

**REGENERATION OF THE
HEART WITH GENE AND
CELL THERAPY**

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PhD

***Dedicated to the memory of my beautiful mother Brenda Green
'Madmo' 05.12.1939 - 11.07.2011 and for my gorgeous son Mahé***



Brave, beautiful, and an inspiration to all that knew you. I miss you so much and wish you could see that I eventually finished! You were an amazing person; kind, caring, selfless, always optimistic, positive and cheerful. You were exceptionally intelligent, far brighter than either of your children, and yet without the amazing opportunities we have had in life.

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Declaration of originality

I would like to confirm that this Thesis is composed of original work. Chapters three and four and parts of the cell therapy segment of the introduction in chapter one were previously published in papers and a book chapter I wrote, and are referenced below.

Evaluation of the porcine ameroid constrictor model of myocardial ischemia for therapeutic angiogenesis studies.

Radke PW, Heintz-Green A, Frass OM, Post MJ, Sato K, Geddes DM, Alton EW. Endothelium. 2006 Jan-Feb;13(1):25-33.

The efficacy of a 'master switch gene' HIF-1 alpha in a porcine model of chronic myocardial ischaemia.

Heintz-Green A, Radke PW, Munkonge FM, Frass O, Zhu J, Vincent K, Geddes DM, Alton EW.

Eur Heart J. 2005 Jul;26(13):1327-32.

Cardiac Repair Clinical Trials Amanda Green and Eric Alton, Chapter 33 In Advances in Tissue Engineering. Polak, Julia M, Mantalaris, Sakis, Harding, Sian E. Imperial College Press 2008.

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ABSTRACT

Cardiovascular disease is the largest cause of morbidity and mortality in the industrialised world. Many patients with a longstanding history of coronary artery disease, multiple coronary interventions and a number of prior coronary events are no longer suitable for conventional revascularisation strategies and are often referred to as 'no-option' patients. Despite intensive medical treatment, many present with clinical signs of heart failure and/or angina in the presence of underperfused, viable myocardium. I investigated two new strategies proposed to treat this patient population; angiogenesis induced by gene therapy in a pre-clinical model of myocardial ischaemia and regenerative therapy with autologous bone marrow derived putative stem cells in a Phase IIA pilot clinical trial.

After establishing the ameroid porcine model of myocardial ischaemia, the efficacy of epicardially delivered Hypoxia Inducible Factor-1 alpha (HIF-1 α) was assessed with adenovirus (AdHIF-1 α) and two plasmid constructs. AdHIF-1 α was found to significantly increase myocardial blood flow and left ventricular function in this model. In a further study electromechanical mapping of ischaemic territory and endocardial delivery with AdHIF-1 α demonstrated similar trends.

I present the first clinical trial to demonstrate the feasibility of retrograde coronary venous delivery of putative stem cells to the heart. The inclusion of ¹¹¹In oxide labelled cells demonstrated a sustained myocardial signal up to 21 days. Retention of cells within the myocardium is likely to be critical in allowing a biologically important paracrine function or stem cell differentiation. In the six-month follow-up, there were no major safety concerns, although only hypothesis generating efficacy endpoints were included, significant improvements were seen in Quality of life, New York Heart Association (NYHA) class and myocardial oxygen consumption (MVO₂). A positive correlation was seen with the number of CD34+ cells delivered and change in MVO₂. Further randomised placebo controlled trials are required to elucidate further any perceived improvements in efficacy.

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ABBREVIATIONS

7-aminoactinomycin D (7ADD)
Acute myocardial infarction (AMI)
Alanine aminotransferase (ALT)
Alkaline phosphatase (ALP)
Allogeneic human mesenchymal stem cells (hMSC)
Aspartate aminotransferase (AST)
Aspartate- amino- transferase (ASAT),
Autologous bone marrow cells (ABMC)
Autologous bone marrow mononuclear cells (ABMMC)
B-type natriuretic peptide (BNP)
Body surface area (BSA)
Bone marrow mononuclear cells (BMMNC)
C-reactive protein (CRP)
Canadian Cardiovascular Society (CCS)
Cardiac magnetic resonance (CMR)
Cardiac output (CO)
Cardiac resynchronisation therapy (CRT)
Cardiac resynchronisation therapy with defibrillation (CRTD)
Cardiovascular disease (CVD)
Chronic heart failure (CHF)
Circulating progenitor cells (CPC)
Cluster differentiation cell marker 31 (CD31)
Computer tomography (CT)
Coronary artery bypass grafts (CABG)
Coronary artery disease (CAD)
Coronary sinus (CS)
Creatine phosphokinase MB isoenzyme (CKMB)
Delayed enhancement cardiac magnetic resonance (DE CMR)
Deoxyribonucleic acid (DNA)
Developmentally regulated endothelial locus-1 (Del-1)
Diaminobenzidine (DAB)
Dilated cardiomyopathy (DCM)

Echocardiography (ECHO)
 Ejection time (ET)
 Electromechanical mapping system (NOGA)
 End diastolic volume index (EDVi)
 End systolic volume index (ESVi)
 Endothelial Progenitor Cells (EPC)
 Fetal calf serum (FCS)
 Fibroblast growth factor (FGF)
 Fibroblast growth factor 1 Acidic (FGF1)
 Fibroblast growth factor 2 Basic (bFGF) or (FGF2)
 Filling time (FT)
 Fluorescence-activated cell sorting (FACS)
 Food and drug administration (FDA)
 Full blood count (FBC)
 Full blood count (FBC)
 Gamma-glytaryl transpeptidase (Gamma GT)
 Glyceryl Trinitrate (GTN)
 GOSH (The Hospital for Sick Children Great Ormond Street)
 Granulocyte cell stem cell factor (G-CSF)
 Haematopoietic stem cells (HSC),
 Haematoxylin and eosin (HE)
 Heart rate (HR)
 Horseradish peroxidase kit (HRP)
 Hypoxia-inducible factor 1 alpha (HIF-1 α)
 Hypoxia-inducible factor-1 beta (HIF-1 β)
 Indium-111 (^{111}In -oxide)
 Inducible nitric oxide synthase (iNOS)
 Institute of Medical Technology, Magdeburg, Germany (IMTM)
 Insulin-like growth factor (IGF-2)
 Internal cardiac defibrillators (ICD)
 Intramuscular (IM)
 Ischaemic cardiomyopathy (ICM)
 Ischaemic heart disease (IHD)

Isovolumic contraction time (IVCT)
Isovolumic relaxation time (IVRT)
Left anterior descending (LAD)
Left atrial pressure (LAP)
Left circumflex coronary artery (LCX)
Left ventricle (LV)
Left ventricular assist device (LVAD)
Left ventricular diameter (LVD) diameter
Left ventricular diastolic volume (LVDV)
Left ventricular ejection fraction (LVEF)
Left ventricular end diastolic diameter (LVED)
Left ventricular end systolic volume (LVESV)
Left ventricular end systolic volume (LVESV)
Left ventricular function (LVF)
Log to the base e (LN)
Magnetic resonance imaging (MRI)
Major Adverse Cardiac Events (MACE)
Mean blood pressure (MBP)
Mesenchymal stem cells (MSC)
Messenger RNA (mRNA)
Mitral regurgitation (MR)
Multipotential adult progenitor cells (MAPC)
Myocardial blood flow (MBF)
Myocardial infarction (MI)
Myocardial oxygen consumption (MVO₂)
National Institute for Health and Clinical Excellence (NICE)
New York Heart Association functional class (NYHA)
Nitric oxide (NO)
Nitric oxide synthase (NOS)
Nitro-L-arginine methyl ester hydrochloride (L-NAME)
Nuclear factor kappa-light-chain-enhancer of activated β cells (NF κ β)
Off-pump CABG (OPCABG),
Percutaneous coronary intervention (PCI)

Peripheral bone marrow cells (PBMC)
 Peripheral circulating progenitor cells (PCPC)
 Phosphate buffered saline (PBS)
 Platelet derived growth factor (PDGF)
 Platelet derived growth factor beta (PDGF- β)
 Positron emission tomography (PET)
 Premature ventricular contraction (PVC)
 Propidium iodide (PI)
 Quality of life (QOL)
 Regions of interest (ROIs)
 Respiratory exchange ratio (RER)
 Right coronary artery RCA
 Royal Brompton Hospital (RBH)
 Side population (SP)
 Single photon emission computed tomography (SPECT)
 Skeletal myoblasts (SM)
 ST elevated myocardial infarction (STEMI)
 Thrombolysis in myocardial infarction (TIMI)
 Total isovolumic time (t-IVT)
 Transactivation domain from the herpes simplex virus (HSV) (VP16)
 Transforming growth factor beta (TGF)
 Transforming growth factor beta (TGF β)
 Troponin I (TrI)
 Tumour necrosis factor alpha (TNF- α)
 Type-1 vascular endothelial growth factor receptor (VEGF-1/flt-1)
 Type- 2 vascular endothelial growth factor receptor (VEGF-2 /flk-1)
 Vascular endothelial growth factor (VEGF)
 Ventricular fibrillation (VF)
 Ventricular tachycardia (VT)
 Weight to aortic size (W/A)
 Weight/aortic diameter ratio (WADR)
 White cell count (WCC)
 World health organisation (WHO)

CHAPTER 1

1.1 INTRODUCTION

a. What is ischaemic heart disease?

Cardiovascular disease is the largest cause of morbidity and mortality in the industrialised world, and remains the leading cause of death within Europe, nearly half resulting from coronary artery disease (CAD) (Bleumink *et al.*, 2004). The World Health Organization (WHO) estimates this situation will worsen and predicts by 2020 over 25 million deaths from cardiovascular disease (CVD) worldwide (Mackay *et al.*, 2004). Chronic myocardial ischaemia represents an imbalance between myocardial oxygen supply and demand, often arising from atherosclerotic CAD, which prevents the native coronary arteries from providing an adequate blood supply to the heart. In some patients, growth of coronary collateral vessels occurs during prolonged ischaemia and can limit infarction of myocardial tissue if the stenosis occurs slowly enough (Schaper and Ito, 1996). The human endogenous angiogenic response is often incomplete (Klagsbrun and D'Amore, 1991) and in most patients inadequate; the large number of revascularisation procedures undertaken each year attest to this. More people are surviving post myocardial infarction (MI), but the resulting irreversible loss of heart function has led to an increased incidence of heart failure (Kocher *et al.*, 2007).

The primary clinical manifestations of chronic myocardial ischaemia are angina pectoris, left ventricular dysfunction and sudden cardiac death usually from an arrhythmia. Management focuses firstly on evaluating the extent and severity of disease, particularly ventricular function as this is the strongest predictor of outcome (Simons and Laham, 1999a). Therapy is then directed at controlling symptoms and improving prognosis. The therapeutic goal is to restore the balance between oxygen demand and blood supply and to maintain ventricular function, as this is the main determinant of prognosis. Prevention of myocardial infarct is by revascularisation strategies, such as percutaneous coronary interventions (PCI) and coronary artery bypass grafts (CABG), whilst treatment of left ventricular dysfunction is achieved by a combination of medical management, and implantable devices such as bi-ventricular pacing.

b. Is there a need for a new treatment option?

There have been many advances in cardiology, importantly including the development of drug eluting stents which have decreased significantly the problem of recurrent restenosis (Waugh and Wagstaff, 2004) and the introduction of cardiac resynchronisation therapy with defibrillation (CRTD) which has reduced the mortality of patients with heart failure specifically New York Heart Association (NYHA) functional class III and IV (Bristow *et al.*, 2004). In cardiac surgery, the advent of off-bypass (CABG) procedures and minimally invasive robotic surgery has increased the armamentarium for tackling complex, high-risk patients (Sellke *et al.*, 2010). However a study of 2860 patients with multi-vessel coronary artery disease who received bypass grafts for the first time demonstrated that 28% resulted in incomplete revascularisation (Jones and Weintraub, 1996). Evidence has shown that the outcome for patients with complete revascularisation is superior to those with incomplete revascularisation (Jones and Weintraub, 1996). Therefore territories not amenable to conventional treatment require a new revascularisation strategy. However this is somewhat controversial, as more recently others have found that PCI did not decrease the incidence of major cardiovascular outcomes in patients with stable coronary artery disease (Boden *et al.*, 2009). Despite this new evidence many in cardiology clinical practice continue to undertake procedures to open occluded coronary arteries in patients with stable disease.

In addition many patients with a longstanding history of coronary artery disease, multiple coronary interventions and a number of prior coronary events are no longer suitable for conventional revascularisation strategies such as PCI or CABG because of the advanced nature of their disease, diffuse coronary disease, small distal vessels, or significant co-morbidities (Mukherjee *et al.*, 1999). These are often referred to as ‘no-option’ patients. Despite intensive medical treatment, many of these patients present with clinical signs of heart failure and or angina in the presence of underperfused, viable myocardium (Auerbach *et al.*, 1999), and remain with a bleak outlook, both in terms of life expectancy and quality of life. In a cardiac catheterisation study of 500 patients (Mukherjee *et al.*, 1999) it was found that 11.6% with symptomatic coronary artery disease were classified as ‘no-option patients’; if these figures are applied to the one million cardiac catheter cases performed in Europe each year this would mean that around 115,000 European

patients could be classified in this way. Estimates from the USA similarly classify over 100,000 of these patients every year (Lenzen *et al.*, 2005).

The loss of LV function whether due to ischaemic heart disease, loss of contractile tissue from myocardial infarction or non-ischaemic causes, leads to a common pathway of impaired cardiac output, increased wall stress and dilatation of the left ventricular (LV) chamber. To maintain an adequate cardiac output at a cellular level there is adaptive up-regulation of sympathetic nervous system pathways to elevate blood pressure, stimulate stroke volume and heart rate (Triposkiadis *et al.*, 2009). Whilst in the short-term this compensatory mechanism is useful in maintaining LV function, in the longer term it becomes maladaptive leading to chronic up-regulation of neurohormonal signaling pathways which results in a loss of receptor and end-organ responsiveness, which then stimulates further up-regulation of neurohormonal activation. In terms of treatment, lowering blood pressure by blocking activation of the rennin-angiotensin system with ACE inhibitors has been successful (Swedberg *et al.*, 1992), which together with blocking the chronic overstimulation of the adrenergic system with β -blockers has improved cardiac function, functional status and reduced mortality (Packer *et al.*, 2001). Suppression of aldosterone signaling has led to additional improvements in morbidity and mortality (Pitt *et al.*, 1999). Unfortunately this has represented the final salvo to date in attempts to 'normalise' chronically up-regulated pathways (Povsic and O'Connor, 2010) and thus there is a need to find alternative strategies to treat chronic heart failure.

Estimates for the annual mortality rate of a population presenting with severe heart failure NYHA class III and IV may be derived from the CONSENSUS-I trial (1987) (36% for the ACE inhibitor arm), or from those on transplantation waiting lists (30%) (Deng *et al.*, 2000). The importance of establishing a new treatment is emphasised by the prevalence of ischaemic heart failure that is rising worldwide. The lifetime risk of developing heart failure is now estimated to be one in five with a five year mortality rate that rivals many cancers (Bui *et al.*, 2011).

In economic terms, a cost of illness study estimates resources used in disease prevention, detection and treatment and is undertaken to aid priority setting in health

care research. Most studies within the UK have concentrated on direct health care costs, but substantial 'indirect costs' are associated with the burden on family and friends of caring for heart disease patients. Economically there is also a loss of productivity, because patients and those that care for them, may no longer be in paid employment, with resultant loss of working days and an increased requirement for incapacity benefits. Using this model in 1999, coronary artery disease cost £1.73 billion to the UK health system in direct costs. In addition, it is estimated that there was a further £2.42 billion in informal care costs and £2.91 billion in estimated productivity loss. Thus the total annual cost of all coronary artery disease related burden was £7.06 billion, the highest of all diseases in the UK (Liu *et al.*, 2002).

If reperfusion and regeneration strategies were available to treat these patients, it may be possible to limit or avoid irreversible myocardial injury to ischaemic viable territory, improve symptoms, function, prognosis and the associated economic costs. In patients with more advanced disease with predominantly LV dysfunction, therapy focuses on regenerating myocardial tissue in an attempt to improve contractibility and function. Thus, I propose that chronic ischaemic heart disease is a relevant patient group to target with novel treatments.

1.2 ANGIOGENESIS

Therapeutic angiogenesis, the up-regulation of endogenous angiogenic factors by gene or protein therapy, has been suggested as a potential new treatment. Thus it is postulated that by increasing blood vessel formation it may be possible to improve perfusion to ischaemic tissues.

The initial thesis chapters describe the development of a pre-clinical programme of angiogenic gene therapy, designed as a direct prelude to a clinical trial for patients with myocardial ischaemia and left ventricular dysfunction who were no longer candidates for conventional revascularisation procedures. In order to appreciate fully the effects of administering these growth factors it is worth reviewing what is currently known about normal blood vessel formation, and the endogenous collateralisation resulting from ischaemic heart disease. Rather than reviewing the angiogenesis literature as a whole, a brief overview, is followed by evidence related

to three key aspects of designing a clinical trial.

- i. Which patient population is most likely to benefit?
- ii. What is the best angiogenic substance to use?
- iii. Why have previous Phase II trials been negative?
- iv. How to deliver therapy?

Finally, the rationale for the design of the pre-clinical programme is outlined.

a. Normal vascular growth

There are three forms of vascular growth; the term *Vasculogenesis* the in-situ development of large muscular vessels from precursor cells relates to the development of large blood vessels by angioblasts *in situ*, during fetal development (Risau and Flamme, 1995). *Angiogenesis* refers to the sprouting of capillaries from pre-existing capillaries or venules (Dumont *et al.*, 1995). *Neoarteriogenesis* describes the remodeling of pre-existing arterioles leading to the formation of mature vessels with a fully developed tunica media (Schaper *et al.*, 1999).

i. Post natal normal blood vessel formation

The first steps in this process are the production of endothelial cell proteases, which allow the endothelial cells to migrate and proliferate, by dissolution of the basement membrane forming a tube-like structure (Engler, 1996). This process is under the influence of critical survival factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). It is important to note that this process produces 'immature' vessels, which do not possess the normal vessel architecture, and are often leaky as a result. Many term this stage angiogenesis.

The newly formed endothelial cells need to be 'stabilised' and achieve this by secreting a number of factors such as platelet derived growth factor (PDGF) and transforming growth factor beta (TGF β), which recruit pericytes to the outer surface of the endothelium (Darland and D'Amore, 1999). Contact of the pericyte with the endothelium also switches off VEGF production.

The final stage is 'wrapping' of the pericyte around the developing vessel and the formation of a basement membrane around the vessel as a long-term signal for

vessel stabilisation. These processes are governed by angiopoietin with angiopoietin-1 inducing this process, whilst angiopoietin -2 can reverse this, destabilising the vessel. Thus, mice deficient in angiopoietin-1 present with abnormal vessels lacking smooth muscle cells or pericyte recruitment (Suri *et al.*, 1996).

ii. Collateral circulation of the heart

In an experimental occlusion model in the rabbit hind limb, new collateral arteries are visible within three days. These collateral vessels contain smooth muscle cells as well as endothelial cells and extend to distances of up to 5 cm. It is unlikely that the source of this dramatic response is due to sprouting capillaries, but is more likely a rapid adaptive response of pre-existing arterioles (Schaper *et al.*, 1999). It is thought that angiogenesis and neoarteriogenesis are responsible for coronary collateral vessel growth in the adult heart (Schaper *et al.*, 1999). However it is clearly neoarteriogenesis that is likely to be the most efficient mechanism of delivering blood flow to an ischaemic region (Lewis *et al.*, 1997). The ability to recruit collaterals may depend on the severity and duration of ischaemia. In patients with symptoms of angina of less than three months duration, there is less collateral development than those patients with angina extending beyond three months (Piek *et al.*, 1997).

iii. Mechanisms of collateral growth

Acute tissue ischaemia, and hence hypoxia, up-regulates VEGF messenger RNA (mRNA) (Sharma *et al.*, 1992), likely through the VEGF promoter having a sequence recognised by the transcription factor hypoxia-inducible factor 1 alpha (HIF-1 α) (Ikeda *et al.*, 1995). However although it is not disputed that coronary artery disease leads to ischaemia, there are important discrepancies between the presence of tissue ischaemia and vascular growth. Thus, muscular collaterals which develop to supply an ischaemic territory, originate from areas of the myocardium which are non-ischaemic, and so the site of ischaemia can be dissociated from the site of collateral formation (Schaper and Ito, 1996).

Others (Schaper and Ito, 1996) also suggest that ischaemia and angiogenesis are linked by an inflammatory process. Thus, collateral growth can be seen as a local remodeling phenomenon promoted by inflammatory cytokines, produced by infiltrating monocytes up-regulating receptors for growth factors.

Remodeling of vessels by stress may also play an important role. Thus, small vessels with chronically increased blood flow tend to become wider via remodeling, whereas large vessels with a low flow will tend to stenose (Schaper and Ito, 1996). Pressure differences between arterioles and large vessels lead to an increase in endothelial adhesion molecules, which attract monocytes. These produce VEGF, tumour necrosis factor alpha (TNF- α) and stimulate the endothelium to produce bFGF and PDGF. In turn these all lead to endothelial and smooth muscle cell mitosis (Ando *et al.*, 1987).

iv. Transfer of Genes to Humans

Human gene transfer is a clinical strategy by which the genetic makeup of a cell is modified for therapeutic purposes. An expression cassette made up of one or more genes, and the sequences controlling their expression, are delivered to target cells. *Ex vivo* delivery involves the cassette being transferred to cells in the laboratory, after which the modified cells are then administered to the recipient. *In vivo* gene transfer involves the expression cassette being transferred directly to the cells of the recipient. In both strategies transfer can be directed to an intracellular site with the addition of a vector (Crystal, 1995). There are three major categories of gene transfer, namely viral vectors such as adenoviruses (Lewis *et al.*, 1997), cationic liposome or the use of naked deoxyribonucleic acid (DNA). Some of the potential advantages and disadvantages of these vectors for use in angiogenesis studies are summarised in (Table 1).

Table 1: Gene transfer vectors

	GENE TRANSFER VECTORS	
Vector	Advantages	Disadvantages
Plasmid	Good safety profile Episomal location Easy to produce	Low transfection efficiency Short transgene expression
Adenovirus	High transfection efficiency Episomal location Easy to produce Large expression cassette	Transient duration of expression Inflammatory reaction Difficult to repeat
Adeno associated virus	Good transfection efficiency Episomal location Longer transgene expression	Difficult to produce Small expression cassette Difficult to repeat
Retrovirus	Chromosomal integration Long transgene expression Low titers	Transfection limited to divided cells Long transgene expression Production of replication competence Difficult to repeat

Table 1: This table summaries the potential advantages and disadvantages of gene transfer vectors for angiogenesis application Adapted from (Laham *et al.*, 2001)

b. Gene therapy vectors

i. Adenovirus vector design and gene transfer

Adenoviruses are DNA viruses in which the ability to replicate is critically dependent on its E1 sequence (Crystal, 1995). For gene therapy purposes the E1 sequence is deleted, rendering the virus incapable of replication, and the expression cassette is then inserted into the viral genome. The adenovirus is placed in contact with the cell, enters through receptor-mediated binding and is taken into cytoplasmic endosomes. From here the virus is able to break out of the endosomal structure and deliver the DNA with its expression cassette into the nucleus, where it functions in an epichromosomal fashion to direct the expression of its product (Crystal, 1995).

ii. Cationic liposome and naked plasmid DNA

The positively charged liposome is attracted to the negatively charged plasmid, which contains the expression cassette. The complex, when placed in contact with the cell, fuses with the plasma membrane and is taken up by an endocytic process. Much of the exogenously added material is destroyed by the cytoplasmic organelles,

whilst the plasmid that reaches the nucleus functions in an epichromosomal fashion as for the adenovirus. Such liposome-DNA complexes, and plasmid DNA, are simple vectors, but targeting is limited and efficiency is low whilst expression is transient. Adenoviral vectors are more efficient for gene transfer in cell types, which possess the appropriate receptor, but again expression is limited in duration. Although they are unable to replicate because of deletion of E1, late viral genes trigger an immunological reaction, which on repeated administration neutralises the vector. Adenoviral vectors are therefore suitable for short-term one-off expression of proteins. Since it is thought that only a short-term stimulus is required to promote angiogenesis, adenoviral based vectors may be appropriate for this purpose (Lewis *et al.*, 1997).

Table 2: Differences between gene and protein therapy

Differences	Gene Therapy	Protein Therapy
Exposure	Sustained	Finite
Dose response	Unpredictable	Defined
Administration	Single?	Repeated
Slow release	Yes	No unless combined with other substances
Inflammatory response	Yes	No, unless effect of protein
Foreign material	Yes	No
Duration of effect	Long	Short
Influence of patient serology	Yes	No

Table 2: The advantages and disadvantages associated with protein and gene therapy. Adapted from (Post *et al.*, 2001)

c. A summary of the clinical angiogenesis literature

i. Brief overview

Numerous papers have shown, in a wide range of animal models, that angiogenic factors induce the generation of new vessels (Simons and Laham, 1999b). Based on studies ranging from the ischaemic rabbit hind limb to the ameroid pig model of myocardial ischaemia, there is a considerable body of evidence that growth factors such as VEGF, bFGF and many others can induce the formation of endothelial-lined vessels, generally smaller than 200 µm diameter. In turn these vessels have been shown to augment perfusion of viable, underperfused territories, and improve myocardial function as judged by magnetic resonance imaging (MRI) and other techniques. A number of clinical trials have been conducted either with protein or gene therapy to induce angiogenesis in the presence of chronic ischaemic coronary artery disease (Table 3). Five of these focused on administration of angiogenic factors given as an adjunct to bypass grafting (Schumacher et al., 1998); (Sellke et al., 1998); (Laham et al., 1999b); (Rosengart et al., 1999); (Ruel et al., 2008), three via thoracotomy (Rosengart et al., 1999); (Symes et al., 1999); (Fortuin et al., 2003), three via endocardial injection and NOGA mapping (Vale et al., 2001); (Kastrup et al., 2005); (Stewart et al., 2009) and a further five were administered via the coronary artery (Henry et al., 2003); (Laham et al., 2000); (Simons et al., 2002); (Unger et al., 2000); (Hedman et al., 2009). NOGA mapping is described in detail in the methods section but in essence by maneuvering a magnetic tipped deflectable catheter within the left ventricle whilst having low emitting magnetic pads under the patient, the precise spatial localisation of the catheter tip within the left ventricle can be determined. As the catheter is sequentially moved and placed against a number of points on the endocardial surface of the heart, the local unipolar electrogram and tip motion during the cardiac cycle are recorded, and a three dimensional map of unipolar voltage and local wall shortening is constructed, thus allowing real-time visualisation of ischaemic viable tissue (Laham et al., 2005). Eight used angiogenic genes, VEGF¹⁶⁵ or VEGF-2, delivered as naked DNA, and two-used VEGF delivered using an adenovirus. Six studies utilised recombinant proteins FGF1 and FGF2 (Table 3). There are important limitations in interpreting these studies. The effects of surgery will create an altered baseline against which to compare the

angiogenic response. This can be circumvented either by using a placebo control group, reassessing the baseline immediately after surgery, or preferably both. Early gene therapy studies were not placebo controlled and did not reassess baseline values after CABG (Schumacher et al., 1998); (Rosengart et al., 1999). Despite these limitations preliminary results were promising and fairly consistent between the studies, reporting improvements in assessment of myocardial perfusion (assessed by MRI and SPECT), angina class, exercise tolerance and angiographic scores, some of which have extended to 12 months following treatment. The first study using the percutaneous route to deliver to the myocardium was uncontrolled and of small sample size, but essentially presented similar encouraging findings (Vale et al., 2001). Later randomised placebo controlled studies reported inconsistent findings. (Kastrup et al., 2005) did not demonstrate any improvement in perfusion but did see improvements in regional wall motion in the VEGF group, whilst (Stewart et al., 2009) could not demonstrate any additional improvement between the group treated with VEGF compared to the large placebo effect seen in the control group. Intravenous and intracoronary deliveries of angiogenic factors, although appearing to be safe, have proved disappointing regarding efficacy (Henry et al., 2003); (Simons et al., 2002).

ii. Which patient population?

All clinical studies of myocardial angiogenesis have selected patients, presenting primarily with symptoms of angina. Close examination of this heterogeneous group indicates that the patients with the poorest left ventricular function appear to benefit the most from angiogenic therapy (Simons et al., 2000). During the development of myocardial hypertrophy, angiogenesis does not match the increase in size of the hypertrophied myocardium. The resultant reduction in capillary density leads to reduced myocardial perfusion. The patient population selected for my study are classified 'no-option', primarily presenting with symptoms of heart failure, but with evidence of a form of metabolic adaptation to low flow known as hibernation. This is characterised by the myocardium becoming akinetic in an attempt to reduce energy expenditure, once blood flow is restored the muscle recovers function (Rahimtoola, 1989). In heart failure hibernating myocardium is often found in patients with severe left ventricular dysfunction (Rahimtoola, 1989). Hibernation is

not a static state, characteristic structural changes include loss of contractile material, numerous small mitochondria, irregular nuclei, glucogen accumulation, and cytoskeletal alterations as well as increased fibronectin, collagen and structural proteins (Vanoverschelde and Melin, 2001). Thus over time there is a reduced ability for functional improvement, and it is therefore important to restore perfusion expediently to prevent this occurring (Rahimtoola, 1989). Importantly this group of patients is likely to have the best risk verses gain ratio.

iii. Why were the Phase II trials negative?

In the larger placebo controlled studies VEGF was administered as either a protein, which is limited by its systemic vasodilator effects or as a plasmid, which is severely limited by poor transfection efficiency (Henry et al., 2003); (Stewart et al., 2009); (Kastrup et al., 2005). In my opinion the only way VEGF gene therapy will be successful is if a more potent vector such as an adenoviral vector accompanies it. Similarly in the placebo controlled studies of bFGF (Simons et al., 2002) in addition to sub-optimal vectors, the incorporation of an intracoronary delivery may also have contributed to the negative result, as the contact time is extremely short resulting in a reduction in transfection efficiency compared to intramyocardial approaches. It may also be that a single growth factor is not the answer and that a ‘master switch gene’ such as HIF1 α may be more efficacious although a direct head to head trial of single verses multiple growth factors has not been completed. As yet I do not believe that the correct gene therapy trials have been undertaken to determine if angiogenic growth factors are capable of augmenting perfusion and or function in a patient group with chronic ischaemic heart disease. To achieve this I would recommend a randomised double blind placebo control design with an adenoviral vector and intramyocardial delivery. The formation of blood vessels is a complex process. Evidence has shown that the effects of VEGF and FGF2 are synergistic (Asahara et al., 1995). Thus my bias would be to focus initially on ‘master switch’ genes such as HIF1 α as I believe these may be more effective than the application of a single growth factor.

Table 3: A Summary of Angiogenesis Clinical Trials

Author	Year	n	Phase	Placebo Controlled	Patient Group	Growth Factor/Dose	Delivery route	Outcome
(Schumacher <i>et al.</i> , 1998)	1998	40	I/ II	Yes	Adjunct to CABG	Protein aFGF 0.01µg/kg or placebo	Intramyocardial	Improved Angiography
(Sellke <i>et al.</i> , 1998)	1998	8	I	No	Adjunct to CABG	Protein bFGF 1µg &10 µg	Epicardial Slow release Heparin Alginate beads	Nuclear Perfusion scans improved in 3 patients
(Losordo <i>et al.</i> , 1998)	1998	5	I	No	CAD Sole Therapy	Gene plasmid VEGF ¹⁶⁵ 125 µg in 4 x 2ml	Intramyocardial	Improved Nuclear perfusion scans & Angiography
(Laham <i>et al.</i> , 1999b)	1999	24	I	Yes	Adjunct to CABG	Protein bFGF 10µg 100µg Placebo	Epicardial Slow release Heparin Alginate beads	Improved Nuclear perfusion and MRI ↓ ischaemic territory in 100 µg group
(Rosengart <i>et al.</i> , 1999)	1999	21	I	No	Adjunct to CABG & Sole Therapy	Gene Adeno 10 ¹⁰ VEGF ¹²¹	Epicardial	Improved Angina class and exercise time in sole therapy group
(Symes <i>et al.</i> , 1999)	1999	20	I/II	No	CAD Sole Therapy	Gene plasmid VEGF ¹⁶⁵ 125 & 250 µg	Epicardial Thoracotomy	↓Angina, Improved nuclear scans

Author	Year	n	Phase	Placebo Controlled	Patient Group	Growth Factor/Dose	Delivery route	Outcome
(Henry <i>et al.</i> , 2003) (VIVA Trial)	2003	178	I/II	Yes	CAD Sole Therapy	Gene Plasmid VEGF ¹⁶⁵ 17 or 50 (IC) mg/kg/min 17 or 50(IV) µg/kg/min days 3,6,9	Intracoronary plus Intravenous Dose	No effect Exercise time or Symptoms Nuclear perfusion scans ↑ In placebo group
(Laham <i>et al.</i> , 2000)	2000	52	I	No	CAD Sole Therapy	Protein bFGF 0.33, 0.65, 2 6, 12, 24, 36 48 µg/kg	Intracoronary	↓Angina frequency ↑Exercise tolerance Improved LV function and perfusion MRI
(Simons <i>et al.</i> , 2002) (FIRST Trial)	2002	337	I/II	Yes	CAD Sole Therapy	Protein bFGF 0.3,3,30 µg/kg or placebo	Intracoronary	No effect Exercise time ↑In placebo group
(Unger <i>et al.</i> , 2000)	2000	25	I	Yes	CAD Sole Therapy	Protein bFGF 3/100 µg/kg	Intracoronary	Well tolerated in the 3/30 mg/kg range
(Vale <i>et al.</i> , 2001)	2001	6	I	Cross over control	CAD	Gene plasmid VEGF-2 or placebo	Endocardial	↓Angina Improved nuclear scans
(Fortuin <i>et al.</i> , 2003)	2003	30	I	Open label	CAD	VEGF-2	Thoracotomy and intramyocardial injection	No angiographic evidence of angiogenesis
(Kastrup <i>et al.</i> , 2005)	2005	80	II	RDBPCT	CAD	0.5 mg of phVEGF ¹⁶⁵ / placebo	Endocardial Injections (NOGA mapping)	No improvement in perfusion but improved regional wall motion in the VEGF group.

Author	Year	n	Phase	Placebo Controlled	Patient Group	Growth Factor/Dose	Delivery route	Outcome
(Ruel <i>et al.</i> , 2008)	2008	19	I	Randomised 2 x 2	CAD CABG	VEGF 2000 µg/ Placebo + L-arginine or placebo	CABG, intramyocardial injection and oral L-arginine or placebo	VEGF + L-arginine improved wall motion
(Stewart <i>et al.</i> , 2009)	2009	93	II	RDBPCT	CAD	Plasmid VEGF 2000 µg /placebo	Endocardial NOGA	No difference between groups large placebo effect
(Hedman <i>et al.</i> , 2009)	2009	103	II	Open label	CAD	Adenoviral/ Plasmid/liposome /placebo VEGF	Intracoronary during stenting	Local Intracoronary VEGF gene transfer is safe over an 8 year follow-up

iv. Which gene?

Many growth factors are capable of inducing angiogenesis. In the late 1980s VEGF was identified as producing endothelial cell mitogenesis and became the predominant growth factor studied for the purpose of angiogenesis. VEGF and other angiogenic factors exert their influence by coupling to cell surface receptors, and subsequently triggering endothelial cells through signaling cascades. Interest has concentrated on VEGF, as endothelial cells are the only differentiated cell type, which respond mitogenically to this factor (Thomas, 1996). Other growth factors such as FGF are also mitogenic for smooth muscle cells, and could cause unwanted smooth muscle cell hyperplasia (Banai et al., 1991). Angiogenesis is a feature of normal tissue repair in which VEGF has been known to play a prominent part. Further importance for the role of VEGF has come from studies assessing the effects of its removal. (Ferrara et al., 1996) disrupted the VEGF gene in embryonic stem cells of mice. The loss of VEGF was found to be lethal between days 11 and 12 of gestation. (Shalaby et al., 1995) disrupted the type- 2 receptor of VEGF (VEGF-2 /flk-1) and found this interfered with the differentiation of endothelial cells resulting in death between eight and nine days of gestation. Further, (Fong et al., 1995) disrupted the type 1 VEGF receptor (VEGF-1/flt-1) and demonstrated that differentiation of endothelial cells still occurred, but that the vessels had thin walls and abnormally large lumens resulting in the death of the embryo at nine days. Thus, a large body of work suggests that VEGF is central to new vessel formation and hence this growth factor has been the focus of gene therapy efforts to produce new vessels.

Growth factors are potent and there have been reports that too much expression of VEGF may be deleterious, and lead to angioma formation (Schwarz et al., 2000). VEGF often produces vessels, which are leaky; with the addition of angiopoietin -1 this effect is less pronounced (Thurston et al., 2000). Refractory hypotension arising from a massive nitric oxide release (Lopez et al., 1997) has hampered intravenous administration of VEGF and bFGF. However, elevated uncontrolled expression of VEGF has also been implicated in malignant tumour growth (Carmeliet and Jain, 2000), enhancing atherosclerotic plaque progression (Celletti et al., 2001) and the increased vascular growth in the retina in diabetic retinopathy (Aiello et al., 1994).

As previously mentioned the formation of blood vessels is a complex process. Although previous studies have been encouraging it is unlikely that effective therapy will be achieved merely with the upregulation of a single growth factor. Evidence has shown that the effects of VEGF and FGF2 are synergistic (Asahara et al., 1995). Mature blood vessel formation requires the interplay of a number of different cell types such as macrophages, endothelial cells, smooth muscle cells, and pericytes.

It may be key to differentiate angiogenesis from arteriogenesis at this point. The former produces capillary proliferation with the vessels lacking a developed media. Arteriogenesis refers to mature vessels with a developed media, such as the collaterals visible at angiography in advanced coronary artery disease. It is likely that arteriogenesis needs to be the aim to elicit sustained clinical benefit (Simons et al., 2000). The factors that mediate arteriogenesis are numerous and complex but include combinations of growth factors, upregulation of their receptors and inhibition of angiogenesis inhibitors (Simons et al., 2000). It is unlikely that any one growth factor can mimic this process and therefore at present there are three options:

- i) Use one factor and hope that it has a disproportionately large influence on angiogenesis compared to the myriad of other factors.
- ii) Combine factors to begin to mimic the endogenous process of forming mature vessels.
- iii) Identify a 'master switch' factor, which is proximally enough placed within the cascade of events to initiate mature vessel growth.

Based on this evidence using hypoxia-inducible factor-1 alpha, which is high in the cascade of pro-angiogenic factors and has been described as the 'master switch' gene (Simons et al., 2000), appeared the most practical and realistic approach. HIF-1 α is an inducible transcription regulatory factor, which in combination with constitutively expressed HIF-1 β plays a principal role in the regulation of gene expression in response to hypoxia. Through control of a number of genes including inducible nitric oxide synthase (iNOS), VEGF, angiopoietins, platelet derived growth factor beta (PDGF- β), transforming growth factor alpha and beta and a number of receptors, HIF-1 α orchestrates adaptation to hypoxia at a systemic, tissue and cellular level (Guillemin and Krasnow, 1997). Transcriptional regulation by HIF-1 α is mediated by

binding of the heterodimeric transcription factor to hypoxia responsive elements on the target gene (Semenza et al., 1997). Two transactivation domains are present in HIF-1 α ; one is located in the middle of the protein (amino acid 530 to 580) and the other at the extreme carboxyl-terminus (amino acid 780 to 826). HIF-1 α protein and binding activity are increased markedly in response to hypoxia, but once cellular oxygen levels increase to normal levels, HIF-1 α protein levels decay rapidly via the ubiquitin-proteasome pathway (Jewell et al., 2001). HIF-1 α is a potent factor and has been reported to lead to plaque rupture and subsequent cardiovascular events (Luque et al., 2008), thus the safety profile needs to be closely monitored.

I designed the pre-clinical protocols and decided that I would like to investigate HIF-1 α to determine the safety profile together with the efficacy to augment perfusion and function in a large animal model of myocardial ischaemia prior to commencing a clinical trial. I thus approached Genzyme, who owned the patent for HIF-1 α and had developed it for angiogenic purposes. It is important to stress at this point that this was a completely academic driven and funded programme and that Genzyme's sole involvement was to provide the HIF-1 α constructs

Genzyme Corporation developed HIF-1 α for angiogenesis, selectively deleting the gene regions between the 390 to 826 amino acid of HIF-1 α and replacing it with a transactivation domain from the herpes simplex virus (HSV) VP16 or from nuclear nuclear factor kappa-light-chain-enhancer of activated β cells (NF κ B), which allow for sustained expression of genes involved in the cellular response to hypoxia expression but which were importantly hypoxia-independent (Vincent et al., 2000). Angiogenesis can be induced by direct application of an angiogenic protein or by application of a gene encoding for the protein. There are advantages and disadvantages to both these approaches (Table 2). However gene therapy is the only option for certain transcription factors such as HIF-1 α since in its protein form it is very rapidly degraded (Li et al., 2000). In post hypoxic tissue culture HIF-1 α is degraded in less than five minutes (Jewell et al., 2001) but this probably over estimates the half-life as it does not account for the time it takes for oxygen to diffuse out of the tissue culture; in isolated perfused and ventilated lung models the half-life of HIF-1 α is less than one minute (Yu et al., 1998). I also wanted to assess both

plasmid and adenoviral constructs as these had not been compared in a randomised placebo controlled study. Adenoviral vectors are thought to offer an increased transfection efficiency (Table 1) as compared to that achieved by naked plasmid DNA. However, this increased angiogenic response may be offset by an increased inflammatory reaction associated with the administration of adenovirus (Wright et al., 2001). My objectives were therefore to assess the safety and efficacy of both constructs in a large animal model.

1.3 CHOOSING A PRE-CLINICAL MODEL

It was important to determine the animal model most likely to be predictive of human chronic myocardial ischaemic. The large animal models currently available for angiogenesis studies are outlined below.

a. Fixed stenosis model

This is usually achieved by banding the coronary artery to a fixed dimension. (Chen et al., 1996) using silk placed around the left anterior descending coronary artery (LAD) to reduce resting flow by 40%, but others have used occluders with a fixed internal diameter (Fallavollita et al., 1997). The main advantages of this model are that it results in a relatively uniform degree of ischaemia and interestingly the myocardial blood flow is reduced at rest postulated to be a result of a reduction in collateral vessel formation with a lesser degrees of occlusion (Fallavollita et al., 1997). The major disadvantage is that it is technically challenging to produce a chronic stable coronary stenosis, which has lead to the lack of widespread use of the model.

b. Hydraulic occluder Model

Periodic ischaemia is achieved by remote activation of a pneumatic occluder cuff, which is chronically instrumented via a thoracotomy onto a coronary artery. The proximal end of the occluder is externalised and then is inflated either with fluid or air once the animal has recovered from the surgery and when any coronary spasm associated with the manipulation of the coronary artery has subsided, which is an advantage over other models. Another advantage is that because of the flow probe a more uniform degree of stenosis and therefore ischaemia may be achieved. The degree of stenosis can be manipulated as needed, unlike the fixed stenosis models where postoperative adjustments are not feasible. As with the fixed stenosis model myocardial blood flow in the target territory is reduced at rest. However the optimal frequency of occlusion is two minutes every 32 minutes (Mohri et al., 1989), which means that this technique is more suited to dogs which are easier to approach and handle than pigs. The main disadvantages of this model are that it is technically demanding, it requires extensive instrumentation and due to the externalised hardware animal maintenance is difficult, and there is often a lack of cuff durability (Meszaros et al., 1999).

c. Microembolisation with calibrated beads

Injection of different sizes of plastic beads in the left atrium, or selected coronary arteries causes occlusion of different areas of the vascular bed. The size of the beads can be varied together with the frequency of application until the desired level of occlusion is achieved. The advantages are that it only requires cardiac catheterisation, which is far less invasive than a thoracotomy; it results in occlusion of smaller vessels and is suitable for both dogs and pigs. The disadvantages are that there tends to be a greater variability in ischaemia between animals, and it often requires an extended time course for collateral growth compared to the ameroid model discussed below (Meszaros et al., 1999).

d. Myocardial infarct model

This is a method where one coronary artery is ligated, providing a simple way to guarantee occlusion. The disadvantages of this model are that this procedure is often fatal in pigs, and in the few animals that survive, it results in a large area of infarct.

e. The Ameroid Model

The most widely used porcine model of chronic ischaemia is the ameroid constrictor. Progressive coronary artery occlusion can be achieved by use of an ameroid occluder, which, is surgically placed onto a coronary artery (Figure 1). Ameroid occluders consist of a ring of modified casein material known as ameroid. When the device is implanted around the artery the casein absorbs water and slowly expands, the inflexible stainless steel ring around the ameroid material, directs the expansion inwards, compressing the artery. If the internal diameter of the ameroid occluder and the quantity of ameroid are sufficient then this leads to total occlusion over a period of 14- 30 or more days. In the pig the ameroid is usually placed on the left circumflex artery (LCX). The gradual occlusion of the LCX results in a pathophysiological stable model of exercise-induced ischaemia (White et al., 1992) with the development of collateral vessels in some ischaemic animals (Figure 2).

Figure 1: Ameroid Occluders

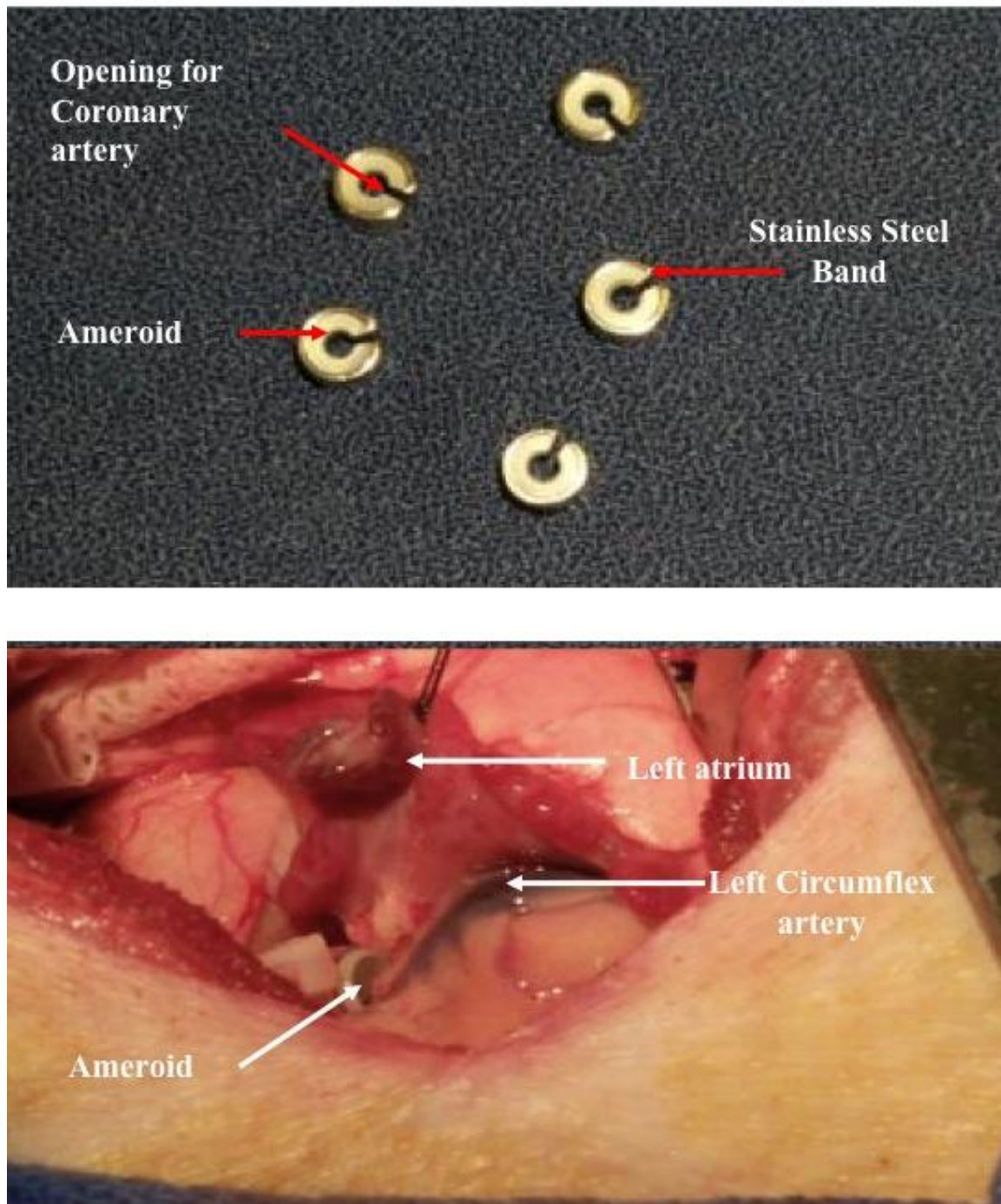


Figure 1: A photograph of an ameroid constrictors 2.25 mm in diameter above, and below in situ on a left circumflex coronary artery of a pig.

Figure 2: Collateral formation after ameroid placement on the Left Circumflex artery of a swine

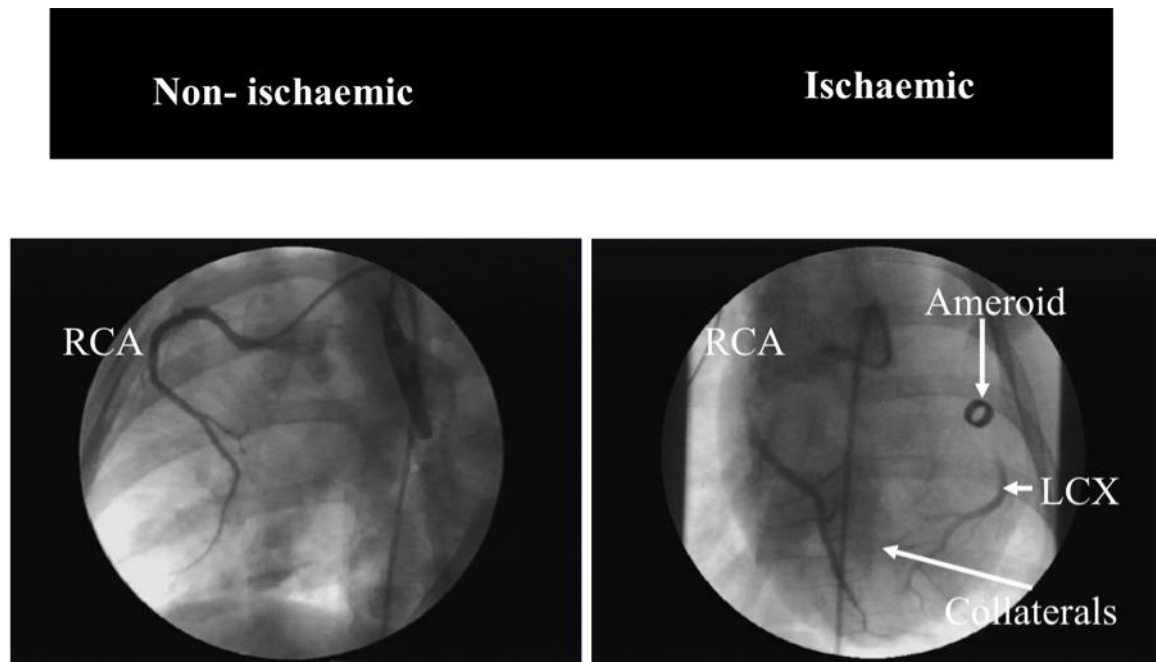


Figure 2: RCA right coronary artery, LCX left circumflex coronary artery. The angiogram on the left was taken of the right coronary artery in a swine prior to ameroid occluder placement on the LCX. The angiogram on the right is of a right coronary artery angiogram three weeks post an ameroid occluder placed proximally on the LCX. There are clear collaterals between the RCA and LCX resulting in distal filling of the LCX.

f. Which species?

Most angiogenesis studies in large animal models have been performed in the pig or dog. In the canine model, occlusion of the LCX produces a dramatic angiogenic response, with the formation of numerous large mature collaterals, containing normal amounts of medial smooth muscle. This large angiogenic response completely restores the obstructed blood flow within four to six weeks (White et al., 1992). The collaterals formed in the dog are physiologically and pharmacologically responsive (Meszaros et al., 1999). In the pig, occlusion of the LCX results in small uniform infarcts with minimal contractile dysfunction. The pathophysiological response provides a model of chronic exercise induced myocardial ischaemia. The collateral vessels, which develop are immature with reduced smooth muscle cell deposition in addition to a lack of autoregulatory properties (White et al., 1992). Revascularisation is much more limited and this model lacks a coronary reserve, closely resembling the response seen in humans (Meszaros et al., 1999).

1.4 DELIVERY ROUTES FOR GENE THERAPY

Angiogenic growth factors are very potent, and may lead to detrimental side effects as discussed previously. Therefore the aim of all delivery strategies should be to deliver a high level of growth factors to the target area whilst producing very little systemic exposure. Many delivery strategies have been employed in angiogenesis trials.

a. Intravascular and Intracoronary

At the time of conducting these studies three percutaneous catheter delivery strategies were available. Intracoronary delivery has been the most popular method to date being employed in five clinical trials Refer to (Table 3). Intravenous delivery has been used in combination with intracoronary delivery in the VIVA study (Henry et al., 2003). It is now appreciated that the rapid blood flow within the myocardium results in a very short contact time between the growth factors and the target cells and poor transfection efficiency. This rapid wash out effect leads to a large systemic re-circulation, and potential exposure of non-target organs to growth factors (Laham et al., 1999a). Biodistribution studies have shown that a single intracoronary or intravenous administration of bFGF protein leads to deposition in ischaemic myocardium of approximately 1.5% of the dose one hour post delivery and at 24 hours this decreases to 0.01%. The levels are even lower in non-ischaemic myocardium (Laham et al., 1999a). Phase II study results proved disappointing (Henry et al., 2003); (Simons et al., 2002). The initial appeal of the simple intravenous and intracoronary delivery routes now appears to be overshadowed by lack of demonstrable efficacy because of the issue of reduced contact time.

b. Retrograde perfusion

Retrograde perfusion via the coronary venous system has also been investigated (Boekstegers et al., 2000). The anterior intraventricular vein is selectively catheterised through the coronary sinus; retroinfusion of transgene is delivered at pressures 20 mmHg higher than right atrial pressure through the venous system. This will be discussed in more detail later in association with cell delivery (1.8D).

c. Epicardial

Epicardial delivery of angiogenic growth factors has been administered as an adjunct to CABG (Schumacher et al., 1998, Rosengart et al., 1999) with documented improvements in angina and increased vessel development. The effects of surgery will create an altered baseline against which to compare the angiogenic response. This can be circumvented either by using a placebo control group, reassessing the baseline immediately after surgery, or preferably both. Neither of the above studies were placebo controlled or reassessed baseline values. Others have administered epicardial delivery as sole therapy, via a thoracotomy (Losordo et al., 1998); (Symes et al., 1999) documenting similar improvements as noted for adjunct therapy above in addition to improvements in perfusion assessed by nuclear perfusion imaging. Because a thoracotomy is an invasive approach, this form of delivery poses ethical constraints on the inclusion of a control group. These are important limitations, and mean that interpreting these studies becomes difficult due to the profound effects from CABG and the large placebo effect associated with angiogenesis therapy (Simons et al., 2002); (Henry et al., 2003).

d. Endocardial

The introduction of a percutaneous technique utilising transendocardial injection has overcome the ethical issues of including a placebo group. One study has reported the use of VEGF-2 as naked DNA administered percutaneously to the myocardium, as sole therapy (Vale et al., 2000). This study reported a reduction in angina and glyceryl trinitrate (GTN) use in addition to improvements in perfusion demonstrated by nuclear perfusion scanning. There were difficulties of interpretation since it was a cross over design, single blinded and of small sample size (n=6). Evidence from pre-clinical studies however highlighted an issue of inactivation of adenoviral vectors by constituents of some delivery catheters (Marshall et al., 2000). It is now recommended that bioreactivity of catheters are assessed with respect to any angiogenic substance. The large volumes of injectate used in the early study of around two ml per injection (Losordo et al., 1998) may lead to significant local tissue damage. Further studies have elucidated these large volumes are less efficacious than smaller volumes, postulated to be due to a loss of injectate with systolic contraction (Laham et al., 2005). This biodistribution study in a porcine model indicated that endocardial

delivery resulted in retention of $18 \pm 4\%$ of the product within the ischaemic myocardium one hour post delivery and declined to 12% 24 hours after the injection and 2% seven days later (Laham et al., 2005). In a later randomised double blind placebo controlled trial (RDBPCT) (Stewart et al., 2009) could not detect any difference between the active group receiving plasmid VEGF via NOGA mapping and endocardial injections compared to the large placebo effect seen in the control group, again verifying the need for adequate controls when assessing efficacy.

e. Slow release protein

Another novel delivery strategy is to combine proteins, with substances such as heparin alginate beads, which can prolong their very short half-life. (Sellke et al., 1998) published details of these heparin alginate beads combined with bFGF that were placed into the epicardial fat in a territory not amenable to surgery at the time of CABG. This study was followed by an elegantly designed placebo controlled study (Laham et al., 1999b), which, resulted in improved perfusion as demonstrated by MRI in the high dose group.

f. Pericardial

The concentration of VEGF and bFGF in the pericardial fluid are considerably higher in patients with ischaemic heart disease than in patients with non-ischaemic heart disease (Fujita et al., 1996). This increase in endogenous angiogenic growth factors within the pericardial fluid suggests a physiological role for these factors in patients with ischaemic heart disease. It was therefore hypothesised that by augmenting these levels of growth factors and by bathing the whole heart it may be possible to upregulate the endogenous process to produce therapeutic levels of angiogenesis. In a porcine model of chronic myocardial ischaemic, using a percutaneous subxyphoid pericardial delivery with fluoroscopic guidance, this technique was shown to increase perfusion, collateral vessels and improve endothelial and myocardial function (Lopez et al., 1998a). However the fact that over 90% of the subjects who are referred for angiogenesis trials have already been subjected to CABG and therefore do not have intact pericardial sacs has discouraged this delivery route for current clinical studies (Simons et al., 2000).

1.5 CELL THERAPY

Heart failure is defined as inability of the heart to keep up with the demands on it and, specifically, failure of the heart to pump blood with normal efficiency. There are many causes of heart failure including coronary artery disease, leading to myocardial infarction, dilated cardiomyopathy from bacterial and viral infections or toxins such as prolonged alcohol exposure. Other causes are primary valve disease, hypertension, hyperthyroidism, vitamin deficiency, and excessive amphetamine use.

Irrespective of the aetiology, heart failure is characterised by cardiac hypertrophy, insufficient vascularisation and cardiomyocyte loss and fibrosis. The key events, leading to heart failure, are a loss of a critical quantity of functioning myocardial cells after prolonged stress or injury with CAD still remaining the main cause (Gheorghiade and Bonow, 1998). Once the heart is unable to function as an efficient pump, blood flow to other organs is affected such as the brain, liver and kidneys. Although cumulative evidence has now demonstrated that cardiomyocytes are not terminally differentiated (Anversa and Nadal-Ginard, 2002), the natural regenerative process is inadequate to replace the massive number of up to one billion cardiomyocytes lost after an acute myocardial infarction (AMI), and thus resulting in chronic heart failure (CHF).

The heart is now viewed as a self-renewing organ in which myocyte regeneration occurs throughout life with cardiac stem cells replacing apoptotic cells or programmed cell death. However, evidence from sex-mismatched heart transplant recipients has shown that small numbers of cells of recipient origin and therefore from a non-cardiac source, have become engrafted into the donor heart (Bertolini and Pruneri, 2002). Animal studies have shown a naturally occurring reparative process that consists of up-regulation of progenitor cell release from bone marrow after myocardial infarction, homing of these cells to the injured tissue, and differentiation of these progenitor cells into endothelium and cardiac myocytes within the infarcted tissue. However in humans, it appears that this process does not naturally occur with high frequency, and thus the rate of repair is insufficient for clinical restoration of function. The goal of cell therapy is to increase the magnitude, speed and frequency of this process. Regeneration of functional myocardial tissue after an ischemic insult

could, therefore, be achieved either by implanting exogenous cells, which may transdifferentiate into the relevant tissues, or by secretion of cytokines which stimulate the endogenous mature cardiomyocytes and resident cardiac stem cells. The ultimate aims of treatment are to generate contractile cells that integrate functionally and structurally in the viable myocardium and to develop a network of capillaries and large size blood vessels for supply of nutrients and oxygen to the injured region. Angiogenesis trials have excluded patients with large areas of myocardial infarction, resulting in myocyte death, necrosis and scar formation because these changes are not reversible with improving myocardial perfusion (Di Carli, 1998). Thus stem cell therapy hypothetically had several significant advantages over current gene therapy strategies by aiming not only to ameliorate but also regenerate lost tissue; the quintessential challenge of heart failure is the underlying loss of contractile tissue. Reduced LVEF remains the main prognostic indicator for reduced mortality (Somaratne et al., 2009) and therefore therapies are concentrated on trying to preserve or increase LVEF; thus any therapy capable of abating this is desirable as clearly there would be no benefit in restoring a blood supply via angiogenesis or arteriogenesis to dead tissue. Cell therapy did not depend on viral vectors to enter the myocardium, and lastly the use of autologous cells is unlikely to lead to rejection or inflammation.

a. Current device therapy for advanced heart failure

One quarter to one third of patients with heart failure have an intraventricular conduction abnormality that results in a prolonged QRS duration on an electrocardiogram (Farwell et al., 2000) and which is associated with a worse prognosis (Baldasseroni et al., 2002). Dyssynchronous electrical activation results in dyssynchronous contraction, which is mechanically inefficient, leading to an increased regional workload and a potential for regional disruption in blood flow. The resultant reduction in left ventricular ejection fraction (LVEF) and decrease in cardiac output worsens the heart failure. To treat dyssynchrony, cardiac resynchronisation therapy (CRT) has been developed. This attempts to coordinate contraction of both the left and right ventricles via simultaneous biventricular pacing and improve LV function. Leads are placed in the right atrium and ventricle and a LV lead is placed into an intracoronary vein via access through the coronary sinus. In many patients suitable for CRT it is also appropriate to place an internal cardiac defibrillator then

referred to as CRTD. Both CRT and CRTD have been shown to improve clinical status and reduce mortality (Abraham et al., 2002); (Young et al., 2003); (Saxon et al., 2006); (Lam and Owen, 2007).

Left ventricular assist devices (LVAD) have also been used as a bridge to heart transplantation and as destination therapy in the very severely ill patient population (Slaughter et al., 2011). A trial of LVAD in moderately sick patients is currently ongoing (Baldwin and Mann, 2010) but as yet this therapy is only applicable for a minority of heart failure patients.

b. A new treatment option

Whilst mortality associated with CAD may be decreasing, morbidity is increasing as more people survive an acute myocardial infarction due to improvements in treatment strategies; the major underlying aetiology of heart failure is now survival post-AMI rather than hypertension or valve disease (Kocher et al., 2007). There are two main patient populations who have been targeted with stem cell therapy. Firstly, those who have sustained an AMI, in an attempt to salvage ischaemic tissue and prevent the devastating LV-remodeling affects which are responsible for the development of heart failure. Secondly, in those patients presenting with chronic heart failure, therapy is aimed at restoring hibernating myocardium into functional myocardial tissue. Although described as two distinct patient populations in reality there is often significant overlap between these groups. Because of the success of pre-clinical studies particularly the pivotal but controversial paper by (Orlic et al., 2001), translation from ‘bench to bedside’ has been rapid.

The first clinical trials were conducted with skeletal myoblasts (SM) but bone marrow still remains the most frequent source of cells used in clinical trials, which are either harvested directly from the bone marrow itself or are stimulated with drugs such as granulocyte-colony stimulating factor (G-CSF) and then harvested from peripheral blood. Bone marrow contains a complex assortment of progenitor cells, including haematopoietic stem cells (HSC), side population (SP) cells, mesenchymal stem cells (MSC) and stromal cells, a subset of which are multipotential adult progenitor cells (MAPC). The following paragraphs summarise the clinical trials to date and have

been broken down into cell type and into acute and chronic patient populations and are summarised in (Table 4).

Table 4: Stem Cell Clinical Trial Summary

Study	N	Design and Numbers	Time post MI	Cell type	Cell preparation	Culture	Mean cell no.	Delivery	Safety	Efficacy
ACUTE MI										
(Strauer et al., 2002)	10	Open label 10 BMC 10 Control refusing cell therapy	7 ± 2 days post AMI	BMC	Ficoll 40 ml	Overnight	2.8 x 10 ⁷	Intracoronary	None	3 months ↑Wall movement ↓Infarct size ↑SV ↓LVESV ↑Perfusion
(Fernandez-Aviles et al., 2004)	20	Open label	13.5 ± 5.5 days	BMC	Ficoll 50 ml	Overnight	7.8 ± 4.1 x 10 ⁷	Intracoronary	No adverse events	6 months ↑LVEF ↓LVESV ↑Regional wall thickness
(Schachinger et al., 2004) TOPCARE – AMI	59	Randomised Open label 30 CPC 29 BMC	4.9 ± 1.5 days	CPC BMC	250 ml Blood 50 ml Ficoll	3 days None	Not stated 5.5 ± 3.9 x 10 ⁶ CD34/CD45+	Intracoronary	No adverse events	1 Year ↑LVEF ↓Infarct size
(Wollert et al., 2004) BOOST *(Meyer et al., 2006)	60	Randomised controlled 30 medical management 30 BMC	4.8 ± 1.3 days	BMC	130 ml gelatin-polysuccinate sedimentation	None	2.5 x 10 ⁹ (SD 9.4 x 10 ⁸)	Intracoronary	No adverse events	6 months ↑LVEF *18 months follow-up no change in LVEF
(Janssens et al., 2006)	67	RDBPCT 34 Control 33 BMC	24 hours post AMI and PCI + STENT	BMC	Ficoll	None	3 x 10 ⁸ (SD 1.3 x 10 ⁸) nucleated cells 1.7 x 10 ⁸ (SD 7.2 x 10 ⁷) Mononuclear Cells	Intracoronary	1 death	4 months No change in LVEF

Study	N	Design and Numbers	Time post MI	Cell type	Cell preparation	Culture	Mean cell no.	Delivery	Safety	Efficacy
(Schachinger et al., 2006) REPAIR AMI	204	RDBPCT 103 Placebo 101 BMC	3 to 7 days	BMC	Ficoll 50 ml	BMC	2.4×10^8	Intracoronary	None	1 year BMC group ↓Major adverse cardiovascular events after AMI (Death, recurrence of MI, or Re-hospitalisation)
(Lunde et al., 2006) ASTAMI *(Beitnes et al., 2009)	100	Randomised controlled 50 Medical management 50 BMC	6 days post AMI	BMC	Ficoll	None	6.8×10^7 median interquartile range 5.4×10^7 to 1.3×10^8	Intracoronary	None	6 months No change LVEF LVEDV Infarct size *3 years Small ↑ in exercise time
(Ge et al., 2006) TCT STAMI	20	Randomised controlled 10 BMC 10 Bone Marrow supernatant	24 hours post AMI and PCI + STENT	BMC	Ficoll 40 ml	None	4×10^7	Intracoronary	None	6 months ↑LVEF ↑Perfusion No change in LVED diameter
(Huikuri et al., 2008) FINCELL	80	RDBPCT 40 BMC 40 Control	AMI and PCI 2 to 6 days	BMMC	80 ml Ficoll	None	3.6×10^8	Intracoronary	None	↑ LVEF No ↑ in restenosis or arrhythmia
(Meluzin et al., 2008)	66	Randomised Controlled 22 High Dose (HD) 22 Low dose (LD) 22 Medical Control	AMI post PC1	BMMC	Buffy coated density gradient centrifugation	Overnight	High Dose 1×10^8 Low Dose 1×10^7	Intracoronary	1 thrombus 1 dissection 1 infected bone marrow sample	3 months ↑Regional wall motion in dose related fashion 12 months High Dose ↑LVEF maintained No change in Regional Wall

Study	N	Design and Numbers	Time post MI	Cell type	Cell preparation	Culture	Mean cell no.	Delivery	Safety	Efficacy
(Yousef et al., 2009) BALANCE	124	Open label 62 BMC 62 Control	AMI 7 ± 2 days	BMMC	80 to 120 ml Ficoll	None	6.1 ± 3.9 x10 ⁷	Intracoronary	None	3 months ↑ LVEF and ↓ Infarct size 8% Improvements maintained for 5 years with ↓ mortality compared to control
(Krause et al., 2009)	20	Open label	10.5 days	BMMC	SPECTRA	None	1 x 10 ⁶ CD45 ^{DIM} CD34 ^{H1}	Endocardial with NOGA mapping	No Adverse effects	12 months ↑ LVEF and electromechanical parameters on NOGA
(Plewka et al., 2009)	60	Open label Randomised 2: 1 40 BMMC 20 Control	7 days	BMMC	Ficoll 100 ml	None	Not stated	Intracoronary		6 months ↑ LVEF ↑ LV systolic and diastolic function
(Tendera et al., 2009) REGENT	200	Randomised Controlled trial 1:1:1 a) BMMC 80 b) CD34+ CXCR4+ 80 c) Control 40	7 range (3 to 12) days after PCI	Unselect ed BMMC (UNSEL) CD34+ CXCR4+ (SEL)	Ficoll 50-70ml in unselected Ficoll 100-120 ml from selected cell patients two-step immuno- magnetic selection	None	Not stated 1.9 x 10 ⁶	Intracoronary	No adverse events	6 months No significant change in LVEF trend for improvements in poorer LVEF and delayed revascularisation
(Wohrle et al., 2010)	42	RDBPCT 2:1 29 BMMC 13 Placebo	5 to 7 days	BMMC	Ficoll	None	3.8 x 10 ⁸	Intracoronary	No adverse events	6 months No change in LVEF or systolic and diastolic function

Study	N	Design and Numbers	Time post MI	Cell type	Cell preparation	Culture	Mean cell no.	Delivery	Safety	Efficacy
(Hirsch et al., 2011) HEBE Trial	200	Randomised Controlled trial 1:1:1 a) BMMC 69 b) PBMC 66 c) Control 65	3 to 8 days	BMMC	Ficoll	None	BMC $3 \pm 1.6 \times 10^8$ PBMC $2.9 \pm 1.4 \times 10^8$	Intracoronary	No adverse events	4 months No change in Regional or global LV Function
(Hare et al., 2009)	53	RDBPCT 2:1	1 to 10 days	Allogenic hMSC	Bone marrow from single unrelated donor isolated Mesenchymal stem cells	Yes to produce Provacel	0.5, 1.6 and 5×10^5	Intravenous	No adverse events	6 months ↑ LVEF ↓ Symptoms
(Ince et al., 2005)	50	Open label Randomised 25 control 25 GSCF	AMI post PC1	GSCF	10 µg/kg GCSF 6 days	None	20 x increase In MNC	Systemic	No adverse events	4 months ↑LVEF ↑Regional wall thickness and movement ↓LVEDV
(Kuethe et al., 2005)	23	Open label Randomised 9 control refused GCSF 14 GSCF	48 hours AMI post PC1	GSCF	10 µg/kg GCSF 7 days	None	Stopped when leucocytes above $5.0 \times 10^{10}/L$	Systemic	No adverse events	3 months ↑ Wall thickness and movement ↑Perfusion
(Zohlhofer et al., 2006) REVIVAL-2	114	RDBPCT	0.5 days	GSCF	10 µg/kg 5 days	None	5 fold increase WBC compared to placebo 14 x CD34+ cells	GCSF mobilised	Bone pain & muscle discomfort	4 to 6 months No change LVEF Restenosis Infarct size

Study	N	Design and Numbers	Time post MI	Cell type	Cell preparation	Culture	Mean cell no.	Delivery	Safety	Efficacy
(Ripa et al., 2006a) STEMMI	78	RDBPCT	0.5 days	GCSF	10 µg/kg 6 days	None	5 x ↑CD45- /CD34- 2 x ↑ CD45- /CD34- /CXCR4+ 10 x ↑ CD34- /CD34- VEGFR-2+	GCSF mobilised	None	No change LVEF Restenosis Infarct size
(Engelmann et al., 2006) GCSF-STEMI	44	RDBPCT	5 days	GCSF	10 µg/kg 5 days	None	4 x increase leucocytes compared to placebo. 23- 29 fold EPC's	GCSF mobilised	1 death 1 MI Requires careful monitoring	3 months No change in LVEF or Restenosis
(Leone et al., 2006)	41	Open label Randomised 1: 2 14 GCSF 27 Control	5 days	GCSF	10 µg/kg GCSF 5 days	None	10 fold increase in CD34+ Cells compared to the control	GCSF mobilised	None	5 months ↑ LVEF

Study	N	Design and Numbers	Time post MI	Cell type	Cell preparation	Culture	Mean cell no.	Delivery	Safety	Efficacy
CHRONIC										
(Menasche et al., 2003)	10	Open label	6.2 ± 1.9 years	SM	Muscle biopsy with complex processing	3 weeks	8.7 x 10 ⁸ myoblasts	Epicardial with CABG	Arrhythmias Required ICD	11 months ↑LVEF ↓NYHA
(Smits et al., 2003)	5	Open label	Chronic	SM	Muscle biopsy and complex processing	17 days	3 ± 2 x 10 ⁸	NOGA Endocardial	1/5 VT and ICD placement	3 months ↑LVEF ↑Wall thickness
(Pagani et al., 2003)	5	Open label	Chronic LVAD bridge to transplant and SM	SM	Muscle biopsy and complex processing	3 weeks	3.0 x 10 ⁸	Epicardial + LVAD	I death	3 transplant SM survival and differentiation into mature myofibers
(Siminiak et al., 2004)	10	Open label	Minimum of 90 days post MI	SM	Muscle biopsy with complex processing	3 weeks	4 x 10 ⁵ - 5 x 10 ⁷ Myoblasts	Epicardial with CABG	Arrhythmias	12 months ↑LVEF ↓NYHA
(Chachques et al., 2004)	20	Open label		SM	Muscle biopsy with complex processing	3 weeks in human serum	3 x 10 ⁸	Epicardial with CABG	No safety concerns	14 months No increase in arrhythmias ↑LVEF ↓NYHA
(Siminiak et al., 2005) POZNAN	10	Open label	Minimum of 90 days post MI	SM	Muscle biopsy with complex processing	3 weeks	Up to 10 ⁸ Myoblasts	Trans coronary venous	Arrhythmias	6 months ↑LVEF ↓NYHA
(Menasche et al., 2008) MAGIC	97	RDBPCT 33 low dose 34 high dose 30 Control	Minimum 4 weeks post AMI	SM	Muscle biopsy with complex processing	3 weeks	4 x 10 ⁸ 8 x 10 ⁸	Epicardial with CABG	Arrhythmias but no ↑ in cardiac events	6 months No change in LVEF ↓ In LV volumes in high dose group

Study	N	Design and Numbers	Time post MI	Cell type	Cell preparation	Culture	Mean cell no.	Delivery	Safety	Efficacy
(Patel et al., 2005)	20	Randomised 10 CABG 10 CABG + cells	Chronic	AC133+ BM	500-600mL	None	2.2×10^7 CD34+	Epicardial With CABG	None	6 months ↑LVEF
(de la Fuente et al., 2007)	10	Open label	Chronic	BMC	50mls Ficoll	None	8.6×10^7	Endocardial helical needle	None	12 months ↑LVEF
(Goussetis et al., 2006)	8	Open label	Chronic	CD133+ CD133- CD34+ 300 ml BM	Labelled with 99m Tc- hexamethyl- propylenamin eoxime	None	$0.8 \pm 0.3 \times 10^7$ CD133+ $0.8 \pm 0.2 \times 10^7$ CD133- CD34+	Intracoronary	None	1 hour distribution n= 8 $9.2\% \pm 3.6$ 24 hour distribution n= 4 $6.8\% \pm 2.4$
(Losordo et al., 2007)	24	RDBPCT Dose escalation 3 active doses and Control	Chronic	CD34+	5μg/kg GCSF 5 days	Leukapheresis followed by overnight storage at 4 degrees C	CD34+ cell/kg 5×10^4 1×10^5 5×10^5	NOGA and endocardial injections	13 pts angina with GCSF	6 months Trends for ↓Angina ↓GTN ↓CCC ↑Exercise tolerance No dose response
(Klein et al., 2007)	10	Open label	Chronic	CD133+	Separated CliniMACS	None	$1.5-9.7 \times 10^6$	Epicardial with CABG	None	9 months ↓NYHA ↓CCC ↑LVEF
(Huttmann et al., 2006)	12	Open label Historical controls	Chronic ICM 9 DCM 7	CD34+	4 x 10 days dose sufficient to raise leukocyte count	None	Leukocyte count to 45,000- 50,000/l until day 10	Systemic	One death from ventricular fibrillation Angina Dyspnea	6 months ↓NYHA ↑6 minute walk test

Study	N	Design and Numbers	Time post MI	Cell type	Cell preparation	Culture	Mean cell no.	Delivery	Safety	Efficacy
(Manginas et al., 2007)	24	Open label Retrospective medical management	Chronic	CD133+ CD133- CD34+	Ficoll	BMMNC incubated with antibody against CD133 followed by CD34	CD133+ $1.7 \pm 0.5 \times 10^7$ CD133- CD43+ $8 \pm 4 \times 10^6$	Intracoronary	1/12 Restonosis 1/12 Increased atherosclerosis no increase compared to control	4-6 months ↑LVEF ↓LVEDV ↓LVESV ↑Perfusion
(Stamm et al., 2003)	6	Open label	Within 90 days	AC133+ From bone marrow aspirate	Ficoll Incubated ferrite conjugated monoclonal antibody against AC133+	Overnight at 4 degrees C in 0.9% NaCl	AC133+ 1.5×10^6	Epicardial With CABG	Local inflammation	3-9 months 4/6 ↑LVEF 5/6 ↑Perfusion 6/6 ↓NYHA
(Stamm et al., 2007)	55	Open label 15 safety patients. 20 CABG + cells 20 CABG control	MI at least 14 Days previous	CD133+ BMC	Ficoll Incubated Ferrite conjugated monoclonal antibody against CD133+	Overnight at 4 degrees C in 0.9% NaCl	3×10^7 CD34+ 5.8×10^6 AC133+	Epicardial With CABG	None	6 months ↑LVEF ↑Perfusion
(Fuchs et al., 2003)	10	Open label	Chronic	BMC	Not described	None	$3.3 \pm 2.8 \times 10^6$	NOGA and endocardial injection	2 re-admitted to hospital for recurrent chest pain	3 months ↓CCC ↓Stress induced ischaemia
(Tse et al., 2003)	8	Open label	Chronic	BMMC	Ficoll	None	10^7 x cells/ml	NOGA and endocardial injection	None	3 months Improved symptoms ↑Perfusion ↑LVEF

Study	N	Design and Numbers	Time post MI	Cell type	Cell preparation	Culture	Mean cell no.	Delivery	Safety	Efficacy
(Perin et al., 2003)	21	Open label 14 BMMC 7 medical control patients	Chronic	BMMC	Ficoll	None	3×10^7	NOGA and endocardial injection	1 sudden Cardiac death	4 months ↑LVEF ↑SPECT ↑MVO ₂
(Mocini et al., 2006)	36	Open label 18 CABG+BM C 18 CABG	Chronic MI < 6 months	BMC	400 ml	None	5.2×10^8	Epicardial With CABG	↑Troponin-I	3 months ↑LVEF
(Strauer et al., 2005) (Strauer et al., 2010)	18	Open label 18 BMC Historic controls	Chronic	BMC	80 ml Ficoll	Overnight	1.5 to 2.2 $\times 10^7$	Intracoronary	3/135 local dissection of coronary artery	3 months ↑LVEF ↑ Wall movement ↓ Infarct size ↑ VO ₂ Max ↑ Metabolic activity * 5 Year ↑LVEF ↑QOL ↑Survival
(Yao et al., 2008)	47	Open label Randomised 24 BMC 23 Control	12 hours	BMMC	Ficoll	None	1.2×10^7	Intracoronary	No Adverse effects	6 months No change in LVEF, infarct size or perfusion
(Assmus et al., 2006) TOPCARE-CHD	75	Randomised Controlled cross over 23 no cell infusion 24 CPC 28 BMC	< 90 days	BMC CPC	50 ml Ficoll 270 ml	None 3 days	2.1×10^8 - 1.1×10^8 BMC 2.2×10^7 - 1.1×10^7 CPC	Intracoronary	3 of 135 infusion procedures local dissection of coronary artery	3 months BMC ↑ LVEF No change with CPC

Study	N	Design and Numbers	Time post MI	Cell type	Cell preparation	Culture	Mean cell no.	Delivery	Safety	Efficacy
(Archundia et al., 2005)	5	Open label compared to 10 matched controls receiving CABG	> 1 year	CD34+	300 µg G-CSF for 5 days	Apheresis selection of CD34+ cells Frozen for 2 days	4 x 10 ⁸ 6 x 10 ⁵ CD34+	Epicardial with CABG	None	6 months ↑ LVEF ↑ LVEDV ↓ LVESV ↑ LV wall thickness 2/5 pts ↓ NYHA
(Beeres et al., 2007)	20	Open label	Chronic	BMMNC	Ficoll	None	9.4 x 10 ⁷	NOGA Endocardial injections	None	12 months ↓ CCS ↑ QOL ↑ LVEF ↑ Improved wall motion
(van Ramshorst et al., 2009)	50	RDBPCT	Chronic	BMMNC	Ficoll	None	1.0 x 10 ⁸	NOGA Endocardial injections	None	3 months ↓ CCS ↑ QOL ↑ LVEF ↑ SPECT
(Zhao et al., 2008)	36	RCT	Chronic	BMMNC	Ficoll	None	7 x 10 ⁸	Epicardial injection with CABG	2 Pts with ventricular arrhythmias	6 months ↓ NYHA ↑ Regional wall movement
(Wang et al., 2005)	29	Open label 13 GCSF 16 Medical controls	Chronic	GCSF	5 µg/kg GCSF 6 days And exercise induced ischaemia	None	4 poor mobilisers CD34+ 5000 ± 700/mL blood 9 mobilisers 28,900 ± 5,100 ml blood	GCSF mobilised	Possible Adverse effect on LVEF	2 months GCSF group ↓ CCC ↓ Angina ↓ GTN No change Perfusion

Study	N	Design and Numbers	Time post MI	Cell type	Cell preparation	Culture	Mean cell no.	Delivery	Safety	Efficacy
(Boyle et al., 2006)	5	Open label	Chronic	CD34+	G-CSF	10 µg/kg 4 days and leukapheresis	CD43+ $6.7 \pm 1.8 \times 10^7$	Intracoronary	Bone pain 1/5 prolonged headache 1/5 ACS	12 months ↓CCC ↑QOL ↑Collateral blood flow
(Erbs et al., 2005)	26	RDBPCT	Chronic Occlusion Post PCI 10 day later cell or control	CPC	300 µg GCSF 4 days	4 days	$6.9 \pm 1.4 \times 10^7$	Intracoronary	None	3 months ↑LVEF ↑Flow reserve ↓Hibernation ↓Infarct size
(Kang et al., 2006) MAGIC Cell-3-DES (Drug eluting stent)	96	Randomised controlled AMI PCI + DES 25 AMI Cell infusion 25 AMI Control 16 old MI Cell infusion 16 Old MI Control	Old and new MI	PBSC	10 µg/kg GCSF 3 days	Apheresis	$1.4 \pm 0.5 \times 10^9$ Mononuclear cells	Intracoronary	None	6 months AMI PBSCs ↑LVEF ↓LVEDV Old MI No change LVEF
(Kang et al., 2007) MAGIC CELL Terminated early	30	Randomised Controlled 10 control Medical management 10 GCSF 10 GCSF & PBSC	Old and new MI	GCSF GCSF & PBSC	10 µg/kg GCSF 4 days	Apheresis For PBSC	PBSC $1 \times 10^9 \times 3$ patients 1.7×10^9 remaining patients	PBSCs intracoronary Infusion	Terminated early GCSF alone Restenosis	6 months PBSC ↑LVEF Gone by 2 years GCSF alone ↑Restenosis

Study	N	Design and Numbers	Time post MI	Cell type	Cell preparation	Culture	Mean cell no.	Delivery	Safety	Efficacy
(Kang et al., 2008)	50	Randomised controlled AMI Sirolimus eluting stent (SES) or Paclitaxel eluting stent (PES) 19 cells 17 medical management	Old and new MI	PBSCs	10 µg/kg G-CSF 3 days	Apheresis	1-2 x 10 ⁹ mononuclear cells	Intracoronary	No neointimal hyperplasia but may peri-stent tissue growth at stented segment especially with PES	6 months No adverse effects or de novo lesions May↑peri-stent tissue growth at stented segment especially with PES
(Ripa et al., 2006b)	16	Historical control 32	Chronic	VEGF ⁻¹⁶⁵ & G-CSF	0.5mg VEGF ⁻¹⁶⁵ 10 µg/kg G-CSF 6 days	None	4 poor mobilisers CD34+ cells < 15.000/ml blood 60 ± 11 /ml blood	NOGA and endocardial VEGF ⁻¹⁶⁵ G-CSF	None	3 months No effect

Table 4 Shaded rows represent Randomised Double-Blind Placebo-Controlled Trials (RDBPCT), Skeletal Myoblasts (SM), Circulating Progenitors Cells (CPC), Peripheral Bone Marrow Stem Cells (PBMC) Autologous Bone Marrow cells (BMC), Autologous Bone Marrow Mononuclear Cells (ABMMC), Granulocyte-colony stimulating factor (G-CSF), Bone Marrow (BM), Vascular Endothelial Growth Factor (VEGF), = Increased = Decreased, Left Ventricular End Systolic Volume (LVESV), Left Ventricular Ejection Fraction (LVEF), Stroke Volume (SV), Left Ventricular End Diastolic Volume (LVEDV), Left ventricular end systolic volume (LVESV) Acute Myocardial Infarction (AMI), Myocardial Infarction (MI), Glyceryl Trinitrate GTN, New York Heart Association Class (NYHA), Canadian Cardiovascular Class (CCC), Myocardial Oxygen Consumption (MVO₂), Ischaemic cardiomyopathy (ICM), Dilated cardiomyopathy (DCM). Drug eluting stent (DES), Sirolimus eluting stent (SES), Paclitaxel eluting stent (PES). ↑= increased, ↓=decreased

1.6 ACUTE MYOCARDIAL INFARCTION (AMI)

Mortality after AMI improves with early reperfusion of the infarct-related artery. However, this is not always achieved and necrosis, scar formation and left ventricular remodeling often occur, which adversely affects the left ventricular function (LVF) and prognosis. The aim of cell therapy in this patient population is to reduce these detrimental effects and replace lost myocardium with functional tissues. A number of different cell types as well as delivery routes have been utilised in this setting

a. Autologous bone marrow cells

(Ge et al., 2006) administered autologous bone marrow mononuclear cells (ABMMC) via the intracoronary route in ten patients within 24 hours of an AMI and successful PCI and stent implantation. Six months later, compared to the control group who received intracoronary instillation of supernatant (n=10), the ABMMC treated group had significant improvements in LVEF from a mean 53.8 (SD 9.2%) to 58.6 (9.9%) and unlike the control group they did not have an increase in left ventricular diameter (LVD). Myocardial perfusion defect scores improved from 21 ± 11 to 13 ± 10 ($p < 0.01$) in the ABMMC, group but were unchanged in the control group 20 ± 14 to 17 ± 15 .

(Schachinger et al., 2004) in the Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI) Phase 1 trial studied 59 patients post-AMI and successful stenting of the culprit artery. Patients were treated with either intracoronary delivery of bone-marrow derived progenitor cells consisting of haematopoietic progenitor, side populations and stromal cells or circulating blood-derived progenitor cells which were predominantly endothelial progenitor cells 4.9 $\pm$ 1.5 days post-AMI. At the four month follow-up LVEF significantly increased ($50 \pm 10\%$ to $58 \pm 10\%$, $p < 0.001$), left ventricular end systolic volume (LVESV) decreased (54 ± 19 ml to 44 ± 20 ml, $p < 0.001$); at one year the improvement in LVEF was maintained.

The Bone Marrow Transfer to Enhance ST Elevation Infarct Regeneration (BOOST) trial (Wollert et al., 2004) was the first randomised controlled trial using autologous bone marrow. After sustaining an AMI and following an acute reperfusion PCI, 60 patients were randomised either to receive optimal medical treatment or intracoronary

unselected autologous bone marrow cells (BMC) 4.8 days (SD 1.3) later into the target artery. Six months later LVEF increased significantly in patients receiving BMCs from $50 \pm 10\%$ to $56 \pm 7\%$ versus $51.3 \pm 9.3\%$ to $52 \pm 12.4\%$ in the control group ($p = 0.003$). There was no reported incidence of arrhythmias, or increase in restenosis. These changes, however, were not sustained when patients were reviewed at 18 months, when the LVEF had increased by 0.7% in the control group and 3.1% in the group receiving BMCs (Meyer et al., 2006). Two possible explanations for this are that the initial improvements in the BMC group may have arisen as a result of a marked placebo effect which has previously been noted in angiogenesis trials (Henry et al., 2003, Simons et al., 2002) or that the long-term viability of transplanted cells is questionable and improvements are only temporary and do not translate into permanent myocardial repair.

(Meluzin et al., 2008) in a dose-escalation study, randomised 66 patients into three groups post-AMI and PCI to either intracoronary delivery of high (10^8 ABMMC) or low dose (10^7 ABMMC) or control (no instillation). Three months later they found that both cell groups had improvements in regional wall motion compared to the control in a dose-related fashion, at 12 months this difference was no longer apparent. However the improvement in LVEF in the high dose group of 6% at 3 months was maintained and was 7% at 12 months (Meluzin et al., 2008).

In the largest study to date (Schachinger et al., 2006) reported the results of the Reinfusion of Enriched Progenitor cells and Infarction Remodeling in Acute Myocardial Infarction (REPAIR AMI) trial. They randomised 204 patients with successful reperfused AMI to receive intracoronary infusion of BMC, or placebo, three to seven days later. At 12 months in the BMC group compared to the control there were significant improvements in rates of the pre-specified cumulative endpoint of death, recurrence of MI and re-hospitalisation for heart failure ($p = 0.006$). At four months quantitative LV angiography was performed to assess LVEF; a significant if modest 2.5% improvement in LVEF ($p = 0.02$) was detected. It has been suggested that an improvement of at least 5% is required to predict improvements in symptoms or mortality (Cintron et al., 1993). Subgroup analysis showed that those patients with lower baseline LVEF gained the most improvement.

(Krause et al., 2009) published the first percutaneous electromechanically guided (NOGA) endocardial injection open-label study in which patients received 1.0×10^6 CD45^{dim}/CD34^{hi} stem cells into their target coronary artery ten days post AMI. 12 months post treatment they reported a 7% improvement in LVEF, and significant improvements in high voltage areas and a reduction in low voltage areas as assessed by NOGA. However the latter is dependent on the quality of the electromechanical maps produced, and the reproducibility of map acquisition is questionable (Sarmiento-Leite et al., 2003).

(Plewka et al., 2009) randomised 60 patients post ST elevated myocardial infarction (STEMI) with a LVEF < 40% treated successfully with a PCI in an open-label 2:1 ratio to either BMC 7 days after a STEMI or medical control. The active group received intracoronary delivery of BMC into the culprit coronary artery and the control group received no sham procedure. Six months post procedure the BMC group demonstrated an absolute improvement in LVEF of 10% compared to 5% in the control group and improvements in LV systolic and diastolic function. Although encouraging the open-label design and lack of a sham invasive procedure biases the results in favour of the BMC group.

In contrast (Lunde et al., 2006) reported negative results from their Autologous Stem Cell Transplantation in Acute Myocardial Infarction (ASTAMI) randomised controlled trial. Forty- seven patients received intracoronary injections of ABMMC six days after an AMI and these were compared to a control group who received no infusion. Three non-invasive imaging modalities of SPECT, echocardiography (ECHO) and MRI were used. The study was powered to have an 80% chance of detecting a 5% increase in LVEF. At six months there was no significant difference between groups, and with arguably the most sensitive measure (MRI) the change in LVEF ($p= 0.05$) favoured the control group.

(Janssens et al., 2006) also did not detect an improvement in LVEF assessed by MRI at the four month follow-up of their double-blind placebo-controlled trial comparing intracoronary BMC ($n = 33$) to infusion of placebo ($n = 34$) in patients' 24 hours post-AMI and successful PCI and stenting. However, in secondary outcome measures the

BMC group, compared to controls, had a significant reduction of 28% in MI size ($p = 0.04$) and improvements in regional systolic function. There were no differences between the groups for perfusion and metabolism as assessed by positron emission tomography (PET). This group concluded that although cell therapy did not improve LV function after AMI it could have had favourable effects on post-infarct remodeling. Interestingly this study administered cells 24 hours after optimal reperfusion. In the REPAIR AMI (Schachinger et al., 2006) study it was found that patients receiving cells five to seven days after MI had a greater improvement in LVEF than in those patients that received cells at an earlier time point. It has been suggested that the milieu immediately post-AMI may adversely affect cell homing and retention within the myocardium potentially impacting on the efficacy in this trial. Clearly further studies are required to determine the optimal time, post-AMI, at which to deliver cells, and the most efficient delivery route, together with the longer term sustainability of any noted improvements.

(Wohrle et al., 2010) also in a later randomised placebo controlled trial of 42 patients receiving intracoronary delivery of either 3.8×10^8 BMC or a rigorous placebo containing erythrocytes importantly visually indistinguishable from the BMC preparation reported no improvement in LVEF or systolic or diastolic function six months post treatment. This was a well-conducted study, whether unselected BMC or this particular dose of BMC or the delivery route was ineffective requires further clarification.

(Huikuri et al., 2008) in the FINCELL trial randomised 80 patients to receive either BMC or medium two to six days post STEMI. At the six month follow-up the patients in the BMC group had an increased LVEF of 5.9 % and 5.1 % compared to the placebo group measured by angiography and 2D echocardiography respectively. There were no safety problems noted.

(Yousef et al., 2009) in the BALANCE trial reported on their open label controlled study of 62 patients treated with BMC 7 ± 2 days after an STEMI. In the initial three month follow-up they reported an increase in LVEF and a reduction in infarct size of 8% and no safety concerns. Although open label and not including a placebo control they did have a control group of 62 patients that had comparable LVEF and diagnosis.

The initial improvements reported in the BMC group were maintained up to five years post treatment and surprisingly in such a small study mortality was also significantly reduced. Clearly the open label design means that all results should be viewed with caution, but it was encouraging to see the benefits had extended up to five years including mortality and was unlikely to be due to the placebo effect at this late time point.

(Tendera et al., 2009) in the REGENT trial has been the only group to compare head to head unselected and a specific sub-population of BMC. They randomised patients to either unselected BMC or a sub-population of CD34+ / CXCR4+. After six months LVEF increased by 3% in both cell treated groups compared to a medical control; however there were no significant differences in absolute changes in LVEF between the groups. There was a trend to improved LVEF in the unselected BMC group with the most impaired baseline LVEF, as was seen in the other trials (Landmesser, 2009).

(Hirsch et al., 2011) reported on the HEBE trial of 200 patients randomised to receive BMC, peripheral bone marrow cells (PBMC), or standard therapy three to eight days post sustaining their first large AMI. Global and regional LV function was assessed four months post treatment by cardiac magnetic resonance (CMR). They found no difference between either of the treatment groups or control. Nor were there any changes in infarct size or rate of clinical events in any of the groups. This is one of the largest studies of intracoronary delivery of cells, so why do the results differ from REPAIR AMI? (Schachinger et al., 2006). There were a number of factors; this was a multi-centered trial and there was inhomogeneity between the numbers of patients randomised amongst the two treatment arms and controls between centres. However even when centres with recruitment below ten patients were excluded the primary endpoint of improvement in LV function was not changed. There was no standardisation of dose of cells given with consequent marked variability in this parameter. They had initially powered the study to see a 6% improvement in LVEF, which in light of other recent publications is probably too high, and thus it was underpowered to pick up the 3% improvement seen in other trials. The median time for revascularisation in this trial was rapid, within 3.3 hours. In a post hoc analysis of the REGENT trial the investigators saw the greatest improvement in those patients with the longest period between onset of symptoms and reperfusion (Tendera et al.,

2009). There was a difference in the cell processing procedures in this trial compared to that in the REPAIR AMI. Unlike in the REPAIR AMI where cells were stored overnight; in this trial cells were delivered on the same day as harvest. However if anything this should have improved efficacy as it has been reported that storage overnight can result in a reduction in cell functionality (Seeger et al., 2007). It could have been other alterations in cell processing; in the REPAIR AMI study it was found that red cell contamination of cell injectate impairs the improvement in LVEF (Assmus et al., 2010). It may be that intracoronary delivery was not optimal, but why this should differ from REPAIR AMI remains unresolved. It was a shame that this trial was not double blind with a control group receiving placebo instillation, however, despite this there was not a difference between groups and if anything the lack of a true control should have increased the placebo effect seen in the treated groups compared to the control group that received standard therapy.

b. Allogenic Human mesenchymal stem cells (hMSC)

Recently (Hare et al., 2009) published the first study to deliver allogeneic human mesenchymal stem cells (hMSC). These cells have the hypothetical advantage of being able to be infused intravenously as they home to injured areas of the myocardium. Importantly they can also be given allogeneically because they lack various major histocompatibility and co-stimulatory cell-surface antigens, whilst secreting anti-inflammatory cytokines. It has been noted that with increasing age and disease the potency of stem cells decreases; thus the use of cells from a young healthy individual may be advantageous. They also may be an enriched population for angiogenesis and myogenesis. However there are potential safety concerns, such as blockage of microvasculature, tumourgenesis or ectopic tissue formation. Hare *et al* designed a randomised double blind study to address some of these potential safety concerns. The hMSC were isolated from a single individual and prepared into Prochymal which is a human mesenchymal stem cell product produced by (Osiris Therapeutics Inc; Baltimore Maryland). Patients were randomised into three cohorts in a 2:1 ratio active or placebo infusion and doses were escalated from 0.5 to 5×10^6 hMSCs/kg body weight. All patients had their first MI within ten days of randomisation. Evaluation six months post treatment showed no difference in safety between the placebo and active groups with trends towards efficacy improvements in

the hMSC groups. Importantly Hare *et al* clinically validated hMSC homing. Further studies are required to explore the potential benefit of these cells in the treatment of cardiovascular disease.

c. Granulocyte cell stem cell factor (G-CSF)

After AMI there is enhanced spontaneous mobilisation of bone marrow derived stem cells. The extent and duration of this mobilisation has also been shown to correlate with improvements in LVEF. Thus, manipulating the number of circulating EPCs at the time of AMI may be a novel therapeutic target to salvage ischaemic damage. A number of small open-label uncontrolled studies have reported favourable effects on LVEF with the administration of G-CSF post-AMI (Kang et al., 2004); (Valgimigli et al., 2005); (Ince et al., 2005); (Kuethe et al., 2005). However, some safety concerns were noted including an increase in restenosis rates, which may be explained by the rise in leukocytes numbers to leukaemia-like levels. Thus, it may be preferable to use strategies which do not induce massive inflammation, and hence which could lead to plaque growth or destabilisation.

(Numaguchi et al., 2006) assessed 51 patients who had had an AMI treated with primary PCI and successful stent implantation. They quantified the number of endothelial progenitor cells (EPC) identified as $CD45^{Low}$, $CD34^{+}$, $CD133^{+}$ and $VEGFR2^{+}$ by flow cytometry; the potential for differentiation was also shown by an up-regulation of CD31 and VEGF2. After seven days of culture they separated patients into two groups; those with > 15 cells with $CD45^{Low}$, $CD34^{+}$ $CD133^{+}$ $VEGFR2^{+}/10^7$ leukocytes (differentiated group) and those with < 15 cells with $CD45^{Low}$, $CD34^{+}$ $CD133^{+}$ $VEGFR2^{+}/10^7$ leukocytes (non-differentiated group). Patients in the differentiated group, with higher numbers of circulating EPCs compared to the undifferentiated group, had a larger area of myocardial salvage ($p = 0.009$), a decrease in end-systolic volume ($p = 0.01$), and better recovery of LVEF ($p = 0.01$).

(Kang et al., 2006) in the Myocardial Regeneration and Angiogenesis in Myocardial Infarction with G-CSF and Intra-Coronary Stem Cell (MAGIC CELL) study investigated the effects of G-CSF alone or G-CSF with intracoronary delivery of

peripheral circulating progenitor cells (PCPC) compared with a medical control. At six months the PCPC group had an increase in LVEF, although at two years this improvement was not maintained. The study was terminated early due to the increased rate of restenosis in the group receiving G-CSF alone (Kang et al., 2004).

Three large randomised placebo-controlled studies have subsequently reported that although G-CSF appeared safe, it did not improve LVEF or reduce infarct size (Zohlnhofer et al., 2006); (Ripa et al., 2006a); (Engelmann et al., 2006) despite successfully increasing the number of circulating stem cells. These studies consistently showed that in the placebo group there was an improvement in LVEF from the time of primary PCI to follow-up three to six months later. This observed improvement ranged from 2% to 8% probably due to recovery of stunned myocardium. Importantly, this highlights the critical need to include a true placebo group in all protocol designs where efficacy is reported as an outcome measure. So why, when it has been reported that an increased number of circulating stem cells have beneficial effects on LVEF, were these studies negative? It may be that the timing was wrong. (Zohlnhofer et al., 2006) have suggested that the milieu five days post-AMI adversely affect the homing signals for cells. In the REPAIR-AMI trial the most beneficial effects were evident in those patients receiving stem cell transplantation more than five days post-AMI and there were no improvements in LVEF in those patients receiving stem cells within four days of their infarct (Schachinger et al., 2004). Secondly, the wrong dose may have been used. Alternatively the type of cells mobilised may not be what is required. Thus, (Ripa et al., 2006a) reported that patients randomised to G-CSF over seven days were exposed to 25×10^9 G-CSF mobilised CD34⁺ cells compared with 3×10^9 cells in the placebo group; in contrast these values were respectively 4.9×10^{11} and 2.0×10^{11} for mesenchymal stem cells. The fraction of CD34⁺ cells/leucocytes increased during G-CSF treatment compared to placebo, whereas the fraction of putative mesenchymal stem cells/leucocytes decreased because the total leucocyte number had increased more in the G-CSF group. An inverse association between the numbers of circulating mesenchymal stem cells and LVEF was found.

(Leone et al., 2006) randomized 41 patients to receive either 10 mg G-CSF or conventional therapy in a 2:1 ratio. After a five month follow-up they found patients

treated with G-CSF had an improvement in LVEF and a reduction in LV remodeling. A recent meta-analysis of stem cells mobilized by G-CSF from ten trials including 445 patients treated post AMI concluded that there was no evidence of efficacy to support their use (Zohlnhofer et al., 2008).

1.7 CHRONIC ISCHAEMIC HEART DISEASE

These patients will generally have run through standard medical and surgical options and remain with a bleak outlook, both in terms of life expectancy and quality of life. The presenting symptom of some of these patients is recalcitrant angina, but in a large proportion the focus is heart failure of increasing severity.

a. Skeletal myoblasts

(Menasche et al., 2003) conducted the first Phase 1 trial in ten patients with an open label, uncontrolled design, utilising autologous skeletal myoblasts (SM) delivered epicardially as an adjunct to CABG. All patients had a history of myocardial infarction with a left ventricular injection fraction < 35% and a residual akinetic scar. The age of infarct was very heterogeneous from one month to 19 years, and thus the regenerative capability and homing milieu were likely to be significantly different. Each patient received between 27 and 57 injections of cells, the dose varying from 5.0 to 11.5×10^8 cells. These were directed in and around the scar in an area distinct from grafts. Initial follow-up was 10.9 ± 4.5 months. LVEF increased significantly from $24 \pm 1\%$ preoperatively to $32 \pm 1\%$ postoperatively ($p < 0.02$). NYHA functional class improved significantly from 2.7 ± 0.2 preoperatively to 1.6 ± 0.1 postoperatively ($p < 0.0001$). There was one death, which occurred early after the operation, and was not attributed to cell therapy. However a significant adverse event was the development of multiple sustained ventricular arrhythmias on 24 hour Holter monitoring despite prophylactic amiodarone, and the trial was stopped. Five of these patients went on to have internal cardiac defibrillators (ICD) implantation. Long-term follow-up of these patients (median 52 (range 18 to 58) months) after CABG and SM transplantation (Hagege et al., 2006) demonstrated that the improvements in NYHA class and LVEF were maintained. It was also noted that there was a low incidence of hospitalisation for heart failure (0.13/patient/year) and the arrhythmic risk could be managed albeit by medical and or ICD implantation.

In a small pilot study of five patients with symptomatic heart failure post-MI (Smits et al., 2003) delivered SM cells as sole therapy via NOGA electromechanical mapping and endocardial injections. Three months later one patient needed an ICD for non-sustained ventricular tachycardia (VT). Angiographic assessment of LVEF increased

from $36 \pm 11\%$ to $41 \pm 9\%$ $p = 0.009$; this improvement was not seen by nuclear or MRI assessments. At six months both angiographic and MRI assessments showed non-significant trends in improvement, but with such small patient numbers it is hard to draw conclusions.

(Pagani et al., 2003) injected SM at the time of left ventricular assist device insertion as a bridge to transplantation. In the four patients transplanted, examination of the native heart revealed engraftment of the SM within the infarcted myocardium. Both these were open-label, uncontrolled studies.

(Siminiak et al., 2004) also conducted an open-label, uncontrolled study of SM administered as an adjunct to CABG in ten patients with viable under-perfused myocardium. There was an increase in LVEF from 35.2% (25% to 40%) preoperatively to 42% (29% to 47%), four months postoperatively which was maintained at the 12 month follow-up. Although this group also noted ventricular arrhythmias these were managed medically without the need for ICD implantation. As for the Menasché study, it is unknown whether detected improvements in LVEF and NYHA class resulted from the application of SM cells or from the revascularisation resulting from surgery. However in the subsequent POZNAN trial an additional ten patients received percutaneous transcatheter delivery of SM as sole therapy (Siminiak et al., 2005). All patients were post-MI with a demonstrated area of non-viable myocardium, eight of which had previously undergone CABG. Again there was a large variability in the time from infarct and the dose of cells delivered. LVEF increased 3 to 8% in six of the ten patients, with no change seen in the remaining subjects. In patients treated with amiodarone, only single ventricular extrasystoles were seen; however in a patient not receiving amiodarone but with an ICD *in situ*, sustained episodes of VT occurred.

The clinical experience with SM cells remains limited; apart from the Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) study (Menasche et al., 2008) all have been very small and uncontrolled. The first randomised placebo-controlled study of the use of SM cells the (MAGIC) study (Menasche et al., 2008) included 97 patients undergoing CABG after MI with moderate to severely reduced LVEF. All patients had prophylactic ICDs implanted. There were low and high dose

groups together with a control; the study was ended prematurely because the treatment was not superior to placebo for the primary outcome measure of improvement in LVEF at six months. There were trends for improvement in LV volumes in the high dose group. Although there were a high number of arrhythmias in the myoblast treated groups there was no increase in serious adverse cardiac events between the pooled myoblast groups compared to control.

These studies have demonstrated the feasibility of SM cell delivery via a number of different routes. In the majority of these studies SM have been administered in combination with CABG and therefore any improvements in LV function are difficult to interpret and cannot be attributable to the haemodynamic effects of SM cells alone. They also raised significant safety concerns regarding potential arrhythmogenic effects despite widespread use of amiodarone and prophylactic placement of ICD's (Menasche et al., 2008). There is no evidence of the formation of gap-junctions between the SM and resident cardiomyocytes; thus any reported improvements likely result from the transplanted cells strengthening the post-infarction scar with amelioration of LV remodeling rather than direct effects on contractility. The lack of electromechanical coupling may have also inadvertently lead to areas of electrical instability and the reported incidence of VT. Alternatively (Chachques et al., 2004) hypothesised that the arrhythmicity of smooth-muscle cells may be due to the conditioning from bovine serum during *ex- vivo* expansion, which may form an inflammatory substrate from an antigen response. Support for this theory comes from his small study where expansion of SM cells was undertaken in autologous donor serum where they reported no incidence of increased arrhythmias; despite this the support for the use of SM remains controversial with the potential risk of arrhythmias and sudden death still requiring clarification for this cell type.

b. Autologous bone marrow mononuclear cells

A number of studies have been undertaken with unselected autologous bone marrow. (Tse et al., 2003) studied eight patients with stable angina, refractory to maximal medical therapy. 10^7 cells/ml were delivered percutaneously with endocardial injections using the Myostar catheter and NOGA mapping system. After three months patients reported improvements in symptoms, as well as more objective changes in myocardial perfusion. LVEF remained unchanged, but there was a 12% improvement in wall thickening, and a 6% improvement in target wall motion determined by MRI.

(Fuchs et al., 2003) also administered unfractionated autologous bone marrow in ten patients not eligible for conventional revascularisation, but with evidence of reversible ischaemia. At three months there was a significant reduction in stress-induced ischaemia in the injected territories. In addition symptomatic angina was also significantly reduced.

(Perin et al., 2003) reported on 21 patients with more severe chronic heart failure (mean LVEF 20%) who were enrolled in a non-randomised open label study (the first 14 patients received autologous bone marrow whilst the last seven patients had no intervention and were used as controls). At two months there was a significant reduction in total reversible defect (assessed by SPECT) and improvement in global left ventricular function within the treatment group and between groups, together with improvements in myocardial oxygen consumption (MVO_2). At four months there was an improvement in LVEF from 20% to 29%, a 31% relative increase. Encouragingly at 12 months SPECT had improved ($p=0.01$) and the significant improvements in MVO_2 were maintained.

(Strauer et al., 2005) performed a cohort study of 18 consecutive patients, five months to 18.5 years following myocardial infarction, comparing them with a control group who fulfilled the entry criteria, but had refused cell therapy. Three months after coronary artery delivery of mononuclear cells, they reported a 30% reduction in infarct size, an improvement in LVEF of 15%, and an improvement of 57% in infarct wall movement velocity. They also noted an 11% improvement in MVO_2 , a 15% improvement in regional ^{18}F -fluor-desoxy-glucose uptake in the infarct tissues, and

concluded that the application of mononuclear cells had led to functional and metabolic regeneration of infarcted and ischaemic tissue. In a recent five year follow-up BMC were not associated with adverse effects, and the group who agreed to BMC had improved LV function, quality of life (QOL) and survival compared to the group who didn't agree to BMC (Strauer et al., 2010). The composition of the control group may have biased their results.

In a larger randomised crossover trial (TOPCARE CHD), (Assmus et al., 2006) reported their results in 75 patients who had an MI at least three months previously. Patients were randomised to no cell infusion (n = 23), circulating progenitor cells (CPC, n = 24) or BMC (n = 28). After three months the patients who were initially randomised to no cell infusion were subsequently randomised to receive either CPC or BMC and the patients who initially received CPC or BMC crossed over to BMC and CPC respectively. The LVEF significantly increased (2.9%) in patients receiving BMC compared to those receiving CPC (-0.4%, $p = 0.003$) or no infusion (-1.2%, $p < 0.001$). There was also an increase in regional contractility in the area targeted by BMC. The crossover phase revealed that intracoronary infusion of BMC was associated with this improvement of LV function and contractility regardless of whether BMC were received as their primary or secondary therapy.

(Yao et al., 2008) also reported on their open-label study of 47 patients with a previous history of recovered MI over a year before treatment in which a successful PCI and stent was implanted within 12 hours of chest pain occurring. 24 patients were randomised to receive 1.2×10^7 BMC based on their decision to consent to active or placebo treatment on their consent form, the control group received an intracoronary infusion of saline in the previously stented vessel. Six months post infusion the BMC group demonstrated no improvement over the control group regarding LVEF, infarct size or perfusion. This negative result may have resulted from the mode of delivery via the intracoronary route in a chronic group of patients where the acute inflammatory milieu may have been less favourable to diffusion of BMC through the gap junctions of the endothelium.

(Beeres et al., 2007) reported on 20 patients with refractory angina who received endocardial injection via NOGA guiding to areas of stress-induced ischaemia

previously detected on gated SPECT. CCS class and quality of life both improved at 12 months and there was also a reduction in ischaemic segments, and a 6% increase in LVEF. Although open-label this study demonstrated promising results.

In a later randomised placebo controlled study (van Ramshorst et al., 2009) studied 50 patients with refractory angina, randomised to receive either 100×10^6 BMC or placebo via endocardial injection with NOGA mapping. At an early time point of 3 months post treatment, patients in the BMC group had significantly, albeit moderately improved summed stress score on SPECT imaging the first reported in a double blind placebo controlled trial. There was an absolute improvement in LVEF assessed by MRI in the BMC group of 3%; 95% CI, (0.5% to 4.7%) vs. -0.1% 95% CI, (-2.1% to 1.1%) in the placebo group. They also reported improvements in CCS class, quality of life and exercise time compared to the placebo group.

(Zhao et al., 2008) compared 18 patients with CABG with 18 patients with CABG in addition to BMC injected into territory not suitable for grafting. Six months post treatment the BMC group had an improvement in LVEF and NYHA class, however ventricular fibrillation was detected in two patients in the BMC group, one was treated successfully with amiodarone whilst the other patient had refractory VF and died 3 days post procedure. This is the first study to document ventricular arrhythmias with the use of BMC, however it is difficult to extrapolate with such small n numbers the effects of CABG from that of the BMC. Further larger studies and meta-analysis have been unable to detect the emergence of arrhythmias with the use of BMC.

In addition to unfractionated BM a number of groups have also isolated specific cells. (Stamm et al., 2007) delivered epicardial CD133+ cells as an adjunct to CABG in 20 patients, compared to 20 patients in whom only CABG was performed. The CABG plus cell therapy group had significantly higher LVEF at six months compared to the CABG control ($37.4\% \pm 8.4\%$ preoperatively to $50.2\% \pm 8.5\%$ at six months and $47.1\% \pm 6\%$ at 18 months, compared to $37.9\% \pm 10.3\%$ to $41.3\% \pm 9.1\%$ at six months in the CABG only group). Between groups comparison showed an improvement in the cell group compared to the CABG group ($p = 0.03$). Perfusion improved more in the CABG plus cell therapy patients.

(Klein et al., 2007) reported on ten patients with ischaemic cardiomyopathy, where CD133+ cells were injected epicardially. All patients had improvements in cardiac function, both in NYHA class, and LVEF.

(Patel et al., 2005) randomised 20 patients into two groups; one received off-pump CABG (OPCABG) (the control), the other OPCABG and epicardial injection of CD34+ ABMMC. They used a large volume (30 ml in 1ml aliquots) with a special needle with side holes to prevent leakage. Six months later, those patients receiving the addition of putative stem cells had improvements in LVEF from preoperative levels of $29\% \pm 6\%$ to $46.1\% \pm 1.9\%$ compared to the control of $30.7\% \pm 2.5\%$ to $37.2\% \pm 3.4\%$. The direct injections may have been a confounding variable, however in a preceding open-label pilot study this group had demonstrated a superior outcome with stem cells injections and OPCABG compared to serum injections and OPCABG. Although encouraging, cell transplant was performed at the time of CABG in these studies so it is difficult to interpret the effects of cell therapy alone.

(Manginas et al., 2007) reported on 24 patients with an old MI; 12 received intracoronary delivery of CD133+, CD133- CD34+ progenitor cells and 12 were followed up medically. In the treatment group left ventricular end diastolic diameter (LVED) and left ventricular end systolic volume (LVESV) were reduced, LVEF at rest improved from $27.2 \pm 6.8\%$ to $29.7 \pm 7.3\%$, and perfusion was also improved. One patient developed restenosis and one progression of atherosclerosis at the site of cell delivery, but after 28 ± 8.7 months only one patient had deterioration in heart failure. In the control group there was deterioration in end diastolic and end systolic volumes, a non-significant reduction in LVEF and no change in perfusion.

In a meta-analysis it was found that efficacy was only achieved with cells numbers of 1×10^9 (Martin-Rendon et al., 2008), clearly the majority of trials to date have been below this number.

c. G-CSF in chronic ischaemic heart disease

(Wang et al., 2005) found that treatment with G-CSF in patients with severe ischaemic heart disease improved symptoms but did not alter myocardial ischaemia. Indeed on MRI and SPECT, although not with Echo, there was a reduction in LVEF. This reduction may have been due to an adverse affect of G-CSF on the myocardium, or because there was a large variability of LVEF assessed by the three different assays utilised. Caution is required in future trials to assess whether there is a negative effect of G-CSF on LVEF in patients with severe ischaemic heart disease (IHD).

(Losordo et al., 2007) administered a five day course of GCSF and then separated the CD34+ cells via leukapheresis. No-option patients were randomised in a 3:1 ratio to receive CD34+ cells or placebo. Patients underwent NOGA electromechanical mapping followed by endocardial injections. All patients had an ICD or wore a life vest defibrillator. In the six month follow-up there were no life threatening ventricular arrhythmias. Angina, GTN, Canadian Cardiovascular Society (CCS) classification and exercise times showed trends for improvement in the CD34+ group compared to control; a larger Phase II trial is underway.

(Huttmann et al., 2006) administered G-CSF in two groups of heart failure patients, one with ischaemic cardiomyopathy (ICM) and another presenting with dilated cardiomyopathy (DCM). G-CSF was administered over a ten-day period interrupted by treatment-free intervals of equal length. Peak CD34+ levels remained constant throughout treatment periods. Nine of the 12 patients received the intended four full cycles and had statistically significant improved six minute walk tests and an improvement of one in their NYHA class, compared to a historical control with ICM. There was one death, and occasional episodes of dyspnoea and angina.

(Archundia et al., 2005) mobilised peripheral mononuclear CD34+ rich cells and injected them epicardially in the peri-infarct zone at the time of CABG in patients who had an MI more than one year previously. Compared to a matched group of patients just undergoing CABG there appeared to be beneficial effects of the addition of CD34+ enriched mononuclear cells. The cell group had improved LVEF, and decreased left ventricular diastolic volume (LVDV), improvements in NYHA class,

and LV wall thickness was seen to increase significantly in two of the five patients.

(Boyle et al., 2006) administered a G-CSF mobilised CD34+ enriched mononuclear cells intracoronary in five patients with chronic ischaemic heart failure. Although they documented improved 12 month follow-up of collateral blood flow on angiography, there were two complications, one patient developed acute coronary syndrome and the other *lentigo maligna*. Clearly with only five patients it is difficult to draw any conclusions.

(Ripa et al., 2006b) investigated three groups of 16 ‘no-option’ patients. One group received intramyocardial injection of plasmid VEGF¹⁶⁵ followed a week later by six days of G-CSF, one group received just intramyocardial injection of plasmid VEGF¹⁶⁵ and the last group received injection of placebo. Although combined therapy resulted in a ten fold increase in circulating CD34+ cells there was no improvements in perfusion, LVEF, exercise time, CCS classification, angina frequency or GTN use. Whether higher doses of VEGF₁₆₅ or increasing the time of expression over one week would lead to benefit remain unanswered questions.

Although initial clinical results appear consistently encouraging, they must be interpreted with a certain degree of caution as to date the majority of clinical trials in the no-option patient population have been uncontrolled and involved small patient numbers. However, no adverse events related to the administration of bone marrow derived cells have been reported. However there are number of key clinical questions that remain unanswered. The number of cells delivered may be insufficient, of those cells delivered most probably die, cells may not be incorporated into the heart and once there they may not function. These unanswered questions have lead some to conclude that the move into the clinic has been too rapid and until these questions are resolved and cell therapy is further optimised that clinical trials should be ceased and further pre-clinical research should be undertaken to try and address these concerns. I believe that to make progress clinical and pre-clinical studies should be carried out in parallel, as invaluable information gained in early clinical trials often provides key directions for further pre-clinical studies.

1.8 WHICH IS THE OPTIMAL METHOD OF DELIVERY?

No matter how good the cell, if not delivered to the target organ then therapy is unlikely to be successful. A number of different delivery routes have been used in the clinical trials published to date. Each method has advantages and disadvantages and each may lend itself more readily to a different patient population. For chronic patients with extensive ischaemia the routes used have been split into epicardial (Pagani et al., 2003); (Patel et al., 2005); (Klein et al., 2007); (Stamm et al., 2003); (Stamm et al., 2007); (Mocini et al., 2006); (Archundia et al., 2005); (Zhao et al., 2008), endocardial (Smits et al., 2003); (de la Fuente et al., 2007); (Losordo et al., 2007); (Fuchs et al., 2003); (Tse et al., 2003); (Perin et al., 2003); (Ripa et al., 2006b); (Beeres et al., 2007); (van Ramshorst et al., 2009) and intracoronary (Goussetis et al., 2006); (Manginas et al., 2007); (Strauer et al., 2005); (Assmus et al., 2006); (Boyle et al., 2006); (Erbs et al., 2005); (Kang et al., 2006).

a. Epicardial

Epicardial delivery has been used in patients with chronic disease and is most likely to guarantee delivery, as injections can be directly visualised and allows delivery of large cells such as SM, which cannot be delivered via the intracoronary route. In addition it may avoid the obstacle of micro-vascular obstruction, which has been reported to have an incidence of up to 50% in patients post-AMI (Janssens et al., 2006) and can be a significant obstacle to intracoronary delivery. Clearly, however, as sole therapy it makes the inclusion of a control group difficult because of ethical constraints. The alternative of delivering stem cells at the time of a planned CABG, allows controls to be included, but is bedeviled by the large proven confounding factor of the surgical revascularisation. The injections themselves may also be pro-angiogenic and, depending on volumes used, may also lead to local myocyte damage.

b. Intracoronary

Intracoronary delivery has been used as the delivery route of choice in patients' post-AMI. Although a number of the open-label studies have reported positive results (Strauer et al., 2002); (Schachinger et al., 2004); (Wollert et al., 2004); (Ge et al., 2006); (Meluzin et al., 2008) results from larger placebo-controlled trials have been variable with both positive (Schachinger et al., 2006) and negative outcomes

(Janssens et al., 2006). This follows a familiar pattern seen in early Phase I angiogenesis gene therapy clinical trials, reporting positive results not borne out by larger placebo controlled phase II studies. One explanation for these negative results is that the rapid first passage through the coronary artery system provides a very short contact time between the product and the target cells and therefore reduces efficiency of delivery and retention. It is likely that to be effective cells must exit the vasculature into the myocardial interstitium.

(Goussetis et al., 2006) performed an interesting pilot study where they labelled CD133+, CD133- and CD34+ cells with 99m Tc-hexamethylpropylenamineoxime and infused these into the infarct-related coronary artery of eight patients. Scintigraphic images at one and 24 hours post-delivery showed uptake of 9% and 7 % of the delivered cells respectively. However other clinical data suggests that after intracoronary delivery only approximately 2% of infused BMC are retained in the heart (Hofmann et al., 2005). The timing of delivery may also be critical after an AMI, the resultant secretion of growth factors such as VEGF and other cytokines cause vessels to become more leaky. This in turn might allow cells to cross the endothelial barrier more easily, and may explain homing of bone marrow cells to areas of infarction. However, the optimal time interval between an ischaemic event and cell delivery has not been clarified. As noted earlier, in the REPAIR AMI study it was found that patients receiving cells five to seven days after MI had a greater improvement in LVEF than in those patients that received cells at an earlier time point. It has been suggested that the milieu and inflammation immediately post-AMI may adversely affect cell homing, retention and survival within the myocardium. However, in 'no-option' patients two obvious differences exist to the acute setting. By definition these patients have inoperable coronary artery disease and it is, therefore, unlikely that their coronary arteries will be sufficiently patent to allow passage of an angioplasty catheter for delivery. Secondly, in this chronic setting the cytokine milieu is unlikely to be as active, and the vessels are consequently less likely to be permeable to cells. Additionally low target zone cell concentration, and biological redistribution (Hofmann et al., 2005) made intracoronary delivery an unlikely route for successful cell transfers in our patients.

c. Endocardial

Endocardial injections have also been utilised in association with electromagnetic mapping of the left ventricle. This technique has the theoretical advantages of targeting ischaemic, but viable myocardium. However delivery of cells is limited to a small radius around each needle track with little lateral diffusion (Laham et al., 2005), underscoring the need to ‘pepper’ the injections over the desired area. A three-dimensional map is constructed using electromagnetic triangulation, which allows accurate localisation of the magnetic tip of the deflectable catheter within the left ventricle, relative to three magnetic pads underneath the subject emitting low intensity magnetic fields. As the deflectable tip of the catheter is moved along a series of points on the endocardial surface of the myocardium, local unipolar electrogram and tip motion during the cardiac cycle are recorded. This information allows calculation of unipolar voltage and local wall shortening at each point. The three-dimensional map that is constructed from this information can be freely rotated in space to view all aspects of the myocardium (Figure 3) The delivery technique is technically challenging, and unless the needle is exactly perpendicular to the endocardial surface there is likely to be a loss of cells both into the left ventricle and via backflow along the needle path. In a porcine study the frequency of missed injections was found to be between 10 to 27%, presumably resulting in intracavity injections and thus a resultant slightly higher liver uptake than epicardial injections (Laham et al., 2005). In my experience the use of NOGA maps, as a surrogate measure of improvements over time in ischaemia and LV function is fraught with the challenges of the reproducibility of maps. Although anatomical reference points used are consistent, the majority of free LV wall points used to construct a map are variable between maps. The higher the number of points in the map, the more accurate the information, but this has to be balanced against the increased time required to acquire the map. The software used to construct the map calculates ischaemia and wall motion based on the information between adjacent points. Thus on repeat studies, information is reliant on the similarity of points taken on subsequent maps. Whilst endocardial injections have been utilised in the clinical trial setting, their efficacy has not been established in large randomised placebo controlled trials.

Figure 3: NOGA Electromagnetic mapping

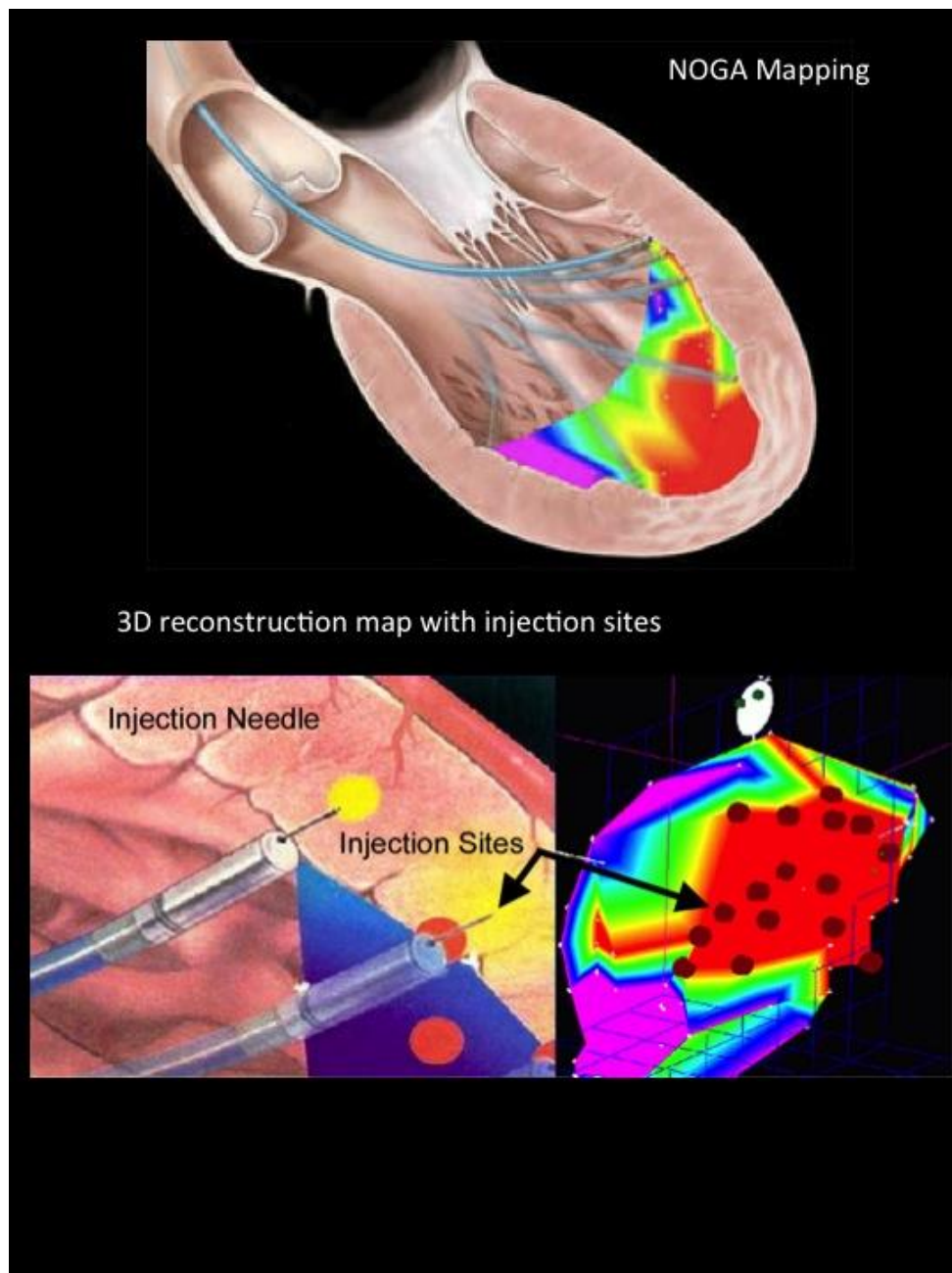


Figure 3: Above the electromechanical NOGA map created by the signal from the magnetic tip contacted with the endocardial surface. For injection in the images below the mapping catheter is replaced with an injection catheter. Once the injection has successfully been delivered a black mark appears on the map to enable placement to be grouped in a zone.

d. Retrograde Coronary venous delivery

Although probably the least well studied part of myocardial anatomy the coronary sinus (CS) and coronary venous network have historically been used for a variety of therapeutic purposes. Synchronised diastolic coronary retroperfusion was utilised to oxygenate acutely ischaemic myocardium by accessing the myocardial capillaries retrogradely. This technique has been used in patients with acute myocardial infarction, unstable angina, and cardiogenic shock and during high risk PCI (Pohl et al., 2004). However due to the complexities of this technique it has never been established in widespread clinical practice. Pharmacological agents such as antiarrhythmic drugs and fibrinolytic agents (Miyazaki et al., 1990) have been delivered via the CS where the arterial system is severely restricted. Cardiac surgeons have also used the coronary venous system to administer cardioplegic solution during surgical procedures (Frater, 1999). Coronary venous delivery increases regional delivery, tissue binding and thus myocardial concentrations compared to coronary artery delivery due to the substantially increased contact time (Boekstegers et al., 2000), and thus it may also be a promising delivery route for cell therapy. More recently electrophysiologists have cannulated the CS and its tributaries during arrhythmia mapping and pacing wire placement for resynchronisation therapy (De Martino et al., 2005). All of the above mentioned techniques strictly regulate the infusion pressure relative to the coronary venous pressure to avoid end venocapillary disruption.

i. Coronary venous anatomy

With the advent of increased interest in the coronary venous system as a delivery route for cell therapy, a detailed knowledge of the coronary venous system is a prerequisite for coronary sinus cannulation, targeting of selective coronary veins and interpretation of the venous phase of coronary angiograms used in the diagnosis of hypoxic myocardium (Gensini et al., 1965). Coronary venous anatomy is variable in man and the reported terminology is inconsistent and confusing; for a comprehensive review please see (von Ludinghausen, 2003). Post mortem studies performed on 350 hearts have assisted in describing in detail the coronary venous anatomy (von Ludinghausen, 1987). There are three coronary venous networks, which are described according to how they open into the right atrium, namely a) tributaries of the coronary

sinus, b) anterior cardiac veins and c) atrial cardiac veins (von Ludinghausen, 1987). Their openings are arranged in a circle between the ostia of both caval veins just above the tricuspid valve. For delivery to the left ventricle the coronary sinus and its tributaries will be the main area of interest.

Techniques involving delivery through coronary sinus cannulation will only be successful if the majority drain into the coronary sinus; this is found in approximately 85% of patients (von Ludinghausen, 1987). In the remaining group, some of these veins connect directly with the right atrium, and may mean that certain myocardial territories are difficult to access. In 99% of patients the anterior and posterior intraventricular (middle cardiac vein) veins are present in addition to at least one additional prominent vein. In 51% of patients with ischaemic heart disease there are three main veins, in 46% of patient there are four and in 2% there are more than four veins (Meisel et al., 2001). The anterior intraventricular vein (great cardiac vein) usually originates in the middle to lower third of the anterior ventricular groove and drains blood from the left anterior descending (LAD) territory. At the base of the heart it turns posteriorly at the antioventricular groove where it enters the great cardiac vein. The posterior intraventricular vein (middle cardiac vein) originates near the apex of the heart and extends along the posterior intraventricular groove towards the base, draining the circumflex coronary artery territory, the posterior lateral left ventricular wall and posterior third or two thirds of the intraventricular septum into the coronary sinus close to the ostium in the right atrium. In 75% of cases it presents as a single vessel and in 24% as two vessels. The left marginal vein originate in the lateral or posterior regions of the left ventricle and drains the right coronary artery into the great cardiac vein or CS (von Ludinghausen, 2003). There are also small cardiac veins (right coronary veins), which are present in around 36% of patients and drain the right coronary artery territory when present (von Ludinghausen, 1987).

ii. Coronary venous delivery techniques

Two methodologies have been described for delivery of products via the coronary venous system. Firstly low pressure delivery, which aims to increase contact time, within the vessels, without endothelial disruption of the vein (Murad-Netto et al., 2004); (Boekstegers et al., 2000); (von Degenfeld et al., 2003). Secondly high

pressure delivery which causes disruption of the endothelial tight junctions so that cells are mechanically driven across from the venocapillary vasculature into the myocardial interstitium resulting in a biological reservoir' (Herity et al., 2000); (Raake et al., 2004); (Hou et al., 2003) (Figure 4). Both techniques involve catheterisation of the subclavian vein or femoral artery, cannulation of the CS and then of a sub-selective coronary vein.

A number of studies have been undertaken in a porcine model. In early feasibility studies it was found that total occlusion of coronary veins for up to 30 minutes did not appear to result in myocardial ischaemia, arrhythmias chest pain or systolic dysfunction (Herity et al., 2000). Localised myocardial staining followed injections of diatrizoate or Evans Blue in all animals. Injection into the anterior intraventricular vein resulted in staining of the anterior left ventricular wall, apex, and intraventricular septum. Injection into the posterior intraventricular vein resulted in staining of the inferior left ventricular wall, posterior septum and posterior walls of the left and right ventricles. At 30 minutes post injection, 20% of the initial signal intensity was still present whilst 50% of the maximal signal clearance had occurred after four minutes. It may be that the exact time and percentages of cell retention may be different from that seen with dyes.

(Boekstegers et al., 2000) demonstrated that adenoviral gene transfer via a pressure-regulated retrograde coronary venous route during ischaemia leads to increased transfection efficiency as compared to an antegrade intracoronary route; however in the absence of ischaemia there was no difference (Boekstegers et al., 2000). In a chronic myocardial ischaemia model, FGF-2 significantly improved regional perfusion and myocardial function when delivered by retroinfusion as compared to antegrade infusion (von Degenfeld et al., 2003). The same group then sought to compare pressure-regulated retrograde coronary venous delivery with epicardial and endocardial injections in an ischaemia reperfusion model. Seven days after administration of an adenoviral mediated beta-galactosidase reporter gene, expression was significantly higher in the retrograde venous group. In addition this group showed widespread transmural transfection, in contrast to distinct local transfection, which occurred after intramyocardial injections (Raake et al., 2004). Subsequently, a pressure-regulated retrograde coronary venous approach was used, to deliver Nuclear

Factor kappa- β decoy oligonucleotides immediately prior to reperfusion in a porcine model of ischaemia reperfusion injury. This produced a reduction in infarct size, and preservation of regional myocardial function seven days after ischaemia as compared to controls (Kupatt et al., 2002).

(Hou et al., 2003) using high-pressure retrograde coronary venous delivery of a plasmid encoding developmentally regulated endothelial locus-1 (Del-1) in a porcine model demonstrated efficient widespread regional delivery as assessed by messenger RNA. (Fearon et al., 2004) investigated high-pressure retrograde FGF-2 protein delivery in a porcine model of chronic myocardial ischaemia; one hour post delivery they reported that significantly more FGF remained in the left ventricle after retrograde venous compared to intracoronary delivery. Four weeks after treatment resting left anterior descending (LAD) blood flow improved slightly in both the retrograde and intracoronary groups compared to controls. The lack of efficacy may have been related to the delivery of protein, which is rapidly degraded.

The porcine coronary venous system differs substantially from humans (Ghoshal, 1975). The main tributary of the porcine coronary sinus, the left azygos vein is not present in man. In addition the porcine vessels tend to be of a larger caliber, have increased collateralisation and are less tortuous than in humans. Thus the catheter systems designed for effective delivery in pigs may not be appropriate for clinical use.

Figure 4: High Pressure Retrograde coronary venous delivery

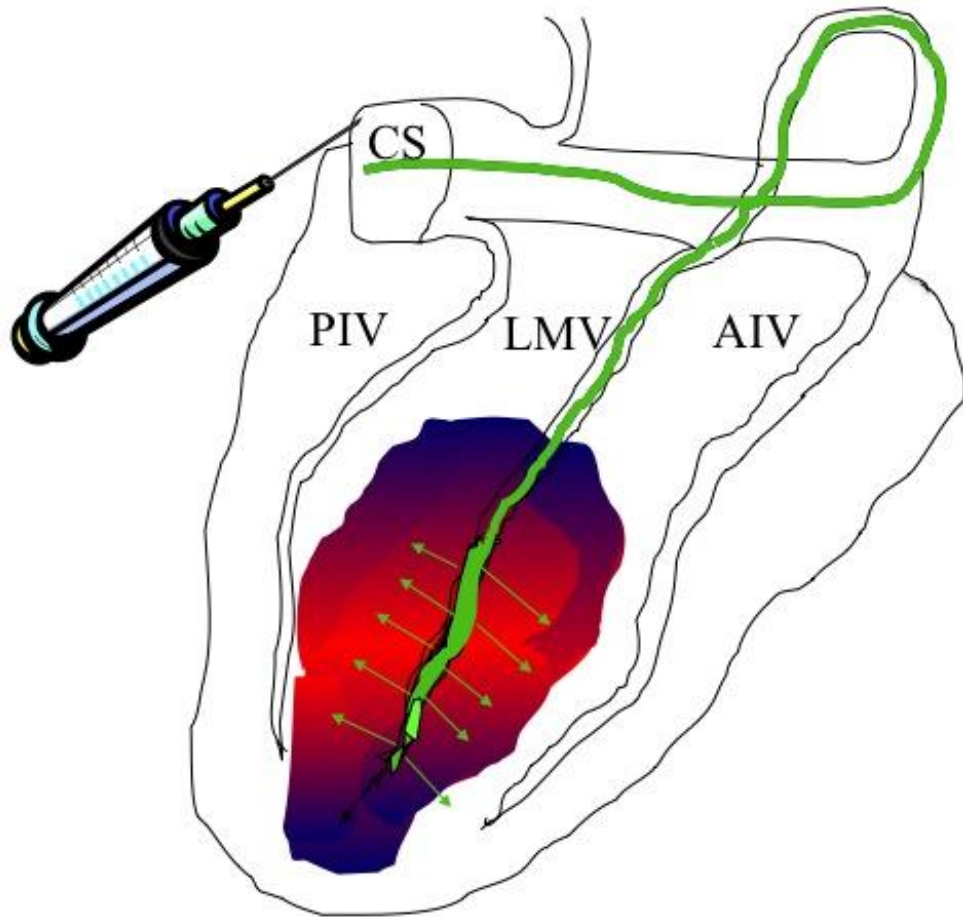


Figure 4: CS= Coronary sinus, PIV= posterior intraventricular vein = (Middle cardiac vein), LMV= Left marginal vein, AIV=anterior intraventricular vein = (Great cardiac vein). In this diagram the left marginal vein is cannulated through the coronary sinus. Contrast is injected at high pressure shown in green resulting in endothelial disruption represented by green arrows, resulting in a sustained myocardial blush shown in red and blue.

iii. Clinical studies of retrograde coronary delivery

Although large animal data appear to support retrograde coronary delivery, controlled clinical trials have not yet been undertaken. (Murad-Netto et al., 2004) published the first case report of autologous bone marrow CD34+ cell infusion via the coronary veins in a patient fourteen days after acute myocardial infarction. No complications were reported and the patient was discharged forty eight hours after treatment.

1.9 RATIONALE FOR DESIGN OF THE PROGRAMME

a. PRE-CLINICAL

In order to take angiogenic therapy forward into a clinical trial I needed to design a pre-clinical programme to address some of the key questions. After reviewing the evidence of all the large animal models the porcine ameroid model was selected because it was the most widely utilised model, which most closely mimicked human chronic myocardial ischaemia and it was less technically demanding and expensive. This enabled studies incorporating larger n numbers, a prerequisite for the assessment of efficacy, which would not have been feasible with other more sophisticated large animal models.

Attempts were made to undertake the research programme in London, but no facility was able to offer a cardiac catheter laboratory, operating theatre and the capability of housing the large number of animals needed for these studies. I contacted one of the leading groups in this field led by Mike Simons in Boston who had extensive experience with the ameroid model, which was in day-to-day use in their department. Investigations were undertaken to see if it was feasible to conduct studies there. However, they did not have the capability to support a relatively large study over an extensive period of time needed to address these issues. Further, because of their urban location, costs of large animal maintenance were high. Therefore, an alternative means of undertaking this work was investigated.

An outstanding facility for large animal myocardial studies at the Institute of Medical Technology, Magdeburg, Germany (IMTM) was identified (Figure 5). This commercial organisation was developed in conjunction with the Department of Cardiology at the University of Magdeburg (Prof. Dr. H. Klein, Director, Department

of Cardiology and Prof. Dr. C. Huth, Director, Department of Cardiothoracic Surgery). The IMTM had state-of-the-art facilities and had completed over 300 experiments in large animals with more than 200 pigs undergoing cardiac catheterisation. The key advantages of this facility were the professional experience with myocardial pig models, and the cost of the studies, which was approximately one fifth of similar facilities in the UK or the USA. The competitive costing in part reflects the rural location of IMTM, which also allowed animals to be housed in their natural surroundings on a working farm. The IMTM had no experience of the ameroid pig model and we therefore made use of our collaboration with the Boston group to transfer the technology. Professor Huth visited the Boston facility with our team to familiarise himself with the procedures, and members of the Boston team visited the IMTM during the initial studies to ensure a smooth transfer of expertise. Despite our best attempts to minimise the 'learning-curve' the ameroid model is a complex one and thus an evaluation study was undertaken over 3 months to validate the model (60 pigs), including endpoint assays, in our hands. Perfusion was assessed with stable isotope labelled microspheres, myocardial function with echocardiography, neovascularisation with angiography and immunohistochemistry and VEGF proteins by Western Blott analysis. This time period, and the number of animals needed, were based on the experience of the Boston group, and were in accord with IMTM Ethical Committee specifications regarding the development of animal models.

Figure 5: Institute of Medical Technology Magdeburg (IMTM)



Figure 5: Depicts the rural setting and housing of pigs in contrast to the sophisticated and advanced catheter laboratory and operating suite on site at the IMTM.

I designed and wrote the protocols for the entire preclinical programme as a direct prelude to a planned angiogenesis clinical trial and so the main aims in each chapter were to gain specific information in order to achieve this.

i. The hypothesis for model development

- Could I establish a pre-clinical large animal model of chronic myocardial ischaemia?
- Would it be possible to formulate the mortality and non-occlusion rates of the ameroid model?
- Would it be possible to establish inclusion criteria that would reliably predict the level of ischaemia present at rest and under stress conditions?
- Was it possible to establish the safety and efficacy outcome measure to assess the effects of angiogenic gene delivery on new vessel formation, myocardial blood flow and left ventricular function during resting and stress conditions?

An evaluation of the ameroid model forms the basis of Chapter 3.

ii. The hypothesis for the Epicardial study

- Is the administration of HIF-1 α safe in an ameroid model?
- Is there any difference in safety and toxicology between different constructs (plasmid or Adenoviral) or dose of plasmid compared to an empty vector Adenoviral control?
- Does any construct of HIF-1 α improve myocardial blood flow, function or blood vessel development compared to a control in an ameroid model under rest and stress conditions?
- If HIF-1 α does demonstrate efficacy over a control, is there any difference between different constructs or doses?

HIF-1 α had never been administered to a large animal model so the aims were to establish the optimal construct and dose and to establish the safety and toxicology profile of HIF-1 α in the ameroid model. I decided to incorporate an epicardial delivery route even though I intended to use a catheter-based in the clinical trial to preclude the confounding variables of catheter-based techniques.

The epicardial delivery of HIF-1 α in an ameroid model forms the basis for Chapter 4.

iii. The hypothesis for the Endocardial study

- Is it feasible to map ischaemia and deliver the optimal construct of HIF-1 α via a clinically relevant electromagnetic mapping and endocardial delivery system (NOGA)?
- Is there any difference in safety and toxicology between the optimal dose and construct of HIF-1 α and an empty vector adenoviral control?
- Does endocardial delivered HIF-1 α improve myocardial blood flow, function and blood vessel development compared to an adenoviral control under rest and stress conditions in an ameroid model?
- What are the mechanisms of any improvements in myocardial blood flow and function?

The endocardial delivery of HIF-1 α in an ameroid model forms the basis for Chapter 5. Although I had been on a NOGA training course with Dr Jonathan Clague in Hamburg Germany at the Biosense facility, an additional aim of the endocardial study was to eliminate any 'learning curve' associated with the use of the NOGA system of delivery in our pre-clinical model prior to planned clinical delivery. Chapter 5 reports on the optimal construct and dose of HIF-1 α with this clinically relevant percutaneous catheter.

b. CLINICAL

After completing the angiogenesis pre-clinical programme and after some significant delays due to negotiations regarding catheter use, the strategic programme at Genzyme changed and they were unable to provide us the necessary clinical grade HIF-1 α to be taken forward into a clinical trial. We were not in a position to produce a clinical grade HIF-1 α ourselves, so at this time I investigated the possibility of utilising another gene. However at this time I did not believe a suitable clinical grade gene therapy product was available. Fortunately at the same time stem cell therapy emerged as a potential treatment strategy for our 'no-option population'. My main aim was to investigate a product capable of improving myocardial blood flow and thus function in a group of 'no-option' patients with IHD. Autologous bone marrow

derived stem cells hypothetically at this point in time not only appeared to have the potential to increase myocardial blood flow via angiogenesis but also function by transdifferentiation or paracrine effects. From a practical perspective the use of autologous stem cells which were not patented by any pharmaceutical company also enabled me far more academic freedom to deliver the optimal ‘product device combination’. I therefore decided to concentrate our clinical efforts on pursuing stem cell therapy. In addition the only CE marked percutaneous catheter available was the Biosense Webster Myostar catheter used in conjunction with the NOGA system. This had several practical and scientific limitations (Psaltis and Worthley, 2009). At the time the Myostar catheter was not commercially available and therefore its use would have involved collaboration with the company who were already working with the Texas Heart Institute. I was also concerned that the global nature of ischaemia in our patient population would be better served by using a delivery route that offered more extensive coverage, thus I decided to pursue a pioneering novel retrograde coronary venous delivery route.

i. Delivery

As emphasised earlier one of the key determinants for success is efficient delivery to the heart. The rationale for utilising the coronary veins as a delivery route for cell therapy was based on a number of factors. Firstly percutaneous delivery obviates the risks associated with a general anaesthetic and invasive open approach to the heart, whilst ethically enabling a true placebo group. Although epicardial delivery is probably the most reliable delivery modality as injections can be directly visualised, the confounding effects of coronary artery bypass grafting at the time of cell delivery makes interpretation of data more difficult (Stamm et al., 2003). Secondly total coronary artery occlusion, a near ubiquitous finding in the ‘no-option’ patient population, often precludes intracoronary delivery and together with the limited contact time and resultant reduction in delivery efficiency seen with intracoronary delivery made this an unattractive choice. The pro-angiogenic regeneration milieu in the setting of chronic myocardial ischaemia may also be markedly different from that found immediately following an acute myocardial infarction, and the homing signals for cells to travel across the endothelial barrier are likely to be much reduced. It is therefore unlikely that the optimal delivery route for the ‘no-option’ patient population will be intracoronary delivery. Intramyocardial delivery via endocardial

injection overcomes these issues and has been used in the clinical setting (Fuchs et al., 2003); (Perin et al., 2003). However in a porcine model it was found to lead only to small localised areas of transfection, and thus is likely to be more applicable in the treatment of discrete rather than global ischaemia, which is often a predominant feature of the 'no-option' patient population. In contrast, retrograde coronary venous delivery results in transmural distribution, and selective cannulation of the anterior intraventricular vein has demonstrated that 25% of the left ventricle can be transfected with a single infusion in a porcine model (Hou et al., 2003).

Although the porcine ameroid model is a good one, my preliminary studies investigating retrograde coronary venous delivery at Dartmouth University USA in this model indicated some significant limitations. Firstly although the anatomy of coronary artery system is relatively similar between pig and human, as described earlier the porcine coronary venous system differs substantially from humans (Ghoshal, 1975). Thus any catheter systems designed for effective delivery in pigs may not be appropriate for clinical use. The dilemma faced was whether to spend a lot of time and focus on optimising this delivery route in the porcine model. Even if this was successful it would not confidently be translatable to humans because of the anatomical differences. The alternative was to move directly into the clinic thus focusing efforts on demonstrating the feasibility of this technique in man, but with the additional problem of then having two unproven hypotheses; of whether retrograde coronary venous delivery actually delivers cells to the heart and once there do bone marrow putative stem cells demonstrate efficacy in the 'no-option' patient population.

To address these issues I designed pilot studies to assess the efficiency and feasibility of retrograde coronary venous delivery before embarking on a large randomised double-blind placebo controlled study. In the Phase IIA study a sub-population of cells were labelled with ^{111}In -oxide so that the distribution and time course of residence in the myocardium of delivered cells could be assessed in man. A number of key variables needed to be addressed with this technique. The optimal delivery pressure, volume and infusion time were yet to be defined. At present in animal models high-pressure delivery relies on hand control, reinforced by fluoroscopic feedback of a myocardial blush. It aims to cause endothelial disruption and thus interstitial delivery without rupturing the epicardial veins or dissecting the coronary

sinus (CS). In addition it is still not established whether biological activity is greater with a small area that has a high concentration of cells or whether a larger area with a lower or equal cell concentration is required. As a consequence the number of veins to be targeted had not been established. Technical difficulties of cannulating tortuous CS tributaries may also mean that certain areas of the myocardium may not be accessible. Further, the ability to recannulate selective coronary veins, which had been used for cell delivery for pacing lead placement, also needed to be assessed. Although there is extremely limited published clinical data on delivery of cells via the coronary venous route, percutaneous cannulation and sub-selection of coronary veins is a well established technique for electrophysiological mapping of arrhythmias and biventricular pacing lead placement. The incidence of coronary sinus dissection during lead placement in a consecutive series of patients was reported to be 6.8% (de Cock et al., 2004). Clinical follow-up was uneventful in all reported cases. There were no signs of pericardial effusion or tamponade and long-term angiographic follow-up demonstrated no significant vessel damage or remodeling. Our experience and others (Gras et al., 2002) is of < 1% of coronary sinus dissection. As the field of cell therapy advances the optimal delivery route needs to be identified. Thus by pioneering this novel technique and addressing the clinical feasibility of this technique, I hoped I would be able to advance the field of regenerative medicine, with the ultimate aim of demonstrating a new effective delivery route.

CHAPTER 2: METHODS

2.1 AMEROID MODEL

a. Location of animal studies

All animal work was conducted at the Institute of Medical Technology, Magdeburg, German (Figure 5) according to the Guide for the Care and Use of Laboratory Animals (NIH Publication No 85-23, revised 1996). The ethics committee of the state Sachsen-Anhalt R P Halle, Germany granted ethical approval.

b. Anaesthesia and monitoring

The study was undertaken in three stages. All procedures were performed under general anaesthesia. The heart rate, a three lead ECG, oxygen saturation and temperature were continuously monitored (Biopac Systems Inc., Santa Barbara, CA, USA). At each stage animals were sedated with a combination of intramuscular (IM) ketamine hydrochloride 600-1200 mg, xylazine hydrochloride 60-120 mg, and atropine 0.5 mg; doses were calculated on a mg/kg basis. Propofol was given as required (30-140 mg) prior to intubation. Isotonic solution combined with fentanyl (0.5 mg) was given during operative procedures. General anaesthesia was induced with 1.5% isoflurane, combined with nitrous oxide/oxygen (ratio 2:1) (Dräger System Iris, Amemone and Romulus 800V Drägerwerk, AG Lübeck Germany). Each animal received (IM) injections of carprofen (100-150 mg), benzylpenicillin (1000 mg) and dihydrostreptomycinesulphate (1250 mg) prior to operative procedures and for two days post operatively.

c. Ameroid placement

A left lateral thoracotomy was performed through the fourth or fifth intercostal space. The pericardium was opened and an ameroid constrictor (2.00, 2.25, 2.50 mm inner diameter, Research Instruments, Escondido, USA) was placed around the LCX in the most proximal position possible (Figure1). The surgeon, based on visual assessment of the outer vessel diameter, chose the ameroid size. The position of the ameroid was described as 'proximal' if placed between the bifurcation and the atrial branch, 'intermediate' if placed distal to the atrial branch and proximal to the first marginal branch of the LCX and as 'distal' if placed distal to the first marginal branch. After

finding a stable position for the constrictor, the pericardium and muscle layers were re-adapted and closed. The ribs were re-opposed and the skin was closed with staples.

d. Transthoracic echocardiography

A single operator performed all echocardiography at rest. Standard two-dimensional and M mode transthoracic images were used (750 Sonotron, 3.25 MHz transthoracic transducer, VINGMED, Horten, Norway). From a right parasternal approach, short axis, mid papillary views were obtained. All data were stored on VHS videotape and analysis of the data was performed off-line in a blinded fashion. Regional myocardial function was assessed using fractional systolic thickening of the posterolateral wall [$Th = (\text{systolic-diastolic thickness} / \text{diastolic thickness}) \times 100$]. Global cardiac function was assessed using fractional LV shortening calculated as [$FS = (\text{diastolic LV diameter} - \text{systolic LV diameter}) / \text{diastolic diameter} \times 100$].

e. Cardiac catheterisation: Screening and entry criteria

Three weeks after ameroid placement animals were placed in a supine position and a 9F arterial sheath was inserted into a femoral artery. This arterial access was used for pressure monitoring, insertion of the cardiac catheters, and blood sampling. Left heart catheterisation was performed by standard techniques, (Philips Angio Diagnost 2U Optimus M200, Holland) and catheters (e.g. Judkins right coronary catheters). After administration of a heparin bolus (100U/kg), angiograms of the left and right coronary arteries were obtained. The flow in the LCX coronary artery was graded according to the TIMI criteria (1988): TIMI 0, no flow within the native vessel; 1, faint, slow filling of the native vessel, without opacification of the distal vessel; 2, slow filling of the entire vessel length and 3, brisk, normal flow within the entire vessel. Ipsi- and contralateral collateral flow was assessed using the Rentrop score (Rentrop et al., 1985): 0, no visible collateral vessels; 1, faint filling of side branches of the main epicardial vessel; 2, partial filling of the main epicardial vessel and 3, complete filling of the main vessel. The highest score (ipsi- or contralateral) was recorded. Animals meeting one or more of the following inclusion criteria were selected for measurements of myocardial blood flow:

1. The LCX was totally occluded
 2. The LCX had a 95% flow limiting stenosis and a ($TIMI \leq 2$ flow).
 3. There was evidence of right-to-left (contralateral) collaterals (Rentrop class ≥ 1).
- Animals not fulfilling these criteria were terminated with

administration of pentobarbital (1600-3400 mg). All angiograms were recorded digitally and stored on CD. An experienced angiographer in a blinded fashion performed analysis of the angiographic data 'off-line'.

f. Assessment of myocardial blood flow

A dedicated angiography catheter was advanced into the left atrium (LA). Pressure recordings within the LA and a test injection of contrast dye verified correct positioning. Reference arterial blood was withdrawn for two minutes at a constant speed of 5 ml/min from the femoral access by a blood withdrawal pump (Harvard Apparatus, Bedford, MA, USA). This was commenced 10 seconds before 4 ml of microspheres (10^6 , per ml 15 μ m diameter Biopal, Worcester, USA) were injected into the left atrium over a period of 30 seconds at rest and under stress. Stress was defined by a dobutamine continuous infusion (6 to 64 μ g/kg) sufficient to increase resting heart rate by 100%. Samarium and gold labelled microspheres were administered at rest and stress prior to gene delivery, whilst three weeks after gene delivery lutetium and iridium labelled microspheres were used. Following an overdose of pentobarbital, the heart was harvested and cut into transverse sections of approximately 1 cm thickness, from the apex to the base the mid-papillary myocardial ring was subdivided into 15 segments (Figure 9). Tissue samples were washed in Sansaline (Biopal, Worcester, USA), weighed and placed in sodium and contamination-free tubes (Biopal Worcester USA). Blood and tissues samples were dried in an oven at 70 centigrade for twenty four hours and then sent to a core laboratory for neutron activation (Biopal, Worcester USA) (Reinhardt et al., 2001). Values are expressed as the number of disintegration per minute (dpm). To calculate the blood flow per unit gram of tissue I used: (tissue (dpm) / tissue weight) x blood volume/ blood sample (dpm) expressed as ml/min/g.

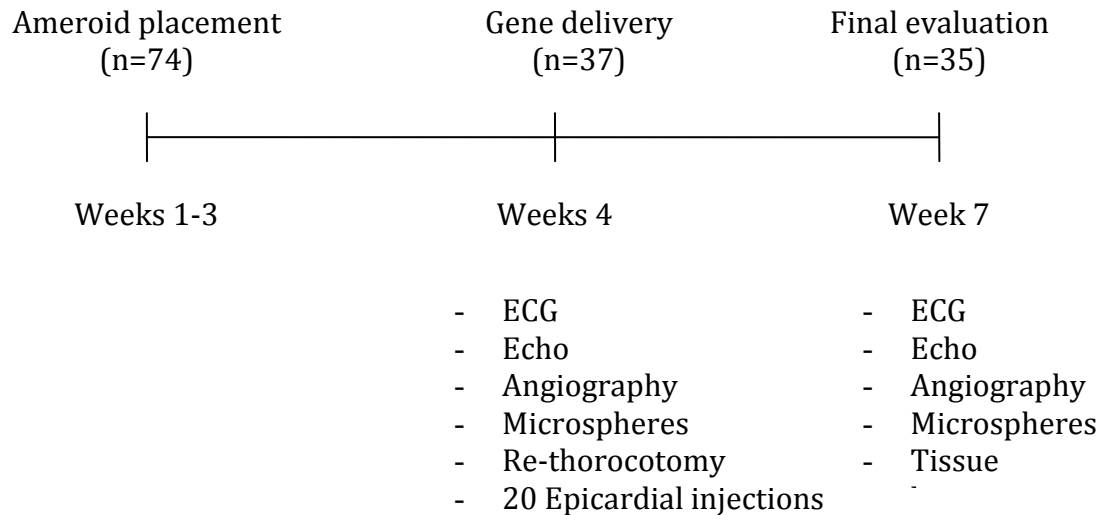
g. Analysis of ischaemia

Absolute regional myocardial blood flow (MBF) was calculated for each segment from dpm measurements corrected for tissue weight as follows: MBF (ml/min/g tissue) = [withdrawal rate (ml/min)/ tissue sample weight (g)] x [dpm (tissue sample)/ dpm (blood sample)]. The segments with the lowest absolute MBF in the lateral wall were identified (ischaemic area) and the blood flow in the ischaemic area was

compared to the segments with the highest MBF (acting as control area). The level of ischaemia was expressed as: $\text{MBF ischaemic segments} / \text{MBF control segments} \times 100$.

2.2 EPICARDIAL DELIVERY OF A “MASTER SWITCH GENE” HIF-1 α SAFETY AND EFFICACY IN A PORCINE MODEL OF CHRONIC MYOCARDIAL ISCHAEMIA

EPICARDIAL HIF-1 α PROTOCOL



Animals were assessed for entry into the study three weeks post ameroid implementation. If all inclusion criteria were satisfied they were randomised to receive gene (MID STUDY). Three weeks post gene delivery they underwent (FINAL STUDY).

a. Randomisation and gene delivery

Pigs meeting the entry criteria outlined in (2.1e) were randomised in a blinded fashion to receive either:

- Plasmid (pHIF-1 α NF κ B 500 μ g/animal)
- Plasmid (pHIF-1 α NF κ B 2500 μ g/animal)
- Adenovirus (Ad2/HIF-1 α VP16 10¹⁰ particles)
- Adenovirus empty vector control (Ad2/CMV-EV 10¹⁰ particles)

Randomisation was performed by telephone to Genzyme USA who gave me a code corresponding to one of the four groups. Following randomisation a resting transthoracic echocardiogram was performed to assess left ventricular function. Regional myocardial blood flow measurements were undertaken with stable isotope-labelled microspheres at rest and during stress. A re-thoracotomy was then performed in the sixth to seventh space. The LCX territory was identified and 20 injections

(each, 50 µl) of randomised substance were injected evenly into a 6.25 cm² (2.5 cm x 2.5 cm) area with a 29-G needle to a depth of 5 mm. Two 5.0 prolene stitches were applied to the epicardial fat at the transverse boundaries of the injected area, to assist with subsequent identification during tissue harvesting.

b. Evaluation of toxicity and efficacy

Three weeks after gene delivery repeat echocardiography, angiography and microsphere studies were performed. Animals were then terminated under anaesthesia (pentobarbital 1600- 3400 mg), the heart excised and the target area identified using the stitches placed in the epicardial fat. This area was then scored with a blade on the epicardial surface and the heart cut into five transverse sections from apex to base (1 to 5). The middle ring, anatomically located at the mid-papillary level was selected for determination of myocardial blood flow. The fourth ring was used for protein and immunohistochemistry assays.

Figure 6: Epicardial Delivery

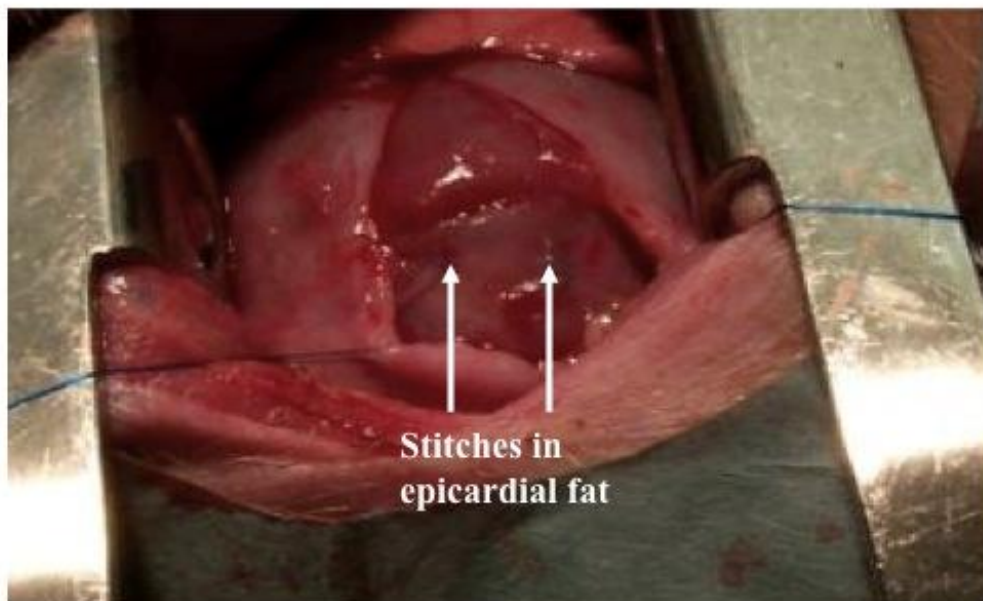


Figure 6: A stopper was applied to the syringe to maintain the correct length. On completion of injections two small stitches were placed in the epicardial fat prior to closing the pericardium to enable location of injection site after termination.

c. Vectors

Ad2/HIF-1 α /VP16 is a recombinant replication-deficient adenovirus in which the virus backbone is E1-, E3+, E4- (but retaining E4/ORF6) and contains the pIX gene insertion in the E4 region (Armentano et al., 1995). The deleted E1 region has been replaced with an expression cassette encoding the chimeric cDNA for HIF-1 α /VP16 (Vincent et al., 2000). Ad2/CMVEV is similar to Ad2/HIF-1 α /VP16 except that it lacks a transgene. A second constitutively active version of HIF-1 α contains amino acids 1-390 of HIF-1 α fused to amino acids 350 to 550 of the human NF κ B p65 subunit.

i. Production of Recombinant plasmid

The full-length (amino acid 1 to 826) HIF-1 α gene was isolated by polymerase chain reaction from a HeLa cell DNA Library. Gene expression was controlled by the cytomegalovirus immediate early enhancer/promoter. The HIF-1 α / VP16 hybrid was constructed by truncating HIF-1 α at amino acid 390 and then joining the transaction domain HSV VP16 downstream (Vincent et al., 2000).

ii. Production of Ad2/ HIF-1 α /VP16

Ad2HIF is a replication-deficient adenovirus of serotype two where the gene insert consists of the DNA-binding and dimerization domains of the HIF-1 α subunit combined with the VP16 transactivation domain from herpes simplex virus. A cytomegalovirus promoter and the polyA signal from SV40 controlled gene expression. Production of the material was initiated by infection of primary human embryonic kidney 293 cells with Ad2HIF. These cells were harvested, followed by cell lyses by DNA digestion and filtration. Purification was achieved using column chromatography and filtration to concentrate vector concentration and final sterile filtration and final dilution in phosphate-buffered saline in 10% sucrose (Kilian et al., 2010).

d. Histological analysis of myocardium

A three mm thick transmural section was removed from the fourth transverse ring (Figure 7) fixed in 4% paraformaldehyde and placed in phosphate buffered saline (PBS) prior to fixation in a paraffin block. Three 5 µm sections, separated by 50 µm intervals, were stained with haematoxylin and eosin (HE) to assess morphology and inflammation. The proportion of the myocardium showing inflammation was assessed by point counting on HE sections using a (Olympus BH-2 Japan) light microscope, fitted with an eyepiece graticule containing 100 crosses (x 10 magnification) (Aherne and Dunnill, 1982). Jie Zhu a consultant histopathologist undertook this well established staining technique and cutting of the tissues blocks.

e. Histological analysis of non-target organs

Samples from the liver, heart, spleen, lung, and kidneys were placed in 10% formalin and processed as above to assess potential toxicity.

f. Immunohistochemistry

Three 5 µm sections, separated by 50 µm intervals, were immunostained using mouse anti-human Von Willebrand Factor (Dako M0616) and anti-human CD31 (Dako M0823) monoclonal antibodies for identification of vascular endothelial cells. The EnVision™ plus horseradish peroxidase kit (HRP) was used to enhance the immuno signal (Sabattini et al., 1998). HRP was detected using diaminobenzidine (DAB) positive labelling of endothelial cells demonstrated by a dark brown pigment. To minimise sampling errors the cumulative mean was established as ten (Figure 8) and the number of capillaries was assessed using a squared eyepiece graticule to avoid repeat counts (x 200 magnification). The data were expressed as number of capillaries / field. Jie Zhu a consultant histopathologist performed all the staining and cutting of tissue blocks and I undertook all the counting with her guidance.

Figure 7: Porcine Tissue Preparation

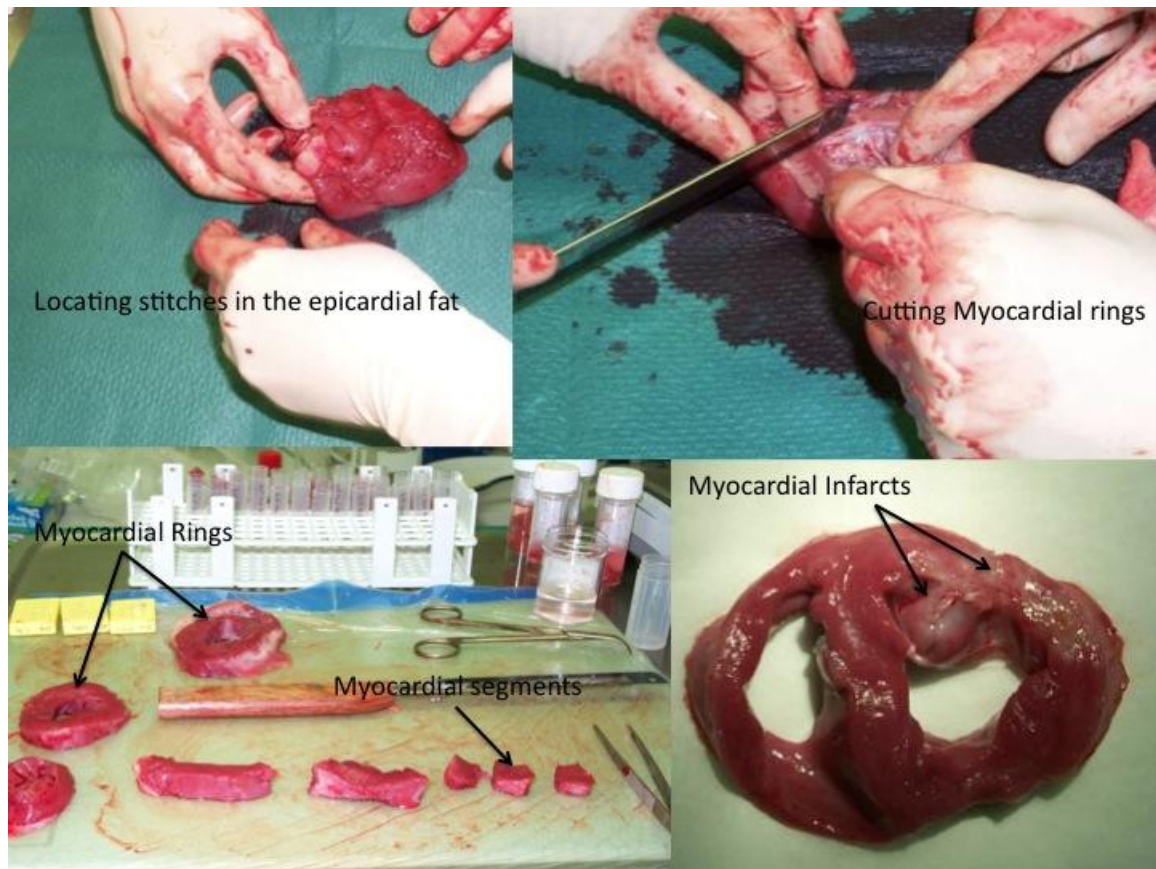


Figure 7: (Top left) after the heart was removed and the stitches were located in the epicardial fat their position was scored to define the target zone where the injections were placed. The heart was then cut into transverse sections (top right). (Bottom left) how the heart was divided for microsphere analysis. (Bottom right) the pale areas pointed to with arrows shows myocardial infarction resulting from ameroid placement.

Figure 8: Cumulative Point count

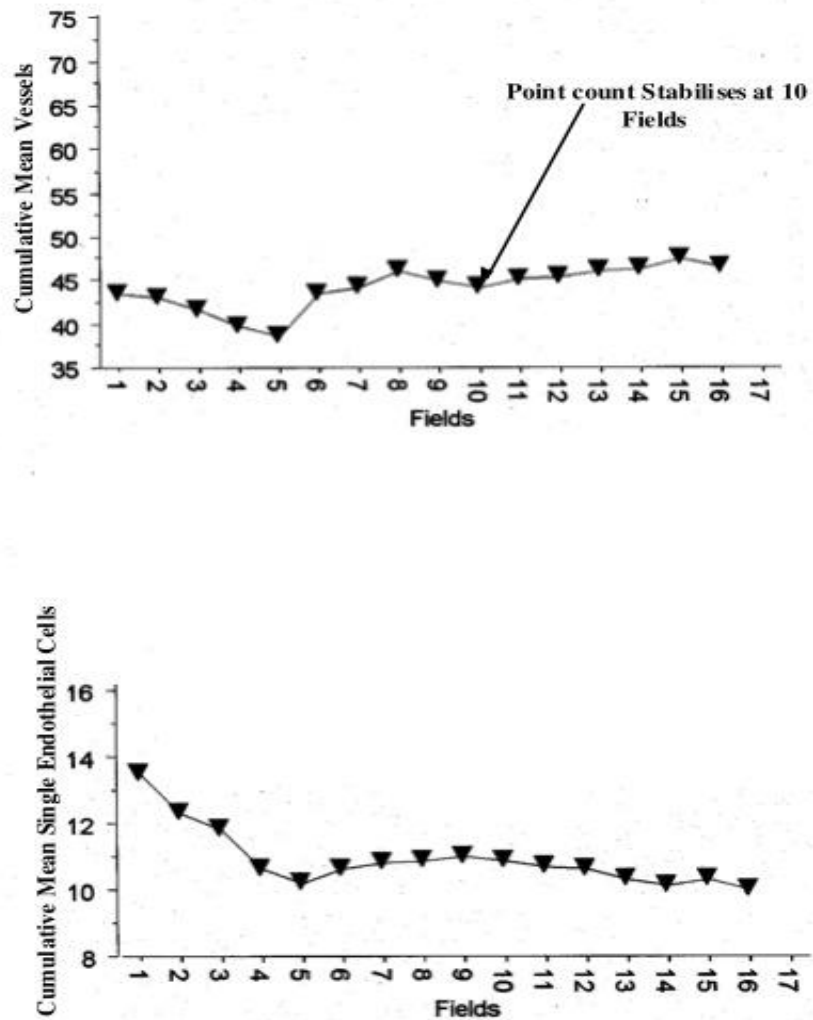


Figure 8: The cumulative point count was calculated above for vessels and below for endothelial cells and found to stabilise at 10 for vessels and endothelial cells.

g. Western blot protein estimation

Epicardial and endocardial surfaces were removed from the target zone segment, so that only the mid- myocardial area was analysed. All samples for protein analysis were snap frozen and stored in liquid nitrogen until analysis by standard techniques (Peterson, 1977). As a positive control 100 µg of E.coli- derived recombinant human VEGF (Autogen Bioclear, Wiltshire UK) was applied (Figure 15).

2.3 ENDOCARDIAL DELIVERY OF HIF-1 α - SAFETY AND EFFICACY IN A PORCINE MODEL OF CHRONIC MYOCARDIAL ISCHAEMIA.

a. Background

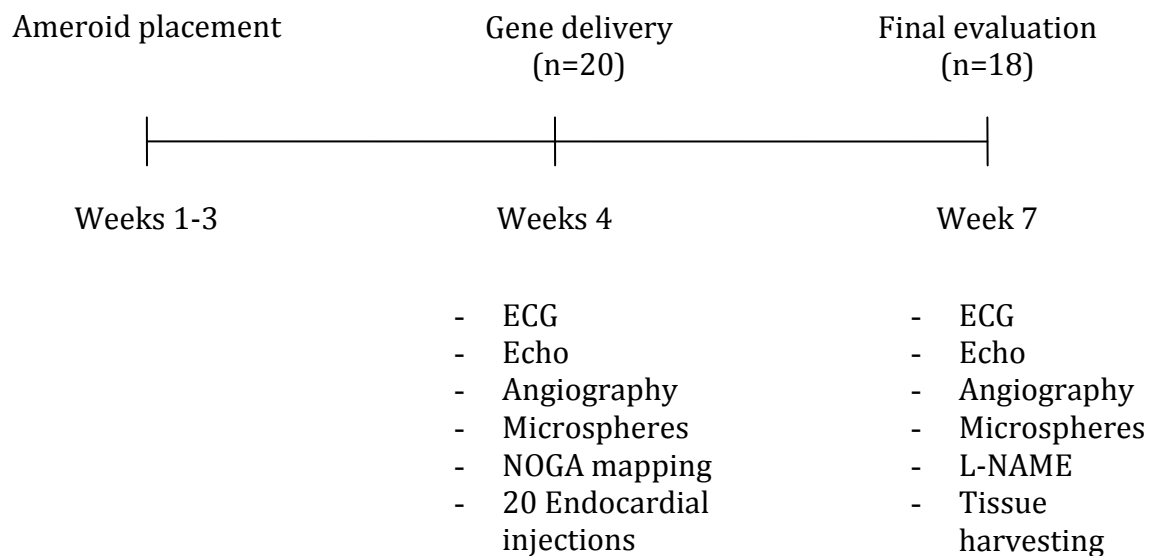
The previous epicardial delivery of AdHIF-1 α /VP16 gene in a porcine model of myocardial ischaemia established the safety, efficacy and the optimal construct and dose to take forward for further studies. The focus of design of the next phase of pre-clinical studies was to mimic the clinical situation (Shyu et al., 2002); (Vale et al., 2000) where less invasive percutaneous delivery strategies, not requiring general anaesthesia or a thoracotomy were desirable. A magnetic guidance percutaneous navigational system (NOGA system, Biosense Webster, USA) had already been shown to effectively transfect designated myocardial sites and had recently been used in a clinical angiogenesis gene therapy trial (Gyongyosi et al., 2005). The aim of this study was, therefore, to evaluate the safety and efficacy of percutaneous, intramyocardial AdHIF-1 α /VP16 gene delivery by the use of a percutaneous catheter system in a porcine model of chronic myocardial ischaemia.

Experience from the epicardial study had shown that it was not possible to detect changes in vessel number with the use of immunohistochemistry. I was also reassured to see there did not appear to be an increased level of local inflammation with the use of an adenoviral vector. Percutaneous delivery even with the assistance of NOGA mapping and fluoroscopic guidance is not as reliable at targeting a pre-specified area compared with directly visualised epicardial injections. I was also unable to mark the injected zone as before with stitches in the epicardial fat. I therefore decided to sacrifice collecting one myocardial ring for immunohistochemistry and histology in favour of collecting two rings for my primary outcome measure of myocardial blood flow. By doing this I hoped that I would minimise any sampling area.

The protocol and methodology for the endocardial delivery study in essence mirrored that of the epicardial study and is outlined below. However aside from the different delivery route there were a few additional aspects to this study. In order to evaluate whether, or to what extent, changes in vessel endothelial function could also contribute to an improvement in perfusion as a result of angiogenic therapy, I

performed an additional set of myocardial blood flow measurements using nitro-L-arginine methyl ester hydrochloride (L-NAME), an irreversible inhibitor of nitric oxide synthase (NOS). All animals on completion of this study also underwent a full necropsy in order to achieve a comprehensive toxicology assessment as this was designed as a direct prelude to a clinical trial.

ENDOCARDIAL HIF-1 α PROTOCOL



Animals were assessed for entry into the study three weeks post ameroid implementation. If all inclusion criteria were satisfied they were randomised to receive gene (MID STUDY). Three weeks post gene delivery they underwent (FINAL STUDY)

b. Experimental model and vector

A total of 20 animals with chronic myocardial ischaemia of the left lateral wall received Ad2/HIF-1 α VP16 (therapeutic gene, 10^{10} particles, 20 injections of 50 μ l, n= 10 animals) or Ad2/CMV-EV (empty vector, 10^{10} particles, 20 injections of 50 μ l, n= 10 animals) in a pre-specified area of the left lateral wall in a randomised, blinded, placebo controlled fashion using an electromechanical navigation and injection system. As for the epicardial study the active Ad2/HIF-1 α /VP16 and an Ad2/CMVEV that was similar to Ad2/HIF-1 α /VP16 but lacking a transgene were utilised as the control.

c. Direct intramyocardial gene delivery using a percutaneous non-fluoroscopic mapping and injection system

After completion of the myocardial blood flow measurements, direct intramyocardial gene delivery was accomplished by the use of an electromechanical mapping and injection system connected to a NOGA console (Biosense-Webster, Johnson and Johnson, CA, USA). An endocardial electromechanical map of the left ventricle was obtained as previously described (Vale et al., 2000) (Figure 3). Three low emitting magnetic pads were placed under the pig, a magnetic tip on the end of the deflectable myostar catheter then made contact with a series of points on the endocardial surface. By using electromagnetic triangulation, an accurate on-line localisation of the tip of the catheter within the ventricle was established. As the mapping catheter made sequential contact with a number of points on the endocardial surface local unipolar electrograms were generated along with tip motion during the cardiac cycle establishing local wall shortening. A three-dimensional map was then constructed with this information that could be rotated freely in space to view all areas of normal ischaemic and infarcted tissue. After completion of the LV map, the mapping catheter was replaced by an injection catheter, which incorporated a retractable 27G needle at the distal tip. A total of 20 injections (50 μ l volume each) were directed into the ischaemic left lateral wall aiming to target a 2.5 cm x 2.5 cm area. Injections were graded as successful when the catheter tip movement during the cardiac cycle (loop stability) was less than 2 mm and the tip was perpendicular to the endocardial surface as defined by Biosense guidance and fluoroscopy. Penetration into the LV wall was established by fluoroscopy and premature ventricular beats recorded on ECG during advancement of the needle. The needle length was adjusted to 5 mm depending on the left lateral wall thickness as assessed by transthoracic echocardiography. Injections were performed in a randomised, blinded; placebo-controlled fashion after a randomisation code which was received by telephone by Genzyme USA who had supplied all the constructs. The two groups were:

- Ad2/HIF-1 α VP16, therapeutic gene, 10^{10} particles,
- Ad2/CMV-EV (empty vector), 10^{10} particles.

d. Assessment of endothelial function

Animals were allowed to recover for 15 minutes after final MBF measurements under dobutamine infusion. Thereafter, 10 mg/kg L-NAME (Sigma-Aldrich, Dorset, UK) in saline was administered through the ear vein. Ten minutes after the L-NAME bolus, another set of microspheres was injected into the left atrium under resting and under pharmacological stress conditions. Haemodynamic parameters heart rate (HR), mean blood pressure (MBP) and left atrial pressure (LAP) were monitored and recorded throughout the procedure. After completion of the L-NAME measurements, animals were sacrificed. Administration of L-NAME has a profound effect on arterial blood pressure. To distinguish between effects of L-NAME on the systemic and the coronary vasculature, the coronary vascular resistance was determined by means of the following formula: Resistance (mmHg/ml/min/g) = mean arterial pressure/ myocardial blood flow. Changes in resistance as a result of L-NAME administration were calculated for each group and compared between the treatment groups.

e. Measurements of regional myocardial blood flow

After sacrifice, the heart was harvested and two complete transverse mid-myocardial sections of the left ventricle at two different levels (basal and apical) were obtained and divided into 26 separate segments. Based on the segmental model, myocardial areas were defined for a complete and systematic evaluation of myocardial blood flow. Calculations of MBF were performed separately for the following areas:

- Target area (segments 4 to 9);
- Target center area (segments 5 to 7);
- Anterior wall (segments 1 and 2);
- Septum (segments 10 to 13).

Analysis of the tissue and blood vials using neutron activation and calculations of myocardial blood flow were performed in a blinded fashion.

f. Safety evaluation: Pathology examination and blood analysis

All animals receiving intramyocardial gene delivery (test article or control) underwent a complete necropsy by a pathologist blinded to the treatment modality. Initially, all

macroscopic findings were recorded. Representative samples of all organs/ tissues were dissected free and fixed in 10% neutral buffered formalin for histology examination. Blood samples were taken at mid-studies (before gene delivery) and at final studies in all animals. The following serum parameters were analysed using standard techniques: white and red blood cell count, aspartate- amino- transferase (ASAT), C-reactive protein (CRP), Troponin I (TrI).

g. Statistics

i. Evaluation of the porcine ameroid constrictor model of myocardial ischaemia

Data were tested for normality of distribution and expressed as mean $\pm$ SEM. Comparison of means between groups was performed using the appropriate Students t-test (paired or unpaired) for continuous variables and the chi-square test for categorical variables. In order to examine the relation between cardiac performance and haemodynamic measurements or myocardial blood flow, correlation coefficients were calculated. The relation between the categorical Rentrop score and other outcome variables (myocardial blood flow, echocardiography) were assessed using a one-way analysis of variance. The null hypothesis was rejected at $p < 0.05$.

ii. Epicardial delivery of a “master switch gene” HIF-1 α - Safety and Efficacy in a porcine model of chronic myocardial ischaemia

For sample size consideration we used a previously published study (Sato et al., 2001) to calculate that n numbers of eight could detect an angiogenic effect. Samples were coded and all data analysed in a blinded fashion, using SPSS version ten. All data are presented as mean $\pm$ SEM. Group means were compared with ANOVA and post hoc Bonferroni correction; all tests were two-sided. The null hypothesis was rejected at $p < 0.05$.

iii. Endocardial delivery of HIF-1 α - Safety and Efficacy in a porcine model of chronic myocardial ischaemia

All data are expressed as mean $\pm$ SEM unless indicated otherwise. Baseline characteristics of the study animals in the two treatment groups were compared using the Mann-Whitney U test for continuous variables and the Fisher's exact test for categorical variables. Myocardial blood flow in the four specified myocardial areas

and the level of ischaemia for the target area were assessed within groups and compared between treatment groups using the Mann-Whitney U statistical test. The null hypothesis was rejected at $p < 0.05$.

CHAPTER 3: EVALUATION OF THE PORCINE AMEROID CONSTRICTOR MODEL OF MYOCARDIAL ISCHAEMIA

3.1 INTRODUCTION

The porcine ameroid model of chronic myocardial ischaemia has been widely used for the evaluation of biochemical, morphological and functional characteristics of the development of coronary collateralisation (White et al., 1992); (Roth et al., 1987). This model has been chosen for preclinical testing of angiogenic proteins and genes in the treatment of myocardial ischaemia (therapeutic angiogenesis) (Harada et al., 1996); (Lopez et al., 1998b); (Mack et al., 1998); (Tio et al., 1999).

Placement of an ameroid occluder device around a coronary artery, typically the left circumflex coronary artery (LCX) does not always result in complete vessel occlusion. Some groups have used the model without specifically determining the degree of stenosis or ischaemia (Mack et al., 1998), whereas others have only used animals with total vessel occlusion (Harada et al., 1996); (Lopez et al., 1998b). Clearly, it would be desirable to predict the presence and degree of myocardial ischaemia, especially in animals without total occlusion of the LCX to prevent non-ischaemic animals being used and to reduce the total number of instrumented animals. Further, detailed knowledge of the regional distribution and variability of myocardial blood flow as an outcome measure, as well as the relationship with morphological (coronary angiography), functional (echocardiography), and haemodynamic measurements is essential for designing study protocols and interpreting experimental results.

Retrospective analysis of a large series of study animals undergoing ameroid placement aims to provide a detailed reference for the porcine model of chronic myocardial ischaemia in the context of therapeutic angiogenesis. 132 domestic pigs (aged three months, 25 ± 1 kg, Agrargenossenschaft Börde, Germany) were scheduled for ameroid placement. These animals were part of two large angiogenesis studies and all available data from the initial experimental stage (ameroid placement) to a baseline evaluation 26 ± 1 (mean $\pm$ SEM) days later were used.

3.2 RESULTS

a. Outcome after ameroid placement

Of the 132 domestic pigs, two animals died during the procedure (cardiac arrest immediately after intubation; cardiac arrest while opening the pericardium) and the remaining 130 animals underwent successful ameroid placement (ameroid size used: 2.0 mm: 26%, 2.25 mm: 62%, 2.5 mm: 12%; mean diameter: 2.21 ± 0.01 mm). A 'proximal' position of the ameroid could be achieved in 104/130 (80%) animals; an 'intermediate' position in 20/130 (15%) and a 'distal' position was necessary in 6/130 (5%) cases. A total of 33/130 (25%) animals died 6 ± 1 days post ameroid placement (range two hours to 28 days), likely from arrhythmias subsequent to abrupt vessel occlusion or coronary spasm. The animal weight/ameroid diameter ratio (WADR) of those animals with sudden deaths was significantly higher than the WADR from surviving animals (11.94 ± 0.25 vs. 11.07 ± 0.20 kg/mm ameroid, $p = 0.025$). There was no difference regarding the frequency of a proximal ameroid position between groups. Three study animals had to be terminated due to clinical signs of severe heart failure, wound infection and severe weight loss, respectively. Therefore, 94/130 (72%) animals reached baseline evaluation 26 ± 1 days post ameroid placement including ECG, transthoracic echocardiography and angiography.

b. Coronary angiography

Of the surviving 94 animals undergoing coronary angiography, 61/94 (65%) animals (Table 5) fulfilled the angiographic inclusion criteria. Of these, 34/61 (56%) showed a complete occlusion of the LCX target vessel. Thus, the percentage of animals scheduled for initial ameroid placement that showed total LCX occlusion was 26% (34/132). A comparison of morphological, haemodynamic and functional baseline characteristics of those animals with total LCX occlusion, and those without, is provided in (Table 6). The WADR was significantly higher in those animals developing a complete LCX occlusion (12.4 ± 0.3 kg/mm vs. 9.7 ± 0.2 kg/mm, $p = 0.0001$). Despite significant ($p < 0.001$) differences in thrombolysis in myocardial infarction (TIMI) grading of antegrade flow in the target vessel, baseline functional and myocardial blood flow measurements including the level of ischaemia were not different between both groups under pharmacological stress conditions. At rest, however, there was a significant ($p < 0.05$) difference in the level of ischaemia.

c. Distribution of myocardial blood flow

Under resting conditions, myocardial blood flow (MBF) was not different between myocardial segments. Pharmacological stress led to a significant increase in MBF in all myocardial segments (Table 5). This increase in MBF, however, was more pronounced in the anterior wall (Figure 9) (segments 1 to 3) as compared to the lateral wall (segments 7 to 10) ($461 \pm 29\%$ vs. $370 \pm 23\%$, $p < 0.05$). As a result, absolute myocardial blood flow was significantly higher in the anterior wall (segments 1 to 3) as compared to a large section of the lateral wall (segments 7 to 10) (2.66 ± 0.12 vs. 2.05 ± 0.09 ml/min/g, $p = 0.0001$). The level of ischaemia in the latter region was $79 \pm 2\%$ under stress conditions indicating an approximately 20% reduction in MBF as a result of ameroid placement. The anterolateral (segments 4 to 6, 2.37 ± 0.12 ml/min/g) and posterolateral (segments 11 and 12, 2.30 ± 0.11 ml/min/g) myocardial areas were characterised by intermediate MBF values with a level of ischaemia of $90 \pm 2\%$ and $89 \pm 3\%$, respectively, indicating an approximately 10% reduction of MBF under stress conditions. In 35/61 animals, epicardial and endocardial sections of the left lateral wall were analysed separately. In those animals, a difference in the ratio between epicardial/endocardial MBF (0.84 ± 0.04 vs. 1.19 ± 0.13 , $p < 0.05$) was observed.

d. Correlation of different outcome parameters

Neither left lateral wall fractional systolic thickening or left ventricular systolic shortening measured by echocardiography correlated with myocardial blood flow, level of ischaemia, heart rate or mean arterial pressure. The Rentrop score was used to quantify the degree of coronary collateralisation. Analysis of variance, however, could not detect any relationship between Rentrop score and LV function or myocardial blood flow assessed by microspheres (Table 7).

Figure 9: Myocardial Blood Flow Assessment

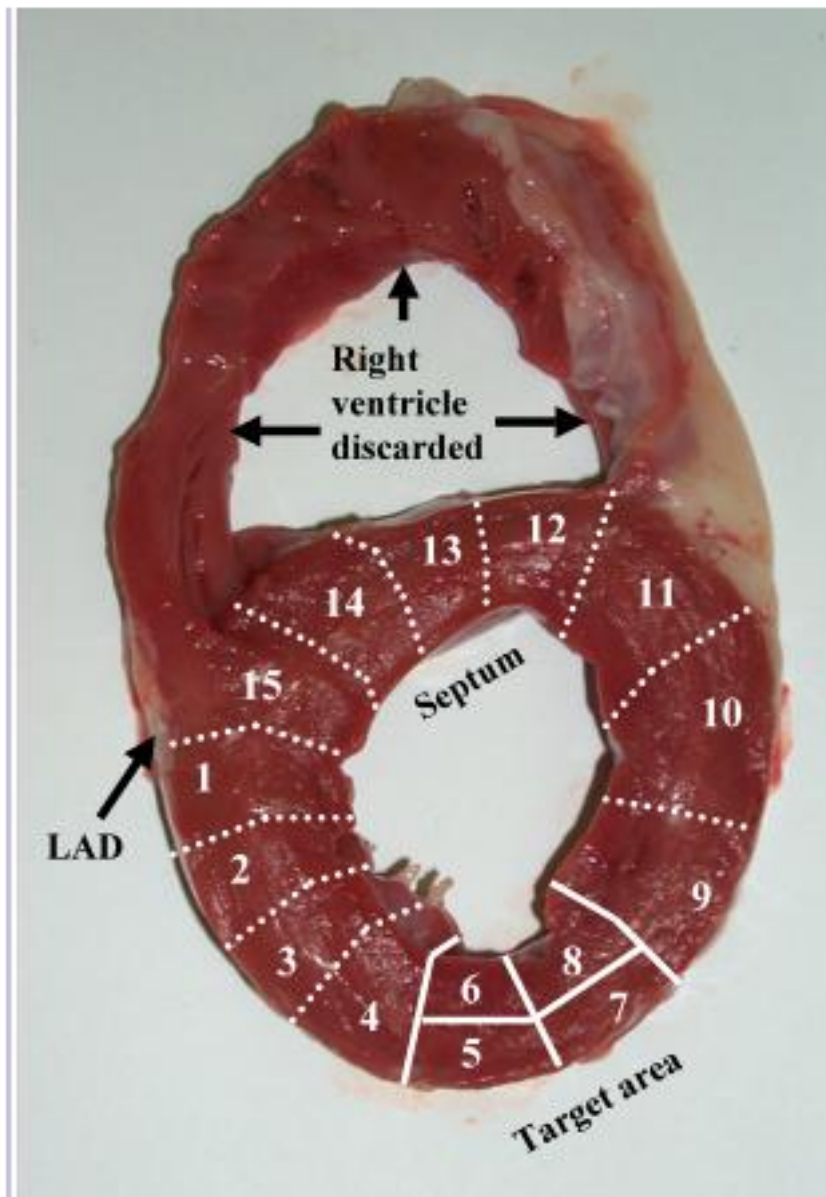


Figure 9: Myocardial Ring with 15 myocardial segments used for systematic microsphere analysis in a transverse mid-papillary section of the left ventricle. The left anterior descending artery (LAD) and the posterior insertion of the right ventricle were used as landmark structures. In 35/61 animals, the left lateral wall was further divided into epicardial and endocardial sections and analysed separately.

Table 5: Summary of angiographic, haemodynamic, echocardiographic and myocardial blood flow measurements

	All study animals (n=61)	
	Rest	Stress
Coronary angiography		
TIMI flow LCX	1.2 ± 0.1	-
Rentrop score	1.0 ± 0.1	-
Haemodynamics (rest/ stress)		
Heart rate (bpm)	85 ± 1	158 ± 2*
Mean arterial pressure (mm Hg)	75 ± 2	142 ± 4*
Echocardiography		
Fractional thickening lateral wall	34 ± 3	-
Fractional shortening left ventricle	22 ± 1	-
Myocardial blood flow (ml/min/g)		
Control area (segments 1-3)	0.53 ± 0.03	2.66 ± 0.12*
Ischaemic area (segments 7-10)	0.50 ± 0.03	2.05 ± 0.09*
Antero-lateral (segments 4-6)	0.50 ± 0.03	2.37 ± 0.12*
Postero-lateral (segments 11-12)	0.48 ± 0.03	2.30 ± 0.11*
Level ischaemia (%)	96 ± 4	79 ± 2 [#]
Ischaemia present (% animals)	61	87 [#]

Table 5: Summary of angiographic, haemodynamic, echocardiographic and myocardial blood flow measurements of 61 animals fulfilling defined angiographic inclusion criteria after LCX ameroid placement.

Bpm, beats per minute; LCX, left circumflex coronary artery; %, percent; ml, milliliters; min, minute; g, gram; TIMI, thrombosis in myocardial infarction

[#] p < 0.01 as compared to rest, * p < 0.00001 as compared to rest.

Table 6: Baseline comparison of morphological, haemodynamic and functional characteristics of animals with and without LCX occlusion

	No Occlusion LCX (n=27)		Occlusion LCX (n=34)	
	Rest	Stress	Rest	Stress
Coronary angiography				
TIMI flow LCX	1.9 ± 0.1	-	0.6 ± 0.2*	-
Rentrop score	0.9 ± 0.2	-	1.1±0.2	-
Haemodynamics (rest/ stress)				
Heart rate (bpm)	88 ± 2	157 ± 3	83 ± 2	159 ± 3
Mean arterial pressure (mmHg)	74 ± 3	138 ± 5	76 ± 4	144 ± 7
Echocardiography				
Fractional thickening lateral wall	31 ± 5	-	36 ± 4	-
Fractional shortening left ventricle	22 ± 1	-	22 ± 1	-
Myocardial blood flow (ml/min/g)				
Control area (segments 1-3)	0.54 ± 0.04	2.78 ± 0.20	0.52 ± 0.04	2.57 ± 0.16
Ischaemic area (segments 7-10)	0.56 ± 0.05	2.22 ± 0.15	0.45 ± 0.04	1.92 ± 0.12
Antero-lateral (segments 4-6)	0.52 ± 0.04	2.41 ± 0.17	0.48 ± 0.04	2.33 ± 0.18
Postero-lateral (segments 11-12)	0.53 ± 0.04	2.45 ± 0.16	0.44 ± 0.04	2.18 ± 0.14
Level ischaemia (%)	106 ± 8	83 ± 4	88 ± 5 [#]	76 ± 3
Ischaemia present (% animals)	52	81	68	91

Table 6: Comparison of morphological, haemodynamic and functional baseline characteristics of animals with total LCX occlusion as compared to non-occluders three weeks after ameroid constrictor placement around the LCX.

Bpm, beats per minute; LCX, left circumflex coronary artery; %, percent; ml, milliliters; min, minute; g, gram; TIMI, thrombosis in myocardial infarction

[#] p < 0.05 and * p < 0.001 as compared to (no occlusion LCX)

Table 7: Relationship between Rentrop score, Echocardiography, and Myocardial Blood Flow after Ameroid placement

Rentrop collateral grading	0 (n=19)	1 (n=26)	2 and 3 (n=16)	p-value
Echocardiography				
Systolic thickening lateral wall (%)	33 ± 6	30 ± 5	41 ± 6	0.56
Systolic shortening left ventricle (%)	21 ± 2	21 ± 1	24 ± 1	0.25
Myocardial blood flow				
Ischaemic area rest (ml/min/g)	0.55 ± 0.07	0.50 ± 0.06	0.44 ± 0.04	0.60
Level ischaemia rest (%)	99 ± 10	98 ± 5	91 ± 9	0.60
Ischaemic area stress (ml/min/g)	2.12 ± 0.15	2.10 ± 0.16	1.89 ± 0.16	0.56
Level ischaemia stress (%)	85 ± 4	78 ± 4	74 ± 4	0.24

Table 7: Relationship between the angiographic, Rentrop score, echocardiographic parameters or myocardial blood flow parameters in 61 animals after placement of an ameroid constrictor around the LCX.

%, percent; ml, milliliters; min, minute; g, gram

Table 8a: Sample sizes calculated from animals with defined angiographic criteria

Effects size	MBF rest	MBF stress	FT lateral wall	FS left ventricle
10%	426	224	1098	156
20%	118	57	203	41
30%	53	27	101	15
50%	21	11	36	7

Table 8b: Sample sizes calculated from animals with complete LCX occlusion

Effects size	MBF rest	MBF stress	FT lateral wall	FS left ventricle
10%	393	190	567	142
20%	122	48	185	36
30%	51	21	75	12
50%	20	9	29	6

Table 8: Sample size calculation indicating the number of animals necessary per treatment group to detect different effect sizes regarding myocardial blood flow and echocardiographic parameters. Calculations are based on data from all animals fulfilling defined angiographic inclusion criteria (**Table 8a**) or based on data from animals with complete LCX occlusion 3 weeks after LCX ameroid placement (**Table 8b**).

MBF, myocardial blood flow; FS, fractional shortening; FT, fractional thickening

3.3 DISCUSSION:

The porcine ameroid model of chronic myocardial ischaemia is widely considered the 'gold standard' for preclinical testing of angiogenic growth factors. This analysis of morphological, functional, and haemodynamic outcome measurements in a series of 132 animals scheduled for ameroid placement increases the understanding of the characteristics of this preclinical model in the context of therapeutic angiogenesis.

a. Model mortality and occlusion rates

Data on periprocedural mortality has not been published to date. In this study (Radke et al., 2006), ameroid placement was associated with a very low mortality (2%) when performed by an experienced cardiac surgeon.

In those animals reaching baseline evaluation, we observed an overall complete occlusion rate of the target vessel induced by the ameroid of 26%. A significant predictor of occlusion was the animal weight to ameroid size (W/A) ratio. Ideally, the coronary artery diameter to ameroid size ratio should have been measured. However due to the proximal positioning of the ameroid constrictors, the diameter of the LCX target vessel could only be measured in a small minority of animals. The W/A ratio, however, was also associated with the occurrence of sudden (most likely) cardiac death after the ameroid procedure. Data on higher overall occlusion rates have been reported with animal weight to ameroid size ratios up to 16 kg/mm (Fuchs et al., 2001b); (White et al., 1992). Clearly, the risk of sudden cardiac death, however, has to be weighed against the potential benefit of higher occlusion rates.

This study suggests that a total occlusion of the LCX target vessel is not an absolute pre-requisite to establish the pathophysiology of myocardial ischaemia during stress. At rest ischaemia of the lateral wall was present only in those animals with a complete occlusion. This contrasts with previous studies suggesting that with complete target vessel occlusion, induction of stress (physical or pharmacological) is necessary to induce ischaemia (White et al., 1992); (Fuchs et al., 2001b); (Fuchs et al., 2001a). Importantly, however, by identified recognition of a set of angiographic criteria allowing identification of those animals with stress-induced ischaemia in the absence of total LCX occlusion. This should allow for both reduced inclusion of animals

without appropriate ischaemia, and increased inclusion of animals deemed non-ischaemic (because of incomplete LCX occlusion) in future trials.

b. Location and severity of myocardial ischaemia

Constriction/occlusion of the proximal left circumflex coronary artery leads to ischaemia of a large area of the left lateral wall. It is worth noting, however, that even with a thoracotomy in the fifth intercostal space, the most ischaemic myocardial area can barely be reached via an epicardial approach without removal of a rib (sixth rib). Alternatively, epicardial delivery can be performed in an area that is directly exposed after thoracotomy in the fifth intercostal space. This more anterolateral area also shows myocardial ischaemia most often represented by segments five to eight under stress conditions. When percutaneous delivery routes are used, however, the most ischaemic areas were accessible and could be targeted from the endocardial surface.

The absolute myocardial blood flow in the target area is significantly reduced by 20% under conditions of pharmacological stress. Any therapeutic effect, therefore, has to be detected within this relatively small window. Clearly, therefore, the method of stress induction needs to be carefully chosen to minimise variability. Interestingly, a four-fold increase in myocardial blood flow under stress conditions remained; indicating only slightly reduced coronary reserve under those conditions.

A > 75% difference in myocardial blood flow between collateral dependent and control areas, however, has been observed as a result of heavy physical exercise using a treadmill test (Roth et al., 1987). This can be performed without the influence of sedatives or narcotic substances with their potential impact on the cardiovascular system. However, this technique requires sophisticated animal facilities and a learning/familiarisation period for the animals, further increasing the complexity of this large animal model. Alternative techniques to increase oxygen demand and to induce myocardial ischaemia include the application of adenosine or rapid pacing. The effects of adenosine in pigs with an ameroid occluder, however, are not clear. Whereas in some studies adenosine infusion induces significant changes in myocardial blood flow (White et al., 1992), other studies failed to show an effect (Fuchs et al., 2001b). Adenosine induces maximal vasodilatation in the coronary and

the systemic circulation with a profound decrease in arterial blood pressure. The coronary resistance, therefore, should be calculated when adenosine is used in order to normalise for systemic haemodynamic effects. Endocardial pacing leads to a reproducible increase in myocardial oxygen demand and has previously been used for detection of treatment effects of therapeutic angiogenesis (Sato et al., 2001); (Giordano et al., 1996). This technique, however, also requires an additional invasive procedure that further increases model complexity. In this study we therefore selected high-dose dobutamine infusion for detection of myocardial ischaemia because of its ease of use and reproducibility. It is worth noting, however, that after stopping the dobutamine infusion, some ischaemic animals experienced profound hypotension with the need for fluid substitution. As a result, we recommend a 10 to 15 minutes recovery period after stopping pharmacological stress prior to further interventions such as re-thoracotomy for myocardial protein/gene delivery. Although it is thought that occlusion occurs due to external compression of the coronary artery this maybe somewhat over simplistic. Application of an ameroid constrictor causes mechanical trauma that may lead to endothelial damage, platelet aggregation and resultant thrombus formation, and may also be influenced by the experience of the operator. O'Konski and colleagues reported a reduction in infarct size in animals receiving aspirin and in those with less vessel manipulation (O'Konski et al., 1987) .

c. Relationship between morphological, functional and haemodynamic outcome measures

Therapeutic angiogenesis aims to improve myocardial blood flow into an ischaemic area as a result of new vessel formation. To be clinically relevant, these effects should translate into changes in regional or even global myocardial function. In principle, changes over time of most of these parameters can be assessed in the porcine ameroid model of myocardial ischaemia. Myocardial perfusion can be measured using invasive microsphere techniques (Reinhardt et al., 2001) or advanced non-invasive imaging modalities like MRI and positron emission tomography (PET) (Sato et al., 2001); (Pearlman et al., 2000); (Udelson et al., 2000). Cardiac function can be assessed using transthoracic echocardiography or by MRI. In addition, angiography-based scoring systems of collateralisation have been used to detect angiogenic treatment effects over time *in vivo*. As shown in this study (Table 7) and in a previous study by (Fuchs et al., 2001b), angiographic assessment of the collateral circulation

does not correlate with myocardial perfusion or function. Further, collaterals formed in response to arterial occlusion and/or therapeutic angiogenesis often have a diameter of $< 200 \mu\text{m}$ (White et al., 1992) and are thus beyond the resolution of conventional angiography and should therefore not be used as a primary outcome measure in therapeutic angiogenesis trials (Fuchs et al., 2001b). In contrast, in studies that specifically address arteriogenesis by using primarily arteriogenic growth factors, angiographic scores may be relevant.

There was no correlation between cardiac function, assessed by transthoracic echocardiography at rest, or myocardial blood flow assessed by microspheres. Interestingly MRI also could not show a significant correlation between myocardial perfusion (delayed blood arrival) and cardiac function (target wall motion) in the same animal model (Mark J Post, unpublished data). Both observations emphasise the impact of further parameters, such as the size of the ischaemic area, extent of myocardial fibrosis, left ventricular geometry, cardiac synchronisation, cardiac pre- and afterload, or the level of anaesthesia on cardiac performance. This also emphasises the need to assess separately flow and function in preclinical and clinical studies of angiogenesis. Echocardiography as utilised in this study showed certain limitations. Echocardiography was only performed at rest and not at stress (i.e. low-dose dobutamine stress echocardiography). Ischaemia, however, was only present during pharmacological stress. The variability of echocardiographic parameters (especially fractional thickening of the lateral wall) in this study showed a coefficient of variation of 10%. In retrospect this variability may have been less apparent if we had conducted stress echocardiography. However this was omitted from this protocol as an additional stress regime was deemed likely to result in excessive mortality in these ischaemic animals.

d. Use of the porcine ameroid model of myocardial ischaemia for therapeutic angiogenesis studies: sample size considerations

Based on the variability of functional endpoint parameters seen in this large series of animals, an estimate of sample size needed to detect defined therapeutic effects can be calculated (Table 8). With regard to perfusion there is relatively little penalty in widening the inclusion criteria beyond total LCX occlusion. Predictably stress approximately halves the number of animals needed to detect a given difference.

Finally, to detect a 30% therapeutic improvement in flow, approximately 25 animals need to be available for final analysis. Allowing for perioperative mortality, adoption of this angiographic inclusion criterion translates into a starting cohort of approximately 50 animals per treatment group. For LV function, measured by echocardiography, there is again relatively little difference when widening inclusion criteria to these suggested values. The effects of stress are proportionally greater (approximately x 5) than is the case for flow. Finally, the numbers required fall within the group sizes noted for flow when considering fractional shortening, but rise to likely unfavourable levels for fractional thickening. It is unlikely, therefore, that the latter should be considered in such studies. Based on the calculations provided in (Table 8), quantitatively large treatment effects or (in a case of small treatment effects) large numbers of study animals are needed for significant results. These observations have important implications in the present environment of concern regarding the efficient use of large animals in biomedical research. In addition, the costs of using this model may be prohibitive.

e. Comparison with other porcine models of myocardial ischaemia

The porcine ameroid constrictor model of myocardial ischaemia has been extensively studied and most widely used (Hughes et al., 2003). Our present knowledge regarding the development of the coronary collateral circulation derives from studies using the ameroid constrictor model (Roth et al., 1987); (Hughes et al., 2003); (White et al., 1992). Furthermore, a large variety of preclinical studies testing proangiogenic substrates like single growth factors (e.g. VEGF, FGF (Mack et al., 1998); (Tio et al., 1999); (Giordano et al., 1996); (Sato et al., 2001)) or autologous bone marrow (Fuchs et al., 2001a) as well as transmyocardial laser revascularization techniques (Hughes et al., 2002) have preceded clinical trials. Ameroid occluders are usually positioned on the LCX, which is the smallest of the coronary vessels, supplying about 20% of the left ventricular myocardium (Roth et al., 1987). It also demonstrates a larger degree of collateralisation than either the LAD or right coronary artery (White and Bloor, 1981). The major advantage of this model is its simplicity, however different degrees of LCX obstruction/occlusion remain an inherent limitation. (O'Konski et al., 1987) reported an average infarct size of $37 \pm 36\%$ of the area of risk in their initial study and yet in follow up studies this was found to be $5 \pm 1\%$, which they attributed to less

manipulation of the vessel. Many animals show normal myocardial blood flow at rest highlighting the need for stress induced myocardial ischaemia and dysfunction which is similar to that observed with clinical exercise induced ischaemia. Other models include the fixed stenosis model, either by banding the coronary artery of interest during surgery (e.g. by a silk tie) or by percutaneous implantation of an obstructing stent (White and Bloor, 1981). The fixed stenosis model has not yet been used for the assessment of angiogenic therapies and is therefore not validated for this indication. This model also results in an acute myocardial infarction rather than a model of stress induced ischaemia. The more uniform degree of flow reduction may, however, be advantageous as compared to the larger inter-animal variability in myocardial blood flow observed in the ameroid constrictor model.

The most advanced porcine model of myocardial ischaemia is the hydraulic occluder model. By placing an adjustable hydraulic occluder around an epicardial coronary artery, the degree of stenosis can be adjusted. Combining a hydraulic occluder with an implanted flow probe distal to the position of the occluder device offers the advantage of a precise adjustment and the magnitude of antegrade flow and thus the degree of ischaemia (Bolukoglu et al., 1992). Like the ameroid model, the hydraulic occluder model has been used to study the pathophysiology of hibernating myocardium and for the evaluation of angiogenic therapies. A major disadvantage as compared to the ameroid occluder model is the complexity of the surgical procedure and animal maintenance due to the externalised hardware. Perioperative mortality rates are also high at 44% thought to result in cardiac arrhythmias from coronary spasm after cuff inflation (Bolukoglu et al., 1992). This methodology is far better suited to the canine rather than the porcine species. In addition, the cost implications mentioned above are even more pronounced using the hydraulic occluder model.

Despite the widespread success of angiogenic factors in the porcine ameroid model, these have not been so impressive when used in man (Henry et al., 2003); (Simons et al., 2002). The reason for this discrepancy is unknown but potentially may be due to differences between animal models and 'no-option' patients. Most translational research is conducted on juvenile animals; at 8 weeks the average weight of the pigs in our studies was 25 ± 1 kg, within 6 weeks these animals weighed over 60 kg despite limitations in diet. Using older animals or developing protocols with longer term

follow-up would be logistically very difficult as within a year a typical farm pig weighs in excess of 200 kg. The model is typically single vessel occlusion in animals where the remaining vessels are normal and thus potentially are more capable of mounting an angiogenic or arteriogenic response than in man. Mini pigs have been suggested as alternative species, and due to their size are an attractive model for long-term studies, however they are extremely expensive and are prone to infection.

In conclusion, this study demonstrated that total occlusion of the left circumflex coronary artery with an ameroid occluder is not a pre-requisite for successfully establishing the pathophysiology of myocardial ischaemia in the porcine model. Additional angiographic criteria such as TIMI flow and baseline collateral index are useful for identifying study animals without total LCX occlusion but with myocardial ischaemia. Angiographic assessment of the collateral flow, however, is not correlated with myocardial blood flow or function and should not be used as a primary outcome measure for therapeutic angiogenesis studies. The majority of published studies use sample sizes, which most probably are too small to detect moderate therapeutic effects.

CHAPTER 4: EPICARDIAL DELIVERY OF A “MASTER SWITCH GENE” HIF-1 α - SAFETY AND EFFICACY IN A PORCINE MODEL OF CHRONIC MYOCARDIAL ISCHAEMIA

4.1 INTRODUCTION

Mature blood vessel formation requires the interplay of a number of different cell types (Darland and D'Amore, 1999), and sequential expression of a number of growth factors together with their receptors. Perhaps not surprisingly, therefore, co-administration for example of VEGF and FGF2 produce synergistic effects (Asahara et al., 1995). Thus, up-regulation of a single growth factor may be insufficient. Hypoxia-inducible factor-1 alpha (HIF-1 α) has been described as a ‘master switch’ gene. HIF-1 α is able to induce production of inducible nitric oxide synthase (iNOS), (VEGF-A), transforming growth factor beta (TGF β), insulin-like growth factor (IGF-2) plasminogen activator inhibitor 1, and erythropoietin in addition to FLT-1, a VEGF receptor. Thus, HIF-1 α acts as an inducible transcription regulatory factor, which, in combination with constitutively expressed HIF-1 β , plays a principal role in the cellular response to hypoxia. To allow for constitutive expression of this factor as a transgene, transactivating domains from either the herpes simplex virus (VP16) or from nuclear factor kappa (NF κ B) can be incorporated (Vincent et al., 2000). Angiogenesis may be induced by application of the candidate protein, or by gene therapy. The latter is, however, the only option for certain transcription factors such as HIF-1 α , since in its protein form it is very rapidly degraded (Li et al., 2000). Gene delivery to the myocardium can be achieved using a number of vectors and delivery routes. Generally adenoviral-mediated gene transfer produces the highest expression levels, but is associated with dose-related inflammation. Plasmid DNA has also proved effective in some studies, and is generally less likely to induce toxicity. For either vector, direct injection into the myocardium via an epicardial approach is most likely to circumvent delivery issues. This study was undertaken to establish the efficacy and safety of plasmid and adenoviral HIF-1 α in a porcine model of chronic myocardial ischaemia.

Previous studies undertaken by Genzyme Corporation (data not shown) suggested that 10^{10} particles was the optimal dose for adenoviral gene transfer to the porcine myocardium using direct intramyocardial injections. Plasmid DNA administration had

been less well studied. I therefore included dose escalation based on available data (Vincent et al., 2000). Practicalities precluded the use of respective control groups for both plasmid and adenoviral arms of the study. Based on existing studies, I hypothesised that an empty adenoviral vector was more likely to be pro-inflammatory than its plasmid counterpart, hence the choice of the control group. The choice of transactivator was governed by manufacturing practicalities. In a preliminary study, I compared a 2500 µg dose of plasmid HIF-1α VP16 with a 2500 µg dose of plasmid HIF-1α NFκB. The mean change in absolute blood flow in the adjacent zone in the plasmid utilising the NFκB transactivator was -0.05 ml/g/min (SEM 0.19 ml/g/min), and in the plasmid with a VP16 transactivator it was 0.22 ml/g/min (SEM 0.16 ml/g/min) $p = 0.7$.

4.2 RESULTS

Seventy-four animals underwent thoracotomy for ameroid placement, of which thirty-seven were available for gene delivery following exclusion criteria and perioperative losses. Table 9 shows there were no significant differences between any of the four groups, for any parameters following randomisation.

Table 9: Randomisation

	CONTROL Ad2/CMVEV	PLASMID HIF-1 α 500 μ g	PLASMID HIF-1 α 2500 μ g	AdHIF- 1 α	P VALUES ANOVA
Number of animal	9	8	10	10	
Weight (kg)	33 (1)	29 (1)	29 (1)	30 (1)	0.1
Resting HR (beats/min)	89 (3)	87 (4)	84 (3)	83 (2)	0.6
Resting MAP (mmHg)	85 (6)	81 (7)	62 (6)	79 (8)	0.1
Resting LAP (mmHg)	17 (2)	16 (2)	18 (2)	17 (2)	0.9
TIMI (0-3)	1.3 (0.4)	1.2 (0.3)	1.8 (0.4)	0.8 (0.3)	0.2
Rentrop (0-3)	0.9 (0.3)	0.4 (0.2)	0.8 (0.2)	0.8 (0.3)	0.6
LV Fractional Shortening (mm)	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)	0.7
MBF at rest LCX/LAD (ml/min/g)	1.1 (0.1)	1.0 (0.1)	1.0 (0.1)	1.2 (0.1)	0.8
MBF at rest LCX/Septum (ml/min/g)	1.0 (0.1)	1.1 (0.1)	1.1 (0.1)	1.2 (0.2)	0.9
MBF Stress Target/LAD (ml/min/g)	0.9 (0.1)	1.0 (0.1)	0.9 (0.1)	0.8 (0.1)	0.4
MBF Stress Target/Septum (ml/min/g)	0.9 (0.1)	1.0 (0.1)	0.9 (0.1)	0.8 (0.1)	0.3

Table 9: Characteristics of the groups at randomisation. Data are represented as means with standard error of the means (SEM). HR = Heart rate, MAP= Mean arterial pressure, LAP = Left atrial pressure, MBF= Myocardial blood flow, LCX= Left circumflex artery territory, LAD = Left anterior descending territory. LV fractional shortening = (diastolic left ventricular diameter-systolic left ventricular diameter)/ diastolic diameter). TIMI = degree of flow in LCX assessed by the TIMI criteria. Rentrop = degree of collateralisation of the LCX assessed by Rentrop criteria. Stress was defined as an increase of 100% of resting heart rate induced with a dobutamine infusion (6 to 64 μ g/kg).

a. Safety and Toxicity

i. Mortality overview

There were two deaths, one in the control group during chest closure post gene delivery and one in the high dose plasmid group after induction of anaesthesia for the final evaluation. Review of baseline data showed that these animals did not differ significantly from the surviving population. Necropsy indicated that both deaths related to cardiorespiratory failure and were judged unrelated to administration of gene therapy.

ii. Inflammation within the myocardium

There were no significant differences in inflammatory foci between groups, either in the target area ANOVA ($p = 0.6$) or septum ($p = 0.8$) (Fig 10).

iii. Toxicity in distant organs

An experienced pathologist detected no gross pathological abnormalities or inflammation. There was no evidence of malignant tumours, or angioma formation side effects, which have been documented in the literature (Schwarz et al., 2000).

Figure 10: Inflammation

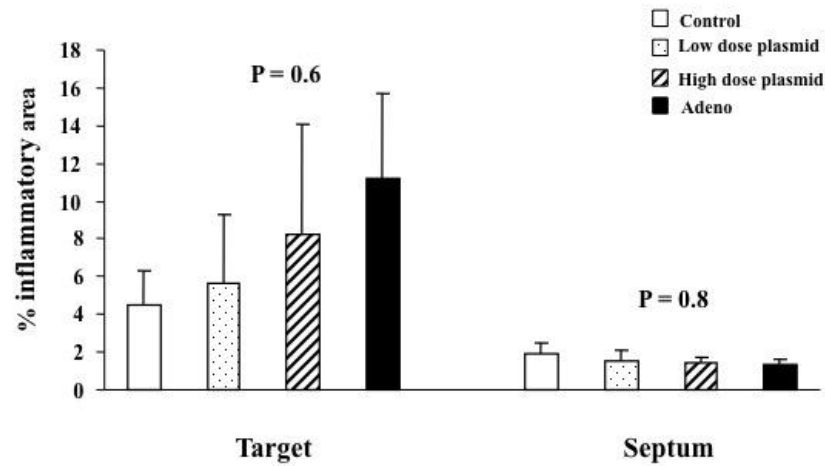


Figure 10 The proportion of the myocardium showing inflammation was assessed by point counting on HE sections using a light microscope, fitted with an eyepiece graticule containing 100 crosses. The graph shows the percentage number of these crosses covered by inflammatory cells in the target zone and septum. Control = Ad2 empty vector 10^{10} particles $n = 8$, Low dose plasmid= HIF-1 α 500 μg $n = 9$, High dose plasmid= HIF-1 α 2500 μg $n = 8$ and Adeno = Ad2 HIF-1 α 10^{10} particles $n = 10$. Error bars indicate SEM.

b. Efficacy

i. Perfusion

The relative (percentage) change in perfusion from gene delivery to final studies is presented for convenience. Absolute differences were also calculated, which gave comparable results. The administration of dobutamine (stress) resulted in four-fold increases in flow as expected. The diastolic blood pressure as the main variable for coronary perfusion was comparable between groups during rest and stress (data not shown). The myocardial ring was separated into separate areas (Figure 11)

At rest no significant (ANOVA) changes in myocardial perfusion were detected in the target area ($p = 0.3$), adjacent ($p = 0.3$), LAD ($p = 0.2$) and septum ($p = 0.2$). However during stress significant differences between groups were seen for the adjacent area (ANOVA $p = 0.02$). Post hoc testing (Bonferroni) demonstrated that the AdHIF-1 α group was significantly ($p = 0.02$) different from the Ad2/Control group. The improvement in flow was 54% (SEM 22%) in the AdHIF-1 α group compared to a reduction of -14% (SEM 11%) in the Ad2/Control. This corresponded to an absolute change in myocardial blood flow of 0.87 ml/g/min (SEM 0.42 ml/g/min) and - 0.48 ml/g/min (SEM 0.35 ml/g/min) respectively. The differences in other myocardial territories were not statistically significant (ANOVA target area ($p = 0.07$), LAD ($p = 0.08$) and septum ($p = 0.1$)). No differences were seen with either plasmid dose in any of the four territories studied (Bonferroni, $p = 1.0$) for all territories (Fig 12).

ii. Left ventricular function

Complete echo data were available for thirty-one animals as the image quality of acquisition was too poor in four cases for an assessment to be made. A core laboratory not involved with the study performed all echo analysis and this laboratory determined decisions pertaining to whether an image was of sufficient quality independently.

There was a significant (ANOVA $p = 0.02$) difference in fractional shortening three weeks after gene delivery. Post hoc (Bonferroni) testing demonstrated that the AdHIF-1 α group was significantly ($p = 0.03$) different from the Ad2/Control. The improvement in the AdHIF-1 α group was 7% (SEM 5%) compared to a reduction of - 6% (SEM 3%) in the Ad2/Control (Fig 13). The latter showed a reduction in shortening, compatible with the known disease progression in this model.

iii. Collateralisation

There were no significant changes in collateralisation of the LCX, assessed by the Rentrop score, for AdHIF-1 α [mean change 0.5 (SEM 0.3)], similarly, there was no change seen in the low dose plasmid HIF-1 α group [mean change 0.0 (SEM 0.2)] or high dose plasmid HIF-1 α [mean change 0.1 (SEM 0.2)] or control group [mean change 0.25 (SEM 0.25)] (ANOVA $p = 0.5$). There was no difference seen in the histological assessment of vessel formation (ANOVA $p = 0.3$) assessed by either Von Willebrand Factor or by a cluster differentiation cell surface marker 31 (CD31) (ANOVA $p = 0.5$) shown in (Figure 14).

iv. Haemodynamics

There were no detectable differences between groups, at rest or during stress, for heart rate, arterial pressure or left atrial pressure.

v. VEGF protein

The half-life of HIF-1 α is too short to assess protein level; we, therefore, investigated a down- stream protein VEGF. No VEGF protein could be detected three weeks after gene delivery by Western blotting in any group. However a single band at an appropriate site was visible after samples were spiked with 100 μg of VEGF protein (Figure 15).

Figure 11: Myocardial Blood Flow Assessment

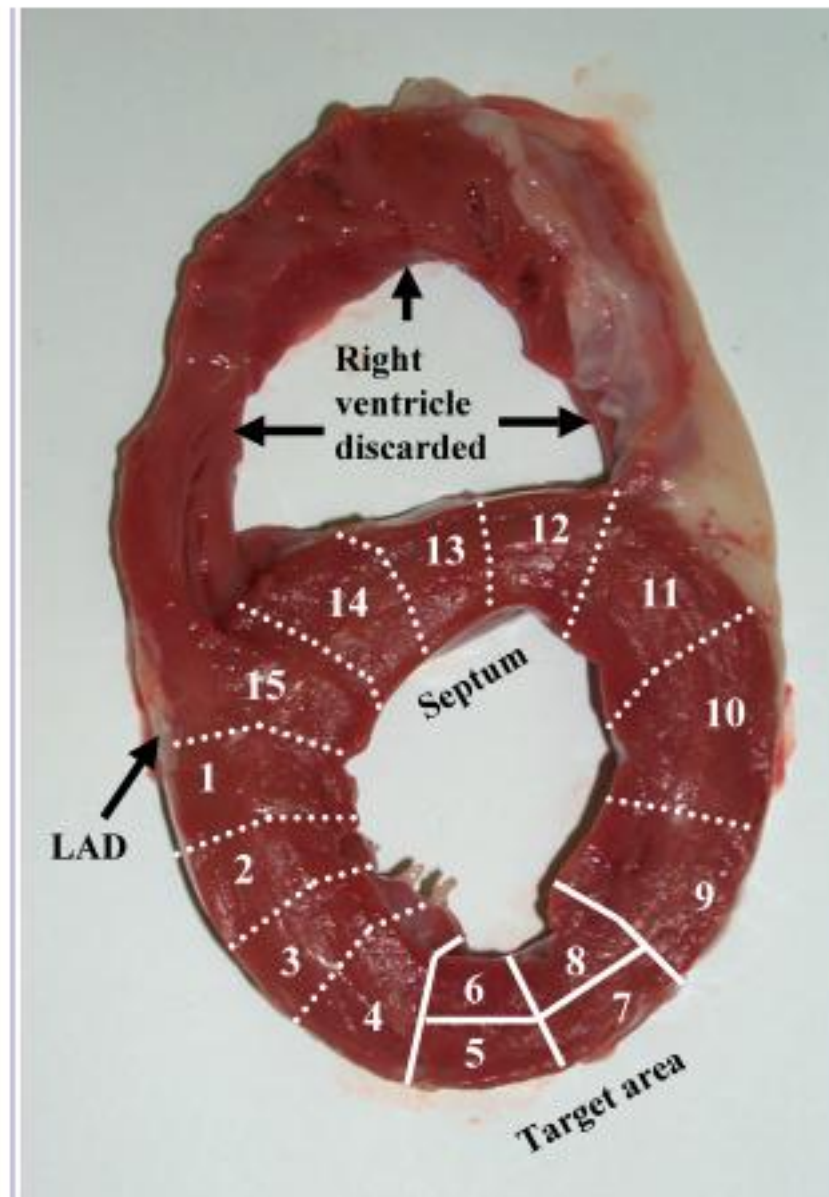


Figure 11: The division of a mid papillary myocardial ring for myocardial blood flow analysis showing the target zone (5 to 8), adjacent zone (4 and 9), left anterior descending artery (LAD 1 and 2) and septum (12 to 15).

Figure 12: Myocardial Blood Flow during Stress

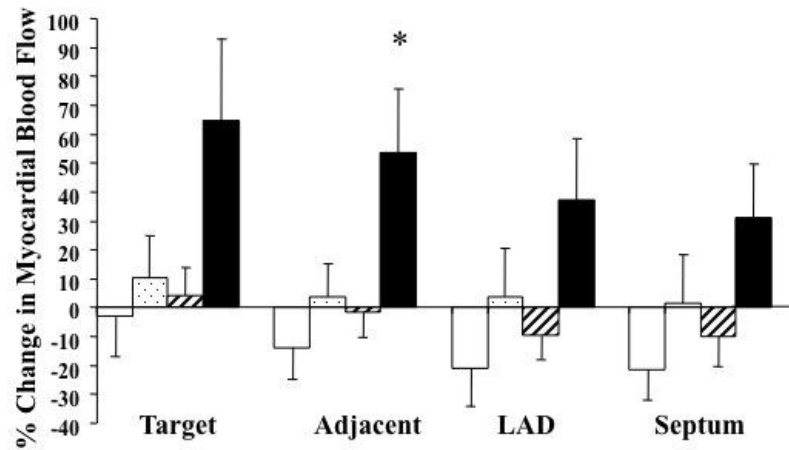


Figure 12: Percentage changes in myocardial blood flow during stress in target zone (5 to 8), adjacent zone (4 and 9), LAD territory (1 and 2) and septum (12 to 15). Significant differences between groups were seen for the adjacent area (ANOVA $p = 0.02$). Post hoc (Bonferroni) demonstrated the Ad2 HIF-1 α 10¹⁰ was significantly ($p = 0.02$) different from Ad2 Control. Control = Ad2 empty vector 10¹⁰ particles $n = 8$, Low dose plasmid = HIF-1 α 500 μ g $n = 9$, High dose plasmid = HIF-1 α 2500 μ g $n = 8$ and Adeno = Ad2 HIF-1 α 10¹⁰ particles $n = 10$. Error bars indicate SEM.

Figure 13: Left Ventricular Function during Rest

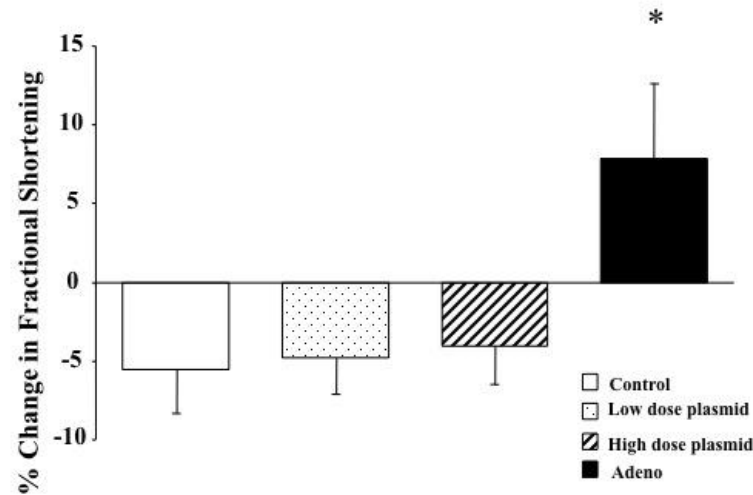


Figure 13: Percentage changes in fractional shortening of the left ventricle = (diastolic left ventricular diameter-systolic left ventricular diameter)/ diastolic diameter x 100. There was a significant (ANOVA $p = 0.02$) difference in resting fractional shortening three weeks after gene delivery. Post hoc (Bonferroni) testing demonstrated that the AdHIF-1 α group was significantly ($p = 0.03$) different from the Ad2/Control. Control = Ad2 empty vector 10^{10} particles $n = 9$, Low dose plasmid = HIF-1 α 500 μg $n = 9$, High dose plasmid = HIF-1 α 2500 μg $n = 8$ and Adeno = Ad2 HIF-1 α 10^{10} particles $n = 8$. Error bars indicate SEM.

Figure 14: Mean number of vessels

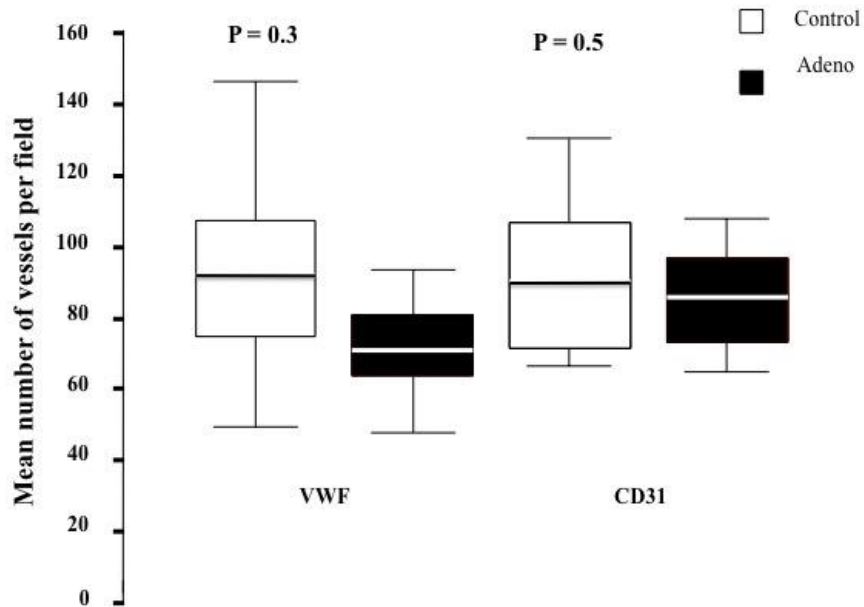


Figure 14: The mean number of blood vessels as assessed by VWF=Von Willebrand Factor and CD31=cluster differentiation cell surface marker 31 in the Control (white box) = Ad2 empty vector 10^{10} particles $n = 9$, and Adeno (Black box) = Ad2 HIF-1 α 10^{10} particles $n = 8$. Error bars indicate SEM.

Figure 15: Immunoblot analysis

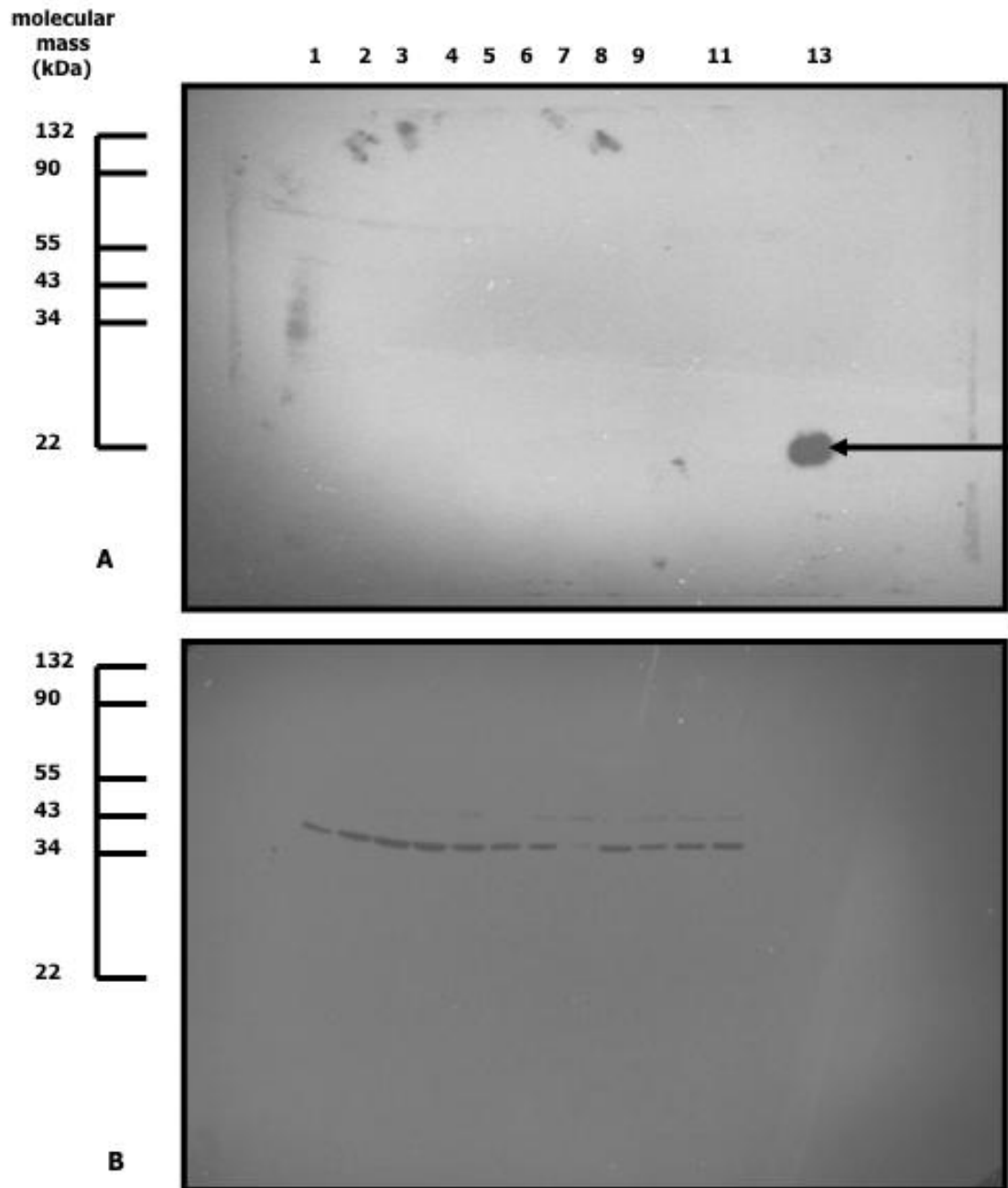


Figure 15: Tissue lysates (100 mg total protein) from the target area harvested at three weeks post gene transfer (Lanes 1 to 12) were resolved by SDS-PAGE (12% gel) and trans-blotted into nitrocellulose membrane. The membrane was first probed with anti-VEGF monoclonal antibody (Panel A), stripped, and finally reprobed with an anti-actin monoclonal antibody as loading control (Panel B). Both the primary antibodies were visualized with a horseradish peroxidase-conjugated secondary immunoglobulin and the enhanced chemiluminescence (ECL) Western blotting kit. Arrow on the right hand side in Panel A indicates the positive control, recombinant human VEGF (500 μ g), run in Lane 13. Mobilities of coelectrophoresed size markers are indicated on the left.

4.3 DISCUSSION

In this study, animals that received Ad2/HIF-1 α demonstrated increased perfusion during pharmacological stress and improved global left ventricular function compared to an Ad2/Control. There were no detectable differences in safety or toxicity between groups.

Plasmid HIF-1 α (500 μ g) has been shown to improve perfusion in a rabbit ischaemic hind limb model (Vincent et al., 2000). More recently plasmid HIF-1 α has been shown to reduce infarct size and increased neovascularisation when injected epicardially at the time of LAD ligation in the rat (Shyu et al., 2002). Although no animal model faithfully mimics myocardial disease in man, ameroid-induced ischaemia in the pig is widely considered the 'gold standard' in which to demonstrate efficacy of novel angiogenic agents. The strict inclusion criteria were used prior to treatment randomisation in an attempt to reduce variability of the outcome measures. Thus, microvessels from chronically ischaemic myocardium show increased reactivity to both FGF-2 and VEGF, whilst upregulation of growth factor receptors has also been demonstrated in this tissue (Sellke et al., 1996). Despite using these criteria the Ad2/HIF-1 α group showed a non-significant increase in the level of baseline ischaemia in the LCX territory compared to the other groups during stress. It is possible therefore that this may have biased the data in favour of a treatment-induced difference. Whether the potential increase in inflammation associated with an adenoviral vector could have mediated the improvements in perfusion were also assessed. However, this was not supported by the histological assessment of myocardial inflammation, which demonstrated no significant difference between Ad2/HIF-1 α , plasmid HIF-1 α or an adenoviral control. It is difficult to assess whether baseline data from the two animals that died differed from the surviving population. Whilst it cannot be excluded as a possibility and this may have biased the results, any effect is likely to have been minimal.

One of the key findings was an improvement in perfusion, apparently in the absence of new capillaries or larger vessels. The same effects have also been seen in a mouse model of myocardial infarction. Clearly, one explanation is a lack of sensitivity of the immunohistological assays, although these were based on previously validated

techniques. Two other potential explanations are improvements in either endothelial or left ventricular function. Growth factor mediated improvements in endothelial function have been documented (Sato et al., 2001). Thus, microvessels from chronically ischaemic myocardium have a greater sensitivity to both VEGF and FGF-2 vasodilation (Sellke et al., 1996) thought to predominantly arise from nitric oxide (NO) release. HIF-1 α may stabilise nitric oxide (NO) or nitric oxide synthase (NOS) or counteract effects of other substances detrimental to vascular function. In keeping with this, pre-treatment of animals with a NOS inhibitor almost completely suppressed VEGF-induced increases in blood flow (Lopez et al., 1997). In a subsequent study (endocardial study) we have demonstrated that nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, does not effect the improvements in perfusion seen during stress in those pigs treated with Ad2/HIF-1 α . A further possible explanation is an improvement in left ventricular function. Studies of FGF-2 in acute ischaemia reperfusion have reported significant salvage of myocardium (Horrigan et al., 1996), which could have resulted from either recruitment of pre-existing collaterals or from myocyte protection by the growth factor or stimulation of resident stem cells.

Although others have reported that HIF-1 α increases levels of VEGF protein (Vincent et al., 2000); (Shyu et al., 2002) in this study no detection of VEGF was seen *in vivo* 21 days after gene delivery. The most likely explanation is the well-documented time course of adenoviral-mediated expression, which peaks over the first week, and is markedly reduced by the end of three weeks (Freedman et al., 2002). Alternatively, additional factors including the time delay between removal of the heart and isolation of the target region may have resulted in VEGF protein degradation or the levels of VEGF protein induced by HIF-1 α may have been below the detection sensitivity of the assay.

Vincent *et al* have demonstrated efficacy with a plasmid HIF-1 α in the peripheral hindlimb model (Vincent et al., 2000). In this study neither dose of pHIF-1 α showed an effect in any of the bioreactivity endpoints. This may reflect differences in gene transfer efficiency in the two organs. Thus, plasmid DNA is well recognised for its efficacy in skeletal muscle, whilst this and others data clearly indicate the superiority

of adenoviral-mediated gene transfer in the myocardium. At least at the titer studied, this advantage in transfection efficiency was not countered by an increase in inflammatory or other toxic effects.

In summary, plasmid p $\text{HIF-1}\alpha$ did not produce any significant changes in bioactivity, Ad2/ $\text{HIF-1}\alpha$ significantly increased myocardial flow during stress as well as significantly improving LV function at rest compared to the control group. However, these improvements could not be correlated with histological or angiographic evaluation of increased vascularity.

CHAPTER 5: ENDOCARDIAL DELIVERY OF HIF-1 α - SAFETY AND EFFICACY IN A PORCINE MODEL OF CHRONIC MYOCARDIAL ISCHAEMIA

5.1 INTRODUCTION

Therapeutic angiogenesis by administration of recombinant genes or growth factors may serve as a treatment strategy for patients with coronary artery disease by enhancing collateral blood supply to the ischemic heart (Freedman and Isner, 2001); (Simons et al., 2000). Clinical studies using vascular endothelial growth factor or fibroblast growth factor gene therapy have shown mixed results (Grines et al., 2003); (Gyongyosi et al., 2005). Angiogenesis and blood vessel maturation are complex and coordinated pathophysiological processes involving a variety of growth factors interacting in sequence (Ware and Simons, 1997); (Darland and D'Amore, 1999). Thus, a clinically meaningful improvement of collateral vessel formation may be best achieved by a combination of growth factors replicating the physiological processes involved in angiogenesis. Alternatively, a 'master switch gene/protein' such as the HIF-1 α located upstream in the angiogenesis cascade could be used (Freedman and Isner, 2001).

HIF-1 α is a heterodimeric transcription factor (HIF-1 α and HIF1- β subunits) regulating gene expression in response to changes in tissue oxygenation, and serves as a key regulator and potent stimulator of angiogenesis (Semenza, 2000). Proteins that increase tissue perfusion such as VEGF, nitric oxide synthase, and erythropoietin are targets of HIF-1 α -mediated transcriptional activation. Initial studies have suggested that a construct consisting of the HIF-1 α DNA-binding and dimerisation domains and the transactivation domain from herpes simplex virus VP16 protein promotes perfusion in small animal models of hindlimb and myocardial ischaemia (Vincent et al., 2000); (Shyu et al., 2002). In addition, I have shown that direct surgically based epicardial intramyocardial injection of Ad2/HIF1 α increases myocardial perfusion and improves left ventricular function in a preclinical model of myocardial ischaemia (Heinl-Green et al., 2005). Clinically, however, less invasive percutaneous gene delivery, without the necessity for general anaesthesia and thoracotomy, would be desirable. A magnetic guidance percutaneous navigational system (NOGA system, Biosense Webster, USA) has already been shown to effectively transfect designated

myocardial sites (Shyu et al., 2002) ; (Semenza, 1999) and has recently been used in a clinical angiogenesis gene therapy trial (Vale et al., 2001); (Gyongyosi et al., 2005) The aim of this study was, therefore, to evaluate the safety and efficacy of percutaneous, intramyocardial AdHIF-1 α /VP16 gene delivery by the use of a percutaneous catheter system in a porcine model of chronic myocardial ischaemia.

5.2 RESULTS

A total of 20 animals underwent intramyocardial gene delivery. One animal in each group died; one during the procedure and one immediately after the procedure. Pathological examination did not reveal any cause related to the gene or the catheter procedure. After treatment, all 18 animals reached final studies. This group of study animals was incorporated into the end- point analysis (Table 10). In those animals, there were no significant differences regarding procedural characteristics (Table 11).

a. Safety

i. Haemodynamics, necropsy and laboratory results

Changes in heart rate, mean arterial blood pressure, and mean left atrial pressure between gene delivery and final studies was comparable in both treatment groups (Table 12). All animals reaching final studies underwent a full necropsy. Macroscopically, mild signs of bronchitis, pleuritis and pericarditis could be found in both groups without significant differences between groups. No signs of inflammation, necrosis or neoplasia could be detected in any of the other organs listed in the (List of organs for toxicology evaluation). Histological signs of subacute or chronic inflammatory reactions due to the experimental approach could not be found in any of the study animals. One animal in each group were noted to have focal lymphocytic infiltrates in the myocardium. Laboratory evaluations did not show any significantly different changes between groups (Table 13). In both groups, CRP and Troponin-I values at final evaluation were below the detection limit.

ii. List of organs for toxicology evaluation

Aorta, brain, cecum, duodenum, oesphagus, eye, gall bladder, heart, ileum, lung, mammary glands, mesenteric lymph nodes, pancreas, rectum, salivary glands, skeletal

muscle, skin/ subcutaneous fat, spleen, thyroid gland, tongue, trachea, urinary tract, bladder, sciatic nerve, knee joint, spinal cord, thymus, liver.

Table 10: Characteristics of treatment groups at randomisation

	Ad2/EV	Ad2/HIF-1 α
Number of animals	9	9
Body weight (kg)	40 $\pm$ 2	39 $\pm$ 1
Resting HR (bpm)	85 $\pm$ 5	88 $\pm$ 4
Resting MAP (mmHg)	153 $\pm$ 7	135 $\pm$ 12
Resting LAP (mmHg)	17 $\pm$ 3	14 $\pm$ 2
TIMI flow grade (0-3)	0.7 $\pm$ 0.2	0.9 $\pm$ 0.3
Rentrop grade (0-3)	1.2 $\pm$ 0.4	1.3 $\pm$ 0.3
LV fractional shortening (%)	0.21 $\pm$ 0.02	0.24 $\pm$ 0.02
LV systolic thickening (%)	0.42 $\pm$ 0.07	0.43 $\pm$ 0.06
MBF at rest TA/septum (ml/min/g)	0.95 $\pm$ 0.08	0.85 $\pm$ 0.07
MBF at stress TA/septum (ml/min/g)	0.85 $\pm$ 0.04	0.79 $\pm$ 0.07

Table 10: HR: heart rate, LAP: left atrial pressure, LV: left ventricle, MBF: myocardial blood flow, TA: target area, TIMI: thrombolysis in myocardial infarction. There were no significant differences between groups. AD2/EV Ad2 empty vector 10¹⁰ particles n = 9, AD2/ HIF-1 α Hypoxia inducible factor alpha n = 9.

Table 11: Procedural characteristics of gene delivery (mean $\pm$ SD)

	Ad2/EV	Ad2/HIF-1 α
Number of animals	9	9
Needle length (mm)	4.9 $\pm$ 0.1	4.8 $\pm$ 0.1
Successful needle injections	180/180	180/180
Mapping time (min)	30 $\pm$ 5	30 $\pm$ 5
Injection time (min)	22 $\pm$ 5	19 $\pm$ 2

Table 11: min: minutes, mm: millimeter. There were no significant differences between groups. AD2/EV Ad2 empty vector 10¹⁰ particles n = 9, AD2/ HIF-1 α Hypoxia inducible factor alpha n = 9.

Table 12: Changes (absolute) in hemodynamic parameters from mid- to final studies

	Rest			Stress		
	Ad2/EV	Ad2/HIF-1 α	p	Ad2/EV	Ad2/HIF-1 α	p
HR (in bpm)	+1 $\pm$ 3	-7 $\pm$ 2	0.07	+9 $\pm$ 7	+3 $\pm$ 4	0.51
MAP (in mmHg)	5 $\pm$ 5	-8 $\pm$ 6	0.11	-5 $\pm$ 8	16 $\pm$ 14	0.11
LAP (in mmHg)	3 $\pm$ 3	5 $\pm$ 3	0.86	2 $\pm$ 3	8 $\pm$ 5	0.66

Table 12: HR: heart rate, LAP: left atrial pressure, MAP: mean arterial pressure
AD2/EV Ad2 empty vector 10^{10} particles n = 9, AD2/ HIF-1 α Hypoxia inducible factor alpha n = 9.

Table 13: Changes in laboratory parameters from mid- to final studies (mean $\pm$ SD)

	Ad2/EV	Ad2/HIF-1 α
Number of animals	9	9
Leucocytes ($10^3/l$)	5.2 $\pm$ 5.0	7.7 $\pm$ 4.1
Erythrocytes ($10^6/l$)	0.2 $\pm$ 0.7	-0.8 $\pm$ 1.3
Haemoglobin (mmol/l)	0.4 $\pm$ 0.8	-0.6 $\pm$ 1.3
Haematocrit (%)	2.4 $\pm$ 4.0	-3.1 $\pm$ 6.9
ASAT (μ mol/l)	0.1 $\pm$ 0.3	0.0 $\pm$ 0.4
CRP > 0.5 mg/dl at final studies	0/9	0/9
TrI > 1.5 ng/ml at final studies	0/9	0/9

Table 13: ASAT: aspartate- amino- transferase, CRP: C-reactive protein, TrI: Troponin I. There were no significant differences between groups. AD2/EV Ad2 empty vector 10^{10} particles n = 9, AD2/ HIF-1 α Hypoxia inducible factor alpha n = 9.

b. Efficacy

i. Angiography, myocardial blood flow and function

In neither group, were there any significant changes in angiographic parameters of target vessel or collateral flow from mid to final studies.

There were no differences in myocardial blood flow changes from mid to final studies between treatment groups either at rest or stress (Table 14). There was, however, a trend towards a more pronounced increase in myocardial blood flow in the HIF-1 α group (Figure 16). Systemic administration of L-NAME (10 mg/kg) under rest and stress conditions did not change the response in myocardial blood flow in either group or myocardial area (Table 15).

Changes in regional myocardial function (left lateral wall fractional systolic thickening) from mid- to final studies were not significantly different between groups. However, there was a strong trend towards wall motion recovery in the HIF-1 α group as compared to the control group (Figure 16).

Table 14: Relative changes (in percent) in myocardial blood flow at rest and under pharmacological stress in animals receiving Ad2/HIF-1 α

	Rest			Stress		
	Ad2/EV	Ad2/HIF-1 α	p	Ad2/EV	Ad2/HIF-1 α	p
Anterior wall	52 $\pm$ 37	60 $\pm$ 56	0.60	-6 $\pm$ 13	11 $\pm$ 15	0.41
Target area	61 $\pm$ 43	80 $\pm$ 55	0.67	4 $\pm$ 16	32 $\pm$ 17	0.14
Target center	48 $\pm$ 31	89 $\pm$ 62	0.47	4 $\pm$ 16	35 $\pm$ 19	0.16
Septum	46 $\pm$ 39	49 $\pm$ 39	0.74	-13 $\pm$ 14	-1 $\pm$ 13	0.53

Table 14: All segments are shown below in the endocardial study the target area was not separated into epicardial and endocardial segments Target area (segments 4 to 9), Target center area (segments 5 to 7), Anterior wall (segments 1 and 2), Septum (segments 10 to 13). AD2/EV Ad2 empty vector 10¹⁰ particles n = 9, AD2/ HIF-1 α Hypoxia inducible factor alpha n = 9.

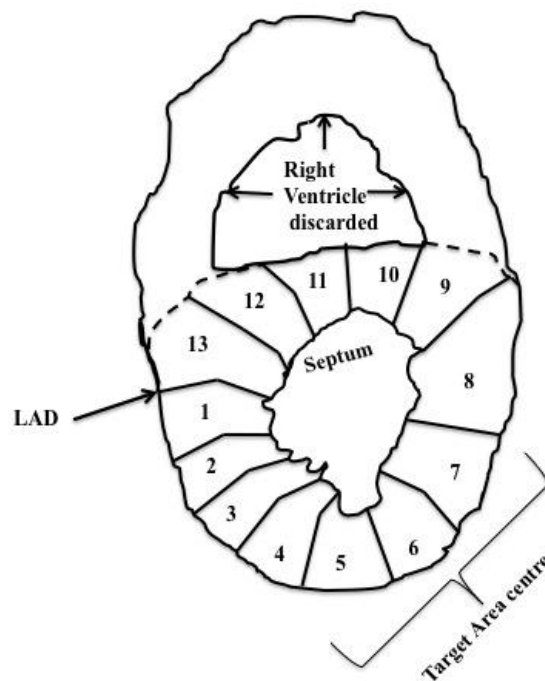


Table 15: Changes in myocardial resistance (in percent) as a result of systemic L-NAME administration (10 mg/kg)

	Rest			Stress		
	Ad2/EV	Ad2/HIF-1 α	p	Ad2/EV	Ad2/HIF-1 α	p
Anterior wall	23 $\pm$ 17	13 $\pm$ 15	0.77	51 $\pm$ 21	42 $\pm$ 14	0.96
Target area	12 $\pm$ 14	17 $\pm$ 18	0.96	56 $\pm$ 23	48 $\pm$ 11	0.96
Target center	14 $\pm$ 11	26 $\pm$ 19	0.77	57 $\pm$ 23	48 $\pm$ 10	1.0
Septum	16 $\pm$ 14	31 $\pm$ 22	0.74	47 $\pm$ 21	44 $\pm$ 14	0.96

Table 15: All segments are shown on Figure 11 in the endocardial study the target area was not separated into epicardial and endocardial segments. Target area (segments 4-9), Target center area (segments 5 to 7), Anterior wall (segments 1 and 2), Septum (segments 10 to 13). AD2/EV Ad2 empty vector 10¹⁰ particles n = 9, AD2/ HIF-1 α Hypoxia inducible factor alpha n = 9.

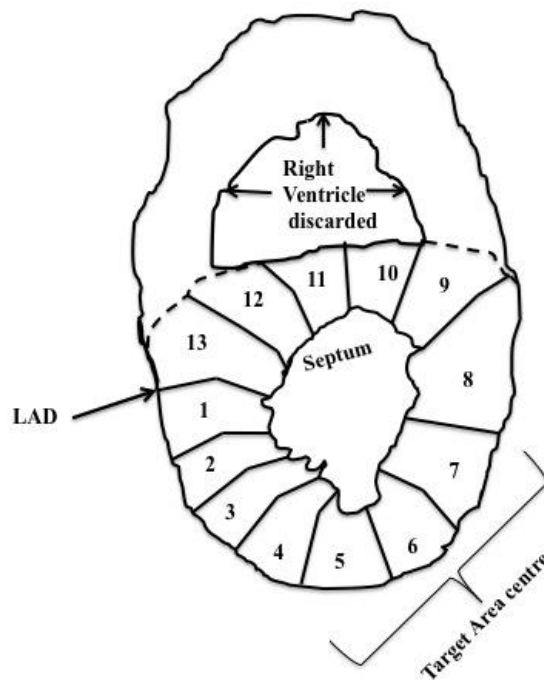


Figure 16: Changes in myocardial blood flow and LV fractional thickening

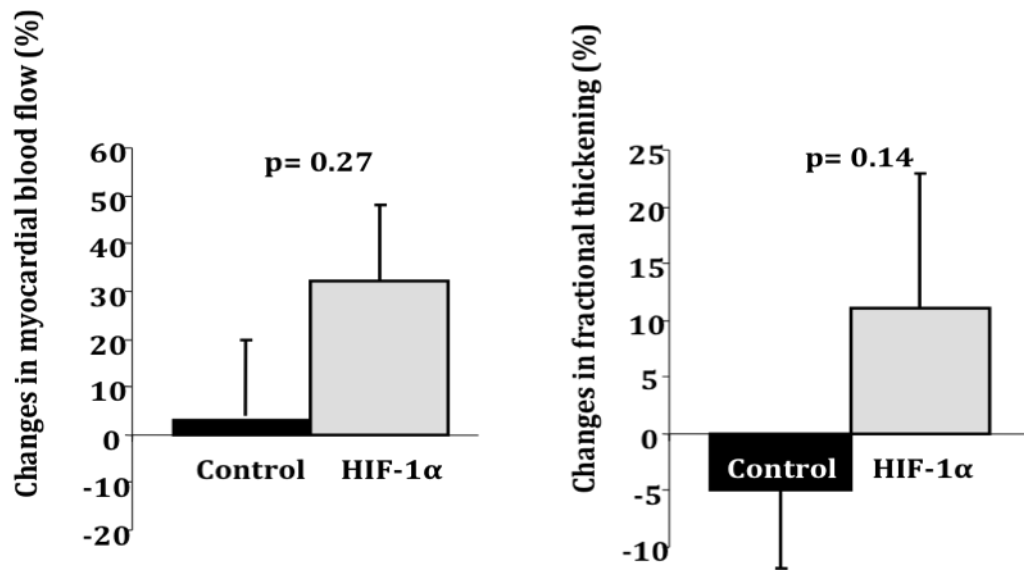


Figure 16: Effect of Ad2/HIF-1 α gene delivery on changes in regional myocardial blood flow (MBF) of the centre target region (segments 5 to 7 Figure 11) under pharmacological stress conditions, and changes in fractional lateral wall thickening under resting conditions (n = 9 per group). Error bars indicate SEM. Control = AD2/EV Ad2 empty vector 10^{10} particles, HIF-1 α = AD2/ HIF-1 α Hypoxia inducible factor alpha.

Figure 17: A comparison of the changes in perfusion in the Epicardial and Endocardial studies during stress.

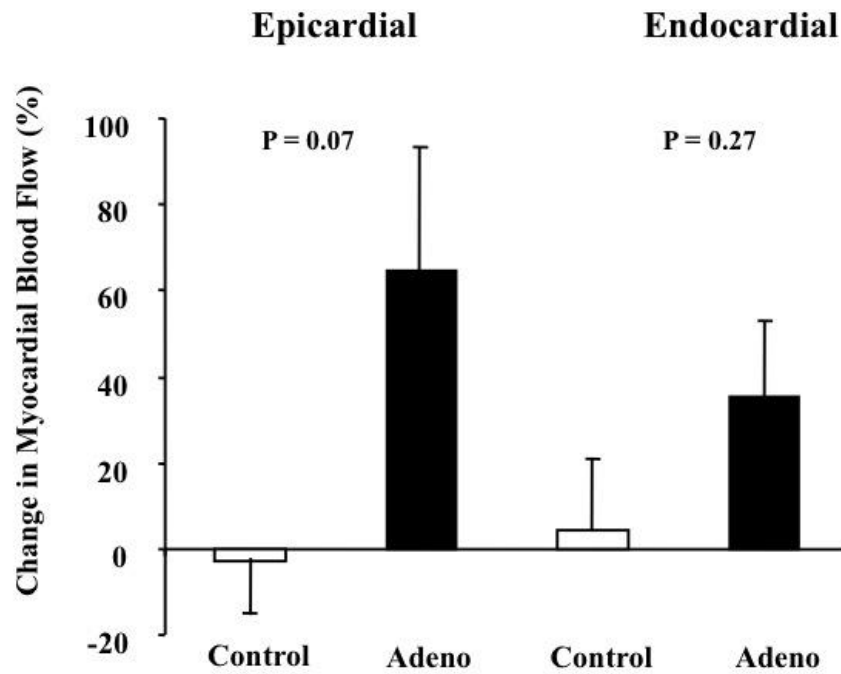


Figure 17: Effect of Ad2/HIF-1 α gene delivery on changes in regional myocardial blood flow in the target area during pharmacological stress conditions Control = Ad2 empty vector 10^{10} particles n = 9, Adeno = Ad2 HIF-1 α 10^{10} particles (n = 8 epicardial, n = 9 endocardial). Error bars indicate SEM.

5.3 DISCUSSION

The aim of this study was to evaluate the safety and efficacy of percutaneous, intramyocardial AdHIF-1 α /VP16 gene delivery by the use of a percutaneous catheter system in a porcine model of chronic myocardial ischaemia.

This study was designed to document both safety and efficacy of intramyocardial AdHIF-1 α /VP16 gene delivery. Due to the complexity of gene delivery and its potential use in man, safety was of paramount interest in this study. A complete necropsy including microscopic analysis of a variety of distant organs was performed in all animals. The observations made in individual study animals (i.e. mild bronchitis, mild pericarditis) were equally distributed between both treatment groups. Laboratory evaluation did not show any differences between groups. The cumulative toxicity data, therefore, support the safety of percutaneous, intramyocardial delivery of Ad2/HIF-1 α VP-16 therapeutic gene (10^{10} particles) or Ad2/CMV-EV empty vector (10^{10} particles).

The analysis of the functional (haemodynamics, myocardial blood flow, echocardiography) and morphological (angiographic collateralisation) study end-points did not show any significant differences between the active and the control group. Moreover, application of an irreversible inhibitor of nitric oxide synthase did not result in a difference in coronary resistance between groups. There were, however, trends towards a larger increase in mean arterial blood pressure at stress, an increased left lateral wall thickness, a measure of regional wall motion, and an improvement in myocardial blood flow in the target areas of the active group (Ad2/HIF-1 α VP16). I had previously shown in a randomised, blinded study using the same animal model and identical therapeutic/ control agents that direct surgically-based transepical intramyocardial injection of Ad2/HIF-1 α increased myocardial perfusion and improved left ventricular function (Heinl-Green et al., 2005). A number of factors may have contributed to the discrepant findings in the endocardial study (Figure 17). First, despite a clearly defined injection process using three independent methods of injection quality assurance (fluoroscopy, NOGA loop stability and necessity for ectopic ventricular beats at the exact moment of injection needle protrusion), complete myocardial retention of the entire injection volume (50 μ l each) is much less

likely using endocardial injections, as compared to injections via a thoracotomy and direct visualisation of the injection site. Preclinical studies have demonstrated that in 5% to 10% of endocardial injections no injection track can be found (Kornowski et al., 2000).

Secondly, in the previous studies, a detailed process of injection site identification was combined with a systematic analysis of the left ventricle (Heinl-Green et al., 2005); (Radke et al., 2006). Thus, the site of injection could be clearly identified at tissue harvest. An 'injection/read out mismatch', however, is much more likely in the case of endocardial injections, as the injection site cannot be reliably marked. To minimise this effect, I analysed the heart according to prespecified guidelines (Heinl-Green et al., 2005) and also incorporated two rather than one myocardial rings; despite this, the maximum effect may still have been missed. Novel non-invasive technologies for quantification of myocardial perfusion (e.g. magnetic resonance imaging) may have overcome this problem (Post et al., 2006); (Gyongyosi et al., 2005). The sensitivity of current protocols, however, may not be sufficient to detect relatively small treatment effects.

Thirdly, recent studies have shown that gene delivery catheters potentially decrease adenovirus vector infectivity (Marshall et al., 2000); (Naimark et al., 2003), thereby decreasing gene transfer efficiency. This phenomenon seems to be related both to the catheter materials used (e.g. nitinol) as well as the contact time of the gene with the inner catheter lumen. Although the catheter system used has been shown to have the potential to decrease vector activity, the mean contact time of the drug and the catheter inner lumen was only around 120 seconds. Attempts were made to undertake *in-vitro* studies of this catheter system with the contact time used in this study, however disappointingly I was unable to negotiate the use of the catheter or AdHIF1 α by the companies involved. Preclinical studies determining the degree of inactivation have used contact times of 30 minutes or more. Furthermore, validation studies of the NOGA injection system have clearly shown successful gene transfer and expression of transgenes (Kornowski et al., 2000).

Finally, and in my view most likely reason, was the study was probably underpowered to reach significance. Prior to initiation of the study power calculations

were undertaken based around the variability of response seen during our surgically-based studies (Heinl-Green et al., 2005). Variability was, however, greater during the present catheter-based approach despite the relatively high number of animals, and in turn cost, involved. Power calculations based on our catheter based findings suggest we would have needed a further two animals per group to show significant changes in the center region under stress conditions. This increase in variability may be important to consider in further pre-clinical, as well as human catheter-based studies.

5.4 CONCLUSION

In an animal model of chronic myocardial ischaemia, percutaneous, intramyocardial AdHIF-1 α /VP16 gene delivery is safe and shows trends towards an improvement in regional myocardial function and perfusion (Figure 17). Improvements in injection technique, perfusion imaging and catheter technology may be necessary to accomplish significant treatment effects using percutaneous gene transfer.

CHAPTER 6: CELL THERAPY PRE-CLINICAL CATHETER STUDIES

6.1 INTRODUCTION

The way in which stem cells are delivered to the heart may be one of the most critical determinants of success in these studies. After reviewing the literature and based on our own delivery technique experiences I designed a study to develop a novel retrograde coronary venous technique in which a catheter is introduced into the veins draining blood from the heart. By applying pressure to a syringe of stem cells attached to the catheter, cells are forced out of the blood vessels and into the heart tissue. Because these veins drain large areas of the heart, it may be possible to deliver the stem cells into a much larger territory than is possible using catheters within the coronary arteries or via needles injected into the heart muscle, an advantage in a patient population presenting with global ischaemia. No clinical trial of stem cells has assessed whether delivery was successfully achieved. Thus, a negative outcome of any trial would not indicate whether the hypothesis is wrong, or whether the cells have not been delivered. To alleviate this factor I thus sought to establish the safety and feasibility of retrograde coronary venous delivery. As with my previous pre-clinical studies I sought other research groups with experience with this technique. Dr Mike Simon's group in Dartmouth USA in collaboration with Keith March in Indianapolis had started preliminary studies of the retrograde route of delivery in animal models. I therefore arranged a visit to Mike Simon's facility in Dartmouth to further investigate this technique. Unfortunately it became quickly apparent that extensive work was required to optimise this approach in the porcine model due to the large coronary venous vessels and extensive collateralisation in this species. No appropriate animal model reliably mimics the anatomy of the human venous network; therefore the prospects of translation to the clinical situation were questionable. Thus at this point I decided an exhaustive period of optimisation in the porcine model would not be useful and that a move to the clinic appeared more appropriate. I therefore set out to establish whether this novel route of coronary venous delivery would be capable of delivering cells in patients with ischaemic heart failure.

After making a decision to move into the clinic a number of questions needed to be addressed in early pilot studies before we could continue, which are outlined below. Because the proposed clinical cardiac catheters had not been used for retrograde

coronary venous delivery of bone marrow derived putative stem cells I sought to establish the effects of pressure, time and contrast on the viability of cells. To determine successful delivery, I also needed a reliable safe method of labelling patients own bone marrow cells. Iron particles had been used with MRI visualisation in animal models but there had been reports that they over-estimated the degree of retention (Terrovitis et al., 2008). The prospect of being the first group to use these clinically was not appealing, as it would have potentially presented an additional confounding variable. Our patient population was also prone to arrhythmias and it was unknown whether iron particles within the heart may exacerbate the likelihood of these events. A rapid and simple way to label the cells with Indium-111 (^{111}In -oxide) a radioactive marker, which has been in clinical use for other purposes for several decades, was thus developed.

6.2 PILOT CLINICAL TRIALS

In preliminary studies patients autologous bone marrow stem cells were labelled with ^{111}In -oxide and cell viability and evidence *in vitro* for a ‘bystander effect’ were assessed on the unlabelled co-cultured cells. The questions I set out to answer were:

a. Hypothesis for the catheter studies

- i.** Does high-pressure delivery through cardiac catheters effect cell viability?
- ii.** Is viability altered by time or co-culture with contrast agents used for angiography?
- iii.** Is it possible to label autologous bone marrow cells and does this affect their viability?

In order to undertake the above studies I needed a supply of bone marrow, ideally from patients with a similar profile to my intended study patients. Bone marrow is usually aspirated either from the sternum or the iliac crest, both are invasive and painful procedures. I therefore wanted to select a patient population that were already having major surgery to these areas; two possibilities existed:

- Patients undergoing hip replacement where it would have been possible to aspirate some bone marrow from the long femur bone or from the iliac crest whilst the patient was under anaesthesia; or

- Patients with IHD scheduled for CABG who would be fully anaesthetised for the procedure and would have their sternum dissected for their operation.

The second option was preferred as it may be that the cell function of patients with IHD is different; some have suggested that bone marrow from chronic IHD patients may be predisposed to a profound lack of neovascularisation (Heeschen et al., 2004). Also, these patients obviously had a similar patient profile as those I ultimately sought to treat.

6.3 METHODS – PRE-CLINICAL CATHETER STUDIES

a. Consent

Patients scheduled for cardiac surgery were approached in pre-admission clinic to see if they were willing to take part in this study. The Royal Brompton and Harefield Hospital Ethics Committee approved this study, and all patients gave written informed consent.

b. Anaesthetic and bone marrow harvest

All patients underwent bone marrow harvesting under sedation, administered by a consultant anaesthetist. All received pre-procedure assessment by an anaesthetist during which the sedation process was fully explained.

The procedure was performed in a standard anaesthetic room. Patients were admitted and identity and consent verified. Standard basic anaesthesia monitoring was applied (electrocardiography, oxygen saturation pulse oximetry and non-invasive blood pressure cuff) and baselines noted.

Intravenous cannulation was performed if not already established and a disposable oxygen mask applied (5 litres/min O₂ flow), with an additional sampling tube connected for respiratory capnography. The anti-emetic ondansetron 4 mg was administered intravenously.

Sedation was administered combining initial intravenous morphine 2.5 – 4.5 mg (0.05 µg/kg) followed by intravenous midazolam titrated as required (usual total dose 2-3 mg). The patient was positioned in supine and the skin on the sternum was swabbed with 2% chlorhexidine and draping commenced. A consultant haematologist thoroughly washed their hands and donned sterile gloves, using a 25-gauge needle 1 ml local anaesthetic solution of 1% or 2% lignocaine were introduced intradermally slightly to the side of the mid-line, approximately 2 cms below the sternomanubrial joint raising a bleb on the skin. The needle was then changed to a 21-gauge needle, and 5 ml of 1% or 2% lignocaine was slowly introduced, into the periosteum. A ‘pricking’ technique was used, introducing small amounts of solution subperiostally

each time the needle was moved over the area. After ascertaining that the anaesthetic had taken effect, the skin over the sternum was held steady, and the aspiration needle with its guard in place and set at 1 cm, and with its stylet locked into position, was carefully pushed through the skin. The needle was steadily pushed through until it was felt to be resting on the underlying bone. The needle was pushed through the cortex of the bone to a depth of approximately 0.5 cm until a slight 'give' was felt, indicating that the marrow space had been entered. The introducer was removed, a 10 ml aspiration syringe containing 0.5 ml of preservative-free heparin 1,000 IU units per ml was placed on the end of the marrow needle and about 5 ml of bone marrow was withdrawn which was quickly handed to me, I injected the marrow into a 600 ml capacity blood transfusion transfer pack containing 3 ml of heparin via the injection port, agitating the contents continuously to ensure that no clots formed.

The bone marrow needle was withdrawn from the bone but not from the skin hole and the introducer was replaced before the needle was again introduced into the underlying bony prominence, about 0.5 cm from the previous site of aspiration. A further 5 ml of marrow was withdrawn into a fresh heparinised syringe and handed to me. Four or five aspirations were made with a single needle before this was replaced. Only one hole in the skin was made and the needle was moved about into the underlying bone for the necessary 10 aspirations of 5 ml each, due to the skin elasticity.

After the procedure, pressure was exerted on the operation site until haemostasis was secured and the site was then cleaned again, the patient was intubated and transferred into the theatre for sternal dissection prior to heart surgery. The port on the transfer pack containing the marrow was closed with the bung and transferred to The Hospital for Sick Children Great Ormond Street (GOSH) for further processing.

Procedure duration from administration of sedation to transfer to theatre was approximately fifteen minutes. During the procedure, patients were either drowsy or sleeping and occasionally responded to the procedure, particularly during aspiration, with local movement or respiratory stimulation.

No adverse effects related to the sedation process were experienced or observed. All patients maintained stable observations throughout the procedure and no cardiovascular or respiratory interventions were required, on follow-up no patient suffered from sternal dehiscence.

c. Transportation to The Hospital for Sick Children Great Ormond Street

I transported all bone marrow personally at room temperature to The Hospital for Sick Children Great Ormond Street (GOSH) via taxi, the time from aspiration to delivery was within one hour. On arrival at GOSH the sample was received by the staff at the Department of Immunology, logged and underwent processing. This process took between three and three and a half hour.

d. Assessment of cell differential and viability

1 ml of bone marrow was removed for the assessment of full blood count (FBC) and differential staining and viability. Cells were stained using the whole blood Lyse wash method. 100 µl of whole blood were added to 5 µl of antibody and incubated for ten minutes at room temperature. Following this cells were mixed with Facslyse™ Becton Dickson, left for 10 minutes at room temperature before being spun and resuspended in Cell Wash™. They were then spun again after which they were added to 500 µl of Cell Fix™, Becton Dickinson supplied all lyse products. After Cell Fix™ was added cells were read on a Facscalibur™ flow cytometer. Four tubes of fluorescent-labelled antibody combinations panels were used ([CD3, NK, CD19 and CD45], [CD3, CD8, CD4 and CD 45], [CD45, CD34 and 7AAD]). All antibodies were supplied by Becton Dickson apart from CD45, which was supplied by Beckman Coulter.

e. Ficoll separation

Bone marrow was red cell depleted and volume reduced by pumping the marrow diluted with 0.9% saline over Lymphoprep (Axis Shield) using the Cobe 2991 cell washer (Caridian BCT). After spinning for 15 minutes at 3000 repetitions/per/minute (rpm), the interface was collected, and washed twice in one litre of wash medium (500 ml 0.9% saline/ 500 ml 4.5% Albumin [Blood Products Laboratory BPL]). The

mononuclear cells were counted, and the count adjusted to the required cell dose and volume diluting with wash medium. All procedures were carried out in sterile conditions. Two syringes each of 10 ml containing 30×10^6 bone marrow mononuclear cells were prepared. The first syringe was used for the catheter experiment below.

f. Assessment of cell differential and viability post-Ficoll

2 ml were removed for FBC and differential staining and viability as described above. Viability was repeated again at different time points between 0 and 24 hours 7-aminoactinomycin D (7ADD alone).

g. Preparation of the catheter

We had initially planned to deliver our cells via an angioplasty catheter so in order to mimic the clinical situation as much as possible the catheter was prepared in the following way. A sterile angioplasty catheter Maverick® Over-the wire Balloon Angioplasty catheter (Boston Scientific Inc) with a Choice® PT Floppy angioplasty guidewire (Boston Scientific Inc) were placed on a sterile drape in an incubator at 37 degrees. A small amount of heparinised saline was trickled into the balloon port and then drawn back. The catheter lumen was then flushed with heparinised saline. The guide wire was then moved up and down inside the catheter three times, and then the wire was removed. The angioplasty balloon was inflated to 14 atmospheres. Following this 10 ml 2:1 saline /contrast solution (Ultravist, Iopromide, Berlix, Germany) at 20 atmospheres with an angioplasty syringe were instilled through the catheter, followed by a flush with 10 ml of heparinised saline.

h. Delivery of cells through the catheter

After the preparation of the catheter 10 ml of 5% Human Serum Albumin (HAS) and saline containing 1.5×10^6 bone marrow mononuclear cells at a pressure of 20 atmospheres were instilled through the catheter. The substrate was collected and separated into three aliquots. Viability was assessed as above at time zero after passage through the catheter and then at four hours and 24 hours with the remaining two aliquots.

The second syringe was separated into four universals, two containing a heparinised saline solution and two containing saline /contrast solutions of variable concentration ranging from 0.1% to 100%. Cells were incubated at room temperature. Two universals were assessed for cell viability at six hours and two at 24 hours.

i. ¹¹¹In-oxide labelling

A sterile vial containing different concentrations of putative stem cells (3 to 30 x 10⁶) in suspension together with a sterile 10 ml vial containing 5% HAS/ 0.9% saline were transported via taxi to University College Hospital Department of Radiopharmacy and transferred to a blood isolator Amercare UK.

¹¹¹In-oxide (Covidien) 0.6 ml was drawn up in a 1 ml syringe and added to 0.3 ml of buffer (used to adjust the PH and ensure the binding of Indium to the oxine). The tube was then inverted several times to mix thoroughly. The stem cells were gently poured into a sterilin pot. ¹¹¹In-oxide /buffer mixture was used to rinse the original pot and added to the stem cells. The pot and syringe were rinsed with 0.2 ml of HSA/saline mix added to the cells and left to stand for 10 minutes. A further 2 ml of HSA/saline mix were added and put in the centrifuge (Amercare UK) at 1000 revolutions for five minutes, after a pellet of cells formed the remaining supernatant liquid was poured off and discarded. A further 10 ml of plasma/saline were added to the cell pellet, mixed and transfer to a nitrogen filled vial. ¹¹¹In-oxide activity was measured in an ionising chamber (Model CRC 15R Supplied in the UK by Southern Scientific made by Capintec Corporation, USA). The isotope sample ionises a gas in the wall of the chamber and is then converted to an electric current. This is measured and determines the radioactive activity ideally we aimed for 10 to 12 MBq activity in the vial. The vial was removed from the isolator, a patient label attached indicating name and MBq activity and then placed in a lead pot with another patient identifying label attached to the pot.

These ¹¹¹In-oxide labelled cells referred to as ‘hot cells’ were then transported together with unlabelled cells referred to as ‘cold cells’ by courier on the same day flights to Glasgow University where they were processed in the following way.

j. Assessing the affects of ¹¹¹In-oxide on the viability of co-cultured bone marrow mononuclear cells.

On arrival in Glasgow hot cells were mixed with cold cells in different concentrations, please see below.

Day -1 (Same day as bone marrow harvested)

- Mixed 3×10^6 hot cells with 30×10^6 cold **(90:10)**
- Mixed 15×10^6 hot cells (from 30×10^6 aliquot) with 15×10^6 cold cells **(50:50)**
- These mixes and cold alone and hot alone (3×10^6 aliquot) re-suspended at 2×10^6 /ml in 10% fetal calf serum (FCS) and left overnight.

Day 0 (24 hours after admixture of cells)

- Cells counted
- FACS analysis for propidium iodide (PI) (dead) and Annexin V (apoptotic)
- Cells harvested, spun down re-suspended at appropriate volume to set mesenchymal stem cells (MSC) assay and methylcellulose haemopoietic assays for the detection of haemopoietic stem cells (HSC).

Day 3

- MSC assays: non-adherent cells poured off and adherent cells washed very gently with PBS x1

Day 13

- MSC sub-confluent (~75% in cold) harvested with trypsin
- Counted
- Analysed by FACS for CD45, CD14, CD90, CD71, CD34 to determine (MSC verses HSC)
- Number of MSC and contaminating macrophages calculated
- Survival calculated

Day 15

- (MSC and HSC) assays counted

6.4 RESULTS

This pilot catheter study was difficult because a) there were several variables, pressure, time, admixture with contrast and ^{111}In -oxide labelling and b) unfortunately only a smallish number of patient samples were able to be assessed. The University of Glasgow had extensive experience and established assays to assess the effects of ^{111}In -oxide labelling on cell viability and function and generously offered to assist us in providing key information regarding the effects of ^{111}In -oxide labelling on BMNC. However understandably their goodwill only extended to a small number of patients, because of time constraints imposed by their own work. Thus, the study clearly had to be descriptive rather than statistically driven. Nevertheless I maximised the information I gained from a relatively small group of patients ($n = 9$) and was able to adequately address the questions I initially set out to answer.

In all the patients 50 ml of bone marrow was aspirated from the sternum under general anaesthesia immediately prior to the patient being taken into theatres to have their sternum dissected for CABG surgery. I assessed viability with 7ADD and differential cell populations ([CD3, NK, CD19 and CD45], [CD3, CD8, CD4 and CD 45], [CD45, CD34 and 7AAD]) We looked at a number of cell markers for completeness, however I was primarily interested in CD34+ cells so I have concentrated on these in the summary below.

In the first patient viability was assessed at four different time points; pre ficol, immediately post ficol, at three hours and at 24 hours. The effects on viability after the passage through a cardiac catheter, and the effects of the contrast agent ultravist non-diluted mixed with the cells. The percentages of CD34+ cells at the same time points and with the same parameters were also assessed.

Pilot 1 Viability

	Time 0	+3 hrs	+24 hrs
Original marrow	99%	99%	99%
Ficol separated	99%	99%	93%
+ contrast med 50/50 (100%)	99%	99%	96%
Post catheter	98%	98%	79%

CD34+

	Time 0	+3 hrs	+24 hrs
Original marrow	0.45%	0.41%	0.43%
Ficol separated	0.56%	0.62%	0.44%
+Contrast med 50/50 (100%)	0.85%	0.30%	1.43%
Post catheter	0.88%	0.77%	0.30%

For the second patient in addition to the above conditions, I also wanted to assess the effects of different concentrations of contrast agent after they had passed through a cardiac catheter to mimic as closely as possible the clinical situation. Initially I had not assessed the effects of contrast after the passage through the cardiac catheter, as I did not want to have two confounding variables. There were conflicting results for the effects on cells of passage through cardiac catheters followed by co-culture with contrast on CD34+ counts. At some of the higher concentrations the cell counts appeared to increase with time and with lower concentrations the counts appeared to drop.

Pilot 2 Viability

	Time 0	T + 3	T + 24
Original post ficoll	98%	98%	94%
Post Catheter	98%	98%	94%
PC + 100% contrast 50:50	99%	95%	96%
PC + 75% contrast	99%	97%	94%
PC + 50% contrast	99%	97%	91%
PC + 40% contrast	99%	97%	98%
PC + 30% contrast	99%	97%	97%
PC + 20% contrast	99%	98%	96%

CD34+

	Time 0	T+3	T+24
Original post ficoll	3.36%	2.51%	3.35%
Post Catheter	2.52%	2.64%	2.91%
PC + 100% contrast 50:50	1.44%	1.49%	1.25%
PC + 75% contrast	1.27%	1.72%	2.16%
PC + 50% contrast	1.11%	1.36%	1.17%
PC + 40% contrast	1.79%	0.8%	1.10%
PC + 30% contrast	1.30%	1.09%	0.80%
PC + 20% contrast	1.61%	1.54%	0.79%

Looking at the these first two patients it was clear that the first patient had a far lower CD34+ count compared to the second. The viability post passage through a catheter at 24 hours was lower (79%) in the first patient but there was little effect in the second

patient (never under 91%). It was difficult to conclude if there was any affect of contrast on CD34+ counts in the first patient as the results were variable but in the second patient it initially appeared that the admixture with contrast agents dramatically reduced the number of CD34+ cells with all concentrations.

In pilot three and four I repeated exactly the same parameters as in pilot two but just at time zero and at 24 hours.

Pilot 3 Viability

	Time 0	T + 24
Original post ficoll	99%	97%
Post Catheter	97%	95%
PC + 100% contrast 50:50	99%	-
PC + 75% contrast	99%	-
PC + 50% contrast	98%	97%
PC + 40% contrast	98%	96%
PC + 30% contrast	98%	96%
PC + 20% contrast	98%	96%

CD34+

	Time 0	T+24
Original post ficoll	5.13%	4.88%
Post Catheter	4.83%	4.57%
PC + 100% contrast 50:50	1.73%	-
PC + 75% contrast	1.72%	-
PC + 50% contrast	1.48%	1.3%
PC + 40% contrast	1.97%	1.35%
PC + 30% contrast	2.65%	1.23%
PC + 20% contrast	2.93%	0.76%

Pilot 4 Viability

	Time 0	T + 3	T + 24
Original post ficoll	98%	97%	97%
Post Catheter	97%	95%	96%
PC + 100% contrast 50:50	97%	95%	89%
PC + 75% contrast	98%	94%	87%
PC + 50% contrast	97%	96%	88%
PC + 40% contrast	97%	95%	88%
PC + 30% contrast	97%	94%	90%
PC + 20% contrast	98%	97%	92%

Pilot 4 CD34+

	Time 0	T+3	T+24
Original post ficoll	3.48%	0.26%	-
Post Catheter	3.42%	4.19%	4.64%
PC + 100% contrast 50:50	1.18%	1.96%	1.06-2.49%
PC + 75% contrast	1.56%	1.62%	1.45%
PC + 50% contrast	1.63%	1.7%	1.62%
PC + 40% contrast	2.05%	1.76%	1.7%
PC + 30% contrast	2.33%	1.81%	1.27%
PC + 20% contrast	2.33%	1.27%	1.49%

Pilot 5 Viability

	Time 0	T + 3	T + 24
Original marrow	98%	99%	99%
post ficoll/pre catheter	99%	99%	99%
post catheter + 50% contrast	99%	99%	99%
PC + 20%	99%	99%	98%
PC + 10%	99%	99%	99%
PC + 5%	98%	99%	99%
PC + 1%	99%	99%	99%
PC + 0.1%	99%	99%	99%

Pilot 5 CD34

	Time 0	T+3	T+24
Original marrow	0.5%	0.53%	0.49%
post ficoll/pre catheter	0.41%	0.34%	0.35%
post catheter + 50% contrast	0.34%	0.37%	0.31%
PC + 20%	0.39%	0.43%	0.33%
PC + 10%	0.38%	0.49%	0.43%
PC + 5%	0.54%	0.46%	0.51%
PC + 1%	0.58%	0.41%	0.52%
PC + 0.1%	0.51%	0.53%	0.49%

In pilot five I decided to alter the concentrations of contrast and decreased the concentrations to assess the effects from 20% down as low as 0.1%. However at this point once we looked at all the cell plots it became evident that this was an artifact and that the contrast agent appeared to be interfering with the intensity of the Fluorescein isothiocyanate (FITC) and Peridinin Chlorophyll Protein Complex (PerCP) flurochrome staining.

The median viability initially after ficoll separation was 99% (range 98 % to 99 %), at 24 hours the median viability was 97% (range 93 % to 99 %). With the admixture of 50/50 contrast and saline the median viability was 99% and 96% at the same

respective time points. The CD34+ cell percentage after ficoll separation was 3.36 % (range 0.41% to 5.13%), at 24 hours this decreased to 1.94% range (0.35% to 4.88%). With admixture with 50/50 contrast and saline the CD34+ cell count was 1.11 % (range 0.34% to 1.63%) at time zero and at 24 hours was 1.30% (range 0.31% to 1.62%), albeit as stated earlier the contrast agent appeared to interfere with the intensity of cell staining.

After completing the first five studies I wanted to address the second question, which was the effect of ^{111}In -oxide labelling on bone marrow mononuclear cells. This was separated into two subsets, which were the effects on the ^{111}In -oxide labelled cells known as ‘hot cells’ and the effects of co-culture with unlabelled cells known as ‘cold cells’. In order to answer these questions I collaborated with Joanne Mountford at Division of Cancer Sciences & Molecular Pathology, University of Glasgow who had extensive experience of assessing bone marrow mononuclear cell function. I looked at three patients summarised below.

		Patient 1	Patient 2	Patient 3
	24 hour viability (Trypan Blue)			
cold			70%	87%
mixed 30 + 3 10MBq			76%	31%
mixed 60 + 3 10MBq				40%
mixed 30 + 30 14MBq			66%	
mixed 10 + 10 12MBq			30%	
hot 20 14MBq			15%	
hot 3 10MBq				7%
	24 hour FACS of alive cells			
cold		80%	42%	51%
mixed 30 + 3 10MBq		77%	45%	52%
mixed 60 + 3 10MBq				49%
mixed 15 + 15 14MBq		61%		
mixed 30 + 30 14MBq			42%	
mixed 10 + 10 12MBq			24%	
hot 20 14MBq			7%	
hot 3 10MBq		38%		5%

	Total N of MSC's 13-14 days	Patient 1	Patient 2	Patient 3
cold		100%	100%	100%
mixed 30 + 3 10MBq		29%	42%	31%
mixed 60 + 3 10MBq				32%
mixed 15 + 15 14MBq		7.4%		
mixed 30 + 30 14MBq			56%	
mixed 10 + 10 12MBq			58%	
hot 20 14MBq			0%	
hot 3 10MBq		0%		0%
	Total N of Haematopoietic colony assays day 15			
cold		100%	100%	100%
mixed 30 + 3 10MBq		40%	42%	68%
mixed 60 + 3 10MBq			42%	67%
mixed 15 + 15 14MBq		2%		
mixed 30 + 30 14MBq				
mixed 10 + 10 12MBq			8%	
hot 20 14MBq			0%	
hot 3 10MBq		0%		0%

In the first patient, the effects of mixing 3×10^6 hot cells with 30×10^6 cold cells in a (90:10) ratio and 15×10^6 hot cells with 15×10^6 cold cells in a (50:50) ratio with hot and cold cells alone were assessed. When the day 13 MSC assay were analysed, MSCs were present at a much lower percentage than expected in mixed hot and cold culture, indicating a significant by-stander effect. Similarly to MSC at day 15 only 40% of the haematopoietic colony assays were seen in the 90:10 and less than 2% were evident in the 50:50 also indicating a significant effect on non-labelled cells.

In the next patient, 3×10^6 hot cells were mixed with 30×10^6 cold cells in a (90:10) ratio and the cell number increased to 30×10^6 hot cells with 30×10^6 cold cells in a (50:50) ratio and a further (50:50) ratio of 10×10^6 hot cells with 10×10^6 cold cells were analysed, together with a higher concentrations of 20×10^6 hot cells.

In addition to viability assessed by Trypan Blue, cell count FACS was assessed by Propidium iodide (PI) for dead cells and Annexin V for apoptotic cells.

	Alive %	Apoptotic %	Dead %
Cold	42	21	37
90:10	45	21	34
50: 50	42	18	40
10 + 10	24	18	58
Hot	7	7	86

Apoptotic cells were seen as viable by trypan blue counting, thus the sum of percentages is comparable to the survival calculated by cell count. These values were similar and consistent. So, although it appears that 70% of cells were alive in the cold cells, 90:10 and 50:50, in the viability assay the real value is about 40% as many were already undergoing apoptosis. However the labelled cells had no added effect to that seen in cold alone at this point.

Both 10+10 and hot samples were very badly damaged by label. 10+10 had a similar total amount of label (12MBq) as the cells in 50:50 mix (14.8 MBq). Thus, it would appear that the activity per cell might be important.

Based on the above data I wanted to assess the smallest number of labelled cells 3×10^6 with the proposed dose of unlabelled cells 30×10^6 . I also wanted to see if there would be any difference by changing the ratio of unlabelled to labelled cells to 95:5 by mixing 3×10^6 hot cells with 60×10^6 cold cells.

	Alive %	Apoptotic %	Dead%
Cold	51	27	22
30 + 3	52	23	24
60 + 3	49	28	22
Hot	5	10	85

With the admixture of 3×10^6 hot cells with either 30×10^6 or 60×10^6 cold cells there did not appear to be any decrease in viability compared to cold cells.

6.5 DISCUSSION

We had initially planned to deliver cells via an angioplasty catheter, however during these preliminary *in vitro* studies it was evident that the extremely small diameter of the catheter resulted in a ‘trickle’ of BMMC suspended in HAS being expelled from the catheter end, which I deemed would be insufficient to generate the pressure required to drive our putative stem cells across the endothelial barrier. We therefore made a decision to opt for a diagnostic catheter for our clinical trial, which we knew had the capabilities to disrupt the endothelium because of prior evidence that a sustained myocardial blush was occasionally inadvertently seen during a venogram for selection of an appropriate vein for LV lead placement during a biventricular device insertion procedure. Myocardial blush was used as evidence of endothelial disruption. It is not usual clinical practice to aim to cause a myocardial blush and it has been suggested that this may be evidence of myocardial damage. This issue is discussed further in the clinical chapter 7.6.

¹¹¹In-oxine is bound irreversibly to intracellular and nuclear cell components (Hughes, 2003). At the time of undertaking this study two studies were published reporting that labelling was not associated with altered cell viability (Bindslev et al., 2006); (Jin et al., 2005). Importantly, in our preliminary studies we did not see any adverse effect of the ¹¹¹In-oxide labelled cells on the viability of unlabelled cells. We therefore felt confident to move ahead with a co-administration of ¹¹¹In-oxide labelled and unlabelled cells, thus providing individual patient information on whether any change in surrogate measures was matched by evidence for cell delivery. Without confirmation of delivery, a negative outcome in any clinical trial cannot differentiate between inadequate delivery, or lack of stem cell effect. These data should allow for a more objective evaluation of the outcome of therapeutic trials delivering putative stem cells to the myocardium.

CHAPTER 7: CLINICAL TRIAL

7.1 INTRODUCTION

Many clinical studies have assessed the therapeutic effectiveness of stem cell delivery to the myocardium. Amongst the relatively few that have been randomised, controlled and blinded, there are conflicting reports regarding outcome, some positive (Schachinger et al., 2006); (Huikuri et al., 2008); (Hare et al., 2009); (Losordo et al., 2007); (van Ramshorst et al., 2009); (Erbs et al., 2005) and some negative (Janssens et al., 2006); (Wohrle et al., 2010); (Engelmann et al., 2006); (Ripa et al., 2006a); (Zohlhofer et al., 2006). One key variable is likely to be the mode of delivery. The optimal target site for the cells is unclear, but retention of the stem cells within the interstitium of the myocardium would seem to be a reasonable goal. Both intravenous and intra-coronary arterial injections are likely to result in minimal contact time between the stem cells and the vascular endothelium. Further, these cells will need to traverse the endothelium to reach the interstitium. Delivery to the left ventricular myocardium can also be achieved via the endocardial route using modified catheters, originally designed for electrophysiological procedures, which have deflectable tips and a needle to allow intramyocardial penetration. However, the area of myocardium covered by each 100-200 µl is small, some injections may be lost to the left ventricular cavity, and flush back along the needle track has also been documented. Epicardial delivery is only feasible during open heart-surgery, with the attendant additional variable of surgery, or as sole therapy via a minimally invasive approach making the inclusion of a placebo group difficult.

The coronary sinus, and its attendant venous network, has been used for a variety of therapeutic interventions. Coronary venous delivery increases the volume of myocardium that can be targeted, both because of the anatomical distribution of the veins, as well as the substantially increased contact time it offers compared to the arterial circulation. A single infusion of a reporter gene in a pig model produced transfection of approximately 25% of the left ventricle (Hou et al., 2003). We proposed to use this delivery route for the first time in a clinical study. We aimed to deliver putative stem cells to the myocardium of patients with end-stage ischaemic heart disease, and with the inclusion of a subset of ¹¹¹In-oxide labelled cells demonstrate delivery and retention over a prolonged period of time.

No clinical trial of stem cells has assessed whether delivery was successfully achieved. Thus, a negative outcome of any trial would not indicate whether the hypothesis is wrong, or whether the cells have not been delivered. To alleviate this factor I thus sought to establish the safety and feasibility of retrograde coronary venous delivery. I wrote the clinical protocol to address a number of questions listed below;

a. Hypothesis for the clinical trial

- Is retrograde coronary venous delivery of bone marrow derived putative stem cells feasible?
- Is it safe to deliver bone marrow derived putative stem cells via the retrograde coronary venous delivery route?
- Does dose of cells delivered affect safety and efficacy?
- Is it possible to image a sub-population of ¹¹¹In labelled bone marrow derived putative stem cells to establish delivery and retention over a prolonged period of time?
- Can we establish bioactivity assays and surrogate outcome measures for the assessment of efficacy for future clinical trials? I was interested in determining whether it would be possible to detect changes with quality of life questionnaires, exercise testing (MVO₂), NYHA class, perfusion (SPECT), LVEF (SPECT, Echocardiography).

7.2 METHODS

a. CLINICAL TRIAL SUMMARY

Title	Cell therapy for ischaemic heart disease
Study site	Royal Brompton Hospital
Study Objectives	1. Evaluate the feasibility of retrograde coronary venous delivery of autologous bone marrow derived mononuclear cells. 2. Evaluate the safety of a single administration of autologous bone marrow mononuclear cells by retrograde coronary venous delivery. 3. Evaluate the use of potential bioactivity assays and surrogate outcomes for assessing bone marrow mononuclear cell induced myocardial changes.
Study Design	A phase IIA, open label single-centre study of bone marrow mononuclear cells by percutaneous retrograde coronary venous delivery to patients with ischaemic heart failure and no standard revascularisation options scheduled for cardiac resynchronisation therapy with defibrillation (CRTD).
Study Population	Patients with symptomatic ischaemic heart failure, not amenable to conventional revascularisation strategies (PCI, CABG, Left ventricular assist device [LVAD]) or transplantation and scheduled for CRTD.
Inclusion Criteria	1. Written informed consent and agree to attend hospital appointments for one year. 2. Male and females over 18 years of age. 3. Symptomatic ischaemic multi-vessel coronary artery disease that are no longer suitable candidates for standard revascularization procedures such as PCI or CABG, LVAD, or heart transplantation. 4. LVEF < 40% on optimal medical therapy, measured either by echocardiography or SPECT.

	5. NYHA class II- IV patient stable on optimal medical therapy for at least 30 days.
Exclusion Criteria	1. Left ventricular aneurysm or thrombus. 2. Thoracic aortic aneurysm. 3. Congenital Heart disease 4. Acute unstable angina (Defined as dynamic ECG changes with associated Troponin-I rise within 24 hours of delivery), idiopathic cardiomyopathy, life-threatening ventricular arrhythmias, recent (less than six weeks). 5. Contraindication to any study procedure. 6. Presence or history of cancer (except low grade and fully resolved non-melanoma skin malignancy). 7. Any co-morbidity likely to reduce short-term survival. 8. Other conditions (e.g. chronic pain or severe rheumatoid arthritis) which may interfere with functional testing (e.g. exercise tolerance testing MVO₂). 9. Recent myocardial infarction < six months. 10. Cerebral vascular accident < six months. 11. Active hepatitis, receiving immunosuppressive therapy, undergoing haemodialysis. 12. Clinically significant abnormal haematology. 13. Recent history of alcoholism, drug abuse, or severe emotional, behavioural, or psychiatric problems, which may preclude compliance with the study requirements. 14. Fertile women who are pregnant, nursing, or using no form of contraception. 15. Receiving experimental medications or participating in another study within 12 weeks of enrolment into this study.

Independent Eligibility	The results of the screening procedures were compiled and submitted to an independent interventional cardiologist (Professor Kim Fox) and cardiac surgeon (Professor John Pepper) who were not associated with the study for consideration for enrolment. It was the independent reviewer's responsibility to confirm eligibility prior to a patient participating in the study.
Optimisation of Medical Management	Dr Theresa McDonagh or Dr Rakesh Sharma (Heart Failure Consultants) reviewed all patients to optimise and standardise medical management. Patients must have had a period of at least one month between changes in medication and delivery. Any classes of drugs that were omitted such as beta-blockers or statins should be documented and a reason given (eg. intolerance etc). They also reviewed each patient to establish the suitability and eligibility for CRTD.
Total number of patients	18 open label (safety and feasibility)
Investigational Products	Autologous bone marrow mononuclear cells Dose escalation • Three patients received 3×10^6 reconstituted in a volume of 20 ml containing 5% HSA and saline. • Three patients received 3×10^6 with 3×10^6 ^{111}In oxide labelled cells reconstituted in a volume of 20 ml containing 5% HSA and saline. • Three patients received 15×10^6 with 3×10^6 ^{111}In oxide labelled cells reconstituted in a volume of 20 ml containing 5% HSA. • Three patients received 30×10^6 with 3×10^6 ^{111}In oxide labelled cells reconstituted in a volume of 20 ml containing 5% HAS and saline. • Three patients received 60×10^6 with 3×10^6 ^{111}In oxide labelled cells reconstituted in a volume of 20 ml containing 5% HSA and saline.

	Three patients received 100×10^6 with 3×10^{11}-In oxide labelled cells reconstituted in a volume of 20 ml containing 5% HSA and saline.
Route	Retrograde coronary venous delivery The total dose of bone marrow mononuclear cells was divided into two, each administered as a 10 ml bolus into selective coronary veins. Central venous access was achieved via the left subclavian vein and the coronary sinus was cannulated using a coronary sinus delivery system. A diagnostic catheter (R Judkins) was inserted through the coronary sinus delivery system and a coronary sinus venogram was performed. The best lateral coronary vein was selected for left ventricular lead placement and was positioned using standard techniques. The CS delivery system was left in place and the CS was 'double cannulated' leaving the initial CS delivery system in position and through the second CS delivery system a (Judkins Right 5F) angiographic catheter was inserted. An appropriate coronary vein for stem cell delivery was subselected to target, where possible, viable but underperfused myocardium based on a prior SPECT scan. A venogram was then performed with a 1:1 dilution of contrast medium and normal saline using a hand-powered injection through a 10 ml syringe, in order to create an intramyocardial 'blush'. The aim of the instillation was to loosen the endothelial tight junctions to achieve delivery of substances into the interstitium evidenced by a myocardial blush. Presence of a myocardial blush was used to indicate successful passage of contrast to the interstitium. If a blush was not achieved the catheter was repositioned and this procedure was

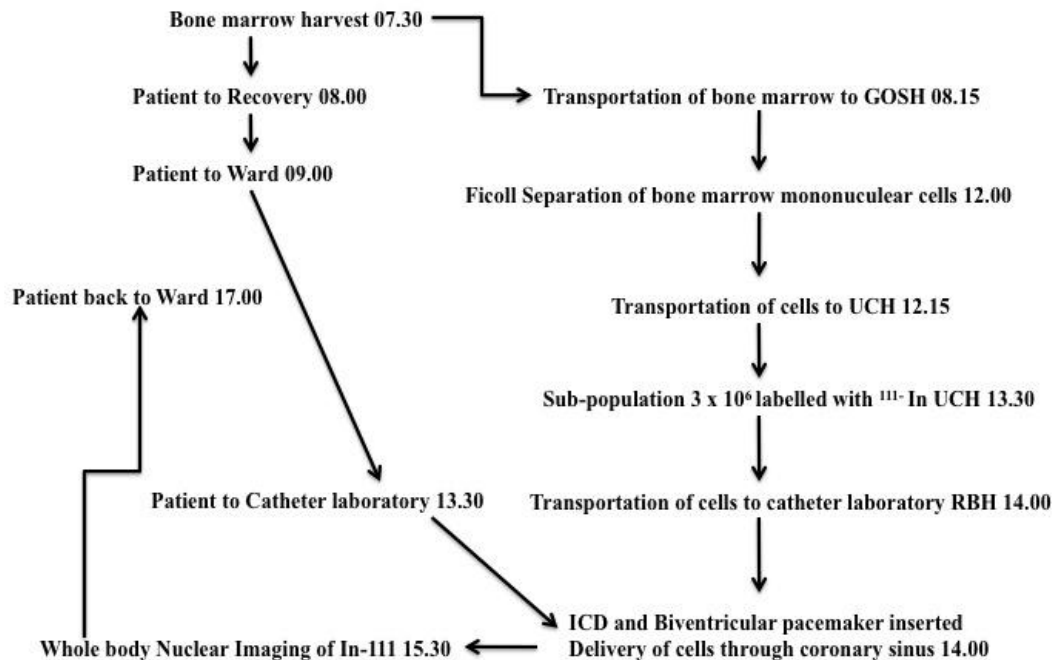
	repeated. Once a blush was achieved this was immediately followed by instillation of cells, using the same pressure. The catheter remained in-situ for ten minutes to increase the potential contact time. This was repeated in a second vein. After the second delivery the second CS delivery system and diagnostic catheter were both removed and the right ventricular defibrillator lead and atrial lead were positioned in the usual fashion. The pacing leads were then connected to the defibrillator and the device was tested in the standard fashion.
Visit Schedule/Study Procedures	Refer to procedure study flow chart After baseline assessments were completed, and the independent reviewers confirmed eligibility, admission was arranged within a three month period, at the Royal Brompton Hospital (RBH). On the morning of the procedure a 50 to 100 ml bone marrow aspirate was taken from the iliac crest with local anaesthetic and sedation by a consultant anaesthetist (RBH) and a consultant haematologist from the Royal Marsden Hospital. This sample was then taken to The Hospital for Sick Children Great Ormond Street (GOSH) and prepared. Mononuclear cells were separated by Ficoll gradient and reconstituted in 5% HSA and Saline.
Evaluation Safety	Peri-procedurally patients were monitored according to PCI guidelines. All patients had continuous ECG monitoring by telemetry for 24 hours after the procedure and remained in the high dependency unit for 48 hours and in hospital for five days to monitor safety and to enable daily ¹¹¹In-oxide scans. Following the treatment procedure, study patients returned for periodic follow up visits to assess the presence of adverse events, clinically significant changes

	in laboratory parameters, onset of arrhythmias or clinically significant ECG changes and deteriorating heart failure.
Evaluation Efficacy Primary	Hypothesis generating outcome measures were assessed prior to and at six months post treatment • Exercise MVO₂ • Quality of Life (SF36, Minnesota) • Perfusion (SPECT) • Function (Echo, Gated SPECT) • NYHA class

b. Procedure Study Flow Chart

Assessments	Screening	0	1	2	5	14 (±3)	21 (±3)	30 (±3)	60 (±7)	90 (±14)	180 (±14)
Medical History	X										
Angiography	X										
Physical Exam (Complete)	X	X									X
Physical Exam (Limited)			X	X		X		X		X	
Concomitant Medication	X	X	X								
Adverse Event Assessment	X	X	X	X	X	X	X	X	X	X	X
Vital Signs	X	X	X	X		X		X		X	X
Clinical Lab Tests (including total CPK, CPK-MB, Troponin I)	X	X	X	X		X					X
ECG Holter monitor or Interrogation of ICD	X	X	X					X			X
BMC Administration		X									
ECG (12 Lead)	X		X	X							
Chest X-ray	X		X								X
Exercise Testing VO₂ Max	X										X
Myocardial Perfusion (SPECT)	X										X
Myocardial Function stress (ECHO)	X										X
New York Heart Association Class	X										X
QOL: Minnesota/ SF-36 Health Surveys	X										X

c. Summary of the Clinical Trial including timings



d. Recruitment

I sent a letter to all the cardiac surgeons and cardiology consultants at the Royal Brompton Hospital (RBH) and also external consultants who refer to the RBH, detailing the trial, including laminated inclusion and exclusion criteria. I also undertook a number of informal presentations to ward staff and registrars to inform them of the study and the inclusions and exclusions. Patients were then referred either by letter to the RBH or directly from the wards or out patients at the RBH for assessment. Baseline assessment consisted of a SPECT scan to determine reversible ischaemia and an estimate of left ventricular ejection fraction, an assessment of peak myocardial oxygen consumption (MVO₂), NYHA class, Quality of life Questionnaires, bloods, a holter monitor or if an ICD was in-situ, this was interrogated. An angiogram was undertaken if the patient had not had one in the previous 12 months, to exclude the possibility of conventional forms of revascularisation. We initially included stress echo as part of the baseline assessments but due to this technique being temporarily withdrawn from clinical use further patients did not have an echo and the last five patients had 2D and 3D echo, to assess

the relative merits of both techniques for use in later trials.

e. Independent eligibility review

The results of the screening procedures were compiled and presented to an independent interventional cardiologist (Professor Kim Fox) and cardiac surgeon (Professor John Pepper) who are not associated with the study for consideration for enrolment. It was the independent reviewer's responsibility to confirm that a patient met all of the study stated inclusion criteria and was not encumbered by any of the stated exclusion criteria prior to determining that a patient may participate in the study. The independent reviewers operated under the following guidelines to determine whether the patient was an appropriate candidate for participation in this study:

1. The independent reviewer utilised the patient's complete medical history in assessing whether the risks associated with study procedures were reasonable. Patients who had a history of cancer (except low grade and fully resolved non-melanoma skin malignancy) were not eligible for this study.
2. The patient had a documented history of symptomatic IHD and was receiving optimal medical therapy.
3. The patient had a LVEF < 40%.
4. The patient had angiographic evidence of multi-vessel CAD that was not amenable to percutaneous or surgical revascularisation, and the patient was not suitable for placement of a LVAD or a suitable recipient for heart transplantation.
5. There was no evidence of neoplasia on the chest X-ray.

The investigators were available to provide assistance regarding the patient's potential as a candidate for the study; however they did not participate in the deliberations of the independent reviewers.

f. Optimisation of medical management and eligibility for CRTD

Dr Theresa Mc Donagh or Dr Rakesh Sharma ensured that heart failure management was optimised and standardised by reviewing all patients. Any suggested changes to medication were documented in the notes. If any changes were made to medications

the patient was stabilised for one month prior to treatment. If any standard class of drug were omitted such as a beta-blocker or a statins this was documented in the notes and a reason given for omission i.e. intolerance.

Although all patients were referred for CRTD, not all individuals fulfilled National Institute for Health and Clinical Excellence (NICE) guidelines; therefore all patients were independently assessed for eligibility for CRTD by independent heart failure consultants Dr Theresa Mc Donagh or Dr Rakesh Sharma prior to enrollment in our study.

g. Consent

Patients scheduled for CRTD who fulfilled all entry criteria and were not encumbered by any exclusion criteria were approached to see if they were willing to take part in this study. The Royal Brompton and Harefield Hospital Ethics Committee approved this study, and all patients gave written informed consent.

h. Anaesthetic

As for sternal bone marrow harvest described before (Clinical Methods 6.3b)

i. Bone marrow harvest

Following administration of morphine, the patient was asked to lie in the left lateral position and skin preparation and draping commenced, intravenous midazolam was commenced. The patient was then positioned on his side with both legs drawn up so that his spine was curved outwards making the posterior iliac crest of the pelvis more prominent. This bony protrusion can usually be easily identified, lateral to the base of the spine near the sacral promontory. A pillow was placed between the patient's knees, and a sheet or blanket placed over his legs and feet. Throughout the procedure it was very important to ensure that the patient did not suffer undue pain and if this was the case, more anaesthetic was introduced.

The skin on the back of the pelvis overlying the posterior iliac crest was swabbed with 2% chlorhexidine. The bony protrusion of the posterior iliac crest was identified with the fingers of one hand, and 1 ml local anaesthetic solution of 1% or 2% lignocaine

were introduced intradermally above this point with a 25-gauge needle, raising a bleb on the skin. The needle was then changed to a 21-gauge needle, and 5 to 10 ml of 1% or 2% lignocaine was slowly introduced, firstly into the subcutaneous fat and then into the periosteum. A 'pricking' technique was used, introducing small amounts of solution subperiostially each time the needle was moved over the area. After ascertaining that the anaesthetic had taken effect, the skin over the bony prominence was held steady, and the aspiration needle with its stylet locked into position was carefully pushed through the skin. The needle was steadily pushed through the subcutaneous tissue until it could be felt resting on the underlying bone. The needle was pushed through the cortex of the bone to a depth of 0.5 cm to not more than 1cm, at which point the introducer was removed. A 10 ml aspiration syringe containing 0.5ml of preservative-free heparin 1,000 IU units per ml was placed on the end of the marrow needle and about 5 ml of bone marrow was withdrawn which was quickly handed to me, I injected the marrow into a 600 ml capacity blood transfusion transfer pack containing 3 ml of heparin via the injection port, agitating the contents continuously to ensure that no clots formed.

The bone marrow needle was withdrawn from the bone but not from the skin hole and the introducer was replaced before the needle was again introduced into the underlying bony prominence, about 0.5 cm from the previous site of aspiration. A further 5 ml of marrow was withdrawn into a fresh heparinised syringe and handed to me. Four or five aspirations were made with a single needle before this was replaced. If only 50 ml of marrow were aspirated in total, only one hole in the skin was made and the needle was moved about into the underlying bone for the necessary 10 aspirations of 5 ml each, due to the skin elasticity. When 100 ml were aspirated an additional skin hole was sometimes required.

After the procedure, pressure was exerted on the operation site until haemostasis was secured and the site was then cleaned again and a Mepore dressing applied. The port on the transfer pack containing the marrow was closed with the bung and transferred to GOSH for further processing.

Procedure duration from administration of sedation to recovery was approximately twenty minutes. During the procedure, patients were either drowsy or sleeping and

occasionally responded to the procedure, particularly during aspiration, with local movement or respiratory stimulation.

On transfer to recovery all patients were responsive and the majority lucid. No patient recalled the actual bone marrow harvesting procedure.

No patient complained of any local discomfort at the harvest site. No adverse effects related to the sedation process were experienced or observed. All patients maintained stable observations throughout the procedure and no cardiovascular or respiratory interventions were required.

j. Transportation to GOSH

As per previous please refer (Clinical methods 6.3 c)

k. Ficoll and pre and post assessment of cell differential and viability

As per previous please refer (Clinical methods 6.3 d, e and f)

l. Dosing

Initially we had planned to undertake a small ($n = 6$) pilot with a dose of 30×10^6 , which had previously been utilised and published by the Texas Heart Institute in a similar group of patients (Perin et al., 2003). However after completion of our first patient (please refer to protocol amendment 7.5 a) dose escalation was incorporated.

Three patients received 3×10^6 BMMNC

Three patients received 3×10^6 BMMNC + 3×10^6 ^{111}In BMMNC

Three patients received 15×10^6 BMMNC + 3×10^6 ^{111}In BMMNC

Three patients received 30×10^6 BMMNC + 3×10^6 ^{111}In BMMNC

Three patients received 60×10^6 BMMNC + 3×10^6 ^{111}In BMMNC

Three patients received 100×10^6 BMMNC + 3×10^6 ^{111}In BMMNC

m. ^{111}In -oxide labelling

As per previous please refer to (Clinical methods 6.3 i)

n. Delivery

Central venous access was achieved via the left subclavian vein and the coronary sinus was cannulated using the Medtronic Attain ® Coronary sinus delivery system. A diagnostic catheter (R Judkins) was inserted through the Attain and a coronary sinus venogram was performed. The best lateral coronary vein was selected for left ventricular lead placement and was positioned using standard techniques. The CS delivery system was left in place and the CS was ‘double cannulated’ leaving the initial Attain in position and through the second Attain a Judkins Right 5F angiographic catheter (Expo ®, Boston) was inserted. An appropriate coronary vein for stem cell delivery was subselected to target, where possible, viable but underperfused myocardium based on a prior SPECT scan. A venogram was then performed with a 1:1 dilution of contrast medium (Ultravist, Iopromide, Berlix, Germany) and normal saline using a hand-powered injection through a 10 ml syringe, in order to create an intramyocardial ‘blush’ (Figure 24). The assumption was this is indicative of endothelial disruption and a ‘mechanical push’ of cells through the endothelium into the myocardium. Putative bone marrow stem cells were then injected using similar force with a 10 ml syringe, and the catheter was left in place for ten minutes. This procedure was repeated in another coronary vein again through the second Attain.

After the second delivery the second Attain and diagnostic catheter were both removed and the right ventricular defibrillator lead and atrial lead were positioned in the usual fashion. Finally, the Attain used to deliver the LV lead was removed (by slitting the Attain catheter). The pacing leads were connected to the defibrillator (Contak Renewal ® 3 RF Boston inc) and the device was tested in the standard fashion. Immediately after the procedure patients were transferred directly to the department of Nuclear-Medicine to assess delivery efficiency.

o. Imaging of cells

All acquisitions were performed using a GE Medical Systems Infinia Hawkeye GP3 dual detector gamma camera system. Whole body acquisitions were performed at various times up to 21 days following the administration of ¹¹¹In-oxide labelled autologous stem cells. Simultaneous anterior and posterior whole body acquisitions were acquired in continuous mode using a medium energy high-resolution collimator

system at a scan speed of 8 cm/min. A dual energy window was employed at 171keV +/-10% and 245keV +/-10%. The scan was set to include the whole of the patient and using the numbered graduations on the scanning bed the patient's position on the bed was recorded and this was used to locate the patient in the same place on the table for each subsequent acquisition. After the whole-body scan patients were transferred back to the ward.

On completion of whole body acquisitions, the homing and bio-distribution of the stem cells was analysed by drawing anterior and posterior regions of interest (ROIs) around the liver, heart (site/s of isotope delivery), spleen, whole body and background. For consistency all the ROIs for each organ were kept the same throughout each patient's study. The counts from each ROI were then entered into a spreadsheet. These were corrected for background and then radioactive decay corrected to the time of the injection. The geometric mean of the counts for each organ was calculated and plotted against time to generate time/activity curves. Bone marrow uptake was calculated by subtracting all the organ readings from the whole body reading and time/activity curves were generated. In addition the radioactivity in each organ was expressed as a percentage of whole body uptake.

At 24 hours post administration of the tracer a tomogram of the thorax with computer tomography (CT) localisation was performed in addition to the whole body scan. The tomogram was acquired with the detectors in a 90° configuration. Acquisition was performed in step and shoot mode in counter clockwise direction over an arc of rotation of 180° (90° per detector) divided into 60 projections (30 per detector) and using a medium energy high- resolution collimator system. The duration of acquisition was set at 40 seconds per projection and the data were recorded into a 64 x 64 matrix with a 1.3 zoom. A triple energy window was employed with windows at 171keV +/-10% and 245keV +/-10% for the ¹¹¹In photo peaks and a 140keV +/- 9% scatter window for scatter correction of the CT acquisition. The emission and CT acquisitions were performed sequentially with automatic repositioning of the patient between the gamma camera detectors and the CT detector between the two stages, the patient was asked not to move during the entire procedure to enable accurate post acquisition registration of the datasets. The emission tomogram of the thorax was acquired with the cardiac area central in the field of view. Then, using the persistence

image, an area was defined within this image to include the whole cardiac area for the CT acquisition – care was taken to ensure that the site or sites of uptake of ^{111}In -oxide stem cells was within the CT field. The number of CT slices and the CT dose were recorded. The CT acquisition was performed with a tube voltage of 140kV, a current of 2.5mA and a rotational velocity of 2 RPM into a 256 x 256 matrix.

The CT data were reconstructed in the transverse, coronal and sagittal planes and then registration with the SPECT data was performed using a dedicated GE Healthcare Xeleris workstation and Volumetrix software tool. Fused CT and SPECT images were used to localise uptake of the tracer within the myocardium by a single blind observer using the standard 17-segment model. Instances of uptake seen within the right ventricle were noted.

Patients were observed on the high dependency unit for 48 hours before moving to the general ward. Patients remained in hospital for five days to monitor safety and for us to undertake daily ^{111}In -oxide scans.

7.3 FOLLOW-UP SAFETY

This study was designed as a safety feasibility study; efficacy end points were included merely for hypothesis generating purposes.

a. Safety Reporting

The first patient we treated resulted in a serious adverse event and was thus reporting both to the DSMB and Ethics committee leading to alterations in the protocol, which are outlined in (7.5 a).

b. Holter

Monitors were applied for 24 hours as part of the patient's baseline assessment apart from those who already had an ICD in situ, which was interrogated at the pacing clinic.

c. X-ray

Routine chest X-rays were undertaken pre-treatment and at six months post treatment.

d. Bloods

The following blood tests were performed:

Routine full blood count (FBC), white cell count (WCC), Potassium, urea, creatinine, glucose, bilirubin, alkaline phosphatase (ALP), Gamma-glytanyl transpeptidase (Gamma GT), alanine aminotransferase (ALT), calcium, aspartate aminotransferase (AST), C-reactive protein (CRP), B-type natriuretic peptide (BNP), Troponin-I, Creatine phosphokinase MB isoenzyme (CKMB).

7.4 FOLLOW-UP EFFICACY

a. Echocardiography

Standard clinical 2D echocardiography as previously described and published (Duncan et al., 2006) and investigational 3D echocardiography were performed at baseline and 6 months post treatment in the last five patients. To minimise variability and to improve reproducibility all studies were performed and analysed by the same consultant cardiologist.

i. 2D Echocardiography

Transthoracic Transthoracic echocardiography was performed using a Phillips Sonos 5500 echocardiograph and a multifrequency transducer (Andover, MA, USA). Standard two-dimensional parasternal long and short axis views and apical two, four and five chamber views were performed. Cross-sectional two-dimensional guided M-mode recordings of the LV minor axis were also obtained. Mitral regurgitation (MR) was recorded using colour-flow Doppler and continuous wave Doppler in the apical four and two chamber views, and its duration, expressed as a percentage of the RR interval, was calculated. Subaortic flow velocity was obtained by pulsed-wave Doppler from the apical five-chamber view, with the sample volume placed in the LV outflow tract, 1 cm below the aortic cusps. LV ejection time (ET) was measured using leading edge methodology as the interval between the onsets of forward aortic flow and aortic valve closure artefact. Stroke distance was calculated as the time integral of aortic velocity, stroke volume as the product of stroke distance and subaortic area, and cardiac output (CO) as stroke volume multiplied by heart rate. Transmitral flow velocities were recorded from the apical four chamber view using pulsed-wave Doppler. Isovolumic relaxation time (IVRT): the interval between A2 and the onset of

mitral flow) and peak E (early diastolic) and A (atrial) velocities were obtained in all patients. Filling time (FT) was measured using leading edge methodology from the onset of the E-wave to the end of the A-wave. Isovolumic contraction time (IVCT) was calculated by subtracting the sum of IVRT (ms), ET (ms), and FT (ms) from RR interval. Total LV ejection and filling periods were derived as the product of the corresponding time interval and heart rate, expressed in s/min. Isovolumic time (t-IVT) is the time in the cardiac cycle when the LV is neither ejecting nor filling. CRT has been found to shorten (t-IVT) (Duncan et al., 2003) and non-responders to CRT have been found to have a prolonged (t-IVT) (Duncan et al., 2006). We were particularly interested on the effect of stem cells on (t-IVT) especially as we had historical data on the effects of CRT used as sole therapy (Duncan et al., 2006).

ii. 3D Echocardiography

Transthoracic 3D Echo was performed using a 2,5 MHz transducer mounted in a handheld rotation device. The cardiac images were recorded from the apex during end-expiratory apnoea within one breath hold, whereby the need for respiratory triggering was abolished. Imaging was performed using tissue harmonic mode using coaxial rotation from the apical position and ECG-triggered recording where each R-wave initiated a 30° stepwise rotation of the transducer. A total of six scanning planes obtained by 30° stepwise rotation of the transducer covered the entire LV. To eliminate disturbances caused by cardiac arrhythmias, only R-R intervals within 20% of the mean were accepted. Ejection fraction was calculated by the formula $EDV - ESV / EDV \times 100$. Body surface area (BSA) was used to calculate End diastolic volume index (EDVi) and End systolic volume index (ESVi) of the LV. A Philips Qlab analysis was used from the apical four and two chambers views to delineate the LV endocardial border, and the LVEF was calculated from the equation $(LV\ EDV - LV\ ESV) / LVEDV$: LVEDV = end-diastolic volume, LVESV = end-systolic volume

b. SPECT

The perfusion imaging was performed with ^{99m}Tc -Tetrofosmin or ^{99m}Tc -Sestamibi with same day stress rest protocol. The imaging was performed prior to and six months after the stem cell injection.

Stress images were acquired over a 180° arc of rotation with 32 projections at 45 seconds per projection. Data were acquired into a 64 x 64 matrix for three energy windows, 140keV +/- 10% for the technetium-99m photopeak, 118keV +/- 6% for the scatter window. Rest acquisition was performed similar to stress studies but with an acquisition time of 60 seconds per projection to allow for gated imaging. Data were acquired into 16-gated bins triggered on the ECG R-wave with a beat rejection window of +/-20% and 1 premature ventricular contraction (PVC) rejection. Post acquisition reconstruction of data was done using an OSEM iterative reconstruction method with a post reconstruction Butterworth filter and cardiac images were re-orientated in the vertical long axis, horizontal long axis and short axis planes using the Cedar Sinai Quantitative Perfusion SPECT (QPS) and Quantitative Gated SPECT (QGS) software.

The images were analysed by two experienced nuclear physicians based on the 17 segment model (Cerqueira et al., 2002). The cardiologist who performed the cell injection predicted the site of stem cell delivery on 17 segment model by viewing the myocardial blush. This area was referred to as the injection area. The rest of the myocardium was referred to as the remote area. The segments were scored for viability (50% uptake in comparison with the maximally perfused area within the entire myocardium) as **V** score, ischaemia **I** score and functional score of wall motion and thickening **F** score. Two experts compared the images before and after the stem cell delivery. The change in terms of improvement (+), no change (=) and deterioration (-) was documented with consensus. All 18 patients were scored with VIF scores.

c. MVO₂

Height and Weight were recorded and three spirometry tests were performed. An ECG and saturation probes and blood pressure cuff were attached and recording taken. The patient was then asked to stand, a mask attached and four minutes of resting measurements were taken, before the treadmill was started.

Stage 1- 1mph at 5%

Stage 2- 1.7mph at 10%

Stage 3- 2.5mph at 12%

Stage 4- 3.4mph at 14%

Stage 5- 4.2mph at 16%

Stage 6- 5mph at 18%

Stage 7- 5.5mph at 20%

Stage 8- 6mph at 22%

The patient was pushed to their maximum and a reason for cessation documented. $\dot{V}O_2$ units were calculated as ml/kg/min, peak respiratory exchange ratio (RER) above 1 indicated that patients had probably reached their maximum capacity whereas values under 1 indicated that patients had not reached their maximum. $\dot{V}E/\dot{V}CO_2$ slopes measured the patient's perfusion efficiency; all measurements were calculated using the Medical Graphics Ultima series.

d. Pacing

Routine biventricular device checks were performed at six weeks and at six months, because all patients had ICD's in situ it was possible to interrogate their devices and arrhythmias, along with any episodes of defibrillation, were noted.

e. Quality of Life Questionnaires

The SF36 version II and the Minnesota living with heart failure questionnaire were undertaken before treatment and six months after treatment. The SF36 version II has a number of questions that can be pooled to give a physical health score and a mental health score, which is how the data is shown.

f. Statistics

The protocol for the clinical trial was discussed with statisticians from the Department of Occupational and Environmental Medicine at the National Heart and Lung Institute, Imperial College, prior to commencement and advice was given for design and analysis. I assessed all data for normality, by plotting histograms and concluded that my data was not normally distributed. I therefore used Wilcoxon Signed Ranks test for non-parametric paired data, Kruskal Wallis for multiple comparisons between dose cohorts and for correlation Spearman's rho. The null hypothesis was rejected when ($P < 0.5$). Median values are shown, with range. Data was analysed by IBM SPSS 19 for microcomputers.

g. Patient Demographics

The baseline demographics of all patients are shown in (Table 16).

Table 16: The Baseline demographics of all Patients

Baseline Patient Demographics	
Age Median (Range)	71 (54 to 84)
Male Gender %	100
Diabetes %	17
Hypercholesterolemia %	100
Smoking %	17
Multivessel Disease %	100
LVEF SPECT	25% (16 to 41)
LVEF ECHO	23% (16 to 40)
MVO ₂ ml/min/kg	11 (4.5 to 18.5)
NYHA	3 (2 to 4)
Previous Myocardial Infarction %	100
Previous PCI %	67
Previous CABG %	78
ICD already in situ %	33

Table 16: Values are median and range or percentage number of patients. All values are calculated from all 18 patients apart from MVO₂, which was calculated from 17/18 patients. New York Heart Association (NYHA) functional class, myocardial oxygen consumption (MVO₂), Left ventricular ejection fraction (LVEF), single photon emission computed tomography (SPECT), echocardiography (ECHO). Myocardial infarction (MI), coronary artery bypass Graft (CABG), percutaneous coronary intervention (PCI), internal cardiac defibrillator (ICD).

7.5 RESULTS

a. Protocol amendments

We had initially planned to undertake a small pilot trial consisting of six patients utilising a dose of 30×10^6 BMMNC; a dose used and published by Emerson Perin at the Texas Heart Institute (Perin et al., 2003). After a largely uneventful procedure, our first patient developed angina on the second evening and associated hypotension. Four days after the procedure she developed signs of infected pulmonary oedema with a rising CRP and white blood cell count (neutrophilia), interstitial oedema on chest X-ray, persistent hypotension and deteriorating respiratory status, she was also noted to have a raised Troponin-I level. Masked CPAP and a Dobutamine infusion were commenced. She continued to have several episodes of unstable angina and clinically deteriorated over the pursuing days and was admitted to our intensive care unit six days after the procedure with a rising CRP and WBC count an elevated Troponin-I and deteriorating cardiac and respiratory status. She was commenced on infusions of Noradrenaline, Milrinone, Teicoplanin Ciprofloxacin and Meropenem. She remained in hospital for three weeks when she was discharged home. This patient made a full recovery and subsequently 12 months later had marked improvements in QOL, and reversible ischaemia on her SPECT scan. However it was concluded after reviewing all the data that a number of factors could have contributed to this patient's clinical deterioration and subsequent intensive care unit admission. Because of the number and complexity of the variables administration of the bone marrow stem cells could not be excluded as a possible contributing factor. The data safety and monitoring board (DSMB) in conjunction with our team therefore made the following alterations to the protocol and amendments to the Ethics proposal.

1. The total cell number delivered was reduced by one log from 30×10^6 to 3×10^6 for at least the next three patients. If no significant safety issues were detected, then the dose was to be increased to 15×10^6 for at least the next three subsequent patients. Finally if no significant safety issues were detected the subsequent patients were to be studied at the original dose of 30×10^6 . The n numbers of patients assigned to the initial open-label, safety and feasibility arm of the study was increased from six patients to nine to twelve to accommodate this dose escalation.
2. ^{111}In -oxide labelling was stopped until the first cohort of patients had been

reviewed without labelled cells, in case the labelled cells were responsible for any of the clinical sequela.

3. The delivery strategy remained the same.
4. The DSMB reviewed data from each of the patient cohorts, and made further recommendations as appropriate.
5. The patient information was altered to reflect these changes, including a summary of the first patient's clinical course.

We noted no safety concerns in the first dosing cohort so we instigated ^{111}In -oxide labelling of 3×10^6 cells for the subsequent cohorts. In this first cohort to receive ^{111}In -oxide labelled cells, one of the two coronary veins selected received the labelled cells whilst the other vein received unlabelled cells. The rationale for this approach was that by injecting unlabelled cells to a different territory to the labelled cells we minimised the potential for any by-stander effect and maximised the chances of any biological effect. The disadvantage of this approach was that we could only potentially visualise one injected territory, which meant that we were unsure of the relative delivery efficiency in the second vein. After discussion with our DSMB it was concluded that all subsequent dosing cohorts would receive an admixture of both ^{111}In -oxide labelled and unlabelled cells into both veins, patients (7 to 18). Finally after completing the above series of patients with no further safety issues anecdotal evidence and discussion with the Texas Heart Institute highlighted that 30×10^6 may be well under the dose response required to see a biological effect and they advised us to increase our dose to be in line with what they were using to 100×10^6 . We opted to do this in two further cohorts; the first of these received 60×10^6 and the second 100×10^6 . Our ethics committee approved this further increase in dose.

The subsequent 18 patients were treated from June 2006 through to February 2009. All following data presented and analysed pertains to this group of patients.

b. Procedural time and Hospital stay

The mean total procedural time including testing of the ICD was 75 minutes. All patients remained in hospital for four days to facilitate ^{111}In -oxide scans. There were no procedural or peri-procedural complications. Two patients had transient

hypotension two days post delivery, associated with raised CRP requiring inotropic support.

c. Adverse events

In the six month follow-up period there were no Major Adverse Cardiac Events (MACE). One patient was readmitted with worsening heart failure, one patient had one appropriate ICD shock; this particular patient had an ICD in situ prior to treatment and had seven previous shocks in the preceding two years. In one patient there was a left ventricular lead displacement. One patient had a PCI and a stent placed in the LCX, and one patient had severe diverticular disease requiring surgery and a temporary colostomy in addition to repositioning of a prominent ICD pulse generator.

d. CRP, Troponin-I and CKMB

We found a consistent pattern of rise in CRP after delivery of cells (Figure 18). This peaked three days after delivery at a median 32 mg/L (range 3 to 74) and returned to baseline values by day nine. The median rise in CRP per group is shown in (Figure 19). Troponin-I peaked at 12 hours post delivery at 1.04 ng/ml (range 0.32 to 14.2) (Figure 20) and was back to baseline levels in the majority of cases by day four. There was no dose response, the median value for each dose are shown in (Figure 21). CKMB also followed the same pattern with a peak at 12 hours 5.4 ng/ml (1.9 to 61.3) (Figure 22), returning to baseline values in the majority of cases by 48 hours, again there was no dose response (Figure 23). The normal reference ranges for the Royal Brompton Hospital were; CRP < 10 mg/l, CKMB (0 to 6.0 µg/l) and Trop-I < 0.04 µg/l.

Figure 18: CRP

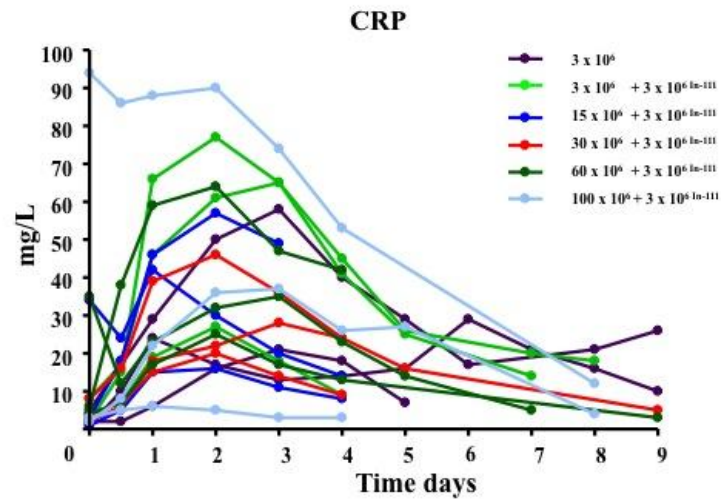


Figure 18: Baseline is at time 0, and then C-reactive protein (CRP) is plotted at 12 hours post delivery and then daily until baseline levels were seen. The majority of patients had a peak in CRP on the third day post delivery. Each dose is colour coded and shown in the legend. N= 18. The normal reference value for the Royal Brompton Hospital for CRP < 10 mg/l.

Figure 19 Median Group CRP

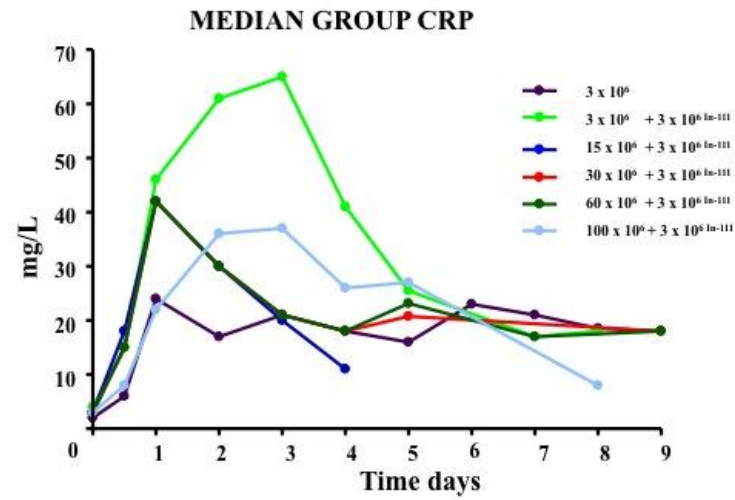


Figure 19: Baseline is at time 0, and then median group C-reactive protein (CRP) level is plotted at 12 hours post delivery and then daily until baseline levels were seen. Each dose is colour coded and shown in the legend. N= 18. The normal reference value for the Royal Brompton Hospital for CRP < 10 mg/l.

Figure 20: Troponin-I

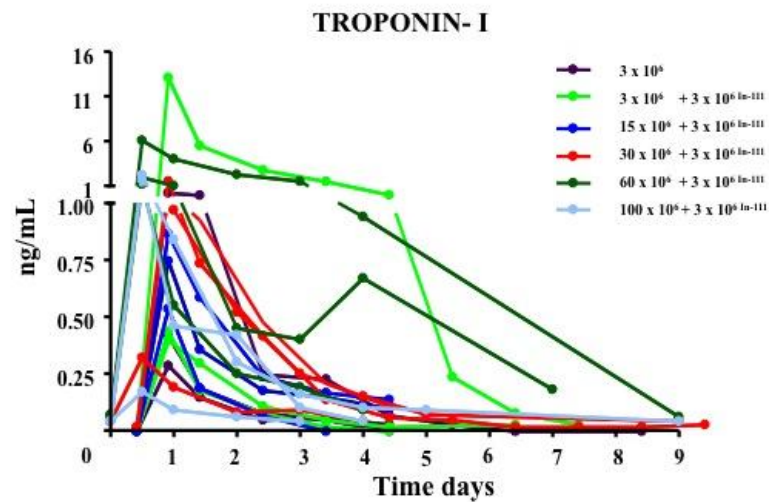


Figure 20: Baseline is at time 0, Troponin-I is plotted at 12 hours post delivery and then daily until baseline levels were seen. The X-axis is split at 1.00ng/ml for ease of review. The bottom axis is from 0 to 1 and the top axis 1 to 16. Each patient is colour coded per dose as shown in the legend. N= 18. The normal reference value for the Royal Brompton Hospital Trop-I < 0.04 µg/l.

Figure 21: Median Group Troponin-I Levels

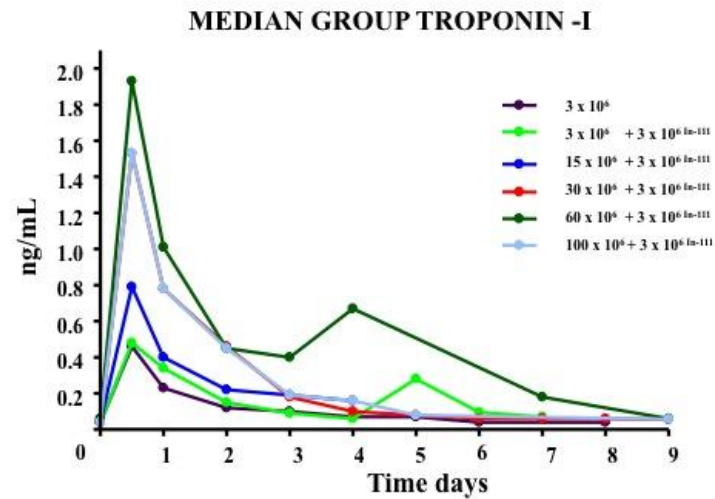


Figure 21: Baseline is at time 0, the median Troponin-I is plotted at 12 hours post delivery and then daily until baseline levels were seen. The median rise in all groups peaked at 12 hours. Each patient is colour coded per dose as shown in the legend. N= 18. The normal reference value for the Royal Brompton Hospital Trop-I < 0.04 µg/l.

Figure 22: CKMB

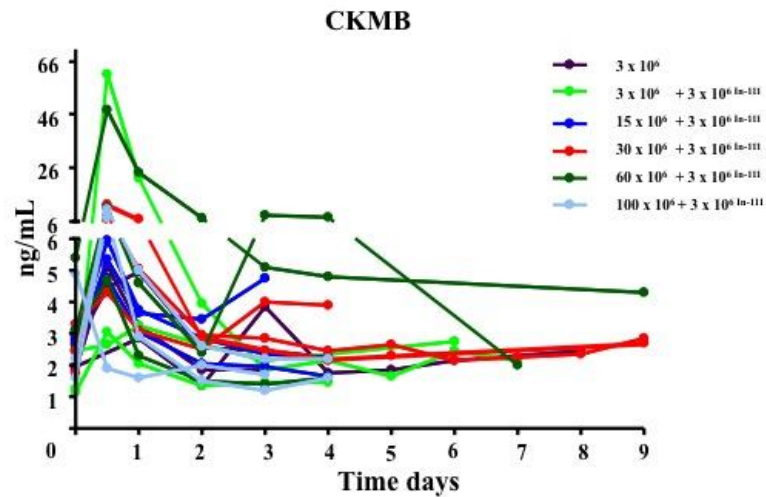


Figure 22: Baseline is at time 0, Creatine Kinase-MB (CKMB) is plotted at 12 hours post delivery and then daily until baseline levels were seen. The X-axis is split at 6 ng/ml for ease of review. The bottom axis is from 0 to 6 and the top axis 6 to 66. Each patient is colour coded per dose as shown in the legend. N= 18. The normal reference value for the Royal Brompton Hospital CKMB (0 to 6.0 µg/l).

Figure 23: Median Group CKMB

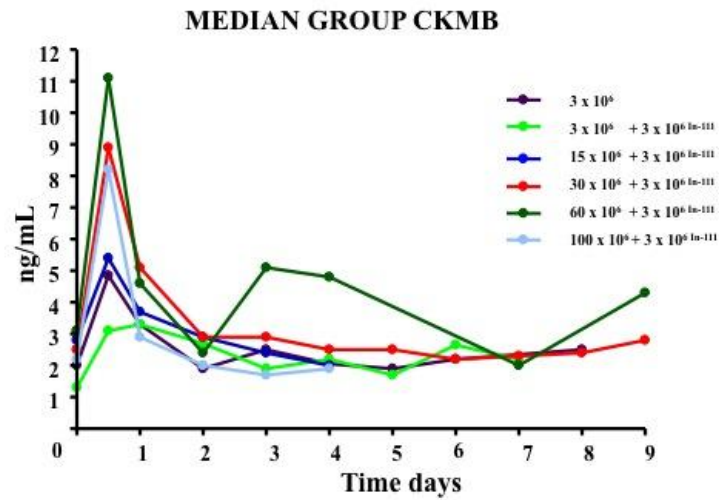


Figure 23: Baseline is at time 0, the median Creatine Kinase-MB (CKMB) is plotted at 12 hours post delivery and then daily until baseline levels were seen. Each patient is colour coded per dose as shown in the legend. N= 18. The normal reference value for the Royal Brompton Hospital CKMB (0 to 6.0 $\mu\text{g/l}$).

e. Pacing review

All patients underwent pacing optimisation with echocardiography guidance 6 weeks after implantation in line with normal clinical practice. One patient was found to have received an appropriate shock for ventricular fibrillation (VF) four days post stem cells and upgrade to CRTD. As stated in the adverse events this particular patient already had an ICD in situ prior to stem cell delivery and had seven previous appropriate shocks in the preceding two years. Pacing interrogation six months after delivery in all other patients was unremarkable with no other episodes of sustained VT, VF or shocks.

f. Delivery

The cellular constituents of the injectate are shown in (Table 17) together with the CD34% /dose in (Table 18A) and the actual number of CD34+ cells given per dosing group in (Table 18B). Following delivery of 3×10^6 unlabelled cells in to each of the first three patients, we studied myocardial cell retention in patients (4 to 18). 3×10^6 cells were labelled with 20 mBq of ^{111}In -oxide, resulting in a median ^{111}In -oxide dose of 8.5 mBq (3.5 to 22.9) and a median labelling efficiency of 2.83 Bq/cell (1.17Bq/cell to 7.6Bq/cell). The ^{111}In -oxide labelled cells were initially injected into one coronary vein ('Target' 1 in Fig 24A), while 3×10^6 unlabelled cells were injected into a separate vein ('Target 2' in Fig 24B), in patients (4 to 6). However to better visualise both injection sites later patients (7 to 18), had an admixture of labelled and unlabelled cells injected into both veins.

Our intention to deliver cells into two coronary veins was achieved in 17 of the 18 patients, in one patient it was difficult to find a secondary target vein and thus we injected both instillations into the same vein with a 10 minute interval between injections. In one of the 18 patients we were unable to achieve a sustained myocardial blush during instillation because of extensive collateralisation with no subsequent ^{111}In -oxide detected in the myocardium on the whole-body scan taken one hour post delivery (Figure 34). In the remaining 17 patients we were able to detect both a myocardial blush on the coronary venogram and ^{111}In oxine within the myocardium at one hour post delivery, which, extended up to ten days in all patients.

Table 17: Median and mean differential cell composition of all patients

Cell	Median %	[Range]	Mean %	SD
CD34	1.1	0.3 to 3.3	1.4	0.9
CD3	68	49 to 78	66.6	8.1
CD19	9.5	1 to 21	8.7	5.18
NK (CD16+56)	15	6 to 22	14	4.5
CD4	41	27 to 54	41.5	7.6
CD8	22	10 to 41	23.5	9.6
Viability	97.5	89 to 99	96.9	2.3

All values are expressed as a percentage (%) of the total cell composition. Note some cells are positive for more than one category; hence the total value exceeds 100%.

Table 18A: Median and mean CD34+ cells per dosing group

Patients	In- ¹¹¹ BMMNC	BMMNC	Median CD34%	[Range]	Mean CD34%	SD
(1-3)	-	3 x 10 ⁶	2.1	2.0 to 2.1	2.1	0.1
(4-6)	3 x 10 ⁶	3 x 10 ⁶	1.4	1.1 to 2.1	1.5	0.5
(7-9)	3 x 10 ⁶	15 x 10 ⁶	0.6	0.3 to 3.3	1.4	1.7
(10-12)	3 x 10 ⁶	30 x 10 ⁶	1	0.8 to 3.0	1.6	1.2
(13-15)	3 x 10 ⁶	60 x 10 ⁶	0.6	0.4 to 0.8	0.6	0.6
(16-18)	3 x 10 ⁶	100 x 10 ⁶	0.8	0.8 to 1.4	1.0	0.4

All values are expressed as percentages (%) of the total cell composition.

Table 18B: Median and mean CD34+ cells given per dosing group

Patients	BMMNC Dose	Median CD34 Given x 10 ⁶	[Range]	Mean CD34 Given x 10 ⁶	SD
(1-3)	3 x 10 ⁶	0.06	0.06 to 0.06	0.06	0
(4-6)	3 x 10 ⁶	0.04	0.03 to 0.06	0.05	0.01
(7-9)	15 x 10 ⁶	0.10	0.04 to 0.50	0.21	0.25
(10-12)	30 x 10 ⁶	0.29	0.24 to 0.89	0.48	0.36
(13-15)	60 x 10 ⁶	0.35	0.25 to 0.48	0.36	0.12
(16-18)	100 x 10 ⁶	0.79	0.77 to 1.39	0.98	0.35

The number of CD34+ cells given per group.

Figure 24: Delivery of Contrast into target coronary veins

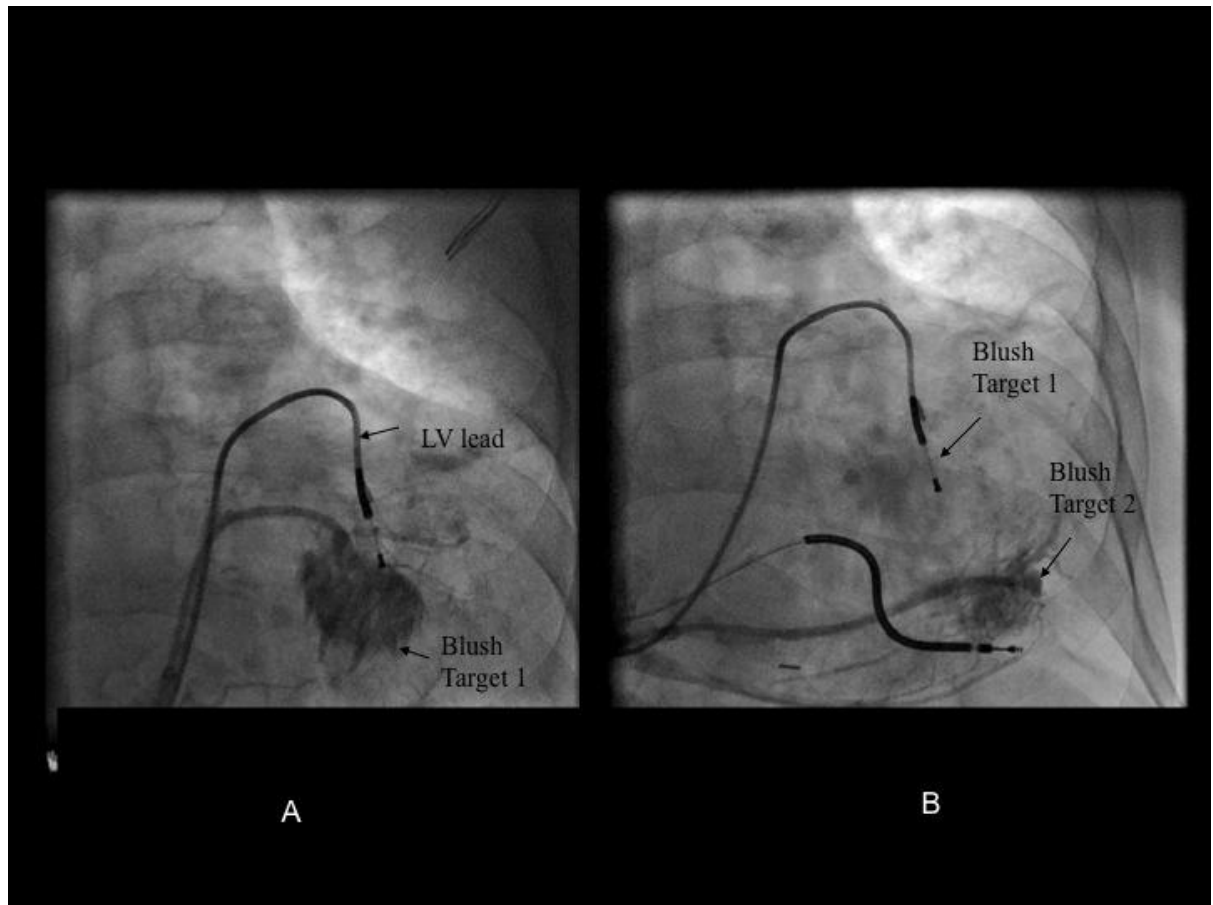


Figure 24: A coronary angiogram demonstrating the delivery of contrast into a coronary vein of a patient immediately prior to delivery of putative stem cells. In image **A** the left ventricular lead (LV) can clearly be seen in situ in a lateral intracoronary vein and a myocardial blush from our first instillation of contrast into the first target vein. On image **B** is a second myocardial blush from instillation of contrast into the second target vein, above this evidence of the sustained first myocardial blush created 15 minutes prior to the second instillation can be visualised.

In order to initially confirm myocardial delivery and retention of ^{111}In oxide labelled cells we also co-administered Tetrofosmin in the first patient. (Figure 25A) shows the distribution of Tetrofosmin in the liver, gut and myocardium. In this patient the ^{111}In oxide signal overlays the Tetrofosmin myocardial ring (Figure 25B) and was also seen in the liver and spleen. In subsequent Hawkeye CT images of the thorax it was clear to see myocardial uptake of ^{111}In oxide (Fig 26). Therefore for all subsequent patients we relied on whole-body scintograms and Hawkeye CT at 24 hours to decrease the radiation burden to patients of co-administering two radioisotopes.

Initial whole-body imaging was performed within two hours after injection, and then daily until radioactive decay precluded diagnostic imaging. Serial whole body scintograms (Figure 27) showed that ^{111}In -oxide activity was clearly localised to the myocardium, liver and spleen and marrow from the first time point of imaging onwards. A representative graph of ^{111}In oxide washout curves, as a percentage of whole body activity for each organ over time are shown in (Figure 28).

The median initial myocardial uptake at two hours was 6.4% range (2.6 to 13), at 24 hours the median retention was 3.6% (range 1.1 to 9.6) of the delivered activity, decaying slowly to approximately 2.5% (range 0.7 to 5.4) by day ten. Note the physical half life of ^{111}In -oxide is 2.8 days, and thus with no biological excretion, in 14 days (5 physical half lives) the signal will have decayed to less than 5 % of its original value. The myocardial washout curves for all patients are shown in (Figure 29) and the myocardial time activity curves expressed as the natural log of exponential decay over time are shown in (Figure 30). Because radioactivity has an exponential rate of decay log to the base e (LN for short) is used. By plotting LN of counts against time it is possible to better visualise how the exponential decay behaves. By plotting the time activity curve for the various regions of interest (ROI) for each patient the decay was seen to be exponential. If the decay was monoexponential then a plot of LN of counts against time should produce a straight line but if it were biexponential then the resultant graph would show two different slopes. Our data appears to have two slopes; a rapid decline in the first 24 hours, followed by a second slower exponential decay phase. One could speculate that the initial fast decline was due to washout of ^{111}In -oxide from the vascular space, and the removal of 'dead cells'. There was a gradual increase in the percentage of the whole

body ^{111}In -oxide signal in the marrow overtime (Figure 28). However there was a decrease over-time in the ^{111}In -oxide signal in the liver and spleen (Figure 31).

Figure 25: Co-administration of Tetrofosmin and ^{111}In oxide

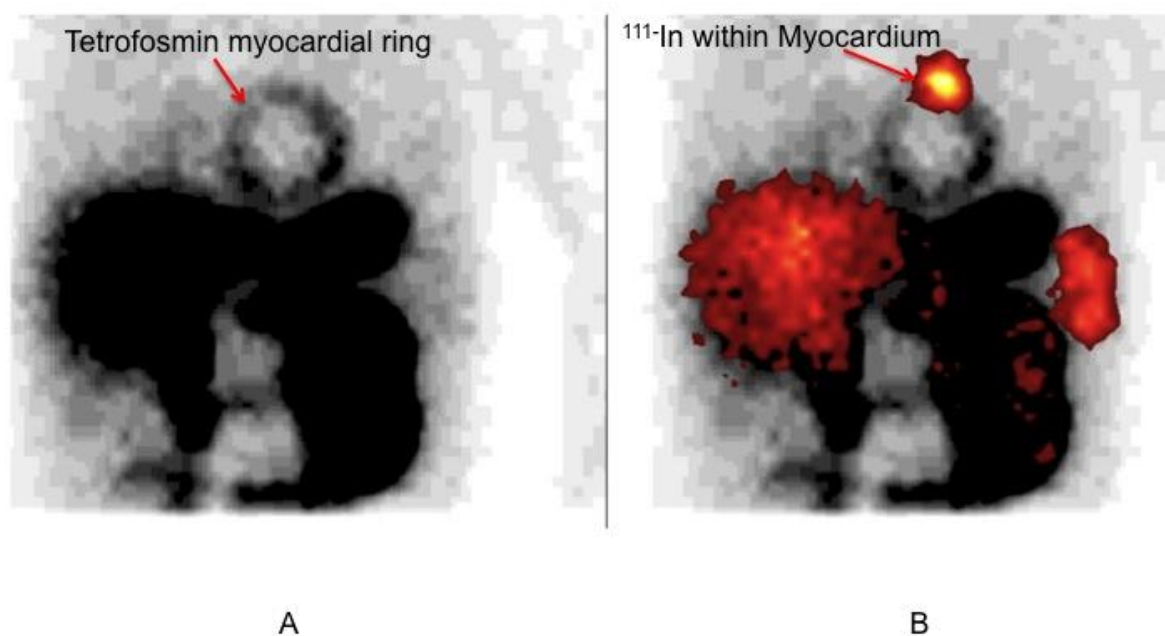


Figure 25: A Tetrofosmin distribution in the Liver, gut and myocardium, shown in black. **B** Co-administration of ^{111}In oxide demonstrating a myocardial signal overlaying the Tetrofosmin shown in red with the highest concentration of signal shown in yellow.

Figure 26: Hawkeye Computer Topographic (CT) Image of the localisation of ^{111}In oxide within the myocardium.

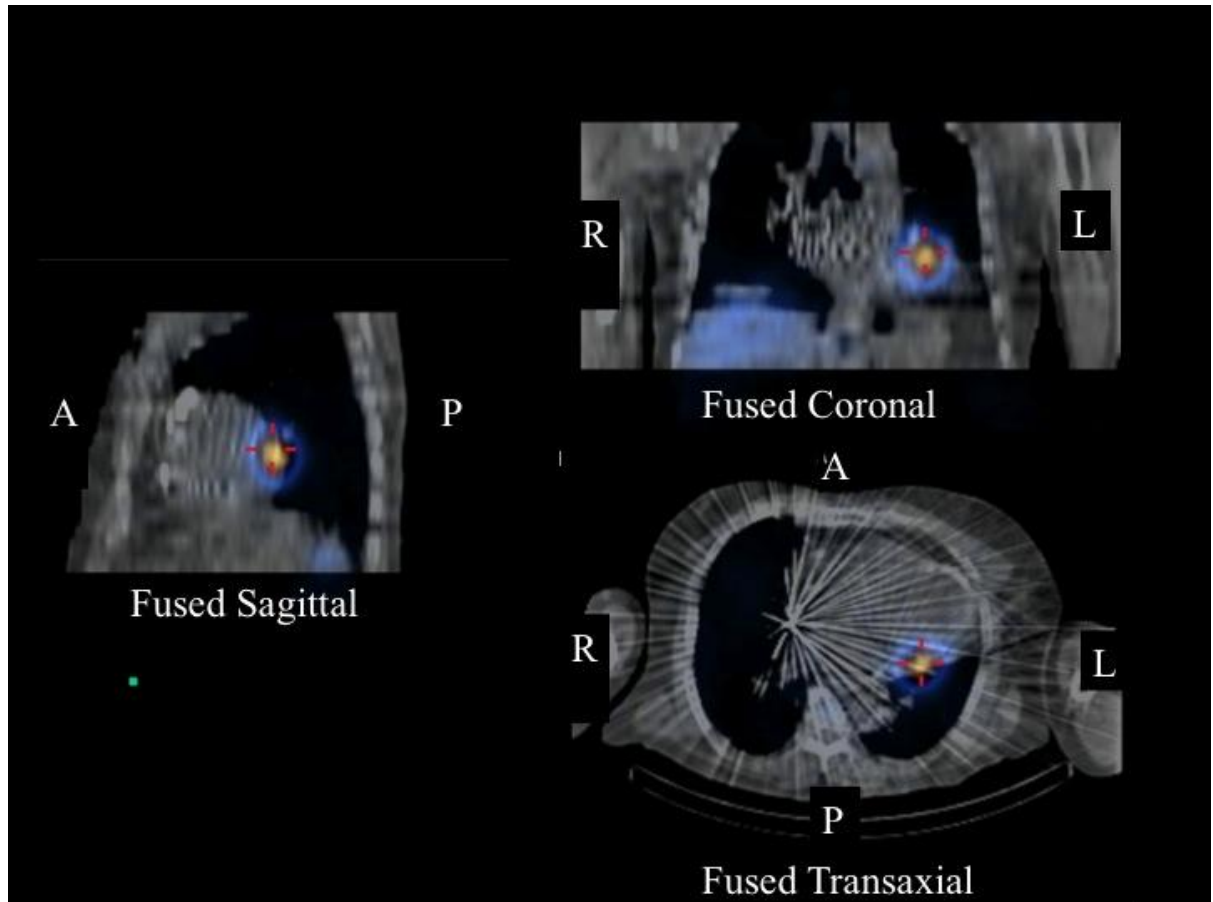


Figure 26: A CT Hawkeye image of the distribution of ^{111}In oxide shown within the myocardium 24 hours post delivery. ^{111}In oxide is shown as a blue signal with yellow showing the highest concentration in coronal, sagittal and transaxial planes. A = anterior, P=posterior, R=right and L=left.

Figure 27: Whole-body scintograms of the Distribution of ^{111}In oxide

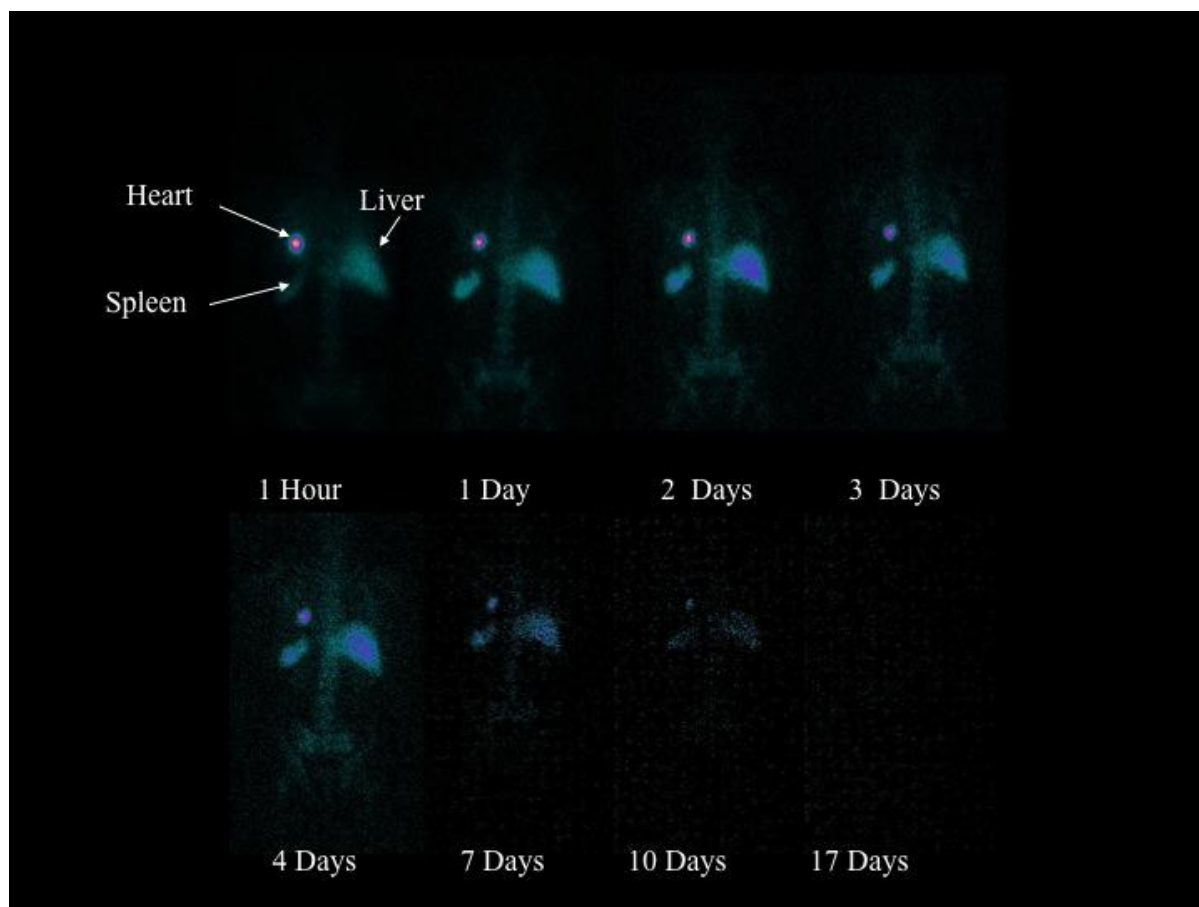


Figure 27: Images were taken from the posterior view. Distribution of ^{111}In oxide throughout the whole-body over a 17 day period is shown. At one hour post delivery arrows indicate ^{111}In oxide signal within the myocardium, liver and spleen. From the first day, there is an increase in signal in the bone marrow, and sustained signal within the myocardium, liver and spleen until the tenth day after this time-point the ^{111}In oxide decay prevents further imaging.

Figure 28: ^{111}In oxide washout in all organs

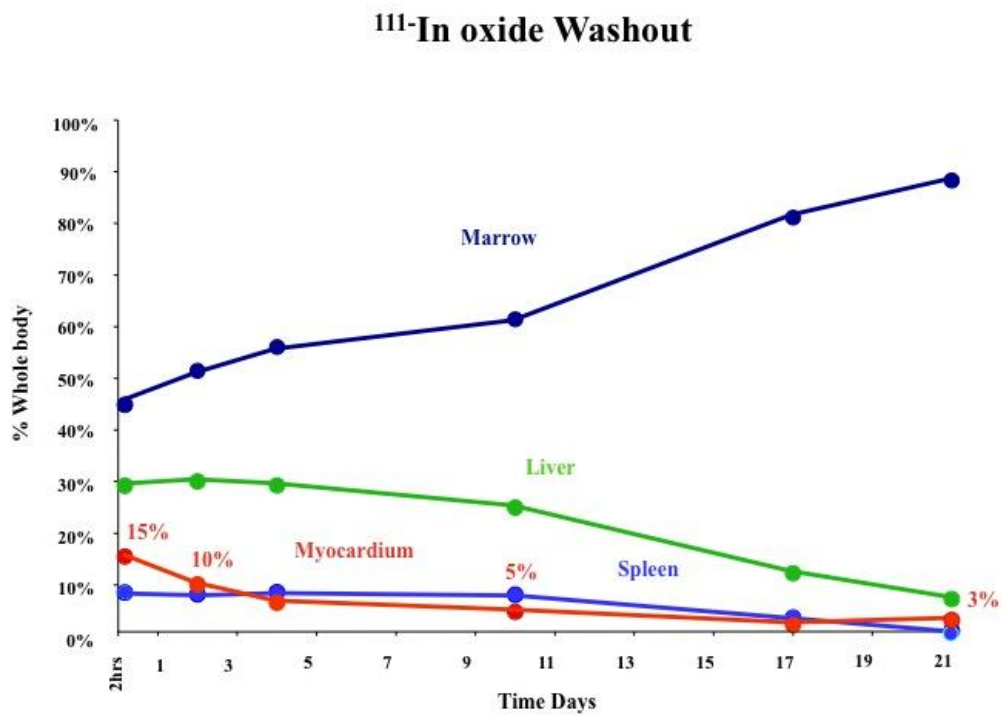


Figure 28: A representative slide of whole-body washout of ^{111}In oxide over-time. The red line and figures represent the myocardial percentage, mid blue is spleen, green is liver, and dark blue is marrow.

Figure 29: Washout curves for Myocardium

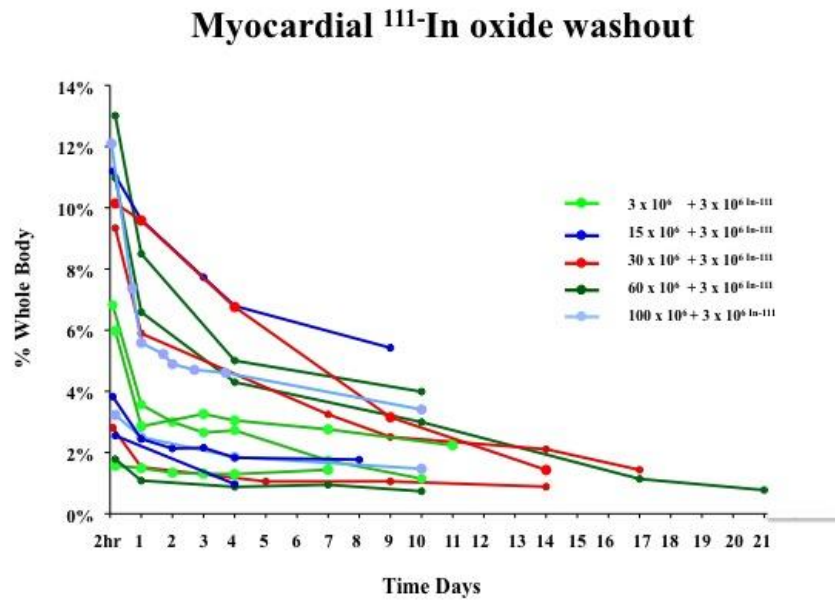


Figure 29: The Myocardial ^{111}In oxide washout as a percentage of whole body activity over time, each patient is colour coded by dose in the legend. One patient in the 100×10^6 had no ^{111}In oxide signal, thus data from the remaining 14 patients are shown above.

Figure 30: Myocardial decay (LN)

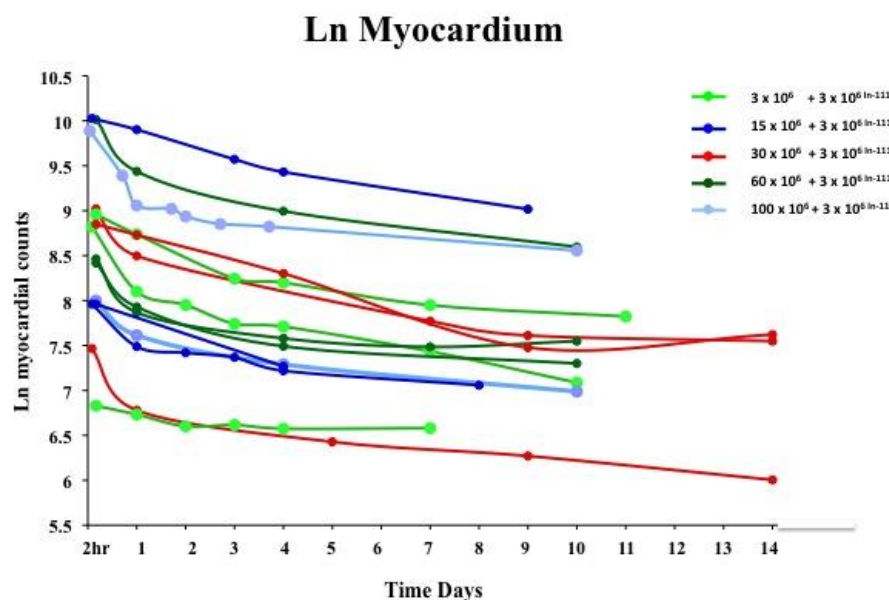


Figure 30:Myocardial time activity curves expressed as the natural log of exponential decay over time. Because radioactivity has an exponential rate of decay then log to the base e (LN) is used. Each patient is colour coded by dose in the legend. One patient in the 100×10^6 had no ${}^{111}\text{In}$ oxide signal, thus data from the remaining 14 patients are shown above.

Figure 31: Washout curves Liver and spleen

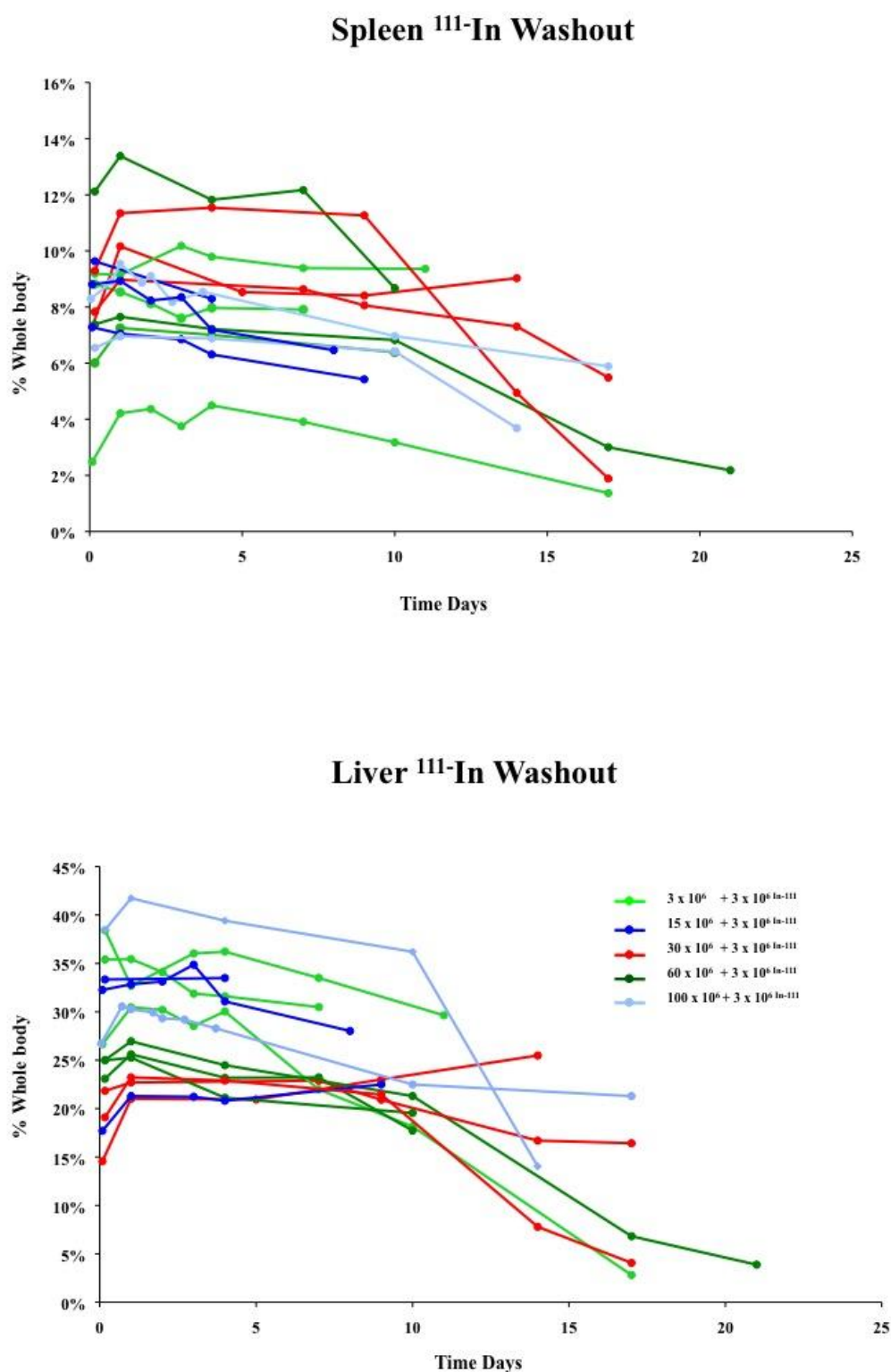


Figure 31: The Liver and spleen ^{111}In oxide washout as a percentage of whole body activity over time, each patient is colour coded by dose in the legend. One patient in the 100×10^6 had no ^{111}In oxide signal, thus data from the remaining 14 patients are shown above.

g. Delivery Maps

All assessments were undertaken in a blinded fashion, each by a single observer, and results plotted on separate standard 17-segment model sheets (Cerqueira et al., 2002). A consultant interventional cardiologist reviewed the patient's delivery venogram and made an assessment of the distribution of myocardial blush. An experienced nuclear radiographer reviewed the combined Fused CT and SPECT and then localised uptake of the ^{111}In oxide tracer within the myocardium. Finally a consultant radiologist documented the ischaemia from the baseline SPECT scan. I collated all the maps and by overlaying the three separate assessments it was possible to view the relative distribution on a 17-segment model of ischaemia, myocardial blush and ^{111}In oxide signal. The first three patients did not receive labelled cells, for the one patient with ischaemia present we were able to see that the blush overlaid the ischaemia. In the following patients 4 to 18 we were able to see a ^{111}In oxide signal in 14 of the 15 patients. In those with reversible ischaemia ($n=7$), two patients had myocardial blush, and a ^{111}In oxide signal overlaying their ischaemic segments (Figure 32). In four patients the myocardial blush and ^{111}In oxide signals were found in the same segments but they were adjacent to the ischaemic territory (Figure 33A and 33B). In one ischaemic patient there was no ^{111}In oxide signal but the transient blush did overlay the ischaemic segment (Figure 34). In the patients with no reversible ischaemia on their baseline SPECT scan who received ^{111}In -oxide labelled cells ($n=8$) the myocardial blush overlaid the ^{111}In -oxide signal in 7 patients (Figure 35A, 35B and 35C) and was in the adjacent segment in one patient (Figure 36). In the thirteen of the fifteen or 87% of patients that received ^{111}In oxide labelled cells, it was found to be in the same segment as the contrast blush on the venogram. The myocardial blush and ^{111}In oxide signal were found to be in the same segment as the ischaemia in two out of seven ischaemic or 29% of patients and in adjacent patients in four out of seven or 57% of patients. The n numbers are too small to make any assumptions regarding whether there is any advantage of targeting the ischaemic or adjacent segments.

Figure 32: Delivery maps in ischaemic patients where the ^{111}In -oxide signal overlays the myocardial blush

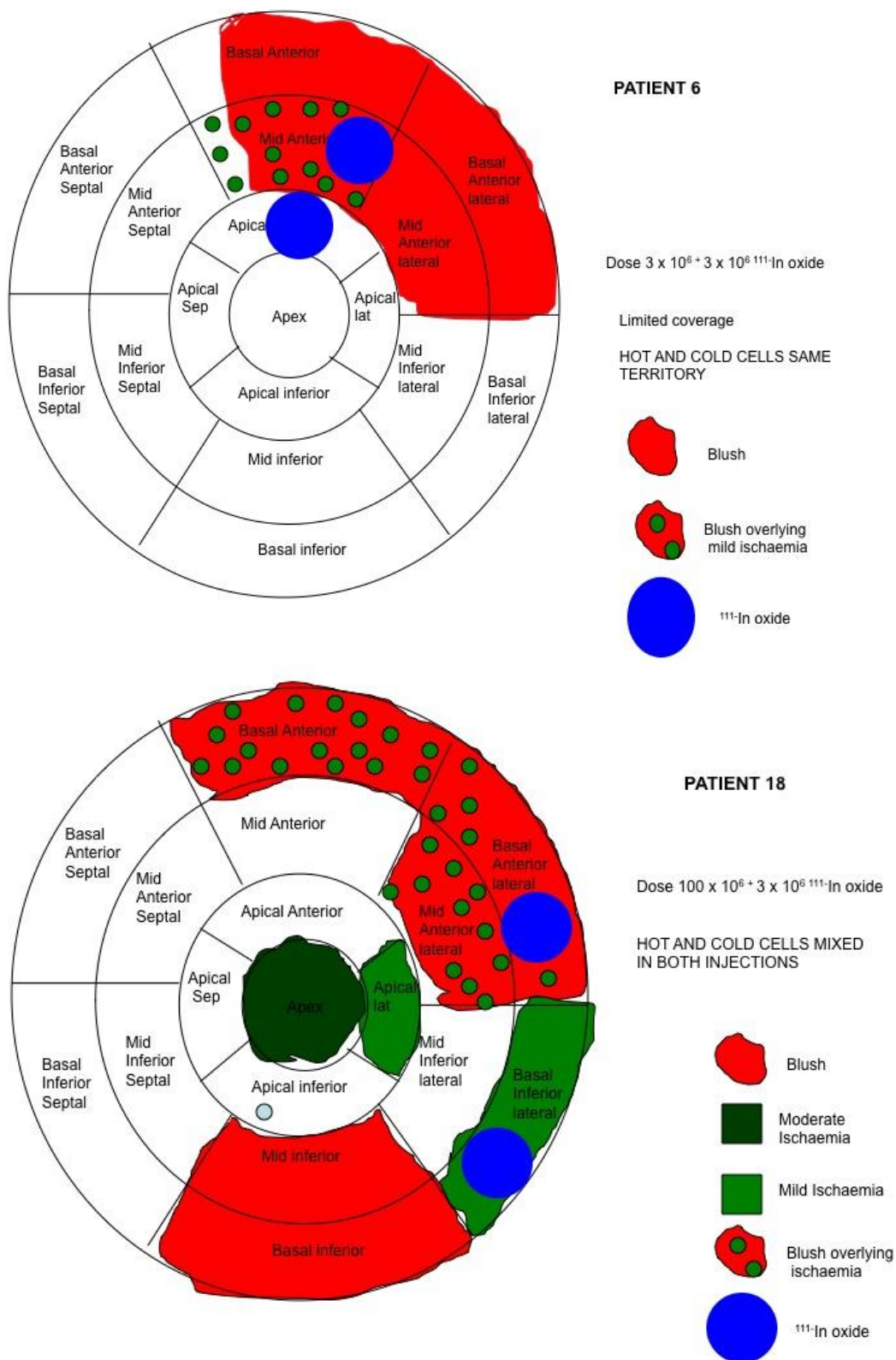


Figure 33A: Delivery maps in ischaemic patients where the ^{111}In -oxide signal overlays the myocardial blush but is adjacent to the ischaemic territory

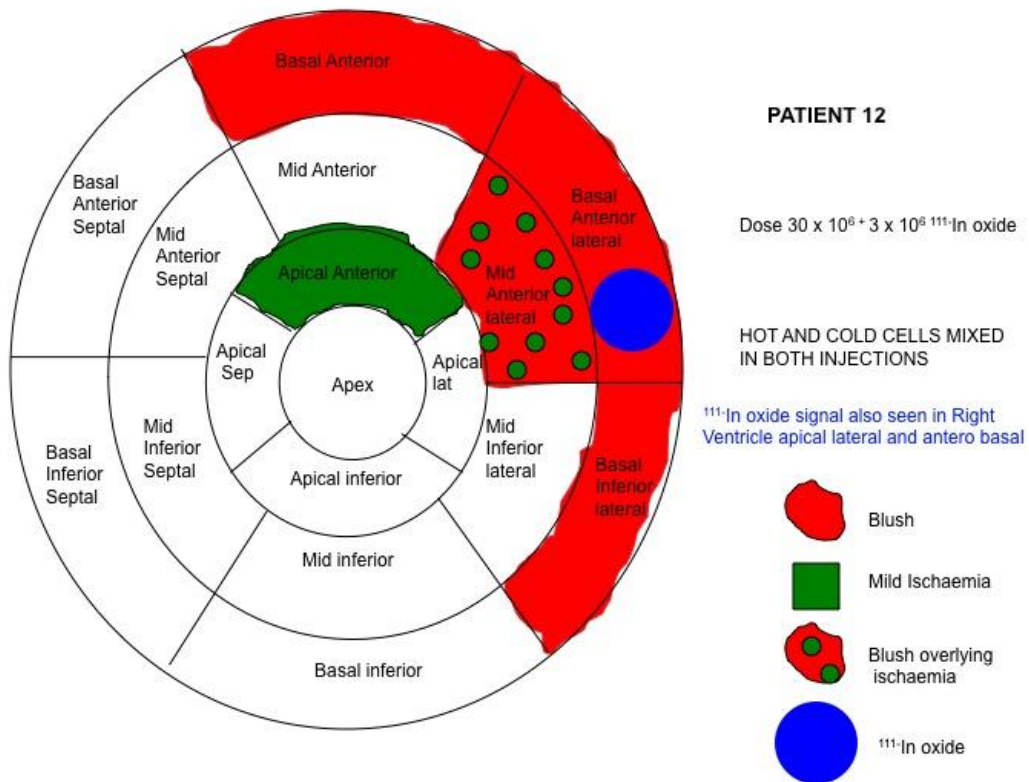
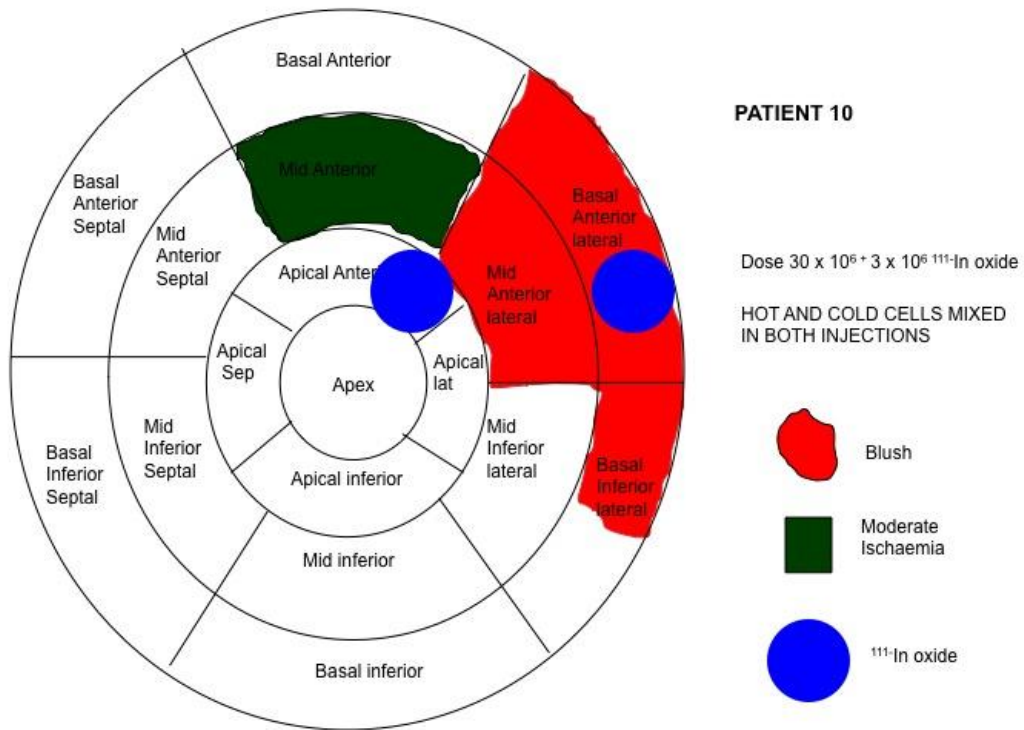


Figure 33B: Delivery maps in ischaemic patients where the ^{111}In -oxide signal overlays the myocardial blush but is adjacent to the ischaemic territory

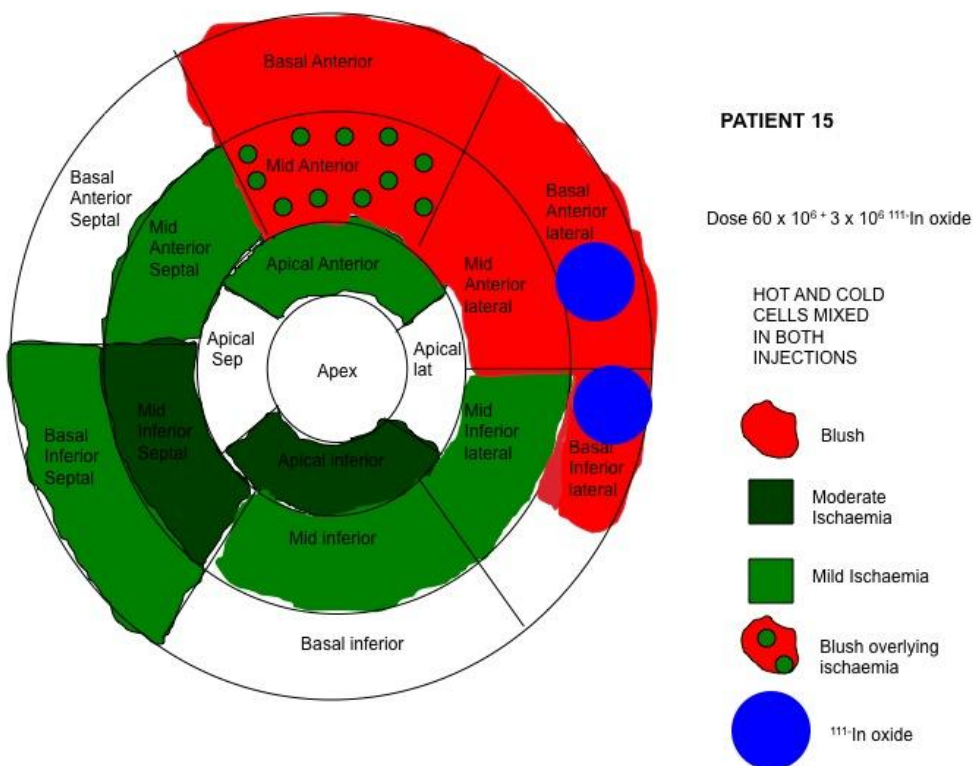
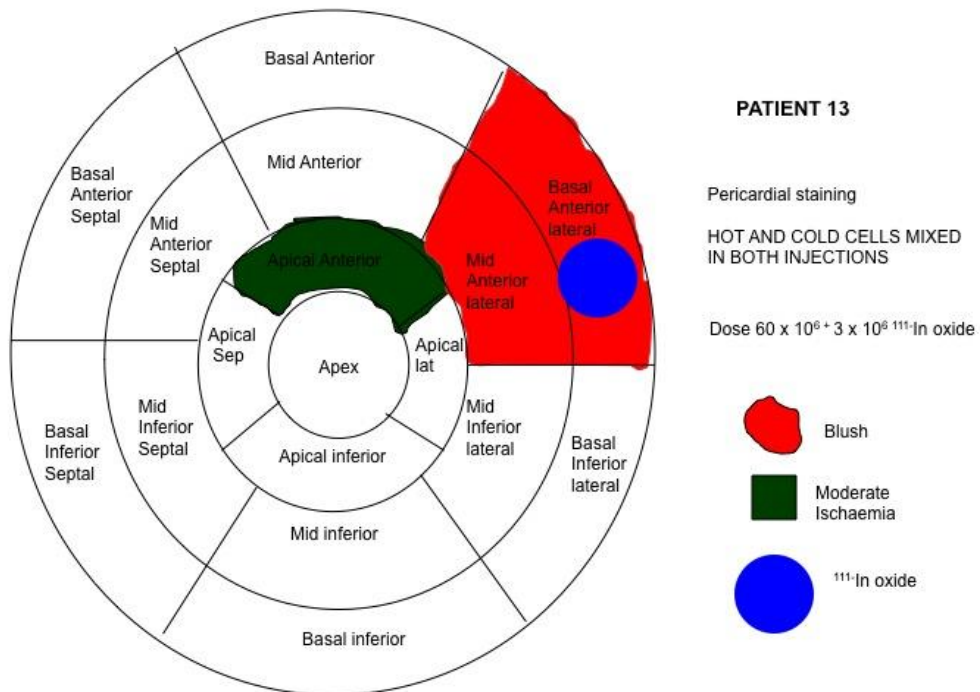


Figure 34: Delivery maps in an ischaemic patients where the myocardial blush overlays the ischaemic territory but there was no ^{111}In -oxide signal

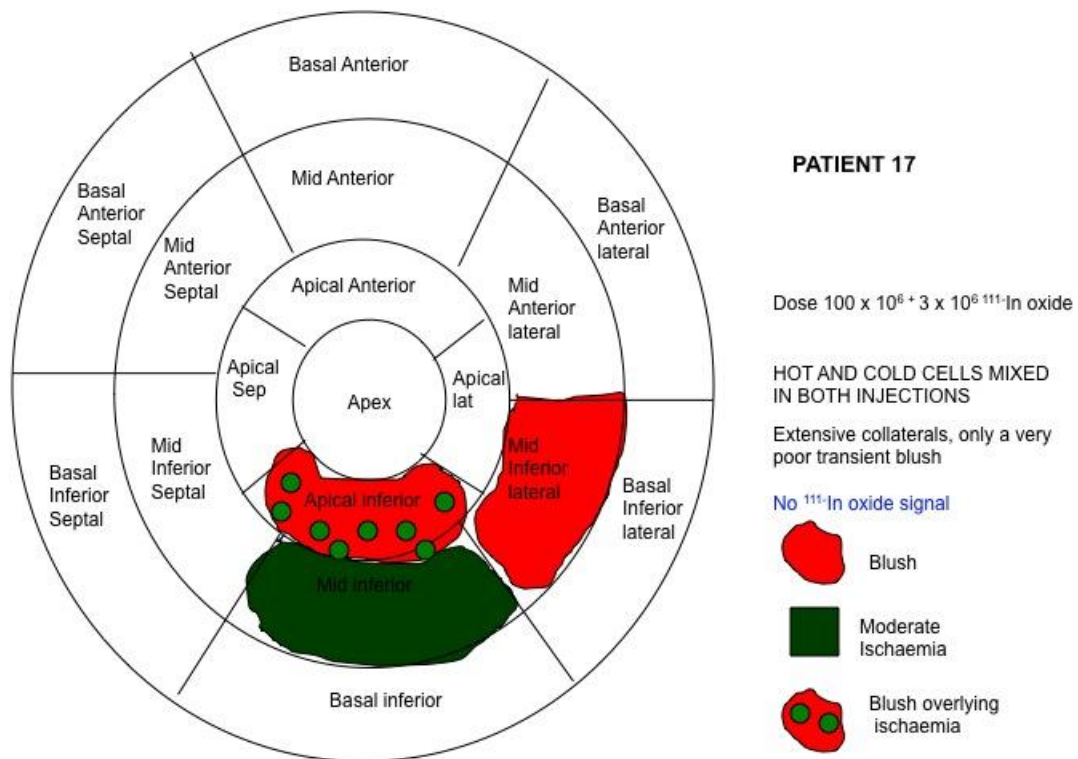


Figure 35A: Delivery maps in non-ischaemic patients where the ^{111}In -oxide signal overlays the myocardial blush

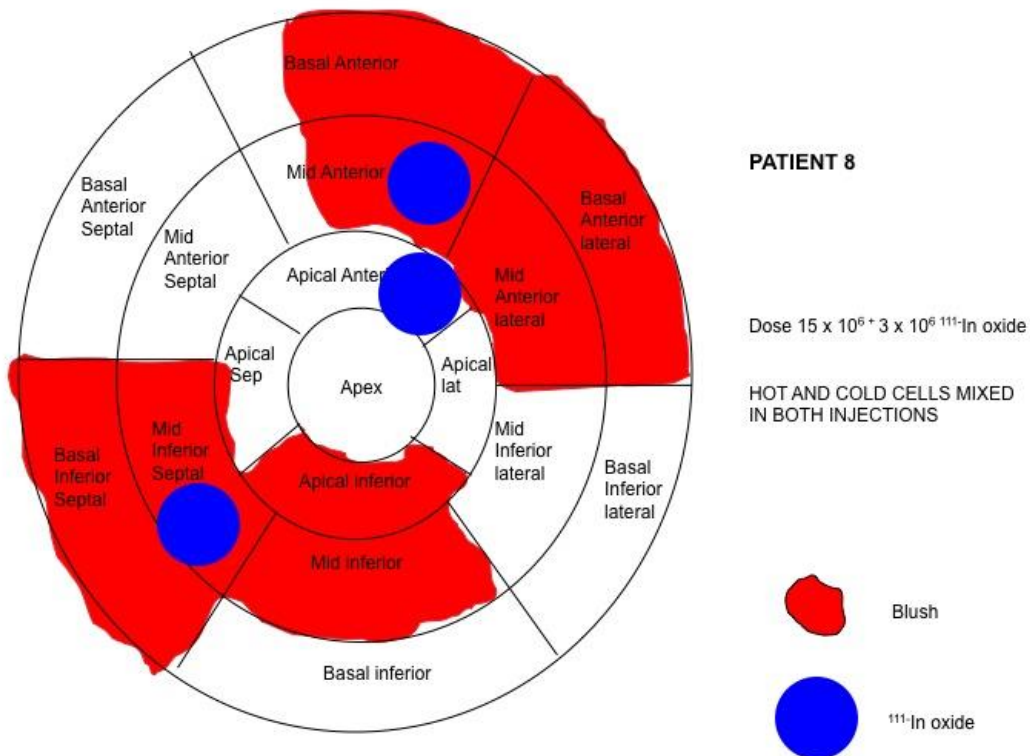
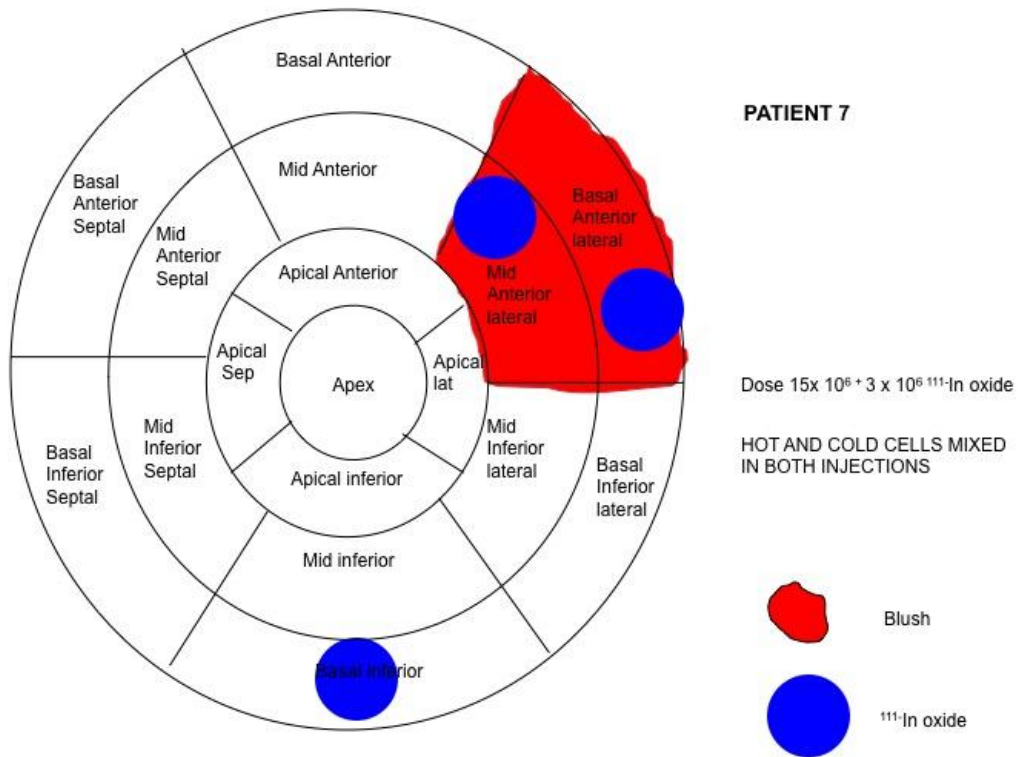


Figure 35B: Delivery maps in non-ischaemic patients where the ^{111}In -oxide signal overlays the myocardial blush

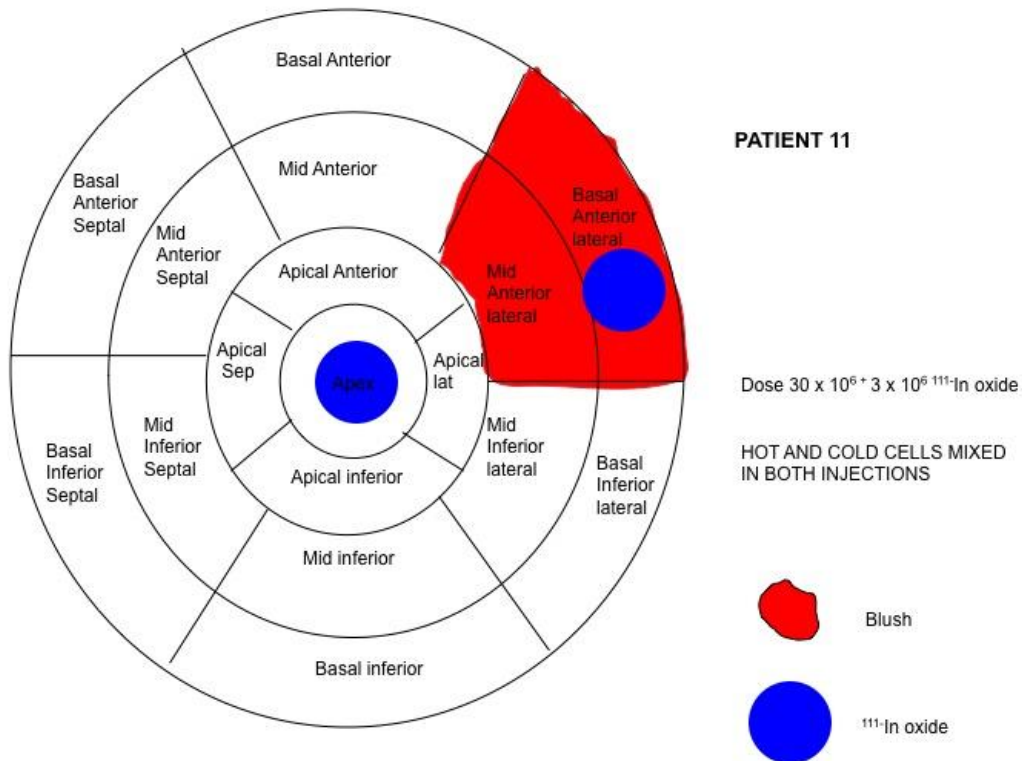
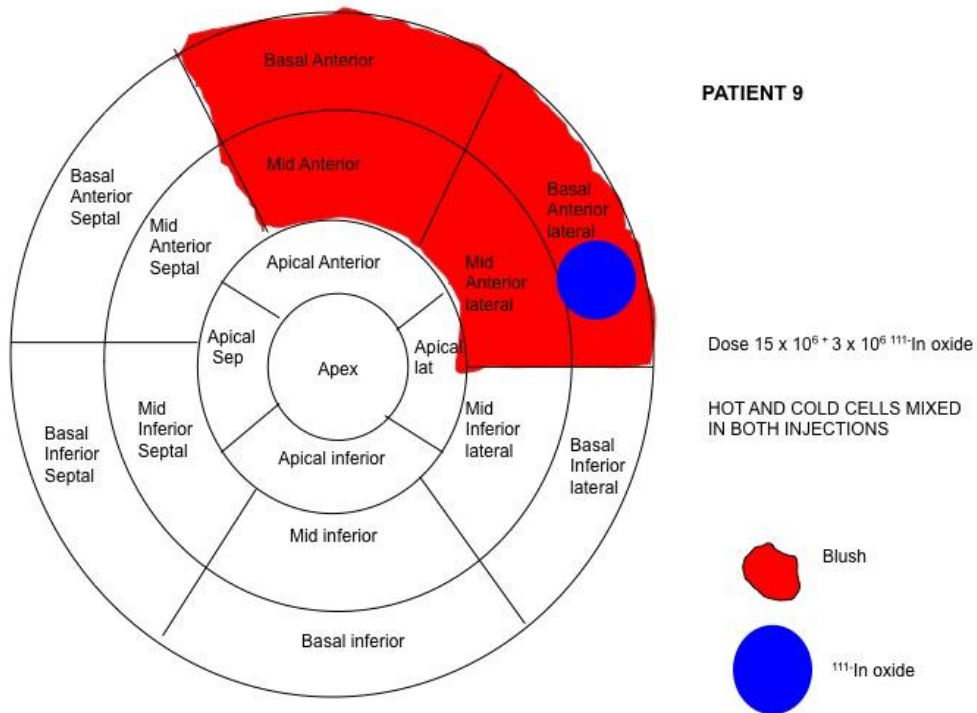


Figure 35C: Delivery maps in non-ischaemic patients where the ^{111}In -oxide signal overlays the myocardial blush

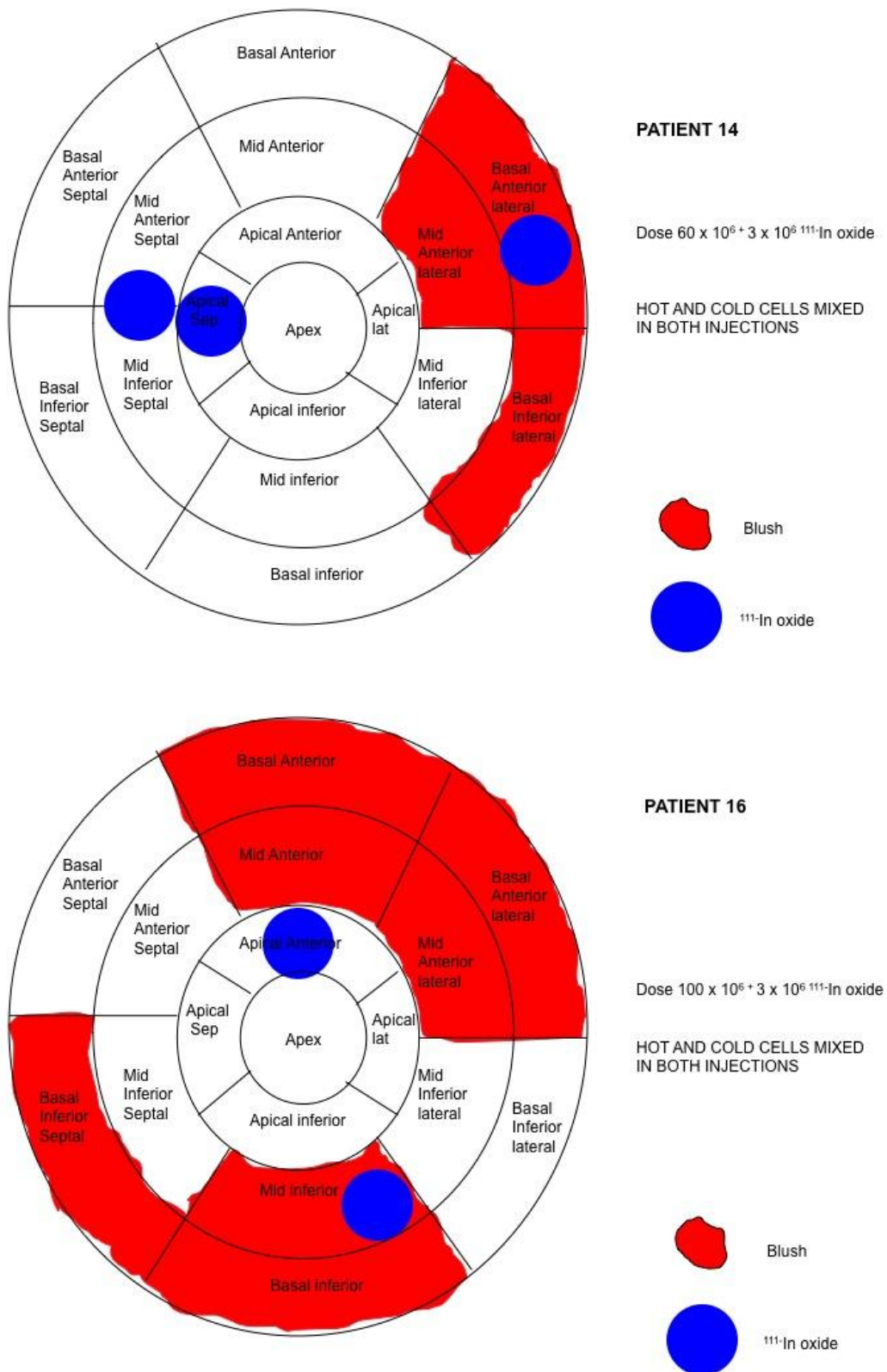


Figure 35D: Delivery maps in non-ischaemic patients where the ^{111}In -oxide signal overlays the myocardial blush

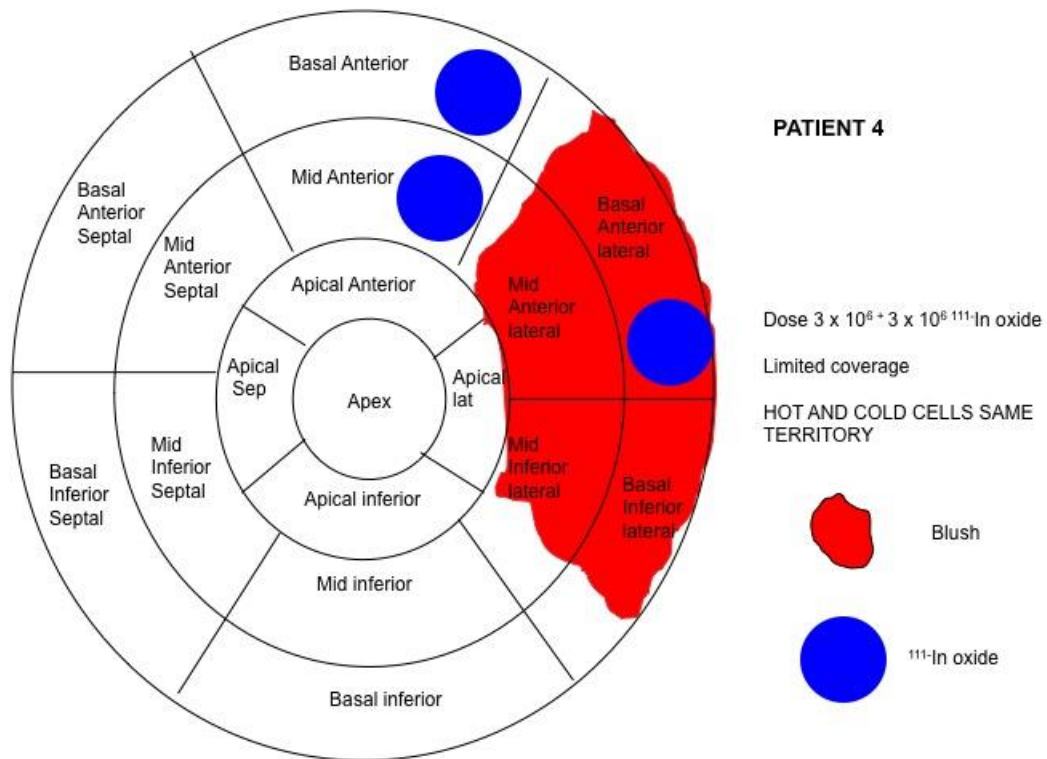
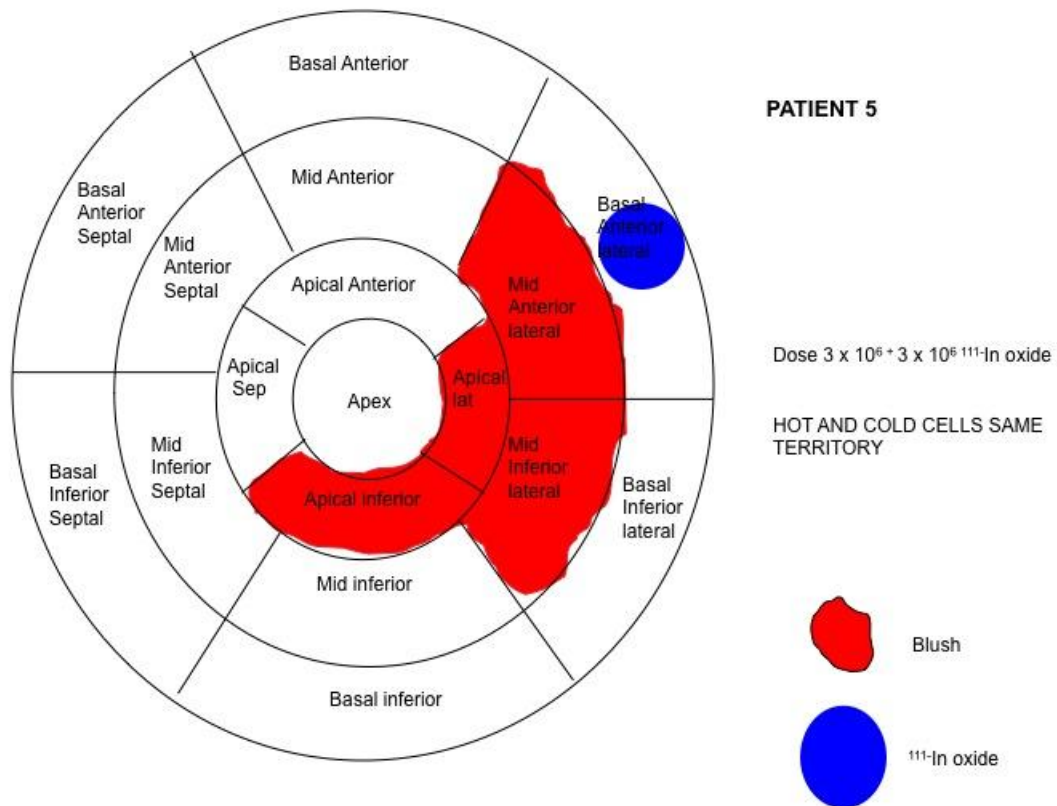


Figure 36: Distribution map of a non-ischaemic patient where the ^{111}In -oxide signal was located adjacent to the contrast myocardial blush



h. MVO₂

Full data was available on 17 of the 18 patients; the pre-delivery and six months post delivery values are shown on (Figure 37). The median MVO₂ at baseline was 10.8 ml/min/kg, which increases significantly to 14.2 ml/min/kg six months post treatment (Figure 38), ($P < 0.001$). For comparison purposes only I have also compared the median improvement in MVO₂ of 2.4 ml/kg/min against that seen in two large studies of CRT (Abraham et al., 2002), and CRTD (Young et al., 2003) respectively of 1.1 ml/kg/min (Figure 39). The controls in both of these studies had biventricular devices inserted which were switched off, giving an indication of a placebo effect of < 0.2 ml/min/kg.

Figure 37: Change in MVO₂

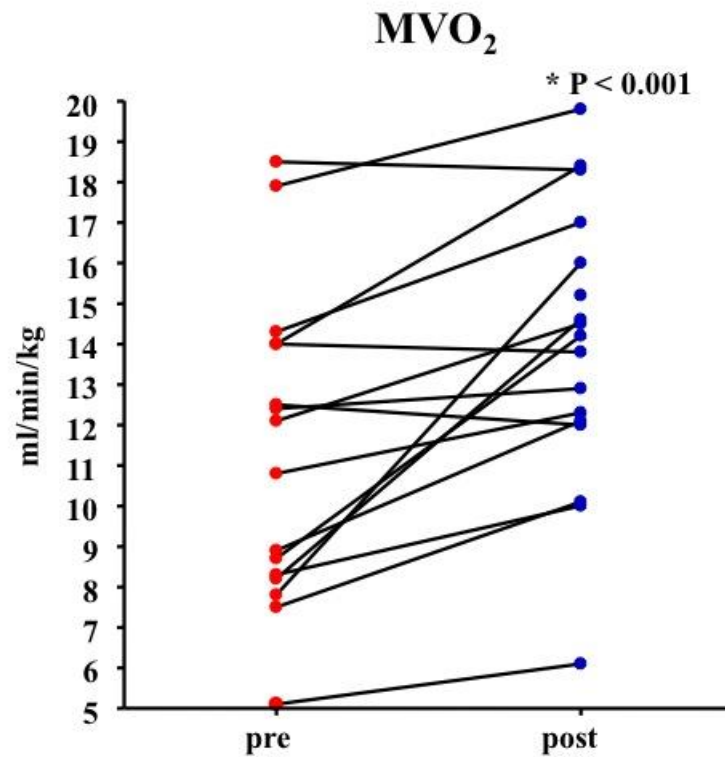


Figure 37:The red circles (pre) represent the baseline value for each patient, the blue circles are each patient's value taken six months after delivery (post). The black lines indicate each patient's change in ml/min/kg over-time. N= 17

Figure 38: The median change in MVO₂

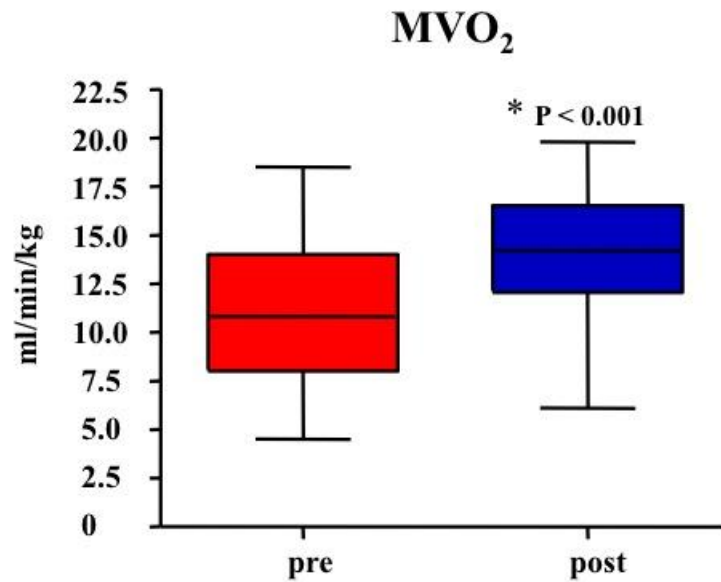


Figure 38: Change in MVO₂ from baseline (pre) to six months after delivery (post). The box-plot shows data from the 75th percentile (top of the box), to the 25th percentile (bottom of the box). The median value is the horizontal line across the box and the vertical lines represent the minimum and maximum levels. N= 17

Figure 39: Comparison of median change in MVO₂ compared to historic data

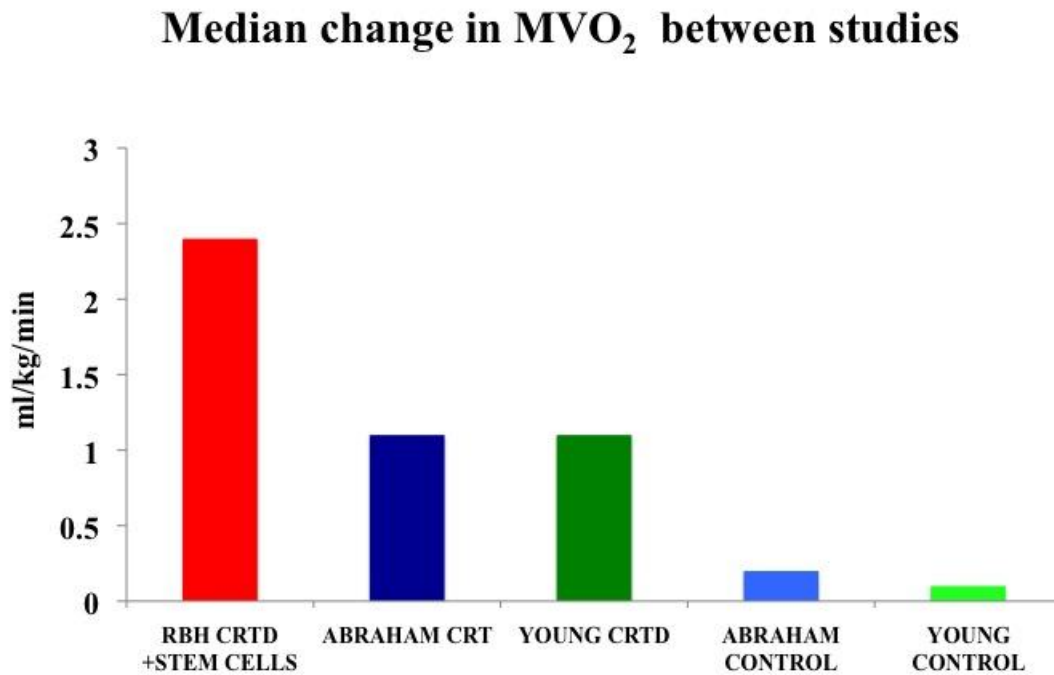


Figure 39: This graph shows the median change in (red) of our trial patients receiving both CRTD and putative stem cells, compared to published historical data of CRT (Abraham et al., 2002) in dark blue and the control group where the biventricular device was switched off in pale blue and CRTD therapy in dark green and their respective control in pale green (Young et al., 2003).

i. New York Heart Association Functional Class (NYHA CLASS)

The baseline and six month post treatment NYHA classes are shown in (Figure 40). All patients improved when reassessed six months later by at least one class, with the median change of class being 1.5 points ($P < 0.0001$) (Figure 41). For comparison, the change in NYHA class is plotted against the historical CRT data from (Abraham et al., 2002) (Figure 42). We had a similar percentage of patients who improved by ≥ 1 functional class 56% compared to Abraham (52%), but we saw a higher rate of patients who improved by ≥ 2 functional classes (44% compared to 16%).

Figure 40: Change in NYHA Class

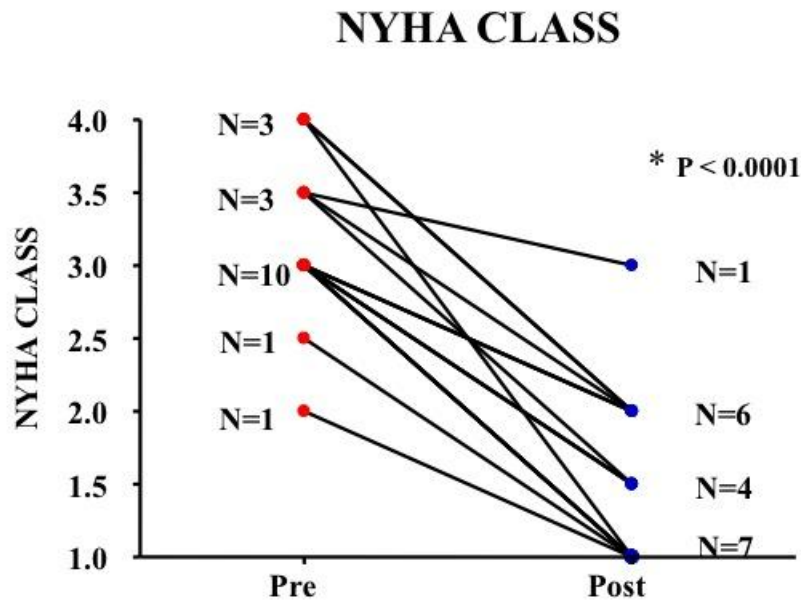


Figure 40: The red circles (pre) represent the baseline NYHA functional class for each patient; the blue circles are each patient's class six months after delivery (post). The black lines indicate each patient's change over-time. N= the number of patients, 18 in total.

Figure 41: The median change in NYHA Class

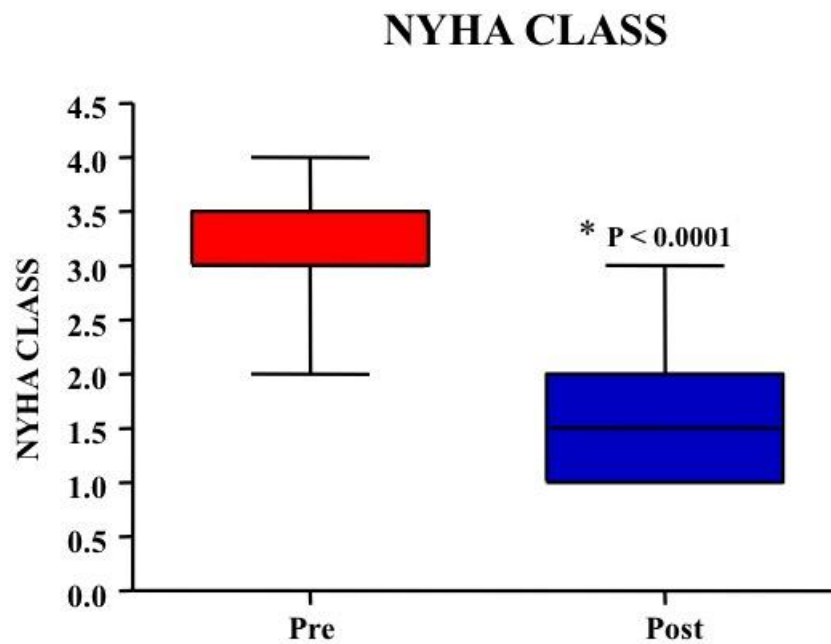


Figure 41:The median change in NYHA functional class from baseline (pre) to six months after delivery (post). The box-plot shows data from the 75th percentile (top of the box), to the 25th percentile (bottom of the box). The median value is the horizontal line across the box and the vertical lines represent the minimum and maximum levels. N= 18.

Figure 42: Comparison of median change in NYHA functional class compared to historic data

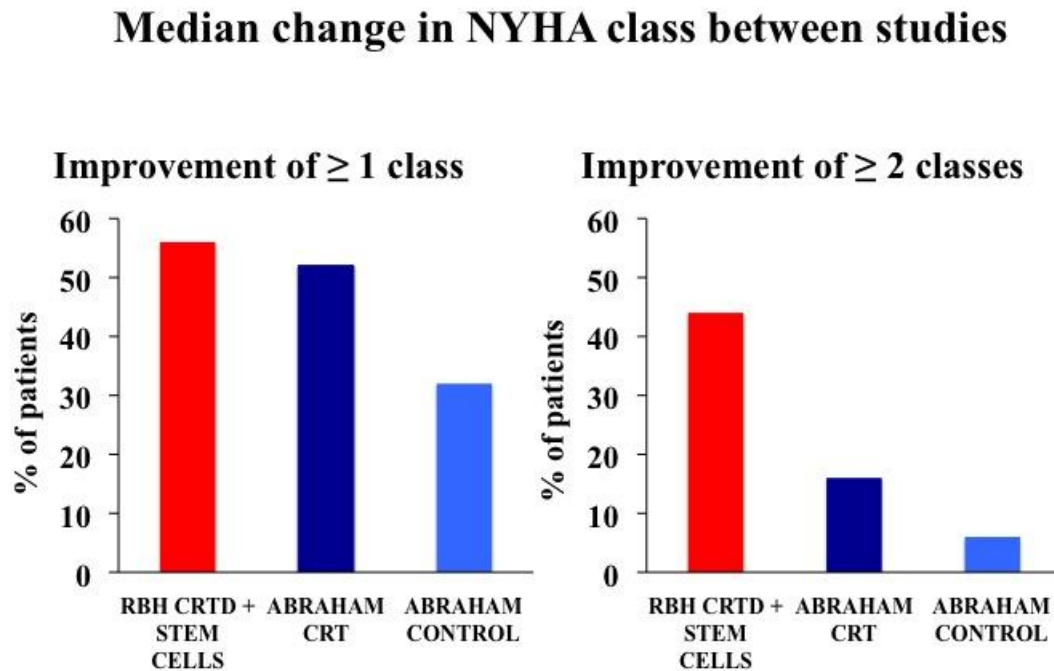


Figure 42: This graph shows the median change in NYHA functional class in (red) of our trial patients receiving both CRTD and putative stem cells, compared to published historical data of CRT (Abraham et al., 2002) in (dark blue) and the control group where the biventricular device was switched off in (pale blue). The graph on the left shows the % number of patients who improved by at least one functional class and the graph on the right demonstrates those who improved by two or more functional classes.

j. Quality of Life (QOL)

i. Minnesota

When viewing the Minnesota living with heart failure questionnaire the lower the quality of life (QOL) value, the less impact heart failure is deemed to have on a patient's QOL. The baseline and six months post treatment scores are shown in (Figure 43). The median baseline value was 54 points that improved dramatically six months post treatment to 15 points ($P < 0.0001$) (Figure 44). For comparison I have plotted our median change in Minnesota QOL (37 points) against that achieved historically with CRT (19 points) (Abraham et al., 2002) and CRTD (17 points) (Young et al., 2003). The improvements in the control groups and indicative of a potential placebo effect were 9 and 11 points respectively (Figure 45).

Figure 43: Change in Minnesota Living with heart failure QOL

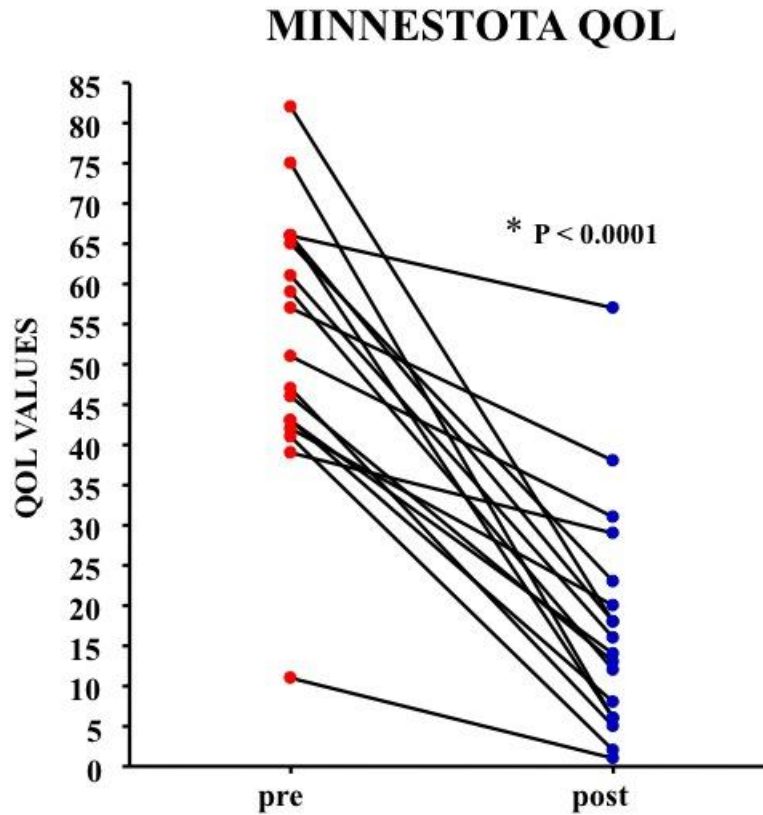


Figure 43: The red circles (pre) represent the baseline Minnesota value for each patient, the blue circles are each patient's value taken six months after delivery (post). The black lines indicate each patient's change in QOL over-time. The lower the score the less heart failure affects the patient. N=18.

Figure 44: The median change in Minnesota QOL

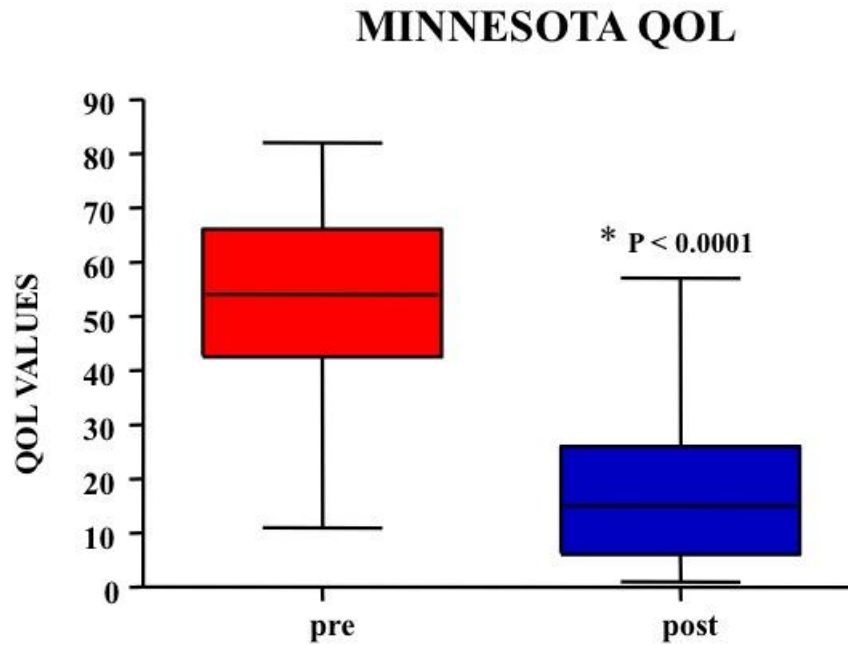


Figure 44: The median change in Minnesota living with heart failure QOL from baseline (pre) to six months after delivery (post). The box-plot shows data from the 75th percentile (top of the box), to the 25th percentile (bottom of the box). The median value is the horizontal line across the box and the vertical lines represent the minimum and maximum values. N= 18.

Figure 45: The median change in Minnesota QOL between Historical Studies

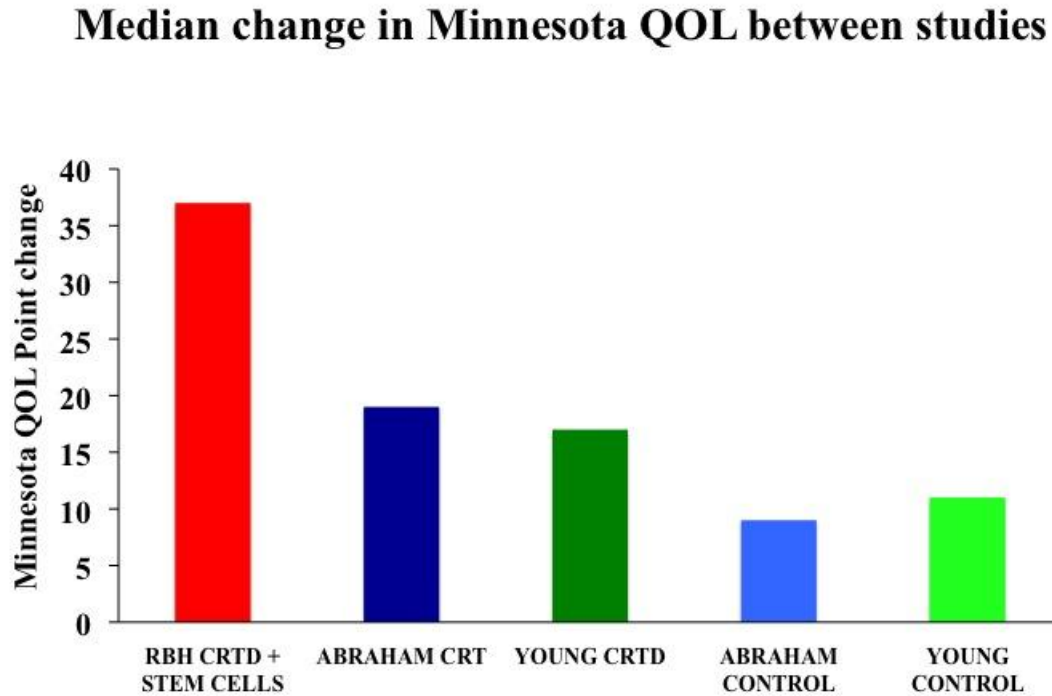


Figure 45: This graph shows the median change in Minnesota QOL (red) of our trial patients receiving both CRTD and putative stem cells, compared to published historical data of CRT(Abraham et al., 2002) in dark blue and the control group where the biventricular device was switched off in pale blue and CRTD therapy in dark green and their respective control in pale green (Young et al., 2003).

ii. SF36 version II

When viewing the SF36 values, disease is assessed to have impacted most on a patient's QOL when they have a lower QOL value. The combined physical and mental scores for each patient are shown in (Figure 46A and 46B). The median improvement from baseline to six months post treatment was 11 points for physical ($P < 0.0001$) (Figure 47A) and 12 points for mental scores ($P < 0.006$) (Figure 47B).

Figure 46A: Combined physical score SF36 version II

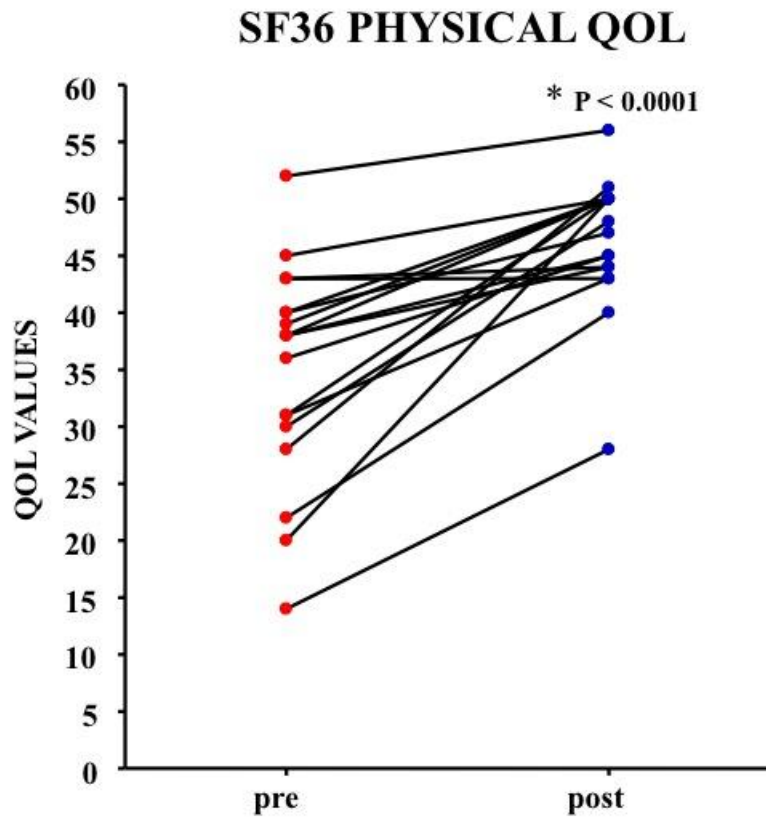


Figure 46A: The red circles (pre) represent the baseline combined physical score, of the SF36 version II QOL value for each patient, the blue circles are each patient's value taken six months after delivery (post). The black lines indicate each patient's change. N= 18.

Figure 46B: The combined mental score SF36 version II

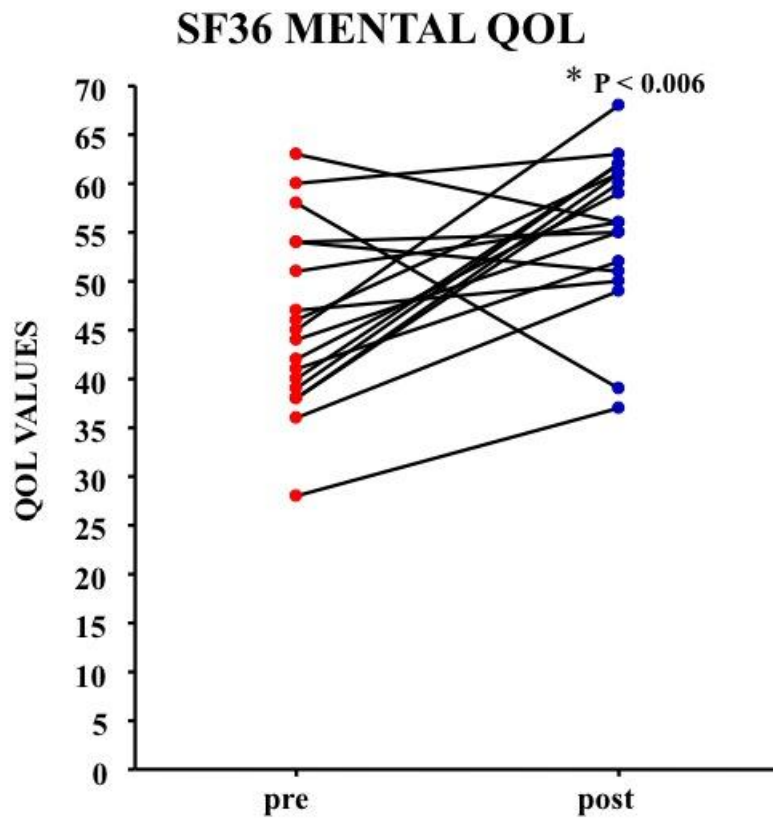


Figure 46B: The red circles (pre) represent the baseline value for each patient combined mental score of the SF36 version II QOL, the blue circles are each patient's value taken six months after delivery (post). The black lines indicate each patient's change. N= 18.

Figure 47A: The median combined physical SF36 score

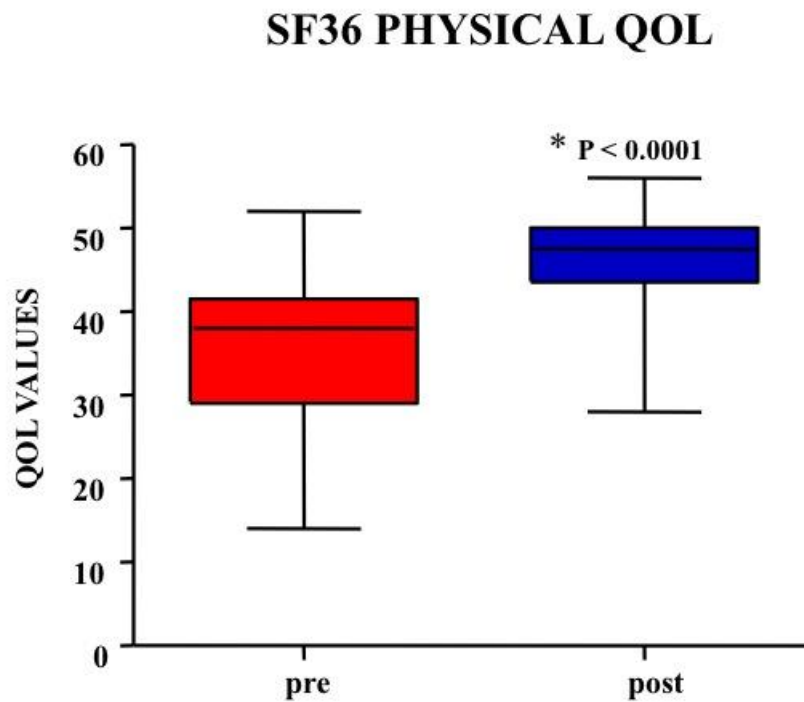


Figure 47A: The median change in combined Physical values of SF36 version II QOL from baseline (pre) to six months after delivery (post). The box-plot shows data from the 75th percentile (top of the box), to the 25th percentile (bottom of the box). The median value is the horizontal line across the box and the vertical lines represent the minimum and maximum values. N= 18.

Figure 47B: The median combined mental SF36 score

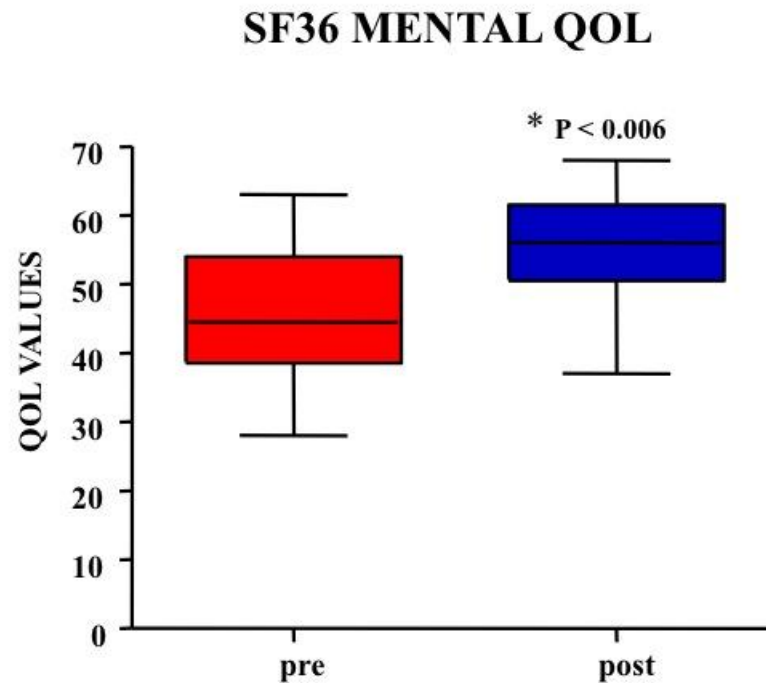


Figure 47B: The median change in combined mental values of SF36 version II QOL from baseline (pre) to six months after delivery (post). The box-plot shows data from the 75th percentile (top of the box), to the 25th percentile (bottom of the box). The median value is the horizontal line across the box and the vertical lines represent the minimum and maximum values. N= 18.

k. SPECT

There was no significant change in viability ($P = 0.38$), ischaemia ($P = 0.25$) or function ($P = 0.17$) between baseline and six months post treatment scans. (Table 19 A, B and C) summaries the findings. Individual variations in viability (V), ischaemia (I) and function (F) scores may be related to the reproducibility of the technique and are unlikely to be clinically important. There was no change in LVEF assessed by SPECT ($P = 0.98$). (Figure 48) shows all the patient data and (Figure 49) shows the median change.

Table 19A: SPECT viability assessment on 17-segment model

Viability Score (V)	V- (Reduced)	V0 (Same)	V+ (Improved)
Injected zone	4	10	4
Remote Zone	4	11	3

Table 19B: SPECT ischaemia assessment on 17-segment model

Ischaemia Score (I)	I- (Reduced)	I0 (Same)	I+ (Improved)
Injected zone	2	8	8
Remote Zone	0	8	10

Table 19C: SPECT Functional assessment on 17-segment model

Function Score (V)	V- (Reduced)	V0 (Same)	V+ (Improved)
Injected zone	4	11	3
Remote Zone	2	14	2

The cardiologist who performed the cell injection predicted the site of the stem cell delivery on the 17-segment model; this area was referred to as the injection area. The rest of the myocardium was referred to as the remote area. The segments were scored for viability **19A** (50% uptake in comparison with the maximally perfused area within the entire myocardium), ischaemia **19B** and functional score of wall motion and thickening **19C**.

Figure 48: LVEF

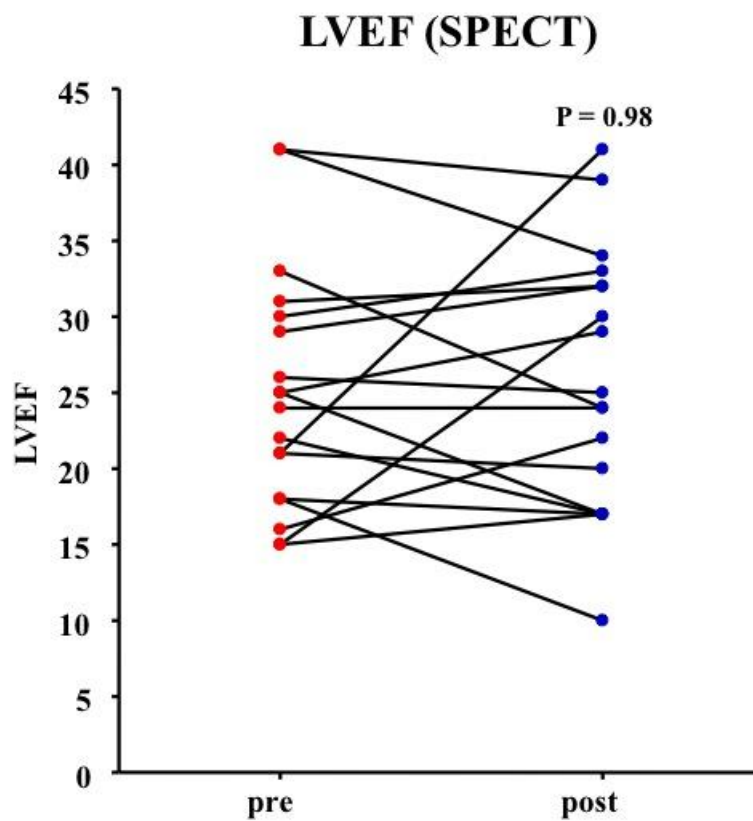


Figure 48: The red circles (pre) represent the baseline % left ventricular ejection fraction (LVEF) for each patient; the blue circles are each patient's values six months after delivery (post). The black lines indicate each patient's change. N= 18.

Figure 49: The median change in LVEF

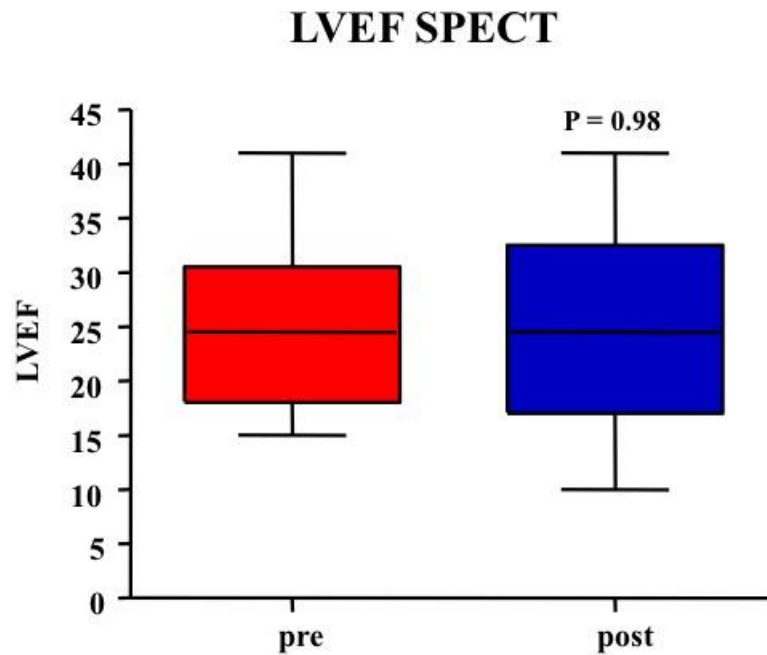


Figure 49: The median change in % left ventricular ejection fraction (LVEF) from baseline (pre) to six months after delivery (post). The box-plot shows data from the 75th percentile (top of the box), to the 25th percentile (bottom of the box). The median value is the horizontal line across the box and the vertical lines represent the minimum and maximum values. N= 18.

I. ECHO

We initially planned to undertake stress echocardiography assessments on all patients at baseline and at six months post delivery. Unfortunately due to infrastructure changes within the RBH this was not clinically available after the first few patients, and no further echocardiographic assessments were performed until the last five patients. These patients received standard clinical 2D and investigational 3D studies. There was variation between the estimated LVEF at baseline and at 6 months post delivery with 2D and 3D echocardiography (Figure 50). There were no improvements in LVEF assessed with either 2D or 3D echocardiography (Figure 51). There was no difference in the median total isovolumic time (t-IVT) a measure of LV desynchronisation between baseline and six months (Figure 52a). However there did appear to be differences in individual patients (Figure 52b). No conclusions can be drawn from this data as the n numbers are too small, but it may be an interesting surrogate marker to include in future trials.

Figure 50: LVEF assessed with 2D and 3D ECHO

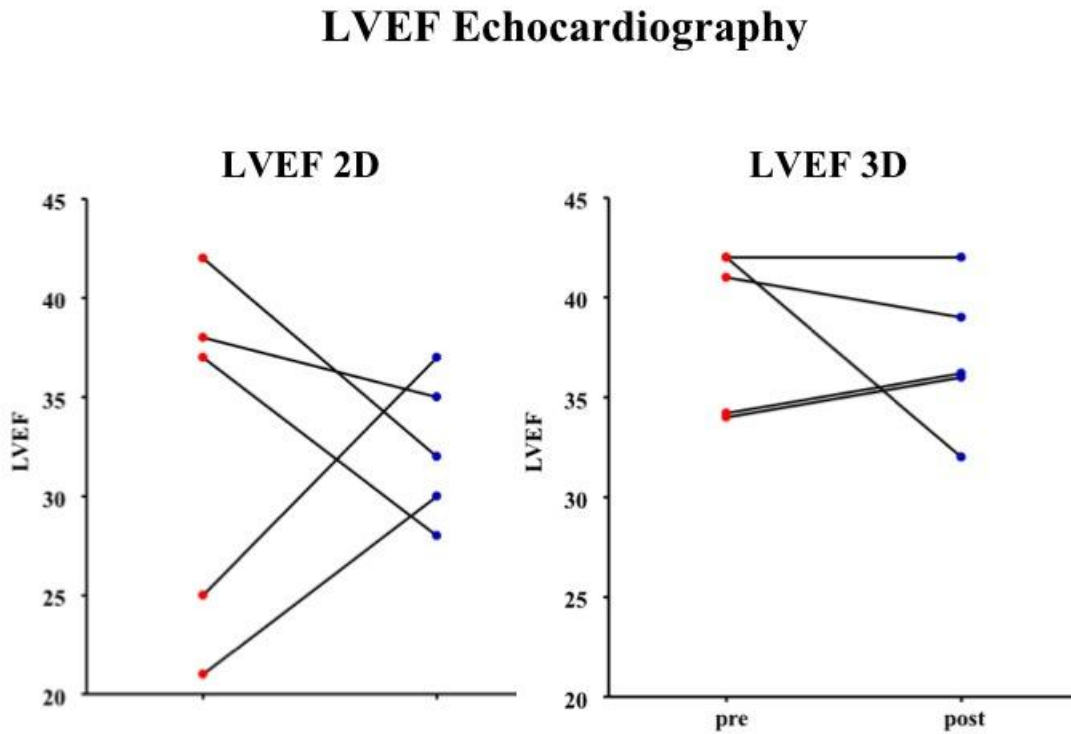


Figure 50: This graph shows the data of a subset of five patients on whom two and three-dimensional (2D) and (3D) echocardiography assessments were performed. The red circles (pre) represent the baseline % left ventricular ejection fraction (LVEF) for each patient; the blue circles are each patient's values six months after delivery (post). The black lines indicate each patient's change.

Figure 51: Median change in LVEF assessed by 2D and 3D echocardiography

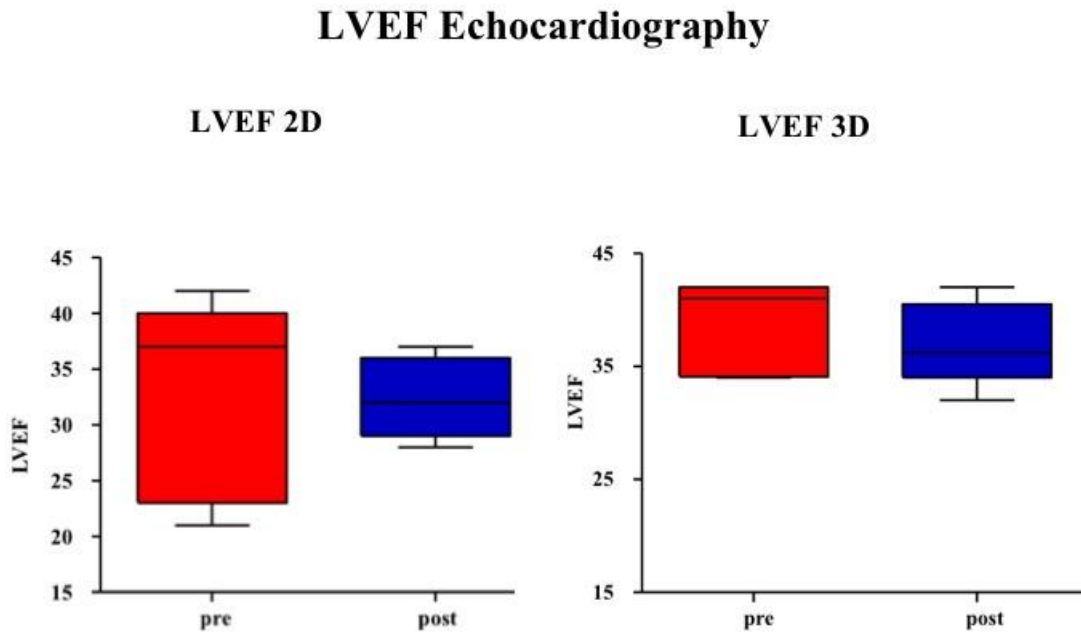


Figure 51: The data of a subset of five patients on whom two and three-dimensional (2D) and (3D) echocardiography assessments were performed. The median change in % left ventricular ejection fraction (LVEF) from baseline (pre) to six months after delivery (post). The box-plot shows data from the 75th percentile (top of the box), to the 25th percentile (bottom of the box). The median value is the horizontal line across the box and the vertical lines represent the minimum and maximum values.

Figure 52: Total isovolumic time Echocardiography

t- IVT Echocardiography

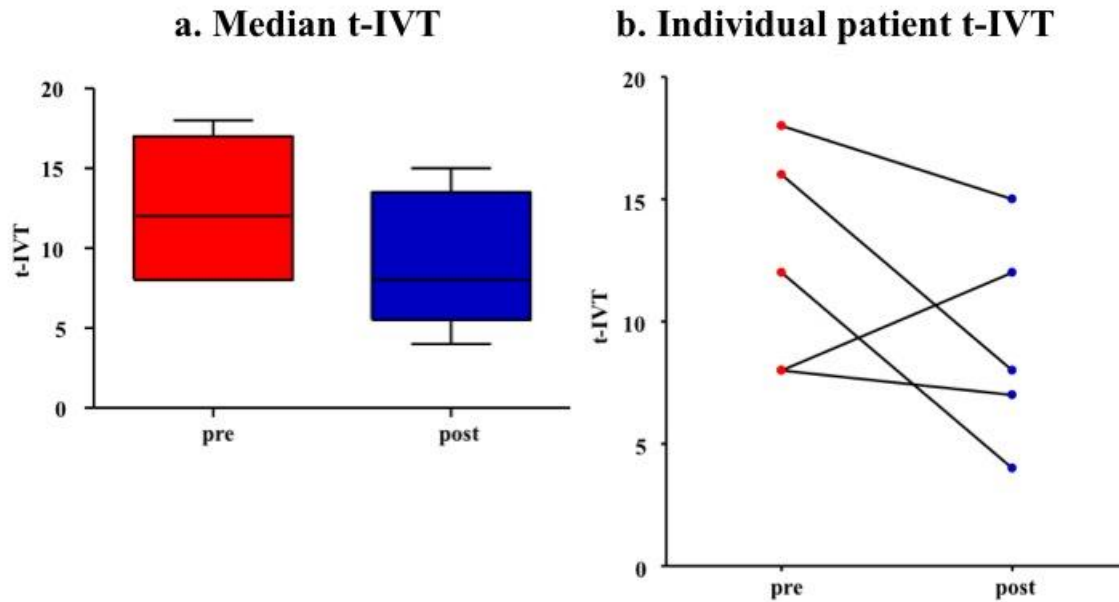


Figure 52: The data of a subset of five patients on whom, total isovolumic time (t-IVT) from baseline (pre) in red to six months after delivery (post) in blue. **52a** The box-plot shows data from the 75th percentile (top of the box), to the 25th percentile (bottom of the box). The median value is the horizontal line across the box and the vertical lines represent the minimum and maximum values. **52b** shows individual changes from baseline in red to six months post delivery in blue.

m. CD34+ Counts

There was a correlation between the percentage number of CD34+ cells injected and the change in MVO₂ ($P = 0.02$) correlation coefficient (0.559) (Figure 53).

Figure 53: The Correlation of CD34+ % and change in MVO₂

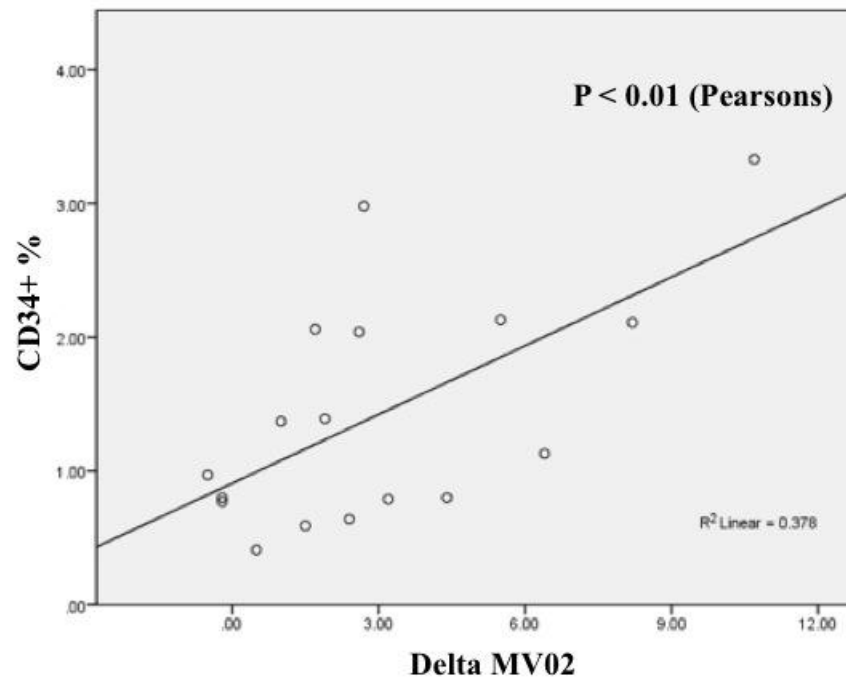


Figure 53:The correlation of the percentage of CD34+ cells given and change in MVO₂ N= 18.

7.6 DISCUSSION

This is the first study to demonstrate the feasibility of retrograde coronary venous delivery of putative stem cells to the heart. The inclusion of the ^{111}In oxide cells demonstrated a sustained signal of over ten days post delivery in all but one patient. Retention of cells within the myocardium is likely to be critical in allowing a biologically important paracrine function or stem cell differentiation. Two studies have shown approximately up to 10% retention of labelled cells following intra-coronary arterial injection at an early time point of 24 hours (Goussetis et al., 2006); (Kurpisz et al., 2007), however this is the first study to report on sustained retention up to 21 days. The defined period of time required to exert a biological effect is yet unknown but is unlikely to be fleeting. To my knowledge no study has documented the feasibility of cell retention within the myocardium over the considerably longer periods likely to be needed for putative stem cell function. We were thus encouraged by the ability of this technique to deliver cells, which appeared to stay within the myocardium for a prolonged period of time.

One question is whether the ^{111}In oxide label could have dissociated from the cells once delivered and therefore the signal we imaged may merely be the ^{111}In oxide label. Despite irreversible binding of ^{111}In oxide to nuclear and cytoplasmic proteins, damaged leucocytes may release more radioactivity and at a higher rate, than intact cells (Roca et al., 2010). In a study of labelled human MSC injected into the intra-osseous cavity of the tibia in athymic rats there was almost complete retention of radioactivity in the implanted site at 48 hours with no evidence of re-distribution to other organs. *In vitro* experiments also demonstrated 61% retention of label at the same time point and any loss of radioactivity was attributable mainly to radioactive decay (Gildehaus et al., 2010).

However (Brenner et al., 2004) in their study of human hematopoietic progenitor cells injected in the left ventricular cavity of nude rats found that there was significant leakage of ^{111}In oxide label from the cells and based on their *in vitro* studies they estimated this to be up to 75% at four days. They reported only 1% of cells were retained within the myocardium at this time point but commented that this was probably an underestimate of

retention because of the leakage. If leakage was compensated for then the actual amount of hematopoietic progenitor cells resident in the myocardium was estimated to be 4%. Within the first 24 hours we did see a decrease in the ^{111}In oxide signal within the myocardium, which may be a sign of ^{111}In oxide leakage from our BMMNC, and thus our estimated myocardium retention of cells of 3.6% at 24 hours may also have underestimated the actual retention of BMMNC within the heart. Evidence that we were imaging viable cells is supported by the prolonged signal within the heart up to 21 days and the absence in all patients of any ^{111}In oxide signal in the kidneys, where we would have expected to see elimination of radiotracer in the event of cell death (Brenner et al., 2004); (Kraitchman et al., 2005).

In a study by (Kraitchman et al., 2005) dogs with an MI injected with free intravenous ^{111}In oxide showed no myocardial deposition in contrast to dogs that received ^{111}In oxide labelled MSC cells that homed to the site of the MI. It is unlikely that we would therefore have seen free unbound ^{111}In oxide within the myocardium.

Imaging of ^{111}In oxide labelled leukocyte distribution is a routine clinical procedure to localise areas of inflammation (Becker and Meller, 2001) and has an established safety record in man. We were however concerned that there may have been some radioactive damage to the labelled cells, as it is known that labelling of mixed leucocytes causes radiation damage to the lymphocytes as a result of self-irradiation by internal conversion of electrons and Auger electrons (Roca et al., 2010). The degree of radiation damage appears to not only be dose dependant (Gildehaus et al., 2010) but also may be species dependant. (Kraitchman et al., 2005) in a study of allogenic canine ^{111}In oxide labelled MSC delivered intravenously to an MI dog model, reported viability was 89%. The labelled cells had a slightly reduced cell proliferation and metabolic activity compared to unlabelled MSC, but homed to the site of injury despite large degrees of mechanical trapping within the lung. In contrast, in a similar study in the porcine model, no homing to the myocardium was detected (Chin et al., 2003).

The cell type may also be important as ^{111}In oxide label resulted in no effect on MSC viability, function or proliferation but a 46% reduction in metabolic function and a significant decrease in migration in a time dependant manner (Gildehaus et al., 2010). Others also reported (Bindslev et al., 2006) and (Jin et al., 2005) no effect on viability of ^{111}In oxide labelling on MSC whereas (Brenner et al., 2004) utilising hematopoietic progenitor cells reported a 30% reduction in viability and no proliferation or differentiation at seven days. We chose to use a non-selective cell population for our study, which had not been reported in animal labelling studies. Our in vitro data indicated some loss of differentiating potential and although we labelled them with a clinical dose usually used for leukocyte distribution scans we couldn't exclude some alteration in any potential 'stem cell' function and thus we pragmatically decided to incorporate both an ^{111}In oxide labelled in addition to an unlabelled population in our patients. It may be that the labelled cells functioned and migrated in a different manner and independently to the unlabelled cells that we were unable to visualise but we felt ethically the combination of potentially radioactively damaged cells should be mixed with a population of unlabelled cells cable of biological activity.

^{111}In oxide labelling adversely affects viability, functionality, migration, and proliferative capacity differently in distinct cell populations as well as species. Further studies are needed to resolve these questions in man but we did not see any difference in safety between the patients who did and those that did not receive labelled cells. Because there is evidence that ^{111}In oxide labelled cells may not have the same migration capabilities as unlabelled cells (Gildehaus et al., 2010), it is plausible that the unlabelled cells may have migrated away from the site of delivery in a different manner than the ^{111}In oxide labelled cells we were able to image, however the increasing numbers returning to the bone marrow would indicate that the ^{111}In oxide labelled cells were viable and retained their migration potential.

There was a peri-procedural rise in Troponin-I, CKMB and CRP in our patients, after delivery of putative stem cells and CRTD, in the absence of clinical signs of myocardial ischaemia, ECG changes or later SPECT evidence of loss of viable tissue or new wall

motion abnormalities. The question is whether these rises are clinically important.

By applying the universal definition of myocardial infarction (Thygesen et al., 2007) our patients did sustain a rise seen with a PCI induced (Type 4a) myocardial infarction as a result of cell delivery and CRTD. The new definitions are controversial and considered too strict by some (De Labriolle et al., 2009). Although cardiac Troponin-I is a sensitive biomarker of myonecrosis, a recent large study found that there was no association with MACE over a one year period in patients with raised Troponin-I in the absence of ischaemia after successful PCI. In this study of stable patients using the new definition of MI resulting from a postprocedural PCI of a raised Troponin-I of $> 3 \times 99^{\text{th}}$ percentile of the normal reference population, calculated at $> 0.30 \text{ ng/ml}$, 23% of patients were considered to have sustained a postprocedural MI (De Labriolle et al., 2009).

An MI resulting from bypass surgery is known as a (Type V) MI, defined with Troponin-I $> 5 \times 99^{\text{th}}$ percentile of the upper reference limit (URL) plus additional Q waves or a new left bundle branch block or new occlusion or imaging evidence of new loss of viable myocardium (Thygesen et al., 2007). In a study assessing the sensitivity of biomarkers to detect effects of CABG on the development of a type V myocardial infarction based on these new definitions it was found that Troponin-I $> 6.6 \mu\text{g/l}$ at 24hours strongly predicted a type V MI on delayed enhancement cardiac magnetic resonance (DE CMR) with a 88% sensitivity and a 97% specificity albeit this was (165 x URL), indicating that $5 \times \text{URL}$ for Troponin-I maybe far too sensitive for detecting CABG related MI (Pegg et al., 2011).

Using this criterion we had one patient in whom the 24 hour Troponin-I was exactly $6.6 \mu\text{g/l}$. In this particular patient there was an intense myocardial blush on the delivery venogram. Close follow-up did not indicate any new changes on ECG, or ischaemia or loss of viability on SPECT. Interestingly (Pegg et al., 2011) found the pattern of Troponin-I and CKMB release was also important in discriminating between those who did and those that did not sustain an MI. In those patients where Troponin-I and CKMB peaked early at one to six hours but decreased by 24 hours there was no evidence of (DE-

CMR) myocyte necrosis, whereas in those patients with evidence of infarction there was a delayed rises (after 24 hours) and sustained release, which they attested to be probably due to the slow active degradation and metabolism of the myofibril apparatus (Martin, 1981). Although our early Troponin-I and CKMB were taken at 12 hours rather than at one to six hours, the graphs demonstrating the increases in CKMB and Troponin over-time in our study follow exactly the patterns seen in the study by (Pegg et al., 2011), in the group with early rises and no evidence of myocardial necrosis on (DE-CMR). (Rahimi et al., 2009) investigated (DE-CMR) in 152 consecutive patients before and 24 hours post a complex PCI or CABG. They found that there was a three-fold increase in incidence in their primary end-point of (death, non fatal MI, ventricular arrhythmias, unstable angina and worsening heart failure) in those patients in whom there was evidence of new hyperenhancement on their DE-CMR scan, even though the myocardial damage was estimated to be relative small, median five grams. Although patients with evidence of hyperenhancement also had post-procedural rises in Trop-I and impaired LV function absolute Trop-I values and change in LVEF after revascularisation were not significantly associated with the primary endpoint. This is an important finding as even relatively small areas of irreversible myocardial injury detected by DE-CMR translate to appreciable increased risk of adverse events (Rahimi et al., 2009) and yet changes in LVEF and rises in Trop-I do not appear to reliably predict these events. Because all our patients had devices in situ, we were unable to undertake CMR studies, and thus it may be that we did not have a sensitive enough measure to detect any delivery related myonecrosis. Further studies of retrograde venous delivery in patients without devices will enable DE-CMR studies to be performed to assess any evidence of hyperenhancement.

Other groups reporting on delivery of bone marrow derived cells via epicardial or endocardial routes have not commented on Troponin-I or CKMB rises (Assmus et al., 2006); (Perin et al., 2003). In a study of CRTD it was found that Troponin-I peaked at 1.79 ng/ml, statistically higher than their control group in whom a transvenous biventricular device or ICD was implanted and they concluded that additional manipulation of the coronary sinus required to insert a CRTD device was causal (Altin et

al., 2007). The peak of 1.04 ng/ml in our patients was well below this. (Stamm et al., 2007) reported a median postoperative CRP level of (41 mg/l range 12-136), with cell delivery via epicardial injections as an adjunct to CABG surgery. It is difficult to extrapolate the effects of cell delivery as it was confounded by CABG surgery, which is known to increase CRP, however the group that had CABG in addition to cell delivery had higher CRP levels than the placebo group where CABG was undertaken without cell delivery (median CRP 26 mg/l (range 3 to 113)). It is difficult to decipher if any additional inflammation results from the epicardial injections or the cells themselves. Our median CRP levels peaked at 32 mg/l (range 3 to 74) and were thus comparable. It may be that a small degree of inflammation is beneficial and pro-angiogenic.

Increases in Troponin-I do arise with PCI (De Labriolle et al., 2009), CABG (Pegg et al., 2011), insertion of a CRTD device (Altin et al., 2007), or testing of an ICD device (Hurst et al., 1999). The combination of CRTD with ICD testing may have contributed to the rises in Troponin-I seen in our trial.

It is not usual clinical practice to aim to achieve a ‘myocardial blush’, some may suspect that the rises in cardiac enzyme may be attributed to this blush and therefore any benefits gained from the delivery of bone marrow derived putative stem cells may be offset from myocardial damage caused by the delivery modality itself. Clearly a RDBPC trial will need to be undertaken to rigorously assess safety and efficacy. However on completion of this trial albeit in an open-label design, preliminary finding would suggest that there has been no clinically significant safety issues arising and some early indicators that there may be some beneficial effects.

We only included efficacy end-points for hypothesis generating purposes however one of the weaknesses when assessing any efficacy end-points in this trial was that it was open label and therefore predisposed to a placebo bias, which we know is powerful in this group of patients. Improvements have not only been reported with symptoms, but also by physiological measures such as exercise time and perfusion (Rana et al., 2005). In

addition it was confounded by CRTD. It is therefore difficult to extrapolate whether any improvements resulted from a placebo effect, the delivery of putative stem cells, or CRTD or a combination of the above. I have therefore compared our results to pivotal studies of CRT and CRTD for comparative purposes only, as our trial was neither designed nor powered to assess efficacy.

In our study the median change in the Minnesota QOL was -37 points (range -9 to -69) whereas in a study of 453 patients receiving CRT alone the improvement was -18 points (-22 to -12). In the control group where a biventricular device was implanted but turned off, the improvement was 9 points (-12 to -5) and thus indicative of the placebo effect of device implantation (Abraham et al., 2002); In the Miracle ICD study the median quality of life score improved by -17 points (-21 to -14) with the control group showing an improvement of -11 points (-16 to -7) (Young et al., 2003). For comparison only I have plotted these on (Figure 35).

The median improvement in our patient's MVO_2 was 2.4 ml/kg/min (-0.5 to 10.7) whereas others have reported only a 1.1 ml/kg/min (0.7 to 1.6) in their CRTD group (Young et al., 2003) and 1.1 ml/kg/min (0.6 to 1.7) for CRT (Abraham et al., 2002) and a negligible placebo effect of 0.1 ml/kg/min and 0.2 ml/kg/min in their respective control groups (Figure 29). Finally 56% of our patients had at least one NYHA functional class change similar to the 52% reported by Abraham, but we also had 44% of patients with more than one class change which was far higher than the 16% reported in this study (Abraham et al., 2002) (Figure 32). Interestingly we saw improvements in all patients whereas they reported no change in 30% of their patients, in line with the well documented non-responder rate for CRT (Auricchio and Prinzen, 2011).

Our study was weakened by not having echo data available on all patients (only a small subset). We had initially investigated the possibility of undertaking Positron emission tomography (PET) scans but the costs were prohibitive for purely exploratory purposes, but may be useful in elucidating any functional improvements in later efficacy trials. CMR is a sensitive parameter to assess small changes in wall motion but was

contraindicated by the inclusion of our biventricular devices. We did include LVEF assessed by SPECT but the reproducibility for this technique is probably below the detection rate of the small improvements in LVEF reported in other trials (Assmus et al., 2006); (Perin et al., 2003). The values of LVEF assessed by 2D and 3D echo were somewhat disparate, although due to the small sample size and the lack of another sensitive measure of LVEF it is difficult to ascertain which one is most accurate for future studies. CT may be a useful alternative to CMR for patients with devices in situ.

We found an interesting correlation between the number of CD34+ cells and change in MVO₂. We have saved samples of bone marrow from all patients so it may be useful to examine these cells for functionality in the future to determine whether those patients with the greatest improvement in MVO₂ also had better migration of CD34+ cells or other measures of functionality such as colony forming units. We also do not know if the increase in CD34+ cells is causal to the improvement seen in MVO₂ or the effect of an improved exercise capacity.

We did not observe a dose response, two possible explanations are that a) we were above the dose response curve or b) There was no effect from the BMMC and any observed changes resulted from another mechanism such as inflammation from the delivery technique itself. A proposed randomised double-blind placebo controlled trial may help to elucidate further the mechanisms responsible for any observed improvements. The maximum number of cells used in our study was 1×10^8 others (Martin-Rendon et al., 2008) have reported in a meta-analysis that improvements are only seen with higher cell numbers such as 1×10^9 . If in future trials we decided to opt for a higher dose within this recommended range then our total cell numbers would indicate this would necessitate the aspiration of larger bone marrow volumes.

One concern we did have with this delivery technique was the feasibility of re-entering the selective coronary veins after delivery of stem cells, as we were concerned that these veins may have become occluded post delivery. In one patient, in whom the LV lead required repositioning twice, we found that there were no evidence of occlusion and there

were no problems with re-cannulating the coronary sinus or the selective vein that had received stem cells for replacement of the LV lead. We also were concerned that in patients in whom there was already a left ventricular lead in situ, it may be difficult to cannulate and select coronary veins without dislodging the LV lead. After the completion of this study we undertook three such patient studies with no delivery related complications and therefore we propose to recruit both patients with and without CRTD for the future Phase IIB trial.

This technique is challenging and probably best suited to electrophysiologists who routinely enter the coronary sinus and sub-select coronary veins. We found that the anterior and lateral walls were relatively easy to target but that the inferior wall and septum were more challenging.

The delivery maps were qualitative rather than quantitative, but they importantly showed that in every patient with a myocardial blush on the venogram there was an ^{111}In signal, which was sustained over a ten day period. The distribution of myocardial blush plotted on a standard 17-segment model was found in the same segment as the ^{111}In oxide in 13 of the 14 patients and in the adjacent segment in one patient. This may be important for future studies where labelled cells are omitted, as the myocardial blush would appear to reliably indicate the region of delivery.

Because this was principally designed as a safety feasibility study patients were recruited with a documented history of ischaemic heart disease but eight of the eighteen had no reversible ischaemia on their SPECT scan. We had planned to recruit patients with at least 10% reversible ischaemia on their SPECT scan for future randomised placebo controlled trials. However we were surprised to see that in patients in whom there appeared to be no reversible ischaemia there was no difference regarding improvements in their respective MVO_2 or QOL from those with ischaemia. It maybe that our measures for detection of ischaemia are too insensitive, or that another mechanism not related to angiogenesis is responsible for the perceived improvements. It does however open the debate for whether reversible ischaemia should be used as entry criteria for future trials.

CHAPTER 8: SUMMARY, FUTURE PLANS AND TRIAL DESIGNS

Through my PhD studies I was able to develop and publish some new inclusion criteria to better define ischaemia at rest and during stress in the porcine ameroid model. After completing this I was the first person to investigate and publish on HIF-1 α gene therapy in a preclinical model of myocardial ischaemia. This work was used as part of the safety and toxicology support for submission to the food and drug administration (FDA) to gain regulatory approval of Ad2HIF-1 α for clinical use to treat patients with ischaemic heart disease. Results from a subsequent trial conducted by another group have recently been published (Kilian et al., 2010). In this trial ten patients after CABG surgery received injections of Ad2HIF-1 α in a territory not suitable for revascularisation. This was designed with three dose escalations up to 1×10^{11} viral particles, three additional control patients received injections of saline. All patients were alive at the one year follow-up and some improvements were noted on PET scans in the injected zone of patients receiving Ad2HIF-1 α . Further larger RDBPC trials are required to establish safety and efficacy of Ad2HIF-1 α . I would like to see Ad2HIF-1 α administered via endocardial injections with the NOGA system and retrograde coronary delivery thus providing evidence of the comparative efficiency of both delivery routes. As stated earlier I do not believe that the correct gene therapy studies have been undertaken to date and it is important that these are completed to prevent a potentially useful therapy being shelved prematurely without the appropriate evidence.

My clinical focus concentrated on the use of autologous bone marrow derived stem cells in a phase IIA pilot clinical trial and specifically investigating a novel method of retrograde coronary venous delivery. This was the first time that this delivery route had been utilised for the delivery of putative stem cells. In those patients receiving a sub-population of ^{111}In labelled bone marrow derived cells I was able to demonstrate delivery and sustained retention for ten days in all but one patient. I was also able to establish the association of distribution of myocardial blush on the coronary venogram with the ^{111}In signal on the Hawkeye CT images. This has important implications for future trial design, as it may be possible to rely on a myocardial blush as evidence of cell delivery rather than

labelling a sub-population of cells for this purpose. The results from this trial have established the early safety and feasibility of this technique and have supported a planned larger Phase IIB randomised double-blind placebo controlled trial planned to start early next year. There are a number of things that I have learned from designing this protocol and planning and undertaking this trial. Some scientific and some practical which have helped me evolve the protocol for the Phase IIB randomised double-blind placebo controlled trial with the following recommendations and suggestions;

1. Continue sending letters to referring consultants with inclusion and exclusion criteria but also include an optimal medication list and request reasons for any omission of a class of drugs if known, prior to referral e.g. intolerance to β -Blockers
2. There should be an upper age limit of 80 for recruitment, to prevent the confounding effects of other morbidities.
3. Better define significant renal disease.
4. Open recruitment to those patients who already have a biventricular device and ICD in situ as well as those referred for a de-novo devices.
5. Make sure the placebo instillation is indistinguishable from the bone marrow cell sample.
6. Review the need to incorporate a sub-population of bone marrow cells to determine delivery, in view of the close correlation with myocardial blush.
7. If a sub-population of cells ¹¹¹In labelled cells are still utilised I suggest changing the imaging time point to 24 hours post delivery. So that patients are not transferred directly from the catheter laboratory, when the likelihood of adverse events may be higher.
8. Reduce length of stay to three days if no ¹¹¹In imaging is to be performed.
9. Change the inclusion criteria to be an established diagnosis of IHD rather than requiring at least 10% reversible ischaemia on the baseline SPECT. This change is because I did not see any difference in any of the efficacy end-points between those who did and those that did not have reversible ischaemia.
10. Change the time point for the assessment of surrogate end-points to 12 months. Although my primary efficacy assessment was conducted at six months, further clinical assessments undertaken at 12 months and two years in some patients

continued to see further improvements in MVO_2 and SPECT at these later time points.

11. Investigate the possibility of using CT in addition to or replacement for echocardiography as a means of assessing LVEF.
12. Make sure baseline bloods are performed at least a week before planned admission.
13. Establish assays to assess the function of patient's cells.
14. Line up two cases at any given time so that if any unforeseen events occur the case does not need to be cancelled.
15. Have a dedicated technician perform all of the MVO_2 studies and consider changing the protocol to the modified Borg in view of the advanced nature and limited exercise capacity of our patient population.
16. Try and gain information on hospital inpatient days for the preceding year.
17. Improve on the design of the clinical record form to gain better information.

From the initiation of my studies to completion the field of regenerative medicine has progressed and yet there are still a number of unanswered questions and challenges for the future.

a. What have we learnt?

At the time of writing there have been a number of different cell types, preparation and doses delivered by a number of different routes to a variety of patients in small, mostly uncontrolled trials, generally with positive outcomes. However, many of the larger, controlled studies have been negative, a similar situation to that experienced by the angiogenesis gene therapy field a number of years ago. It is imperative that we learn lessons from the past, if the challenges for the future are to be overcome. Inadequately designed angiogenesis studies substantially set back progress in arguably what may have been, and may still be, a promising field. Thus, an important factor that emerged from double-blind placebo-controlled angiogenesis gene therapy studies was the large placebo effect, the extent and prevalence of which surpassed expectations. Importantly, this effect was not limited to 'soft' endpoints such as angina frequency, and QOL, but to physiological end-points such as exercise capacity and perfusion (Rana et al., 2005). The lack of a control group and the open label design precluded conclusions on efficacy,

however marked improvement in physiological measures were interpreted as equating to efficacy in these trials. So why do physiological measures improve? From my own observation I think it is likely related to the enhanced will to exercise created by the placebo effect. This strongly suggests that evaluation of efficacy is only possible in a double blind trial format. Yet slightly disappointingly the majority of cell-based studies are designed for safety and feasibility and never powered sufficiently for efficacy, have enthusiastically continued to publish on it. One also suspects that there is possibly a publication bias with preferential publishing of positive data. This has lead to skepticism within the medical profession, as it appears to be following the well-trodden path of incredible results→ miracle cure→sobering disappointment→wouldn't give it to my dog! →Cautious optimism, appropriate for a select group of patients?

Another caveat is the surrogate outcome measures used in the majority of cell regenerative studies have been adopted from cardiology and cardiac surgery studies and may not be sensitive enough to detect the small changes seen in neovascularisation, or LV remodeling. For example SPECT has a spatial resolution of 8 to 10 mm and therefore may not detect small tissue-level changes in perfusion, even though these small changes may be what is required to improve the QOL of 'no-option patients'(Lee et al., 2006). Conversely one could argue that if these affects are too small for detection then they are clinically meaningless. The problem is that surrogate end-points used to keep the cost of running trials from being prohibitive and to look for effects that may have clinical relevance are poorly understood. For example are the small 3 to 5% statistically significant changes in LVEF in the acute MI studies consistently demonstrated by meta-analysis (Abdel-Latif et al., 2007); (Hristov et al., 2006); (Lipinski et al., 2007) ; (Burt et al., 2008) clinically meaningful? Without evidence from large expensive outcome studies designed and powered to investigate hard outcomes this debate continues. In a recent review (Reffellmann et al., 2009) attempted to put bone marrow-derived stem cell transplantation for cardiac regeneration results into perspective with existing key clinical trials. By doing this it was interesting to be reminded that interventions that resulted in relatively small changes in LVEF, actually had significant effects on clinical outcome and thus altered clinical practice (Table 20). We also know that LVEF does not correlate well

with NYHA class. The fact remains that we do not have a good understanding of how changes in surrogate end points correlate with eventual clinical outcome.

Table 20: Summary of changes in LVEF seen in clinical trials that have influenced clinical practice based on their significant effect on clinical outcome.

			Change in LVEF
PCI versus Thrombolysis	PTCA versus streptokinase	(Zijlstra et al., 1993)	+6 % p = 0.004
	PTCA/Stent versus rTPA	(Ribichini et al., 1998)	+7 % p < 0.001
ACE inhibitor	Captopril versus placebo SAVE	(Rutherford et al., 1994)	+1.2 % p = 0.005
	Ramipril versus placebo HEART	(Pfeffer et al., 1997)	+2.5 % p < 0.5
β Blocker	Carvedilol versus placebo CAPRICORN	(Doughty et al., 2004)	+3.9 % p = 0.015

Table 20: Adapted from (Reffellmann et al., 2009); (Lovell and Mathur, 2011)

b. Were clinical trials undertaken prematurely?

Some may argue the move from bench to bedside has been premature, without adequate knowledge of the optimal cell type, method of handling and isolation and basic mechanisms of action. Further unanswered questions include the optimal patient population, timing related to the myocardial event, dose, optimal delivery route and appropriate assays and time points to detect clinically relevant changes. Although sympathetic to this view, much will be learnt by clinical studies proceeding in parallel with the necessary preclinical experiments. Certain answers can only be gained in the clinic, including a) optimal delivery route, b) assay optimisation and c) assessment of safety. An example of the latter is the electrical instability exhibited with skeletal myoblasts in man (Menasche et al., 2003) that was not detected in animal models. Chronic ischaemic heart disease is complex and results from a combination of genetic predisposition, environmental influences, biological and pathological processes. No animal model can adequately replicate endothelial dysfunction, diabetes, hypertension, inflammatory plaque formation and the numerous other aspects associated with human disease, it is therefore not surprising that the promise of stem cells seen in small animal

models has not borne out a similar magnitude of effect in man. The translation from animal to man has a number of other problems, the number of cells per unit of heart muscle is far higher in animals than man, as the number of cells is limited by the clinical bone marrow aspirate and the inability to expand the cells sufficiently between harvest and transplantation in tissue culture facilities. Most animal studies are undertaken with juvenile animals, the most common large animal model for angiogenesis studies is the porcine ameroid constrictor model. Juvenile, pre-pubertal, normocholesterolemic Yorkshire pigs are generally used that have normal endothelial function, and a robust rich source of bone marrow derived stem cells, unlike our patient population where advanced age along with a number of other co-morbidities have resulted in endothelial dysfunction along with an often depleted exhausted source of stem cells (Dimmeler, 2010). In my opinion animal models are vital in progressing the field, but they will not be able to fully answer the mechanistic questions relevant for human disease. The extensive number of variables demonstrates that much can still be gained from small, well thought out and conducted trials focused on exploring mechanism or specific hypotheses. I believe that as long as any reference to efficacy in these trials is taken in the context of a large placebo bias it may assist in improving outcome measures for larger definitive clinical outcome trials. We owe it to our patients who have often entered these trials with a large 'leap of faith' to gain as much information as possible to optimise and progress this field. Through my own PhD studies, we have gained enormous amounts of information, understanding and practical experience of what works and what does not work which will definitely shape our future trial design and could not have been gained solely from pre-clinical models.

c. Which is the best target patient population?

There is no clear indication of the most suitable patient population, or timing of delivery. However 'no-option' patients with a poor prognosis have the best risk-benefit ratio, and sub-group analysis suggests the greatest benefit is seen in patients with the lowest baseline LVEF (Schachinger et al., 2006). The effects of cell therapy seem to be restricted to the infarct-related territory, which may already be receiving adequate early revascularisation (Wollert et al., 2004). Thus, based on the evidence to date, patients with

persistently lower LVEF several days after an AMI infarct may gain the most benefit, and delivery of cells may be optimally administered at slightly later time points than initially thought (Schachinger et al., 2006). Meta-analysis of bone marrow derived stem cells for the treatment of AMI have found a consistent albeit small change in LVEF (Abdel-Latif et al., 2007); (Hristov et al., 2006); (Lipinski et al., 2007) ;(Burt et al., 2008). We are also learning more about the functionality of cells; (Assmus et al., 2007) found in TOPCARE-CHD (Table 4) that in patients with colony forming unit capacity above the median there was a reduction in mortality at follow-up. These findings suggest that in some patients the severity of depletion and exhaustion of their autologous bone marrow cells severely limits potential efficacy unless these deficiencies can be overcome, either by using an allogenic cell source for example of mesenchymal immune privileged cells, or by treatments directed at improving their own cell functionality. Of interest are reports that benefits have also been gained in patients with heart failure of non-ischaemic etiology (Seth et al., 2010) while subendocardial ischaemia has been reported in non-ischaemic cardiomyopathy and angiogenesis could conceivably play a role in the improvements seen in LV function, the dramatic improvements of 5.6% increase in LVEF points to other mechanisms including stimulation of resident stem cells via paracrine signaling or anti-apoptotic effects.

d. Which is the correct cell?

Another key question is what is the optimal cell type? At present there appears to be no clear steer on answering this question. The balance lies between delivering a highly enriched population of a particular favoured cell type, with the risk this is not the one relevant to myocardial benefit, or a heterogeneous population for which the risk is that the key cell type is too diluted. Preliminary data in man comparing a subpopulation of CD34+ CXCR4+ cells against an unselected bone marrow mononuclear cells population would suggest that no additional benefit is gained from using a selected population at this stage (Tendera et al., 2009). A further likely relevant factor is that many in the field believe that even if a sub-population can be defined, these cells may depend on a 'soup' of growth factors secreted by other bone marrow cells. Further research is clearly needed to elucidate the mechanisms by which cells may impart their benefit, and in turn

identifying which is the optimal cell type. Little data are available on how long the effect of stem cells needs to be maintained, thus, repeated delivery may offer additional benefits. Extended follow-up will be important in future trials, to assess whether benefits seen can be maintained; as initial improvements in LVEF were often not seen at later follow-up (Smits et al., 2003); (Kang et al., 2007); (Meyer et al., 2006). Different patient populations will require potentially different combinations of device-biological combinations, as it is naïve to think that a ‘one-fit’ solution will suit all. In AMI it may be sufficient to use a cell source capable of inducing angiogenesis and preventing apoptosis of cardiac myocytes; the unscheduled timing of such an event will lead to cell sources that do not require extensive time consuming processing and manipulation as treatment will be performed in an acute setting. The delivery device required to treat patients after an AMI may be very different from those in the chronic setting. Patients presenting predominantly with heart failure and a loss of functioning myocytes will require a robust cell source capable of regeneration of contractile tissue in an ischaemic environment or stimulating formation of contractile tissue by upregulating the function of resident stem cells. At present the search for cells capable of *de novo* regeneration of functional myocardial tissue continues, cardiac progenitor cells (Beltrami et al., 2001), pluripotent primitive progenitors and induced pluripotent progenitors (Park et al., 2008) are all being investigated. The prospect of using a combination of gene and cell therapy by so called ‘primed cells’ in which cells have been transfected with genes to alter potential for cardiac differentiation, homing and retention, has also undergoing pre-clinical investigation and is currently in clinical trials only time will tell if their promise in pre-clinical models will be replicated in man. As for the story of single growth factors in the angiogenesis trials, it is unlikely a single cell type will be sufficient to regenerate a complex tissue such as the heart. Conceptually the combination of a number of different cells aiming at synergism is an attractive approach

e. Delivery

In addition, different clinical presentations, whether it be AMI or chronic IHD, will require different delivery strategies. Chronic IHD therapy will require a delivery device capable of targeting large areas, bypassing occluded coronary arteries and weakened

cytokine homing signals. At present high retention within the myocardium remains elusive, advances in cell labelling and tracking will assist in optimising delivery and retention within the myocardium and ascertaining in negative trials whether ineffective delivery was responsible for the outcome. Our preliminary results would indicate at this stage that retrograde coronary venous delivery is worthy of further investigation for this treatment group.

f. Problems with Funding?

One major problem with cell therapy is the lack of appropriate funding for large multi-centre studies. Autologous stem cells from a big pharmaceutical prospective are not an attractive proposition; skeptically I question whether the enthusiasm for selective cells is driven by biological superiority or the potential for patents and secure industrial funding? Conversely the use of un-selected bone marrow derived cells in our trial and others is often influenced by the pragmatic ease of use. I am sympathetic to this stance from an academic perspective; stem cell therapy poses unique regulatory and clinical challenges. We experienced first hand the problems arising from trying to get company agreement on a device-biological combination for our angiogenesis trial. It is not always as simple as using the best product with the best device, and at times scientific compromises are made. For stem cells this situation is even more complex, with cell therapy strategies encompassing cell procurement, cell modification, purification and delivery involving a number of companies all negotiating over ownership rights in a tough economic climate. Not only do all these issues need to be optimised and the companies involved agree on a business plan but all the other aspects of trial design such as patient selection and optimisation of cell delivery to different patient populations. It is naïve to think that a ‘one-fit’ solution will suit all, conceptualising cell therapy for each condition may need to be viewed as a treatment strategy rather than merely a pharmaceutical product (Povsic and O'Connor, 2010). Selection of sensitive, meaningful outcome measures, given that pre-clinical models are often non-informative, are challenges we all face, and yet to optimise each step is often cost-prohibitive in small academically driven trials. The European commission has funded much of the German groups work and has recently agreed to fund the pan European 3000 patient outcome study trial of autologous bone

marrow cells in acute myocardial infarction at an estimated cost of six million Euros. Whilst the National Heart and Lung Blood Institute (NHLBI) cardiovascular cell therapy research network has funded a large multi-centre trial in the United States (Traverse et al., 2009), however financial concerns have prevented enrollment in well designed, clinically important protocols that would definitely progress the field. Further collaborative efforts involving governments, industrial and academic partnerships are needed if stem cell therapy is to be advanced.

g. Conclusions

Clearly not all of these questions can be answered in the clinical trial setting and it is imperative that basic science and translational research are conducted simultaneously to ascertain the mechanisms of action, to elucidate further the optimal cell type and help guide clinical research. Well conducted clinical trials need to establish the optimal dose, delivery and timing of administration in the relevant patient populations, and with the recommendations from the European Society of Cardiology stem cell task force, use a consensus approach based on the best evidence to standardise patient selection, and surrogate end-points so that the results from smaller studies can be directly compared (Bartunek et al., 2006). Whilst it is important that the field moves on enthusiastically, it is again worth harking back to the angiogenesis gene therapy studies. In general, these were poorly conducted, without knowledge of the most basic variables such as dose, delivery route and duration of action. The resulting, predictably unfavourable results, dampened enthusiasm for what may be just as, or a more useful approach, than stem cells. In fact the future may well combine the two approaches with cells manipulated and or administered with gene therapy as so called ‘magic bullets’. In order to avoid the same pitfall there is the obvious need for clearly focused translational preclinical research running in parallel with, and well coordinated with, randomised, placebo-controlled trials. Finally, it is unlikely that this will be a ‘quick-fix’; the old adage about 20 years from bench to bedside will likely represent a minimum in this field, and patience will be a key virtue for both the community and the funders if this approach is to succeed.

REFERENCES

- The survival of patients with heart failure with preserved or reduced left ventricular ejection fraction: an individual patient data meta-analysis. *Eur Heart J*.
- (1987) Effects of enalapril on mortality in severe congestive heart failure. Results of the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS). The CONSENSUS Trial Study Group. *N Engl J Med*, 316, 1429-35.
- (1988) Immediate vs delayed catheterization and angioplasty following thrombolytic therapy for acute myocardial infarction. TIMI II A results. The TIMI Research Group. *JAMA*, 260, 2849-58.
- ABDEL-LATIF, A., BOLLI, R., TLEYJEH, I. M., MONTORI, V. M., PERIN, E. C., HORNUNG, C. A., ZUBA-SURMA, E. K., AL-MALLAH, M. & DAWN, B. (2007) Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis. *Arch Intern Med*, 167, 989-97.
- ABRAHAM, W. T., FISHER, W. G., SMITH, A. L., DELURGIO, D. B., LEON, A. R., LOH, E., KOCOVIC, D. Z., PACKER, M., CLAVELL, A. L., HAYES, D. L., ELLESTAD, M., TRUPP, R. J., UNDERWOOD, J., PICKERING, F., TRUEX, C., MCATEE, P. & MESSENGER, J. (2002) Cardiac resynchronization in chronic heart failure. *N Engl J Med*, 346, 1845-53.
- AHERNE, W. A. & DUNNILL, M. S. (1982) *Point Counting and Morphometry*, London, Edward Arnold.
- AIELLO, L. P., AVERY, R. L., ARRIGG, P. G., KEYT, B. A., JAMPEL, H. D., SHAH, S. T., PASQUALE, L. R., THIEME, H., IWAMOTO, M. A., PARK, J. E. & ET AL. (1994) Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med*, 331, 1480-7.
- ALTIN, T., AKYUREK, O., VURGUN, K., BETON, O., SAYIN, T., KILICKAP, M., KARAOGUZ, R., GULDAL, M. & EROL, C. (2007) Effect of transvenous cardiac resynchronization therapy device implantation on cardiac troponin I release. *Pacing Clin Electrophysiol*, 30, 1356-62.
- ANDO, J., NOMURA, H. & KAMIYA, A. (1987) The effect of fluid shear stress on the migration and proliferation of cultured endothelial cells. *Microvasc Res*, 33, 62-70.
- ANVERSA, P. & NADAL-GINARD, B. (2002) Myocyte renewal and ventricular remodelling. *Nature*, 415, 240-3.
- ARCHUNDIA, A., ACEVES, J. L., LOPEZ-HERNANDEZ, M., ALVARADO, M., RODRIGUEZ, E., DIAZ QUIROZ, G., PAEZ, A., ROJAS, F. M. & MONTANO, L. F. (2005) Direct cardiac injection of G-CSF mobilized bone-marrow stem-cells improves ventricular function in old myocardial infarction. *Life Sci*, 78, 279-83.
- ARMENTANO, D., SOOKDEO, C. C., HEHIR, K. M., GREGORY, R. J., ST GEORGE, J. A., PRINCE, G. A., WADSWORTH, S. C. & SMITH, A. E. (1995) Characterization of an adenovirus gene transfer vector containing an E4 deletion. *Hum Gene Ther*, 6, 1343-53.
- ASAHARA, T., BAUTERS, C., ZHENG, L. P., TAKESHITA, S., BUNTING, S., FERRARA, N., SYMES, J. F. & ISNER, J. M. (1995) Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in vivo. *Circulation*, 92, II365-71.

- ASSMUS, B., FISCHER-RASOKAT, U., HONOLD, J., SEEGER, F. H., FICHTLSCHERER, S., TONN, T., SEIFRIED, E., SCHACHINGER, V., DIMMELER, S. & ZEIHNER, A. M. (2007) Transcoronary transplantation of functionally competent BMCs is associated with a decrease in natriuretic peptide serum levels and improved survival of patients with chronic postinfarction heart failure: results of the TOPCARE-CHD Registry. *Circ Res*, 100, 1234-41.
- ASSMUS, B., HONOLD, J., SCHACHINGER, V., BRITTEN, M. B., FISCHER-RASOKAT, U., LEHMANN, R., TEUPE, C., PISTORIUS, K., MARTIN, H., ABOLMAALI, N. D., TONN, T., DIMMELER, S. & ZEIHNER, A. M. (2006) Transcoronary transplantation of progenitor cells after myocardial infarction. *N Engl J Med*, 355, 1222-32.
- ASSMUS, B., TONN, T., SEEGER, F. H., YOON, C. H., LEISTNER, D., KLOTSCH, J., SCHACHINGER, V., SEIFRIED, E., ZEIHNER, A. M. & DIMMELER, S. (2010) Red blood cell contamination of the final cell product impairs the efficacy of autologous bone marrow mononuclear cell therapy. *J Am Coll Cardiol*, 55, 1385-94.
- AUERBACH, M. A., SCHODER, H., HOH, C., GAMBHIR, S. S., YAGHOUBI, S., SAYRE, J. W., SILVERMAN, D., PHELPS, M. E., SCHELBERT, H. R. & CZERNIN, J. (1999) Prevalence of myocardial viability as detected by positron emission tomography in patients with ischemic cardiomyopathy. *Circulation*, 99, 2921-6.
- AURICCHIO, A. & PRINZEN, F. W. (2011) Non-responders to cardiac resynchronization therapy. *Circ J*, 75, 521-7.
- BALDASSERONI, S., OPASICH, C., GORINI, M., LUCCI, D., MARCHIONNI, N., MARINI, M., CAMPANA, C., PERINI, G., DEORSOLA, A., MASOTTI, G., TAVAZZI, L. & MAGGIONI, A. P. (2002) Left bundle-branch block is associated with increased 1-year sudden and total mortality rate in 5517 outpatients with congestive heart failure: a report from the Italian network on congestive heart failure. *Am Heart J*, 143, 398-405.
- BALDWIN, J. T. & MANN, D. L. (2010) NHLBI's program for VAD therapy for moderately advanced heart failure: the REVIVE-IT pilot trial. *J Card Fail*, 16, 855-8.
- BANAI, S., JAKLITSCH, M. T., CASSCELLS, W., SHOU, M., SHRIVASTAV, S., CORREA, R., EPSTEIN, S. E. & UNGER, E. F. (1991) Effects of acidic fibroblast growth factor on normal and ischemic myocardium. *Circ Res*, 69, 76-85.
- BARTUNEK, J., DIMMELER, S., DREXLER, H., FERNANDEZ-AVILES, F., GALINANES, M., JANSSENS, S., MARTIN, J., MATHUR, A., MENASCHE, P., PRIORI, S., STRAUER, B., TENDERA, M., WIJNS, W. & ZEIHNER, A. (2006) 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*, 27, 1338-40.
- BECKER, W. & MELLER, J. (2001) The role of nuclear medicine in infection and inflammation. *Lancet Infect Dis*, 1, 326-33.

- BEERES, S. L., BAX, J. J., ROES, S. D., LAMB, H. J., FIBBE, W. E., DE ROOS, A., VAN DER WALL, E. E., SCHALIJ, M. J. & AT SMA, D. E. (2007) Intramyocardial bone marrow cell transplantation and the progression of coronary atherosclerosis in patients with chronic myocardial ischemia. *Acute Card Care*, 9, 243-51.
- BEITNES, J. O., HOPP, E., LUNDE, K., SOLHEIM, S., ARNESEN, H., BRINCHMANN, J. E., FORFANG, K. & AAKHUS, S. (2009) Long-term results after intracoronary injection of autologous mononuclear bone marrow cells in acute myocardial infarction: the ASTAMI randomised, controlled study. *Heart*, 95, 1983-9.
- BELTRAMI, A. P., URBANEK, K., KAJSTURA, J., YAN, S. M., FINATO, N., BUSSANI, R., NADAL-GINARD, B., SILVESTRI, F., LERI, A., BELTRAMI, C. A. & ANVERSA, P. (2001) Evidence that human cardiac myocytes divide after myocardial infarction. *N Engl J Med*, 344, 1750-7.
- BERTOLINI, F. & PRUNERI, G. (2002) Chimerism of the transplanted heart. *N Engl J Med*, 346, 1410-2; author reply 1410-2.
- BINDSLEV, L., HAACK-SORENSEN, M., BISGAARD, K., KRAGH, L., MORTENSEN, S., HESSE, B., KJAER, A. & KASTRUP, J. (2006) Labelling of human mesenchymal stem cells with indium-111 for SPECT imaging: effect on cell proliferation and differentiation. *Eur J Nucl Med Mol Imaging*, 33, 1171-7.
- BLEUMINK, G. S., KNETSCH, A. M., STURKENBOOM, M. C., STRAUS, S. M., HOFMAN, A., DECKERS, J. W., WITTEMAN, J. C. & STRICKER, B. H. (2004) Quantifying the heart failure epidemic: prevalence, incidence rate, lifetime risk and prognosis of heart failure The Rotterdam Study. *Eur Heart J*, 25, 1614-9.
- BODEN, W. E., O'ROURKE, R. A., TEO, K. K., MARON, D. J., HARTIGAN, P. M., SEDLIS, S. P., DADA, M., LABEDI, M., SPERTUS, J. A., KOSTUK, W. J., BERMAN, D. S., SHAW, L. J., CHAITMAN, B. R., MANCINI, G. B. & WEINTRAUB, W. S. (2009) Impact of optimal medical therapy with or without percutaneous coronary intervention on long-term cardiovascular end points in patients with stable coronary artery disease (from the COURAGE Trial). *Am J Cardiol*, 104, 1-4.
- BOEKSTEGERS, P., VON DEGENFELD, G., GIEHRL, W., HEINRICH, D., HULLIN, R., KUPATT, C., STEINBECK, G., BARETTON, G., MIDDELER, G., KATUS, H. & FRANZ, W. M. (2000) Myocardial gene transfer by selective pressure-regulated retroinfusion of coronary veins. *Gene Ther*, 7, 232-40.
- BOLUKOGLU, H., LIEDTKE, A. J., NELLIS, S. H., EGGLESTON, A. M., SUBRAMANIAN, R. & RENSTROM, B. (1992) An animal model of chronic coronary stenosis resulting in hibernating myocardium. *Am J Physiol*, 263, H20-9.
- BOYLE, A. J., WHITBOURN, R., SCHLICHT, S., KRUM, H., KOCHER, A., NANDURKAR, H., BERGMANN, S., DANIELL, M., O'DAY, J., SKERRETT, D., HAYLOCK, D., GILBERT, R. E. & ITESCU, S. (2006) Intra-coronary high-dose CD34+ stem cells in patients with chronic ischemic heart disease: a 12-month follow-up. *Int J Cardiol*, 109, 21-7.

- BRENNER, W., AICHER, A., ECKEY, T., MASSOUDI, S., ZUHAYRA, M., KOEHL, U., HEESCHEN, C., KAMPEN, W. U., ZEIHNER, A. M., DIMMELER, S. & HENZE, E. (2004) ^{111}In -labeled CD34⁺ hematopoietic progenitor cells in a rat myocardial infarction model. *J Nucl Med*, 45, 512-8.
- BRISTOW, M. R., SAXON, L. A., BOEHMER, J., KRUEGER, S., KASS, D. A., DE MARCO, T., CARSON, P., DICARLO, L., DEMETS, D., WHITE, B. G., DEVRIES, D. W. & FELDMAN, A. M. (2004) Cardiac-resynchronization therapy with or without an implantable defibrillator in advanced chronic heart failure. *N Engl J Med*, 350, 2140-50.
- BUI, A. L., HORWICH, T. B. & FONAROW, G. C. (2011) Epidemiology and risk profile of heart failure. *Nat Rev Cardiol*, 8, 30-41.
- BURT, R. K., LOH, Y., PEARCE, W., BEOHAR, N., BARR, W. G., CRAIG, R., WEN, Y., RAPP, J. A. & KESSLER, J. (2008) Clinical applications of blood-derived and marrow-derived stem cells for nonmalignant diseases. *JAMA*, 299, 925-36.
- CARMELET, P. & JAIN, R. K. (2000) Angiogenesis in cancer and other diseases. *Nature*, 407, 249-57.
- CELLETTI, F. L., WAUGH, J. M., AMABILE, P. G., BRENDOLAN, A., HILFIKER, P. R. & DAKE, M. D. (2001) Vascular endothelial growth factor enhances atherosclerotic plaque progression. *Nat Med*, 7, 425-9.
- CERQUEIRA, M. D., WEISSMAN, N. J., DILSIZIAN, V., JACOBS, A. K., KAUL, S., LASKEY, W. K., PENNELL, D. J., RUMBERGER, J. A., RYAN, T. & VERANI, M. S. (2002) Standardized myocardial segmentation and nomenclature for tomographic imaging of the heart: a statement for healthcare professionals from the Cardiac Imaging Committee of the Council on Clinical Cardiology of the American Heart Association. *Circulation*, 105, 539-42.
- CHACHQUES, J. C., HERREROS, J., TRAININI, J., JUFFE, A., RENDAL, E., PROSPER, F. & GENOVESE, J. (2004) Autologous human serum for cell culture avoids the implantation of cardioverter-defibrillators in cellular cardiomyoplasty. *Int J Cardiol*, 95 Suppl 1, S29-33.
- CHEN, C., CHEN, L., FALLON, J. T., MA, L., LI, L., BOW, L., KNIBBS, D., MCKAY, R., GILLAM, L. D. & WATERS, D. D. (1996) Functional and structural alterations with 24-hour myocardial hibernation and recovery after reperfusion. A pig model of myocardial hibernation. *Circulation*, 94, 507-16.
- CHIN, B. B., NAKAMOTO, Y., BULTE, J. W., PITTENGER, M. F., WAHL, R. & KRAITCHMAN, D. L. (2003) ^{111}In oxine labelled mesenchymal stem cell SPECT after intravenous administration in myocardial infarction. *Nucl Med Commun*, 24, 1149-54.
- CINTRON, G., JOHNSON, G., FRANCIS, G., COBB, F. & COHN, J. N. (1993) Prognostic significance of serial changes in left ventricular ejection fraction in patients with congestive heart failure. The V-HeFT VA Cooperative Studies Group. *Circulation*, 87, VI17-23.
- CRYSTAL, R. G. (1995) Transfer of genes to humans: early lessons and obstacles to success. *Science*, 270, 404-10.
- DARLAND, D. C. & D'AMORE, P. A. (1999) Blood vessel maturation: vascular development comes of age. *J Clin Invest*, 103, 157-8.

- DE COCK, C. C., VAN CAMPEN, C. M. & VISSER, C. A. (2004) Major dissection of the coronary sinus and its tributaries during lead implantation for biventricular stimulation: angiographic follow-up. *Europace*, 6, 43-7.
- DE LA FUENTE, L. M., STERTZER, S. H., ARGENTIERI, J., PENALOZA, E., MIANO, J., KOZINER, B., BILOS, C. & ALTMAN, P. A. (2007) Transendocardial autologous bone marrow in chronic myocardial infarction using a helical needle catheter: 1-year follow-up in an open-label, nonrandomized, single-center pilot study (the TABMMI study). *Am Heart J*, 154, 79 e1-7.
- DE LABRIOLLE, A., LEMESLE, G., BONELLO, L., SYED, A. I., COLLINS, S. D., BEN-DOR, I., PINTO SLOTTOW, T. L., XUE, Z., TORGUSON, R., SUDDATH, W. O., SATLER, L. F., KENT, K. M., PICHARD, A. D., LINDSAY, J. & WAKSMAN, R. (2009) Prognostic significance of small troponin I rise after a successful elective percutaneous coronary intervention of a native artery. *Am J Cardiol*, 103, 639-45.
- DE MARTINO, G., MESSANO, L., SANTAMARIA, M., PARISI, Q., DELLO RUSSO, A., PELARGONIO, G., SANNA, T., NARDUCCI, M. L., CHIRIACO, T., BELLOCCI, F., ZECCHI, P. & CREA, F. (2005) A randomized evaluation of different approaches to coronary sinus venography during biventricular pacemaker implants. *Europace*, 7, 73-6.
- DENG, M. C., DE MEESTER, J. M., SMITS, J. M., HEINECKE, J. & SCHELD, H. H. (2000) Effect of receiving a heart transplant: analysis of a national cohort entered on to a waiting list, stratified by heart failure severity. Comparative Outcome and Clinical Profiles in Transplantation (COC PIT) Study Group. *BMJ*, 321, 540-5.
- DI CARLI, M. F. (1998) Predicting improved function after myocardial revascularization. *Curr Opin Cardiol*, 13, 415-24.
- DIMMELER, S. (2010) Regulation of bone marrow-derived vascular progenitor cell mobilization and maintenance. *Arterioscler Thromb Vasc Biol*, 30, 1088-93.
- DOUGHTY, R. N., WHALLEY, G. A., WALSH, H. A., GAMBLE, G. D., LOPEZ-SENDON, J. & SHARPE, N. (2004) Effects of carvedilol on left ventricular remodeling after acute myocardial infarction: the CAPRICORN Echo Substudy. *Circulation*, 109, 201-6.
- DUMONT, D. J., FONG, G. H., PURI, M. C., GRADWOHL, G., ALITALO, K. & BREITMAN, M. L. (1995) Vascularization of the mouse embryo: a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. *Dev Dyn*, 203, 80-92.
- DUNCAN, A., WAIT, D., GIBSON, D. & DAUBERT, J. C. (2003) Left ventricular remodelling and haemodynamic effects of multisite biventricular pacing in patients with left ventricular systolic dysfunction and activation disturbances in sinus rhythm: sub-study of the MUSTIC (Multisite Stimulation in Cardiomyopathies) trial. *Eur Heart J*, 24, 430-41.
- DUNCAN, A. M., LIM, E., CLAGUE, J., GIBSON, D. G. & HENEIN, M. Y. (2006) Comparison of segmental and global markers of dyssynchrony in predicting clinical response to cardiac resynchronization. *Eur Heart J*, 27, 2426-32.

- ENGELMANN, M. G., THEISS, H. D., HENNIG-THEISS, C., HUBER, A., WINTERSPERGER, B. J., WERLE-RUEDINGER, A. E., SCHOENBERG, S. O., STEINBECK, G. & FRANZ, W. M. (2006) 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*, 48, 1712-21.
- ENGLER, D. A. (1996) Use of vascular endothelial growth factor for therapeutic angiogenesis. *Circulation*, 94, 1496-8.
- ERBS, S., LINKE, A., ADAMS, V., LENK, K., THIELE, H., DIEDERICH, K. W., EMMRICH, F., KLUGE, R., KENDZIORRA, K., SABRI, O., SCHULER, G. & HAMBRECHT, R. (2005) Transplantation of blood-derived progenitor cells after recanalization of chronic coronary artery occlusion: first randomized and placebo-controlled study. *Circ Res*, 97, 756-62.
- FALLAVOLLITA, J. A., PERRY, B. J. & CANTY, J. M., JR. (1997) 18F-2-deoxyglucose deposition and regional flow in pigs with chronically dysfunctional myocardium. Evidence for transmural variations in chronic hibernating myocardium. *Circulation*, 95, 1900-9.
- FARWELL, D., PATEL, N. R., HALL, A., RALPH, S. & SULKE, A. N. (2000) How many people with heart failure are appropriate for biventricular resynchronization? *Eur Heart J*, 21, 1246-50.
- FEARON, W. F., IKENO, F., BAILEY, L. R., HIATT, B. L., HERITY, N. A., CARTER, A. J., FITZGERALD, P. J., REZAEE, M., YEUNG, A. C. & YOCK, P. G. (2004) Evaluation of high-pressure retrograde coronary venous delivery of FGF-2 protein. *Catheter Cardiovasc Interv*, 61, 422-8.
- FERNANDEZ-AVILES, F., SAN ROMAN, J. A., GARCIA-FRADE, J., FERNANDEZ, M. E., PENARRUBIA, M. J., DE LA FUENTE, L., GOMEZ-BUENO, M., CANTALAPIEDRA, A., FERNANDEZ, J., GUTIERREZ, O., SANCHEZ, P. L., HERNANDEZ, C., SANZ, R., GARCIA-SANCHO, J. & SANCHEZ, A. (2004) Experimental and clinical regenerative capability of human bone marrow cells after myocardial infarction. *Circ Res*, 95, 742-8.
- FERRARA, N., CARVER-MOORE, K., CHEN, H., DOWD, M., LU, L., O'SHEA, K. S., POWELL-BRAXTON, L., HILLAN, K. J. & MOORE, M. W. (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature*, 380, 439-42.
- FONG, G. H., ROSSANT, J., GERTSENSTEIN, M. & BREITMAN, M. L. (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature*, 376, 66-70.
- FORTUIN, F. D., VALE, P., LOSORDO, D. W., SYMES, J., DELARIA, G. A., TYNER, J. J., SCHAER, G. L., MARCH, R., SNELL, R. J., HENRY, T. D., VAN CAMP, J., LOPEZ, J. J., RICHENBACHER, W., ISNER, J. M. & SCHATZ, R. A. (2003) One-year follow-up of direct myocardial gene transfer of vascular endothelial growth factor-2 using naked plasmid deoxyribonucleic acid by way of thoracotomy in no-option patients. *Am J Cardiol*, 92, 436-9.
- FRATER, R. W. (1999) Retrograde cardioplegia. *J Heart Valve Dis*, 8, 118-9.

- FREEDMAN, S. B. & ISNER, J. M. (2001) Therapeutic angiogenesis for ischemic cardiovascular disease. *J Mol Cell Cardiol*, 33, 379-93.
- FREEDMAN, S. B., VALE, P., KALKA, C., KEARNEY, M., PIECZEK, A., SYMES, J., LOSORDO, D. & ISNER, J. M. (2002) Plasma vascular endothelial growth factor (VEGF) levels after intramuscular and intramyocardial gene transfer of VEGF-1 plasmid DNA. *Hum Gene Ther*, 13, 1595-603.
- FUCHS, S., BAFFOUR, R., ZHOU, Y. F., SHOU, M., PIERRE, A., TIO, F. O., WEISSMAN, N. J., LEON, M. B., EPSTEIN, S. E. & KORNOWSKI, R. (2001a) Transendocardial delivery of autologous bone marrow enhances collateral perfusion and regional function in pigs with chronic experimental myocardial ischemia. *J Am Coll Cardiol*, 37, 1726-32.
- FUCHS, S., SATLER, L. F., KORNOWSKI, R., OKUBAGZI, P., WEISZ, G., BAFFOUR, R., WAKSMAN, R., WEISSMAN, N. J., CERQUEIRA, M., LEON, M. B. & EPSTEIN, S. E. (2003) Catheter-based autologous bone marrow myocardial injection in no-option patients with advanced coronary artery disease: a feasibility study. *J Am Coll Cardiol*, 41, 1721-4.
- FUCHS, S., SHOU, M., BAFFOUR, R., EPSTEIN, S. E. & KORNOWSKI, R. (2001b) Lack of correlation between angiographic grading of collateral and myocardial perfusion and function: implications for the assessment of angiogenic response. *Coron Artery Dis*, 12, 173-8.
- FUJITA, M., IKEMOTO, M., KISHISHITA, M., OTANI, H., NOHARA, R., TANAKA, T., TAMAKI, S., YAMAZATO, A. & SASAYAMA, S. (1996) Elevated basic fibroblast growth factor in pericardial fluid of patients with unstable angina. *Circulation*, 94, 610-3.
- GE, J., LI, Y., QIAN, J., SHI, J., WANG, Q., NIU, Y., FAN, B., LIU, X., ZHANG, S., SUN, A. & ZOU, Y. (2006) Efficacy of emergent transcatheter transplantation of stem cells for treatment of acute myocardial infarction (TCT-STAMI). *Heart*, 92, 1764-7.
- GENSINI, G. G., DIGIORGI, S., COSKUN, O., PALACIO, A. & KELLY, A. E. (1965) Anatomy of the Coronary Circulation in Living Man; Coronary Venography. *Circulation*, 31, 778-84.
- GHEORGHIADE, M. & BONOW, R. O. (1998) Chronic heart failure in the United States: a manifestation of coronary artery disease. *Circulation*, 97, 282-9.
- GHOSHAL, N., G (1975) *Porcine heart and arteries*, Philadelphia, Saunders.
- GILDEHAUS, F. J., HAASTERS, F., DROSSE, I., WAGNER, E., ZACH, C., MUTSCHLER, W., CUMMING, P., BARTENSTEIN, P. & SCHIEKER, M. (2010) Impact of Indium-111 Oxine Labelling on Viability of Human Mesenchymal Stem Cells In Vitro, and 3D Cell-Tracking Using SPECT/CT In Vivo. *Mol Imaging Biol*.
- GIORDANO, F. J., PING, P., MCKIRNAN, M. D., NOZAKI, S., DEMARIA, A. N., DILLMANN, W. H., MATHIEU-COSTELLO, O. & HAMMOND, H. K. (1996) Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile function in an ischemic region of the heart. *Nat Med*, 2, 534-9.

- GOUSSETIS, E., MANGINAS, A., KOUTELOU, M., PERISTERI, I., THEODOSAKI, M., KOLLAROS, N., LEONTIADIS, E., THEODORAKOS, A., PATERAKIS, G., KARATASAKIS, G., COKKINOS, D. V. & GRAPHAKOS, S. (2006) Intracoronary infusion of CD133+ and CD133-CD34+ selected autologous bone marrow progenitor cells in patients with chronic ischemic cardiomyopathy: cell isolation, adherence to the infarcted area, and body distribution. *Stem Cells*, 24, 2279-83.
- GRAS, D., CEBRON, J. P., BRUNEL, P., LEURENT, B. & BANUS, Y. (2002) Optimal stimulation of the left ventricle. *J Cardiovasc Electrophysiol*, 13, S57-62.
- GRINES, C. L., WATKINS, M. W., MAHMARIAN, J. J., ISKANDRIAN, A. E., RADE, J. J., MARROTT, P., PRATT, C. & KLEIMAN, N. (2003) A randomized, double-blind, placebo-controlled trial of Ad5FGF-4 gene therapy and its effect on myocardial perfusion in patients with stable angina. *J Am Coll Cardiol*, 42, 1339-47.
- GUILLEMIN, K. & KRASNOW, M. A. (1997) The hypoxic response: huffing and HIFing. *Cell*, 89, 9-12.
- GYONGYOSI, M., KHORSAND, A., ZAMINI, S., SPERKER, W., STREHBLOW, C., KASTRUP, J., JORGENSEN, E., HESSE, B., TAGIL, K., BOTKER, H. E., RUZYLLO, W., TERESINSKA, A., DUDEK, D., HUBALEWSKA, A., RUCK, A., NIELSEN, S. S., GRAF, S., MUNDIGLER, G., NOVAK, J., SOCHOR, H., MAURER, G., GLOGAR, D. & SYLVEN, C. (2005) NOGA-guided analysis of regional myocardial perfusion abnormalities treated with intramyocardial injections of plasmid encoding vascular endothelial growth factor A-165 in patients with chronic myocardial ischemia: subanalysis of the EUROINJECT-ONE multicenter double-blind randomized study. *Circulation*, 112, I157-65.
- HAGEGE, A. A., MAROLLEAU, J. P., VILQUIN, J. T., ALHERITIERE, A., PEYRARD, S., DUBOC, D., ABERGEL, E., MESSAS, E., MOUSSEAUX, E., SCHWARTZ, K., DESNOS, M. & MENASCHE, P. (2006) Skeletal myoblast transplantation in ischemic heart failure: long-term follow-up of the first phase I cohort of patients. *Circulation*, 114, I108-13.
- HARADA, K., FRIEDMAN, M., LOPEZ, J. J., WANG, S. Y., LI, J., PRASAD, P. V., PEARLMAN, J. D., EDELMAN, E. R., SELLKE, F. W. & SIMONS, M. (1996) Vascular endothelial growth factor administration in chronic myocardial ischemia. *Am J Physiol*, 270, H1791-802.
- HARE, J. M., TRAVERSE, J. H., HENRY, T. D., DIB, N., STRUMPF, R. K., SCHULMAN, S. P., GERSTENBLITH, G., DEMARIA, A. N., DENKTAS, A. E., GAMMON, R. S., HERMILLER, J. B., JR., REISMAN, M. A., SCHAEER, G. L. & SHERMAN, W. (2009) A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. *J Am Coll Cardiol*, 54, 2277-86.
- HEDMAN, M., MUONA, K., HEDMAN, A., KIVELA, A., SYVANNE, M., ERANEN, J., RANTALA, A., STJERNVALL, J., NIEMINEN, M. S., HARTIKAINEN, J. & YLA-HERTTUALA, S. (2009) Eight-year safety follow-up of coronary artery disease patients after local intracoronary VEGF gene transfer. *Gene Ther*, 16, 629-34.

- HEESCHEN, C., LEHMANN, R., HONOLD, J., ASSMUS, B., AICHER, A., WALTER, D. H., MARTIN, H., ZEIHNER, A. M. & DIMMELER, S. (2004) Profoundly reduced neovascularization capacity of bone marrow mononuclear cells derived from patients with chronic ischemic heart disease. *Circulation*, 109, 1615-22.
- HEINL-GREEN, A., RADKE, P. W., MUNKONGE, F. M., FRASS, O., ZHU, J., VINCENT, K., GEDDES, D. M. & ALTON, E. W. (2005) The efficacy of a 'master switch gene' HIF-1 α in a porcine model of chronic myocardial ischaemia. *Eur Heart J*, 26, 1327-32.
- HENRY, T. D., ANNEX, B. H., MCKENDALL, G. R., AZRIN, M. A., LOPEZ, J. J., GIORDANO, F. J., SHAH, P. K., WILLERSON, J. T., BENZA, R. L., BERMAN, D. S., GIBSON, C. M., BAJAMONDE, A., RUNDLE, A. C., FINE, J. & MCCLUSKEY, E. R. (2003) The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis. *Circulation*, 107, 1359-65.
- HERITY, N. A., LO, S. T., OEI, F., LEE, D. P., WARD, M. R., FILARDO, S. D., HASSAN, A., SUZUKI, T., REZAEI, M., CARTER, A. J., YOCK, P. G., YEUNG, A. C. & FITZGERALD, P. J. (2000) Selective regional myocardial infiltration by the percutaneous coronary venous route: A novel technique for local drug delivery. *Catheter Cardiovasc Interv*, 51, 358-63.
- HIRSCH, A., NIJVELDT, R., VAN DER VLEUTEN, P. A., TIJSSEN, J. G., VAN DER GIESSEN, W. J., TIO, R. A., WALTENBERGER, J., TEN BERG, J. M., DOEVENDANS, P. A., AENGEVAEREN, W. R., ZWAGINGA, J. J., BIEMOND, B. J., VAN ROSSUM, A. C., PIEK, J. J. & ZIJLSTRA, F. (2011) 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*.
- HOFMANN, M., WOLLERT, K. C., MEYER, G. P., MENKE, A., ARSENIJEV, L., HERTENSTEIN, B., GANSER, A., KNAPP, W. H. & DREXLER, H. (2005) Monitoring of bone marrow cell homing into the infarcted human myocardium. *Circulation*, 111, 2198-202.
- HORRIGAN, M. C., MACISAAC, A. I., NICOLINI, F. A., VINCE, D. G., LEE, P., ELLIS, S. G. & TOPOL, E. J. (1996) Reduction in myocardial infarct size by basic fibroblast growth factor after temporary coronary occlusion in a canine model. *Circulation*, 94, 1927-33.
- HOU, D., MACLAUGHLIN, F., THIESSE, M., PANCHAL, V. R., BEKKERS, B. C., WILSON, E. A., ROGERS, P. I., COLEMAN, M. C. & MARCH, K. L. (2003) Widespread regional myocardial transfection by plasmid encoding Del-1 following retrograde coronary venous delivery. *Catheter Cardiovasc Interv*, 58, 207-11.
- HRISTOV, M., HEUSSEN, N., SCHOBEL, A. & WEBER, C. (2006) Intracoronary infusion of autologous bone marrow cells and left ventricular function after acute myocardial infarction: a meta-analysis. *J Cell Mol Med*, 10, 727-33.
- HUGHES, D. K. (2003) Nuclear medicine and infection detection: the relative effectiveness of imaging with ¹¹¹In-oxine-, ^{99m}Tc-HMPAO-, and ^{99m}Tc-stannous fluoride colloid-labeled leukocytes and with ⁶⁷Ga-citrate. *J Nucl Med Technol*, 31, 196-201; quiz 203-4.

- HUGHES, G. C., BISWAS, S. S., YIN, B., BAKLANOV, D. V., ANNEX, B. H., COLEMAN, R. E., DEGRADO, T. R., LANDOLFO, C. K., LANDOLFO, K. P. & LOWE, J. E. (2002) A comparison of mechanical and laser transmyocardial revascularization for induction of angiogenesis and arteriogenesis in chronically ischemic myocardium. *J Am Coll Cardiol*, 39, 1220-8.
- HUGHES, G. C., POST, M. J., SIMONS, M. & ANNEX, B. H. (2003) Translational physiology: porcine models of human coronary artery disease: implications for preclinical trials of therapeutic angiogenesis. *J Appl Physiol*, 94, 1689-701.
- HUIKURI, H. V., KERVINEN, K., NIEMELA, M., YLITALO, K., SAILY, M., KOISTINEN, P., SAVOLAINEN, E. R., UKKONEN, H., PIETILA, M., AIRAKSINEN, J. K., KNUUTI, J. & MAKIKALLIO, T. H. (2008) 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*, 29, 2723-32.
- HURST, T. M., HINRICHS, M., BREIDENBACH, C., KATZ, N. & WALDECKER, B. (1999) Detection of myocardial injury during transvenous implantation of automatic cardioverter-defibrillators. *J Am Coll Cardiol*, 34, 402-8.
- HUTTMANN, A., DUHRSEN, U., STYPMANN, J., NOPPENNEY, R., NUCKEL, H., NEUMANN, T., GUTERSOHN, A., NIKOL, S. & ERBEL, R. (2006) 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*, 101, 78-86.
- IKEDA, E., ACHEN, M. G., BREIER, G. & RISAU, W. (1995) Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. *J Biol Chem*, 270, 19761-6.
- INCE, H., PETZSCH, M., KLEINE, H. D., SCHMIDT, H., REHDERS, T., KORBER, T., SCHUMICHEN, C., FREUND, M. & NIENABER, C. A. (2005) Preservation from left ventricular remodeling by front-integrated revascularization and stem cell liberation in evolving acute myocardial infarction by use of granulocyte-colony-stimulating factor (FIRSTLINE-AMI). *Circulation*, 112, 3097-106.
- JANSSENS, S., DUBOIS, C., BOGAERT, J., THEUNISSEN, K., DEROOSE, C., DESMET, W., KALANTZI, M., HERBOTS, L., SINNAEVE, P., DENS, J., MAERTENS, J., RADEMAKERS, F., DYMARKOWSKI, S., GHEYSENS, O., VAN CLEEMPUT, J., BORMANS, G., NUYTS, J., BELMANS, A., MORTELMANS, L., BOOGAERTS, M. & VAN DE WERF, F. (2006) Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial. *Lancet*, 367, 113-21.
- JEWELL, U. R., KVIETIKOVA, I., SCHEID, A., BAUER, C., WENGER, R. H. & GASSMANN, M. (2001) Induction of HIF-1alpha in response to hypoxia is instantaneous. *FASEB J*, 15, 1312-4.
- JIN, Y., KONG, H., STODILKA, R. Z., WELLS, R. G., ZABEL, P., MERRIFIELD, P. A., SYKES, J. & PRATO, F. S. (2005) Determining the minimum number of detectable cardiac-transplanted ¹¹¹In-tropolone-labelled bone-marrow-derived mesenchymal stem cells by SPECT. *Phys Med Biol*, 50, 4445-55.

- JONES, E. L. & WEINTRAUB, W. S. (1996) The importance of completeness of revascularization during long-term follow-up after coronary artery operations. *J Thorac Cardiovasc Surg*, 112, 227-37.
- KANG, H. J., KIM, H. S., KOO, B. K., KIM, Y. J., LEE, D., SOHN, D. W., OH, B. H. & PARK, Y. B. (2007) Intracoronary infusion of the mobilized peripheral blood stem cell by G-CSF is better than mobilization alone by G-CSF for improvement of cardiac function and remodeling: 2-year follow-up results of the Myocardial Regeneration and Angiogenesis in Myocardial Infarction with G-CSF and Intra-Coronary Stem Cell Infusion (MAGIC Cell) 1 trial. *Am Heart J*, 153, 237 e1-8.
- KANG, H. J., KIM, H. S., ZHANG, S. Y., PARK, K. W., CHO, H. J., KOO, B. K., KIM, Y. J., SOO LEE, D., SOHN, D. W., HAN, K. S., OH, B. H., LEE, M. M. & PARK, Y. B. (2004) 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*, 363, 751-6.
- KANG, H. J., KIM, Y. S., KOO, B. K., PARK, K. W., LEE, H. Y., SOHN, D. W., OH, B. H., PARK, Y. B. & KIM, H. S. (2008) Effects of stem cell therapy with G-CSF on coronary artery after drug-eluting stent implantation in patients with acute myocardial infarction. *Heart*, 94, 604-9.
- KANG, H. J., LEE, H. Y., NA, S. H., CHANG, S. A., PARK, K. W., KIM, H. K., KIM, S. Y., CHANG, H. J., LEE, W., KANG, W. J., KOO, B. K., KIM, Y. J., LEE, D. S., SOHN, D. W., HAN, K. S., OH, B. H., PARK, Y. B. & KIM, H. S. (2006) Differential effect of intracoronary infusion of mobilized peripheral blood stem cells by granulocyte colony-stimulating factor on left ventricular function and remodeling in patients with acute myocardial infarction versus old myocardial infarction: the MAGIC Cell-3-DES randomized, controlled trial. *Circulation*, 114, I145-51.
- KAstrup, J., JORGENSEN, E., RUCK, A., TAGIL, K., GLOGAR, D., RUZYLLO, W., BOTKER, H. E., DUDEK, D., DRVOTA, V., HESSE, B., THUESEN, L., BLOMBERG, P., GYONGYOSI, M. & SYLVEN, C. (2005) Direct intramyocardial plasmid vascular endothelial growth factor-A165 gene therapy in patients with stable severe angina pectoris A randomized double-blind placebo-controlled study: the Euroinject One trial. *J Am Coll Cardiol*, 45, 982-8.
- KILIAN, E. G., SADONI, S., VICOL, C., KELLY, R., VAN HULST, K., SCHWAIGER, M., KUPATT, C., BOEKSTEGERS, P., PILLAI, R., CHANNON, K., HETZER, R. & REICHART, B. (2010) Myocardial transfection of hypoxia inducible factor-1alpha via an adenoviral vector during coronary artery bypass grafting. - A multicenter phase I and safety study. *Circ J*, 74, 916-24.
- KLAGSBRUN, M. & D'AMORE, P. A. (1991) Regulators of angiogenesis. *Annu Rev Physiol*, 53, 217-39.
- KLEIN, H. M., GHODSIZAD, A., MARKTANNER, R., POLL, L., VOELKEL, T., MOHAMMAD HASANI, M. R., PIECHACZEK, C., FEIFEL, N., STOCKSCHLAEDER, M., BURCHARDT, E. R., KAR, B. J., GREGORIC, I. & GAMS, E. (2007) Intramyocardial implantation of CD133+ stem cells improved cardiac function without bypass surgery. *Heart Surg Forum*, 10, E66-9.

- KOCHER, A. A., SCHLECHTA, B., GASPAROVICOVA, A., WOLNER, E., BONAROS, N. & LAUFER, G. (2007) Stem cells and cardiac regeneration. *Transpl Int*, 20, 731-46.
- KORNOWSKI, R., LEON, M. B., FUCHS, S., VODOVOTZ, Y., FLYNN, M. A., GORDON, D. A., PIERRE, A., KOVESDI, I., KEISER, J. A. & EPSTEIN, S. E. (2000) Electromagnetic guidance for catheter-based transendocardial injection: a platform for intramyocardial angiogenesis therapy. Results in normal and ischemic porcine models. *J Am Coll Cardiol*, 35, 1031-9.
- KRAITCHMAN, D. L., TATSUMI, M., GILSON, W. D., ISHIMORI, T., KEDZIOREK, D., WALCZAK, P., SEGARS, W. P., CHEN, H. H., FRITZGES, D., IZBUDAK, I., YOUNG, R. G., MARCELINO, M., PITTENGER, M. F., SOLAIYAPPAN, M., BOSTON, R. C., TSUI, B. M., WAHL, R. L. & BULTE, J. W. (2005) Dynamic imaging of allogeneic mesenchymal stem cells trafficking to myocardial infarction. *Circulation*, 112, 1451-61.
- KRAUSE, K., JAQUET, K., SCHNEIDER, C., HAUPT, S., LIOZNOV, M. V., OTTE, K. M. & KUCK, K. H. (2009) Percutaneous intramyocardial stem cell injection in patients with acute myocardial infarction: first-in-man study. *Heart*, 95, 1145-52.
- KUETHE, F., FIGULLA, H. R., HERZAU, M., VOTH, M., FRITZENWANGER, M., OPFERMANN, T., PACHMANN, K., KRACK, A., SAYER, H. G., GOTTSCHILD, D. & WERNER, G. S. (2005) Treatment with granulocyte colony-stimulating factor for mobilization of bone marrow cells in patients with acute myocardial infarction. *Am Heart J*, 150, 115.
- KUPATT, C., WICHELS, R., DEISS, M., MOLNAR, A., LEBHERZ, C., RAAKE, P., VON DEGENFELD, G., HAHNEL, D. & BOEKSTEGERS, P. (2002) Retroinfusion of NFkappaB decoy oligonucleotide extends cardioprotection achieved by CD18 inhibition in a preclinical study of myocardial ischemia and retroinfusion in pigs. *Gene Ther*, 9, 518-26.
- KURPISZ, M., CZEPCZYNSKI, R., GRYGIELSKA, B., MAJEWSKI, M., FISZER, D., JERZYKOWSKA, O., SOWINSKI, J. & SIMINIAK, T. (2007) Bone marrow stem cell imaging after intracoronary administration. *Int J Cardiol*, 121, 194-5.
- LAHAM, R. J., CHRONOS, N. A., PIKE, M., LEIMBACH, M. E., UDELSON, J. E., PEARLMAN, J. D., PETTIGREW, R. I., WHITEHOUSE, M. J., YOSHIZAWA, C. & SIMONS, M. (2000) Intracoronary basic fibroblast growth factor (FGF-2) in patients with severe ischemic heart disease: results of a phase I open-label dose escalation study. *J Am Coll Cardiol*, 36, 2132-9.
- LAHAM, R. J., POST, M., REZAEI, M., DONNELL-FINK, L., WYKRZYKOWSKA, J. J., LEE, S. U., BAIM, D. S. & SELLKE, F. W. (2005) Transendocardial and transepical intramyocardial fibroblast growth factor-2 administration: myocardial and tissue distribution. *Drug Metab Dispos*, 33, 1101-7.
- LAHAM, R. J., REZAEI, M., POST, M., SELLKE, F. W., BRAECKMAN, R. A., HUNG, D. & SIMONS, M. (1999a) Intracoronary and intravenous administration of basic fibroblast growth factor: myocardial and tissue distribution. *Drug Metab Dispos*, 27, 821-6.

- LAHAM, R. J., SELLKE, F. W., EDELMAN, E. R., PEARLMAN, J. D., WARE, J. A., BROWN, D. L., GOLD, J. P. & SIMONS, M. (1999b) Local perivascular delivery of basic fibroblast growth factor in patients undergoing coronary bypass surgery: results of a phase I randomized, double-blind, placebo-controlled trial. *Circulation*, 100, 1865-71.
- LAHAM, R. J., SIMONS, M. & SELLKE, F. (2001) Gene transfer for angiogenesis in coronary artery disease. *Annu Rev Med*, 52, 485-502.
- LAM, S. K. & OWEN, A. (2007) Combined resynchronisation and implantable defibrillator therapy in left ventricular dysfunction: Bayesian network meta-analysis of randomised controlled trials. *BMJ*, 335, 925.
- LANDMESSER, U. (2009) Bone marrow cell therapy after myocardial infarction. What should we select? *Eur Heart J*, 30, 1310-2.
- LEE, S. U., WYKRZYKOWSKA, J. J. & LAHAM, R. J. (2006) Angiogenesis: bench to bedside, have we learned anything? *Toxicol Pathol*, 34, 3-10.
- LENZEN, M. J., BOERSMA, E., BERTRAND, M. E., MAIER, W., MORIS, C., PISCIONE, F., SECHTEM, U., STAHL, E., WIDIMSKY, P., DE JAEGERE, P., SCHOLTE OP REIMER, W. J., MERCADO, N. & WIJNS, W. (2005) Management and outcome of patients with established coronary artery disease: the Euro Heart Survey on coronary revascularization. *Eur Heart J*, 26, 1169-79.
- LEONE, A. M., RUTELLA, S., BONANNO, G., CONTEMI, A. M., DE RITIS, D. G., GIANNICO, M. B., REBUZZI, A. G., LEONE, G. & CREA, F. (2006) Endogenous G-CSF and CD34+ cell mobilization after acute myocardial infarction. *Int J Cardiol*, 111, 202-8.
- LEWIS, B. S., FLUGELMAN, M. Y., WEISZ, A., KEREN-TAL, I. & SCHAPER, W. (1997) Angiogenesis by gene therapy: a new horizon for myocardial revascularization? *Cardiovasc Res*, 35, 490-7.
- LI, J., POST, M., VOLK, R., GAO, Y., LI, M., METAIS, C., SATO, K., TSAI, J., AIRD, W., ROSENBERG, R. D., HAMPTON, T. G., SELLKE, F., CARMELIET, P. & SIMONS, M. (2000) PR39, a peptide regulator of angiogenesis. *Nat Med*, 6, 49-55.
- LIPINSKI, M. J., BIONDI-ZOCCAI, G. G., ABBATE, A., KHIANEY, R., SHEIBAN, I., BARTUNEK, J., VANDERHEYDEN, M., KIM, H. S., KANG, H. J., STRAUER, B. E. & VETROVEC, G. W. (2007) 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*, 50, 1761-7.
- LIU, J. L., MANIADAKIS, N., GRAY, A. & RAYNER, M. (2002) The economic burden of coronary heart disease in the UK. *Heart*, 88, 597-603.
- LOPEZ, J. J., EDELMAN, E. R., STAMLER, A., HIBBERD, M. G., PRASAD, P., THOMAS, K. A., DISALVO, J., CAPUTO, R. P., CARROZZA, J. P., DOUGLAS, P. S., SELLKE, F. W. & SIMONS, M. (1998a) Angiogenic potential of perivascularly delivered aFGF in a porcine model of chronic myocardial ischemia. *Am J Physiol*, 274, H930-6.

- LOPEZ, J. J., LAHAM, R. J., CARROZZA, J. P., TOFUKUJI, M., SELLKE, F. W., BUNTING, S. & SIMONS, M. (1997) Hemodynamic effects of intracoronary VEGF delivery: evidence of tachyphylaxis and NO dependence of response. *Am J Physiol*, 273, H1317-23.
- LOPEZ, J. J., LAHAM, R. J., STAMLER, A., PEARLMAN, J. D., BUNTING, S., KAPLAN, A., CARROZZA, J. P., SELLKE, F. W. & SIMONS, M. (1998b) VEGF administration in chronic myocardial ischemia in pigs. *Cardiovasc Res*, 40, 272-81.
- LOSORDO, D. W., SCHATZ, R. A., WHITE, C. J., UDELSON, J. E., VEERESHWARAYYA, V., DURGIN, M., POH, K. K., WEINSTEIN, R., KEARNEY, M., CHAUDHRY, M., BURG, A., EATON, L., HEYD, L., THORNE, T., SHTURMAN, L., HOFFMEISTER, P., STORY, K., ZAK, V., DOWLING, D., TRAVERSE, J. H., OLSON, R. E., FLANAGAN, J., SODANO, D., MURAYAMA, T., KAWAMOTO, A., KUSANO, K. F., WOLLINS, J., WELT, F., SHAH, P., SOUKAS, P., ASAHARA, T. & HENRY, T. D. (2007) Intramyocardial transplantation of autologous CD34+ stem cells for intractable angina: a phase I/IIa double-blind, randomized controlled trial. *Circulation*, 115, 3165-72.
- LOSORDO, D. W., VALE, P. R., SYMES, J. F., DUNNINGTON, C. H., ESAKOF, D. D., MAYSKY, M., ASHARE, A. B., LATHI, K. & ISNER, J. M. (1998) Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. *Circulation*, 98, 2800-4.
- LOVELL, M. J. & MATHUR, A. (2011) Cardiac stem cell therapy: progress from the bench to bedside. *Heart*, 96, 1531-7.
- LUNDE, K., SOLHEIM, S., AAKHUS, S., ARNESEN, H., ABDELNOOR, M., EGELAND, T., ENDRESEN, K., ILEBEKK, A., MANGSCHAU, A., FJELD, J. G., SMITH, H. J., TARALDSRUD, E., GROGAARD, H. K., BJORNERHEIM, R., BREKKE, M., MULLER, C., HOPP, E., RAGNARSSON, A., BRINCHMANN, J. E. & FORFANG, K. (2006) Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. *N Engl J Med*, 355, 1199-209.
- LUQUE, A., TURU, M., JUAN-BABOT, O., CARDONA, P., FONT, A., CARVAJAL, A., SLEVIN, M., IBORRA, E., RUBIO, F., BADIMON, L. & KRUPINSKI, J. (2008) Overexpression of hypoxia/inflammatory markers in atherosclerotic carotid plaques. *Front Biosci*, 13, 6483-90.
- MACK, C. A., PATEL, S. R., SCHWARZ, E. A., ZANZONICO, P., HAHN, R. T., ILERCIL, A., DEVEREUX, R. B., GOLDSMITH, S. J., CHRISTIAN, T. F., SANBORN, T. A., KOVESDI, I., HACKETT, N., ISOM, O. W., CRYSTAL, R. G. & ROSENGART, T. K. (1998) Biologic bypass with the use of adenovirus-mediated gene transfer of the complementary deoxyribonucleic acid for vascular endothelial growth factor 121 improves myocardial perfusion and function in the ischemic porcine heart. *J Thorac Cardiovasc Surg*, 115, 168-76; discussion 176-7.
- MACKAY, J., MENSAH, G. A., MENDIS, S., GREENLUND, K. & WORLD HEALTH ORGANIZATION. (2004) *The atlas of heart disease and stroke*, Geneva, World Health Organization.

- MANGINAS, A., GOUSSETIS, E., KOUTELOU, M., KARATASAKIS, G., PERISTERI, I., THEODORAKOS, A., LEONTIADIS, E., PLESSAS, N., THEODOSAKI, M., GRAPHAKOS, S. & COKKINOS, D. V. (2007) Pilot study to evaluate the safety and feasibility of intracoronary CD133(+) and CD133(-) CD34(+) cell therapy in patients with nonviable anterior myocardial infarction. *Catheter Cardiovasc Interv*, 69, 773-81.
- MARSHALL, D. J., PALASIS, M., LEPORE, J. J. & LEIDEN, J. M. (2000) Biocompatibility of cardiovascular gene delivery catheters with adenovirus vectors: an important determinant of the efficiency of cardiovascular gene transfer. *Mol Ther*, 1, 423-9.
- MARTIN, A. F. (1981) Turnover of cardiac troponin subunits. Kinetic evidence for a precursor pool of troponin-I. *J Biol Chem*, 256, 964-8.
- MARTIN-RENDON, E., BRUNSKILL, S., DOREE, C., HYDE, C., WATT, S., MATHUR, A. & STANWORTH, S. (2008) Stem cell treatment for acute myocardial infarction. *Cochrane Database Syst Rev*, CD006536.
- MEISEL, E., PFEIFFER, D., ENGELMANN, L., TEBBENJOHANN, J., SCHUBERT, B., HAHN, S., FLECK, E. & BUTTER, C. (2001) Investigation of coronary venous anatomy by retrograde venography in patients with malignant ventricular tachycardia. *Circulation*, 104, 442-7.
- MELUZIN, J., JANOUSEK, S., MAYER, J., GROCH, L., HORNACEK, I., HLINOMAZ, O., KALA, P., PANOVSKEY, R., PRASEK, J., KAMINEK, M., STANICEK, J., KLABUSAY, M., KORISTEK, Z., NAVRATIL, M., DUSEK, L. & VINKLARKOVA, J. (2008) Three-, 6-, and 12-month results of autologous transplantation of mononuclear bone marrow cells in patients with acute myocardial infarction. *Int J Cardiol*, 128, 185-92.
- MENASCHE, P., ALFIERI, O., JANSSENS, S., MCKENNA, W., REICHENSPURNER, H., TRINQUART, L., VILQUIN, J. T., MAROLLEAU, J. P., SEYMOUR, B., LARGHERO, J., LAKE, S., CHATELLIER, G., SOLOMON, S., DESNOS, M. & HAGEGE, A. A. (2008) The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation. *Circulation*, 117, 1189-200.
- MENASCHE, P., HAGEGE, A. A., VILQUIN, J. T., DESNOS, M., ABERGEL, E., POUZET, B., BEL, A., SARATEANU, S., SCORSIN, M., SCHWARTZ, K., BRUNEVAL, P., BENBUNAN, M., MAROLLEAU, J. P. & DUBOC, D. (2003) Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction. *J Am Coll Cardiol*, 41, 1078-83.
- MESZAROS, G. L., BRUNTON, L. L. & BLOOR, C. M. (1999) *Animal Models of Angiogenesis in Cardiovascular Tissues*, New York, Oxford University Press.
- MEYER, G. P., WOLLERT, K. C., LOTZ, J., STEFFENS, J., LIPPOLT, P., FICHTNER, S., HECKER, H., SCHAEFER, A., ARSENIIEV, L., HERTENSTEIN, B., GANSER, A. & DREXLER, H. (2006) Intracoronary bone marrow cell transfer after myocardial infarction: eighteen months' follow-up data from the randomized, controlled BOOST (BOne marrOw transfer to enhance ST-elevation infarct regeneration) trial. *Circulation*, 113, 1287-94.

- MIYAZAKI, A., HATORI, N., TADOKORO, H., RYDEN, L., CORDAY, E. & DRURY, J. (1990) More rapid thrombolysis with coronary venous retroinfusion of streptokinase compared with intravenous administration. An experimental study in canines. *Eur Heart J*, 11, 936-44.
- MOCINI, D., STAIBANO, M., MELE, L., GIANNANTONI, P., MENICHELLA, G., COLIVICCHI, F., SORDINI, P., SALERA, P., TUBARO, M. & SANTINI, M. (2006) Autologous bone marrow mononuclear cell transplantation in patients undergoing coronary artery bypass grafting. *Am Heart J*, 151, 192-7.
- MOHRI, M., TOMOIKE, H., NOMA, M., INOUE, T., HISANO, K. & NAKAMURA, M. (1989) Duration of ischemia is vital for collateral development: repeated brief coronary artery occlusions in conscious dogs. *Circ Res*, 64, 287-96.
- MUKHERJEE, D., BHATT, D. L., ROE, M. T., PATEL, V. & ELLIS, S. G. (1999) Direct myocardial revascularization and angiogenesis--how many patients might be eligible? *Am J Cardiol*, 84, 598-600, A8.
- MURAD-NETTO, S., MOURA, R., ROMEO, L. J., MANOEL NETO, A., DUARTE, N., BARRETO, F., JENSEN, A., VINA, R. F., VRASLOVIK, F., OBERDAN, A., BENETTI, F., SASLAVSKY, J., VINA, M. F. & AMINO, J. G. (2004) Stem cell therapy with retrograde coronary perfusion in acute myocardial infarction. A new technique. *Arq Bras Cardiol*, 83, 352-4; 349-51.
- NAIMARK, W. A., LEPORE, J. J., KLUGHERZ, B. D., WANG, Z., GUY, T. S., OSMAN, H., MOAINIE, S. L., GORMAN, R. C., REED, G., GORMAN, J. H., 3RD, PALASIS, M., PARMACEK, M. S. & WILENSKY, R. L. (2003) Adenovirus-catheter compatibility increases gene expression after delivery to porcine myocardium. *Hum Gene Ther*, 14, 161-6.
- O'KONSKI, M. S., WHITE, F. C., LONGHURST, J., ROTH, D. & BLOOR, C. M. (1987) Ameroid constriction of the proximal left circumflex coronary artery in swine. A model of limited coronary collateral circulation. *Am J Cardiovasc Pathol*, 1, 69-77.
- ORLIC, D., KAJSTURA, J., CHIMENTI, S., JAKONIUK, I., ANDERSON, S. M., LI, B., PICKEL, J., MCKAY, R., NADAL-GINARD, B., BODINE, D. M., LERI, A. & ANVERSA, P. (2001) Bone marrow cells regenerate infarcted myocardium. *Nature*, 410, 701-5.
- PACKER, M., COATS, A. J., FOWLER, M. B., KATUS, H. A., KRUM, H., MOHACSI, P., ROULEAU, J. L., TENDERA, M., CASTAIGNE, A., ROECKER, E. B., SCHULTZ, M. K. & DEMETS, D. L. (2001) Effect of carvedilol on survival in severe chronic heart failure. *N Engl J Med*, 344, 1651-8.
- PAGANI, F. D., DERSIMONIAN, H., ZAWADZKA, A., WETZEL, K., EDGE, A. S., JACOBY, D. B., DINSMORE, J. H., WRIGHT, S., ARETZ, T. H., EISEN, H. J. & AARONSON, K. D. (2003) Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation. *J Am Coll Cardiol*, 41, 879-88.
- PARK, I. H., ZHAO, R., WEST, J. A., YABUUCHI, A., HUO, H., INCE, T. A., LEROU, P. H., LENSCH, M. W. & DALEY, G. Q. (2008) Reprogramming of human somatic cells to pluripotency with defined factors. *Nature*, 451, 141-6.

- PATEL, A. N., GEFFNER, L., VINA, R. F., SASLAVSKY, J., URSCHER, H. C., JR., KORMOS, R. & BENETTI, F. (2005) Surgical treatment for congestive heart failure with autologous adult stem cell transplantation: a prospective randomized study. *J Thorac Cardiovasc Surg*, 130, 1631-8.
- PEARLMAN, J. D., LAHAM, R. J. & SIMONS, M. (2000) Coronary angiogenesis: detection in vivo with MR imaging sensitive to collateral neocirculation--preliminary study in pigs. *Radiology*, 214, 801-7.
- PEGG, T. J., MAUNSELL, Z., KARAMITSOS, T. D., TAYLOR, R. P., JAMES, T., FRANCIS, J. M., TAGGART, D. P., WHITE, H., NEUBAUER, S. & SELVANAYAGAM, J. B. (2011) Utility of cardiac biomarkers for the diagnosis of type V myocardial infarction after coronary artery bypass grafting: insights from serial cardiac MRI. *Heart*.
- PERIN, E. C., DOHMANN, H. F., BOROJEVIC, R., SILVA, S. A., SOUSA, A. L., MESQUITA, C. T., ROSSI, M. I., CARVALHO, A. C., DUTRA, H. S., DOHMANN, H. J., SILVA, G. V., BELEM, L., VIVACQUA, R., RANGEL, F. O., ESPORCATTE, R., GENG, Y. J., VAUGHN, W. K., ASSAD, J. A., MESQUITA, E. T. & WILLERSON, J. T. (2003) Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. *Circulation*, 107, 2294-302.
- PETERSON, G. L. (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal Biochem*, 83, 346-56.
- PFEFFER, M. A., GREAVES, S. C., ARNOLD, J. M., GLYNN, R. J., LAMOTTE, F. S., LEE, R. T., MENAPACE, F. J., JR., RAPAPORT, E., RIDKER, P. M., ROULEAU, J. L., SOLOMON, S. D. & HENNEKENS, C. H. (1997) Early versus delayed angiotensin-converting enzyme inhibition therapy in acute myocardial infarction. The healing and early afterload reducing therapy trial. *Circulation*, 95, 2643-51.
- PIEK, J. J., VAN LIEBERGEN, R. A., KOCH, K. T., PETERS, R. J. & DAVID, G. K. (1997) Clinical, angiographic and hemodynamic predictors of recruitable collateral flow assessed during balloon angioplasty coronary occlusion. *J Am Coll Cardiol*, 29, 275-82.
- PITT, B., ZANNAD, F., REMME, W. J., CODY, R., CASTAIGNE, A., PEREZ, A., PALENSKY, J. & WITTES, J. (1999) The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *N Engl J Med*, 341, 709-17.
- PLEWKA, M., KRZEMINSKA-PAKULA, M., LIPIEC, P., PERUGA, J. Z., JEZEWSKI, T., KIDAWA, M., WIERZBOWSKA-DRABIK, K., KORYCKA, A., ROBAK, T. & KASPRZAK, J. D. (2009) Effect of intracoronary injection of mononuclear bone marrow stem cells on left ventricular function in patients with acute myocardial infarction. *Am J Cardiol*, 104, 1336-42.
- POHL, T., GIEHRL, W., REICHART, B., KUPATT, C., RAAKE, P., PAUL, S., REICHENSPURNER, H., STEINBECK, G. & BOEKSTEGERS, P. (2004) Retroinfusion-supported stenting in high-risk patients for percutaneous intervention and bypass surgery: results of the prospective randomized myoprotect I study. *Catheter Cardiovasc Interv*, 62, 323-30.

- POST, M. J., LAHAM, R., SELLKE, F. W. & SIMONS, M. (2001) Therapeutic angiogenesis in cardiology using protein formulations. *Cardiovasc Res*, 49, 522-31.
- POST, M. J., SATO, K., MURAKAMI, M., BAO, J., TIRZIU, D., PEARLMAN, J. D. & SIMONS, M. (2006) Adenoviral PR39 improves blood flow and myocardial function in a pig model of chronic myocardial ischemia by enhancing collateral formation. *Am J Physiol Regul Integr Comp Physiol*, 290, R494-500.
- POVSIC, T. J. & O'CONNOR, C. M. (2010) Cell therapy for heart failure: the need for a new therapeutic strategy. *Expert Rev Cardiovasc Ther*, 8, 1107-26.
- PSALTIS, P. J. & WORTHLEY, S. G. (2009) Endoventricular electromechanical mapping-the diagnostic and therapeutic utility of the NOGA XP Cardiac Navigation System. *J Cardiovasc Transl Res*, 2, 48-62.
- RAAKE, P., VON DEGENFELD, G., HINKEL, R., VACHENAUER, R., SANDNER, T., BELLER, S., ANDREES, M., KUPATT, C., SCHULER, G. & BOEKSTEGERS, P. (2004) Myocardial gene transfer by selective pressure-regulated retroinfusion of coronary veins: comparison with surgical and percutaneous intramyocardial gene delivery. *J Am Coll Cardiol*, 44, 1124-9.
- RADKE, P. W., HEINL-GREEN, A., FRASS, O. M., POST, M. J., SATO, K., GEDDES, D. M. & ALTON, E. W. (2006) Evaluation of the porcine ameroid constrictor model of myocardial ischemia for therapeutic angiogenesis studies. *Endothelium*, 13, 25-33.
- RAHIMI, K., BANNING, A. P., CHENG, A. S., PEGG, T. J., KARAMITSOS, T. D., CHANNON, K. M., DARBY, S., TAGGART, D. P., NEUBAUER, S. & SELVANAYAGAM, J. B. (2009) Prognostic value of coronary revascularisation-related myocardial injury: a cardiac magnetic resonance imaging study. *Heart*, 95, 1937-43.
- RAHIMTOOLA, S. H. (1989) The hibernating myocardium. *Am Heart J*, 117, 211-21.
- RANA, J. S., MANNAM, A., DONNELL-FINK, L., GERVINO, E. V., SELLKE, F. W. & LAHAM, R. J. (2005) Longevity of the placebo effect in the therapeutic angiogenesis and laser myocardial revascularization trials in patients with coronary heart disease. *Am J Cardiol*, 95, 1456-9.
- REFFELMANN, T., KONEMANN, S. & KLONER, R. A. (2009) 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*, 53, 305-8.
- REINHARDT, C. P., DALHBERG, S., TRIES, M. A., MARCEL, R. & LEPPA, J. A. (2001) Stable labeled microspheres to measure perfusion: validation of a neutron activation assay technique. *Am J Physiol Heart Circ Physiol*, 280, H108-16.
- RENTROP, K. P., COHEN, M., BLANKE, H. & PHILLIPS, R. A. (1985) Changes in collateral channel filling immediately after controlled coronary artery occlusion by an angioplasty balloon in human subjects. *J Am Coll Cardiol*, 5, 587-92.
- RIBICHINI, F., STEFFENINO, G., DELLAVALLE, A., FERRERO, V., VADO, A., FEOLA, M. & USLENGHI, E. (1998) Comparison of thrombolytic therapy and primary coronary angioplasty with liberal stenting for inferior myocardial infarction with precordial ST-segment depression: immediate and long-term results of a randomized study. *J Am Coll Cardiol*, 32, 1687-94.

- RIPA, R. S., JORGENSEN, E., WANG, Y., THUNE, J. J., NILSSON, J. C., SONDERGAARD, L., JOHNSEN, H. E., KOBER, L., GRANDE, P. & KASTRUP, J. (2006a) 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*, 113, 1983-92.
- RIPA, R. S., WANG, Y., JORGENSEN, E., JOHNSEN, H. E., HESSE, B. & KASTRUP, J. (2006b) Intramyocardial injection of vascular endothelial growth factor-A165 plasmid followed by granulocyte-colony stimulating factor to induce angiogenesis in patients with severe chronic ischaemic heart disease. *Eur Heart J*, 27, 1785-92.
- RISAU, W. & FLAMME, I. (1995) Vasculogenesis. *Annu Rev Cell Dev Biol*, 11, 73-91.
- ROCA, M., DE VRIES, E. F., JAMAR, F., ISRAEL, O. & SIGNORE, A. (2010) Guidelines for the labelling of leucocytes with (111)In-oxine. Inflammation/Infection Taskgroup of the European Association of Nuclear Medicine. *Eur J Nucl Med Mol Imaging*, 37, 835-41.
- ROSENGART, T. K., LEE, L. Y., PATEL, S. R., SANBORN, T. A., PARIKH, M., BERGMAN, G. W., HACHAMOVITCH, R., SZULC, M., KLIGFIELD, P. D., OKIN, P. M., HAHN, R. T., DEVEREUX, R. B., POST, M. R., HACKETT, N. R., FOSTER, T., GRASSO, T. M., LESSER, M. L., ISOM, O. W. & CRYSTAL, R. G. (1999) Angiogenesis gene therapy: phase I assessment of direct intramyocardial administration of an adenovirus vector expressing VEGF121 cDNA to individuals with clinically significant severe coronary artery disease. *Circulation*, 100, 468-74.
- ROTH, D. M., MARUOKA, Y., ROGERS, J., WHITE, F. C., LONGHURST, J. C. & BLOOR, C. M. (1987) Development of coronary collateral circulation in left circumflex Ameroid-occluded swine myocardium. *Am J Physiol*, 253, H1279-88.
- RUEL, M., BEANLANDS, R. S., LORTIE, M., CHAN, V., CAMACK, N., DEKEMP, R. A., SUURONEN, E. J., RUBENS, F. D., DASILVA, J. N., SELLKE, F. W., STEWART, D. J. & MESANA, T. G. (2008) Concomitant treatment with oral L-arginine improves the efficacy of surgical angiogenesis in patients with severe diffuse coronary artery disease: the Endothelial Modulation in Angiogenic Therapy randomized controlled trial. *J Thorac Cardiovasc Surg*, 135, 762-70, 770 e1.
- RUTHERFORD, J. D., PFEFFER, M. A., MOYE, L. A., DAVIS, B. R., FLAKER, G. C., KOWEY, P. R., LAMAS, G. A., MILLER, H. S., PACKER, M., ROULEAU, J. L. & ET AL. (1994) Effects of captopril on ischemic events after myocardial infarction. Results of the Survival and Ventricular Enlargement trial. SAVE Investigators. *Circulation*, 90, 1731-8.
- SABATTINI, E., BISGAARD, K., ASCANI, S., POGGI, S., PICCIOLI, M., CECCARELLI, C., PIERI, F., FRATERNALI-ORCIONI, G. & PILERI, S. A. (1998) The EnVision++ system: a new immunohistochemical method for diagnostics and research. Critical comparison with the APAAP, ChemMate, CSA, LABC, and SABC techniques. *J Clin Pathol*, 51, 506-11.

- SARMENTO-LEITE, R., SILVA, G. V., DOHMAN, H. F., ROCHA, R. M., DOHMAN, H. J., DE MATTOS, N. D., CARVALHO, L. A., GOTTECHALL, C. A. & PERIN, E. C. (2003) Comparison of left ventricular electromechanical mapping and left ventricular angiography: defining practical standards for analysis of NOGA maps. *Tex Heart Inst J*, 30, 19-26.
- SATO, K., WU, T., LAHAM, R. J., JOHNSON, R. B., DOUGLAS, P., LI, J., SELLKE, F. W., BUNTING, S., SIMONS, M. & POST, M. J. (2001) Efficacy of intracoronary or intravenous VEGF165 in a pig model of chronic myocardial ischemia. *J Am Coll Cardiol*, 37, 616-23.
- SAXON, L. A., BRISTOW, M. R., BOEHMER, J., KRUEGER, S., KASS, D. A., DE MARCO, T., CARSON, P., DICARLO, L., FELDMAN, A. M., GALLE, E. & ECKLUND, F. (2006) Predictors of sudden cardiac death and appropriate shock in the Comparison of Medical Therapy, Pacing, and Defibrillation in Heart Failure (COMPANION) Trial. *Circulation*, 114, 2766-72.
- SCHACHINGER, V., ASSMUS, B., BRITTEN, M. B., HONOLD, J., LEHMANN, R., TEUPE, C., ABOLMAALI, N. D., VOGL, T. J., HOFMANN, W. K., MARTIN, H., DIMMELER, S. & ZEIHNER, A. M. (2004) Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction: final one-year results of the TOPCARE-AMI Trial. *J Am Coll Cardiol*, 44, 1690-9.
- SCHACHINGER, V., ERBS, S., ELSASSER, A., HABERBOSCH, W., HAMBRECHT, R., HOLSCHERMANN, H., YU, J., CORTI, R., MATHEY, D. G., HAMM, C. W., SUSELBECK, T., WERNER, N., HAASE, J., NEUZNER, J., GERMING, A., MARK, B., ASSMUS, B., TONN, T., DIMMELER, S. & ZEIHNER, A. M. (2006) Improved clinical outcome after intracoronary administration of bone-marrow-derived progenitor cells in acute myocardial infarction: final 1-year results of the REPAIR-AMI trial. *Eur Heart J*, 27, 2775-83.
- SCHAPER, W. & ITO, W. D. (1996) Molecular mechanisms of coronary collateral vessel growth. *Circ Res*, 79, 911-9.
- SCHAPER, W., MUNOZ-CHAPULI, R., WOLF, C. & ITO, W. (1999) *Collateral circulation of the heart*, New York, Oxford University Press.
- SCHUMACHER, B., PECHER, P., VON SPECHT, B. U. & STEGMANN, T. (1998) Induction of neoangiogenesis in ischemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease. *Circulation*, 97, 645-50.
- SCHWARZ, E. R., SPEAKMAN, M. T., PATTERSON, M., HALE, S. S., ISNER, J. M., KEDES, L. H. & KLONER, R. A. (2000) Evaluation of the effects of intramyocardial injection of DNA expressing vascular endothelial growth factor (VEGF) in a myocardial infarction model in the rat--angiogenesis and angioma formation. *J Am Coll Cardiol*, 35, 1323-30.
- SEEGER, F. H., TONN, T., KRZOSSOK, N., ZEIHNER, A. M. & DIMMELER, S. (2007) Cell isolation procedures matter: a comparison of different isolation protocols of bone marrow mononuclear cells used for cell therapy in patients with acute myocardial infarction. *Eur Heart J*, 28, 766-72.
- SELLKE, F. W., CHU, L. M. & COHN, W. E. (2010) Current state of surgical myocardial revascularization. *Circ J*, 74, 1031-7.

- SELLKE, F. W., LAHAM, R. J., EDELMAN, E. R., PEARLMAN, J. D. & SIMONS, M. (1998) Therapeutic angiogenesis with basic fibroblast growth factor: technique and early results. *Ann Thorac Surg*, 65, 1540-4.
- SELLKE, F. W., WANG, S. Y., STAMLER, A., LOPEZ, J. J., LI, J. & SIMONS, M. (1996) Enhanced microvascular relaxations to VEGF and bFGF in chronically ischemic porcine myocardium. *Am J Physiol*, 271, H713-20.
- SEMENZA, G. L. (1999) Regulation of mammalian O₂ homeostasis by hypoxia-inducible factor 1. *Annu Rev Cell Dev Biol*, 15, 551-78.
- SEMENZA, G. L. (2000) Surviving ischemia: adaptive responses mediated by hypoxia-inducible factor 1. *J Clin Invest*, 106, 809-12.
- SEMENZA, G. L., AGANI, F., BOOTH, G., FORSYTHE, J., IYER, N., JIANG, B. H., LEUNG, S., ROE, R., WIENER, C. & YU, A. (1997) Structural and functional analysis of hypoxia-inducible factor 1. *Kidney Int*, 51, 553-5.
- SETH, S., BHARGAVA, B., NARANG, R., RAY, R., MOHANTY, S., GULATI, G., KUMAR, L., AIRAN, B. & VENUGOPAL, P. (2010) The ABCD (Autologous Bone Marrow Cells in Dilated Cardiomyopathy) trial a long-term follow-up study. *J Am Coll Cardiol*, 55, 1643-4.
- SHALABY, F., ROSSANT, J., YAMAGUCHI, T. P., GERTSENSTEIN, M., WU, X. F., BREITMAN, M. L. & SCHUH, A. C. (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature*, 376, 62-6.
- SHARMA, H. S., WUNSCH, M., BRAND, T., VERDOUW, P. D. & SCHAPER, W. (1992) Molecular biology of the coronary vascular and myocardial responses to ischemia. *J Cardiovasc Pharmacol*, 20 Suppl 1, S23-31.
- SHYU, K. G., WANG, M. T., WANG, B. W., CHANG, C. C., LEU, J. G., KUAN, P. & CHANG, H. (2002) Intramyocardial injection of naked DNA encoding HIF-1 α /VP16 hybrid to enhance angiogenesis in an acute myocardial infarction model in the rat. *Cardiovasc Res*, 54, 576-83.
- SIMINIAK, T., FISZER, D., JERZYKOWSKA, O., GRYGIELSKA, B., ROZWADOWSKA, N., KALMUCKI, P. & KURPISZ, M. (2005) Percutaneous trans-coronary-venous transplantation of autologous skeletal myoblasts in the treatment of post-infarction myocardial contractility impairment: the POZNAN trial. *Eur Heart J*, 26, 1188-95.
- SIMINIAK, T., KALAWSKI, R., FISZER, D., JERZYKOWSKA, O., RZEZNICZAK, J., ROZWADOWSKA, N. & KURPISZ, M. (2004) Autologous skeletal myoblast transplantation for the treatment of postinfarction myocardial injury: phase I clinical study with 12 months of follow-up. *Am Heart J*, 148, 531-7.
- SIMONS, M., ANNEX, B. H., LAHAM, R. J., KLEIMAN, N., HENRY, T., DAUERMAN, H., UDELSON, J. E., GERVINO, E. V., PIKE, M., WHITEHOUSE, M. J., MOON, T. & CHRONOS, N. A. (2002) Pharmacological treatment of coronary artery disease with recombinant fibroblast growth factor-2: double-blind, randomized, controlled clinical trial. *Circulation*, 105, 788-93.
- SIMONS, M., BONOW, R. O., CHRONOS, N. A., COHEN, D. J., GIORDANO, F. J., HAMMOND, H. K., LAHAM, R. J., LI, W., PIKE, M., SELLKE, F. W., STEGMANN, T. J., UDELSON, J. E. & ROSENGART, T. K. (2000) Clinical trials in coronary angiogenesis: issues, problems, consensus: An expert panel summary. *Circulation*, 102, E73-86.

- SIMONS, M. & LAHAM, R. J. (1999a) Chronic myocardial ischemia: contemporary management strategies. *Drugs Today (Barc)*, 35, 667-84.
- SIMONS, M. & LAHAM, R. J. (1999b) *Therapeutic Angiogenesis in myocardial Ischemia*, New York, Oxford University Press.
- SLAUGHTER, M. S., MEYER, A. L. & BIRKS, E. J. (2011) Destination therapy with left ventricular assist devices: patient selection and outcomes. *Curr Opin Cardiol*.
- SMITS, P. C., VAN GEUNS, R. J., POLDERMANS, D., BOUNTIOUKOS, M., ONDERWATER, E. E., LEE, C. H., MAAT, A. P. & SERRUYS, P. W. (2003) Catheter-based intramyocardial injection of autologous skeletal myoblasts as a primary treatment of ischemic heart failure: clinical experience with six-month follow-up. *J Am Coll Cardiol*, 42, 2063-9.
- SOMARATNE, J. B., BERRY, C., MCMURRAY, J. J., POPPE, K. K., DOUGHTY, R. N. & WHALLEY, G. A. (2009) The prognostic significance of heart failure with preserved left ventricular ejection fraction: a literature-based meta-analysis. *Eur J Heart Fail*, 11, 855-62.
- STAMM, C., KLEINE, H. D., CHOI, Y. H., DUNKELMANN, S., LAUFFS, J. A., LORENZEN, B., DAVID, A., LIEBOLD, A., NIENABER, C., ZURAKOWSKI, D., FREUND, M. & STEINHOFF, G. (2007) Intramyocardial delivery of CD133+ bone marrow cells and coronary artery bypass grafting for chronic ischemic heart disease: safety and efficacy studies. *J Thorac Cardiovasc Surg*, 133, 717-25.
- STAMM, C., WESTPHAL, B., KLEINE, H. D., PETZSCH, M., KITTNER, C., KLINGE, H., SCHUMICHEN, C., NIENABER, C. A., FREUND, M. & STEINHOFF, G. (2003) Autologous bone-marrow stem-cell transplantation for myocardial regeneration. *Lancet*, 361, 45-6.
- STEWART, D. J., KUTRYK, M. J., FITCHETT, D., FREEMAN, M., CAMACK, N., SU, Y., DELLA SIEGA, A., BILODEAU, L., BURTON, J. R., PROULX, G. & RADHAKRISHNAN, S. (2009) VEGF gene therapy fails to improve perfusion of ischemic myocardium in patients with advanced coronary disease: results of the NORTHERN trial. *Mol Ther*, 17, 1109-15.
- STRAUER, B. E., BREHM, M., ZEUS, T., BARTSCH, T., SCHANNWELL, C., ANTKE, C., SORG, R. V., KOGLER, G., WERNET, P., MULLER, H. W. & KOSTERING, M. (2005) Regeneration of human infarcted heart muscle by intracoronary autologous bone marrow cell transplantation in chronic coronary artery disease: the IACT Study. *J Am Coll Cardiol*, 46, 1651-8.
- STRAUER, B. E., BREHM, M., ZEUS, T., KOSTERING, M., HERNANDEZ, A., SORG, R. V., KOGLER, G. & WERNET, P. (2002) Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation*, 106, 1913-8.
- STRAUER, B. E., YOUSEF, M. & SCHANNWELL, C. M. (2010) The acute and long-term effects of intracoronary Stem cell Transplantation in 191 patients with chronic heart failure: the STAR-heart study. *Eur J Heart Fail*, 12, 721-9.
- SURI, C., JONES, P. F., PATAN, S., BARTUNKOVA, S., MAISONPIERRE, P. C., DAVIS, S., SATO, T. N. & YANCOPOULOS, G. D. (1996) Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell*, 87, 1171-80.

- SWEDBERG, K., HELD, P., KJEKSHUS, J., RASMUSSEN, K., RYDEN, L. & WEDEL, H. (1992) Effects of the early administration of enalapril on mortality in patients with acute myocardial infarction. Results of the Cooperative New Scandinavian Enalapril Survival Study II (CONSENSUS II). *N Engl J Med*, 327, 678-84.
- SYMES, J. F., LOSORDO, D. W., VALE, P. R., LATHI, K. G., ESAKOF, D. D., MAYSKIY, M. & ISNER, J. M. (1999) Gene therapy with vascular endothelial growth factor for inoperable coronary artery disease. *Ann Thorac Surg*, 68, 830-6; discussion 836-7.
- TENDERA, M., WOJAKOWSKI, W., RUZYLLLO, W., CHOJNOWSKA, L., KEPKA, C., TRACZ, W., MUSIALEK, P., PIWOWARSKA, W., NESSLER, J., BUSZMAN, P., GRAJEK, S., BREBOROWICZ, P., MAJKA, M. & RATAJCZAK, M. Z. (2009) Intracoronary infusion of bone marrow-derived selected CD34+CXCR4+ cells and non-selected mononuclear cells in patients with acute STEMI and reduced left ventricular ejection fraction: results of randomized, multicentre Myocardial Regeneration by Intracoronary Infusion of Selected Population of Stem Cells in Acute Myocardial Infarction (REGENT) Trial. *Eur Heart J*, 30, 1313-21.
- TERROVITIS, J., STUBER, M., YOUSSEF, A., PREECE, S., LEPPPO, M., KIZANA, E., SCHAR, M., GERSTENBLITH, G., WEISS, R. G., MARBAN, E. & ABRAHAM, M. R. (2008) Magnetic resonance imaging overestimates ferumoxide-labeled stem cell survival after transplantation in the heart. *Circulation*, 117, 1555-62.
- THOMAS, K. A. (1996) Vascular endothelial growth factor, a potent and selective angiogenic agent. *J Biol Chem*, 271, 603-6.
- THURSTON, G., RUDGE, J. S., IOFFE, E., ZHOU, H., ROSS, L., CROLL, S. D., GLAZER, N., HOLASH, J., MCDONALD, D. M. & YANCOPOULOS, G. D. (2000) Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nat Med*, 6, 460-3.

- THYGESEN, K., ALPERT, J. S., WHITE, H. D., JAFFE, A. S., APPLE, F. S., GALVANI, M., KATUS, H. A., NEWBY, L. K., RAVKILDE, J., CHAITMAN, B., CLEMMENSEN, P. M., DELLBORG, M., HOD, H., PORELA, P., UNDERWOOD, R., BAX, J. J., BELLER, G. A., BONOW, R., VAN DER WALL, E. E., BASSAND, J. P., WIJNS, W., FERGUSON, T. B., STEG, P. G., URETSKY, B. F., WILLIAMS, D. O., ARMSTRONG, P. W., ANTMAN, E. M., FOX, K. A., HAMM, C. W., OHMAN, E. M., SIMOONS, M. L., POOLE-WILSON, P. A., GURFINKEL, E. P., LOPEZ-SENDON, J. L., PAIS, P., MENDIS, S., ZHU, J. R., WALLENTIN, L. C., FERNANDEZ-AVILES, F., FOX, K. M., PARKHOMENKO, A. N., PRIORI, S. G., TENDERA, M., VOIPIO-PULKKI, L. M., VAHANIAN, A., CAMM, A. J., DE CATERINA, R., DEAN, V., DICKSTEIN, K., FILIPPATOS, G., FUNCK-BRENTANO, C., HELLEMANS, I., KRISTENSEN, S. D., MCGREGOR, K., SECHTEM, U., SILBER, S., WIDIMSKY, P., ZAMORANO, J. L., MORAIS, J., BRENER, S., HARRINGTON, R., MORROW, D., LIM, M., MARTINEZ-RIOS, M. A., STEINHUBL, S., LEVINE, G. N., GIBLER, W. B., GOFF, D., TUBARO, M., DUDEK, D. & AL-ATTAR, N. (2007) Universal definition of myocardial infarction. *Circulation*, 116, 2634-53.
- TIO, R. A., TKEBUCHAVA, T., SCHEUERMANN, T. H., LEBHERZ, C., MAGNER, M., KEARNY, M., ESAKOF, D. D., ISNER, J. M. & SYMES, J. F. (1999) Intramyocardial gene therapy with naked DNA encoding vascular endothelial growth factor improves collateral flow to ischemic myocardium. *Hum Gene Ther*, 10, 2953-60.
- TRAVERSE, J. H., HENRY, T. D., VAUGHAN, D. E., ELLIS, S. G., PEPINE, C. J., WILLERSON, J. T., ZHAO, D. X., PILLER, L. B., PENN, M. S., BYRNE, B. J., PERIN, E. C., GEE, A. P., HATZOPOULOS, A. K., MCKENNA, D. H., FORDER, J. R., TAYLOR, D. A., COGLE, C. R., OLSON, R. E., JORGENSEN, B. C., SAYRE, S. L., VOJVODIC, R. W., GORDON, D. J., SKARLATOS, S. I., MOYE, L. A. & SIMARI, R. D. (2009) Rationale and design for TIME: A phase II, randomized, double-blind, placebo-controlled pilot trial evaluating the safety and effect of timing of administration of bone marrow mononuclear cells after acute myocardial infarction. *Am Heart J*, 158, 356-63.
- TRIPOSKIADIS, F., KARAYANNIS, G., GIAMOUZIS, G., SKOULARIGIS, J., LOURIDAS, G. & BUTLER, J. (2009) The sympathetic nervous system in heart failure physiology, pathophysiology, and clinical implications. *J Am Coll Cardiol*, 54, 1747-62.
- TSE, H. F., KWONG, Y. L., CHAN, J. K., LO, G., HO, C. L. & LAU, C. P. (2003) Angiogenesis in ischaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. *Lancet*, 361, 47-9.
- UDELSO, J. E., DILSIZIAN, V., LAHAM, R. J., CHRONOS, N., VANSANT, J., BLAIS, M., GALT, J. R., PIKE, M., YOSHIZAWA, C. & SIMONS, M. (2000) Therapeutic angiogenesis with recombinant fibroblast growth factor-2 improves stress and rest myocardial perfusion abnormalities in patients with severe symptomatic chronic coronary artery disease. *Circulation*, 102, 1605-10.

- UNGER, E. F., GONCALVES, L., EPSTEIN, S. E., CHEW, E. Y., TRAPNELL, C. B., CANNON, R. O., 3RD & QUYYUMI, A. A. (2000) Effects of a single intracoronary injection of basic fibroblast growth factor in stable angina pectoris. *Am J Cardiol*, 85, 1414-9.
- VALE, P. R., LOSORDO, D. W., MILLIKEN, C. E., MAYSKY, M., ESAKOF, D. D., SYMES, J. F. & ISNER, J. M. (2000) Left ventricular electromechanical mapping to assess efficacy of phVEGF(165) gene transfer for therapeutic angiogenesis in chronic myocardial ischemia. *Circulation*, 102, 965-74.
- VALE, P. R., LOSORDO, D. W., MILLIKEN, C. E., MCDONALD, M. C., GRAVELIN, L. M., CURRY, C. M., ESAKOF, D. D., MAYSKY, M., SYMES, J. F. & ISNER, J. M. (2001) Randomized, single-blind, placebo-controlled pilot study of catheter-based myocardial gene transfer for therapeutic angiogenesis using left ventricular electromechanical mapping in patients with chronic myocardial ischemia. *Circulation*, 103, 2138-43.
- VAN RAMSHORST, J., BAX, J. J., BEERES, S. L., DIBBETS-SCHNEIDER, P., ROES, S. D., STOKKEL, M. P., DE ROOS, A., FIBBE, W. E., ZWAGINGA, J. J., BOERSMA, E., SCHALIJ, M. J. & ATSMAS, D. E. (2009) Intramyocardial bone marrow cell injection for chronic myocardial ischemia: a randomized controlled trial. *JAMA*, 301, 1997-2004.
- VANOVERSCHDELDE, J. L. & MELIN, J. A. (2001) The pathophysiology of myocardial hibernation: current controversies and future directions. *Prog Cardiovasc Dis*, 43, 387-98.
- VINCENT, K. A., SHYU, K. G., LUO, Y., MAGNER, M., TIO, R. A., JIANG, C., GOLDBERG, M. A., AKITA, G. Y., GREGORY, R. J. & ISNER, J. M. (2000) Angiogenesis is induced in a rabbit model of hindlimb ischemia by naked DNA encoding an HIF-1 α /VP16 hybrid transcription factor. *Circulation*, 102, 2255-61.
- VON LUDINGHAUSEN, M. (1987) Clinical anatomy of cardiac veins, Vv. cardiacae. *Surg Radiol Anat*, 9, 159-68.
- VON LUDINGHAUSEN, M. (2003) The venous drainage of the human myocardium. *Adv Anat Embryol Cell Biol*, 168, I-VIII, 1-104.
- WANG, Y., TAGIL, K., RIPA, R. S., NILSSON, J. C., CARSTENSEN, S., JORGENSEN, E., SONDERGAARD, L., HESSE, B., JOHNSEN, H. E. & KASTRUP, J. (2005) Effect of mobilization of bone marrow stem cells by granulocyte colony stimulating factor on clinical symptoms, left ventricular perfusion and function in patients with severe chronic ischemic heart disease. *Int J Cardiol*, 100, 477-83.
- WARE, J. A. & SIMONS, M. (1997) Angiogenesis in ischemic heart disease. *Nat Med*, 3, 158-64.
- WAUGH, J. & WAGSTAFF, A. J. (2004) The paclitaxel (TAXUS)-eluting stent: a review of its use in the management of de novo coronary artery lesions. *Am J Cardiovasc Drugs*, 4, 257-68.
- WHITE, F. C. & BLOOR, C. M. (1981) Coronary collateral circulation in the pig: correlation of collateral flow with coronary bed size. *Basic Res Cardiol*, 76, 189-96.

- WHITE, F. C., CARROLL, S. M., MAGNET, A. & BLOOR, C. M. (1992) Coronary collateral development in swine after coronary artery occlusion. *Circ Res*, 71, 1490-500.
- WOHRLE, J., MERKLE, N., MAILANDER, V., NUSSER, T., SCHAUWECKER, P., VON SCHEIDT, F., SCHWARZ, K., BOMMER, M., WIESNETH, M., SCHREZENMEIER, H. & HOMBACH, V. (2010) Results of intracoronary stem cell therapy after acute myocardial infarction. *Am J Cardiol*, 105, 804-12.
- WOLLERT, K. C., MEYER, G. P., LOTZ, J., RINGES-LICHTENBERG, S., LIPPOLT, P., BREIDENBACH, C., FICHTNER, S., KORTE, T., HORNIG, B., MESSINGER, D., ARSENIJEV, L., HERTENSTEIN, B., GANSER, A. & DREXLER, H. (2004) Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. *Lancet*, 364, 141-8.
- WRIGHT, M. J., WIGHTMAN, L. M., LILLEY, C., DE ALWIS, M., HART, S. L., MILLER, A., COFFIN, R. S., THRASHER, A., LATCHMAN, D. S. & MARBER, M. S. (2001) In vivo myocardial gene transfer: optimization, evaluation and direct comparison of gene transfer vectors. *Basic Res Cardiol*, 96, 227-36.
- YAO, K., HUANG, R., QIAN, J., CUI, J., GE, L., LI, Y., ZHANG, F., SHI, H., HUANG, D., ZHANG, S., SUN, A., ZOU, Y. & GE, J. (2008) Administration of intracoronary bone marrow mononuclear cells on chronic myocardial infarction improves diastolic function. *Heart*, 94, 1147-53.
- YOUNG, J. B., ABRAHAM, W. T., SMITH, A. L., LEON, A. R., LIEBERMAN, R., WILKOFF, B., CANBY, R. C., SCHROEDER, J. S., LIEM, L. B., HALL, S. & WHEELAN, K. (2003) Combined cardiac resynchronization and implantable cardioversion defibrillation in advanced chronic heart failure: the MIRACLE ICD Trial. *JAMA*, 289, 2685-94.
- YOUSEF, M., SCHANNWELL, C. M., KOSTERING, M., ZEUS, T., BREHM, M. & STRAUER, B. E. (2009) 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*, 53, 2262-9.
- YU, A. Y., FRID, M. G., SHIMODA, L. A., WIENER, C. M., STENMARK, K. & SEMENZA, G. L. (1998) Temporal, spatial, and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung. *Am J Physiol*, 275, L818-26.
- ZHAO, Q., SUN, Y., XIA, L., CHEN, A. & WANG, Z. (2008) Randomized study of mononuclear bone marrow cell transplantation in patients with coronary surgery. *Ann Thorac Surg*, 86, 1833-40.
- ZIJLSTRA, F., DE BOER, M. J., HOORNTJE, J. C., REIFFERS, S., REIBER, J. H. & SURYAPRANATA, H. (1993) A comparison of immediate coronary angioplasty with intravenous streptokinase in acute myocardial infarction. *N Engl J Med*, 328, 680-4.
- ZOHLNHOFFER, D., DIBRA, A., KOPPARA, T., DE WAHA, A., RIPA, R. S., KASTRUP, J., VALGIMIGLI, M., SCHOMIG, A. & KASTRATI, A. (2008) Stem cell mobilization by granulocyte colony-stimulating factor for myocardial recovery after acute myocardial infarction: a meta-analysis. *J Am Coll Cardiol*, 51, 1429-37.

ZOHLNHOFFER, D., OTT, I., MEHILLI, J., SCHOMIG, K., MICHALK, F., IBRAHIM, T., MEISETSCHLAGER, G., VON WEDEL, J., BOLLWEIN, H., SEYFARTH, M., DIRSCHINGER, J., SCHMITT, C., SCHWAIGER, M., KASTRATI, A. & SCHOMIG, A. (2006) Stem cell mobilization by granulocyte colony-stimulating factor in patients with acute myocardial infarction: a randomized controlled trial. *JAMA*, 295, 1003-10.