AUTOLOGOUS BONE MARROW STEM CELLS IN PAEDIATRIC HEART FAILURE

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

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ABSTRACT

Stem cell treatment for heart failure is a topic that generates much interest. Although heart failure is much less common in children than in adults, the social and economic implications in paediatric care are significant[1]. The bone marrow mononuclear cell fraction has been used experimentally as therapy after myocardial infarction in adults; it appears safe and has a modest effect on ventricular function[2-4]. Studies in adults with dilated cardiomyopathy have also shown positive results[5, 6]. Paediatric bone marrow is usually more cellular than that of adults and the patient environment possibly more receptive to potential repair and regeneration. As modest improvements are seen in adults after stem cell injections we anticipated similar injections in children to be potentially more effective.

A case series has been described of stem cell therapy in children with heart failure[7], but no randomised studies exist. The lack of randomised studies has led to few centres considering such therapy.

This randomised, crossover, placebo controlled pilot study was designed to primarily determine the safety and feasibility of stem cell intracoronary therapy in children. Secondary end points were ventricular volumes, ejection fraction and N Terminal pro B-type Natriuretic Peptide (NTproBNP). Cardiac magnetic resonance imaging was used for the assessment of cardiac volumes and function and allowed a considerable reduction in the patient numbers required to investigate the hypothesis[8].

This is the first randomised controlled trial of the use of autologous bone marrow derived stem cells in paediatric dilated cardiomyopathy. This study demonstrated that the use of stem cells in children to address the condition of heart failure is both feasible and acceptable. No mortality or morbidity was experienced throughout the protocol, and no adverse events were reported. Left ventricular volumes were reduced at three months following stem cell injection compared with placebo, but no significant change in systolic function and NTpro-BNP were found.

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CHAPTER 1

INTRODUCTION

1.1 Rationale for the study

This research was prompted by the increasingly long waiting time of children on the heart transplant list, and the declining offers of organ donation for transplantation. Such discrepancies between organ supply and demand highlight the urgent need to investigate alternative therapies for heart failure either as definitive treatment or to increase the length of time a child's condition may be maintained, with either stable or improved cardiac function, thus reducing the pressure on organ availability for cardiac transplantation.

In adults bone marrow derived stem cells have shown promise as such an intervention[1-8]. Meta-analysis has concluded that a modest improvement is usually seen with intra-coronary injection of stem cells, and that the technique is safe. However, the paediatric bone marrow is usually more cellular and the patient environment possibly more receptive to such potentials of repair and regeneration, and so it is felt that results of similar injections in children could be more marked.

Great Ormond Street Hospital (GOSH) is one of the few centres in the world capable of this paediatric research project because of the unique patient group it hosts, the cardiac catheter and investigation technologies, transplant and mechanical heart expertise and bone marrow preparation facilities all being available on one site.

This study commenced in 2008 and the first administration of autologous stem cells to a paediatric cardiac patient took place on the 12th September that year. This intramyocardial injection was performed on a child-awaiting transplant at

the time of surgical insertion of a paediatric left ventricular support device. Since then ten children with significant heart failure have received intracoronary injections of autologous stem cells, completing our pilot study.

1.2 Heart Failure: Background

Heart failure may be defined as inadequate cardiac output to meet the metabolic demands of the vital organs. It occurs with either an acute or chronic onset or as an acute exacerbation of an already recognised chronic state. There are many anatomical and patho-physiological aetiologies of this condition, which can affect any age group. Heart failure has a significant mortality and morbidity associated with it, and as a consequence it places a huge medical, emotional and financial burden on society. Although most commonly affecting the elderly, as a consequence of coronary artery disease and hypertension, heart failure also has a significant impact when presenting in childhood.

Adult patients presenting for the first time with clinical heart failure have a median age of 76 years, and for many life expectancy is poor. A quarter die within three months, over a third by one year, and nearly half are dead by two years[9]. The incidence of heart failure in London studies was 1.3 cases per 1000 population per year for those aged 25 years and over. Incidence increased from 0.02 cases per 1000 population per year in those aged 25–34 years to 11.6 in those aged 85 years and over[10]. Within the paediatric population alone it is estimated that approximately 100 new cases of heart failure from heart muscle disease are identified across the UK annually, with a corresponding incidence of 0.87/100,000 population <16 years[11]. The main causes of heart failure in

children include abnormal myocardial function, congenital cardiac lesions and cardiac dysrhythmias.

Although heart failure is much less common in children than in adults, the social and economic implications in paediatric care are significant. Children more frequently require longer hospital admissions with greater duration of intensive care support[11], utilising costly surgical[12] or catheter based interventions as part of the management of their heart failure. Increasing numbers of children are also surviving longer with their cardiac condition thanks to the improving therapies and interventions and in addition to these children others with congenital cardiac lesions are also surviving due to improved surgical techniques permitting better long-term outcome. The social and economic impact of children with chronic illnesses, including heart failure, is considerable. Long periods of hospitalisation are not only distressing for children and their families but also disruptive to the family unit. Additional economic strain is placed on the family by disruption of parental earnings and increased living costs while their child is in hospital. A child whose illness prevents them from working in adult life or who dies as a result of their illness also has a negative impact economically through the costs of supportive care and loss of contributory earnings. For these reasons and others heart failure in children is therefore a significant public health issue[13].

The main symptoms of heart failure, dyspnoea, anasarca (the build-up of fluid previously termed "dropsy") and cachexia have been well documented historically, although they are also attributable to a wide range of other conditions. In the seventeenth century William Harvey recognised the heart as

being the organ responsible for pumping blood around the body rather than just a source of heat generation, and consequently began the association of these symptoms with the failing of the heart in its function. He documented his findings in "de Motu Cordis" otherwise known as " On the Motion of the Heart and Blood" published in 1628. Albertini, a century later, was the first to document the association of a failing heart with peripheral and pulmonary oedema[14].

Early documentation of heart failure only appears to review adults with the condition; it was not until the late 1890s that descriptions of childhood heart disorders were recorded in medical texts, infection at this time being the main cause of heart related illness. The most common cause of cardiac failure in children remained rheumatic fever until the mid twentieth century when an apparent decrease in virulence of streptococci, with a concomitant decrease in incidence of scarlet fever and rheumatic fever occurred resulting in congenital cardiac disorders becoming more widely recognised as causing failure in infancy and early childhood. Recognition that an abnormal cardiac structure could cause heart failure by means of volume overload, pressure or flow disturbances even when the heart muscle was of normal function provided a different aetiology compared with adults where heart muscle damage via infection and infarction was the primary cause of heart failure.

The main causes of heart failure in children are now understood to include high volume load, excessive pressure load, abnormal myocardial function, congenital cardiac lesions and cardiac dysrhythmias. Outside of infancy where congenital cardiac lesions result in the majority of cases of heart failure, intrinsic

myocardial disease is the commonest form of cardiac failure and is the main indication for heart transplant in children.

Treatment strategies for children with heart failure were extrapolated from adult therapies and have subsequently evolved through experience and observation of responses and outcomes. Such observational experience has guided therapy until recent times, with few randomised clinical trials of therapies in children being undertaken. It was only in 2007 that the first multicentre, randomised trial of a therapeutic agent in children with heart failure was performed by Shaddy *et al*. He investigated the use of carvedilol in cardiac failure[15]. It has remained difficult to gain approval for such randomised studies involving children and in addition, recruitment of children to such studies is more difficult than comparable study protocols with adults. Despite the difficulty in planning and conducting randomised controlled studies in children, they are essential before therapies can be safely transferred to the wider paediatric population. Therapeutic options for children with heart failure are limited, and if medical management is not successful, invasive surgical strategies, including cardiac transplantation become essential. The high mortality rate for children with heart failure, when evaluated in conjunction with the inadequate supply of donor organs for transplantation, provided the justification for this potentially high-risk protocol.

1.3 Assessment of heart failure

Heart failure is assessed in the clinic setting by several means. A thorough history taken from either the child or his family permits initial assessment of the child's functional status. The New York Heart Association (NYHA) Functional

Classification of heart failure (Appendix 1) provides a simple tool for classifying the extent of heart failure and is utilised both within the adult and paediatric settings. This classification provides a means of categorising patients based on their limitations of physical activity, degree of breathlessness and cardiac symptoms. The NYHA functional classification grades patients from classes I to IV depending on the severity of their presentation. Those patients in class I are asymptomatic and have no limitation to ordinary physical activity, whereas those in class IV are severely limited and may even experience symptoms of heart failure while at rest. Another similarly used classification is the Ross Classification of heart failure. Such classifications enable clinicians to categorise patients easily and reproducibly and also to note improvement or deterioration in clinical function.

1.4 Patient assessment

History and examination form the foundation for assessment of a patient with a cardiac disorder. The information that is determined at this stage allows selection of the most appropriate investigations followed by focused and timely management of the condition encountered.

1.4 i. History

The patient history details should include antenatal and gestation information covering factors such as maternal disease, medications and environmental exposures to drugs and alcohol for example. Birth history, gestation and birth weight provide important indicators for both cardiac and non-cardiac disorders. Prematurity is associated with persistent patent ductus arteriosus, birth asphyxia with pulmonary hypertension and myocardial dysfunction for example.

Family history of cardiac disorders, miscarriage, early or sudden unexplained death of family members or atypical seizure should be enquired after.

Cardiac symptoms should also be detailed; cyanosis, breathlessness, poor feeding, growth issues, reduced exercise tolerance, stridor, wheeze, grunting, orthopnoea, chest pain, palpitations, fits, faints and funny turns can all have cardiac significance and should be explored in detail. A full systems enquiry may also reveal associated symptoms of cardiac disease. Current and previous medication details can provide insight into management of related symptoms and disorders as well current therapeutic strategies.

The social history should explore the impact of the cardiac illness on the child in terms of growth, schooling and social isolation or stigmatisation. The impact of illness in a child will additionally have emotional and financial impact on the parents and potentially affect siblings and future children. Coping strategies and contingency plans can be discussed and support, medical, social and educational, offered when necessary.

1.4 ii. Examination

Initial inspection gives a good indication as to the severity of the presenting illness and the need for resuscitation or rapid early management. Dysmorphic features and general growth are also easily observed. The remainder of the full examination follows the standard protocol of inspection, palpation, percussion, and auscultation. All children should have their height and weight plotted on an appropriate growth chart.

1.4 iii. Investigations

History and examination of the patient will determine which investigations, if any, should most appropriately be performed. Traditionally electrocardiograms (ECG) and chest X rays (CXR) are the initial investigations performed in most circumstances but with the growing availability of echocardiograms (Echo) these are being increasingly utilised.

a) Chest X-ray (CXR)

The CXR can be used to establish the position, basic anatomy and abnormalities of the heart, major vessels, lungs, thoracic and upper abdominal structures. The cardiothoracic ratio is usually < 50% (55-60% in neonates); a large heart may be seen in large shunt lesions, dilated cardiomyopathy, or pericardial effusion.

b) Electrocardiogram (ECG)

The ECG is useful for assessing the cardiac rate, rhythm, conduction abnormalities, chamber enlargement, muscular hypertrophy and strain. The ECG should always be evaluated within the clinical context, also taking into consideration the age of the patient as certain parameters assessed by the ECG change as the child grows. ECGs are readily available and do not require expensive or complicated equipment.

c) Echocardiogram (Echo)

Echocardiography is a non-invasive technique that can be used at the bedside to investigate cardiac conditions and is particularly suited to assessing structural congenital lesions. Echocardiography has replaced assessment by cardiac

catheterisation and angiography in most situations. As well as examining cardiac structure, echocardiography can be used to assess cardiac function, monitor haemodynamics within the chambers and great vessels and diagnose conditions such as effusion or tamponade. Dilated cardiomyopathy (DCM) is frequently diagnosed using echocardiography. Systolic dysfunction and progressive left ventricular (LV) dilatation are characteristics of dilated cardiomyopathy. Echocardiography is also the primary source of information for monitoring the condition. Measures of systolic versus diastolic diameter (fractional shortening) or volume (ejection fraction) are routinely collected to monitor LV systolic function[16].

For children, echocardiographic values are usually normalised for age and body surface area. The values obtained are expressed as Z-scores, or the number of standard deviations (SDs) above or below the mean of a normal population. Values more than 2 SDs or Z scores more than +/-2 are unusual and often indicate an abnormal cardiac structure or function. The walls of the left ventricle can become thin in dilated cardiomyopathy, leading to a reduction in the LV posterior wall thickness Z score.

d) Blood investigations

Routine haematology and chemical pathology tests are sent to obtain general information regarding physical status. Particular attention should be paid to the liver function tests, which may become deranged at the extremes of system failure.

Of particular note for children with heart failure is the assessment of B natriuretic peptide (BNP). In clinic, this blood test is frequently performed to monitor the degree of cardiac incompetence. Natriuretic peptides (NP) are a group of hormones responsible for the modification of sodium and fluid balance within the body. There are three major classes of natriuretic peptides, A, B and C, which all have a 17-amino acid ring structure. Through their effects on sodium and fluid homeostasis they act to protect the cardiovascular system from fluid overload. A type natriuretic peptide (ANP) and B type natriuretic peptide (BNP) are released predominantly by the heart, A type being synthesized mainly by the atria and B type by the ventricles. C type natriuretic peptide (CNP) is also synthesized by the cardiac tissue but its primary origin is the endothelial cells[17].

ANP and BNP are released from cardiac myocytes when they undergo increased tension as a result of volume overload; their levels alter measurably in the blood in response to the mechanical stress placed on the myocardium. When the ventricular myocyte is stretched by the increased cardiovascular load in heart failure, pre-proBNP is enzymatically cleaved to proBNP and released in the form of the active hormone BNP, along with the inactive fragment of the pro-hormone, N terminal portion of the pro- B type natriuretic peptide (NTproBNP)[18]. BNP binds with receptors which consequently act to reduce systemic vascular resistance and central venous pressure, in addition to inducing natriuresis[19].

In heart failure BNP levels in the plasma are noted to increase, high plasma levels indicating a poor prognosis (Table 1.1). Both BNP and NTproBNP have evolved to be useful biomarkers of cardiac function as well as prognosis in heart

failure[20]. BNP however has a short half-life but as it is released with NTproBNP, which is a much more stable peptide in serum, NTproBNP is measured preferentially[21]. Studies directly comparing BNP and NTproBNP have also advocated the use of NTproBNP as a superior indicator of both mortality and morbidity in terms of re-hospitalisation[22,23].

Table 1.1: Relationship between BNP and the degree of heart failure

BNP level (pg/ml)	Degree of heart failure
<100	No heart failure
100-300	Heart failure present
300-600	Mild heart failure
600-900	Moderate heart failure
>900	Severe heart failure

BNP is an important prognostic marker currently used in the assessment of paediatric heart failure. Studies have shown that a strong link exists between elevated levels of circulating BNP in children with heart failure and their clinical status, reflected by abnormal clinical and echocardiogram indices[24]. BNP levels are also indicative of prognosis and morbidity in children with left ventricular failure[25], high levels being used to identify those children at higher risk of poor outcome[26].

e) Cardiac catheterisation

Echocardiography and magnetic resonance imaging (MRI) have replaced cardiac catheterisation and angiography for anatomical assessment, and MRI is

beginning to replace it for haemodynamic assessment. However, it remains an essential tool for the cardiologist, particularly as it can be combined with therapeutic interventional procedures. Angiography is particularly useful in assessing the highly complex anatomy for example in pulmonary atresia with major aorto-pulmonary collaterals where individual vessel injections are needed.

f) Interventional cardiac catheterisation:

Percutaneous or minimally invasive approaches are steadily replacing open cardiac surgery procedures. Vascular access is secured percutaneously, via the femoral artery or vein usually; in neonates the umbilical vessels may be accessed instead. For nearly 50 years balloon septostomy has been performed to improve oxygenation in transposition of the great arteries, balloon dilatation of valves and re-coarctation became widespread in the 1980's. In the 1990's atrial septal defect (ASD) occlusion devices were widely adopted. As many other lesions have continued to be added to the list of percutaneous procedures, including patent ductus occlusion, shunt occlusion, stent insertion in narrowed arteries, and ductal stenting, a new paediatric speciality has developed of paediatric interventional cardiology. It has even become possible to insert valves percutaneously and perform complex palliation of single ventricle anatomy in conjunction with surgeons as a "hybrid" operation.

g) Cardiac magnetic resonance imaging (MRI)

Although MRI is not a portable, bedside or clinic technique like echocardiography, it is being increasingly used for detailed cardiac assessment. MRI is non-invasive and uses no radiation routinely. This modality can create

detailed three dimensional imaging allowing accurate visualisation of the cardiac and vascular structures as well as facilitating calculation of blood flow, ventricular volumes and muscle mass. For accurate imaging the child needs to remain still for the duration of the scan. Patients also need to be able to follow commands to perform breath-holds for optimum data collection; smaller children may therefore require a general anaesthetic or sedation for this procedure. The cardiac parameters measured from the MRI images can be used to evaluate cardiac function. Ejection fraction, which determines the amount of blood pumped from the ventricles with every cardiac contraction, is commonly used as a valuable measure of function. An ejection fraction (EF) between 55-70% is normal, although it is possible to have an ejection fraction within this range, but still have heart failure (Heart failure with preserved ejection fraction, HFpEF). EF of 45-55% indicates cardiac damage and often mild heart failure. EF less than 40% may be evidence of heart failure, and less than 30% signifies severe heart failure. An EF of more than 75% may indicate a condition such as hypertrophic cardiomyopathy.

As MRI scanners use powerful magnets to form the images precautions must be taken with metal items near the scanner. Unfixed metal objects are not permitted in the vicinity of an MRI scanner and fixed metal items need to be checked. Patients with cardiac pacemakers should not be allowed near the scanner as their internal metal pacemaking wires may become dislodged due to the magnetic force, although some new pacemakers are MRI compatible. Surgical clips or fixed internal prostheses may however be permitted but can interfere with the quality of images obtained. Increasingly MR imaging is combined with cardiac catheterization in hybrid laboratories with the facility to

perform the imaging and intervention in one room and slide the patient through to the MR scanner. This facility is also useful for combined assessment of pressure and flow to calculate pulmonary vascular resistance in children with suspected pulmonary hypertension.

1.5 Pathologies associated with heart failure

1.5 i. Heart muscle disease

Heart muscle disease is less of a problem than congenital heart disease with an incidence of around 0.4/1,000 for the most common lesion, dilated cardiomyopathy.

a) Dilated cardiomyopathy (DCM)

In this lesion the heart is dilated and contracts poorly. DCM can be the end stage of metabolic disease, or result from toxins such as anthracyclines, or from chronic tachycardias, or from nutritional deficiency such as vitamin D deficiency and hypocalcaemia, but most cases are probably sporadic or inherited mutations of cardiac structural proteins. Inheritance varies with many cases considered to be isolated lesions, but all modes of inheritance are described and the genetic lesions associated so varied that routine screening is rarely available. Some more common lesions that can be screened for include Barth syndrome (X linked with cyclical neutropenia) and lamin A/C mutations (often associated with skeletal myopathy and abnormal rhythms). The cardiomyopathy associated with Duchenne and Becker muscular dystrophy rarely causes symptoms before later teenage years. In most cases of DCM symptoms can be controlled with medical therapy for many years, but a few cases do deteriorate and it is the most common indication for paediatric heart transplantation in

Europe. The cornerstone of long-term treatment for heart failure is currently with angiotensin converting enzyme inhibitors, beta-blockers (particularly carvedilol) and spironolactone. Other treatments are more geared to symptom control such as diuretics and perhaps digoxin (if the latter is used in low dose with low levels to reduce the risk of sudden death).

b) Myocarditis

Myocarditis can cause confusion with dilated cardiomyopathy as the echocardiographic appearances are similar. Typical acute myocarditis tends to have less dilatation and lacks the thin walls of DCM. Many aetiologies of myocarditis have been described. Viruses are the most common cause, particularly entero-viruses (these can be very destructive to the myocardium) but parvovirus is also common, adeno-virus, influenza, chickenpox and Epstein-Barr virus and many others are all know to cause myocardial inflammation. Other causes need to be considered in endemic areas such as Chagas' disease, HIV, hepatitis C, dengue fever and diphtheria. Treatment for myocarditis is largely symptomatic as little evidence for immunosuppression exists. Severe cases may need mechanical circulatory support.

c) Pericarditis

The causes of myocarditis are also implicated in pericarditis, although an isolated pericardial effusion may also be bacterial and purulent or related to autoimmune disease (systemic lupus erythematosus /juvenile rheumatoid arthritis). Post cardiac surgery/pericardotomy pericardial effusion is well recognised in children and can be very severe causing tamponade, particularly after ASD surgery.

d) Hypertrophic cardiomyopathy (HCM)

In contrast to DCM, the heart muscle in this condition is very thickened; the heart contracts strongly, often the outflow is obstructed and frequently a restrictive physiology is seen. Most commonly it is associated with sarcomeric protein mutations and is inherited in an autosomal dominant manner. Sudden death is well recognized and high-risk cases are candidates for implantable cardioverter defibrillators. In children, dysmorphic syndromes that affect components of RAS-mitogen* activated protein kinase such as Noonan, Leopard, Costello and neurofibromatosis type 1 are associated with hypertrophic cardiomyopathy (*RAS, or rat sarcomere, are a family of proteins expressed in all cells that are involved in transmitting signals within cells to control growth, differentiation and survival). Metabolic disease associated with HCM include Pompe syndrome with severe hypertrophy in the neonatal period and mitochondrial disease often with impaired systolic function and hypertrophy.

e) Restrictive Cardiomyopathy RCM

This uncommon cardiomyopathy is a physiological diagnosis with impaired cardiac filling. Usually the prognosis is poor with complications including arrhythmias, thromboembolic disease, pulmonary hypertension and heart failure. Treatment is very difficult. It can be associated with some neuromuscular diseases such and myofibrillary problems.

f) Arrhythmogenic right ventricular cardiomyopathy (ARVC or AC)

This is very rare in the first decade of life and tends to be a problem of teenagers and young adults. There is a high risk of sudden death. Although

predominantly a disease of the right ventricle the left can also be involved. It is autosomal dominantly inherited but genetically heterogeneous.

1.6 Management of heart failure

Therapeutic strategies to manage paediatric heart failure have historically been extrapolated from those used in adult medicine, the low prevalence of heart failure in children meaning that extensive studies are difficult to conduct in children. As we know, however, the causes of heart failure in children are often different from the causes in adults, for this reason the use of adult protocols in children may not be entirely appropriate. Whereas the treatment of cardiac failure due to congenital cardiac lesions is generally surgical repair, treatment of heart muscle dysfunction is generally medical. The American College of Cardiologists and the American Heart Association have produced protocols for the management of heart failure. These protocols involve the use of diuretics, angiotensin-converting enzyme (ACE) inhibitors, digoxin and beta blockade[27,28]. Historically medical management has been focused on improving symptoms. Over the last 20 years however the goal of treatment has not just been limited to treating the symptoms, but has also aimed to reverse the remodelling process that is linked to the progression of heart failure[16]. Those patients that do not respond adequately to such medications may require a step up in their treatment protocol. Escalation of heart failure therapy includes the use of intravenous inotropes, cardiac resynchronisation therapy with implantable devices, mechanical support and cardiac transplantation.

1.7. Heart failure outcome

Approximately one third of children with new onset heart failure due to heart muscle disease require transplantation or have died within one year of

presentation[11]. A further third may improve clinically, some even normalise their cardiac function, and the remaining third remain stable with their condition[11].

Table 1.2: Summary of the major therapeutic agents used in heart failure[27]

Medication	Use
Diuretics	First line treatment for congestive cardiac failure Main classes used in children: -loop e.g. furosemide -mineralocorticoid/glucocorticoid receptor antagonists eg spironolactone -thiazide diuretics e.g. metolazone Must be used in combination with drugs such as ACE inhibitor or beta-blocker to prevent clinical worsening of symptoms via vicious cycle of sympathetic and renin angiotensin aldosterone system activation
ACE inhibitors	Causes vasodilatation and afterload reduction May prevent myocardial thickening and fibrosis
Digoxin	Positively inotropic: increases intracellular sodium concentration hence increases calcium ions available to sarcoplasmic reticulum for contraction Negatively chronotropic: reduces sympathetic activation by reduction in norepinephrine, renin and aldosterone concentrations May facilitate introduction of β blocker
β blockers	Long term use may prevent abnormal cardiac remodelling Potentially reverses down-regulation of adrenergic receptors Use best delayed until heart failure symptom management achieved
Oral phosphodiesterase inhibitors	Not recommended for general use eg enoximone; used with caution in specific circumstances eg weaning IV inotropes, or on establishing β blocker therapy
Anticoagulation	To address increased risk of thrombus formation in dilated ventricles of DCM or atria in RCM. Warfarin preferable in older children, aspirin in younger
Intravenous inotropes	*In extremis*, patient requiring intensive support May be a maintenance step prior to mechanical support or cardiac transplantation

1.7 i Mechanical support

Mechanical circulatory support is being used increasingly in the bridging of paediatric patients to transplant, the success of the technique resulting in a self-perpetuating demand as experience in its use increases. Currently there are two main strategies of mechanical bridging to transplant for children and adults. These two methods are extra-corporeal membrane oxygenation, abbreviated to ECMO, and the use of ventricular assist devices, VADs. Ventricular assist devices may be paracorporeal or intracorporeal offering either uni-ventricular or bi-ventricular cardiac support by means of continuous or pulsatile flow. Intra aortic balloon pumps are also used commonly as temporary cardiac support in adults. The selection of which device is most suitable in a given situation is dependent on several clinical and logistical factors. Each support modality has its own range of indications, risks and benefits that must be factored into the clinical scenario.

Mechanical circulatory support provides a crucial role in the bridging of end stage heart failure patients to transplant. Each of the current modes of mechanical support offer the benefit of increasing the time that an individual patient may be able to wait for a suitable donor organ at the cost of significant risk of morbidity and mortality to the individual. It cannot be underestimated however that in situations where a child is *in extremis* the possibility of any life preserving intervention is appealing and undoubtedly artificial cardiac support has prevented many patient deaths. The major risk factors associated with this type of therapy include consequences of bleeding diatheses, end organ failure, infection and embolic events. Certain of these factors can be monitored and addressed but others are more unpredictable and an unfortunate but inherent

risk of the therapeutic modality. As devices evolve, these risk factors may also change and hopefully diminish the risks associated with this vital therapy.

As already described, when maximal medical therapy is insufficient to support a patient with a failing heart, cardiac transplantation offers a therapeutic option. With its associated risks of morbidity and mortality, this life limiting therapy is far from ideal, but often the only possible management option available. At a critical time for the heart transplant service, as donor organs are in such low supply, but demands in all age groups remain high, serious thought must be given as to how we may redress the balance. Mechanical cardiac support at present offers the capacity for bridging a patient until a donor organ is available, but this intervention too is associated with significant risk.

1.7 ii Heart transplantation

Heart failure patients form the majority of children who require a heart transplant over the age of 1 year, around the world[11]. During the financial year 2010-2011 131 heart transplants were performed in the UK, 40 of those for children, 18 of which were performed at GOSH. This number has been gradually reducing year on year and in the first six months of 2012 only 3 had been carried out. Sadly this figure does not reflect the full demand for organs, and as a result many children remain listed for a transplant, some of whose needs are extremely urgent, and the number of children who die on the active transplant list whilst waiting for a heart is also significantly increasing. During the calendar year April 2012 to 2013, 31 children were listed for cardiac transplantation at GOSH alone; by the end of that year 9 had been transplanted, 8 remained on the waiting list, 4 had improved and were consequently removed from the list and 10 children had died waiting[a]. Only 50% of our paediatric patients are alive two

years after their time of listing, either having received a transplant or remaining alive while still listed[a].

Although heart transplants, like other organ transplants, provide a treatment option, they are by no means curative. Despite advances in medicines and technology, patients post heart transplant are significantly life limited. Cumulative data collected since 2000 from our institution indicates that following survival of the transplant procedure our patients have a 95% chance of survival to 30 days, 92% to 1 year and 84% to 5 years. The median survival of those that do live to one year is 20 years post transplant.

Transplant carries the risk of significant morbidity and mortality both directly through the procedure itself and indirectly as a consequence of side effects of essential drug therapies, immuno-suppression, malignancy and further cardiovascular disease in addition to the psychological and social impact on the child and their family caring for a chronic condition which necessitates daily ingestion of drugs, regular hospital follow-up and often disruption to "normal" family life, routines and education.

The surgical technique of cardiac transplantation was first described in 1960, followed seven years later by the first successful human heart transplant. The procedure has subsequently evolved to provide a hugely important treatment modality to manage cardiac conditions, which have either exhausted medical management strategies or would otherwise prove terminal. Although transplant is a valuable treatment, with the benefits of improving quality and duration of life, it does not offer a cure in the conventional sense as it is currently unable to

restore a fully normal life expectancy. The use of transplantation is restricted by rigorous selection criteria of the transplant programme, assessment of the presenting condition, underlying pathology and co-morbidities being essential for full evaluation of the patient as to their suitability for transplant. The programme is limited however by the availability of donor organs, resulting in an increasing mismatch between supply and demand within the transplant service. As a result even with stringent assessment of those deemed suitable for transplant, additional consideration must be given to finding the most suitable recipient for the scarce and precious donor organ when it does become available.

Conditions for which transplantation may be an option are similar in both adult and paediatric populations, however the proportion of acquired heart disease is understandably higher in older age groups. In general heart transplantation is considered in individuals with end stage heart failure, with a life expectancy of less than 18 months, where other medical therapy has been exhausted or ineffective. There are absolute and relative contraindications to cardiac transplantation, including significant medical, infective and psychiatric co-morbidities in addition to physiological status at the time of assessment. These restrictions are imposed to ensure the most effective and appropriate allocation of scarce donor organs.

With an increasing demand on the supply of organs available for heart transplant, alternative strategies are being sought to maintain patients with end stage cardiac failure for longer periods of time. Devices which support a failing heart to allow cardiac recovery in some cases or more commonly to accommodate the time to source a suitable donor heart for transplantation

provide crucial therapeutic options, however alternative management strategies need to be investigated as a matter of urgency. Conventional therapies generally aim to prevent further cardiac deterioration and symptomatically optimise the clinical condition of the patient. The majority of management strategies currently available are unable to address the underlying pathology by reversing or repairing cardiac myocyte damage and hence are not able to improve the long-term cardiac function that may influence overall outcome for the patient. Investigation of alternative treatment options could potentially focus on myocardial recovery.

One option is to promote the repair of the native cardiac muscle in situ. This may possibly be achieved in two ways; invoking innate repair mechanisms or supplying cells or additional substrate to encourage repair and regeneration locally. Therapies such as these have the potential to support the failing heart in the short term or allow healing and long-term functional improvement.

1.8 Cardiac regeneration

Until recently it was believed that the heart was a terminally differentiated organ with limited capacity for regeneration. Research has revealed that the heart does in fact undergo some cell turnover and hence does have the ability to repair itself, however slowly[29].

Surveillance cardiac biopsies are routinely performed after cardiac transplantation to assess potential signs of immunological rejection; this biopsy tissue also offers us a unique opportunity to assess cardiac tissue in other ways. Male recipients of female donor hearts at cardiac transplantation have

been noted on such routine cardiac biopsy surveillance to have cells containing Y-chromosomes within the myocardium. Clearly, at the time of transplantation, the female donor heart tissue is composed of cells with only X chromosomes within the nuclei. Cells containing any Y chromosome are by definition male in origin and it can reasonably be speculated that they have therefore migrated to and integrated within the donor graft after transplant. The Y chromosomes are detected easily by fluorescence in situ hybridisation (FISH) techniques [30,31,32]. Those cells with Y-chromosomes can be investigated further to identify primitive stem cell markers such as c-kit, MDR-1 and Sca-1. In 2002, Quaini et al[33] observed by such methods that in male recipients of female donor hearts, high levels of cardiac chimerism is caused by the migration of primitive cells from the recipient to the grafted heart.

The origin and fate of the Y containing cells found within the donor graft is unclear. As yet there is no specific evidence that these cells go on to develop into new functional myocardial tissue; these cells may however contribute to cardiac remodelling. These cells may work in combination with other circulating endothelial and progenitor cells to colonise and regenerate regions of myocardium. If such events are possible within the transplanted myocardium it is logical to assume that such occurrences are also feasible within normal cardiac tissue of individuals who have not received a transplant. Ischaemia or injury may provide the cues for such relocation of cells to occur. Inflammatory mediators may attract new cells to regions of cardiac damage along chemical mediator gradients. Interestingly most Y positive cardiomyocytes in males with female allograft hearts have been detected in regions of acute rejection[34].

The extent of chimerism seen within cardiac biopsies has also been noted to have a time-dependent relationship, more chimeric cells being generally noted as time from transplant increases[35]. This supports the more modern theory that cardiac cell turnover does occur but only at slow rates. Under normal circumstances this slow cell turnover may be adequate to maintain good cardiac function, but in situations of damage or injury it is likely to be insufficient. If methods could be devised to augment and increase the rate of this naturally occurring turnover it is possible that new auto-therapeutic strategies could evolve for cardiac conditions of heart muscle damage.

Systemic chimerism is also seen in female host recipients of male organs and within the graft organs of male recipients of female tissue. Such bidirectional movement of cells indicates a possible state of flux of cells between tissues. The markers detected on the surfaces of these chimeric cells indicate the primitive nature of these mobile cells. This has opened up a new potential therapeutic avenue to explore for cardiac pathologies, the role of stem cells.

In addition to stem cells homing in on the damaged myocardium from distant sites within the body, resident cardiac progenitor cells have also recently been detected.

Cardiac progenitor cells (CPCs) have been noted to exist in several animal populations including humans[36,37,38] and their discovery not only counters the traditional view of the heart as a non mitotic organ but also offers a potential source of self repair. These cardiomyocyte-like cells that are able to undergo mitosis were first observed in humans following acute ischaemic insults[39] and

since then several sub-populations have been categorised depending on their distinct cell surface markers. The marker profiles of these cells has enabled classification of the cardiac progenitor cell type, their differentiation potential and those factors which stimulate their expansion. The cardiac progenitor phenotypes reported to date are side population (SP) cells, c-kit + cardiac progenitor cells, Sca-1+ cardiac progenitor cells, Islet 1+ cardiac progenitor cells, SSEA1+ cardiac progenitor cells, "cardiosphere" or "cardiosphere"-derived cardiac progenitor cells[40].

CPCs have only been reported in relatively small numbers in vivo, while to enable them to be of therapeutic value, substantial numbers would be required. Their therapeutic potential therefore relies on the ability of these cell populations to be expanded either in vivo or in vitro. CPCs have been obtained from cardiac biopsy tissue and methods developed to expand them in vitro[36]. Clinical safety trials of their use have also taken place[41,42]. CPCs also have the potential to be stimulated to undergo mitosis in vivo if the correct factors are applied. Substantial information is still to be determined regarding the specific characteristics of CPCs, so although they may appear to be an ideal candidate for use as a cardiac therapy, much information is yet to be determined regarding their specific origin and homeostatic mechanisms.

1.9 Stem cells background

1.9 i The use of stem cells in heart failure

Experimental data on the potential for cardiac tissue to regenerate prompted several investigations into the potential use of stem cells in adult populations. These studies used adult patients, the majority with either acute ischaemic damage or chronic post-infarction heart failure. To date stem cells have been

used with modest effect in these patients with myocardial damage[43] (Table 1.3). Stem cells, obtained from a variety of sources including bone marrow, peripheral circulating blood, adipose, skeletal or cardiac tissue, were harvested and re-introduced into the coronary circulation or cardiac tissue directly. Those studies using bone marrow derived cells utilised the mononuclear cell fraction (CD34+/ CD133+), some further refined the cell populations used according to specific cell surface markers. Early studies using skeletal myoblasts were less successful than those using bone marrow derived mononuclear cells.

The exact mechanism of action of stem cells in these studies has remained unclear. It is thought that they may initiate repair via several concurrent mechanisms; when introduced into the failing heart they may augment inflammatory and healing processes post infarction, influencing scar formation, new vessel and tissue growth, minimising damage and apoptosis, hence reducing levels of resultant heart failure and offering functional cardiac improvement. As the mechanism of repair is unclear, a pragmatic approach was taken in the majority of these studies, to use the unrefined mononuclear cell population, so as not to potentially exclude a possible key stem cell subtype.

These studies used a variety of different investigation tools to monitor cardiac function. Generally function was assessed in terms of contractility, end systolic volume and ejection fraction. The majority used cardiac MRI assessments to investigate their patients.

Stem cells were transferred to the patient by different routes including direct intramyocardial injection, intracoronary injection and peripheral perfusion with varying degrees of success, the selection, harvesting, processing and storage

of the stem cells possibly influencing the final outcome. Meta analysis of the studies has reported a small but significant improvement in cardiac function[45].

Table 1.3: Randomised controlled trials of myocardial regeneration, evaluating changes in left ventricular ejection fraction in adults with acute myocardial infarction [44]

Study	*n*	Therapeutic approach	Time of therapy and duration of follow-up	Δ LVEF	Quantification method	*P* value
REPAIR-AMI[29]	204	Intracoronary BMC or placebo	4 days to 4 months	+5.5% vs +3.3%	Angiography	0.02
ASTAMI[28]	101	Intracoronary BMC or placebo	6 days to 6 months	+1.2% vs +4.3%	MRI	0.05
Janssens S *et al.*[29]	67	Intracoronary BMC or placebo	1 day to 4 months	+2.2% vs +3.3%	MRI	0.36
BOOST[30]	60	Intracoronary BMC or control	5 days to 18 months	+5.9% vs +3.1%	MRI	0.27
Bartunek J *et al.*[31]	35	Intracoronary BMC or control	11 days to 4 months	+7.1% vs +4.3%	Angiography	NS
Chen SL *et al.*[32]	69	Intracoronary BMC or placebo	18 days to 6 months	+18% vs +6%	Ultrasound	0.01
MAGIC Cell-3-DES[33]	50	Intracoronary BMC plus subcutaneous G-CSF or control	7 days to 6 months	+5.1% vs −0.2%	MRI	0.047
FIRSTLINE-AMI[34]	50	Subcutaneous G-CSF or control	1.5 h to 4 months	+6.0% vs −4.0%	Ultrasound	0.01
REVIVAL-2[35]	114	Subcutaneous G-CSF or placebo	5 days to 6 months	+0.5% vs +2.0%	MRI	0.14
STEMMI[36]	78	Subcutaneous G-CSF or placebo	1 day to 6 months	+8.5% vs +8.0%	MRI	0.9

Abbreviations: ASTAMI, Autologous Stem cell Transplantation in Acute Myocardial Infarction; BMC, bone marrow cells; BOOST, bone marrow transfer to enhance ST-elevation infarct regeneration; FIRSTLINE-AMI, Front Integrated Revascularization and Stem cell Liberation in Evolving Acute Myocardial Infarction; G-CSF, granulocyte-colony-stimulating factor; MAGIC Cell-3-DES, Myocardial regeneration and Angiogenesis in myocardial infarction with G-CSF and Intra-Coronary Stem Cell Infusion-3-DES; NS, not significant; REPAIR-AMI, Reinfusion of Enriched Progenitor cells And Infarct Remodeling in Acute Myocardial Infarction; REVIVAL-2, Regenerate Vital Myocardium by Vigorous Activation of Bone Marrow Stem Cells; STEMMI, STEM cells in Myocardial Infarction; Δ LVEF: change in left ventricular ejection fraction.

The heart is composed of several cell types functioning on an intricate structural matrix. Its performance is reliant on the complicated relationship between muscle cells sited on a structurally competent framework contracting in a coordinated fashion as a result of an intact and precise electrical pathway. Cardiac incompetence results from abnormal structural development, damage to the normal structural matrix or damage to the cardiac cells. The clinical consequence of this incompetence may be mild, moderate, severe or incompatible with life. With this in mind stem cell therapy to repair such defects must be appropriately tailored to address the underlying disorder and needs to establish effectively a complicated physiological function.

Cellular myoplasty involves the introduction of stem cells *in vivo* directly into the heart[46] or its blood supply. Cellular myoplasty aims to produce functional improvement in the damaged myocardium by several means; some of these processes are not fully understood and will be discussed later in this summary. Stem cells are used to re-populate damaged regions of heart muscle with functionally competent cells. Different stem cell populations have been studied and have been demonstrated to produce variable degrees of functional improvement when used in this way.

Stem cells all possess the ability of self renewal and are also able to develop specific cell lines which then allow tissues and organs both to grow and repair. The range of new cell types that a stem cell is able to produce is described as its potency. Totipotent stem cells are able to form all types of embryonic and extraembryonic cells; pluripotent cells are derived from totipotent cells and whilst slightly lineage restricted are still able to form the majority of cell types. Multipotent cells are able to differentiate into fewer cell lines than pluripotent cells, oligopotent fewer still and unipotent stem cells can only produce their own cell type.

Stem cells used for therapeutic purposes may be autologous, derived from the individual to whom they will be administered, or allogeneic, donated or cultured from an external source, the advantage of autologous cells being that there are no immunological complications in terms of rejection reactions associated with their use. Clearly cells harvested from another individual have the potential to initiate an immune response. It is recognised that the more lineage specific the stem cell or the lower the potency, the greater the potential immune reaction.

Pluripotent embryonic cells elicit the least reaction and are the most immuno-privileged cells available. As these cells are still not recognised as self by the host a degree of immune activation may still be encountered and immunotherapy may be indicated with their administration. A disadvantage of autologous cells is that they need to be harvested, processed and sometimes cultured before use, all time consuming processes that may preclude their use in acute settings. Allogeneic cells however may be manipulated and stored for utilisation on demand.

In 2012, John Gurdon from the United Kingdom and Shinya Yamanaka from Japan were awarded the Nobel Prize for Physiology or Medicine in recognition of their many years of work, which finally identified that mature cells could be transformed into pluripotent stem cells. Prior to this research, most scientists believed that mature adult cells were already terminally committed to their specialist role and lineage. Gurdon however showed that essentially all cells contained the same genes, and so hold all the information needed to make any tissue. The potential to develop any cell line clearly offers exciting possibilities within the field of regenerative medicine. These cells, which are reprogrammed to permit pluripotency, are known as iPSCs or induced pluripotent stem cells.

Induced pluripotent stem cells are developed by transfecting donor cells in culture, with pluripotency- associated genes such as Oct4, Sox2, c-Myc and Klf4. Viral vectors are typically used to insert these reprogramming factors into the cells. Cell colonies then develop that have pluripotent stem cell characteristics. Specific cell types can be selected, by means of morphology or surface marker expression, and further cultured for use. iPSCs offer several

advantages over other cell types as they provide pluripotency without the need for destruction of the original donor, as is frequently the case with embryonic pluripotent cells. In addition, the cells derived can also be patient specific; cells can potentially be harvested from a patient, be reprogrammed and then re-introduced. The process of creating induced pluripotent stem cells is slow and ineffective in terms of functional cell yield, however the end product has huge potential value.

1.9 ii Stem cell types previously investigated for use as a cardiac therapy

Skeletal muscle cells have been used in cell based therapies in the heart[47]. Skeletal myoblasts are readily available and can be effectively amplified *in vitro*. Early animal studies looking at the potential effect of cell injections on the failing heart used skeletal muscle cells. Research suggested functional cardiac improvement in animal studies, however this improvement was not fully replicated when trialled in adults post myocardial infarction[47]. The innate difference between skeletal muscle cells and the resident heart muscle cells also meant that their incorporation did not result in fully normal cardiac functioning and arrhythmias were frequently encountered.

1.9 iii Stem cell types currently utilised clinically for heart failure

Adult stem cells are found in the bone marrow, circulating blood and adipose tissue and can be harvested in different ways. These stem cells are slightly more lineage dedicated rather than retaining full pluripotent capacity of embryonic stem cells; they still however retain their capacity for renewal. Although referred to as adult stem cells they are also present in children and may be more accurately described as somatic stem cells, or those cells with

regenerative capacity, that remain present after birth. Adult type stem cells with greater potency than most other types of adult stem cells are found in umbilical cord blood, and are being increasingly harvested and stored for later potential use.

Bone marrow is a rich source of stem cells. As with skeletal muscle derived stem cells they can be relatively easily harvested and processed to obtain selective populations of stem cells if necessary. Stem cells are recognisable by their cell surface markers and can be separated accordingly. The bone marrow mononuclear cell fraction includes mesenchymal stem cells, haematopoietic stem cells and endothelial progenitor cells. Granulocyte colony-stimulating factor (GCSF) has been used in some studies[44] to mobilise stem cells from the bone marrow, increasing the number of circulating stem cells in the individual and optimising the potential number of cells for peripheral harvesting. Although administration of bone marrow derived stem cells has resulted in modest functional cardiac improvement in clinical trials in adults, it is unclear by what mechanisms this improvement is achieved.

Endothelial progenitor cells derived from the bone marrow may improve the general cardiac environment by initiating vasculogenesis, new vessel formation allowing improved perfusion and function of the damaged myocardium, increasing cytokine and stem cell distribution to areas of injury and permitting subsequent cardiac recovery by paracrine activation and new cell regeneration.

It should be noted that several stem cell types have been identified but were not considered for use in this study protocol. Certain stem cell types have been

deemed inappropriate for clinical use at present, either because they are unable to accurately replicate the desired physiological response or for reasons such as ethical unacceptability or concerns over potential adverse effects.

1.9 iv Administration of cells

Different methods of stem cell delivery have been researched[48]. To date cells have been introduced into the damaged heart by direct intramyocardial injection[49] or infusion into the circulation either locally to the heart[43,5]or more peripherally[50,51].

It is believed that when several types of stem cells are administered together they exert synergistic effects advantageous to their survival and function. The injected stem cells may directly repopulate the myocardium, but have also been observed to fuse with resident damaged myocardial cells, resulting in recovery of host cell function. Such cell chimerism has already been reported in transplanted organs[33].

The injected cells are likely to be attracted to areas of damage by inflammatory cytokines. The cells will follow the cytokine gradient to home in on the region of damage and integrate into local tissue. Damaged host cells release chemical mediators, acting to attract stem cells, encourage integration of the cells and activate their innate programmed cell function to induce and optimise repair and regeneration. Heart failure is associated with a variety of pro-inflammatory cytokines and inflammatory mediators, such as TNFα (Tumour necrosis factor alpha) and IL6 (Interlekin 6). These markers can serve as indicators of disease severity and progression[52]. Pro-inflammatory cytokines are known to cause

systolic dysfunction, myocardial hypertrophy, disturbance of the myocardial extracellular matrix, apoptosis and endothelial dysfunction. They also regulate the expression of adhesion molecules such as VCAM1 (vascular cell adhesion molecule) and ICAM-1 (intracellular adhesion molecule) expressed on the surface of cells[52]; their presence is recognised to be associated with cardiovascular disease. Intercellular communication may also trigger the activation of resident dormant cardiac stem cells within the myocardium.

Scar formation and tissue remodelling, seen after a cardiac insult, is similarly regulated by chemical modulators. Interference with the scarring process may limit resultant long-term damage to the myocardium. The ability to control scar tissue formation is an important factor in healing and repair processes in most tissues including the heart. Scar formation is influenced by age, the young being better able to minimise the effects of scarring, possibly due to their higher levels of circulating stem cells and their subsequent regenerative ability. Age has also been recognised as a factor in stem cell survival in cardiomyoplasty techniques, with survival potential of stem cells declining with age[53].

Stem cell infusions can be relatively easily administered and can be performed repeatedly at time intervals indicated by the clinical status of the patient. Serial injections have the potential to offer long-term support for the patient with minimal risk as most procedures can be performed without the need for an anaesthetic.

1.10 Summary of this study

With an understanding of the research already conducted in this field and an appreciation of the need to find alternative therapies for children with heart failure it was decided to initiate a pilot study to investigate whether autologous intracoronary stem cell therapy is safe and feasible for children.

This first assessment of stem cell therapy for children with heart disease was an extension of the work previously conducted in adults with similar cardiomyocyte destruction and impaired systolic contraction of the ventricles. The preceding disease processes and co-morbidities leading to heart damage in children are generally different from those in adults. Rather than ischaemia and aging, in children it is more usually secondary to congenital heart disease, inflammatory, immune or cytotoxic mechanisms, but the final cellular damage is comparable across the age range. Pilot studies in adults with dilated cardiomyopathy have produced encouraging results[4,5,54]. This randomised, blind, crossover placebo controlled trial sought to evaluate the potential use of intracoronary injections of autologous stem cells in the management of paediatric heart failure.

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a. GOSH data compiled and stored by Dr M Fenton, Consultant Cardiologist and Paediatrician, Great Ormond Street Hospital, London

CHAPTER 2

MATERIALS AND METHODS

2.1 Introduction

Based on the adult stem cell studies described previously [1, 2, 3] a protocol was devised for a pilot study to assess the feasibility of similar work in the paediatric population, an age group where such therapy had not previously been undertaken. This pilot study was designed to investigate whether autologous intracoronary stem cell therapy was safe and feasible for children with heart failure. The present chapter describes the methods utilised in this pilot study.

2.2 Ethical Approval

Ethical Approval was obtained for this project in April 2008 from the Great Ormond Street Hospital Research and Ethics Committee, prior to commencement of the study (REC 08/H0713/37).

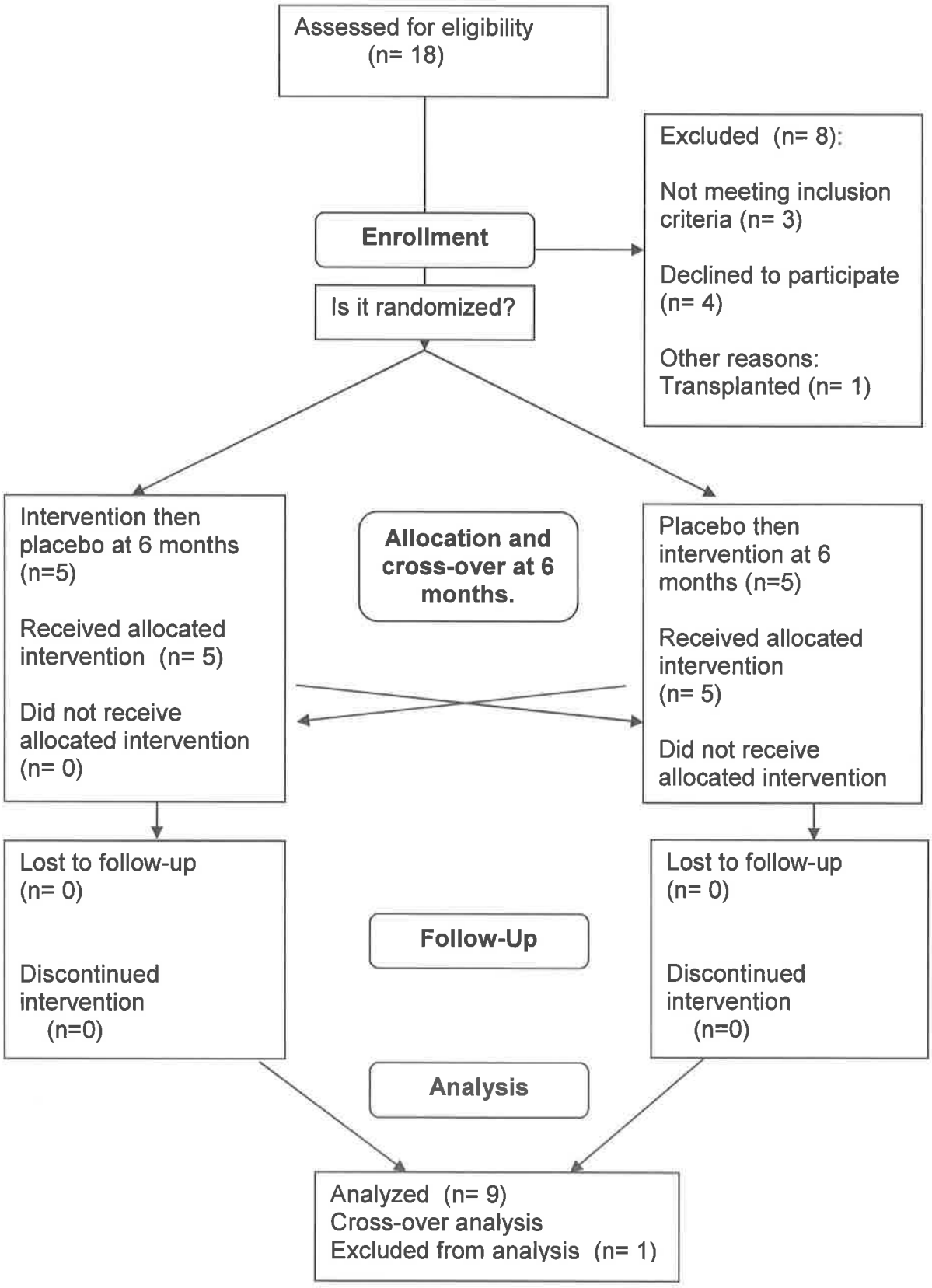
2.3 Study Design

This pilot study was of a randomised, crossover, placebo controlled design. The Consolidated Standards of Reporting Trials (CONSORT) statement was used to guide the development of the protocol[4].

2.4 Statistical advice

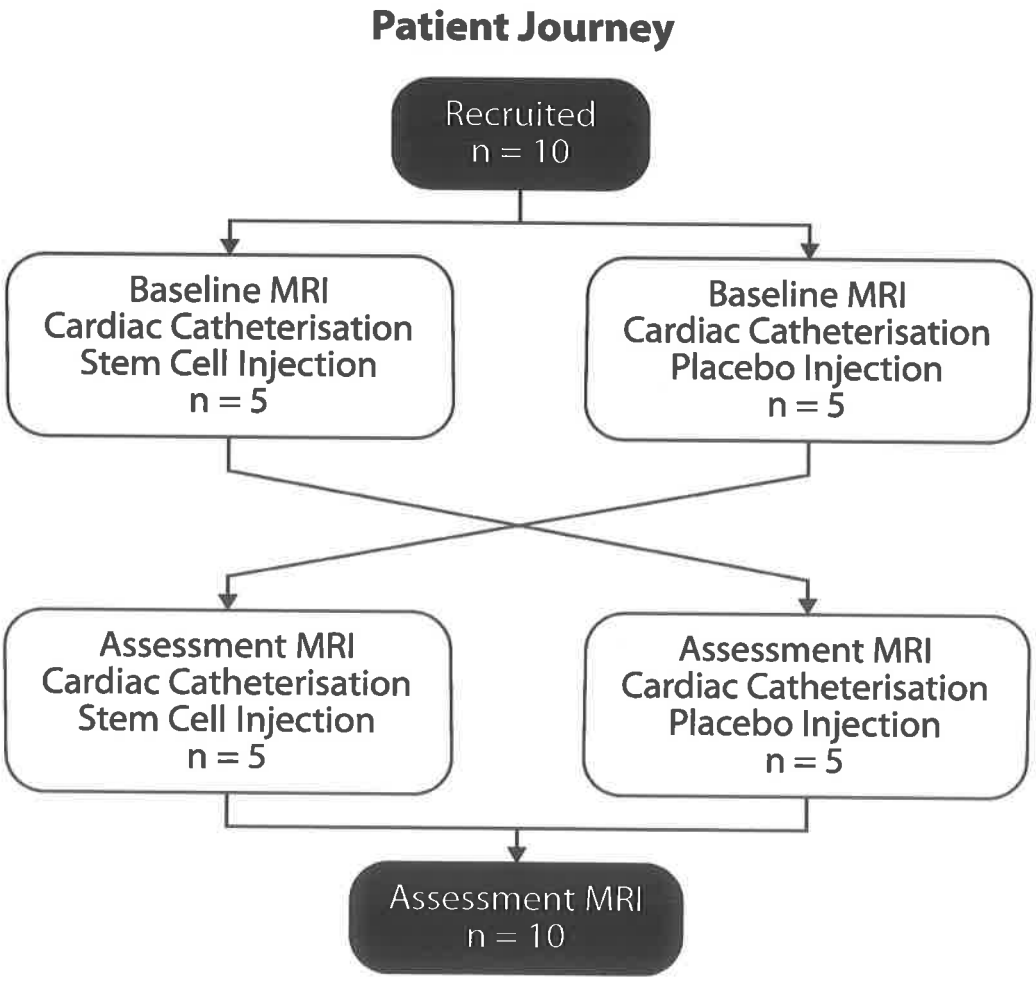
A CONSORT flow diagram was produced (Figure 2.1) and a project plan for a crossover study designed (Figure 2.2).

Figure 2.1 CONSORT diagram for study



Statistical review of the available literature[1,5,6] showed that a study population of 10 (n=10) would be sufficient to detect a 3% change in ejection fraction (EF) as measured by magnetic resonance imaging (MRI) with 80% power and 5% significance with a standard deviation (SD) of difference of 3%, in a crossover design. For all calculations it was assumed that the distribution of differences was Normal. Of note this would be a reduction in study numbers compared with those needed when analysing standard echo measurements. Calculation of a sample size for the crossover study required a definition of the minimum difference to be detected.

Figure 2.2: Patient journey illustrating the cross-over study design



2.5 Eligibility criteria

Children aged 1 year to 16 years at review, either attending heart failure clinic at Great Ormond Street Hospital (GOSH) or referred for acute hospital admission and management of their heart failure at GOSH were invited to participate in the study. Heart failure was either previously diagnosed or newly diagnosed by a consultant paediatric cardiologist on the basis of history, examination and investigation findings.

The cause of impaired cardiac function in our study population was selected to be heart muscle disease. Shortening fraction on echocardiogram was within the approximate range 15-20%. The functional status of the recruited patients was class 3 or 4 as defined by the NYHA or Ross classification (Appendix 1). The lower age limit was selected to permit coronary artery catheterisation to be feasibly performed taking into account the size of the patient. Exclusion criteria included the need for high dependency or intensive care and congenital heart disease.

Potential patients were identified from clinical case note review, and they and their families were approached either by telephone or at outpatient clinic review to evaluate their interest in participation. Any patients who, after preliminary briefing, appeared interested in participating in the study were given age and language appropriate written information (Appendix 2); they were also given a contact telephone number for the Research Fellow. Each family was given the opportunity to discuss the study with the research team before consent for inclusion was obtained (Appendix 3).

2.6 Protocol

Ten patients who met the eligibility criteria were recruited for this study. Each patient was randomised at entry to the study to determine whether they would receive stem cells at Stage One or Stage Two of the protocol. Randomisation was performed using a computer-generated table obtained from Graphpad (Internet: http://www.graphpad.com/quickcalcs/RandMenu.cfm).

Table 2.1: Randomisation table used:

Subject #	Group Assigned
1	A
2	B
3	B
4	B
5	B
6	A
7	A
8	B
9	A
10	A

On recruitment, patients were allocated study numbers sequentially and their corresponding crossover study protocol was determined. Those allocated to Group A received placebo at their first intervention followed by bone marrow aspiration and intra-coronary injection of bone marrow derived mononuclear cells at their second stage. Those allocated to Group B received bone marrow derived mononuclear cells via intra-coronary injection at their first intervention before receiving placebo at their second stage.

Following recruitment and randomisation, patients had their individual admission time-line planned. Those patients who were to receive stem cells at their first study admission were reviewed within the 28 days preceding that admission. At this review, screening for infection, as per stem cell laboratory protocol, was performed, following full written consent.

Table 2.2: Infection screening required prior to stem cell laboratory handling of bone marrow aspirate

Anti-HIV1&2	Anti-HTLV-1 & 2	Toxoplasmosis
HBsAg	VZV IgG antibody	Herpes 1 and 2
Anti-HBc	EBV	Rubella IgG
Anti-HCV-ab	CMV	Measles IgG
Syphilis		

On the day of the procedure the patient was admitted to the cardiac day-care facility for baseline observations and clinical assessment. The study procedure was performed in the Cardiac MRI theatre under general anaesthetic. Blood samples were taken at the time of anaesthesia for routine investigation (Full blood count, urea, electrolytes, liver function and NTproBNP).

Those patients receiving intracoronary stem cells at this admission had 20mL of bone marrow aspirated from the posterior iliac crest under aseptic conditions immediately after induction of anaesthesia.

2.7 Bone marrow aspiration technique

Those patients on anticoagulation therapy were highlighted in advance of all procedures and admissions, and haematology advice was sought. Individual cases were discussed with a consultant haematologist and management plans constructed to safeguard the patient with optimal coagulation management prior to, during and following the bone marrow aspiration.

All bone marrow processing was undertaken at the Great Ormond Street Hospital stem cell processing facility. This was booked in advance, for which patient identifiers and infection screening results were required.

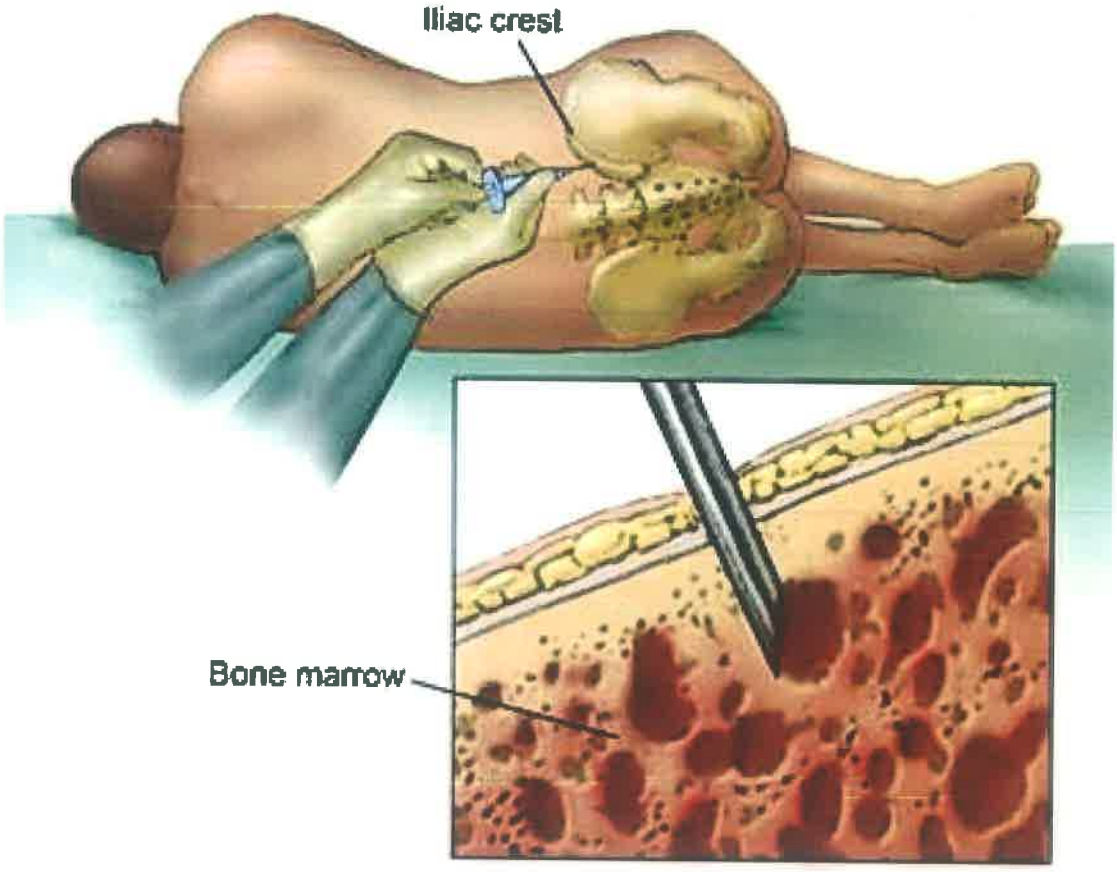
Written, informed consent for bone marrow aspiration was required prior to the procedure. This was logged on the general consent form used for all the procedures involved in this study at each admission event.

The patient's identity was confirmed prior to being taken for the anaesthetic and again at this point, patient identifiers were double checked from the patient's wristband before the bone marrow was aspirated.

Following anaesthetic induction and the securing of a stable airway the patient was positioned to permit bone marrow aspiration. As the posterior iliac crest is used as the standard site for bone marrow aspiration, the patient was placed in the left lateral decubitus position with the knees flexed. The posterior iliac crest was identified and the overlying skin cleaned as for any surgical incision using 10% povidone-iodine solution (Betadine). Following skin cleaning the patient was covered with a sterile surgical drape with a fenestrated opening placed

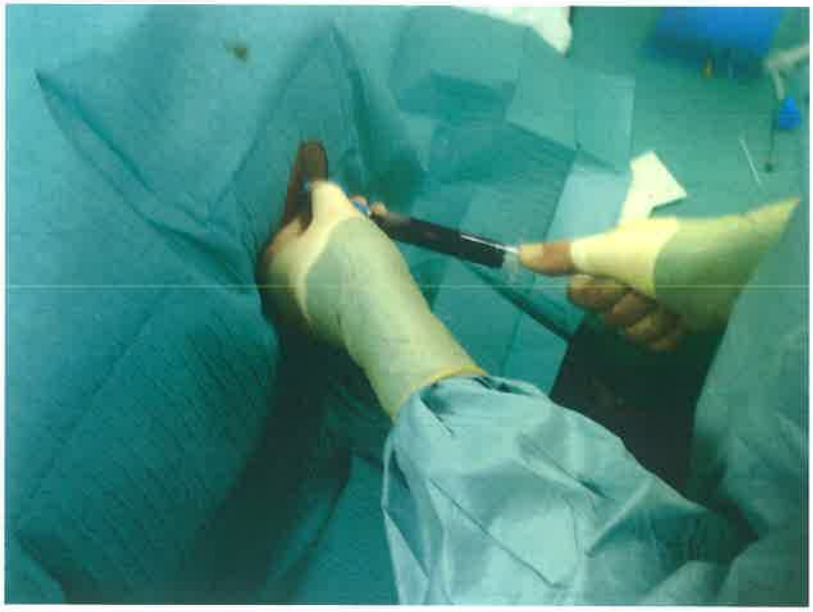
over the intended site of bone marrow harvest. The Research Fellow observed full aseptic conditions throughout the procedure.

Figure 2.3: Illustration of bone marrow aspiration from the posterior iliac crest



A paediatric bone marrow trochar and needle were inserted into the posterior iliac crest using constant pressure and a slight twisting motion until the bone marrow cavity was entered. 20 mL of bone marrow was aspirated from a single needle puncture site using heparinised 10mL syringes attached to the bone marrow needle following removal of the central trochar. The aspirated bone marrow was transferred to sterile heparinised universal specimen bottles.

Figure 2.4: clinical bone marrow aspiration



Following the aspration the bone marrow needle was withdrawn and disposed of safely. Using a sterile gauze, pressure was maintained over the skin puncture site to secure haemostasis. The site was then dressed and the patient cleaned and un-draped for the next stage of the protocol.

The heparinised universal specimen pots holding the harvested bone marrow were labelled with the patient's name, date of birth, hospital number and the date of the procedure. The universal pots were then placed in a specimen bag with the required paperwork before being taken to the stem cell facility for processing. All bone marrow samples received by the facility required handwritten confirmation of the patient details, name and designation of the clinician who had harvested the sample, details of the clinician responsible for and requesting the laboratory services, the laboratory processing services

required and details of the intended recipient of the blood product obtained from the bone marrow sample.

Figure 2.5: Aspirated bone marrow in heparinised universal containers prior to processing

Immediately after aspiration the bone marrow sample was taken to the stem cell laboratory for mononuclear cell separation using standard techniques of gradient centrifugation.

2.8 Mononuclear cell separation protocol

Although the Research Fellow was trained in the mononuclear cell separation technique, for speed of processing on the day of each procedure the bone marrow samples were all processed by Mrs M Brocklesby, clinical immunology technician.

The preservative free heparinised universal specimen bottles were prepared in the immunology laboratory prior to each procedure and issued to the Research Fellow on the morning of the procedure. The samples were booked into the Blood Bank system and products issued through the blood bank, following the Standard Operating Procedure: Separation of stem cells for injection into the heart. CTRE001 (Appendix 4). The research fellow contacted the immunology laboratory when the patient was called for anaesthetic, the clinical scientist then went to the Cell Therapy Laboratory (CTL) to sign in and prepare the facility.

Following Standard Operating Procedure CTLSOP 004 (Gowning in the CTL) the technician switched on and cleaned the cabinet by following the CTLSOP 001 (Cabinet Cleaning). Daily plates were placed around the facility as per protocol for later evaluation of sterility of the environment following CTLSOP 005 (Monitoring SOP).

On receiving the fresh bone marrow sample the volume of the haematopoietic cell (HSC) donation was measured against the bottle graduations and recorded in the bone marrow register (BMR).

2.8 i Methods of making reagents required

To make Lymphoprep™: sterile solution for isolation of human mononuclear cells

1. The required items were sprayed into the cabinet from the tray.
2. The silver cover was peeled off the Lymphoprep™ bottle and swabbed with a sterile wipe.
3. A 20mL syringe and needle were used to draw 20mL of Lymphoprep™ into the syringe.
4. The needle was removed and 10mL of Lymphoprep™ ejected into each of two sterile universal specimen containers. The universal containers were labelled with their contents and placed into the rack.

To make Wash Solution

5. The connector line (tubing) on the 150mL bag was sealed near to the bag. The line joining the bag and the bottle at the second sealed joint was broken.
6. A sample site coupler was placed into the 150mL bag and swabbed with a sterile gauze.
7. The lid of the 100mL 4.5% albumin bottle was removed and the rubber stopper swabbed.
8. Using a needle and 50mL syringe 50mL of albumin was removed from the bottle and injected into the 150mL bag.
9. The sample site port on the 500mL bag of saline was swabbed.
10. Using a needle and 50mL syringe 50mL of saline was removed from the bag and injected into the 150mL bag.

11. The contents of the 150 mL bag were mixed thoroughly. The bag was labelled as: Wash Solution, Saline/Albumin 50/50w/v and place into a white tray.

2.8 ii Red cell depletion process

12. The universals containing the Lymphoprep™ were labelled with the patient's details.
13. The sample site on the 150mL bag of saline/albumin was swabbed.
14. Using a needle and 20mL syringe 10-15mL of wash solution was removed, the needle discarded, and a small volume ejected into the universal pot containing the bone marrow sample. The bone marrow sample was diluted with an approximately equal volume of saline/albumin and mixed gently.
15. The Lymphoprep™ was swirled around the filled universal container to coat the sides.
16. The diluted bone marrow/saline mixture was carefully layered onto the Lymphoprep™, with minimal mixing.
17. The solution was centrifuged at room temperature for 15 minutes at 2800 rpm (800G) with the centrifuge brake switched off.
18. While cells were spinning, blood bank labels were sealed into an overwrap bag, and a pocket for the syringe was made using the multivac, leaving a gap to slide the syringe into later.
19. The spun samples were placed into the universal rack and, using a sterile Pasteur pipette, the mononuclear cells were removed from the interface between the Lymphoprep™ and the plasma/saline layers and placed in another two labelled universals containers.
20. The sample site on the 150mL bag of wash solution was swabbed.

21. Using a needle and 20mL syringe, 20mL of wash solution was removed, the needle discarded, and the wash solution ejected to top up the universals containing the separated mononuclear cells; the solution was mixed gently.
22. The solution was centrifuged at room temperature for 7 minutes at 1300 rpm (200G) with the centrifuge brake on.
23. Once the mononuclear layer had been spun to a pellet the supernatant was discarded using a "kwill" and a 20 mL syringe. The kwill was then replaced with a needle and the supernatant ejected into a '**discard**' universal container.
24. The cell pellet was re-suspended by flicking the bottom of the universal container with a forefinger.
25. The sample site on the 150mL bag of wash solution was swabbed.
26. Using a needle and 20mL syringe, 20mL of wash solution was removed and ejected to top up one of the universals containing the separated mononuclear cells. The solution was mixed gently and pooled with the second universal, and again mixed gently. A balance universal pot was made up to the same volume as the pooled universal using saline/albumin.
27. The solution and balance universal containers were centrifuged at room temperature for 5 minutes at 1300 rpm (200G) with the centrifuge brake on.
28. Once the mononuclear layer had been spun into a pellet, a kwill and 30mL syringe were used to remove the supernatant and eject it into the 'discard universal'.
29. The cell pellet was re-suspended by flicking the bottom of the universal with a forefinger.
30. The sample site on the 150mL bag of wash solution was swabbed.

31. Using a needle and 2.5mL syringe, 2mL of saline/albumin was removed and ejected into the re-suspended cell pellet. The solution was gently mixed.

2.8 iii To prepare the product for administration

32. Using a 2.5mL syringe, 1mL of product was aspirated and the syringe sealed with a syringe cap; the capped syringe was sealed in the pre-prepared overwrap bag with blood bank labels.
33. The product was issued to the waiting research fellow.

2.8 iv Sterility checking

34. The sample site on the 150mL discard bag was swabbed.
35. The plastic covers on the blood culture bottles were removed and the injection ports swabbed.
36. Using a 10mL syringe and needle, 10mL of discard solution was removed and 5mL injected into each of the blood culture bottles. The blood culture bottles were labelled with patient information, date and time.
37. Finger impression cultures were performed.
38. When all sterile manipulations had been completed, the cabinet was cleared and cleaned as per CTLSOP 001 'Cabinet cleaning SOP'. All contaminated waste was transferred to an autoclave bag. All other paper and plastic waste was placed into a clear domestic waste bag. The finished product was placed together with any unused consumables into tray two and CTLSOP 002 followed 'Cleaning and transferring items through the entry hatch into the cell therapy laboratory and into class II cabinets' to remove all items (excluding waste bags) out through the "out" hatch; this included the sample

for counting and staining. All waste was taken out through the exit doors when leaving the clean room.

39. All waste was disposed of appropriately following satisfactory completion of cell counts.

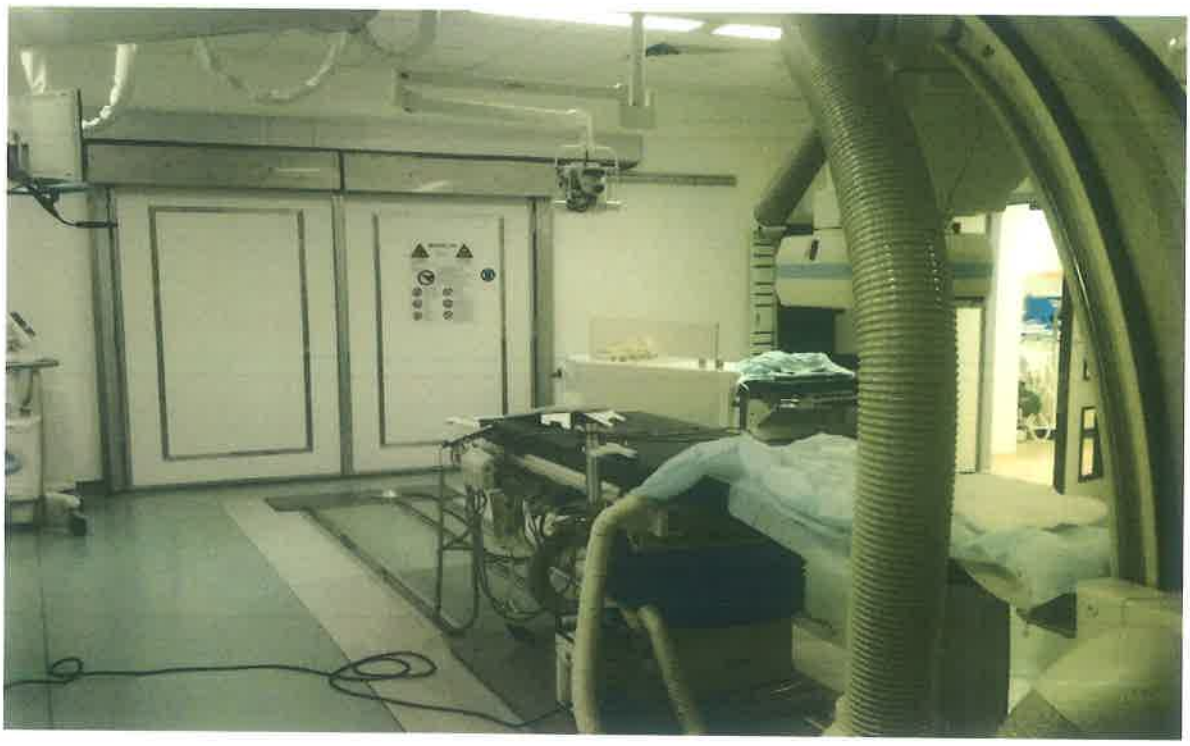
40. The sample suspension in the universal container was used for cell count and immunophenotyping. A white cell count was required for the issued stem cells and staining.

Figure 2.6: stem cell processing laboratory at GOSH

The red cell depletion and mononuclear cell separation protocol took approximately 90 minutes to complete. The bone marrow sample was processed to provide 1mL for coronary injection and 1mL, which was cryogenically frozen for further phenotypic analysis at a later date. The immunophenotyping process is described in Chapter 5. An aliquot of stem cell suspension was analysed to obtain total mononuclear cell counts per mL. This cell count represented the total number or mononuclear cells injected via intra-coronary catheterisation. In addition cell viability was also assessed.

Whilst waiting for the prepared stem cells, the cardiac MRI scan was performed using a breath hold protocol for image acquisition. Following the MRI scan the patient was transferred by means of the mechanised sliding table back into the angiography suite.

Figure 2.7: mechanised table leading to and from the MRI scanner behind the closed doors

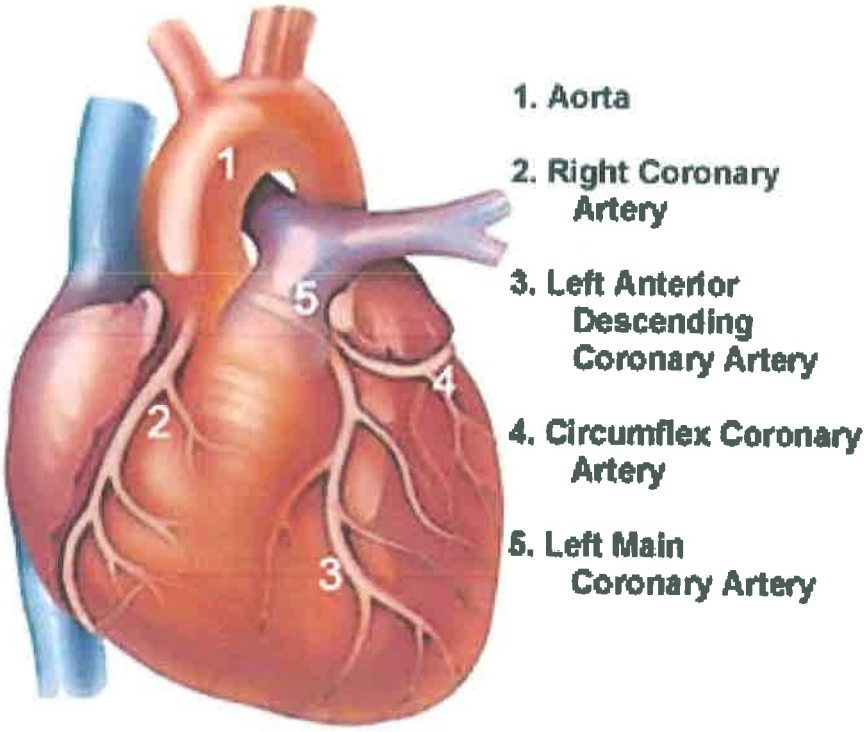


On return to the angiography suite the patient was maintained under general anaesthetic until notice was given by the stem cell laboratory that the stem cell processing was nearing completion. Following contact with the stem cell laboratory an arterial sheath was placed in the femoral artery of the study patient and advanced until its tip was sited in the left main coronary artery. Partial occlusion of the coronary artery was observed as demonstrated by ST segment changes and damping of the pressure trace was routinely monitored during such procedures.

When the stem cell suspension was delivered back to the angiography suite brief angiography was performed to confirm anatomy and patency of the left main coronary artery. The 1mL volume was slowly injected via the coronary artery catheter into the left main coronary artery followed by a 1mL 0.9% saline flush over a period of 2 minutes.

Figure 2.8: 1mL injectable suspension of mononuclear cells

Figure 2.9: Diagram showing the heart and the main blood vessels



Following the intracoronary injection of stem cell suspension the cardiac catheter was removed and haemostasis at the skin entry site achieved. The patient was then woken and recovered in a standard manner.

Patients were admitted to the cardiac ward overnight with routine monitoring of the arterial catheterisation access site and bone marrow aspiration site; cardiac care observations were continued throughout. Patients were discharged the following day and electively reviewed in the outpatients clinic at three months post-procedure.

Cross-over of interventions occurred at six months with the stem cell group receiving placebo via the cardiac catheterisation (no bone marrow harvest was

required for the placebo arm of the study) on the second occasion. The same admission, monitoring and follow up was arranged as for the stem cell stage of the study. The placebo used for the intracoronary injection used was 1mL 0.9% saline, consistent with the placebo solution used in adult studies. The 1mL of placebo was followed with a 1mL 0.9% saline flush for correlation with the stem cell arm of the study. An interim clinic review was again arranged at three months post procedure and a follow up cardiac MRI scan at six months.

Those patients randomly allocated placebo at stage one of the study crossed over to receive stem cells at stage two. Interim and final follow up arrangements were the same for the stem cell and placebo groups.

2.9 Primary outcome measures

The primary outcome measures were freedom from death and transplantation.

2.10 Secondary outcome measures

NTproBNP and cardiac MRI measured EF were the main secondary outcome measures. In addition functional status was assessed by the New York Heart Association classification or the Ross classification in younger children. The absolute change in the heart function was assessed by MRI scan. Left ventricular ejection fraction (how much blood the heart pumps out with each beat measured as a percentage change in the volume of the left sided pumping chamber) was assessed using cardiac MRI scan. Other variables relating to the left ventricle (left ventricular mass, left ventricular end diastolic volume and left ventricular end systolic volume) were also determined.

2.11 Patient management

Each patient involved in the study had open access to the Research Fellow throughout the duration of the study. The holistic care of the patients was of paramount importance and additional support required by any patient, either relating to their research involvement or incidental to their cardiac management, was facilitated by the Research Fellow. Where possible the medical management of the study population was maintained as constant throughout the duration of the study; if however clinical need arose whereby medication or management alterations were required in the best interest of the patient, these were made accordingly. Any incidental findings made as a result of the research were managed according to standard medical care.

2.12 Monitoring

The safety monitoring committee included a cardiologist from Great Ormond Street Hospital not involved in the trial, an external cardiologist and a lay representative. They were informed of all procedures undertaken. Any adverse events were to be reported to the committee. Any significant or frequent adverse events encountered were analysed, and serious events may have resulted in the suspension or end of the study. The committee was empowered to halt the trial.

2.13 Data

Data collected for this study were stored securely as agreed by the Research Ethics Committee. Paper documents were filed and stored in a locked cupboard in a restricted access office entered using a code key. Data stored on computer databases were stored under password protection. It was agreed that all data be stored for ten years before being destroyed.

2.14 References

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CHAPTER 3

IMMUNOPHENOTYPING

3.1 Introduction

This chapter describes the processes used to label and subsequently identify the cell populations acquired from the bone marrow aspirates of the ten paediatric patients recruited to the study. The cell-surface marker labelling system is described and those markers utilised within this study are explained in more detail. The methods utilised to count, label and subsequently analyse the cell samples are described.

3.2 Background

Bone marrow was used as a source of haematopoietic stem cells (HSCs) and progenitor cells in this study. The mononuclear cell fraction of the autologous bone marrow aspirate was used for intracoronary injection. Characterisation of these stem cells was required to provide additional information for this study and to potentially allow extrapolation of data to deduce the effects of certain cell populations on the myocardial function. HSCs and progenitor cells express unique cell surface markers that can be identified and used to identify and quantify cell subset populations. The cell surface markers and their combinations present on the surface of specific cell types have been previously well researched[1]. This information was utilised to analyse the cells injected for this study. Flow cytometry was used to identify the cell surface markers and polychromatic flow cytometry allowed multiple markers to be identified within a single cell sample. In this way flow cytometry was used to characterise and enumerate the cell populations used in this study.

Flow cytometry is an established method of immunophenotyping of HSCs and progenitor cell subpopulations. Flow cytometry uses the principle of light scatter

caused by particles passing through a laser beam, and excitation and fluorescence emission of the fluorochromes bound to specific molecules either inside or on the surface of the particles to identify, count and in some instances sort the particles. Clinically this technique is used to analyse cells and in this protocol all the cell molecules labelled via antibody were extracellular markers of mononuclear cells derived from bone marrow. The initial cell population to be assessed was suspended in a medium and passed in a continuous stream of one cell thickness through the flow cytometer. Each cell passed individually through the beam of one or more lasers, depending on the number of fluorochromes and hence cell surface markers to be evaluated. The manner in which each fluorochrome scattered the light energy was detected and evaluated. Light scattered in a forward direction was focused, by a confocal lens, towards a light detector that converted the light energy into electrical energy. This electrical signal was digitalised and recorded as the parameter Forward Scatter (FSC) and provided information regarding size and shape of the cell. Light scattered to the side was similarly focused onto a detector and transformer. This Side Scatter (SSC) signal provided information regarding granularity of the cell. Using FSC and SSC allowed basic identification of cell types within a population. Additional detectors that read fluorescence, called photomultiplier tubes, were used to detect the light energy produced by excitation of attached fluorochromes as they passed through the laser. Each fluorochrome emitted a defined wavelength of light that was detected and digitalised to be counted by the cytometer's associated computer software to be recorded as a single event.

Multiparametric analysis was performed when cells were labelled with several different fluorochromes and were hence analysed according to several parameters. This protocol utilised a multiparametric flow cytometry technique to analyse the bone marrow derived mononuclear cells.

3.3 Immunophenotyping

Immunophenotyping was performed in order to identify the classes of cells that were obtained from the bone marrow aspirate and subsequently administered to each patient. The markers of interest were selected initially based on the adult protocols already discussed. Additional novel markers of interest were also included in the analysis to identify different populations of stem cells and progenitor cells[2] including haematopoietic stem cells, multipotent progenitor cells (MPP), common lymphoid progenitor cells (CLP), common myeloid progenitor cells (CMP), granulocyte macrophage progenitor (GMP), megakaryocyte erythroid progenitor cells (MEP), regulatory T cells (T-reg), mesenchymal stem cells (MSC) and cardiac stem cells (CSC).

3.3 i Cell surface markers assessed in this study

The cluster of differentiation (CD) naming system of cell-surface markers was established at the first International Workshop and Conference on Human Leucocyte Differentiation Antigens (HLDA) held in Paris in 1982[1]. Initially intended to name monoclonal antibodies the system has subsequently expanded to encompass all other cell-surface molecules. These cell-surface molecules have numerous functions such as receptors, ligands, cell signalling and cell adhesion. The cell-surface markers may be used individually or in combination to identify cell types. In this way cells may be identified and

classified, a process known as immunophenotyping. Cell populations are defined as either positive or negative depending on whether they express or lack the specific CD molecule under investigation. The CD surface molecules evaluated in this study are described below and their combinations and the cell populations they represent are described later, summarised in Table 3.1.

CD3: the signalling component of the T cell receptor (TCR) complex, the CD3/T-cell antigen receptor complex is found on 70% to 80% of normal human peripheral blood lymphocytes.

CD4: a co-receptor for MHC class II which is present on T-helper/inducer lymphocytes and monocytes. The subset of helper/inducer T-lymphocytes comprises 28% to 58% of normal peripheral blood lymphocytes. The CD4 antigen is also a receptor used by HIV to enter T cells.

CD8: a transmembrane glycoprotein that acts as a co-receptor for the TCR specific for MHC class I. CD8 is predominantly expressed on the surface of cytotoxic T cells and is also found on natural killer cells (NK) and subsets of myeloid dendritic cells, cortical thymocytes. It is most frequently lost from most T cell neoplasms except T cell lymphoblastic lymphoma.

CD16: a low affinity Fc (constant region of IgG) receptor found on the surface of the NK cells, neutrophil polymorphonuclear leucocytes, monocytes and macrophages.

Table 3.1: Cell surface markers to be labelled using specific fluorochrome-conjugated antibodies to identify cell populations within each tube

Fluoro-chrome	FITC	PE	PerCP Cy5.5	PE CY5	PE Cy7	APC	APC Cy7	V450	V500	AF700	Cell populations to be identified
Panel 1	CD10	CD135		CD90		CD45RA		CD38	CD45	CD 34	HSC, MPP, CLP, CMP, GMP, MEP
Panel 2	CD127	CD25				CD45RA	CD4		CD45		T Reg
Panel 3	CD71	CD105		CD90	CD34		CD44	CD73	CD45		MSC
Panel 4	CD3	CD16 CD56	CD45		CD4	CD19	CD8				TBNK
Panel 5	CD144			CD34 PerCP	CD184	CD133 CD309 AF647			CD45		Cardiac SC, VEGFR, endothelial & neural SC

CD19: the B-lymphocyte surface antigen, a component of the B-cell co-receptor is almost exclusively found on B-cells. CD19 is present on B-cells from early in development to B-cell blasts but is lost on maturation to plasma cells.

CD25: a type I transmembrane protein present on activated T-cells, activated B-cells, some thymocytes, myeloid precursors and oligodendrocytes. In combination with CD122 it can form a high affinity receptor for IL-2. A large proportion of resting memory T-cells in humans also express CD25. CD25 is expressed in most B-cell neoplasms, subacute non-lymphocytic leukaemias, neuroblastomas, and tumour infiltrating lymphocytes. Its soluble form, called *sIL-2R* may be elevated in these diseases and is occasionally used to track disease progression.

CD34: expressed on HSCs (hence MPPs, CLPs, CMPs, GMPs and MEPs), vascular endothelium and some tissue fibroblasts. CD34 is believed to be a target for phosphorylation by activated protein kinase C and may therefore play a role in signal transduction as well as adhesion of specific antigens to endothelium.

CD38: antigen expressed on all pre-B lymphocytes, plasma cells and thymocytes. CD38 is also present on activated T-lymphocytes, NK cells, myeloblasts and erythroblasts. CD38 is expressed in the early stages of T and B cell differentiation, is lost during the intermediate stage of maturation and then reappears during the final stages. CD38 is expressed on 90% of CD34+ cells and is not expressed on pluripotent stem cells. Co-expression of CD38 and CD34 indicates cell lineage commitment.

CD44: expressed on leucocytes, erythrocytes, epithelial cells and weakly on platelets. CD44 is also called extracellular matrix receptor type III and has functional roles in cell migration, lymphocyte homing and adhesion during haematopoiesis and lymphocyte activation.

CD45: this transmembrane protein called the leucocyte common antigen (LCA) is present on all human leucocytes including lymphocytes, monocytes, granulocytes, eosinophils, and thymocytes but is absent from circulating erythrocytes, platelets and mature erythroid cells of bone marrow and non haematopoietic tissues.

CD45RA: this isoform of the human LCA is found on approximately 40 - 50% of peripheral CD4+ T cells, 50% of peripheral CD8+ cells and on a proportion of B cells and monocytes. The T cells expressing this antigen are naïve T cells. CD45RA antibodies are useful for the study of suppressor/inducer subpopulations of CD4+ lymphocytes.

CD56: neural cell adhesion molecule (NCAM) CD56 is a homophilic binding glycoprotein expressed on NK cells and some lymphocytes as well as neurons, glia and skeletal muscle.

CD71: the human transferrin receptor CD71 mediates the cellular uptake of iron. CD71 antigen is expressed in low proportions on normal resting lymphocytes and is expressed on all cells upon activation. The transferrin receptor is essential for iron transport into proliferating cells.

CD73: is expressed on bone marrow derived multipotent mesenchymal stem cells (MSCs) and is one of the minimum criteria for identifying MSCs. CD73 is also expressed on subsets of T and B lymphocytes, follicular dendritic cell, epithelial cells, endothelial cells, fibroblasts, cardiomyocytes, neurons, osteoblasts and trophoblasts.

CD90: a molecule expressed on 1 to 4% of human fetal liver cells, cord blood cells and bone marrow cells.

CD105: an integral membrane protein found on vascular endothelial cells, MSCs, activated macrophages and synctiotrophoblasts of the placenta, CD105 is also weakly expressed on stromal fibroblasts.

CD127: CD127 or the interleukin-7 receptor has been shown to play a crucial role in the development of immune cells. CD127 is present on naïve and memory T cells. Defects in this cell-surface protein are associated with severe immunodeficiency pathologies, such as severe combined immunodeficiency (SCID).

CD133: CD133 is a transmembrane cell-surface antigen; it is a haematopoietic and neural stem cell marker. In the haematopoietic system CD133 expression is restricted to a subset of CD34 ^{bright} stem and progenitor cells in human fetal liver, bone marrow, cord blood and peripheral blood. Additionally, CD133 is expressed by a small population of CD34- cells in these tissues and circulating endothelial progenitor cells.

CD135: CD135 plays the role of growth factor receptor for early haematopoietic progenitors. It is expressed on multipotent, myelomonocytic and primitive B-cell progenitors. The more primitive haematopoietic progenitor cells express low levels of CD135.

CD144: also known as VE (vascular endothelium) cadherin, CD144 is a calcium-dependent cell-cell adhesion glycoprotein. It is likely to play an important role in endothelial cell biology through control of the cohesion and organisation of the intercellular junctions. CD144 is essential for a proper vascular development and maintenance of newly formed vessels.

CD184: CD184 also known as Fusin is expressed on a wide variety of CD4+ and CD4- cells; it is involved in mesenchymal stem cell homing and migration.

CD309: CD309 is also known as vascular endothelial growth factor 2 (VEGFR-2). CD309 is a higher affinity receptor for VEGF and plays a role in haematopoiesis. It is also involved in angiogenesis both during embryogenesis and as a homeostatic mechanism during pathological events such as vascularisation of tumours. It has also been identified on a subset of haematopoietic stem cells, endothelial progenitor cells (EPCs) as well as on their common progenitor, the haemangioblast. CD309 is also found on mature endothelial cells.

Table 3.2: Summary of surface markers used to identify cell types

Cell type		Marker Definition
Haematopoietic stem cell	**HSC**	$CD34^{+}CD38^{-}CD90^{+}CD45RA^{-}$
Multipotent progenitor	**MPP**	$CD34^{+}CD38^{-}CD90^{-}CD45RA^{-}$
Common lymphoid progenitor	**CLP**	$CD34^{+}CD10^{+}$
Common myeloid progenitor	**CMP**	$CD34^{+}CD38^{+}CD135^{+}CD45RA^{-}$
Granulocyte macrophage progenitor	**GMP**	$CD34^{+}CD38^{+}CD135^{+}CD45RA^{+}$
Megakaryocyte erythroid progenitor	**MEP**	$CD34^{+}CD38^{+}CD135^{-}CD45RA^{-}$
Regulatory T cell	**T-reg**	$CD4^{+}CD25^{+}CD45RA^{+/-}$
Mesenchymal stem cell	**MSC**	$CD45^{-}CD34\ CD73^{+}CD105^{+}\ CD90^{+}$
T cell		$CD45^{+}CD3^{+}$
T helper cell		$CD45^{+}CD3^{+}CD4^{+}$
Cytotoxic T cell		$CD45^{+}CD3^{+}CD8^{+}$
Natural killer cell	**NKcells**	$CD45^{+}CD16^{+}\ CD56^{+}$
B cell		$CD45^{+}CD19^{+}$
Endothelial progenitor cell	**EPC**	$CD34^{+}CD144^{+}$
Haematopoietic and neural stem cell		$CD34^{+}CD133^{+}$
Cardiac stem cell	**CSCs**	$CD34^{+}CD45^{+}CD309^{+}CD184^{+}$

Combinations of the cell surface molecules were grouped to identify specific cell types. These groupings were called antibody panels. The cell-surface markers to be identified via each panel of antibodies allowed optimal assessment of the cell populations within the research samples of bone marrow derived mononuclear cells. A summary of those cell populations and corresponding markers is shown in Table 3.2.

3.4 Panel 1

The panel of antibodies used in tube 1 permitted identification of haematopoietic stem cells (HSCs: CD34+ CD38- CD90+ CD45RA-), multipotent progenitor cells (MPPs: CD34+ CD38- CD90- CD45RA-), common lymphoid progenitor cells (CLPs: CD34+ CD10+), common myeloid progenitor cells (CMPs: CD34+ CD38+ CD10- CD135+ CD45RA-), granulocyte macrophage progenitor cells (GMPs: $CD34^{+}$ $CD38^{+}$ $CD135^{+}$ $CD45RA^{+}$) and megakaryocyte erythroid progenitor cells (MEPs: $CD34^{+}$ $CD38^{+}$ $CD135^{-}$ $CD45RA^{-}$). Table 3.3 shows the specific surface markers investigated and their corresponding fluorochromes.

Table 3.3: Panel 1 markers and antibodies

Fluorochrome	FITC	PE	PE Cy5	APC	V450	V500	AF700
Cell surface marker	CD10	CD135	CD90	CD45RA	CD38	CD45	CD34

3.5 Panel 2

The panel of antibodies used in tube 2 permitted evaluation of regulatory T-cells (T-reg) within the sample. CD45+ cells also expressing CD4 need to be identified and further evaluated for the presence of CD25 and $CD127^{low}$ surface markers. This population corresponds to T-reg cells ($CD4^+$ $CD25^+$ $CD127^{low}$ $CD45RA^{+/-}$) which can be further divided into naïve (CD45RA+) and memory (CD45RA-) subsets.

Table 3.4: Panel 2 markers and antibodies

Fluorochrome	FITC	PE	APC	APC Cy7	V500
Cell surface marker	CD127	CD25	CD45RA	CD4	CD45

3.6 Panel 3

Panel 3 investigated the MSC population determined according to their known surface markers. MSCs being $CD45^-$ $CD34^-$ $CD73^+$ $CD105^+$ $CD90^+$.

Table 3.5: Panel 3 markers and antibodies

Fluorochrome	FITC	PE	PE Cy5	PECy7	APCH7 (APC Cy7)	V450	V500
Cell surface marker	CD71	CD105	CD90	CD34	CD44	CD73	CD45

3.7 Panel 4

This panel analysis utilised a pre-made reagent kit to assess lymphocyte subsets within the bone marrow sample. The BD multi test six colour TBNK reagent™ determined the absolute counts and percentages of mature T ($CD45^+CD3^+$), B ($CD45^+CD19^+$), NK ($CD45^+CD16^+$ $CD56^+$) lymphocyte populations. The BD multitest six colour reagent used the following antibodies to identify and enumerate the different lymphocyte subsets:

Table 3.6: Panel 4 markers and antibodies

Fluorochrome	FITC	PE	PerCP Cy5.5	PE Cy7	APC	APC Cy7
Cell surface marker	CD3	CD16 CD56	CD45	CD4	CD19	CD8

3.8 Panel 5

The panel of antibodies used in tube 5 were selected to evaluate the sample for populations of cardiac stem cells (CSCs, $CD34^+$ $CD45^+$ $CD309^+$ $CD184^+$), endothelial precursors ($CD34^+$ $CD144^+$), haematopoietic and neural precursors ($CD34^+$ $CD133^+$) and vascular endothelial growth factor receptor (CD309). This novel panel structure included some cell surface antigens measured in previous adult studies in combination with recently recognised markers of cardiac stem cells. This combination was created to provide original data for this study.

Table 3.7: Panel 5 markers and antibodies

Fluorochrome	FITC	PerCP (PE Cy5)	PE Cy7	APC	V500	AF647 (APC)
Cell surface marker	CD144	CD34	CD184	CD133	CD45	CD309

3.9 Measurement of cell counts and calculation of cell suspension volumes needed for optimal immunostaining

3.9 i Controls

1. The first control for this protocol was fresh whole blood, on which mononuclear cell extraction, count and immunostaining were performed. Fresh whole blood was drawn from a healthy adult volunteer with no links to this study. Performing this process on the whole blood allowed any protocol difficulties to be highlighted prior to commencing analysis of the limited bone marrow derived mononuclear cell samples. Peripheral blood mononuclear cells were extracted from the whole blood using an identical density centrifugation separation technique to that described above for extraction of mononuclear cells from bone marrow.

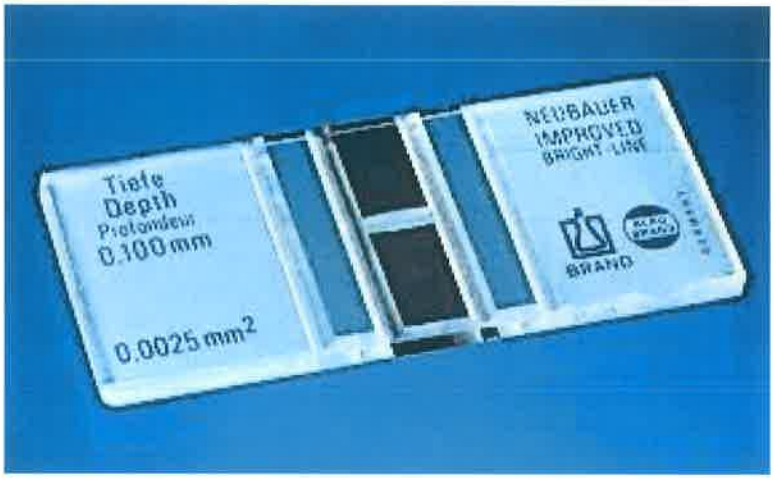
2. The second control was bone marrow derived mononuclear cells. The full research protocol was performed on the second control to test the methodology and evaluate preliminary results using the same cell types as would subsequently be used for the study. Peripheral blood monocytes while similar to bone marrow derived monocytes are known to have different proportions of subpopulations, therefore the second control analysed the identical cell sample type. The bone marrow derived mononuclear cells used as this control were taken from a spare frozen aliquot of cells in excess of the volume needed to complete the remainder of the study.

3.9 ii Sample processing

1. This protocol was designed for the labelling of 0.5-2x10^6 cells using the panels described above.
2. Prior to commencing the immunostaining protocol the number of cells to be stained was calculated. This count was performed to establish the maximum number of cells in each tube and hence calculate the appropriate amount of antibody solution required for each assay. As certain cell populations being evaluated were rare all cells available within each sample had to be labelled with antibody for counting.
3. Fetal calf serum (FCS) was defrosted in a water bath at 37°C until warm.
4. The clean work cabinet was switched on by first opening the glass window, then turning on the fan at the top of the cabinet, and turning off the alarm. The cabinet fan was allowed to reach maximum flow rate.
5. Fluorescence activated cell sorting (FACS) tubes were labelled. (Table 3.9)
6. 10mL of warm FCS was transferred into a 15mL falcon tube
7. The stored cell sample to be analysed was removed from the -40°C freezer and defrosted quickly in a 37°C water bath.
8. The warmed cells were taken to the cabinet and a Pasteur pipette used to transfer the cells into the 10mL warm FCS. The suspension was gently mixed with the Pasteur pipette.
9. The suspension was centrifuged at 1000 RPM for 5 min.
10. The supernatant (S/N) was discarded.
11. The remaining cell pellet was resuspended in 5mL warm R10 solution.

12. Cells were counted using a counting chamber haemocytometer. The cell count obtained was used to determine the cell suspension concentration and hence the volume needed for the staining protocol.

Figure 3.1: A Neubauer haemocytometer



3.9 iii Use of the haemocytometer

i. 10μL of cell suspension was transferred to a 96 well plate.

ii. 10μL of Trypan Blue was added to the cell suspension on the 96 well plate

iii. The haemocytometer (Figure 3.1) cover slip was correctly applied to the counting chamber of the haemocytometer

iv. 10μL of the mixture from the 96 well plate was applied to the edge of the coverslip on the haemocytometer to be sucked into the counting chamber void by capillary action

Figure 3.2: Application of cell suspension mixed with Trypan Blue to the cover slip of the counting chamber

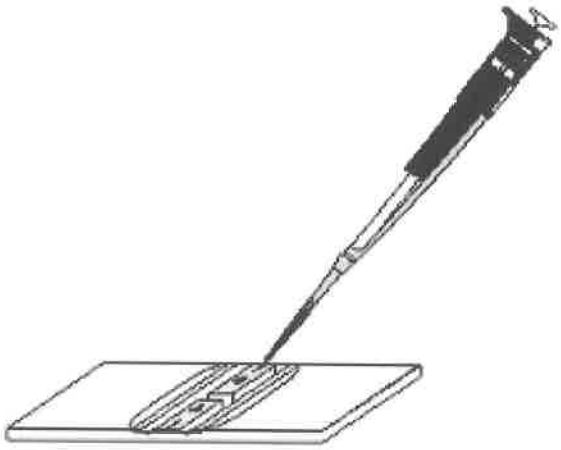
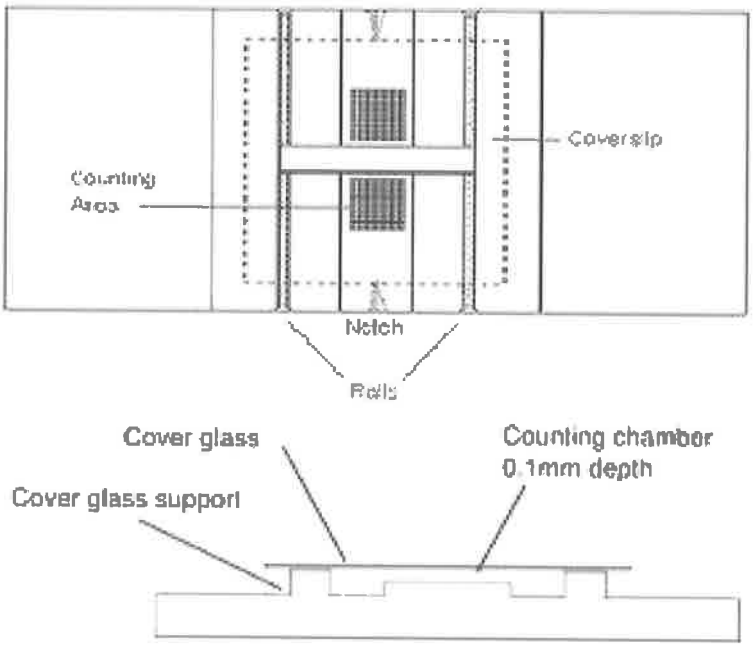
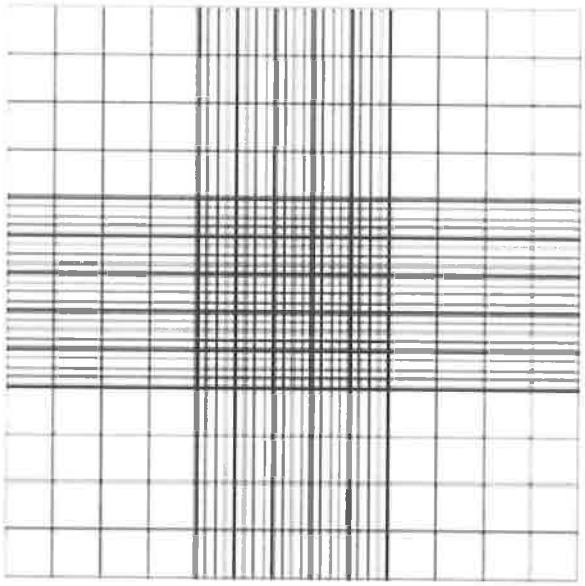


Figure 3.3: Diagram of the haemocytometer from above and in profile



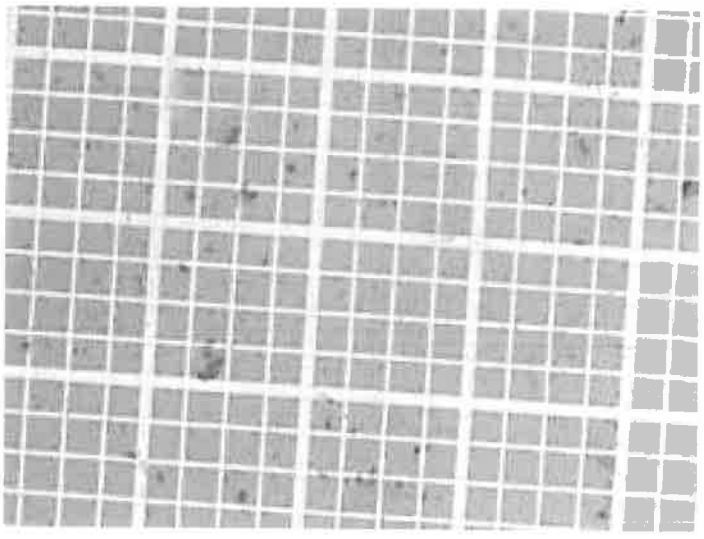
v. The counting chamber of the haemocytometer was placed under a light microscope and viewed at 40x magnification to visualise the Neubauer rulings and the cells to be counted

Figure 3.4: Diagram of the counting chamber of the haemocytometer



vi. Cells within the 5 x 5 Neubauer rulings grid were counted

Figure 3.5: Typical light microscopy view of a Neubauer haemocytometer counting chamber with cell suspension applied



vii. The number of cells within 1mL of the original suspension was calculated using the equation:

Number of cells in (5 x5) grid x dilution factor x 10^4

(For this protocol dilution factor = 2)

viii. To obtain the cell count within the original cell suspension the obtained cell count per mL was multiplied by the original suspension volume

(For this protocol original cell suspension volume = 10mL)

13. The cell suspension was centrifuged again at 1000 RPM for 5 min. The resulting supernatant was discarded.
14. The cell pellet obtained was resuspended in 500 µL of FACS wash buffer in preparation for staining.
15. The suspended cells were taken out of the clean cabinet and transferred to the main laboratory for staining.
16. 1 x 10^6 cells were transferred to the FACS tube in preparation for staining (calculation of volume of cell suspension required from each patient sample is shown in table 3.8)

Table 3.8: Cell counts obtained and calculated cell suspension volumes required from the bone marrow derived cell samples

Patient number	Cell count from a single stored frozen sample phial (variable no. of phials stored for each patient)	Volume of cell suspension used to provide 1 x 10^6 cells for staining (µL)	Volume of R10 used to make up solution for staining to 5µl (µL)
001	10 x 10^6	2.5	2.5
002	9 x 10^6	2.7	2.3
003	4.5 x 10^6	All available	
004	22.3 x 10^6	1.2	3.8
005	8.9 x 10^6	2.8	2.2
006	4.0 x 10^6	All available	
007	4.8 x 10^6	All available	
008	12.2x 10^6	2	3
009	30.9 x 10^6	0.8	4.2
010	3 x 10^6	All available	

3.9 iv Immunostaining of mononuclear cells

1. The appropriate amount of fluorochrome-conjugated antibody required for staining was added to the cell suspension tube as per manufacturer's instructions. A fresh tip was used to transfer each separate antibody into the tube to avoid contamination.
2. The cells were stained for 10 minutes at room temperature in the dark.
3. The cells were washed once with 4mL FACS wash and centrifuged at 1000 RPM for 5 min.
4. The cells were resuspended in 300µL 4% PFA and covered with foil.
5. The cells were kept at 4°C in the dark until transferred to the FACS facility for processing and data acquisition.
6. In preparation for analysis of patient samples the flow cytometer was calibrated and compensation settings acquired for the fluorochrome-conjugated antibody panels assessed in this protocol using control beads in place of patient samples. The beads used for compensation were OneComp eBeads (eBioscience, San Diego, CA, USA). The beads, spherical particles that could be stained with individual fluorochrome-conjugated antibodies, were used as single-colour compensation controls. Each drop of beads contained two populations of particles, a positive population able to bind with antibody and a negative population that would not. When the fluorochrome-conjugated antibody was added to the bead solutions both positive and negative control populations were obtained. This bimodal distribution of populations allowed single colour controls in a multi colour flow cytometry protocol.

   The beads were stained with the individual antibodies used for the study and with the combinations of antibodies used in each panel plus for the

full set fluorescence minus one (FMO). FACS tubes were labelled for each staining condition required for the protocol as well as for FMO (as listed in Table 3.9).

Table 3.9: FACS tube labelling key- for individual fluorochromes, FMO and full panel combinations as used for control and compensation studies

FACS tube	Fluorochrome	FACS tube	Fluorochrome
1	Neg/unstained	17	FMO-PE Cy5
2	FITC	18	FMO-PerCP Cy5.5
3	PE	19	FMO-PE Cy7
4	PE Cy5	20	FMO-V450
5	PerCP- Cy5.5	21	FMO-V500
6	PE Cy7	22	FMO-APC
7	V450	23	FMO-AF700
8	V500	24	FMO-AF647
9	APC	25	FMO-APC Cy7
10	AF700	26	FMO-APC H7
11	AF647	27	Panel 1
12	APC Cy7	28	Panel 2
13	APC H7	29	Panel 3
14	ALL ANTIBODIES	30	Panel 4
15	FMO-FITC	31	Panel 5
16	FMO-PE		

7. To each FACS tube one drop of control beads was added.
8. 4mL of FACS wash was added to each tube to wash the beads.
9. The suspension of beads and FACS wash was centrifuged at 1200RPM for 5 minutes following which the resultant supernatant was discarded.

10. 5μL of the required antibody was added to the appropriate tube as listed
11. The control beads were wrapped in foil and allowed to combine with the antibodies for 10 minutes at room temperature in the dark.
12. 4mL of FACS wash was added to each tube and the suspension was centrifuged for 5 minutes at 12000 RPM following which the resultant supernatant was discarded.
13. The control beads were resuspended in 300μL of FACS wash and covered with foil.
14. The control bead solutions were kept at 4°C in the dark until transferred to the FACS facility for processing and data acquisition.
15. The bone marrow derived mononuclear cells from each of the 10 study patients were stained with the fluorochrome-conjugated antibodies required in each panel using the same method of labelling as both the beads and control sample (peripheral blood derived mononuclear cells). Only labelling with the antibodies required for each panel was performed on the study samples; individual fluorochrome staining and FMO were not required.
16. Following labelling, the bone marrow derived mononuclear cell samples were kept at 4°C in the dark until transferred to the FACS facility for processing and data acquisition.

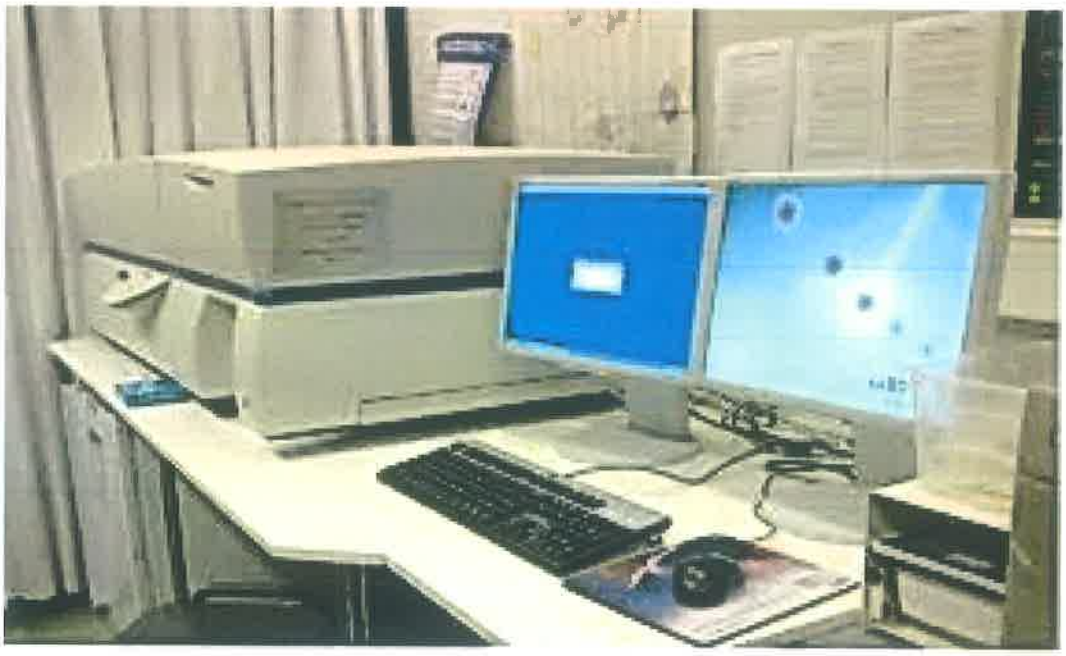
3.9 v Flow cytometry

Following the antibody labelling process, standard flow cytometry methods were used to identify the cell types under investigation. A BD LSR II™ flow cytometer (BD Biosciences, San Jose, CA, USA) was used for this protocol.

3.9 v a) Compensation

Compensation was required as more than one colour fluorochrome was utilised in this protocol. When more than one colour, multiparametric, analysis is used the colours of the fluorochromes have the potential to overlap as their wavelengths are not discrete but instead each form a part of the continuous colour spectrum. Compensation allows identification of potential overlap of the colours used and permits the data to be recorded in these regions more discretely, so separating the markers of interest being identified.

Figure 3.6: The BD LSR II flow cytometer and workstation used at the flow cytometry facility, Institute of Child Health, University of London



1. Compensation was determined using the OneComp eBeads after fluorochrome-conjugated antibody labelling as described above. After labelling of the samples, compensation was performed. BD FACS Diva software compensation setup tool was used for compensation and subsequent compensation bead sample analysis.
2. Compensation could be performed either before or during data acquisition (online) or after running the study samples (offline), as the compensation setting could be saved and applied at any time to any acquired data.
3. The fluorochromes to be used were selected on the software parameters.
4. A Compensation Control Worksheet was created within the FACS Diva menu.
5. Within the Compensation Worksheet an individual tab for each fluorochrome was created.
6. The cytometer was assessed before use for adequate functioning and that it had been left prior to and between uses with FACS wash within its tubing system.
7. The cytometer was set to "RUN"
8. The FACS wash tube was removed from the sample injection probe (SIP) and the tube support arm was left open.
9. The cytometer was set to "LO" flow rate and the first sample loaded onto the SIP, the support arm was quickly replaced to its working position, holding the FACS tube on the SIP firmly to create a vacuum seal between the top of the SIP and the open neck of the FACS tube.

10. With the "RUN" button still on, the "Acquire data" button on the FACS Diva software desktop screen was clicked. Data acquisition plots commenced collating on the desktop screen.
11. The data collected from the cytometer were observed on the desktop screen and FSC and SSC voltages adjusted as required using the parameter tab within the cytometer window of the desktop screen to allow the events to register clearly within the dot plot.
12. Using the same method the fluorescent emission signal for the colours of the fluorochromes was adjusted in the respective histograms until the signal appeared normally distributed within the first log.
13. " Stop acquisition" was pressed on the cytometer once all voltages had been adjusted.
14. The cytometer was left on "STANDBY".
15. The unstained sample was run first to provide baseline data. FSC and SSC voltages were established.
16. The data from the unstained beads were recorded after gating of the cell population was performed. Gating the cell population permitted exclusion of debris and dead cells from the analysis, reducing auto-fluorescence.
17. Beads labelled with each individual fluorochrome were then run on the cytometer in the same manner to analyse the corresponding single stains.
18. Once all single stain analyses had been performed "Calculate Compensation" was selected from the menu bar on the desktop.
19. All subsequent specimens using the same fluorochromes were analysed using the same compensation settings automatically.

3.9 v b) Data acquisition

1. To record new sample experiments and analyses a "Global Worksheet" was created and the plots required for acquisition selected.
2. Following compensation, the control sample of mononuclear cells derived from peripheral blood was run on the cytometer.
3. As with the compensation beads, the control sample of peripheral blood derived mononuclear cells was analysed with single fluorochrome stains, FMO and for each full panel of antibodies.
4. Following the first control, the second control of bone marrow derived mononuclear cells was performed for each full panel of antibodies. .
5. After both control samples had been run, the research samples were acquired sequentially (Panel 1 was run with each of the ten patient samples in order, followed by panel 2 etc).
6. For the first control sample (blood derived mononuclear cells) a stopping gate was applied to the dot plots within the acquisition dashboard on the desktop; this allowed the number of events required for analysis to be selected. Each event represented a single cell. For the control sample a stopping gate of 100,000 events was used. For the bone marrow derived mononuclear cells no effective stopping gate was used (stopping gate of 10,000,000 was used as this would exceed the number of cells within the analysis samples and hence the whole volume would be utilised) as the entire sample was acquired to allow detection of rare cell populations.
7. The cytometer was switched from "STANDBY" to "RUN" and "Record Data" on the acquisition dashboard was selected. The acquisition of data occurred on the run sample until the pre-defined stop limit was reached or the sample was run to completion.

8. The data obtained from each sample were saved under a corresponding file name and the process repeated for each subsequent sample.
9. Subsequent samples were acquired using the same settings as established on the initial control acquisitions and data saved under a single experiment name.
10. On completion of the sample processing and data acquisition the cytometer was cleaned and shut down according to the manufacturer's instructions (See Appendix 5).

3.9 v c) Data gating

The acquired data were individually assessed using gating to isolate cell populations carrying specific fluorochrome-conjugated antibodies to surface markers. Combinations of these markers permitted isolation of discrete cell populations. FLOWJo® FACS analysis software (Tree Star Inc.) was used to perform the data gating and permitted calculation of absolute cell type and number.

Gating involved visual selection of cell populations to be analysed further by their cell markers. A gate was drawn around the population on the dot plot before that population was investigated further according to the presence or absence of surface markers. All cells were initially evaluated with respect to FSC and SSC. The cell population to be further assessed was gated then evaluated with respect to two other fluorochromes representing each surface marker. Each resultant dot plot was analysed in four quadrants, each indicating the presence or absence of each of the two fluorochromes.

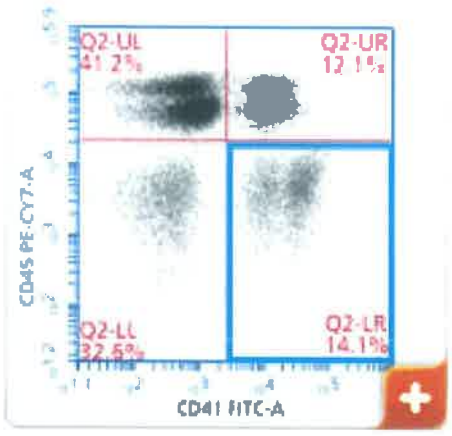


Figure 3.7: Identification and gating of peripheral blood populations

Upper left quadrant: 41.2%

CD 45+CD41-

Upper right quadrant: 12.1%

CD45+ CD41+

The gating strategies utilised in this protocol are outlined below:

Panel 1:

Part 1

CD45+ population identified and gated (two populations: monocytes and lymphocytes)

↓

SSC plotted against CD34

CD45+ CD34+ population gated

↓

SSC re-plotted against CD45

CD34+ CD45low= true CD34+ cells

Part 2

CD45+ population identified and gated (two populations: monocytes and lymphocytes)

↓

SSC plotted against CD34

CD45+ CD34+ population gated

↓ ↓

↓ CD38 plotted against CD34

↓ CD34+ CD38- gated

↓ ↓

↓ CD90 plotted against CD45RA

↓ CD90+ CD45RA- =HSC

↓ CD90- CD45RA- =MPP

CD10 plotted against CD38

CD34+ CD10+ =CLP

CD10- CD38+ population gated

↓

CD135 plotted against CD45RA

CD135 + CD45RA- = CMP

CD135 + CD45RA+ = GMP

CD135 - CD45RA- = MEP

Panel 2:

CD45+ population identified and gated

↓

CD45+ CD4+ population gated

↓

CD127 plotted against CD25

CD127 low CD25+ population gated

↓

CD45RA plotted against CD4

CD45RA+= naïve T-Reg

CD45RA-= memory T-Reg

Panel 3:

CD34 plotted against CD45

CD34- CD45- population gated

↓

CD105 plotted against CD73

CD105+ CD73+ population gated

↓

CD90 plotted against CD45

CD90+ CD45-= MSC

Panel 4:

CD3+ population identified and gated= T lymphocytes

↓ ↓

↓ CD3+ CD4+=T helper/ inducer cells

CD3+ CD8+= T suppressor/ cytotoxic T cells

CD19+= B lymphocytes

CD16+ CD56+= NK cells

Panel 5:

CD45+ population identified and gated

↓

CD34+ subpopulation identified and gated

↓

CD34+ CD45 low subpopulation identified and gated

↓ ↓ ↓

↓ ↓ CD45+ CD34+ CD144+ = Endothelial progenitors

↓ CD45+ CD34+ CD133/ CD309 + = VEGFR

CD45+ CD34+ CD133/309+ CD184+= cardiac stem cells

3.10 References

1. Zola, H. & Swart, B. The human leucocyte differentiation antigens (HLDA) workshops: the evolving role of antibodies in research, diagnosis and therapy. *Cell research* **15**, 691–4 (2005).
2. Wang, X., Shook, J., Edinger, M., Warner, N. & Bush-Donovan, C. Multiparametric Immunophenotyping of Human Hematopoietic Stem Cells and Progenitor Cells by Flow Cytometry. *BD Biosciences* 1–16 (2012).

CHAPTER 4

RESULTS 1: PRIMARY END POINTS

4.1 Introduction

This chapter reviews the data profiles of the study participants, the baseline data and assesses the primary outcome measure for this study, namely freedom from death or transplant.

4.2 Results

Ten children (mean age 7.2y, range 2.2-14.1y, 6 male) with dilated cardiomyopathy were recruited from the Heart Failure clinic at Great Ormond Street Hospital. Patient profiles were obtained from the history taken either from the patient or their family supported with data taken from their individual case notes. Patient profiles on recruitment are shown below and were updated throughout the study.

The underlying heart muscle pathology causing the cardiomyopathy of each patient is listed with their additional individual data below (Table 4.1). On entry to the study all ten patients were in New York Heart Association or Ross classification of heart failure group 3 or 4 indicating moderate to severe illness. The NTproBNP mean was 1173 pg/ml (normal range 29-206 pg/m as per GOSH laboratory reference range). Their baseline data are summarised in Table 4.2.

4.2 i Primary end points

All 10 patients completed the entire one year study without complication, the primary end point, freedom from death or transplant, was achieved. No withdrawals from the study, morbidity or mortality of study participants or adverse events occurred. Nine patients completed the original protocol, the tenth patient had Duchenne Muscular Dystrophy and as general anaesthesia

was deemed to carry a significantly increased risk for him he completed an otherwise identical study design under sedation. This deviation from the original study protocol resulted in his data being excluded from the secondary end point analysis (patient reference 006) but is included below, as it did not affect the primary end points of freedom from death or transplant.

Table 4.1: Individual baseline patient data

Patient number	Age	Sex	Diagnosis	NTpro BNP pg/ml
001	03y11m18d	M	Inferior MI	860
002	06y01m28d	F	Anthracycline induced	6280
003	12y08m01d	F	Anthracycline induced	699
004	14y00m27d	M	Anthracycline induced	542
005	02y02m21d	M	Post enteroviral myocarditis	1293
006	13y10m21d	M	Duchenne muscular dystrophy	215
007	03y09m12d	F	Familial	99
008	02y05m25d	M	DCM ?post viral	518
009	09y00m12d	M	Neonatal enteroviral myocarditis	442
010	03y09m22d	F	Idiopathic DCM,?vit D deficiency	780

Table 4.2: Patient Data Summary

Category	Value
Age	2.2y-14.1y (mean 7.2y)
NYHA/Ross Classification	3-4
End systolic volume on MRI (indexed, ml/m^2)	43.69-165.59 (mean 74.73)
End diastolic volume on MRI (indexed, ml/m^2)	81.3-283.78 (mean128.51)
NT pro BNP (pg/ml)	99-6280 (mean 1172.8)
Sex	6 male 4 female

4.3 Anonymised patient profiles at time of recruitment

4.3 i Patient 001

Age: 3y11m18d

Weight: 15.6 kg

Height: 102.7 cm 26/04/11

Gender: Male

Diagnoses

1. Supra-mitral membrane
2. Resection of supramitral membrane with ligation of arterial duct 30/03/2007

3. Subsequent severe mitral regurgitation
4. Mitral valve replacement 17 mm St Jude mechanical prosthesis 29/05/2007
5. Suspected compromise of circumflex coronary artery resulting from inferior myocardial infarction
6. Borderline raised pulmonary vascular resistance index
7. Listed for cardiac transplantation
8. Horseshoe kidney (diagnosed July 2009)
9. Ladd's procedure for gut malrotation
10. Gastrostomy fed
11. Mild scoliosis- left shoulder elevated compared with right

Summary

Antenatally 001's mother experienced a vaginal bleed at 28/40- no cause was found, and she experienced no other complications. 001 was born by elective LSCS at term (previous LSCS), he had mild grunting after delivery but was not admitted to SCBU.

At five weeks of age poor feeding and poor weight gain was noted, he was diagnosed with a viral infection. At ten weeks he had bronchiolitis and was admitted to his local hospital after a cyanotic episode. A barium swallow was performed to investigate suspected tracheo-oesophageal fistula. This revealed malrotation and a Ladd's procedure was performed. 001 was partially NG fed subsequently. At seventeen weeks he had pneumonia; on examination a cardiac murmur was detected; echo demonstrated a supra-mitral membrane. Resection of the membrane with ligation of the arterial duct was performed on

30/03/07, unfortunately he subsequently suffered with severe mitral valve regurgitation. A mitral valve replacement, using 17mm St Jude's mechanical prosthesis, was performed on 29/05/07. During the procedure he had suspected compromise of circumflex artery resulting in an inferior MI. 001 had ECG changes and regional wall abnormalities on echo, a myocardial perfusion scan confirmed the presence of an established MI in the posterior and inferior segments of the left ventricle. 001 suffered with poor cardiac function subsequently.

001 was assessed and listed for transplant July 2009. He had an incidental finding of a horseshoe kidney on abdominal ultrasound July 2009. 001 had a gastrostomy feeding tube inserted in October 2010.

Developmentally his motor milestones were delayed; he crawled at over 1y, walked late, had an in-toeing gait. 001 required Piedro boots for ankle stability. His speech was normal and there were no cognitive concerns.

001 had frequent chest infections requiring IV antibiotics. He had also previously demonstrated poor growth. When referred for transplant assessment in January 2008 001's growth had fallen from below the 0.4^{th} centile despite high calorie NG feeds. On optimisation of 001's heart failure medications and PEG insertion 001 plotted $<25^{th}$ centile for weight and $> 9^{th}$ centile for height.

Status on recruitment

001 was the second of three male children, his younger brother had pulmonary stenosis. 001 was described by the family as tiring easily and having episodes

of breathlessness, occasionally being sweaty. On clinical review 001 appeared generally well. He had a constellation of dysmorphic features including upslanting palpebral fissures with epicanthic fold right eye, mild 5th finger clinodactyly but no unifying diagnosis following review by the genetics team at Great Ormond Street.

Medications

1. Captopril 5mg tds (three times daily)
2. Carvedilol 3.125mg bd (twice daily)
3. Frusemide 12 mg tds
4. Warfarin 3 to 4 mg od (once daily) to maintain INR 3.5-4.5
5. Augmentin Duo 2.5ml bd

Up to date with immunisations

Allergy: duoderm

Investigations

Echocardiogram

Date:	**FS%:**	**Comment:**
04.03.08	11%	LV dilated with inferior lateral akinesis; RV size and function normal; trace of TR Doppler vel 2.9m/s, St Jude's well sited, no regurgitation. LV internal dimension 36mm, Z score 3.76
03.03.09	16%	Increased mitral valve inflow velocity, mean gradient 14mmHg, max vel. of 2.8m/s. Systolic function sig impaired with reduced systolic velocities

		at mitral valve annulus on Doppler. LVD 3.8cm. Marked dyskinesis
23.04.09	11%	Significantly impaired LV function, LV dilated, LVDd 41mm; mild TR peak vel 4.0m/s, mitral valve restricted, peak inflow 2.4m/s mean gradient 9mmHg
12.11.09	15%	Significantly ventricular impairment, gradient across mitral valve 27mmHg. LVDd 42mm
15.06.10	13%	LVDd 43mm, gradient across mitral valve 14mmHg, max vel 2.5m/s, increased flow in pulmonary veins

Blood investigations

Chemical pathology

NT-pro-Brain Natriuretic Peptide 860H pg/ml (29 - 206)

Sodium	145 mmol/L (133 - 146)	Potassium	3.0 mmol/L (3.5 - 5.5)
Urea	2.6 mmol/L (2.5 - 6.0)	Creatinine	25 umol/L (20 - 39)
Calcium	1.74 mmol/L (2.22 - 2.51)	Magnesium	1.03mmol/L (0.66 - 1.0)
Phosphate	1.39 mmol/L (1.20 - 1.8)	Albumin	40 g/L (35 - 52)
Bilirubin	4 umol/L (<18)		

Alkaline Phosphatase 198 U/L (150-380)

Alanine Transaminase <6 U/L (10 - 25)

Haematology

Blood Group:	O Rh +ve antibody screen negative
Haemoglobin	11.3 g/dL (11.5 - 14.5)
Platelet Count	286 x10^9/L (150 - 450)
White Cell Count	4.51 x10^9/L (5.0 - 15.0)
Prothrombin Time	14.7 Seconds (9.6 - 11.8)

APTT	76.3 Seconds (26.0 - 38.0)
Thrombin Time	35.5 Seconds (9.2 - 15.0)
Fibrinogen	2.5 g/L (1.7 - 4.0)
INR	1.4

Virology

Cytomegalovirus IgG	positive
EBV VCA IgG Antibody	negative
HB surface antigen	negative
HCV	negative
HTLV1 & 2	negative
Measles IgG Antibody	positive
HSVG	negative
Rubella IgG Antibody	81IU/ml
VZV IgG Antibody	positive
HIV	negative
TOXOG	negative
VDRL	negative

Incidental findings

Patient 001 was noted to have an extensive spinal cord syrinx detected incidentally on his first cardiac MRI. The cardiac MRI revealed a large spinal cord syrinx extending through cervical, thoracic and lumbar levels. As this was an incidental finding on a cardiac MRI scan, the views obtained were insufficient to exclude spinal dysraphism, but 001 and 001's images were reviewed by a Consultant Neurologists at Great Ormond Street Hospital. Further care of this condition was agreed by the neurology and neurosurgical teams. Subsequent

investigation and monitoring concluded that the condition was not evolving. 001 continues with regular reviews by these teams.

4.3 ii Patient 002

Age: 6y1m28d

Weight: 12.5 kg

Height: 94.2cm

Gender: Female

Diagnosis

1. Neonatal AML treated with chemotherapy to remission
2. Relapse at 16 months of age treated with chemotherapy and a matched unrelated peripheral blood stem cell transplant
3. GvHD of the skin April 2006
4. May 2006 cardiorespiratory arrest secondary to poor cardiac function
5. Cardiomyopathy secondary to anthracycline toxicity

Summary

002 presented at the age of three weeks with multiple subcutaneous bruises and nodules, and oral thrush. At four weeks of age 002 was noted to have generalised lymphadenopathy and hepatomegaly. A full blood count showed leucocytosis and circulating myeloid blasts. 002 was referred to Great Ormond Street where a bone marrow aspirate confirmed M5 AML (acute monocytic leukaemia); 002's CSF was clear. Due to 002's age initial treatment was with at 75% dose, this was tolerated well but with no resulting reduction in subcutaneous nodules or marrow disease. 002 went on to receive an

alternative chemotherapy regime at 60% dose, which resulted in cytogenetic remission. 002 was received further chemotherapy with three doses of triple intrathecal therapy. 002 tolerated these courses reasonably well apart from one, which was complicated by severe mucositis and sepsis.

002 completed therapy at the end of December 2004 at which time cardiac function was normal. 002 remained reasonably well for most of 2005. It was noted in October 2005 that blood counts were low, but bone marrow was normal. In December 2005, sixteen months from initial diagnosis, 002's mother noticed that 002 was becoming pale and lethargic, and 002 subsequently developed fever with diarrhoea. 002's white cell count at this time was 36. A repeat bone marrow aspirate at Great Ormond Street confirmed relapse with no cytogenetic clonal evolution. 002 was treated with two courses of chemotherapy with no major problems and the subsequent BMA was in morphological remission.

Following initial treatment for the relapse 002 remained well and commenced on a course of Cytarabine whilst awaiting bone marrow transplant. A repeat BMA carried out on the 5th April 2006 demonstrated good morphological remission. 002 developed a line infection in April 2006 culturing *neisseria* and *S. viridans* which was treated effectively with IV teicoplanin and cefotaxime and line removal.

002 underwent a matched unrelated peripheral blood stem cell transplant in April 2006 and subsequently had stage II GvHD of the skin. During therapy, on

the 17th May 2006, 002 suffered a cardiorespiratory arrest and was transferred to PICU for further management.

002 had significant pulmonary oedema and was oscillated immediately. An echo showed poor LV function with an ejection fraction of 20% with evidence of cardiomyopathy. 002 also had a high adenovirus count. A CT head was performed which showed a small area of high attenuation seen in the region of the right cerebral peduncle. It was unclear whether this was an area of haemorrhage or artefact and an MRI was recommended. 002 was extubated on 21.5.06, however this attempt failed and 002 had to be re-intubated. An EEG showed diffuse slowing indicative of non-specific cerebral dysfunction. 002 was started on a hydralazine and milrinone infusion which was slowly weaned off. Subsequent hypertension was controlled with increasing doses of captopril. A cardiac MRI was performed on 2.6.06, results suggested that myocarditis was unlikely, however there was reduced global LV function and marked hypokinesia of the septum. 002 was extubated on 2.6.06 and remained well subsequently.

Haematologically, an engraftment from 27th September showed 88% donor chimerism in the whole blood. A repeat taken on 2nd October showed 75% donor in whole blood, 76% in MNCs and 88% in granulocytes. Cell counts at this time remained normal. 002 underwent donor lymphocyte infusion in October 2006 to increase engraftment.

Cardiovascularly, 002 was referred for further management by the Heart Failure team. 002 was medically managed and responded well initially to medications,

but subsequent progressive worsening of the cardiac function resulted in assessment for cardiac transplantation in February 2008. Serial Fractional shortening measurements markedly reduced over the preceding 6 months (38% June '06, 28% Aug '06, 20 % May '07, 12% Jan '08). At this time 002 suffered with recurrent leg pain, and was only able to walk for approximately five minutes. On examination 002 had 8cm hepatomegaly. 002 made some gradual clinical improvement over the next six months and was taken off the active transplant list later the same year.

Status on recruitment

002 lived at home with her mother and older brother. 002 had remained reasonably well over the previous two years on medical management of heart failure. 002 attended mainstream school but had fallen behind her peers due to the chronic illness therefore educational assistance had been requested. 002 was able to mobilise independently but with reduced exercise tolerance. It was noted in May 2010 that 002 was becoming more tired and was suffering with frequent chest infections, one requiring hospitalisation. 002 had poor general growth.

Medications

1. Captopril 5mg tds
2. Carvedilol 3.125mg bd
3. Aspirin 37.5mg od
4. Frusemide 10mg tds
5. Penicillin 125mg od

Allergies: nil known

Investigation results

Cardiac MRI

Scan performed 4th June 2007:

Indexed ventricular volume measurements

	EDV (mL/ m^2)	Normal EDV	ESV (mL/ m^2)	Normal ESV	Ventricular CI (L/min/m^2)
LV	87	41 - 81	69	12 - 21	3.2
RV	79	48 - 87	58	11 - 28	3.6

Conclusion The patient was tachycardic under general anaesthetic.

Small, circumferential pericardial effusion

The left ventricle is moderately dilated with severe, global impairment of systolic function (EF 22%).

Echocardiogram

Date:	**FS%:**	**Comment:**
03.08.10	18%	Dilated LV with mild/moderately impaired systolic function, dilated RV with reasonable function, mild TR and MR, no effusions on echo.

Blood Investigations

Chemical pathology

NT-pro-Brain Natriuretic Peptide 6280 pg/ml (29 – 206)

Sodium	140 mmol/L (133 – 146)	Potassium	4.4 mmol/L (3.5 - 5.5)
Urea	4.2 mmol/L (2.5 - 6.0)	Creatinine	48 µmol/L (27 – 48)
Calcium	2.55 mmol/L (2.22 - 2.51)	Magnesium	0.95 mmol/L (0.66 - 1.00)

Phosphate	1.56 mmol/L (1.2 - 1.8)	Albumin	53 g/L (37 – 56)

Total bilirubin 10 umol/L (<18)

Alkaline Phosphatase 274 U/L 175 - 420

Alanine Transaminase 17 U/L 10 - 35

Haematology

Blood Group	A Rh -ve
Haemoglobin	12.4 g/dL (11.5 - 14.5)
Platelet Count	226 x10^9/L (150 - 450)
White Cell Count	10.36 x10^9/L (5.0 - 15.0)
Prothrombin Time	12.4 Seconds (9.6 - 11.8)
APTT	44.4 Seconds (26.0 - 38.0)
Thrombin Time	12.0 Seconds (9.2 - 15.0)
Fibrinogen	2.2 g/L (1.70 - 4.0) 50:50

Prothrombin Time Mix 11.1 Seconds

50:50 APTT Mix 37.2 Seconds (31.5 - 40.5)

Comments: Prolonged APTT which corrects with normal plasma. This suggests an underlying factor deficiency although inhibition, possibly caused by a "Lupus" inhibitor cannot be excluded.

Virology

Cytomegalovirus IgG	positive
EBV VCA IgG Antibody	positive
HB surface antigen	negative
HCV	negative
HTLV 1 & 2	negative
Measles IgG Antibody	negative
HSVG	negative

Rubella IgG Antibody	negative
VZV IgG Antibody	negative
HIV	negative
TOXOG	negative
VDRL	negative

4.3 iii Patient 003

Age:	12y8m1d
Weight:	34.05 kg
Height:	131.7cm
Gender:	Female

Diagnoses

1. Trisomy 21.
2. Previous AML with treatment completed in February 2000.
3. Dilated cardiomyopathy secondary to chemotherapy.
4. Dec 2008: thromboembolic event
5. Progressive non-scarring alopecia areata

Summary

003 was diagnosed with AML in early childhood, completing chemotherapy at the age of two years. Dilated cardiomyopathy developed secondary to chemotherapy, although cardiac function remained within the normal range for some time (fractional shortening 30-33% until 2004). As a result of the cardiac deterioration in 2005, 003 was converted to enalapril and started on carvedilol, stabilising the fractional shortening to the mid 20's. Medication was briefly changed to candesartan because of a suspicion that the alopecia was related to

her ACE inhibitor therapy, however cardiac function continued to deteriorate and medications were changed back to enalapril.

003 remained stable with a fractional shortening of 20-22% until 2008 when she presented with a thrombo-embolic event associated with acute clinical deterioration. 003 had an episode of transient facial weakness, reported to the GP, followed three weeks later by a more significant systemic thrombus causing loss of peripheral pulses in both lower limbs. This episode was successfully treated with thrombolysis. The acute cardiac deterioration at this time did not improve despite augmentation of medications. 003 was referred to the Heart Failure Clinic at Great Ormond Street for further review, fractional shortening was found to be 16% and exercise tolerance was reduced at 200m.

During 2009 003 continued to have a number of problems related to poor cardiac function, taking increasing amounts of time off school. 003's parents described that the exercise tolerance worsened and that she managed to only walk approximately 100m before becoming tired and unable to continue. 003's appetite remained reasonable. 003 was still able to sleep well at night, lying flat in bed and had no symptoms of palpitations or breathlessness. She started secondary school in 2009, attending a school for children with special educational needs.

During 2009 and early 2010 003 deteriorated clinically. Echo parameters reduced and 003 became more unwell. Following an admission to hospital for abdominal pain 003 was diagnosed as irritable bowel syndrome but the presentation may have instead been related to poor gut perfusion. The

reduction in fractional shortening and poor nutrition may have had some affect on the poor echo parameters. 003 was listed for cardiac transplantation in November 2009.

Status on recruitment

003 was reasonably well from a cardiac perspective and whilst still not able to attend school on a regular basis due to practical reasons, was managing reasonably well on the prescribed cardiac medications. 003 lived at home with both parents and older brother.

Medications

1. Captopril 18.75mg tds
2. Carvedilol 12.5mg bd
3. Spironolactone 10mg od
4. Frusemide 10mg od
5. Digoxin 100mcg od
6. Warfarin 2.5-3mg od to maintain INR 2-3

Allergies: nil known

Investigation results

Echocardiogram

Date:	**FS%:**	**Comment:**
15/09/09	9%	Moderately dilated LV with severely reduced systolic function. Moderately dilated LA
10/11/09	12%	Moderately dilated LV with moderate to severe impairment
09/02/10	23%	No significant change

03/08/10	26%	Mildly impaired LV systolic function, mild MR/AR, Normal RV function, dilated LA, no effusions

Blood investigations

Chemical pathology

NT-pro-Brain Natriuretic Peptide 699 pg/ml (29 – 206)

Sodium	143 mmol/L (133 – 146)	Potassium	4.3 mmol/L (3.5 - 5.5)
Urea	6.7 mmol/L (2.5 - 6.0)	Creatinine	56 umol/L (34 – 67)
Calcium	2.26 mmol/L (2.19 - 2.66)	Magnesium	0.82 mmol/L (0.70 - 0.95)
Phosphate	1.08 mmol/L (1.1 - 1.75)	Albumin	42 g/L (37 – 56)

Total bilirubin 5 umol/L (<18)

Alkaline Phosphatase 233 U/L (105 – 420)

Alanine Transaminase <6 U/L (10 – 55)

Haematology

Blood Group	O Rh +ve
Haemoglobin	12.6 g/dL (11.5 - 15.5)
Platelet Count	241 $x10^{-9}$/L (150 - 450)
White Cell Count	3.92 x10^9/L (4.5 - 13.5)
Neutrophil	1.90 $x10^{-9}$/L (1.8 - 8.0) 48.5 %
Prothrombin Time	13.8H seconds (9.6 - 11.8)
APTT	33.8 Seconds (26.0 - 38.0)
Thrombin Time	13.1 Seconds (9.2 - 15.0)
Fibrinogen	2.6 g/L (1.70 - 4.0)
INR	1.3

Virology

Cytomegalovirus IgG	negative
EBV VCA IgG Antibody	positive
HB surface antigen	negative
HCV	negative
HTLV 1 & 2	not detected
Measles IgG Antibody	negative
HSVG	negative
Rubella IgG Antibody	negative
VZV IgG Antibody	positive
HIV	negative
TOXOG	negative
VDRL	negative

4.3 iv Patient 004

Age:	14y0m27d
Weight:	39.6 kg
Height:	161.3 cm
Gender:	Male

Diagnoses

1. Post chemotherapy cardiomyopathy.
2. Mesoblastic nephroma with left nephrectomy, April 1997
3. Recurrence of abdominal mass, July 1997 (undifferentiated sarcoma) Completion of treatment, June 1998.
4. Post chemotherapy cardiomyopathy diagnosed 2008 in Greece

5. Left middle cerebral artery thrombosis, October 2008.
6. PCR parvovirus positive.

Summary

004 initially presented to urologists at the age of six months having been referred from his local hospital with a left renal mass discovered on review for recurrent urinary tract infection. On the 23rd April 1997 he was admitted for exploration of the renal mass and eventually a left nephrectomy. At that stage the initial histopathology suggested a congenital mesoblastic nephroma which was completely excised. In June of the same year the histology was reviewed and it was felt that in fact it had been incompletely excised with the tumour extending to the excision margins.

004 was followed up routinely in the Oncology Outpatients Clinic where in July 1997 following imaging it was believed that the tumour had recurred. 004 was commenced on chemotherapy, vincristine protocol 9101. 004 received four doses of vincristine and one dose of actinomycin D via a central line. The tumour continued to enlarge despite therapy and a further surgical exploration was performed on 24/09/97. Again there was macroscopic excision of the recurrence, but pathology showed this tumour to be substantially different from the first. Although it was still a spindle-cell sarcoma it had now undergone chondroblastic differentiation as well as cellular atypia and relatively high number of mitoses. Because of the change in histology, therapy was altered to the sarcoma protocol. The next course of chemotherapy consisted of vincristine, actinomycin D and cyclophosphamide but ultrasound scan revealed another tumour recurrence in the left renal bed. This was treated with surgery (the

tumour was adherent to the splenic flexure of the colon and to the spleen) and with six months of chemotherapy- vincristine, etoposide, carboplatin, epirubicin, actinomycin D and cyclophosphamide, followed by radiotherapy. The last chemotherapy was given on 02/04/1998.

Following therapy 004 developed Factor II deficiency (secondary to lupus anticoagulant, under the care of GOS haematologists), renal osteodystrophy, osteopenia with subsequent fractured left femur (November 1998) and fractured right humerus (April 2000) and hypocalcaemic hypermagnesaemic tetany as a result of renal tubulopathy.

004 and his family moved to Greece in 2002 and in 2008 he was noted to have developed cardiomyopathy, secondary to chemotherapy. 004 suffered a left middle cerebral artery thrombus in October 2008 and was commenced on warfarin as a result. In January 2009 004 moved back to the UK and began being reviewed by the heart failure team at Great Ormond Street hospital. There were no clinical signs of heart failure at this stage, but fractional shortening was 14%. This steadily improved with alteration to cardiac medications, and as a result warfarin was discontinued in March 2009.

Status on recruitment

004 was well, he was in year 10 of mainstream school and lived in the UK with his mother; an older brother lived with their father in Greece. On examination he appeared well, warm and well perfused, JVP was not raised and the liver was not enlarged, cardiovascular examination was normal, fractional shortening on echo was 26%.

Medications

1. Carvedilol 3.125 mg bd
2. Digoxin 62.5 mcg od
3. Aspirin 32.5mg od
4. Lisinopril 5mg od

Allergies: Nil known

Investigation results

Cardiac MRI: 19/12/2008

Indexed ventricular volume measurements

	EDV (mL/m^2)	Normal EDV	ESV (mL/m^2)	Normal ESV	Ventricular CI ($L/min/m^2$)
LV	95	47 - 92	66	13 - 30	3.0
RV	56	55 - 105	30	15 - 45	2.8

Conclusion The left ventricle is dilated and thin-walled with severe, global impairment of systolic function (EF 30%).

Low LV mass.

No evidence of large areas of myocardial fibrosis or scarring.

Echocardiogram

Date:	**FS%:**	**Comment:**
03/03/09	18%	Dilated LV, visually moderate to severe global Impairment, reduced RV free wall motion
26/05/09	22%	Dilated LV, moderately reduced systolic function RV function satisfactory

28/07/09	29%	Dilated LV, reduced LV systolic function RV function satisfactory
20/10/09	29%	Mild-mod dilated LV with moderately reduced systolic Function, reasonable RV function
23/03/10	22%	Dilated LA, moderately impaired LV function, good RV systolic function, mild PR
21/09/10	26%	Dilated LA/LV, mild to mod impaired systolic function

Bloods investigations

Chemical pathology

NT-pro-Brain Natriuretic Peptide: 542 pg/ml (29 – 206)

Sodium	143 mmol/L (133 – 146)	Potassium	4.0 mmol/L (3.5 - 5.5)
Urea	7.2 mmol/L 2.5 - 7.5	Creatinine	69 umol/L (42 – 79)
Calcium	2.38 mmol/L 2.19 - 2.66	Magnesium	0.88 mmol/L (0.70 - 0.95)
Phosphate	1.34 mmol/L 1.1 - 1.75	Albumin	46 g/L (37 – 56)

Total bilirubin <2 umol/L <18

Alkaline Phosphatase 212 U/L 130 - 525

Alanine Transaminase 13 U/L 10 - 45

Digoxin 0.52 nmol/L 1 - 2.6

Haematology

Blood Group:	O Rh +ve antibody screen negative
Haemoglobin	11.8 g/dL (13.0 - 16.0)
White Cell Count	6.52 $x10^{-9}$/L (4.5 - 13.5)
Platelet Count	312 $x10^{-9}$/L (150 – 450)
Prothrombin Time	10.6 Seconds (9.6 - 11.8)

APTT	29.0 Seconds (26.0 - 38.0)
Thrombin Time	11.3 Seconds (9.2 - 15.0)
Fibrinogen	2.8 g/L (1.7 - 4.0)

Virology

Cytomegalovirus IgG	negative
EBV VCA IgG Antibody	negative
HB surface antigen	negative
HCV	negative
HTLV 1&2	not detected
Measles IgG Antibody	positive
HSVG	positive
Rubella IgG Antibody	108 IU/mL
VZV IgG Antibody	positive
HIV	negative
TOXOG	negative
VDRL	negative

4.3 v Patient 005

Age:	2y2m21d
Weight:	10.6 kg
Height:	84 cm
Gender:	Male

Diagnoses

1. Post enteroviral myocarditis with chronic left ventricular failure.
2. Chylothorax managed with bilateral chest drains- resolved

3. Jaundice and liver dysfunction probably related to TPN- resolved
4. Candida sepsis- resolved
5. Tracheostomy 20/09/2008- decannulated 2010
6. Stent inserted in the atrial septum to decompress the left atrium 9/11/2008
7. Multiple admissions with the infection/dehydration
8. Renal failure August 2009 - likely related to reaction to Augmentin, requiring forty-eight hours of dialysis
9. Captopril therapy stopped because of renal failure
10. Nasogastric fed
11. Recent admission with exacerbation of cardiac failure.

Summary

005 was diagnosed with neonatal adenoviral myocarditis, spending the first nine months of life hospitalised, following adenoviral meningitis at the age of 8 days; 005 also suffered with candida sepsis.

Secondary to adenoviral infection 005 developed myocarditis; early cardiac function was very poor, necessitating a prolonged period of mechanical ventilation. The left atrial pressure was high resulting in chronic pulmonary oedema, 005 therefore had an atrial septostomy in August 2008 followed by insertion of a stent into the atrial septum (via transhepatic route) 09/11/08 after which 005 began to clinically improve.

005 was referred for transplant assessment in November 2009 at which time an echo showed moderate contractility of the left ventricular septum, lateral wall

and anterior wall. The postero-inferior wall was infarcted, thin and hypokinetic, particularly towards the base. FS was 24%, mild tricuspid regurgitation was present.

005's myocardial function deteriorated over the following weeks, and although quantitative assessment of function was impaired due to the infarction of the posterior left ventricular wall, FS appeared to reduce to 7% (much of the assessment was qualitative at this stage).

005 received long-term pressure support via tracheostomy (inserted 20/09/08) and subsequently weaned from pressure support. The tracheostomy was decannulated in early 2010, although a residual sinus remained.

Following treatment with Augmentin in August 2009 005 had a severe allergic reaction, developing target lesions. He presented to the local hospital at which time acute renal failure was diagnosed. 005 required 48h of dialysis; renal function subsequently normalised.

005 was reviewed regularly at Great Ormond Street by the Heart Failure team and his cardiac function as determined by FS improved (23% August 2010). 005 had an admission to hospital with an exacerbation of cardiac failure in September 2010. His mother reported that 005 was becoming increasingly symptomatic, but subsequently he stabilised. 005's carvedilol was discontinued and captopril commenced which was tolerated well.

005 received TPN initially during his illness but had bilateral chylothoraces requiring chest drains. 005 also developed jaundice and liver dysfunction, attributed to TPN; this resolved on discontinuing TPN. 005 was subsequently fed via NG tube and remained nasogastrically fed (900ml neocate/day) with suspected cow's milk intolerance. In the past 005 had suffered with significant periods of vomiting; although this improved, an aversion to oral feeding remained.

Status on recruitment

005 lived with both parents and two older siblings, his mother was expecting their fourth child.

Neurologically there were no concerns, he was developmentally appropriate for his age. 005 was active, walked well, enjoyed bouncing on the trampoline and had a good quality of life.

On examination 005 seemed reasonably well, he was warm and well perfused. He had mild recession, mild tachypnoea and good bilateral air entry. Heart sounds were normal with no gallop and the liver was palpable 3 cm below the costal margin. Blood pressure measured 114/76 mmHg and heart rate was 98 beats per minute.

Medications

1. Aspirin 37.5mg od
2. Enoximone 10mg tds
3. Captopril 8mg tds
4. Digoxin 50mcg bd

5. Frusemide 10mg tds
6. Spironolactone10mg bd
7. Omeprazole 10mg od

Allergies: augmentin, plasters

Intolerant of cow's milk

Investigation results

Echocardiogram

Date:	**FS%:**	**Comment:**
09/02/10	14%	
03/08/10	23%	Dilated LV with mild-to-moderately impaired systolic function. The LA was dilated with MR, no other valvular regurgitation or stenosis. TR not present, RV systolic function was good. The LV measured 40 mm (Z+4.5) with a systolic dimension of 30. Diastolic function also significantly affected with dilated atria.
26/08/10	21%	

Blood investigations

Chemical pathology

NT-pro-Brain Natriuretic Peptide 1293 pg/ml (29 – 206)

Sodium	136 mmol/L (133 - 146)	Potassium 4.0 mmol/L(3.5 - 5.5)
Urea	6.4 mmol/L (2.5 - 6.0)	Creatinine 36 umol/L (17 - 37)
Calcium	2.35 mmol/L (2.17 - 2.44)	Magnesium 0.89mmol/L (0.66-1.0)
Phosphate	1.39 mmol/L (1.2 - 2.1)	Albumin 33 g/L (34 - 42)
Bilirubin	4 umol/L (<18)	
Alkaline Phosphatase	196 U/L (145 - 320)	

Alanine Transaminase	25 U/L (5 - 45)

Haematology

Blood Group:	A Rh +ve antibody screen negative
Haemoglobin	10.8 g/dL (10.5 - 13.5)
Platelet Count	210 $x10^{-9}$/L (150 - 450)
White Cell Count	5.76 $x10^{-9}$/L (5.0 - 15.0)
Prothrombin Time	11.1 Seconds (9.6 - 11.8)
APTT	36.0 Seconds (26.0 - 38.0)
Thrombin Time	11.7 Seconds (9.2 - 15.0)
Fibrinogen	2.6 g/L (1.7 - 4.0)

Virology

Cytomegalovirus IgG	negative
EBV VCA IgG Antibody	negative
HB surface antigen	negative
HCV	negative
HTLV 1&2	not detected
Measles IgG Antibody	positive
HSVG	negative
Rubella IgG Antibody	354IU/ml
VZV IgG Antibody	negative
HIV	negative
TOXOG	negative
VDRL	negative

4.3 vi Patient 006

Age: 13y10m21d

Weight: 45.8 kg

Height: 148.4 cm

Gender: Male

Diagnosis

1. Duchenne muscular dystrophy with significant cardiac impairment.

Summary

006 was born by vaginal delivery at 36/40 to parents who are first cousins of Egyptian background. There were no perinatal concerns. Developmentally he stood with support at 11-12 months, walked at 14-15 months with some toe-walking, and an abnormal gait. He climbed up stairs at 18-24 months and climbed up stairs one foot per step at 24 months. 006 had one word speech at 2 years and put two words together 3 years.

006 was referred for medical review after his mother had concerns regarding slow speech development. On review at the age of 4y 6m, 006 was noted to have an abnormal gait with occasional toe-walking, mild speech delay and to generally tire easily. On examination 006 had bilateral calf hypertrophy, suggestive of Duchenne Muscular Dystrophy. CK was elevated and muscle biopsy confirmed the diagnosis of Duchenne Muscular Dystrophy, at the milder end of the clinical spectrum for this disorder.

006 was referred for specialist neuromuscular management at Great Ormond Street (The Dubowitz neuromuscular centre, Great Ormond Street Hospital).

006 had progressive Achilles tendon tightening and had bilateral tendo achilles release in October 2005. 006 was poorly compliant with wearing night splints and achilles tightening again became a problem.

In keeping with his diagnosis, 006 had a steady decline of muscle strength. 006 developed problems climbing the stairs and standing from sitting. 006 received physiotherapy and occupational therapy input locally.

Cardiomyopathy was diagnosed in 2004; 006 initially had mildly impaired LV systolic function with inferoposterior hypokinesis. This was initially managed on perindopril locally, but he was subsequently referred to the Heart Failure clinic at Great Ormond Street. 006 was commenced on steroids (prednisolone) in 2007.

006 was otherwise well, with no episodes of chest infections or nocturnal hypoventilation.

Status on recruitment

006 lived with both parents and an older brother. He was in Year 9 at school. He enjoyed attending school and was able to use a lift there instead of climbing stairs. Unable to participate in sports lessons, 006 used this time to do stretching exercises. 006 had a statement of special educational needs.

006 and his family were referred to a psychologist to help them cope with the diagnosis. 006 was unaware of his diagnosis at the start of the study.

At thirteen years old 006 was reasonably well with generally good skeletal muscle strength. 006 did not require the wheelchair provided and did not have any symptoms of cardiac failure. 006 walked independently but became tired after about 15 minutes, he would fall about twice a week, and suffered with back discomfort relieved by rest. 006 was able to ride a bike, kick a ball and swam once a week. On examination he was clinically otherwise well, his heart sounds were normal, there was no murmur.

Medications

1.	Perindopril	4mg od
2.	Carvedilol	3.125mg od started 12/11/10
3.	Prednisolone	25mg od 10/7 on 10/7 off
	Allergies:	Nil known

Investigation results

Echocardiogram

Date:	**FS%:**	**Comment:**
12.12.10	15%	Poor left ventricular systolic function with a mildly dilated left ventricular cavity. Left ventricular end diastolic diameter was 51 (Z +2.6). The images were difficult but show deterioration in cardiac function.

Lung Function 23/11/10

FEV1	1.77L	77% predicted- possibly underestimated
FVC	2.26L	82% predicted- possibly underestimated
FEV1 % FVC	78.67	92% predicted
PEF	3.73 L/s	77% predicted

Bloods investigations

Chemical pathology

NT-pro-Brain Natriuretic Peptide 215 pg/ml (29 – 206)

Sodium	140 mmol/L (133 – 146)	Potassium	3.9 mmol/L (3.5 - 5.5)
Urea	3.4 mmol/L (2.5 - 7.5)	Creatinine	31 umol/L (37 – 71)
Calcium	2.47 mmol/L (2.19 - 2.66)	Magnesium	0.95 mmol/L (0.70 - 0.95)
Phosphate	1.50 mmol/L (1.1 - 1.75)	Albumin	43 g/L (37 – 56)
Bilirubin	<2 umol/L (<18)		
Alkaline Phosphatase	115 /L 130 - 525		
Alanine Transaminase	225 U/L 10 - 45		

Haematology

Blood Group:	B Rh +ve no antibodies
Haemoglobin	13.9 g/dL (13.0 - 16.0)
Platelet Count	373 $x10^{-9}$/L (150 – 450)
White Cell Count	9.54 $x10^{-9}$/L (4.0 - 11.0)
Prothrombin Time	10.6 Seconds (9.6 - 11.8)
APTT	33.6 Seconds (26.0 - 38.0)
Thrombin Time	11.2 Seconds (9.2 - 15.0)
Fibrinogen	3.5 g/L (1.7 - 4.0)

Virology

Cytomegalovirus IgG	negative
EBV VCA IgG Antibody	negative
HB surface antigen	negative
HCV	negative
HTLV 1&2	not detected
Measles IgG Antibody	positive

HSVG	positive
Rubella IgG Antibody	69 IU/mL
VZV IgG Antibody	positive
HIV	negative
Toxoplasma	negative
VDRL	negative

4.3 vii Patient 007

Age:	3y9m12d
Weight:	15.03 kg 09/11/10
Height:	96 cm 09/11/10
Gender:	Female

Diagnosis

1. Familial dilated cardiomyopathy, MYH7 gene isolated

Summary

007 was diagnosed with cardiomyopathy in December 2008 after presenting to a GP with wheeze and cough. As symptoms were not resolving a chest XR was performed which revealed cardiomegaly and 007 was referred for local cardiology review. At the time of referral LV function was quite impaired with significant LV dilatation. LV diastolic dimension 5.6cm, FS <10%. Medical management stabilised 007's condition, symptomatically she remained reasonably well on therapy.

Two other members of 007's family had been diagnosed with cardiomyopathy. 007's father and aunt were both under review with cardiologists locally. All three screened positive for MYH7 gene. 007's father was diagnosed with non-compaction of the left ventricle and underwent ICD insertion in March 2010.The aunt had been enrolled in the adult stem cell study in London.

Status on recruitment

007 was clinically well, attending nursery three days per week. On examination she was warm and well perfused, had normal first and second heart sounds with 3/6 systolic murmur, especially intense in the apex, which radiated all over the chest. There was no gallop rhythm and the chest was clear with no added sounds. Her abdomen was soft and non-tender with no hepatomegaly. 007 was not tachypnoeic or tachycardiac and looked well, she had no oedema and her JVP was not raised.

Medications

1. Lisinopril 5 mg od
2. Aspirin 75 mg od
3. Carvedilol 3.5 mg bd

Allergies: nil

Investigation results

Echocardiogram

Date:	**FS%:**	**Comment:**
11.2009	13%	Mildly dilated LA, severely dilated LV, severely impaired LV systolic function, 'rocking' motion

		myocardium, no obvious EFE on echo, normal appearance of papillary muscles
11.2010	14%	Moderate MR secondary to poor coaptation, dilated left ventricle with depressed LV function but synchronous; moderate to severe MR. Normal mitral inflow flow; no significant TR, no aortic regurgitation or pulmonary regurgitation and good flow was seen in LVOT and PA branches.

Blood investigations

Chemical pathology

NT-pro-Brain Natriuretic Peptide: 99 pg/ml (29 – 206)

Sodium	138 mmol/L (133 - 146)	Potassium	3.7 mmol/L (3.5 - 5.5)
Urea	3.8 mmol/L (2.5 - 6.0)	Creatinine	20 umol/L (20 - 39)
Calcium	2.34 mmol/L (2.22 - 2.51)	Magnesium	0.67 mmol/L (0.66 - 1.00)
Phosphate	1.77 mmol/L (1.2 - 1.8)	Albumin	45 g/L (35 - 52)
Bilirubin	4 umol/L (<18)		
Alkaline Phosphatase	251 U/L (150 - 380)		
Alanine Transaminase	29H U/L (10 - 25)		

Haematology

Blood Group:	O Rh +ve antibody screen negative
Haemoglobin	12.1 g/dL (11.5 - 14.5)
Platelet Count	317 $x10^{-9}$/L (150 - 450)
MPV	9.3 fL (7.8 - 11.0)
White Cell Count	22.05 $x10^{-9}$/L (5.0 - 15.0)

Comments: Neutrophil Leucocytosis

Prothrombin Time	10.8 Seconds (9.6 - 11.8)
APTT	36.5 Seconds (26.0 - 38.0)
Thrombin Time	11.2 Seconds (9.2 - 15.0)
Fibrinogen	2.4 g/L (1.70 - 4.0)

Virology

Cytomegalovirus IgG	negative
EBV VCA IgG Antibody	positive
HB surface antigen	negative
HCV	negative
HTLV 1 & 2	not detected
Measles IgG Antibody	positive
HSVG	equivocal
Rubella IgG Antibody	159 IU/ml
VZV IgG Antibody	negative
HIV	negative
TOXOG	negative
VDRL	negative

4.3 viii Patient 008

Age:	2y5m25d
Weight:	11.3 kg on 21/12/10 (9^{th} centile)
Height:	87 cm (25^{th} centile)
Gender:	Male

Diagnosis:

1. Dilated cardiomyopathy- suspected post viral

Summary

008 presented to hospital at the age of 9 weeks with breathlessness following a one-week history of upper respiratory tract infection. Initially 008 was thought to have bronchiolitis, but a chest x-ray demonstrated cardiomegaly and an echocardiogram confirmed poor function due to a dilated left ventricle. 008 was transferred for further management at Great Ormond Street Hospital after stabilisation with diuretics.

On admission, 008 was tachycardic, pale, blue lipped and quiet. His liver was 2 cm below the costal margin. 008 had an apical pansystolic murmur. The chest was clear on auscultation. Echocardiogram confirmed the diagnosis of left ventricular dysfunction and dilatation. The coronary artery origins were seen with normal flow. The arch was patent and there was no coarctation. The rest of the scan was normal.

In view of his clinical condition, 008 was managed on the Intensive Care Unit with oxygen, diuretics, and intravenous milrinone. Following the introduction of captopril, milrinone was weaned and care continued on the cardiology ward. A full series of investigations were performed to ascertain the aetiology of the cardiomyopathy; the differential diagnoses included myocarditis or a primary inherited or metabolic cardiomyopathy. No positive viral cultures or positive PCR investigations for common cardiomyopathy inducing viral infections were obtained. 008 was discharged on furosemide 6 mg tds, spironolactone 6 mg bd, captopril 2 mg tds, ranitidine 6 mg bd, and aspirin 27.5 mg a day. Captopril was increased to 2.5 mg per dose and later carvedilol was started at 50 mcg/kg/dose twice a day.

008 was subsequently reviewed locally and at Great Ormond Street over the following two years; cardiac function remained consistently poor despite optimal medical management. 008 had poor growth and weight gain despite dietician involvement, he required early NG feeding support.

Status on recruitment

008 lived at home with both parents and five year old brother. Cardiac function on echo remained poor and echocardiographic appearances showed that the heart had become more dilated, although he remained apparently completely asymptomatic and had reasonable exercise tolerance. Feeding and growth had also stabilised. On examination, blood pressure was 74/33, oxygen saturation 100% in air. Heart sounds were normal with no murmurs, and there was no gallop rhythm. Chest was clear and liver was not enlarged.

Medications

1. Captopril 5 mg tds.
2. Spironolactone 11 mg od
3. Furosemide 11 mg bd
4. Digoxin 50 mcg od
5. Aspirin 60 mg od
6. Clarithromycin prophylaxis 250mg od
7. Carvedilol discontinued

Allergies: nil known

Investigations

Echocardiogram

Date:	FS%:	Comment:
21.12.10	13.5%	Cardiac function poor, heart more dilated; severely dilated left ventricle with impaired left ventricular systolic function. Moderate-to-severe mitral regurgitation and a very globular appearance of the left ventricle. The left atrium seemed to be increasing in size most likely related to worsening mitral regurgitation. The left ventricular end diastolic diameter measured 53.2 (Z +14.1)

Blood investigations

Chemical pathology

NT-pro-Brain Natriuretic Peptide 518 pg/ml (29 – 206)

Sodium	134 mmol/L (133 -146)	Potassium 4.4 mmol/L (3.5- 5.5)
Urea	6.7 mmol/L (2.5 - 6.0)	Creatinine 24 umol/L (17 - 37)
Calcium	2.28 mmol/L (2.17 - 2.44)	Magnesium 0.96mmol/L(0.6-1.0)
Phosphate	1.46 mmol/L (1.2 - 2.1)	Albumin 35 g/L (34 - 42)

Haematology

Haemoglobin	10.1 g/dL (10.5 - 13.5)
Platelet Count	219 $x10^{-9}$/L (150 - 450)
MPV	9.0 fL (7.8 - 11.0)
White Cell Count	5.23 $x10^{-9}$/L (5.0 - 15.0)
Prothrombin Time	10.9 Seconds (9.6 - 11.8)
APTT	34.7 Seconds (26.0 - 38.0)

Thrombin Time	11.5 Seconds (9.2 - 15.0)
Fibrinogen	1.8 g/L (1.7 – 4.0)

Virology

Cytomegalovirus IgG	negative
EBV VCA IgG antibody	negative
HB surface antigen	negative
HCV	negative
HTLV 1 & 2	negative
Measles IgG antibody	positive
HSV 1 & 2	negative
Rubella IgG	60 IU/mL
VZV IgG antibody	negative
HIV	negative
TOXOG	not detected

4.3 ix Patient 009

Age:	9y0m12d
Weight:	27.45kg 07/03/2011
Height:	132.6cm 07/03/2011
Gender:	Male

Diagnoses:

1. Enteroviral myocarditis aged seven days with prolonged intubation and ventilation
2. Septal infarct thought to be secondary to hypotension during myocarditis
3. Subsequent subglottic stenosis requiring tracheostomy

4. Laryngotracheal reconstruction and decannulation 2003
5. Adenoidectomy
6. Supraglottoplasty September 2004
7. Division of aryepiglottic folds and arytenoid mucosal trimming 2009

Summary

009 suffered with neonatal enteroviral myocarditis requiring prolonged intubation and ventilation. During the course of the acute myocarditis 009 had a period of hypotension resulting in a septal infarct (subsequent coronary angiography confirmed normal coronary arteries and anatomy supporting the diagnosis of septal infarct secondary to hypotension.)

As a result of prolonged intubation, 009 suffered with subglottic stenosis requiring a tracheostomy to be formed in 2002. 009 had numerous ENT reviews with several operative procedures to optimise his airway.

Status on recruitment

009 remained under the care of respiratory and ENT consultants for his airway and respiratory management. Respiratory symptoms were unchanged, stridor on exertion persisted. His sleep study was normal although no episodes of stridor were noted during the reading. Respiratory function tests were stable (2010 results FEV1 64% FVC 69%).

Cardiac function remained stable and 009 has reasonable exercise tolerance. On examination his heart sounds were normal, no murmurs, no hepatomegaly and no other obvious signs of heart failure.

Medications

1.	Lisinopril	10mg od
2.	Carvedilol	9.3mg bd
3.	Spironolactone	12mg bd
	Allergies:	Ametop

Investigation results

Echocardiogram

Date:	FS%:	Comment:
26/09/2007	26%	mildly impaired LV function, LV dilatation, LVEDd 46.9mm, LVESd 34.8mm
25/03/2008	15%	Dilated LV and LA, mild to mod systolic dysfunction, mild MR, LVEDd 46.5mm
17/08/10	28%	Dilated LV, reasonable function, mild MR, small perimembranous VSD with L→ R shunt, dilated LA
22/02/11	18%	Mild MR, dilated LA, mod LV dilatation LVEDd 46.8, LVESd 38.2
19/04/2011	20%	Dilated LV, EDd 46mm, LA 3.9cm, bright area in anterior septum of old infarct scarring which is akinetic.

Coronary angiography

12/06/06: under GA, no complications, normal coronary arteries and anatomy

Blood investigations

Chemical pathology

NT-pro-Brain Natriuretic Peptide 442 pg/ml (29 – 206)

Sodium	136 mmol/L (133 - 146)	Potassium 4.2 mmol/L (3.5- 5.5)
Urea	6.9 mmol/L (2.5 - 6.0)	Creatinine 40 umol/L (30 - 57)
Calcium	2.23 mmol/L (2.22 - 2.51)	Magnesium0.83mmol/L(0.6-1.0)
Phosphate	1.60 mmol/L (1.20 - 1.8)	Albumin 37 g/L (37 - 56)

Total bilirubin 3 umol/L (<18)

Alkaline Phosphatase	159 U/L (135 - 530)
Alanine Transaminase	18 U/L (10 - 35)

Haematology

Blood Group:	A Rh +ve
Haemoglobin	10.7 g/dL (11.5 - 15.5)
Platelet Count	263 $x10^{-9}$/L (150 - 450)
White Cell Count	5.08 $x10^{-9}$/L (4.5 - 13.5)
Prothrombin Time	11.2 Seconds (9.6 - 11.8)
APTT	29.8 Seconds (26.0 - 38.0)
Thrombin Time	12.1 Seconds (9.2 - 15.0)
Fibrinogen	2.0 g/L (1.7 - 4.0)

Virology

Cytomegalovirus IgG	negative
EBV VCA IgG Antibody	negative
Hepatitis B surface antigen	negative
HCV antibody	negative
HTLV 1 & 2	negative
Measles IgG Antibody	positive

HSVG	negative
Rubella IgG Antibody	98 IU/ml
HIV	negative
TOXOG	negative
VDRL	negative

4.3 x Patient 010

Age:	3y9m22d
Weight:	16 kg
Height:	102.7 cm
Gender:	Female

Diagnoses

1. Dilated cardiomyopathy
2. Afebrile seizures

Summary

010 was diagnosed with dilated cardiomyopathy at the age of six months (June 2008) after presenting to a local hospital with increasing respiratory distress, fever and vomiting. 010 was transferred to the Evelina PICU where Vitamin D deficiency and hypocalcaemia were noted. At presentation fractional shortening was 16% on echo. 010 was discharged seven days later on frusemide, captopril, aspirin, Calcium-Sandoz and Vitamin D. Cardiomyopathy screen at the time indicated enteroviral IgM but no other abnormalities. 010 was re-admitted six weeks later to the local hospital with a further episode of

respiratory distress and was transferred to CICU at Great Ormond Street where IV milrinone was commenced. FS at this time was approximately 10%. 010 gradually stabilised and was discharged home two weeks later. 010 was subsequently under review by the Heart Failure team.

010 overcame early feeding and growth difficulties that required prolonged NG feeding, to subsequently feed normally with reasonable growth. The diagnosis of vitamin D deficiency related cardiomyopathy was queried as despite the Vitamin D and calcium levels stabilising with supplementation, significant cardiac impairment persisted. 010 was classified as an idiopathic cardiomyopathy.

010 experienced two generalised afebrile seizures, the first in August 2010 the second in October. The seizure in October was prolonged lasting 45 minutes and was treated with two doses of rectal diazepam. The seizures described by her mother appeared to be related to falling asleep. EEG results were consistent with a focal onset seizure, a prominent abnormality was seen over the left temporoparietal region. 010 was commenced on oral sodium valproate and remained under local neurology review. A unifying diagnosis may link 010's cardiomyopathy and seizures.

Status on recruitment

010 was reported by her mother to be reasonably active, although tired easily. 010 was eating and growing well. Her mother felt that there was no difference in exercise capacity and her cardiac symptoms were stable. 010 lived with her parents, one year old sister and nine year old brother.

On clinical examination her heart rate was 91bpm, oxygen saturation 100% in air, blood pressure 91/62mmHg. 010 was warm and well perfused, with normal capillary refill time. She had no oedema. Her heart sounds were normal with a 3/6 ejection systolic murmur best heard at the apex but radiated all over the precordium, the chest was clear, abdomen was soft with no hepatomegaly

Medications

1.	Captopril	7 mg tds
2.	Aspirin	70 mg once day
3.	Carvedilol	6.25 mg twice a day
4.	Spironolactone	14mg bd
5.	Azithromycin	200mg od Mon/ Wed/ Fri
6.	Sodium Valproate	100mg bd
7.	Frusemide	10mg bd

Allergies: Nil known

Investigation results

Echocardiogram

Date:	**FS%:**	**Comment:**
23.03.11	19%	Echo stable showing a grossly dilated LV and LA with LV systolic impairment. There was moderate to severe MR as previously, trace of PR and left ventricle Z-score in diastole today was +8.03 and left ventricle systolic Z-score was +11.3.

19.04.11	19%	Dilated LV with reduced systolic function. LV diastolic dimension 59.4mm. LV systolic dimension 48.2mm

Blood investigations

Chemical pathology

NT-pro-Brain Natriuretic Peptide: 780 pg/ml (29 – 206)

Sodium	145 mmol/L (133 - 146)	Potassium	5.1 mmol/L (3.5 - 5.5)
Urea	2.2 mmol/L (2.5 - 6.0)	Creatinine	35 umol/L (20 - 39)
Calcium	2.52 mmol/L (2.22 - 2.51)	Magnesium	0.96 mmol/L (0.66 - 1.00)
Phosphate	1.49 mmol/L (1.2 - 1.8)	Albumin	47 g/L (35 - 52)

Total bilirubin 9 umol/L (<18)

Alkaline Phosphatase	202 U/L (135 - 530)
Alanine Transaminase	12 U/L (10 - 35)

Haematology

Haemoglobin	11.3 g/dL (11.5 - 14.5)
Platelet Count	246 $x10^{-9}$/L (150 - 450)
White Cell Count	4.63 $x10^{-9}$/L (5.0 - 15.0)
Prothrombin Time	11.4 Seconds (9.6 - 11.8)
APTT	34 Seconds (26.0 - 38.0)
Thrombin Time	12.4 Seconds (9.2 - 15.0)
Fibrinogen	2.0 g/L (1.7 - 4.0)

Virology

Cytomegalovirus IgG	positive
EBV VCA IgG antibody	positive
Hepatitis B surface antigen	negative

Hepatitis C antibody	negative
HTLV 1 & 2 antibody	not detected
Measles IgG antibody	positive
Herpes simplex virus 1 & 2 IgG	negative
Rubella IgG antibody	>400 IU/ml
VZV IgG antibody	positive
HIV 1&2 antibody by EIA	negative
TOXOG	antibody not detected
VDRL	negative

CHAPTER 5

RESULTS 2: SECONDARY END POINTS

5.1 Introduction

This chapter describes the assessment of the secondary end points of this study. The secondary end points of ejection fraction (EF), left ventricular (LV) end diastolic volume (EDV), LV end systolic volume (ESV), LV mass, stroke volume (SV), cardiac output (CO) and aortic (Ao) flow were measured using standard techniques from the MRI images. Anonymised MRI images were evaluated blindly at the end of the study to eliminate bias that may otherwise have interfered with the scientific significance of the data collected.

5.2 MRI image acquisition

Each patient underwent a cardiac MRI under general anaesthetic except patient 006 who as previously discussed had his procedures performed under sedation. Continuity of anaesthetic protocol permitted better reproducibility for analysis of cardiovascular parameters on MRI.

The MRI scans were performed on a 1.5-T MR scanner (Avanto, Siemens, Erlangen, Germany) using a 12-element phased-array coil for signal reception and the body coil for signal transmission. A vector ECG system was used for cardiac gating. Multi-slice black blood sequences were used in all but one of the patients. The single patient that did not have the multi-slice black blood imaging instead had a real-time radial k-t sensitivity encoding sequence used instead; a different protocol was required for this patient due to the presence of clinically significant cardiac arrhythmias that would not permit reliable evaluation with the multi-slice black blood protocol. For all imaging the patients were placed supine in the scanner and the ICH heart protocol was used. An ECG gated breath-hold protocol was used to obtain the vertical long axis (two chamber) and horizontal

long axis (four chamber) images that were then used to identify the cardiac short axis. The whole heart was then imaged in the short axis plane from ventricular apex to base using steady-state free precession (SSFP) cines. The data acquisition slices varied depending on the size of the patient and were 5 mm, 6mm or 8 mm. All cardiac MRI images used for the study were acquired using 10 to 15 second breath-holds, achieved by the anaesthetist, and data were stored digitally for later off-line analysis. Off-line analysis consisted of measurement of cardiac volumes, mass and function. Data were stored securely within the hospital's computer system for clinical use and were also stored in an anonymised form for research purposes. Patient identifiers were removed from the anonymised images to allow unbiased analysis later on. Data stored on computer databases were stored under password protection.

The cardiac MRI scans were evaluated and reported as any other clinical scan, as per hospital protocol, shortly after acquisition of the data, by cardiac MRI physicians. Clinical data were reported and stored in the same manner as any other clinical scan, to allow medical review or input as required or deemed in the best interest of the patients.

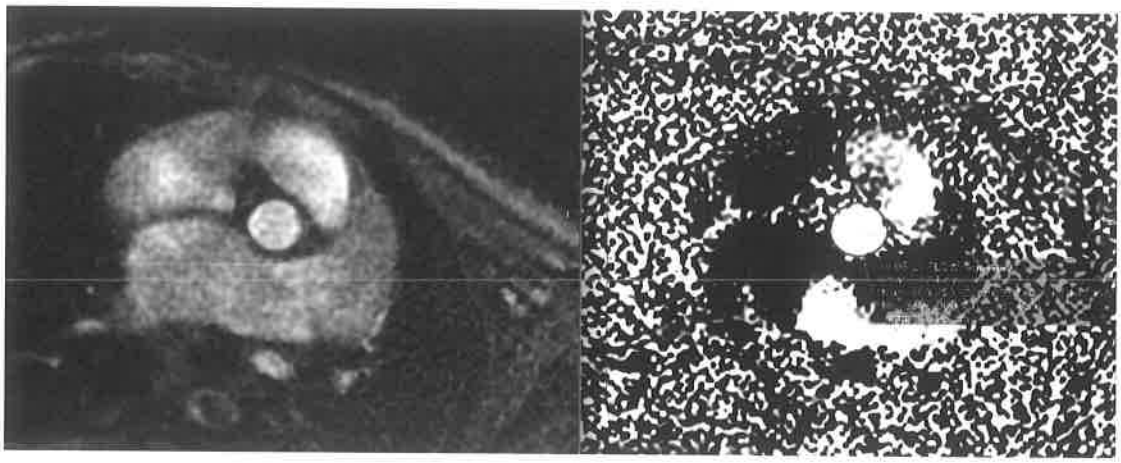
The cardiac MRI scans were also stored with patient identifiers removed from them. This was to permit the researcher to analyse the data blinded to any patient history or information. In this way unbiased image analysis was performed. All image processing was performed using in-house plug-ins for the open-source Osirix DICOM (digital imaging and communications in medicine) software. Off-line analysis of the data was performed using Osirix software on either a clinical workstation or the Research Fellow's own laptop. The

researcher received training in the use of the Osirix software and on the measurement of the cardiac indices required for the study. As a second check of these data the researcher's results were evaluated, again blind to patient identifiers, by a Consultant in Cardiac MRI radiology. The key to the blinding of the MRI data was devised, applied and stored securely by an MRI technician not otherwise involved in the study. On completion of the MRI analyses the study was un-blinded and the data obtained were linked to the correct research patient for review.

5.3 Measurement of aortic flow

Aortic flow was measured by analysing concurrently the aortic flow cine image and the phase contrast aortic flow image (Figure 5.1). Both MRI image sequences were viewed at the same time using the 2D viewer. The aortic circumference was traced using the drawing tool in the Osirix software. The highlighted circumference of the aorta, or the region of interest (ROI) was saved and then propagated to all other aortic images in the same sequence. The propagated aortic circumferences were intended to highlight the aorta as the ROI. The MRI images were checked on the cine sequence to ensure that the circumference of the aorta was accurately determined. Using the Osirix plug-in to determine quantification of each ROI in the sequence, the phase contrast images were used to measure aortic flow.

Figure 5.1: Concurrent views of the aorta in both cine and phase contrast images with the aortic circumference highlighted in blue



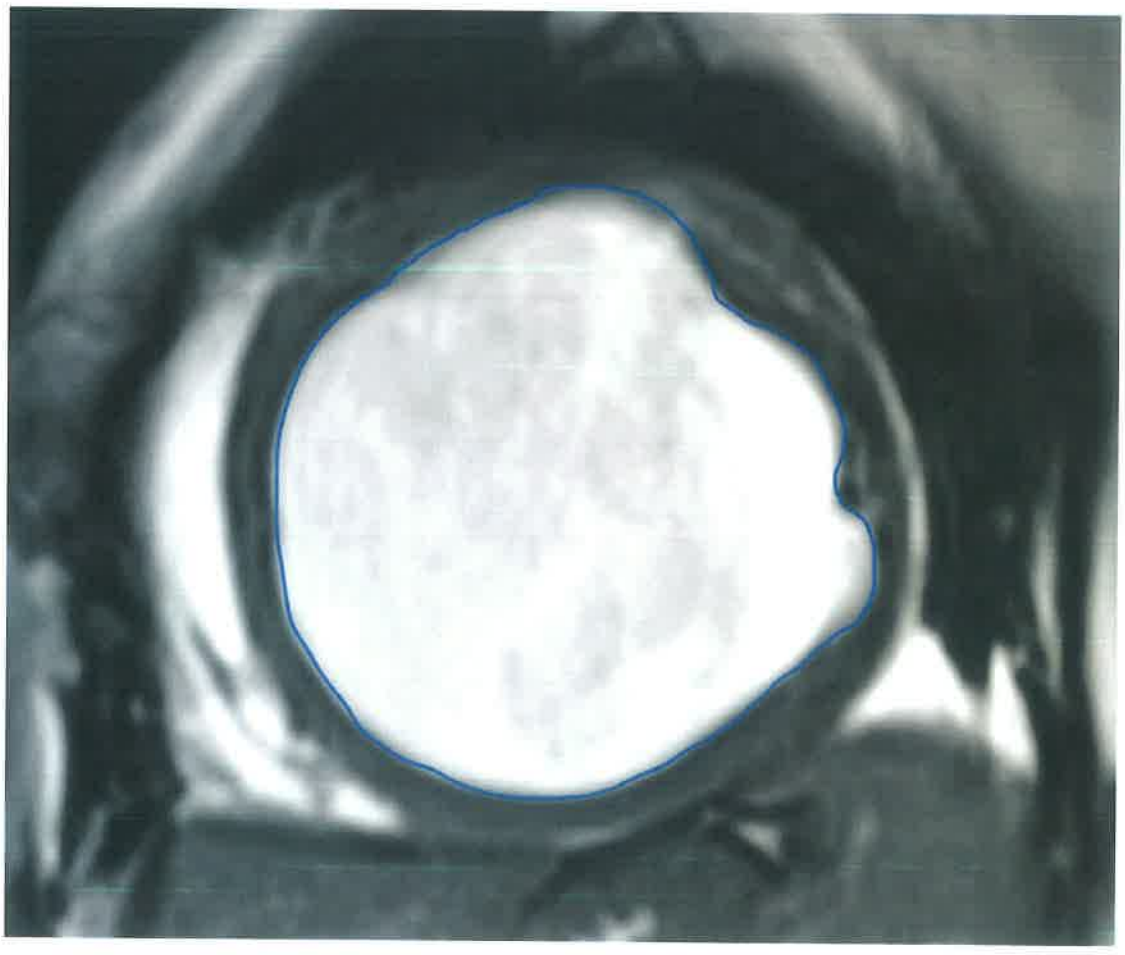
5.4 Measurement of LV indices

Left ventricular (LV) volumes were measured from contiguous short-axis steady state free precession cines covering both ventricles (7–13 slices, depending on the size of the child). Each slice was acquired in a single 10 to 15 second breath hold as previously described. The long axis views were utilised to plan the short axis "slices" that were used to analyse LV volumes and mass. The LV end-diastolic volume (EDV) and end-systolic volume (ESV) were measured by manual segmentation of the endocardial borders in the short-axis data. Careful segmentation of the basal slices in conjunction with 4-chamber and LV long-axis views were performed to overcome problems with delineating the mitral valve. The cardiac images or segments were taken 5 to 8mm apart along the long axis of the heart. By tracing around, or contouring, the cardiac borders to be assessed using the drawing tool in Osirix the volume of that cardiac parameter was calculated for each individual slice. Simple summation of these slice volumes allowed the total volume to be calculated depending on the contour drawn

5.4.i Measurement of LV EDV

The LV EDV is the maximum filled volume of the LV immediately prior to the onset of systole. The end diastolic phase of the cardiac cycle was determined by watching the cine images in the 4D viewer of the Osirix software. End diastole was identified when the mitral valve had just closed. The endocardial contour, the innermost cardiac muscle layer of the LV, excluding the papillary muscles and trabeculae, was traced in diastole from the most apical to the most basal slice of the short axis image stack of segments (Figure 5.2). The area contoured, and hence volume when each slice width was applied, was calculated. Summation of the endocardial volume in diastole of the left ventricle produced the total LV volume in diastole. The LV outflow tract was also included in the endocardial contouring.

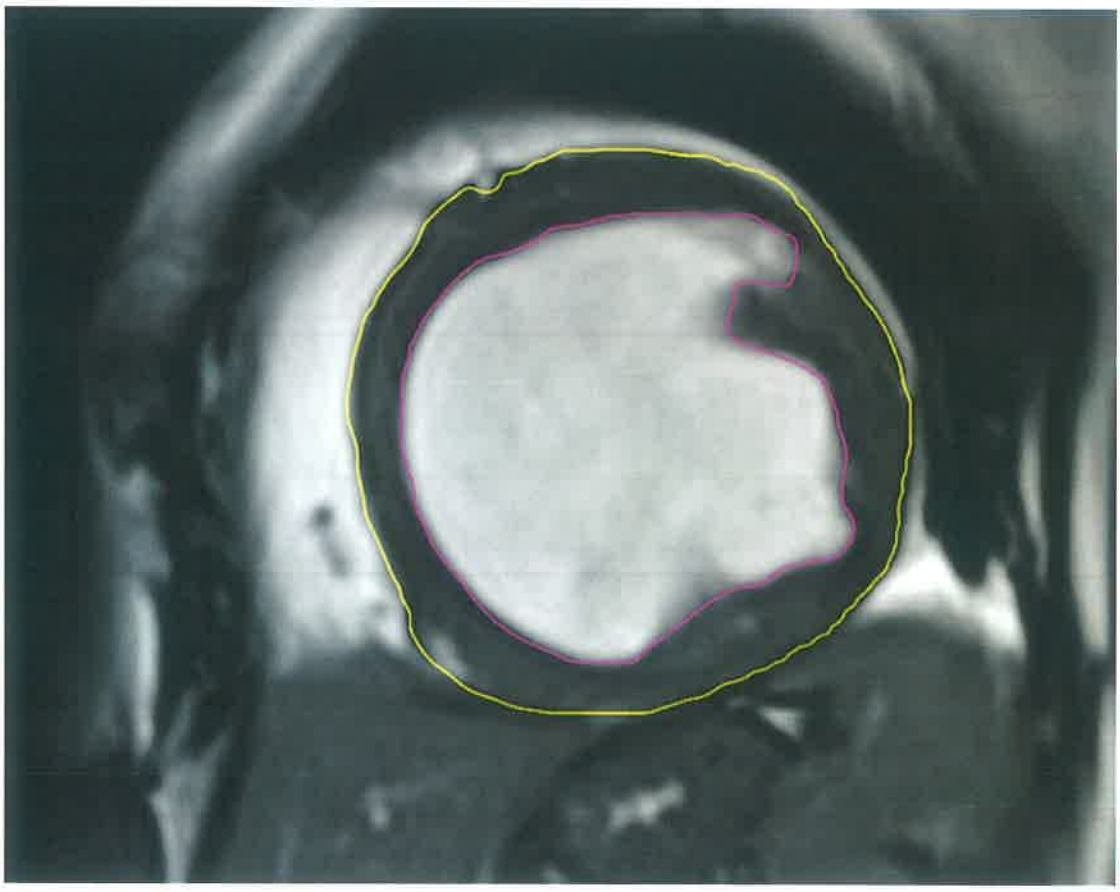
Figure 5.2: LV in diastole: blue contouring of endocardium.



5.4 ii Measurement of LV ESV

The LV ESV is the residual volume of the LV at the extreme of systole, after maximum ejection. The end systolic phase was determined by watching the cine images in the 4D viewer of the Osirix software. End systole was identified at maximal myocardial contraction and closure of the aortic valve. The endocardial contour, the innermost cardiac muscle layer of the LV, excluding the papillary muscles and trabeculae, was traced in systole from the most apical to the most basal slice of the short axis image stack of segments (Figure 5.3). The area contoured, and hence volume when each slice width was applied, was calculated. Summation of the endocardial volume in systole of the left ventricle produced the total LV volume in systole. The LV outflow tract was also included in the endocardial contouring.

Figure 5.3: LV in systole: yellow contouring of the epicardium, pink contouring of the endocardium.



5.4.iii Measurement of LV myocardial mass

The LV epicardium, the outermost layer of cardiac tissue surrounding the LV, was contoured on each segment during systole. The epicardial contour included the LV septum and had to encompass the endocardial contours if both epicardial and endocardial contours were visible on the same segment. The LV mass was calculated by Osirix by subtraction of the endocardial volume from the epicardial volume. The resulting volume after multiplication with the specific density of myocardium, 1.05 g/cm^3, provided the myocardial mass.

5.5 Calculation of stroke volume, cardiac output and ejection fraction

Stroke volume, cardiac output and ejection fraction were calculated from the data obtained on segmentation of the MRI scans. The UCL plug-in performed measurement of the required variables based on the data obtained on image analysis.

LV Stroke volume (SV):

The LV stroke volume is the difference between the LV EDV and LV ESV and is the amount of blood that the left ventricle pumps out in a single contraction.

$$SV=EDV-ESV$$

Cardiac output (CO):

The amount of blood that the heart pumps out in one minute is called the cardiac output and is the product of the stroke volume and the heart rate.

$$CO= SV \times HR$$

LV Ejection fraction (EF):

The LV EF is the proportion of blood pumped out of the LV on each contraction, expressed as a proportion of the LV maximal volume, the LV EDV. It is usually measured as a percentage of the LV EDV. A normal value for LV EF is considered to be 55-70%.

$$\text{LV EF} = \frac{\text{LV EDV} - \text{LV ESV}}{\text{LV EDV}} \times \frac{100}{1} = \frac{\text{LV SV}}{\text{LV EDV}} \times \frac{100}{1}$$

The results obtained were checked by a Consultant Radiologist specialising in paediatric cardiac MRI who was also blinded to patient identifiers and clinical history.

5.6 Results summary

For comparison of the data only indexed values were used for the cardiac indices evaluated. Indexed values corrected the data for body surface area and permitted true comparisons to be made between the series of studies of the same individual and between different individuals. Even within an individual, the series of three MRI scans taken over the course of one year would not be directly comparable due to the fact that the patient's parameters such as growth would have altered over that time. Such variability is more significant between individuals, hence indexed values calculated by the Osirix software were considered more significant for assessment.

For each of the secondary end points, EDV, ESV and EF, comparisons between treatments (Table 5.1) were made on an intention to treat basis. A crossover analysis on post treatment measures was conducted using an analysis of variance model, considering sequence, period and treatment effects.

Carry-over effect was tested first and where there was no evidence of a significant effect, the treatment effect was tested. A subsequent generalized estimating equation (GEE) model was fitted which allowed for an adjustment for baseline measures.

Where the distribution of the data was clearly non-Normal we used a logarithmic transformation. Stata was used for all statistical analyses and all tests were two-sided. A P value < 0.05 was considered significant.

Table 5.1: Actual cell number injected into the left main coronary artery of each patient. (Mean cell count 51.65 x 10^6; * patient with Duchenne Muscular Dystrophy)

Patient number	Number of cells
001	115.1 x 10^6
002	34.7 x 10^6
003	11.8 x 10^6
004	31.5 x 10^6
005	34.2 x 10^6
006*	26.9 x 10^6
007	64.5 x 10^6
008	73.5 x 10^6
009	35.5 x 10^6
010	88.8 x 10^6

Table 5.2: Secondary outcome measures before and after stem cell injection:

	Before stem cells				After stem cells			
Patient number	**NT pro BNP**	**EDV**	**ESV**	**EF**	**NT pro BNP**	**EDV**	**ESV**	**EF**
001	1626	130.9	86.23	34	1934	124.29	80.31	35
002*	6285	90.55	52.87	42	4055	76.48	47.36	38
003*	699	92.14	46.9	49	989	92.38	57.43	38
004*	542	81.33	43.69	46	2389	77.73	41.76	46
005*	1293	109.83	74.09	33	1912	111	76.82	31
006	302	118.5	81.68	31	399	138.73	101.57	27
007	180	142.43	81.93	42	74	128.29	79.84	38
008*	518	283.78	165.59	42	403	275.73	173.41	37
009	419	99.58	57.33	42	557	92.14	43.65	53
010	300	162.46	106.89	34	22	122.12	69.29	43

Table 5.3: Secondary outcome measures before and after placebo injection:

	Before placebo				After placebo			
Patient number	**NT pro BNP**	**EDV**	**ESV**	**EF**	**NT pro BNP**	**EDV**	**ESV**	**EF**
001	860	115.35	73.42	36	1626	130.9	86.23	34
002*	4055	76.48	47.36	38	4340	79.72	45.75	43
003*	989	92.38	57.43	38	531	89.39	48.98	45
004*	2389	77.73	41.76	46	1005	79.71	45.53	43
005*	1912	111	76.82	31	4194	125.41	89.61	29
006	215	100.41	50.33	50	302	118.5	81.68	31
007	99	130.88	83.36	36	180	142.43	81.93	42
008*	403	275.73	173.41	37	300	254.06	166.62	34
009	442	110.96	58.58	48	419	99.58	57.33	42
010	780	169.84	98.47	42	300	162.46	106.89	34

*patients that received stem cell arm of study prior to placebo

As noted in Chapter 4, nine patients completed the original protocol (Tables 5.2 and 5.3), the tenth patient had Duchenne muscular dystrophy and as general anaesthesia was deemed to carry a significantly increased risk for him he completed an otherwise identical study design under sedation. This deviation from the original study protocol resulted in his data being excluded from the subsequent secondary end point analysis (patient reference 006 excluded).

Table 5.4: Summary of secondary outcome measures before and after stem cell intra-coronary injection*:

	Before stem cells				After stem cells			
	NT pro BNP (pg/ml)	**EDV (ml)**	**ESV (ml)**	**EF (%)**	**NT pro BNP (pg/ml)**	**EDV (ml)**	**ESV (ml)**	**EF (%)**
Mean	1318	132.6	79.5	40	1371	122.24	74.31	40
Range	180-6285	81.33-283.78	43.69-165.59	33-49	22-4055	77.73-275.73	41.76-173.41	46-53

Table 5.5: Summary of secondary outcome measures before and after placebo intra-coronary injection*:

	Before placebo				After placebo			
	NT pro BNP (pg/ml)	**EDV (ml)**	**ESV (ml)**	**EF (%)**	**NT pro BNP (pg/ml)**	**EDV (ml)**	**ESV (ml)**	**EF (%)**
Mean	1325	128.9	78.96	39	1433	129.3	80.99	38
Range	99-4055	76.48-275.73	41.76-173.41	31-48	180-4340	79.71-254.06	45.53-166.62	29-45

* Patient with Duchenne Muscular Dystrophy excluded from statistical analyses

For the secondary end points EDV, ESV and EF there was no evidence of carryover effect or period effect (Tables 5.4 and 5.5).

For EDV and ESV results within +/- 3% difference were assessed as unchanged. Indexed EDV 6 months post cell infusion (for n=9) was reduced in 6 patients and unchanged in 3 (Figure 5.4, Tables 5.3 and 5.6); EDV increased in 4 patients following placebo (Figure 5.4, Table 5.3 and 5.7). The ratio of the geometric means for treatment effect, adjusting for baseline = 0.93, 95% CI (0.88, 0.99), P = 0.01, indicating EDV was on average 7% lower in patients after stem cell treatment compared with time after placebo. EDV reduced in all patients with an injected cell count >34.2 x 10^6. ESV decreased or stayed the same in 6 patients following cell injection (Figure 5.5, Tables 5.3 and 5.6). The ratio of geometric means for treatment effect, adjusting for baseline = 0.90, 95% CI (0.82, 1.00), P = 0.05, indicating ESV was on average 10% lower after stem cell treatment compared with placebo.

Table 5.6: Change in cardiac parameters after stem cell injection for n=9

	Decrease	No change	Increase
EDV	6	3	0
ESV	5	1	3
EF	5	2	2
BNP	4	0	5

Table 5.7: Change in cardiac parameters after placebo injection for n=9

	Decrease	No change	Increase
EDV	4	1	4
ESV	3	2	4
EF	6	0	3
BNP	5	0	4

Figure 5.4: EDV before and after placebo and stem cell injections

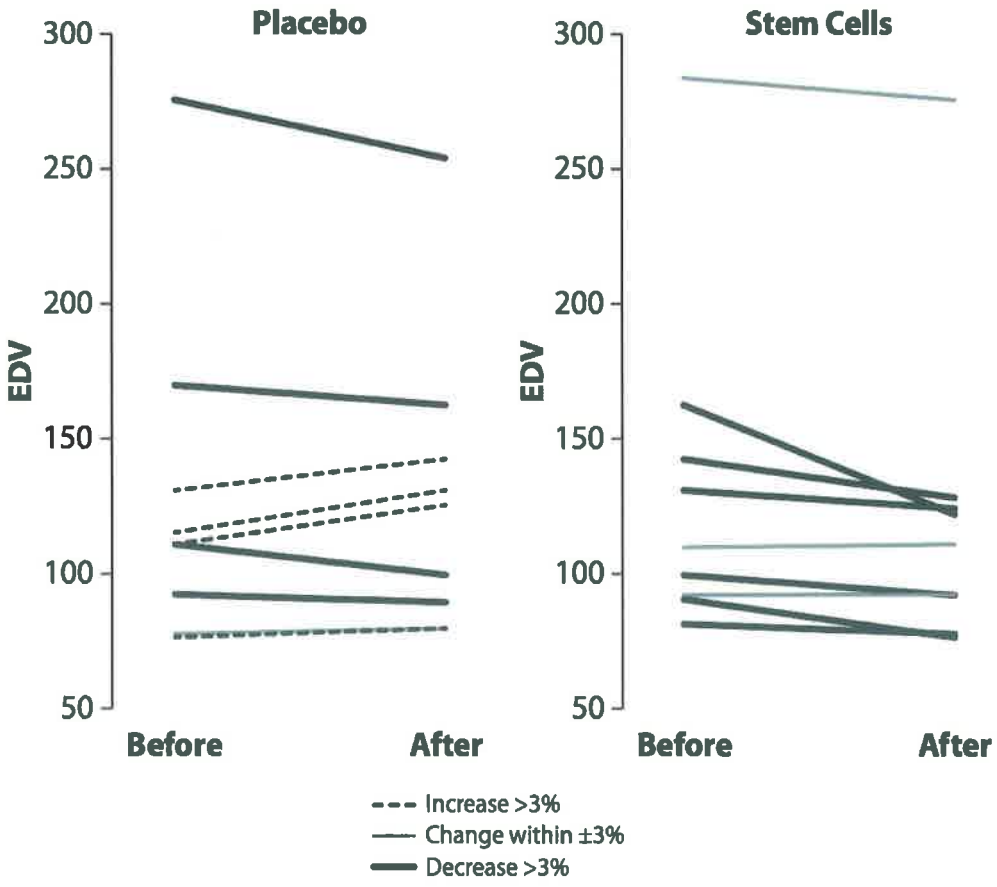
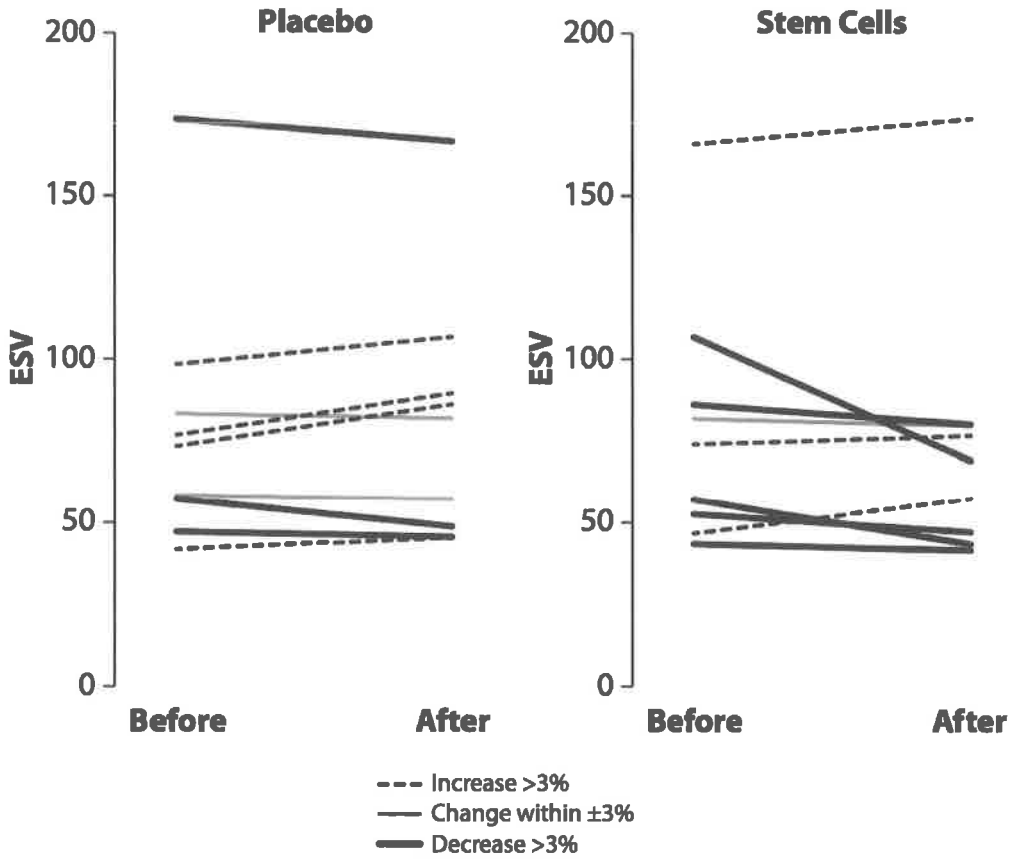


Figure 5.5: ESV before and after placebo and stem cell injections



For EF and NT pro BNP there was no evidence of a difference in treatments; mean difference for EF, adjusted for baseline =1.73, 95% CI (-2.01, 5.46), P = 0.37 and for NT pro BNP -0.29, 95% CI (-0.95, 0.37) P=0.39 (Figures 5.6 and 5.7, Tables 5.3 and 5.6).

Figure 5.6: EF before and after placebo and stem cell injections

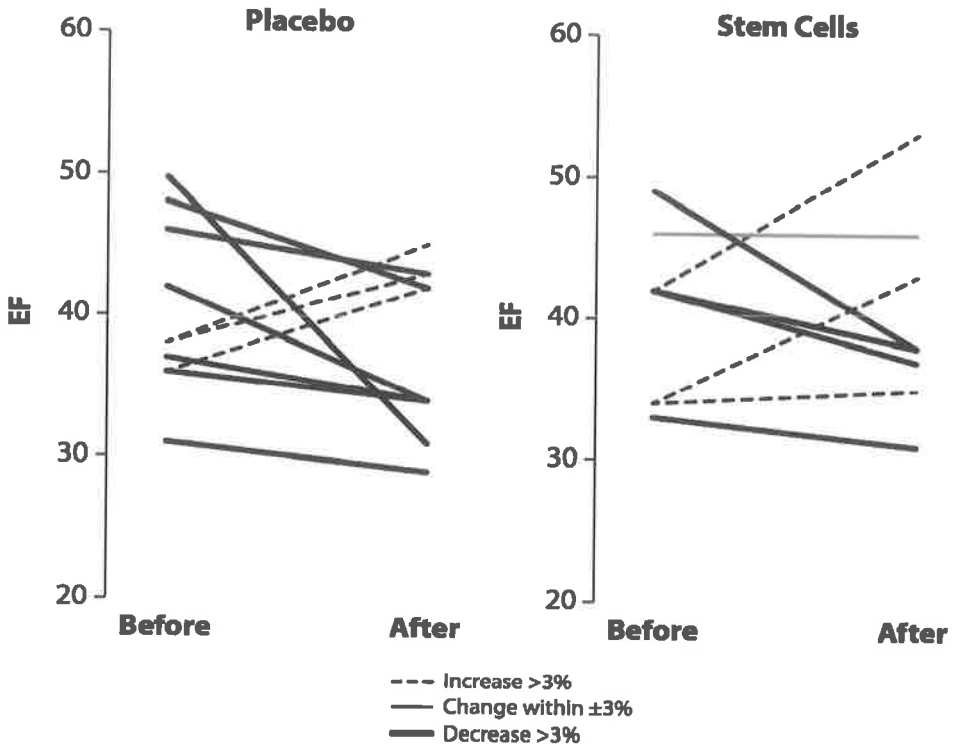
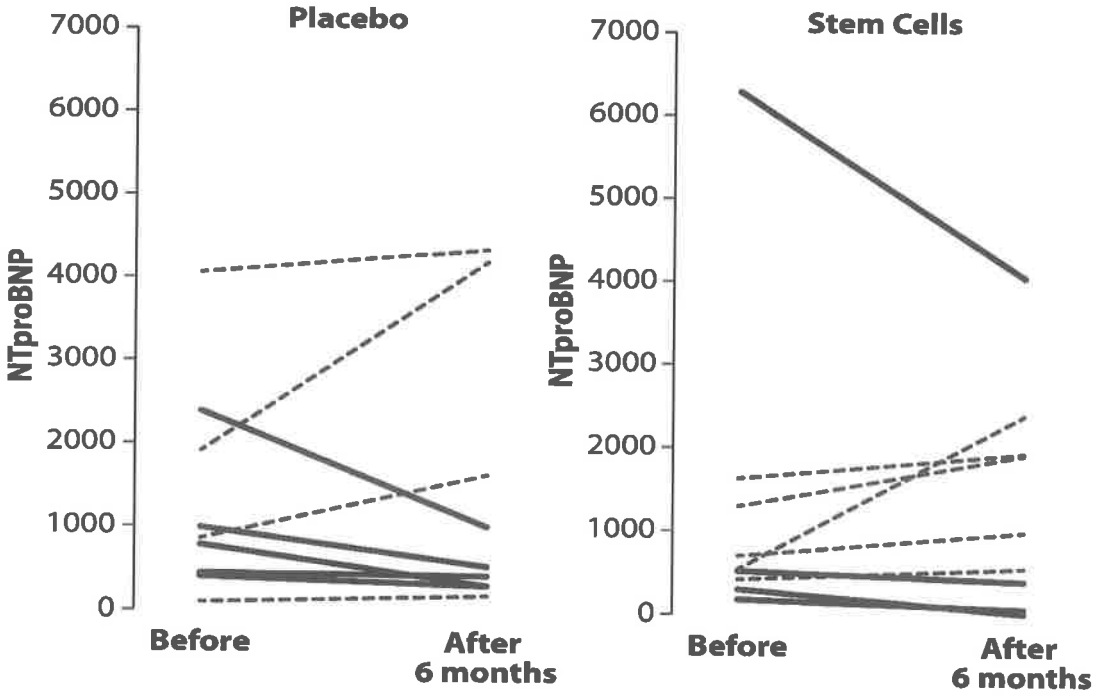


Figure 5.7: NTproBNP before and after placebo and stem cell injections



The subpopulations of cells calculated from the immunophenotyping gating protocol are described in Table 5.8. Little is known about the exact proportions of cell populations expected from the bone marrow of healthy individuals. Bone marrow is generally not available for non-therapeutic purposes, hence evaluation of normal ranges is difficult to establish. In addition, subpopulations of cells may well vary with age, gender, ethnicity and acute or chronic illness. At Great Ormond Street Hospital the Cell Therapy Laboratory does process samples from healthy children who are acting as donors for siblings requiring bone marrow transplantation. In this instance, the patient is assessed clinically and peripheral blood investigations are analysed. If these assessments are normal it is assumed that the bone marrow is potentially usable (if appropriate matching is determined), the bone marrow itself is not analysed before use, as following harvesting all of the cells obtained are administered to the sibling recipient. Although bone marrow cell counts are taken, this is for therapeutic calculation purposes only, and to date no database of these results exists.

Even from the same individual donor, sampling techniques may result in different proportions of cell types being obtained. Bone marrow harvests should utilise the technique of frequent, small aliquot sampling from different sites; too large a bone marrow aspirate taken from a single site results in dilution of the bone marrow sample with the blood, which fills the aspirate cavity on removal of the bone marrow cells. With such recognised variability in bone marrow cell subpopulation counts, estimates of normal ranges are difficult to confirm, hence the significance of the data obtained from this study is difficult to extrapolate.

	001		002		003		004		005		006		007		008		009		010	
	%	x10 ^{6}	%	x10 ^{6}	%	x10 ^{6}	%	x10 ^{6}	%	x10 ^{6}	%	x10 ^{6}	%	x10 ^{6}	%	x10 ^{6}	%	x10 ^{6}	%	x10 ^{6}
Cell Count		115.1		34.1		11.8		31.5		34.2		26.9		64.5		73.5		35.5		88.8
Lymph	43.1	49.6	73.2	25	59.3	7	85.1	26.8	71.1	24.3	65.9	17.7	80.6	52.0	78.3	57.6	58.1	20.6	65.1	57.8
mono	6.8	7.8	6.4	2.18	7.2	0.8	5.5	1.7	14.2	4.9	5.5	1.48	6.6	4.3	8.7	6.4	10.0	3.6	7.7	6.8
CMP	0.40	0.5	0.20	0.07	0.09	0.01	0.07	0.02	0.40	0.14	0.50	0.13	0.30	0.19	0.40	0.29	0.50	0.18	0.10	0.09
GMP	1.10	1.3	0.40	0.14	0.30	0.04	0.20	0.06	1.00	0.34	1.00	0.27	0.70	0.45	0.90	0.66	1.30	0.46	0.10	0.09
MEP	0.30	0.35	0.30	0.10	0.20	0.02	0.20	0.06	0.20	0.07	0.20	0.05	0.20	0.13	0.20	0.15	0.30	0.11	0.30	0.27
HSC	0.20	0.23	0.10	0.03	0.07	0.008	0.05	0.02	0.20	0.07	0.07	0.02	0.05	0.03	0.09	0.07	0.10	0.04	0.30	0.27
MPP	0.03	0.04	0.009	0.003	0.00	0	0.005	0.002	0.03	0.01	0.006	0.002	0.003	0.002	0.003	0.002	0.01	0.003	0.08	0.007
CLP	1.10	1.2	14.00	4.8	0.30	0.04	0.30	0.09	0.50	0.17	0.20	0.05	0.30	0.2	0.90	0.66	1.20	0.43	0.40	0.36
naïve Treg	0.55	0.63	1.07	0.36	2.26	0.27	2.20	0.69	1.58	0.54	0.65	0.17	1.79	1.2	1.82	1.34	1.12	0.04	0.29	0.26
mem Treg	0.23	0.26	0.35	0.12	0.80	0.09	1.24	0.39	0.46	0.16	0.25	0.07	0.71	0.46	0.55	0.4	0.44	0.16	0.26	0.23
MSC	0.02	0.02	0.007	0.002	0.04	0.005	0.005	0.002	0.002	0.0007	0.01	0.003	0.004	0.002	0.001	0.0007	0.001	0.0004	0.01	0.009
B cell	2.44	2.8	7.61	2.6	2.20	0.26	15.60	4.9	9.12	3.12	10.40	2.8	5.01	3.2	7.60	5.6	7.65	2.7	7.80	6.9
T help	7.10	8.2	21.30	7.3	1.24	0.15	19.50	6.14	20.10	6.84	6.36	1.71	39.30	25.3	38.40	28.2	18.30	6.5	7.22	6.4
T sup.	0.31	0.36	9.32	3.18	0.32	0.04	5.50	1.7	5.34	1.81	0.95	0.26	12.70	8.2	5.41	3.98	5.08	1.8	0.96	0.85
NK	0.24	0.28	5.02	1.71	0.56	0.07	5.12	1.6	3.01	1.03	6.16	1.66	5.87	3.8	6.28	4.61	1.58	0.56	2.36	2.1
NKT	0.027	0.03	0.11	0.04	0.07	0.008	0.065	0.02	0.097	0.03	0.028	0.008	0.12	0.08	0.14	0.1	0.098	0.035	0.039	0.03
T cell	1.69	1.9	37.90	12.9	1.90	0.22	24.30	7.7	29.60	10.1	8.33	2.24	56.40	36.4	45.90	33.7	25.20	8.9	9.71	8.6
EPC	0.20	0.23	0.09	0.03	0.20	0.02	0.06	0.02	0.05	0.02	0.09	0.02	0.02	0.01	0.03	0.02	0.30	0.11	0.06	0.05
VEGFR 2, HSC, NSC	3.00	3.5	1.60	0.55	0.90	0.11	0.30	0.09	2.30	0.79	1.30	0.35	1.80	1.2	2.10	1.54	3.30	1.2	3.30	2.9
CSC	2.50	2.9	1.30	0.44	0.80	0.09	0.30	0.09	1.80	0.62	1.20	0.32	1.40	0.9	1.80	1.3	2.70	0.96	3.00	2.7

Table 5.8 Cell subpopulation counts from gating strategies

CHAPTER 6

PATIENT EXPERIENCES

6.1 Introduction

So far, this thesis has explored the scientific potential of the use of stem cells in paediatric heart failure. As a pilot study it sought to meet certain criteria of safety and feasibility, and although only a small patient cohort was studied it was hoped that a clinically identifiable and measurable effect of our protocol on the patients involved could be demonstrated. The scientific data and primary and secondary end points of this protocol have been discussed in previous chapters, but this chapter explores the additional information that has been gathered throughout the study. While not scientifically robust or quantifiable, any parent and caregiver to children would believe that this information is significant and should be recognized even if not reportable to professional research fora.

This chapter explores the experience of our patients as they underwent the year long research protocol. Ten individuals and their respective families gave their time and effort to be a part of this work, and without them this study would have been both impossible and pointless. It is therefore important that their stories are heard. The information reported in this chapter is taken from contemporaneous notes and direct feedback from the families themselves. Patient correspondence has been anonymised but is otherwise unedited.

6.2 Patient 001

Patient 001 was 3 years and 11 months old when he commenced on this trial protocol. During resection of a congenital supra-mitral membrane at the age of six months his own mitral valve was damaged necessitating replacement with a St Jude mechanical prosthesis two months later. As a consequence of compromise of blood flow through the circumflex coronary artery he suffered an inferior myocardial infarction during the surgical placement of the prosthetic valve. He had poor cardiac function following this insult and was listed for transplant in 2008. Rising pulmonary vascular resistance had become an increasing concern in this patient, and this was being closely monitored, significantly elevated PVR had the potential to exclude him from consideration for heart transplant. Prior to recruitment to this study he experienced frequent chest infections requiring hospital intravenous therapy and his growth was poor (< 0.4^{th} centile for height and weight at time of listing). His parents described how he tired easily, having frequent episodes of breathlessness and becoming sweaty on minimal exertion.

During the six months following his intracoronary injection he had no admissions for treatment of chest infections and appeared to his family to be more active, less breathless and less sweaty. Clinically his condition had greatly improved, as had his cardiac function to the point that he was discussed at our Joint Clinical Conference with regards to de-listing him. His weight and height at this stage had improved such that he had outgrown his 17mm St Jude device. At this meeting it was however concluded that replacement of the device for a

larger one would be too high risk and that his best future management would be to remain listed for transplant.

001 remains well one year after completing the study and is still waiting for his heart transplant.

6.3 Patient 002

002 was six years old when she was recruited to the study. She had suffered with AML in the neonatal period and was treated with chemotherapy. Due to a relapse she required further chemotherapy in addition to a stem cell transplant. 002 suffered with cardiomyopathy as a consequence of the anthracycline treatment that she received and subsequently remained under the care of the GOSH heart failure team. At the time of her enrolment in the study 002 suffered with frequent chest infections which often required hospital treatment with IV antibiotics. Her mother described that she tired easily, had poor exercise tolerance and her growth was also noted to be for poor, plotting consistently below the 0.4^{th} centile. 002 had fallen behind considerably with her peers at school due to the amount of early years education she had missed as a result of illness, hospital admissions and hospital follow up. Her mother requested support for an application for her daughter to be permitted to stay down an academic year.

Following her injection of mononuclear cells 002 had another brief chest infection but did not require hospital therapy. She proceeded to recover well and her mother described greatly improved exercise tolerance. Her mother

described how on several occasions she had asked her daughter to 'slow down' with her activity, something she had previously not needed to do, and how also she felt that her daughter only tired from being physically unfit after many years of relative inactivity rather than her cardiac condition limiting her exercise. As time after her intracoronary injection went on and her activity increased her mother felt that 002's physical stamina also improved.

002 tolerated remaining with her peer group at full-time school with the help of a classroom assistant. During the course of the study 002 remained well, her mother wrote the following email to me following the conclusion of her involvement in the study:

Hello Sian,

Thanks for your letter.
002, Xxxxxxx and I are well.
Both children are doing well at Full Time school.
002 continues to strive.

After 002 recieved her Stem Cells, she became quite tired and a little unwell for a short while, but, after this period, 002 became a lot more active. It was as if the Stem Cells were taking over and then adapted and settled.

002 continues to do well, but, as time goes on, especially Winter time, you can see more and more signs of Heart Failure, especially blue lips and she becomes colder alot quicker than the other children at her school.

Hoping this helps out. Hope all is well with you and your family.

Xxxxx, Xxxxxx and 002 Xxxxxx.

6.4 Patient 003

003 had Trisomy 21 and developed AML in early childhood completing her chemotherapy successfully at the age of two years. She went on to develop dilated cardiomyopathy as a result of her chemotherapy with significant cardiac impairment. Despite medical management she had an episode of acute cardiac deterioration that resulted in a significant thrombo-embolic event in 2008. Her cardiac function remained poor affecting her school attendance considerably. At the time of enrolment her exercise tolerance was limited to approximately 100m on flat ground, she was able to mobilize independently around her home but would use a wheelchair at school and while out.

Following her injection of mononuclear cells 003's exercise tolerance over the winter months improved significantly, despite this being the time of year that most of our heart failure patients tolerate less well. At her three-month review her family showed me video footage of her in her local park. 003 had walked to the park in the snow and was shown playing ball with her dog before running away. Her parents were thrilled at the time with her activity, and 003 has continued to do well, her exercise tolerance remaining good. She is now in full time school and loves to dance.

Her mother wrote the following letter to me:

Hi Sian,

Lovely to hear from you hope you are well.

Not sure if this is what you need, let me know if not, here goes.

Pre Stem Cell:

Unable to attend school anymore, unable to climb stairs, get into bath etc.. Lost a lot of weight as no appetite, constant stomach cramps. (You get the idea!)

Post Stem Cells:

Couple of months after noted that when we took the dog out for a walk 003 wanted to get out of her chair and walk the dog herself, would go 200 yards or so, but then say her legs hurt (Not that she was out of breath). Over time she did a little more and that's when you saw the footage of her running in the park in the snow with the dog. She has done that on a few occasions.

Took a dip around (roughly Feb 2011) function dropped stomach cramps came back - up shot of that her frusemide was increased in Cardiff. Picked up soon after.

003 started back to school part time she is now full time and has started after school club on a Monday.
By the end of the week 003 is tired.
003 had attended Parties and Discos and dances full on for about 15 mins or so then she needs to sit down.
She also attends a low key dance group once a week (Touch Trust)

003 still needs her chair for long distances and she takes it to school but teachers say she does not use it all day. Some days not at all.
She has days better than others

Not had as many illnesses as she used to, she has colds etc but before they would seem to go to her chest but (touchwood) has not had one for a while.
003 was on antibiotics for one thing and another regularly. Can't remember the last lot of antibiotics...

I know medication has been juggled etc but I think a combination of that and the Stem Cell transplant has really helped.
She still has a dilated heart of course but her function in her last echo at GOSH July 2012 was 30%.

We still pace ourselves as a family as 003 does get tired and if she overdoes things it does show, but we are so grateful for this new lease of life that she has.

Hope this has helped, if you need anything else don't hesitate to ask.

Yours Sincerely

Xxxx and Xxxx Xxxxxx

6.5 Patient 004

Patient 004 was six months old when he was diagnosed with mesoblastic nephroma necessitating a left nephrectomy. He had a recurrence of his abdominal mass and two months later was diagnosed with an undifferentiated sarcoma requiring chemotherapy. The tumour was unresponsive to chemotherapy and further surgical resection was required. Pathological examination of the removed mass indicated a spindle cell sarcoma now with chondroblastic differentiation. 004 required prolonged chemotherapy and subsequently developed cardiomyopathy. Secondary to his poor cardiac function he suffered a left middle cerebral artery stroke in October 2008, which left him with residual right-sided weakness. His heart failure therapy was subsequently managed at GOSH.

At recruitment 004 was 14 years old and was managing well with his heart failure. He attended mainstream school full time, participating in most activities, but he specifically reported difficulty walking to school due to a hill he would encounter en route. He was accepting that he was unable to cycle to school with his peers.

004 tolerated the research procedures without complaint and was indifferent in his response when questioned about any changes in his activity during the course of the study. On indirect questioning however he revealed that at his review six months after the injection of mononuclear cells he had been attending an advanced cycling course and had been cycling for the preceding four days for eight hours each day. On commenting by the Research Fellow and anaesthetist speaking to him at the time, he conceded that this equated to an improvement in exercise tolerance. Following his injection of mononuclear cells 004 began routinely cycling to school daily. 004 continued to do well clinically and started a college course in September 2012.

6.6 Patient 005

005 was diagnosed with enteroviral myocarditis in the neonatal period and remained hospitalized for approximately 9 months. He was first referred to GOSH at the age of one year for a transplant assessment, when he became increasingly symptomatic despite maximal medical therapy. Although not requiring transplant listing at that time, his cardiac function remained poor. On recruitment to the study at the age of two years, his growth was poor as was his general exercise tolerance. Throughout the course of the study he did not deteriorate any further in fact he demonstrated a moderate increase in his activity levels, although this was not maintained for any significant period of time. Of note however he tolerated several other interventional procedures during this period and had fewer intercurrent illnesses and associated hospital admissions.

His mother wrote to me at the end of the study:

Dear Sian,

Am always really disappointed that I can't join Xxxxx and 005 and miss all the good people!

For your anecdotal addendum:
For a few fleeting months he seemed to be enjoying greater energy and stamina and certainly wasn't nearly as ill during the winter months that ensued. Our local cardiologist announced that 005s function was the best he'd ever seen - and he saw it at the beginning, when he described it as a 'bag of worms'. I like to believe that the stem cells gave 005 some sort of cardiac function boost, but it's fair to say that 'belief' and 'faith' are very flimsy evidences in the scientific world! Perhaps it is a therapy that would need to be repeated regularly, or done closer to the time of initial damage to heart tissues. For 005 I think 2.5 years after the myocarditis was too late.

However, we appreciate every drop of effort and care that occurred during 005's journey - especially your warmth and personal touches. I've enjoyed the experience more because of that.

With much affection
Xxxxx

You got a bit of my soul with that

6.7 Patient 006

006 was 13 years old when he was recruited to the study. He had been diagnosed at the age of 4 1/2 years with Duchenne muscular dystrophy, and at the age of 7 years with cardiomyopathy. His clinical condition was in a state of gradual decline despite maximal medical therapy. 006 attended mainstream

school full time despite his restricted mobility. His mobility was impaired primarily due to his muscular dystrophy rather than his cardiac function.

006 was unaware of his diagnosis at the time of recruitment to this study, an issue that became paramount during the course of attempting to discuss this research with them. 006 declined to use his wheelchair in public or at school if at all possible, he rarely used his orthotic splints and declined to follow his exercise regime, possibly because he did not fully appreciate the need to do so as he was unaware of his diagnosis.

Although 006's family was extremely keen for him to be included in the study, they had not shared his diagnosis and its implications with him despite him being an articulate and intelligent teenager. On this basis the Research Fellow felt that it would be inappropriate to include this patient in the study without his full awareness of the reasons for his involvement. This was carefully explained to 006's parents who remained extremely eager to participate in this work. His family over the course of the following few months decided to share his diagnosis with him and approached the Research Fellow to request that they could discuss it further. His parents explained to him the nature of this study. On that basis it was agreed that we could openly discuss the possibility of this research with 006, his family and the research team.

006 was keen to be included in the study and his parents, now more openly communicative about his condition remained enthusiastic. The dynamics within the family appeared to change throughout the research period, particularly as

006's mother was now more accepting of discussing the condition both with and without 006 being present, and her attendance at clinics with the family increased considerably.

006's condition continued to deteriorate throughout the study, his mobility worsening particularly. He did however become more accepting of the assistance available to him and was more content to use his wheelchair, particularly at school.

6.8 Patient 007

007 was diagnosed with cardiomyopathy at eighteen months of age following an episode of wheeze and cough which was investigated with a chest x-ray, which noted cardiomegaly. Subsequently two other members of her family, her father and her paternal aunt, were also diagnosed with cardiomyopathy. All three members of this family share a genetic mutation as the cause of their familial cardiomyopathy. Despite her poor cardiac function 007 was able to attend nursery three days per week at the time of her recruitment to the study, at the age of three years nine months. Her family did not describe any specific clinical improvements throughout the course of the study however it was noted that she increased her time at nursery to 5 full days per week and she had a reduced number of illnesses during this period. 007 now attends primary school full time and remains well. Over the year that she was involved in the study, 007's weight climbed from the 50^{th} to between the $50\text{-}75^{th}$ centiles and her height increased from just above the 25^{th} to the 50^{th} centile.

Her family wrote:

30th November 2012

Dear Sian,

Apologies for not responding to your letter sooner – we have moved house and changed our home telephone number. For your records, our new address and home telephone number are as above.

With reference to your request for information about our experience, we're happy to help.

When we first received your letter inviting 007 to take part in the trial, our initial reaction was no. There were a couple of reasons behind this! When 007 was diagnosed with DCM we were told amongst other things, that a general anaesthetic would be risky for her due to her heart being weak. In addition, my cousin had died many years before when she was just 7 years old, after being put under general anaesthetic and never regaining consciousness, so were very nervous at the prospect of 007 having a GA, which could be seen as 'unnecessary'. At that point, the most invasive procedure 007 had ever had was a blood test, so the thought of her having a cardiac catheter was quite daunting for us.

However, at one of our previous consultations at GOSH, we recalled that were told that the best options for a 'recovery' (of sorts) for 007, given that her DCM is genetic, would either be transplantation, which comes with a whole new set of issues, or stem cell treatment, which could be a long way off. We also thought, if this were offered to Xxxx (dad), we would definitely say yes... his sister was taking part in a similar stem cell trial (although we were not sure if she had been given a placebo or stem cells) and all this considered, it seemed rash of us to just dismiss something which could potentially be beneficial to 007, so we decided to put our questions to you in an email. Your answers were reassuring, and we realised that 007 would be in safe hands.

Our experience of being admitted to day care and onto the ward went smoothly on all occasions. 007 was particularly fond of the kitchen and toys in the day ward! It was very difficult for us to pass her over in the anaesthetic room, but the anaesthetist was

so reassuring and caring - we would like to have him on hand for any procedure that 007 might need in the future! I can remember that we were not prepared for how grumpy 007 would be when she came around from the GA! Her throat hurt from the tube and with the first catheter she was quite bruised as the first attempt to go in through her left femoral artery was unsuccessful (this was a one off – every other catheterisation barely left a trace!). She was feeling quite sorry for herself. A blood test showed she possibly had an infection brewing and she was prescribed antibiotics before we left. We really appreciated the follow-up call you made the next day to check how 007 was doing, and we felt that you were very thorough in looking after her and in keeping us informed whilst we were at the hospital. We are lucky that 007 really loves it at the hospital, so even though she was going for 'an operation' she was never reluctant.

At the time, we did not notice a particular change in 007's energy levels or activity – however, she coped well with increasing her days at nursery, which could possibly have been due to effects from the stem cells, as she did not have any changes in medication over that period. Also, her echo results showed that her Fractional Shortening at one point was 22%, which is the best result she has ever had. Her results have since declined very slightly, her latest echo in November 2012, showed her FS to be around 18%, but still, this is a good improvement from what she was, which was tending to be around 13-14%. I remember that we were told that because the stem cells were taken from 007's own bone marrow, they would still contain the genetic mutation which has caused her DCM, so any positive effects we saw may only last, perhaps 18 months, as that was when her DCM was diagnosed. In our non-medical opinion, it appears that the stem cells may well have had a beneficial effect on 007's heart function, even if it was only temporary. She is also managing to gain weight and get taller, which is great – she seems to never stop eating and we know she is not feeling herself when she loses her appetite!

007 is now doing five days at school, in Year 1. She does get tired towards the end of the week and particularly towards the end of term, but this I think is normal for most 5yr olds! She is always ready for the weekend to come so she can have a break from school – but on her days off we think she does seem to have a bit more energy than she did before. 007 does find it hard to keep up with her friends in PE, but she asks her teacher if she can stop and she sits out activities if she gets out of breath or doesn't feel up to it. She has had a few days off school this year when she has been poorly with chest infections, but there have not really been any new cardiac symptoms we can recall in the last few months. We are really pleased that at the moment she is

managing to keep up with the five days at school in Year 1 as she did struggle a bit in Reception.

We have observed that 007 is still not quite as active as her cousins who are a similar age to her - sometimes she does struggle to keep up when they're playing boisterous games and she cannot run as fast as them - but does not seem to complain as much of tiredness as she did a while ago. It is difficult to say whether this is due to the stem cell treatment or whether she is just becoming more aware of her own limitations as she gets older.

Unfortunately we managed to mix up our last appointment date in GOSH, so ended up cancelling and being seen at Alder Hey instead. We will rearrange the GOSH review - we are keen to keep in touch with the team there as we value their expert opinion and if we hadn't been seen there, we would never have had the opportunity to take part in this trial.

I am currently 25 weeks pregnant and due in March 2013, so we will try to arrange the appointment in GOSH for after the new baby arrives. We'd really love to hear more about how the study has gone when you have written your reports up - please get in touch with us if you need any further information from us.

Take care and have a great Christmas,

Xxxxxx, Xxxxx and 007 Xxxxxxx

6.9 Patient 008

Patient 008 was diagnosed with dilated cardiomyopathy at the age of nine weeks. He initially presented to his GP with signs of bronchiolitis but DCM was diagnosed after cardiomegaly was noted on a chest X-ray. He remained clinically symptomatic with poor cardiac function despite medical management. He fed poorly and had poor weight gain, requiring NG feeding support. On initial contact with this family, 008's mother was notably distressed about his diagnosis. His function remained consistently poor and his mother was

consequently extremely distressed and tearful about his condition, communication was generally via 008's father. His parents reported however that 008 always appeared well to others and did not generally demonstrate the clinical condition that would usually be associated with his poor cardiac function.

Following his intracoronary injection however 008's clinical condition appeared to improve. His parents, and his mother particularly, became animatedly enthusiastic about his exercise tolerance. At his review three months after his injection of mononuclear cells, his parents showed me video footage of 008 cycling in the lane where he lived. His mother reported that he would do this all day if given the opportunity and that he appeared to have greater energy that his older brother who was entirely well. His mother commented that she was no longer as distressed about attending his clinic reviews as she felt that she could see such an improvement in his condition that the figures and values measured at his clinic assessments were now of secondary importance to his actual physical state.

His father wrote to me on behalf of his family:

6th Floor Main Nurses Home
Great Ormond Street Hospital
London
WC1N 3JH

Hello Sian hope you are well, many thanks for your letter which we received a few weeks ago, sorry for not getting back to you until now, but we have all been very busy.

First of all myself and Xxxxxx would like to say a very BIG THANKS for all your help you have given us both while we have been coming to great Ormond Street, and a very BIG THANK YOU for thinking about 008.

For the stem cell research, when you first approached myself and Xxxxxx regarding the stem cell treatment we was both very nervous at first, Xxxxxx and I have heard only a little about stem cell treatment but I would like to say thanks for explaining the stem cell treatment to us both in a way we could easily understand.

We as parents made the discussion to let 008 have the stem cell treatment, because we both felt we had nothing to lose and we did not want to lose the chance if there was a way to help 008.

I can say for the both of us that the information which we received was very satisfactory and if we had any question they was all ways answered in a way which we could understand.

Admissions were very helpful; the day care ward was also very good because they kept us informed at all times while we were on the ward.

008 is doing very well at the moment, he is running around like a very health 4 year old child, 008 loves riding his bike which he could do all day, I will send you over a short video of 008 playing outside on his bike.

008 has started school full time now and is doing very well, he gets great support from all his teachers, all 008's teachers have all said how great he looks for someone with a heart condition.

I can re-call before 008 had the stem-cell treatment he used to sweat a lot, which now to me he does not as much, as 008's Daddy I will always believe that we made such a great decision to let 008 have the treatment, and I will always believe deep in my heart that one day his heart will get better.

Once again many thanks from all the Xxxxxx Family

6.10 Patient 009

009 suffered with neonatal enteroviral myocarditis requiring prolonged intensive care support. His cardiac function remained poor despite medical therapy and secondary to an acute cardiac deterioration causing severe hypotension he suffered a septal infarct. 009 tolerated the procedures of the year-long protocol very well. He attended mainstream school full time throughout.

6.11 Patient 010

010 was diagnosed with dilated cardiomyopathy at the age of six months. At the time of diagnosis it was believed that her DCM was secondary to vitamin D deficiency although this diagnosis is now in question, no other specific cause has been found. At the time of her initial presentation 010 required intensive care therapy and IV management of her heart failure. Her condition was slow to stabilize on medical therapy, with associated poor growth and difficulty feeding. On recruitment at 3 years and 9 months of age, 010 was clinically stable. Her weight was above the 50th centile and her height on the 75th. Over the course of study 010 made a steady clinical improvement. At her review three months after the intracoronary injection 010's mother was pleased with her activity level as she was able to attend nursery school five mornings each week. Her growth was good during the study period, her height at the end of the year long study had climbed to the 91st centile, and her weight to between the 75th and 91st centiles. 010 began full time school in September 2012.

6.12 Summary

The majority of the patients involved in this study had positive experiences. There was no clinical morbidity or mortality, none of the individual patients or their families fed back any negative consequence of the procedure, admission or general involvement in this work and most discussed with me the positive benefits of taking part in this study. On the basis of the information highlighted in this chapter I believe that it would be possible to conduct Quality of Life (QoL) style interviews and data reporting on this study population. We actively considered performing QoL interviews as part of this work but as our protocol was not blinded for the stem cell procedure the results would not have been recognized as scientifically significant. As a testimony to this study, the majority of participants vocalized their interest in being involved with subsequent similar research or treatment.

I appreciate that the information I have presented in this chapter is crude and anecdotal, however I believe that it still holds clinical significance. It is plausible that a trained psychologist or an individual following an approved interview proforma may be able to extract statistically significant and relevant information regarding this study. More rigorous evaluation of the information obtained from such interviews could provide substantial data that would stand up to scientific scrutiny.

CHAPTER 7

DISCUSSION

7.1 Evolution of this research

At the early planning stage of this study, it was hoped that a double-blind protocol could be followed. This could have been achieved if all patients had bone marrow harvested at Stage One of the protocol, with one cohort subsequently receiving intracoronary injection of fresh autologous mononuclear cells and the second cohort having their cells frozen for later administration. Investigation of the freezing procedure revealed that significant cell numbers could be lost during the process, both by direct cellular damage as a consequence of low temperature storage, and cell loss through the washing process essential to remove all traces of the solutions required to freeze cells safely.

Peripheral blood stem cell transplants are frequently performed using stem cells that have previously been frozen and stored at -80°C, but cryopreservation solutions are required to minimise cellular damage at these cold temperatures. Dimethysulphoxide (DMSO) is a cryopreservation fluid frequently used in the United Kingdom to protect haematopoietic stem cells from the damaging effects of ice crystal formation within the cells when low temperature storage is used. DMSO is the cryopreservation agent used at GOSH for cell storage.

Prior to the clinical use of frozen HSCs, the suspensions are washed thoroughly to remove all traces of DMSO. The process of freezing cells inevitably causes some degree of damage and ultimately reduces the viable cell count available for clinical use. In the experience of our laboratories approximately 30% of the original cell count may be lost during the freeze-store-thaw process. The washing procedure also results directly in cell loss; the more washing cycles

that are undertaken, the more cells that are lost but the less potential cryopreservative contaminant remains within the solution. A careful balance must therefore be struck between the appropriate number of washes that should be performed to remove maximal contaminants whilst retaining an optimal cell count.

It is inevitable that traces of DMSO remain within the cell suspensions even after washing, and this would be transferred with the cells when used therapeutically. Studies have been performed to observe the cardiovascular effects of cryopreserved cells when infused peripherally into patients[1]. In 2009 Horacek et al monitored one cohort of patients who were infused peripherally with HSCs that had been cryopreserved using DMSO; the second cohort received the same volume of fresh cells when no preservative had been used. The patients who received the cryopreserved cells were noted to have significantly raised systolic BP, diastolic BP and heart rate during the procedure compared with those who received fresh cells. Although these cardiovascular changes were observed to be only transient and required no intervention in the patient group studied, such effects had the potential to cause unnecessary complications in our patients with pre-existing cardiac pathology.

The potential effect of DMSO would also have been difficult to counter with any placebo. For these reasons it was concluded that it would be most appropriate to use only fresh cell suspensions so as to optimise the cell count available therapeutically and minimise the potential side effects of any contaminants. Additional analysis of a previously frozen cell suspension could be performed to evaluate a new cell count and to investigate, perhaps by mass spectroscopy, for

the trace levels of DMSO but this would have involved usage of a portion of the clinical sample, further reducing the available cell count for injection.

The possibility of using granulocyte-colony stimulating factor (GCSF) to boost the yield of mononuclear cells harvested from the bone marrow was also considered. On discussion with teams at GOSH that use GCSF routinely however, it was considered to be an unnecessary step. The bone marrow transplant team at GOSH revealed that, in their experience, children who were administered GCSF and subsequently went on to have a bone marrow harvest commented that the GCSF injections were far more uncomfortable than the effects of the bone marrow harvest itself. Children frequently complained that the regular subcutaneous injections of GCSF, required to stimulate bone marrow production of stem cells, caused an unpleasant stinging sensation, and some children refused to complete the course of GCSF therapy. As our heart failure patients were being recruited to a pilot study primarily to assess the safety and feasibility of intracoronary mononuclear cell injections it was felt unnecessary to expose them to this additional, unpleasant procedure.

Unlike previous similar studies that involved only adult patients, our research was investigating a paediatric population. It was expected that the cellularity of the bone marrow in children would be greater than that in adults and hence no augmentation would be required to produce sufficient cell counts from a relatively small harvest.

The placebo arm of this randomised, placebo-controlled trial replicated precisely the mononuclear cell injection protocol in every way, other than the application

of the autologous cells. In so doing it was possible to establish directly any benefit or adverse risk of the stem cell intracoronary injection. To further support the elimination of the placebo arm of this study, no significant clinical improvement was reported following the administration of placebo in these patients. As both placebo and stem cell arms of the study were well tolerated, it should be possible to eliminate the need for a placebo injection in future protocols.

The study population of 10 patients, whilst sufficient to provide statistically significant cardiac data for a pilot study, could be expanded in the future to allow more subtle changes to be measured. Increasing the study cohort population would increase the validity of any changes seen. Randomisation was used in this pilot to assess the feasibility and robustness of the design prior, potentially, to developing a larger multi-centre protocol. A larger scale study following this present protocol would need to be located at institutions such as GOSH with the required facilities available on site to permit a single anaesthetic procedure. Such extensive facilities are limited in number as they are high in expense. Modification of the protocol could however open up the possibility of its utilisation in other institutions. Two aspects of our protocol that could be amenable to alteration and ultimately permit its incorporation into a more standard service are the general anaesthetic and the stem cell processing.

The highest risk element of this protocol was related to the anaesthetising of our paediatric patients with heart failure. Studies[2,3] have demonstrated considerable risk of morbidity and mortality when performing anaesthetics on similar patients. Our protocol utilised the skills of a single, highly experienced anaesthetist for

the entire study. Light anaesthesia was administered and no adverse events were encountered. The general anaesthetic protocol used for our patients was consistent, and hence minimised cardiovascular variability secondary to the use of different agents. Intravenous induction of anaesthesia was achieved using propofol, followed by neuromuscular blockade with atracurium to facilitate tracheal intubation. The opioid analgesic, fentanyl, was used concomitantly to reduce the dose requirement of the other drugs required during anaesthesia. This process of total intravenous anaesthesia was monitored closely and tailored to the requirements of each patient.

The study by Kipps *et al*[2]. indicated that although their mortality rate with general anaesthesia was low, the complication rate was high especially in patients similar to ours with severe ventricular dysfunction. Peri-procedural morbidity was noted to be 38% in this study and 30-day mortality was 3%. A retrospective study by Murphy *et al* [3] indicated that in their series of 21 patients receiving a total of 28 anaesthetics, the overall mortality was 29%. In both of these studies anaesthesia was achieved via different means, either inhalation or intravenous, and practice was not standardised. The indication from each anaesthetic provided another variable, some anaesthetics being provided within an emergency setting.

Our outcomes of 0% morbidity and mortality were highly clinically significant. On that basis it should not be presumed that our anaesthetic protocol should be viewed as a recommended standard to be utilised for all children with dilated cardiomyopathy, instead our success should be reviewed within the context of our specific study. All of our procedures were elective, the patients were fully

screened and evaluated before undergoing their anaesthetic and if any concerns were highlighted during that screening, the research team had the capacity and opportunity to cancel or postpone the planned study. Clinical summaries on all of the research patients were updated and circulated prior to each admission. This permitted the clinical history to be scrutinised by all team members and any concerns to be discussed and evaluated prior to general anaesthetic. Instead of general endorsement of the drug protocol used for anaesthesia within our study the significance of the roles of careful planning, close monitoring, skilled anaesthesia and close teamwork should also be emphasised.

One method of ultimately minimising the risk of anaesthesia would be to avoid a general anaesthetic altogether. As highlighted previously, one of our patients had cardiomyopathy as a result of his neuromuscular disorder, Duchenne muscular dystrophy. Patients with Duchenne muscular dystrophy are at increased risk of some anaesthesia-related hazards such as rhabdomyolysis, fever and hyperkalaemia[4]. Following recruitment his case was discussed with the research team, and most pertinently, with the anaesthetist responsible for anaesthetising all of the patients in the study. It was concluded that the risk of anaesthetising this patient outweighed the potential benefits of the pilot study, so it was decided that he could not follow the agreed study protocol. The patient and his family were extremely keen to continue their involvement in this work, so it was agreed to allow him to undergo the interventions under sedation. This was agreed with the ethics committee and documented accordingly. This deviation from study protocol did result in his data being excluded from the group analysis.

If the study protocol could be performed under sedation rather than anaesthesia the risk would be ultimately reduced for all participants. In many parts of Europe cardiac catheterisation and bone marrow aspiration are performed under sedation, even in children. In the UK it is common practice to carry out these procedures under anaesthetic but questioning this standard would be reasonable. The pain perception of the procedure of bone marrow aspiration is one of great discomfort. None of the patients in this study however vocalised any concerns regarding discomfort at any point after the procedure, despite direct questioning. The majority of the children instead vocalised greater concern regarding discomfort of the intravenous cannula siting and securing. The bone marrow extraction performed for our protocol involved the withdrawal of only a small volume of cells. Larger aspirations may possibly be associated with greater discomfort post procedure. For the general acceptance of stem cell harvest without anaesthetic, it may be necessary to explore alternative methods however.

Stem cells may be obtained from blood more easily and less painfully than from the bone marrow. If it were possible to devise a method to obtain suitable volumes of stem cells from peripheral blood then this protocol may be adapted to harness such peripheral blood filtration. A process as simple as blood donation would be less intrusive and complicated for the patients involved and may therefore be more acceptable. Stem cells could be isolated from this peripheral blood to obtain those cells required for re-injection. Obtaining the stem cells from peripheral blood by simple venepuncture may allow stem cell processing to be performed prior to the elective cardiac catheterisation and reinjection.

Venepuncture is not routinely performed under sedation or anaesthetic. If the stem cell collection could be performed via vascular access, independently of the cardiac catheterisation, it is reasonable to assume that the sedation or anaesthetic required for cardiac catheterisation would consequently be shorter than if both procedures were performed together. It may even be considered more acceptable to perform the cardiac catheterisation without anaesthetic, but under sedation alone. This would clearly reduce the risks to the patients involved.

It is likely that peripheral venesection would not provide stem cells in high enough numbers to be extracted and re-administered at a later date in therapeutically sufficient volumes. One possible alternative is to use a circuit such as a vas-cath for continuous removal of circulating stem cells. A vas-cath is a central venous catheter device most commonly used for renal dialysis. It connects the circulating blood to a filtration system that usually extracts waste products but may be tailored for extraction of stem cells. Such a central catheter could be inserted under general anaesthetic, but could then remain *in situ* long term to be accessed as required for stem cell harvesting. The stem cells collected could then be re-administered, fresh, when the required volume is obtained.

The method used for isolation of mononuclear cells from bone marrow appears to have a significant effect on both the yield, the function and consequently the clinical effect of the injected cells as observed in previous adult studies. Many investigations in the adult population have noted the effects of different subsets of bone marrow derived progenitor cells, circulating endothelial progenitor cells

and stem cells derived from tissue sources such as muscle or adipose tissue on the myocardium. Seeger *et al* [5] observed that two of the adult studies, investigating the use of bone marrow cells following acute myocardial infarction (MI), had very different outcomes. Further investigation into the different protocols used suggested that the outcome difference might have been attributable to the preparation and storage media used for the bone marrow cells. In the cases referenced, the ASTAMI (Lymphoprep separation and 0.9% sodium chloride plus plasma storage) and REPAIR-AMI (Ficoll separation and X-vivo plus plasma storage) trials, the storage of cells in 0.9% saline with plasma in the ASTAMI protocol appeared to be the major factor leading to a subsequent impairment of cell function, despite cell viability being unaffected. This highlighted the fact that cell viability and cell number may not entirely reflect the functional capacity of the cells when transferred in vivo, this having a significant effect on outcomes [5]. Our study utilised Lymphoprep separation methods with cell suspension in 0.9% saline and albumin solution, as this was the GOSH policy. This however may be open to review in future studies.

The majority of adult studies have investigated the potential effects of mononuclear cells on myocardial damage secondary to infarction. It should be noted at this stage that the underlying pathology of the patients involved in the adult studies is markedly different from the pathology encountered in the paediatric population of heart failure sufferers. The adult patients are notably older than the paediatric patients, frequently in their sixth and seventh decades of life. These patients have encountered numerous pathologies and the general consequences of old age, before recruitment to such trials. The effects of co-morbidities, poly-pharmacy, old age, the effects of smoking,

hypercholesterolaemia and menopause account for substantial differences between the adult and paediatric populations. Potentially the absence of these factors in the paediatric population of children with heart failure could optimise any beneficial effect of stem cell therapy in our patients. Another positive influence on our paediatric patients is that, under normal circumstances, they would be expected to experience more rapid cell turnover during the process of growth. It is likely that their bone marrow is more cellular and their host environment more receptive to the potential benefits of stem cells. This theory of bone marrow cellularity is supported by our findings, as we obtained similar bone marrow derived mononuclear cell counts from relatively small bone marrow harvests, as compared with the adult studies. The mean cell count used in this protocol was 51.59 x 10^6, obtained from a 20 mL aspirate of bone marrow. The cell counts used in the adult studies with similar protocols ranged from 87 x 10^6 to 304 x 10^6 obtained from BM harvests of 50-130ml. The methods and cell counts of several of the adult studies are shown in Table 7.1.

The volume of harvested cells, the populations of administered cells and the frequency of intracoronary injections could also be investigated further. Although the stem cell numbers used in this study were comparable with the cell counts administered in the adult studies, a relatively small volume of bone marrow was aspirated by comparison. It is not unrealistic to postulate that greater stem cell numbers injected into the coronary vasculature could yield greater effect, although that cannot be concluded from the relatively small amount of data obtained from this study. The same rationale can be extended to suggest that repeated stem cell injections could also be beneficial, producing a more sustained cardiac response. This pilot protocol was well tolerated by

both patients and their families, hence incorporating several regular stem cell harvests and injections in future studies could realistically be considered especially if clinical benefit was proven.

The cell surface markers used to identify the key stem cells that may be involved in cardiac repair and regeneration, CD 184 and CD 309, were determined from previous studies and immunological appreciation of the role of these cells[6]. Further, more detailed evaluation is required to draw any significant conclusions from these data, and was beyond the scope of this study.

The cardiac follow up for this study was elective at three months (a clinical review and an echocardiogram) and six months after intra-coronary injection (a clinical review and cardiac MRI), after both Stages 1 and 2 of the study. Final follow up was at study completion, at 1 year. It is possible that six months was not the optimal period for assessment following BMC or placebo injection, as cardiac changes may be seen either before or after this time and may be missed with this single point of assessment. Although serial MRI scans at more regular intervals could be performed to assess functional change in a small number of patients, it is likely that there would be individual variability as to when the peak effect, if any, was noted for any single patient. If a demonstrable clinical cardiac improvement was identifiable in the majority of patients receiving intracoronary stem cell injections, serial MRI scanning may not be required, instead assessment may be made more routinely on clinical review alone. By comparison follow-up in the adult studies ranged from one day to 18 months and comprised angiography, MRI or ultrasound depending on the protocol.

Table 7.1: Summary of the adults stem cell trials[7,8]

Study	Pathology	Cell type	Cell preparation: (Aspirate vol Separation Culture)	Mean cell count	Assessment
Strauer et al	Acute MI	BMC	• 40mL • Ficoll •Teflon- overnight	28 x 10^6	LV angiography
BOOST	Acute MI	BMC	• 150mL •gelatin/ polysuccinate sedimentation • none	2460 x 10^6	LV EF: Cardiac MRI
TOPCARE-AMI	Acute MI	CPC BMC	• 250 mL • Blood • 3 days • 50ml • Ficoll • none	16 x 10^6 213 x 10^6	Cardiac MRI and LV angiography
Fernández-Avilés *et al*	Acute MI	BMC	• 50mL • Ficoll •Teflon- overnight	78 x 10^6	Cardiac MRI
Janssens *et al*	Acute MI	BMC	• 130ml • Ficoll • none	304 x 10^6	LV EF: cardiac MRI
ASTAMI	Acute MI	BMC	• 50ml • Lymphoprep • none	87 x 10^6	LV EF: SPECT
REPAIR-AMI	Acute MI	BMC	• 50ml • Ficoll • vivo 10 medium and serum	236x10^8	LV EF: angiography
STEMMI	Acute MI	G-CSF	• circulating blood • none • none Circ[9] CD34+ cells	↑ to 21.8x10^9	LV systolic wall thickness
Tse *et al*	Chronic CHD	BMC	• 40mL • Ficoll • none	Not reported	Wall motion, wall thickening
Fuchs *et al*	Chronic CHD	BMC	• filtered • none•	78.3x10^6	Angina score
Perin *et al*	Chronic CHD	BMC	• 50mL • Ficoll • none	30 x 10^6	Contractility, ESV: SPECT

Quality of Life (QoL) assessment was initially planned to assess the patient experience of this pilot study. Our protocol was amended at an early stage such that the patient and their family were aware of the timing of the mononuclear cell injection. As a result, QoL interviews were not performed in this pilot study as the protocol was not double blind, each patient and their family being aware of the timing of the bone marrow aspiration and subsequent stem cell injection. If improved methods for cell storage were available it would become feasible to perform a larger bone marrow harvest and store sample aliquots of stem cells for serial injection. Alternatively, if a vas-cath could be used to harvest stem cells peripherally, a double blind protocol could be devised so that although stem cells could be harvested, they may not be administered. A double blind placebo controlled trial could be performed in this situation and QoL could realistically be considered to review patient progress.

Investigations have previously been performed to identify the destination of bone marrow derived mononuclear cells after injection into the coronary arteries[9,10]. These studies have primarily labelled CD133+ cells. Research by Forest *et al* [9] highlights that intravenous bone marrow mononuclear cell injection was ineffective to target myocardium however intracoronary injections were more effective. Her study used bone marrow mononuclear cells labelled with a fluorescent marker and/or technetium radiolabelling. Her protocol, using a porcine model of acute MI, indicated that intracoronary administration of BMCs distributed within the heart and lungs, intravenous injections promoting lung homing rather than cardiac engraftment. The work of Forest could be applied to the enhanced immunophenotyping protocol used in this study, to evaluate the fate of the additional cell groups identified as being injected.

Of note, there was similar bio distribution of bone marrow mononuclear cells in the animals with or without MI. Previous work has indicated that acute ischaemia or MI is followed by local inflammatory reactions involving up-regulation of chemokine receptors and adhesion molecules. These chemical responses are likely to facilitate adhesion and infiltration of cells into the damaged tissue. The ability of mononuclear cells to migrate in this fashion is essential in regenerative medicine. Although Forest's study indicated no difference in the response to cell migration and adhesion in the presence or absence of MI, she did report that ST segment elevation was noted on ECG at the time of coronary catheterisation and cell injection using angioplasty balloon inflations. They did not evaluate the intensity of ischaemia but their results indicated the likelihood of an alteration to the local microenvironment downstream to the injection rendering it more receptive to cell homing. This clinical scenario was replicated in our own study where ST-segment changes were recognised during the intracoronary injection procedure caused by the partial occlusion of the coronary artery by the catheter.

The protocol used in this pilot study involved the injection of mononuclear cells or placebo into the left main coronary artery via a catheter device. Previous studies in adults had used catheters with balloon occlusion devices inflated prior to the stem cell injections. Balloon occlusion temporarily obstructs blood flow in the respective vessel, allowing stem cells to remain within the cardiac vasculature for as long as possible. The intracoronary injection in our protocol was a slow procedure lasting approximately 2 minutes. We elected not to use balloon occlusion of the left main coronary artery, as we believed it would be safer for our patients not to experience this. Other centres have previously

encountered adverse events when using occlusion catheters in small children in similar case studies. It was felt that due to the small size of the patients involved in our study and the relative close fitting of catheters to the vessel intima, a significant amount of vessel occlusion would occur without the need for a balloon catheter. As already discussed pressure traces observed throughout the procedure in our patients indicated that ischaemia was caused as a consequence of the catheterisation process supporting our decision.

It is possible that the ischaemia caused by a close-fitting intracoronary catheter and by balloon inflation of a catheter device would not only cause ischaemia but trigger the physiological and chemical cascade seen in ischaemia-reperfusion reactions elsewhere in the body. The effect of such an ischaemia-reperfusion reaction and its consequent chemical cascade was evaluated in isolation by the placebo arm of this trial. The placebo arm followed an identical protocol and similarly caused ST segment changes on injection of the saline solution. As no cells were injected in the placebo arm of the study, any clinical response to the injection could be attributable to the procedure alone and the ischaemia-reperfusion cascade it had triggered.

Chemokine gradients induced by inflammation or other cellular damage may favourably promote the recruitment of stem cells in damaged tissue such as the myocardium. The cytokine interleukin-1 (IL-1) is known to play an important regulatory role within immune and inflammatory responses. There is increasing evidence to suggest that the balance between IL-1 agonists and antagonists plays an essential role in a variety of cardiovascular conditions such as the formation of atheromatous lesions, enhancement of the vascular inflammation,

and the triggering of plaque destabilisation. Following acute MI, IL-1 also regulates the inflammatory response and the remodelling, which finally produces scar tissue. IL1 has also been implicated in the pathogenesis of heart failure by suppression of cardiac contractility, promotion of myocardial hypertrophy and induction of cardiomyocyte apoptosis[11]. Inflammatory cascades related to different pathologies are likely to vary. For example following acute ischaemia seen in MI, acute-phase responses will be paramount, whereas in chronic heart failure other inflammatory mediators will dominate. Viral and autoimmune causes of heart failure will also present with different chemical mediators and chemoattractants. Identification of the chemical mediators would provide valuable information and possibly novel strategies for targeted therapies to address the underlying condition[12,13].

Y-chromosome positive cardiomyocytes in males with female allograft hearts have been detected in regions of acute rejection[14]. This supports the postulated theory that stem cells have the ability to home in on sites of acute damage, requiring repair. The chemical mediators produced in response to stress and damage may be the same as those that attract cells capable of repair, and they may additionally be the same factors that retain these cells *in situ.*

The exact mechanism of action of stem cells in damaged tissue is unclear. Once located in a region of damage it is possible that stem cells may fuse with resident, damaged tissue, augmenting the action of the host cells and hence restoring function; stem cells may repopulate the host tissue with normally functioning cells of the same origin and type, in so doing inducing repair; alternatively vasculoneogenesis may occur, improving local circulation at the

site of damage, increasing the resultant access potential of other repair mediators. It is likely however that a combination of all of these factors comes into play, as is seen in other physiological situations of repair and regeneration.

Chemical mediators are known to play a significant part in the remodelling of living tissue. This remodelling is likely to be achieved by stem cells. Remodelling of the myocardium may be measurable by observed changes in cardiac mass or structure, particularly identifiable on cardiac MRI. This protocol assessed functional change in the myocardium; however, remodelling could also be assessed via cardiac MRI. It was possible to determine LV mass the significance of this data is yet to be established via the MRI imaging obtained. This was done as part of the study however the significance of these values is yet to be determined.

The parameters used for cardiac assessment in this study were LV end-diastolic volume, end-systolic volume and ejection fraction. These variables were measured blind by the Research Fellow, trained in basic MRI analysis, and the results double-checked by a Consultant Radiologist specialising in Paediatric Cardiac MRI imaging. Using a single assessor of these cardiac MRI scans minimised inter-observer variability of the study. Intra-observer variability was also reduced by following strict rules while assessing the regions of interest within each scan, and repeatedly performing analyses of each of the 30 MRI scans performed to ensure consistency of the results. A larger study including a greater number of patients would likely necessitate evaluation of the MRI scans by more than one assessor. The greater the number of scans to be analysed,

the more robust the findings would be as the effect of human error would be minimised.

Cardiac improvement could be assessed by additional means. Quality of Life (QoL) assessment could be performed, which would be especially valuable if blinding of the BMC injection stage could be achieved. This non-invasive means of assessment, although time-consuming, may be more acceptable to patients and their families than assessments requiring anaesthesia or hospital in-patient stay. Angiography was used as a means of assessment in several of the adult studies. This invasive procedure would require, at a minimum, heavy sedation or preferably an anaesthetic when performed in children, and hence would have similar acceptance as the MRI and catheter procedure combined. Echocardiograms provide another non-invasive means of cardiac assessment. However, echo derived ejection fraction is difficult to measure in children and echo measurements are much more varied than those derived from MRI.

This protocol utilised BMCs and their potential effect when administered via intracoronary injection. The subpopulations within the BMC solution were analysed immuno-phenotypically to provide additional novel data. Other cell types have been utilised in similar protocols to assess their potential roles and it should be noted that additional stem cell types exist although they are not apparently deemed appropriate for clinical use, such as embryonic stem cells and induced pluripotent stem cells.

Embryonic stem cells are pluripotent, being able to develop all the cell types required by the developing embryo such as muscle, skin, bone, blood, nervous

system and gut. These multiple cell lines are able to grow into a functioning independent organism when placed in an appropriate developmental environment. Embryonic stem cells are derived from the inner cell mass of the developing blastocyst and may be expanded in culture *in vitro*. Although they offer a very promising potential resource for a complete range of tissue generation, their use is associated with ethical issues and potential long-term complications. The ethical issues regarding the use of cells obtained from an embryo have been thoroughly debated[15,16,17]. While nobody disputes the therapeutic potential of stem cells, the moral dilemma of using embryonic stem cells is also clear. The process of harvesting embryonic stem cells usually destroys the human embryo and raises issues regarding the value of life, questioning a process that eliminates a potential human life to treat another. The pluripotency of embryonic stem cells also offers the potential of human cloning from these cell lines. These contentious issues have been debated internationally. In October 2011 a European Union ruling refused to allow patenting of the procedures that are used to obtain embryonic stem cells, and in the United States Federal funding of research on embryonic cell lines obtained after August 9th 2001 was removed [18].

Whilst the ability of embryonic stem cells to develop into a wide range of tissues offers great therapeutic potential, this cellular versatility also conveys the possible risk of inappropriate or unwanted tissue types developing. Problems have been encountered in some studies when certain cell lines have been used in animal models. The possibility of tumour formation, abnormal calcification and cardiac arrhythmias has been reported and has the potential to cause significant morbidity *in vivo*[19]. Long term follow up studies will be required to

evaluate this further. Abnormal calcification is known to be the end stage of many pathological processes including degeneration and dysmorphic development. With this in mind it would be interesting to understand the preceding environment that has been created when abnormal calcification secondary to the use of embryonic stem cells is observed.

Induced pluripotent stem cells (iPSCs) are derived from a previously non-pluripotent cell line, typically a somatic cell, which has been genetically manipulated by the transduction of transcription [20]. These artificially created pluripotent cells retain the ability to produce the various cell types derived from all three germ cell layers with unlimited capacity[21]. Their pluripotency may lead to similar risks of teratogenicity[22] as with other pluripotent cell lines such as embryonic stem cells. Viral vectors are commonly used to integrate new genes into cell lines to induce pluripotency. Use of viral vectors to induce pluripotency may cause additional risks to a recipient by additionally triggering activation of oncogenes[23]. IPSCs can be developed from the somatic cells of individual patients for autologous use thereby reducing immune activation. Their clinical application however is restricted to use as non-acute therapy by the time it takes to harvest and generate these cells. Forcing pluripotency of old somatic cells also increases the risk of passing damaged genes from the original cell into subsequent generations of progeny.

Certain congenital cardiac disorders such as atrioventricular septal defect, tetralogy of Fallot and familial congenital heart disease have recognised genetic associations, as do certain syndromes with specific cardiac defects such as Holt Oram, Alagille and Noonan syndromes. Inherited forms of cardiomyopathy exist

in addition to cardiomyopathy associated with inherited neuromuscular conditions, such as Duchenne Muscular Dystrophy. Use of autologous iPSCs as therapy for these genetically based cardiac conditions may be sub-optimal as autologous cell lines would transmit the same genetic abnormality as the original parent cell from which they were cultured. The same may be true of any autologous stem cell therapy where a genetic defect is recognised.

Genetic engineering may permit gene repair or insertion into iPSCs to create patient-specific therapies. Alternatively stem cells for therapeutic use in situations where a genetic component to the disorder is already recognised or suspected may be better obtained from an allogeneic source, a healthy donor, a non-affected cell line being therefore used to address the genetic abnormality of the patient.

The presence of genetic aberrations of the patient being identifiable in iPSCs may be advantageous as it could permit the investigation of the genetic component of the phenotypic variant. Sufficient iPSCs could be cultured and used to create a disease model of the disorder. *In vitro* analysis, providing valuable information, may be obtained allowing additional understanding of the mechanisms causing the defect. Genetic variants, developmental and environmental influences may be explored to provide new information on which novel therapeutic strategies can be focused.

The role of iPSCs in the future of stem cell therapy needs to be carefully researched. It appears however that using current technology only pluripotent

stem cells such as embryonic stem cells and induced pluripotent stem cells can be expanded in sufficient quantities to provide clinically useful tissue[24].

7.2 Future roles of stem cells

Other stem cell types being actively investigated for clinical use include cardiac stem cells. The identification of cardiac stem cells within the myocardium offers a great opportunity for the ultimate cardiac stem cell repair. Cardiac stem cells maintain mitotic ability to create cardiac specific progeny. They offer significant potential advantage over other adult type stem cells as they are already committed to the appropriate cardiovascular cell lineages and are therefore most appropriate for the myocardial environment[25]. Cardiac progenitor cells are only present in the native myocardium in relatively small numbers, therefore harnessing their therapeutic potential will rely on adequate harvesting and cell expansion. As with other stem cell types it may be possible to stimulate cardiac progenitor proliferation by applying growth factors and mediators[26]. An understanding of the factors that stimulate dormant resident cardiac progenitors and activate their proliferation would assist in the development strategies to effectively use these cells therapeutically. Clarity in understanding of the origin of these cells would be helpful to utilise their full potential. Cardiac progenitor cells may develop during fetal life, remaining dormant in regions of the myocardium as yet unidentified, and becoming active upon stimulation either homeostatically or in response to triggers produced as a consequence of damage. Alternatively they may derive from an extra cardiac source, again activating in response to similar postulated factors and mechanisms[25]. Understanding where exactly they reside and how they are activated and controlled would clearly be beneficial as these cells are so precisely dedicated

to a cardiac lineage as potentially to be the most useful source of specific cardiovascular repair cells.

Amniotic stem cells are multipotent stem cells harvested from the amniotic fluid[27]. They have not been associated with abnormal tumour formation and are not linked with the same ethical deliberations as embryonic stem cells, but are still able to differentiate into a wide range of cell lineages and so maintain significant clinical potential.

Adipose tissue is a source of mesenchymal stem cells which have the ability to differentiate into bone, muscle, fat and cartilage cell lines[22]. Functional cardiomyocyte type cells have been cultured from adipose stromal cells and exhibit typical contractility and pacemaker activity. Adipose tissue being so readily available may provide a useful potential source of cardiac progenitors[28].

To date, stem cell therapy has relied on the ability to isolate appropriate progenitor cells for clinical use. The current method of identification of the stem cell-surface protein markers is the application of labelled antibody, followed by the identification of the labelled antibody by flow cytometry. Many cell-surface proteins however remain undetectable. The relatively new method of proteomics is able to establish the presence of a particular protein in a specific location on a cell without the use of antibody. Quantitative proteomics has the potential to be useful in helping identify, quantify and localise novel cell-surface protein markers and may have a place in future stem cell studies[29].

7.3 Cardiac tissue engineering

Tissue engineering processes are used across several fields of medicine and have the same common objective of creation of biological substitutes that can restore, maintain or improve tissue formation[27]. In the cardiac domain the aim is to repair damaged myocardium using cultured cells and artificial scaffolds that are created *in vitro* and inserted directly into the heart[30]. As with stem cell injections, cardiac tissue engineering needs ideally to be able to produce functionally viable material that is capable of conduction of electrical impulses and co-ordinated rhythmic contraction whilst supporting new vessel formation and allowing adequate oxygenation at a cellular level of its component parts. The native heart is a complex construction of several cell types, each component having a critical and individual role in the overall functioning of the organ. This intricate natural arrangement is clearly difficult to construct artificially.

7.3 i *In situ* cardiac tissue engineering

Studies have investigated the potential use of integrating stem cells into an injectable matrix with a view to improving the retention of cells at the site of cardiac injection. It has also been suggested that a gel type biomatrix could be effective in supporting stem cell survival after injection[30]. In addition to cellular components of these biomaterials, growth factors can be infused into the structures to improve survival, growth and repair of the tissue. Injectable solutions can be delivered into the damaged myocardium in the same way as stem cell suspensions, without the need for major surgical procedures and may therefore be similarly utilised to address cardiac muscle pathology. Several types of materials are available to suspend and deliver cells to target areas,

including fibrin, collagen, alginates and synthetic structures[31]. Such matrices simply supply a transfer medium for cells and therapeutic agents but often do little to mimic or support the native extracellular environment. This may be a disadvantage as cardiac function may need to be optimised by repairing both cardiac structure and cell loss. Some studies have however suggested that a degree of myocardial support is offered by the injected biomaterial and that although some biomechanical support is advantageously conveyed through the matrix it rarely provides appropriate flexibility to permit synchronicity of motion[14]. The biogels invariably degrade over time limiting the period for which the cell therapy is effective. Using gels in this injectable form also poses the risk of vascular occlusion if the gel does not adhere at the correct site and instead lodges in the vascular bed[32].

7.3 ii *In vitro* development of cardiac replacement tissue

With underlying matrix:

Replacement tissue can be constructed by seeding harvested or cultured stem cells on a non-immunogenic structural scaffold. The scaffold is used to replicate the region of damaged cardiac tissue and to provide an adherent surface on which the cells can function. Aside from the potential complications associated with different types of stem cells, the architectural scaffold can introduce complicating factors into the host environment. To minimise these complications the matrix used should as far as possible meet certain requirements. The material used for such constructs should be sterilisable, non-toxic, durable and appropriate to meet the physiological and mechanical demands that will be placed upon it in the long term[33]. Ideally the tissue utilised should also be able

to support potential growth and movement of the tissue or organ in line with that encountered physiologically.

As with injectable matrices, the scaffold can be made of naturally occurring or synthetic compounds. Natural scaffolds include engineered collagen, fibrin and other connective tissue structures. Heart valves and tissue patches are being developed for surgical implantation and may prove useful in patients with congenital cardiac disorders. For example at present the demand for pulmonary valve homografts exceeds supply, hence there is a real need to provide alternatives. Stem cell engineered constructs that have the potential to grow and adapt with the patient will avoid complications such as calcification and stenosis seen long term with current grafts. However significant risk is associated with any surgical procedure in patients with congenital heart disease and needs to be recognised when evaluating suitability of procedures for this patient population.

Studies have commenced looking at the potential of cellular stripping of complete or partial cadaveric donated tissue. This method will potentially provide an anatomically complete and correct matrix onto which stem cells may be populated. Different cell types would be required to reproduce a functioning heart with sufficient power of contractility, coordinated muscle action and propagation and conduction of electrical stimulation. The appropriate implantation of this range of cells will be challenging.

Although the technique appears to be feasible *in vitro* difficulties exist with inducing competent vasculature to allow sufficient perfusion of the multiple cell

layers, tissue patches or indeed the entire new organ. Currently survival of implanted structures is significantly limited by poor vascularity and is reliant primarily on perfusion alone for support. This is sub-optimal for longevity of graft tissue survival and minimises the potential for the inserted tissue to grow and adapt with the heart.

Without underlying matrix:

Stem cells are also being cultured *in vitro* for use without any supportive transfer medium or matrix. Stem cell engineering has been used to culture sheets of replacement tissue suitable for insertion into the heart. These sheets of cells, one layer thick, can be layered on top of one another to create a three dimensional structure. The cells used are beneficial for cardiac recovery but lack the essential vascularity to provide oxygenation and nutrition[24], processes which must therefore occur by diffusion. For this reason layered structures such as these cannot exceed the critical dimensions, approximately three or four cell layers[30] or 50 to 100 μm[20].

Cell sheets have been developed from cardiac progenitor cells obtained from cardiac biopsies, these cultured cells being collectively called cardiospheres. Cardiosphere derived cells (CDCs) are multipotent and have been used successfully in animal studies to improve myocardial function after infarction. These cardiospheres are scaffold-free and appear to efficiently deliver the appropriate cells to the damaged myocardium and promote cardiac repair by neovascularisation and cardiomyogenesis. CDCs are cardiogenic *in vitro,* they promote regneration and improve heart function in mouse models of infarction[34]. Trials of intracoronary injections of CDCs have taken place; intracoronary

administration of autologous CDCs did not raise significant safety concerns. Preliminary indications of bioactivity include decreased scar size, increased viable myocardium, and improved regional function of infarcted myocardium at 1 year post-treatment. These results, which are consistent with therapeutic regeneration, merit further investigation in future trials[35,36].

7.4 Future perspectives

In the near future it is likely that stem cell therapy will be recognised as a safe and effective means of producing improved cardiac function and incorporated into common clinical use. The longevity of its effectiveness may depend on the underlying disorder and co-morbidities, the route of administration and the type of cells used. The role of stem cells in heart muscle disease is already being recognised and its place as a treatment is likely to be confirmed. In situations where anatomical repair of the heart is required the utilisation of stem cell administration is still being explored. As processes of biomechanical engineering progress we can anticipate exciting developments in its application. Methods of improving oxygenation and nutrition to engineered cardiac structures are being researched, these include promotion of blood vessel formation, use of growth factors and specialised scaffolds and cell sheet layers[22].

At present the use of stem cells as a cardiac therapy has focused on conditions where secondary muscle damage has occurred, for example as a result of ischaemic, inflammatory and cytotoxic insult. Stem cells have been administered into the local or peripheral circulation or by direct surgical implantation into the damaged myocardial tissue. The processes which follow,

and subsequently result in functional improvement, are not clearly defined but are likely to be multi-factorial and interactive.

Individual case studies have highlighted the potential use of stem cells within the paediatric population, and this first randomised cross over placebo-controlled trial has extended that application and assessment further. Our first experience of the use of bone marrow derived mononuclear cells in the management of a child with heart failure was in 2008 when we performed our first administration of autologous stem cells to a child awaiting cardiac transplantation. This procedure was performed to coincide with the insertion of a Berlin Heart left ventricular assist device (LVAD). The child, an eight year old boy with heart failure secondary to unconfirmed viral myocarditis, was already sedated and ventilated on our paediatric intensive care unit. Bone marrow aspiration was performed under aseptic technique as a surgical procedure on the unit immediately prior to the child being taken to our cardiac theatres in preparation for insertion of the left ventricular assist device. The bone marrow was processed and stem cells obtained and suspended in a 2mL volume for intramyocardial injection under direct visualisation. The stem cell suspension was divided into ten injectable aliquots by the injecting cardiac surgeon and a suture placed at each injection site to mark the injection points over the left ventricle. The patient tolerated the procedure well and went on to receive a cardiac transplant 14 days later, at which time his explanted heart was analysed with particular attention paid to the stem cell injection sites. This was the first of our experiences with such techniques, and indicated to us that our main study protocol would be feasible within our establishment. Case reports from other institutions have also described the use of stem cells in a young child with

dilated cardiomyopathy[37] and another with hypoplastic left heart syndrome and right heart failure[38].

Patients with failing heart muscle secondary to their congenital condition are likely to respond to stem cell therapy in a similar way to those with a primary heart muscle disorder, but at present stem cells are not available for use as treatment of congenital abnormalities. Stem cells have not been used to refine or re-order cardiac structures that already exist *in vivo. In vitro* stem cell studies have involved culturing cell lines that are able to function in place of cardiac myocytes and seeding them either directly into the heart or onto a non-immunogenic matrix that replicates an anatomically correct cardiac structure. Such tissues can be inserted into a congenitally malformed heart in an attempt to produce structural normality or functionality. These studies are in their early stages but offer exciting potential.

7.5 Future development of this study

As this study has achieved its primary outcome measure of safety and feasibility, it has the potential to be implemented in the centres designated by the National Commissioning Group for mechanical support and bridge to transplantation. After safety and efficacy are established in these centres it can be disseminated to the twelve regional paediatric cardio-thoracic centres including Belfast, Glasgow and Dublin (the latter centre closely collaborates with the UK and refers children to London for mechanical support and heart transplantation). This would develop a new service delivery, which would be easier and more accessible than organ transplantation and mechanical support. The treatment could provide an extended period of improved health and well

being for children with heart muscle disease. It may be over optimistic to consider this therapy as a cure but it could ameliorate heart failure symptoms and defer transplant surgery for some years and may reduce dependency on mechanical circulatory support. These would be positive developments in paediatric heart failure management.

Stem cell therapies provide options over and above conventional methods of cardiac treatment used to date. In patients where maximal pharmaceutical management is insufficient, surgical strategies such as repair, augmentation or transplant are often the only remaining option. Stem cell therapy can be used in several ways, from repair and regeneration of resident tissue to complete replacement of abnormal structures. Cellular myoplasty has the potential capacity to preserve and restore cardiac function. It can be initiated rapidly in acute situations providing a definitive therapeutic strategy or allowing functional improvement sufficient to bridge to recovery or an alternative conventional treatment. Engineered tissues and constructs can be individually tailored to meet the requirements of each patient. Surgical engraftment of tissue may ultimately reduce the need for other more traditional surgical interventions such as transplant, and their consequent associated complications.

This is the first randomised placebo controlled study of autologous stem cells in paediatrics. In paediatrics, invasive interventions often require general anaesthesia, which complicates study organisation in fragile heart failure patients, but it was possible to complete both arms in all our patients. We have shown that infusion of the cells was safe and feasible with a single anaesthetic allowing time to harvest the marrow, prepare cells, perform MRI and infuse

them into the coronary arteries. The availability of cardiac MRI in the angiography suite enabled accurate assessment of ventricular volumes and interestingly the data show that infusion of cells was associated with decreased left ventricular volumes compared to placebo. The improvements in ejection fraction did not reach significance, although reversing remodelling and improving ventricular volumes may be more important in chronic heart failure[39,40].

Recently expression of concern has been published regarding one publication[41]. Criticism is even harsher in paediatrics where non-randomised treatments are often developed because invasive placebo limbs are so difficult. Even in non-invasive studies of paediatric heart failure there is perhaps only a single high quality randomized placebo-controlled drug study[42]. Our study may help to reassure doubters that complex randomised placebo-controlled studies of stem cell therapy can be achieved safely in paediatric patients. Our data show a reduction in ventricular volumes, which is broadly comparable with adult data on autologous stem cells suggesting reversal of remodelling[43]. However it is important to be realistic, even though this safety and feasibility study was not designed to formally assess functional outcome, the MRI would have been reliable to detect a small (3%) change in EF but this was not detected and NT proBNP did not improve.

Research into the use of stem cells as a potential cardiac therapy is advanced enough to provide data supporting the safety and feasibility of several techniques[44]. The procedures for harvesting different cell types are established, as are the methods of processing. Administration of the stem cells also follows

standard protocols for peripheral or cardiac vascular insertion or surgical implantation. Combining the most appropriate protocols for each step is paramount in optimising the use of stem cells effectively *in vivo*. Understanding the processes involved in tissue engraftment and its long-term survival *in vitro* will provide essential insight to maximise the effect of their use. The therapeutic potential of stem cells is real; the fine-tuning of their application is now open for investigation.

7.6 References

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CHAPTER 8

CONCLUSION

8.1 Conclusion

The primary objective of this pilot study was to develop a protocol that was safe, reproducible and well tolerated by patients and their families. This was achieved.

This study demonstrated that the use of stem cells in children to address the condition of heart failure is both feasible and acceptable. No mortality or morbidity was experienced throughout the year-long protocol, and no significant adverse events were reported. The procedures utilised within the protocol were both safe and acceptable to the patients, their families and the operators. Of particular note the patients reported no discomfort from the procedures undertaken. Inconvenience for the patients and their families was minimised as the research review and admission stages were integrated, where possible, into the clinical care pathway for each individual. A single patient successfully underwent the procedures under sedation rather than general anaesthetic. If a similar protocol could be extended to other patients, the recognised major risk of this study, general anaesthetic, would be removed and the protocol could ultimately become more acceptable and available.

The secondary endpoints, the assessment of cardiac function, indicated a positive effect of intracoronary injection of autologous mononuclear cell injections. The majority of patients demonstrated a reduction in the end-systolic volume and the end diastolic volumes as measured on cardiac MRI imaging. Interpretation of numerical data in isolation was difficult when viewed out of context of the entire clinical picture. Quantification of cardiac function was difficult to evaluate accurately using EF alone. When both ESV and EDV were

reduced EF typically remained unchanged despite the functional improvement suggested by the reduced ESV and EDV. This was clearly the result of EF being a calculated ratio of these two parameters that synchronously reduced. However, in isolation, an unchanged EF could potentially be incorrectly interpreted as no alteration in function.

On the small amount of data collated on these 10 patients regarding the stem cells administered, it was difficult to extrapolate any particular clinical effect of any individual cell populations on cardiac function. As previously postulated, it is most likely that the cell populations administered *en masse* interact in a synergistic fashion to influence their environment, in this case the myocardium.

The methods of flow cytometry and FACS analysis provided data that permitted an interesting insight into the cell populations present within this patient cohort. This novel investigation protocol has the potential to be utilised further in the future. Similar analyses of such stem cell injections have the potential to provide more significant data if expanded to a wider population group.

The protocol was facilitated by the availability of all required departments being within the same hospital site. For this study, in-patient facility, cardiac catheterisation suite, MRI scanner and stem cell laboratory were required to make the single anaesthetic protocol feasible.

Several of the families involved in this research expressed an interest to be included in further similar studies. Anecdotally, some felt that the injection of mononuclear cells had had a beneficial effect, even if only temporary. Many

families were interested in the evolution of the study protocol. This supports the conclusion that research into the study of the effects of stem cells in children with heart failure should be explored further.

The primary end points of freedom from death or transplant were achieved in this study. The secondary end-point of improved cardiac function, as demonstrated on cardiac MRI, was achieved with respect to reduced EDV and ESV. The additional anecdotal evidence from the patients and families involved in the study endorsed the process and the potential beneficial outcome.

As an initial pilot study in this field, this research provides an exciting grounding from which further studies may be planned. The protocol utilised on this occasion has the potential to be modified in the ways previously discussed to make it more acceptable to a wider population of patients and more available for use in other clinical centres.

GLOSSARY OF TERMS

Ab	antibody
ACE	angiotensin converting enzyme
Ag	antigen
AML	acute myeloid leukaemia
ANP	A-natriuretic peptide
Ao	aorta
APTT	activated partial thromboplastin time
ARVC	arrhythmogenic right ventricular cardiomyopathy
ASD	atrial septal defect
ASTAMI	trial acronym: Autologous Stem-cell Transplantation in Acute Myocardial Infarction
BMA	bone marrow aspirate
BM	bone marrow
BMC	bone marrow cells
BMR	Bone Marrow Registry
BNP	B-natriuretic peptide
BP	blood pressure
BD	twice daily
BW	birth weight
CD	Cluster of Differentiation: classification of cell surface markers
CDC	cardiosphere derived cell
CHD	congenital heart disease
CICU	cardiac intensive care unit

CK	creatine kinase
CLP	common lymphoid progenitor cell
CMP	common myeloid progenitor cell
CMV	cytomegalovirus
CNP	C-natriuretic peptide
CO	cardiac output
CoAo	coarctation of aorta
CONSORT	Consolidated Standards of Reporting Trials
CPC	cardiac progenitor cell
CSC	cardiac stem cell
CSF	cerebrospinal fluid
CT	computerised tomography
CXR	chest X-Ray
CTL	Cell Therapy Laboratory
DCM	dilated cardiomyopathy
DMSO	dimethyl sulphoxide
EBV	Epstein-Barr virus
ECG	electrocardiogram
ECMO	extra-corporeal membrane oxygenation
EDV	end-diastolic volume
EEG	electro-encephalogram
EF	ejection fraction
EFE	endocardial fibro-elastosis
ENT	ear nose and throat
EPC	endothelial progenitor cell
ESV	end-systolic volume

FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FEV1	forced expiratory volume in one second
FISH	fluorescence in situ hybridisation
FMO	fluorescence minus one
FS	fractional shortening
FSC	forward scatter light signal in flow cytometer
FVC	forced vital capacity
GOSH	Great Ormond Street Hospital
GCSF	granulocyte colony stimulating factor
GMP	granulocyte macrophage progenitor cell
GvHD	graft versus host disease
HBc	hepatitis B core antigen
HBs	hepatitis B surface antigen
HBV	hepatitis B virus
HCM	hypertrophic cardiomyopathy
Hct	haematocrit
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HLDA	human leucocyte differentiation antigen
HR	heart rate
HSC	haematopoietic stem cell
HSV	herpes simplex virus
HSOP	hospital standard operating procedure
HTLV	human thymo-lymphotropic virus

ICD	implantable cardiovertor-defibrillator
ICH	Institute of Child Health
Ig	immunoglobulin
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-1	interleukin-1
IL-6	interleukin-6
INR	international normalised ratio
iPSCs	induced pluripotent stem cells
IV	intravenous
IVC	inferior vena cava
JVP	jugular venous pressure
LA	left atrium
LV	left ventricle
LCA	leucocyte common antigen
LSCS	lower segment caesarean section
LV	left ventricle
LVAD	left ventricular assist device
LVOT	left ventricular outflow tract
LVD	left ventricular diameter
LVDd	left ventricular diameter in diastole
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
MEP	megakaryocyte erythroid progenitor cell
MHC	major histocompatibility complex

MI	myocardial infarction
MLB	microlaryngoscopy and bronchoscopy
MNC	mononuclear cell
MPP	multipotent progenitor cell
MR	mitral regurgitation
MRI	magnetic resonance imaging
MSC	mesenchymal stem cell
NCAM	neural cell adhesion molecule
NG	naso-gastric
NK	natural killer cells
NP	natriuretic peptide
NT-pro-BNP	N-terminal pro-B type natriuretic peptide
NYHA	New York Heart Association
OD	once daily
PA	pulmonary artery
PBS	phosphate buffered solution
PCR	polymerase chain reaction
PEF	peak expiratory flow
PEG	percutaneous endoscopic gastrostomy
PFA	paraformaldehyde
PICU	paediatric intensive care unit
PR	pulmonary regurgitation
PVR	peripheral vascular resistance
QoL	quality of life
RA	right atrium
RBC	red blood cell count

RCM	restrictive cardiomyopathy
REPAIR-AMI	trial acronym: Re-infusion of Enriched Progenitor cells and Infarct Remodelling in Acute Myocardial Infarction
ROI	region of interest on MRI scan
RPM	revolutions per minute
RV	right ventricle
RVOT	right ventricular outflow tract
SCBU	special care baby unit
SCID	severe combined immunodeficiency
SD	standard deviation from the mean
SLE	systemic lupus erythematosus
S/N	supernatant
SOP	standard operating procedure
SP	side population
SPECT	single positron emission computed tomography
SSC	side scatter light signal in flow cytometer
SSFP	steady-state free precession cines
SV	stroke volume
SVC	superior vena cava
SVD	spontaneous vertex delivery
TCR	T-cell receptor
TDS	three times daily
TNFα	tumour necrosis factor alpha
TOXO G	toxoplasma gondii antibody
TPN	total parenteral nutrition

TR	tricuspid regurgitation
Treg	regulatory T-lymphocyte
VAD	ventricular assist device
VDRL	Venereal Disease Research Laboratory
VE	vascular endothelium
VEGF	vascular endothelial growth factor
VZV	varicella Zoster virus