The effects of cytokine and progenitor cell therapy on clinical and biochemical status in patients with non-ischaemic dilated cardiomyopathy

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I, Samer Arnous, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Background A small number of open-label or pilot studies have assessed the benefit of stem cell therapy in dilated cardiomyopathy (DCM). I report the findings of the REGENERATE-DCM trial, a double-blind, randomised, placebo-controlled trial of bone-marrow derived mononuclear cell and adjunctive granulocyte colony stimulating factor (G-CSF) administration in patients with DCM.

Methods 60 patients with DCM were randomized into four treatment groups: intracoronary stem cell, intracoronary serum, peripheral G-CSF and peripheral placebo (saline). Apart from the placebo group, all patients received 5-days of G-CSF, with bone marrow harvest performed on Day 6 in the intracoronary group. Primary endpoint was change in left ventricular ejection fraction (LVEF) assessed by advanced cardiac imaging at 3 months.

Results There was little or no difference in baseline characteristics between the groups. At 3 months, intracoronary stem cell therapy was associated with a 5.37% increase in LVEF (38.30 ±12.97 from 32.93 ±16.46 p= 0.014). This increase in LVEF in cell treated patients was associated with clear evidence of decrease in NYHA classification and improved exercise capacity. No evidence of a change in LVEF was seen in the other two treatment groups.

Conclusion The novel combination of G-CSF and intracoronary cell therapy led to an improvement in cardiac function and symptoms at 3 months.

Contents

Abbreviations

DCM	Dilated cardiomyopathy
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
AMI	Acute myocardial infarction
IHD	Ischaemic heart disease
MI	Myocardial infarction
CHF	Congestive heart failure
SCF	Stem cell factor
BMC	Bone marrow mononuclear cells
MSC	Mesenchymal stem cells
HSC	Haematopoietic stem cells
EPC	Endothelial progenitor cells
CFR	Coronary flow reserve
LVEF	Left ventricular ejection fraction
LVSD	Left ventricular systolic dysfunction
SCF	Stem cell factor
ISR	In-stent restenosis
IVUS	Intravascular ultrasound
CAD	Coronary artery disease
NYHA	New York Heart Association
CCS	Canadian Cardiovascular Society

BNP	Brain-Natriuretic peptide
RCT	Randomised controlled trial
PCI	Percutaneous coronary intervention
CRP	C - reactive protein
IL-1	Interleukin-1
IL- 1Ra	Interleukin-1 receptor antagonist
IL-6	Interleukin-6
IL-10	Interleukin-10
MCP	Monocyte chemoattractant protein-1
TNF-alpha	Tumour necrosis factor alpha
LVNC	Left ventricular non-compaction

1. INTRODUCTION:

1.1. Dilated Cardiomyopathy

1.1.1. Incidence

Dilated cardiomyopathy (DCM) is a leading cause of heart failure and remains the most common indication for cardiac transplantation worldwide[1,2]. Dilated cardiomyopathy is defined as the presence of depressed fractional shortening (<25%) or reduced LVEF (< 45%), and a dilated LV (end-diastolic diameter of > 117% of the corrected predicted value)[3], in the absence of coronary artery disease or abnormal loading conditions (such as hypertension or valve disease) sufficient to cause global systolic impairment[4]. Right ventricular dysfunction and dilation may be present but are not necessary for the diagnosis. The estimated incidence of DCM in North American and European populations is estimated at 6-8 per 100,000[5,6] and the prevalence at 36.5 per 100,000[5]. It remains a leading cause of heart failure under the age of 35 and the most common indication for cardiac transplantation worldwide[1,2]. The reported frequencies probably represent an underestimate, however, since most studies focus on index cases presenting with clinical heart failure. A primary diagnosis of heart failure represents 1% of all inpatient cases in men and 0.4% in women. The average length of stay for a patient admitted to hospital with heart failure is over 13 days, three times the average length of stay for all patients. In the last ten years, the number of hospital admissions for heart failure in England has increased by around 5% in men and 4% in women aged 45 years and over. In England, hospital admissions for heart failure are projected to increase by over 50% over the next 25 years, from 74,500 in 2000/01 to 113,000 in 2026/27.

Previously, the majority of patients in any DCM cohort presented with symptoms of high pulmonary venous pressure and a low cardiac output. Increasingly, DCM is diagnosed as an incidental finding in asymptomatic individuals during routine examination or family screening[7-9]. Heart failure occurs as a consequence of continuous neurohormonal stimulation, which results in arteriolar vasoconstriction, sodium and water retention, endothelial dysfunction and fibrosis. In index cases, the diagnosis of DCM is made in the presence of depressed fractional shortening (<25%) or reduced LVEF (< 45%), and a dilated LV (end-diastolic diameter of > 117% of the corrected predicted value)[3].

1.1.2. Prognosis

The prognosis of DCM is highly variable. Earlier studies reported 5-year mortality rates of 50%, which have declined to 20% in more recent reports. This improvement in survival reflects both early disease detection and advances in heart failure therapy. However the prognosis and quality of life in symptomatic heart failure patients remains worse than many malignancies and serious chronic conditions such as arthritis and chronic lung disease[10].

Prognostic markers of increased mortality in DCM include the degree of LV dysfunction, higher NYHA functional class and high Brain-natriuretic peptide (BNP) levels. BNP is a powerful predictor of all cause mortality in heart failure[11] and may also predict the response to therapy[12]. Patients with DCM may have impaired coronary flow reserve (CFR) and microvascular obstruction[13] despite normal epicardial coronary arteries. Impaired CFR is also associated with an increase in mortality and progression of heart failure[14].

1.1.3. Pathogenesis

A- Genetic

Over 40 disease causing genes have been identified to date[15,16] most of which encode proteins of the sarcolemma[17-19], cytoskeleton[20], sarcomere[21-24], nuclear envelope (e.g. Lamin[25-27]) and energy generation[28] (Table 1). The structural and functional consequences of mutations in cytoskeleton, sarcomere, sarcolemma and nuclear envelope include impairment of myocardial force generation, force transmission and cell survival (figure 1). DCM is inherited as an autosomal dominant trait in 90% of families. This mode of transmission is often associated with incomplete and age-related penetrance, although onset by the fourth decade of life is typical[9]. Expression is also variable and frequently incomplete; although symptomatic disease may not be present, cardiac evaluation may reveal unexplained ECG and/or echocardiographic abnormalities. Autosomal dominant forms of DCM may be associated with conduction disease[29] or skeletal myopathy[30,31].

Other modes of inheritance include autosomal recessive, X-linked recessive, and mitochondrial. In autosomal recessive DCM, patients usually present at a younger age compared with the autosomal dominant form. The disease course is characterised by more rapid progression to death or cardiac transplantation[9]. X-linked inheritance is characterised by the absence of male to male transmission[4,9]. Women may be affected but usually express a milder form of disease expression and are affected at an older age. Affected patients usually, but not always, have an increase in CK (MM) isoform level, e.g. mutations in dystrophin which also cause Duchenne and Beckers muscular

dystrophy. Matrilineal inheritance is usually associated with signs of mitochondrial-related phenotype such as lactacidaemia, hypoacusia, palpebral ptosis, myopathy with ragged red fibres, ophtalmoplegia, encephalopathy, or retinitis pigmentosa. In this form of inheritance the mother, son or daughter may be affected, but the affected males do not transmit the disease to their offspring.

Incomplete phenotypic expression is common among relatives of subjects with DCM, contributing to under-recognition of familial disease. Nevertheless, nearly a third of asymptomatic relatives of patients with DCM have echocardiographic abnormalities on screening (such as depressed fractional shortening and left ventricular enlargement), and over a quarter of these patients develop overt DCM[7]. Furthermore, cardiac specific autoantibodies were present in over 30% of asymptomatic relatives of patients with DCM[32,33], and are weak independent predictors of developing DCM at 5 year follow-up[33,34]. In a longitudinal study of families with DCM, 23% of 767 asymptomatic relatives were found to have echocardiographic evidence of depressed fractional shortening or LV dilation, and these patients were 8 times more likely to develop overt DCM than those with normal echocardiograms[35]. Additional studies in the asymptomatic relatives revealed that the 25% of relatives with LV enlargement without systolic dysfunction had histological findings consistent with DCM[36] and had a significant reduction in exercise capacity[7,37]. This suggests that LVE represents subclinical disease, with incomplete penetrance. In spite of low penetrance, incomplete and variable expression, and small nuclear families commonly evaluated in clinical practice, familial disease can be confirmed in up to 50% of DCM cases.

Figure 1: The structure of the cardiac myocyte, showing the sarcomere, the cytoskeletal network, calcium channels, nuclear proteins, lysosomes, mitochondria, and AMP-activated protein kinase (AMPK). Mutations in the genes encoding most of the protein components of these structures lead to cardiomyopathies (reproduced from Arnous et al Genetics of Dilated Cardiomyopathy: Risk of Conduction Defects and Sudden Cardiac Death; cardiac electropyshiology,2010 [15]).

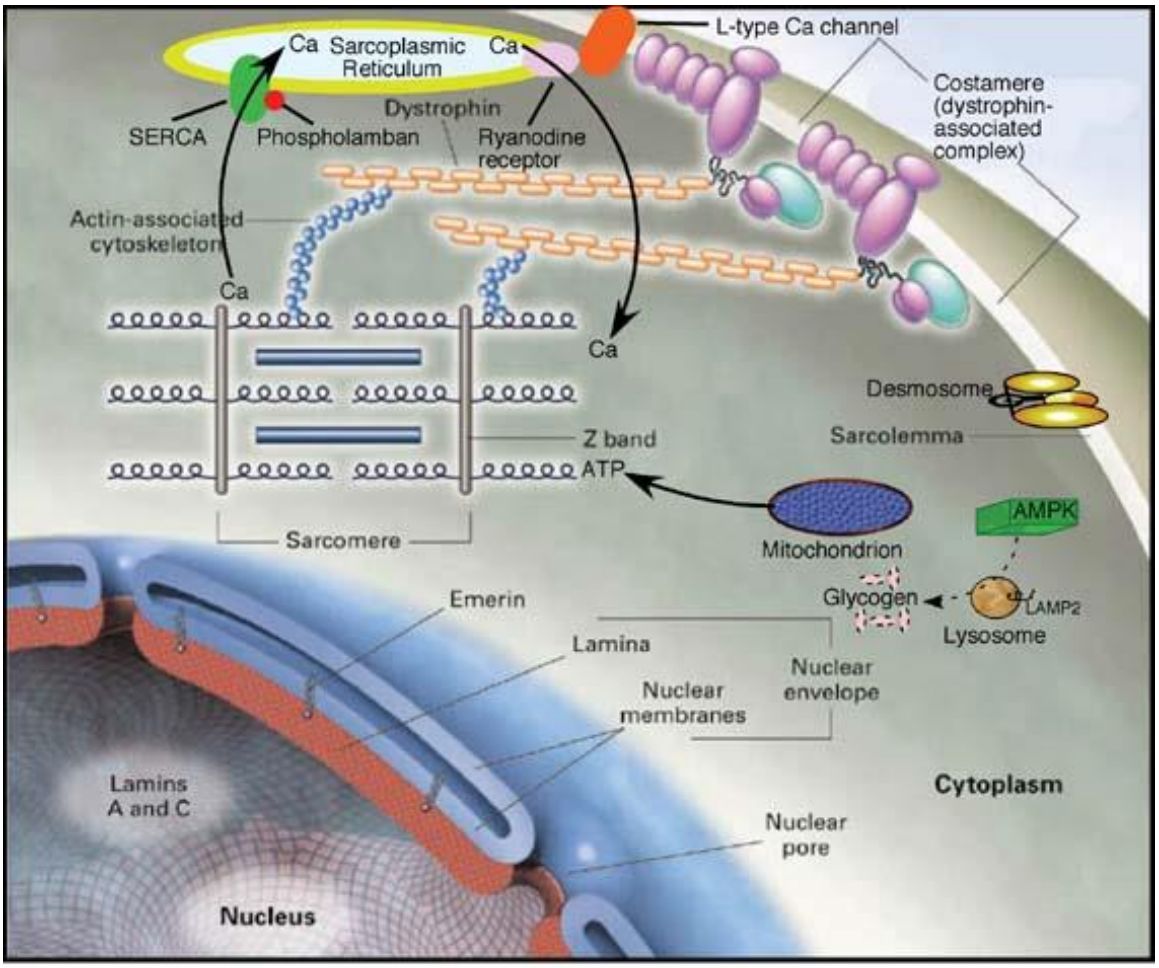


Table 1: Gene mutations associated with dilated cardiomyopathy

Protein	Gene	Locus	Mode of Transmission	Associated phenotype	References
Sarcomeric					
Actin	ACTC	15q14	AD	None	[21]
α-tropomyosin	TPM1	15q22	AD	None	[21]
β-myosin heavy chain	MYH7	14q12	AD	None	[22,38-40]
α-myosin heavy chain	MYH6	14q12	AD	None	[41]
Troponin T	TNNT2	1q32	AD	None	[22,24,42]
Troponin C	TNNC1	3p21-p14	AD	None	[24]
Troponin I	TNNI3	19q13	AD, AR	None	[43,44]
Myosin-binding-protein-C	MYBPC3	11p11	AD	None	[45]
Sarcomere and Z-disc related					
Titin	TTN	2q31	AD	None	[46]
Telethonin	TCAP	17q12	AD	None	[47,48]
Muscle LIM protein	CSRP3	11p15	AD	None	[47]
Metavinculin	VCL	10q22-q23	AD	None	[49]
Cypher/ZASP	LDB3	10q22-q23	AD	CD, LVNC	[50]
α-Actinin 2	ACTN2	1q42-q43	AD	None	[51]

Table 1: Gene mutations associated with dilated cardiomyopathy (continued)

Protein	Gene	Locus	Mode of Transmission	Associated phenotype	References
PDZ and LIM domain Protein 3	PDLIM3	4q35	AD	None	[52]
Myopallidin	MYPN	10q21	AD	None	[53]
Four-and-a-half LIM protein 2	FHL2	2q12-q14	AD	None	[54]
Nexilin	NEXN	1p31	AD	None	[55]
Cytoskeletal					
Desmin	DES	2q35	AD	CD, SM	[20,56]
Dystrophin	DMD	Xp21	XLR	SM	[19,57]
δ-sarcoglycan	SGCD	5q33	AD	SD	[17]
αß-Crystallin	CRYAB	11q22-q23	AD	None	[58]
Nuclear					
Lamin A/C	LMNA	1q21	AD	CD, SD, SM	[25,26,31,59-64]
Thymopoeitin	TMPO	12q22	AD#	None	[65]
Emerin	STA/EMD	Xq28	XLR	Emery-Dreifuss muscular dystrophy	[66]

Table 1: Gene mutations associated with dilated cardiomyopathy (continued)

Protein	Gene	Locus	Mode of Transmission	Associated phenotype	References
Ion channel and ion channel related					
Sulphonylurea receptor-2 (SUR2)	ABCC9	12p12.1	AD	None	[67]
Desmosomal					
Desmoplakin	DSP	6p24	AR	Carvajal syndrome	[68]
Other					
Phospholamban	PLN	6q22	AD	None	[69-71]
Tafazzin	TAZ/G4.5	Xq28	XLR	Neutropenia, Short stature	[72]
M2 muscarinic receptor	CHRM2	7q35	AD#	CD, SD	[73]
Eya4	EYA4	6q23	AD#	SHL	[74]
LAMP2	LAMP2	Xq24	AD#	CD, SM	[75]
Laminin -α4	LAMA4	6q21	AD#	None	[76]
Integrin-linked kinase	ILK	11p15	AD#	None	[76]
Presenilin 1	PSEN1	14q24	AD#	None	[77]
Presenilin 2	PSEN2	1q31-q42	AD#	None	[77]
Cardiac ankyrin repeat protein (CARP)	ANKRD1	10q23	AD	None	[78]
RNA binding motif protein 20	RBM20	10q25	AD	None	[79]

B- Acquired

A list of acquired causes of dilated cardiomyopathy and putative mechanisms of action is summarised in table 2.

1- **Viral cardiomyopathy** – viral infection can cause myocarditis and cardiomyopathy via a direct cytotoxic effect on the myocardium, or an adverse autoimmune response due to persistent viraemia. Causative agents include Coxackie B, influenza virus and human immunodeficiency virus (HIV).

2- **Alcohol** – excessive alcohol consumption can lead to cardiomyopathy in susceptible individuals. Although the exact proposed mechanism is unknown, alcohol is thought to cause myocardial dysfunction via oxygen free radicals and defects in cardiac protein synthesis. Abstinence can often lead to dramatic improvement in cardiac function.

3- **Peripartum cardiomyopathy** – This is a rare cause of cardiomyopathy that occurs in late pregnancy and within the first 5 months of the postpartum period, and is strongly associated with gestational hypertension and twin pregnancy[4]. Prognosis depends on the degree of LV dysfunction at the time of diagnosis. Even if patients recover, there is a risk of recurrence in subsequent pregnancies so patients are advised against further pregnancies.

4- **Medications –** The most common medications implicated in cardiomyopathy include anthracycline, doxorubicin, adramyacin and cyclophosphamide. Other drugs associated with DCM include antiretroviral agents (Lamivudine), antipsychotics (clozapine), and antidepressants (amitriptyline and clomipramine)[80].

4-5- Left ventricular non-compaction

Left ventricular non-compaction (LVNC) is a disorder of the myocardium defined by the presence of prominent trabeculations on the luminal surface of the left ventricle and the presence of deep inter-trabecular recesses that extend into the ventricular wall[81]. Diagnosis of LVNC can be difficult as fine trabeculations are a feature of the normal ventricle. However recent advances in imaging modalities such contrast echocardiography and cardiac magnetic resonance imaging (MRI) have lead to an improvement in visualising the trabeculations. Patients with LVNC often have an associated dilated cardiomyopathy and present with symptoms of systolic heart failure. Management of the heart failure is no different to that in DCM.

Table 2: Acquired causes of DCM and putative mechanism of disease

AETIOLOGIES OF DCM	PUTATIVE MECHANISM OF DISEASE
VIRAL INFECTION (COXSACKIE B, INFLUENZA, HIV)	CHRONIC INFLAMMATION, VIRAL PROTEIN PERSISTENCE
PREGNANCY	MYOCARDITIS, AUTOIMMUNE
METABOLIC DISORDERS (HAEMOCHROMATOSIS)	IRON DEPOSITION, MYOCYTE DYSFUNCTION.
EOSINOPHILIC (CHURG STRAUSS SYNDROME)	EOSINOPHILIC ENDOMYOCARDITIS, INTERSTITIAL FIBROSIS
KAWASAKI DISEASE	CORONARY ANEURYSM AND RECURRENT THROMBO-EMBOLI
ALCOHOL	DIRECT TOXIC EFFECT ON THE MYOCARDIUM
ENDOCRINE (HYPOPHOSPHETAEMIA, HYPOCALCAEMIA, THIAMINE)	IMPAIRMENT OF MYOCARDIAL CONTRACTILITY
DRUGS (DOXORUBICIN, ADRIAMYCIN, CYCLOPHOSPHAMIDE)	FREE RADICALS AND INCREASED OXIDATIVE STRESS
• Tachycardia induced cardiomyopathy	Left ventricular remodelling, dilation and electrical disturbances

1.1.4. Current Treatment guidelines

Dilated cardiomyopathy represents the final common morphologic outcome of various biologic insults resulting in myocardial necrosis and chronic fibrosis. The proposed mechanisms involved in the pathogenesis of DCM include genetic predisposition, viral infection, myocardial ischemia and antibody-mediated cytotoxicity and apoptosis of cardiomyocytes. Currently patients with DCM are treated in accordance with the European Society of Cardiology (ESC) guidelines for the management of heart failure, which are backed by clinical trials[82] (Table 3). There remains, however, a significant knowledge gap. The optimal approach to forestalling progression in relatives with early disease is unresolved. Furthermore, there is an unmet need to individualise standard therapies, which do not factor in the influence of underlying aetiology on treatment responsiveness. Recent studies suggest that this might result in sub-optimal or inappropriate therapy in some patients[83-85]. Furthermore, current conventional therapies in DCM do not correct the underlying defects in cardiac muscle. The only therapeutic option that currently addresses fibrosis and cardiomyocyte loss is heart transplantation. Heart transplantation has a significant impact on mortality with a survival rate following a heart transplant of 83% at 1 year and 72% at 5 years[86]. However, due to stringent selection criteria and chronic shortage of donor hearts, the vast majority of patients are deemed unsuitable or never receive a transplant. Left ventricular assist device (LVAD) has been shown to improve the short-term survival as a bridge to transplantation or in

patients who are not suitable for cardiac transplant[87,88]. However there are many limitations to LVAD including the risk of stroke, device infection and device failure[87,88]. Therefore, preventing this progression of myocardial dysfunction is a major challenge requiring novel therapeutic strategies, such as stem cell transplantation, to improve the prognosis and quality of life for these patients.

Table 3: Pharmacological therapy in systolic heart failure

Pharmacological agent	**Effect**
Angiotensin Converting Enzyme Inhibitors (ACEIs)	Improves mortality, symptoms, exercise tolerance, quality of life, and exercise performance[89].
ß-Blockers	Improves ventricular function and patient well-being, reduces hospital admission, and increases survival[90].
Aldosterone Antagonists	Reduce hospital admission and improve survival[91].
Digoxin	No effect on survival. Improves ventricular function and patient well-being, reduces hospital admission[92].
Diuretics	Improve symptoms and relief pulmonary and venous congestion[93].
LCZ696 angiotensin II receptor blocker and neprilysin	More effective in reducing the risk of death from cardiovascular causes or hospitalization for heart failure than was ACE inhibition with enalapril[94]

2. CELL THERAPY

The human hearts own self-renewal capabilities are unable to overcome the massive loss of cardiomyocytes, up to one billion cells, seen in acute myocardial infarction and heart failure. Regenerative medicine is an emerging interdisciplinary field of research, which hopes to use the properties of embryonic stem cells and adult stem cells in clinical applications focusing on the repair and regeneration of cells, tissues, or organs. One of the many remaining unanswered questions is which type of stem/progenitor cell is the best candidate for cardiac regeneration. Stem cells are defined by two unique characteristics: they are unspecialized cells capable of unlimited self-renewal, and they can differentiate into more specialized cells and organs.

Stem cells can be broadly divided into those that are derived from the embryo (embryonic stem cells - ESCs) and those that are found in adult tissue (adult stem cells). ESCs are pluripotent, which means having the ability to develop into cells from all three germinal layers (i.e., mesoderm, endoderm and ectoderm) and produce all the tissue types needed to form a functional organism. Developed adult organs also contain undifferentiated stem cells but in far fewer numbers: these are called adult (somatic) stem cells (ASC). These cells were thought to be limited in differentiation potential and only able to replenish and repair injured tissue in the organ in which they resided. Stem cells can be further divided according to the number of differentiated cell types they can produce[95].

1- ***Totipotent*****:** Cells able to form all fully differentiated cells of the body and trophoblastic cells of the placenta. The only cells considered to be totipotent are the embryo, zygote and the descendants of the first two cell divisions.
2- ***Pluripotent:*** Cells able to differentiate into almost all cells that arise from the three germ layers. They are unable to give rise to placental cells. An example of pluripotent cells is embryonic cells that form the inner cell mass of the blastocyst. These cells are formed at around 5 days following fertilisation.
3- ***Multipotent:*** For example adult stem cells are capable of forming a limited range of differentiated cell lineages.

2.1. Allogeneic stem cells

2.1.1. Embryonic stem cells

Embryonic stem cells (ES), the most primitive of stem cells, are derived from the inner cell mass. ES are pluripotent, meaning that they are able to transform into any type of cell including cardiomyocytes and can replicate indefinitely[95]. However ethical issues, their potential to form teratomas and the need for immunosuppressive therapy have hindered their use in clinical trials.

2.1.2. Foetal cardiomyocytes

Foetal cardiomyocytes were one of the first cell types to be investigated as potential candidates for cardiac repair. However, the use of fetal cardiomyocytes has similar concerns to ESCs, including availability, immunogenicity, and ethics.

2.1.3. Human umbilical cord derived stem cells

Umbilical cord blood is blood which remains in the placenta and in the attached umbilical cord after childbirth. It has been shown to be a potent source of hematopoietic stem cells. Cord blood contains a large number of non-hematopoietic stem cells which rarely express HLA class II antigens and appear to be immunologically naive, thus reducing the risk of rejection. In animal models of acute myocardial infarction, injection of human umbilical cord blood mononuclear cells is associated with significant reductions in infarct size.

2.2. Autologous cell types

A number of adult cell types have been shown to induce cardiac repair or functional improvement. These include bone marrow derived mononuclear cells (BMCs) that consist of haematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and endothelial progenitor cells (EPCs). Other adult derived progenitor cells that have been extensively investigated for regenerative capacity include skeletal myoblasts, cardiac resident stem cells, umbilical cord blood stem cells and inducible pluripotent stem cells.

2.2.1. Adult stem cells

Adult stem cells are present in already developed tissue. They are an attractive option to explore for transplantation as they are autologous, but their differentiation potential is more restricted than embryonic stem cells. They are multipotent, which means they can only differentiate into a limited range of cell types. They can transdifferentiate into specialised cells that are outside their lineage commitment[96]. Adult stem cells are divided into the following depending upon their origin:

2.2.2. Bone marrow-mononuclear cells

Currently, the major sources of adult cells used for basic research and in clinical trials originate from the bone marrow. The bone marrow mononuclear subset is heterogeneous and comprises mesenchymal stem cells, haematopoietic progenitor cells and endothelial progenitor cells. The differentiation capacity of different populations of bone marrow-derived stem cells into cardiomyocytes has been studied intensively.

2.2.3. Haematopoietic stem cells

Haematopoietic stem cells (HSC) are defined as cells that transdifferentiate along the blood lineage. They do not have the ability to transdifferentiate into cardiomyocytes in vitro. However, in a mouse model, treatment with Granulocyte colony stimulating factor (G-CSF) and stem cell factor (SCF) after a myocardial infarction enhanced the mobilisation and homing of HSC from the bone marrow. This led to cardiomyogenesis and vasculogenesis and an improvement in survival and cardiac function[97]. In contrast, in non-human primate models, infusion of G-CSF and SCF post MI was not associated

with cardiomyogenesis or improvement in LV function but did promote angiogenesis[98]. The first human trial was conducted on patients with severe CAD not amenable to coronary artery bypass. The authors demonstrated an improvement in collateral flow with short -term administration of Granulocyte-macrophage colony stimulating factor (GM-CSF). More studies are needed to assess the beneficial effect of HSC mobilisation on cardiac regeneration.

2.2.4. Mesenchymal stem cells

Mesenchymal stem cells (MSC) are ubiquitous in various tissues including bone marrow, skin, adipose tissue and muscle. They have the potential to differentiate into cells and tissue of mesenchymal origin including muscle, bone, cartilage and adipose tissue. Unlike haematopoietic progenitor cells, MSC are negative for CD34 and CD45 and they stain positively for cardiac, endothelial specific markers and gap junction proteins. MSC can transdifferentiate into cardiomyocytes and endothelial cells in vivo when transplanted into the heart in MI models[99], and in the setting of dilated cardiomyopathy their transplantation in animal models has been shown to improve cardiac function[100]. Their regenerative potential and ability to facilitate myocardial and vascular repair make them an attractive option for regenerative cell therapy, however further research is needed to provide a better understanding into their basic physiology and therapeutic potential.

2.2.5. Endothelial progenitor cells

Endothelial progenitor cell (EPCs) are derived from the bone marrow or the peripheral circulation (CEPCs) and play an important role in vasculogenesis in myocardial

infarction and limb ischemia. EPCs and CEPCs differ phenotypically and functionally from mature endothelial cells by expressing the marker AC133[101,102]. AC133 is expressed on cells with pluripotent stem cell properties. In animal models of MI, transplantation of EPCs or CEPCs causes a significant increase in capillary density, regional blood flow and cardiac function in the ischaemic heart[103,104]. This improvement in cardiac function has been translated in small non-randomised controlled human trials. Transplantation of EPC or CEPCs in the setting of acute MI or ischaemic cardiomyopathy has led to an increase in cardiac function (improvement in EF by 7-9%) and improvement in myocardial perfusion[105-110].

2.2.6. Skeletal myoblasts

Skeletal myoblasts (also called satellite cells) form a reservoir of regenerative cells located at the basal lamina of skeletal muscle tissue. These cells were one of the first to be used for regenerative cell therapy in cardiac disease. They have a high potential for self renewal after muscle injury. Their high resistance to ischemia and ability to survive in an environment with poor vascularisation has made skeletal myoblasts an attractive option for cardiac repair. One of the major characteristics of skeletal myoblasts is that they are committed to the myogenic lineage and therefore when engrafted into infarcted myocardium they do not electrically couple with cardiomyocytes or express cardiomyocytes specific markers. On the one hand this lineage restriction provides a reassuring safe guard against tumour formation; however it also prevents the myogenic cells to differentiate into cardiomyocytes. Since myoblasts do not readily transdifferentiate into a new generation of cardiac cells, it is likely that they exert their beneficial effect on the LV through their contractile properties and paracrine effects.

Preclinical animal studies have demonstrated that, after transplantation into infarcted myocardium, skeletal myoblasts form myotubules and enhance cardiac function[111]. A preliminary clinical trial demonstrated that engraftment of myoblasts into the heart is feasible and is associated with an improvement in LV function[112]. However the increase in the incidence of ventricular tachycardia and life-threatening arrhythmias in early studies halted their use in clinical trials[113]. This observation made the prophylactic use of implantable cardioverter-defibrillator mandatory before conducting further clinical trials using skeletal myoblasts. However in the largest randomised controlled trial to date (the MAGIC trial)[114], an internal cardioverter defibrillator was implanted in every patient in the treatment and placebo group. This did not reveal a significant difference in the incidence of arrhythmias between the two groups. However there was no significant improvement in LV function between the 2 groups. Skeletal myoblasts cannot as yet be used as an established cell therapy due to their inability to transdifferentiate into cardiomyocytes, and lack of evidence on the beneficial effect on LV function.

2.2.7. Cardiac stem cells (cardiospheres)

Until recently the accepted paradigm was that the human heart is a post mitotic and terminally differentiated organ and the only adaptive response to cellular injury is myocardial hypertrophy. A study examining hearts post myocardial infarction indicated that a subpopulation of myocytes was not terminally differentiated and the number of dividing myocytes was significantly larger in the peri-infarct zone than in the distant myocardium[115]. The presence of Lin^- $C\text{-}Kit^+$ cells with myogenic stem cell properties

within the rat myocardium suggests that the heart contains stem cells with regenerative capacity[116]. These cells gave rise to myocytes, smooth muscle and endothelial vascular cells when injected into an ischemic heart. The injected cells regenerated 50% of the contractile myocytes normally present in the myocardium in a matter of few weeks. This has raised the exciting possibility that these cells can be coached to home on the infarcted or fibrosed myocardium in vivo and promote functional recovery without the need for introducing exogenous cells. Several clinical trials are underway examining the safety and efficacy of autologous cardiac stem cells and their effect on cardiac repair and clinical outcome.

2.2.8. Inducible-pluripotent stem cells (iPS)

Cells that more closely resemble embryonic stem cells in their regenerative potential without the ethical issues would ultimately provide an important future direction. A cell type that comes close, and is on the horizon of being tested for potential clinical application, is the inducible pluripotent stem cells (iPS). iPS can be reprogrammed back to an undifferentiated pluripotent state by inserting four genes, Oct3/4, Sox2, KL4 and c-Myc, into differentiated somatic cells similar to ES in morphology, proliferation and teratomas formation[117-119]. The biggest obstacles preventing iPS cells from hitherto being used in clinical trials are the difficulty to produce sufficient cells for clinical use and their ability to form cancer cells. Tumour formation potential is due to the fact that iPS cell lines are derived by inserting putative oncogenes using integrating retroviruses into the host genome. A study suggested that up to 20% of the iPS cell offspring developed tumours[120]. These issues need to be overcome before iPS cells can be used in clinical trials. Until then, it is important to establish whether the simple unfractionated bone

marrow cell approach has a clinical benefit, given the large number of studies that have been performed using this cell type without providing a clear answer.

2.2.9. Thymosin Beta4

Thymosin Beta4 is a G-actin monomer binding protein (a peptide) that plays an important role in protection, regeneration and remodelling of injured tissues. It has the ability to minimize myocardial loss after a MI[121]. The epicardium is a protective layer of connective tissue that covers the heart and delivers signals that can induce the growth of foetal cardiac muscle and coronary vessels that migrate into the myocardium. Treatment with Thymosin Beta4 has been shown to induce adult embryonic epicardial cells to form vascular precursors for angiogenesis and vasculogenesis[122,123]. A recent study showed that pre-treatment with Thymosin Beta4 in mice for 7 days prior to MI activated the resident epicardial stem cells[124]. This resulted in a significant improvement in ejection fraction (EF) at 28 days post infarction (mean EF 23% in placebo arm vs. 41% in treatment arm). There was also a reduction in end systolic and diastolic diameters and a reduction in infarct size. Although this has generated a lot of excitement, methods of optimising the efficiency of Thymosin Beta4 will need to be established before this resident progenitor cell based therapy becomes a realistic option in humans.

2.3. Bone marrow cell (BMC) therapy in clinical trials

2.3.1. BMC therapy in acute MI

A landmark animal study by Orlic et al demonstrated that intramyocardial injection of bone marrow derived stem cells (BMC) after acute MI in murine models led to

cardiomyogenesis and neoangiogenesis resulting in a significant recovery of the myocardium[125]. Since then numerous animal studies using stem cell transplantation in acute and chronic myocardial ischemia demonstrated improvements in cardiac function and reduction in infarct size[126]. This led to various clinical trials assessing the effect of BMC on cardiac function post myocardial infarction (table 4).

A comprehensive systematic review which included 13 trials with a total of 811 patients, showed an improvement in LVEF by 2.99% in the BMC group compared to standard reperfusion therapy[127]. A previous met-analysis by Lipinski et al which included 10 trials with AMI, showed that intracoronary stem cell therapy (within the first 14 days after infarction) was associated with a small but significant (3.0%) improvement in LV systolic function compared to standard medical therapy[128]. It was also associated with a non-significant reduction in death and rehospitalisation from heart failure. Although they found no significant association between the benefits of intracoronary cell injection and the number of injected cells, there was a trend toward a statistically significant association with the injected volume, suggesting the possible presence of a dose-response relationship. The improvement in LVEF was observed in both bone marrow and peripheral mononuclear cells. Similar conclusions were reached in the meta-analysis by Abdel-Latif et al, which included 18 studies and showed that stem cell therapy significantly increased LVEF by 3.66%[129].

In contrast to animal models, the improvement in LV function in most clinical trials is at best modest. However these end points were used as surrogate markers looking for any evidence of efficacy

It should also be noted that several of our established therapies that have an impact on prognosis in patients with MI and a reduced LV function, such as angiotensin-converting enzyme (ACE) inhibitors, β-blockers[130], thrombolytic therapy and percutaneous coronary intervention[131,132], are associated with similar improvements in LVEF. It is likely that adult stem cells exert their benefit on cardiac remodelling through an 'indirect' paracrine effect, and that the small functional benefit seen with this therapy may translate into significant long term improvement in exercise tolerance and survival[133].

The main surrogate markers used as an end-point have been ejection fraction and perfusion defects, which correlate poorly with prognosis and quality of life[134,135]. Therefore, in the future, the validation of progenitor cell therapy for clinical use may depend on the demonstration of a benefit with regard to clinical outcomes such as improvement in prognosis, quality of life[136], NYHA functional class and exercise capacity.

2.3.2. BMC therapy in DCM

Intramyocardial injection of BMC in animal studies was associated with enhanced LV remodelling and an improvement in survival[137], as well as induction of angiogenesis and inhibition of myocardial fibrosis[100].

Promising results from these animal studies in DCM led to the publication of small pilot studies showing that stem cell transplantation led to an improvement in cardiac function, symptoms and ventricular remodelling after intramyocardial and intracoronary

injection[138-141] (Table 5). A pilot study of intracoronary stem cell implantation in patients with DCM showed a significant improvement in ejection fraction by 5.4% and an improvement in NYHA functional class in the treatment group at 6 month follow-up[142]. A case report of intracoronary injection of autologous bone marrow progenitor cells resulted in a reduction of NYHA class from IV to I, an improvement of EF from 20% to 45% and a significant reduction in plasma BNP levels at 6 month follow-up[143]. In the TOPCARE-DCM trial, intracoronary administration of BMC was associated with regional and global improvement in LVEF at 3 month follow-up, associated with an improvement in microvascular function[144]. More recently, a pilot randomised trial showed that intracoronary delivery of autologous CD34+ cells improved left ventricular function and exercise tolerance and potentially improved survival[145]. Although the results of these studies are encouraging, it is difficult to draw any conclusive evidence regarding the efficacy of stem cell therapy as most of these trials were not randomised and did not include a control group. Therefore, there is clearly a need for large randomised trials to address the efficacy of stem cell therapy in heart failure secondary to DCM, and to provide a better understanding of the various unanswered questions with this novel therapy.

Table 4 Clinical trials of BMC therapy in acute myocardial infarction

Study reference	Study design	N	Timing post infarction	Adjunct procedure	Outcome
Meluzin et al[146] (2006)	RCT	66	5-9 days	PCI	Dose dependent Improvement of regional myocardial function by PET
Fernandez-Aviles et al[108] (2004)	Pilot	20	13.5±5.5 days	PCI	Decrease in end systolic volume, improvement in regional and global LV function
Janssens et al[147] (2006)	RCT	69	1 day	PCI	No effect on global LVEF, but may favourably affect infarct remodelling
Lunde et al[148] (2006)	RCT	97	6 days	PCI	No effect on global LVEF
Schächinger et al[149] (2006)	RCT	204	3-7 days	PCI	Improvement in global LVEF

Table 4 Clinical trials of BMC therapy in acute myocardial infarction (continued)

Study reference	Study design	N	Timing post infarction	Adjunct procedure	Outcome
Ge J et al[150] (2006)	RCT	20	Immediately	PCI	Improvement in LVEF
De Lezo JS et al[151] (2007)	RCT	30	7 days	IV fibrinolytics +PCI	Improvement in LVEF
Wollert et al[110] (2004)	RCT	60	4.8 days	PCI	Improvement in LV systolic function
Lipiec et al[152] (2009)	Pilot	39	4-11 days	PCI	Improvement in myocardial perfusion with no effect on global LVEF
Huikuri et al[153] (2008)	RCT	80	2-6 days	IV thrombolysis + PCI	Improvement in global LVEF
Assmus et al[105] (2002)	Pilot	59	3-7 days	PCI	Improvement in LVEF
Schächinger et al[109] (2004)	Pilot	59	3-7 days	PCI	Improvement in EF

~~Study reference~~	~~Study design~~	~~n~~	~~Timing post infarction~~	~~Adjunct procedure~~	~~Outcome~~

Table 4 Clinical trials of BMC therapy in acute myocardial infarction (continued)

Study reference	Study design	N	Timing post infarction	Adjunct procedure	Outcome
Strauer et a[154]l (2002)	Pilot	20	5-9 days	PCI	Reduction in infarct region
Bartunek et al[155] (2005)	Pilot	35	11.6 days	PCI	Improvement in LVEF
Hirsch et al[156] (2010)	RCT	200	3-8 days	PCI	No effect on global or regional LV function
Kuethe et al[157] (2004)	Pilot	5	6 days	PCI	No improvement in LV function
Penicka et al[158] (2007)	Pilot	27	9 days	PCI	No improvement in LV function

RCT; randomised controlled trial, PCI; percutaneous coronary intervention, LV; left ventricular, LVEF; left ventricular ejection fraction.

Study	Patient number	Type of Study	Route of Delivery	Outcome	Ref
Arguero et al (2006)	5	Pilot	I.M via thoracotomy	Improvement in LV function and NYHA	[139]
Ghodsizad et al (2006)	1	Case report	I.M minimally invasive trans epimyocardial	Improvement in LV function	[138]
Seth et al (2006)	24	Pilot	I.C	Improvement in LV function and NYHA	[142]
Fischer-Rasokat et al (2009)	33	Pilot	I.C	Improvement in LV function and microvascular function	[144][146]
Vrtovec et al (2011)	55	Pilot	I.C	Improvement in LV function and exercise tolerance	[145]

Table 5 Summary of pilot studies of BMC therapy in dilated cardiomyopathy

3. CYTOKINE THERAPY

Endogenous G-CSF is a potent haematopoietic cytokine that affects progenitor cell proliferation, maturation and functional activation[159] and enhances the mobilisation and recruitment of stem cells from the bone marrow to the circulation[160]. Stem cells could be mobilised from the bone marrow in mouse models of myocardial infarction using a combination of the cytokines stem cell factor (SCF) and granulocyte colony stimulating factor (G-CSF)[97].Treatment with these cytokines for 5 days prior to coronary artery ligation and for 3 days afterwards resulted in reduction in mortality, infarct size, and an improvement in left ventricular function.

3.1. Stem Cell mobilisation with G-CSF in humans

G-CSF produced by recombinant DNA technology has historically been used to treat chemotherapy-induced myelosuppression. Recently the cytokine has been used for treatment of post-transplantation neutropenia, chronic severe congenital or acquired neutropenia, and mobilization of haematopoietic progenitor stem cells into the circulation for collection by leukapheresis. Determination of the concentration of cells expressing the CD34 cell surface protein in the peripheral blood by flow cytometry is accepted as an accurate marker of stem cell mobilization. With a standard G-CSF dose of 10 μg/kg/day given subcutaneously for 5 days, at least a 10-fold increase in circulating CD34+ cells, a population that contains long-term repopulating stem cells, can be achieved. The majority of CD34+ cells are lineage-committed haematopoietic precursor cells, but stem cells with multi-lineage engraftment abilities are mobilised concurrently. In this regard, multiple studies have documented a close relationship

between the quantity of CD34+ cells administered to patients, whose own marrow has been ablated, and the rapidity and quality of haematopoietic reconstitution[161]. It seems possible that measurement of the quantity of CD34+ mobilised with G-CSF administration can serve as a surrogate for effects of this cytokine on primitive cells that are capable of localising to ischaemic regions of the heart and regenerating myocardial and vascular cell lines. Administration of exogenous G-CSF and SCF mobilizes pluripotent Lin^- c-*kit*$^+$ cells from the bone marrow to the peripheral blood[162]. This increase in mobilisation into the circulation may create a favourable microenvironment for stem cell homing to the myocardium, enhance survival and induce angiogenesis[163]. Pre-treatment with G-CSF has also been shown to have a direct anti-apoptotic effect on cardiomyocytes[164] (Table 2). G-CSF has a direct protective effect on the myocardium including reducing fibrosis and adverse cardiac remodelling, inhibiting apoptosis and inflammation and enhancing cardiac function and myocyte regeneration. There is data to support the safety and efficacy of exogenous G-CSF as a treatment strategy for heart failure[165,166], and there are no reported long-term side effects following short courses of G-CSF in DCM. Self limiting side effects of G-CSF include bone pain, transient elevation in cardiac and liver enzymes[165], and transient impairment of renal function[166]. Caution should therefore be exercised when G-CSF is given to patients with pre-existing renal impairment.

3.1.1. G-CSF therapy in AMI

Orlic and co-workers[97] were able to demonstrate that primitive cells could be mobilised from the bone marrow in a mouse model of myocardial infarction using a combination of the cytokine SCF and G-CSF. Treatment with these cytokines for 5 days prior to

coronary artery ligation and for 3 days afterwards resulted in reduction in mortality, infarct size, magnitude of cavity dilatation and diastolic stress at 27 days post-infarct compared with saline-injected animals. Left ventricular ejection fraction, as measured by echocardiography, was greater in cytokine-treated animals compared with controls. Although phase 1 clinical trials after myocardial infarction showed that G-CSF treatment was safe and associated with possible beneficial effects on left ventricular systolic function[167,168], a subsequent randomised placebo controlled study of subcutaneous G-CSF after primary PCI for AMI infarction showed no additional improvements in left ventricular function when compared to placebo[169]. This was confirmed in a meta-analysis on the effect of G-CSF in AMI which showed no overall benefit although subset analysis showed there may be benefit limited to patients with LV systolic dysfunction and if the infusion is started early[170]. There has also been concerns raised regarding the potential of G-CSF to cause in-stent restenosis (ISR) as it the increases the level of circulating neutrophils which may accelerate the process of neointimal proliferation[171]. One of the initial clinical trials reported an unexpectedly high rate of ISR in 10 patients treated with G-CSF following an AMI[172]. However, a more recent trial involving patients with large infarcts and late revascularisation did not show any increased incidence of ISR[173]. Furthermore, Jorgensen et al demonstrated by using intravascular ultrasound (IVUS), 5 months after stent insertion for AMI, no increased in-stent neointimal hyperplasia in the G-CSF treatment group compared to placebo[174]. It remains to be determined if G-CSF treatment could be an effective part of a treatment strategy combining several cytokines and/or local stem cell delivery.

3.1.2. G-CSF therapy in IHD

G-CSF treatment in patients with ischaemic cardiomyopathy (ICM) and DCM given as four 10-day treatment periods interrupted by treatment-free intervals of equal length, resulted in an improvement by one New York Heart Association (NYHA) class and a statistically significant increase in six-minute walking distance compared to historical controls[166]. In this study, patients with ICM experienced an increase in episodes of dyspnoea or angina and one of the subjects had an episode of fatal ventricular fibrillation, although this may have been due to the presence of underlying severe coronary artery disease. Another study showed that administering 5μg/kg/day for 5 days to patients with ICM and advance heart failure (EF<35% and NYHA III-IV) resulted in a significant improvement in LVEF[165]. Interestingly G-CSF significantly increased the plasma levels of the anti-inflammatory cytokine IL-10 without an effect on pro-inflammatory cytokines levels. Other studies have demonstrated that G-CSF inhibits pro-inflammatory cytokines like interleukin 1, tumour necrosis factor-α and interferon-γ[164]. This positive effect on the anti/pro inflammatory cytokine ratio may confer a benefit in patients with heart failure. In a more recent study, 10 μg/kg/day of G-CSF was administered for 5 days to severely symptomatic patients with ICM not amenable to revascularisation (CCS and NYHA ≥ III). This resulted in a significant reduction in CCS and NYHA from IV to III and III to I at 4 month follow-up[175].

These trials demonstrate that G-CSF therapy in patients with heart failure due to ICM is safe and feasible and maybe associated with an improvement in the clinical and biochemical status.

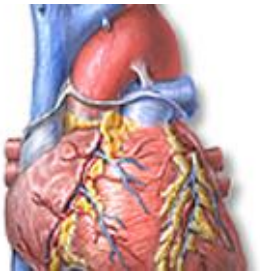
3.1.3. G-CSF therapy in DCM

Endogenous G-CSF is a potent hematopoietic cytokine that affects progenitor cell proliferation, maturation and functional activation[159,176], and enhances the mobilization and recruitment of stem cells from the bone marrow to the circulation[160,177]. Administration of exogenous G-CSF and stem cell factor mobilizes pluripotent *Lin*-c-*kit*+ cells from the bone marrow to the peripheral blood[162,178]. This increase in mobilization into the circulation may create a favorable microenvironment for stem cell homing to the myocardium, enhance survival and induce angiogenesis[163,178]. Pretreatment with G-CSF has also been demonstrated to have a direct anti-apoptotic effect on cardiomyocytes. There are data to support the safety and efficacy of exogenous G-CSF as a treatment strategy for heart failure[145,166], and there are no reported long-term side effects following short courses of G-CSF in DCM. Self-limiting side effects of G-CSF include bone pain, transient elevation in cardiac and liver enzymes, and transient impairment of renal function[166,179]

In animal studies, stem cell and cytokine therapy in DCM led to an improvement in cardiac function[137,180]. In the non-ischemic heart failure model, significant increases in the survival rate and cardiac function were achieved by cell therapy and cytokine therapy inhibited the fibrosis and apoptosis of the cardiomyocytes[137]. G-CSF receptors are expressed on cardiomyocytes in idiopathic dilated cardiomyopathy[181]. In doxorubicin- induced cardiomyopathy, early administration of G-CSF enhanced the migration of bone marrow cells into the heart, and attenuated the cardiotoxicity of doxorubicin[180]. G-CSF has an 'indirect effect' on bone marrow to mobilize cells to

migrate into diseased heart and a 'direct' effect on cardiomyocytes through G-CSF receptors resulting in increase in homing of stem cells into the myocardium and an improvement in cardiac function (Figure 2). The improvement in LV function in DCM animal models was not translated in the small pilot study using G-CSF in heart failure patients[165]. It remains to be determined if G-CSF treatment could be an effective part of a treatment strategy combining several cytokines and/or local stem cell delivery.

Figure 2: Mechanism of action of G-CSF in DCM



Cardio protective Effect

- Anti-apoptotic effect
- Reduction of Fibrosis
- Myocyte generation
- Direct anti-inflammatory effect
- Angiogenesis
- Improve LV function
- Reduce LV remodelling

Enhance Progenitor cell

- Proliferation
- Maturation
- Activation
- Mobilisation

4. CLINICAL TRIAL

4.1.1. Rationale

A number of preclinical trials and pilot studies have demonstrated the safety and efficacy of stem cell therapy in DCM (summarised below). However, there are no randomised control trials in this field. We therefore aimed to conduct the first randomised control trial to assess the role of intracoronary injection of bone marrow derived stem cells compared with placebo in patients with DCM pre-treated with G-CSF, and to evaluate the effect of G-CSF administration on cardiac function and symptoms in patients with idiopathic dilated cardiomyopathy.

4.1.2. Summary of previous trials:

Intramyocardial injection of mesenchymal stem cells (MSCs) in a mouse model was associated with enhanced LV remodelling and an improvement in survival[137], as well as induction of angiogenesis and inhibition of myocardial fibrosis[100]. In a swine model of heart failure with normal coronary arteries, transplantation of autologous MSCs over expressing VEGF prevented LV decompensation, induced neovascularisation and improved myocardial blood flow[182]. Promising results from these animal studies in DCM led to the publication of small pilot studies creating further excitement by showing that stem cell transplantation led to an improvement in cardiac function, symptoms and ventricular remodelling after intramyocardial and intracoronary injection (table 3). A pilot study of intracoronary stem cell implantation in patients with DCM showed a significant improvement in ejection fraction of 5.4% and an improvement in NYHA functional class

in the treatment group at a 6-month follow-up[142]. A case report of intracoronary injection of autologous bone marrow progenitor cells resulted in a reduction of NYHA class from IV to I, an improvement of ejection fraction from 20 to 45% and a significant reduction in plasma BNP levels at 6-month follow-up[143]. In the Transplantation Of Progenitor Cells And Recovery of Left Ventricular Function in Patients with non ischemic Dilated Cardiomyopathy (TOPCARE-DCM) trial, intracoronary administration of BMC was associated with regional and global improvement in LV ejection fraction at 3 months follow-up associated with an improvement in microvascular function[144]. More recently, a pilot randomized trial demonstrated that intracoronary delivery of autologous CD34**+** cells improved LV function and exercise tolerance, and potentially improved survival[145]. Although the results of these studies are encouraging, it is difficult to draw any conclusive evidence regarding the efficacy of cytokine and stem cell therapy as most of these trials were not randomized and did not include a control group.

4.1.3. Study background and hypothesis

The Bone Marrow Derived Adult Stem Cells for Dilated Cardiomyopathy (REGENERATE-DCM) trial is to my knowledge the first randomized, placebo controlled trial worldwide to investigate the role of granulocyte-colony stimulating factor (G-CSF) and autologous bone marrow derived progenitor cells to improve cardiac function in patients with non ischemic DCM. The main objectives of the study were:

To conduct an appropriately powered, randomized, placebo-controlled trial to evaluate the role of intracoronary injection of BMSCs compared with placebo infusion in patients with DCM, pre-treated with G-CSF;

Evaluate the effect of G-CSF administration on cardiac function and symptoms in patients with idiopathic DCM;

Assess whether direct coronary injection of autologous bone marrow-derived stem cells will confer an additional improvement in cardiac function and symptoms above that derived from G-CSF injection alone.

4.1.4. Study hypothesis:

1- Administration of G-CSF to patients with idiopathic dilated cardiomyopathy will lead to an increase in circulating progenitor cells as measured by peripheral CD34+ positive cell counts.

2- Cardiac function and symptoms will improve in patients in whom the peripheral CD34+ counts increase in response to G-CSF administration.

3- Direct coronary injection of autologous bone marrow derived stem cells will confer an additional improvement in cardiac function and symptoms above that derived from G-CSF injection alone.

This multicenter study was conducted at the London Chest hospital (Barts and the London NHS Trust, UK), The Heart hospital (University College London Hospitals NHS Trust, UK) and the Royal Brompton hospital (Royal Brompton and Harefield NHS Trust, UK). Patients were recruited from hospitals and heart failure clinics throughout the UK. The patient population of interest are symptomatic patients (NYHA

II–III) with a confirmed diagnosis of DCM, who are on optimal heart failure treatment and have no other treatment options.

4.1.5. Methods and study design

The study was powered to the primary end point of a within group change in LVEF at 3 months as measured by advanced cardiac imaging. The sample size was calculated to detect an improvement in LVEF of 3.5%, as demonstrated by a contemporary meta-analysis of previous cell therapy Phase I/II trials[183].

Analysis was based on the intention-to-treat principle. Baseline demographic and clinical variables were summarised for each group of the study. Continuous variables are presented as means ± SD and categorical variables are presented as percentages. 95% confidence intervals (CI) are given. P-values are two-sided with a value of < 0.05 considered to indicate statistical significance. Statistical analyses were performed using SPSS version 19 (IBM Corp. Armonk, NY, USA) and graphs produced using Graphpad Prism version 5.0 (GraphPad Software, San Diego, CA).

Prior to recruitment, a full clinical history and examination was made to confirm that the patient met the inclusion criteria and had none of the exclusion criteria. A recent coronary angiogram was required on all patients confirming that there was no significant coronary disease to account for LV dysfunction.

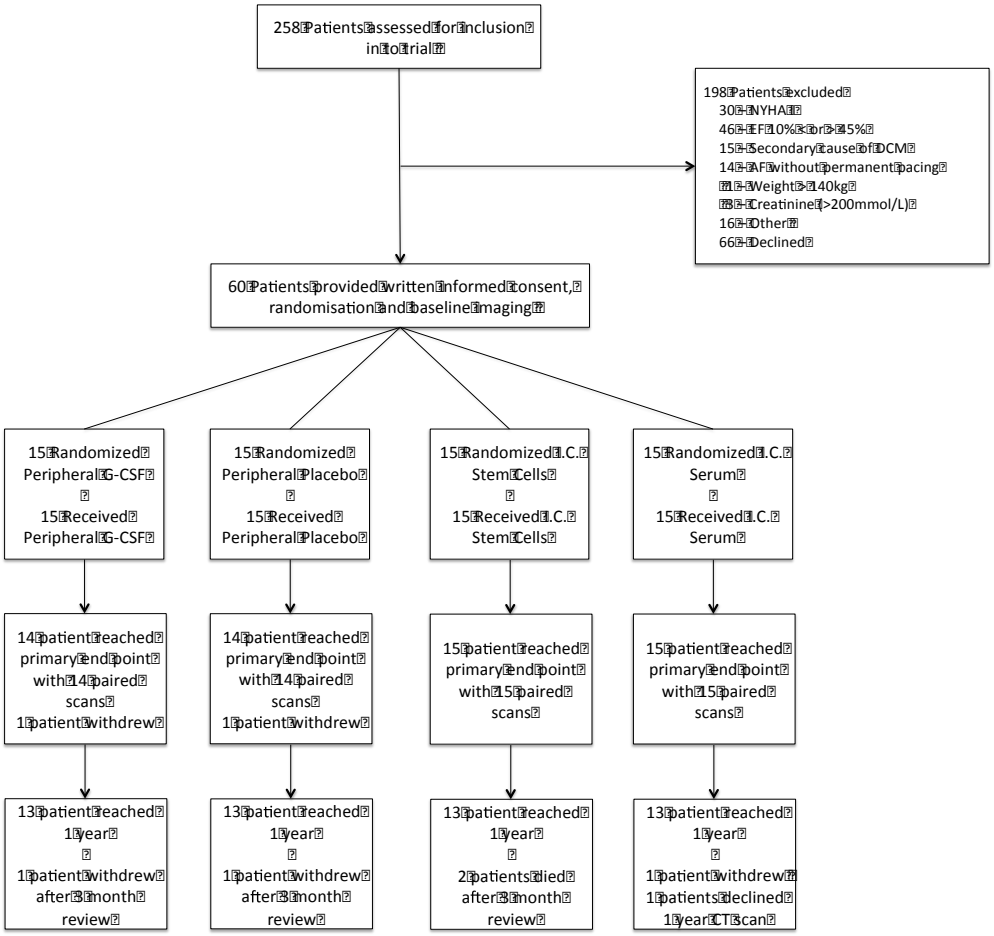
A 12 lead electrocardiogram was recorded at first assessment. Blood samples were taken for measurement of full blood count, renal function, creatinine kinase (CK), Troponin T, C-reactive protein, liver and thyroid function tests, HbA1c, glucose and total cholesterol. Blood samples are also taken for HIV, Hep B & C, HTLV and syphilis.

The study was conducted in full accordance with the principles of the Declaration of Helsinki and was approved by the Local Research Ethics Committee. Informed consent was obtained from all patients prior to inclusion in the study. A total of 60 patients were randomly assigned to one of the 2 arms of the trial: (Figure 1)

I) Peripheral mobilization with G-CSF or placebo injections (peripheral arm)

II) Intracoronary delivery of bone marrow-derived stem/progenitor cells or serum (placebo) following G-CSF injections (intervention arm). (See diagram of study design)

Figure 3: Study design of the REGENERATE-DCM trial



Randomisation took place on a web based database created for the study. A baseline exercise test with VO2 max and a cardiac MR/CT was performed on all subjects at baseline. In addition, overall quality of life is assessed using EQ-5D quality of life questionnaire whilst quality of life related to heart disease was specifically measured using the Kansas City Cardiomyopathy Questionnaire. Half of the subjects in the non-intervention arm were randomised to placebo injection and half to receive a standard G-CSF dose of 10 μg/kg/day (263 microgram in 1ml) given subcutaneously for 5 days. All patients randomised to the interventional arm of the study had 100 ml of bone marrow aspirated following 5 days of G-CSF subcutaneous injection. The dose of G-CSF we used was 10 μg/kg/day (263 microgram in 1ml). This dose is similar to doses used for stem cell mobilisation in advanced chronic heart failure, and was shown to be sufficient to mobilise progenitor cells into the peripheral blood in adequate numbers[166]. The bone marrow samples are processed and analysed for cellular composition and function in a fully compliant Good Manufacturing Practice (GMP) laboratory. The cell suspension or placebo is infused using a stop-flow technique through an over-the-wire percutaneous balloon catheter method. The placebo solution consists of patient's own serum.

Bone Marrow Harvest and isolation of BMMNC

Following written consent, bone marrow samples were obtained from patients from the posterior superior iliac spine using a 15-gauge bone marrow aspiration needle. A total of 5 ml of bone marrow was aspirated into a 10 ml syringe containing 1 ml heparin sodium.

A total of 100 ml of bone marrow was aspirated equally into 20 × 10 ml heparin-treated syringes. The bone marrow aspirates was then kept at room temperature whilst awaiting transfer to the stem cell laboratory. The Ficoll-Paque density gradient media was used for cell isolation as follows: Bone marrow aspirates from the heparin-treated syringes were pooled into a single transfusion bag. The entire volume (100 ml) was passed through a blood component transfusion set with a 200µm filter. A total of 25 ml of this volume was then layered on 50 ml of Ficoll-Paque (density gradient: 1.077 ± 0.001 g/ml) and the remaining 50 ml of bone marrow layered on 25 ml of Lymphoprep. Both preparations were centrifuged in a Sorvall RT Legend centrifuge at 2500 rpm for 30 min with the brake off. The BMMNC layer from each preparation was extracted and spun again at 2500 rpm for 10 min with the brake off. The resulting cell pellets were re-suspended and underwent two cycles of washing in 0.9% saline. The final cell pellets were re-suspended in 2 ml autologous serum. Serum was obtained from 6 × 7 ml plain red-top vacutainers of peripheral blood, which had been allowed to clot and centrifuged in a Sorvall RT Legend centrifuge at 2500 rpm for 5 min to yield autologous serum. Each cell suspension was passed through a 100µm filter which was rinsed through with 0.5 ml autologous serum.

4.1.6. Patient population

The patient population of interest are symptomatic patients (NYHA II-III) with a confirmed diagnosis of dilated cardiomyopathy who are on optimal heart failure treatment including device therapy (biventricular pacemaker & internal cardio defibrillator) , and have no other treatment options. Exclusion criteria include: Documented latest ejection fraction >45% on echocardiogram, the presence of

cardiogenic shock or acute decompensated heart failure, severe left ventricular dysfunction (ejection fraction <10%), congenital cardiac disease, cardiomyopathy in association with a neuromuscular disorder, previous cardiac surgery, serious known concomitant disease with a life expectancy of less than one year, the presence of atrial fibrillation unless paced, renal impairment (Creatinine >200mmol/L), neoplastic disease without documented remission within the past 5 years (see table below).

Table 6: REGENERATE-DCM: Inclusion/Exclusion criteria for the trial

Inclusion criteria	Exclusion criteria
• Symptomatic patients (NYHA II-III) with a confirmed diagnosis of DCM with an ejection fraction <45% who are on optimal medical therapy for at least 6 months with no further treatment options	• Left ventricular ejection fraction < 10% • Cardiogenic shock • Congenital cardiac disease • Cardiomyopathy due to a reversible cause • Cardiomyopathy in association with a neuromuscular disorder (eg: Duchenne's muscular dystrophy) • Previous cardiac surgery • Contra-indication to bone marrow aspiration • Known active infection • Chronic active inflammatory disease • Serious known concomitant disease with a life expectancy of < 1 year • Atrial fibrillation (unless paced) • Renal impairment (Creatinine >200 mmol/L)

	• Neoplastic disease without documented remission within the past 5 years

4.1.7. Safety endpoints and outcome measurements

Follow-up assessments were performed at 3 and 12 months. All primary end point measurements were performed in a core laboratory by investigators who remained blinded to treatment assignment. Importantly, the safety end point of major adverse cardiac events was closely monitored by an independent safety committee. The primary end point is change in LV ejection fraction as measured by cardiac MR or CT at 3 months. The secondary end points are as follows:

- Change in Concentrations of NT-proBNP (cardiac enzyme),
- Changes in $V0_2$ max (exercise capacity)
- Changes in ejection fraction, ventricular dimensions as measured by cardiac MRI/ CT at 12 months.
- Functional class changes according to NYHA and quality of life (QoL - EQ-5D & Kansas City) questionnaires,
- Occurrence of a Major Adverse Cardiac Event (MACE) defined as cardiac death, myocardial infarction (CK / CK-MB over 2 times the upper limit of normal) &
- Hospitalisation for Heart failure & the occurrence of major arrhythmias defined as symptomatic ventricular tachycardia or survived sudden death

4.1.8. Cardio-pulmonary exercise testing

In chronic heart failure, cardio-pulmonary exercise testing is established as the mainstay of objective measurement of functional ability. One of the key parameters measured is peak oxygen consumption (peak VO2) and VE/VCO2 slope which have been shown to be important prognostic markers[176,184,185]. Peak VO2 represents the highest rate of oxygen uptake as oxygen consumption rises incrementally during exercise. A variety of factors may result in reduction of peak VO2 level including low cardiac output, reduced peripheral blood flow, impaired skeletal muscle metabolism or early termination of the test. Although left ventricular ejection fraction does not predict peak VO2, patients with low peak VO2 have a higher mortality rate. Studies have shown that mortality of patients with a peak VO2 of 14.5 mL/kg per minute or less was double that of patients whose VO2 exceeded this value[177,186]. Normally, peak VO2 decreases with age, declining 5% to 10% per decade of life. At any age, peak VO2 is 10% to 20% higher in men than women, in part because of a higher hemoglobin concentration, a larger muscle mass, and a greater stroke volume in men. As a result, the measured VO2max will be compared with a predicted value generated from empirically derived formulas based on age, sex, and height. The peak VO2 would be considered decreased if the measured value is less than 85% of predicted[178,186].

The major link between the circulatory and ventilatory responses to exercise is carbon dioxide production. Ventilatory efficiency is defined by linking ventilation relative to carbon dioxide production, resulting in the matching of ventilation and perfusion[178,187]. VE/VCO2 is the ventilation- carbon dioxide production ratio and the VE/VCO2 slope is the rate of increase in ventilation per unit increase in carbon dioxide production. This is

greater in patients with chronic heart failure compared to normal individuals, and higher than normal values are associated with poor exercise tolerance and a worse prognosis.

5. BIOMARKER SUB-STUDY

5.1. Background

Patients with heart failure have an increased level of circulating brain natriuretic peptide (BNP) and inflammatory cytokines (e.g. IL-6 and TNF-α). Over expression of these cytokines are associated with the progression of LV dysfunction and worsening of heart failure symptoms[188]. A number of trials have indicated that inflammation is a strong predictor of future death and CHF in patients with acute coronary syndrome (ACS)[189-191]. In particular, a more recent study showed that increased production of IL-6, MCP-1, and NT-proBNP after an ACS are at greater risk for subsequent HF and death[192]. Elevation of CRP, an inflammatory marker, is a predictor of development of heart failure and death after a MI[193,194]. In the acute setting, CRP is a sensitive pro-inflammatory biomarker but is not specific to cardiovascular disorders. More specific pro-inflammatory biomarkers such as IL-6, MCP and BNP have more recently been shown to be independent and powerful predicators of death and heart failure in patient s with ACS[192] (see figure). In a randomized, placebo-controlled trial of patients with ST segment elevation MI, daily treatment with interleukin Ra (IL-Ra), a naturally occurring Il-1 receptor, for 14 days after angioplasty resulted in a reduction in left ventricular remodelling and a reduction in CRP[195]. Furthermore, after 18 months, stage IV heart failure developed in 60% of the placebo group, compared to none in the ILRa-treated patients. Atherosclerosis, the underlying cause of CAD and myocardial infarction, is now known to be heavily influenced by inflammation. The expression of inflammatory molecules, such as BNP, IL-6, IL-10 and TNF-α, are increasingly recognised as markers of underlying disease activity and are likely to be future therapeutic targets.

For example, established heart failure therapies such as B-blockers[196] and ACEI[197] are associated with a significant reduction in inflammatory cytokines.

5.2. Biomarker sub-study Hypothesis

The hypothesis of this sub-study is:

1- Administration of G-CSF to patients with idiopathic dilated cardiomyopathy will lead to a reduction in plasma BNP levels and pro-inflammatory cytokines.

2- Cardiac function and symptoms will improve in patients in whom the levels of plasma BNP and pro-inflammatory cytokines have decreased in response to G-CSF administration.

3- Direct coronary injection of autologous bone marrow derived stem cells will confer an additional reduction in the levels of plasma BNP and pro-inflammatory cytokines above that derived from G-CSF injection alone.

4- A reduction in plasma BNP and pro-inflammatory cytokines has a positive correlation with improvements in cardiac function and symptoms.

5.3. Brain Natriuretic Peptide

Brain natriuretic peptide (BNP) is produced as a pro-hormone (Pro-BNP) from the ventricular and atrial myocardium in response to pressure overload and ventricular dilatation. It was first identified in pig brains in 1988. BNP has been established as a valuable marker for the diagnosis, prognosis and treatment of heart failure. In the setting of acute heart failure, BNP measurement has been found to be superior to clinical judgment for providing an accurate diagnosis[198]. A serum level of BNP > 100 pg/ml has a sensitivity of 90 percent and a specificity of 76 percent for the diagnosis of CHF, and levels of <50 pg/ml have a negative predictive value of 96%[198]. In patients

presenting with dyspnoea to the emergency department, a BNP value of 480 pg/ml had a sensitivity of 68%, specificity of 88%, and an accuracy of 85% for predicting a subsequent heart failure end point. Patients with BNP levels > 480 pg/ml had a 51% 6-month cumulative probability of a heart failure event, whereas those with BNP levels < 230 pg/ml had an excellent prognosis with only a 2.5% incidence of heart failure end points[199].

BNP has diuretic, natriuretic and anti-hypertensive effects by inhibiting the renin-angiotensin-aldosterone system, and exhibit a paracrine effect that inhibits fibrosis and hypertrophy and enhance diastolic function[199]. Studies have demonstrated that high BNP levels have a high correlation with the degree of left ventricular dysfunction, symptom severity and mortality in patients with congestive heart failure (CHF)[200-203]. BNP has also been shown to independently predict the morbidity and mortality in asymptomatic left ventricular systolic dysfunction[204] and in mild to moderate CHF[205]. In a prospective study, evaluating over 3000 patients without heart failure from the Framingham Heart Study to determine the usefulness of BNP in predicting the risks for all-cause mortality, CHF, acute coronary syndrome, atrial fibrillation, and stroke, researchers found that high levels of BNP were associated with a 27% increase in risk of all-cause mortality, a 28% increase in risk for a first cardiovascular event, and a 77% increase in the risk of CHF[203]. Medications used to treat CHF decrease serum levels of BNP. Increased or persistent elevation of BNP despite treatment indicates progression of disease or lack of response to therapy, and is an indication for the need to intensify the treatment. In the Val-HeFT trial, patients with the greatest decline in BNP from baseline after treatment had the lowest mortality rates, whereas patients with greatest

increase in BNP had the highest[11]. Several trials have compared BNP guided treatment and clinically guided treatment on clinical outcomes. In these studies there were significantly fewer events – death or heart failure hospitalization – in the BNP group than in the clinical group[206,207]. BNP guided treatment was associated with a 25% reduction in the median level of total treatment cost and hospital stay[208]. Furthermore, BNP has been shown to be a strong independent predictor of sudden death in patients with CHF[209]. The study by Gardner et al demonstrated that in patients with advanced heart failure, BNP levels above the median value was the only independent predictor of mortality and the combined endpoint of mortality or urgent transplantation[210]. Therefore measuring BNP levels would aid in deciding which of the advanced heart failure patients may benefit from the scarce resources of a cardiac transplant[210]. BNP provides important prognostic information in CHF that is independent of hemodynamic parameters such as pulmonary –capillary wedge pressure and LVEF[211]. Therefore, these findings suggest that plasma BNP concentration is a sensitive biomarker of left ventricular impairment or dysfunction.

This section summarises the hypothesis of the biomarker sub-study and the methodology I used to measure the pro-inflammatory cytokines using the Randox Biochip technology.

5.4. Randox Biochip technology

The Evidence Investigator™ Biochip Array technology is used to perform simultaneous quantitative detection of multiple analytes from a single patient sample.

The core technology is the Randox Biochip, a solid-state device containing an array of discrete test regions of immobilised antibodies specific to different cytokines and growth factors. A sandwich chemiluminescent immunoassay is employed for the cytokine array. Increased levels of cytokine in a specimen will lead to increased binding of antibody labelled with horseradish peroxidase (HRP) and thus an increase in the chemiluminescence signal emitted. The light signal generated from each of the test regions on the biochip is detected using digital imaging technology and compared to that from a stored calibration curve. The concentration of analyte present in the sample is calculated from the calibration curve. Several different immunoassay based multi-analyte arrays have been developed for use on Evidence Investigator™. The Evidence Investigator™ Cytokine Array will quantitatively test for IL-1α, IL-1ϑ, IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFNγ, EGF, MCP-1 and TNFI simultaneously.

I used patients' serum that has been stored frozen in small aliquots at -80 C for the Evidence Investigator™ Cytokine & Growth Factors Array.

Sample preparation was carried out in accordance with the collection tube manufacturer's recommendations (collection devices were durable, leak-proof and constructed of non-absorbing plastics). Samples were diluted using working strength wash buffer.

Test procedures:

- CALIBRATION

A nine-point calibration was performed using the Randox Evidence Investigator™ Cytokine & Growth Factors calibrators, which cover the measuring range of all assays. A total of 6 biochip carriers were assayed simultaneously, and a new calibration curve was constructed for each assay series.

- Quality control

Randox Cytokine Multianalyte Controls, level I, II, III were used for quality control in the monitoring of accuracy and precision. The controls should be assayed at intervals defined by the user. Control results should fall within acceptable limits, otherwise corrective action should be taken as established by laboratory guidelines.

- Materials

All materials were equilibrated to room temperature prior to use. The required number of biochip carriers was removed from their packaging. The handling tray supplied with the Investigator thermo shaker unit was placed onto the working surface. Each carrier was inserted into the handling tray, ensuring they are flat and secure by clicking into position. All sample and reagent additions, washing and incubations were performed using the handling tray and carriers were removed from this for the final signal addition and imaging stage of the procedure. The thermo shaker was equilibrated to 37°C for 30 minutes prior to use.

5.4.1. Interleukin 1α (IL-1α):

The Evidence Investigator™ Interleukin-1 alpha (IL-1α) test has been designed for the quantitative measurement of IL-1α in human serum and plasma samples. Interleukin-1 (IL-1) is a regulatory and inflammatory cytokine, which exists in two forms, IL-1α and IL-1β, which share 25% homology at amino acid level. IL-1α is produced as a biologically active 31-kDa precursor, which undergoes proteolytic cleavage yielding a 17-kDa protein of 159 amino acids. There are 2 cell surface binding proteins for IL-1 expressed on various cells, IL-1R1 and IL-1R2, and one non-binding receptor accessory protein IL-1-Racp. IL-1α and IL-1β bind to both receptor types but IL-1α binds better to IL-1R1 than IL-1R2. IL-1RI is an 80 kDa glycoprotein, which has been isolated from T-cells, fibroblasts, keratinocytes, endothelial cells, synovial lining cells, chondrocytes and hepatocytes and IL-1R2 has been found in B-cells, neutrophils and bone marrow cells[212]. In healthy people cells of the central nervous system, some epithelial cells and skin keratinocytes produce IL-1α. It is also produced by macrophage and many other cell types in response to stimuli by inflammatory agents, infections or microbial endotoxins[212]. IL-1 has been shown to induce synthesis of endothelial cell-surface factors and activate endothelial cells. In the liver, it initiates an increase in hepatic protein synthesis and decreased albumin production. IL-1 also induces collagenase production and has been found to be chemotactic and stimulatory for neutrophils. IL-1 also affects the endocrine system and is mitogenic for B-lymphocytes. It also plays an important role in immune functions by activating macrophages, natural killer cells and T-cells and by inducing B-cell proliferation and maturation and LAK production. IL-1 is not commonly found circulating except during severe disease where the cytokine is

released from dying cells. Colonic tissue levels of IL-1α correlate to severity of inflammatory bowel disease. IL-1 production is increased in sepsis, rheumatoid arthritis, leukaemia, diabetes and atherosclerosis[212,213]. The assay range for IL-1α is 0-500 pg/ml, with <1% cross-reactivity with other cytokines.

5.3.4 Interleukin 1ß (IL-1ß):

IL-1β is synthesised as a biologically inactive precursor of 269 amino acids with a molecular mass of 31 kDa, which undergoes proteolytic cleavage by IL1β converting enzyme, which yields a 17kDa protein of 153 amino acids. IL-1β plays a central role in acute and chronic inflammation, both locally and systemically. It is produced by monocytes, macrophages, astrocytes, oligodendroglia, adrenal cortical cells, natural killer cells, endothelial cells, keratinocytes, platelets, neurons, neutrophils, osteoblasts, Schwann cells, trophoblasts and fibroblasts and affects nearly every cell type. IL-1 activates macrophages, induces fibroblast proliferation, and induces expression of matrix metalloproteinases, leading to extracellular matrix degradation, monocyte migration and degradation of IL-1β, which acts as a negative feedback mechanism. IL-1 has a cytotoxic effect on normal cells and the IL-1β form is growth-inhibitory and cytocidal for certain melanoma cells. IL-1β has been shown to inhibit insulin release and therefore may play a role in the development of autoimmune insulin-dependent diabetes mellitus. It is also involved in bone remodelling by stimulating bone resorption and inhibiting bone collagen synthesis. The production of IL-1β is increased in sepsis, rheumatoid arthritis, leukaemia, diabetes and atherosclerosis[214].The Evidence Investigator™ IL-1β assay that we used in our study is a sandwich chemiluminescent

immunoassay that detects IL-1β in human serum and plasma. The biochip substrate contains immobilised monoclonal antibody to IL-1β. The assay range for IL-1β is 0-250 pg/ml, with <1% cross-reactivity with other cytokines.

5.4.2. Interleukin-2 (IL-2):

IL-2 is a glycoprotein with a molecular mass of 15-18 kDa that has varying degrees of glycosylation accounting for the observed molecular weight range. IL-2 is synthesised as a precursor protein of 153 amino acids from which 20 amino acids are cleaved to produce the active form[215]. It has a single disulphide bond that is essential for biological activity. The IL-2 receptor consists of three distinct membrane associated units that bind IL-2 with high affinity: a 55kDa I chain, a 70-75 kDa α chain and a 64 kDa γ chain. Soluble IL-2RI and IL-2Rα have been found in serum. IL-2 is a lymphokine that is synthesised and secreted primarily by T-cells following their activation by mitogens or antigen-activated T-lymphocytes. Transformed T-cells and B-cells, leukaemia cells, lymphokine activated killer cells and natural killer cells also secrete IL-2 in small amounts. Due to its effects on T-cells and B-cells IL-2 is a central regulator of immune responses[216]. It stimulates proliferation of T-cells, activated B-cells, natural killer cells, monocytes, thymocytes and lymphokine activated killer cells. It causes differentiation of activated B-cells, monocytes and oligodendrocytes. It also stimulates the cytolytic activity and cytokine production by activated B-cells, monocytes, natural killer cells and thymocytes. IL-2 supports the induction and secretion of immunoglobulins[217]. It plays a role in anti-inflammatory reactions and in tumour surveillance. IL-2 stimulates the synthesis of IFNγ and IL-4 by T-cells as well as inducing secretion of IL-1 and TNF-α.

IL-2 is also a possible neuromodulator and growth regulator of glial cells, and has been found to damage the blood brain barrier. IL-2 and IL-2R are elevated in Hodgkins disease, multiple sclerosis, rheumatoid arthritis, type 1 diabetes, AIDS, severe burn trauma and allograft rejection. Antibodies against IL-2 and IL-2R may suppress immune responses and prevent rejection. IL-2 has also shown some promise as an anti-cancer drug due to the ability to activate tumour-attacking LAK and TIL cell but problems have arisen with toxicity[218]. The Evidence Investigator™ IL-2 assay that we used in our study is a sandwich chemiluminescent immunoassay that detects IL-2 in human serum and plasma. The assay range for IL-2 is 0-3000 pg/ml, with <1% cross-reactivity with other cytokines.

5.4.3. Interleukin 4 (IL-4):

IL-4 is a glycoprotein synthesised as a precursor protein of 153 amino acids. The first 24 amino acid residue signal peptide is cleaved to produce a 129 amino acid 15-19 kDa protein. IL-4 has two glycosylation sites at two arginine residues and contains six cysteine residues involved in disulphide bond formation, which are essential for biological activity. It exerts biological activity through a high affinity receptor expressed by cells of haemopoietic lineage. The IL-4 receptor is produced as a 825 amino acid precursor glycoprotein that is cleaved to produce a mature protein of 800 amino acids which in turn has a 24 amino acid transmembrane domain, a 569 amino acid intracellular domain and a 207 amino acid extracellular domain. IL-4 is produced by activated T-cells, mast cells, eosinophils and peripheral basophils and its synthesis is induced by IL-2 and inhibited by TGFα. IL-4 induces T cell differentiation and inhibits T cell cytokine production. It also enhances the proliferation and differentiation of

activated B-cells. A further role of IL-4 is in the induction of IgE synthesis by B cells and the expression of class II MHC antigens. IL-4 has also been shown to inhibit activation and proliferation of natural killer cells induced by IL-2 and inhibits the IL-2 dependant generation of LAK cells. IL-4 also inhibits the synthesis of IL-1, IL-6, IL-8, IL-10, IL-12 and TNFI in macrophages[219] and promotes proliferation of mast cells and enhances respiratory burst and phagocytic properties of neutrophils[220]. IL-4 is a chemoattractant for fibroblasts and induces the production of extracellular matrix proteins and also induces proliferation of capillary endothelial cells[221,222]. IL-4 levels are elevated in disorders such as asthma and Hodgkin's lymphoma, and may also play an important role in the treatment of chronic inflammatory diseases, such as rheumatoid arthritis, and autoimmune disorders[223].

The assay range for IL-4 in the The Evidence Investigator™ that we used in our study is 0-900 pg/ml, with <1% cross-reactivity with other cytokines.

5.4.4. Interleukin-6 (IL-6):

Interleukin (IL-6) is a pleiotropic cytokine with a broad range of humoral and cellular immune effects relating to inflammation, host defence, and tissue injury[224]. IL-6 is a central mediator of the acute-phase response and a primary determinant of hepatic production of C-reactive protein[225]. IL-6 is synthesised as a precursor protein of 212 amino acids. The N-terminal 28 amino acid residue signal peptide is cleaved to produce a 21kDa protein. It has two potential N-glycosylation sites that have no effect on bioactivity. Different post-translational alterations such as glycosylation and phosphorylation give various forms of IL-6 with molecular masses of 21.5-28 kDa. The IL-6 receptor is a strongly glycosylated 80 kDa protein of 449 amino acids. Two different

forms of the receptor have been described that bind IL-6 with differing affinities, a soluble form of the IL-6 receptor has also been described. The IL-6 receptor is expressed on T cells, mitogen activated B cells, peripheral monocytes and some macrophage and B cell derived tumour cell types. IL-6 influences antigen-specific immune responses and inflammatory reactions. It is mainly produced by stimulated monocytes, fibroblasts and endothelial cells, but also by macrophages, T-cells, B-lymphocytes, hepatocytes, granulocytes, smooth muscle cells, eosinophils, chondrocytes, osteoblasts, mast cells, glial cells and keratinocytes after stimulation[226]. IL-6 stimulates differentiation and antibody secretion of B-cells and also initiates IL-2 production and IL-2 receptor expression. It also activates T cells and in the presence of IL-2 induces differentiation. Production of acute phase proteins by hepatocytes is also stimulated by IL-6 and colony-stimulating activity on hematopoietic stem cells[227]. Additional bioactivities include induction of neuronal cell differentiation, induction of the maturation of megakaryocytes and inhibition of the growth and induction of terminal differentiation of M1 myeloid leukaemia cells. Measurement of IL-6 serum levels may be useful in monitoring the activity of myelomas and to calculate tumour cell masses. Excessive over-production of IL-6 has been observed in rheumatoid arthritis, multiple myeloma, Lennert syndrome, Kawasaki disease, Castlemans disease, cardiac myxomas and liver cirrhosis[228]. Baseline levels of the inflammatory cytokine IL-6 are significantly elevated among apparently healthy men at risk for future myocardial infarction[229], suggesting a role for cytokine-mediated inflammation in the early stages of atherosclerosis.. The assay range for IL-6 in the Evidence Investigator™ that we used in our study is 0-900 pg/ml, with <1% cross-reactivity with other cytokines

5.4.5. Interleukin-8 (IL-8):

IL-8 is a member of a structurally similar family of cytokines called chemokines, which demonstrate chemotactic activity for neutrophils. IL-8 is a non-glycosylated protein of 8 kDa protein and consists of 99 amino acids with a 22 residue signal peptide that is cleaved to generate a 77 amino acid. Proteases yield N-terminal variants. It can form non-covalent dimers, but they are not necessary for biological activity. Two classes of IL-8 receptors have been identified; CXCR-1 and CXCR-2, which are 7-transmembrane, G-protein-coupled receptors. One has high affinity for IL-8 and other chemokines and one has high affinity for IL-8 and low affinity for other chemokines[230]. IL-8 is produced in response to pro-inflammatory stimuli. It is produced by monocytes, macrophages, fibroblasts, endothelial cells, keratinocytes, melanocytes, hepatocytes, chondrocytes, T-cells, neutrophils, and astrocytes[231,232]. Il-8 activates neutrophil granulocytes and antagonises IgE production by B-cells. It possesses anti-inflammatory activities and acts as a chemotactic for all known types of migratory immune cells. It enhances metabolism of reactive oxygen species, increases chemotaxis and enhances expression of adhesion molecules. The assay range for IL-8 in the The Evidence Investigator™ that we used in our study is 0-3000 pg/ml, with <1% cross-reactivity with other cytokines.

5.4.6. Interleukin 10 (IL-10):

Interleukin-10 (IL-10) is a homodimeric protein with a molecular weight of 18 kDa. It is produced as a 178 amino acid residue precursor, which is cleaved to give a mature protein of 160 amino acids. It also has two intrachain disulphide bonds, which are

required for activity[233]. Receptors for IL-10 are all transmembrane glycoproteins about 110 kDa in size. The IL-10 receptor binds to its ligand with high affinity most haemopoietic cells express them at low levels. IL-10 primarily acts as an anti - inflammatory agent, inhibiting cytokine production by T cells and natural killer cells caused by activation of monocytes/macrophages[234]. IL-10 strongly inhibits the production of IL-1α, IL-6, IL-8, IL-10, IL-12, GM-CSF, G-CSF, M-CSF, TNF-α, MIP-1α by activated monocytes/macrophages. It also inhibits the ability of monocytes/macrophages to modulate turnover of extracellular matrix[235]. It initiates growth and differentiation of B cells and induces histamine release by mast cells. IL-10 also expresses an anti-inflammatory response on neutrophils and an immunosuppressive effect on dendritic cells[236,237]. Serum levels of IL-10 are decreased in HF and correlate negatively with severity[238]. IL-10 suppresses the synthesis of TNF-α and is thought to have a cardioprotective effect in CHF patients[239]. In patients with DCM baseline IL-10 and TNF α are elevated at baseline. Certain tumour cell lines including melanomas and a variety of carcinomas produce IL-10 and it is also produced during septicaemia and septic shock. IL-10 is involved in development of inflammatory bowel disease. Expression of IL-10 is detected in patients with multiple sclerosis and it has been suggested that it could potentially be a useful therapeutic agent for this disease. IL-10 also contributes to immunosuppression seen in many infectious diseases such as Leprosy, Malaria and HIV, which show increased IL-10 production. IL-10 alone and synergistically with IL-4 has been seen to reduce various symptoms observed with acute and chronic arthritis and daily administration of IL-10 inhibits spontaneous onset of insulin dependent diabetes mellitus in mice. The assay range for IL-8 in the Evidence

Investigator™ that we used in our study is 0-1000 pg/ml, with <1% cross-reactivity with other cytokines.

5.4.7. Vascular Endothelial Growth Factor (VEGF):

Vascular endothelial growth factor (VEGF) is secreted as a glycosylated homodimeric protein of 46 kDa that is made up of two 24-kDa subunits linked by disulphide bonds. Various molecular variants of VEGF have been identified depending on splicing of the mRNA: 121, 145, 165, 189 and 206 amino acids[240]. There are three receptors in the VEGF family; VEGFR1, expressed on endothelial cells, trophoblasts and macrophages mediates motility and vascular permeability. VEGFR2, expressed on endothelial cells, haemopoietic stem cells, megakaryocytes and retinal cells is essentially involved in proliferation. VEGFR3 is expressed specifically on lymphatic endothelium and is important for lymphatic proliferation. VEGF is expressed by vascularised tissue such as pituitary, brain, lungs, kidneys, heart and adrenal glands, although it is assumed that all tissues have the potential to produce the growth factor[241]. VEGF is a specific mitogen and survival factor for endothelial cells and a key promoter of angiogenesis[240,242]. It also causes vasodilation, stimulates cell migration and inhibits apoptosis[243]. Synthesis of VEGF is stimulated when cells become deficient in oxygen or glucose or under inflammatory conditions[240].

An increase in VEGF production has been observed in patients with preeclampsia, ischemic heart disease, sickle cell anaemia, psoriasis, diabetes, rheumatoid arthritis, POEMS syndrome and Kawasaki disease[244,245]. Increased serum concentrations of VEGF have been observed in various types of cancer. Drug development to block angiogenesis by interfering with VEGF function has been successful in many tumour

treatments. VEGF has also been used successfully in therapeutic angiogenesis in patients with end-stage coronary artery disease[246].

The assay range for VEGF in the Evidence Investigator™ that we used in our study is 0-3000 pg/ml, with <1% cross-reactivity with other cytokines.

5.4.8. Interferon-γ (IFN-γ):

Biologically active interferon gamma (IFNγ) is a 20 or 25 kDa glycoprotein depending on its glycosylation state. This lymphokine is synthesised as a 166 amino acid sequence but is cleaved to give a 143 amino acid residue. It binds to a single chain 90 kDa glycoprotein receptor that has a high degree of specificity and is expressed on all types of human cells. Binding of IFNγ to its receptor induces expression of various proteins specific to IFNγ stimulation. IFNγ is produced by mitogen activated T lymphocytes and natural killer cells and its main role is its involvement in the regulation of immunological functions essential to host defense mechanisms. It has anti viral and anti-parasitic properties. It modulates growth and differentiation of T lymphocytes and influences cell-mediated mechanisms of cytotoxity. It increases macrophage-mediated killing of extra-cellular parasites and inhibits viral replication. IL2, bFGF and EGF induce IFNγ synthesis in T lymphocytes. Knock out studies have shown that IFNγ is not essential for the development of the immune response but is essential for resistance to viral infections[247]. IFNγ decreases clinical symptoms in severe atopic dermatitis. It also decreases joint pain and is effective in the treatment of chronic polyarthritis. Treatment of malignancies by IFNγ has been disappointing but combination treatment with other interferons has been more successful. IFNγ may also be useful in the treatment of

infections in immunosuppressed patients. Decreased levels of IFNγ are observed in acute and asymptomatic asthma and are associated with severe airway obstructions. Cells derived from the skin and peripheral blood supply of cutaneous T cell lymphoma patients have depressed levels of IFNγ. Studies have also observed an age related increase in IFNγ production[248]. The assay range for IFNγ in the The Evidence Investigator™ that we used in our study is 0-1500 pg/ml, with <1% cross-reactivity with other cytokines.

5.4.9. Epidermal Growth Factor (EGF):

Human EGF is produced as a long precursor protein of 1207 amino acids, which is released by protoelytic cleavage to give a globular protein of 6.4 kDa consisting of 53 amino acids. EGF contains three intramolecular disulfide bonds, which are essential for its biological activity. EGF is produced by various organs including the brain, kidney, salivary gland and stomach, and is found in nearly all bodily fluids at low levels and at higher levels in saliva, milk, semen and urine.

EGF production is stimulated by progesterone and inhibited by oestrogen and is stored in platelets and released on degranulation. It is mitogenic and chemotactic for epidermal and epithelial cells and also mitogenic for basal cells of olfactory epithelium and for endothelial cells and therefore plays an important role in angiogenesis[249]. EGF controls and stimulates proliferation of epidermal and epithelial cells such as fibroblasts, kidney epithelial cells, human glial cells, ovary granulosa cells and thyroid cells. It has been shown to act as a differentiation factor and increases turnover and synthesis of extra cellular matrix such as fibronectin, collagens, laminin and glycosaminoglycans. It may also influence the activity of GABA-ergic and dopaminergic neurons. EGF has various

therapeutic applications such as the healing of burns, venous and diabetic ulcers, skin graft donor sites, corneal wounds, tympanic membrane perforations, gastric and duodenal ulcers and increasing sensitivity of malignancies to cytotoxic drugs[250]. EGF has been shown to be elevated in patients with brain tumours and to induce differentiation in tumour cell lines. Also many epithelial cancers over-express the EGF receptor. Tumour aggressiveness is also associated with increased expression of EGF receptors. This expression is also high in invasive and disseminated tumours therefore many strategies to block the EGF receptor have been studied to inhibit tumour proliferation. It has also been proposed that EGF plays an important role in male infertility, as there is a correlation between the level of circulating EGF, and the number of spermatids in the testis.

The assay range for EGF in the Evidence Investigator™ that we used in our study is 0-900 pg/ml, with <1% cross-reactivity with other cytokines.

5.4.10. Tumor Necrosis Factor –α (TNF-α):

Tumour necrosis factor alpha (TNF-α) is a 157 amino acid 26-kDa transmembrane protein which is secreted as a soluble mature 233 amino acid homotrimer of 17 kDa by proteolytic cleavage. Both forms of TNF-α are biologically active. There are two distinct structurally homologous TNF receptors, which bind to TNF-α with high affinity; TNFR1 and TNFR2. At least one receptor is expressed on nearly all cell types and soluble forms have been found in serum and urine. TNF-α has a role in host resistance to infection as a mediator of immune and inflammatory responses. Various immune cells

such as macrophages, monocytes, neutrophils, T cells and natural killer cells, following stimulation by lipopolysaccharide, secrete TNF- α[251]. Synthesis of TNF- α is induced by interferons, IL-2, GM-CSF, bradykinin, immune complexes and inhibitors of cyclooxygenase. TNF- α production is inhibited by IL-6, TGFβ, VitD3, prostaglandin E2, dexamethasone and cyclosporin A. Increased production of TNF- α leads to cachexia, septic shock following infection by gram-negative bacteria, autoimmune disorder and meningococcal septicaemia[252]. Increased levels of TNF- α are also seen in multiple sclerosis, rheumatoid arthritis, brain injury, meningococcal meningitis, HIV, and Alzheimer's disease[253]. Patients with advanced heart failure also exhibit high levels of TNF-α[254]. TNF- α is involved in the growth of malignant tumours and has been investigated as an antitumor drug but its use has been limited due to its systemic toxic side effects. Anti TNF- α has been shown to decrease inflammation in ulcerative colitis and is also useful in the treatment of sepsis and rheumatoid arthritis[252]. TNF-α is derived from macrophages, endothelial and smooth muscle cells. TNF- α levels are significantly elevated in advanced heart failure[255]and post MI[256]. Furthermore persistent elevation of TNF– α for > 3 months post MI is associated with up to a 3 fold increase in recurrent MI or coronary death[257]. The assay range for TNF- α in the Evidence Investigator™ that we used in our study is 0-1500 pg/ml, with <1% cross-reactivity with other cytokines.

6. RESULTS

6.1. Baseline characteristics

A total of 60 patients were recruited for the trial. The patient baseline characteristics were well matched between the 4 groups (tables below). There were no significant differences with regards to age, sex, hypertension, ICD/pacemaker device therapy or treatment with disease modifying agents such as ACEi/ARB, B-blockers, diuretics, Aldosterone antagonists and Digoxin. All patients were on maximum tolerated medical therapy; Over 98% of patients were on ACEi or ARB and 88% were on B-blockers. The baseline characteristics of this cohort of patients with idiopathic dilated cardiomyopathy are in keeping with previously published trials[145,186]. Of note, the prevalence of hypertension in our cohort was < 10% all of which were well controlled with medication.

The baseline New York Heart Association (NYHA) classification for heart failure symptoms and Canadian Cardiovascular Society (CCS) classification for angina were similar between the groups.

	Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value	ANOVA
	n = 15	n = 15		n = 15	n = 15		
Age - years (mean;SEM)	52.87 ± 3.07	56.07 ± 2.55	0.4298	51.67 ± 3.12	54.87 ± 3.70	0.52	0.76
BMI - kg/m2 (mean;SEM)	29.19 ± 1.39	29.71 ± 1.25	0.7845	27.23 ± 1.12	28.26 ± 1.8	0.636	0.6214
Male gender - %	66.6	86.6	0.2084	66.6	60	0.7165	0.4307
Hypertension - %	6.6	13.3	0.5589	13.3	6.6	0.5589	0.8728
Hyperlipidemia - %	13.3	19.9	0.6383	0	6.6	0.3259	0.3081
Diabetes - %	6.6	13.3	0.5589	13.3	6.6	0.5589	0.8728
Active smoker - %	6.6	13.3	0.5589	13.3	13.3	>0.9999	0.9279
Family History of CAD - %	6.6	13.3	0.5589	13.3	13.3	>0.9999	0.36
Diagnosis to therapy duration - years (mean;SEM)	7.3 ± 1.95	5.4 ± 0.91	0.3677	4.9 ± 0.96	8.00 ± 1.61	0.10	0.31

SEM; standard error of the mean

Table 7: Baseline characteristics of the REGENERATE-DCM trial

	Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value	ANOVA
	n = 15	n = 15		n = 15	n = 15		
Medical Therapy							
Statins	46.6	53.3	0.7263	26.6	46.6	0.2712	0.6213
ACEi	39.9	59.9	0.2893	73.3	73.3	>0.9999	0.2008
ARB	59.9	33.3	0.1534	26.6	26.6	>0.9999	0.1876
ACEi/ARB	100	93.3	0.3259	100	100	>0.9999	0.3997
Aspirin	26.6	39.9	0.4560	26.6	33.3	0.7025	0.8486
B-Blockers	79.9	100	0.0719	86.6	86.6	>0.9999	0.3963
Diuretics	59.9	59.9	>0.9999	73.3	53.3	0.2712	0.7349
Aldosterone Antagonists	53.3	79.9	0.1299	66.6	79.9	0.4265	0.3556
Digoxin	33.3	13.3	0.2084	39.9	26.6	0.456	0.4282
Device Therapy							
ICD	33.3	19.9	0.4265	26.6	26.6	>0.9999	0.8859
Biventricular Pacemaker	0	6.60	0.3259	13.3	13.3	>0.9999	0.5113
CRT-D	26.6	39.9	0.456	46.6	19.9	0.1299	0.4077
Overall device therapy	26.6	46.6	0.2712	59.9	33.3	0.1534	0.2676

ACEi; Angiotensin converting enzyme inhibitors, ARB; Angiotensin receptor blockers, ICD; implantable cardiac defibrillators, CRT-D; cardiac resynchronisation therapy - defibrillator

Table 8: Baseline characteristics of the REGENERATE-DCM trial

	Peripheral G-CSF; Mean (SEM)	Peripheral Placebo; Mean (SEM)	IC Stem Cells; Mean (SEM)	IC Serum; Mean (SEM)	p-value
Hb (g/dL)	13.78 (0.2566)	13.99 (0.2396)	13.60 (0.2649)	13.05 (0.2702)	0.077
Platelets (x10^ 9/L)	236.8 (13.46)	223.8 (13.20)	226.9 (14.61)	239.7 (14.69)	0.8276
WCC (x10^ 9/L)	7.020 (0.5264)	7.253 (0.6728)	7.447 (0.5242)	6.760 (0.5129)	0.8412
Urea (mmol/L)	7.447 (0.5714)	7.413 (0.7157)	8.247 (1.018)	7.193 (0.6636)	0.7762
Creatinine (umol/L)	96.60 (5.107)	99.80 (6.472)	99.87 (6.945)	92.40 (7.421)	0.8323
eGFR (mL/min)	70.27 (4.217)	71.73 (5.745)	71.67 (6.629)	75.80 (6.903)	0.9228
CRP	6.800 (1.212)	6.867 (0.8776)	6.600 (1.341)	6.867 (0.7149)	0.9979
CK	121.3 (13.60)	181.9 (40.90)	162.6 (29.96)	140.1 (17.38)	0.4405
Trop	0.0215 (0.0078)	0.0153 (0.0031)	0.0145 (0.0028)	0.0137 (0.0038)	0.6491
Bilirubin (umol/L)	11.27 (2.126)	15.53 (4.031)	10.67 (1.190)	11.53 (1.404)	0.4908
ALT (IU/L)	25.33 (1.443)	31.13 (4.052)	25.47 (2.160)	29.33 (4.042)	0.4759
ALP (IU/L)	60.13 (5.710)	74.73 (6.320)	61.87 (3.690)	75.33 (9.765)	0.4934
GGT (IU/L)	37.93 (5.319)	54.20 (11.33)	52.00 (10.68)	55.00 (13.51)	0.6403
Cholesterol (mmol/L)	5.113 (0.2916)	5.460 (0.5119)	4.887 (0.2646)	5.133 (0.2830)	0.7197
Glucose (mmol/L)	7.264 (1.819)	6.280 (0.7742)	5.427 (0.2773)	5.460 (0.2476)	0.5075
T4	17.13 (0.6994)	16.70 (1.143)	17.42 (0.8865)	18.45 (1.483)	0.7334
TSH (IU/L)	3.275 (1.258)	5.123 (2.525)	1.953 (0.3042)	1.825 (0.2237)	0.3619
BNP	1640 (508.1)	1304 (318.6)	964.7 (240.0)	1474 (566.7)	0.7054
HbA1c	6.640 (0.6436)	6.387 (0.3733)	5.967 (0.1609)	6.113 (0.1633)	0.6260

Table 9: REGENERATE-DCM trial: Baseline bloods

NYHA	Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value	ANOVA
II -%	73.3	60.0	0.7408	46.7	66.7	0.2425	0.3586
III - %	26.7	40.0		46.7	33.3		
IV - %	0	0		6.7	0		
CCS	Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value	ANOVA
0 - %	93.3	66.7	0.2615	60.0	80.0	0.2919	0.4192
I - %	6.7	13.3		6.7	6.7		
II -%	0	13.3		20.0	0.0		
III - %	0	6.7		13.3	6.7		

NYHA; New York Heart Association, CCS; Canadian Cardiovascular Society, IC; Intracoronary

Table 10: Baseline characteristics of the REGENERATE-DCM trial

6.2. Primary Endpoint: Change in Ejection Fraction

The baseline ejection fraction (EF) was similar between groups. The mean baseline EF in the peripheral placebo group was 28.9% compared to 36% in the peripheral G-CSF group (P = 0.09). The mean baseline EF in the intracoronary serum group was 41.7% compared to 32.9% in the intracoronary stem cell group (P = 0.14).

At 3 months there was a significant improvement in mean EF in the intracoronary stem cell group from 32.9% to 38.4% (P = 0.0138). This represents a within group (Paired t-test) change of 5.4% in EF. There was no corresponding significant improvement in EF in the remaining 3 groups. In the intracoronary serum group, EF reduced from 41.7% to 40.6% (within group reduction of 1.1%) (P = 0.46). In the peripheral G-CSF group the EF improved from 36% to 36.6% (within group improvement of 0.6%), and in the peripheral placebo group the EF reduced from 28.9% to 28.68% (within group reduction of 0.22%).

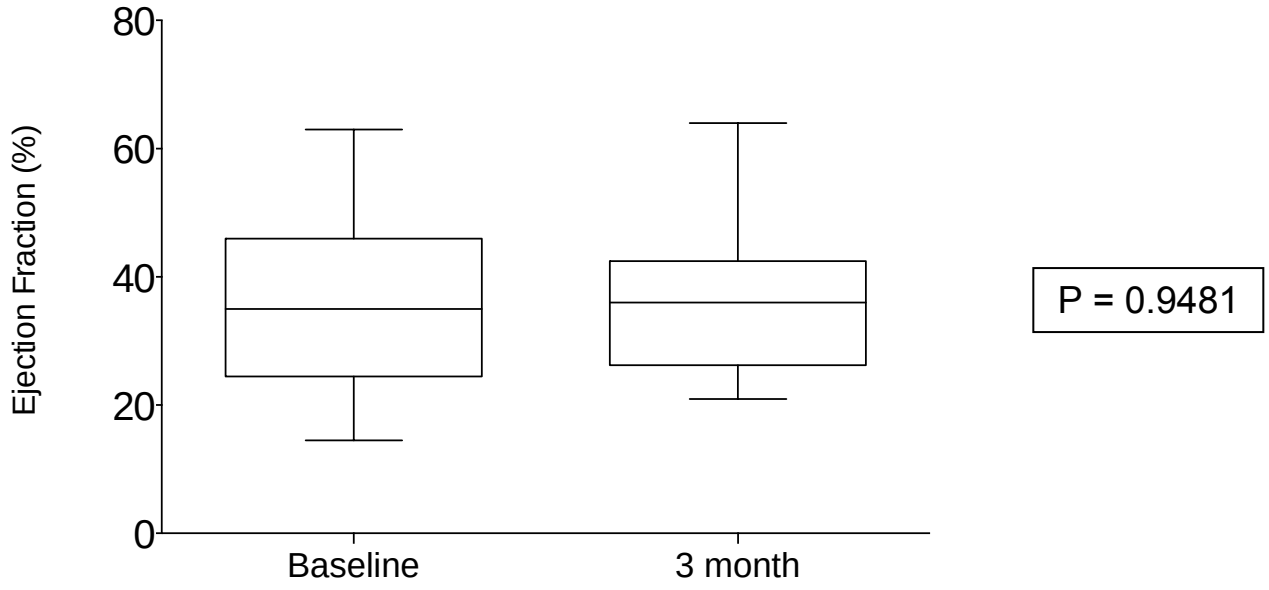
6.3. LV mass, LV dimensions and stroke volume

We found no significant improvement in LV mass, end-systolic volumes, end-diastolic volumes or stroke volume in any of the 4 groups (tables 14,15,16,17). Ejection fraction is influenced by a number of confounding variables including afterload, preload, electrical activation pattern and neurohormonal parameters, and therefore changes of some of these factors may become more apparent before the remodeling changes that affect left ventricular volumes.

		Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value	ANOVA
Ejection Fraction (%)								
Baseline	Mean (SEM)	36.0 (3.423)	28.90 (2.373)	0.0944	32.93 (4.250)	41.70 (3.938)	0.1411	0.8722/0.1679
3 month	Mean (SEM)	36.64 (3.212)	28.68 (2.312)	0.0546	38.30 (3.349)	40.60 (3.301)	0.6285	0.0635/0.8360
p-value (Paired t test)		0.9481	0.6294		0.0138	0.4634		

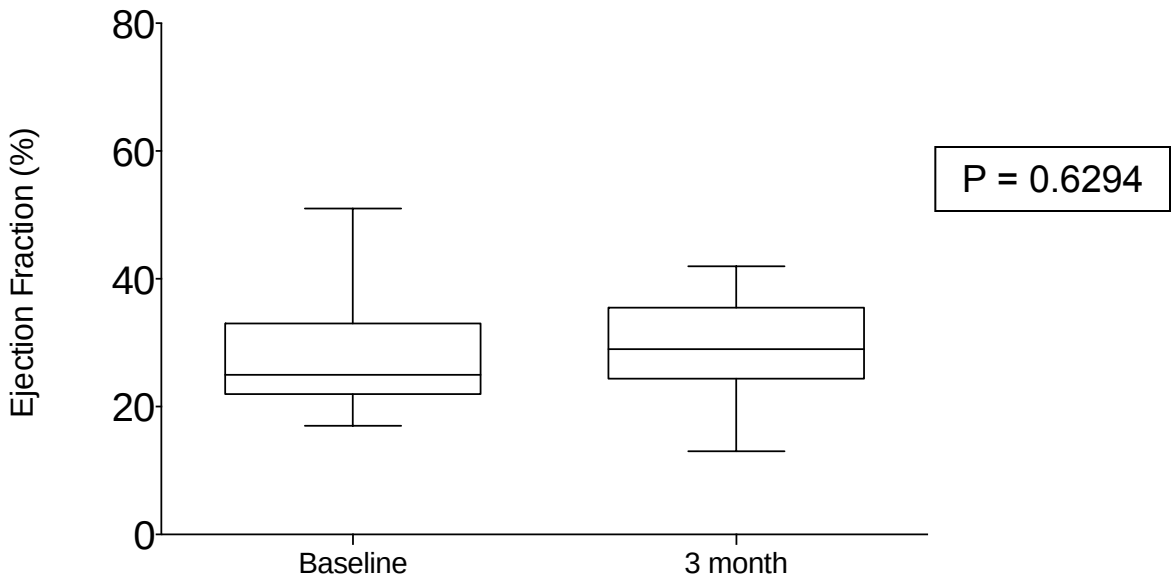
G-CSF; Granulocyte colony-stimulating factor, SEM; standard error of mean, IC; intracoronary

Table 11: Primary endpoint results of the REGENERATE-DCM trial: change in ejection fraction



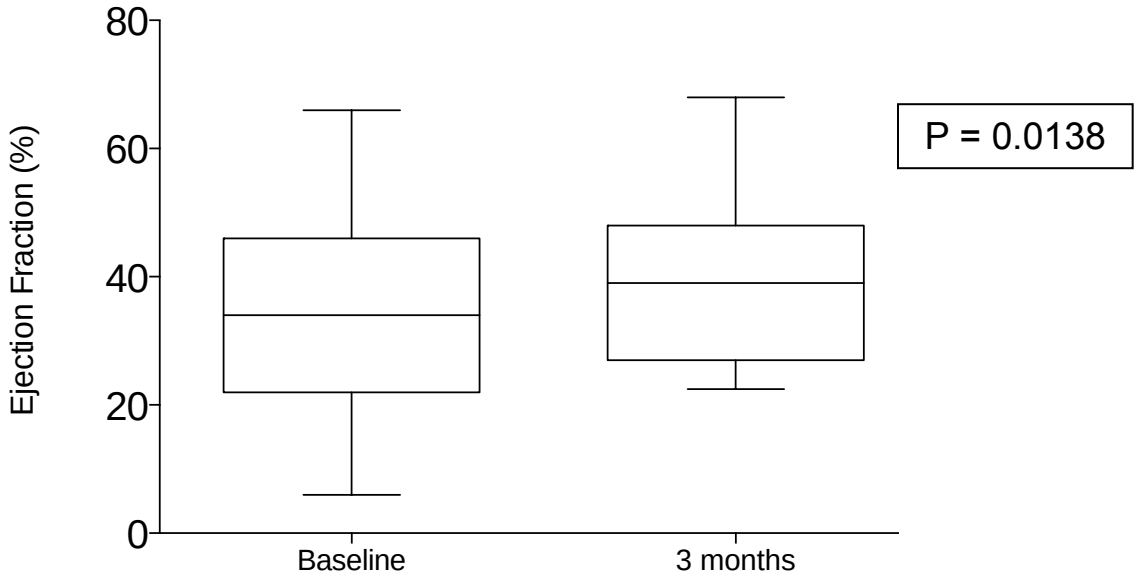
Peripheral G-CSF

Figure 4: Change in ejection fraction in peripheral G-CSF arm



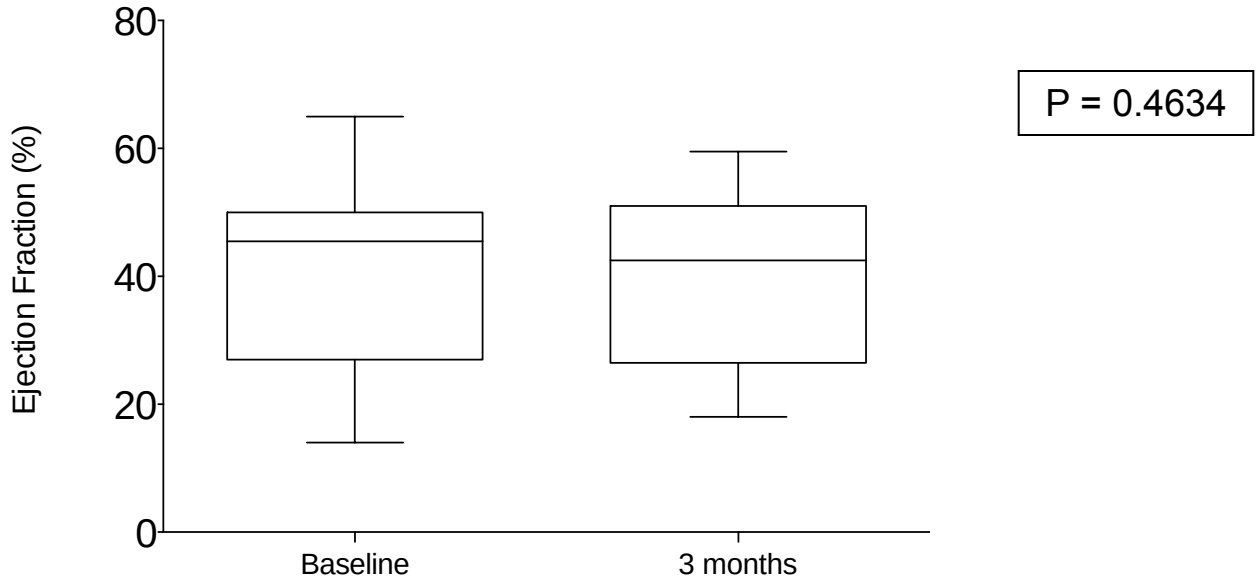
Peripheral Placebo

Figure 5: Change in ejection fraction in peripheral placebo arm



Intracoronary stem cells

Figure 6: Change in ejection fraction in intracoronary cell arm



Intracoronary serum

Figure 7: Change in ejection fraction in intracoronary serum arm

		Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value	ANOVA	ANOVA
Difference in Ejection Fraction (%)									
Difference	Mean (SEM)	0.1429 (2.151)	-1.071 (2.168)	0.6942	5.367 (1.908)	-1.100 (1.459)	0.0119	0.0433	0.0382

Table 12: REGENERATE DCM trial: Within group difference in ejection fraction

		Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value	p-value
End Diastolic Volume (mls)								
Baseline	Mean (SEM)	285.1 (31.80)	274.0 (16.90)	0.7616	253.8 (31.83)	226.7 (21.12)	0.5799	0.4202
3 month	Mean (SEM)	278.1 (35.38)	278.6 (15.58)	0.9883	254.2 (30.16)	247.3 (30.24)	0.8734	0.8156
p-value	Paired t test	0.2653	0.9828		0.9793	0.1236		

Table 14: REGENERATE-DCM trial: Change in end-diastolic volume at 3 months

		Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value	p-value
End Systolic Volume (mls)								
Baseline	Mean (SEM)	193.3 (29.51)	195.1 (14.11)	0.9573	176.1 (27.7)	139.5 (22.25)	0.3114	0.3401
3 month	Mean (SEM)	185.5 (29.24)	198.3 (12.33)	0.6912	164.7 (25.53)	156.9 (28.52)	0.8393	0.636
p-value	Paired t test	0.2207	0.8210		0.2290	0.0971		

Table 15: REGENERATE-DCM trial: Change in end-systolic volume at 3 months

		Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value	p-value
Stroke Volume (mls)								
Baseline	Mean (SEM)	91.77 (6.953)	79.03 (7.596)	0.2266	82.03 (11.81)	87.07 (8.134)	0.7282	0.7506
3 month	Mean (SEM)	92.57 (8.875)	80.39 (8.425)	0.3288	89.53 (7.192)	93.07 (6.062)	0.7100	0.6329
p-value	Paired t test	0.8728	0.8282		0.4076	0.2752		

Table 16: REGENERATE-DCM trial: Change in stroke volume at 3 months

		Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value	p-value
Myocardial Mass (g)								
Baseline	Mean (SEM)	196.3 (17.52)	226.7 (11.09)	0.1541	203.6 (18.65)	179.9 (20.35)	0.3965	0.2938
3 month	Mean (SEM)	214.7 (19.09)	236.5 (14.54)	0.3714	210.1 (18.57)	199.3 (21.91)	0.7106	0.5670
p-value	Paired t test	0.2152	0.439		0.5707	0.0126		

Table 17: REGENERATE-DCM trial: Change in myocardial mass at 3 months

6.4. Progenitor cell (CD34+) mobilisation with G-CSF

The bone marrow mononuclear cell fraction contains a heterogeneous population of cell types which includes hematopoietic progenitor (HSC) cells which are identified by the CD34 + marker[179,186]. The CD34 + HSCs as well as endothelial progenitor cells are thought to be the cell types most likely to promote cardiac repair through a number of mechanisms including neoangiogenesis, cell fusion and paracrine effect[184,185].

We have previously described how we performed flow cytometry and colony-forming unit analysis[186]. Briefly, HSC populations were identified by incubating cells with the

fluorescein isothiocyanate (FITC)-labeled antibody against human CD45 (BD Biosciences) and the phycoerythrin (PE)-labeled antibody against human CD34 (BD Biosciences) for 15 min at room temperature. EPCs were analyzed by initially incubating samples with mouse serum IgG (Sigma) for 15 min at 4 °C with a cocktail of antibodies comprising the allophycocyanin-labeled antibody to CD133 (Miltenyi Biotec) and the PE-labeled antibody to VEGFR-2 (R&D Systems) to characterize EPCs and FITC labeled monoclonal antibodies to CD2, CD13, and CD22 (Beckman Coulter) to identify, and therefore, eliminate inclusion of lineage-negative non-progenitor cells. To ensure exclusion of nonviable cells in the final EPC count, cells were also incubated with a PerCP-Cy5-labeled 7AAD stain (BD Biosciences). Cells were then incubated for 15 min at room temperature with 2mL of the Pharm Lyse **-** buffer (BD Biosciences) to lyse red blood cells. Samples were washed once in phosphate-buffered saline and 20 **m**L of Accucount flow cytometry beads (Saxon Europe) were added before analysis.

Day 0 PB MNCs (2 x 10^5 per dish), and Day 6 PB MNCs (2 x 10^4 per dish) were seeded, in triplicate preparations, in methylcellulose plates (Methocult H4534, including stem cell factor, granulocyte-macrophage colony stimulating, and interleukin-3, Stem cell Technologies). Plates were studied under phase contrast microscopy, and granulocyte-macrophage colony- forming units (CFU-GM; colonies > 50 cells) were counted after 14 days of incubation. Results were taken from the mean of the triplicate results and presented as a ratio of CFU per CD34 cell plated.

Effects of G-CSF on mobilization

Recombinant human G-CSF (Granocyte_; Chugai Pharma) was administered subcutaneously at a dose of 10 **m**g/Kg/day for 5 consecutive days to patients enrolled in the REGENERATE-DCM. Patients in the control group received saline injections. A peripheral blood sample was obtained on days 0, 1, 2, 3 and 6 for estimation of peripheral progenitor cell counts.

G-CSF treatment led to a significant increase in the peripheral concentration of CD34+ cells (day 6 concentration of 56.79 CD34U/**m**L from baseline of 3.9 CD34U/**m**L; P < 0.0001) (figure 5). There was no significant increase in cell concentration in patients who received saline injection (day 6 concentration of 4.79 CD34U/**m**L from baseline of 4.64 CD34U/**m**L; P = 0.514) (figure 6). In the G-CSF group, the mean difference in CD34+ concentration between day 0 and day 6 was 52.53 CD34U/**m**L compared to 0.3629 CD34U/**m**L in the placebo group (P < 0.0001) (table 6).

Effects of disease state on CD34+ function and number

When compared to patients with ischemic heart disease (IHD), DCM patients had a higher concentration of peripheral blood CD34+ (3.4 ± 2.1 CD34 + U/ μL vs. 2.6 ± 1.9 CD34 + U/ μL, P = 0.033). The increase in CD34+ concentration post 5 days of G-CSF injection was comparable in DCM and IHD patients[186]. This is consistent with previous reports showing circulating EPCs inversely correlate with number of risk factors for coronary artery disease[187]. Although there was a higher concentration of circulating CD34 + cells in DCM patients, the CFU analysis suggested that there was no difference

in functional capability[186,258-260].

In both DCM and IHD patients, we found an inverse relationship between age and peripheral blood CD34 + cell concentration. Older patients had a lower concentration of CD34 + cells in the bone marrow following mobilization with G-CSF (75.49 – 64.49 CD34 U/**m**L vs. 113.5 – 61.03, P = 0.0023). There was also a trend (P = 0.09) toward lower percentage increase in circulating CD34 + cells (day 6 compared to day 0) with G-CSF in those aged above 64 years[186,261].

The difference in circulating progenitor cells between IHD and DCM has not previously been demonstrated. Our findings suggest that the atherosclerotic process rather than chronicity or severity of disease is the important determinant of the progenitor cell concentration.

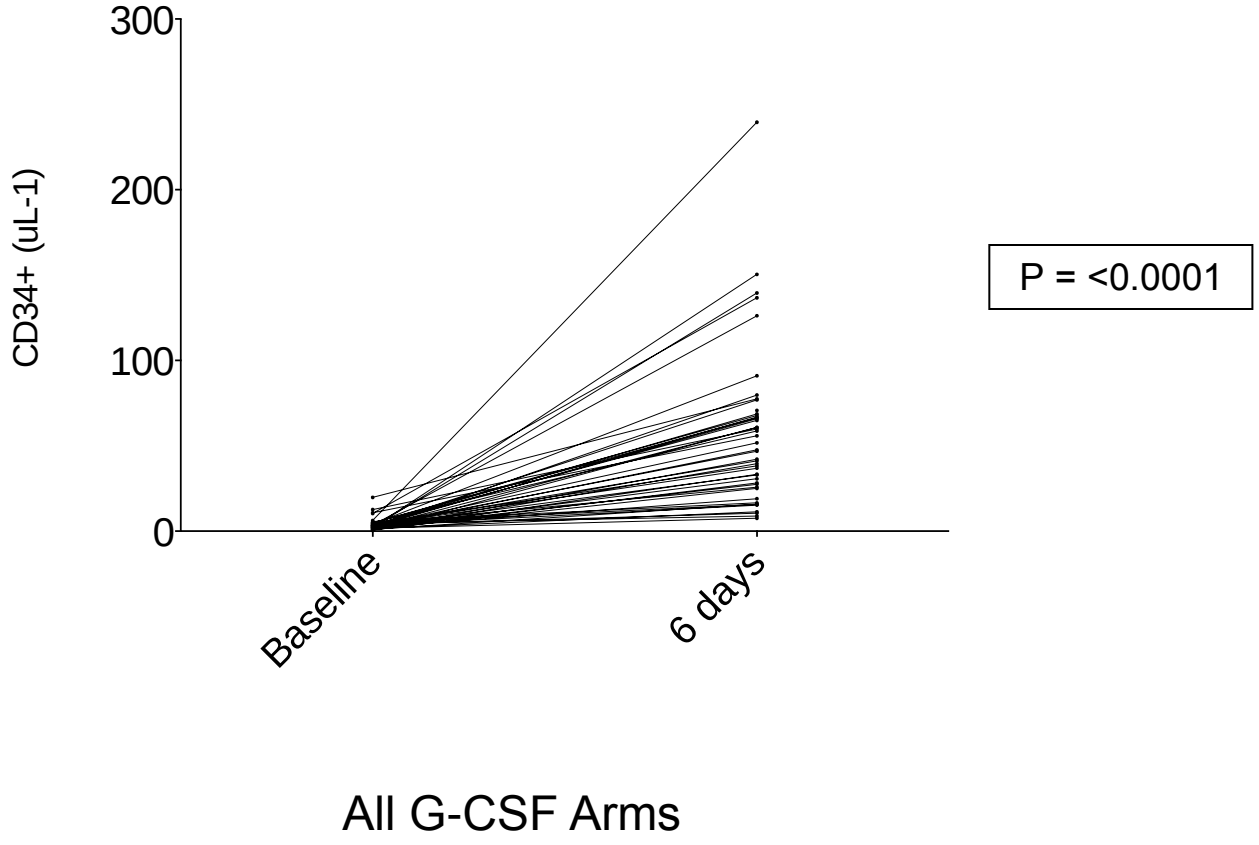


Figure 8: REGENERATE-DCM trial: Change in CD34^{+} in the peripheral and intervention G-CSF arm

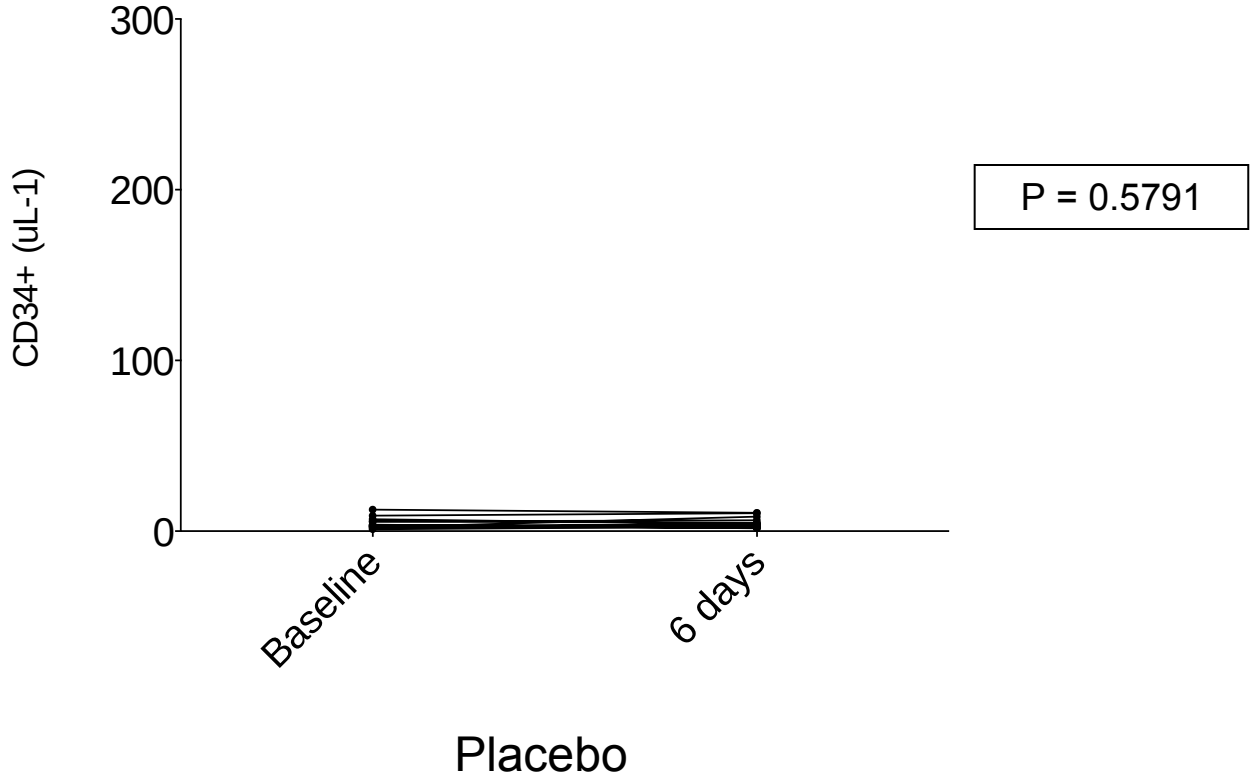


Figure 9: REGENERATE-DCM trial: Change in $CD34^+$ in the placebo arm

		All G-CSF Arms	Placebo	p-value
CD34$^+$ (uL-1)				
Baseline	Mean (SEM)	3.939 (0.5340)	4.637 (0.8549)	0.5140
6 days	Mean (SEM)	56.79 (6.658)	4.795 (0.7835)	< 0.0001
P-value	Paired t test	< 0.0001	0.5791	
CD34$^+$ (uL-1)				
Difference	Mean (SEM)	52.53 (6.695)	0.3629 (0.6378)	< 0.0001

SEM; Standard error of mean

Table 13: REGENERATE-DCM trial: CD34+ concentration at baseline and 6 days in the G-CSF and placebo arms

6.5. Effect of intracoronary stem cell therapy on heart failure and angina symptoms

The change in New York Heart Association (NYHA) heart failure symptoms at 3 months in the 4 groups is summarised in tables 12 and 13. There was no significant difference in NYHA functional classification at baseline between the 4 groups (Mean 2.07, P = 0.2). There was no significant change in NYHA functional class at 3 months in the peripheral placebo, peripheral G-CSF and intracoronary serum groups. In the peripheral placebo group, the mean NYHA increased from 2.4 at baseline to 2.429 at 3 months (P=0.08), and in the peripheral G-CSF group decreased from 2.267 at baseline to 2.0 at 3 months (P=0.3). In the intracoronary serum group, the mean NYHA was 2.3 at baseline and 3 month follow-up (P>0.99). There was however, a significant improvement in heart failure symptoms in the intracoronary stem cell group with a reduction of NYHA functional class from 2.6 at baseline to 2.067 at 3 months (P=0.0061). Six patients (40%) in the intracoronary stem cell group had an improvement by 1 stage in NYHA functional classification (5 patients improved from NYHA 3 to 2, and one patient form NYHA 3 to1). None of the patients in the intracoronary stem cell group had worsening of their symptoms at 3 month follow-up. In the intracoronary placebo group, 1 (6.7%) of the patients had a reduction in NYHA functional class from 2 to1, and 4 (26.7%) patients had worsening of their symptoms from NYHA 2 to 3. None of the patients in the peripheral placebo group had an improvement in functional class, while 1 (7.1%) patient's symptom worsened from NYHA 2 to 3. By comparison, 3 (21.4%) patients NYHA functional class improved (2

patients improved from NYHA 2 to 1, and 1 patient from NYHA 3 to 2), and 1 patients symptoms worsened form NYHA 2 to 3.

There was a significant within group improvement in symptoms in the intracoronary stem cell group that was not seen in any of the other groups.

A significant proportion of patients had angina at baseline (6.7%, 33.3%, 40% and 20% in the peripheral G-CSF, peripheral placebo, intracoronary stem cells and intracoronary serum respectively; P= 0.29) (table 3). There was a significant within group improvement in angina symptoms (mean reduction of CCS classification of 0.33) in the intracoronary stem cell group compared to the 3 other groups (P= 0.029) (table 14).

Figure 10: REGENERATE-DCM trial: Change in NYHA functional class

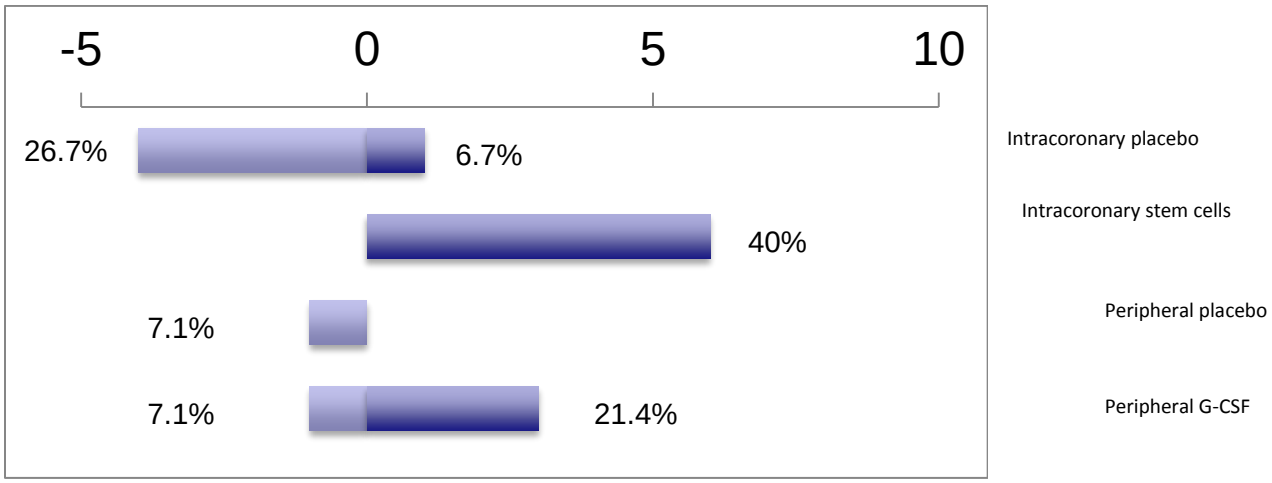


Figure legend: Total number (15 in each group; -5 to 10) and % of patients that had a change in NYHA functional class in each group.

Table 18: REGENERATE-DCM trial: Mean change in NYHA functional class

		Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem cell	IC Serum	p-value	ANOVA
NYHA								
Baseline	Mean (SEM)	2.267 (0.1182)	2.400 (0.1309)	0.456	2.600 (0.1633)	2.333 (0.1260)	0.2066	0.5125
3 month	Mean (SEM)	2.000 (0.1482)	2.429 (0.1373)	0.0436	2.067 (0.2063)	2.333 (0.2108)	0.3736	0.3007
	p-value	0.3356	0.0823		0.0061	> 0.9999		
NYHA							**p-value IC arms**	
Difference	Mean (SEM)	-0.1429 (0.1429)	0.07143 (0.07143)		-0.4667 (0.1652)	0.2000 (0.1447)	0.0052	

Table 19: REGENERATE-DCM trial: Mean change in CCS functional class

		Peripheral G-CSF	Peripheral Placebo	IC Stem cell	IC Serum	Chi Sq
CCS						
Improved	Number (%)	0 (0%)	1 (7.1%)	3 (20%)	0 (0%)	0.1234
Unchanged	Number (%)	13 (92.9%)	10 (21.4%)	12 (80%)	12 (80%)	10.03, 6
Worsened	Number (%)	1 (7.1%)	3 (21.4%)	0 (0%)	3 (20%)	
CCS						**P-value IC arms**
Difference	Mean (SEM)	0.1333 (0.1333)	0.07143 (0.1951)	-0.3333 (0.2108)	0.2667 (0.1533)	0.029

6.6. Change in BNP at 3 months

The baseline bloods were similar between the 4 groups (table 15). The mean plasma B-type Natriuretic Peptide (BNP) at baseline was 1346 pg/ml with no significant difference between the 4 groups (P=0.7). The high level of BNP at baseline is a marker of the severity of heart failure in this cohort of patients. Despite an improvement in ejection fraction, there was no significant change in BNP at 3 months in the intracoronary stem cell group (964.7 baseline, 1106 at 3 months; P=0.67). However, in one of the patients, the BNP increased from 1548 to 6050. This patient was admitted to hospital with an

acute exacerbation of Gout and deranged liver function prior to his 3 month follow-up, which likely would have accounted for the otherwise unexplained increase in BNP. His ejection fraction increased from 20% to 25% at 3 months. When this patient is removed from the BNP analysis, we found a trend towards a significant reduction in 3 month BNP in the intracoronary stem cell group from 964.7 to 752.7 (P= 0.0565), and an inverse correlation between plasma BNP levels and ejection fraction (figure 7).

		Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value	ANOVA	ANOVA
BNP									
Baseline	Mean (SEM)	1640 (508.1)	1303 (318.6)	0.5792	964.7 (240)	1474 (566.7)	0.4039	0.8163	0.6447
3 month	Mean (SEM)	1411 (517.8)	1424 (400.7)	0.9834	1106 (408.1)	1124 (394.7)	0.9745	0.9994	0.8432
p-value	Paired t test	0.1447	0.7619		0.6665	0.2094			

Table 20: REGENERATE-DCM trial: Change in plasma B-type Natriuretic Peptide (BNP)

		Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value	ANOVA	ANOVA
BNP									
Baseline	Mean (SEM)	1640 (508.1)	1303 (318.6)	0.5792	964.7 (240)	1474 (566.7)	0.4039	0.8163	0.6447
3 month	Mean (SEM)	1411 (517.8)	1424 (400.7)	0.9834	752.7 (219.7)	1124 (394.7)	0.427	0.4254	0.7584
p-value	Paired t test	0.1447	0.7619		0.0565	0.2094			

Table 21: REGENERATE-DCM trial: Change in plasma B-type Natriuretic Peptide (BNP) after removal of outlier in the IC stem cell group

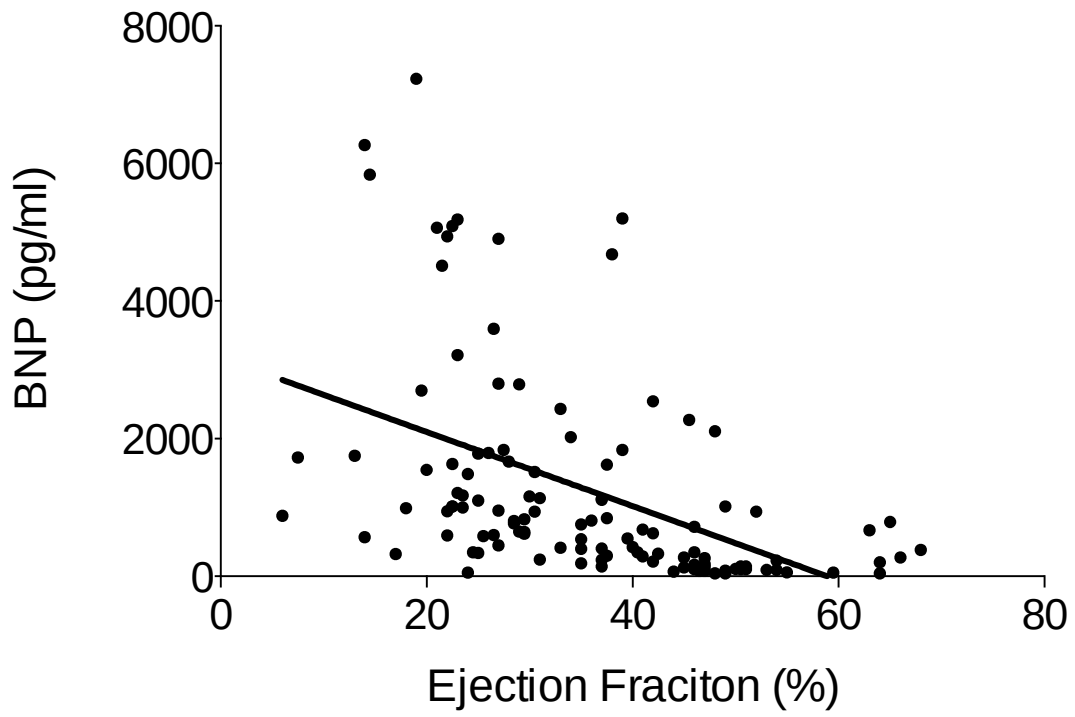


Figure 11: REGENERATE-DCM trial: Ejection fraction correlation with B-type Natriuretic Peptide (BNP)

6.7. Changes in exercise functional capacity at 3 months

Patients with chronic heart failure have a reduced maximal exercise capacity characterized by the reduction in oxygen consumption at peak exercise[262].

Determination of VO2 max (which determines the limits of the cardiopulmonary system) during exercise has been shown to predict prognosis in these patients [263].

When VO2max was ≤14.5 ml/kg per min, survival rates were significantly less than in patients with VO2max >14.5 ml/kg per min[263], and patients who achieve > 50% predicted VO2 max have an excellent short-term prognosis[264].

The mean peak VO_2 in our cohort of patients at baseline was 18.7 ml/kg/min. This is probably due to the fact that the patients were young (mean age 54) and therefore had a good exercise threshold. The mean peak VO_2 increased to 19.2 ml/kg/min at 3 months with no significant difference observed between the 4 groups. However, 13 patients failed to reach peak exercise threshold at baseline and 6 patients failed to reach peak exercise threshold at 3 months, an additional 2 patients withdrew and 1 patient was not allowed to perform the test due to clinical issues. Therefore a total of 22 patients (36.6%) failed to have appropriate paired testing. The baseline mean peak VO2 in the peripheral G-CSF group was 18.8 (64.47% predicted), which increased to 19.16 (66.85 % predicted) at 3 months, and the mean baseline peak VO2 in the peripheral placebo group was 18.9 (66.21 % predicted), which increased to 17.9 (73.64 % predicted) at 3 months (P=NS). The mean baseline peak VO2 in the IC stem cell group was 17.6 (60.21 % predicted), which increased to 19.1 (63.33 % predicted) at 3 months, and the mean baseline peak VO2 in the IC serum group was 19.5 (69.93% predicted), which increased to 20.6 (73.64 % predicted) at 3 months (P=NS) (Tables 17 and 18).

The mean baseline VE/VCO2 in the peripheral G-CSF group was 32.4 which increased to 31.3 at 3 months, and the mean baseline VE/VCO2 in the peripheral placebo group was 36.1 which increased to 38.2 at 3 months; P=NS. The mean baseline VE/VCO2 in the IC stem cell group was which increased to 39.6 at 3 months, and the mean baseline VE/VCO2 in the IC serum group was 32.9 which increased to 34 at 3 months; P=NS. Furthermore, comparison of the changes in the exercise parameters at baseline and 3 months showed no significant difference between the 4 groups.

As maximum effort is not always achieved during exercise testing, a widely used submaximal index of exercise capacity is the anaerobic threshold (AT). AT usually occurs between 45%-65% of measured peak VO2, but it occurs lower in patients with heart failure[265]. Measurement of anaerobic threshold (AT) during exercise testing is based on the principle that energy production shifts from an aerobic metabolism to a metabolism that combines both anaerobic and aerobic patterns during a progressively increasing workload exercise. The value of oxygen uptake at AT has been used to assess the severity of heart failure[266] as it is independent of patients motivation and exercise duration[267]. However, AT is not identifiable in a large proportion of patients with heart failure[268], and this is associated with a worse prognosis[269].

The mean baseline anaerobic threshold (AT) in the peripheral G-CSF group was 12.8 which increased to 14.1 at 3months, and the mean baseline AT in the peripheral placebo group was 12.8 which reduced to 12.4 at 3 months; P=NS. The mean baseline AT in the IC stem cell group was 12.8 which reduced to 12.2 at 3 months, and the mean baseline AT in the IC stem cell group was 13.99 which reduced to 12.3; P=NS.

Achievement of ≥ 85% of maximal age-predicted target heart is a well-recognized indicator of sufficient subject effort during exercise. However, the variability of maximal heart rate and the wide use of B-blockers in heart failure population undermine the effectiveness of to gauge subject effort by their heart response alone. The peak respiratory exchange ratio (RER), defined as carbon dioxide production divided by oxygen consumption, is used to quantify exercise exertion level and obviates the need to assess the subjects heart rate.. It is the most accurate and reliable gauge of exercise effort[266]. A peak RER of ≥ 1.10 is considered a universal indicator that reflects maximal

exertion independent of patient characteristics such as age, sex, fitness, and disease state. Achievement of a peak RER <1.00 that is terminated by subject request, in the absence of any electrocardiographic or hemodynamic abnormalities, generally reflects submaximal cardiovascular effort[266]. A significant change in exercise capacity after specific intervention with similar peak RER values suggests that the observed changes are secondary to the intervention used. Exercise duration (i.e. the number of minutes the subject can continue on the exercise treadmill) is a good measure of the functional capacity and is a predictor of mortality. In fact, of the prognostic variables measured during exercise treadmill testing, exercise duration is the strongest[270,271]. Exercise duration decreases with age and is lower in women compared to men, but it retains its prognostic value after adjusting for age and sex. Functional capacity can be measured by measuring oxygen uptake during exercise, which can be converted to metabolic equivalents (METs): 1 MET = 3.5 mL O_2/kg/min. In patients with ischemic heart disease post revascularization, exercise capacity has been found to be the most powerful predictor of subsequent outcome, particularly mortality. For every 1MET increase there was a 27% reduction in mortality[272].

In the REGENERATE-DCM trial, there was a significant improvement in exercise duration at 3 months in the intracoronary stem (IC) cell group compared to the intracoronary serum group. The mean baseline exercise time (ET) at baseline in the IC serum group was 477.1 seconds (s) compared to 424.1s in the IC stem cell group (P = 0.43). At 3 months, the mean ET in the IC serum group increased to 506.7s, compared to 504s in the IC stem cell group. The mean increase in ET was significantly higher in the IC stem cell group (P= 0.0146). There was no significant difference in the mean ET

between the 2 peripheral groups; baseline mean ET 476.9s and 458.3s in the GCSF and placebo groups respectively, which increased to 460.4s and 402.9s at 3 months (P= 0.1368). The difference in oxygen consumption at 3 months was -1.9, -4.9, -3.5 and +8.2 METs in the peripheral G-SCF, peripheral placebo, IC serum and IC stem cells respectively. Although there was a trend towards to an improvement in oxygen consumption (MET) in the IC stem cell group, this did not reach statistical significance.

Peak VO_2 (ml/kg/min)		**Peripheral G-CSF**	**Peripheral Placebo**	**p-value**	**IC Stem Cells**	**IC Serum**	**p-value**
Baseline	Mean (SEM)	18.83 (1.400)	18.99 (0.9087)	0.9226	17.67 (1.488)	19.55 (1.816)	0.4316
3 month	Mean (SEM)	19.16 (1.642)	17.97 (0.9487)	0.5398	19.13 (1.940)	20.61 (2.442)	0.638
P-value	Paired t test	0.5834	0.2181		0.1677	0.2571	

Table 22: REGENERATE-DCM trial: Change in VO2 max at 3 months

		Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value
VO_2 % predicted (%)							
Baseline	Mean (SEM)	64.47 (5.142)	66.21 (4.648)	0.8038	60.21 (4.302)	69.93 (4.638)	0.1375
3 month	Mean (SEM)	66.85 (6.676)	63.54 (4.646)	0.6879	63.33 (6.159)	73.64 (6.377)	0.255
P-value	Paired t test	0.9444	0.4303		0.3437	0.1211	

Table 23: REGENERATE-DCM trial: Change in VO2% predicted at 3 months

		Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value
VE/VCO_2							
Baseline	Mean (SEM)	32.47 (1.788)	36.17 (2.592)	0.2443	34.86 (2.469)	32.93 (1.572)	0.5107
3 month	Mean (SEM)	31.29 (1.450)	38.23 (3.207)	0.0539	39.60 (4.232)	34.00 (1.541)	0.2371
p-value	Paired t test	0.4297	0.1114		0.1359	0.2042	

Table 24: REGENERATE-DCM trial: Change in VE/VCO2 at 3 months

		Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value
Anaerobic Threshold (mls/kg/min)							
Baseline	Mean (SEM)	12.82 (0.9203)	12.86 (0.9869)	0.9791	12.81 (0.7933)	13.99 (1.215)	0.4477
3 month	Mean (SEM)	14.12 (1.354)	12.43 (1.478)	0.4127	12.21 (1.326)	12.34 (1.506)	0.9479
p-value	Paired t test	0.3796	0.4855		0.9522	0.0952	

Table 25: REGENERATE-DCM trial: Change in Anaerobic Threshold at 3 months

		Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value
Saturations (%)							
Baseline	Mean (SEM)	97.80 (0.3928)	98.00 (0.3780)	0.7173	98.93 (0.2282)	97.93 (0.1533)	0.0011
3 month	Mean (SEM)	97.43 (0.3882)	97.69 (0.2083)	0.5635	98.53 (0.3065)	98.36 (0.3249)	0.6961
p-value	Paired t test	0.6198	0.5016		0.2711	0.2123	

Table 26: REGENERATE-DCM trial: Change in Oxygen saturation at 3 months

	Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value ANOVA	ANOVA
Peak VO_2 (ml/kg/min)							
Mean *Difference* (SEM)	0.5143 (0.9145)	-1.092 (0.8404)	0.2097	1.460 (1.003)	0.9143 (0.7712)	0.6729	0.0961	0.8861
VO2 % predicted (%)							
Mean *Difference* (SEM)	0.2308 (3.239)	-2.462 (3.016)	0.5487	3.857 (3.925)	3.929 (2.368)	0.9877	0.308	0.9998
VE/VCO2							
Mean *Difference* (SEM)	-0.6429 (0.7887)	2.046 (1.191)	0.0678	7.067 (3.592)	1.071 (0.8015)	0.1264	0.1813	0.0851
Anaerobic Threshold (mls/kg/min)							
Mean *Difference* (SEM)	0.8727 (0.9494)	-0.8917 (1.235)	0.2765	-0.07273 (1.184)	-1.821 (1.012)	0.2712	0.8479	0.4551
Saturations (%)							
Mean *Difference* (SEM)	-0.2143 (0.4216)	-0.1538 (0.2221)	0.9023	-0.4000 (0.3491)	0.4286 (0.3267)	0.0957	0.8508	0.1668

Table 27: REGENERATE-DCM trial: Differences in Peak VO2, VO2% predicted, VE/VCO2, Anaerobic Threshold and oxygen saturation between baseline and 3 months

Exercise time		Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value	ANOVA	ANOVA
Baseline	Mean (SEM)	476.9 (39.39)	458.3 (35.23)	0.7275	424.1 (47.30)	477.1 (47.60)	0.4368	0.8181	0.6212
3 month	Mean (SEM)	460.4 (52.41)	402.9 (53.89)	0.451	504.0 (61.70)	506.7 (59.17)	0.9753	0.383	0.9993
p-value	Paired t test	0.6836	0.1368		0.0146	0.4471			

Exercise time difference		Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value	ANOVA	ANOVA
Difference	Mean (SEM)	-16.53 (39.73)	-55.40 (35.10)	0.4696	79.87 (28.68)	29.60 (37.84)	0.2988	0.0188	0.5405

s

Table 28: REGENERATE-DCM trial: The difference in exercise duration at baseline and 3 month

6.8. Secondary Endpoint: Quality of life

I used the EQ5D and the VAS questionnaires to assess our patients Quality of life at baseline and at 3 months. The EuroQOL five dimensions questionnaire (EQ-5D) is one of the most commonly used generic questionnaires to measure health-related quality of life, which encompasses both positive (well-being) and negative aspects (illness). It consists of a questionnaire and a visual analogue scale (EQ-VAS). The EQ-5D is a self-reported description of the subject's current health in 5 dimensions that include mobility, self-care, usual activities, pain/discomfort and anxiety/depression. Each dimension has 3 levels: no problems, some problems, severe problems. The respondent is asked to indicate his/her health state by ticking in the box against the most appropriate statement in each of the 5 dimensions. This decision results in a 1-digit number expressing the level selected for that dimension. The EQ-VAS records the subject's perceptions of their own current overall health and can be used to monitor changes with time. It records the respondent's self-rated health on a vertical, visual analogue scale where the endpoints are labeled 'Best imaginable health state' and 'Worst imaginable health state'. This information can be used as a quantitative measure of health outcome as judged by the individual respondents.

In the REGENERATE-DCM trial, we found no significant change in the quality of life as judged by the EQ-5D and VAS questionnaires (tables 23 and 24).

EQ5D Index Score		Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value	p-value	p-value
Baseline	Mean (SEM)	0.4915 (0.08969)	0.6753 (0.08192)	0.1415	0.5748 (0.06290)	0.7311 (0.05716)	0.0762	0.5761	0.2728
3 month	Mean (SEM)	0.6665 (0.07500)	0.6847 (0.09520)	0.8849	0.5492 (0.07536)	0.6901 (0.06313)	0.1605	0.3962	0.3439
p-value	Paired t test	0.1194	0.8408		0.6541	0.5056			
		Peripheral G-CSF	**Peripheral Placebo**	**p-value**	**IC Stem Cells**	**IC Serum**	**p-value**	**p-value**	**p-value**
EQ5D Index Score									
Difference	Mean (SEM)	0.1132 (0.06647)	0.01315 (0.06409)	0.2923	-0.03008 (0.06546)	-0.0410 (0.06000)	0.9029	0.8666	0.9902

Table 29: REGENERATE-DCM trial: EQ5D Index score at baseline and 3 months

		Peripheral G-CSF	Peripheral Placebo	p-value	IC Stem Cells	IC Serum	p-value	p-value	
VAS									
Baseline	Mean (SEM)	55.07 (5.464)	57.27 (4.096)	0.7497	53.21 (3.997)	56.93 (4.126)	0.5238	0.7773	0.5941
3 month	Mean (SEM)	67.73 (4.489)	57.08 (3.574)	0.0735	58.46 (5.948)	60.07 (4.100)	0.8222	0.8815	0.8154
p-value	Paired t test	0.2553	0.4900		0.4308	0.3139			
		Peripheral G-CSF	**Peripheral Placebo**	**p-value**	**IC Stem Cells**	**IC Serum**	**p-value**	**p-value**	**p-value**
VAS									
Difference	Mean (SEM)	7.636 (6.328)	2.385 (3.348)	0.4515	4.231 (5.189)	3.133 (3.000)	0.8514	0.9469	0.9795

Table 30: REGENERATE-DCM trial: EQ5D Index score at baseline and 3 months

6.9. Results of the Biomarker sub-study

I measured the pro-inflammatory cytokines detailed in this section in all 4 groups at day 0 and day 6. There was no significant change in the plasma levels of the cytokines at these time points (graphs below).

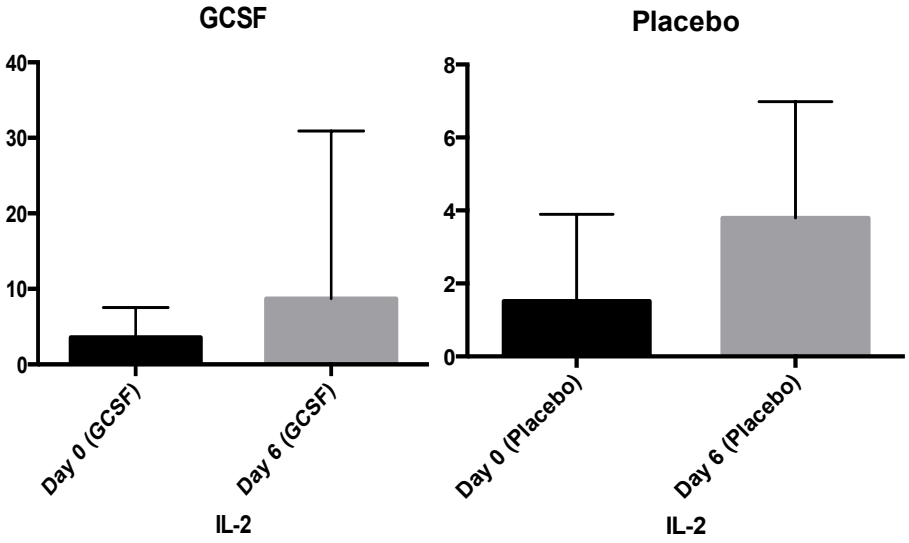


Figure 12A: REGENERATE-DCM trial: Change in plasma IL-2 at day 0 and day 6 in the peripheral G-CSF and placebo group

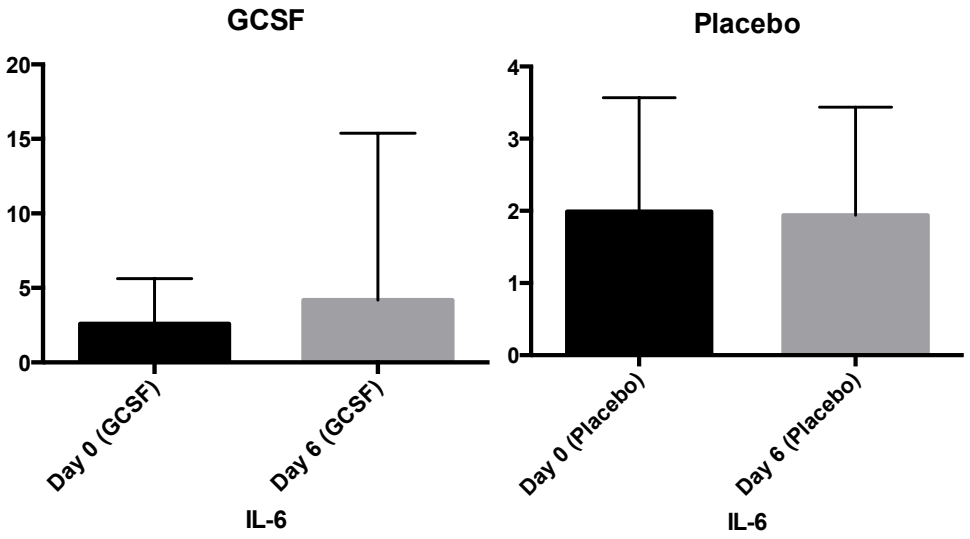


Figure 12B: REGENERATE-DCM trial: Change in plasma IL-6 at day 0 and day 6 in the peripheral G-CSF and placebo group

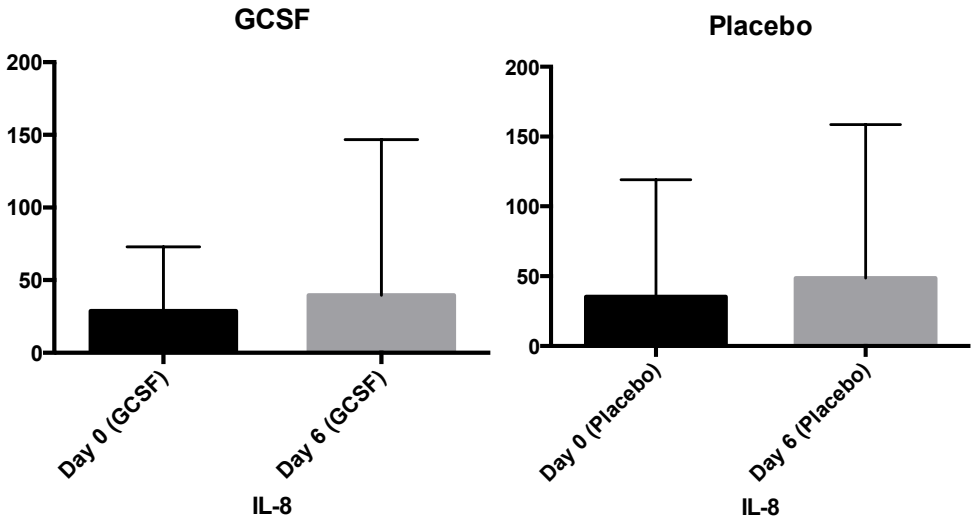


Figure 12C: REGENERATE-DCM trial: Change in plasma IL-8 at day 0 and day 6 in the peripheral G-CSF and placebo group

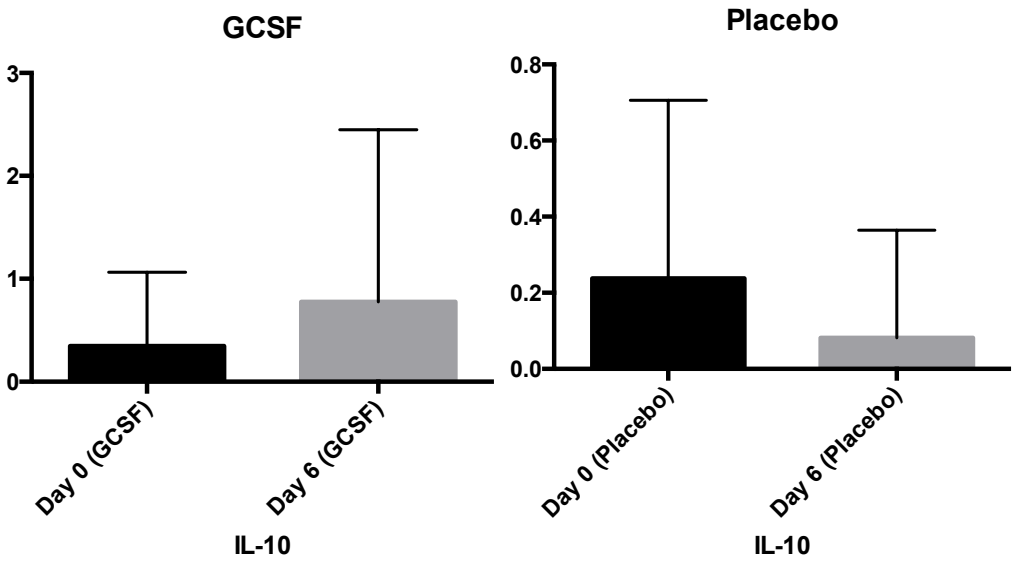


Figure 12D: REGENERATE-DCM trial: Change in plasma IL-10 at day 0 and day 6 in the peripheral G-CSF and placebo group

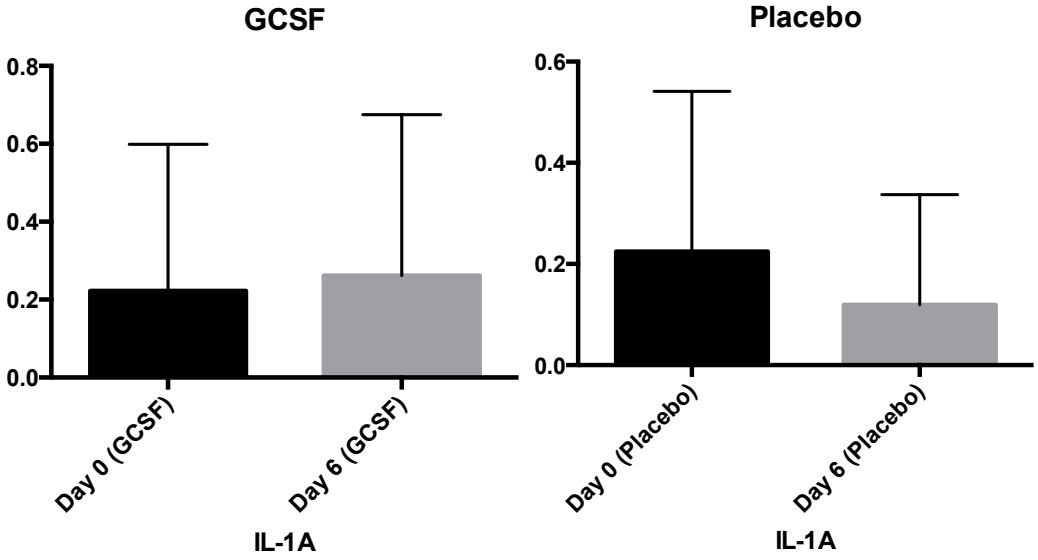


Figure 12E: REGENERATE-DCM trial: Change in plasma IL-1A at day 0 and day 6 in the peripheral G-CSF and placebo group

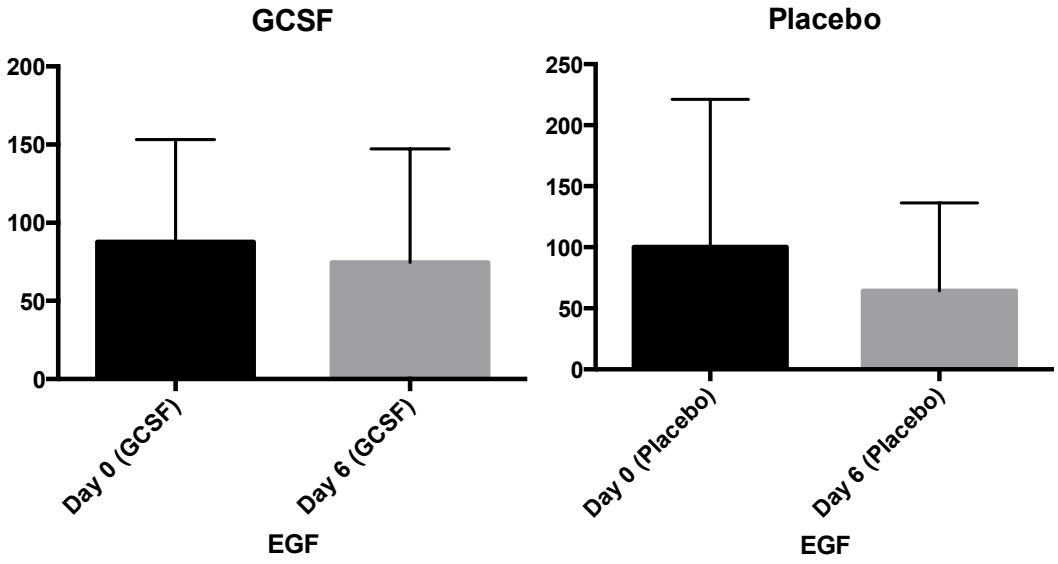


Figure 12E: REGENERATE-DCM trial: Change in plasma EGF at day 0 and day 6 in the peripheral G-CSF and placebo group

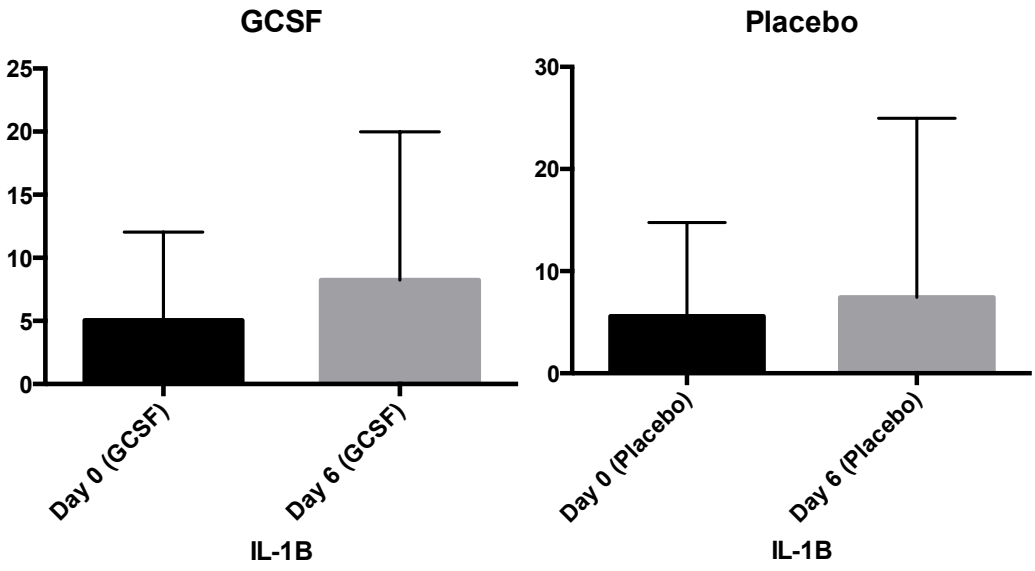


Figure 12F: REGENERATE-DCM trial: Change in plasma IL-1B at day 0 and day 6 in the peripheral G-CSF and placebo group

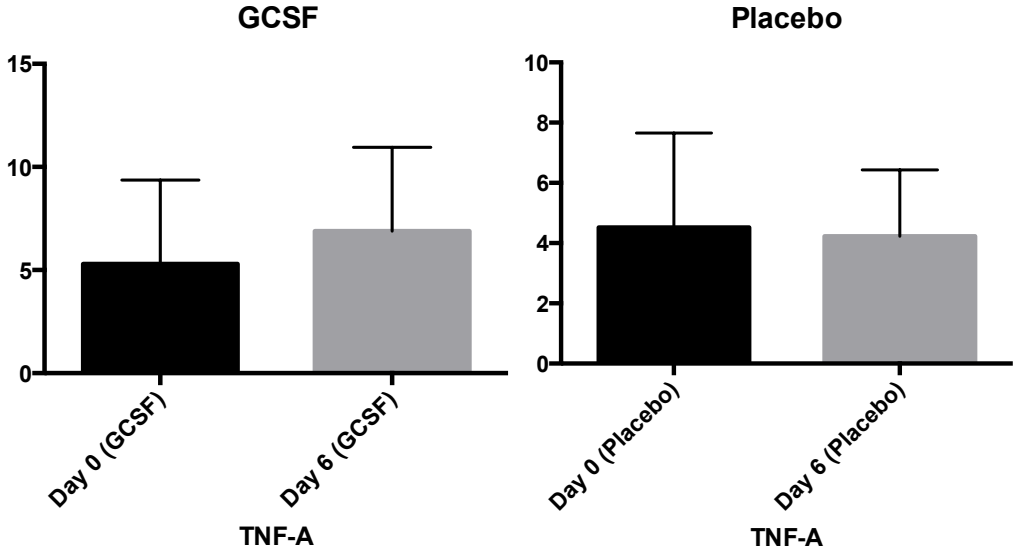


Figure 12G: REGENERATE-DCM trial: Change in plasma TNF-A at day 0 and day 6 in the peripheral G-CSF and placebo group

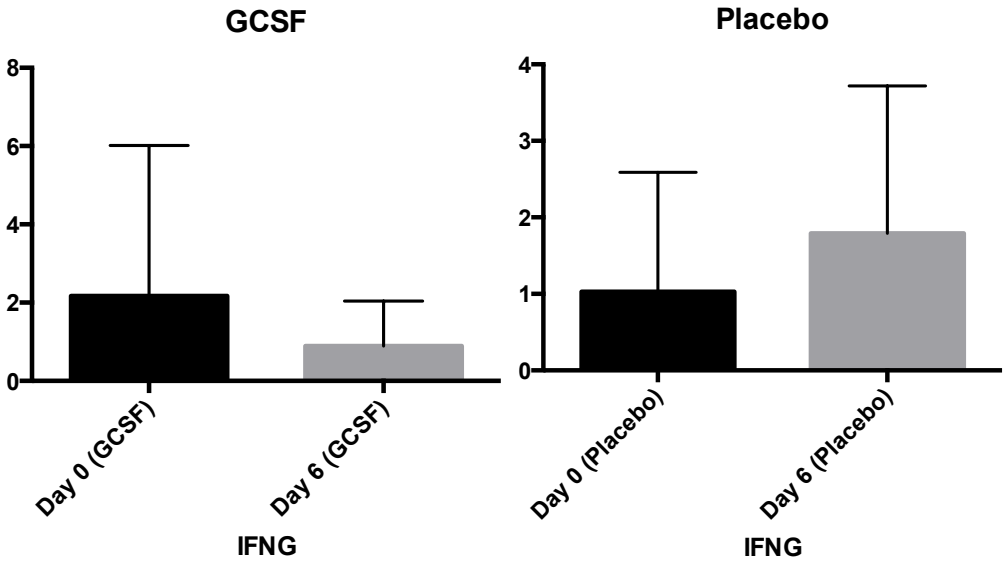


Figure 12H: REGENERATE-DCM trial: Change in plasma IFNG at day 0 and day 6 in the peripheral G-CSF and placebo group

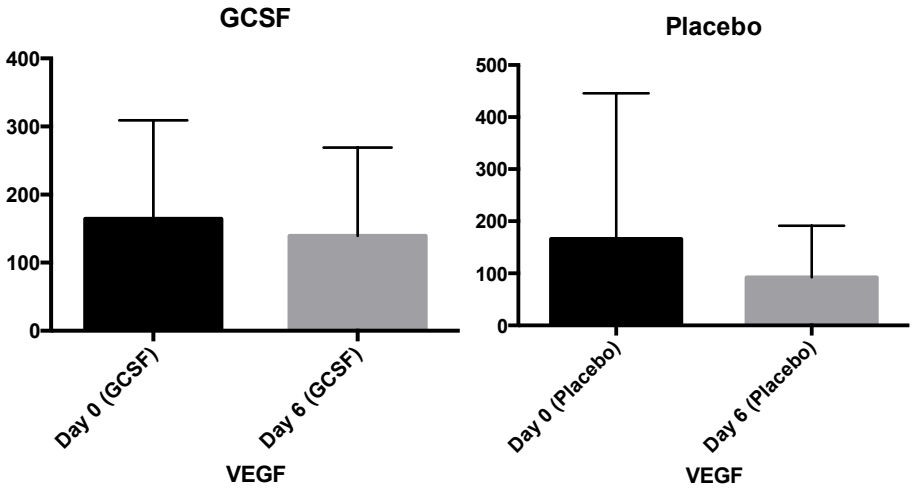


Figure 12 I: REGENERATE-DCM trial: Change in plasma VEGF at day 0 and day 6 in the peripheral G-CSF and placebo group

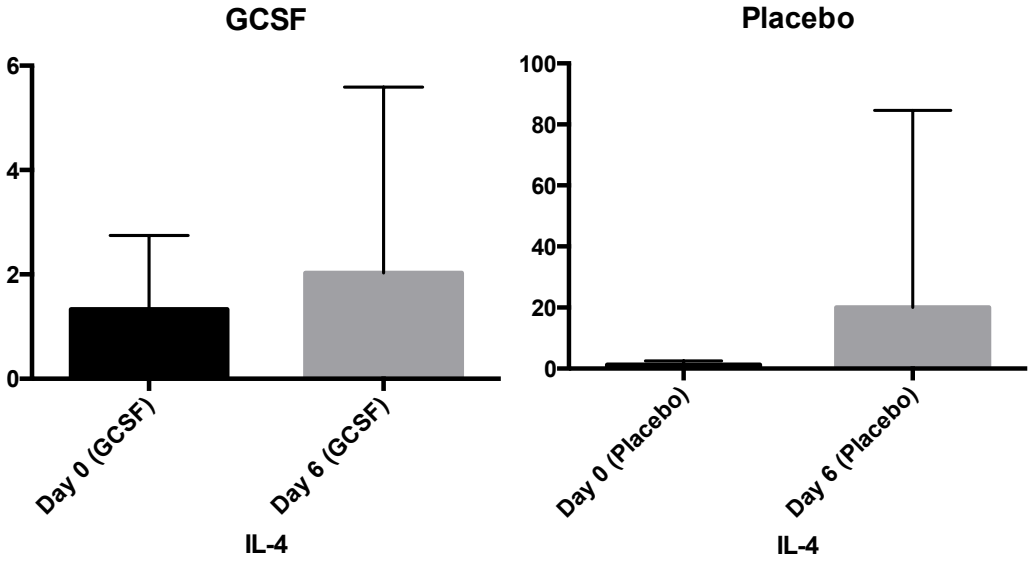


Figure 12 J: REGENERATE-DCM trial: Change in plasma IL-4 at day 0 and day 6 in the peripheral G-CSF and placebo group

6.10. Trial Safety:

There were no MACE events recorded during intracoronary injection of stem cells or saline. One patient experienced chest pain and ECG changes and one patient experienced dissection of the right coronary artery during intracoronary infusion of stem cells. This required treatment with a coronary stent with no further adverse sequelae. Both intracoronary events did not reach protocol stated rise in CK to be ruled as myocardial infarction. There were no significant differences in reported serious adverse events, adverse events or hospital admissions between the 4 groups.

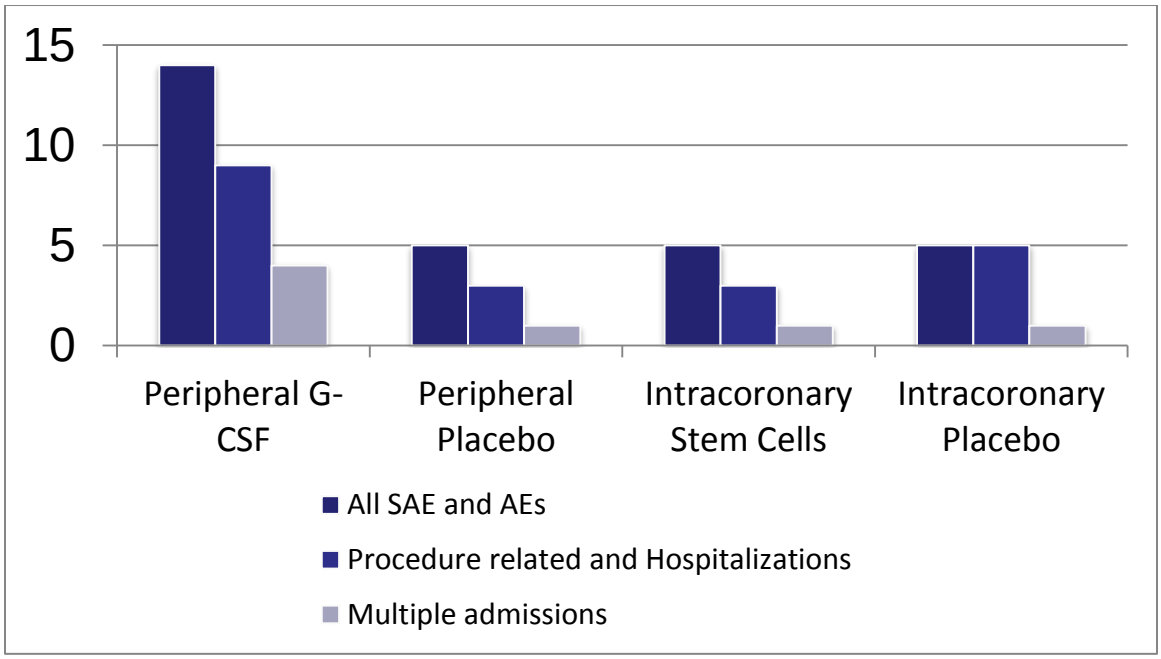


Figure 12: REGENERATE-DCM trial: Serious adverse events (SAE), adverse events (AE) and hospital admissions

7. DISCUSSION

This is the first randomised trial in DCM assessing the use of bone marrow derived stem cells and adjunctive G-CSF to demonstrate a significant increase in cardiac function both within the intracoronary stem cell treated patients and in comparison to an interventional placebo control. The trial is also unique in that compared to the interventional control group it demonstrated a 5.47% increase in LVEF in the intracoronary stem cell group at 3 months. This LVEF improvement was associated with significant improvements in the clinical parameters of NYHA class and exercise capacity. The remaining treatment groups (intracoronary serum, G-CSF alone and peripheral placebo) showed no evidence of improvement in any of the end-points at 3 months. These results therefore demonstrate the beneficial effects across multiple clinical and intermediate parameters of autologous cell and adjunctive cytokine therapy in a randomised control trial of patients suffering from dilated cardiomyopathy.

Similar beneficial effects on cardiac function with BMC therapy have been shown in other early phase studies[144,273], with the most recent demonstrating improvements out to 5 years post therapy[274]. However no study has been performed with an interventional control group blinded to the investigators. Previous studies have also used G-CSF as a cell mobilising factor but few have controlled for the possibility that this cytokine may have a direct effect on cardiac function as has been previously suggested[165,275]. Early preclinical and initial Phase I trials suggested that G-CSF administration was safe and possibly associated with improvements in LVEF[168]. The REGENERATE DCM is therefore the first trial designed with a separate interventional and cytokine control group.

Dilated cardiomyopathy is characterized by ventricular dilatation and reduced myocardial-wall thickness which results in an increase in myocardial-wall stress [159,162,276]. Left-ventricular end-diastolic volumes did not decrease; indicating that transfer of bone-marrow-cells did not improve left-ventricular remodeling at 3 months. In patients with heart failure, cardiac dysfunction can be progressive even in the absence of change in symptoms. A fall in ejection fraction (EF) is an independent predictor of mortality. The Vasodilator-Heart Failure Trials (V-HeFT I and V-HeFT II) identified EF as a powerful predictor of all-cause mortality in HF patients[263,277], and improvement in EF has ben linked to improved prognosis[258-260,278]. Even patients with asymptomatic LV dysfunction are at an increased risk of sudden cardiac death[261]. While serial measurement of EF provides important prognostic information[277], other additional effects of therapy should be taken into consideration such as changes in plasma BNP, cardiac remodeling and improvement in exercise functional capacity. Preventing chronic progression of heart failure by slowing or reversing the remodeling process should be a target for novel therapies of heart failure[278]. Heart failure therapies that had an impact on morbidity and mortality, such as B-blockers and ACE inhibitors, had a positive impact on LV remodeling and EF[163,166,279-283]. However these changes were only apparent after 6 months of initiating therapy[283,284]. A sub-study from CIBIS I showed that although Bisoprolol improved EF and reduced end-systolic dimensions after 5 months therapy, it had no significant effect on LV end- diastolic dimensions[285].

Changes in cardiac remodeling is a slow process and should be assessed after at least 4 months of initiating therapy[278]. This, partly, explains the lack of improvement of LV end-systolic and end-diastolic volumes in the REGENERATE-DCM trial despite the

improvement in EF.

BNP significantly correlates with NYHA classification. BNP was more closely correlated with mortality than NYHA functional class and ejection fraction. Koglin et al studied 78 CHF patients in a multivariable prognostic model to monitor the clinical course and outcomes for 398 days.[18] Parameters assessed included BNP and variables used to develop the heart failure survival score (HFSS), which include LVEF, resting heart rate, mean blood pressure and intraventricular conduction volume. Changes in BNP level were significantly related to changes in physical activity and prediction of survival status and was as powerful as the HFSS. BNP has also been used to identify heart failure patients at high risk of death or hospitalization[286]. Plasma BNP concentrations are higher in patients with more severe symptoms and in those with more severe cardiac damage[287], and is an independent predictor of death in patients with chronic heart failure [211]. BNP correlates well with cardiopulmonary exercise capacity and with composite measures of heart failure severity, such as the Heart Failure Survival Score[288]. After initiating treatment, there is a correlation between an improvement in functional status and the reduction in plasma BNP levels. BNP also plays an important role in risk stratifying patients with advanced heart failure selected for heart transplantation[288]. In patients taking B-blockers, BNP is a better prognostic predictor than more traditional parameters such as peak VO2[210]. Data on 128 consecutive patients who were taking beta-blockers and were awaiting transplantation show that the only independent predictor of all cause mortality was an NT-proBNP value above the median of 1498 pg/ml. A study involving 452 patients with left ventricular dysfunction with a high rate of sudden death found that the BNP concentration was the only

independent predictor of sudden death[209]. However, there is a wide variation of plasma BNP levels as it relies on various factors such as age, renal function, left ventricular ejection fraction and left ventricular filling pressure. Furthermore, renal function tends to deteriorate with progression of heart failure and stepping up diuretic therapy, which results in a progressive rise in plasma BNP levels[289]. Therefore using serial BNP as a guide to response to therapy is complicated and should take into consideration other factors that raise plasma BNP such as worsening renal function. Furthermore, in a Cleveland Clinic study, a wide variation of plasma BNP levels were observed in patients admitted for decompensated heart failure requiring haemodynamically guided therapy. In particularly some of the patients with idiopathic dilated cardiomyopathy had BNP levels in the lower ranges (≤ 100 pg/mL)[290]. A more recent observational study has shown that 6-month post-discharge BNP was closely associated with long-term outcome, but BNP at 3 months as well as at discharge was not predictive of it[291]. In this study readmitted patients, in contrast to event-free patients, showed an increase in BNP levels despite clinical stabilization and had no improvement in echocardiographic parameters during the 6 months follow-up. The authors concluded that an increase in 6-month (but not 3-month) post-discharge BNP before clinical evidence of decompensation in part reflects a poor reverse LV remodeling, and therefore BNP measurement at 6-mponth post treatment may have a role as a therapeutic guide[291].

In the REGENERATE-DCM trial, the mean plasma B-type Natriuretic Peptide (BNP) at baseline was 1346 pg/ml. The high level of BNP at baseline is a marker of the severity of heart failure in this cohort of patients. Despite an improvement in ejection fraction, there was a trend towards a significant reduction in 3 month BNP in the intracoronary

stem cell group, but this did not reach statistical significance. The small sample size and short time frame (3 months) may have accounted for lack of significant change in plasma BNP levels. We hypothesized that administration of G-CSF to patients in the REGENERATE-DCM trial will lead to a reduction in pro-inflammatory cytokines, and injection of autologous BMMNC will confer an additional reduction above that derived from G-CSF injection alone. However, there was no significant reduction in the cytokines at day 6 in either the G-CSF or stem cell group compared to placebo. Unfortunately, I was unable to measure the plasma pro-inflammatory cytokines at 3 month follow-up. Given the lack of significant reduction in BNP levels at 3 months, it would seem unlikely that G-SCF or intracoronary stem cells would have resulted in a reduction in the pro-inflammatory biomarkers during the same timeframe.

The study population enrolled in REGENERATE DCM is typical of a DCM population, with similar baseline characteristics and medical therapy to patients in other published. The biochemical markers indicating severity of heart failure were similar across all groups with a NT-proBNP level greater than 1000 pg/ml suggestive of significant left ventricular dysfunction. The REGENERATE DCM patient population had high levels of optimal medical therapy in all groups and were stabilised prior to randomisation. The improvement in cardiac function and symptoms in the cell treated group was thus unlikely to be due to differences in medical therapy between the groups. The REGENERATE DCM patient population also had appropriate levels of ‘device therapy’ in keeping with current American Heart Association guidelines and consistent between groups. The results of the REGENERATE-DCM trial demonstrate a significant improvement in left ventricular function and an improvement in symptoms and exercise

capacity. Further investigation using similar methodology in a Phase III trial is needed to assess whether cell therapy lead to a prognostic benefit in these patients to accompany the functional changes seen.

8. CONCLUSION:

The intracoronary infusion of autologous bone marrow derived mononuclear cells in combination with G-CSF therapy in patients with DCM appears to be safe and is associated with an improvement in LVEF 3 months after therapy. These functional differences were accompanied by improvement in a panel of biochemical and symptom related outcomes supporting a real clinical effect.

9. CONCLUSIONS AND FUTURE DIRECTIONS

There has been quite a rapid translation of stem/progenitor cell research from preclinical to human trials, which answered many questions but also highlighted the current limitations. This rapid translation is partly because BMC transplantation is easy for clinical application owing to its simplicity and autologous model, negating the problems of ethics and immune rejection. The REGENERATE-DCM in conjunction with other trials has demonstrated the safety and efficacy of stem cell and cytokine therapy in the treatment of myocardial cell loss. However, criticism of the rapid translation of this novel therapy has been the lack of precise characterization of the cells injected, measurement of the efficiency of cell uptake and engraftment by the heart and assessment of bio-distribution and elimination of cells from the body. Hence, further work aimed specifically at defining bio-distribution and cellular kinetics will be crucial to the rational design of future clinical trials and the eventual safe application of cell therapy in the clinical setting.

Great progress has been made but the field remains unclear regarding cell type, cell number and injection method as well as understanding fully which groups of patients to target. The situation has not been helped by the heterogeneity of trial design and the diversity of techniques used. There is no universally accepted standardized method to process cells (although regulatory issues apply) and the only consensus document regarding trial design was published by the ESC task force in 2006[292]. The question of standardization for BMSC processing should be addressed in the next five years following the EU award of funding to conduct a 3,000 patient mortality trial of BMSC in

AMI (BAMI Consortium). Standardization of techniques is a key component of this award. It is hoped that this Phase III trial will answer the questions which have arisen from the smaller Phase I/II clinical trials of BMSC that have been published to date. Its primary endpoint will address whether BMSC therapy reduces mortality in AMI.

Strategies are also needed to understand the mechanisms involved in stem cell and cytokine therapy, and the interactions between the stem cells and the local environment to improve the cells' homing abilities and enhance their survival. Both animal and clinical studies in DCM have demonstrated that stem cells do not transdifferentiate into cardiomyocytes, suggesting that a paracrine effect with stimulation of vasculogenesis and cell proliferation as a potential important mechanism of stem cells in cardiac remodeling and improvement in cardiac status. In conjunction with basic science research, further large randomized trials are needed to assess the efficacy of this treatment and its long-term effect on cardiac function, survival and quality of life.

Many challenges lie ahead, including the need to identify the right patient who is likely to benefit from this therapy. The REGENERATE-DCM trial, which included patients with no other treatment option, was a good logical starting point as these the subset of patient that are most likely to benefit from this therapy. We have demonstrated the safety of cell therapy for intracoronary administration of BMSCs in the dilated cardiomyopathy population where no increased incidence of serious adverse effects compared to standard therapy over short and long-term follow-up. A similar trial to the BAMI consortium is needed to address whether the benefits we have shown in the REGENERATE-DCM trial translate to mortality benefit in this subset of patients.

Another limitation has been the debate over the use of surrogate endpoints in trials. It is now becoming crucial to develop an understanding of surrogate endpoints in clinical trials and their relevance to eventual outcome studies. The complexities of translational research mean that there is an unmet need for surrogate endpoints which can be used to reduce study size and duration and which have widely accepted meaning when it comes to hard clinical endpoints. Trials of innovative new therapies are expensive and, without robust surrogate endpoints that can reduce cost, they will remain out of reach of academia and perhaps represent too much risk for industry. Added to these difficulties are the ever-increasing regulatory issues which are clearly indicated but which need to be implemented in a way that allows the field to flourish rather than to flounder. Investment in infrastructure needs to accompany the creation of appropriate regulation. That said, the number of clinical trials continues to grow. Now, with the beginning of Phase III trials, a new page has been turned in this exciting story of discovery.

Reference List

1 ***Manolio TA, Baughman KL, Rodeheffer R, et al.*** **Prevalence and etiology of idiopathic dilated cardiomyopathy (summary of a National Heart, Lung, and Blood Institute workshop. *Am J Cardiol* 1992; 69: 1458–66.**

2 **Hertz MI, Aurora P, Christie JD, *et al.* Scientific Registry of the International Society for Heart and Lung Transplantation: introduction to the 2009 Annual Reports. *J Heart Lung Transplant* 2009; 28: 989–92.**

3 **Henry WL, Gardin JM, Ware JH. Echocardiographic measurements in normal subjects from infancy to old age. *Circulation* 1980; 62: 1054–61.**

4 **Elliott P, Andersson B, Arbustini E, *et al.* Classification of the cardiomyopathies: a position statement from the European Society Of Cardiology Working Group on Myocardial and Pericardial Diseases. *Eur Heart J* 2008; 29: 270–6.**

5 **Codd MB, Sugrue DD, Gersh BJ, Melton LJ. Epidemiology of idiopathic dilated and hypertrophic cardiomyopathy. A population-based study in Olmsted County, Minnesota, 1975-1984. *Circulation* 1989; 80: 564–72.**

6 **Rakar S, Sinagra G, Di Lenarda A, *et al.* Epidemiology of dilated cardiomyopathy. A prospective post-mortem study of 5252 necropsies. The Heart Muscle Disease Study Group. *Eur Heart J* 1997; 18: 117–23.**

7 **Baig MK, Goldman JH, Caforio AL, Coonar AS, Keeling PJ, McKenna WJ. Familial dilated cardiomyopathy: cardiac abnormalities are common in asymptomatic relatives and may represent early disease. *J Am Coll Cardiol* 1998; 31: 195–201.**

8 **Gavazzi A, Repetto A, Scelsi L, *et al.* Evidence-based diagnosis of familial non-X-linked dilated cardiomyopathy. Prevalence, inheritance and characteristics. *Eur Heart J* 2001; 22: 73–81.**

9 **Mestroni L, Rocco C, Gregori D, *et al.* Familial dilated cardiomyopathy: evidence for genetic and phenotypic heterogeneity. Heart Muscle Disease Study Group. *J Am Coll Cardiol* 1999; 34: 181–90.**

10 **Hobbs FDR, Kenkre JE, Roalfe AK, Davis RC, Hare R, Davies MK. Impact of heart failure and left ventricular systolic dysfunction on quality of life: a cross-sectional study comparing common chronic cardiac and medical disorders and a representative adult population. *Eur Heart J* 2002;**

23: 1867–76.

11 Anand IS, Fisher LD, Chiang Y-T, *et al.* Changes in brain natriuretic peptide and norepinephrine over time and mortality and morbidity in the Valsartan Heart Failure Trial (Val-HeFT). *Circulation* 2003; 107: 1278–83.

12 Latini R, Masson S, Anand I, *et al.* The comparative prognostic value of plasma neurohormones at baseline in patients with heart failure enrolled in Val-HeFT. *Eur Heart J* 2004; 25: 292–9.

13 Canetti M, Akhter MW, Lerman A, *et al.* Evaluation of myocardial blood flow reserve in patients with chronic congestive heart failure due to idiopathic dilated cardiomyopathy. *Am J Cardiol* 2003; 92: 1246–9.

14 Neglia D, Michelassi C, Trivieri MG, *et al.* Prognostic role of myocardial blood flow impairment in idiopathic left ventricular dysfunction. *Circulation* 2002; 105: 186–93.

15 Arnous S, Syrris P, Sen-Chowdhry S. Genetics of Dilated Cardiomyopathy: Risk of Conduction Defects and Sudden Cardiac Death. *Cardiac Electrophysiology …* 2010.

16 Seidman JG, Seidman CE. The genetic basis for cardiac remodeling. *Annu Rev Genomics Hum …* 2005.

17 Tsubata S, Bowles KR, Vatta M, *et al.* Mutations in the human delta-sarcoglycan gene in familial and sporadic dilated cardiomyopathy. *J Clin Invest* 2000; 106: 655–62.

18 Barresi R, Di Blasi C, Negri T, *et al.* Disruption of heart sarcoglycan complex and severe cardiomyopathy caused by beta sarcoglycan mutations. *J Med Genet* 2000; 37: 102–7.

19 Arbustini E, Diegoli M, Morbini P, *et al.* Prevalence and characteristics of dystrophin defects in adult male patients with dilated cardiomyopathy. *J Am Coll Cardiol* 2000; 35: 1760–8.

20 Taylor MRG, Slavov D, Ku L, *et al.* Prevalence of desmin mutations in dilated cardiomyopathy. *Circulation* 2007; 115: 1244–51.

21 Olson TM, Michels VV, Thibodeau SN, Tai YS, Keating MT. Actin mutations in dilated cardiomyopathy, a heritable form of heart failure. *Science* 1998; 280: 750–2.

22 Kamisago M, Sharma SD, DePalma SR, *et al.* Mutations in sarcomere protein genes as a cause of dilated cardiomyopathy. *N Engl J Med* 2000; 343: 1688–96.

23 Kaski JP, Burch M, Elliott PM. Mutations in the cardiac Troponin C gene are a cause of idiopathic dilated cardiomyopathy in childhood. *Cardiol Young* 2007; 17: 675–7.

24 Mogensen J, Murphy RT, Shaw T, *et al.* Severe disease expression of cardiac troponin C and T mutations in patients with idiopathic dilated cardiomyopathy. *J Am Coll Cardiol* 2004; 44: 2033–40.

25 Fatkin D, MacRae C, Sasaki T, *et al.* Missense mutations in the rod domain of the lamin A/C gene as causes of dilated cardiomyopathy and conduction-system disease. *N Engl J Med* 1999;

341: 1715–24.

26 Arbustini E, Pilotto A, Repetto A, *et al.* Autosomal dominant dilated cardiomyopathy with atrioventricular block: a lamin A/C defect-related disease. *J Am Coll Cardiol* 2002; 39: 981–90.

27 Bonne G, Di Barletta MR, Varnous S, *et al.* Mutations in the gene encoding lamin A/C cause autosomal dominant Emery-Dreifuss muscular dystrophy. *Nat Genet* 1999; 21: 285–8.

28 Arbustini E, Diegoli M, Fasani R, *et al.* Mitochondrial DNA mutations and mitochondrial abnormalities in dilated cardiomyopathy. *Am J Pathol* 1998; 153: 1501–10.

29 Kass S, MacRae C, Graber HL, *et al.* A gene defect that causes conduction system disease and dilated cardiomyopathy maps to chromosome 1p1-1q1. *Nat Genet* 1994; 7: 546–51.

30 Messina DN, Speer MC, Pericak-Vance MA, McNally EM. Linkage of familial dilated cardiomyopathy with conduction defect and muscular dystrophy to chromosome 6q23. *Am J Hum Genet* 1997; 61: 909–17.

31 Brodsky GL, Muntoni F, Miocic S, Sinagra G, Sewry C, Mestroni L. Lamin A/C gene mutation associated with dilated cardiomyopathy with variable skeletal muscle involvement. *Circulation* 2000; 101: 473–6.

32 Caforio AL, Keeling PJ, Zachara E, *et al.* Evidence from family studies for autoimmunity in dilated cardiomyopathy. *Lancet* 1994; 344: 773–7.

33 Caforio ALP, Mahon NG, Baig MK, *et al.* Prospective familial assessment in dilated cardiomyopathy: cardiac autoantibodies predict disease development in asymptomatic relatives. *Circulation* 2007; 115: 76–83.

34 Mestroni L, Maisch B, McKenna WJ, *et al.* Guidelines for the study of familial dilated cardiomyopathies. Collaborative Research Group of the European Human and Capital Mobility Project on Familial Dilated Cardiomyopathy. Eur. Heart J. 1999; 20: 93–102.

35 Mahon NG, Murphy RT, MacRae CA, Caforio ALP, Elliott PM, McKenna WJ. Echocardiographic evaluation in asymptomatic relatives of patients with dilated cardiomyopathy reveals preclinical disease. *Ann Intern Med* 2005; 143: 108–15.

36 Mahon NG, Madden BP, Caforio ALP, *et al.* Immunohistologic evidence of myocardial disease in apparently healthy relatives of patients with dilated cardiomyopathy. *J Am Coll Cardiol* 2002; 39: 455–62.

37 Mahon NG, Sharma S, Elliott PM, *et al.* Abnormal cardiopulmonary exercise variables in asymptomatic relatives of patients with dilated cardiomyopathy who have left ventricular enlargement. *Heart* 2000; 83: 511–7.

38 Daehmlow S, Erdmann J, Knueppel T, *et al.* Novel mutations in sarcomeric protein genes in dilated cardiomyopathy. *Biochem Biophys Res Commun* 2002; 298: 116–20.

39 Villard E, Duboscq-Bidot L, Charron P, *et al.* Mutation screening in dilated cardiomyopathy:

prominent role of the beta myosin heavy chain gene. *Eur Heart J* 2005; 26: 794–803.

40 Kärkkäinen S, Heliö T, Jääskeläinen P, *et al.* Two novel mutations in the beta-myosin heavy chain gene associated with dilated cardiomyopathy. *Eur J Heart Fail* 2004; 6: 861–8.

41 Carniel E, Taylor MRG, Sinagra G, *et al.* Alpha-myosin heavy chain: a sarcomeric gene associated with dilated and hypertrophic phenotypes of cardiomyopathy. *Circulation* 2005; 112: 54–9.

42 Hanson EL, Jakobs PM, Keegan H, *et al.* Cardiac troponin T lysine 210 deletion in a family with dilated cardiomyopathy. *J Card Fail* 2002; 8: 28–32.

43 Murphy RT, Mogensen J, Shaw A, Kubo T, Hughes S, McKenna WJ. Novel mutation in cardiac troponin I in recessive idiopathic dilated cardiomyopathy. *Lancet* 2004; 363: 371–2.

44 Carballo S, Robinson P, Otway R, *et al.* Identification and functional characterization of cardiac troponin I as a novel disease gene in autosomal dominant dilated cardiomyopathy. *Circ Res* 2009; 105: 375–82.

45 Hershberger RE, Norton N, Morales A, Li D, Siegfried JD, Gonzalez-Quintana J. Coding sequence rare variants identified in MYBPC3, MYH6, TPM1, TNNC1, and TNNI3 from 312 patients with familial or idiopathic dilated cardiomyopathy. *Circ Cardiovasc Genet* 2010; 3: 155–61.

46 Gerull B, Gramlich M, Atherton J, *et al.* Mutations of TTN, encoding the giant muscle filament titin, cause familial dilated cardiomyopathy. *Nat Genet* 2002; 30: 201–4.

47 Knöll R, Hoshijima M, Hoffman HM, *et al.* The cardiac mechanical stretch sensor machinery involves a Z disc complex that is defective in a subset of human dilated cardiomyopathy. *Cell* 2002; 111: 943–55.

48 Hayashi T, Arimura T, Itoh-Satoh M, *et al.* Tcap gene mutations in hypertrophic cardiomyopathy and dilated cardiomyopathy. *J Am Coll Cardiol* 2004; 44: 2192–201.

49 Olson TM, Illenberger S, Kishimoto NY, Huttelmaier S, Keating MT, Jockusch BM. Metavinculin mutations alter actin interaction in dilated cardiomyopathy. *Circulation* 2002; 105: 431–7.

50 Vatta M, Mohapatra B, Jimenez S, *et al.* Mutations in Cypher/ZASP in patients with dilated cardiomyopathy and left ventricular non-compaction. *J Am Coll Cardiol* 2003; 42: 2014–27.

51 Mohapatra B, Jimenez S, Lin J-H, *et al.* Mutations in the muscle LIM protein and alpha-actinin-2 genes in dilated cardiomyopathy and endocardial fibroelastosis. *Mol Genet Metab* 2003; 80: 207–15.

52 Arola AM, Sanchez X, Murphy RT, *et al.* Mutations in PDLIM3 and MYOZ1 encoding myocyte Z line proteins are infrequently found in idiopathic dilated cardiomyopathy. *Mol Genet Metab* 2007; 90: 435–40.

53 Duboscq-Bidot L, Xu P, Charron P, *et al.* Mutations in the Z-band protein myopalladin gene and idiopathic dilated cardiomyopathy. *Cardiovasc Res* 2008; 77: 118–25.

54 **Arimura T, Hayashi T, Matsumoto Y, *et al.* Structural analysis of four and half LIM protein-2 in dilated cardiomyopathy. *Biochem Biophys Res Commun* 2007; 357: 162–7.**

55 **Hassel D, Dahme T, Erdmann J, *et al.* Nexilin mutations destabilize cardiac Z-disks and lead to dilated cardiomyopathy. *Nat Med* 2009; 15: 1281–8.**

56 **Li D, Tapscoft T, Gonzalez O, *et al.* Desmin mutation responsible for idiopathic dilated cardiomyopathy. *Circulation* 1999; 100: 461–4.**

57 **Towbin JA, Hejtmancik JF, Brink P, *et al.* X-linked dilated cardiomyopathy. Molecular genetic evidence of linkage to the Duchenne muscular dystrophy (dystrophin) gene at the Xp21 locus. *Circulation* 1993; 87: 1854–65.**

58 **Inagaki N, Hayashi T, Arimura T, *et al.* Alpha B-crystallin mutation in dilated cardiomyopathy. *Biochem Biophys Res Commun* 2006; 342: 379–86.**

59 **Jakobs PM, Hanson EL, Crispell KA, *et al.* Novel lamin A/C mutations in two families with dilated cardiomyopathy and conduction system disease. *J Card Fail* 2001; 7: 249–56.**

60 **Vytopil M, Benedetti S, Ricci E, *et al.* Mutation analysis of the lamin A/C gene (LMNA) among patients with different cardiomuscular phenotypes. *J Med Genet* 2003; 40: e132.**

61 **Taylor MRG, Fain PR, Sinagra G, *et al.* Natural history of dilated cardiomyopathy due to lamin A/C gene mutations. *J Am Coll Cardiol* 2003; 41: 771–80.**

62 **Sylvius N, Bilinska ZT, Veinot JP, *et al.* In vivo and in vitro examination of the functional significances of novel lamin gene mutations in heart failure patients. *J Med Genet* 2005; 42: 639–47.**

63 **Sébillon P, Bouchier C, Bidot LD, *et al.* Expanding the phenotype of LMNA mutations in dilated cardiomyopathy and functional consequences of these mutations. *J Med Genet* 2003; 40: 560–7.**

64 **Burkett EL, Hershberger RE. Clinical and genetic issues in familial dilated cardiomyopathy. *J Am Coll Cardiol* 2005; 45: 969–81.**

65 **Taylor MRG, Slavov D, Gajewski A, *et al.* Thymopoietin (lamina-associated polypeptide 2) gene mutation associated with dilated cardiomyopathy. *Hum Mutat* 2005; 26: 566–74.**

66 **Bione S, Small K, Aksmanovic VM, *et al.* Identification of new mutations in the Emery-Dreifuss muscular dystrophy gene and evidence for genetic heterogeneity of the disease. *Hum Mol Genet* 1995; 4: 1859–63.**

67 **Bienengraeber M, Olson TM, Selivanov VA, *et al.* ABCC9 mutations identified in human dilated cardiomyopathy disrupt catalytic KATP channel gating. *Nat Genet* 2004; 36: 382–7.**

68 **Norgett EE, Hatsell SJ, Carvajal-Huerta L, *et al.* Recessive mutation in desmoplakin disrupts desmoplakin-intermediate filament interactions and causes dilated cardiomyopathy, woolly hair and keratoderma. *Hum Mol Genet* 2000; 9: 2761–6.**

69 Schmitt JP, Kamisago M, Asahi M, *et al.* Dilated cardiomyopathy and heart failure caused by a mutation in phospholamban. *Science* 2003; 299: 1410–3.

70 Haghighi K, Kolokathis F, Pater L, *et al.* Human phospholamban null results in lethal dilated cardiomyopathy revealing a critical difference between mouse and human. *J Clin Invest* 2003; 111: 869–76.

71 Haghighi K, Kolokathis F, Gramolini AO, *et al.* A mutation in the human phospholamban gene, deleting arginine 14, results in lethal, hereditary cardiomyopathy. *Proc Natl Acad Sci USA* 2006; 103: 1388–93.

72 D'Adamo P, Fassone L, Gedeon A, *et al.* The X-linked gene G4.5 is responsible for different infantile dilated cardiomyopathies. *Am J Hum Genet* 1997; 61: 862–7.

73 Zhang L, Hu A, Yuan H, *et al.* A missense mutation in the CHRM2 gene is associated with familial dilated cardiomyopathy. *Circ Res* 2008; 102: 1426–32.

74 Schönberger J, Wang L, Shin JT, *et al.* Mutation in the transcriptional coactivator EYA4 causes dilated cardiomyopathy and sensorineural hearing loss. *Nat Genet* 2005; 37: 418–22.

75 Taylor MRG, Ku L, Slavov D, *et al.* Danon disease presenting with dilated cardiomyopathy and a complex phenotype. *J Hum Genet* 2007; 52: 830–5.

76 Knöll R, Postel R, Wang J, *et al.* Laminin-alpha4 and integrin-linked kinase mutations cause human cardiomyopathy via simultaneous defects in cardiomyocytes and endothelial cells. *Circulation* 2007; 116: 515–25.

77 Li D, Parks SB, Kushner JD, *et al.* Mutations of presenilin genes in dilated cardiomyopathy and heart failure. *Am J Hum Genet* 2006; 79: 1030–9.

78 Moulik M, Vatta M, Witt SH, *et al.* ANKRD1, the gene encoding cardiac ankyrin repeat protein, is a novel dilated cardiomyopathy gene. *J Am Coll Cardiol* 2009; 54: 325–33.

79 Brauch KM, Karst ML, Herron KJ, *et al.* Mutations in ribonucleic acid binding protein gene cause familial dilated cardiomyopathy. *J Am Coll Cardiol* 2009; 54: 930–41.

80 Montastruc G, Favreliere S, Sommet A, *et al.* Drugs and dilated cardiomyopathies: A case/noncase study in the French PharmacoVigilance Database. *Br J Clin Pharmacol* 2010; 69: 287–94.

81 Pantazis AA, Elliott PM. Left ventricular noncompaction. *Curr Opin Cardiol* 2009; 24: 209–13.

82 Dickstein K, Cohen-Solal A, Filippatos G, *et al.* ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: the Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2008 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association of the ESC (HFA) and endorsed by the European Society of Intensive Care Medicine (ESICM). Eur. Heart J. 2008; 29: 2388–442.

83 Meune C, Van Berlo JH, Anselme F, Bonne G, Pinto YM, Duboc D. Primary prevention of sudden

death in patients with lamin A/C gene mutations. *N Engl J Med* 2006; 354: 209–10.

84 Pasotti M, Klersy C, Pilotto A, *et al.* Long-term outcome and risk stratification in dilated cardiolaminopathies. *J Am Coll Cardiol* 2008; 52: 1250–60.

85 Van Berlo JH, de Voogt WG, van der Kooi AJ, *et al.* Meta-analysis of clinical characteristics of 299 carriers of LMNA gene mutations: do lamin A/C mutations portend a high risk of sudden death? *J Mol Med* 2005; 83: 79–83.

86 Taylor DO, Edwards LB, Boucek MM, Trulock EP, Keck BM, Hertz MI. The Registry of the International Society for Heart and Lung Transplantation: twenty-first official adult heart transplant report--2004. *J Heart Lung Transplant* 2004; 23: 796–803.

87 Miller LW, Pagani FD, Russell SD, *et al.* Use of a continuous-flow device in patients awaiting heart transplantation. *N Engl J Med* 2007; 357: 885–96.

88 Slaughter MS, Rogers JG, Milano CA, *et al.* Advanced heart failure treated with continuous-flow left ventricular assist device. *N Engl J Med* 2009; 361: 2241–51.

89 McAlister FA, Stewart S, Ferrua S, McMurray JJJV. Multidisciplinary strategies for the management of heart failure patients at high risk for admission: a systematic review of randomized trials. *J Am Coll Cardiol* 2004; 44: 810–9.

90 Hjalmarson A, Goldstein S, Fagerberg B, *et al.* Effects of controlled-release metoprolol on total mortality, hospitalizations, and well-being in patients with heart failure: the Metoprolol CR/XL Randomized Intervention Trial in congestive heart failure (MERIT-HF). MERIT-HF Study Group. *JAMA* 2000; 283: 1295–302.

91 Pitt B, Zannad F, Remme WJ, *et al.* The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *N Engl J Med* 1999; 341: 709–17.

92 Lader E, Egan D, Hunsberger S, Garg R, Czajkowski S, McSherry F. The effect of digoxin on the quality of life in patients with heart failure. *J Card Fail* 2003; 9: 4–12.

93 Faris R, Flather M, Purcell H, Henein M, Poole-Wilson P, Coats A. Current evidence supporting the role of diuretics in heart failure: a meta analysis of randomised controlled trials. *Int J Cardiol* 2002; 82: 149–58.

94 McMurray JJV, Packer M, Desai AS, *et al.* Angiotensin-neprilysin inhibition versus enalapril in heart failure. *N Engl J Med* 2014; 371: 993–1004.

95 Lovell MJ, Mathur A. The role of stem cells for treatment of cardiovascular disease. *Cell Prolif* 2004; 37: 67–87.

96 Poulsom R, Alison MR, Forbes SJ, Wright NA. Adult stem cell plasticity. *J Pathol* 2002; 197: 441–56.

97 Orlic D, Kajstura J, Chimenti S, *et al.* Mobilized bone marrow cells repair the infarcted heart,

improving function and survival. *Proc Natl Acad Sci USA* 2001; 98: 10344–9.

98 Norol F, Merlet P, Isnard R, *et al.* Influence of mobilized stem cells on myocardial infarct repair in a nonhuman primate model. *Blood* 2003; 102: 4361–8.

99 Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* 2002; 105: 93–8.

100 Nagaya N, Kangawa K, Itoh T, *et al.* Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. *Circulation* 2005; 112: 1128–35.

101 Yin AH, Miraglia S, Zanjani ED, *et al.* AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood* 1997; 90: 5002–12.

102 Miraglia S, Godfrey W, Yin AH, *et al.* A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. *Blood* 1997; 90: 5013–21.

103 Kawamoto A, Gwon HC, Iwaguro H, *et al.* Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. *Circulation* 2001; 103: 634–7.

104 Kamihata H, Matsubara H, Nishiue T, *et al.* Improvement of collateral perfusion and regional function by implantation of peripheral blood mononuclear cells into ischemic hibernating myocardium. *Arterioscler Thromb Vasc Biol* 2002; 22: 1804–10.

105 Assmus B, Schächinger V, Teupe C, *et al.* Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). *Circulation* 2002; 106: 3009–17.

106 Stamm C, Westphal B, Kleine HD, *et al.* Autologous bone-marrow stem-cell transplantation for myocardial regeneration. *Lancet* 2003; 361: 45–6.

107 Chen S-L, Fang W-W, Ye F, *et al.* Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. *Am J Cardiol* 2004; 94: 92–5.

108 Fernández-Avilés F, San Román JA, García-Frade J, *et al.* Experimental and clinical regenerative capability of human bone marrow cells after myocardial infarction. *Circ Res* 2004; 95: 742–8.

109 Schächinger V, Assmus B, Britten MB, *et al.* Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction: final one-year results of the TOPCARE-AMI Trial. *J Am Coll Cardiol* 2004; 44: 1690–9.

110 Wollert KC, Meyer GP, Lotz J, *et al.* Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. *Lancet* 2004; 364: 141–8.

111 Taylor DA, Atkins BZ, Hungspreugs P, *et al.* Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. *Nat Med* 1998; 4: 929–33.

112 Menasché P, Hagège A, Scorsin M, *et al.* [Autologous skeletal myoblast transplantation for

cardiac insufficiency. First clinical case]. *Arch Mal Coeur Vaiss* 2001; 94: 180–2.

113 Hagège AA, Marolleau J-P, Vilquin J-T, *et al.* Skeletal myoblast transplantation in ischemic heart failure: long-term follow-up of the first phase I cohort of patients. *Circulation* 2006; 114: I108–13.

114 Menasché P, Alfieri O, Janssens S, *et al.* The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation. *Circulation* 2008; 117: 1189–200.

115 Beltrami AP, Urbanek K, Kajstura J, *et al.* Evidence that human cardiac myocytes divide after myocardial infarction. *N Engl J Med* 2001; 344: 1750–7.

116 Beltrami AP, Barlucchi L, Torella D, *et al.* Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* 2003; 114: 763–76.

117 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; 126: 663–76.

118 Takahashi K, Tanabe K, Ohnuki M, *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; 131: 861–72.

119 Aasen T, Raya A, Barrero MJ, *et al.* Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nat Biotechnol* 2008; 26: 1276–84.

120 Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature* 2007; 448: 313–7.

121 Bock-Marquette I, Saxena A, White MD, Dimaio JM, Srivastava D. Thymosin beta4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. *Nature* 2004; 432: 466–72.

122 Smart N, Risebro CA, Melville AAD, *et al.* Thymosin beta4 induces adult epicardial progenitor mobilization and neovascularization. *Nature* 2007; 445: 177–82.

123 Bock-Marquette I, Shrivastava S, Pipes GCT, *et al.* Thymosin beta4 mediated PKC activation is essential to initiate the embryonic coronary developmental program and epicardial progenitor cell activation in adult mice in vivo. *J Mol Cell Cardiol* 2009; 46: 728–38.

124 Smart N, Bollini S, Dubé KN, *et al.* De novo cardiomyocytes from within the activated adult heart after injury. *Nature* 2011; 474: 640–4.

125 Orlic D, Kajstura J, Chimenti S, *et al.* Bone marrow cells regenerate infarcted myocardium. *Nature* 2001; 410: 701–5.

126 Yoon Y-S, Lee N, Scadova H. Myocardial regeneration with bone-marrow-derived stem cells. *Biol Cell* 2005; 97: 253–63.

127 Martin-Rendon E, Brunskill SJ, Hyde CJ, Stanworth SJ, Mathur A, Watt SM. Autologous bone

marrow stem cells to treat acute myocardial infarction: a systematic review. *Eur Heart J* 2008; 29: 1807–18.

128 Lipinski MJ, Biondi-Zoccai GGL, Abbate A, *et al.* Impact of intracoronary cell therapy on left ventricular function in the setting of acute myocardial infarction: a collaborative systematic review and meta-analysis of controlled clinical trials. *J Am Coll Cardiol* 2007; 50: 1761–7.

129 Abdel-Latif A, Bolli R, Tleyjeh IM. Adult bone marrow-derived cells for cardiac repair: A systematic review and meta-analysis. *Arch Intern Med* 2007; 167: 989–97.

130 Reffelmann T, Könemann S, Kloner RA. Promise of blood- and bone marrow-derived stem cell transplantation for functional cardiac repair: putting it in perspective with existing therapy. *J Am Coll Cardiol* 2009; 53: 305–8.

131 Stone GW, Grines CL, Cox DA, *et al.* Comparison of angioplasty with stenting, with or without abciximab, in acute myocardial infarction. *N Engl J Med* 2002; 346: 957–66.

132 Montalescot G, Barragan P, Wittenberg O, *et al.* Platelet glycoprotein IIb/IIIa inhibition with coronary stenting for acute myocardial infarction. *N Engl J Med* 2001; 344: 1895–903.

133 Yousef M, Schannwell CM, Köstering M, Zeus T, Brehm M, Strauer BE. The BALANCE Study: clinical benefit and long-term outcome after intracoronary autologous bone marrow cell transplantation in patients with acute myocardial infarction. *J Am Coll Cardiol* 2009; 53: 2262–9.

134 Curtis JP, Sokol SI, Wang Y, *et al.* The association of left ventricular ejection fraction, mortality, and cause of death in stable outpatients with heart failure. *J Am Coll Cardiol* 2003; 42: 736–42.

135 Tribouilloy C, Rusinaru D, Mahjoub H, *et al.* Prognosis of heart failure with preserved ejection fraction: a 5 year prospective population-based study. *Eur Heart J* 2008; 29: 339–47.

136 Sharif F, Bartunek J, Vanderheyden M. Adult stem cells in the treatment of acute myocardial infarction. *Catheter Cardiovasc Interv* 2011; 77: 72–83.

137 Li T-S, Mikamo A, Takahashi M, *et al.* Comparison of cell therapy and cytokine therapy for functional repair in ischemic and nonischemic heart failure. *Cell Transplant* 2007; 16: 365–74.

138 Ghodsizad A, Ruhparwar A, Marktanner R, *et al.* Autologous transplantation of CD133+ BM-derived stem cells as a therapeutic option for dilatative cardiomyopathy. *Cytotherapy* 2006; 8: 308–10.

139 Argüero R, Careaga-Reyna G, Castaño-Guerra R, Garrido-Garduño MH, Magaña-Serrano JA, de Jesús Nambo-Lucio M. Cellular autotransplantation for ischemic and idiopathic dilated cardiomyopathy. Preliminary report. *Arch Med Res* 2006; 37: 1010–4.

140 Martino HF, Oliveira PS, Assunção E, *et al.* [Cell therapy in dilated cardiomyopathy]. *Arq Bras Cardiol* 2006; 86: 390–2.

141 Zeinaloo A, Zanjani KS, Bagheri MM, Mohyeddin-Bonab M, Monajemzadeh M, Arjmandnia MH.

Intracoronary administration of autologous mesenchymal stem cells in a critically ill patient with dilated cardiomyopathy. *Pediatr Transplant* 2011; 15: E183–6.

142 Seth S, Narang R, Bhargava B, *et al.* Percutaneous intracoronary cellular cardiomyoplasty for nonischemic cardiomyopathy: clinical and histopathological results: the first-in-man ABCD (Autologous Bone Marrow Cells in Dilated Cardiomyopathy) trial. *J Am Coll Cardiol* 2006; 48: 2350–1.

143 Rupp S, Bauer J, Tonn T, *et al.* Intracoronary administration of autologous bone marrow-derived progenitor cells in a critically ill two-yr-old child with dilated cardiomyopathy. *Pediatr Transplant* 2009; 13: 620–3.

144 Fischer-Rasokat U, Assmus B, Seeger FH, *et al.* A pilot trial to assess potential effects of selective intracoronary bone marrow-derived progenitor cell infusion in patients with nonischemic dilated cardiomyopathy: final 1-year results of the transplantation of progenitor cells and functional regeneration enhancement pilot trial in patients with nonischemic dilated cardiomyopathy. *Circ Heart Fail* 2009; 2: 417–23.

145 Vrtovec B, Poglajen G, Sever M, *et al.* Effects of intracoronary stem cell transplantation in patients with dilated cardiomyopathy. *J Card Fail* 2011; 17: 272–81.

146 Meluzín J, Mayer J, Groch L, *et al.* Autologous transplantation of mononuclear bone marrow cells in patients with acute myocardial infarction: the effect of the dose of transplanted cells on myocardial function. *Am Heart J* 2006; 152: 975.e9–15.

147 Janssens S, Dubois C, Bogaert J, *et al.* Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial. *Lancet* 2006; 367: 113–21.

148 Lunde K, Solheim S, Aakhus S, *et al.* Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. *N Engl J Med* 2006; 355: 1199–209.

149 Schächinger V, Erbs S, Elsässer A, *et al.* Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. *N Engl J Med* 2006; 355: 1210–21.

150 Ge J, Li Y, Qian J, *et al.* Efficacy of emergent transcatheter transplantation of stem cells for treatment of acute myocardial infarction (TCT-STAMI). *Heart* 2006; 92: 1764–7.

151 de Lezo JS, Herrera C, Pan M, *et al.* Regenerative therapy in patients with a revascularized acute anterior myocardial infarction and depressed ventricular function. *Rev Esp Cardiol (Engl Ed)* 2007; 60: 357–65.

152 Lipiec P, Krzemińska-Pakuła M, Plewka M, *et al.* Impact of intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction on left ventricular perfusion and function: a 6-month follow-up gated 99mTc-MIBI single-photon emission computed tomography study. *Eur J Nucl Med Mol Imaging* 2009; 36: 587–93.

153 Huikuri HV, Kervinen K, Niemelä M, *et al.* Effects of intracoronary injection of mononuclear bone marrow cells on left ventricular function, arrhythmia risk profile, and restenosis after

thrombolytic therapy of acute myocardial infarction. *Eur Heart J* 2008; 29: 2723–32.

154 Strauer BE, Brehm M, Zeus T, *et al.* Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation* 2002; 106: 1913–8.

155 Bartunek J, Vanderheyden M, Vandekerckhove B, *et al.* Intracoronary injection of CD133-positive enriched bone marrow progenitor cells promotes cardiac recovery after recent myocardial infarction: feasibility and safety. *Circulation* 2005; 112: I178–83.

156 Hirsch A, Nijveldt R, van der Vleuten PA, *et al.* Intracoronary infusion of mononuclear cells from bone marrow or peripheral blood compared with standard therapy in patients after acute myocardial infarction treated by primary percutaneous coronary intervention: results of the randomized controlled HEBE trial. *Eur Heart J* 2011; 32: 1736–47.

157 Kuethe F, Richartz BM, Sayer HG, *et al.* Lack of regeneration of myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans with large anterior myocardial infarctions. *Int J Cardiol* 2004; 97: 123–7.

158 Penicka M, Horak J, Kobylka P, *et al.* Intracoronary injection of autologous bone marrow-derived mononuclear cells in patients with large anterior acute myocardial infarction: a prematurely terminated randomized study. *J Am Coll Cardiol* 2007; 49: 2373–4.

159 Avalos BR. Molecular analysis of the granulocyte colony-stimulating factor receptor. *Blood* 1996; 88: 761–77.

160 Anderlini P, Donato M, Chan KW, *et al.* Allogeneic blood progenitor cell collection in normal donors after mobilization with filgrastim: the M.D. Anderson Cancer Center experience. *Transfusion* 1999; 39: 555–60.

161 Bender JG, To LB, Williams S, Schwartzberg LS. Defining a therapeutic dose of peripheral blood stem cells. *J Hematother* 1992; 1: 329–41.

162 Bodine DM, Seidel NE, Gale MS, Nienhuis AW, Orlic D. Efficient retrovirus transduction of mouse pluripotent hematopoietic stem cells mobilized into the peripheral blood by treatment with granulocyte colony-stimulating factor and stem cell factor. *Blood* 1994; 84: 1482–91.

163 Kurdi M, Booz GW. G-CSF-based stem cell therapy for the heart-unresolved issues part A: paracrine actions, mobilization, and delivery. *Congest Heart Fail* 2007; 13: 221–7.

164 Harada M, Qin Y, Minamino T, Toko H. G-CSF Prevents Cardiac Remodeling after Myocardial Infarction by Activating Jak/STAT in Cardiomyocytes (Vascular Regeneration (M), The 69th Annual …. *… journal: official journal …* 2005.

165 Joseph J, Rimawi A, Mehta P, *et al.* Safety and effectiveness of granulocyte-colony stimulating factor in mobilizing stem cells and improving cytokine profile in advanced chronic heart failure. *Am J Cardiol* 2006; 97: 681–4.

166 Hüttmann A, Dührsen U, Stypmann J, *et al.* Granulocyte colony-stimulating factor-induced

blood stem cell mobilisation in patients with chronic heart failure--Feasibility, safety and effects on exercise tolerance and cardiac function. *Basic Res Cardiol* 2006; 101: 78–86.

167 Kuethe F, Figulla HR, Herzau M, *et al.* Treatment with granulocyte colony-stimulating factor for mobilization of bone marrow cells in patients with acute myocardial infarction. *Am Heart J* 2005; 150: 115.

168 Ince H, Petzsch M, Kleine HD, *et al.* Prevention of left ventricular remodeling with granulocyte colony-stimulating factor after acute myocardial infarction: final 1-year results of the Front-Integrated Revascularization and Stem Cell Liberation in Evolving Acute Myocardial Infarction by Granulocyte Colony-Stimulating Factor (FIRSTLINE-AMI) Trial. *Circulation* 2005; 112: I73–80.

169 Ripa RS, Jørgensen E, Wang Y, *et al.* Stem cell mobilization induced by subcutaneous granulocyte-colony stimulating factor to improve cardiac regeneration after acute ST-elevation myocardial infarction: result of the double-blind, randomized, placebo-controlled stem cells in myocardial infarction (STEMMI) trial. *Circulation* 2006; 113: 1983–92.

170 Abdel-Latif A, Bolli R, Zuba-Surma EK, Tleyjeh IM, Hornung CA, Dawn B. Granulocyte colony-stimulating factor therapy for cardiac repair after acute myocardial infarction: a systematic review and meta-analysis of randomized controlled trials. *Am Heart J* 2008; 156: 216–9.

171 Falanga A, Marchetti M, Evangelista V, *et al.* Neutrophil activation and hemostatic changes in healthy donors receiving granulocyte colony-stimulating factor. *Blood* 1999; 93: 2506–14.

172 Kang H-J, Kim H-S, Zhang S-Y, *et al.* Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial. *Lancet* 2004; 363: 751–6.

173 Engelmann MG, Theiss HD, Hennig-Theiss C, *et al.* Autologous bone marrow stem cell mobilization induced by granulocyte colony-stimulating factor after subacute ST-segment elevation myocardial infarction undergoing late revascularization: final results from the G-CSF-STEMI (Granulocyte Colony-Stimulating Factor ST-Segment Elevation Myocardial Infarction) trial. *J Am Coll Cardiol* 2006; 48: 1712–21.

174 Jørgensen E, Ripa RS, Helqvist S, *et al.* In-stent neo-intimal hyperplasia after stem cell mobilization by granulocyte-colony stimulating factor Preliminary intracoronary ultrasound results from a double-blind randomized placebo-controlled study of patients treated with percutaneous coronary intervention for ST-elevation myocardial infarction (STEMMI Trial). *Int J Cardiol* 2006; 111: 174–7.

175 Leone AM, Giannico MB, Bruno I, *et al.* Safety and efficacy of G-CSF in patients with ischemic heart failure: the CORNER (Cell Option For Recovery in the Non-Eligible Patients for Revascularization) study. *Int J Cardiol* 2011; 150: 75–8.

176 Francis DP, Shamim W, Davies LC, *et al.* Cardiopulmonary exercise testing for prognosis in chronic heart failure: continuous and independent prognostic value from VE/VCO(2)slope and peak VO(2). *Eur Heart J* 2000; 21: 154–61.

177 Cohn JN, Johnson G, Ziesche S, *et al.* A comparison of enalapril with hydralazine-isosorbide dinitrate in the treatment of chronic congestive heart failure. *N Engl J Med* 1991; 325: 303–10.

178 Milani RV, Lavie CJ, Mehra MR, Ventura HO. Understanding the basics of cardiopulmonary exercise testing. *Mayo Clin Proc* 2006; 81: 1603–11.

179 Peichev M, Naiyer AJ, Pereira D, *et al.* Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. *Blood* 2000; 95: 952–8.

180 Tomita S, Ishida M, Nakatani T, *et al.* Bone marrow is a source of regenerated cardiomyocytes in doxorubicin-induced cardiomyopathy and granulocyte colony-stimulating factor enhances migration of bone marrow cells and attenuates cardiotoxicity of doxorubicin under electron microscopy. *J Heart Lung Transplant* 2004; 23: 577–84.

181 Hamamoto M, Tomita S, Nakatani T, *et al.* Granulocyte-colony stimulating factor directly enhances proliferation of human troponin I-positive cells derived from idiopathic dilated cardiomyopathy through specific receptors. *J Heart Lung Transplant* 2004; 23: 1430–7.

182 Wang X, Hu Q, Mansoor A, *et al.* Bioenergetic and functional consequences of stem cell-based VEGF delivery in pressure-overloaded swine hearts. *Am J Physiol Heart Circ Physiol* 2006; 290: H1393–405.

183 Abdel-Latif A, Bolli R, Tleyjeh IM, *et al.* Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis. *Arch Intern Med* 2007; 167: 989–97.

184 Murry CE, Soonpaa MH, Reinecke H, *et al.* Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* 2004; 428: 664–8.

185 Mathur A, Martin JF. Stem cells and repair of the heart. *Lancet* 2004; 364: 183–92.

186 Mozid AM, Jones D, Arnous S, *et al.* The effects of age, disease state, and granulocyte colony-stimulating factor on progenitor cell count and function in patients undergoing cell therapy for cardiac disease. *Stem Cells Dev* 2013; 22: 216–23.

187 Vasa M, Fichtlscherer S, Aicher A, *et al.* Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 2001; 89: E1–7.

188 Hocking DC, Phillips PG, Ferro TJ, Johnson A. Mechanisms of pulmonary edema induced by tumor necrosis factor-alpha. *Circ Res* 1990; 67: 68–77.

189 Lindmark E, Diderholm E, Wallentin L, Siegbahn A. Relationship between interleukin 6 and mortality in patients with unstable coronary artery disease: effects of an early invasive or noninvasive strategy. *JAMA* 2001; 286: 2107–13.

190 Jernberg T, Lindahl B, Siegbahn A, *et al.* N-terminal pro-brain natriuretic peptide in relation to inflammation, myocardial necrosis, and the effect of an invasive strategy in unstable coronary artery disease. *J Am Coll Cardiol* 2003; 42: 1909–16.

191 James SK, Lindahl B, Timmer JR, *et al.* Usefulness of biomarkers for predicting long-term mortality in patients with diabetes mellitus and non-ST-elevation acute coronary syndromes (a GUSTO IV substudy). *Am J Cardiol* 2006; 97: 167–72.

192 Kavsak PA, Ko DT, Newman AM, *et al.* Risk stratification for heart failure and death in an acute coronary syndrome population using inflammatory cytokines and N-terminal pro-brain natriuretic peptide. *Clin Chem* 2007; 53: 2112–8.

193 Suleiman M, Khatib R, Agmon Y, *et al.* Early inflammation and risk of long-term development of heart failure and mortality in survivors of acute myocardial infarction predictive role of C-reactive protein. *J Am Coll Cardiol* 2006; 47: 962–8.

194 Kavsak PA, MacRae AR, Newman AM, *et al.* Elevated C-reactive protein in acute coronary syndrome presentation is an independent predictor of long-term mortality and heart failure. *Clin Biochem* 2007; 40: 326–9.

195 Abbate A, Kontos MC, Grizzard JD, *et al.* Interleukin-1 blockade with anakinra to prevent adverse cardiac remodeling after acute myocardial infarction (Virginia Commonwealth University Anakinra Remodeling Trial [VCU-ART] Pilot study). *Am J Cardiol* 2010; 105: 1371–1.

196 Ohtsuka T, Hamada M, Hiasa G, *et al.* Effect of beta-blockers on circulating levels of inflammatory and anti-inflammatory cytokines in patients with dilated cardiomyopathy. *J Am Coll Cardiol* 2001; 37: 412–7.

197 Gullestad L, Aukrust P, Ueland T, *et al.* Effect of high- versus low-dose angiotensin converting enzyme inhibition on cytokine levels in chronic heart failure. *J Am Coll Cardiol* 1999; 34: 2061–7.

198 McCullough PA, Nowak RM, McCord J, *et al.* B-type natriuretic peptide and clinical judgment in emergency diagnosis of heart failure: analysis from Breathing Not Properly (BNP) Multinational Study. *Circulation* 2002; 106: 416–22.

199 Moe GW. B-type natriuretic peptide in heart failure. *Curr Opin Cardiol* 2006; 21: 208–14.

200 Lubien E, DeMaria A, Krishnaswamy P, *et al.* Utility of B-natriuretic peptide in detecting diastolic dysfunction: comparison with Doppler velocity recordings. *Circulation* 2002; 105: 595–601.

201 Maisel AS, Krishnaswamy P, Nowak RM, *et al.* Rapid measurement of B-type natriuretic peptide in the emergency diagnosis of heart failure. *N Engl J Med* 2002; 347: 161–7.

202 Maisel AS, McCord J, Nowak RM, *et al.* Bedside B-Type natriuretic peptide in the emergency diagnosis of heart failure with reduced or preserved ejection fraction. Results from the Breathing Not Properly Multinational Study. *J Am Coll Cardiol* 2003; 41: 2010–7.

203 Wang TJ, Larson MG, Levy D, *et al.* Plasma natriuretic peptide levels and the risk of cardiovascular events and death. *N Engl J Med* 2004; 350: 655–63.

204 Tsutamoto T, Wada A, Maeda K, *et al.* Plasma brain natriuretic peptide level as a biochemical

marker of morbidity and mortality in patients with asymptomatic or minimally symptomatic left ventricular dysfunction. Comparison with plasma angiotensin II and endothelin-1. *Eur Heart J* 1999; 20: 1799–807.

205 Hülsmann M, Berger R, Sturm B, *et al.* Prediction of outcome by neurohumoral activation, the six-minute walk test and the Minnesota Living with Heart Failure Questionnaire in an outpatient cohort with congestive heart failure. *Eur Heart J* 2002; 23: 886–91.

206 Jourdain P, Jondeau G, Funck F, *et al.* Plasma brain natriuretic peptide-guided therapy to improve outcome in heart failure: the STARS-BNP Multicenter Study. *J Am Coll Cardiol* 2007; 49: 1733–9.

207 Troughton RW, Frampton CM, Yandle TG, Espiner EA, Nicholls MG, Richards AM. Treatment of heart failure guided by plasma aminoterminal brain natriuretic peptide (N-BNP) concentrations. *Lancet* 2000; 355: 1126–30.

208 Mueller C, Scholer A, Laule-Kilian K, *et al.* Use of B-type natriuretic peptide in the evaluation and management of acute dyspnea. *N Engl J Med* 2004; 350: 647–54.

209 Berger R, Huelsman M, Strecker K, *et al.* B-type natriuretic peptide predicts sudden death in patients with chronic heart failure. *Circulation* 2002; 105: 2392–7.

210 Gardner RS, Ozalp F, Murday AJ, Robb SD, McDonagh TA. N-terminal pro-brain natriuretic peptide. A new gold standard in predicting mortality in patients with advanced heart failure. *Eur Heart J* 2003; 24: 1735–43.

211 Tsutamoto T, Wada A, Maeda K, *et al.* Attenuation of compensation of endogenous cardiac natriuretic peptide system in chronic heart failure: prognostic role of plasma brain natriuretic peptide concentration in patients with chronic symptomatic left ventricular dysfunction. *Circulation* 1997; 96: 509–16.

212 Dinarello CA. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood* 2011; 117: 3720–32.

213 Dinarello CA. Interleukin-1 and interleukin-1 antagonism. *Blood* 1991; 77: 1627–52.

214 Dinarello CA, Wolff SM. The role of interleukin-1 in disease. *N Engl J Med* 1993; 328: 106–13.

215 Taniguchi T, Matsui H, Fujita T, *et al.* Structure and expression of a cloned cDNA for human interleukin-2. *Nature* 1983; 302: 305–10.

216 Pericle F, Liu JH, Diaz JI, *et al.* Interleukin-2 prevention of apoptosis in human neutrophils. *Eur J Immunol* 1994; 24: 440–4.

217 Nakanishi K, Hirose S, Yoshimoto T, *et al.* Role and regulation of interleukin (IL)-2 receptor alpha and beta chains in IL-2-driven B-cell growth. *Proc Natl Acad Sci USA* 1992; 89: 3551–5.

218 Rubin JT. Interleukin-2: its biology and clinical application in patients with cancer. *Cancer Invest* 1993; 11: 460–72.

219 Hart PH, Vitti GF, Burgess DR, Whitty GA, Piccoli DS, Hamilton JA. Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tumor necrosis factor alpha, interleukin 1, and prostaglandin E2. *Proc Natl Acad Sci USA* 1989; 86: 3803–7.

220 Boey H, Rosenbaum R, Castracane J, Borish L. Interleukin-4 is a neutrophil activator. *J Allergy Clin Immunol* 1989; 83: 978–84.

221 Postlethwaite AE, Holness MA, Katai H, Raghow R. Human fibroblasts synthesize elevated levels of extracellular matrix proteins in response to interleukin 4. *J Clin Invest* 1992; 90: 1479–85.

222 Toi M, Bicknell R, Harris AL. Inhibition of colon and breast carcinoma cell growth by interleukin-4. *Cancer Res* 1992; 52: 275–9.

223 Obiri NI, Hillman GG, Haas GP, Sud S, Puri RK. Expression of high affinity interleukin-4 receptors on human renal cell carcinoma cells and inhibition of tumor cell growth in vitro by interleukin-4. *J Clin Invest* 1993; 91: 88–93.

224 Van Snick J. Interleukin-6: an overview. *Annu Rev Immunol* 1990; 8: 253–78.

225 Heinrich PC, Castell JV, Andus T. Interleukin-6 and the acute phase response. *Biochem J* 1990; 265: 621–36.

226 Kishimoto T, Akira S, Taga T. Interleukin-6 and its receptor: a paradigm for cytokines. *Science* 1992; 258: 593–7.

227 Wong GG, Witek-Giannotti JS, Temple PA, *et al.* Stimulation of murine hemopoietic colony formation by human IL-6. *J Immunol* 1988; 140: 3040–4.

228 Bhardwaj N, Santhanam U, Lau LL, *et al.* IL-6/IFN-beta 2 in synovial effusions of patients with rheumatoid arthritis and other arthritides. Identification of several isoforms and studies of cellular sources. *J Immunol* 1989; 143: 2153–9.

229 Ridker PM, Rifai N, Stampfer MJ, Hennekens CH. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation* 2000; 101: 1767–72.

230 Holmes WE, Lee J, Kuang WJ, Rice GC, Wood WI. Structure and functional expression of a human interleukin-8 receptor. *Science* 1991; 253: 1278–80.

231 Oppenheim JJ, Zachariae CO, Mukaida N, Matsushima K. Properties of the novel proinflammatory supergene 'intercrine' cytokine family. *Annu Rev Immunol* 1991; 9: 617–48.

232 Matsushima K, Baldwin ET, Mukaida N. Interleukin-8 and MCAF: novel leukocyte recruitment and activating cytokines. *Chem Immunol* 1992; 51: 236–65.

233 Windsor WT, Syto R, Tsarbopoulos A, *et al.* Disulfide bond assignments and secondary structure analysis of human and murine interleukin 10. *Biochemistry* 1993; 32: 8807–15.

234 Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR. Interleukin-10. *Annu Rev Immunol* 1993; 11: 165–90.

235 Lacraz S, Nicod LP, Chicheportiche R, Welgus HG, Dayer JM. IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human mononuclear phagocytes. *J Clin Invest* 1995; 96: 2304–10.

236 Wang P, Wu P, Anthes JC, Siegel MI, Egan RW, Billah MM. Interleukin-10 inhibits interleukin-8 production in human neutrophils. *Blood* 1994; 83: 2678–83.

237 Wang B, Zhuang L, Fujisawa H, *et al.* Enhanced epidermal Langerhans cell migration in IL-10 knockout mice. *J Immunol* 1999; 162: 277–83.

238 Stumpf C, Lehner C, Yilmaz A, Daniel WG, Garlichs CD. Decrease of serum levels of the anti-inflammatory cytokine interleukin-10 in patients with advanced chronic heart failure. *Clin Sci* 2003; 105: 45–50.

239 de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* 1991; 174: 1209–20.

240 Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. *Endocr Rev* 1997; 18: 4–25.

241 Berse B, Brown LF, Van de Water L, Dvorak HF, Senger DR. Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. *Mol Biol Cell* 1992; 3: 211–20.

242 Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 1995; 146: 1029–39.

243 Yang R, Thomas GR, Bunting S, *et al.* Effects of vascular endothelial growth factor on hemodynamics and cardiac performance. *J Cardiovasc Pharmacol* 1996; 27: 838–44.

244 Seko Y, Imai Y, Suzuki S, *et al.* Serum levels of vascular endothelial growth factor in patients with acute myocardial infarction undergoing reperfusion therapy. *Clin Sci* 1997; 92: 453–4.

245 Terai M, Yasukawa K, Narumoto S, Tateno S, Oana S, Kohno Y. Vascular endothelial growth factor in acute Kawasaki disease. *Am J Cardiol* 1999; 83: 337–9.

246 Freedman SB, Isner JM. Therapeutic angiogenesis for coronary artery disease. *Ann Intern Med* 2002; 136: 54–71.

247 Weck KE, Dal Canto AJ, Gould JD, *et al.* Murine gamma-herpesvirus 68 causes severe large-vessel arteritis in mice lacking interferon-gamma responsiveness: a new model for virus-induced vascular disease. *Nat Med* 1997; 3: 1346–53.

248 Rea IM, McNerlan SE, Alexander HD. CD69, CD25, and HLA-DR activation antigen expression on

CD3+ lymphocytes and relationship to serum TNF-alpha, IFN-gamma, and sIL-2R levels in aging. *Exp Gerontol* 1999; 34: 79–93.

249 Schreiber AB, Winkler ME, Derynck R. Transforming growth factor-alpha: a more potent angiogenic mediator than epidermal growth factor. *Science* 1986; 232: 1250–3.

250 Schultz G, Rotatori DS, Clark W. EGF and TGF-alpha in wound healing and repair. *J Cell Biochem* 1991; 45: 346–52.

251 Vilcek J, Lee TH. Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. *J Biol Chem* 1991; 266: 7313–6.

252 Waage A, Halstensen A, Espevik T. Association between tumour necrosis factor in serum and fatal outcome in patients with meningococcal disease. *Lancet* 1987; 1: 355–7.

253 Sharief MK, Hentges R. Association between tumor necrosis factor-alpha and disease progression in patients with multiple sclerosis. *N Engl J Med* 1991; 325: 467–72.

254 Dutka DP, Elborn JS, Delamere F, Shale DJ, Morris GK. Tumour necrosis factor alpha in severe congestive cardiac failure. *Br Heart J* 1993; 70: 141–3.

255 Levine B, Kalman J, Mayer L, Fillit HM, Packer M. Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. *N Engl J Med* 1990; 323: 236–41.

256 Başaran Y, Başaran MM, Babacan KF, *et al.* Serum tumor necrosis factor levels in acute myocardial infarction and unstable angina pectoris. *Angiology* 1993; 44: 332–7.

257 Ridker PM, Rifai N, Pfeffer M, Sacks F, Lepage S, Braunwald E. Elevation of tumor necrosis factor-alpha and increased risk of recurrent coronary events after myocardial infarction. *Circulation* 2000; 101: 2149–53.

258 Likoff MJ, Chandler SL, Kay HR. Clinical determinants of mortality in chronic congestive heart failure secondary to idiopathic dilated or to ischemic cardiomyopathy. *Am J Cardiol* 1987; 59: 634–8.

259 Parameshwar J, Keegan J, Sparrow J, Sutton GC, Poole-Wilson PA. Predictors of prognosis in severe chronic heart failure. *Am Heart J* 1992; 123: 421–6.

260 Gradman A, Deedwania P, Cody R, *et al.* Predictors of total mortality and sudden death in mild to moderate heart failure. Captopril-Digoxin Study Group. *J Am Coll Cardiol* 1989; 14: 564–70–discussion571–2.

261 Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. *Circulation* 1990; 81: 1161–72.

262 Weber KT, Kinasewitz GT, Janicki JS, Fishman AP. Oxygen utilization and ventilation during exercise in patients with chronic cardiac failure. *Circulation* 1982; 65: 1213–23.

263 Cohn JN, Johnson GR, Shabetai R, *et al.* Ejection fraction, peak exercise oxygen consumption,

cardiothoracic ratio, ventricular arrhythmias, and plasma norepinephrine as determinants of prognosis in heart failure. The V-HeFT VA Cooperative Studies Group. *Circulation* 1993; 87: VI5–16.

264 Stelken AM, Younis LT, Jennison SH, *et al.* Prognostic value of cardiopulmonary exercise testing using percent achieved of predicted peak oxygen uptake for patients with ischemic and dilated cardiomyopathy. *J Am Coll Cardiol* 1996; 27: 345–52.

265 Bensimhon DR, Leifer ES, Ellis SJ, *et al.* Reproducibility of peak oxygen uptake and other cardiopulmonary exercise testing parameters in patients with heart failure (from the Heart Failure and A Controlled Trial Investigating Outcomes of exercise traiNing). *Am J Cardiol* 2008; 102: 712–7.

266 Balady GJ, Arena R, Sietsema K, *et al.* Clinician's Guide to cardiopulmonary exercise testing in adults: a scientific statement from the American Heart Association. Circulation. 2010; 122: 191–225.

267 Agostoni P, Bianchi M, Moraschi A, *et al.* Work-rate affects cardiopulmonary exercise test results in heart failure. *Eur J Heart Fail* 2005; 7: 498–504.

268 Myers J, Goldsmith RL, Keteyian SJ, *et al.* The ventilatory anaerobic threshold in heart failure: a multicenter evaluation of reliability. *J Card Fail* 2010; 16: 76–83.

269 Agostoni P, Corrà U, Cattadori G, *et al.* Prognostic value of indeterminable anaerobic threshold in heart failure. *Circ Heart Fail* 2013; 6: 977–87.

270 Gibbons RJ, Balady GJ, Bricker JT, *et al.* ACC/AHA 2002 guideline update for exercise testing: summary article: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to Update the 1997 Exercise Testing Guidelines). Circulation. 2002; 106: 1883–92.

271 Arena R, Myers J, Williams MA, *et al.* Assessment of functional capacity in clinical and research settings: a scientific statement from the American Heart Association Committee on Exercise, Rehabilitation, and Prevention of the Council on Clinical Cardiology and the Council on Cardiovascular Nursing. *Circulation* 2007; 116: 329–43.

272 Valeur N, Clemmensen P, Saunamäki K, Grande P, DANAMI-2 investigators. The prognostic value of pre-discharge exercise testing after myocardial infarction treated with either primary PCI or fibrinolysis: a DANAMI-2 sub-study. *Eur Heart J* 2005; 26: 119–27.

273 Seth S, Bhargava B, Narang R, *et al.* The ABCD (Autologous Bone Marrow Cells in Dilated Cardiomyopathy) trial a long-term follow-up study. *J Am Coll Cardiol* 2010; 55: 1643–4.

274 Vrtovec B, Poglajen G, Lezaic L, *et al.* Effects of intracoronary CD34+ stem cell transplantation in nonischemic dilated cardiomyopathy patients: 5-year follow-up. *Circ Res* 2013; 112: 165–73.

275 Iwanaga K, Takano H, Ohtsuka M, *et al.* Effects of G-CSF on cardiac remodeling after acute myocardial infarction in swine. *Biochem Biophys Res Commun* 2004; 325: 1353–9.

276 Richardson P, McKenna W, Bristow M, *et al.* Report of the 1995 World Health Organization/International Society and Federation of Cardiology Task Force on the Definition and Classification of cardiomyopathies. Circulation. 1996; 93: 841–2.

277 Cintron G, Johnson G, Francis G, Cobb F, Cohn JN. Prognostic significance of serial changes in left ventricular ejection fraction in patients with congestive heart failure. The V-HeFT VA Cooperative Studies Group. *Circulation* 1993; 87: VI17–23.

278 Cohn JN, Ferrari R, Sharpe N. Cardiac remodeling--concepts and clinical implications: a consensus paper from an international forum on cardiac remodeling. Behalf of an International Forum on Cardiac Remodeling., 2000: 569–82.

279 Waagstein F, Bristow MR, Swedberg K, *et al.* Beneficial effects of metoprolol in idiopathic dilated cardiomyopathy. Metoprolol in Dilated Cardiomyopathy (MDC) Trial Study Group. *Lancet* 1993; 342: 1441–6.

280 Colucci WS, Packer M, Bristow MR, *et al.* Carvedilol inhibits clinical progression in patients with mild symptoms of heart failure. US Carvedilol Heart Failure Study Group. *Circulation* 1996; 94: 2800–6.

281 A randomized trial of beta-blockade in heart failure. The Cardiac Insufficiency Bisoprolol Study (CIBIS). CIBIS Investigators and Committees. *Circulation* 1994; 90: 1765–73.

282 Konstam MA, Rousseau MF, Kronenberg MW, *et al.* Effects of the angiotensin converting enzyme inhibitor enalapril on the long-term progression of left ventricular dysfunction in patients with heart failure. SOLVD Investigators. *Circulation* 1992; 86: 431–8.

283 Greenberg B, Quinones MA, Koilpillai C, *et al.* Effects of long-term enalapril therapy on cardiac structure and function in patients with left ventricular dysfunction. Results of the SOLVD echocardiography substudy. *Circulation* 1995; 91: 2573–81.

284 Doughty RN, Whalley GA, Gamble G, MacMahon S, Sharpe N. Left ventricular remodeling with carvedilol in patients with congestive heart failure due to ischemic heart disease. Australia-New Zealand Heart Failure Research Collaborative Group. *J Am Coll Cardiol* 1997; 29: 1060–6.

285 Lechat P, Escolano S, Golmard JL, *et al.* Prognostic value of bisoprolol-induced hemodynamic effects in heart failure during the Cardiac Insufficiency BIsoprolol Study (CIBIS). *Circulation* 1997; 96: 2197–205.

286 Cowie MR, Jourdain P, Maisel A, *et al.* Clinical applications of B-type natriuretic peptide (BNP) testing. *Eur Heart J* 2003; 24: 1710–8.

287 Valli N, Georges A, Corcuff JB, Barat JL, Bordenave L. Assessment of brain natriuretic peptide in patients with suspected heart failure: comparison with radionuclide ventriculography data. *Clin Chim Acta* 2001; 306: 19–26.

288 Koglin J, Pehlivanli S, Schwaiblmair M, Vogeser M, Cremer P, vonScheidt W. Role of brain natriuretic peptide in risk stratification of patients with congestive heart failure. *J Am Coll Cardiol* 2001; 38: 1934–41.

289 Cataliotti A, Malatino LS, Jougasaki M, *et al.* Circulating natriuretic peptide concentrations in patients with end-stage renal disease: role of brain natriuretic peptide as a biomarker for ventricular remodeling. *Mayo Clin Proc* 2001; 76: 1111–9.

290 O'Neill JO, Bott-Silverman CE, McRae AT, *et al.* B-type natriuretic peptide levels are not a surrogate marker for invasive hemodynamics during management of patients with severe heart failure. *Am Heart J* 2005; 149: 363–9.

291 Nishii M, Inomata T, Takehana H, *et al.* Prognostic utility of B-type natriuretic peptide assessment in stable low-risk outpatients with nonischemic cardiomyopathy after decompensated heart failure. *J Am Coll Cardiol* 2008; 51: 2329–35.

292 Bartunek J, Dimmeler S, Drexler H, *et al.* The consensus of the task force of the European Society of Cardiology concerning the clinical investigation of the use of autologous adult stem cells for repair of the heart. *Eur Heart J* 2006; 27: 1338–40.

Appendix

Publications

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

Bone marrow mononuclear cells and acute myocardial infarction
Arnous S, Mozid A, Martin J, Mathur A.
Stem Cell Research and Therapy. 2012 Jan 17;3(1):2.

Stem Cell Therapy for Heart Diseases
Mozid AM, Arnous S, Sammut EC, Mathur A
British Medical Bulletin, 2011;98:143-59

Safety and feasibility of intramyocardial versus intracoronary delivery of autologous cell therapy in advanced heart failure: the REGENERATE-IHD pilot study.
Mozid A, Yeo C, Arnous S, Ako E, Saunders N, Locca D, Brookman P, Archbold RA, Rothman M, Mills P, Agrawal S, Martin J, Mathur A.
Regen Med. 2014 May;9(3):269-78

The Effects of age, disease state and granulocyte-colony stimulating factor on progenitor cell count and function in patients undergoing cell therapy for cardiac disease.
Mozid A, Jones D, Arnous S, Saunders N, Wragg A, Martin J, Agrawal S, Mathur A.
Stem Cells and Development. 2013 Jan 15;22(2):216-23

The long-term survival and the risks and benefits of implantable cardioverter defibrillators in patients with hypertrophic cardiomyopathy.
O'Mahony C, Lambiase PD, Quarta G, Cardona M, Calcagnino M, Tsovolas K, Al-Shaikh S, Rahman SM, Arnous S, Jones S, McKenna W, Elliott P.
Heart. 2012 Jan;98(2):116-25.

Book Chapters

Genetics of dilated cardiomyopathy: Risk of conduction defects and sudden cardiac death
Samer Arnous, Petros Syrris, Srijita Sen-Chowdhry, William J.McKenna
Cardiac Electrophysiology clinics 2(2010) 599-609

Cell base regenerative therapy
Mozid A, Arnous S, Lovell M, Mathur A.
EuroPCR Textbook of Interventional Cardiology 2012 : Chapter 3.49

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