δ-0.29; 95% CI -0.70 to 0.13; p=0.169) at 1 year. In the IC BMC group, mean NYHA changed from 2.47 to 2.21 (Δ-0.25; 95% CI-0.69 to 0.18; p=0.249) at 6 months and from 2.47 to 2.13 (Δ-0.33; 95% CI -0.76 to 0.09; p=0.124) at 1 year.

In the IM BMC group, there was a significant improvement in NYHA class at six months from 2.57 to 2.00 (Δ-0.57; 95% CI -1.07 to -0.08; p=0.025), which was maintained at one year (2.57 to 2.07; Δ -0.50; 95% CI -1.00 to 0.00; p=0.048). In the IM serum group, a significant reduction in NYHA was seen at six months from 2.27 to 1.80 (Δ-0.47; 95% CI -0.84 to -0.09; p=0.016) but this was not sustained at one year (2.27 to 2.08; Δ-0.15; 95% CI -0.58 to 0.20; p=0.451) (Figure 3.7).

At 2 years, the IM BMC group showed a continued, significant reduction in NYHA class from 2.53 to 1.80 (Δ-0.73; 95% CI -1.26 to -0.21; p=0.007). No change was seen in any other group (IM placebo group-2.27 to 2.14; Δ-0.13; 95% CI -0.62 to 0.37; p=0.615; IC BMC group-2.47 to 2.08; Δ-0.38; 95% CI -0.82 to 0.06; p=0.087; IC placebo group-2.67 to 2.50; Δ-0.17; 95% CI 0.63 to 0.30; p= 0.473; peripheral G-CSF group-2.40 to 2.33; Δ-0.07; 95% CI -0.63 to 0.50; p= 0.813 and the peripheral placebo group-2.40 to 2.11; Δ -0.29; 95% CI -0.87 to 0.30; p=0.326) (figure 3.8).

Figure 3.7. NYHA class at 1 year. Bar charts showing mean New York Heart Association (NYHA) class at baseline, 6 months and 1 year shown for each of the treatment groups (A-F). A significant reduction in NYHA class was noted in the IM BMC group. A-peripheral placebo; B - peripheral G-CSF only; C – peripheral G-CSF + IC serum; D - peripheral G-CSF + IC BMC; E - peripheral G-CSF + IM serum; F - peripheral G-CSF + IM BMC. IC-intracoronary; IM- intramyocardial; G-CSF- granulocyte-colony stimulating factor; BMC-bone marrow derived cells * denotes p<0.05; Significance assessed using one-way ANOVA with Bonferroni correction for multiple comparisons
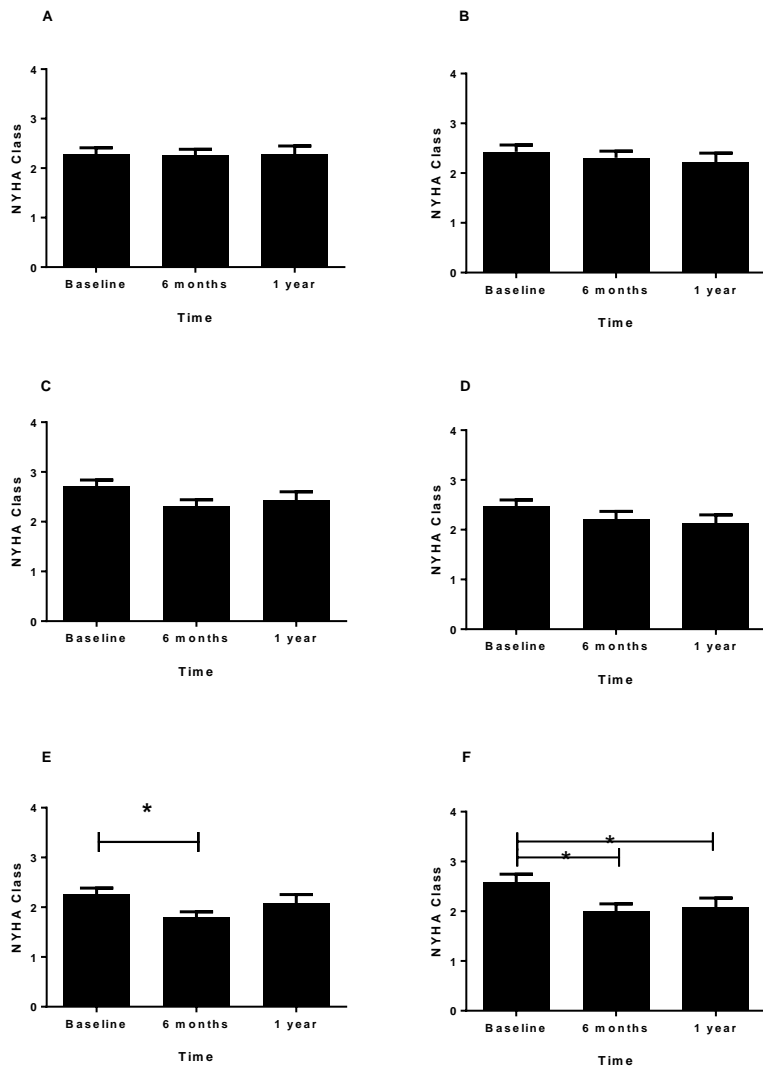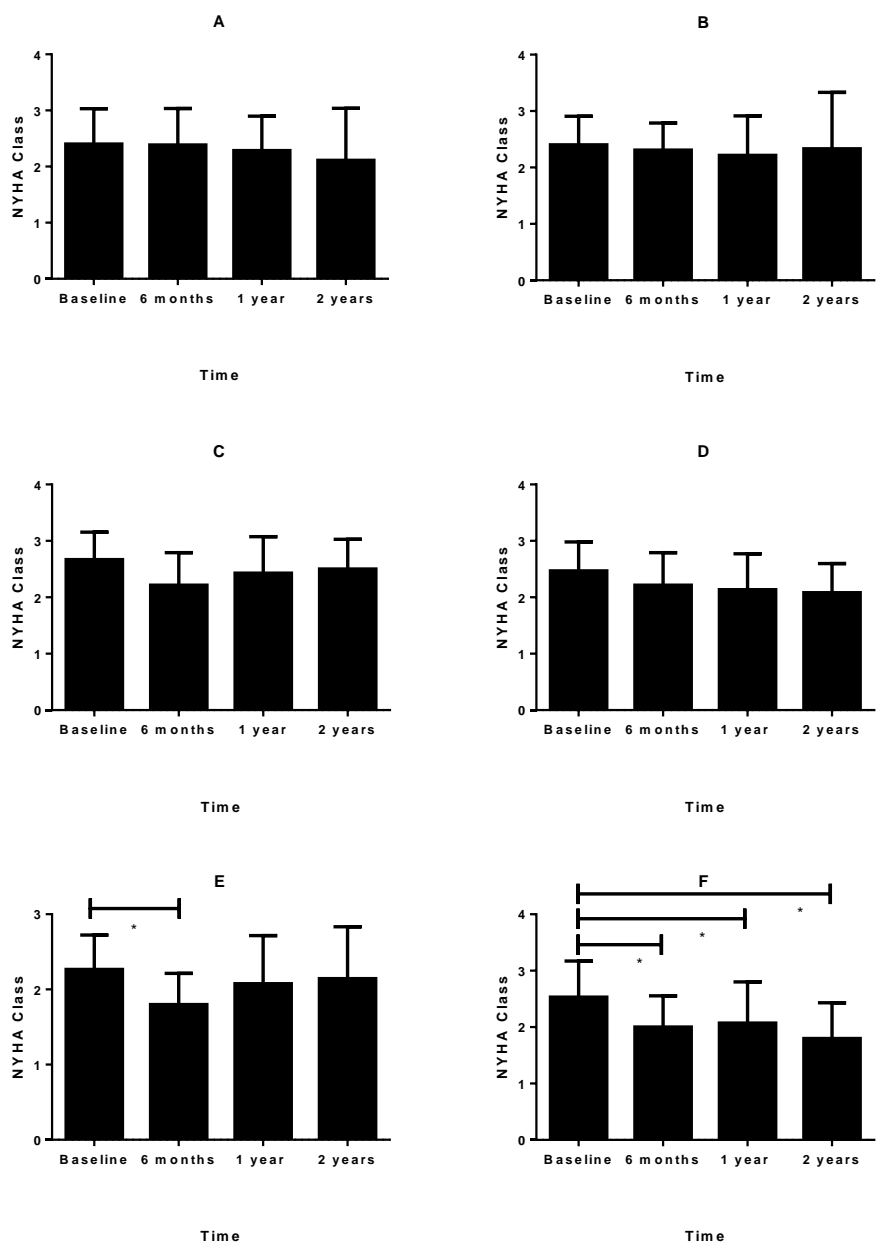
Figure 3.8 NYHA class at 2 years. Mean New York Heart Association (NYHA) class at baseline, 6 months, 1 year and 2 years

shown for each of the treatment groups (A-F). A significant reduction in NYHA class was noted in the IM BMC group at all time

points. A-peripheral placebo; B - peripheral G-CSF only; C – peripheral G-CSF + IC serum; D - peripheral G-CSF + IC BMC; E - peripheral

G-CSF + IM serum; F - peripheral G-CSF + IM BMC. IC-intracoronary; IM-intramyocardial; G-CSF- granulocyte-colony stimulating

factor; BMC-bone marrow derived cells * denotes p<0.05; Significance assessed using one-way ANOVA with Bonferroni correction

for multiple comparisons

3.2.5 Canadian Cardiovascular Society score

There was no significant change in Canadian Cardiovascular Society (CCS) angina class seen in any

of the treatment groups either at 6 months, 1 year (Figures 3.9) or 2 years.
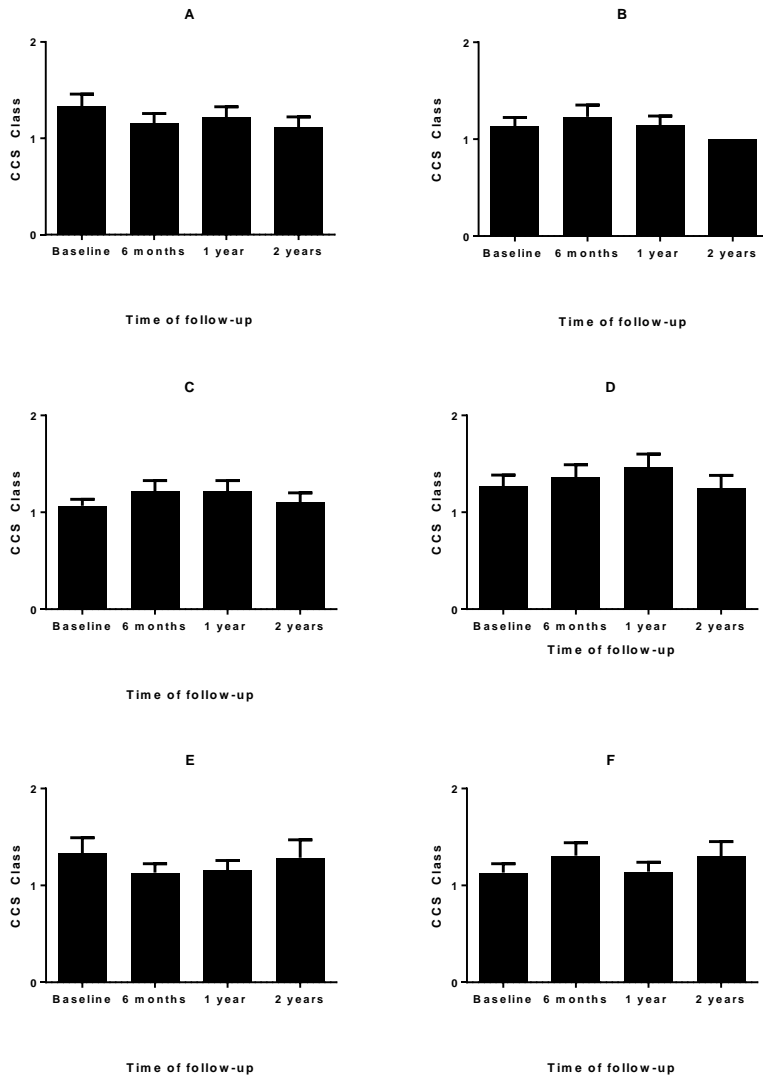
Figure 3.9. Canadian Cardiovascular Society (CCS) angina score. Bar charts of CCS score at baseline, 6 months and 1

year shown in each group A-F. No significant changes were noted in any group. A-peripheral placebo; B-G-CSF only; C-IC serum; D-IC

BMC; E- IM serum; F-IM BMC. IC-intracoronary; IM-intramyocardial; BMC= Bone marrow derived cells. G-CSF =granulocyte colony

stimulating factor. Significance assessed using one-way ANOVA with Bonferroni correction for multiple comparisons

3.2.6 NT pro-BNP

At 6 months, the NT pro-BNP level showed a non-significant increase from 775.7±235.7 to 907.9±376.3 (p=0.685) in the peripheral placebo group. Similarly, the change in NT pro-BNP was also non-significant in the peripheral G-CSF group (1498±602.8 to 1644±639.3; p=0.638). In the IC arm, neither group showed any significant change in NT pro-BNP. In the IC placebo group, the NT pro-BNP level changed negligibly from 1180±298.3 to 1198±334.2 (p=0.608) and in the IC BMC group, it decreased from 440.5±58.6 to 396.9±69.7 (p=0.15). The IM serum group demonstrated a trend towards a fall in NT pro-BNP at 6 months (572.0±113.5 to 408.6±80.9; p=0.050) although this was not significant. At six months, only the IM BMC group showed a significant fall in NT pro-BNP (977.5±231.7 to 768.4±201.6; p=0.018) (figure 3.10). All values were log transformed for within-group analyses.

3.2.7 Change in global LVEF measured using transthoracic echocardiography

In the peripheral placebo group, there was a reduction in LVEF of -3.0% (95% CI -16.0 to 10.0; p=0.590) at 1 year as compared to baseline. In the G-CSF only group, the LVEF did not change at 1 year when compared to baseline. The IC serum group showed a -0.5% (95% CI -10.6 to 9.7; p=0.927) decrease. The IC BMC group demonstrated an almost negligible change in LVEF (0.2%; 95% CI -9.6 to 10.0; p=0.973). In the IM serum group, there was a -0.8% (95% CI -10.1 to 8.5; p=0.859) reduction at 1 year. In contrast, the IM BMC group showed a 3.5% improvement (95% CI -3.2 to 10.2; p=0.272) - the highest improvement in LVEF out of all six groups (figure 3.11). At 2 years, the change in LVEF in the IM BMC group persisted (+4.0%; 95% CI -9.8 to 17.8; p=0.515) (figure 3.12). All other groups showed a reduction or marginal increase (+0.70% in IC placebogroup) at 2 years.
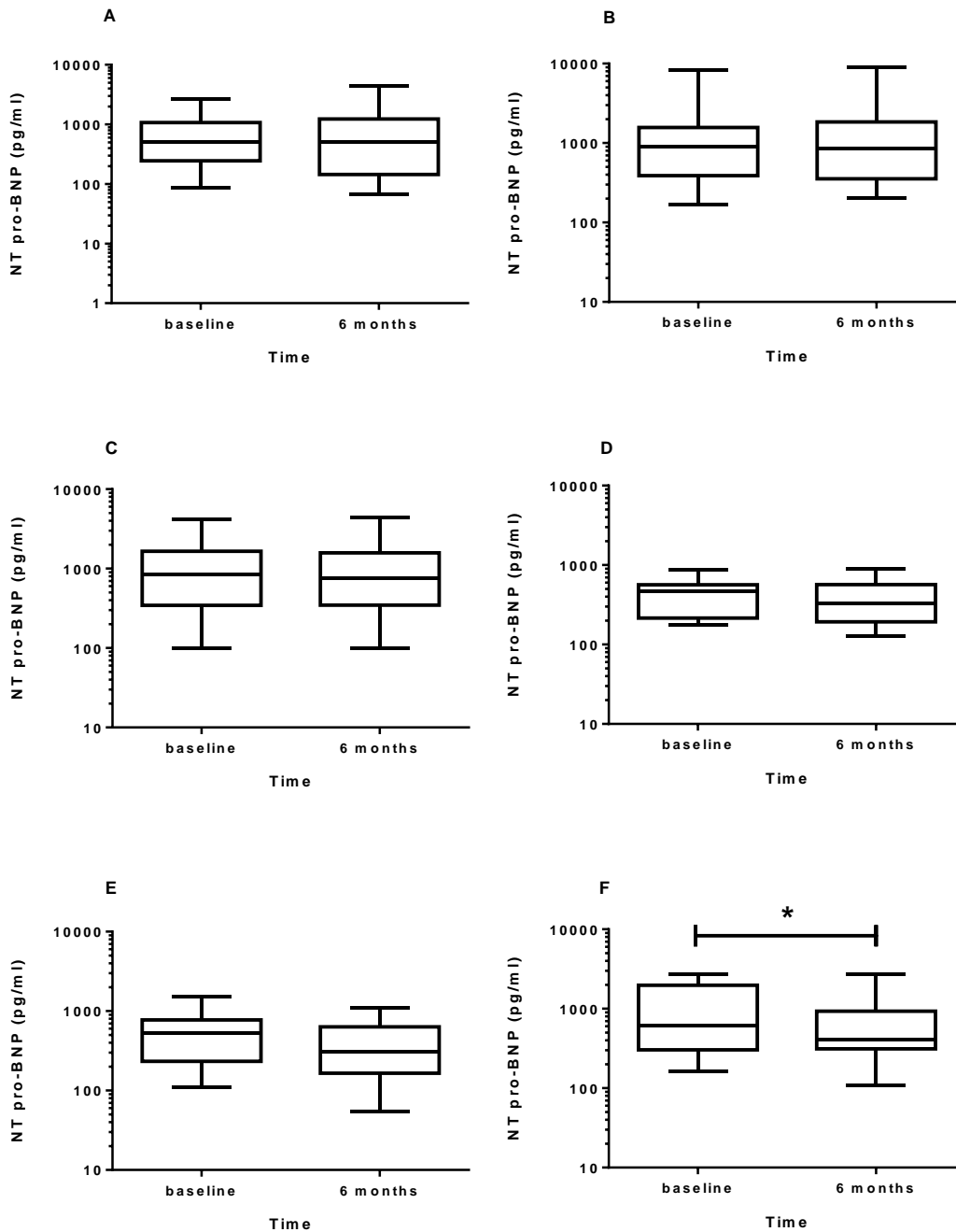
Figure 3.10. Change in NT pro-BNP. Box and whisker plots showing NT pro-BNP (pg/ml) at baseline and 6 months (median and range on a logarithmic scale). NT pro-BNP was significantly reduced in the IM BMC group. A-peripheral placebo; B - peripheral G-CSF only; C – peripheral G-CSF + IC serum; D - peripheral G-CSF + IC BMC; E - peripheral G-CSF + IM serum; F - peripheral G-CSF + IM BMC. IC-intracoronary; IM-intramyocardial; G-CSF- granulocyte-colony stimulating factor; BMC-bone marrow derived cells. * denotes p<0.05 paired t-test

3.2.8 Change in LVEF as measured using quantitative left ventriculography

Quantitative left ventriculography (QLV) was performed at baseline and 6 months in the IC and IM arms only. In the IC BMC group, LVEF decreased by -1.8% (95% CI -6.7 to 3.2; p=0.448) and in the IC serum group, the magnitude of LVEF reduction was similar (-1.9%; 95% CI -9.1 to 5.3; p=0.504). The change in LVEF was not significantly different between the IC serum and BMC groups (p=0.969). In the IM BMC group, there was a -1.8% reduction in LVEF (95% CI -5.2 to 1.7; p=0.288). In the IM serum group, there was, a 1.2% increase in LVEF (95% CI -3.0 to 5.5; p=0.546). However, none of the changes in LVEF were statistically significant. Furthermore, the difference between the change in LVEF in the two IM groups was not significant (p= 0.260).
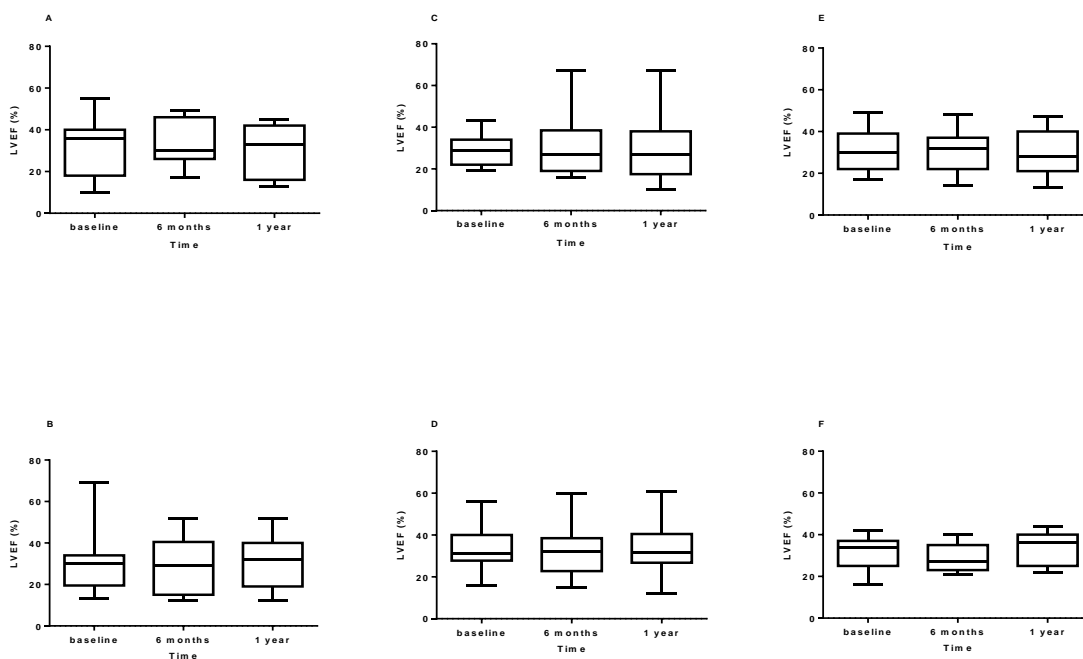
Figure 3.11. Change in LVEF as measured using transthoracic echocardiography at 1 year. Box and whisker plots of LVEF derived using contrast transthoracic echocardiography at baseline, 6 months and 1 year in each of the treatment groups are shown in this figure. No significant changes were noted at 1 year although a 3.5% improvement in LVEF was noted in the IM BMC group. A-peripheral placebo; B-G-CSF only; C-intracoronary serum; D-IC BMC; E- IM placebo; F-IM BMC. IC-intracoronary; IM-intramyocardial; BMC-bone marrow derived cells; LVEF-left ventricular ejection fraction. *p<0.05. Significance assessed using one-way ANOVA with Bonferroni correction for multiple comparisons
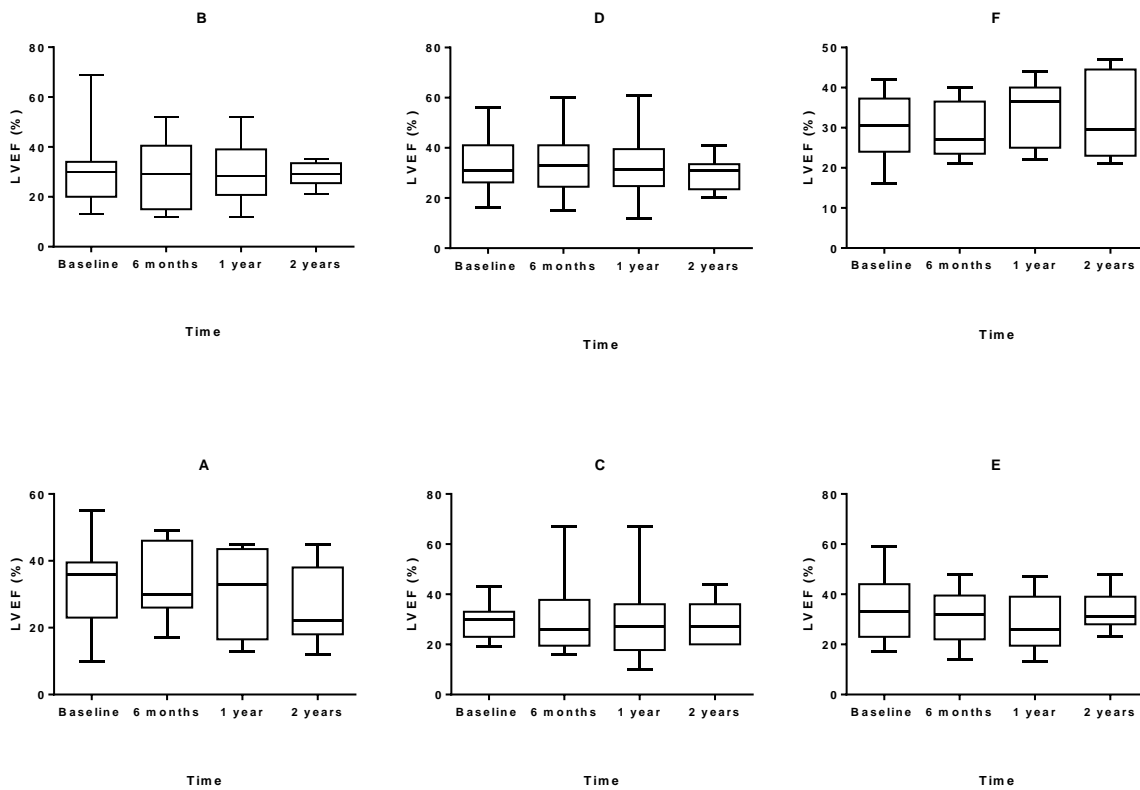
Figure 3.12. Change in LVEF as measured using transthoracic echocardiography at 2 years. Box and whisker plots showing mean LVEF at baseline, 6 months, 1 year and 2 years shown for each of the treatment groups (A-F). The trend towards improvement in LVEF in the IM BMC group at 1 year was sustained at 2 years. A-peripheral placebo; B - peripheral G-CSF only; C – peripheral G-CSF + IC serum; D - peripheral G-CSF + IC BMC; E - peripheral G-CSF + IM serum; F - peripheral G-CSF + IM BMC. IC-intracoronary; IM-intramyocardial; G-CSF - granulocyte-colony stimulating factor; BMC-bone marrow. *p<0.05. Significance assessed using one-way ANOVA with Bonferroni correction for multiple comparisons

3.2.9 Quality of Life questionnaires

MacNew

Only the IC BMC group showed a significant improvement in the MacNew QoL questionnaire at 1 year. At 6 months, the IM BMC group showed a significant improvement in the MacNew score but this was not sustained at 1 year. All other groups showed no significant change in MacNew scores at 1 year (table 3.5). Both the IC groups - IC BMC and placebo- showed significant improvements in

the MacNew questionnaire at 2 years. The remaining groups showed no significant change in the

MacNew questionnaire (Table 3.6)

Table 3.5. Quality of life questionnaire scores at 1 year

	MacNew		EQ5D Index/ VAS		SF36 Physical		SF36 Mental	
Group	Baseline	1 year	Baseline	1 year	Baseline	1 year	Baseline	1 year
Peripheral								
Placebo (n=14)	4.54±1.59	4.74±1.14	0.62±0.26/52.7±29.1	0.65±0.19/50.0±24.6	24.0±11.1	28.2±11.9	47.9±14.3	49.0±13.5
G-CSF (n=13)	3.96±0.87	4.30±1.41	0.61±0.26/51.1±20.4	0.47±0.32/50.4±25.7	21.8±10.5	25.9±12.9	45.1±12.4	42.5±12.5
Intracoronary								
Placebo (n=12)	4.53±1.16	4.64±1.32	0.63±0.26/52.4±18.9	0.65±0.26/59.6±19.2	23.7±8.0	32.3±12.9[*]	54.0±12.0	47.7±13.6
BMC (n=13)	4.29±1.40	4.73±1.33[*]	0.50±0.36/49.6±19.8	0.66±0.22/59.5±27.3	24.7±12.1	28.9±9.9	50.6±14.8	51.0±13.2
Intramyocardial								
Placebo (n=13)	4.15±0.98	4.95±1.47	0.55±0.29/49.8±12.8	0.63±0.26/55.0±24.5	31.0±9.8	34.7±10.6	40.2±17.7	48.8±15.4
BMC (n=12)	3.80±1.03	4.09±1.34	0.55±0.26/45.5±22.0	0.53±0.41/46.9±26.0	25.5±13.7	29.9±15.4[*]	44.1±9.5	44.0±12.6

EQ5D

In the EQ5D questionnaire, only the IC placebo group showed a significant improvement in the EQ5D visual

analogue score at 6 months. None of the other groups showed any significant improvement in QoL, using

the EQ5D, at 1 or 2 years (tables 3.5 and 3.6).

SF36

At 6 months, the peripheral placebo and IC placebo groups showed significant increase in SF36 physical

wellbeing score. At 1 year, the IC placebo group and IM BMC group showed significant improvement in the

physical wellbeing score. None of the other groups showed any significant change in SF36 at 1 year.

Table 3.6. Quality of life questionnaire scores at 2 years

	MacNew		EQ5D Index/VAS		SF36 Physical		SF36 Mental	
Group	Baseline	2 years	Baseline	2 years	Baseline	2 years	Baseline	2 years
Peripheral								
Placebo (n=9)	5.01±1.69	4.96±1.20	0.71±0.14/52.6±26.2	0.72±0.17/54.3±16.2	26.9±11.3	34.4±10.9*	51.4±11.3	47.3±12.4
G-CSF (n=9)	3.77±0.88	3.86±1.13	0.55±0.32/52.3±16.5	0.44±0.33/47.3±17.4	22.0±11.2	26.7±9.9	41.5±10.9	42.7±11.4
Intracoronary								
Placebo (n=8)	4.49±1.24	4.88±0.99*	0.71±0.16/54.7±25.8	0.75±0.14/50.4±33.4	25.9±8.0	33.7±12.0*	52.0±12.9	48.6±13.2
BMC (n=11)	3.98±1.29	4.66±1.23*	0.44±0.35/45.9±18.7	0.57±0.27/57.2±28.7	23.4±12.1	24.8±10.7	48.0±14.4	53.4±12.9
Intramyocardial								
Placebo (n=6)	4.03±0.79	4.90±1.35	0.44±0.25/45.0±6.5	0.52±0.26/56.0±22.6	32.5±11.6	32.0±11.6	36.2±20.5	49.9±17.0
BMC (n=8)	3.82±0.70	4.42±0.93	0.57±0.19/40.3±16.0	0.47±0.32/50.4±20.4	24.7±11.8	26.8±11.2	45.1±9.6	50.9±10.8

At 2 years, the peripheral placebo and IC placebo groups showed significant improvement in the SF-36 physical summary score. None of the other groups showed any significant change in the SF-36 scores. The QoL scores at 1 year and 2 years are shown in table 3.5 and 3.6 respectively.

3.3 Safety

3.3.1 Adverse effects related to G-CSF administration

The main adverse effect noted was bone pain (12 of 14 recorded events; 85.7% of adverse events recorded) (table 3.7). The incidence of adverse effects to G-CSF administration were not significantly different across the groups (p=0.686).

Table 3.7. Incidence of adverse effects secondary to G-CSF administration across the groups

Group	Bone pain	Other adverse effects	
Intramyocardial			
IM Serum	5	0	
IM BMC	0	2 (1 splenomegaly and 1 chest pain)	
Intracoronary			**P=0.686 (Fisher's exact test)**
IC Serum	2	0	
IC BMC	2	0	
Peripheral			
G-CSF	3	0	

3.3.2 Procedural complications

In the IC arm, there were 4 procedural complications. These were: femoral artery pseudoaneurysm, femoral dissection, coronary dissection (managed conservatively), and a small femoral access site haematoma. In the IM arm, there were 4 patients who developed small, femoral access site haematomas. One patient developed a major access site bleed and required vascular surgery. Three patients had arrhythmias-one patient developing AF, one patient went into VT requiring DC cardioversion and one patient had an episode of non-sustained VT. One patient developed a small pericardial effusion, which was managed conservatively. There was no significant difference in procedural complication rates between the two arms (p=0.209).

3.3.3 Cardiac enzymes

A small increase in post-procedural troponin was detected in the IM arm (0.02±0.04; p=0.007) (Figure 3.13a). However analysis by treatment group, revealed no significant difference in troponin increase between the two groups. Clinically this rise was insignificant and was not associated with a

significant rise in creatine kinase (85.1 to 129.5; p=0.102) (figure 3.13b). Importantly, no clinical events occurred during the procedures. No significant increases in either CK or troponin were seen in any other treatment arm.
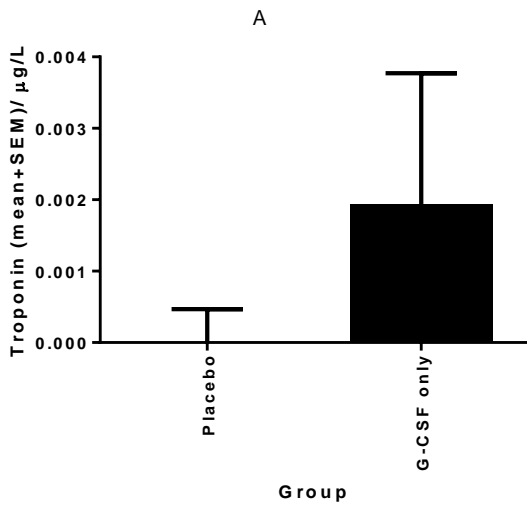
Figure 3.13a. Change in troponin. Diagram showing change in troponin between day 0 and day 7 (post-intervention) in the A-Peripheral arm B- Intracoronary arm and C-intramyocardial arm*p<0.05. BMC-bone marrow derived cells

A
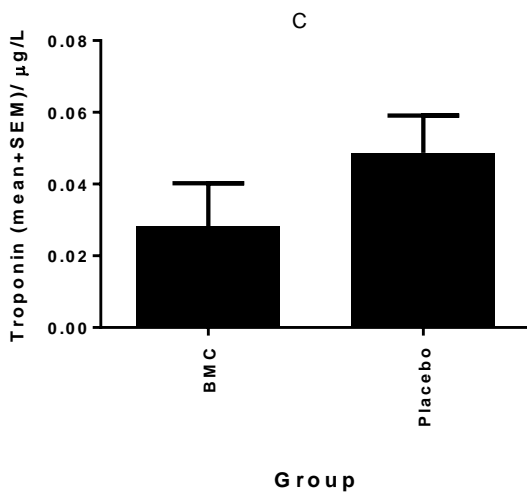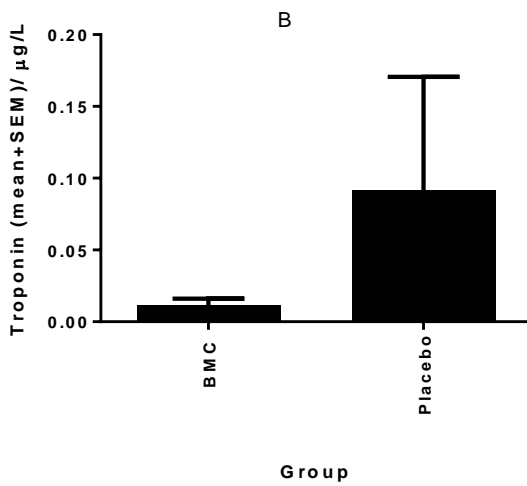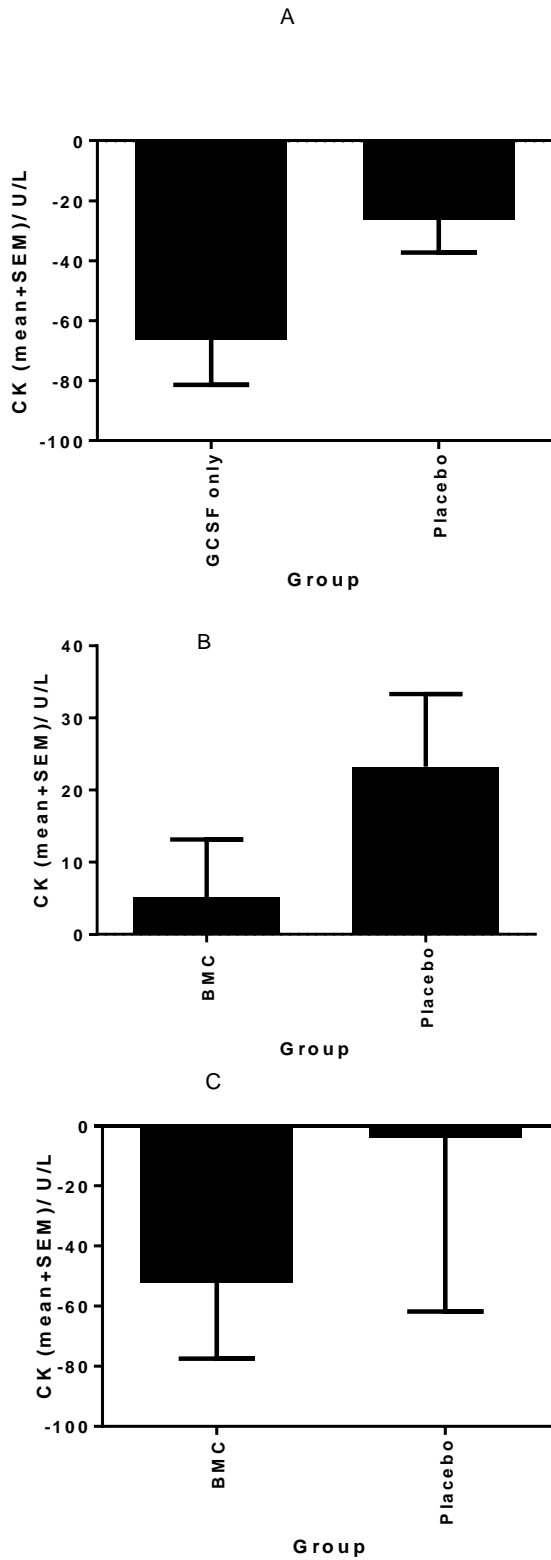
Figure 3.13b. Change in creatine kinase. Bar chart showing change in creatine kinase between day 0 and day 7 (post-intervention) in A-Peripheral arm B-Intracoronary arm and C-intramyocardial arm *p<0.05. BMC-bone marrow derived cells

B

C

3.3.4 MACE

1 year

Low rates of MACE were seen at 1 year in all treatment groups with no difference between them (Logrank p=0.539). There was one cardiac death in the G-CSF only group (6.7%) and one MI in the IC placebo group (6.7%).

2 years

The MACE rates were as follows: 2 in peripheral placebo group (2 MI; 13.3%), 2 in G-CSF group (2 cardiac deaths; 13.3%) and 2 in the IC placebo group (1 MI with PCI and 1 cardiac death; 13.3%) and 2 in the IM BMC group (2 cardiac deaths: 13.3%) (Figure 3.14). The MACE rates were not significantly different between the groups (Logrank p=0.514).

Figure 3.13 Kaplan-Meier MACE curves. Major adverse cardiovascular events (MACE) shown for each of the groups to 2 years. No significant differences were seen in event rates between groups (Logrank p=0.514). MACE defined as cardiac death, MI, CABG or PCI. PP-peripheral placebo; IC-intracoronary; IM-intramyocardial; BMC-bone marrow derived cells

3.3.5 Serious arrhythmias

No significant difference was noted in the incidence of serious arrhythmias (as defined in chapter 2) between the groups at 1 year (Fisher's exact test; p=0.89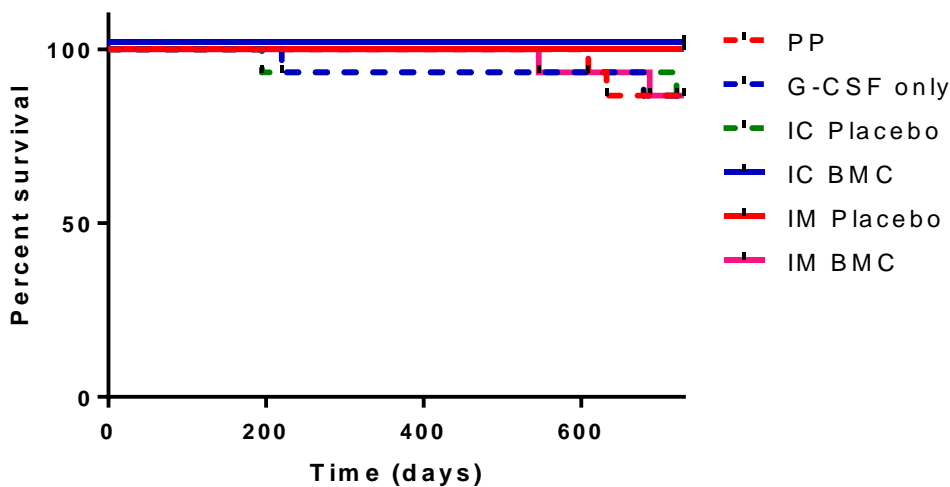6). There was 1 serious arrhythmia in each of the IM and IC groups and 2 in the G-CSF only group. Between 1 and 2 years, an additional 1 serious arrhythmia occurred in each of the IC groups. At 2 years, there were a total of 8 serious arrhythmias (Table 3.8). There was no significant difference in the incidence of serious arrhythmias at 2 years across the groups (Fisher's exact test; p=0.865).

Table 3.8. Incidence of serious arrhythmias across the groups at 1 year.

	0-1 year	1-2 years	Total
Peripheral			
G-CSF only	2	0	2
Placebo	0	0	0
Intracoronary			
IC BMC	1	1	2
IC placebo	1	1	2
Intramyocardial			
IM BMC	1	0	1
IM placebo	1	0	1

3.3.6 Hospitalisation for heart failure exacerbation

Rates of hospitalisation for heart failure were low across the groups (table 3.9), with no significant differences between the groups (p=0.982). Interestingly the IM BMC group had no admissions with

heart failure over the first year. At 2 years, a total of 8 patients had admissions with heart failure-4 in the peripheral arm (3 in G-CSF group and 1 in placebo group). 2 patients in the IC BMC group and 1 in the IC placebo group required admission. The IM BMC group again had no patients requiring admission with heart failure at 2 years. There was no significant difference between the number of patients requiring hospitalisation with heart failure across the groups at 2 years (Fisher's exact test; p=0.664).

Table 3.9. Hospitalisation for heart failure at 1 year.

Group	0-1 year	1-2 years	Total
Peripheral			
GCSF only	2	1	3
Placebo	1	0	1
Intracoronary			
IC BMC	1	1	2
IC placebo	1	0	1
Intramyocardial			
IM BMC	0	0	0
IM placebo	1	0	1

3.3.7 Mortality

There were total of 2 deaths in the whole trial at 1 year {1 non-cardiac (pneumonia), and 1 cardiac (MI)} (Table 3.9), with no difference between treatment groups. There were a total of 7 deaths out of the 90 participants (7.8%) at 2 years. Of these, there were 5 cardiac deaths-2 in peripheral G-CSF group, 1 in the IC placebo group and 2 in the IM BMC group (Table 3.10).

Table 3.10. Mortality at 1 and 2 years

Group	All cause deaths at 1 year	Cardiac deaths at 1 year	All cause deaths at 2 years	Cardiac deaths at 1-2 years
Peripheral				
Placebo	0	0	0	0
G-CSF	1	1	2	1
Intracoronary				
IC Placebo	0	0	2	1
IC BMC	0	0	0	0
Intramyocardial				
IM Placebo	1	0	1	0
IM BMC	0	0	2	2

3.4 Summary

- Administration of intramyocardial and intracoronary stem cells appear safe in patients with ischaemic cardiomyopathy

- Only the IM BMC group met the primary endpoint with a 4.99% improvement in LVEF at 1 year as measured using CT/CMR

- At 2 years, none of the groups showed a significant change in LVEF. The IM BMC group showed a sustained increase in LVEF.

- Change in LVEF was greater in patients with a lower LVEF at baseline (< median LVEF)

- The IM BMC group showed a significant reduction in NYHA class at 6 months, 1 year and 2 years; whilst the IM placebo group showed a significant reduction in NYHA class at 6 months only. None of the other groups showed any significant change in NYHA class.

- None of the groups showed any significant change in CCS class at any time point

- The IM BMC group showed a significant reduction in NT pro-BNP at 6 months

- None of the groups demonstrated a significant change in LVEF at 1 year or 2 years using transthoracic echocardiography or QLV.

- There was no significant difference in the incidence of adverse events related to G-CSF administration or procedural complication across the groups

- MACE rates were low across the groups at both 1 year and 2 years

Chapter 4 The relationship between cytokine levels and cardiac function

4.1 Background

Activation of inflammatory pathways has been linked to the progression of heart failure in several studies as detailed in section 1.1.2.3. Heart failure is associated with changes in many different cytokines. Since the suggestion of a link between inflammation and heart failure by Levine et al. following the discovery of elevated TNF- α in heart failure patients, several studies have shown that a number of pro-inflammatory cytokines and chemokines are elevated in HF.[222] These include TNF-α, interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), IL-1, IL-2, IL-18 and interferon-γ (IFN-γ) [32]. These pro-inflammatory mediators are released from the myocardium and other extra-cardiac tissues in response to injury and although their primary purpose is to aid in the repair process, sustained and overexpression of these cytokines and chemokines exerts deleterious effects on the heart. These effects include but are not limited to myocyte hypertrophy, oxidative stress, increased matrix metalloproteinase secretion by fibroblasts and extracellular matrix degradation as well as increased myocyte apoptosis, necrosis and fibrosis. To counteract these pro-inflammatory mediators, anti-inflammatory cytokines such as IL-10 are released into the circulation. However, it appears that in HF patients, this response is blunted with reduced responses of anti-inflammatory cytokines compared to pro-inflammatory cytokine levels. [49] Thus it appears that the balance between pro- and anti-inflammatory cytokines and chemokines is tilted towards the pro-inflammatory state in patients with HF. Several of these cytokines have been linked to disease severity and prognosis e.g. TNF-α, IL-6, etc. In this cytokine sub-study, the relationship between a panel of cytokines consisting of pro- and inflammatory cytokines as well as proangiogenic cytokines was studied in the context of G-CSF and autologous bone marrow derived cell therapy to assess any relationships with cardiac function and heart failure progression.

4.2 Hypothesis

The administration of G-CSF and autologous bone marrow derived cells leads to an improvement in cardiac function in patients with ischaemic cardiomyopathy by modulating cytokine levels

4.3 Specific aims

1. To measure levels of IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, TNF-α, IFN-γ, IL-1a, IL-1b and MCP-1 over time in patients with ischaemic cardiomyopathy and correlate these to LVEF

2. To measure the effect of G-CSF alone and in combination with autologous BMC on the levels of IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, TNF-α, IFN-γ, IL-1a, IL-1b and MCP-1 in patients with ischaemic cardiomyopathy

For methods-see section 2.2

4.4 Results

The 11 cytokines measured were: IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, TNFα, IFNγ, MCP-1, IL-1a and IL-1b. A total of 50 patients had paired serum samples at baseline and 1 year stored at -80°C that were suitable for analysis (figure 4.1). The remainder of the samples were either unsuitable due to having undergone multiple freeze-thaw cycles during equipment transfers, incorrect labelling, insufficient sample volume or were missing. The inter-assay coefficients of variation (CV) for the 11 cytokines were within acceptable limits, with the majority of CV's <15%.
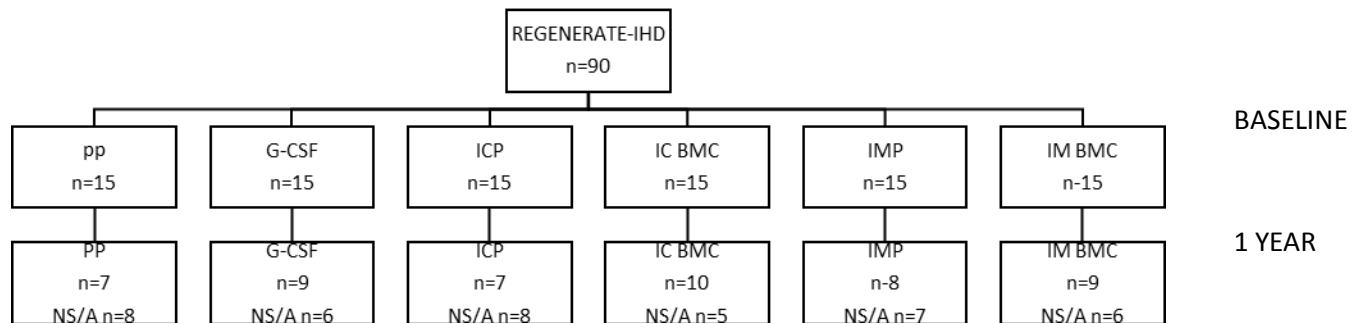

Figure 4.1. **Cytokine analysis flowchart.** Diagram depicting the distribution of cytokine samples in each group. PP-peripheral placebo; ICP- intracoronary placebo; BMC- Bone marrow derived cells; IMP- intramyocardial placebo NS/A-samples not available or suitable (due to multiple freeze-thaw cycles during equipment transfers, incorrect labelling or insufficient sample volume)

4.4.1 Change in cytokines associated with adverse outcomes in heart failure and association with LVEF at 1 year

Interleukin-8

The interleukin-8 (IL-8) level at baseline was 4.01±4.8pg/ml and increased to 5.03±6.5pg/ml. The mean increase of 1.02 pg/ml was statistically significant (p=0.042) (Fig 4.2). IL-8 showed a negative correlation with LVEF (r^2 =0.05; p=0.024) (Fig 4.3). In the peripheral placebo group, IL-8 significantly increased by 0.92pg/ml (95% CI 0.05 to 1.79; p=0.042). In the G-CSF only group, IL-8 increased by 1.95pg/ml (95% CI -2.90 to 6.80; p=0.373). IL-8 increased by 0.48pg/ml (95% CI -2.72 to 3.69; p=0.725) in the IC placebo group and by 0.83pg/ml (95% CI -0.93 to 2.58; p=0.315) in the IC BMC group. In the IM placebo group, IL-8 increased by 0.74pg/ml (95% CI -0.78 to 2.26; p=0.289). Only in the IM BMC group did the mean IL-8 level decrease (-0.21pg/ml; 95% CI -1.38 to 0.97) although this was not significant (p=0.695) (Fig 4.4). However, the change in IL-8 between the groups was not significant (one-way ANOVA p=0.427).
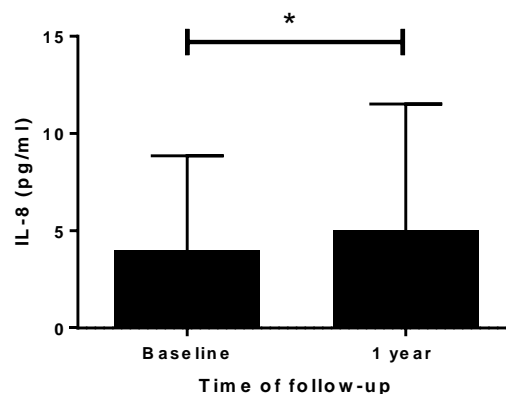
Figure 4.2. Change in IL-8 in the entire cohort. Bar charts showing mean (+SEM) IL-8 level at baseline and 1 year. * p<0.05. IL-8- interleukin-8

r^2=0.05

p=0.024

Figure 4.3. IL-8 and LVEF. Correlation curve between IL-8 and LVEF. A significant correlation was observed between IL-8 and

LVEF. LVEF-left ventricular ejection fraction. IL-8 interleukin 8

Figure 4.4. IL-8 level by study group. Bar charts showing change in IL-8 levels in each group at baseline and 1 year (mean+SEM). A-peripheral placebo B-G-CSF only C-IC placebo D-IC BMC E-IM placebo F-IM BMC. *p<0.05. G-CSF-granulocyte colony stimulating factor. IC-intracoronary. IM-intramyocardial. BMC-bone marrow cells. IL-8 interleukin 8.

Interferon-γ

The mean interferon-γ (IFNγ) level for the entire cohort did not change significantly between baseline and 1 year (0.20±0.2pg/ml to 0.23±0.2pg/ml; p=0.413) (Fig 4.5). There was a significant negative correlation between IFNγ and LVEF (r^2=0.06; p=0.015) (Fig 4.6). IFNγ did not change significantly in any of the six groups. In the peripheral placebo group, IFNγ increased by 0.10pg/ml (95% CI -0.09 to 0.28; p=0.245). In the G-CSF only group, IFNγ increased by 0.15pg/ml (95% CI -0.11 to 0.42; p=0.209). There was negligible change in IFNγ in the IC placebo group (-0.01pg/ml; 95% CI -0.02 to 0.04; p=0.664) and the IC BMC group (-0.002pg/ml; 95% CI -0.03 to 0.03; p=0.883). Similarly, in the IM placebo group, IFNγ change was negligible (0.003 pg/ml; 95% CI -0.09 to 0.09; p=0.948). In the IM BMC group, IFNγ levels fell by a mean of -0.09pg/ml (95% CI -0.36 to 0.19; p=0.492) (Fig 4.7). The change in IFNγ at 1 year was not significantly different across the groups (one-way ANOVA p=0.323)
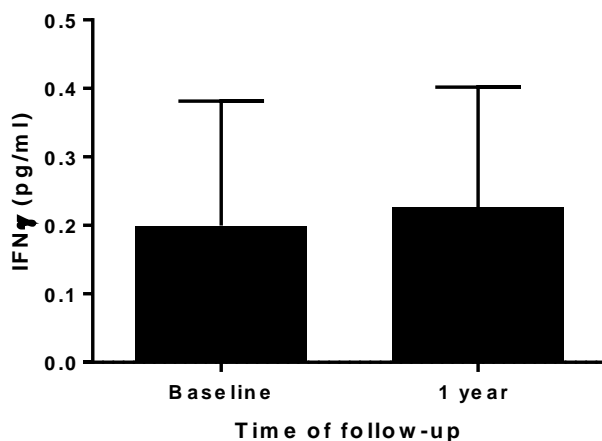
Figure 4.5. Change in IFN-γ in the entire cohort. Bar charts showing mean (+SEM) IFN-γ level at baseline and 1 year. * p<0.05. IFN-γ-interferon gamma
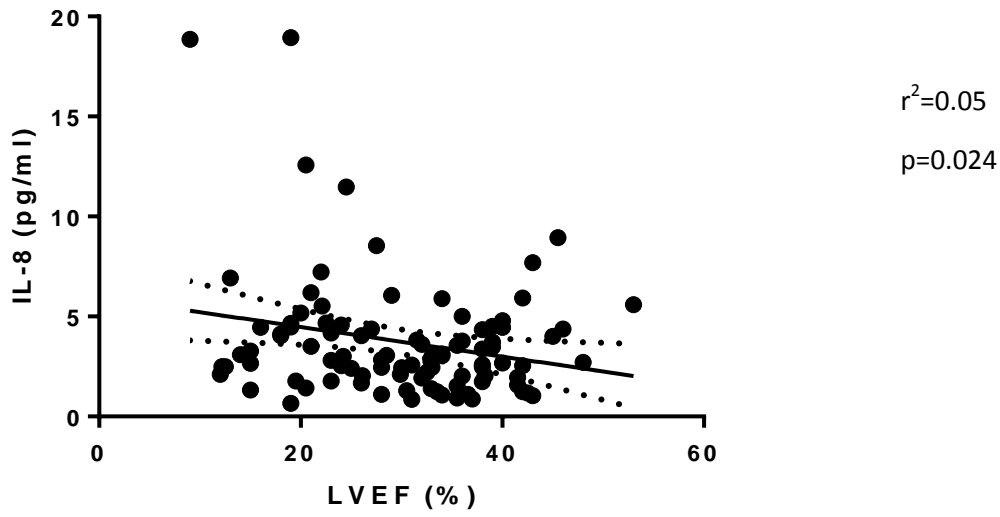
Figure 4.6. IFN-γ and LVEF. Correlation curve between IFN-γ and LVEF. A significant correlation was observed between IFN-γ

and LVEF. LVEF-left ventricular ejection fraction. IFN-γ interferon gamma
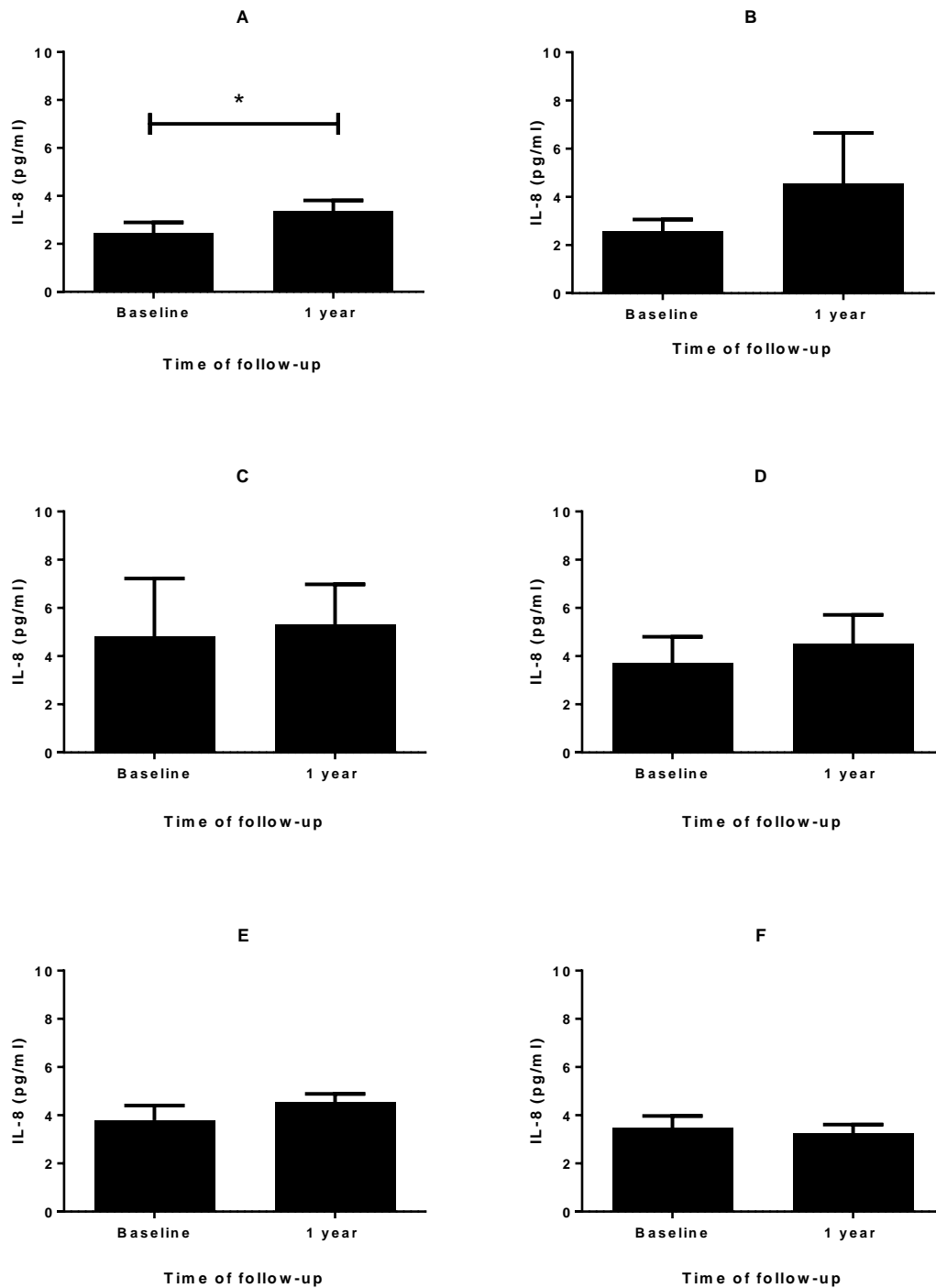
Figure 4.7. IFNγ level by study group. Bar charts showing change in IFNγ levels in each group at baseline and 1 year (mean+SEM). A-peripheral placebo B-G-CSF only C-IC placebo D-IC BMC E-IM placebo F-IM BMC. *p<0.05. G-CSF-granulocyte colony stimulating factor. IC-intracoronary. IM-intramyocardial. BMC-bone marrow cells. IFNγ-interferon gamma.

Interleukin-6

The baseline interleukin-6 (IL-6) level was 2.35±1.6pg/ml and increased more than two fold to 5.90±16.0pg/ml, an increase of 3.6pg/ml, although this was not significant (p=0.127) (Fig 4.8). IL-6 level was not significantly correlated with LVEF (r^2=0.01; p=0.284). IL-6 increased significantly in the IM placebo group (1.22pg/ml; 95% CI 0.05 to 2.40; p=0.044). In the remainder of the groups there was no significant change in IL-6 levels. In the peripheral placebo group, IL-6 levels were virtually unchanged (mean change 0.01pg/ml; 95% CI -1.07 to1.09; p=0.988). In the G-CSF only group, IL-6 increased by 3.28pg/ml (95% CI -0.33 to 6.88; p=0.070). IL-6 also increased in both IC groups- in the IC placebo group by 0.85pg/ml (95% CI -1.51 to 3.21; p=0.410) and in the IC BMC group by 0.67pg/ml (95% CI -1.36 to 2.70; p=0.472). In the IM BMC group, IL-6 increased by 1.49pg/ml (95% CI -0.60 to 3.54; p=0.136) (Fig 4.9).

Figure 4.8. Change in IL-6 in the entire cohort. Bar charts showing mean (+SEM) IL-6 level at baseline and 1 year. * p<0.05. IL-6- interleukin-6
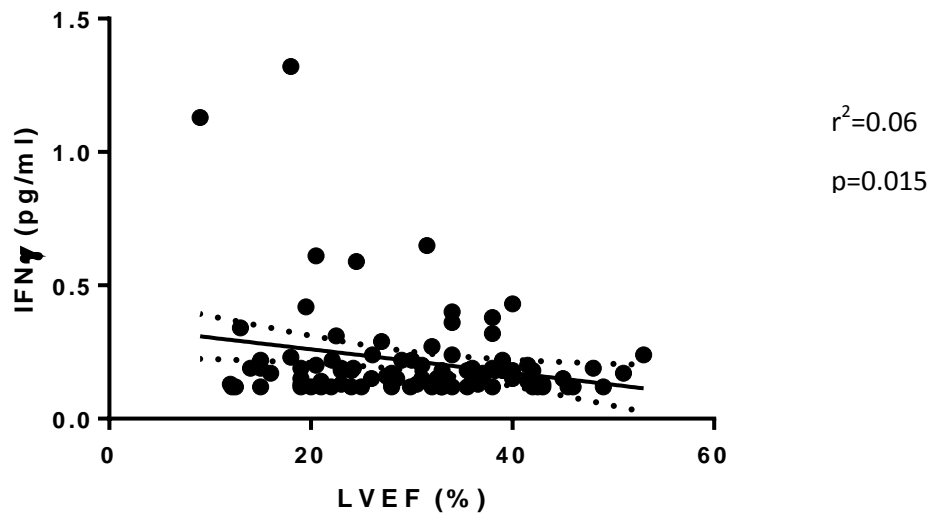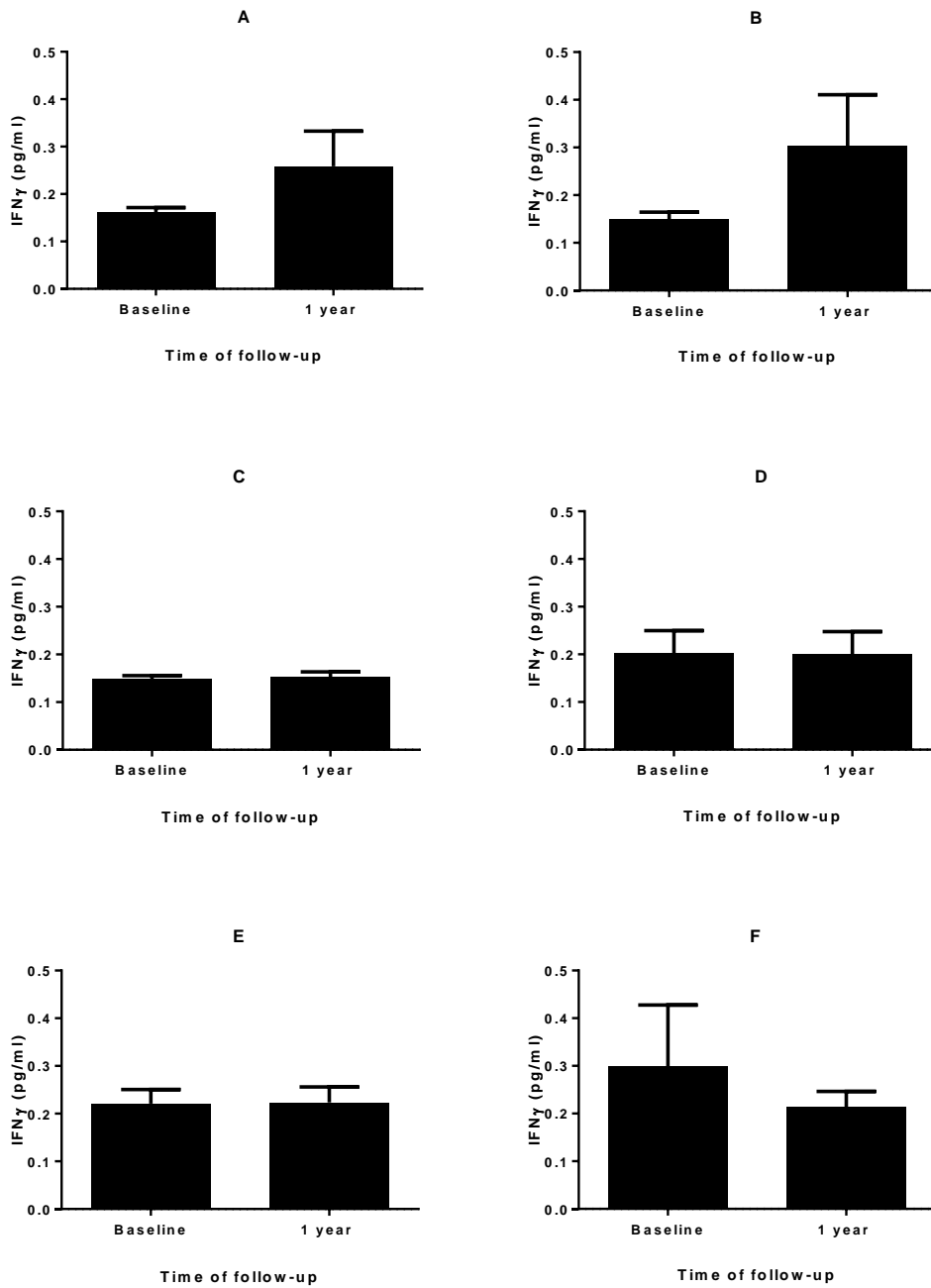
Figure 4.9. IL-6 level by study group. Bar charts showing change in IL-6 levels in each group at baseline and 1 year (mean+SEM). A-peripheral placebo B-G-CSF only C-IC placebo D-IC BMC E-IM placebo F-IM BMC. *p<0.05. G-CSF-granulocyte colony stimulating factor. IC-intracoronary. IM-intramyocardial. BMC-bone marrow cells. IL-6 interleukin 6.

Tumour Necrosis Factor-α

The tumour necrosis factor-α (TNF-α) level for the entire cohort was not significantly changed between baseline and 1 year (2.54±0.9pg/ml to 2.78±1.0pg/ml; p=0.089) (Fig 4.10). No significant association between TNF-α and LVEF was seen in our cohort (r^2 =0.004; p=0.557). TNF- α did not change significantly in any of the groups. In the peripheral placebo group, TNF-α decreased by -0.39pg/ml (95% CI -1.07 to 0.29; p=0.210) while in the G-CSF only group, by 1.00pg/ml (95% CI -0.25 to 2.25; p=0.103). TNF-α increased by 0.22pg/ml in the IC placebo group (95% CI -0.50 to 0.94; p=0.478) and by 0.22pg/ml (95% CI -0.15 to 0.60; p=0.211) in the IC BMC group. TNF-α increased by 0.25pg/ml in the IM placebo group (95% CI -0.25 to 0.74; p=0.279) but was virtually unchanged (-0.007pg/ml; 95% CI -0.60 to 0.58; p=0.980) in the IM BMC group (Fig 4.11).
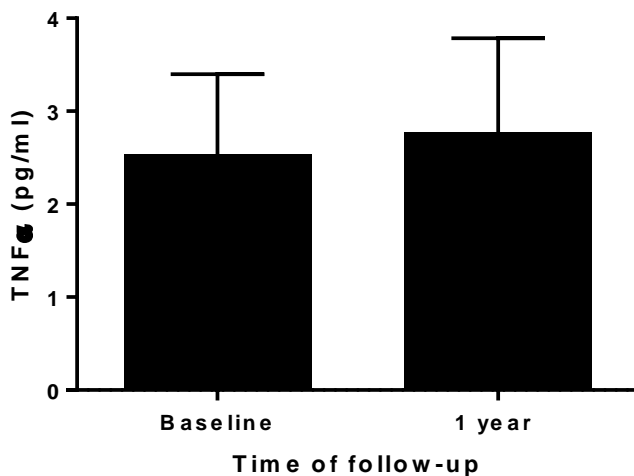
Figure 4.10. Change in TNF-α in the entire cohort. Bar charts showing mean (+SEM) TNF-α level at baseline and 1 year. * p<0.05. TNF-α –Tumour necrosis factor alpha

Figure 4.11. TNFα level by study group. Bar charts showing change in TNFα levels in each group at baseline and 1 year (mean+SEM). A-peripheral placebo B-G-CSF only C-IC placebo D-IC BMC E-IM placebo F-IM BMC. *p<0.05. G-CSF-granulocyte colony stimulating factor. IC-intracoronary. IM-intramyocardial. BMC-bone marrow cells.TNF-α Tumour necrosis factor alpha.

Interleukin-1a

The mean interleukin-1a (IL-1a) level did not change significantly at 1 year (44±0.49pg/ml to 0.47±0.33pg/ml; p=0.616) (Fig 4.12). No association between IL-1a and LVEF was observed in the cohort (r^2=0.02; p=0.164). IL-1a levels did not change significantly at 1 year in any of the groups. In the peripheral placebo group, IL-1a increased by 0.27pg/ml (95% CI -0.08 to 0.62; p=0.110) while in the G-CSF only group, IL-1a increased by 0.12pg/ml (95% CI -0.10 to 0.33; p=0.250). In the IC placebo group, the change in IL-1a was minimal (-0.02pg/ml; 95% CI -0.18 to 0.12; p=0.689) as was also seen in the IC BMC group (0.05pg/ml; 95% CI -0.04 to 0.14; p=0.211). IL-1a decreased in the IM placebo group by -0.26pg/ml (95% CI -0.73 to 0.22; p=0.238). IL-1a level was virtually unchanged in the IM BMC group (-0.002pg/ml; 95% CI -0.08 to 0.08; p=0.949) (Fig 4.13).
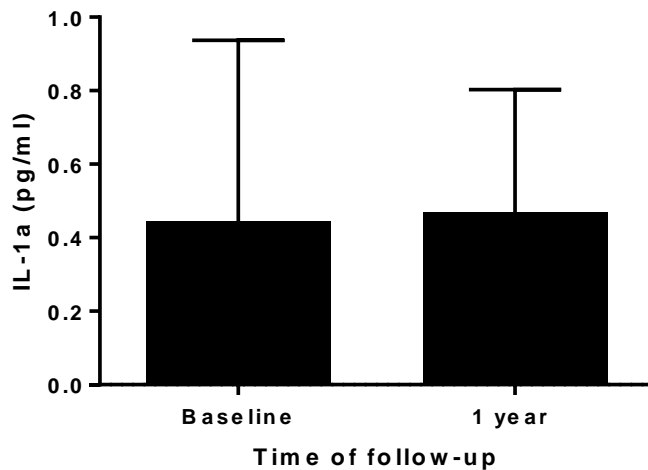
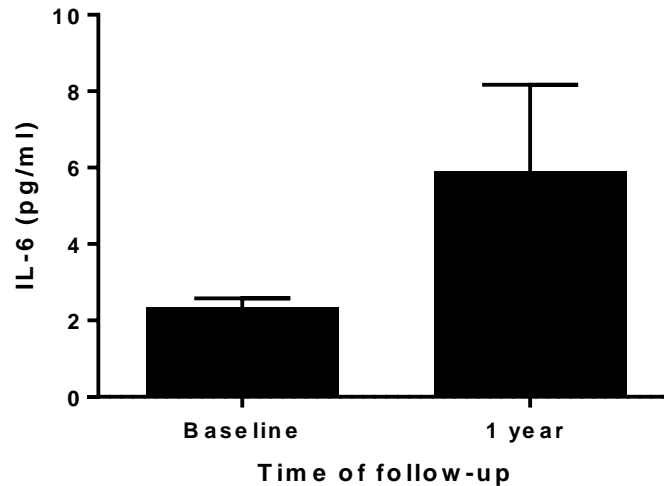**Figure 4.12. Change in IL-1α in the entire cohort.** Bar charts showing mean (+SEM) IL-1-α level at baseline and 1 year. * p<0.05. IL-1α –Interleukin 1 alpha.
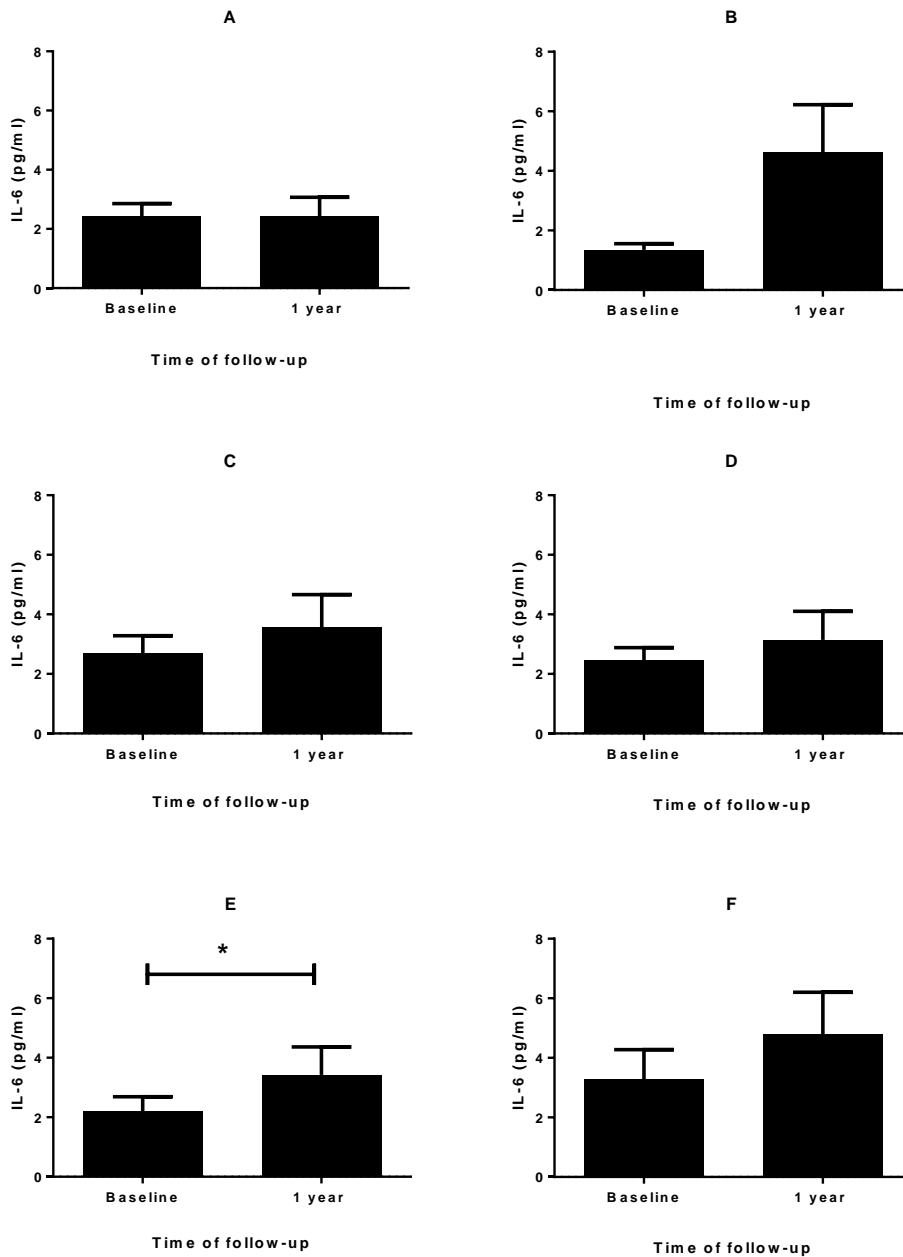
Figure 4.13. IL-1a level by study group. Bar charts showing change in IL-1a levels in each group at baseline and 1 year (mean+SEM). A-peripheral placebo B-G-CSF only C-IC placebo D-IC BMC E-IM placebo F-IM BMC. *p<0.05. G-CSF-granulocyte colony stimulating factor. IC-intracoronary. IM-intramyocardial. BMC-bone marrow cells. IL-1a-interleukin 1 alpha.

Interleukin-1b

The baseline interleukin-1b (IL-1b) level in the entire cohort was 1.20±1.11pg/ml and changed, non-significantly, to 1.81±5.26pg/ml, an increase of 0.61pg/ml (p=0.431) (figure 4.14). No association was observed between IL-1b and LVEF in our cohort (r^2 =0.02; p=0.145). In the peripheral placebo group, mean IL-1b increased by 0.62pg/ml (95% CI -0.66 to 1.91; p=0.282) while it increased by 0.36pg/ml (95% CI -1.66 to 2.38; p=0.691) in the G-CSF only group. IL-1b increased by 0.20pg/ml (95% CI -0.68 to 1.08; p=0.603) in the IC placebo group and changed marginally (0.05pg/ml; 95% CI -0.04 to 0.14; p=0.211) in the IC BMC group. In the IM placebo group, IL-1b decreased significantly by -1.27pg/ml (95% CI -2.44 to -0.11; p=0.036). IL-1b also decreased in the IM BMC group, albeit non-significantly, by -0.88pg/ml (95% CI -1.88 to 0.11; p=0.075) (figure 4.15) . However, the change in IL-1b at 1 year was not significantly different across the groups (one-way ANOVA p=0.128).

Figure 4.14. Change in IL-1b in the entire cohort. Bar charts showing mean (+SEM) IL-1β level at baseline and 1 year. *

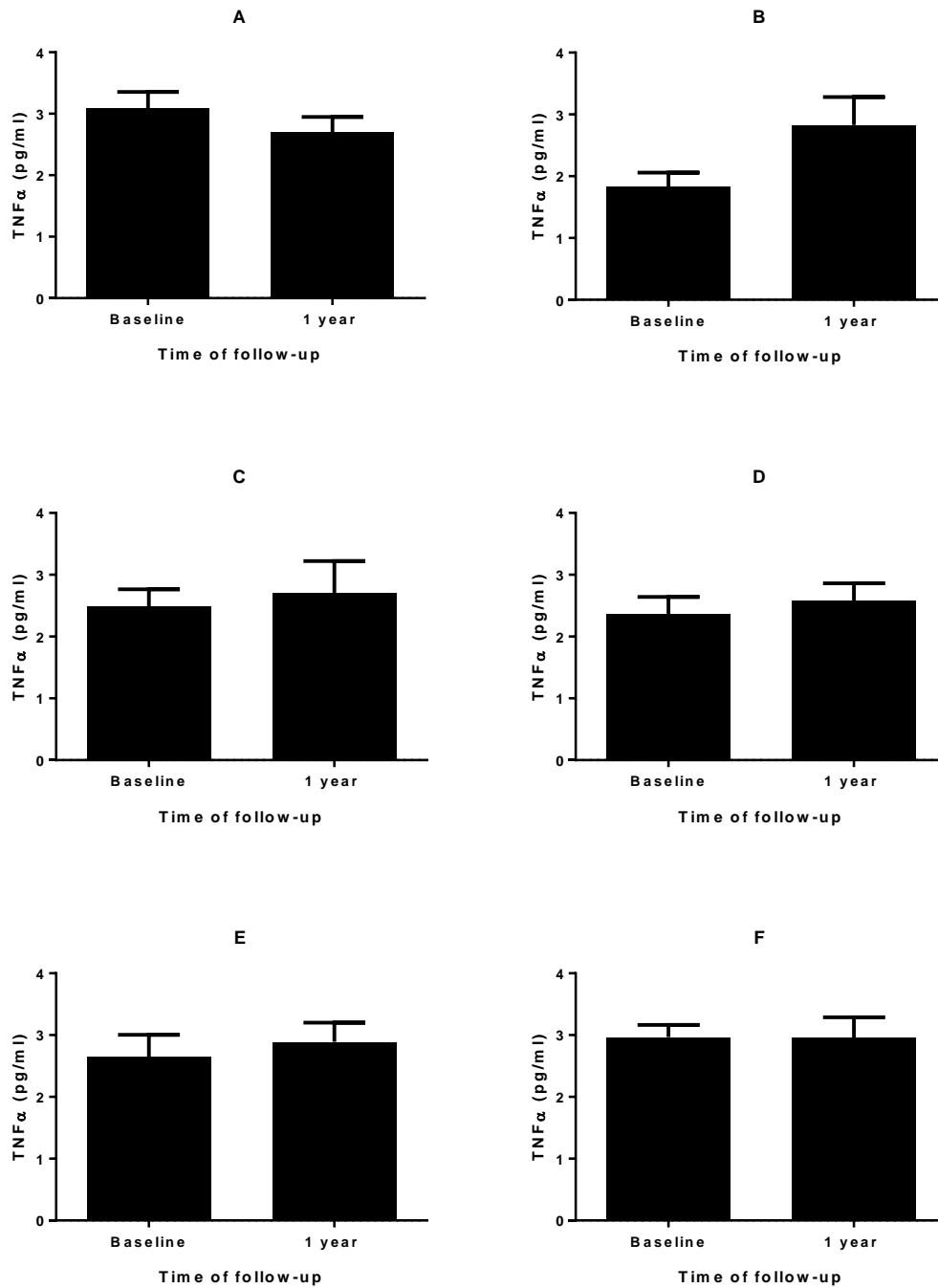p<0.05. IL-1b–Interleukin 1 beta.

Figure 4.15. IL-1b level by study group. Bar charts showing change in IL-1b levels in each group at baseline and 1 year (mean+SEM). A-peripheral placebo B-G-CSF only C-IC placebo D-IC BMC E--IM placebo F-IM BMC. *p<0.05. G-CSF-granulocyte colony stimulating factor. IC-intracoronary. IM-intramyocardial. BMC-bone marrow cells. IL-1b interleukin 1 beta.

Monocyte Chemoattractant Protein-1

The mean baseline monocyte chemoattractant protein-1 (MCP-1) level in the entire cohort was 108.4±61.4pg/ml and increased, non-significantly, at 1 year to 124.0±56.7pg/ml, a mean increase of 15.5pg/ml (p=0.071) (Fig 4.16). No association between MCP-1 and LVEF was observed in our cohort (r^2=0.01; p=0.379). In the G-CSF only group, MCP-1 increased significantly by 33.97pg/ml (95% CI 4.34 to 63.60; p=0.030) (figure 4.17). In the IM BMC group MCP-1 decreased non-significantly, by 15.79pg/ml (95% CI -77.42 to 45.84; p=0.571). The remainder of the groups showed non-significant increases in MCP-1. In the peripheral placebo group, MCP-1 increased by 3.82pg/ml (95% CI -3.82 to 11.46; p=0.267). In the IC placebo group, MCP-1 increased by 25.93pg/ml (95% CI -24.93 to 76.79; p=0.259) and by 30.64pg/ml (95% CI -32.88 to 94.16; p=0.304) in the IC BMC group. In the IM placebo group, MCP-1 increased by 12.24pg/ml (95% CI -10.00 to 34.47; p=0.234) (Fig 4.17). The change in MCP-1 at 1 year was not significantly different across the groups (one-way ANOVA p=0.495).
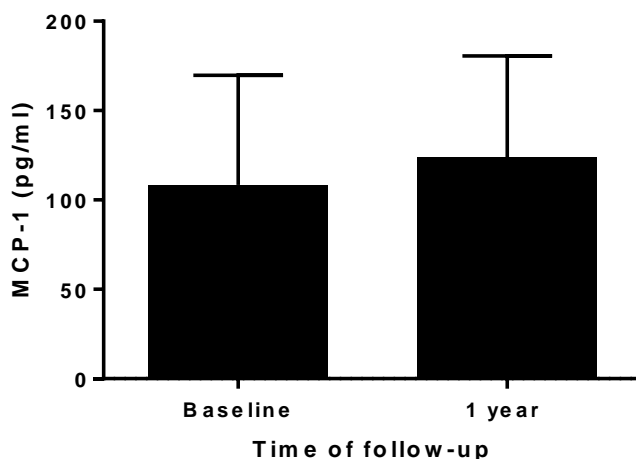
Figure 4.16. Change in MCP-1 in the entire cohort. Bar charts showing mean (+SEM) MCP-1 level at baseline and 1 year.

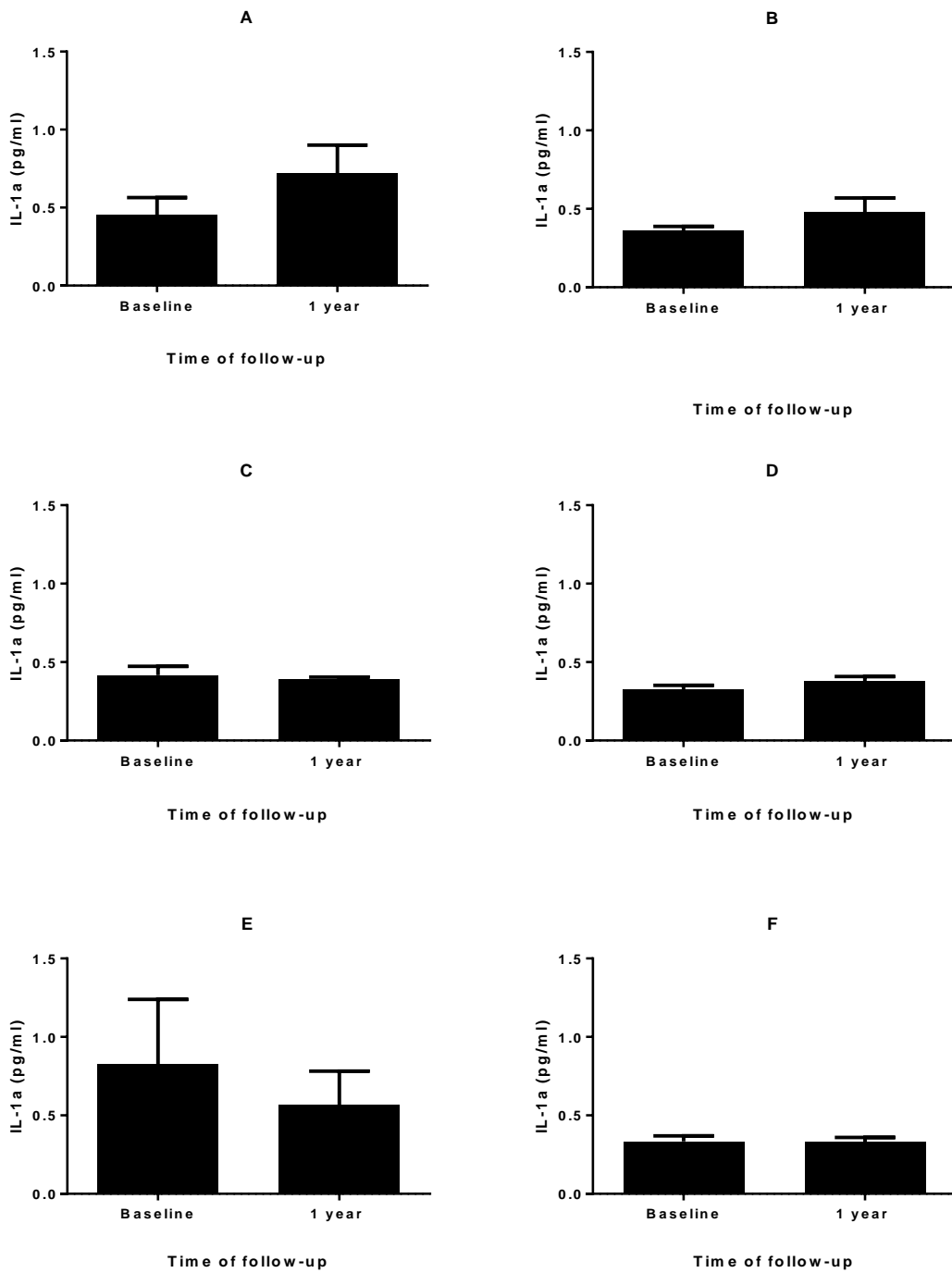* p<0.05. MCP-1 monocyte chemoattractant protein-1.
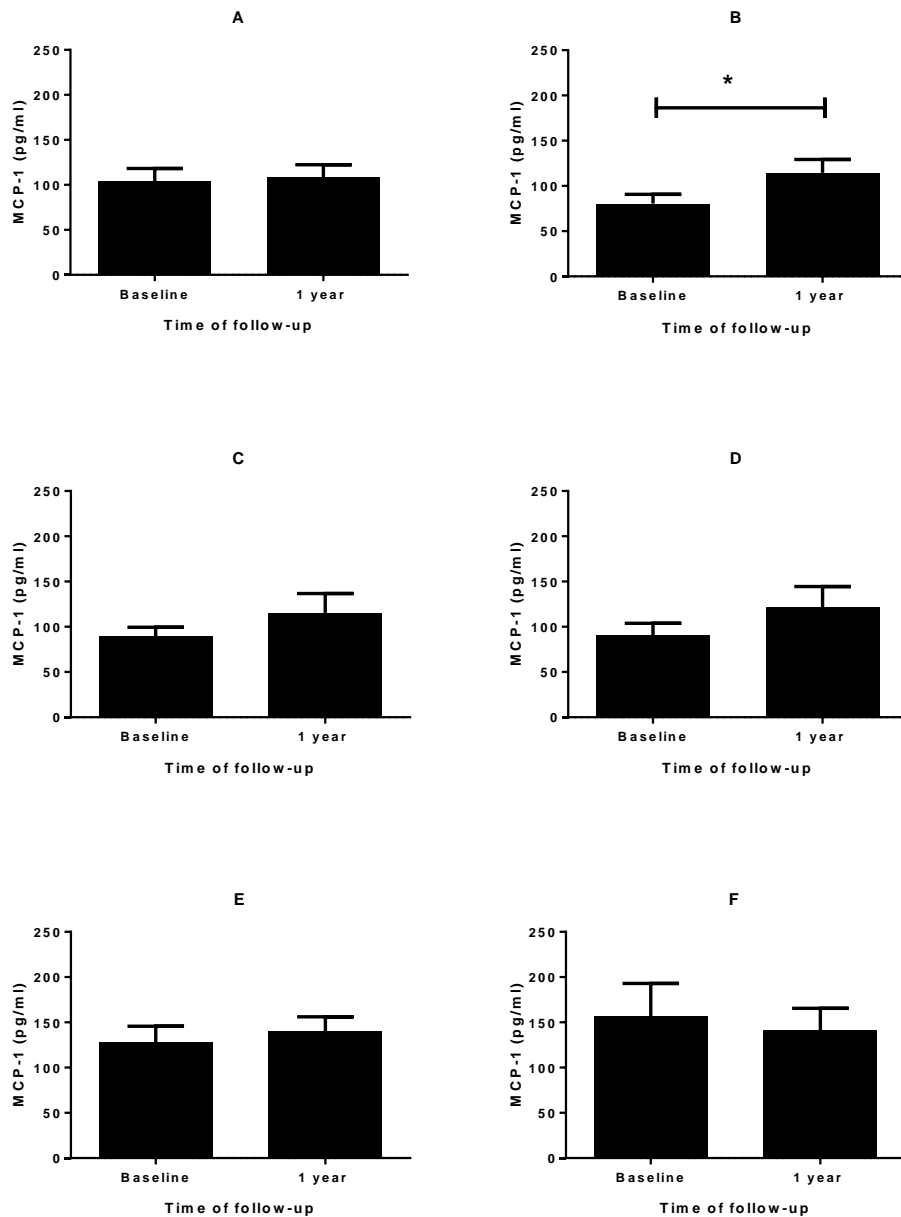
Figure 4.17. MCP-1 level by study group. Bar charts showing change in MCP-1 levels in each group at baseline and 1 year (mean+SEM). A-peripheral placebo B-G-CSF only C-IC placebo D-IC BMC E-IM placebo F-IM BMC. *p<0.05. G-CSF-granulocyte colony stimulating factor. IC-intracoronary. IM-intramyocardial. BMC-bone marrow cells. MCP-1 Monocyte chemoattractant protein-1.

Interleukin-2

In the whole cohort, mean interleukin-2 (IL-2) level at baseline was 2.49±0.4pg/ml compared to 4.2±6.6pg/ml (p=0.078) at 1 year (Fig 4.18). There was no association seen with LVEF (r^2= 0.00003, p=0.961). When splitting the IL-2 levels by treatment group no significant change in mean levels were seen at 1 year compared to baseline in any group {peripheral placebo group, 1.21pg/ml (95% CI -0.30 to 2.7; p=0.097); G-CSF only group, 2.34pg/ml (95% CI -2.95 to 7.64; p=0.337); IC placebo group, 0.45pg/ml (95% CI -0.95 to 1.85; p=0.461); IC BMC group, -0.05pg/ml (95% CI -0.51 to 0.41; p=0.808); IM placebo group, 5.11pg/ml (95% CI -7.125 to 17.35; p=0.356), and IM BMC group, 1.12pg/ml (95% CI -0.60 to 2.84; p=0.172)} (Fig 4.19).
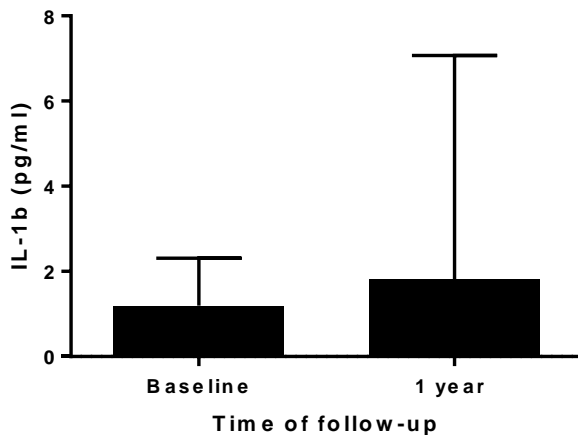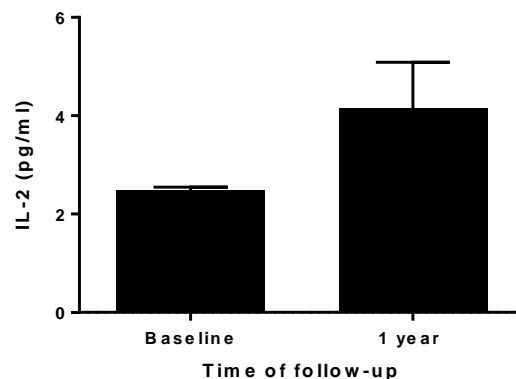

Figure 4.18. Change in IL-2 in the entire cohort. Bar charts showing mean (+SEM) IL-2 level at baseline and 1 year. * p<0.05
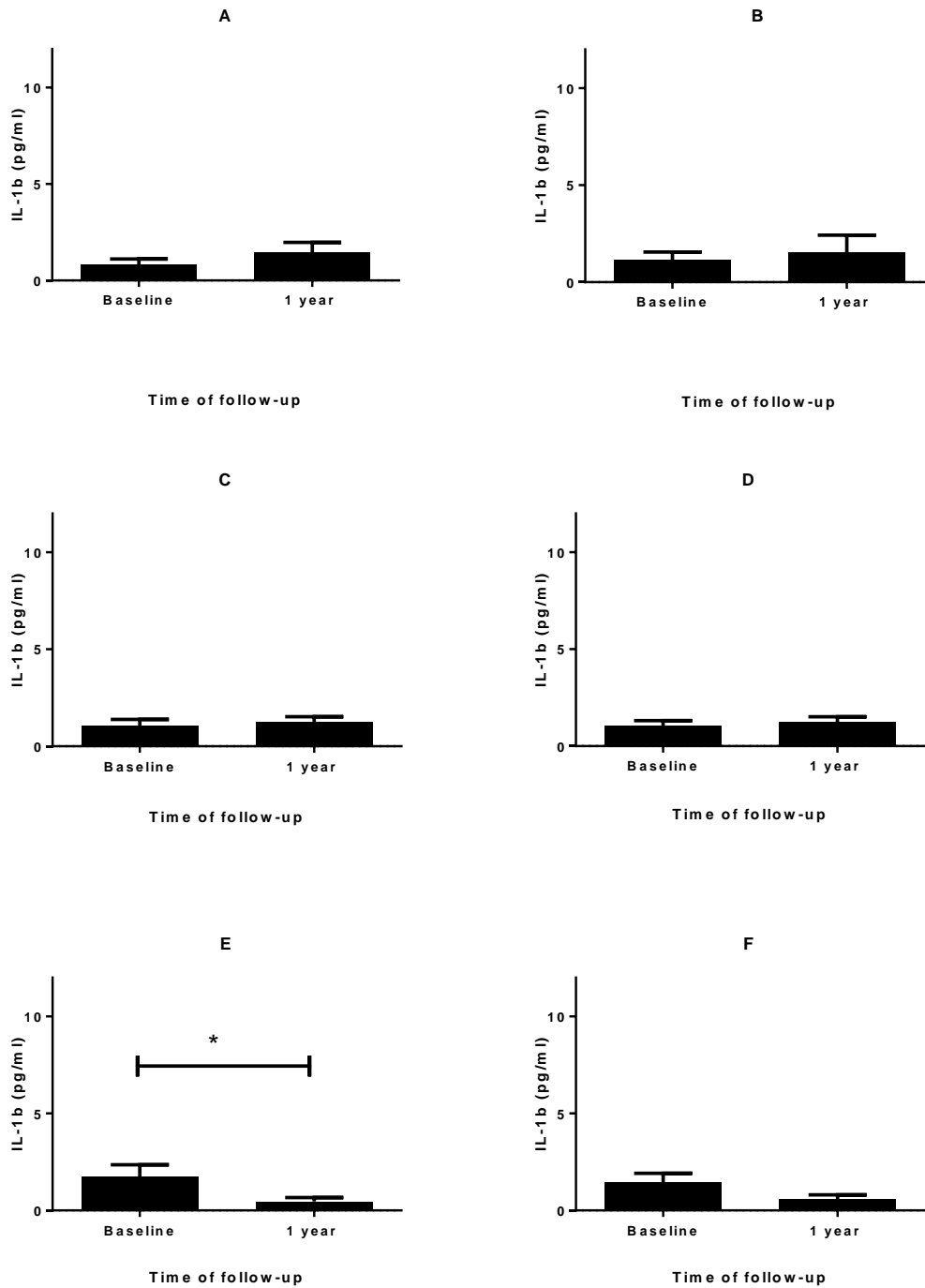
Figure 4.19. IL-2 level by study group. Bar charts showing change in IL-2 levels in each group at baseline and 1 year (mean+SEM). A-peripheral placebo B-G-CSF only C-IC placebo D-IC BMC E-IM placebo F-IM BMC. *p<0.05 (paired t-test). G-CSF-granulocyte colony stimulating factor. IC-intracoronary. IM-intramyocardial. BMC-bone marrow cells. IL-2 interleukin 2.

Interleukin-4

The baseline interleukin-4 (IL-4) level was 2.16±0.3 pg/ml. At 1 year, the IL-4 level did no change

significantly (2.34±0.7pg/ml; p=0.105) (Fig 4.20). IL-4 levels showed no significant association with

LVEF (r^2 = 0.01; p=0.296). IL-4 did not change significantly in any of the six groups. In the PP group,

IL-4 levels increased by 0.43pg/ml (95% CI -0.95 to 1.81; p=0.476) while in the G-CSF only group, IL-

4 increased by 0.37pg/ml (95% CI -0.32 to 1.06; p=0.250). IL-4 level changed negligibly in the IC

placebo group (0.08pg/ml; 95% CI -0.20 to 0.36; p=0.509) and in the IC BMC group (-0.03pg/ml;

95% CI -0.27 to 0.22; p=0.825).   The IMP group also had a marginal decrease in IL-4 levels (-

0.09pg/ml; 95% CI -0.31 to 0.14; p=0.407) while the IM BMC group had a 0.31pg/ml increase in IL-4

levels (95% CI -0.22 to 0.85; p=0.210) (Fig 4.21).
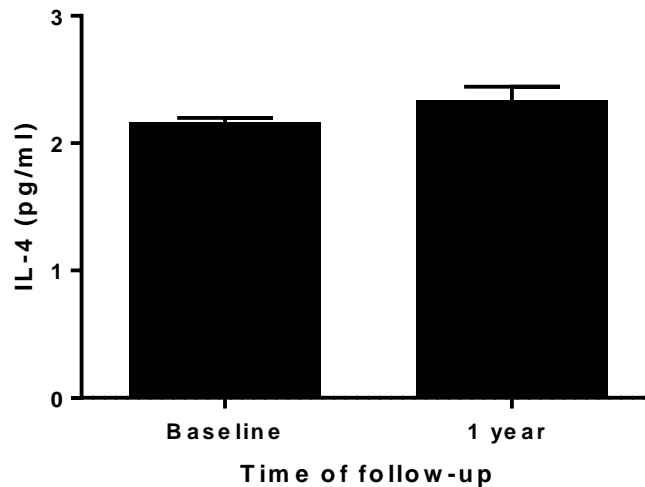
Figure 4.20. Change in IL-4 in the entire cohort. Bar charts showing mean (+SEM) IL-4 level at baseline and 1 year. *

p<0.05. IL-4-interleukin-4.
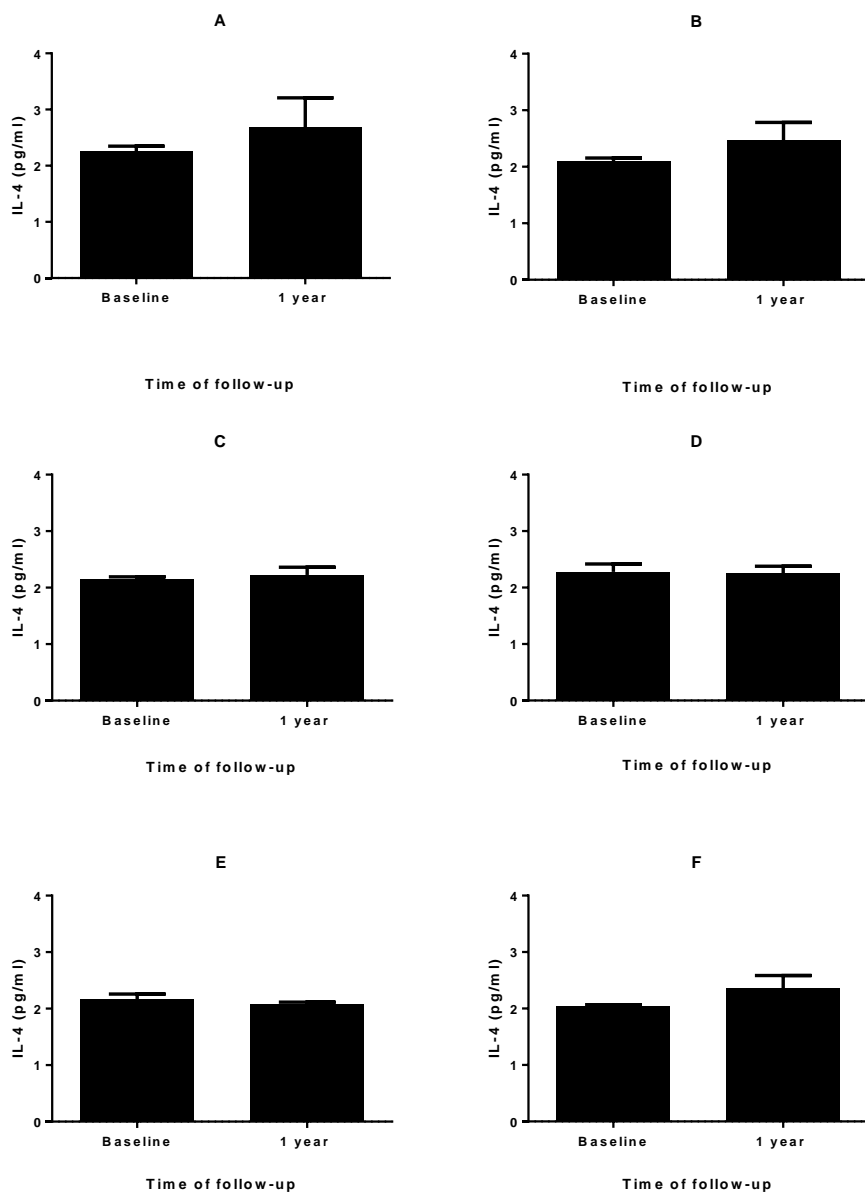
Figure 4.21. IL-4 level by study group. Bar charts showing change in IL-4 levels in each group at baseline and 1 year (mean+SEM). A-peripheral placebo B-G-CSF only C-IC placebo D-IC BMC E-IM placebo F-IM BMC. *p<0.05. G-CSF-granulocyte colony stimulating factor. IC-intracoronary. IM-intramyocardial. BMC-bone marrow cells. IL-4 interleukin 4.

4.4.2 Change in cytokines associated with positive outcomes in heart failure and association with LVEF at 1 year

Interleukin-10

The mean interleukin-10 (IL-10) level at baseline was 0.82±0.3pg/ml and increased significantly to 1.77±2.8pg/ml, a mean increase of 0.9pg/ml (p=0.020) (Fig 4.22). There was a trend towards a positive association between IL-10 levels and LVEF (r^2 = 0.04; p=0.062) (Fig 4.23). No significant change in IL-10 levels were noted in any of the groups. In the peripheral placebo group, IL-10 increased by 0.46pg/ml (95% CI -0.10 to 1.02; p=0.093). In the G-CSF only group, IL-10 also increased by 0.22pg/ml (95% CI -0.21 to 0.65; p=0.267). IL-10 changed negligibly by -0.07pg/ml (95% CI -0.53 to 0.39; p=0.678) in the IC placebo group and remained virtually unchanged in the IC BMC group (0.05pg/ml; 95% CI -0.13 to 0.23; p=0.536). In the IM placebo group, IL-10 changed marginally by 0.05pg/ml (95% CI -0.77 to 0.87; p=0.880). In the IM BMC group the change in IL-10 was more pronounced at 0.49pg/ml (-0.55 to 1.53; p=0.301) (Fig 4.24).
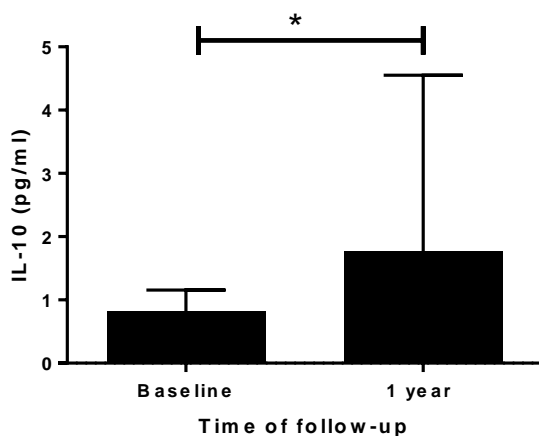
Figure 4.22. Change in IL-10 in the entire cohort. Bar charts showing mean (+SEM) IL-10 level at baseline and 1 year. * p<0.05. IL-10-interleukin 10

$r^2=0.04$

p=0.062
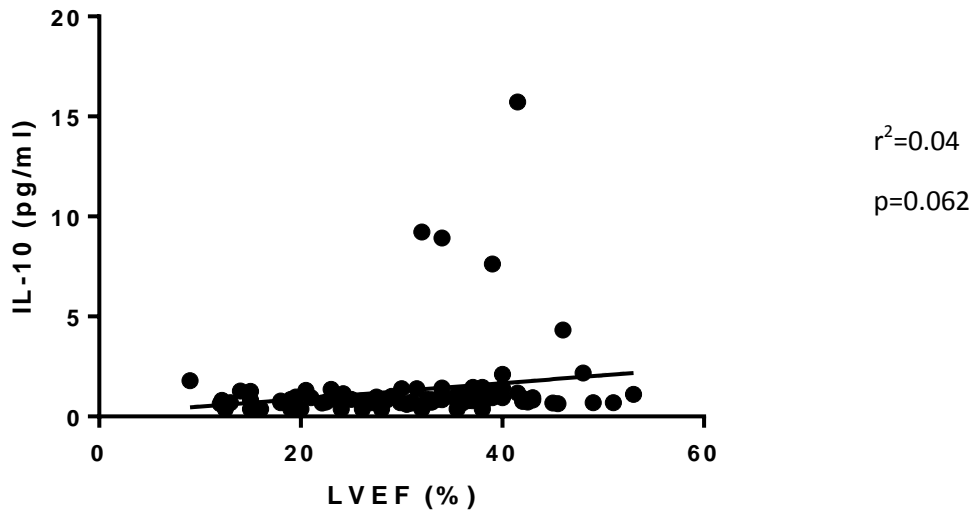
Figure 4.23. IL-10 and LVEF. Correlation curve between IL-10 and LVEF. A significant correlation was observed between IL-10

and LVEF. LVEF-left ventricular ejection fraction. IL-10 interleukin 10
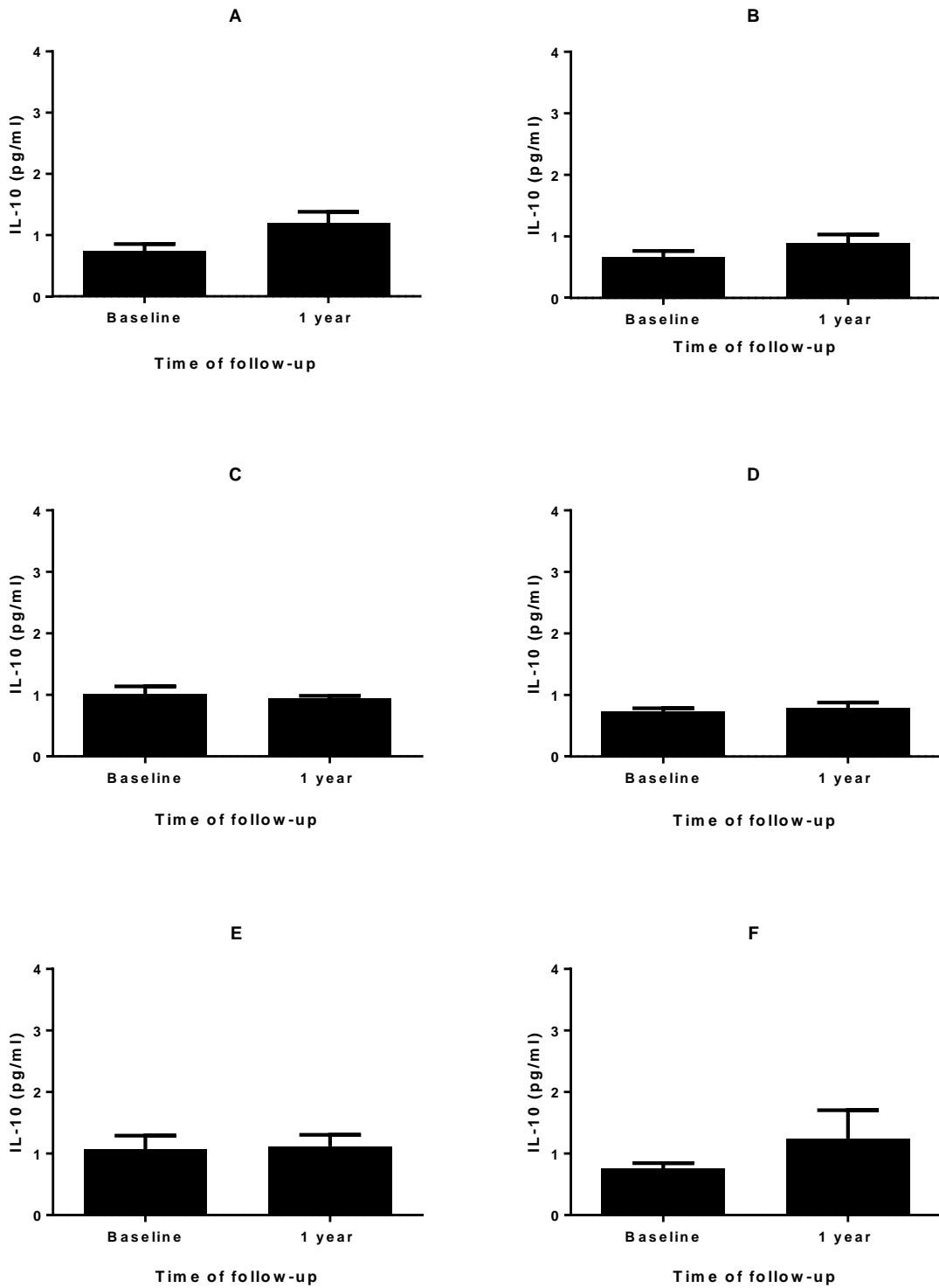
Figure 4.24. IL-10 level by study group. Bar charts showing change in IL-10 levels in each group at baseline and 1 year (mean+SEM). A-peripheral placebo B-G-CSF only C-IC placebo D-IC BMC E-IM placebo F-IM BMC. *p<0.05. G-CSF-granulocyte colony stimulating factor. IC-intracoronary. IM-intramyocardial. BMC-bone marrow cells. IL-10 interleukin 10

Vascular Endothelial Growth Factor

The mean vascular endothelial growth factor (VEGF) level at baseline was 17.16±17.0pg/ml and increased, non-significantly to 22.07±32.1pg/ml, a mean increase of 4.91pg/ml (p=0.292) (figure 4.25). There was no association between IL-10 levels and LVEF (r^2 =0.01; p=0.307). None of the groups showed any significant change in VEGF levels (figure 4.26). In the peripheral placebo group, VEGF increased by 2.08pg/ml (95% CI -7.86 to 12.02; p=0.627). In the G-CSF only group, VEGF decreased by -1.01pg/ml; 95% CI -8.02 to 5.99; p=0.747). In the IC placebo group, VEGF level was marginally decreased by -0.39pg/ml (95% CI -7.31 to 6.53; p=0.890). In contrast, VEGF levels increased by 11.65pg/ml (95% CI -9.72 to 33.03; p=0.249) in the IC BMC group although this increase was not significant. In the IM placebo group, VEGF levels decreased by 2.16pg/ml (95% CI -8.11 to 3.80; p=0.420). In contrast to the IM placebo group, VEGF increased in the IM BMC group (4.90pg/ml; 95% CI -8.34 to 18.14; p=0.410). The change in VEGF at 1 year was not significantly different across the groups (one-way ANOVA p=0.511).
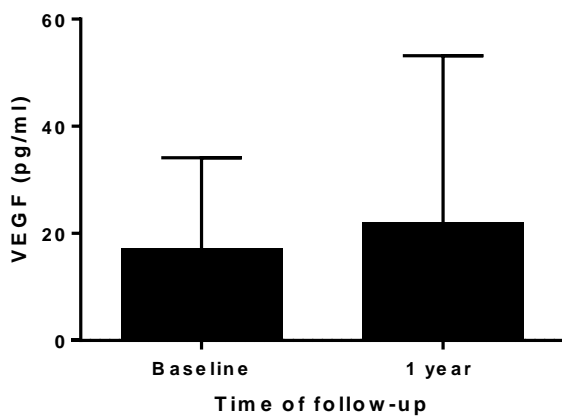
Figure 4.25. Change in VEGF in the entire cohort. Bar charts showing mean (+ SEM) VEGF level at baseline and 1 year. * p<0.05 VEGF-vascular endothelial growth factor. SEM-standard error of mean.
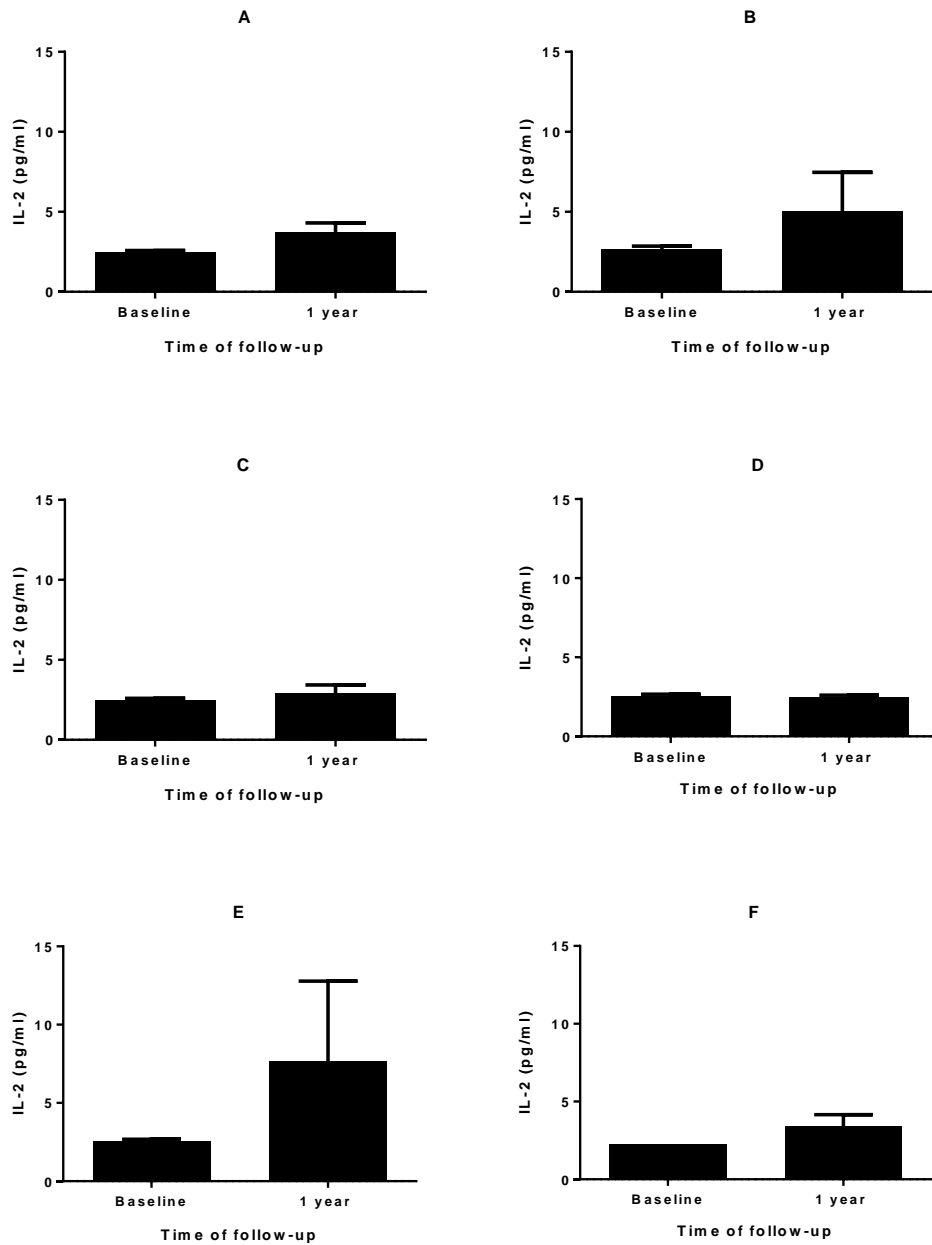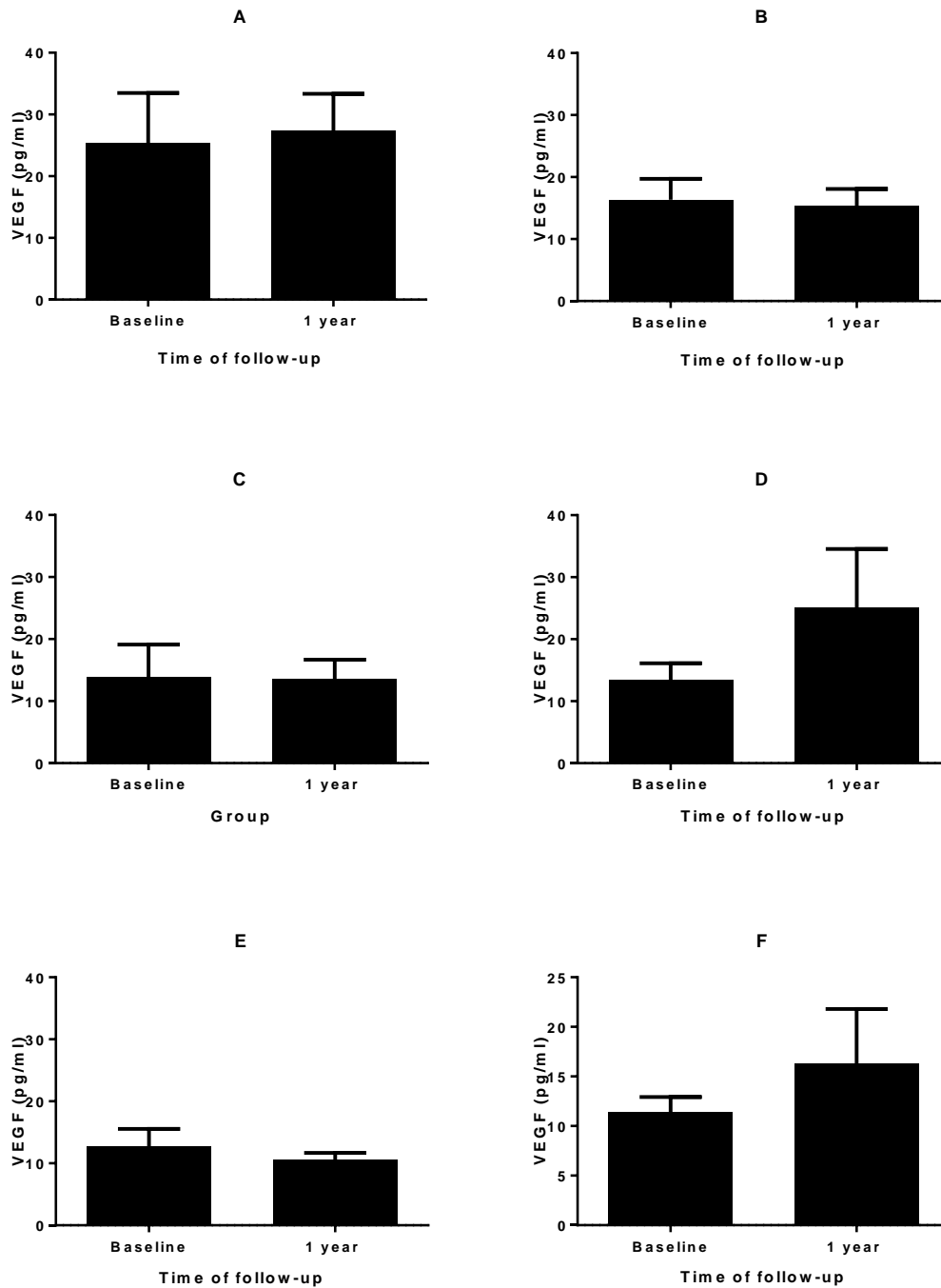
Figure 4.26. VEGF level by study group. Bar charts showing change in VEGF levels in each group at baseline and 1 year

(mean+SEM). A-peripheral placebo B-G-CSF only C-IC placebo D-IC BMC E-IM placebo F-IM BMC. *p<0.05. G-CSF-granulocyte colony

stimulating factor. IC-intracoronary. IM-intramyocardial. BMC-bone marrow cells. VEGF-vascular endothelial growth factor.

4.5 Summary

Change in cytokines associated with adverse outcomes in heart failure

- **IFN-γ and IL-8 showed a negative correlation with LVEF while IL-10 showed a positive correlation with LVEF**

- **IL-8 increased significantly in the peripheral placebo group and non-significantly in all other groups except the IM BMC group in which there was a non-significant decrease.**

- **No significant change in IFN-γ levels were noted in any of the six groups. IFN-γ decreased in the IM BMC group and showed an increase or negligible change in the remainder of the groups.**

- **IL-1b decreased significantly in the IM placebo group and showed a trend towards significant reduction in the IM BMC group. All other groups showed an increase or marginal change in IL-1b levels.**

- **MCP-1 increased significantly in the G-CSF only group and non-significantly in all the other groups except the IM BMC group in which it non-significantly decreased.**

- **IL-2 was not changed significantly in any of the six groups and showed no correlation with LVEF**

- **IL-4 was not changed significantly in any of the six groups and showed no correlation with LVEF**

- **IL-6 increased significantly in the IM placebo group. None of the other groups showed any significant change.**

- **TNF-α was not changed significantly in any of the six groups and showed no correlation with LVEF**

- **IL-1a was not changed significantly in any of the six groups and showed no correlation with LVEF**

Change in cytokines associated with a positive outcome in heart failure

- **IL-10 increased significantly in the entire cohort at 1 year. IL-10 did not change significantly in any of the six groups.**

- **VEGF increased in the IC BMC and IM BMC groups while VEGF levels decreased in the IC and IM placebo groups but none of the changes were significant.**

Chapter 5 . Mechanistic insights into the effect of cell therapy in ischaemic cardiomyopathy- the relationship between cells and scar, cytokines, and diastolic function.

5.1 Background

Despite several previous studies assessing the effect of cell therapy on changes in left ventricular function in patients with ischaemic cardiomyopathy (see section 1.2.5.4), few have assessed other parameters such as scar burden, diastolic function or looked at relationships between cell counts and outcomes. Some trials using cardiac stem cells have reported reduction in scar size including CADUCEUS and SCIPIO.[114, 116] However, no trial yet has looked at scar size in patients receiving G-CSF alone or in combination with bone marrow derived cells, as well as the relationship between changes in scar burden and cytokine levels, NT pro-BNP and left ventricular volumes.

The vast majority of cell therapy trials have focused primarily on change in systolic LV function. However, almost an equal number of patients exist in the community who have heart failure with preserved ejection fraction likely secondary to diastolic dysfunction.[223, 224] Furthermore, coexistent diastolic impairment with systolic impairment is not uncommon. Only one trial has looked at the effect of cell therapy on diastolic function and reported an improvement in diastolic function in patients receiving bone marrow derived cells. [151] No trial has looked into the effect of G-CSF and autologous BMC on diastolic function when administered via different routes.

Aspects of the relationship between progenitor cell count/function and outcomes have been studied in a limited number of studies, including the FOCUS-HF, FOCUS-CCTRN and POSEIDON trial.[145, 146, 155] In the FOCUS-HF trial, CD34+ cell counts as well as CFU-GM and CFU-F (colony

forming unit fibroblast) analysis were performed and correlated with demographics and outcomes. It has also been observed that cell counts and their functional characteristics deteriorate in patients with advanced heart failure.[154, 225] The age of the patient and comorbidities such as diabetes can affect the cell count. This chapter aims to assess relationship between cell therapy and scar burden, diastolic function and looks at the relationship between CD34+, endothelial progenitor cells (EPC) and mononuclear cells (MNC) counts and function and change in the primary endpoint (left ventricular ejection fraction-LVEF).

5.2 Hypothesis

1. Myocardial scar reduction is associated with improvement in LVEF, cytokine levels and cell/G-CSF therapy

2. Bone marrow derived cell therapy and G-CSF are associated with improved diastolic function

3. Increased peripheral and bone marrow cell counts and improved cell function are associated with improved LVEF

5.3 Specific aims

1. To assess the mechanisms by which improvements in LVEF may have occurred specifically assessing myocardial scar size, cytokine levels and diastolic function.

2. To assess the relationship between cell characteristics and change in LVEF e.g. numbers of cells delivered and CFU-GM colonies

5.4 Results

5.4.1 Myocardial scar

Myocardial scar was measured in 27 patients at baseline and 1 year and in 11 patients at 2 years. (Figure 5.1) The remainder of the patients did not have CMR based myocardial scar measurements because of contraindications to CMR or due to delays in getting approval to initiate 2 year follow-ups.
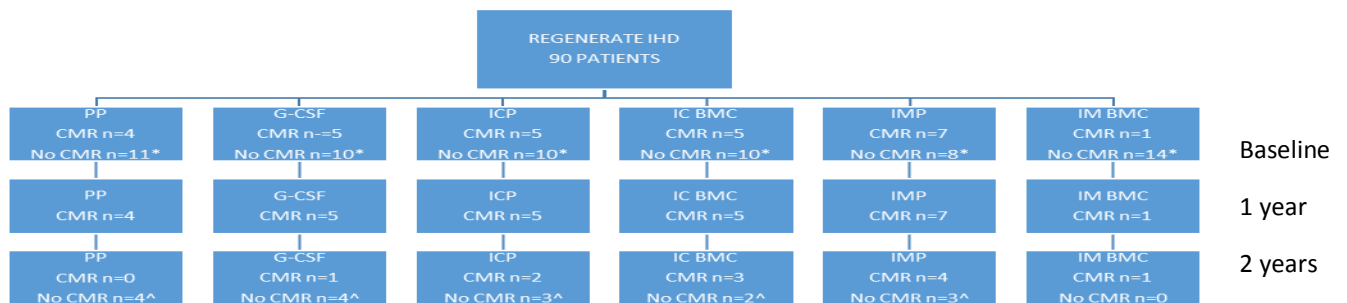
Figure 5.1. Diagram showing number of patients eligible for scar analysis using CMR. CMR-cardiac magnetic resonance imaging. PP-peripheral placebo. IC-intracoronary IM-intramyocardial BMC-bone marrow cells; G-CSF-granulocyte colony stimulating factor. *No CMR-due to MR incompatible device; ^No CMR-logistical reasons; delayed commencement of 2 year follow-up (see text)

Scar size in the entire patient cohort

In the whole cohort, the mean scar burden (as percentage of total myocardial mass) was 10.2±1.2% at baseline and 9.4±0.9% at 1 year i.e. a change of -0.8±0.8% (p=0.311). A weak trend to a negative correlation was observed between percentage change in scar and an increase in LVEF difference (r^2=0.07; p=0.172) at the same time points (figure 5.2). At 2 years, no statistically significant correlation was found between scar burden and LVEF (r^2=0.04; p=0.556) (fig 5.3). The low number of patients at 2 years undergoing CMR meant that no further sub-analyses of the data was possible.
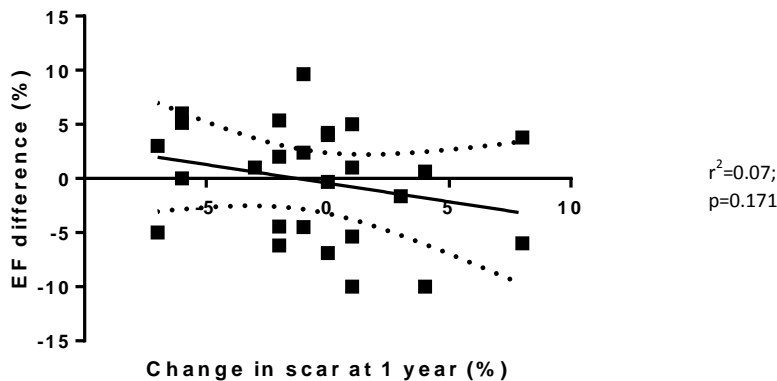
Figure 5.2. Change in scar (%) versus change in LVEF (%) at 1 year. Correlation curve showing lack of correlation between scar burden and LVEF.

Scar size and change in LVEF

In patients who had an improvement in LVEF (defined as an increase or no change in LVEF) (henceforth, referred to as responders) at 1 year, the mean scar burden decreased, albeit non-significantly, from 10.4±1.4% to 8.7±1.7% i.e. -1.7±1.0 (95% CI -3.9 to 0.5; p=0.127). In comparison, in those patients who had a decline in LVEF (henceforth, referred to as non-responders), there was an increase in scar burden from 10.0±1.9% to 10.5±1.7% i.e. an increase of 0.5±1.2% (95% CI -2.1 to

3.0; p=0.702). The change in means between the two groups was not significant (p=0.188), the difference between the mean changes in the responders (-1.7±1.0%) versus the non-responders (0.5±1.2%) was 2.1±1.4% (95% CI -1.1 to 5.4) (figure 5.4).
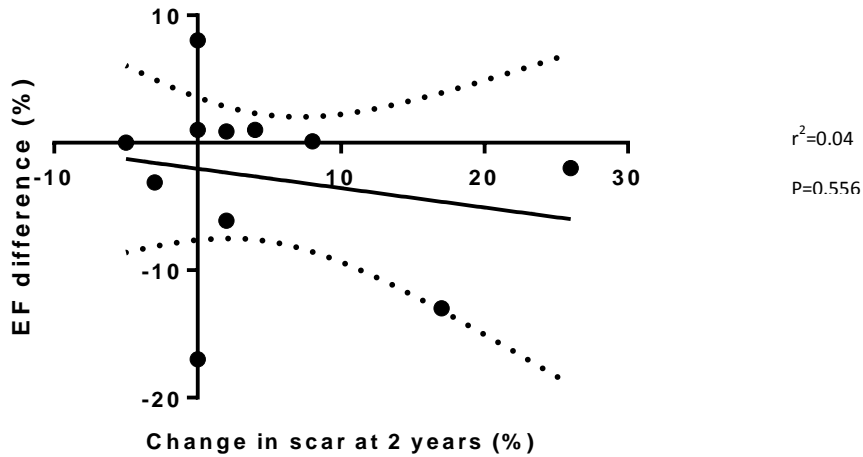
Figure 5.3. Change in scar (%) versus change in LVEF (%) at 2 years. Correlation curve showing lack of correlation between scar burden and LVEF.
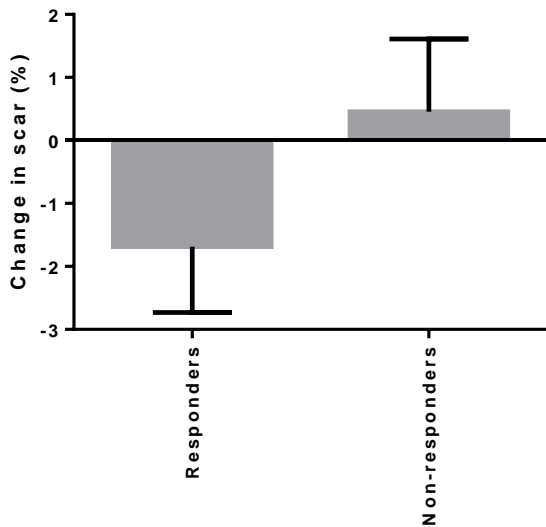
Figure 5.4. Change in scar in LVEF improvers (responders) versus decliners (non-responders). Responders-no decline in LVEF. Non-responders-decline in LVEF. LVEF-left ventricular ejection fraction

Scar size and correlation with left ventricular volumes and NT pro-BNP in responders

In responders, the mean end-diastolic volume (EDV) increased at 1 year by 14.1±8.2mls (p=0.105). The end systolic volume (ESV) also increased by 2.3±5.5 (p=0.682). The myocardial mass (MM) increased by 2.3±3.0g (p=0.452). All the above changes were statistically non-significant. The median (IQR) NT pro-BNP decreased from 334.5 (120.3 to 904.8) to 279.0 (146.0 to 858.5) (p>0.999). No significant correlation was found between change in EDV, ESV or MM and change in scar at 1 year. Although non-significant, a weak trend towards a positive correlation was observed between change in scar and change in NT pro-BNP i.e. a reduction in scar size was associated with a reduction in NT pro-BNP (r^2=0.08; p=0.277) (figure 5.5).

LV volumes and NT Pro-BNP in responders with a reduction in scar size compared to those with an increase in scar size

As expected from the above correlation curves, no significant difference, between those with a scar reduction and those with increased scar, was seen in a) EDV (18.5±11.2 v 6.8±11.9; p=0.505) b) ESV (6.3±8.0 v -4.3±6.5; p=0.371) c) MM (1.3±3.4 v 4.1±6.1; p=0.673) d) NT pro-BNP (-36.3±103.9 v -36.5±46.3; p=0.999).

A)

r²=0.00; p=0.912

B)

r²=0.01; p=0.765

C)

r²=0.01; p=0.653

D)

r²=0.08 p=0.277
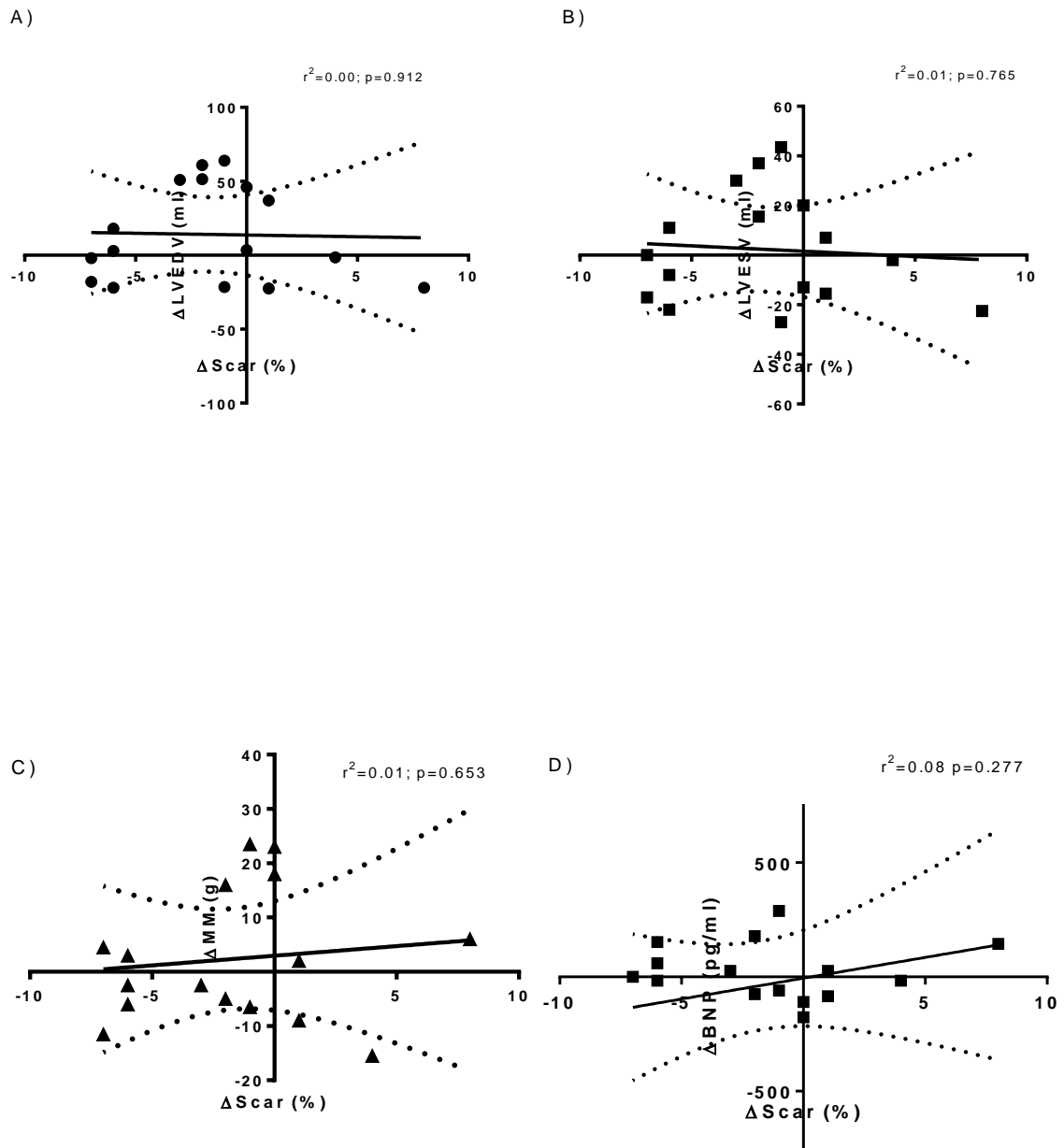
Figure 5.5. Correlation between left ventricular volumes, myocardial mass, NT pro-BNP and change in scar in responders. Graphs show a lack of any significant association between LVEDV, LVESV, MM or NT pro-BNP and myocardial scar burden expressed as a percentage of myocardial mass. LVEDV-left ventricular end-diastolic volume. LVESV-left ventricular end-systolic volume. MM-myocardial mass.

Change in scar size and cell (and G-CSF) therapy

A significant reduction in scar size was noted in the intracoronary cell therapy group when compared to the true placebo (i.e. saline only) group (-2.6±1.2% v 1.0±1.1%; p=0.032). No significant difference in scar size was noted between the intracoronary cell therapy group when compared to the intracoronary placebo group (-2.6±1.2% v -3.0±1.8%; p=0.706). Furthermore, when compared to the true placebo (i.e. saline) group, the change in scar size in the intracoronary placebo group was not significant (p=0.111). In the G-CSF group, a greater increase in scar size was noted when compared to the peripheral placebo group, although these changes were not significant (1.4±1.7% v 1.0±1.1%; p=0.881). Insufficient patient distribution in the IM groups (only one patient who underwent cardiac MR in the IM BMC group) meant that it was not possible to make a comparison between or within IM cell therapy and placebo groups. Figure 5.6 summarises the changes in scar size with cell/G-CSF therapy.

Change in cytokine levels in patients with a change in scar size in entire group

A total of 8 patients had 11 different cytokines (IL2, IL4, IL6, IL8, IL10, VEGF, INF-γ, TNF-α, IL1a, IL1b, MCP-1) measured at baseline and 1 year. No significant correlation was found between change in scar burden and change in any of the cytokine levels at 1 year. No significant changes in any of the cytokines were noted in either the scar reduction or scar increase groups. Table 5.1 summarises the key changes in the cytokines/chemokines. Figures 5.7 and 5.8 show the changes in mean levels of individual cytokines/chemokines in the scar reduction and scar increase groups respectively.
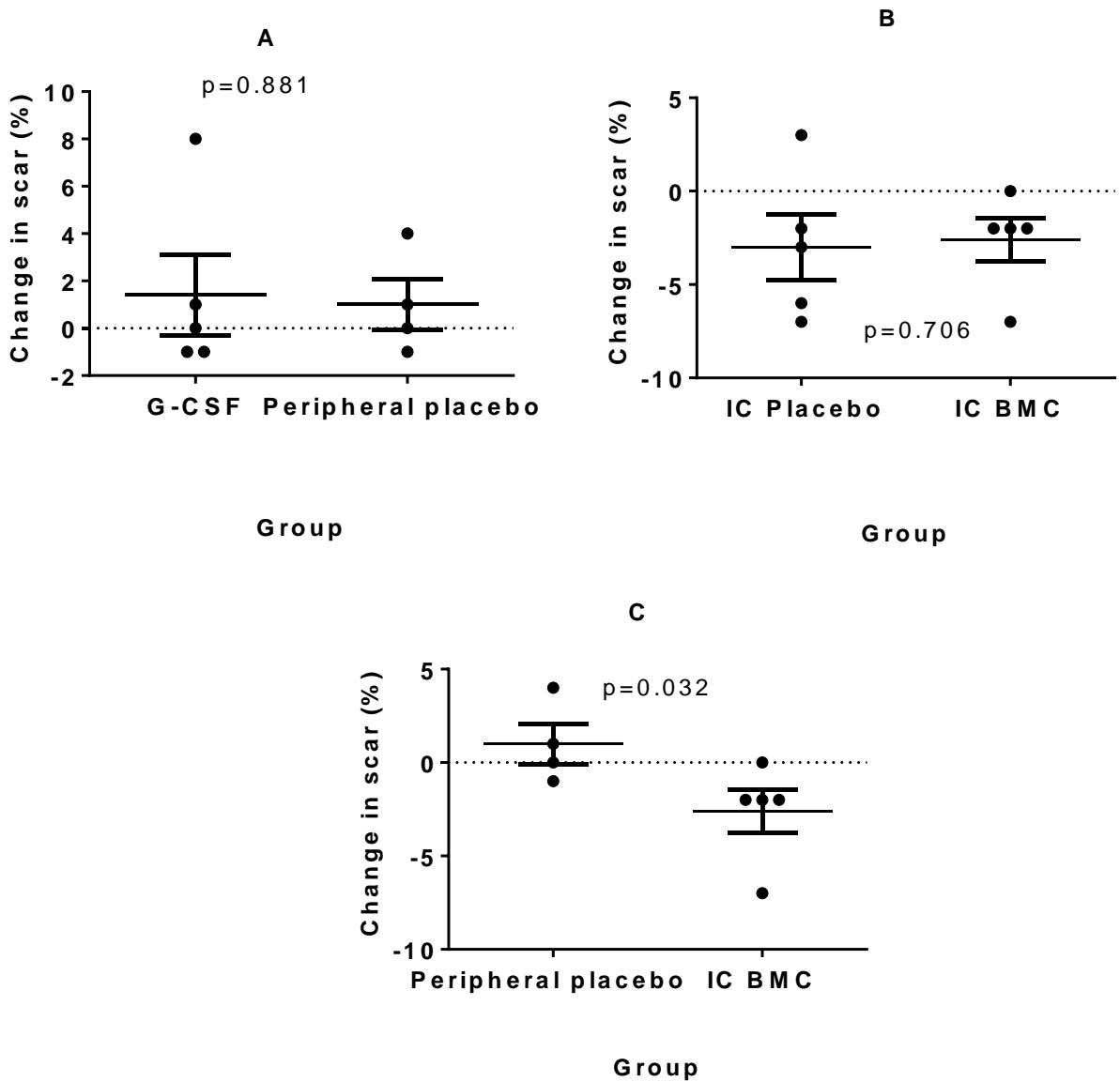
Figure 5.6. Change in scar size in the different trial groups. A significant reduction in scar size in the IC

BMC group was seen when compared to the peripheral placebo group. A-G-CSF v peripheral placebo; B-IC placebo v

IC BMC; C-IM BMC v peripheral placebo; IC-intracoronary. G-CSF-granulocyte colony stimulating factor; BMC-bone

marrow derived cells

CYTOKINE/CHEMOKINE	REDUCED SCAR	INCREASED SCAR
IL-2	+	+
IL-4	-	+
IL-6	+	+
IL-8	+	-
IL-10	+	-
VEGF	+	-
TNF	-	+
MCP-1	+	+
IL-1	IL1a - IL1b -	IL1a - IL1b+

Table 5.1. Change in cytokines/chemokines with change in scar. + signifies any increase; - signifies any reduction
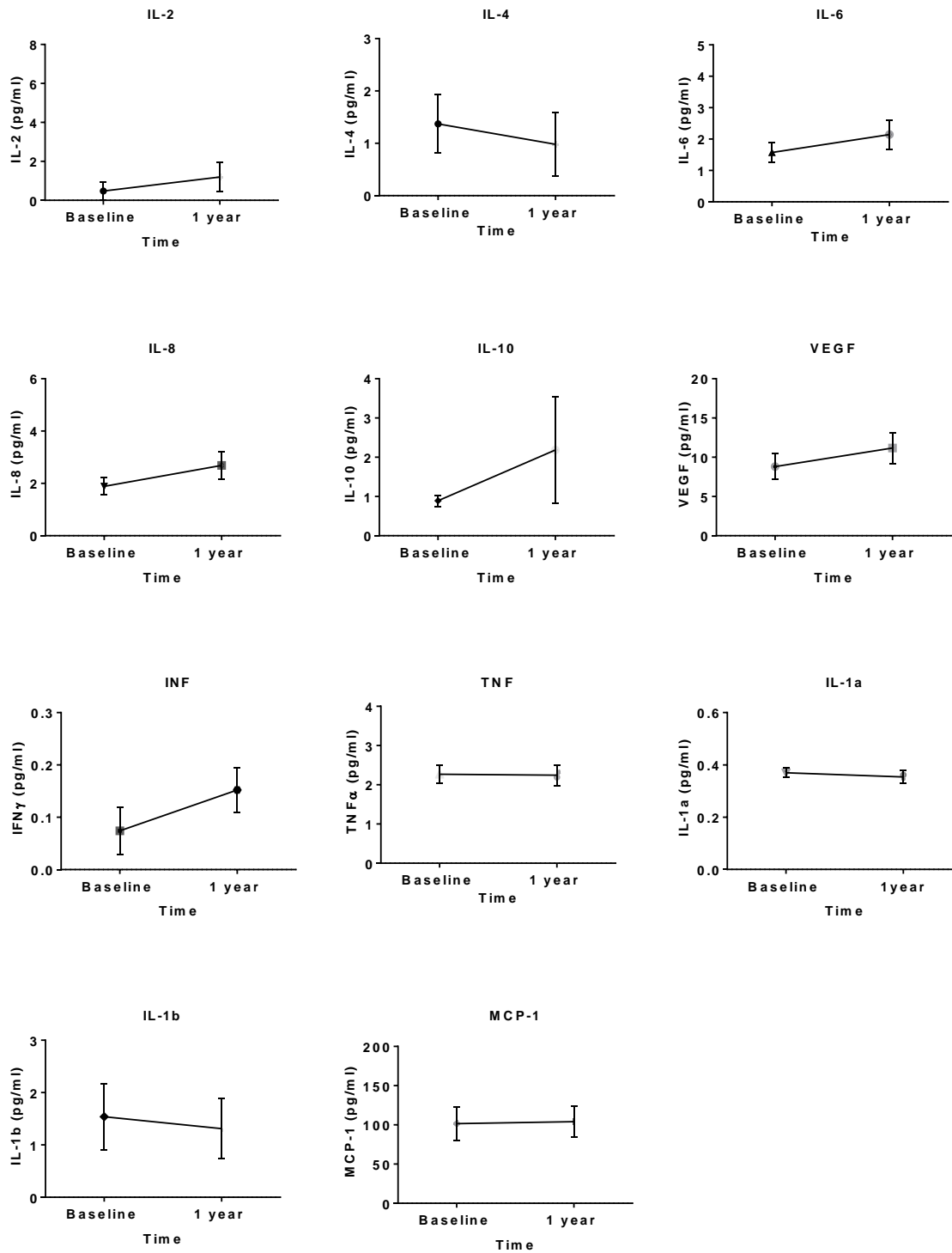
Figure 5.7. Change in mean (±SEM) cytokine levels in the reduced scar group. None of the changes in cytokine levels were significant. A. IL-2 B. IL-4 C. IL-6 D. IL-8. E. IL-10 F. VEGF G. IFN H. TNF I. IL-1a J. IL-1b K. MCP-1 *p<0.05
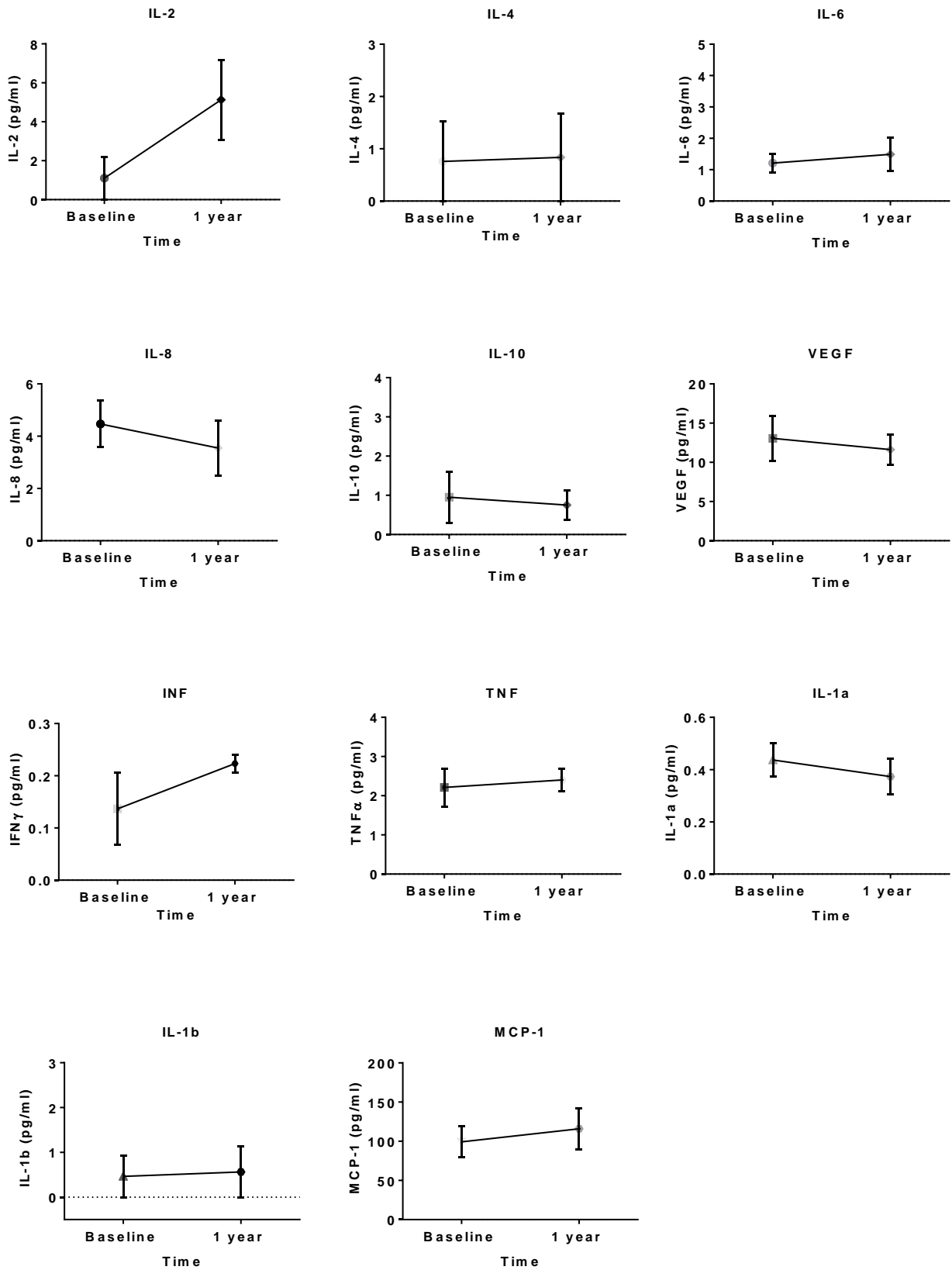
Figure 5.8. Change in mean (±SEM) cytokine levels in the increased scar group. None of the changes in cytokine levels were significant. A. IL-2 B. IL-4 C. IL-6 D. IL-8. E. IL-10 F. VEGF G. IFN H. TNF I. IL-1a J. IL-1b K. MCP-1 *p<0.05

5.4.2 Diastolic function

A total of 68 patients had paired sets of data on all 4 parameters measured for diastolic function at both baseline and 1 year. The spread of data across the six groups is shown in figure 5.9.
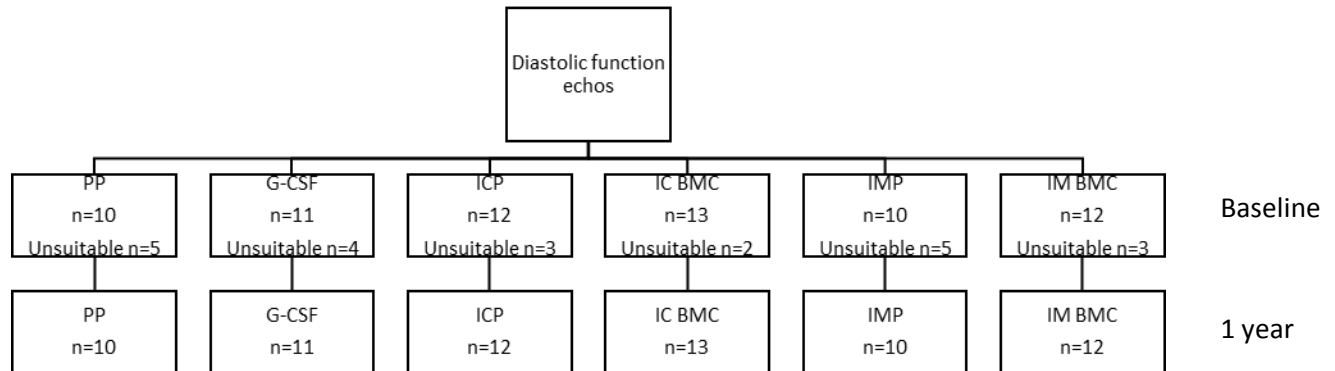
Figure 5.9. Diastolic function. Diagram depicting the number of patients in whom diastolic function was assessed by measuring e:a ratio, deceleration time, E:E' and left atrial dimensions in each group. IC-intracoronary; IM-intramyocardial; BMC-bone marrow derived cells; G-CSF- granulocyte colony stimulating factor; Unsuitable-due to insufficient images, poor image quality, unable to retrieve

Peripheral arm

In the peripheral placebo group, none of the measured diastolic parameters changed significantly. The E:A ratio (fig 5.10) changed by 0.41±1.36 (p=0.362), the deceleration time (DT) (fig 5.11) by 8.0±48.0 (p=0.611), E:E' by 3.31±6.00 (p=0.115) and LA size by -0.10±0.52 (p=0.559). In the peripheral G-CSF group, the E:A ratio did not change significantly (1.02±0.69 to 1.29±1.19; p=0.184). The deceleration time (DT) also did not change significantly (-28.4±117.5; p=0.442). Neither was there any significant change in the E:E' (-1.50±4.18; p=0.260) or the left atrial (LA) size

(-0.02±0.59; p=0.921). The change in E:E' in the G-CSF only group when compared to the peripheral placebo group was significant (Δ-4.81±2.24; p=0.045). None of the other parameters were significantly different between the two groups.

Intracoronary arm

None of the diastolic parameters changed significantly in the IC placebo group. The E:A ratio (fig 5.10) changed by 0.33±1.21 (p=0.369), DT (fig 5.11) by 4.08±146.2 (p=0.925), E:E' by -1.56±6.19 (p=0.401) and LA size by -0.01±0.44 (p=0.949). Similar to the IC placebo group, none of the diastolic parameters changed in the IC BMC group either. The E:A ratio changed by 0.14±0.31 (p=0.132), DT by 32.7±121.9 (p=0.353), E:E' by 1.16±6.26 (p=0.518) and LA size by -0.18±0.67 (p=0.361). None of the parameters were significantly different between the two groups.

Intramyocardial arm

No significant change in diastolic parameters were found in either the IM placebo or IM BMC groups. In the IM placebo group, E:A (fig 5.10) changed by 0.19±0.52 (p=0.273), DT (fig 5.11) by 33.5±69.9 (p=0.164), E:E' by -0.48±5.42 (p=0.785) and LA by -0.14±0.78 (p=0.586). In the IM BMC group, E:A changed by 0.03±0.45 (p=0.818), DT by -0.58±52.8 (p=0.970), E:E' by -0.74±2.65 p=0.356) and LA by 0.16±0.49 (p=0.286). The change in E:E' in the IM BMC group was significantly different to that in the peripheral placebo group (Δ-4.04±1.92; p=0.048). The rest of the parameters were not significantly different to the peripheral placebo group and none of the parameters were significantly different between the IM BMC and IM placebo groups.
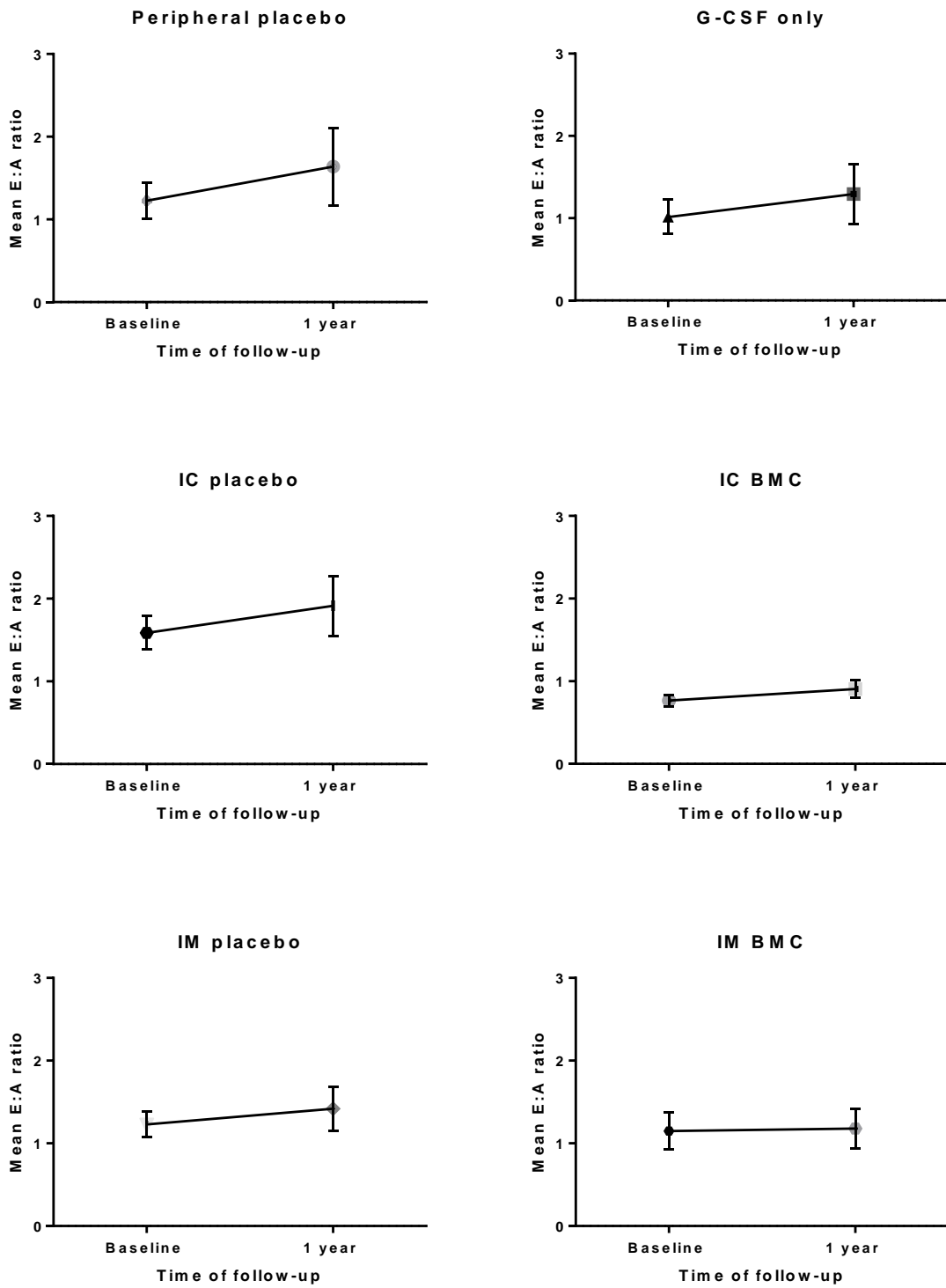
Figure 5.10. Change in mean E:A ratio. Diagram depicting the change in mean (±SEM) E:A ratio at 1 year in each of the six groups. No significant change in E:A was noted in any group. IC-intracoronary; IM-intramyocardial; BMC-bone marrow derived cells; G-CSF- granulocyte colony stimulating factor.
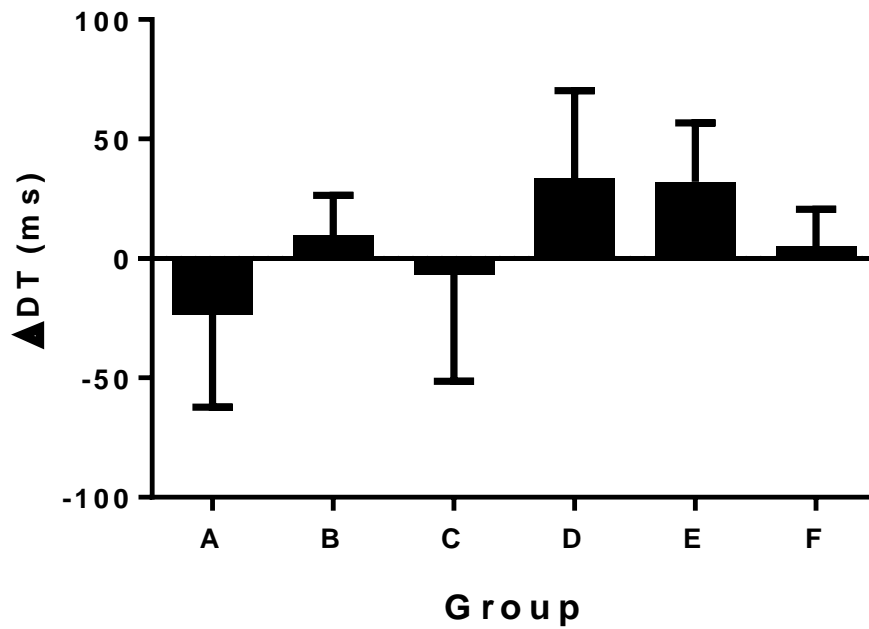
Figure 5.11. Deceleration time. Diagram depicting the mean (±SEM) change in deceleration time (DT) at 1 year in each of the six groups. No significant change in DT was noted in any group. A-peripheral placebo; B- G-CSF only; C- IC placebo; D-IC BMC; E- IM placebo; F-IM BMC; IC-intracoronary; IM-intramyocardial; BMC-bone marrow derived cells; G-CSF- granulocyte colony stimulating factor.

Diastolic function and scar reduction

In patients who had a reduction in scar burden, there was no significant change in any of the diastolic function parameters measured. The E:a ratio changed from 0.90±0.28 to 1.08±0.76 (p=0.495). The DT changed from 238.4±52.7 to 242.0±126.0 (p=0.935). The E:E' increased from 9.95±4.78 to 10.91±4.52 (p=0.651). The LA diameter also increased from 4.42±0.64 to 4.55±0.85 (p=0.703).

5.4.3 Cell count and function analyses

Baseline cell distribution

Peripheral blood

The mean (±SD) peripheral blood CD34+ cell counts in the entire cohort at baseline was $2.90 \times 10^6 \pm 2.03$/l cells, the EPC count was 0.08×10^6/l (±0.30) and the peripheral mononuclear cell (MNC) count was 2.31×10^9/l (±0.74). There was no difference in the baseline counts between the groups: CD34+ (one-way ANOVA p=0.538), EPC (one-way ANOVA p=0.361) and MNC (one-way ANOVA p=0.641).

Response to granulocyte colony stimulating factor (G-CSF)

All five groups that received G-CSF showed an adequate response in cell counts to G-CSF. The mean (±SD) day 6 peripheral blood CD34+ count was 57.51×10^6/l (±70.75), an approximately x20 increase in cell count. The mean EPC count at day 6 was 0.39×10^6/l (±0.54), a 5-fold increase in cell count. The mean MNC count went up 3.4 fold to 7.80×10^9/l (±8.46) in response to G-CSF (Figure 5.12). There was no difference in day 6 cell counts between the groups that had received G-CSF when compared using one-way ANOVA: CD34+ (p=0.153), EPC (p=0.195) and MNC (p=0.179).

Bone marrow

The mean (±SD) CD34+ cell count in the bone marrow reinfused in the interventional groups (i.e. IC and IM groups) was 2.92×10^6 (±1.74) with a mean EPC count of 136.30×10^2 (±380.20) and a mean bone marrow MNC count in the BM infusate of 109.30×10^6 (±93.50). There was no difference in cell counts across the groups when compared using one-way ANOVA: CD34+ (p=0.282), EPC (p=0.294) and MNC (p=0.740).
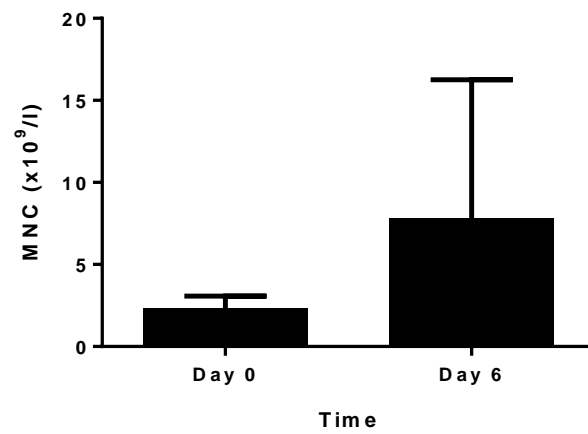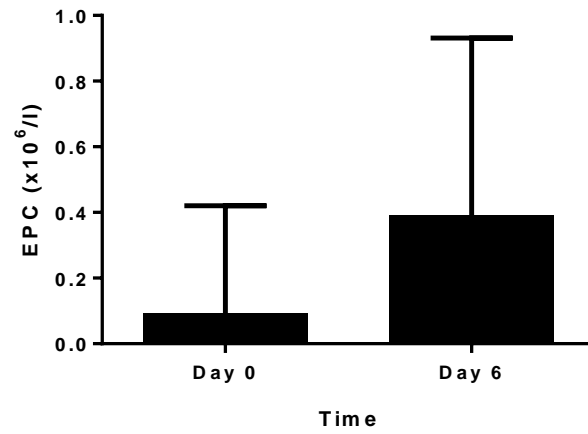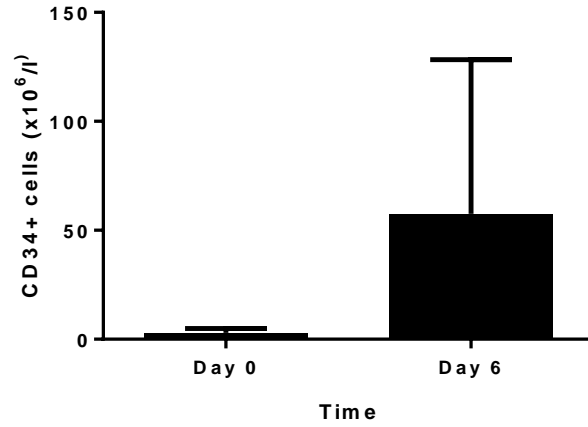
Figure 5.12 Mobilisation of cells after G-CSF. Box and whisker plots showing change in peripheral blood cell counts between day 0 and day 6 in response to G-CSF. G-CSF-Granulocyte colony stimulating factor. EPC-endothelial progenitor cell. MNC-mononuclear cells

Colony forming units granulocyte monocyte (CFU-GM)

The mean CFU-GM count derived from the bone marrow samples in the intervention arms (IC and IM) was 13.1 (±10.2) colonies/plate. The mean CFU-GM count in the IC BMC and IM BMC groups was 10.9 (±9.5) colonies/plate. The mean CFU-GM in the IC BMC group was 16.4 (±11.5) colonies/plate. The mean CFU-GM in the IM BMC group was 9.0 (±8.7) colonies/plate. There was no difference in CFU-GM count between the four groups when compared using one-way ANOVA (p=0.808).

Cell and CFU counts and outcome

Correlation between peripheral blood cell counts and LVEF

There was a trend towards a correlation between CD34+ cell count at baseline (day 0) and change in LVEF in the whole cohort (Pearson r^2=0.04; p=0.058) (figure 5.13) but no significant correlation was seen in any of the six groups. Neither was there any correlation between the change in CD34+ cell count between day 0 and day 6 in response to the G-CSF (i.e. all groups except peripheral placebo), and change in LVEF (r^2=0.04;p=0.100).
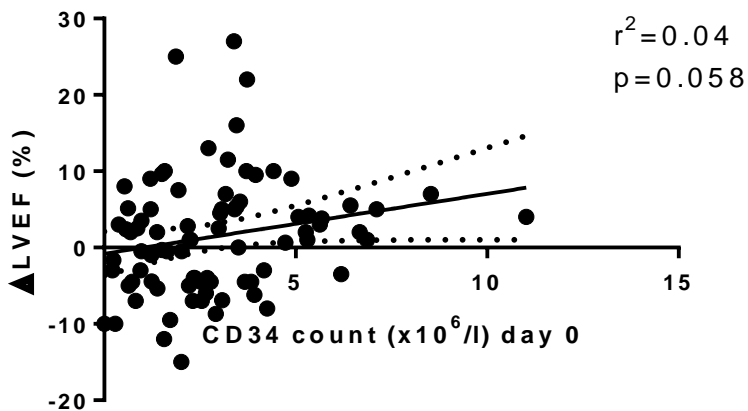
Figure 5.13. Correlation between CD34 count and change in LVEF. Correlation curve showing trend towards significant correlation between day 0 CD34 peripheral blood count and change in LVEF at 1 year in the entire cohort.

No association was found between EPC day 0 cell count and change in LVEF in the whole cohort (r^2=0.001; p=0.799). Neither was any association found between EPC day 0 cell count and change in LVEF in any of the groups. However, an association was observed between change in EPC count between day 0 and day 6 and the change in LVEF (r^2=0.24; p=0.0003) in the G-CSF receiving groups (figure 5.14).
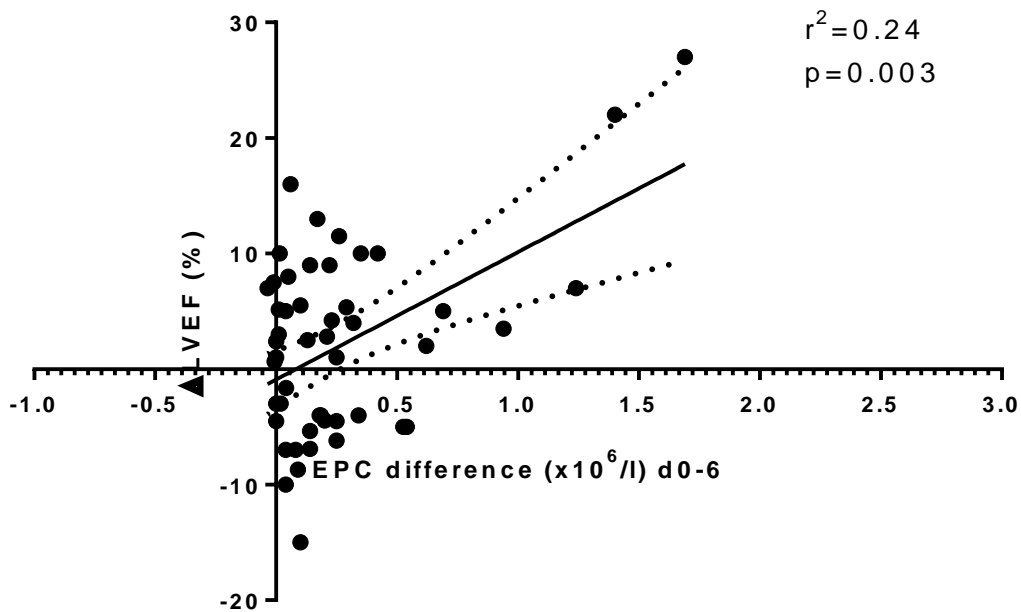
Figure 5.14.

Correlation between mobilisation in EPC and change in LVEF. Correlation curve showing a significant correlation between change in EPC count in peripheral blood between day 6 and day 0 in response to G-CSF and change in LVEF at 1 year. G-CSF- granulocyte colony stimulating factor. LVEF-left ventricular ejection fraction

No association was found between MNC day 0 cell count and change in LVEF in the whole cohort (r^2=0.00; p=0.587). The MNC day 0 counts in each of the groups did not show any association either with the change in LVEF in the respective groups. There was, however, a significant association between change in MNC count between day 0 and day 6 and the change in LVEF (r^2=0.05; p-0.043) (figure 5.15).
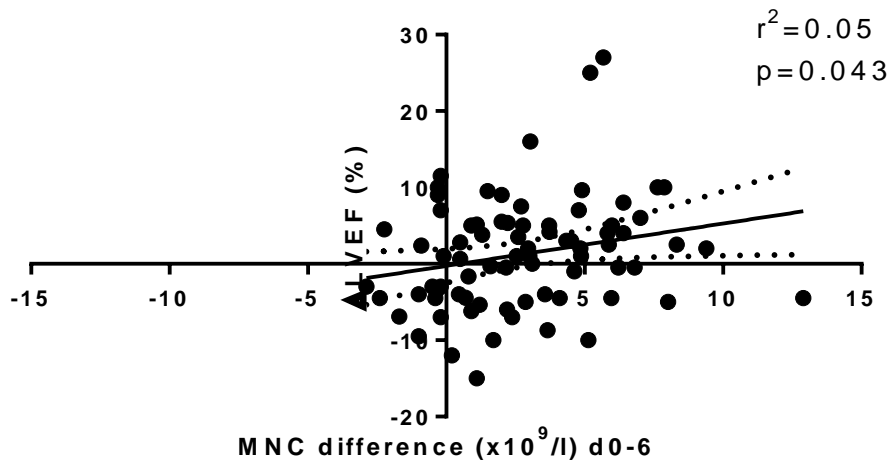
Figure 5.15. Correlation between mobilisation in MNC and change in LVEF. Correlation curve showing a significant correlation between change in MNC count in peripheral blood between day 6 and day 0 in response to G-CSF and change in LVEF at 1 year. G-CSF- granulocyte colony stimulating factor. LVEF-left ventricular ejection fraction. MNC- mononuclear cells

Correlation between bone marrow cell counts and LVEF

No association was observed between the BM infusate CD34+ cell count (r^2=0.00 p=0.760), EPC count (r^2=0.15; p=0.150) or reinfusate bone marrow MNC (BMMNC) count (r^2=0.02; p=0.474) and change in LVEF in the two cell therapy groups combined i.e. IC BMC and IM BMC (r^2=0.00 p=0.760). Analysing the groups individually, neither was any association found between the BM reinfustate CD34+ count, EPC count or BMMNC count and change in LVEF in the IC BMC group (CD34+ r^2=0.00; p=0.888; EPC r^2=0.03; p=0.641; BMMNC r^2=0.04; p=0.506) or the IM BMC group (CD34+ r^2=0.09; p=0.293; EPC r^2=0.04; p=0.622; BMMNC r^2=0.01; p=0.777).

Correlation between CFU-GM and LVEF

A significant association between CFU-GM and change in LVEF was observed in the IM BMC group (r^2=0.62; p=0.020). (Figure 5.16). However, in the IC BMC group, no association was found between CFU-GM and change in LVEF (r^2=0.03; p=0.898).
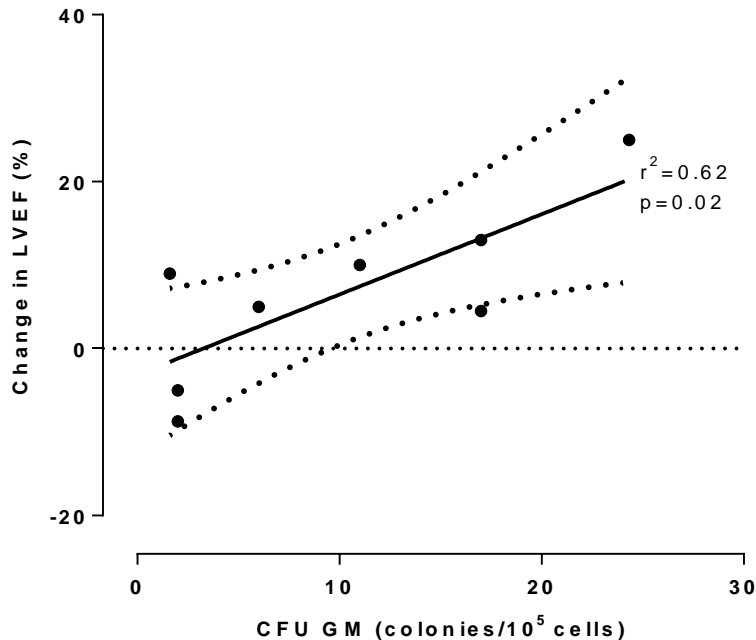
Figure 5.16. Colony forming unit granulocyte-monocyte (CFU GM) count and change in LVEF. The figure

depicts a significant, linear relationship between the CFU GM count in the bone marrow and the change in LVEF in the

intramyocardial cell therapy group suggesting a link between the functional capacity of the haematopoietic stem cells and change in

LVEF. LVEF- left ventricular ejection fraction. Significance shown for correlations determined using Pearson's linear regression of best

fit ±95% confidence intervals

DIABETES, AGE AND CELL/CFU COUNTS

The presence of diabetes mellitus did not affect the baseline peripheral CD34+ (p=0.437), EPC

(p=0.176) or MNC (p=0.131) cell counts. Neither was there any difference in the mobilisation of

CD34+ (p=0.155), EPC (p=0.082) or MNC (p=0.596) at day 6 between diabetics and non-diabetics.

The CD34+ (p=0.843), EPC (p=0.923) and BMMNC (p=0.418) cell counts in the bone marrow re-

infusate were also unaffected by the diabetes status as was CFU-GM (p=0.371) and viability

(p=0.370).

Age was not associated with baseline, peripheral CD34+ (r^2=0.02; p=0.156), EPC (r^2=0.01; p=0.482) or MNC (r^2=0.01; p=0.288) cell counts. Neither was there any association between mobilisation of CD34+ (r^2=0.03; p=0.175), EPC (r^2=0.01; p=0.565) or MNC (r^2=0.01; p=0.375) at day 6 and age. The EPC count in the BM re-infusate was significantly, negatively associated with the age of the patient at admission (r^2=0.13; r= -0.36; p=0.021). However, the CD34+ (r^2=0.04; p=0.150) and BMMNC (r^2=0.01; p=0.610) cell counts in the bone marrow re-infusate showed no correlation with age. Neither was there any association between age and viability (r^2=0.01; p=0.535) or CFU-GM (r^2=0.03; p=0.384).

5.5 Summary

- **No significant association between change in scar and change in LV volumes, myocardial mass or NT pro-BNP was observed**

- **In patients with an improvement in LVEF, a non-significant reduction in scar was seen with a non-significant increase in scar seen with lower LVEFs**

- **No significant association between change in scar and cytokine levels were observed; however, certain trends were noted:**

  - **TNF-α, IL-4 and IL-1b decreased in the group with reduced scar as opposed to an increase in the group with an increase in scar burden**

  - **IL-10 and VEGF increased in the group with reduced scar as opposed to an decrease in the group with an increase in scar burden**

- **In the IC BMC group, a significant reduction in scar was observed as compared to the true placebo group**

- **No significant association between the parameters measured for diastolic function and G-CSF/cell therapy was observed**

- **No significant association between diastolic function and scar was noted**

- **A good response in cell mobilisation was seen in response to G-CSF**

- **The increase in peripheral blood EPC and MNC counts at day 6, compared to day 0, were significantly associated with change in LVEF at 1 year**

- **No significant association was noted between bone marrow counts of CD34+, EPC or BMMNC and LVEF change**

- **CFU-GM was significantly associated with change in LVEF in the IM BMC group**

- **Only the EPC count in the bone marrow was significantly, negatively associated with age.**

This thesis reports the first-in-man randomised controlled trial to investigate the effect of G-CSF alone or in combination with autologous BMC in ischaemic cardiomyopathy. Heart failure is a major cause of morbidity and mortality and there is no doubt that further improvement in treatment is needed. Novel strategies are required and stem cell therapy has long been regarded as one method, which may be successful in accomplishing this.

This thesis suggests that G-CSF therapy alone does not result in improved cardiac function or changes in functional or biochemical outcomes in patients with ischaemic cardiomyopathy. However, the administration of G-CSF together with autologous BMC therapy appears to result in a significant improvement in LVEF, NYHA class at 1 year and NT pro-BNP levels at 6 months but only when the cells were delivered via the intramyocardial route. Furthermore, this beneficial effect on LVEF and NYHA class appears to be sustained in the intermediate term i.e. 2 years. Importantly, no safety concerns were evident at 1 or 2 years. Subsequent sections report the findings of the mechanistic sub-studies looking at the association between cytokines, myocardial scar, cell numbers/function, and diastolic function with G-CSF/cell therapy and outcomes.

Three key aims were set out by this thesis, which will be discussed in detail during this discussion.

The 1ˢᵗ aim in this MDRes was to ascertain if the administration of G-CSF monotherapy resulted in a beneficial effect on cardiac function in ischaemic cardiomyopathy patients and whether it was safe in this patient group

The complex and expensive nature of cell therapy trials entails putting the patient through invasive procedures, including bone marrow aspiration and arterial access to deliver the cells, and thus, exposing them to the potential risks associated with these procedures. Therefore, the appeal of "pharmaco-regenerative" medicine is evident. The concept of pharmaco-regenerative medicine - that an established drug or cytokine can activate stem and progenitor cells or act as paracrine mediators considered to be the effectors of cell therapy - is attractive. G-CSF is a prime example of the former i.e. an activator of mobilisation of bone marrow derived cells. The concept that a few days of subcutaneous injections of G-CSF could potentially perform the same function as "manually" delivering cells to the heart and result in an improvement in cardiac function and symptoms led us to test the safety and efficacy of G-CSF as a sole intervention. In this thesis, I showed that although G-CSF therapy was safe it did not result in an improvement in cardiac function or symptoms.

Although the lack of effect of G-CSF alone is disappointing, particularly in view of the ease of administration of G-CSF, three questions remain: 1. why did G-CSF not work in the current study cohort? 2. Are these results in keeping with other clinical data? and 3. Does this data add to existing knowledge?

There are several possibilities to explain why G-CSF did not work in this study cohort. G-CSF is a potent haematopoietic cytokine that is known to mobilise CD34+ cells as well as non-CD34+ cells, including MSCs, from the bone marrow into the circulation. One possibility is that the cell mobilisation response to G-CSF is blunted in patients with advanced heart failure and the older patient population, however, given the good response in circulating cell numbers seen in our study after G-CSF administration and the previous similar findings in other studies, this explanation is highly unlikely. Rather, the failure of G-CSF is more likely secondary to functional impairment of the

mobilised cells resulting in impaired homing and engraftment. It has already been shown that the functional capacity of BMCs in older patients and in those with advanced ischaemic cardiomyopathy patients is impaired. [154, 226] G-CSF has been suggested to further impair the function of these cells. The exact mechanism of action of G-CSF is still not clear but it appears to interact with the SDF-1/CXCR-4 axis. SDF-1 is present in the bone marrow stroma and the receptor CXCR-4 is present on the surface of BMC. SDF-1/CXCR-4 interaction plays a role in the retention of BMC in the bone marrow and the homing of cells to the heart. G-CSF is known to activate proteases that cleave CXCR-4, as explained in section 1.2.7. Furthermore, G-CSF downregulates SDF-1 levels in the bone marrow.[227] The net result is a reduced SDF-1/CXCR4 interaction. This aids in the mobilisation of the cells from the bone marrow. However, the attraction of cells towards the cardiac target requires chemotactic signalling via the SDF-1/CXCR-4 axis. Unfortunately, the cleavage of CXCR-4 by G-CSF induced proteases is irreversible. Thus, although G-CSF promotes mobilisation of cells into the peripheral blood, it impedes the homing of the cells towards the cardiac target. Furthermore, Ripa et al. have documented similar levels of SDF-1 in the chronic ischaemia population as compared to the normal population as opposed to the increased SDF-1 levels observed after an acute MI.[228] Thus it appears that in chronic heart failure patients, there is no increased chemotactic stimulus to aid the homing of the G-CSF mobilised cells. Theiss et al. have demonstrated in mice acute MI models that administration of G-CSF together with sitagliptin, a drug used in managing diabetes but also known to stabilise SDF-1, improves cardiac homing of cells and leads to improved cardiac function and reduction in short-term mortality. This has led to the ongoing SITAGRAMI phase III trial looking at the effect of combined G-CSF and sitagliptin in acute MI patients.[229] A similar trial in chronic HF patients is probably warranted. Further proof that G-CSF impairs the functional capacity of cells comes from work by Dimmeler et al. They studied the

mobilisation and functional capacity of EPC's in response to G-CSF and found that although the mobilisation was adequate in terms of the quantification of mobilised EPC's, the functional capacity of the EPC's, as characterised by looking at the migratory capacity of the cells in response to VEGF and SDF-1, was reduced.[230]

The optimal G-CSF dose and length of administration is as yet unclear. There is no literature that compares different doses of G-CSF or length of administration in ischaemic cardiomyopathy patients. In the TOPCARE-G-CSF trial, both 5µg/kg/day and 10µg/kg/day of G-CSF were used.[231] As the level of mobilisation of peripheral CD34+ cells were comparable, no further sub-analysis was undertaken between the two groups and the groups were pooled. Whether, a lower dose of G-CSF, enough to mobilise but yet minimally disrupt the homing of the cells, remains to be tested. This would require a direct dose comparison study.

The results of my thesis are in keeping with the majority of studies performed using G-CSF. G-CSF was shown to be safe in this study with the main reported adverse event being bone pain constituting 85.7% of adverse events. This figure is in keeping with the reported figure of 84% by Anderlini et al.[158] No significant difference in the rate of MACE was noted in the G-CSF only group or in the other groups that had received G-CSF. Two previous studies had raised concern regarding the possibility of increased progression of atherosclerosis and an increased rate of in-stent restenosis after G-CSF in acute MI patients. However, this was not supported in the study by Ripa et al in the STEMMI trial. The findings, in my thesis, of no increased rate of MI or cardiac death in

the G-CSF groups extends and supports Ripa's findings in the chronic ischaemic cardiomyopathy setting.

With respect to efficacy, the lack of benefit is consistent with the literature. In the study by Wang et al, 13 patients with severe CAD and a mean baseline LVEF of 39% were treated with a lower dose of G-CSF than used in most trials (5µg/kg/day) for 6 days and, in fact, found a reduction in LVEF, assessed by MRI, of 5% in the G-CSF group. [157] In the study by Hill et al., they treated 16 patients with chronic coronary artery disease with the higher 10µg/kg/day dose of G-CSF for 5 days and found no significant change in LVEF. [156] However, both these studies were small and not placebo controlled. In the TOPCARE-G-CSF trial by Assmus et al, G-CSF was compared against G-CSF plus IC circulating EPC's (CEPC) with no significant effects of G-CSF or G-CSF mobilised CEPC reinfusion on cardiac function.

In summary, despite the theoretical benefit of G-CSF in terms of bone marrow cell proliferation and mobilisation, I found no evidence of G-CSF, as monotherapy, being beneficial in chronic ischaemic heart failure patients. The novelty and strength of this trial compared to the previous trials was the presence of an appropriate comparator arm consisting of saline only and the presence of other study arms allowing us to assess the efficacy and safety of G-CSF both alone and in combination with BMC therapy. No other trial has, to date, compared G-CSF, G-CSF plus BMC therapy and cell delivery route, all in the same trial with appropriate control arms.

The 2nd aim in this MDRes was to ascertain if the administration of G-CSF in combination with autologous BMC resulted in a beneficial effect on cardiac function in ischaemic cardiomyopathy patients.

The findings that the combination of G-CSF and IM BMC therapy resulted in a 4.99% improvement in LVEF at 1 year along with a reduction in NT pro-BNP at 6 months and a sustained reduction in NYHA class at 1 and 2 years are promising. The above findings suggest two interpretations: 1. G-CSF, in combination with autologous BMC are both safe and beneficial to both cardiac function and patients' functional outcomes and 2. The benefit of autologous BMC is seen when the BMC are delivered intramyocardially. These two interpretations bring to the forefront several questions which will be addressed in the following sections. They are:

1. Are autologous BMC delivered safe?

2. Does G-CSF combined with autologous BMC improve cardiac function in ischaemic cardiomyopathy patients?

3. Is the IM route superior to the IC route?

4. If so what is the mechanism behind the lack of effect with the IC route and the observed effect with the IM route?

5. What is the physiological basis of the improvement in LVEF seen?

6. What are the implications of the findings in the context of current clinical practice and in guiding further research in the field?

Are autologous BMC delivered safe?

This thesis has shown that G-CSF alone and in combination with BMC are safe in the acute procedural period as well as at 1 and 2 year follow-up, with no observed increase in G-CSF and autologous BMC related safety end-points during this period. . These findings are in keeping with the existing safety data from recent meta-analyses including that by Kandala et al and two by Doree et al. [199, 201] [191] Most cell therapy trials to date have reported follow-up at 6 months or 1 year. The data in this thesis extends to two years and shows a persistent safety record of G-CSF and BMC during this longer follow-up period.

Does G-CSF combined with autologous BMC improve cardiac function in ischaemic cardiomyopathy patients AND is the IM route superior to the IC route?

The design and findings of the study necessitate that these two questions are discussed together. The magnitude of the significant improvement in LVEF seen in the IM BMC group but not in the IC BMC group suggests efficacy of G-CSF combined with autologous BMC and superiority of the IM route. The lack of effect seen with the IC route has multiple possible explanations. The delivery of cells via the IC route is reliant on both the presence of a patent vessel for the cells to be infused via, the extravasation of the cells from the coronary artery into the ischaemic and peri-ischaemic tissue and the integration of the cells into the target tissue i.e. engraftment. Theoretically, IC delivery is an attractive strategy, as it should allow uniform distribution of the cells into the target, peri-infarct, ischaemic tissue as long as there is a patent epicardial vessel for the cells to travel through. Although, epicardial vessel patency can be well assessed on coronary angiography, undertaken pre-cell infusion, the presence of microvascular disease (MVD) cannot be assessed reliably with simple coronary angiography. MVD is associated with traditional coronary heart disease (CHD) risk factors.

Cigarette smoking[232], hypercholesterolaemia[233], hyperglycaemia[234] and hypertension[235] have all been shown to increase coronary microvascular resistance and microvascular dysfunction. In our cohort, all 15 patients in the IC BMC group had ≥1 of the following risk factors: hypertension, diabetes, hypercholesterolaemia or smoking (either current or ex-smoker). Thus, although not directly assessed for, it can be postulated that a significant number of these patients had a degree of MVD that might have prevented adequate numbers of cells reaching the target tissue. Secondly, the method of IC reinfusion of BMC used was the stop-flow technique. This involves, as previously described, balloon inflation to transiently occlude proximal coronary flow with the aim of preventing rapid or early cell washout. The cells were delivered in divided doses with three balloon inflations, with balloon deflation in between, and 3.3mls of cell suspension being infused during each balloon inflation. The question arises as to whether the rate of cell suspension delivery via this method could have resulted in clumps of cells causing microvascular obstruction (MVO) and micro-coronary infarcts and that modifications of the method would be necessary with single, higher pressure infusions over shorter durations to reduce the risk of this phenomenon occurring, as has been shown in animal models.[236] However, the phenomenon of MVO and micro-coronary infarcts is mainly seen with larger cell types e.g. MSC's[237] or CSC's and not with unfractionated BMC and is therefore unlikely to explain the lack of effect seen with IC BMC therapy. This was evident from the lack of features on coronary angiography suggestive of MVO post-cell infusion and is further supported in the analyses by Assmus et al. who looked at 775 patients undergoing IC BMC delivery using the stop-flow technique and found very low rates of slow-flow. [194] Lastly, the cells have to travel once they have exited the artery to get to the target peri-infarct zone. The efficacy of this process will not only depend on flow in the coronary vessels but also on appropriate chemotactic signals and the distance from the last patent vessel exit point to the target tissue. Furthermore, as

previously mentioned, G-CSF is known to cleave CXCR-4 and this effect would lead to lower migration and engraftment of the cells and could further explain the lack of effect of IC BMC therapy seen here compared to other trials which have not used G-CSF. In summary, the delivery of cells via the IC route can be considered "indirectly" delivered as opposed to the "directly" delivered cells with the IM route (see below). The larger number of variables influencing the process of cells migration and engraftment probably reduces the chances of the cells reaching their target.

The observed efficacy with the IM route is very encouraging. This efficacy of the IM route over the IC route could be explained by several possible explanations. In contrast to the IC route, the IM route does not require patent epicardial vessels and can be directly delivered to the area of interest. With regards to identification of the area of interest, the use of electromechanical mapping technology allows the targeting of viable myocardium in the peri-infarct area. This allows optimal, controlled delivery of the cells to areas of the myocardium which would theoretically derive the greatest benefit. In contrast, the delivery of the cells via the IC route allows no such controlled delivery. Furthermore, cell retention at the target site has been a persistent problem in cell therapy trials, however, pre-clinical studies, have demonstrated that the IM route had four times greater cell retention compared to the IC route in a swine ischaemic model.[195] [192] Although direct evidence of increased cell retention in humans is difficult, the results of the pre-clinical study imply that the IM route would be more efficacious. However, the results of the IM placebo group in our trial does raise the question of whether the IM injections per se cause a local, "beneficial" inflammatory reaction in the myocardium which triggers an unexplained, reparative process and thus, demonstrates the 4.15% improvement in LVEF. However, there are several reasons this may not be the case. Firstly, there was no significant improvement in LVEF seen in the IM placebo

group. Secondly, there was no significant improvement in NT pro-BNP at 6 months or NYHA class at 1 year in the IM placebo group. Thus, the conclusion that can be derived is that this is likely secondary to the small number of patients in each group and that a larger scaled, phase III trial would be necessary to show a more clear lack of effect of the IM injection process alone.

What is the physiological basis of the improvement in LVEF seen?

The next question that arises is what is the plausible physiological mechanism of the improvement in LVEF seen in the IM group i.e. how does one explain the improvement in LVEF in terms of LV volumes and contractility? LVEF is calculated by expressing the stroke volume (SV) as a percentage of the LVEDV. The SV and thus the LVEDV and LVESV are determined primarily by three factors- preload, afterload and contractility. The preload is a reflection of the loading state of the heart prior to contraction. According to the Frank-Starling law, increasing preload increases the force of contractility and hence, ejects out a greater SV with each beat. Contractility (or inotropy) is determined primarily by the autonomic nervous system. In the failing heart, as inotropy declines, the Frank Starling curve shifts downwards as shown in the figure 6.1, i.e. for a certain LV end-diastolic pressure (LVEDP, a measure of preload), a lower SV is ejected in the failing heart compared to the normal heart and conversely, for a certain SV, a higher LVEDP is needed in the failing heart compared to the normal heart, as an attempt to compensate for the reduced inotropy, as per the Frank-Starling mechanism i.e. an increase in preload. Two varying patterns were observed in the IM BMC group at the two time-points i.e. 1 year and 2 years. At 1 year, the LVESV decreased but the LVEDV increased marginally, albeit non-significantly. This was accompanied by an increase in myocardial mass.
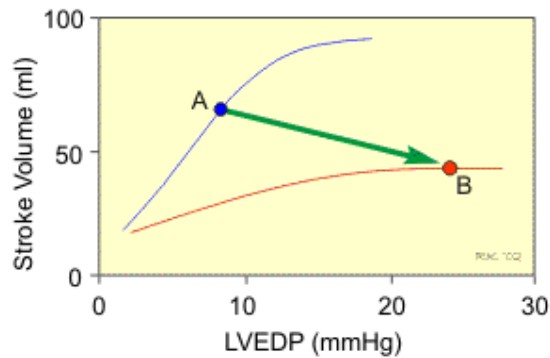
Figure 6.1 Relationship between SV and LVEDP. Curve A represents a normal heart and curve B represents a heart with ventricular systolic dysfunction. Reproduced with permission from www.cvphysiology.com

At 2 years, the LVEDV as well as the LVESV decreased together with a significant increase in myocardial mass. The natural progression of ischaemic HF would involve initial eccentric hypertrophy to minimise wall stress. However, the increased wall stress would eventually result in adverse remodelling resulting in continued increase in LVEDV and LVESV. Therefore, it appears, that the use of G-CSF and IM BMC in combination halts this adverse remodelling and reverses this process-"reverse remodelling". This was evidenced by the reduction in LV volumes seen initially as a reduction in LVESV at 1 year and then both LVESV and LVEDV at 2 years. Whether the increase in myocardial mass is mainly accounted for by myocardial regeneration rather than solely by compensatory hypertrophy is difficult to distinguish without histological evidence.

What are the implications of the findings in the context of current clinical practice and in guiding further research in the field?

The finding of an improvement in LVEF with IM delivered autologous BMC is in keeping with data published in the most recent meta-analysis by Fisher et al. of autologous bone marrow stem cells in chronic heart disease, which showed a 5.95% mean difference in LVEF in the sub-set of patients with heart failure and a 5.30% mean difference in the IM group (which was statistically significant

when compared to the 3.19% improvement in the IC group, suggesting superiority of the IM route)[191]. The findings are also in agreement with three other recent meta-analyses of cell therapy and heart failure: Kandala et al. (5.13% via IM route) [199], Jeevanantham et al. (3.96%; not route specific) [200] and Fisher et al. (3.47%; 8 of 9 trials via IM route) [201]. Importantly, although the change in LVEF would appear to be relatively low in these analyses and in this thesis, the magnitude of effect is similar to that seen in other trials of heart failure therapies (e.g. angiotensin converting enzyme inhibitor), which are now part of standard practice [238] In terms of comparing to previous cell therapy trials, the improvement in LVEF seen in the IM arm of this study agrees with a few of them including that by Beeres et al., Perin et al. and Pokushalov et al.[140, 176, 239] However, the more recent FOCUS trials by Perin et al. showed a lack of improvement in LVEF with autologous BMC.[145, 146] The latter trials did not use G-CSF as an adjunct for cell mobilisation. Furthermore, the study used an automated cell separation technique which is different to the manual Ficoll-Hypaque method utilised in our study. However, the larger FOCUS-CCTRN trial did show a significant improvement of 3.1% in LVEF in the under 62 years age group. In contrast, the results of my thesis failed to corroborate that of recent intracoronary cell therapy trials. The IC arm results in my thesis are in keeping with studies done by Yao et al. and against the findings of the large IC cell therapy trial-STAR-HEART. [143, 151] However, the results of the STAR-HEART trial are now under question due to scrutiny of the methodology used in the trial. In summary, all inference on efficacy of BMC therapy and superiority of route to date has been based on meta-analysis of single route trials. Furthermore, all the trials to date have either been non-randomised, not placebo-controlled or did not use advanced cardiac imaging. This is the first trial using autologous BMC in ischaemic cardiomyopathy patients that has allowed a direct comparison to be made between IC

and IM injection of autologous cells and confirms the results of the meta-analyses suggesting that the IM route is most effective in this group of patients.

So what are the implications of the findings of my thesis for the patient with ischaemic cardiomyopathy? The improvement of 4.99% in LVEF in the IM BMC group occurred, not in isolation, but in combination with an improvement in NYHA class and a reduction in NT pro-BNP levels. The change in LVEF of 4.99% is comparable to some of the landmark trials in heart failure which have led to the establishment in clinical practice of the commonly used medications used in heart failure. In the Val-HeFT trial (investigating the use of the angiotensin receptor blocker Valsartan in heart failure), the improvement in LVEF with Valsartan was 4.0%. [240] In the randomised trial by Fisher et al. looking at the effect of metoprolol in ischaemic cardiomyopathy, an increase of 4.0% was observed in the treatment group.[241] Similarly, in the RESOLVD pilot study, metoprolol increased LVEF by 2.4% in chronic heart failure patients.[242] In the CAPRICORN (Carvedilol Post-Infarct Survival Control in Left Ventricular Dysfunction) echocardiography sub-study, LVEF improved by 5.0% in the carvedilol group (at 6 months) as opposed to 1.0% in the placebo group.[243] Thus, it appears that the magnitude of improvement in LVEF seen in in this thesis is in keeping with that observed in landmark heart failure trials. In addition, a number of these trials showed significant improvements in NYHA class as well e.g. Val-HeFT, CONSENSUS, MERIT-HF (Effect of metoprolol CR/XL in chronic heart failure: Metoprolol CR/XL Randomised Intervention Trial in Congestive Heart Failure)[41], Fisher et al.[240, 241] An improvement in NYHA class, a well-established prognostic indicator in chronic heart failure, was also observed in this study. [244]

Together with the improvement in LVEF and NYHA class, a reduction in NT pro-BNP was also noted at 6 months in the IM BMC group. NT pro-BNP has been shown to be a prognostic marker in heart

failure and a rising level implies worse outcomes, including increased hospitalisation, arrhythmia and pump-failure related death, in patients with heart failure. [61] In fact, trials and meta-analyses have shown that BNP guided tailoring of heart failure therapy improves outcomes in chronic heart failure patients. In a meta-analysis by Li et al., BNP guided heart failure therapy resulted in improved mortality.[58] The trigger for the release of BNP appears to be cardiomyocyte stretch. Thus, the reduction in LV volumes (albeit non-significant) together with the improved LVEF, would, at least partially, account for the reduction in NT pro-BNP observed in the IM BMC group. As discussed in section 1.1.6, LVEF is associated with mortality risk in heart failure. The REGENERATE-IHD trial being a phase II trial, it was not possible to analyse mortality benefit. The improvement in three different prognostic factors in heart failure-LVEF, NYHA and NT pro-BNP-in the IM BMC group is certainly encouraging and would necessitate larger, phase III trials to assess mortality benefit. Most of the landmark trials of drug therapy mentioned above showed reduction in mortality and it will not be surprising if phase III cell therapy trials show a similar mortality benefit. Once the phase III trials are over, and if confirmatory, the challenges of implementing such a procedure as an established treatment modality will require substantial amounts of funding and training. On average the cost of treating a single patient with IM BMC therapy is around £4-6k. However, considering the potential for savings in terms of reductions in hospital readmissions, GP visits and medication costs, the savings to healthcare economies are likely to far outweigh the costs of the procedure. Considering the large number of patients with ischaemic cardiomyopathy in the UK and worldwide, this is likely to result in significant financial savings for the NHS and other healthcare systems. .

For advanced heart failure patients, the most important concerns are not always based on mortality but rather on whether a new trial intervention will bring symptomatic relief and well-

being and whether the intervention will not make the patient worse i.e. safety. On that note, the findings in my thesis have successfully shown an improvement in patients' functional outcomes as well as confirmed the safety record to date of BMC therapy in the ischaemic cardiomyopathy population.

The 3rd aim was to look at the mechanistic aspects of improvement in left ventricular function by studying change in cytokine levels, myocardial scar, diastolic function and cell quantity and function.

Does the administration of G-CSF and autologous bone marrow derived cells lead to an improvement in cardiac function in patients with ischaemic cardiomyopathy by modulating cytokine levels?

Analysis of specific cytokines was undertaken with the aim of gaining further insight into the association between G-CSF/cell therapy and cytokines with respect to changes in cardiac function. As far as I am aware, my work is the first to have analysed such a wide array of cytokines in a cell therapy trial in ischaemic cardiomyopathy patients. The ideal result would have been that G-CSF together with cell therapy, when delivered intramyocardially, increases the level of "protective" cytokines and reduces the effect of "harmful" cytokines and thus, suggests a mechanistic link between cytokines and improvement in LVEF. However, such clear conclusions cannot be derived for a number of reasons. Firstly, the number of paired blood samples (at baseline and 1 year) in each group were small, bearing in mind this was a phase II study. The study was not powered to detect significant changes in cytokine levels. Secondly, each cytokine is not a lone mediator of effect but rather part of a complex network of effectors. Thus, any association found between cytokine levels and cardiac outcomes would purely be, as mentioned above, an "association" rather than a cause and effect relationship. Bearing these two important limitations in mind, certain trends in cytokine levels are evident from the findings. Firstly, the important "positive" findings will be discussed. IL-8 was noted to increase in all groups apart from the IM BMC group at

1 year with the highest rise seen in the peripheral placebo group. IL-8 is known to be a potent mediator of the inflammatory micro-environment involved in the pathogenesis of atherosclerosis.[245] In the Controlled Rosuvastatin Multinational Trial in Heart Failure (CORONA) trial, the cytokine sub study revealed that IL-8 was a significant predictor of all-cause or cardiovascular mortality and the composite end-point of hospitalisation from heart failure or cardiovascular mortality in multivariate analysis. The study was undertaken in patients with chronic ischaemic cardiomyopathy aged ≥60 years and NYHA II-IV. Thus, the patient population was similar to our cohort. The finding that the group with the greatest increase in LVEF in my thesis has a reduction in IL-8 levels would certainly raise the possibility of an anti-inflammatory mechanism of action of G-CSF and IM BMC therapy as a means of improvement of cardiac function and symptoms. However, why the IC BMC group fails to show a reduction in IL-8 levels is unclear. One possibility is that the greater retention of cells in the IM BMC group allows time for paracrine activation of anti-inflammatory pathways to counter the effect of IL-8. From a basic science point of view, detailed analysis of the link between cell therapy and IL-8 would require measurement of downstream as well as upstream mediators of IL-8 action as well as the effects of IL-8 inhibition and outcomes. However, from a clinical point of view, the results of the CORONA sub study and our findings suggest that IM BMC therapy is associated with a reduction of a pro-inflammatory cytokine which is known to be a negative prognostic indicator in chronic heart failure.

MCP-1 is a polypeptide known to attract monocytes and macrophages as well as lymphocytes that can lead to enhanced fibrosis and adverse remodelling in chronic heart failure. Overexpression of MCP-1 has been shown to result in myocarditis and heart failure in transgenic mice and MCP-1 inhibition using gene therapy resulted in improved survival and attenuation of LV volumes and

improvement in cardiac function.[246] [39] Furthermore, high levels of MCP-1 in chronic heart failure patients have been shown to correlate with lower LVEF. In that study, the level of MCP-1 was highest in those patients with ischaemic cardiomyopathy. [247] In this thesis a similar trend to IL-8 was observed in levels of MCP-1 in the IM BMC group. Only in this IM BMC group was there a reduction in MCP-1, albeit non-significantly. All other groups showed an increase in MCP-1, with the highest increase in the G-CSF only group. Are the reductions in IL-8 and MCP-1 seen in the IM BMC group mere observations or is there a mechanistic link between the two? One study has investigated the role of mechanical stretch on various different cytokine levels and found that cyclic mechanical stretch upregulated the production of IL-8 and MCP-1 in human endothelial cells. [248] The cohort of patients in our trial are patients with coronary artery disease. The process of atherosclerosis is triggered and propagated by various stimuli including high blood pressure which contributes to the cyclic mechanical stretch undergone by endothelial cells. Thus it is possible that the effect of cell therapy is the secretion of paracrine factors which inhibit the downstream mediators of mechanical stretch and thus a reduction in IL-8 and MCP-1 and subsequent attenuation in adverse remodelling with improvement in symptoms and cardiac function. However, to delineate the exact effect and mechanism of IL-8 and MCP-1 reduction in the context of G-CSF and cell therapy will need further pre-clinical studies.

VEGF is a potent pro-angiogenic cytokine involved in neovascularisation that also has anti-apoptotic properties and is involved in homing of stem cells from the bone marrow to ischaemic myocardium. With progressive heart failure, the level of VEGF decreases and as a result there is decreased microvascular growth and hence, reduced capillary density. My findings showed an increase in VEGF levels in both the IC and IM BMC groups whereas a decrease in VEGF was seen in

the IC and IM placebo groups. Although non-significant, these results suggest that upregulation of VEGF is seen in association with administration of BMC together with G-CSF but not with G-CSF alone. This supports the paracrine hypothesis and suggests that these cells either secrete VEGF directly or secrete factors which in turn trigger upregulation of VEGF. However, as to why VEGF upregulation results in improvement in LVEF in the IM BMC group but not in the IC BMC group implies that this improvement is an end-result of a complex interaction of a multitude of paracrine factors acting in an optimal milieu.

Another observation in the cytokine sub-study was that IL-1b and IFN-γ levels both decreased in the IM BMC group. IL-1b is a pro-inflammatory cytokine that is known to be a myocardial depressant and associated with declining functional status. Van Tassell et al. were the first group to conduct a clinical trial using IL-1b blockade in HF patients. They noted an improvement in the median peak VO2 consumption on cardiopulmonary exercise testing. However, the study lacked a placebo group. [249] Interestingly, in that study, blockade of IL-1b receptor resulted in a reduction in IL-1b levels. It thus appears that deliver of BMC via the IM route might result in the secretion of an IL-1b receptor blocker which in turn results in a reduction in the negative prognostic marker IL-1b. The reduction in IL-1b seen in the IM BMC group is certainly in keeping with the improvement in functional status seen in patients in this group. The exact role of IFN-γ on cardiac function is a matter of controversy. Contradicting research suggests IFN-γ to be both detrimental to cardiac function as well as a protective agent.[37] Our study appears to support the former observation in that the group with the largest improvement in LVEF and functional status showed a reduction in IFN-γ levels in comparison to no decrease in the remainder of the groups. However, until the role

of IFN-γ in heart failure is clearer, it is difficult to extrapolate on the importance of the reduction in IFN-γ seen in the IM BMC group.

In summary, the cytokine sub-study was a hypothesis generating analysis of cytokine levels in response to G-CSF and BMC therapy in ischaemic cardiomyopathy patients. Important signals which warrant larger, appropriately powered studies are seen. Namely, that a reduction in the detrimental, pro-inflammatory cytokines MCP-1, IL-8, IL-1b and IFN-γ levels and an increase in the beneficial, pro-angiogenic cytokine VEGF were seen in the group with the greatest improvement in cardiac function is certainly noteworthy.

Is myocardial scar reduction associated with improvement in LVEF, cytokine levels and cell/G-CSF therapy?

CMR with late gadolinium assessment allows the accurate quantification of myocardial scar. However, most of the cell therapy studies to date have used non-CMR imaging modalities to assess cardiac function. The presence of CMR incompatible devices in advanced heart failure patients as well as the lack of widespread availability of CMR have been key reasons for the decreased use of CMR in cell therapy trials. As a result, change in scar burden in patients receiving BMC therapy and G-CSF has not been very widely characterised. Although MRI was used in this study, the expected low number of patients undergoing CMR, due to implanted devices, was a limitation. As a result, the distribution of CMR scans across the groups was uneven and hence, prevented a detailed analysis of scar burden by group. Furthermore, no analysis of the IM BMC group could be undertaken. Hence, analysis of the relationship between scar and outcomes was carried out on the pooled group of CMR recipients and then further analysed amongst subgroups with reduction in

scar and improvement in LVEF. This was done in an attempt to gain some insight into the mechanisms leading to the cardiac outcomes rather than into the mechanism of action of route, specific cell delivery or G-CSF therapy. Bearing in mind these limitations, a few noteworthy observations were made. In the patients with an improvement in LVEF, a reduction in scar was noted in contrast to an increase in scar size in those with a reduction in LVEF. Survival in HF patients is inversely related to LVEF. In a study by Kwon et al., higher myocardial scar burden was associated with higher mortality in severe ischaemic cardiomyopathy.[250] Thus, the decrease in scar burden seen in the group with an improvement in LVEF would imply a better prognosis in this group. Whether this reduction in scar is mediated by an increase in viable myocardium through myocardial regeneration is still unclear. A few cell therapy trials have analysed myocardial scar burden. In the trial by Ang et al., injection of BMC into scarred myocardium during CABG did not result in a reduction in scar size. [180] However, in the CADUCEUS trial, which used cardiac stem cells, a reduction in scar size was noted.[116] Further to the CADUCEUS study, the same group used a porcine model of convalescent MI and demonstrated, using CMR and histology, that MR quantification of change in scar was accurate and that the reduction in scar and increase in viable myocardium was explained for by cardiomyocytes hyperplasia hence implying activation of endogenous regenerative mechanisms.[251] Although still not a definitive proof of myocardial regeneration in humans, the finding of a reduction in scar burden in those with an improvement in LVEF is certainly intriguing and suggests, albeit indirectly, a myocardial regenerative process. However, direct evidence for myocardial regeneration would require histological studies as well as using radio-labelled cells. The barrier to the former in humans is obvious while radiolabelling cells raises concerns regarding impaired cell survival and proliferation.[252]

Assessment of the relationship between myocardial scar and cytokine levels was the first ever performed in a cell therapy trial to date. Although the number of patients with cytokines analysed who also underwent CMR was small, there were sufficient samples to make a few interesting observations. In the group with scar reduction, the pro-inflammatory cytokine IL-1b decreased and the increase in MCP-1 was much less pronounced compared to the scar increase group. In comparison, an increase in pro-angiogenic cytokine VEGF was seen in the scar reduction group as opposed to a reduction in VEGF in the scar increase group. Along with VEGF, IL-10, an anti-inflammatory cytokine, also increased in the scar reduction group. Although, these findings suggest a mechanistic association between scar reduction and IL-1b, VEGF, MCP-1 and IL-10, the exact relationship between scar and cytokines is unclear and would necessitate a further study specifically designed/powered to address this issue. Furthermore, the above reported associations between scar and LVEF or between scar and cytokines are, as mentioned above, "associations" only. Due to the limited sample size, these associations provide no insight into the role of cytokines in executing the effect of G-CSF and intramyocardial or intracoronary cell therapy. However, they provide important signals which should be the focus of future studies.

Is bone marrow derived cell therapy and G-CSF associated with improved diastolic function?

This thesis did not show any significant effect of either G-CSF alone or in combination with cell therapy on diastolic function. The majority of studies have understandably focused on LV systolic function and very few studies have looked at diastolic function. The findings in this thesis are in contrast to the study by Yao et al. who reported an improvement in LV diastolic indices as measured using echocardiography. In that study, IC delivery of autologous BMC resulted in an improvement in diastolic function but not in systolic function. This reported improvement in

diastolic function was reported at 6 months follow-up however it would have been interesting to see if this improvement was persistent at 12 months. The finding of systolic improvement only and no diastolic improvement in the IM BMC group implies that the functional improvements noted in NYHA class as well as the reduction in NT pro-BNP were attributable to the improvement in systolic function

Are increased peripheral and bone marrow cell counts and improved cell function associated with improved LVEF?

The correlation observed between CFU-GM and LVEF in the IM BMC group implies that the functional capacity of the cells is intricately linked to outcomes in cardiac function. This provides further proof to the concept of the link between functional capacity of the infused cells and outcomes in cell therapy trials as described by Assmus et al. in the TOPCARE-CHD trial.[231] In that trial, a link between CFU-GM and mortality risk was observed. Furthermore, as has been described before[154, 225] and more recently in the FOCUS-HF trial, the functional capacity of BMC is reduced, as measured using CFU-GM, in advanced HF.[146] The above trials provided evidence that the functional capacity of infused BMC, although impaired in advanced HF, are inversely associated with mortality risk. This thesis results add further to the existing literature suggesting that the functional capacity of autologous BMC is not only linked to mortality outcomes but also to outcomes in cardiac systolic function in patients with severe left ventricular dysfunction.

The proliferation and mobilisation of EPC and MNC in peripheral blood in response to G-CSF was shown to be associated with change in LVEF at 1 year. These are important observations which suggest that EPC's in peripheral blood i.e. CEPC's, might be involved in neovascularisation as a mechanistic role in the improvement of LVEF. Furthermore, the link between the proliferation and mobilisation of MNC, but not CD34+ cells into the peripheral blood, with change in LVEF also suggests that the CD34- MSCs might have a role to play in the improvement in cardiac function. The FOCUS-HF trial did measure CFU-F to look at the functional capacity of MSCs. However, the focus in that trial was to analyse for any variation in CFU-F with respect to age and not cardiac function. Future trials to both quantify and qualitatively characterise EPC and MSC and correlate result with cardiac outcome are necessary to identify the "key player" amongst the subsets of cell types present in the adult bone marrow. No trial has yet done a head-to head comparison of CEPC v MSC v HSC v unfractionated BMC in relation to cardiac function.

LIMITATIONS

The study was powered based on within group rather than between group comparisons. However, it is important to remember that this study involved invasive procedures with multiple follow-ups and investigations for a patient population who are limited in functional capacity from their advanced heart failure. Thus, it is very difficult to recruit such patients in vast numbers over a specified period of time. Secondly, the measurement of NT pro-BNP at 6 months but not at the time of the primary outcome endpoint at 1 year was undertaken for logistic reasons and to reflect early remodelling. It would have been ideal to have measured NT pro-BNP at 6 months, 1 year and 2 years. Thirdly, one might question the use of both CT and CMR interchangeably when measuring

LVEF. However, although this could be seen as a potential limitation, two points need to be made. Firstly, the correlation between CT and CMR in measuring LVEF is good.[213] Secondly, as each individual participant underwent a single imaging modality at all time points, thus allowing comparison of measurements between baseline and 1 year or baseline and 2 years, a within group analysis was used.

Three different QoL questionnaires were used, however, if the trial was being conducted again it would be better to use a combination of a general QoL questionnaire and a disease specific QoL, preferably the combination of the SF-36 and Minnesota living with heart failure (MLHF) questionnaires as they have some of the best validity for use in heart failure patients. [253] The latter is a heart failure specific questionnaire. None of the QoL questionnaires used in the study were heart failure specific QoL questionnaires. Furthermore, it would have been advisable to use the Seattle HF model prognosis predictor to identify any suggestions of improved mortality risk in the patient cohort over time. Furthermore, the study was not powered to detect significant changes in QoL scores and hence, interpretation of the QoL changes was difficult.

 The mechanistic sub-studies were again limited by the low sample size, particularly in the CMR scar study, this made it difficult to conduct within arm comparisons of change in scar. However, this limitation is not one unique to this trial and has been a recurring issue with trials on patients with advanced HF who have implanted devices. Other limitations in the mechanistic sub studies include the lack of acquisition of image frames on echocardiogram that would have enabled me to measure diastolic function more comprehensively e.g. interventricular relaxation time, pulmonary venous flow. In the cell sub-study, cell quantification of MSC's and cell characterisation of EPC and

MSC using CFU-EPC and CFU-F would have added additional information on cell characteristics and outcomes.

FUTURE DIRECTIONS

The demand for novel therapies in heart failure is enormous. Recently, new pharmacological therapy has shown promise. The PARADIGM-HF trial assessed the efficacy and safety of a new class of drug called neprilysin inhibitors. Neprilysin is an endopeptidase that cleaves natriuretic peptides and other vasoactive peptides. In the PARADIGM-HF trial, neprilysin inhibitor, in combination with valsartan, reduced the risk of death and hospitalisation for heart failure as compared to enalapril alone. [68] However, newer pharmacological therapies in the field of heart failure have been few and far between.

In the field of cell therapy, several unanswered questions remain. The shift from preclinical to clinical work had been relatively quick. Trial designs have been heterogeneous with a lack of standardised protocols and endpoints. Most studies have looked at global LVEF as a primary end point. It is probable that surrogate end-points as opposed to individual tests will best represent activity that will translate into harder clinical end-points.

The optimal cell type remains to be determined. Eduardo Marban's group compared bone marrow derived MSCs, CDCs, ADSCs and BMMC in animal studies. The results suggest the superiority of CDC's in terms of myogenic and angiogenic potency and improvement in cardiac function.[113]

Cell function and proliferation capacity can vary secondary to patient characteristics and comorbidities. This could imply 'testing' a patient's cells before deciding on patient suitability - hence allogeneic products. It is possible that stem cell therapy will become individualised in the future based on the underlying pathophysiology and choice of best cell type to stimulate the

necessary mechanism e.g. angiogenic cells to improve symptoms of angina vs. cardiopoietic cell types to treat heart failure.

Ultimately cells alone may not be enough to overcome the overwhelming pathology that accompanies myocardial dysfunction. The field of tissue engineering combines regenerative cells with bioactive materials (scaffolds) in combination with growth factors to create a structural complex or "cardiac patch" that helps support cell survival and proliferation. Various synthetic and natural biomaterials have already been used as scaffolds e.g. collagen, fibrin. More recently, scaffold-free cell sheet technology involving stacking of single layers of cell sheets to create three-dimensional tissue. Initial results in animal models are promising. [254] Ultimately tissue engineering may create a total heart based on a bio structure that has been repopulated with a patients' own stem cells thereby avoiding tissue rejection. Currently this theory is being explored using de-cellularised hearts[255].

Other exciting developments include genetic and pharmacologic manipulation of stem cells to improve their therapeutic efficiency. For example, a study overexpressing GSK 3beta (glycogen synthase, a serine/threonine kinase) in MSC's and then transplanting them into murine hearts showed significant improvement in left ventricular function and mortality.[256] Small, non-coding RNA's (miRNA's) appear to regulate gene expression by blocking mRNA translation or inducing degradation of mRNA. Expression of miRNAs influences stem cell differentiation and miRNAs are potential adjuncts in improving the efficiency of cell therapy. Other approaches include combining cell therapy with mechanical support devices e.g. LVAD. Reports have shown significant clinical improvement in patients with ischaemic cardiomyopathy administered stem cells while on LVAD [257] and cell therapy may provide a useful 'bridge' in transplant medicine to allow maintenance of myocardial function whilst a suitable donor is found.

Cell therapy has a big future in heart failure but I don't believe in its current state. My vision of stem failure treatment will involve individualised, "tailor-processed" cells which would be first sub-selected to get the optimal cell type (suited to the pathology being treated), functionally tested, sub-functional cells discarded, the cells genetically modified and/or cultured in appropriate "cardiogenic cocktails" and then injected into the myocardium in the correct dose using electromechanical mapping. Cell therapy in its current state is quite unselected and crude-a "one size fits all" approach. The foreseen advances, however, are a long way away. The findings of this research work highlight important "signals" which need further study in phase III trials.

SUMMARY

The findings of my thesis have shown that G-CSF and intramyocardially delivered autologous bone marrow derived cells significantly improve cardiac function, patients' symptoms and biochemical parameters. This is the first time a randomised placebo-controlled trial has explored and shown a positive effect of G-CSF in combination with autologous BMC. Furthermore, the mechanistic sub-studies have investigated the link between cytokines, cell numbers/function, myocardial scar and diastolic function and G-CSF± autologous BMC. The results have provided important signals including, reduction in pro-inflammatory cytokines with BMC, reduction in scar with improvement in LVEF and a positive relationship between CFU-GM and LVEF. The low sample numbers have prevented reaching statistical significance. However, these encouraging signals should trigger the design of appropriately powered phase II trials to clarify these mechanistic hypothesis.

Furthermore, the positive improvement in LVEF with functional and biochemical parameters along with a consistent safety record warrant the design and conduct of phase III trials to provide more

definitive answers on efficacy. Although the magnitude of the improvement in LVEF might not appear significant in clinical terms, most of the trials of established pharmacotherapy in heart failure have shown similar magnitudes of improvement in LVEF and subsequent mortality benefit. The challenges will be to conduct large scale, phase III trials, bearing in mind the invasive nature of the procedures, the need for highly trained staff able to operate electromechanical mapping systems and the limitations imposed by the morbidity of patients with advance heart failure in attending long term follow-up. Overcoming these limitations, and provided phase III trials show a significant benefit, G-CSF and autologous BMC administration could revolutionise the treatment of advanced ischaemic cardiomyopathy patients.

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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 Your Health and Well-Being

This survey asks for your views about your health. This information will help keep track of how you feel and how well you are able to do your usual activities. *Thank you for completing this survey!*

For each of the following questions, please tick the one box that best describes your answer.

1. **In general, would you say your health is:**

Excellent	Very good	Good	Fair	Poor
▼	▼	▼	▼	▼
□₁	□₂	□₃	□₄	□₅

2. **<u>Compared to one year ago</u>, how would you rate your health in general <u>now</u>?**

Much better now than one year ago	Somewhat better now than one year ago	About the same as one year ago	Somewhat worse now than one year ago	Much worse now than one year ago
▼	▼	▼	▼	▼
□₁	□₂	□₃	□₄	□₅

3. **The following questions are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much?**

	Yes, limited a lot	Yes, limited a little	No, not limited at all
a <u>Vigorous activities</u>, such as running, lifting heavy objects, participating in strenuous sports	☐ 1	☐ 2	☐ 3
b <u>Moderate activities</u>, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf	☐ 1	☐ 2	☐ 3
c Lifting or carrying groceries	☐ 1	☐ 2	☐ 3
d Climbing <u>several</u> flights of stairs	☐ 1	☐ 2	☐ 3
e Climbing <u>one</u> flight of stairs	☐ 1	☐ 2	☐ 3
f Bending, kneeling, or stooping	☐ 1	☐ 2	☐ 3
g Walking <u>more than a mile</u>	☐ 1	☐ 2	☐ 3
h Walking <u>several hundred yards</u>	☐ 1	☐ 2	☐ 3
i Walking <u>one hundred yards</u>	☐ 1	☐ 2	☐ 3
j Bathing or dressing yourself	☐ 1	☐ 2	☐ 3

4. **During the <u>past 4 weeks</u>, how much of the time have you had any of the following problems with your work or other regular daily activities <u>as a result of your physical health</u>?**

	All of the time	Most of the time	Some of the time	A little of the time	None of the time
a Cut down on the <u>amount of time</u> you spent on work or other activities	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
b <u>Accomplished less</u> than you would like	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
c Were limited in the <u>kind</u> of work or other activities	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
d Had <u>difficulty</u> performing the the work or other activities (for example, it took extra effort)	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

5. During the <u>past 4 weeks</u>, how much of the time have you had any of the following problems with your work or other regular daily activities <u>as a result of any emotional problems</u> (such as feeling depressed or anxious)?

	All of the time ▼	Most of the time ▼	Some of the time ▼	A little of the time ▼	None of the time ▼
a Cut down on the <u>amount of time</u> you spent on work or other activities	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
b <u>Accomplished less</u> than you would like	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
c Did work or other activities <u>less carefully than usual</u>	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

6. During the <u>past 4 weeks</u>, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbours, or groups?

Not at all ▼	Slightly ▼	Moderately ▼	Quite a bit ▼	Extremely ▼
☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

7. How much <u>bodily</u> pain have you had during the <u>past 4 weeks</u>?

None ▼	Very mild ▼	Mild ▼	Moderate ▼	Severe ▼	Very severe ▼
☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	☐ 6

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

Not at all	A little bit	Moderately	Quite a bit	Extremely
▼	▼	▼	▼	▼
☐1	☐2	☐3	☐4	☐5

9. These questions are about how you feel and how things have been with you <u>during the past 4 weeks</u>. For each question, please give the one answer that comes closest to the way you have been feeling. How much of the time <u>during the past 4 weeks</u>…

		All of the time	Most of the time	Some of the time	A little of the time	None of the time
		▼	▼	▼	▼	▼
a	Did you feel full of life?	☐1	☐2	☐3	☐4	☐5
b	Have you been very nervous?	☐1	☐2	☐3	☐4	☐5
c	Have you felt so down in the dumps that nothing could cheer you up?	☐1	☐2	☐3	☐4	☐5
d	Have you felt calm and peaceful?	☐1	☐2	☐3	☐4	☐5
e	Did you have a lot of energy?	☐1	☐2	☐3	☐4	☐5
f	Have you felt downhearted and low?	☐1	☐2	☐3	☐4	☐5
g	Did you feel worn out?	☐1	☐2	☐3	☐4	☐5
h	Have you been happy?	☐1	☐2	☐3	☐4	☐5
i	Did you feel tired?	☐1	☐2	☐3	☐4	☐5

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

All of the time	Most of the time	Some of the time	A little of the time	None of the time
▼	▼	▼	▼	▼
☐₁	☐₂	☐₃	☐₄	☐₅

11. How TRUE or FALSE is <u>each</u> of the following statements for you?

	Definitely true	Mostly true	Don't know	Mostly false	Definitely false
	▼	▼	▼	▼	▼
a I seem to get ill more easily than other people	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
b I am as healthy as anybody I know	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
c I expect my health to get worse	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
d My health is excellent	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

Thank you for completing these questions!

EQ - 5D

Health Questionnaire

English version for the UK
(validated for use in Eire)

1

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 about	❑
I have some problems in walking about	❑
I am confined to bed	❑

Self-Care

I have no problems with self-care	❑
I have some problems washing or dressing myself	❑
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	❑
I have some problems with performing my usual activities	❑
I am unable to perform my usual activities	❑

Pain/Discomfort

I have no pain or discomfort	❑
I have moderate pain or discomfort	❑
I have extreme pain or discomfort	❑

Anxiety/Depression

I am not anxious or depressed	❑
I am moderately anxious or depressed	❑
I am extremely anxious or depressed	❑

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**

Best
imaginable
health state

100

9 0

8 0

7 0

6 0

5 0

4 0

3 0

2 0

1 0

0

Worst
imaginable
health state

230

Because all replies are anonymous, it will help us to understand your answers better if we have a little background data from everyone, as covered in the following questions.

1. Have you experienced serious illness?   Yes   No
    - *in you yourself* ❑ ❑
    - *in your family* ❑ ❑   PLEASE TICK APPROPRIATE BOXES
    - *in caring for others* ❑ ❑

2. What is your age in years ? ▭

3. Are you: Male   Female
    ❑ ❑   PLEASE TICK APPROPRIATE BOX

4. Are you:
    - a current smoker ❑
    - an ex-smoker ❑   PLEASE TICK APPROPRIATE BOX
    - a never smoker ❑

5. Do you now, or did you ever, work in   Yes   No
    health or social services? ❑ ❑   PLEASE TICK APPROPRIATE BOX

    If so, in what capacity? ....

6. Which of the following best describes
    your main activity?
    - *in employment or self employment* ❑
    - *retired* ❑
    - *housework* ❑   PLEASE TICK APPROPRIATE BOX
    - *student* ❑
    - *seeking work* ❑
    - *other (please specify)* ❑ ....

7. Did your education continue after   Yes   No
    the minimum school leaving age? ❑ ❑   PLEASE TICK APPROPRIATE BOX

8. Do you have a Degree or equivalent   Yes   No
    professional qualification? ❑ ❑   PLEASE TICK APPROPRIATE BOX

9. If you know your postcode, would you please write it here ▭

231

Publications related to stem cell research work during MDRes study

- **Choudhury T**, Mathur A. The birth of 'regenerative pharmacology': a clinical perspective. *Br J Pharmacol.* 2013 May; 169(2):239-46

- **Choudhury T**, Hamshere S, Mathur A. Recent advances in biological therapies for cardiovascular disease (Book chapter) in *Recent Advances in Cardiology* 16th ed. 2013

- Hamshere S, **Choudhury T**, Jones DA. Et al. A randomised double-blind control study of early intracoronary autologous bone marrow cell infusion in acute myocardial infarction (REGENERATE-AMI). *BMJ Open*. 2014. Feb 18; 4(2):e004258

- Mozid AM, Holstensson M, **Choudhury T**, Ben-Haim S, Allie R, Martin J, Sinusas AJ, Hutton BF, Mathur A. Clinical feasibility study to detect angiogenesis following bone marrow stem cell transplantation in chronic ischaemic heart failure. *Nucl Med Commun*. 2014 Apr 24

- Hamshere S, Arnous S, **Choudhury T**, Choudry F, Mozid A, Yeo C, et al. Randomized trial of combination cytokine and adult autologous bone marrow progenitor cell administration in patients with non-ischaemic dilated cardiomyopathy: the REGENERATE-DCM clinical trial. *European heart journal*. 2015 Sep 2. PubMed PMID: 26333366

- **Choudhury T**, Mozid A, Hamshere S et al. Safety and efficacy of G-CSF and autologous, bone marrow-derived cells in ischaemic cardiomyopathy: Results of the REGENERATE-IHD Phase II trial. (submitted-under review)

Addendum

(Amendments as per viva outcome recommendations)

In section 2.1.2, the first paragraph in the section titled "*Randomisation*" should read:

"Patients were randomised in a sequential manner with the first 30 recruited patients allocated to the IM arm as the aim was to complete this arm first. This was done as treatment in this arm required the presence of expensive equipment for electromechanical mapping (NOGA®) as well the presence of senior clinicians and technical staff trained in the use of such equipment. The next 60 patients were randomised in a 1:1 manner to the other 2 arms (peripheral or IC) (fig 2.1) Randomisation was performed using a dedicated trial software system (HD Clinical, Bishops Stortford, Herts, UK)."

instead of:

"Patients were randomised to 1 of 3 arms: peripheral, intracoronary (IC) or intramyocardial (IM) (figure 2.1). The first 30 patients were allocated to the IM arm as the aim was to complete this arm first. This was done as treatment in this arm required the presence of expensive equipment for electromechanical mapping (NOGA®) as well the presence of senior clinicians and technical staff trained in the use of such equipment.   The remainder of the 60 patients were randomised in a 1:1 manner to the other 2 arms (peripheral or IC), using a dedicated trial software system (HD Clinical, Bishops Stortford, Herts, UK)."

In section 2.1.2, the first paragraph in the section titled *"Patient selection"* should read:

Patients were referred to the trial with a confirmed diagnosis of HF from local HF clinics. Patients underwent screening by the research nurse or physician prior to inclusion in the study to ensure the inclusion criteria were met and that no exclusion criteria were present. Screening for potential trial participants started in February 2005 and last patient recruitment was in April 2012. All potential

trial participants received detailed information regarding the objectives and methodology of the trial and the post-trial follow-up arrangements. A detailed patient information sheet (PIS) was provided to each patient by the research nurse. The potential participant was given ample time to think about the pros and cons of participating in the trial. Following this period, the research team answered any further questions raised by the patient. Once the patient was happy to go ahead, the participant was asked to sign a consent form to indicate informed consent. The participant was informed of his/her right to change their mind at any point in the trial. The participant also consented to the use of his/her data gathered during the trial as well as any remaining bone marrow and stored blood sample for future trial related research.

instead of:

Patients were referred to the trial with a confirmed diagnosis of HF from local HF clinics. Patients underwent screening by the research nurse or physician prior to inclusion in the study to ensure the inclusion criteria were met and that no exclusion criteria were present. All potential trial participants received detailed information regarding the objectives and methodology of the trial and the post-trial follow-up arrangements. A detailed patient information sheet (PIS) was provided to each patient by the research nurse. The potential participant was given ample time to think about the pros and cons of participating in the trial. Following this period, the research team answered any further questions raised by the patient. Once the patient was happy to go ahead, the participant was asked to sign a consent form to indicate informed consent. The participant was informed of his/her right to change their mind at any point in the trial. The participant also consented to the use of his/her data gathered during the trial as well as any remaining bone marrow and stored blood sample for future trial related research.

Table 3.1 should show:

	Saline (n=15)	G-CSF (n=15)	IC serum (n=15)	IC BMC (n=15)	IM serum (n=15)	IM BMC (n=15)
Age, years (mean ± SD)	63.3±9.3	63.1±8.2	62.8±10.7	62.1±9.7	60.4±11.2	65.3±9.4
Sex M/F n	14/1	13/2	14/1	14/1	15/0	15/0
BMI (kg/m^2) (mean±SD)[#]	29.5±4.3	31.4±6.0	31.7±6.5	29.7±4.8	29.6±3.7	30.8±4.0
Medical History, n (%)						
Hypertension	3 (20.0)	1 (6.7)	2 (13.3)	3 (20.0)	5 (33.3)	5 (33.3)
Diabetes	4 (26.7)	5 (33.3)	2 (13.3)	2 (13.3)	4 (26.7)	4 (26.7)
CABG	5 (33.3)	4 (26.7)	3 (20.0)	7 (46.7)	6 (40.0)	4 (26.7)
MI	13 (86.7)	12 (80.0)	14 (93.3)	13(86.7)	13 (86.7)	13 (86.7)
Hypercholesterolaemia	4 (26.7)	6 (40.0)	5 (33.3)	5 (33.3)	8 (53.3)	4 (33.3)
Smoker/ex-smoker,	12 (80.0)	8 (53.3)	13 (86.7)	11(73.3)	14 (93.3)	11 (73.3)
Time from last MI, days median (IQR)	1307 (1064-5443)	2527 (966-4928)	2856 (1278-6041)	1805 (896-3855)	2406 (706-5402)	2684 (706-5402)
LVEF (%) (mean ± SD)	34.7±10.1	27.9±12.4	31.6±7.4	31.7±8.8	29.0±9.2	28.6±10.2
Devices number,n (%)						
CRT-D	4 (26.7)	5 (33.3)	4 (26.7)	4 (26.7)	3 (20.0)	7 (46.7)
CRT-P	1 (6.7)	1 (6.7)	0 (0)	0 (0)	0 (0)	0 (0)
ICD only	7 (46.7)	2 (13.3)	4 (26.7)	5 (33.3)	5 (33.3)	6 (40.0)
Medication history, n (%)						
Statin	12 (80.0)	13 (86.7)	13 (86.7)	13(86.7)	14 (93.3)	13 (86.7)
ACEi/ARB	14 (93.3)	14 (93.3)	13 (86.7)	14(93.3)	15 (100)	15 (100)
B-blocker	15(100)	14 (93.3)	12 (80.0)	15(100)	11 (73.3)	14 (93.3)
Aldosterone antagonist	9 (60.0)	13 (86.7)	9 (60.0)	12(80.0)	9 (60.0)	12 (80.0)
Diuretics	10 (66.7)	11 (73.3)	13 (86.7)	12(80.0)	8 (53.3)	12 (80.0)
NYHA at baseline, n (%)						
II	10 (66.7)	9 (60.0)	5 (33.3)	8 (53.3)	11 (73.3)	8 (53.3)
III/IV	5 (33.3)	6 (40.0)	10 (66.7)	7 (46.7)	4 (26.7)	7 (46.7)

Instead of:

	Saline (n=15)	G-CSF (n=15)	IC serum (n=15)	IC BMC (n=15)	IM serum (n=15)	IM BMC (n=15)	p-value
Age, years (mean ± SD)	63.3±9.3	63.1±8.2	62.8±10.7	62.1±9.7	60.4±11.2	65.3±9.4	0.841
Sex M/F n	14/1	13/2	14/1	14/1	15/0	15/0	0.896
BMI (kg/m^2) (mean±SD)[#]	29.5±4.3	31.4±6.0	31.7±6.5	29.7±4.8	29.6±3.7	30.8±4.0	0.739
Medical History, n (%)							
Hypertension	3 (20.0)	1 (6.7)	2 (13.3)	3 (20.0)	5 (33.3)	5 (33.3)	0.414
Diabetes	4 (26.7)	5 (33.3)	2 (13.3)	2 (13.3)	4 (26.7)	4 (26.7)	0.748
CABG	5 (33.3)	4 (26.7)	3 (20.0)	7 (46.7)	6 (40.0)	4 (26.7)	0.653
MI	13 (86.7)	12 (80.0)	14 (93.3)	13(86.7)	13 (86.7)	13 (86.7)	0.949
Hypercholesterolaemia	4 (26.7)	6 (40.0)	5 (33.3)	5 (33.3)	8 (53.3)	4 (33.3)	0.715
Smoker/ex-smoker,	12 (80.0)	8 (53.3)	13 (86.7)	11(73.3)	14 (93.3)	11 (73.3)	0.150
Time from last MI, days median (IQR)	1307 (1064-5443)	2527 (966-4928)	2856 (1278-6041)	1805 (896-3855)	2406 (706-5402)	2684 (706-5402)	0.964
LVEF (%) (mean ± SD)	34.7±10.1	27.9±12.4	31.6±7.4	31.7±8.8	29.0±9.2	28.6±10.2	0.385
Devices number,n (%)							
CRT-D	4 (26.7)	5 (33.3)	4 (26.7)	4 (26.7)	3 (20.0)	7 (46.7)	0.781
CRT-P	1 (6.7)	1 (6.7)	0 (0)	0 (0)	0 (0)	0 (0)	0.999
ICD only	7 (46.7)	2 (13.3)	4 (26.7)	5 (33.3)	5 (33.3)	6 (40.0)	0.485
Medication history, n (%)							
Statin	12 (80.0)	13 (86.7)	13 (86.7)	13(86.7)	14 (93.3)	13 (86.7)	0.995
ACEi/ARB	14 (93.3)	14 (93.3)	13 (86.7)	14(93.3)	15 (100)	15 (100)	0.896
B-blocker	15(100)	14 (93.3)	12 (80.0)	15(100)	11 (73.3)	14 (93.3)	0.079
Aldosterone antagonist	9 (60.0)	13 (86.7)	9 (60.0)	12(80.0)	9 (60.0)	12 (80.0)	0.351
Diuretics	10 (66.7)	11 (73.3)	13 (86.7)	12(80.0)	8 (53.3)	12 (80.0)	0.363
NYHA at baseline, n (%)							
II	10 (66.7)	9 (60.0)	5 (33.3)	8 (53.3)	11 (73.3)	8 (53.3)	0.376
III/IV	5 (33.3)	6 (40.0)	10 (66.7)	7 (46.7)	4 (26.7)	7 (46.7)	

Table 3.2 should show:

	Saline (n=9)	G-CSF (n=9)	IC serum (n=10)	IC BMC (n=12)	IM serum (n=7)	IM BMC (n=10)
Age, years (mean ± SD)	65.3±12.2	64.0±9.8	65.4±10.3	58.7±8.9	56.8±9.7	61.8±7.5
Sex M/F n	8/1	8/1	9/1	11/1	7/0	10/0
BMI (kg/m^2) (mean±SD)$^{\#}$	30.0±4.2	30.3±5.2	30.0±5.7	30.9±4.7	31.4±3.7	30.8±4.6
Medical History, n (%)						
Hypertension	2 (22.2)	0 (0.0)	1 (10.0)	2 (16.7)	4 (57.1)	2 (20.0)
Diabetes	1 (11.1)	2 (22.2)	1 (10.0)	2 (16.7)	1 (14.3)	1 (10.0)
CABG	2 (22.2)	4 (44.4)	2 (20.0)	6 (50.0)	2 (28.6)	2 (20.0)
MI	8 (88.9)	8 (88.9)	9 (90.0)	11 (91.7)	7 (100.0)	8 (80.0)
Hypercholesterolaemia	2 (22.2)	3 (33.3)	3 (30.0)	4 (33.3)	5 (71.4)	4 (40.0)
Smoker/ex-smoker,	6 (66.7)	6 (66.7)	8 (80.0)	10 (83.3)	6(85.7)	6 (60.0)
Time from last MI, days median (IQR)	3593 (5420)	2788 (4634)	2808 (2288)	1731 (3402)	1894 (5336)	3993 (4957)
LVEF (%) (mean ± SD)	34.0±10.8	27.4±12.9	31.7±7.3	31.3±8.4	28.1±12.3	29.3±11.7
Devices number,n (%)						
CRT-D	4 (26.7)	4 (33.3)	3 (26.7)	3 (26.7)	0 (20.0)	4 (46.7)
CRT-P	1 (6.7)	1 (6.7)	0 (0)	0 (0)	0 (0)	0 (0)
ICD only	4 (46.7)	2 (13.3)	4 (26.7)	5 (33.3)	3 (33.3)	4 (40.0)
Medication history, n (%)						
Statin	7 (80.0)	8 (86.7)	9 (86.7)	12 (86.7)	7 (93.3)	10 (86.7)
ACEi/ARB	8 (93.3)	8 (93.3)	9 (86.7)	12 (93.3)	7 (100)	10 (100)
B-blocker	9 (100.0)	9 (93.3)	7 (80.0)	12 (100.0)	5 (73.3)	10 (93.3)
Aldosterone antagonist	4 (60.0)	7 (86.7)	6 (60.0)	11 (80.0)	4 (60.0)	9 (80.0)
Diuretics	5 (66.7)	5 (73.3)	8 (86.7)	10 (80.0)	4 (53.3)	7 (80.0)
NYHA at baseline, n (%)						
II	9 (66.7)	6 (60.0)	3 (33.3)	6 (53.3)	6 (73.3)	5 (53.3)
III/IV	0 (33.3)	3 (40.0)	7 (66.7)	6 (46.7)	1 (26.7)	5 (46.7)

Instead of:

	Saline (n=9)	G-CSF (n=9)	IC serum (n=10)	IC BMC (n=12)	IM serum (n=7)	IM BMC (n=10)	p-value
Age, years (mean ± SD)	65.3±12.2	64.0±9.8	65.4±10.3	58.7±8.9	56.8±9.7	61.8±7.5	0.626
Sex M/F n	8/1	8/1	9/1	11/1	7/0	10/0	0.945
BMI (kg/m^2) (mean±SD)$^{\#}$	30.0±4.2	30.3±5.2	30.0±5.7	30.9±4.7	31.4±3.7	30.8±4.6	0.990
Medical History, n (%)							
Hypertension	2 (22.2)	0 (0.0)	1 (10.0)	2 (16.7)	4 (57.1)	2 (20.0)	0.122
Diabetes	1 (11.1)	2 (22.2)	1 (10.0)	2 (16.7)	1 (14.3)	1 (10.0)	0.980
CABG	2 (22.2)	4 (44.4)	2 (20.0)	6 (50.0)	2 (28.6)	2 (20.0)	0.574
MI	8 (88.9)	8 (88.9)	9 (90.0)	11 (91.7)	7 (100.0)	8 (80.0)	0.967
Hypercholesterolaemia	2 (22.2)	3 (33.3)	3 (30.0)	4 (33.3)	5 (71.4)	4 (40.0)	0.504
Smoker/ex-smoker,	6 (66.7)	6 (66.7)	8 (80.0)	10 (83.3)	6(85.7)	6 (60.0)	0.871
Time from last MI, days median (IQR)	3593 (5420)	2788 (4634)	2808 (2288)	1731 (3402)	1894 (5336)	3993 (4957)	0.894
LVEF (%) (mean ± SD)	34.0±10.8	27.4±12.9	31.7±7.3	31.3±8.4	28.1±12.3	29.3±11.7	0.783
Devices number,n (%)							
CRT-D	4 (26.7)	4 (33.3)	3 (26.7)	3 (26.7)	0 (20.0)	4 (46.7)	0.368
CRT-P	1 (6.7)	1 (6.7)	0 (0)	0 (0)	0 (0)	0 (0)	0.373
ICD only	4 (46.7)	2 (13.3)	4 (26.7)	5 (33.3)	3 (33.3)	4 (40.0)	0.946
Medication history, n (%)							
Statin	7 (80.0)	8 (86.7)	9 (86.7)	12 (86.7)	7 (93.3)	10 (86.7)	0.328
ACEi/ARB	8 (93.3)	8 (93.3)	9 (86.7)	12 (93.3)	7 (100)	10 (100)	0.659
B-blocker	9 (100.0)	9 (93.3)	7 (80.0)	12 (100.0)	5 (73.3)	10 (93.3)	0.012
Aldosterone antagonist	4 (60.0)	7 (86.7)	6 (60.0)	11 (80.0)	4 (60.0)	9 (80.0)	0.108
Diuretics	5 (66.7)	5 (73.3)	8 (86.7)	10 (80.0)	4 (53.3)	7 (80.0)	0.612
NYHA at baseline, n (%)							
II	9 (66.7)	6 (60.0)	3 (33.3)	6 (53.3)	6 (73.3)	5 (53.3)	0.017
III/IV	0 (33.3)	3 (40.0)	7 (66.7)	6 (46.7)	1 (26.7)	5 (46.7)	

The last paragraph in the *"Limitations"* section in Ch 6 should read:

The cytokines sub-study was limited by the small sample size and statistical issues with multiple comparisons. Each of the 11 cytokines were compared between 6 different groups as well as for within group change. Despite using Bonferroni correction and with small sample size, the inherent probability of chance findings with multiple comparisons is unavoidable and a significant limitation of the cytokine sub-study. The mechanistic sub-studies were again limited by the low sample size, particularly in the CMR scar study, this made it difficult to conduct within arm comparisons of change in scar. However, this limitation is not one unique to this trial and has been a recurring issue with trials on patients with advanced HF who have implanted devices. Other limitations in the mechanistic sub studies include the lack of acquisition of image frames on echocardiogram that would have enabled me to measure diastolic function more comprehensively e.g. interventricular relaxation time, pulmonary venous flow. In the cell sub-study, cell quantification of MSC's and cell characterisation of EPC and MSC using CFU-EPC and CFU-F would have added additional information on cell characteristics and outcomes.

Instead of:

The mechanistic sub-studies were again limited by the low sample size, particularly in the CMR scar study, this made it difficult to conduct within arm comparisons of change in scar. However, this limitation is not one unique to this trial and has been a recurring issue with trials on patients with advanced HF who have implanted devices. Other limitations in the mechanistic sub studies include the lack of acquisition of image frames on echocardiogram that would have enabled me to measure diastolic function more comprehensively e.g. interventricular relaxation time, pulmonary venous flow. In the cell sub-study, cell quantification of MSC's and cell characterisation of EPC and MSC using CFU-EPC and CFU-F would have added additional information on cell characteristics and outcomes.

A new section between 3.2.2 and 3.2.3 has been added which reads:

The figure below (fig A) shows the inter-observer variability for CT analysis of LVEF, the primary

endpoint, between myself and the second operator. Where a difference of >5% was observed

between the two readers, the scan was sent to a level III, consultant imaging cardiologist specialising

in CT to re-analyse the scan.

Bias -1.95 SD of bias 4.1
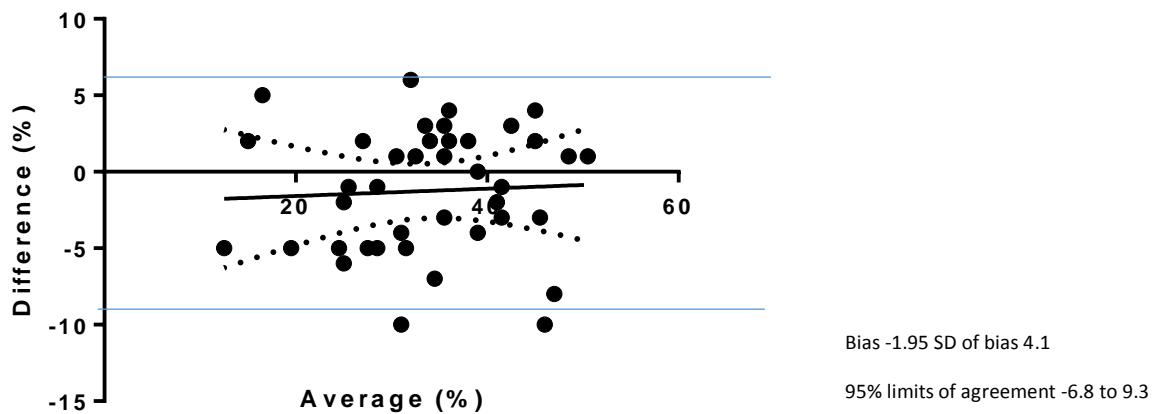
95% limits of agreement -6.8 to 9.3

Figure A. Bland-Altman plot showing inter-observer variability. The Bland-Altman plot shows the

inter-observer variability for measurement of the primary endpoint of left ventricular ejection

fraction as measured using computed tomography. The blue lines indicate the 95% limits of

agreement.

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