Clinical and laboratory studies of mesenchymal stem cells in long bone fracture nonunion

Tarek A. A. R. A. Roshdy

Submitted in accordance with the requirements for the degree of Doctor of Medicine

The University of Leeds
School of Medicine
August, 2011

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

ACKNOWLEDGEMENTS

I would like to acknowledge and thank my Supervisors, Professor Peter Giannoudis, Professor Dennis McGonagle, and Dr. Elena Jones for their constant support, encouragement, constructive discussion and valuable advice they have given me throughout my study period. Both Professor Giannoudis and Professor McGonagle have supervised and helped me with the clinical aspect of this research. Dr. Elena Jones has been of great help in setting up my experimental studies and has supervised each and every part of my research, including laboratory experiments and without her help and input this work was not going to be possible.

I wish to acknowledge the Kuwaiti Civil Service Commission (CSC) in my country, the State of Kuwait and the Kuwait Cultural Office in London for financially supporting all aspects of my education, which includes University tuition fees, bench fees and all other expenses related to my stay in the United Kingdom.

I would like to thank Mr. G.K.Sefton, Consultant Orthopaedic Surgeon in Harrogate District Hospital who has been my mentor and Clinical Tutor throughout my training period in the United Kingdom.

I wish to express my gratitude for Professor Robert West, Professor in Health Statistics, Division of Epidemiology and Biostatistics, Leeds Institute of Genetics, Health and Therapeutics, for his great help in the statistical analysis of the results.

I acknowledge the help of my colleague, Dimitrios Kouroupis with the flow cytometry investigations of this work.

Last but not least, I wish to thank my family and friends for their ongoing support and understanding during the entire period of this work. Their love and support has always been and always will be of great value to me.

ABSTRACT

Treatment of atrophic long bone fracture nonunion is challenging with current therapeutic interventions including bone morphogenetic proteins (BMPs) or autologous mesenchymal stem cells (MSCs). In this work it was hypothesised that, total MSC numbers and their responsiveness to BMPs in the nonunion setting were compromised, leading to poor healing. Additionally, the rationale for systemic injection of MSCs to repair bone is based on a contentious concept of their widespread circulation. To address the number and functional competence of iliac crest bone marrow MSCs in nonunion, this study employed colony forming unit fibroblast and osteoblast assays (CFU-F, CFU-O) and flow cytometry enumeration of the $CD45^{low}CD271^{+}$ cell population, to compare nonunions (n=11) with united long bone fracture patients (n=11). Unexpectedly, total number of MSCs, was higher in nonunion; however their proliferative capacity, was lower. No response to BMP-7, assessed by CFU-F, CFU-O and calcium deposition assays, was found in both nonunion and union study groups. Possible mechanical translocation of MSCs into the venous circulation was investigated using matched antecubital venous blood from the upper limb (UL) and lower limb femoral venous blood (LL) samples from nonunion patients undergoing reaming with reamer irrigation aspiration (RIA) (n=12) and other non-reaming procedures (NR, n=12) with control groups including UL from early rheumatoid arthritis (RA, n=11) and healthy controls (n=12). CFU-F assay results revealed the presence of MSC colonies in LL at higher frequencies than UL samples in RIA and NR, (8LL, 2PB) and (5LL, 1PB) respectively. None were detected in the UL of RA and controls.

Altogether, these results indicate a functional defect in proliferative capacity of MSCs in nonunion. MSCs however are unlikely to circulate and contribute to reduced healing at fracture sites. This points towards a generalized systemic effect of the nonunion state on MSC dynamics, which should be further explored in future.

CONTENTS

FIGURES

TABLES

ABBREVIATIONS

µl	microlitre
µM	micromolar
7AAD	7-Amino-actinomycin D
ALP	alkaline phosphatase
ANA-2	antinuclear antibody -2
As2P	ascorbic-2 Phosphate
BM	bone marrow
BM MSC	bone marrow mesenchymal stem cell
BMA	bone marrow aspirate
BMAC	bone marrow aspiration concentrate
BMP	bone morphogenetic protein
BSA	bovine serum albumin
CFU-F	colony forming unit fibroblast
CFU-O	colony forming unit osteoblast
CPC	cresolphtalein complexone
CRP	C reactive protein
CV	coefficient of variation
DMEM	Dulbecco's modified Eagle's Medium
DMSO	dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ESR	erythrocyte sedimentation rate
EX	culture expanded
FACS	fluorescence-activated cell sorting
FBS	foetal bovine serum
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FGF	fibroblast growth factor
FSC	forward scatter
HDE	humanitarian device exemption
hMSC	human mesenchymal stem cells
IC	Iliac crest

IGF	insulin-like growth factor
IL-1	interleukin 1
IL-6	interleukin 6
ISS	Injury severity score
LGI	Leeds General Infirmary
LIMM	Leeds Institute of Molecular Medicine
LN2	liquid nitrogen
MEA	aminoethanol buffer
mg	milligram
ml	millilitre
MNCs	mononuclear cells
MSC	mesenchymal stem cell
MSCs	mesenchymal stem cells
ng	nanogram
NH	nonhematopoietic
Nr	number
NSAIDS	non-steroidal anti-inflammatory drugs
OD	optical density
ORIF	open reduction and internal fixation
p0	passage zero
PB	peripheral blood
PBS	Dulbecco's phosphate buffered saline
PD	population doubling
PDGF	platelet derived growth factor
PDs	population doublings
PDT	population doubling time
RA	rheumatoid arthritis
rcf	relative centrifugal force
RF	rheumatoid factor
RIA	reamer irrigator aspirator
RT	room temperature
RTA	road traffic accident
SCID	severe combined immune deficiency

SD	standard deviation
SSC	side scatter
TGF-β	transforming growth factor beta
TNF-α	tumour necrosis factor alpha
w/v	weight/volume

CHAPTER 1

FRACTURE HEALING AND FRACTURE NONUNION IN LONG BONES

1.1 INTRODUCTION

Bone is a metabolically active connective tissue under constant regulation by a variety of biochemical, biomechanical, cellular and hormonal factors. Under normal conditions, these factors play an important role in the maintenance of bone integrity and are key players in the regulation of fracture repair [1]. Due to its rigid nature, bone is vital for mechanical support, locomotion and protection of vital body organs. Its high calcium content makes it a reservoir for calcium and hence is extremely important for calcium homeostasis. Bone marrow (BM) present inside bone medullas is the main site of hematopoiesis in adults [2].

A bone fracture is a disruption in the continuity of bone. This can range from microscopic discontinuity to a multi-fragmented (comminuted) break. As the spectrum of fractures is vast, classification systems have been used to accurately describe them. Fracture classification systems provide a unified language to accurately describe fractures, facilitate the placement of guidelines for optimal fracture management and help in predicting fracture outcomes.

Numerous classification systems have been proposed to describe long bone fractures in adults. They maybe be classified based on their anatomical location within a bone (metaphyseal, diaphyseal, or epiphyseal). Based on fracture geometry they may be referred to as displaced or undisplaced, transverse, spiral, segmental or comminuted. Fractures inside joints are known as intra-articular fractures whereas those not involving joints are described as extra-articular.

A fracture maybe classified as "open" or "closed". An open fracture is one which has a wound that connects the fracture site to the outside environment. These fractures are at a higher risk of infection and nonunion. In a closed fracture, the skin remains intact and therefore there is no communication between the fracture site and the outside environment [3]. One of the most commonly used systems to describe open fractures is the Gustilo and Anderson classification (Table 1.1) [4].

Table 1.1: The Gustilo and Anderson classification for open fractures

Grade	Description
Grade I	Clean wound ≤ 1cm, usually from inside out. Minimal soft tissue damage
Grade II	Moderately contaminated wound >1cm. Slight or moderate soft tissue damage
Grade III	Extensive soft tissue damage, usually due to high velocity injury and / or severe crush injury
Grade IIIa	Soft tissue coverage of the fractured bone is adequate
Grade IIIb	Extensive soft tissue injury requiring a flap to cover the exposed bone
Grade IIIc	Any open fracture that is associated with arterial injury necessitating repair.

Based on their aetiology, fractures maybe classified into four groups [5].

1- **Fractures caused only by a sudden injury.** These are the most common types of fractures. They occur in healthy bone when a strong force exceeding the strength of the affected bone is applied. This force may be direct (when the force hits the bone directly) or indirect (when the force is transmitted along the bone to cause fracture elsewhere in the bone).

2- **Fragility fractures.** These fractures are associated with generalised bone weakness (e.g. osteoporosis). Since the affected bone is already weakened by the disease, less force is required to cause the fracture.

3- **Fatigue (or stress) fractures.** These result from frequent repeated stresses on a bone leading to an eventual fatigue of the bone and fracture. This commonly occurs in athletes and military recruits. The lower limb is a common site for such fractures, mainly the metatarsals, fibula and neck of femur.

4- **Pathological fractures.** These can occur secondary to bone lesions that weaken the bone e.g. bone tumours. A minor trauma can initiate the fracture and at times no trauma at all is necessary to induce it.

1.2 NORMAL FRACTURE HEALING

Fracture repair is a physiological process key to the restoration of bone integrity and function [6]. The reparative event in bone is unique in that it heals with bone without scarring. Two main types of fracture healing are known; indirect (or secondary) and direct (or primary).

1.2.1 Indirect fracture healing

This is the most common type of fracture healing and is characterised by the production of callus. It simulates both intramembranous and endochondral bone formation that is also seen in the embryo during skeletal development [7]. During endochondral ossification, mesenchymal stem cells (MSCs) differentiate into chondrocytes, which in turn lay down a cartilaginous frame that becomes ossified to form bone. In contrast, during intramembranous ossification, bone is formed directly from mesenchymal tissue without the initial cartilaginous model [2].

This type of healing can be seen following non operative fracture management or following fixation of fractures using implants that allow micro motion at the fracture site (e.g. intramedullary nails) [8, 9]. Secondary fracture healing occurs over 3 overlapping stages; 1) the early inflammatory stage; 2) the repair stage (chondrogenic and osteogenic); and 3) the late remodelling stage [1] (Figure 1.1).

1.2.1.1 Inflammatory stage

Immediately following an acute fracture, a haematoma is formed from the bleeding ends of bone and surrounding soft tissues and an acute inflammatory response is triggered. At the molecular level, this is a very complex and coordinated process which involves the release of multiple sets of molecules: pro-inflammatory cytokines, growth and differentiation factors, metalloproteinases and angiogenic factors [10]. The acute inflammatory response peaks in the first 24 hours and reaches an end within one week [11].

Phagocytic cells and macrophages in particular are attracted to the site of repair clearing away the debris and dead tissue that were formed as a result of the insult. This is in preparation for healthy granulation tissue formation, neovascularisation and attraction of MSCs to the site of repair [1].

The release of inflammatory mediators and cytokines mainly prostaglandins, tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-11(IL-11) and interleukin-18 (IL-18) promotes the recruitment of inflammatory cells and angiogenesis at the site of fracture [12]. Administration of non-steroidal anti-inflammatory drugs (NSAIDS) at this stage of the healing process can delay or even prevent fracture healing [13].

Bone needs osteogenic cells to regenerate and these are thought to be derived from the differentiation of MSCs residing in neighbouring BM, bone cortex, periosteum and adjacent soft tissue [14]. Bone marrow MSCs (BM MSCs) are highly proliferative stromal cells that are clonogenic and are capable of trilineage differentiation into bone, cartilage and fat. In addition to local recruitment, it has also been suggested that MSCs are possibly systemically recruited via the circulation from sites distant to the fracture [15-17]. However, this is a contentious assertion and is not supported by other studies [18-20].

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor beta (TGF-β) superfamily which consists of at least 34 members; they are key molecules involved in the induction of differentiation, growth, apoptosis and morphogenesis of different types of cells [21, 22]. BMPs 2 – 7, 9 and 14 are known to be responsible for MSC recruitment and osteoblastic differentiation during fracture healing [22, 23]. During the inflammatory stage, BMP-2 and 4 seem to be the main players in recruitment of MSCs to the site of repair and later in the repair process; they promote the differentiation of MSCs into chondrocytes which form the cartilaginous soft callus. During the osteogenic phase of fracture healing BMP-7 together with other BMPs is highly expressed with an exclusive peak at day 14 and 21 of BMP-7 and BMP-8A respectively [22].

1.2.1.2 Repair stage

During this stage of the fracture healing process a cartilaginous frame known as "soft callus" is formed by intramembranous bone formation which connects the fracture ends together hence provides temporary stability. Under the effect of BMPs, undifferentiated MSCs proliferate and differentiate into chondroblasts which in turn become chondrocytes [24]. These secrete type II collagen and proteoglycans which form the soft callus [23]. For fracture healing to continue, a blood supply needs to be established for the developing callus [25]. An initial phase of chondrocyte resorption and degradation is essential to allow for healthy vascularisation of the callus. This is followed by chondrocyte hypertrophy with the subsequent release of angiogenic growth factors including angiopoietins and a vascular endothelial growth factor (VEGF) [26]. Calcification of the extracellular matrix then takes place [27].

Concomitantly, healing by intramembranous bone formation takes place. MSCs from the surrounding periosteum undergo osteogenic differentiation producing the "hard callus" directly without the cartilaginous frame noted in endochondral bone formation [28].

1.2.1.3 Remodelling stage

During this stage, coordination between the resorption of callus by osteoclasts and the deposition of lamellar bone by osteoblasts coexists. Cytokines including IL-1 and TNF-α orchestrate this stage and at the same time, the expression of BMPs is considerably reduced [29].

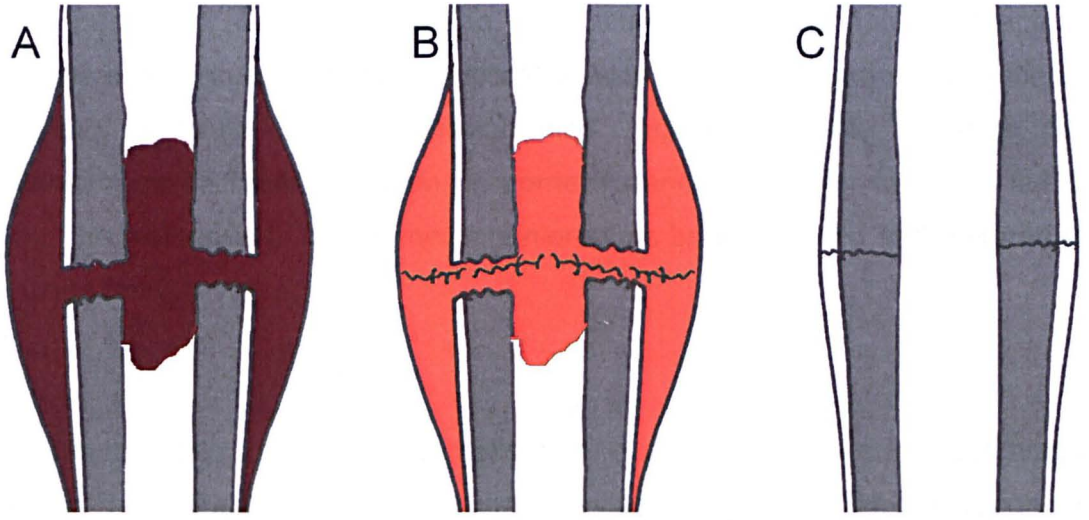

Figure 1.1: Stages of secondary fracture healing

A: Inflammatory stage, haematoma formation at the fracture site. B: Repair stage, soft callus is developing at the fracture ends and C: Remodelling stage, callus has been resorbed and bone is being gradually restored to its original shape.

1.2.2 Direct fracture healing

Direct (primary) fracture healing occurs in cortical bone, when there is almost absolute stability at the fracture site and minimal gapping of fracture ends (e.g. following rigid internal fixation of fractures with compression plates). In simple terms, osteoclasts working together in groups known as "cutting cones" cause

bone resorption, and these are followed by osteoblasts that lay down new bone. This process aims at restoring the functional unit of cortical bone known as the Haversian system or osteon [6]. Characteristically, there is no callus formation during this process and bone is healed "directly" by bone [30].

1.3 LONG BONE FRACTURE NONUNION

Whereas fracture healing is a physiological process, and theoretically all bones are expected to heal, this scenario is far from true in orthopaedic practice. Fracture nonunion is a serious complication with severe implications on patients including prolonged pain, suffering and reduced mobility. Despite the advancement of fracture fixation, in some instances however fractures fail to heal. The incidence of long bone nonunions has been reported in the literature to range from 5-10% [31].

In order to tackle this serious complication, one has to pose some seemingly obvious questions; when can a fracture be considered as "healed"? Secondly, when can it be described as a "nonunion"? There is no consistent definition of fracture union and nonunion in the literature, but one can describe 2 types of fracture union, clinical and radiological. Clinical union is characterised by the absence of motion and / or tenderness at the fracture site upon application of physiological pressure on the fracture site (directly or indirectly e.g. by weight bearing). Radiological union is diagnosed when callus is found bridging the fracture site at least 3 out of 4 cortices on 2 different plain x-ray views [32]. The Food and Drug Administration (FDA), in 1988, defined nonunion as "a fracture that is over 9 months old and that has not shown radiographic signs of progression toward healing for 3 consecutive months" [33]. Delayed union lies in the "grey zone area" between the expected time to union and the development of an established nonunion. There is no delineating line between delayed and nonunion, and this can be a source of great misunderstanding and debate amongst treating physicians. Chronic or synovial pseudoarthrosis develops as an end stage of a longstanding nonunion [34].

1.3.1 Risk factors

Various risk factors are known to contribute to fracture nonunion. Some of these factors are related to the general health state of the patient whereas other factors are affected by the local fracture environment.

1.3.1.1 General risk factors

Factors like age, dietary insufficiency and diabetes can cause health problems of varying degrees of severity and that may contribute to the development of nonunion. In osteoporosis, there is reduction in healing with reported reduction in osteoblasts and reduced amounts of callus produced and hence the time to union maybe prolonged [35-37].

As already stated, age is also another important risk factor for nonunion. Fractures in children and young adults heal much quicker than older individuals. This is due to the increase in both the vascularity and number of osteoblasts in the children's periosteum [38]. The frequency of MSCs in human BM aspirates has been reported to decline with age [39], which was subsequently confirmed in the laboratory where the current work was undertaken [40]. Furthermore, MSCs from older donors appear to be less proliferative [41] and less capable of osteogenic and chondrogenic differentiation [40].

Diabetes is a chronic disease known to cause neuropathy and vasculopathy. An association between diabetes and delayed fracture healing has been reported in the literature [42]. Bone marrow mesenchymal stem cells (BM MSCs) from patients with type 2 diabetes appear to be functional so the deleterious effects of diabetic background on bone healing is likely to be related to vascular deficiency rather than MSC/osteoblast deficiency [43] .

Smoking negatively affects fracture healing and this has been studied at the molecular level by investigating nicotine which is a prominent component of cigarette smoke. Nicotine was found to reduce osteoblast proliferation during fracture healing and cause vasoconstriction reducing tissue perfusion and

oxygenation [44]. Functional nicotinic receptors are present on MSCs therefore nicotine can also negatively affect cellular processes involving MSCs [45]. In both human and animal studies, nicotine reduced bone healing rates following long bone fractures [46, 47] and after spine fusion [48, 49].

Non steroidal anti-inflammatory drugs (NSAIDs) have long been used for pain relief. However, they reduce osteoblastic activity by the inhibition of prostaglandin synthesis [50]. A recent study from the laboratory in which this work was carried out has shown that MSC chondrogenesis in particular is affected by NSAIDs and this is related to the inhibition of cyclooxygenase-1 (COX-1) activity and prostaglandin E2 (PGE2) synthesis [51]. In a study on 240 mice by Murnaghan et al [52], they reported an increased volume of soft callus and cartilage together with a delay in bone formation in the group of mice on NSAIDs. In a retrospective study of patients with diaphyseal fractures of the femur, treated with intramedullary nailing, Giannoudis et al [50], reported a significant increase in nonunion with patients receiving NSAIDs, in particular those who received such treatment for over 4 weeks.

Corticosteroids are an important class of drugs widely used systemically to treat various disease conditions including autoimmune diseases (including rheumatoid arthritis, RA), systemic inflammatory disorders and cancers. The main untoward effects on bone include, delayed bone healing [53, 54], osteoporosis [55] and avascular necrosis of the femoral head [56].

Finally, excessive alcohol drinking and poor diet are risk factors for nonunion. Alcohol inhibits new bone formation and mineralization [57], as it has a negative effects on both MSCs [58] and endothelial cells [59]. During fracture repair, there is high demand for proteins, calcium, phosphorus and vitamin D, therefore a balanced diet is important to ensure sound healing [60].

1.3.1.2 Local risk factors

Local factors include the anatomical site of the fracture, degree of fracture comminution, severity of the trauma sustained, extent of soft tissue damage and periosteal stripping, the absence or presence of infection, and the vascular state of the affected limb or region (both pre and post injury) and finally, the adequacy and stability of fixation used for treatment [37].

The degree of bone comminution and displacement of the fracture ends are governed by the magnitude of the causative trauma and initial strength of the bone prior to injury. Periosteal stripping and soft tissue damage are more likely to occur with high energy accidents [61]. Therefore, more healing complications can be anticipated following high energy incidents.

The vascular state of the affected limb and bone is of utmost importance for the healing process. Since healing requires an adequate blood supply any reduction in blood flow will have a negative effect on the fracture healing process. The blood supply to the bone maybe reduced acutely with bone breakage, or it may be part of a pre-existing chronic vascular insufficiency (e.g. diabetic ischemia) [62].

Mechanical stability at the fracture site affects the size and volume of callus produced. For secondary healing to occur, micro motion should be present at the fracture site, however, when this motion becomes excessive, this can be a contributing factor for the development of nonunion [8, 9].

Good bone to bone contact is another key factor in bone repair. The larger the gap between fracture ends, the less stable the fixation becomes and the more challenging it becomes for callus to bridge this gap. Soft tissue interposition and bone loss reduces bone contact and hence affects the healing process adversely [63, 64].

Fractures occurring in cancellous bone heal in a shorter period of time compared to those occurring in cortical bone. This is attributed to the fact that cancellous bone is more vascular than the latter. Due to their proximity to cancellous bone, fractures in the metaphysial region of long bones heal quicker than those in the diaphysis [38, 65].

1.3.2 Epidemiology

Tibial diaphyseal fractures are the most common type of long bone fractures encountered by the orthopaedic surgeon. On average, there are about 26 tibial diaphyseal fractures per 100,000 of the population per year [66]. The commonest cause of tibial diaphyseal fractures is road traffic accidents (RTA). Soccer is the second most common cause [67].

Fractures of the tibia maybe treated operatively or non-operatively. Babis et al [68], reported that out of 48 patients suffering from distal tibia fractures and treated with hybrid external fixation, 5 developed nonunions. Collinge et al [69], reported a 5% reoperation rate for distal tibia nonunions following internal fixation with minimally invasive plates. In a systematic review by Lam et al [70], nonunion of the tibia following reamed intramedullary nails was reported to range from 7- 20%. These reports indicate that nonunion of the tibia remains a significant problem despite the different treatment modalities.

Similarly, femoral shaft fractures usually result from high energy trauma and are associated with varying degrees of soft tissue damage [71]. Results of operative treatment are far superior to non operative treatment and they allow early mobilization hence reduce complications such as pressure sores, muscle wasting and respiratory insufficiency [72]. However, femoral nonunions remain problematic with nonunion rates of 1.6% reported with biological plates [73], 12% with external fixation [74] and 1.9% with unreamed intramedullary nailing [75].

Fractures of the shaft of the humerus represent 20% of all humeral fractures and account for 1% to 3% of all fractures [76]. Sarmiento et al [77], reported nonunion in 3% of his series of 465 closed humerus fractures treated with functional bracing. McKee et al [78], reported a 3% nonunion rate following fixation with dynamic plates.

Despite the enormous progress made during recent decades in the treatment of long bone fractures, fracture healing is still hindered by complications, nonunion being one of the most serious. Nonunion represent a particular challenge, and the difficulties surrounding its management are frequently under estimated [72].

1.3.3 Classifications of nonunion

Broadly speaking, according to the presence or absence of infection, nonunions can be divided into infected and aseptic. Based on the amount of callus at the fracture site, aseptic nonunion is further classified into hypertrophic (hypervascular, viable or vital) and atrophic (avascular, nonviable, avital) [79].

Radiographically, hypertrophic nonunions are characterised by an abundance of callus around the fracture ends which fails to cross the fracture site and unite the bone (Figure 1.2). The presence of callus proves that the fracture ends are viable and well vascularised and that the patients' fracture healing response is not the reason behind the nonunion. However, there is an ongoing mechanical instability issue that is the underlying cause of nonunion. Patients suffering this type of nonunion will benefit from revision of their fixation with more stable implants [80].

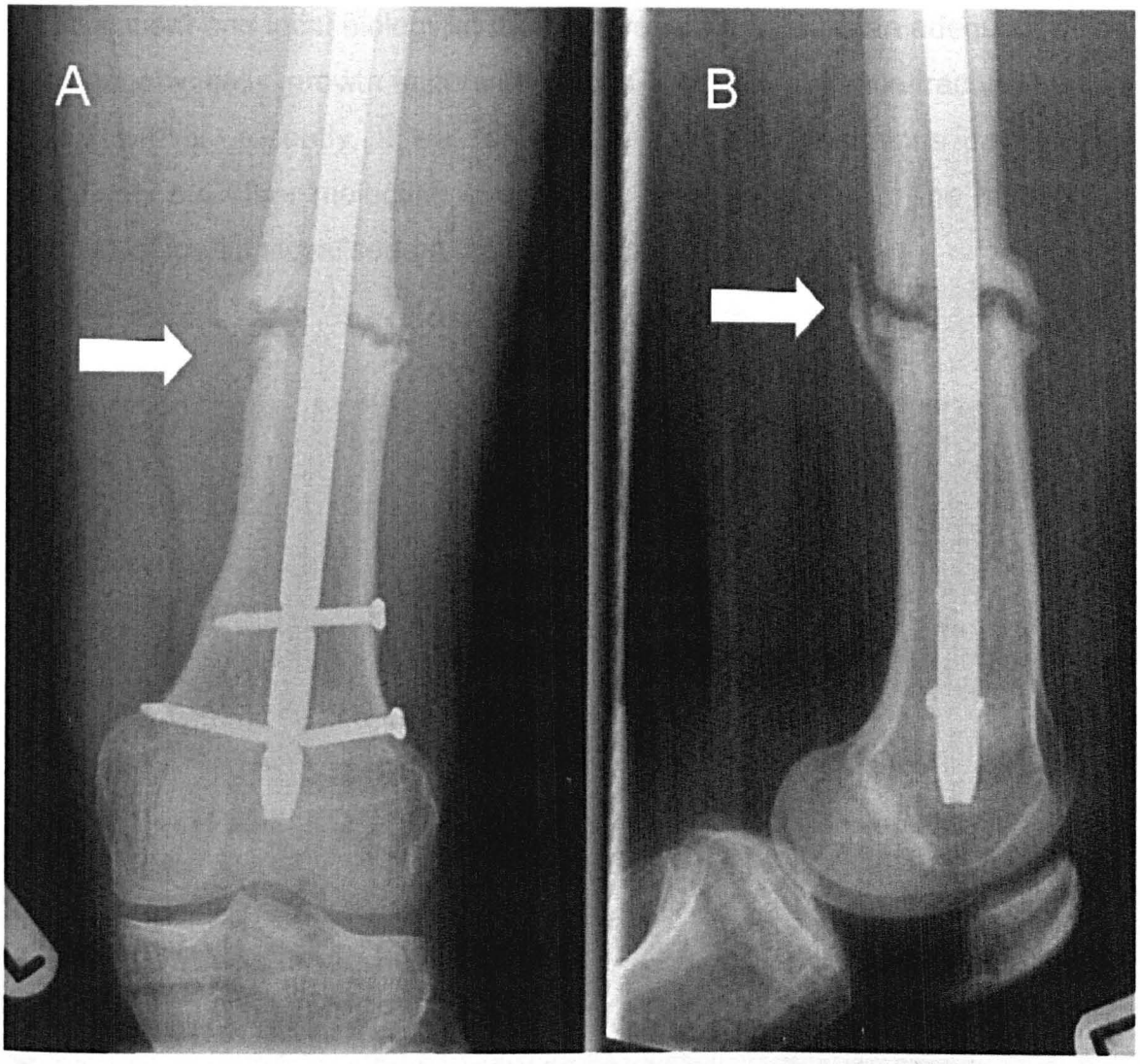

Figure 1.2: (A) Anteroposterior and (B) lateral view radiographs of the distal femur fixed with an intramedullary nail showing signs of hypertrophic nonunion
The arrows point at the hypertrophic nonunion site, note the excessive callus formation and the persistent fracture gap.

On the other hand, atrophic nonunion is characterised by minimal or absent callus formation on x-ray. The fracture ends look avascular and osteopenic and in later stages may become sclerosed (Figure 1.3). This reflects an inadequacy of blood supply or an inadequacy of osteogenic cells reaching the fracture site. This can happen after high velocity injuries where a great deal of periosteal stripping and soft tissue damage has occurred. In atrophic nonunion, the fracture ends are nonviable or avascular and this makes treatment of such patients very challenging. The treating physician should aim at stimulating the bone to be more biologically active and to improve the general health condition

of the patient and local biology at the fracture site to ensure an adequate inflow of osteogenic cells, growth factors and blood supply to enhance fracture healing [80]. However, recently, it was shown that atrophic nonunions do not lack vascularity but other molecular events may be responsible for the radiological features of inertia visualised on the radiographs [81].

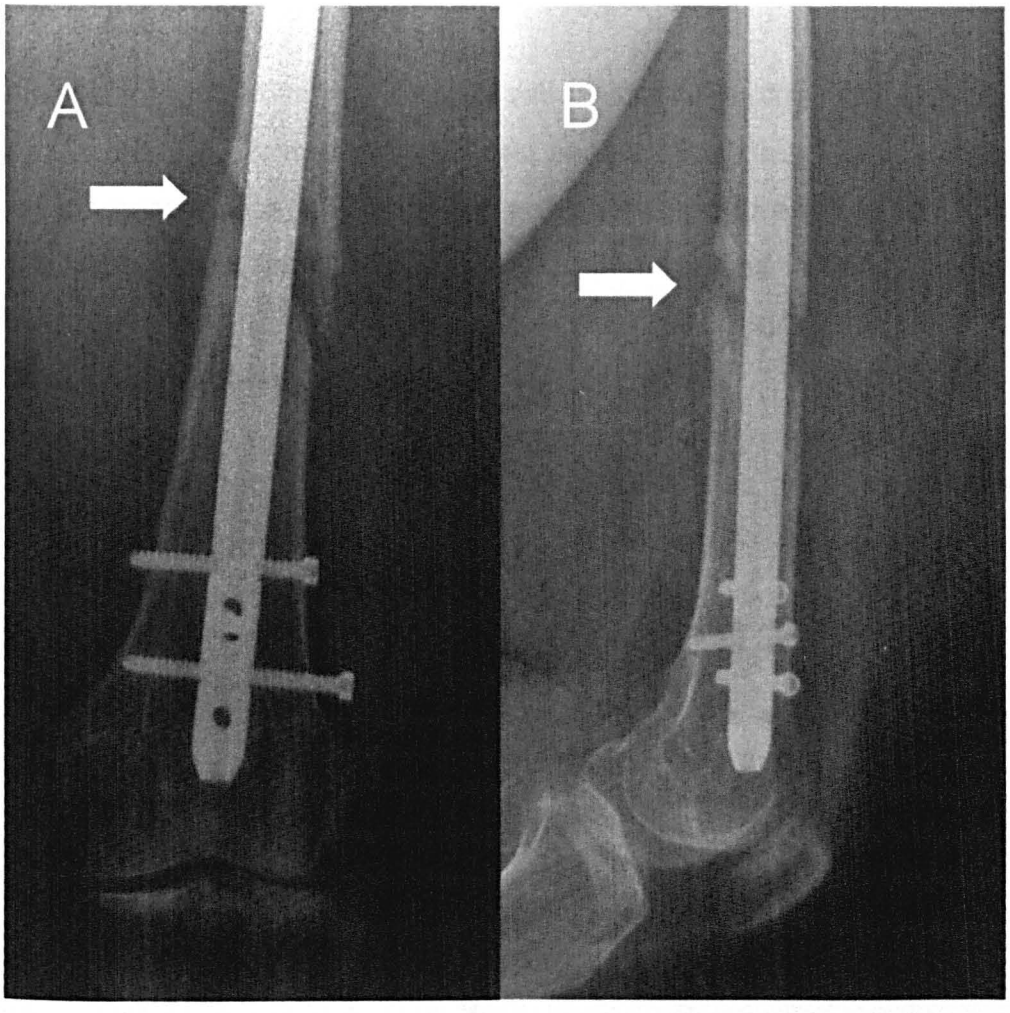

Figure 1.3: (A) Anteroposterior and (B) lateral view radiographs of the femur shaft fixed with an intramedullary nail showing signs of atrophic nonunion

The arrows point at the atrophic nonunion site, note the absence of callus formation and the persistent fracture gap.

1.3.4 The diamond concept

Giannoudis et al [7], proposed that four equally important factors are desirable for a successful bone repair process: adequate mechanical stability, growth factors (osteoinductive agent), osteoconductive scaffolds (autologous, allogeneic or synthetic) and osteogenic cells. This approach, known as 'the diamond concept' has received great attention lately (Figure 1.4) [7].

When a treating physician is faced with a nonunion case, it is wise to investigate and to plan further treatment for that patient along the 4 routes emphasised by the diamond concept. This offers a systematic approach to the diagnosis and treatment of the case. Bearing the diamond concept in mind, together with the known risk factors of nonunion, a treatment plan maybe put forward to properly address the problem.

The study of mechanical aspects and scaffolds as possible treatment options for nonunion are beyond the scope of this study, however in the following chapter, the roles of MSCs and BMPs will be discussed.

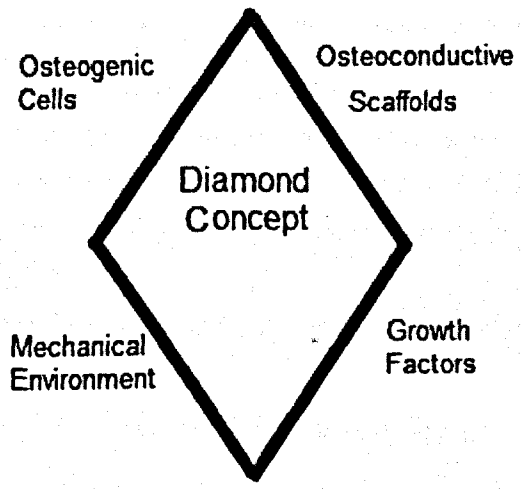

Figure 1.4: The Diamond Model of Bone Fracture Healing Interactions
(Image adapted from Giannoudis et al, 2007) [7].

1.4 SUMMARY

This chapter set out the basic biology of fracture repair at the cellular and molecular levels. It also described the biological basis for nonunion and touched on the cellular and mechanical factors contributing to this. The next chapter will cover current strategies for the augmentation of fracture repair, some deficiencies in the knowledge pertaining to this and then sets out a series of research questions to explore fracture repair.

CHAPTER 2

BIOLOGICAL ENHANCEMENT OF FRACTURE HEALING

2.1 INTRODUCTION

Atrophic fracture nonunion develops from "poor biology" at the fracture site. The fracture ends are biologically inactive and hence treatment should be geared towards biologically enhancing the fracture union. Using the four prerequisites for sound healing defined by the diamond concept, a need for osteogenic cells and growth factors appears to be essential for treatment of this condition. In this chapter, enhancement of fracture healing using MSCs and BMP-7 will be discussed.

2.2 MESENCHYMAL STEM CELLS

By definition, a human bone marrow MSC is a highly proliferative, non-haematopoietic stromal cell capable of differentiating and forming tissue of mesenchymal origin; bone, cartilage, and fat (Figure 2.1).

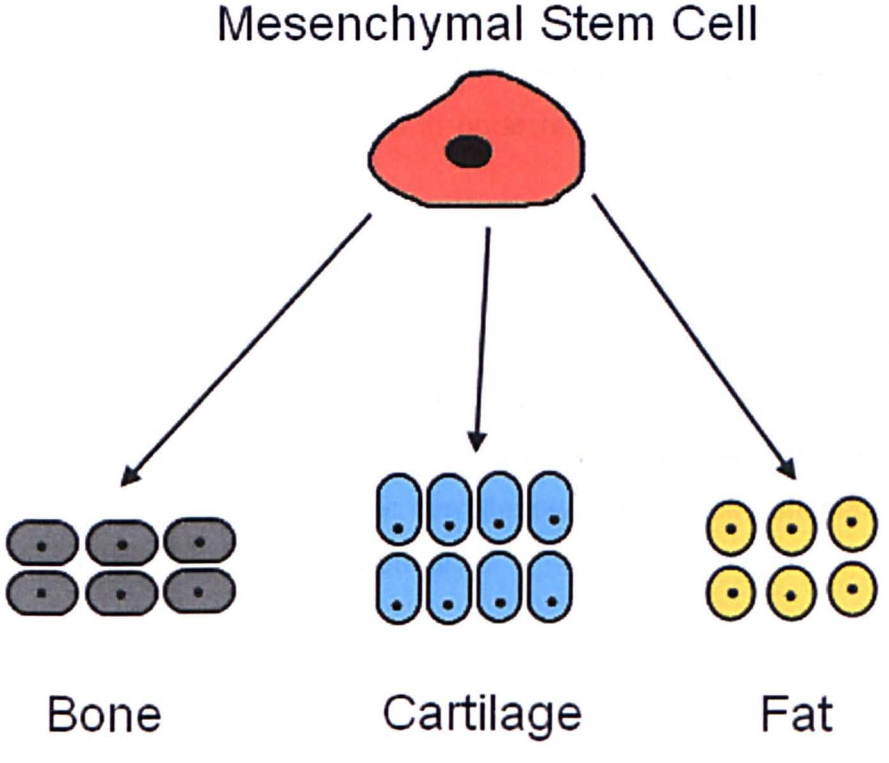

Figure 2.1: Schematic of the 3 main lineages that MSCs can differentiate to.

MSCs are regarded as reservoirs of regenerative cells with known tissue specific characteristics [82]. They are thought to have the ability to migrate and home to sites of injury and under specific signals, differentiate into cells of the appropriate connective tissue lineage. These signals maybe caused by tissue damage in conditions such as trauma, fracture, inflammation, necrosis and tumour [83].

Due to their proliferative capacity and ability to differentiate into tissues of the mesenchymal lineage, MSCs have attracted increasing attention in the field of musculoskeletal regenerative medicine. They have the potential to regenerate and produce tissues such as bone and cartilage and are capable of producing growth factors and cytokines that aid in the repair and regeneration processes [82]. Currently, multiple orthopaedic conditions exist that have no satisfactory solution, an important example of which is atrophic nonunion of bone following fractures. It is believed that MSCs may provide a more definitive answer for fracture repair by the local augmentation of MSC numbers at fracture sites. Numerous *in vitro and in vivo* studies have been carried out to better understand the biology of MSCs, including their phenotypic characterisation and methods for their enrichment [84, 85]. Clinical trials have also been carried out to study the potential use of MSCs in bone repair including fracture nonunion [86-88] .

The idea that MSCs circulate in humans is contentious and has not been shown by all working in the field [20]. Given that most stromal tissues appear to have an MSC reservoir then the need for a circulating MSC pool is further questioned [89]. However, the fact that MSCs can engraft after their systemic infusion has been demonstrated after allogeneic hematopoietic stem cell transplantation [90] and following injury in animal models [91].

2.2.1 The discovery of mesenchymal stem cells

The history of the field goes back over 140 years with the original description of putative MSCs in 1867 by a German pathologist named Clonheim. He noticed that BM contained fibroblasts that appeared to be capable of forming collagen at distant sites of wound repair in animals [92].

The Russian born histologist, Alexander Maximow (1874 – 1928), developed the unitarian theory of hematopoiesis or origination of blood elements from a common precursor cell. He also stated that the stromal/connective tissue environment in the marrow controlled haematopoiesis, a highly contentious statement during his career, but now dogma. He was also first to develop *in vitro* cell culture technique [93]. By then BM fibroblasts or hematopoietic-supporting stromal cells have not been called MSCs, since their differentiation to mesenchymal lineages had not yet been proven experimentally.

In the 1970s, a Russian scientist Alexander Friedenstein and co-workers conducted further *in vitro* experiments studying the function of BM fibroblasts. They plated whole BM in plastic culture dishes for about 4 hours. They then discarded the non adherent cells and culture expanded the remaining adherent ones. They noticed that the adherent cells remained quiescent for 2-4 days and then began rapid division to form colonies. The resultant colonies were fibroblastic in appearance and where capable of forming bone or cartilage when transplanted in diffusion chambers in Figure 2.2. It is from this work that the term colony forming unit fibroblast (CFU-F) arose [89, 94].

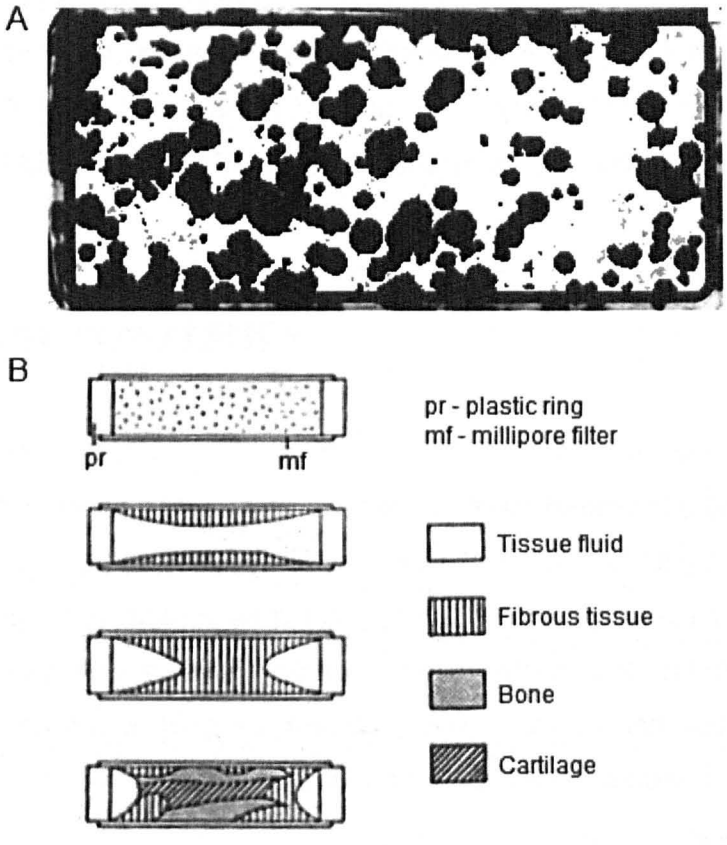

Figure 2.2: Diffusion chamber experiments originally described by Friedenstein
Image adopted from Friedenstein,1987 [95]. A: CFU-F colonies grown in tissue culture flask. B: diffusion chamber experiments.

The term mesenchymal stem cell was later coined by Arnold Caplan and colleagues who further expanded on a concept of multi-lineage differentiation of CFU-Fs [96]. Currently, the term MSC denotes a BM derived stromal cell that is highly proliferative and capable of undergoing trilineage differentiation into bone, cartilage and fat [97]. However, there is still some uncertainty as to whether MSCs can really be true stem cells. This is because their life-long self-renewal capacity has not yet been demonstrated *in vivo* [98]. Therefore, MSCs have also been designated as multipotential marrow stromal cells or simply marrow stromal cells by some investigators [98, 99]. In 1999 Pittenger and colleagues [100] published a key article in stem cell research, cited over 9400 times on Google Scholar at the time of writing, showing the amazing proliferative and clonogenic potential of BM MSCs.

While MSCs were originally defined in the BM [92], populations of MSCs were later derived from virtually every site in the skeleton [82] including the synovial membrane [101], joint fat pad [102] and joint synovial fluid [103], the role of these different niches of MSCs in fracture repair is incompletely defined.

2.2.2 Characteristics of MSCs

There is no single marker that can identify MSCs in all the tissues; therefore a combination of several parameters is used to define these cells both *in vitro and in vivo*. These include cell surface phenotype, morphology and function. Morphologically, they appear as spindle-shaped fibroblastic cells in culture [92]. However, freshly-isolated cells have a more flattened morphology and long cytoplasmic projections [84]. Numerous authors have used flow cytometry to phenotype MSCs [84, 104] and multiple markers were proposed to characterise them [85].

To ensure clarity and to standardize the nomenclature, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy was convened and issued the following statement to define an MSC; "First, MSC must be plastic-adherent when maintained in standard culture conditions. Second, MSC must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. Third, MSC must differentiate to osteoblasts, adipocytes and chondroblasts *in vitro*" [105]. This definition remains the gold-standard for all researches currently working in the field.

Flow cytometry is a valuable tool used to determine the surface molecules on MSCs and has been used by numerous researches to characterise MSC populations. CD271 molecule is a low affinity nerve growth factor receptor that specifically selects for BM CFU-Fs [106]. CD271-positive BM stromal cells have the CD45-/low phenotype and express "classic" MSC markers CD73, CD105 and CD90, as previously shown in the laboratory in which this work was done

[84, 85]. Therefore, in addition to standard CFU-F assay for MSC quantification, these cells can be also enumerated using flow cytometry [85], which is much less time consuming (2-3 hours instead of 14 days for a simple CFU-F assay). Enumeration of BM MSCs in nonunion patients using parallel CFU-F and flow cytometry assays has not previously been reported.

2.2.3 *In vitro* differentiation of MSCs

Part of the definition of an MSC is its ability to form bone, cartilage and fat. Functional assays to test the trilineage ability of MSCs *in vitro* have been developed and optimized [100] and differentiation assays were used as a measure of their proliferative capacity. Adipogenic and chondrogenic differentiation assays are outside the scope of this work however it is worth mentioning briefly the rationale behind these assays. With regards to adipogenesis, it is commonly induced by chemical and biological compounds, primarily insulin, using densely-packed confluent MSCs. Chondrogenic differentiation assays are performed in tri-dimensional "pellets", to mimic chondrogenic condensations occurring *in vivo* and are induced by the addition of TGF-β3 [100].

Osteogenic differentiation of MSCs is commonly performed using confluent cultures exposed to a mixture of osteoinductive agents (dexamethasone, ascorbic acid and glycerophosphate). Cells begin to mineralise during the second week of culture, leading to the formation of functional osteoblasts by week 3 [100]. Osteogenic progression is measured using alkaline phosphatase (ALP) and calcium assays at week 2 and 3, respectively [100, 107]. Often osteogenic differentiation assay is also performed at clonogenic level; this assay is called colony-forming unit-osteoblast (CFU-O). The only difference between CFU-F and CFU-O is the composition of media used (standard expansion media versus osteoinductive media).

As mentioned in the above sections, the CFU-F assay was originally described by Friedenstein et al [108]. This test highlights the ability of MSCs to form colonies and hence is used as an indirect measure of MSC proliferation. Cells are seeded at low densities in plates, fed for 2 weeks after which they are fixed with formaldehyde and then stained with crystal violet. In humans, each MSC forms one colony, whereas in mice or rats, an MSC is capable of forming more than one colony due to its ability to detach [109]. This assay has become "traditional" and is widely used in many researches due to its ease and its value. The number of colonies found per dish is equivalent to the number of MSCs plated at the start of the assay. The colonies maybe examined under the microscope to confirm their fibroblast like morphology. In the studies undertaken in this thesis, both CFU-F and CFU-O assays will be used to measure the number and osteogenic capacity of MSCs in health and nonunion.

2.2.4 Clinical applications of MSCs

Human mesenchymal stem cells (hMSCs) are a very attractive cell therapeutic as they can be isolated from patients with relative ease, capable of *in vitro* expansion, can theoretically be genetically engineered to produce target genes, safe in that they do not carry the risk of tumour formation (unless subjected to excess stress or expansion) and are physiologically present in injury sites as part of the healing process [110]. In contrast, embryonic stem cells can form teratomas following *in vivo* implantation in animal models [111].

The successful use of MSCs in bone repair applications has been described in several pioneering early proof of concept studies [112-114]. In 1989 Connolly et al [114], reported good callus formation in 9 out 10 patients with delayed union of the tibia after injection of BM at the fracture site. In 1999 Horwitz et al [112], infused unmanipulated BM intravenously into 3 patients suffering severe osteogenesis imperfecta and demonstrated improved bone histology together with an increase of total body mineral content with this treatment, however, this work remains contentious. In 2001 Quarto et al [113], used MSCs loaded on hydroxyapatite scaffolds to treat large bone defects in 3 patients and reported

good healing within 6 to 6.5 months post surgery in defects that would have otherwise required 12 to 18 months to heal. Goel et al in 2005 [115], reported healing in 15 out of 20 patients with tibia nonunion after injection of BM at the site of nonunion. Pioneering studies on BM aspirate concentrate (BMAC) by Hernigou et al in 2005 [116], have shown that percutaneously implanted autologous BM MSCs can improve clinical outcomes. Despite this, the use of MSCs in orthopaedic surgery remains patchy because the biology of MSCs *in vivo* in health and following fracture is not well understood. There remains a lack of biological basis for surgeons to use these cells routinely and a great need to better understand their biology, particularly their response to growth factors and whether or not these cells could be systemically homing to fracture sites following injury.

It is well established that bone, periosteum and BM contain osteogenic progenitors and MSCs that can be used to aid bone healing [117]. Iliac crest bone graft has long been used to enhance bone healing in cases of nonunions and spinal fusions, but this was not without complications. Donor site morbidity in the form of chronic pain from the site of the graft has always haunted both surgeon and patient alike and is reported in literature to range from 15-39% of cases for up to 2 years post operatively [118-120].

BM aspiration commonly obtained from the iliac crest is a minimally invasive technique requiring less surgical expertise than the "classic" iliac crest bone graft. Its MSC content offers the osteoinductive properties that iliac crest bone graft offers without the complications that may arise from the donor site. It has been used by numerous authors to enhance fracture nonunion. Moreover, BM is injected percutaneously in the site of the nonunion therefore minimizing the local risks of a second open surgery to introduce the bone graft such as infection and excessive soft tissue damage [116, 121]. Revision surgery to introduce bone graft has been described by Boyd as "bone grafting is primarily a second wounding procedure, in which surgeon hopes that the response of the body will be more favorable than the response following the original trauma" [121].

As mentioned above, Hernigou in 2005 [116] aspirated 20cm^3 of autologous BM from both iliac crests, concentrated them with a BM concentrator, then injected the concentrate into the nonunion site of patients with atrophic nonunions of the tibia. In his series 53 out of 60 patients united with this technique. He evaluated the number of BM MSCs after concentration that were percutaneously injected into the nonunion site using a simple CFU-F assay and was able to prove that the volume of callus produced and the union rate were directly proportional to the amount of MSCs injected. He noted a reduction of MSC numbers in patients with comorbidities like diabetes and in smokers. This study showed that a minimum of 30,000 CFU-Fs were needed at the nonunion site for it to heal [116]. Even though MSCs are known to exist in BM, they are very rare [18].

Seebach et al [122], examined BM samples of 55 individuals divided into 4 groups. The groups were comprised of 13 patients who were involved in poly trauma, 15 patients with only one fracture, 20 suffering atrophic nonunion and 7 healthy volunteers. He found a significant increase in CFU-F numbers in patients with poly trauma as compared to those suffering nonunion and a significant increase in colony density between the poly trauma and the mono fracture group in males [122].

Several animal models have shown that the use of MSCs in conjunction with scaffolds or growth factors is associated with good outcomes in an orthopaedic setting [98, 123]. In a recent review by Jones and Yang [98], the importance of developing an animal model that closely resembles the clinical setting was emphasized. Currently, the subcutaneous implant model (studied most commonly in the immunocompromised "nude" mice) and the diffusion chamber one have their strong points and weaknesses. In the former model, bone resorption has been reported with inadequate mechanical forces applied to the implant together with possible contamination of the study implant with the host's tissues. In the latter model, contamination with host tissue is no longer an issue as the study samples are isolated in a sealed chamber but the lack of mechanical forces and stresses remains a concern [98].

2.2.5 Circulation of MSCs

The traditional concept for the putative role of MSCs in tissue homeostasis and repair comes from the haematological BM stem cell model. In the latter model it is well established that CD34+ BM haematopoietic cells or their more differentiated daughter progeny cells (i.e. precursors for lymphocytes, neutrophils and monocytes) are capable of circulating. Thus, circulating erythrocytes oxygenate the tissues and innate immune cells and lymphocytes likewise egress to their designated tissue environments and carry out effector functions.

The validated model for adult stem cells emanating from this haematological model has also been applied to MSCs [96]. As reviewed by Prockop in 1997 [92], sites of induced tissue injury appeared to accrue cells derived from the marrow in the tissue repair process. In the last couple of decades numerous studies have reported that MSCs circulate in health and disease [17, 19, 20, 124, 125]. Obviously a better understanding of the putative mechanisms of systemic circulation of MSCs could be immensely important for local and systemic strategies aimed at novel approaches to bone repair and nonunions. Several studies have shown that systemically injected MSCs may in some cases have the capacity to home to sites of tissue injury, but the artificial introduction of MSCs into the blood does not prove that they circulate under physiological conditions [18].

Indeed the circulation of MSCs in the blood remains a highly contentious area of debate. Embryologically, mesenchyme arises before a circulation develops and its presence is a prerequisite for a circulatory system [126]. In the last decade, large numbers of tissue resident MSCs have been shown at virtually every skeletal site [127] which suggests endogenous tissue specific MSC pools that challenges the need for a circulatory physiological MSC pool. Furthermore, most of the existing work on circulation MSCs has not used rigorous phenotypic criteria for MSCs [128] and there has been a lack of data showing that putative circulating MSCs are actually highly proliferative and multipotential.

From the biological perspective it is well known that rheumatoid arthritis (RA), is an erosive polyarthritis and one putative mechanism for joint erosion in RA relates to the hypothesis that an abnormal circulating stromal progenitor cell is capable of carrying the erosive phenotype to multiple joints. Indeed this model has had some compelling support from a recent animal model (murine), which showed that transplanted human MSCs translocated from one flank to the other to effect cartilage destruction. RA synovial fibroblasts were thought to be the reason behind the spread of the disease from one joint to another in severe combined immune deficiency (SCID) mice [129, 130].

When rigorous phenotypic and functional criteria for MSCs have been applied several groups have failed to demonstrate evidence of MSCs in the circulation [128, 131]. Jones & McGonagle [89], found no evidence for an MSC population in health and noted that cells that morphologically resembled MSCs had functional properties of tissue macrophages. Likewise several other studies failed to show evidence for MSCs in the circulation [19, 20].

2.2.6 Reamer irrigator aspirator

The association between the increase in intramedullary pressure during reaming or nail insertion and pulmonary complications is well documented in literature [132, 133]. Pape et al [134], found that reamer systems which offer irrigation and aspiration while reaming, reduce the harmful systemic effects of subsequent nailing of femurs in sheep. The Reamer Irrigator Aspirator (RIA) system by Synthes, is designed to reduce both intramedullary pressure and thermal necrosis risk [135] during reaming as it irrigates the canal with saline during the process (Figure 2.3). In theory, this reduction of intramedullary pressure reduces the risk of fat embolism and pulmonary complications.

Figure 2.3: The RIA system
The arrows point to the inflow (irrigation) and outflow (aspiration) of saline to irrigate the medulla during reaming.

The RIA system was found to generate a generous quantity of bone as a byproduct of the reaming process. Researchers examined the resultant bone from reaming as well as waste liquid and found it rich in MSCs [136, 137]. The large amount of graft generated together with the high MSC content, has encouraged its use by various authors as a method for harvesting bone graft material. Additionally, the bone material obtained is very malleable due to the small fragment size and may be very useful for certain applications. This method of graft harvesting is minimally invasive and has less donor site morbidity when compared to the classical iliac crest bone graft and at the same time is of great efficacy [138-140]. Several reports in the literature exist discussing RIA as a satisfactory method of bone graft harvesting for the treatment of nonunions (Figure 2.4) [138, 141-144].

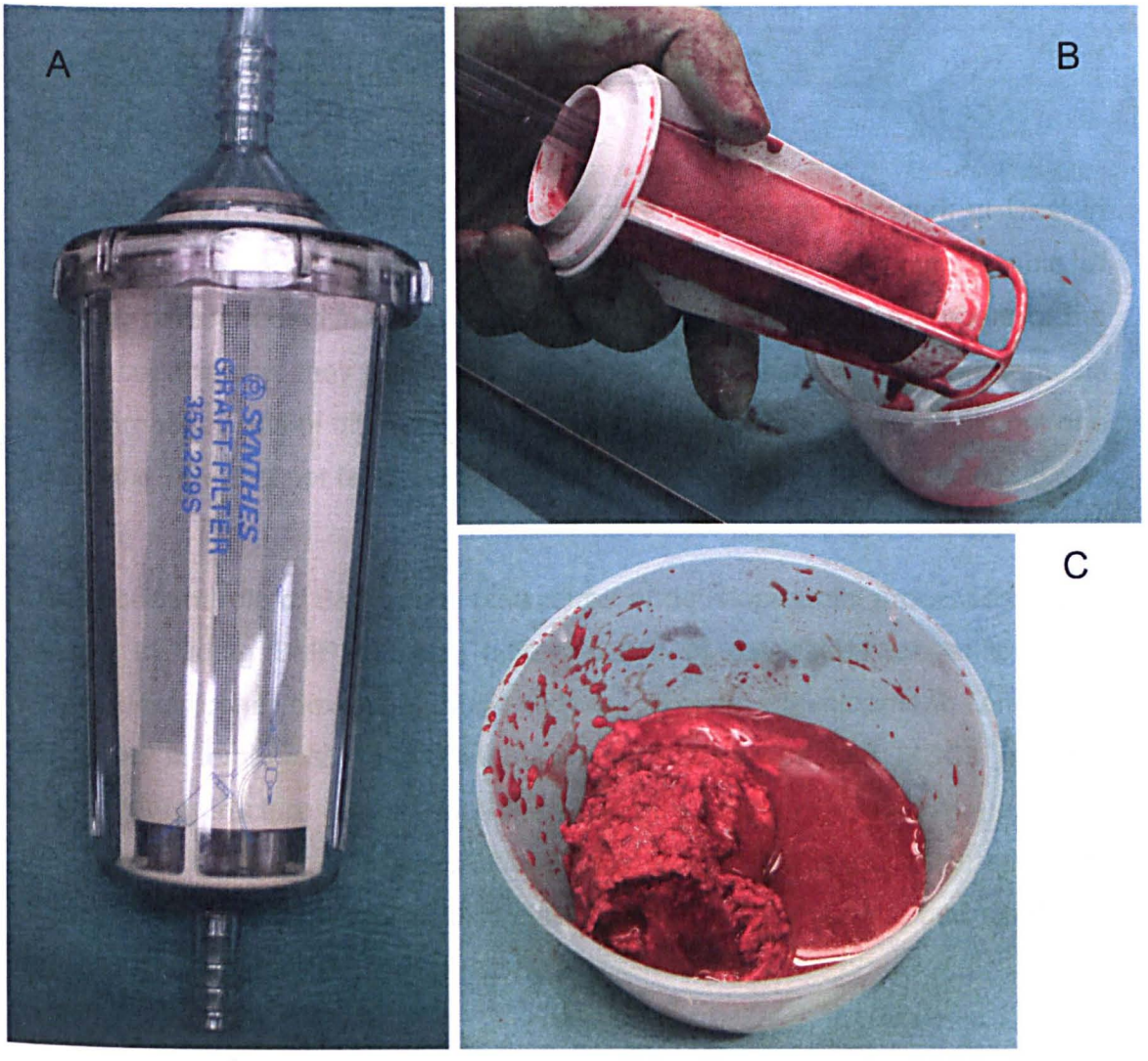

Figure 2.4: RIA as a graft harvester
A: An unused 750µm filter to be attached to the RIA system prior to reaming to collect bone graft. B: Emptying the collected bone graft from the filter after reaming. C: The final bone graft.

Based on the concept that RIA reduces the intramedullary pressure and hence the incidence of fat embolism, it was hypothesized that MSCs would not be mechanically translocated into the deep and superficial venous systems after reaming with RIA, whereas this would be more likely to occur following conventional reaming.

2.3 BONE MORPHOGENETIC PROTEINS

The discovery of BMPs can be traced back to the pioneering work of Urist in 1965 when he showed that ground up matrix from the BM was capable of inducing osteogenesis in ectopic tissues [145]. The BMPs are members of the TGF-β superfamily; they are known to induce differentiation, growth, apoptosis and morphogenesis of different types of cells [146]. BMPs represent almost one third of the TGF-β super family, with at least 34 members already described [22].

The important role of BMPs in a wide range of developmental processes is well known; including BMP induced differentiation of osteoblast progenitors and subsequent bone development in adults, skeletal development, and limb formation in embryos [147]. Osteoblasts, chondrocytes and MSCs produce BMPs during fracture repair which then trigger a series of events that promote the formation of bone and cartilage. On a cellular level, they stimulate chemotaxis, MSC proliferation and differentiation, angiogenesis, and synthesis of extracellular matrix [83].

It is worth noting that BMPs are key players in all 3 stages of fracture healing. During the initial inflammatory stage, BMP-2 and 4 seem to be the main players, their role progresses into the chondrogenic stage of the repair phase of fracture healing together with BMP-3b and BMP-6. During the osteogenic phase of fracture healing, BMP-7, together with other BMPs, is highly expressed, with a peak at day 14 and 21 of BMP-7 and BMP-8 (Figure 2.5) [22].

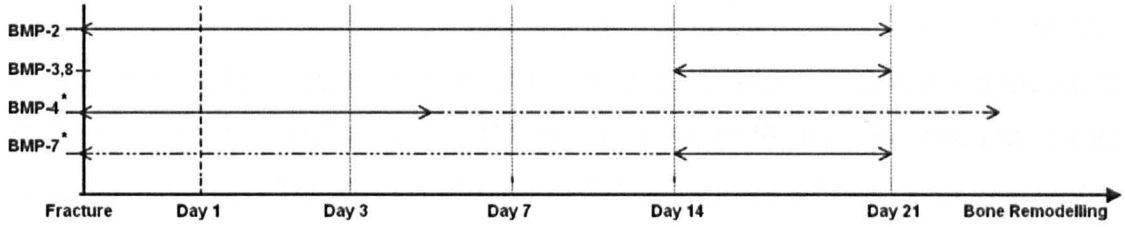

Figure 2.5: Schematic summary of BMP expression patterns during fracture healing
(The dashed line represents a difference of opinion amongst scientists in terms of the timing of expression.) Image modified from Dimitriou et al, 2005 [148].

Initially, numerous animal trials were carried out to test the safety and efficacy of BMPs in induction of osteogenesis [149-151]. As the results were promising, on the 17th October 2001, the Food and Drug Administration (FDA) granted Stryker Biotech approval for its BMP-7 implant under a humanitarian device exemption (HDE). Randomized control trials have shown recombinant human BMP-7 to be associated with improved fracture repair [152, 153]. In a study by Moghaddam et al, [154], BMP-7 has been used for the treatment of nonunions of femur, tibia and humerus, with a success rate of 82%. In these settings, the therapeutically administered BMP-7 is thought to recapitulate normal BMP-7 activity in physiological fracture repair.

The BMPs interact with type I (BMPR1A), and type II (BMPR1B) receptors on the cell surface. Seven type I (ALK1-7) and five type II receptors (TβRII, ACTRIIA, ACTRIIB, BMPRII, AMHRII) have been identified in humans [155]. The BMP signaling propagation is initiated by BMPs binding to high-affinity BMP serine-threonine kinase receptors that transduce signals through Smad and non-Smad signaling pathways. Phosphorylated SMADs form protein complexes and by nuclear translocation activate downstream genes [155]. Interestingly, previous studies showed that BMP signaling transduction is associated with other signaling pathways including Wnt and FGF signaling, an activity that is highly regulated by BMP antagonists [156].

Antagonists of BMP such as Noggin (Nog), Cerberus (Cer1), CAN (Cerberus-DAN), Follistatin, Gremlin (Grem1), Chordin (Chrd), Twisted gastrulation (Twsg1), and Crossveinless 2 (CV2) regulate BMP activity through direct receptor binding [157]. Both BMPs and BMP antagonists have been involved in bone development and angiogenesis. BMPs are able to strongly promote MSCs recruitment and differentiation into bone-forming osteoblasts which can facilitate effective bone regeneration in orthopedic applications [158].

2.4 BONE MARROW CONCENTRATORS AND MSC ENRICHMENT

The iliac crest BM is a known source of MSCs and has been used to promote healing in nonunion cases [159]. In order to enrich for osteogenic progenitors or MSCs, commercial BM concentrator devices have been used. Usually, 30ml to 60ml of bone marrow aspirate (BMA) from the iliac crest is obtained, centrifuged in a cell separator and concentrated to 3ml or 6ml of BM potentially enriched for MSCs. This bone marrow aspiration concentrate (BMAC), can then be injected into the site of nonunion to promote fracture healing.

As previously discussed, Heringou et al [116], reported union in 53 out of 60 patients with tibia nonunion using this technique. They showed that the volume of mineralised callus generated at the nonunion site was proportional to the number of CFU-Fs in the BMAC.

In a recent prospective clinical and laboratory study by Jager et al [160], 75 patients with bone defects greater than 1cm x 1cm were recruited. Half of their defects were filled with autologous cancellous bone graft, and the other half was filled with either BMAC loaded onto hydroxyapatite granules or BMAC loaded onto a porcine collagen sponge. Samples from the BM before and after concentration were obtained and examined for the presence of MSCs with flow cytometry, CFU-F and CFU-O assays. They reported complete bone healing at 17.3 weeks in the hydroxyapatite group compared to 22.4 weeks in the collagen group. They also reported up to 5.2 fold increase in CFU-F numbers post concentration.

The BM concentrator devices thus offer an alternative, minimally invasive strategy for fracture repair using MSCs compared to growth factors or the use of allogeneic culture expanded MSCs that can be procured commercially. As shown by Jager et al [160], they may be combined with a scaffold for superior results.

2.5 SUMMARY AND AIMS

As outlined above, there are several biological strategies to treat nonunion, some involve the use of BMPs and others entail the use of autologous MSCs. It remains unclear which method is likely to be most efficacious. Furthermore, the interaction between MSCs and BMPs in fracture repair is poorly defined and the whole question of how growth factors and MSCs might work in unison to optimise bone regeneration *in vivo* in man is not yet clear.

Finally, the biology of MSCs in blood circulation in humans is not well understood. It is known that MSCs tend to be associated with fat globules in the marrow [85]. Consequently, it might be expected that MSCs would be released in situations where the risk of fat embolism existed. The phenomenon of fat embolism following reaming and or nailing of long bone fractures is well described in the literature with translocation of fat intravascularly by mechanical means [161, 162].

Hypotheses to be tested

This thesis sets to explore the following hypotheses:

1- The numbers and/or proliferative capacity of iliac crest BM MSCs in patients suffering nonunion are lower compared to patients who suffered long bone fractures and healed uneventfully. If this true, the injection of extra MSCs in nonunion sites may be beneficial to induce repair.

2- BMP-7 will increase the *in vitro* MSC potency in patients suffering long bone fracture nonunion in a dose-dependent manner.

3- MSCs are present in the vascular system following reaming with RIA at a lower frequency than in BM and this is a biophysical process. If such a scenario were true then MSC numbers would be expected to be greater in the lower limb femoral venous blood compared to antecubital venous blood from the upper limb due to the trapping of MSCs in the pulmonary and peripheral capillary networks.

To address these hypotheses, this study has the following aims:

1- To enumerate iliac crest BM MSCs and osteoprogenitors in a group of patients with long bone fracture nonunion (nonunion group) in comparison to patients with previous long bone fractures that have healed without delay and/ complications (union group) who were used as "controls". This was done using gold standard assays for MSCs (CFU-F) and osteoprogenitors (CFU-O) or flow cytometry. To evaluate MSC proliferative capacity by measuring the sizes of individual colonies and the numbers of cells per colony.

2- To investigate the effect of BMP-7 on cultured MSCs from nonunion and union patients following osteogenic differentiation and measuring the levels of calcium produced. To investigate the effects of BMP-7 on non-cultured MSCs from both nonunion and union patients, following osteoinduction and measuring the levels of calcium and the numbers and sizes of CFU-F colonies.

3- To enumerate circulating MSCs in upper and lower limb venous blood of nonunion patients immediately following reaming of their sound femur with RIA for bone graft harvesting. To investigate the presence or absence of circulating MSCs in union patients being operated upon for metal work removal. Additionally, to compare the findings from those 2 groups with findings from the peripheral venous blood of normal healthy control patients and with early RA patients.

CHAPTER 3

MATERIALS AND METHODS

3.1 BASIC REAGENTS

Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's Phosphate Buffered Saline (PBS) (1X, without calcium and magnesium), Trypsin/ ethylenediaminetetraacetic acid (EDTA) solution (0.05%/0.02% EDTA), and Penicillin/Streptomycin solution (all from Invitrogen) were used for cell culture experiments. The following reagents and solutions were purchased from Sigma: acetic acid, trypan blue 0.4% w/v (both for counting), β-glycerophosphate, ascorbic acid, alizarin red and dexamethasone (all in powder form to make up osteogenic media), and dimethyl sulfoxide (DMSO, for freezing). Tissue culture plastic, including 15ml centrifuge tubes, 50ml centrifuge tubes, 35mm x 10mm dishes, and pipettes, was from Corning. Prolypropylen microtubes were from Trefflab (0.5ml and 1.5ml), pipette tips were from Gilson. Also, 2.0ml cryovials were from Alpha laboratories. Non-haematopoietic (NH) expansion media was from Miltenyi Biotec and fetal bovine serum (FBS) was from JRH Biosciences.

3.2 PREPARATION OF RECOMBINANT HUMAN BMP-7

Recombinant Human BMP-7 (Peprotech) in vials of 500μg BMP-7 was centrifuged using Eppendorf Centrifuge Model 5810 for 1 minute at 650 rcf. Thereafter, BMP-7 was reconstituted in distilled water to a concentration of 0.1mg/ml (500μg BMP-7 dissolved in 5ml distilled water). A buffer containing carrier protein (0.1% bovine serum albumin, BSA) was made by mixing 0.1g of BSA with 100ml PBS. This mixture was sterile filtered. Forty-five millilitres of the 0.1% BSA was added to the 5ml of 0.1mg/ml BMP-7 solution to make up 10μg/ml stock solution. One hundred microlitre aliquots of the resultant mixture was made (each aliquot contained 1μg=1000 ng/ml BMP-7) and were frozen at -80°C and thawed immediately prior to use.

3.3 PREPARATION OF OSTEOGENIC MEDIA

Osteogenic media was composed of DMEM, 10% FBS, 100μM ascorbic-2 phosphate (As2P), 10mM β-glycerophosphate, and 100nM dexamethasone. To prepare stock solutions from powders, 25mg of dexamethasone was dissolved in 1280μl of 100% ethanol to form 50mM stock solution. This was further aliquoted in 10μl volumes and stored in sterile vials at -20°C. Ten microliters of 50mM solution was added to 1ml of NH medium to give a 500μM solution, this was aliquoted in 20μl aliquots (1:5000 dilution needed for final media). To prepare a 200mM stock solution of As2P, 2.57g was dissolved in 50ml of water. This was aliquoted into 250μl aliquots (1:2000 dilution needed for final media). To prepare 2M stock solution of β2-glycerophosphate, 10g β2-glycerophosphate was dissolved in 23ml of water (1:200 dilution needed for final media). This solution was aliquoted into 1.25ml aliquots and stored in sterile Eppendorf tubes in -20°C.

Osteogenic media was prepared in 500ml DMEM bottles. Fifty milliliters of DMEM was decanted from a fresh bottle, then 50ml of 10% FBS, five 20μl aliquots of dexamethasone, two 1.25ml aliquots of β2-glycerophosphate and one 250μl aliquot of As2P were added. The media was sterile filtered and stored at -20°C.

3.4 BONE MARROW SAMPLE COLLECTION

Under general anaesthesia, with the patient supine, the iliac crest region was sterilized and draped according to regular operating standards. A 2mm stab incision was made over the anterior iliac crest. BM was aspirated with a Stryker 13G match-ground bevel tip introduction needle by the same surgeon (Professor Giannoudis) into a heparinised 10ml syringe (Figure 3.1). To reduce the dilution by peripheral blood, the needle's position was changed after each 4mls of aspirate obtained. Immediately, the aspirate was transferred to 5ml

vacutainer tubes containing EDTA. All samples were collected from the Leeds General Infirmary (LGI) and transported at room temperature to Leeds Institute of Molecular Medicine (LIMM), within 1 hour of collection, and processing was initiated immediately.

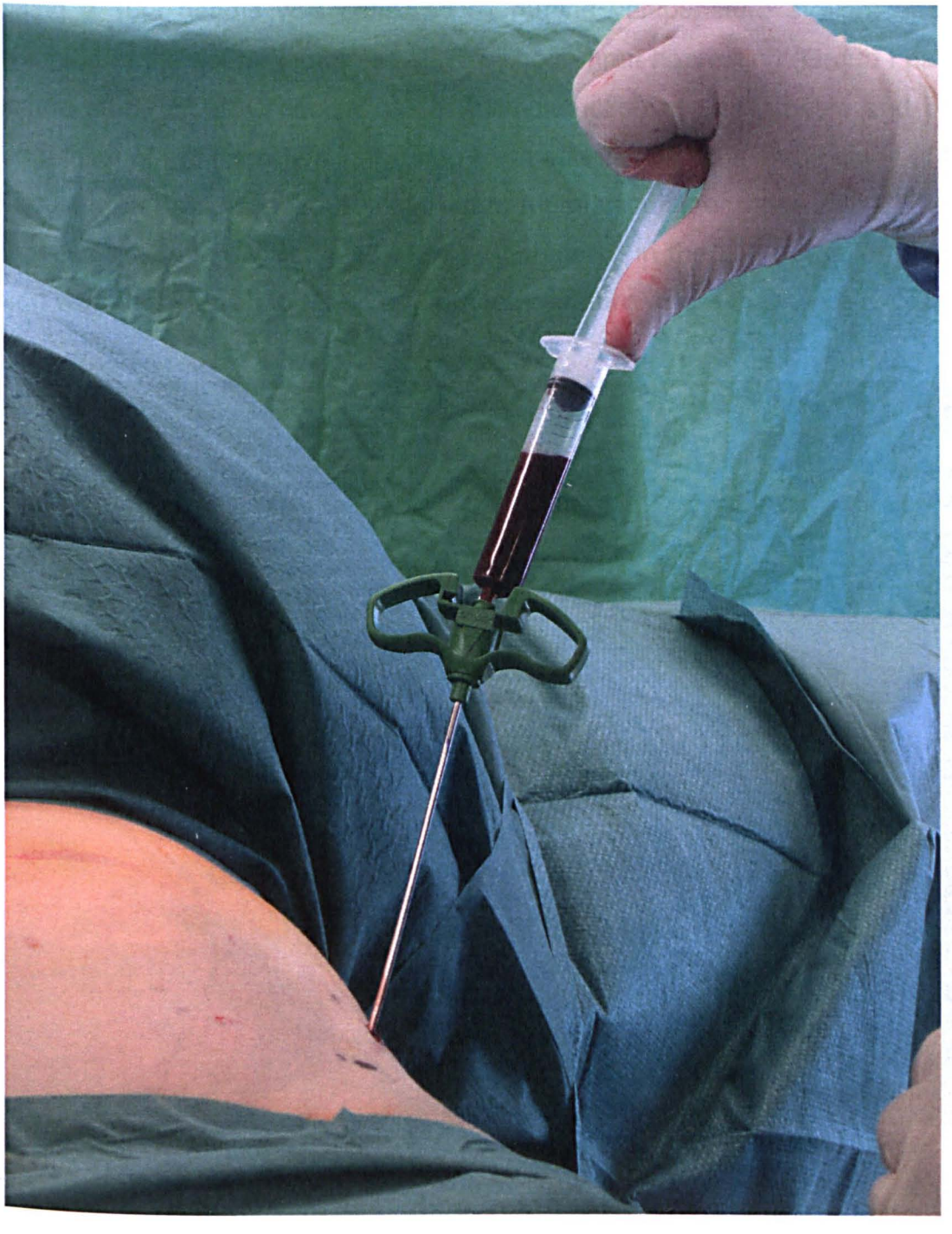

Figure 3.1: Iliac crest bone marrow aspiration technique
Under anaesthesia and sterile conditions, 10ml of BM is aspirated while altering the aspiration site every 4ml to reduce the risk of blood contamination.

3.5 BLOOD SAMPLE COLLECTION

Under sterile conditions and general anaesthesia 10ml of femoral venous blood from the limb ipsilateral to the surgical procedure was obtained (Figure 3.2). Matched 10ml of peripheral venous blood from the contralateral antecubital vein using a 10G needle was collected by routine venipuncture straight after the reaming of the sound femur with RIA for bone graft harvesting (Group I patients in the study of MSC circulation) or after induction of anaesthesia and before beginning of the operation in the non RIA patients (Group II in the study of MSC circulation). Samples were transferred to a 5ml vacutainer tubes with EDTA and labelled accordingly. Samples were all harvested in the LGI operation theatres and were transferred to LIMM at room temperature where they were processed.

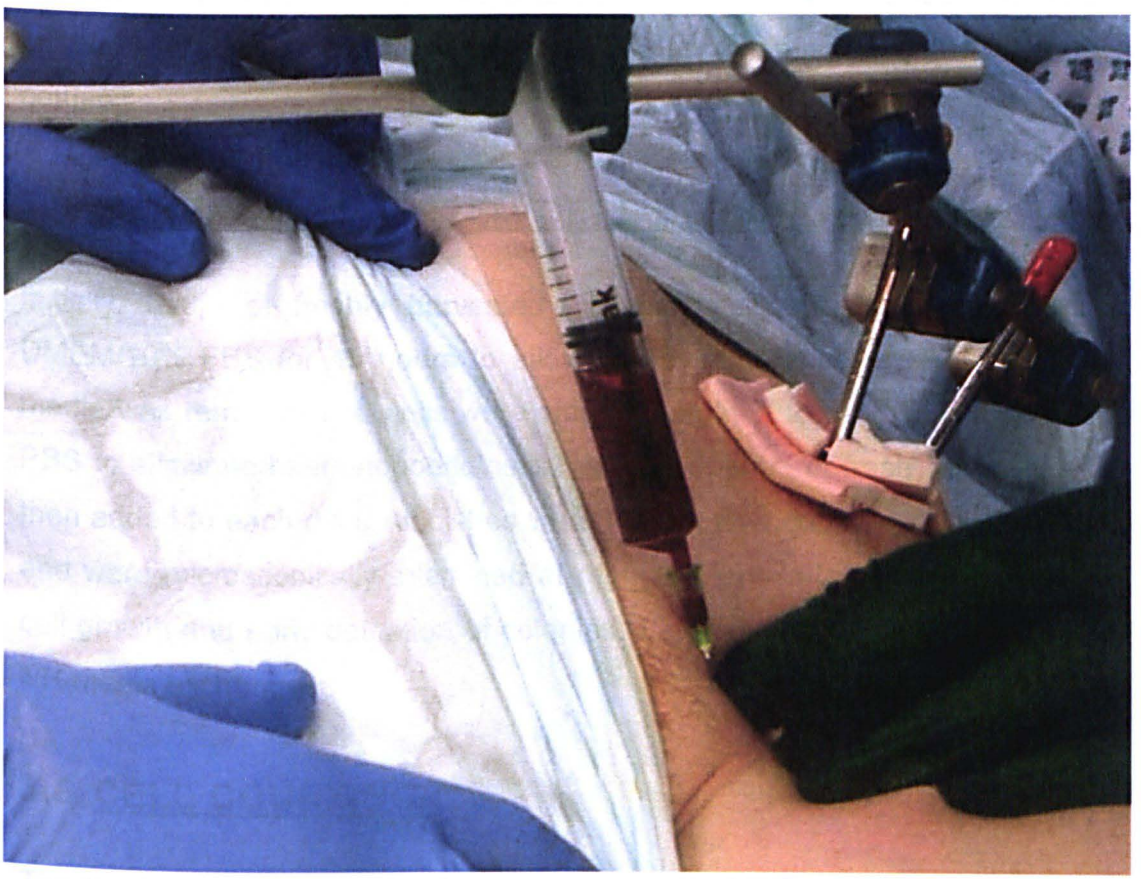

Figure 3.2: Femoral vein aspiration from the femoral triangle

3.6 PROCESSING OF BLOOD SAMPLES

In the tissue culture hood, blood was transferred into 50ml Falcon tube where it was diluted (1:2) with sterile PBS. The diluted blood was then layered on top of equal amount of Lymphoprep solution (Figure 3.3) then centrifuged for 20 minutes at 650 rcf with no brakes. The interface zone (Figure 3.4) containing the MNCs was collected with a sterile pastette and emptied into a clean 50ml Falcon tube. A second centrifugation was performed at 650rcf for 5 minutes (with brakes on). Cell pellet was then re-suspended in 1ml of DMEM media. Cell counting was then performed as described earlier and cells were seeded in 100mm dishes at a density of 10^7cells/dish, in 10ml of NH expansion media. Cultures were fed by the exchange of 5ml of NH media twice weekly. On day 14, colonies were fixed with crystal violet as described in section 3.10 and then counted.

3.7 MESENCHYMAL STEM CELL SEEDING

Initially, 300µl of freshly harvested BM aspirate was seeded in 10ml of DMEM/10% FBS for 48 hours to allow plastic adherence of cells. After that, media was removed and cells were washed gently 2 to 3 times with 10mls of PBS to eliminate haematopoeitc cells. A 10ml volume of fresh NH media was then added to each dish. All dishes were kept in an incubator at 37^0C, 5% Co_2 and were microscopically examined at regular intervals to monitor progress of cell growth and early detection of colonies. Cells were fed once weekly by half NH media exchange.

3.8 CELL COUNTING

The number and the viability of mononuclear cells (MNCs) following lymphoprep isolation was evaluated by diluting the cells with 0.4% w/v trypan blue. For counting cells 1:2 or 1:10 dilutions were performed, depending on the number of cells. For 1:2 dilution 10µl of cells were diluted with 10µl of trypan blue, and for 1:10 dilution 10µl of cells were diluted with 90µl of trypan blue. A volume of 10µl

of the resulting mixture was placed in the haemocytometer chamber. The number of trypan blue negative cells was counted in four large grid squares and the number of MNCs per 1ml was determined by the formula:

Number of cells in 4 large grid squares x 10000 x Dilution factor in trypan blue.

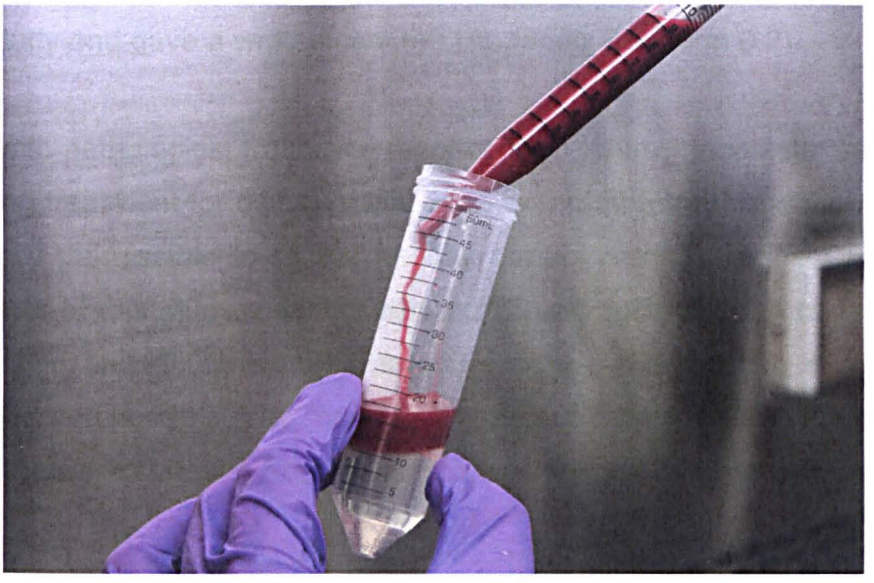

Figure 3.3: Lymphoprep technique
The diluted blood is layered on top of the Lymphoprep solution in the hood prior to centrifugation.

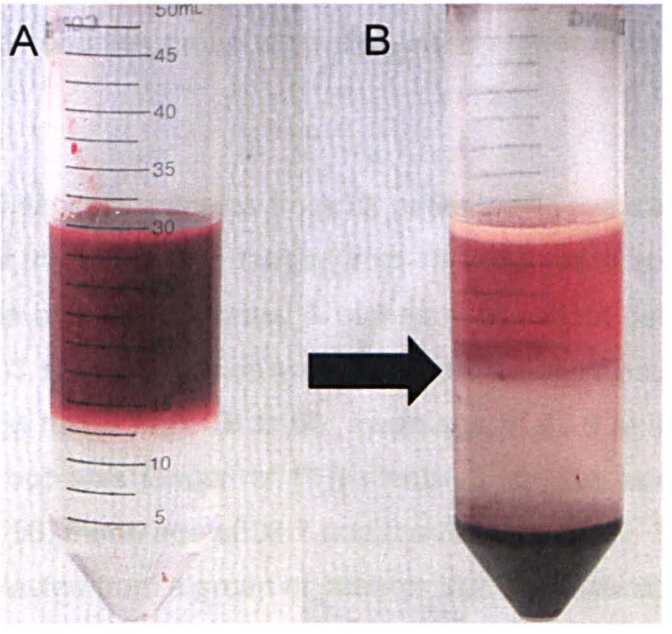

Figure 3.4: Lymphopreped sample (A) before (B) after centrifugation
Arrow denotes the interface zone.

3.9 PATIENT COHORTS

All studies in this work were approved by Leeds (East) Research Ethics Committee Project No: OR06/7651 and Project No: 04/Q1206/107 (Appendix 1 section 9.1). All study subjects were informed regarding to the purposes of the specific study and gave a written consent (Appendix 2 section 9.2).

Patients with pathological fractures, renal or liver failure, neoplastic conditions, or receiving treatment for osteoporosis were excluded from this work. As it was not ethically permissible to recruit healthy control patients in the CFU-F and BMP-7 parts of this study, patients who previously suffered long bone fracture nonunion and united uneventfully (referred to as "union" group in this work) were considered controls. The "nonunion" group of patients in this study refer to patients who were suffering from atrophic long bone fracture nonunion.

3.9.1 Patient cohorts for CFU-F study

The hypothesis under investigation was that the total number and size of CFU-F colonies from the nonunion group were significantly lower in comparison to the union group.

A total of 22 BM aspirate samples from 22 patients (13 females and 9 males) were recruited for this study, age ranged from 18 to 63 years, mean age of 40.1 years and median age of 41.5 years. A summary of patient demographics can be seen in Table 3.1. The nonunion group was formed of 11 patients (5 females and 6 males), age range from 19 to 63, mean age of 40.5 and median age of 43. The union group was formed of 11 patients (8 females and 3 males), age range from 18 to 56, mean age of 39.7 and median age of 41. The union group was randomly selected from a group of patients that were admitted to have their metal work removed following union of the fracture.

Table 3.1: Basic characteristics of patients participated in nonunion versus union CFU-F study

Sample ID	Gender	Age	Diagnosis
TR014	male	31	Nonunion femur
TR016	male	19	Nonunion femur
TR019	female	50	Nonunion femur
TR021	male	22	Nonunion femur
TR024	female	43	Nonunion tibia
TR027	male	33	Nonunion femur
TR031	male	62	Nonunion tibia
TR032	male	63	Nonunion femur
TR038	female	58	Nonunion tibia
TR039	female	44	Nonunion tibia
TR053	female	21	Nonunion tibia
TR018	female	44	Removal of metal work tibia
TR022	female	46	Removal of metal work tibia
TR023	female	41	Removal of metal work femur
TR025	male	33	Removal of metal work femur
TR033	female	39	Removal of metal work tibia
TR035	female	42	Removal of metal work tibia
TR042	female	46	Removal of metal work femur
TR043	male	18	Removal of metal work tibia
TR044	female	56	Removal of metal work tibia
TR045	male	41	Removal of metal work femur
TR046	female	31	Removal of metal work femur

3.9.2 Patient cohorts for enumeration of BM MSCs using flow cytometry

To validate the data from the CFU-F study in the nonunion and union groups, flow cytometry was used. A total of 17 patients were recruited, 7 nonunion and 10 union. Mean age of nonunion group was 42 years, median age 44 years, 4 females and 3 males. In the union group, mean age was 43 years, median 41.5 years, 5 females and 3 males. Patients for this study were both gender and aged matched. A summary of patient demographics can be seen in Table 3.2.

Table 3.2: Characteristics of patients participating in MSC enumeration by flow cytometry

Sample ID	Gender	Age	Diagnosis
TR001	male	50	Nonunion femur
TR014	male	31	Nonunion femur
TR016	male	19	Nonunion femur
TR019	female	50	Nonunion femur
TR024	female	43	Nonunion tibia
TR038	female	58	Nonunion tibia
TR039	female	44	Nonunion tibia
TR020	male	47	Removal of metalwork tibia
TR025	male	33	Removal of metalwork femur
TR035	female	42	Removal of metal work tibia
TR042	female	46	Removal of metal work femur
TR044	female	56	Removal of metalwork tibia
TR045	male	41	Removal of metal work femur
TR046	female	31	Removal of metal work femur
TR051	male	36	Removal of metalwork tibia
TR052	female	60	Removal of metalwork tibia
TR058	male	38	Removal of metalwork femur

3.9.3 Patient cohorts BMP-7 study

The effect of BMP-7 on the calcium production of BM MSCs and the proliferation of CFU-F colonies from BM MSCs from union and nonunion patients was studied using both expanded and non-expanded (native) MSCs. A total of 14 patients participated in this study, 7 females and 7 males, age ranged from 21 to 63 years old, mean age of 46.1 years and median age of 44 years. Demographics of patients can be seen in Table 3.3.

Three different study groups were formed based on the type of sample tested.

- **Group A,** native BM MSCs obtained from patients suffering from long bone nonunion. This group was composed of 6 patients, 3 females and 3 males, age range from 22 to 63 years old, mean and median age of 48.7 years and 51 years respectively.

- **Group B,** experiments were carried out on cultured expanded BM MSCs obtained from patients suffering long bone nonunion. This group was composed of 3 patients, 2 females and 1 male. Age ranged from 44 years to 63 years, mean age of 55 years and median age of 58 years.

- **Group C,** culture expanded BM MSCs from long bone fracture union patients were studied. This group was comprised of 4 patients, 2 males and 2 females. Age ranged from 21 to 62, mean age of 42 years old and median age of 42.5 years old.

In all 3 study groups, the effect of no BMP-7, 100ng BMP-7 and 300ng BMP-7 on calcium production and CFU-F colony proliferation was tested.

Table 3.3: Demographics of patients participating in the study of effect of BMP-7 on MSC proliferation and differentiation

Sample ID	Gender	Age	Diagnosis	Group
TR021	Male	22	Nonunion femur	A
TR024	Female	43	Nonunion tibia	A
TR031	Male	62	Nonunion tibia	A
TR032	Male	63	Nonunion femur	A
TR038	Female	58	Nonunion tibia	A
TR039	Female	44	Nonunion tibia	A
TR032 (EX*)	Male	63	Nonunion femur	B
TR038 (EX*)	Female	58	Nonunion tibia	B
TR039 (EX*)	Female	44	Nonunion tibia	B
TR034 (EX*)	Male	62	Removal of metal work tibia	C
TR035 (EX*)	Female	42	Removal of metal work tibia	C
TR037 (EX*)	Male	21	Removal of metal work tibia	C
TR043 (EX*)	Male	43	Removal of metal work tibia	C

* culture expanded

Table 3.4: Summary of the study groups for the effect of BMP-7 on MSCs

Group designation	Group description	n Female : n Male	(Age Range) Mean, Median
A	Fresh BM MSCS from nonunion patients	3:3	(22 – 63) 48.7, 51
B	Expanded BM MSCs from nonunion patients	1:2	(44 – 63) 55, 58
C	Expanded BM MSCs from Union - patients with long bone fractures that united without healing complications	2:2	(21 - 62) 42, 42.5

3.9.4 Patient cohorts for the study of MSC circulation

For this study, a total of 71 samples from 47 patients were tested. Four different study groups were formed (Table 3.5).

- **Group I,** 12 patients suffering long bone fracture nonunion and are undergoing reaming of the sound femur with RIA for bone graft harvesting together with revision of their initial fixation. Age ranged from 19 to 63, mean and median age was 40.5 years old, 5 females and 7 males. A total of 24 venous blood samples (10ml each of matched lower limb femoral (LL) and upper limb peripheral venous blood (UL), Figure 3.5) were collected from this group straight after the reaming process as this is the peak time where fat embolism is known to occur. Individual patient characteristics for this study can be seen in Table 3.6.

- **Group II,** 12 patients where recent or intra-operative skeletal trauma including reaming did not take place (i.e. cold cases). As it was not ethically permissible to recruit healthy controls, this group served as control. Age ranged from 18 to 67, mean age of 41.5 and median age of 40, 5 females and 7 males (Table 3.7). A total of 24 samples (10ml of femoral venous blood and 10ml of peripheral venous blood) from each patient were collected after induction of anaesthesia and before commencement of surgery (to ensure no surgical skeletal trauma took place). In cases where limb surgery was proposed, the blood samples were collected from the contralateral injured side.

- **Group III,** 11 patients diagnosed as early RA and not under any immunosuppressive treatment. This group were selected on the basis of recent work suggesting that circulating MSCs may have been capable of carrying the disease from joint to joint via the circulation [129, 130]. Age ranged from 37 to 77 years old, mean age of 56 and median age of 53, 8 females and 4 males (Table 3.8). Only 10ml of peripheral venous blood samples (no femoral venous blood samples) were collected from this

group during their visit to the outpatient department. Ideally, matched femoral venous blood should have been collected from these patients, but this is neither ethical nor practical.

- **Group IV,** 12 healthy controls were recruited to further look for circulating MSCs in the peripheral blood circulation. Their age ranged from 24 to 51 years old, mean age of 32 and median age of 32 years old, 4 females and 8 males. As was the case for Group III, only 10ml of peripheral venous blood was collected and no femoral blood samples obtained. Basic characteristics are shown in Table 3.9.

Table 3.5: Study Groups for the circulation of MSCs in the blood

Group Designation	Group description	n Female: n Male	(Age Range) Mean, Median	Samples collected
I	Long bone fracture nonunion undergoing femoral reaming with RIA	5:7	(19 – 63) 40.5, 40.5	LL, PB
II	Patients undergoing various surgical "cold" orthopaedic procedures	5:7	(18 – 67) 41.5, 40.5	LL, PB
III	Early RA and not under any immunosuppressive treatment	7:4	(37 – 77) 56, 53	PB
IV	Healthy controls	4:8	(24 – 51) 32, 32	PB

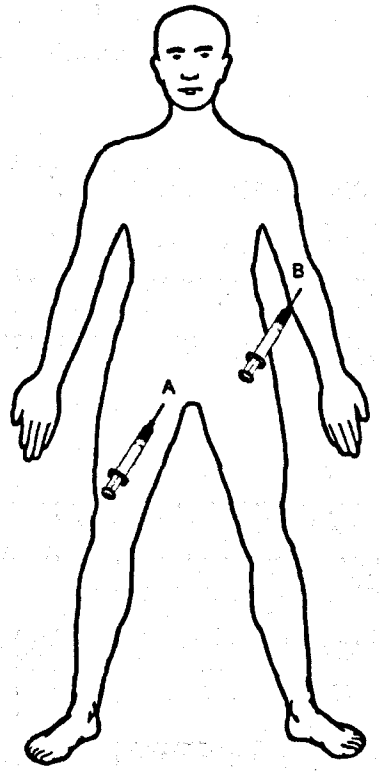

Figure 3.5: Venipuncture sites
A: contralateral mediancubital vein. B: ipsilateral femoral vein (image modified from Giannoudis et al, 2010) [163].

Table 3.6: Basic characteristics of Group I patients participated in the MSC circulation study

Sample ID	Gender	Age	Diagnosis
TR016	Male	19	Nonunion femur
TR019	Female	50	Nonunion femur
TR021	Male	22	Nonunion femur
TR024	Female	43	Nonunion tibia
TR027	Male	33	Nonunion femur
TR028	Male	33	Nonunion femur
TR031	Male	62	Nonunion tibia
TR032	Male	63	Nonunion femur
TR038	Female	58	Nonunion tibia
TR039	Female	44	Nonunion tibia
TR040	Male	38	Nonunion tibia
TR053	Female	21	Nonunion tibia

Table 3.7: Basic characteristics of patients from Group II patients participated in the MSC circulation study

Sample ID	Gender	Age	Diagnosis
TR029	Female	39	Sacroilitis for sacroiliac screws
TR030	Male	67	Removal of metal work ankle
TR034	Male	62	Removal of metal work tibia
TR036	Female	42	Sacroilitis for sacroiliac screws
TR037	Male	21	Removal of metal work tibia
TR041	Male	38	Osteomyletis tibia for debridement
TR047	Female	46	Cocydena for local injection
TR054	Male	18	Osteomyletis tibia for debridement
TR065	Female	56	Sacroilitis for sacroiliac screws
TR077	Male	41	Meniscal tear of knee for arthroscopy
TR078	Female	31	Sacroilitis for sacroiliac screws
TR080	Male	38	Osteomyletis tibia for debridement

Table 3.8: Basic characteristics of early RA patients participated in the study

Sample ID	Gender	Age	CRP*	ESR*	plasma viscosity*
TR066	Female	77	<5.0	11	1.53
TR067	Female	37	27	39	N/A
TR068	Female	41	<5.0	12	N/A
TR069	Female	59	34	42	N/A
TR070	Male	70	<5.0	10	N/A
TR071	Male	54	28	N/A	1.73
TR072	Female	51	<5.0	N/A	1.74
TR073	Male	49	56	N/A	2.03
TR074	Female	73	54	9	N/A
TR075	Male	53	<5.0	9	N/A
TR076	Female	49	<5.0	9	N/A

*inflammatory markers used to assess the degree of inflammation in RA patients

Table 3.9: Basic characteristics of healthy control patients participated in the study

Sample ID	Gender	Age
TR048	Female	24
TR049	Female	30
TR050	Male	46
TR055	Male	37
TR056	Female	35
TR057	Male	32
TR059	Male	31
TR060	Male	37
TR061	Male	40
TR062	Male	27
TR063	Male	51
TR064	Female	27

3.10 CFU-F ASSAY

All CFU-F assays on BM aspirates were performed from freshly harvested samples obtained as outlined in section 3.4. A volume of 300µl of BM sample was initially seeded in 100mm diameter petri-dish with 10ml of DMEM/10% FBS for 48 hours to allow for plastic adherence of cells. Thereafter, media was removed and cells were washed gently 2 to 3 times with 10ml of PBS to eliminate haematopoietic cells. A 10ml volume of fresh NH media was then added to each dish. All dishes were kept in an incubator at 37^0C, 5% Co_2 and were microscopically examined at regular intervals, to monitor progress of cell growth and early detection of colonies. Cells were fed once weekly by half NH media exchange.

The experiment was ended at day 14. Media was removed; cells were gently washed twice with 10ml PBS after which 5ml of 3.7% formaldehyde was added for 15 minutes to each dish to fix the cells. Excess formaldehyde was poured off and 5ml of 1% Crystal Violet stain (w/v) was added for another 15 minutes. Dishes were washed extensively with tap water and left to dry. Dishes were then scanned at 360 dpi (Epson Perfection 3590 Photo Scanner) with images processed using ArcSoft Photostudio version 5.

3.11 CFU-O ASSAY

Colony forming unit osteoblast (CFU-O) assay was performed as a measure of osteogenesis of individual colonies. Three hundred microliters of fresh BM aspirate was plated into a 100mm diameter petri-dish with 10ml of DMEM/10% FBS and incubated at 37°C, 5% CO_2 for 48 hours to allow plastic adherence of cells. Two 10ml PBS washes were done to remove red cells, then 10ml of osteogenic media was added to each dish. Half the volume of osteogenic media was exchanged once weekly for cell feeding. On day 14, ALP staining (see below) of the adherent colonies was performed.

For ALP staining assay, citrate working solution was first made up by the addition of 2ml citrate concentrated solution (Sigma) to 98ml of deionised water. To prepare the fixative, 20ml of citrate working solution was added to 30ml of acetone (VWR International). Fast blue solution was prepared by dissolving the contents of one capsule of fast blue RR salt (Sigma) in 48ml of distilled water, 2ml of Napthol AS-MX phosphate alkaline solution mix (Sigma) was then added, the mixture (called fast blue solution) was covered with foil to protect it from light. Using a Pasteur pipette, the osteogenic media was removed from the dishes. Cells were washed twice with 10ml of PBS after which 2ml of fixative was added gently and left for 30 seconds. Cells were then washed with deionized water. A volume of 5ml of fast blue solution was added to each dish and incubated for 30 minutes in the dark at RT. Cells were then washed twice with 10ml of deionized water. Then, 2ml of Mayers Haematoxylin (Sigma) was

next added for 10 minutes. Finally, cells were washed twice with 10ml of deionized water and left to dry. Blue colouring of colonies occurred with ALP activity which indicates osteogenesis. Samples were left to dry, then scoring and scanning was performed as described above.

3.12 PERFORMING PROLIFERATION AND OSTEOBLASTIC DIFFERENTIATION ASSAYS IN THE PRESENCE OF BMP-7 (FRESH MSCS, GROUP A)

The overwhelming majority of MSC based research is carried out on culture expanded MSCs. However, the functional assessment of freshly isolated MSCs prior to prolonged culture manipulation may be much more representative of what is going on in vivo. For this arm of the study, 300µl of fresh iliac crest BM was seeded in 6 dishes (duplicates) in 10ml of DMEM/10% FBS and incubated at 37^0C, 5% Co_2 for the first 48 hours to allow for plastic adherence. Media was then removed, and cells were gently washed 2-3 times with 10ml of PBS to eliminate non adherent haematopoeitc cells. Ten millilitres of NH media were added to each dish and supplemented with the relevant BMP-7 dose according to the study group. On the 7^{th} day from the change of media to NH, cells were fed by 5ml media change and an additional dose of BMP-7 was added again according to the study group. A week later (day 14), experiments were stopped for CFU-F assay.

3.13 EVALUATION OF COLONY SIZES AND SURFACE AREAS USING A GRID

Using Microsoft Office Word 2007, four 150 x 150mm grids graticuled into 10 x 10mm; 7.5 x 7.5mm; 5 x 5mm and 2.5 x 2.5mm were designed Figure 3.6. The surface area of each colony was measured against these grids. The total colony count and the colony count per group size was noted. The surface area of each

colony was estimated to be equivalent to the surface area of the best fitted grid square. Using Microsoft Office Excel, the total surface area of all colonies was calculated and an average colony surface area was estimated (total surface area of all colonies/colony count).

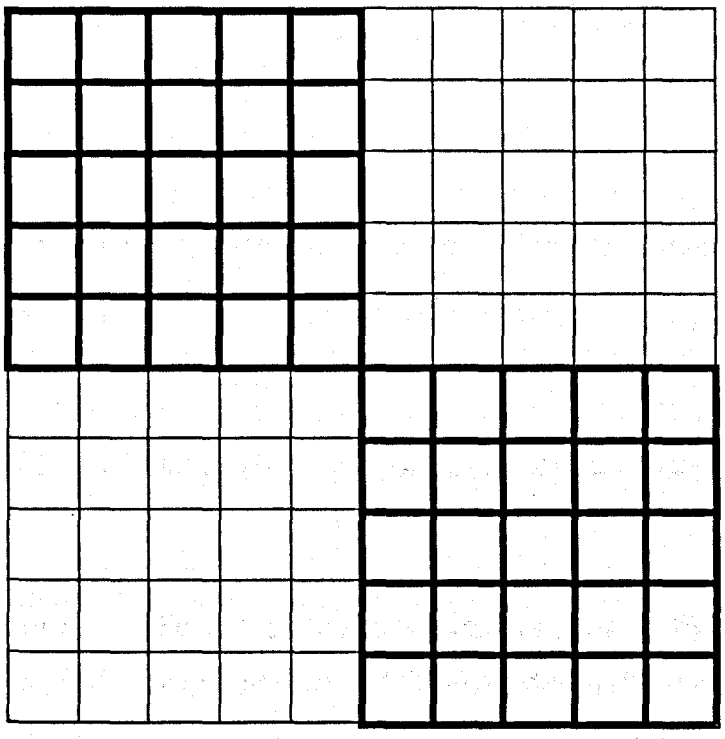

Figure 3.6: Example of a grid used for colony scoring (not to original size) Four grids were made graticuled into 10 x 10mm; 7.5 x 7.5mm; 5 x 5mm and 2.5 x 2.5mm. Dishes were placed on top of these grids and colonies were scored.

3.14 MEASUREMENTS OF CELL GROWTH CHARACTERISTICS AND POPULATION DOUBLING TIME IN OSTEOGENIC CONDITIONS

In these experiments 300µl of fresh BM aspirate was plated into a 100mm diameter petri-dish with 10ml of DMEM/10% FBS and incubated for 48 hours to allow plastic adherence of cells. After that, red cells were removed with two 10ml PBS washes. The remaining cells were left to grow in 10ml of osteogenic media for 14 days with half the media volume was exchanged once weekly for cell feeding. On day 14, cells were trypsinised and then counted using trypan blue staining and haemocytometer. To trypsinise cells, two 5ml washes with 5ml PBS were initially performed, after which 3ml of trypsin was added to facilitate cell lifting and the plates were incubated for 2-3 minutes at 37°C. To deactivate the trypsin, 5ml of DMEM/10% FBS was added to each plate. Cells were examined microscopically to ensure complete cell lifting has occurred. If remaining adherent cells were noted, mechanical lifting was done with the use of a cell scrapper. The cell suspension was transferred to a falcon tube and then centrifuged for 5 minutes at 650rcf. The cell pellet was resuspended in 800µl of DMEM/10% FBS and cells were counted as described earlier. Population doublings (PDs) achieved by MSCs were calculated using the formula:

PDs= $\log_2$ (number of cells at trypsinisation / CFU-O per dish). PD rate (on day 14) was calculated as PDs/14.

Population doubling time (PDT) at day 14 was also calculated as follows :

PDT= (14 $\log_2$) / log number of cells at trypsinisation – log CFU-O per dish

3.15 EXPANSION OF MSCS FOR GROUPS B AND C IN BMP-7 EXPERIMENTS

In these experiments 300µl of fresh BM aspirate was plated into one 100mm diameter petri-dish with 10ml of DMEM/10% FBS and incubated for 48 hours to allow plastic adherence of cells. After that, red cells were removed with two 10ml PBS washes. The remaining cells were left to grow in 10ml of NH media until confluence. In the meantime, half the media volume was exchanged twice weekly for cell feeding. Dishes were examined regularly under the microscope to monitor cell growth. Once confluent, p0 cells were trypsinised and then counted using trypan blue staining and haemocytometer as described in section 3.14.

3.16 THE STUDY OF THE EFFECT OF BMP-7 ON THE OSTEOGENIC DIFFERENTIATION OF EXPANDED MSCS

MSCs cultured to passage 3 from the same patient were counted and seeded in duplicate dishes in osteogenic media as described in section 3.11. Three groups were established: no BMP-7, 100ng/ml BMP-7 and 300ng/ml BMP-7. BMP-7 was added accordingly on the time of seeding. Cells were fed by half media change after one week during which another dose of BMP-7 was added according to the group. On day 14, the osteogenic differentiation process was ended and a colorimetric calcium production assay was performed.

3.17 COLORIMETRIC CALCIUM PRODUCTION ASSAY

Fully functional osteoblasts produce calcium as their final product; this was used as measure of osteoblastic differentiation. On day 14, the osteogenic medium was removed, and the cells were washed twice with 10ml of calcium free PBS. To extract the calcium bound to cells, 3ml of 0.5N Hcl was added in each dish and incubated at room temperature (RT) for 5 minutes. With a cell

scraper, cells were scraped off the surface of the dishes. This suspension was then transferred to two 1.5ml Eppendorf tubes with a 5ml syringe and needle. Cells were allowed to mix in the cold room at 4°C for 4 hours using a rotator, and were subsequently stored at -20°C.

The amount of calcium produced by osteoblasts was quantified based on a direct colorimetric method, Cresolphtalein complexone (CPC) using Sentinel Diagnostics' calcium kit. The kit was composed of 3 reagents: reagent 1, reagent 2 and standard. Reagent 1 was 2-aminoethanol buffer (MEA), reagent 2 was CPC and Calcium standard was supplied as 10 mg/dL(100µg/ml) solution. A round bottom 96 well plate was labelled as dilution plate then 50µl of 0.5N Hcl was added from wells B1-H1, B2- H2, B3 – H3 to prepare dilutions of calcium standard (Figure 3.7A). Then 50µl of standard calcium solution was added from well A1 – A3 and B1 – B3 (Figure 3.7B). Using a multichannel pipette, serial dilution of the standard calcium solution was performed from B1-B3 to H1-H3 (50µl of the contents of each well was removed, then added to the following row and this process was repeated till row H. Figure 3.7C shows the calcium concentrations in each well.

Working reagent solution was prepared by mixing 15ml of reagent 1 with 6ml of reagent 2 (5:2). A flat bottom 96 well plate was then labelled as "reading plate". Two hundred microlitres of the working solution was added to each well in the reading plate. Four microlitres of calcium standard was transferred from the mixing plate to the corresponding well of the reading plate. Three, 4µl aliquots (triplicates), of the sample to be tested was then transferred to the reading plate (Figure 3.7D). The amount of calcium per well was measured photometrically using a 570nm filter on a Mithras LB 940. Standard calcium curves where then plotted using Microsoft Excel 2007 and an equation was produced; this was later used to measure the amount of calcium per well.

A

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

B

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

C

	1	2	3	4	5	6	7	8	9	10	11	12
A	100µl	100µl	100µl									
B	50µl	50µl	50µl									
C	25µl	25µl	25µl									
D	12.5µl	12.5µl	12.5µl									
E	6.25µl	6.25µl	6.25µl									
F	3.13µl	3.13µl	3.13µl									
G	1.56µl	1.56µl	1.56µl									
H	0µl	0µl	0µl									

D

µg/ml	1	2	3	4	5	6	7	8	9	10	11	12
A	100µl	100µl	100µl	Sample 1 (Triplicates)			Sample 9 (Triplicates)			Sample 17 (Triplicates)		
B	50µl	50µl	50µl	Sample 2 (Triplicates)			Sample 10 (Triplicates)			Sample 18 (Triplicates)		
C	25µl	25µl	25µl	Sample 3 (Triplicates)			Sample 11 (Triplicates)			Sample 19 (Triplicates)		
D	12.5µl	12.5µl	12.5µl	Sample 4 (Triplicates)			Sample 12 (Triplicates)			Sample 20 (Triplicates)		
E	6.25µl	6.25µl	6.25µl	Sample 5 (Triplicates)			Sample 13 (Triplicates)			Sample 21 (Triplicates)		
F	3.13µl	3.13µl	3.13µl	Sample 6 (Triplicates)			Sample 14 (Triplicates)			Sample 22 (Triplicates)		
G	1.56µl	1.56µl	1.56µl	Sample 7 (Triplicates)			Sample 15 (Triplicates)			Sample 23 (Triplicates)		
H	0µl	0µl	0µl	Sample 8 (Triplicates)			Sample 16 (Triplicates)			Sample 24 (Triplicates)		

Figure 3.7: Calcium assay procedure

A - Dilution plate, Step 1, preparing to dilute calcium standard. B - Dilution plate, Step 2, adding calcium standard. C - Dilution plate, Step 3, making dilutions of calcium standard. D – Reading plate, Step 4, adding samples and standards to working solution.

3.18 MONONUCLEAR CELL FREEZING

To freeze cells, freezing medium containing 10% DMSO, 45% DMEM and 45% FBS was used. A solution containing 10% DMSO, 45% DMEM, and 45% FBS was prepared in a special falcon tube which was kept on ice. A volume of 1ml of the freezing media was added to the cell pellet (average 10^7 MNCs/ml) and thoroughly mixed. The content of the tube was transferred into a cryovial, which was placed in a cool freezing container (Nalgene containing isopropanol) for 1-2 days at –80 °C. Finally, the tubes were transferred to the Liquid Nitrogen (LN2) tanks for long term storage. MNC stocks were kept in LN2 facility for future use.

3.19 REVIVING FROZEN CELLS FOR FLOW CYTOMETRY

Deoxyribonucleate 5'-oligonucleotido-hydrolase (DNAse), Sigma, was dissolved in 2.75ml of PBS and frozen at -20°C in 40ul aliquots. Forty microliters of DNAse was added to 20ml of thawing media (DMEM / 10% FBS). DNAse was added to defrosting media to avoid cell clumping/cell loss during reviving process.

Samples were held in the water bath at 37°C to defrost for about one minute. The defrosted sample was then emptied into a 50ml Falcon tube. Thawing media (DMEM/10% FCS) containing DNAse was added to the sample very slowly (drop by drop) using a pastette until 20ml of volume was reached. The empty storage vial was rinsed with thawing media containing DNAse and its content emptied into the Falcon tube. A 70-micron cell strainer was sometimes used to filter the solution if clumps developed. The sample was centrifuged for 10 minutes at 650 rcf. The supernatant discarded and the pellet slowly re-suspended in 2ml thawing media. A standard trypan blue cell count was performed and cell number calculated using the aforementioned formula.

3.20 FLOW CYTOMETRY

Flow cytometry was performed to independently validate CFU-F data using a two colour Becton Dickinson FACScan. Jones et al [85], showed that flow cytometry for the $CD45^{low}CD271^{+}$ cell population is a reliable method for detection, enumeration and phenotypic characterisation of BM MSCs in both healthy and diseased individuals.

For flow cytometry experiments, BM aspirates were undergone mononuclear cell (MNC) fractionation using (Lymphoprep). MNCs were frozen at 10^7cells/vial and stored in LN2. Prior to flow cytometry, MNC were de-frosted, as described above and stained with appropriate antibodies. Antibodies and isotope controls used for flow cytometry are shown in Table 3.10 and Table 3.11. Staining was performed as follows. In a 96 well round bottom plate, cells were added into well A1 and A2. The plate was spun down for 1 minute at 650 rcf. Supernatant from both wells was discarded. Remaining pellet in well A1 was stained with the primary conjugated antibodies, wereas pellet in well A2 (control) was stained with isotype controls. Plate was kept in the fridge for 20 minutes at 4°C. Cells were then washed with 150ul of Facsflow (BD) then spun again for 10 seconds at 650 rcf. Supernatant was discarded. Two 5ml polystyrene round bottom tubes (BD) were placed in ice and filled with 0.9ml of Facsflow each. One hundred microliters of Facsflow was added in wells A1 and A2 and contents pipetted into a FACS tube which was labeled accordingly.

As mentioned above, cells were stained with CD45-FITC and CD271-PE (LNGFR) conjugated antibodies to identify $CD45^{low}CD271^{+}$ cell population. 7-AAD staining was incorporated to exclude dead/dying cells. Data were acquired using BD FACScan equipped with CellQuest software version 3.1 (BD Biosciences). Gating strategy for the enumeration of $CD45^{low}CD271^{+}$ cells is shown on Figure 3.8.

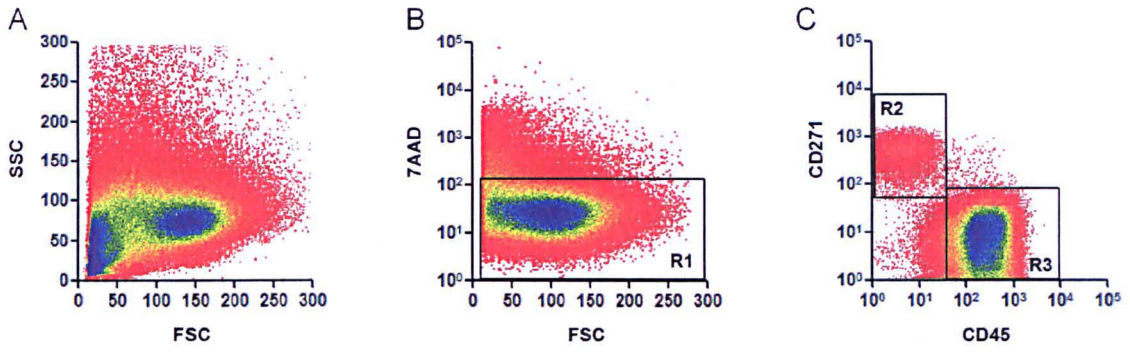

Figure 3.8: Gating strategy for MSC enumeration by flow cytometry
A – cell debris are excluded based on size. B - dead cells excluded based on 7-AAD staining. 7-AAD+ cells are dead/dying, 7-AAD- cells are live. C - $CD45^{low}CD271^{+}$ cell population is identified as a tight cluster (R2) and its frequency is calculated as a percentage of live cells (R3, haematopoietic cells not measured).

Table 3.10: List of antibodies used for flow cytometry

Specificity	Conjugate	Isotype	Clone	Product Number	Manufacturer
CD45	FITC	IgG1, Kappa	T29/33	F0861	Dako
CD271	PE	IgG1, Kappa	C40-1457	557196	BD

Table 3.11: List of isotype controls used for flow cytometry

Conjugate	Isotype	Clone	Product Number	Manufacturer
FITC	IgG1, Kappa	MOPC-31C	550616	BD
PE	IgG1, Kappa	B56	556027	BD

3.21 STATISTICAL ANALYSES

All calculations were done using GraphPad Prism 5 software version 5.04. Normality testing was carried using Shapiro-Wilk test to determine whether the data were parametric or nonparametric. Nonparametric statistics was used as data was found to be nonparametric. Non-matched and donor-matched data were compared using the unpaired (Mann-Whitney) and paired (Wilcoxon) nonparametric tests, respectively. Data were expressed as means (standard deviation) or median (range) as appropriate. The cut-off value for significance was p=0.05.

For studies to explore the circulation of MSCs in the blood in health, orthopaedic procedures and early (section 6.1), statistics were carried out using SPSS 15 (SPSS Inc) software, the odds ratio test was carried to compare findings of MSCs in the upper limb venous blood (UL) and the lower limb venous blood (LL). An assumption was made that samples are indepeIndent based on the extreme rarity of MSC colonies in the blood. The cut-off value for significance was p=0.05.

CHAPTER 4

RESULTS OF CFU-F STUDY

4.1 COMPARISON BETWEEN THE NUMBERS OF MSCS AND OSTEOPROGENITORS IN BM ASPIRATES IN NONUNION AND UNION PATIENTS

The hypothesis under investigation was that the total number and size of CFU-F colonies from nonunion patients will be significantly lower in comparison to long bone fracture union patients. A total of 22 patients were recruited for this study 11 in each study group.

A volume of 10ml of BM from the anterior iliac crest was aspirated from these individuals according to protocol described in section 3.40. A comparison between BM CFU-F and BM CFU-O colony numbers and sizes between these 2 groups was performed. Both assays were carried out in a standardised way (see sections 3.10 and 3.11 respectively) in duplicate or triplicate dishes and experiments were ended on day 14. Colony surface areas were worked out using the grid described earlier (section 3.13).

4.1.1 CFU-F assay validation

CFU-F assay was performed in duplicate or triplicate dishes and it was important to assess the technical variability between the dishes before evaluating differences between patient groups. Using Microsoft Excel 2007, the mean, standard deviation (SD) and coefficient of variation (CV) was calculated for a group of union patients and an average of 23.6% variability between dishes was noted (Table 4.1).

Table 4.1: Variability between CFU-F assay duplicates and triplicates in union group

Sample ID	Colonies per dish			Mean	SD	CV*
	Dish 1	Dish 2	Dish 3			
TR018	15	18		16.5	2.1	12.9
TR023	6	6		6.0	0.0	0.0
TR033	5	6		5.5	0.7	12.9
TR035	17	8		12.5	6.4	50.9
TR025	8	4	7	6.3	2.1	32.9
TR043	19	22	16	19.0	3.0	15.8
TR044	6	5	8	6.3	1.5	24.1
TR022	17	35	15	22.3	11.0	49.3
TR042	32	30	30	30.7	1.2	3.8
TR045	10	15	18	14.3	4.0	28.2
TR046	4	7	5	5.3	1.5	28.6
Average				13.2	3.0	23.6

* CV is calculated as (SD/Mean)x100%

4.1.2 Differences in CFU-F numbers between union and nonunion patient groups

The total CFU-F colonies in the nonunion group were compared to the union (Figure 4.1A). Statistical analysis performed using the Mann Whitney Test, showed that the total CFU-F number in the nonunion group was significantly higher than in the union group (median values 29 and 12.5 colonies respectively, p=0.0488). This was unexpected since it contradicted the initial hypothesis. More donor-to-donor variability was noticed in the nonunion group (range 3 to 139 colonies) compared to the union (range 5 to 31 colonies), but

this could not be simply explained from patient's demographics data, since both groups were age-matched.

It was noted however, that the two groups were not completely gender-matched, female: male ratio was 5:6 in nonunion group and 8:3 in the union group. Therefore, a comparison was between the CFU-F numbers in female and male patients in the union group was carried out and no significant differences were found (12 and 16 colonies for females and males, respectively, p=0.3758). It was therefore concluded that the differences between nonunion and union groups could not be attributed to gender composition of these two groups.

Statistical analysis revealed that the total CFU-O numbers in the nonunion and union groups were not significantly different (p=0.5952). This was contrasting CFU-F data and suggested that the increase of total CFU-F in the nonunion group was not due to increased numbers of osteoprogenitors. To explore these findings further, colonies were scored using the grid mentioned earlier (section 3.13). The results for CFU-F colonies are shown on Figure 4.2 and Figure 4.4.

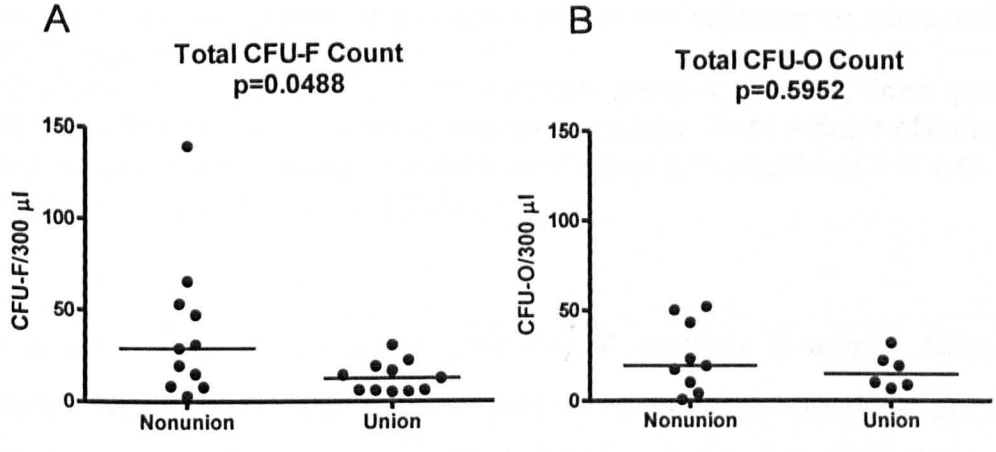

Figure 4.1: The numbers of CFU-F (A) and CFU-O (B) colonies in nonunion and union groups
The increase in CFU-F count in nonunion was not reflected on the CFU-O / osteoprogenitor numbers. Horizontal bars represent median values.

4.1.3 Differences in CFU-F colony sizes between nonunion and union groups

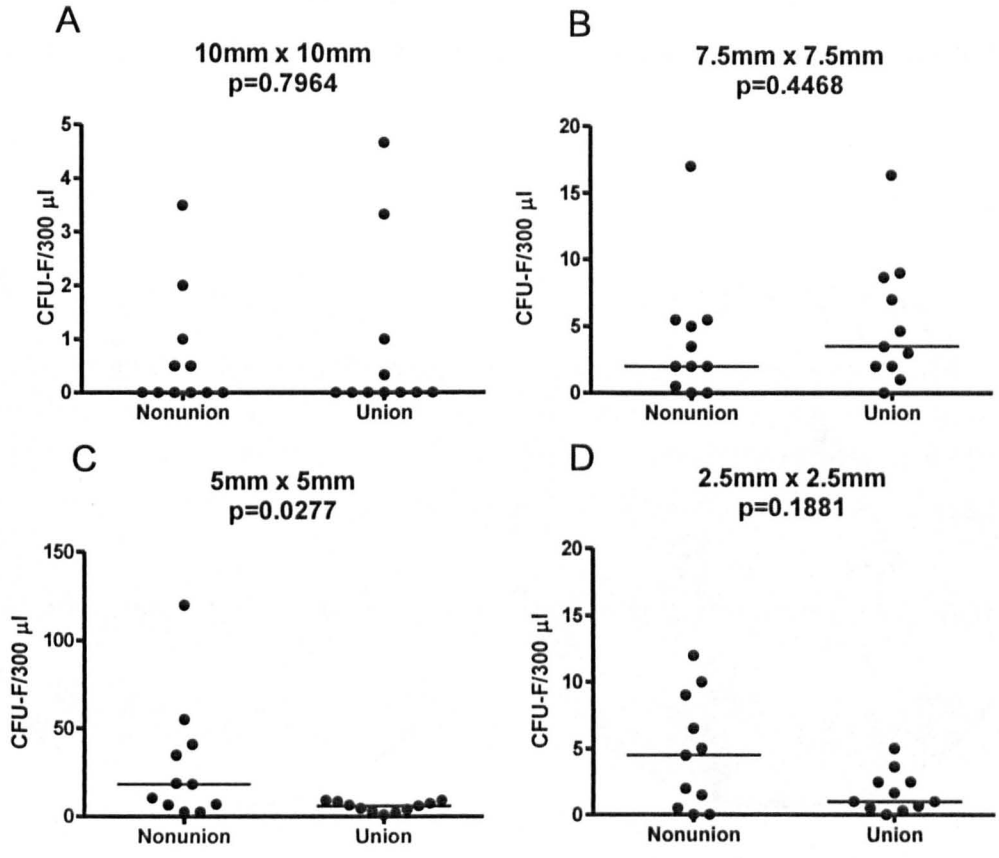

Figure 4.2: Measurement of the sizes of CFU-F colonies in nonunion and union groups
A: 10mm x 10mm group. B: 7.5mm x 7.5mm group. C: 5mm x 5mm group. D: 2.5mm x 2.5mm group. A significant increase of the 5mm x 5mm colonies was noted in the nonunion group. Colonies were stained on day 14 and scored using the grid as described in section 3.13.

There were more of the larger-sized CFU-F colonies (10mm x 10mm and 7.5mm x 7.5mm) in the union group when compared to the nonunion group (not significant, p=0.7964 and p=0.4468, respectively). On the other hand, small sized colonies (5mm x 5mm and 2.5mm x 2.5mm) were increased in the nonunion group as compared to union patients. The difference in colony number between the two groups was of statistical significance in the 5mm x 5mm group (p=0.0277). So, it appeared that CFU-F colonies in the union group were more proliferative, hence the larger size of individual colonies.

Concurrently, the predominance of smaller sized colonies in the nonunion group suggested decreased MSC proliferation or early senescence. Photographs of representative culture dishes are shown on Figure 4.3. It shows the higher number and more frequent small sized colonies in the nonunion group. Fewer colony numbers and a predominance of large sized colonies can be noted in the union group.

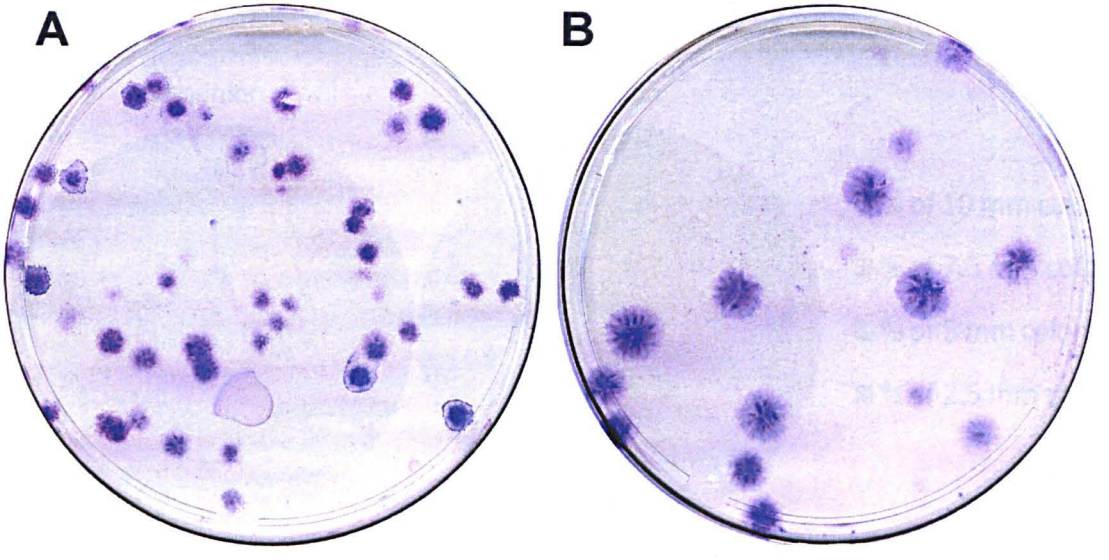

Figure 4.3: Scanned dishes showing numbers and sizes of colonies in representative nonunion and union patients
A. Nonunion. B. Union. Note the more numerous small sized colonies in the nonunion patient's sample and the larger, less numerous ones in the union patient's sample.

The proportions of small colonies as a percentage of total colonies in both patient groups were calculated Figure 4.4. It was found that in the nonunion group, the small sized CFU-F colonies (5mm x 5mm and 2.5mm x 2.5mm) represented an average 83% (67%+18%, respectively) of the total colonies compared to 17% large colonies (10mm x 10mm and 7.5mm x 7.5mm, 5%+12%, respectively). In contrast, in the union group, the small sized colonies represented an average 62% of total colonies (47%+15%) compared to 38% large colonies (4%+34%). These data confirmed that colony sizes in nonunion

patient samples were skewed towards smaller colonies. Altogether, these data showed an increased number of smaller sized CFU-F colonies in the nonunion group. Based on these findings it was speculated that this may represent a hasty attempt by the body to heal itself by producing more mesenchymal progenitors in order to improve its healing power. This could happen however at the expense of producing fully formed colonies that are capable of normal proliferative capacity.

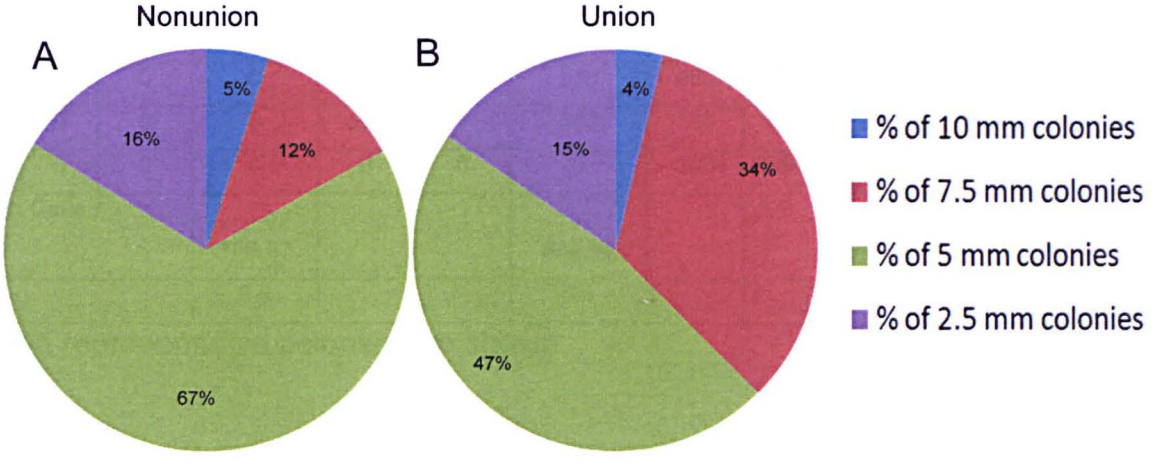

Figure 4.4: Pie charts showing distributions of CFU-F colonies according to size in nonunion and union groups
Total colonies were considered as 100% and different size colonies are shown as a percentage of total.

4.1.4 Evaluation of the number of cells per colony in nonunion and union groups

Colony size represents an indirect measure of progenitor cell proliferation and is determined not only by the number of cells forming each colony, but also by sizes of cells in the colonies and relative distances between the cells. The number of cells in 22 CFU-F colonies representing both patient groups was therefore enumerated and the results are shown in Table 4.2 and Table 4.3. Assuming each CFU-F colony originated from one MSC, population doublings (PDs) were worked out using the formula described in section 3.14.

Table 4.2: Enumeration of number of cells per colony in the nonunion group of patients

Sample ID	10mm x 10mm*		Average	7.5mm x 7.5mm*		Average	5mm x 5mm*		Average	2.5mm x 2.5mm*		Average
TR014	0	0	0	0	0	0	250	150	200	100	100	100
TR016	0	0	0	550	650	600	350	400	375	150	50	100
TR019	800	700	750	500	550	525	150	300	225	100	50	75
TR021	0	0	0	0	0	0	200	250	225	150	100	125
TR024	0	0	0	650	650	650	200	250	225	150	50	100
TR027	800	-	800	450	550	500	200	300	250	150	50	100
TR031	800	700	750	550	350	450	200	150	175	0	0	
TR032	0	0	0	450	600	525	250	350	300	50	50	50
TR038	0	0	0	600	550	575	400	300	350	100	50	75
TR039	0	0	0	550	-	550	150	150	150	100	-	100
TR053	0	0	0	0	0	0	200	250	225	50	50	50
Average Cells / Colony			766.67			546.88			245.45			87.5
PD			9.6			9.1			7.9			6.5

*two representative colonies are shown

Table 4.3: Enumeration of number of cells per colony in union group of patients

Sample ID	10mm x 10mm*		Average	7.5mm x 7.5mm*		Average	5mm x 5mm*		Average	2.5mm x 2.5mm*		Average
TR018	0	0	0	600	650	625	350	250	300	50	100	75
TR022	0	0	0	600	650	625	350	250	300	150	150	150
TR023	0	0	0	350		350	200	300	250	50	100	75
TR025	0	0	0	400	500	450	250	200	225	50	100	75
TR033	0	0	0	0	0	0	0	0	0	50	50	50
TR035	800	850	825	500	600	550	150	100	125	50		50
TR042	0	0	0	0	0	0	300	250	275	50	50	50
TR043	750	700	725	500	600	550	350	400	375	100	150	125
TR044	800	850	825	500	450	475	350	200	275	50	50	50
TR045	0	0	0	600	600	600	350	250	300	50	50	50
TR046	0	0	0	0	0	0	200	300	250	100	50	75
Average Cells / Colony			791.67			528.13			267.5			75
PD			9.6			9			8.1			6.2

* two representative colonies are shown

On analysis of the cell per colony data for the nonunion group of patients (Table 4.2), an average 10mm x10mm colony from those patients consisted of approximately 767 cells, equivalent to approximately 9.6 divisions/population doublings (PDs) of an original colony-forming cell. An average size of the 7.5mm x 7.5mm colonies was 547 cells, the respective values for the 5mm x 5mm, 2.5mm x 2.5mm colonies were 246 cells and 88 cells, the latter equivalent to 6.5 PDs.

Very similar findings were obtained from looking at the cell per colony data for the union group of patients (Table 4.3). An average 10mm x10mm colony from union patients consisted of approximately 792 cells, equivalent to approximately 9.6 PDs. A 7.5mm x 7.5mm, 5mm x 5mm and 2.5mm x 2.5mm colonies consisted of approximately 528, 268 and 75 cells, the latter representing 6.2 PDs.

In both nonunion and union samples, PDs were proportional to the colony size (Table 4.2 and Table 4.3). This demonstrated that the difference in colony sizes between the four different colony size groups was due to the higher number of cells per colony and not to due to an increase in the individual cell size. This additionally confirmed that progenitors forming the largest colonies were indeed the most rapidly growing, whereas those forming smaller colonies were growing much slower.

The combined PD data are shown on Figure 4.5. Altogether they show that the predominance of smaller colonies in the *nonunion group* was not because of the smaller size of cells forming those colonies or their more compacted distribution. Similarly, large colonies in the union group were not because of the larger size of cells forming colonies or higher distances between the cells within the colonies. In the opposite, these data showed that the colony size measurement technique used in this study was adequate to evaluate proliferative capacities of colony-forming progenitors. From this data, it can be concluded with a high degree of confidence that CFU-F progenitors in nonunion patients were in average less proliferative, compared to patients with united fractures.

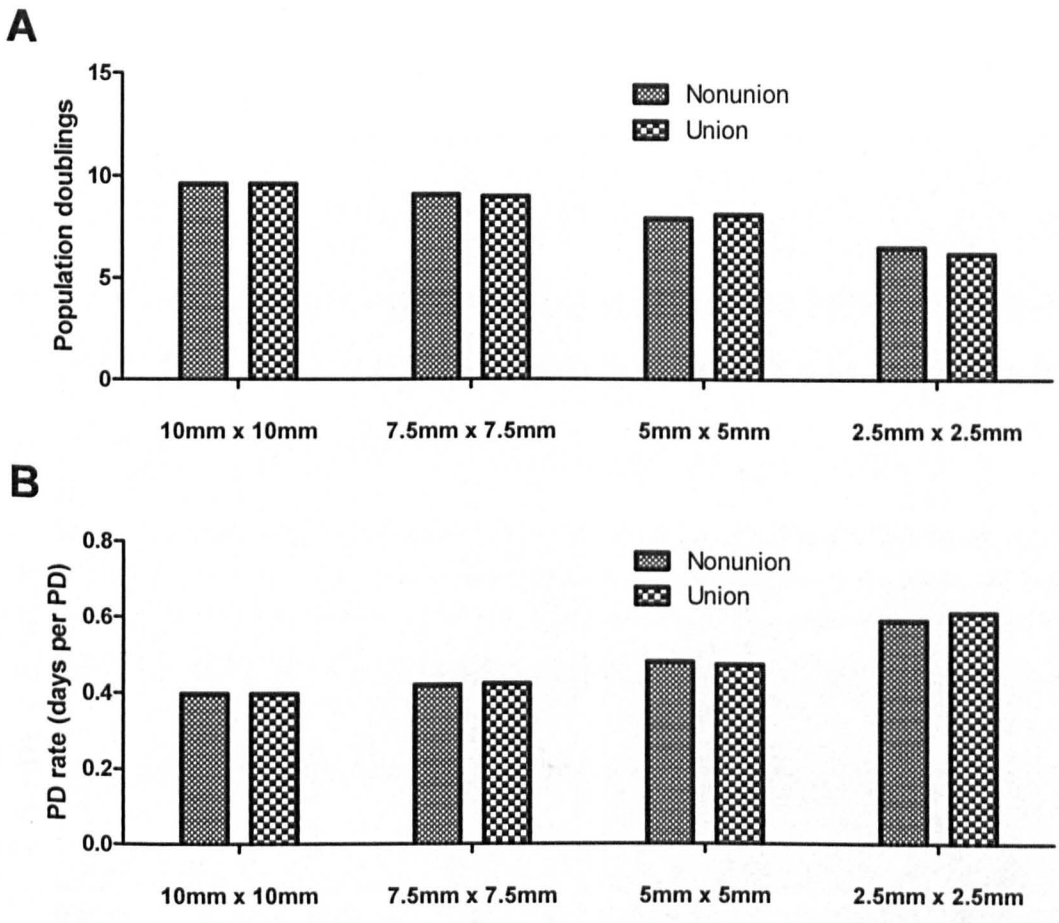

Figure 4.5: Evaluation of cumulative population doublings (A) and population doubling time (PDT) of colony forming cells in nonunion and union groups
Graphs show the same doubling capacity of cells forming small and large colonies in both nonunion and union groups indicating that grid system for measuring colony size was adequate for comparing CFU-F proliferative capacities in these two groups of patients.

All four colony categories from both nonunion and union groups were examined microscopically. There were no differences in colony morphology between nonunion and union in the corresponding groups. Representative microphotographs of colonies of nonunion and union groups are shown on Figure 4.6.

Overall, CFU-F data showed that the BM of nonunion patients was characterised by an increased number of less-proliferative CFU-F progenitors. This possibly represented a body's response to heal the fracture, but this response was not sufficient, since the produced CFU-F progenitors had proliferative capacity that was lower than normal.

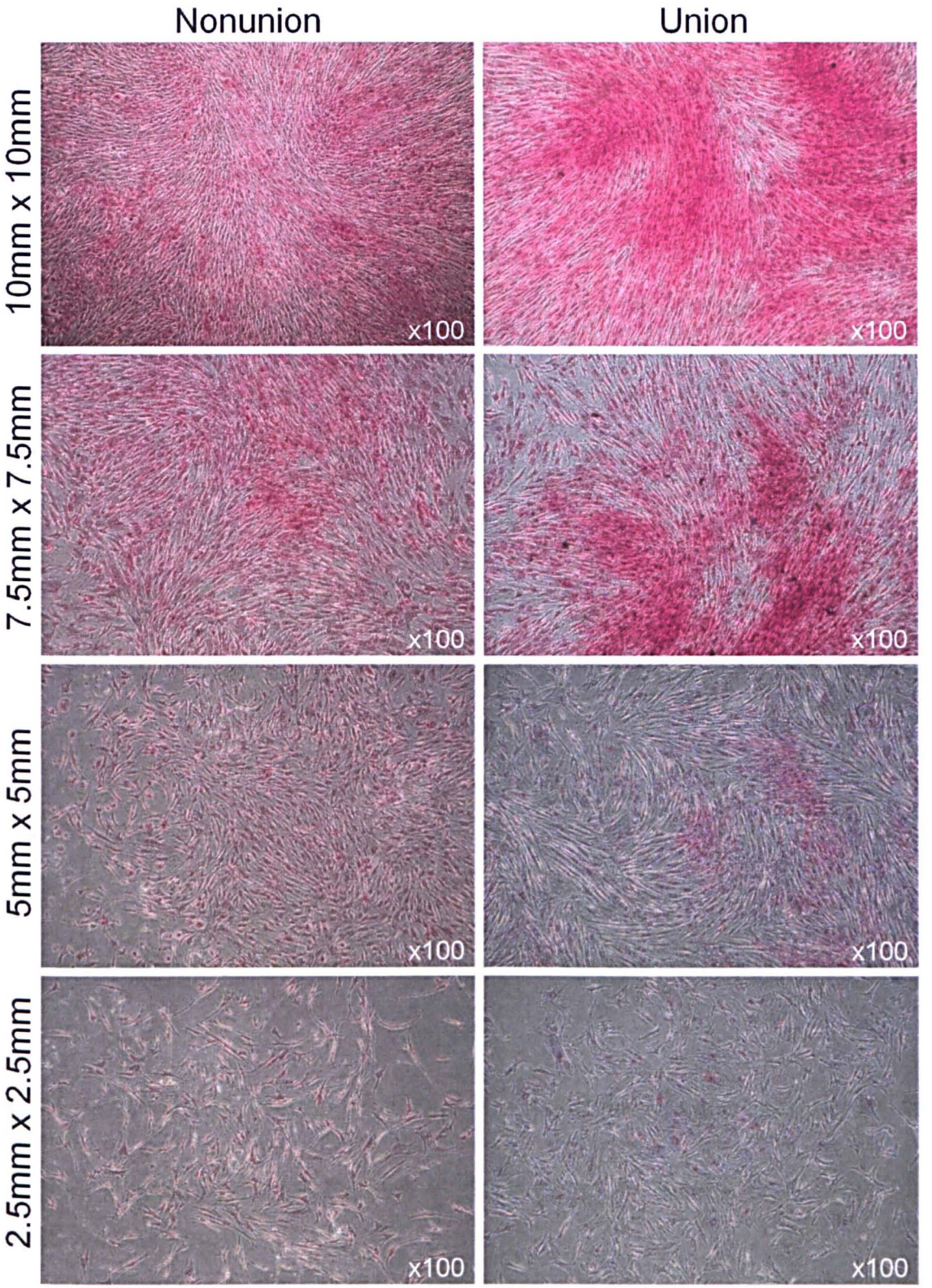

Figure 4.6: Microphotographs of colonies from nonunion and union groups

4.1.5 Evaluation of the sizes of CFU-O colonies in nonunion and union patients

Similar analysis of colony sizes was performed for CFU-O/osteoprogenitors. For this assay dishes were processed and colonies stained as described in section 3.11. Colonies were stained for ALP on day 14 and the results are shown in Figure 4.7.

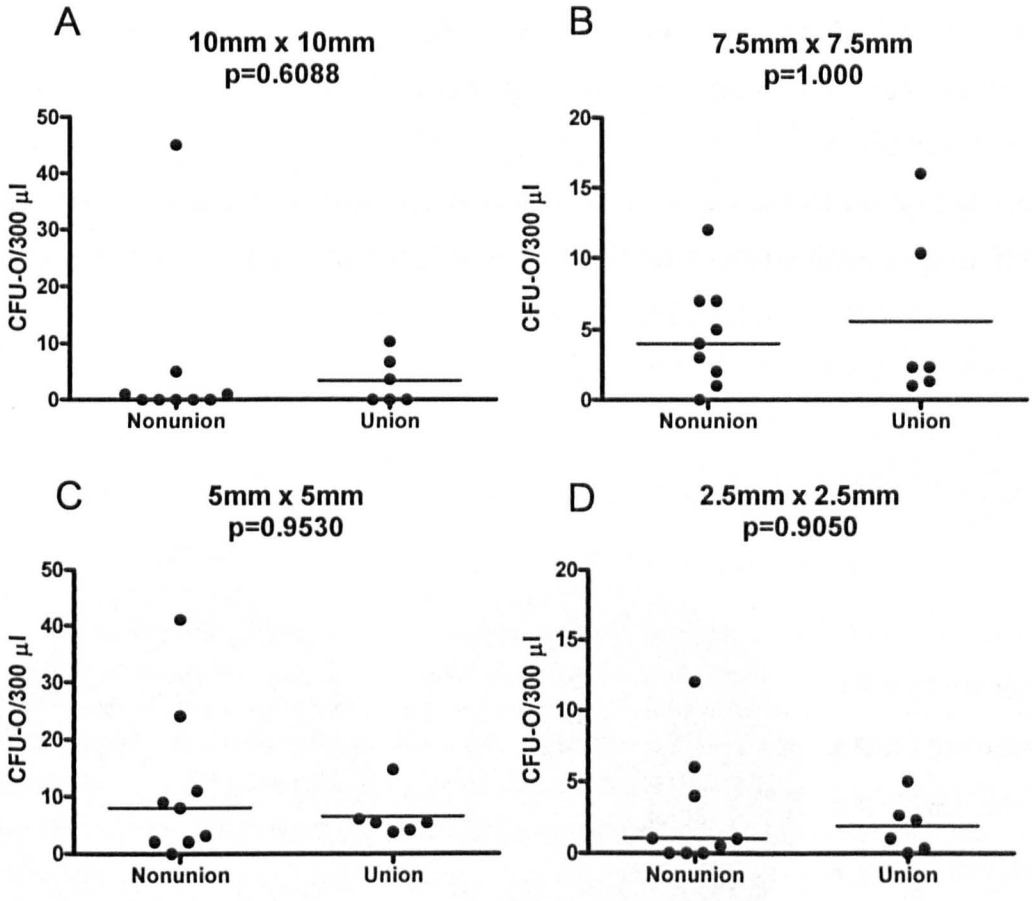

Figure 4.7: CFU-O colony numbers in nonunion group compared to unions

A: 10mm x 10mm group. B: 7.5mm x 7.5mm group. C: 5mm x 5mm group. D: 2.5mm x 2.5mm group.

As mentioned previously, no statistical differences between nonunion and union groups were found for the total number of CFU-O (Figure 4.1B). Using the grid system, the distribution of CFU-O colonies according to size in both groups was analysed. None of those results reached statistical significance, but a general trend of increased number of "bigger" CFU-O colonies in the union group was noted (Figure 4.7 and Figure 4.8). This shows that despite the increase in number of smaller CFU-F colonies in the nonunion group, they were not "osteogenic" (i.e. not CFU-Os). One can speculate that these small colonies have lost their osteogenic capacity or were pre-senescent. This can be the reason behind the nonunion happening in the first place, or it could be the result of the body's hasty response to nonunion. It is hard to immediately resolve these issues as a prospective study needs to be arranged whereby BM samples are collected from all fracture patients at the time of initial fixation and following up BM samples to be collected if they develop atrophic nonunion.

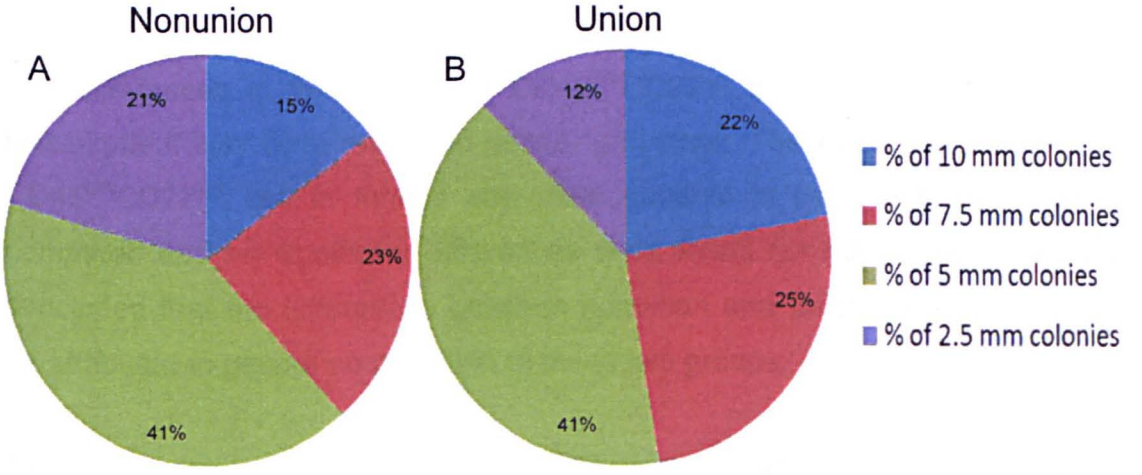

Figure 4.8: Pie chart showing the distribution of CFU-O colonies according to size in nonunion and union groups.

Altogether, these results showed that CFU-O colonies were distributed almost identically in both groups. Based on the significant increase in smaller CFU-F numbers in the nonunion groups, one would have assumed that these small

colonies were osteogenic. However, the result showed that they were not and that increase in total colony number in nonunion patients was not translated into an increase in osteogenic function.

4.1.6 Confirmation of the CFU-F colony data using flow cytometry

In these experiments BM aspirates from 7 patients suffering nonunion and 10 long bone fracture union patients (used as controls) were used. Flow cytometry was performed using frozen samples of MNCs according to protocol described in section 3.20.

The results for MSC enumeration based on $CD45^{low}CD271^{+}$ cell phenotype are shown on Figure 4.9. The frequency of these cells in the nonunion group was slightly higher than the union group (median values of 0.02% and 0.015%, respectively, p=0.8070). Although statistical significance was not reached using flow cytometry testing, these results support the CFU-F findings described earlier.

The differences in frequencies of $CD45^{low}CD271^{+}$ cells were next tested to investigate if they were related to gender groupings. Therefore, the numbers of $CD45^{low}CD271^{+}$ cell in female and male patients in the union group were compared and no significant differences were found (p=1.0). It was therefore concluded that the differences between nonunion and union groups could not be attributed to gender composition of these two groups.

The frequencies of $CD45^{low}CD271^{+}$ cells in BM MNC fraction could not be directly compared to the numbers of CFU-F/ml of aspirate, therefore the flow cytometry data was converted to derive the numbers of $CD45^{low}CD271^{+}$ cells/ml of marrow using the data on the numbers of MNCs obtained from 1ml of aspirate for all patients studied. MNC counts were performed after samples were subjected to Lymphoprep and before the samples were frozen. No significant differences in MNC counts and viabilities after de-frosting were observed between nonunion and union groups (p=1.000 and 0.1143,

respectively). The converted data for the numbers of $CD45^{low}CD271^{+}$ cells/ml of marrow are shown on Figure 4.10.

Statistical analysis revealed that the number of $CD45^{low}CD271^{+}$ cells/ml of marrow in the nonunion group was higher than in the union group (median values 1022 cells/ml and 309 cells/ml, respectively, p=0.7577). Although statistical significance was not reached, this result is consistent with CFU-F findings demonstrating relative increase of MSCs in nonunion patients compared to unions. Although flow cytometry-based enumeration of MSCs was broadly consistent with CFU-F data, it has lesser value since flow cytometry did not provide any information of the proliferative capacity of these MSCs. In contrast, this information is obtainable from CFU-F assays and can be discerned from measuring the sizes of individual colonies.

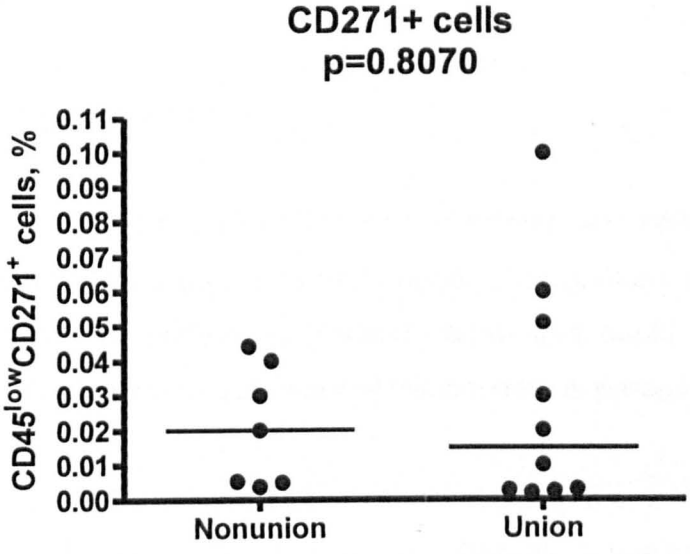

Figure 4.9: Enumeration of $CD45^{low}CD271^{+}$ cells in nonunion and union patients represented as a percentage of $CD45^{low}CD271^{+}$ cells in relation to MNCs

Despite higher average numbers in nonunion patients, statistical significance was not reached. Horizontal bars represent median.

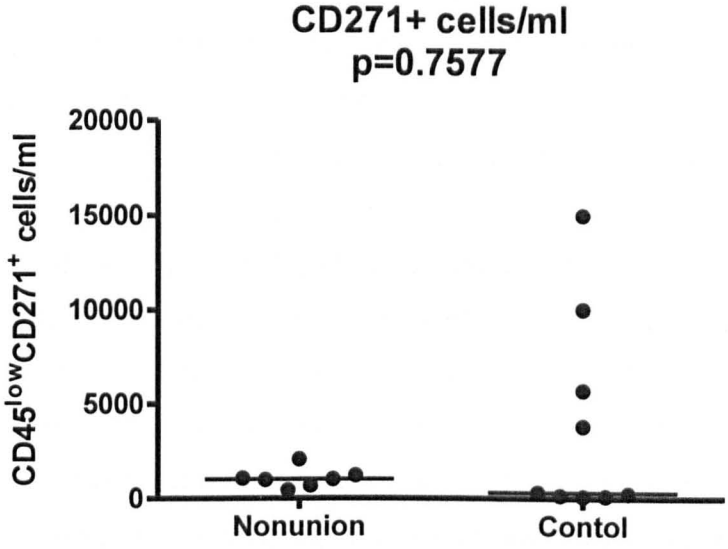

Figure 4.10: Enumeration of $CD45^{low}CD271^{+}$ cells in nonunion and union patients represented as the number of $CD45^{low}CD271^{+}$ cells per millilitre of BM aspirate
Despite higher average numbers in nonunion patients, statistical significance was not reached Horizontal bars represent median.

4.1.7 Clinical correlations

Clinical data relevant to the development of nonunion was collected for patients who participated in the aforementioned studies. Correlations were drawn in an attempt to identify an underlying "clinical" factor that could contribute to the development of nonunion. A summary of clinical data is presented in Table 4.4.

In this study, union was defined by clinical union parameters; the disappearance of pain and movement at the fracture site whereas nonunion was diagnosed when those symptoms persisted for more than 9 months post fracture fixation, together with radiographs showing lack of bridging callus in at least 3 cortices. Time to union was defined as the time between the definitive fracture fixation to the time of clinical union as documented in patents' follow up clinic notes.

For this study, a total of 22 patients were recruited, 11 suffered aseptic atrophic long bone nonunion (nonunion group) and 11 with previous long bone fractures, that were treated operatively and have united without delay (union group). The nonunion group was formed of 5 females and 6 males, age range from 19 to 63 years, mean age of 40.5 years and median age of 43 years and the union group was formed of 8 females and 3 males, age range from 18 to 56 years, mean age of 39.7 years and median age of 41 years. Eight patients from the nonunion group and 5 from the union group were smokers. The time to union in the union group ranged from 6 to 11 weeks, mean of 8.4 weeks and median 8 weeks. Five of the nonunion group patients failed to unite at the time of writing (maximum follow up was 7 months). For the remaining nonunion group patients, time to union ranged from 60 to 264 weeks, mean of 144 weeks and median 136 weeks. Injuries in the nonunion group were caused by road traffic accidents (RTA) in 5 patients, sports injuries in 2 patients, pedestrian accident in 2 patients, motorbike accidents in 1 patient and fall in 1 patient. Injuries in the union group were due to RTA in 3 patients, sports injuries in 1 patient, pedestrian accident in 2 patients, work related injury in 1 patient and fall in 3 patients. None of those patients were on corticosteroid or NSAID treatment; all but three were non alcohol drinkers.

Table 4.4: Clinical demographics of patients participating in the nonunion vs union study.

Sample ID	Gender	Age	Study group	Smoking	Time to union (weeks)	Causative Injury	(Type)& Bone fractured
TR014	male	31	Nonunion	Yes	264	RTA	(Closed) Femur
TR016	male	19	Nonunion	Yes	60	RTA	(Closed) Femur
TR019	female	50	Nonunion	Yes	168	pedestrian	(open GIIIa) Tibia
TR021	male	22	Nonunion	Yes	N/A	RTA	(open GII) Femur
TR024	female	43	Nonunion	Yes	116	Sports	(open GI) Tibia
TR027	male	33	Nonunion	No	100	pedestrian	(Closed) Femur
TR031	male	62	Nonunion	Yes	156	Fall	(Closed) Femur
TR032	male	63	Nonunion	Yes	N/A	RTA	(Closed) Tibia
TR038	female	58	Nonunion	Yes	N/A	RTA	(Closed) Tibia
TR039	female	44	Nonunion	No	N/A	motorbike	(open GII) Tibia
TR053	female	21	Nonunion	No	N/A	Sports	(Closed) Femur
TR018	female	44	Union	Yes	7	RTA	(Closed) Tibia
TR022	female	46	Union	No	8	Fall	(Closed) Tibia
TR023	female	41	Union	No	10	RTA	(Closed) Femur
TR025	male	33	Union	Yes	7	RTA	(Closed) Femur
TR033	female	39	Union	No	8	pedestrian	(open GII) Tibia
TR035	female	42	Union	Yes	9	motorbike	(open GIIIa) Tibia
TR042	female	46	Union	No	8	Fall	(Closed) Femur
TR043	male	18	Union	Yes	6	Work	(Closed) Tibia
TR044	female	56	Union	No	8	Sports	(Closed) Tibia
TR045	male	41	Union	Yes	10	Fall	(Closed) Femur
TR046	female	31	Union	No	11	Pedestrian	(Closed) Femur

N/A - data not available as fracture was not united at the time of writing

The effect of cigarette smoking on the total number and sizes of CFU-F colonies in both nonunion and union groups was studied (Figure 4.11). There was a trend for an increase in the CFU-F numbers in the smokers when compared to the non-smokers in both nonunion and union groups. There was a statistically significant increase in number of the smallest (2.5mm x 2.5mm) colonies in the union group amongst the smokers ($p=0.0219$) and a similar trend was noted in the nonunion group. Due to the small sample size, the remaining results failed to reach statistical significance.

Furthermore, correlations were drawn between the time to union, total CFU-F colonies and colony sizes in the union group. The total CFU-F count and the number of 10mm x 10mm colonies seemed to be inversely proportional to the duration of fracture healing. In other words, the more CFU-F colonies (in particular the large sized 10mm x 10mm ones), the more likely it was for the fracture to heal in a shorter period of time. The opposite was noticed with the 5mm x 5mm colonies. The more of those colonies present, the longer it took for the fractures to heal. Due to the small sample size, no statistical significance was reached, and because this work has not been reported before in the literature, a power calculation was not possible prior commencing this study.

Upon looking back on all the results presented so far in this study, one can notice certain trends and observations. In general, nonunion patients have more of the small sized CFU-F colonies, which may be less proliferative and osteogenic, whereas the union patients have fewer total CFU-F numbers, but more of the larger, more proliferative ones. Similar findings were noticed in patients who smoked. They had more total colonies and a significant increase of the small, 2.5mm x 2.5mm colonies in the union group. When the observation that the time to union was found to be longer with patients who had more of the small sized colonies and was found shortened with those who had more of the large sized ones, more questions maybe asked. How effective are the small sized colonies and how much do they contribute to the fracture healing process? One may even argue that the presence of the small sized colonies pose a threat to union. May these observations be used as a predictor for nonunion in the future to identify the fracture at highest risk for developing

nonunion and hence justify close patient follow up and monitoring and perhaps early surgical intervention if complications were to develop? These questions will be addressed in future studies.

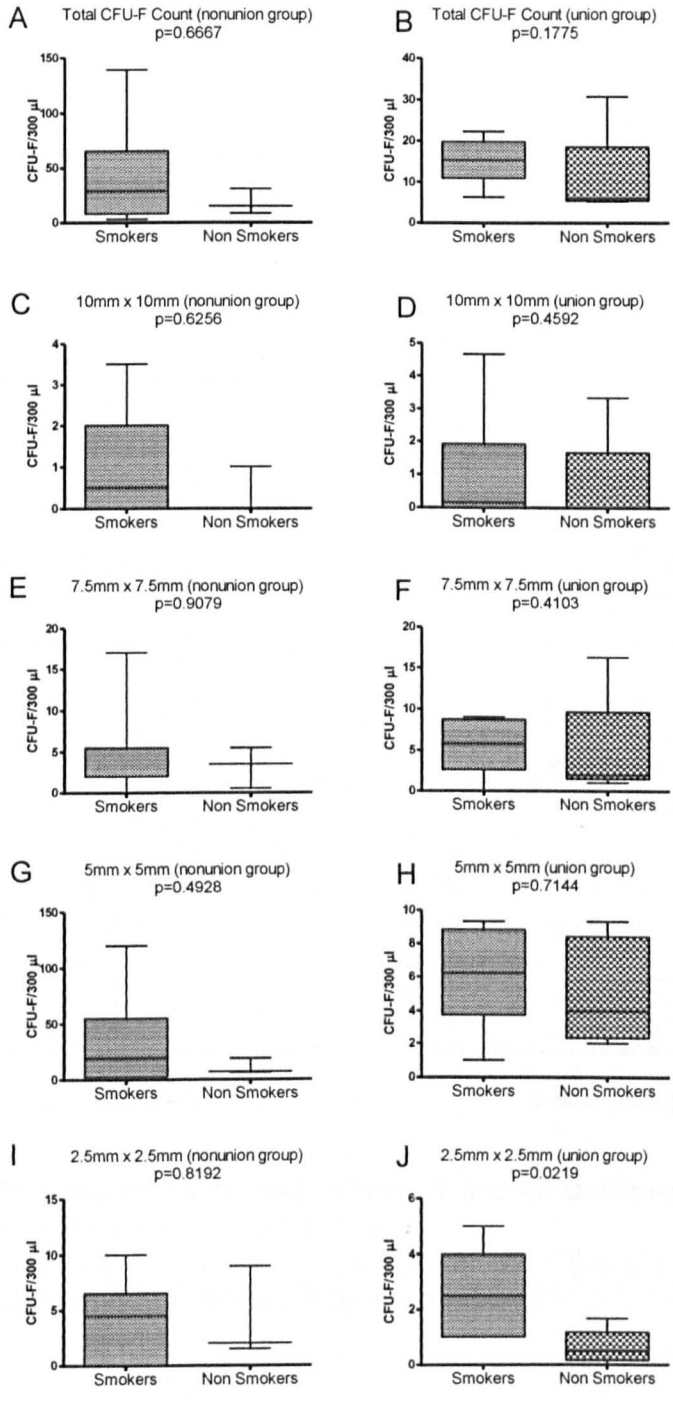

Figure 4.11: Boxplots showing the effect of smoking on CFU-Fs in nonunion and union groups

Graphs on the left are representative of the nonunion group whereas those on the right represent union group. A, B: total CFU-F counts, C, D: 10mm x 10mm group, E, F: 7.5mm x 7.5mm group, G, H: 5mm x 5mm group, I,J : 2.5mm x 2.5mm group.

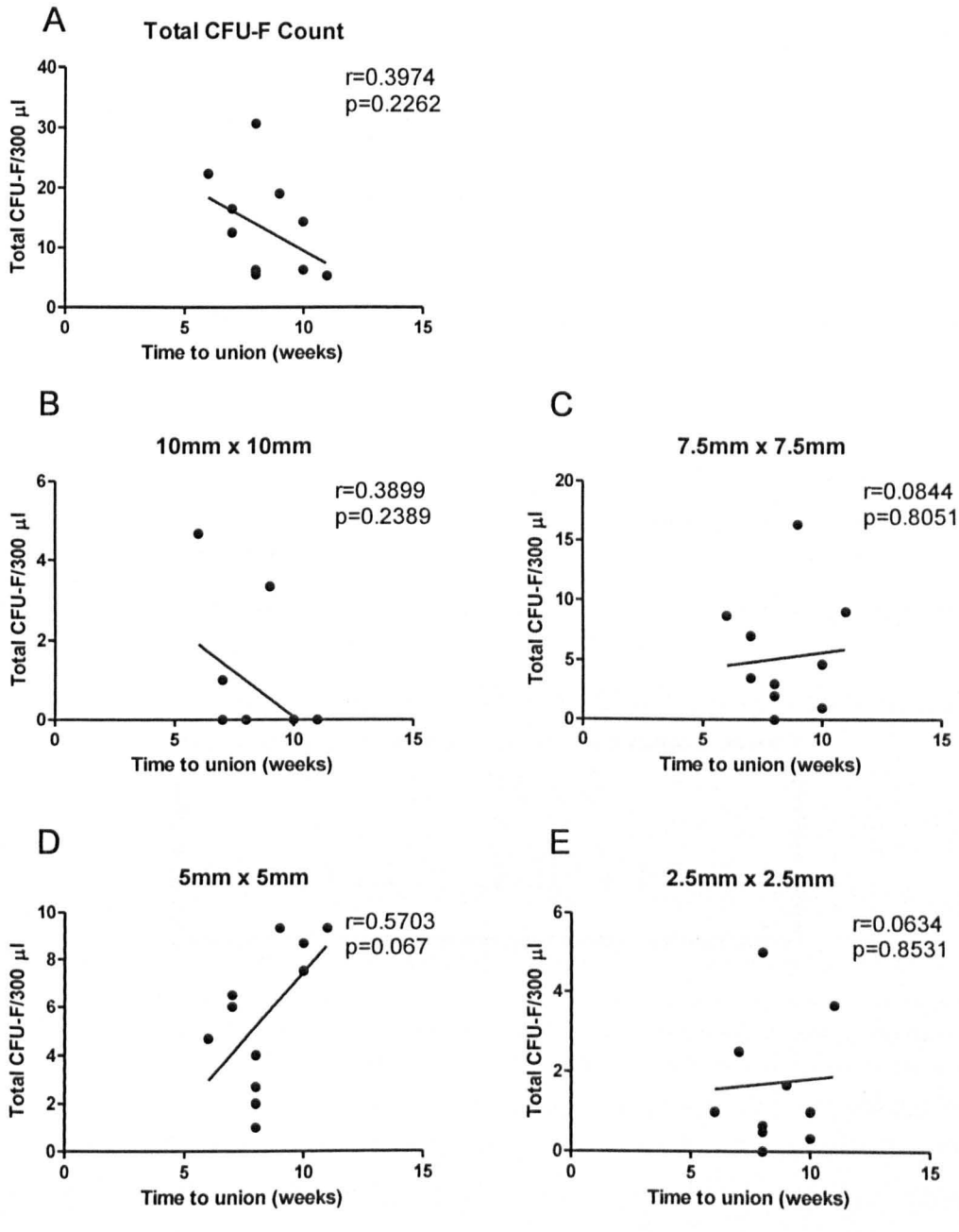

Figure 4.12: Graphs showing the effect of the CFU-F numbers and sizes on the time to union in the union group

A: Total CFU-F count, B: 10mm x 10mm group, C: 7.5mm x 7.5mm group, D: 5mm x 5mm group and E: 2.5mm x 2.5mm group.

CHAPTER 5
RESULTS OF BMP-7 STUDY

5.1 EFFECT OF BMP-7 ON BM MSCS

5.1.1 Validation of osteogenesis assays

Osteogenesis assay was performed in duplicate (duplicate dishes) and calcium assay was performed in triplicate (triplicate wells). Variability between replicates was first assessed to investigate the accuracy of these assays to measure the expected effects.

5.1.2 Calcium assay performance

A standard curve built using Ca^{++} standards from Sigma showed good linearity and consistency between the different experiments. An example of 6 experiments (3 from the nonunion group and 3 from the union group) is shown in Table 5.1.

Table 5.1: Variability between Ca^{++} assay triplicates
Optical density (OD) readings for a random group of experiments, standard deviation (SD) and coefficients of variation (CV) for triplicate wells.

OD 1	OD 2	OD 3	Mean OD	(SD)	(CV)
0.235	0.244	0.24	0.24	0.0045	1.88
0.229	0.228	0.229	0.23	0.0006	0.25
0.258	0.266	0.261	0.26	0.0040	1.55
0.246	0.239	0.247	0.24	0.0044	1.79
0.238	0.24	0.24	0.24	0.0012	0.48
0.24	0.25	0.24	0.24	0.0046	1.90

The results show a very good statistical intra-assay performance which reflects the high degree of pipetting accuracy achieved between Ca^{++} assay triplicates. The CV value ranged from 0.3% to 1.9% with an average of 1.3%, which was significantly below the accepted level of 5%.

5.1.3 Osteogenic assay performance

Osteogenic assay is a 14-day *in vitro* functional assay used as a measure of osteogenesis. The precision of the results depends on the number of replicates set-up for each dose of BMP-7 treatment (duplicate dishes were used). Performing the assay in duplicate dishes produced variable CVs ranging between 1% to 37% and an average CV of 9% (Table 5.2).

Table 5.2: Intra-donor variability between duplicate osteogenic assay dishes for a random group of experiments
Calcium production in duplicate dishes (µg/ml), SD and CV between duplicate dishes.

Calcium (µg/ml) Dish 1	Calcium (µg/ml) Dish 2	Mean	SD	CV
8.22	7.98	8.10	0.17	2.10
8.46	8.34	8.40	0.08	1.01
11.74	13.44	12.59	1.20	9.55
26.01	24.28	25.15	1.22	4.86
12.07	20.52	16.30	5.98	36.67
9.39	9.13	9.26	0.18	1.99

The average CV remained below the acceptable level of variability for this assay (10%, based on previous experience from the laboratory were this work was carried out). Variability could have been further reduced by performing osteogenic assay in triplicates; however this would have required higher volumes of samples and higher cost of osteogenic medium / BMP-7.

5.1.4 Effect of BMP-7 on calcium production by expanded BM MSCs from union patients

Previously-published studies investigated the effect of BMP-7 on culture-expanded BM MSCs and observed enhanced osteogenesis following the addition of BMP-7 [107]. To validate these findings in this experimental system, p0 culture-expanded MSCs from union patients were first treated with 100ng/ml BMP-7 or 300ng/ml BMP-7 and colorimetric calcium assays were used as a measure of osteogenic differentiation. Since it was not possible to use healthy patients as controls in this study, patients previously suffering long bone fractures that have united without healing complications and were re-operated for hardware removal were recruited as controls. During their surgeries, posterior iliac crest BM was aspirated as mentioned earlier. Cells from 4 donors were grown in NH media until confluent (p0), and the resulting expanded MSCs were then placed in osteogenic media where they were treated with no BMP-7, 100ng/ml BMP-7 or 300ng/ml BMP-7. Results for individual donors are shown in Figure 5.1 and for all 4 donors combined on Figure 5.2.

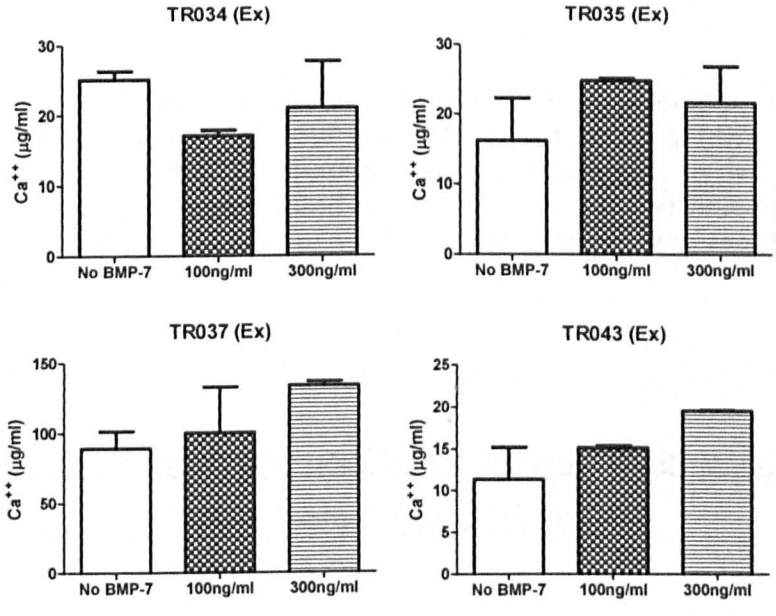

Figure 5.1: The effect of BMP-7 on calcium production by expanded MSCs in the union group (4 donors shown separately).
Error bars represent osteogenic assay duplicates. *Ex – experiments performed with expanded MSCs.

The result showed that BMP-7 had no effect on culture expanded MSCs from union patients with the addition of either 100ng/ml or 300ng/ml (Figure 5.1). Average results for all the 4 donors, depicted on Figure 5.2 highlight high donor-to-donor variability in calcium production by expanded MSCs at baseline and with the addition of BMP-7.

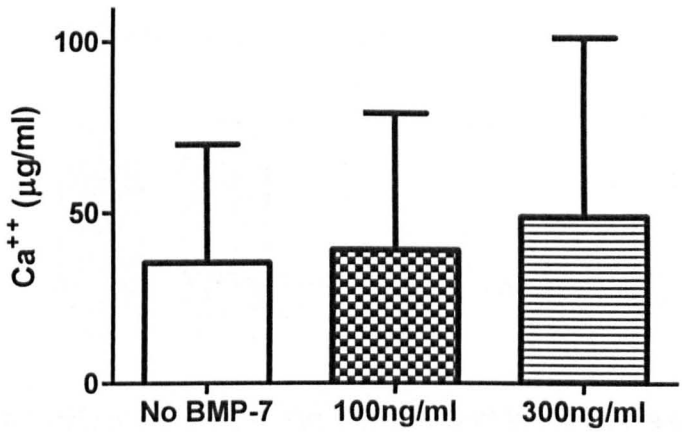

Figure 5.2: The effect of BMP-7 on calcium production from culture-expanded BM MSCs from all four patients in the union group
There was a trend showing increased calcium production by MSCs in a dose dependant manner. Results tested using Wilcoxon ranked non-parametric test showed no statistical significance. Error bars represent variation between donors (SD).

5.1.5 Effect of BMP-7 on proliferation and colony formation by expanded BM MSCs from union patients

These experiments were set-up as described in section 3.12 and total colony numbers and sizes were evaluated (Figure 5.3 and Figure 5.4). Similar to calcium assay results, CFU-F results showed no effect with the addition of either 100ng/ml or 300ng/ml.

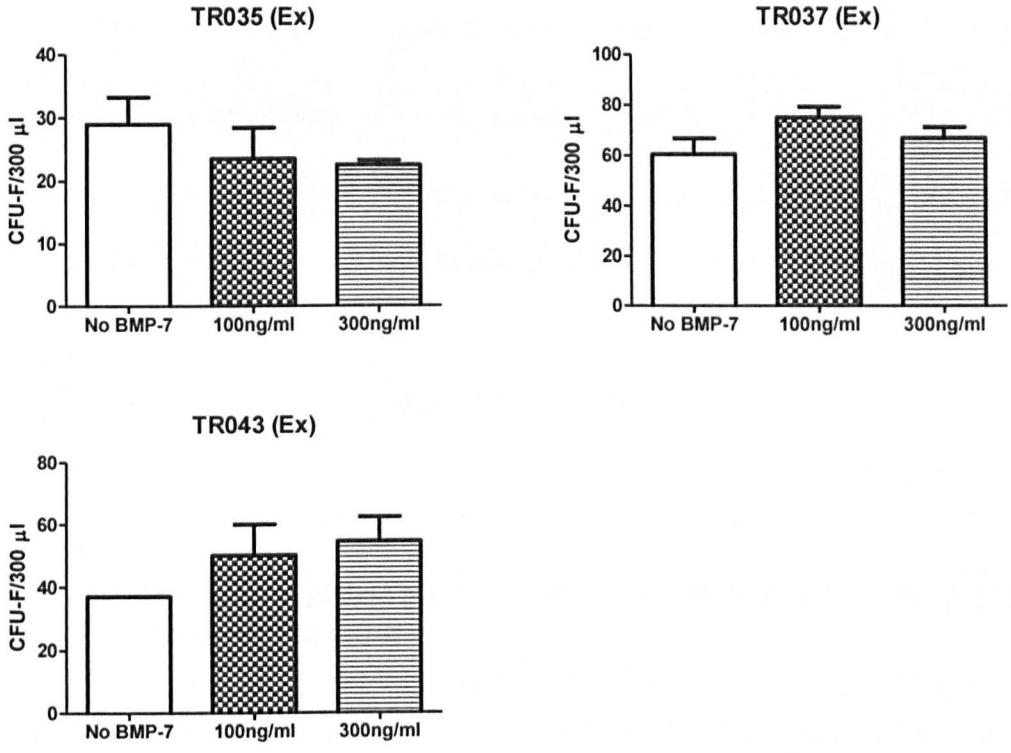

Figure 5.3: The effect of BMP-7 on colony formation by expanded MSCs in the union group (3 donors shown separately)
Error bars represent CFU-F assay duplicates.

The effects of BMP-7 on CFU-F colony sizes were also evaluated and results are presented in Figure 5.4. As mentioned previously, the sizes of colonies reflect the proliferative capacity of colony-forming MSCs.

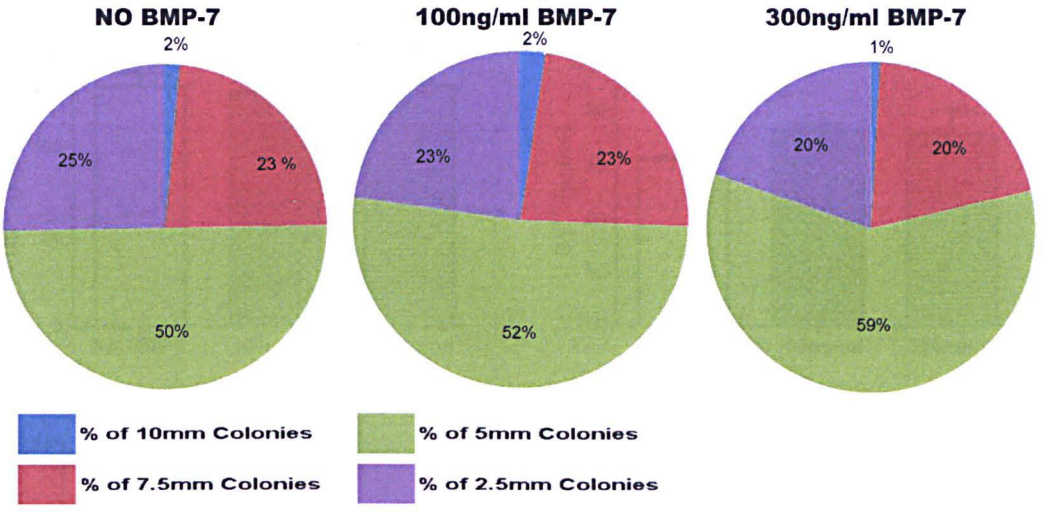

Figure 5.4: Pie chart showing the effect of BMP-7 on colony sizes by culture-expanded MSCs from union patients

Overall, these data showed no effect of addition of either 100ng/ml or 300ng/ml BMP-7 on the proliferation and colony formation by expanded MSCs from union patients.

5.1.6 Effect of BMP-7 on calcium production by expanded BM MSCs from nonunion patients

Similar to union patients, culture-expanded MSCs from nonunion patients were used to assess the effects of BMP-7 on calcium production. Calcium production by MSCs from 3 different donors is shown on Figure 5.5 and for all 3 donors combined is shown on Figure 5.6.

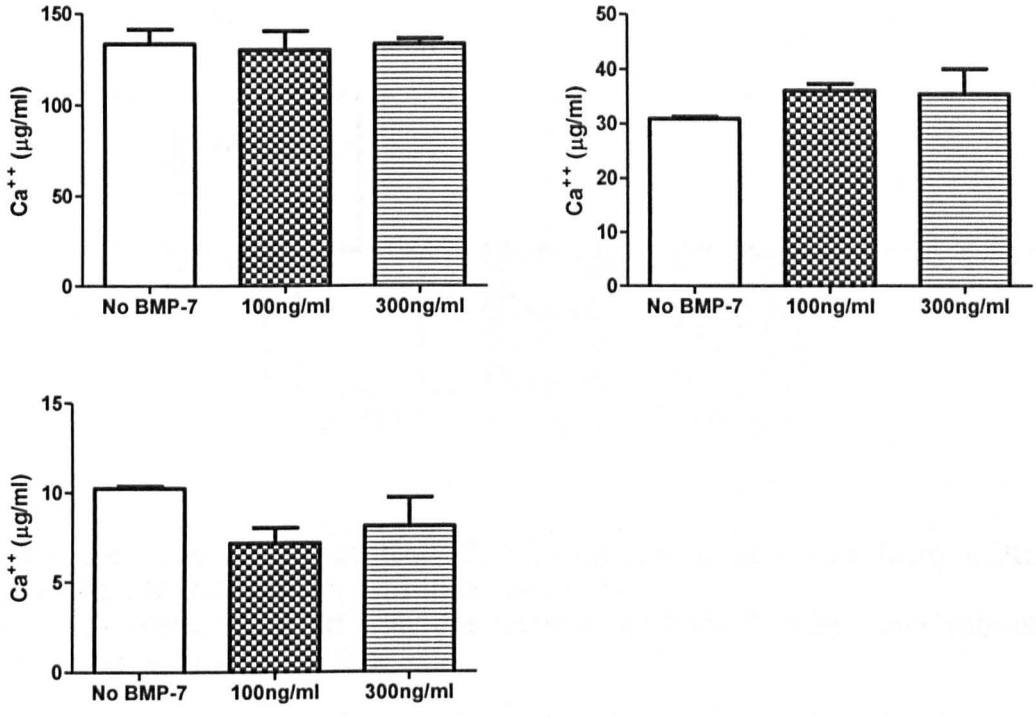

Figure 5.5: The effect of BMP-7 on calcium production by expanded MSCs in the nonunion group (3 donors shown separately)
Error bars represent osteogenic assay duplicates.

The results show that expanded MSCs from nonunion patients failed to respond to BMP-7 at all.

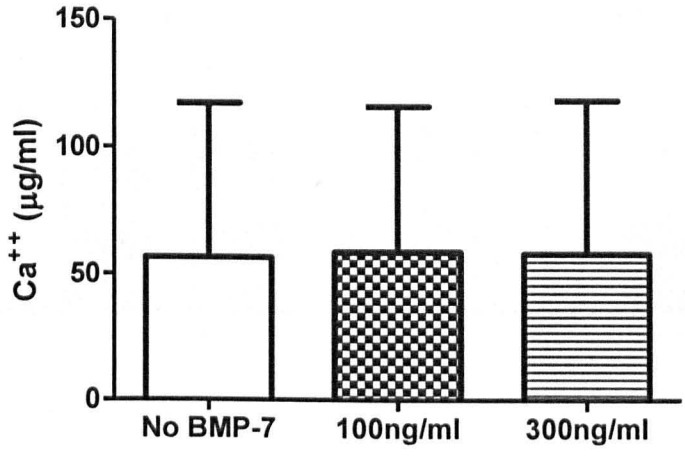

Figure 5.6: The effect of BMP-7 on calcium production from culture-expanded BM MSCs from nonunion patients
No effects were observed with the addition of BMP-7. Error bars represent variation between donors (SD).

5.1.7 Effect of BMP-7 on the proliferation and colony formation by expanded BM MSCs from nonunion patients

As for all similar experiments described in this section, colony formation was assessed in several duplicate dishes and colony sizes were evaluated using scoring grids. Colony number and sizes are presented in Figure 5.7.

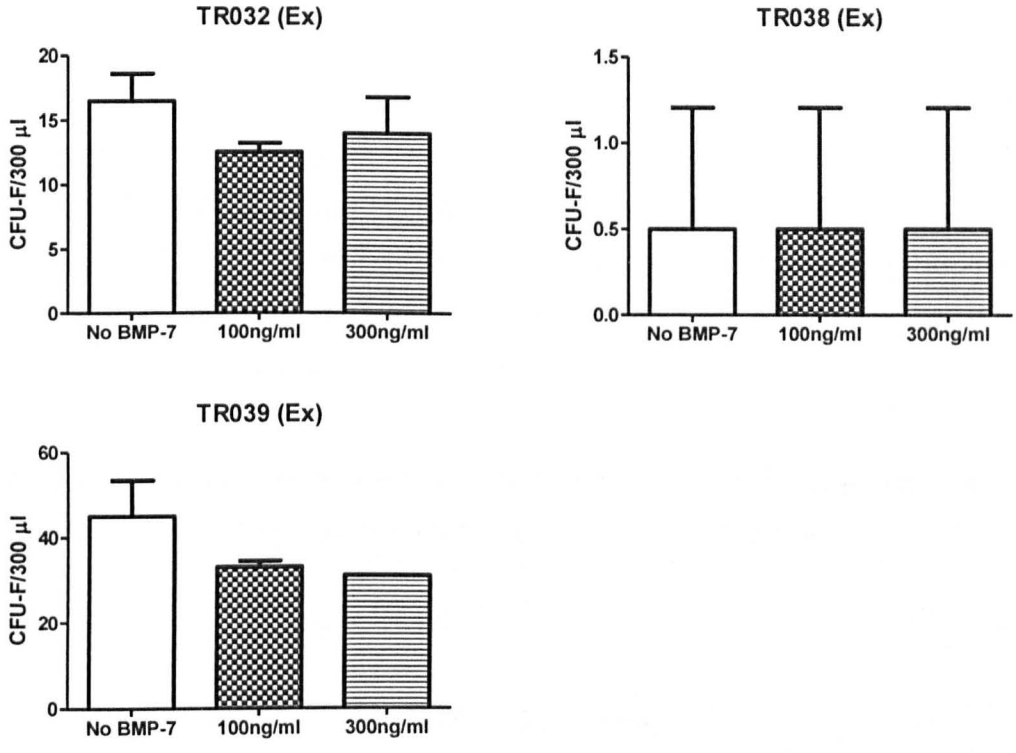

Figure 5.7: The effect of BMP-7 on colony formation by expanded MSCs in the nonunion group (3 donors shown separately)
Error bars represent CFU-F assay duplicates.

The results show that expanded MSCs from nonunion donors failed to respond to BMP-7. This was consistent with calcium data showing that MSCs cultured from BM of nonunion patients were unresponsive to BMP-7.

5.1.8 Effect of BMP-7 on calcium production from fresh uncultured BM MSCs from nonunion patients

Although most investigations are still performed using MSCs expanded in culture [107], it is well-known that culture-adaptation affects MSC characteristics and leads to gradual senescence [164]. Additionally, BMP-7 is acting on native MSCs when used clinically during surgery [152-154]. Based on these considerations and to reflect clinical situations more closely, next set of

experiments was designed to explore the effect of BMP-7 on fresh, uncultured MSCs from nonunion patients. In these experiments, 300µl of fresh BM from long bone fracture nonunion patients (N=6) were seeded in 10ml of DMEM/2% FCS media and then transferred to osteogenic media containing, no BMP-7, 100ng/ml BMP-7 or 300ng/ml BMP-7. End-point assays (calcium assay and CFU-F assay) were performed as described above. Calcium production by MSCs from 6 different nonunion patients is shown on Figure 5.8 and for all 6 donors combined in shown on Figure 5.9.

The results showed no trends for an increased calcium production by fresh MSCs from nonunion patients with the addition of BMP-7 (Figure 5.8).

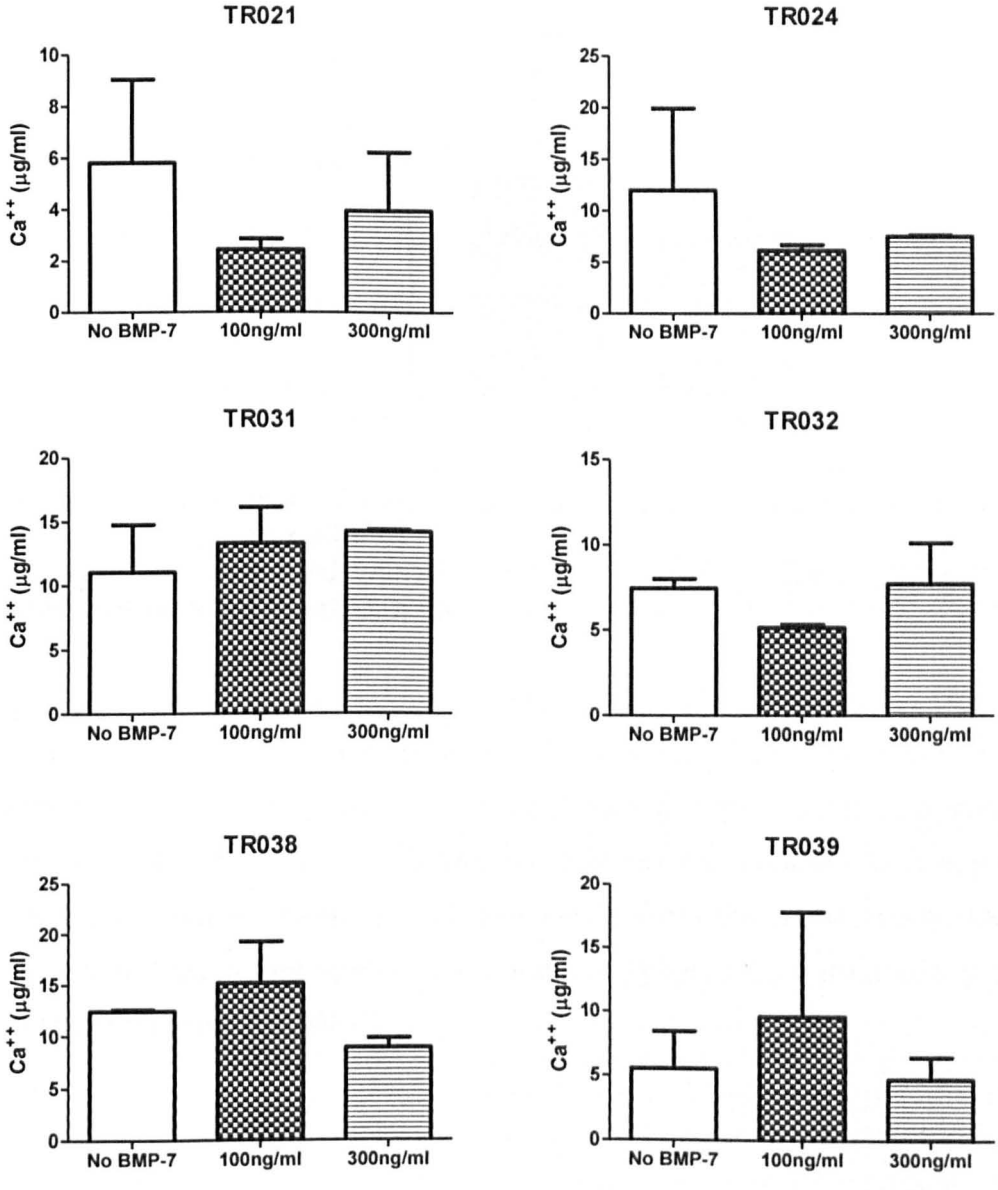

Figure 5.8: The effect of BMP-7 on calcium production by fresh MSCs from 6 nonunion patients
Error bars represent osteogenic assay duplicates.

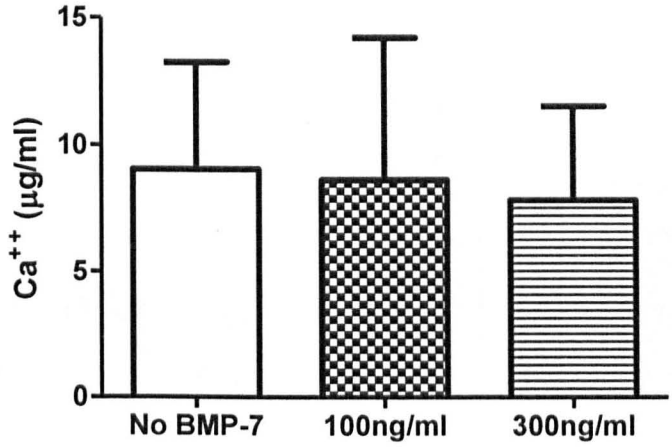

Figure 5.9: The effect of BMP-7 on calcium production from fresh BM MSCs from nonunion patients
No effects were observed with the addition of BMP-7. Error bars represent variation between donors (SD, n=6).

The mean calcium production for all 6 donors depicted on Figure 5.9 show no up-regulation in calcium production by fresh MSCs from nonunion patients with the addition of BMP-7. This is consistent with results obtained from expanded MSCs from nonunion patients and with MSCs from the union group. Overall, these data indicated that MSCs from nonunion patients were unable to produce calcium in response to BMP-7.

5.1.9 Effect of BMP-7 on the proliferation and colony formation by fresh non expanded BM MSCs from nonunion patients

In these experiments, 300µl of fresh BM from long bone fracture nonunion patients were seeded in NH media containing BMP-7. On day 14, colonies were counted and their sizes measured as described earlier. The results for individual donors are shown Figure 5.10.

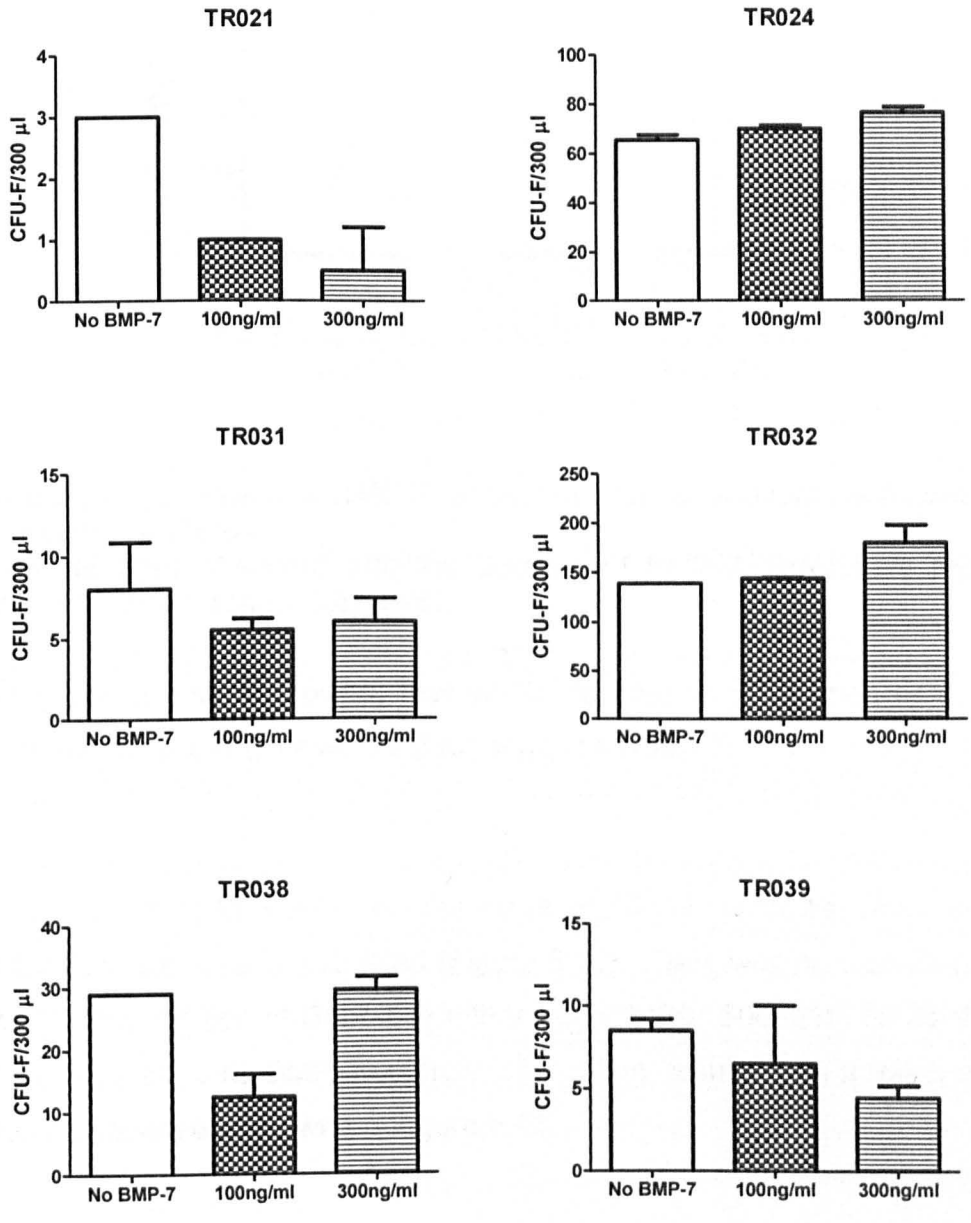

Figure 5.10:The effect of BMP-7 on colony formation by fresh, non-expanded MSCs in the nonunion group (6 donors shown separately)
Error bars represent CFU-F assay duplicates.

The results show that the addition of BMP-7 has not affected colony formation in all donors.

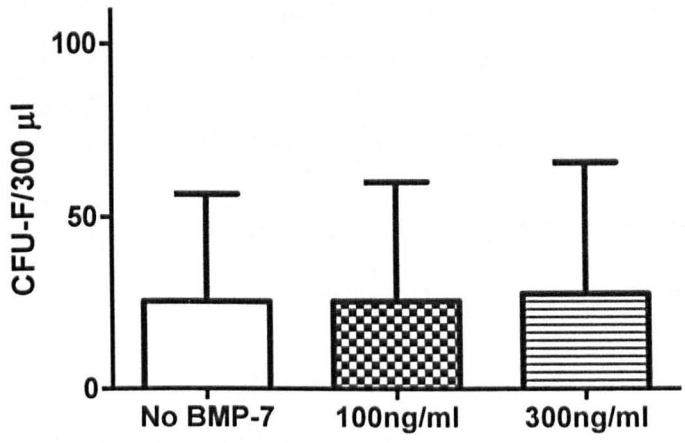

Figure 5.11: The effect of BMP-7 on colony formation from fresh BM MSCs of nonunion patients
No effects were observed with the addition of BMP-7. Error bars represent variation between donors (SD, n=6).

Overall, these results showed, that similar to calcium production data, MSCs from nonunion patients have failed to respond to BMP-7.

Finally, the effect of BMP-7 on the sizes of CFU-F colonies, reflecting their proliferative rate, was investigated (Figure 5.12). There was no significant effect noted by the addition of BMP-7 in either 100ng/ml or 300ng/ml concentrations when compared to no BMP-7 controls. The colony results from a representative nonunion patient are shown on Figure 5.13.

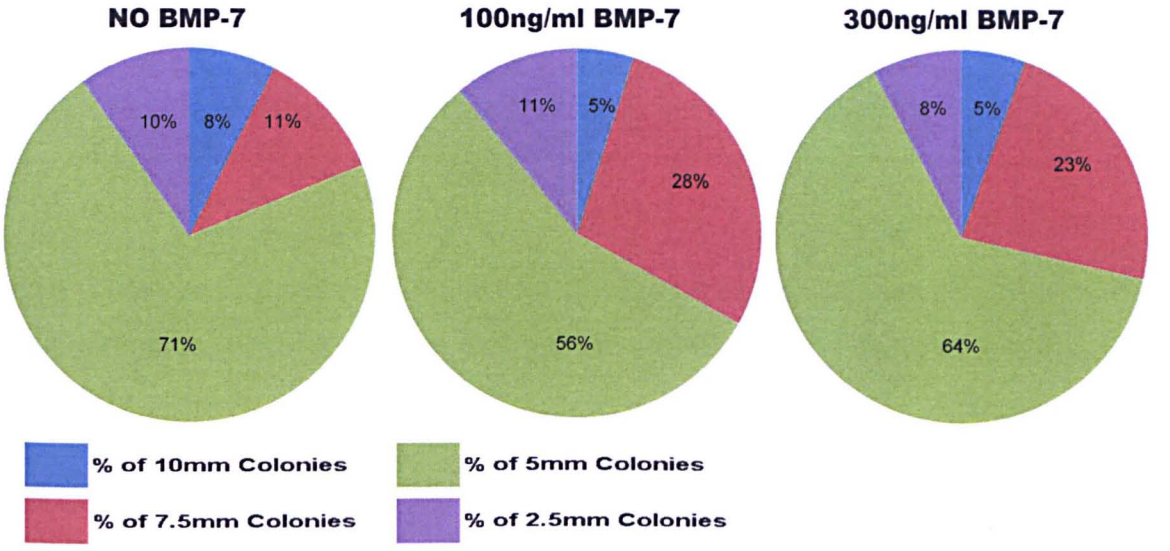

Figure 5.12: Pie chart showing the distribution of MSC colonies according to size by fresh, non-expanded MSCs from nonunion patients

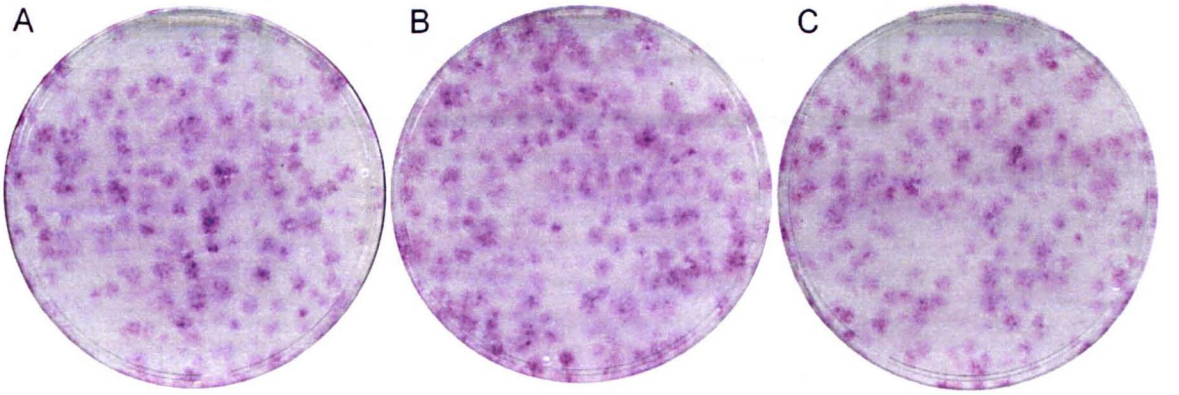

Figure 5.13: Scanned dishes of CFU-F from a representative nonunion patient showing the effect of addition of BMP-7

A: No BMP-7, B: 100ng/ml BMP-7, C: 300ng/ml BMP-7. No significant change was noted between all three dishes illustrating that the addition of BMP-7 does not affect the number of CFU-F colonies from nonunion patients.

CHAPTER 6

RESULTS OF MSC CIRCULATION STUDY

6.1 STUDIES TO EXPLORE THE CIRCULATION OF MSCS IN THE BLOOD IN HEALTH, ORTHOPAEDIC PROCEDURES AND EARLY RA

The presence or absence of circulating MSCs in various orthopaedic conditions, in early rheumatoid arthritis patients not on disease modifying treatment and in healthy controls were studied and results are shown in this chapter. The orthopaedic operating theatre was a unique environment where skeletal manipulation took place and thus provided a ready clinical model to assess whether MSCs circulation may be a biophysical process.

6.1.1 Group I

Group I, was comprised of 12 patients suffering long bone fracture nonunion and undergoing reaming of the sound femur with RIA for bone graft harvesting. Out of the 12 patients, 8 showed evidence of circulating MSC colonies in their lower limb venous blood (66.67%) and in only 2 out of the 12 patients (16.67%) circulating MSCs were identified in their upper limb venous blood.

In all 12 patients, a total of 216.9 x 10^6 MNCs, from an original 120ml of lower limb venous blood collected were seeded and a total of 20 MSCs were identified. An average of one MSC colony was present in every 10.8 x 10^6 MNCs seeded. Volumetrically, total of 120ml of lower limb venous blood was collected from all the patients and only 20 MSC colonies were identified (one MSC colony per 6ml of blood). The number of MSCs found in the lower limb venous blood per MNC seeded for individual participating patients is shown in Table 6.1.

A total of 179.7 x 10^6 MNCs, from an original volume of 120ml of blood, from the upper limb venous circulation from all patients were seeded and only 2 MSC colonies were identified. So, one MSC colony per 89.85 x 10^6 MNCs was found (one MSC per 60ml of blood i.e. 10 fold less than in LL). It is worth mentioning

that those 2 patients have shown evidence of circulating MSCs in their lower limb venous blood at a higher concentration than that in the upper limb venous blood, matched images from one patient are shown in Figure 6.1. This finding supports the evidence that MSCs are filtered in the lungs as they pass from the lower limb into the upper limb venous system. The amount of MNCs and MSCs in the upper limb venous circulation (identified per patient) are summarized in Table 6.1.

Odds ratio statistical testing was carried out comparing the number of samples positive for MSCs in LL compared to UL upon "exposure" to reaming with RIA. Assuming sample independence (justified by the extreme rarity of MSCs in the blood), odds ratio of 10 was found, with a 95% confidence interval and a p value of 0.036. One can conclude that there is an increased chance of finding MSCs in the lower limb venous blood when compared to the upper limb venous blood following reaming with RIA.

It can be deduced that MSCs do circulate in both lower limb and upper limb venous systems of nonunion patients at very low concentration after reaming with RIA. They are present at an even lower concentration in the upper limb venous system as they are possibly filtered in the lung and subsequently filtered in the tissue capillary beds. This can raise the question of how feasible would it be to introduce MSC cellular therapy for intravenous injection. The total number of MSCs in the upper limb venous blood as compared to the lower limb venous blood in each of the 12 patients of this study is shown in Figure 6.2.

Table 6.1: Total number of MNCs seeded and MSC colonies observed in LL and PB of group I patients.

Sample ID	MNCs seeded from LL blood, in 10^6	Total MSC Colony Numbers observed in LL	Frequency in LL, 1 cell per MNC, in 10^6	MNCs seeded from UL blood, in 10^6	Total MSC Colony Numbers observed in UL	Frequency in UL, 1 cell per MNC, in 10^6
TR016	12.4	3	4.1	12.1	1	12.1
TR019	16.0	3	5.3	17	1	17.0
TR021	4.7	0	0	5.5	0	0
TR024	10.4	1	10.4	9.1	0	0
TR027	11.3	0	0	7.1	0	0
TR028	13.7	1	13.7	4.4	0	0
TR031	10.1	1	10.1	16.6	0	0
TR032	8.5	4	2.1	8.5	0	0
TR038	4.1	0	0	4.5	0	0
TR039	37.3	4	9.3	47.6	0	0
TR040	53.9	0	0	27.3	0	0
TR053	34.3	3	11.4	20.5	0	0

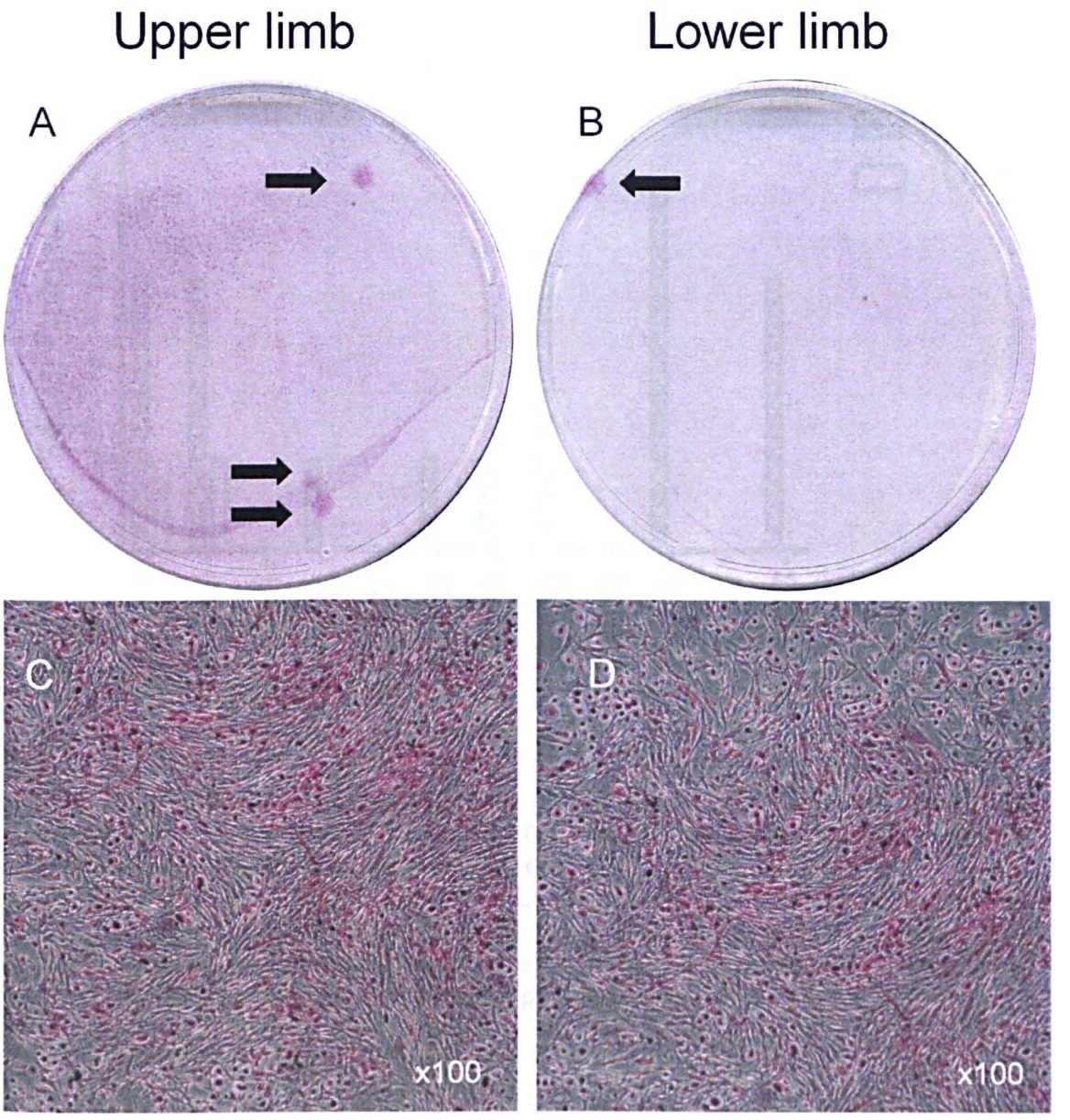

Figure 6.1: Scanned Crystal violet stained CFU-F colonies (A and B) and microscopic images of same colonies (C and D) from the same patient
Arrows pointing at CFU-F colonies. Note 3 CFU-F colonies in the femoral venous blood sample compared to 1 in the peripheral venous blood suggesting possible filtration of MSC colonies in the lung.

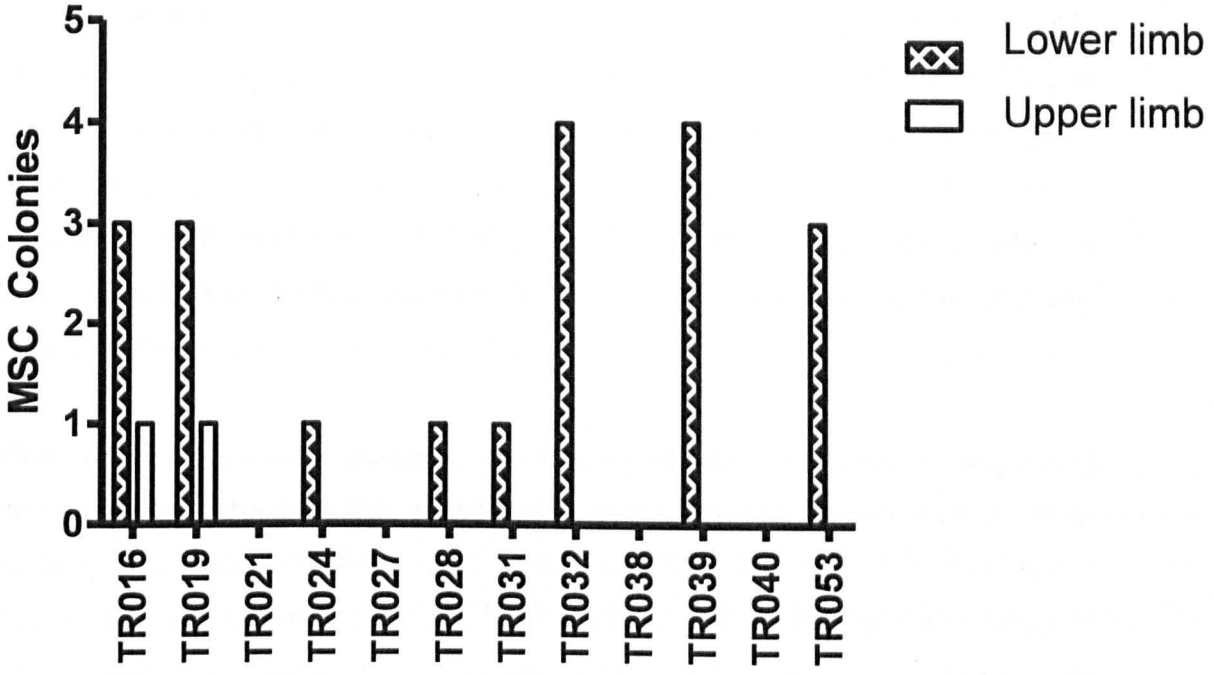

Figure 6.2: Number of MSC colonies in 10ml of lower limb compared to 10ml of upper limb venous blood in all 12 nonunion patients after reaming with RIA for bone graft harvesting (Group I)
More MSC colonies were found in the LL as compared to the same volume of UL. When colonies were evident in the peripheral blood, this was only in the context of being also present, at a higher level in the femoral vein. This likely represents filtration in the lungs and peripheral tissue capillary beds.

6.1.2 Group II

This group was formed of 12 patients where recent or intra-operative skeletal trauma including reaming did not take place and samples were collected before commencement of surgery. A total of 24 samples from 12 patients were collected. Out of the 12 LL samples, 5 patients showed evidence of circulating MSCs (41.67%) and only 1 out of the 12 UL samples showed evidence of circulating MSCs (8.33%). The latter case with UL MSCs also had MSCs evident in the LL femoral vein circulation.

An average of 1 MSC colony was present in every 58.4 x 10^6 MNCs seeded from the femoral venous blood compared to an average of 1 MSC colony per 718 x 10^6 MNCs in peripheral blood. In the 120ml of femoral venous blood collected from all patients, 10 MSC colonies were present, that is an average of 1 MSC colony per 12ml of blood, whereas only 1 MSC colony was identified in the 120ml of PB from the same patients (10 fold less). The total number of MNCs seeded and MSCs identified in both the LL and PB samples for this group of patients is shown in Table 6.2.

Odds ratio statistical analysis of these results was done assuming independence of the samples, which again, was justified by the extreme rarity of MSCs in the blood. An odds ratio of 2.5 was found with a 95% confidence interval, p=0.32. Unlike the results from Group I, these findings have failed to reach statistical significance. Further odds ratio testing was carried out between the LL sample results from both Group I and II; an odds ratio of 4, with a 95% confidence interval and p=0.11.

These findings prove even more that MSC circulation is an extremely rare entity in the orthopaedic setting. The lower limb venous circulation adjacent to lower limb skeletal pathology may contain more MSCs when compared to the upper limb venous system.

Table 6.2: Total number of MNCs and MSC colonies identified in LL and PB in patients undergoing various orthopaedic procedures not involving reaming

Sample ID	MNCs seeded from LL blood, in 10^6	Total MSC Colony Numbers observed in LL	Frequency in LL, 1 cell per MNC, in 10^6	MNCs seeded from PB blood, in 10^6	Total MSC Colony Numbers observed in PB	Frequency in PB, 1 cell per MNC, in 10^6
TR029	20.2	4	5.1	9.4	0	0.0
TR030	16.0	0	0	16.0	0	0.0
TR034	158.1	0	0	114.4	0	0.0
TR036	34.4	0	0	179.2	0	0.0
TR037	7.9	0	0	16.9	1	16.9
TR041	45.3	2	22.7	46.6	0	0.0
TR047	31.5	1	31.5	33.4	0	0.0
TR054	70.2	0	0	70.0	0	0.0
TR065	117.0	1	117.0	128.0	0	0.0
TR077	15.1	0	0	16.5	0	0.0
TR078	15.3	0	0	10.6	0	0.0
TR080	52.5	2	26.3	77.0	0	0.0

When one compares the results from this group to those of Group I, it is noticeable that the volumetric frequency of MSCs in the femoral venous blood has halved (1 MSC colony per 6ml of femoral venous blood versus 1 colony per 12ml of femoral venous blood in Group II). The increase in MSC frequency in Group I is more likely to be the consequence of the mechanical translocation of the MSCs into the deep venous system as sample collection was immediately procured following reaming.

6.1.3 Group III

None of the 11 patients suffering from early RA showed evidence of circulating MSC colonies in their UL venous blood samples. The total number of MNCs seeded in each patient is shown in Table 6.3.

Table 6.3: Number of mononuclear cell seeded compared to the MSC colonies in early RA patients.

Sample ID	Cells seeded (x 10^6)	MSC Colony Number
TR066	14.2	0
TR067	26.6	0
TR068	8.6	0
TR069	8.7	0
TR070	8.6	0
TR071	7.3	0
TR072	22.1	0
TR073	27.6	0
TR074	34.1	0
TR075	25.1	0
TR076	9.4	0
TR066	14.2	0

The failure to identify MSCs in peripheral venous blood of early RA patients argues against the findings of some authors who attributed the spread of RA to circulating synovial fibroblasts [129, 130].

6.1.4 Group IV

This was the healthy control group of volunteers. Upon CFU-F staining of the samples, there was no evidence of MSCs in their UL venous blood. Results can be summarized in Table 6.4. One can argue against the circulation of MSCs in healthy individuals.

Table 6.4: Number of MNC seeded compared to MSC colonies in healthy controls

Sample ID	Cells seeded (x 10^6)	MSC Colony Number
TR048	31.9	0
TR049	44.7	0
TR050	24.1	0
TR055	18.3	0
TR056	35.3	0
TR057	23.0	0
TR059	10.7	0
TR060	19.8	0
TR061	22.9	0
TR062	7.4	0
TR063	27.9	0
TR064	23.3	0

6.1.5 Macrophage like colonies

Numerous studies have argued for the presence of MSCs in the circulation and it is possible that some other cell type may have been erroneously scored as MSC colonies. It was noted that in most of the CFU-F dishes, two distinct

types of colonies were identified. These colonies either had a fibroblast like or macrophage like morphology. Both these colony populations have plastic adherent properties. Fibroblast-like colonies were scored as CFU-Fs/MSCs. Macrophage-like colonies are shown on Figure 6.3 and were not scored due to their clearly distinct differences from the CFU-Fs.

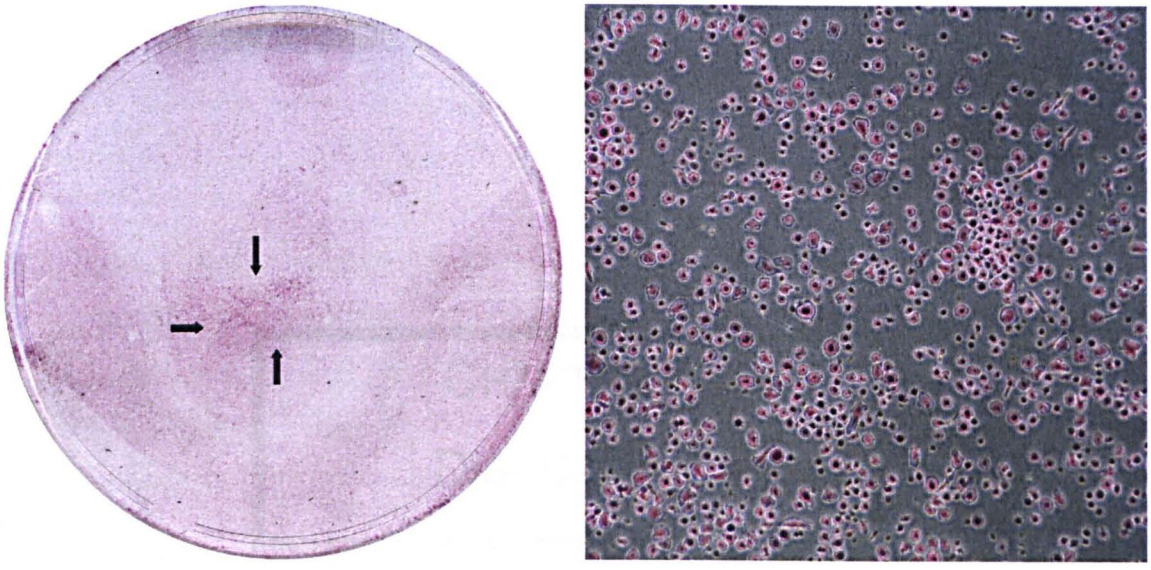

Figure 6.3: Gross and microscopic images of macrophage like colonies
A: scanned dish showing multiple macrophage like colonies (arrows).
B: microscopic image of the same colonies (x100 magnification).

CHAPTER 7
DISCUSSION

7.1 DISCUSSION RELATED TO THE NUMBERS OF MSCS AND OSTEOPROGENITORS IN BM ASPIRATES IN NONUNION AND UNION PATIENTS

The role of MSCs in the aetiology of nonunions is poorly understood. The questions that maybe asked by treating physicians are: do local MSCs in atrophic nonunions have diminished proliferative and osteogenic capacities, explaining why fractures do not unite? Following from this, is this deterioration a systemic phenomenon affecting MSCs not only at the fracture site but also in the BM? This hypothesis is plausible taking into consideration the prevailing views pertaining to the circulation of BM MSCs to the sites of injury as a potential mechanism of repairing fractures.

To address these questions, the first part of this study investigated the number and proliferative capacity of MSCs in patients suffering nonunions in comparison to the union group which was age and gender matched. The union group comprised patients who had previous long bone fractures which subsequently healed uneventfully. The ideal methodology to test this hypothesis would be to prospectively recruit long bone fracture patients and obtain BM aspirates for CFU-F assays from them during their fracture fixation surgery. Thereafter, follow them at regular intervals until either union or nonunion is established (based on FDA criteria for union). This would allow a more robust prospective comparative analysis.

Contrary to the original hypothesis, the studies described herein showed that the reservoir of BM CFU-Fs in patients with atrophic nonunions was not numerically diminished; in fact their number was increased. Their proliferative capacity was however low which may explain poor healing response *in vivo*.

Only one previously published study has tested a similar hypothesis and the results were somewhat different. In Seebach et al study [122], the number of CFU-Fs in the BM of nonunion patients was found to be significantly below normal controls (healthy volunteers). In concordance with the current study,

they found lower proliferative capacity of MSCs from nonunion patients. The discrepancy between Seebach and the present work in respect to CFU-F numbers can be explained by several factors. Firstly, the group of normal volunteers in their study was not perfectly age-matched (mean age range for volunteers 32 and 33 years old for males and females and mean age range for nonunion patients 41 and 42 years, respectively). It is well known that the BM CFU-F capacity declines with age [39], and it is possible that the observed lower CFU-F numbers in nonunion group of patients actually reflected age differences rather than nonunion-related differences.

Secondly, the type of CFU-F assay was also different in Seebach et al study [122]. The authors fractionated BM aspirates first, to obtain MNC fractions, and then plated the cells by cell density. In this study, direct BM plating technique was used; it has an advantage over MNC plating in that minimal manipulations of aspirated marrow are performed, thus minimizing the loss of cells through centrifugation and washing steps. Of note, MNCs from nonunion patients were used for the flow cytometry study in this work and the trends were similar to CFU-F direct plating technique. So on comparison, the discrepancy in the results lies rather in the differences in control groups selected rather than the techniques used.

It is also well-known that the CFU-F content of BM aspirates depends critically on the surgical technique of actual marrow aspiration [165]. In this study the aspiration site was altered every 4ml, hence insuring minimal dilution with blood and the consistence in sample collection for all the patients and groups. No detail is given in Seebach study [122] as to their sample collection protocol and if samples for volunteers and nonunion patients were collected by different surgeons and using different techniques; this could have also affected the results. Intriguingly, these authors have found massive differences in CFU-F numbers between normal males and females – a controversial finding, never documented before, despite numerous published studies on this subject [39, 166] and certainly not observed in this study.

Furthermore, the number of CFU-Fs can be dependent on the media used and serum in particular, which critically influences the cell attachment. Whilst attached though, MSCs begin to proliferate and form colonies. In this respect, findings in this work and Seebach et al [122] findings are very similar. Indeed, the proliferative capacity of MSCs in nonunion patients was found to be inferior to control in both studies. Remarkably, this coherence was found irrespective of the methods used to assess proliferation. Seebach et al [122] measured the sizes of colonies using digitized images and a specialised image software. In this study manual grading of colonies was used, from big 10mmX10mm to small 2.5mmx2.5mm). The average numbers of cell per colony of each size was scored and it was proved that colony sizes were indeed reflective of the number of cell per colony (i.e. the degree of proliferation of colony-forming cells) and not of cell spreading and motility leading to a bigger but less dense colonies. So, it can be said with a strong degree of confidence that MSCs from nonunion patients are indeed less proliferative. Indeed, this lack of proliferative potential raises the question that these cells are no longer true MSCs in that they have lost an intrinsic feature of stem cells, i.e. high proliferative capacity.

The novelty of this study over Seebach et al [122] study also lies in the use of flow cytometry as an independent adjunct method of MSC enumeration and also in the assessment of the PD rate of union and nonunion CFU-Fs in culture. In the present work, it was shown that MSCs forming large colonies have undergone nearly 10 divisions during the 14-day culture period whereas MSCs forming small colonies have divided only 6 times. This emphasizes the immense heterogeneity of MSCs documented in other similar studies [167, 168] and shows that further work is needed to dissect the relationship between the proliferation potential of individual MSCs and their osteogenic properties.

The mechanism underlying poor proliferation of BM MSCs in nonunion patients remains unclear. Given that MSCs were obtained from the iliac crest and not the fracture site then this appears to be, at least in part, a systemic problem. Kwong et al [169], have proposed an imbalance in the production of BMPs and their antagonists, but they have investigated and confirmed the validity of this

hypothesis at the local level, within the cartilaginous intermediate of nonhealing fractures. Hoffman et al [170] have investigated the viability, differentiation and gene expression profiles of osteoblasts at sites proximal to hypertrophic nonunions and found these to be significantly altered, with a notable down-regulation of molecules involved in bone-related signalling pathways. The effect on MSCs in the BM however, which is a distant site to the nonunion, is likely to be mediated via factors acting systemically via circulation. Seebach et al [122], have come to the same conclusion saying that "yet unidentified serum factors", which may affect the number of MSCs in fracture patients, including BM. The levels of IL-6 which are shown to correlate with trauma severity, may be one such factor [122], given the known inhibitory role of pro-inflammatory cytokines on osteogenesis [171]. It is possible that circulating levels of osteogenesis-related growth factors like TGF-β, IGF and PDGF [170] are also involved, as well as inhibitory molecules, this certainly merits further investigation.

Overall, the results of this study support the rationale for using autologous BM MSC injections to augment bone healing in atrophic nonunions [87, 116]. However they advocate caution, showing that local application of autologous poorly-proliferative MSCs may not be the optimal solution. Stimulation of patient's BM MSCs activity prior to harvesting marrow may be a better option. In this respect, the discovery of the serum factors responsible for systemic activation of MSCs is the best way forward. It also suggests that healthy allogenic MSCs might represent an alternative therapy source in this clinical setting.

7.2 DISCUSSION RELATING TO BMP-7

Several clinical studies have demonstrated a beneficial effect of BMP-7 on the healing of nonunions [83, 152, 154, 172, 173] .The mechanism behind the action of BMP-7 in these clinical trials however, remains poorly understood. The second part of this study therefore addressed an issue of responsiveness of MSCs derived from the BM of nonunion patients to BMP-7 in comparison to

united fracture patients. The range of chosen concentrations of BMP-7 tested was within the reported in previously-published literature [107, 123, 174].

Contradictory to the findings of Zhi et al and Shen et al studies [123, 174], no increase in calcium release by union MSCs with the addition of BMP-7 was observed. It should be noted however that to achieve statistical significance in calcium production up-regulation, Zhi et al had to increase the assay duration to 35 days, above the normal assay length, whereas their day-17 results were very similar to the results presented here. Shen et al did not evaluate calcium production quantitatively; only microphotographs of cultures stained with Alizarin Red were provided, making it hard to judge the degree of calcium up-regulation.

Both groups of authors did not find any effects when BMP-7 was added to standard expansion media; only when BMP-7 was added to osteogenic media, the effects on MSCs have become prominent. Based on these findings it can be concluded that BMP-7 acts in synergy with other media components, potentially dexamethasone, leading to better osteoinduction [174]. In concordance, Shea et al showed that BMP-7 activity was enhanced in "nutrient-rich media", suggesting a potential autocrine mechanism of BMP-7 action [175], the idea also proposed by others [176]. In this work, the effect of BMP-7 on calcium production by MSCs grown in standard expansion media was not studied. This was based on the initial pilot results that were carried out prior to the experiments in this work which shown no osteogenic induction in standard media. Similar findings from the work by Shen et al [123] also found no effects, both consistent with Zhi et al study [174]. However, the effects of BMP-7 on MSC proliferation in standard conditions and the CFU-F colony counts were studied in this work and showed no effect on MSC proliferation. Shen et al actually found an inhibition of MSC proliferation with the addition of BMP-7 *in vitro* [123]. In concordance, Lavery et al [176], showed that the addition of BMP-7 transiently attenuated cell cycle progression and proliferation of primary human MSCs. These combined data support an idea that BMP-7 on its own exerts no notable enhancing effects on MSC proliferation and its effects on MSC osteogenic differentiation are dependent on a synergy with other, yet unidentified, osteoinductive molecules [177].

From the literature, it appears that no previous study has explored the responsiveness of MSCs from nonunion patients to BMP-7. In this study, the effects of BMP-7 on control MSCs and MSCs from nonunion patients were not observed at all. This was unexpected with the view of beneficial effects of BMP-7 in treating nonunions in clinical trials [83, 152, 154, 172, 173]. To explore these observations further, more experiments were conducted involving uncultured MSCs from nonunion patients. It is well known that MSCs lose their potency following expansion [178] and it is possible that MSCs from nonunion patients lose their responsiveness to BMP-7 faster than MSCs from union patients, leading to no observed effects in experiments when cultured nonunion MSCs are used. Experiments with fresh MSCs involved no prior cultivation and were performed at the clonogenic level straight after BM plating. As mentioned above, the experiments studying osteogenesis were conducted in osteoinductive media only and experiments exploring proliferation were performed in expansion media only. In these experiments, the overall effects on MSC osteogenesis remained absent. The effects on MSC proliferation were donor-dependent. Those donors who had high CFU-F counts in the first place tended to up-regulate these counts further. When the whole group of patients was assessed for colony distributions however, BMP-7 appeared to have no effect. This effect on MSC proliferation was similar to the effects observed for union MSCs. Given these findings, one can become less optimistic as to the direct value of using BMP-7 in nonunion patients, because not only their MSC osteogenesis was unaffected, but their proliferation also.

One requires to have in mind however, that *in vitro* experiments provide only first initial ideas and testing in other systems including animal models is needed to consolidate knowledge in this area [179, 180]. As an example of limitations of using *in vitro* assays for BMP-7 research, one can highlight the Neumann et all paper, who showed that culturing MSCs in 3-dimentional, rather than 2-dimentional culture, can lead to their enhanced adipogenesis (and not osteo- or chondrogenesis) [181]. The experiments conducted in animal models are much more powerful. The experiments performed in a mouse model of tibial fractures have demonstrated the up-regulation of BMP-7 mRNA at later stages of healing (days 14-21), which can be described as "osteogenic" stages during which

calcified cartilage is resorbed and osteoblastic recruitment is most active [24] . So it is possible that the action of BMP-7 *in vivo* is linked to MSC recruitment activities rather than to their direct differentiation to osteoblasts. This is an attractive idea, but in the contrary to that Lu et al showed that recombinant BMP-7 did not actually recruit MSCs from the BM to the fracture sites in the mouse model used [182]. Interestingly, they showed that BMP-7 had a prominent chondrogenic effect [182], also observed in an *in vitro* study by Shen et al [123]. Another possible explanation for the discrepancy between the reported *in vitro* and *in vivo* studies lies in a more complicated nature of cellular and molecular interactions *in vivo*. It is very likely that BMP-7 exert its effects in cooperation with other BMPs and their inhibitors [179, 183]. The need of using supraphysiological concentration of BMP-7 in clinic may be due to high concentrations of BMP pathway inhibitors such as noggin [184] at the fracture sites. Based on this proposition, one can suggest that the way of reducing the cost of BMP-7 therapy for nonunion patients, which is indeed efficacious, is to interfere with its inhibitors at the fracture sites. This would be very interesting to test in a future *in vivo* study. Another potential solution, as proposed by Zhi et al, is to further explore the synergy of BMP-7 with other pharmacological compounds such as steroids, in order to reduce "costs and complications associated with supraphysiological dosage of rhBMPs in therapeutic protocols" [174]. Synergistic BMP pairs have been proposed as another solution [21].

7.3 DISCUSSION ON MSC CIRCULATION

Historically, it was considered that MSCs were BM resident cells that could circulate to distant sites and lead to tissue repair following injury [92]. Several studies have reported MSCs in the circulation in both animal models and in man [17, 19, 185]. Shirley et al, used autologous culture expanded, fluorescently labelled MSCs from New Zealand White rabbits to study the systemic recruitment capacity of MSCs to sites of bone fracture. MSCs were obtained from the tibia of the rabbits, culture expanded in osteogenic conditions and then fluorescently labelled. Ulnar osteotomy was performed to simulate fracture and 48 hours later the MSCs were injected either intravenously, or into the sound tibia BM cavity, or into the osteotomy gap. Another group was formed in which labelled MSCs were injected into the sound tibia BM cavity 4 weeks prior to ulnar osteotomy and a control group in which no labelled MSCs were injected. They detected the labelled MSCs at the fracture gap in all but the control group 3 weeks after the osteotmy and attributed their presence to the homing capacity of MSCs to site of fracture. Eghbali-Fatourech et al [185], attributed their generous detection of circulating MSCs in normal physiological conditions due to the fact that they have not relied on the plastic adherence property to isolate MSCs, but instead used flow cytometry and FACS to enrich for MSCs. Collectively, these studies might suggest a role for MSCs under normal physiological conditions and during tissue repair following injury, something that could be extremely important for fracture nonunion therapy development strategies.

Moreover, several studies have argued that circulating MSCs or other stromal cells could be responsible for the destructive nature of RA with polyarticular joint erosion. The paradigm assumes that MSCs circulate but that their function in RA is altered and that this leads to severe joint destruction [129, 130].

However several studies have failed to show MSCs in the circulation [18] or detected them at a very low concentration [19]. Jones and McGonagle [18] investigated the presence of circulating MSCs in the peripheral blood from 16

healthy individuals using multiparameter flow cytometry after enrichment for MSCs. Upon use of rigorous flow cytometric phenotypic criteria, they were unable to detect an MSC population in the circulation, but reported phagocytic cells of possible macrophage / monocytic nature.

MSCs have been shown to be closely related to fat globules [85, 186]. It is also interesting to note that patients with major trauma to long bones can experience fat embolism [187], this present data supports the idea that trauma to the skeleton disrupts capillary beds in the bone and facilitates the egress of fat or MSCs or both into the circulation. In a recent study by Kumagai et al, it was demonstrated that MSCs may home to sites of fractured femurs in the parbiotic mice model [188].

In this work, MSCs were not detected in the UL samples of neither the healthy donors nor patients with RA not on immunosuppressive therapy. However, they were detected in the UL and LL venous blood following reaming with RIA or in other cases in which skeletal trauma took place, suggestive of a biophysical translocation of MSCs from their BM niche into the circulation. The concept proposed is similar to fat embolism reported following major long bone fractures and reaming [189] whereby MSCs are likely to enter the circulation in meaningful numbers only at times of skeletal trauma including surgical trauma. In total, the present data argues against recruitment of MSCs into the blood outside the setting of skeletal trauma. Given their low numbers, these circulating MSCs are unlikely to contribute significantly to fracture healing.

7.4 LIMITATIONS OF THIS STUDY

Given the 2 year study period for this work, it was not possible to recruit patients for a prospective observational study of the CFU-F number and distribution of colony sizes following the initial fracture fixation, in anticipation of any healing complications that may arise during follow up.

The biggest potential limitation of this study is the small patient cohorts used in some studies, particularly in the BMP-7 study. In general, patient recruitment into a nonunion study was determined by clinical constraints, mainly the limited number of cases available for recruitment, together with prioritizing trauma patients over "elective cases". Secondly, very stringent excluding criteria, particularly in relation to patients with advanced age were applied in order to carefully age and sex match all the groups under comparisons.

Due to the high cost of recombinant BMP-7, the BMP-7 study had to be very carefully planned, with the important arm being the need to use at least two different concentrations of BMP-7 in order to discern dose-dependent effects. The highest dose of 300ng/ml of BMP-7 had to be used, as previous studies utilizing 100ng/ml had shown discrepancy in the results [123, 174]. Due to high cost of BMP-7, this resulted in the need to scale-down the number of experimental groups to a minimum (expanded union MSCs, expanded nonunion MSCs and uncultured nonunion MSCs). Indeed, BMP-7 was not tested in union non-expanded MSCs. and this would be very interesting to test in a follow-on study. Calcium assay was performed on day 14 post-induction; one very recent study suggested that testing at later time-points (day 35) could have yielded stronger effects [174]. The mere fact, that a longer than normal assay duration in the latter study was needed, fully confirm the data in this study that the effects of BMP-7 on cultured human MSCs are minimal. It has to be acknowledged however, that performing assays at two time-points (at 14 days post-induction and at a longer one) could have better addressed the issue whether or not MSCs from nonunion patients showed a delayed response to BMP-7. This can be also explored in a follow-on study.

In relation to the evaluation of the MSC reservoir in patients with nonunion, the imitations of this study can be described as follows. Firstly, colony size measurements based on a grid system are fairly subjective and an automated analysis would be more desirable. However, utmost effort was made to prove that colony sizes on the grid truly reflected the proliferative potential of colony-forming MSCs. This was done by counting cells in selected colonies and converting these numbers to PDs or divisions of individual cells. Of note, an automated analysis was performed in one previous study [174] and results presented in this study are very comparable. Furthermore, assessment of the MSC numbers by flow cytometry was performed. One may suggest that another type of proliferation assay could be used to validate the colony size findings. These could have included proliferation assays like xtt or Vybrant assays [51], however the latter assays utilise culture-expanded MSCs whereas in this work, experiments were aimed to minimise any artefacts inherent to prolonged culture manipulations. Hence traditional colony assays initiated directly from the BM aspirate were used [190], thus limiting the portfolio of downstream proliferation assays that could be used.

The limitation of the circulation MSCs study can be described as an inability to obtain femoral blood samples from normal volunteers and rheumatoid arthritis patients for the aforementioned reasons. Ideally in the RIA group, blood samples should have been collected before and after reaming, but this was not ethically permissible especially for the femoral venous blood sample due to the possible complications from this procedure. Additionally, due to the extreme scarcity of CFU-F colonies it was not possible to expand them to a sufficient level in order to investigate them further – either by flow cytometry or using functional osteogenesis assays. The current results however, provide a good starting point as to what kind of average femoral blood volume needs to be collected in order to realistically expect sufficient quantities of MSCs to enable these more comprehensive studies (potentially several hundred millimetres which is unethical and impracticable).

7.5 FUTURE DIRECTIONS

This research presented here highlights the strengths and weaknesses of *in vitro* assays to study disease and to model the effects of therapeutic agents on biology of bone cells and their progenitors. In the future, it would very interesting to use these assays to further model the interactions between BMP-7 and other candidate complimentary molecules (like other BMPs or steroids) to see if their combined effects would have enhanced osteogenesis of MSCs from nonunion patients. Additionally, it would be interesting to study similar effects on MSC chondrogenesis and their cytokine release, particularly in relation to those cytokines involved in MSC recruitment at the local level. The preliminary work in this thesis suggests that autologous MSCs obtained from locations distant from the nonunion sites may have defective capabilities. Therefore, it would be worthwhile to conduct a broader study of CFU-F dynamics in fracture patients aiming to using CFU-F colony size measurements as a predictive assay for fracture nonunion. If this stands true, it would be interesting to discover systemic circulating factor(s) responsible for this phenomenon, which would have allowed developing a predictive test based on serum analysis rather than BM aspirate which is much more invasive.

CHAPTER 8

REFERENCES

1. Kalfas, I.H., *Principles of bone healing.* Neurosurg Focus, 2001. 10(4): p. E1.
2. Baroli, B., *From natural bone grafts to tissue engineering therapeutics: Brainstorming on pharmaceutical formulative requirements and challenges.* Journal of pharmaceutical sciences, 2009. 98(4): p. 1317-75.
3. McRae, R. and M.F.E.O.F. Esser, *Practical fracture treatment.* 5th ed. ed2008, Edinburgh: Elsevier/Churchill Livingstone. 447 p.
4. Gustilo, R.B., R.M. Mendoza, and D.N. Williams, *Problems in the management of type III (severe) open fractures: a new classification of type III open fractures.* The Journal of trauma, 1984. 24(8): p. 742-6.
5. Hamblen, D.L., A.H.R.W. Simpson, and J.C.O.o.f.i.j.i. Adams, *Adams's outline of fractures, including joint injuries.* 12th ed. / David L. Hamblen, A. Hamish R.W. Simpson. ed2007, Edinburgh ; New York: Churchill Livingstone Elsevier. vi, 328 p.
6. McKibbin, B., *The biology of fracture healing in long bones.* J Bone Joint Surg Br, 1978. 60-B(2): p. 150-62.
7. Giannoudis, P.V., T.A. Einhorn, and D. Marsh, *Fracture healing: the diamond concept.* Injury, 2007. 38 Suppl 4: p. S3-6.
8. Pape, H.C., et al., *Effects of intramedullary femoral fracture fixation: what is the impact of experimental studies in regards to the clinical knowledge?* Shock, 2002. 18(4): p. 291-300.
9. Perren, S.M., *Evolution of the internal fixation of long bone fractures. The scientific basis of biological internal fixation: choosing a new balance between stability and biology.* The Journal of bone and joint surgery. British volume, 2002. 84(8): p. 1093-110.
10. Bielby, R., E. Jones, and D. McGonagle, *The role of mesenchymal stem cells in maintenance and repair of bone.* Injury, 2007. 38 Suppl 1: p. S26-32.
11. Marsell, R. and T.A. Einhorn, *The biology of fracture healing.* Injury, 2011.
12. Gerstenfeld, L.C., et al., *Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation.* Journal of cellular biochemistry, 2003. 88(5): p. 873-84.
13. Harder, A.T. and Y.H. An, *The mechanisms of the inhibitory effects of nonsteroidal anti-inflammatory drugs on bone healing: a concise review.* Journal of clinical pharmacology, 2003. 43(8): p. 807-15.
14. De Long, W.G., Jr., et al., *Bone grafts and bone graft substitutes in orthopaedic trauma surgery. A critical analysis.* The Journal of bone and joint surgery. American volume, 2007. 89(3): p. 649-58.
15. Kitaori, T., et al., *Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model.* Arthritis and rheumatism, 2009. 60(3): p. 813-23.
16. Granero-Molto, F., et al., *Regenerative effects of transplanted mesenchymal stem cells in fracture healing.* Stem Cells, 2009. 27(8): p. 1887-98.
17. Shirley, D., et al., *Systemic recruitment of osteoblastic cells in fracture healing.* Journal of orthopaedic research : official publication of the Orthopaedic Research Society, 2005. 23(5): p. 1013-21.
18. Jones, E. and D. McGonagle, *Human bone marrow mesenchymal stem cells in vivo.* Rheumatology, 2008. 47(2): p. 126-31.
19. Kuznetsov, S.A., et al., *Circulating skeletal stem cells.* The Journal of cell biology, 2001. 153(5): p. 1133-40.
20. Kuznetsov, S.A., et al., *Circulating connective tissue precursors: extreme rarity in humans and chondrogenic potential in guinea pigs.* Stem Cells, 2007. 25(7): p. 1830-9.
21. Luu, H.H., et al., *Distinct roles of bone morphogenetic proteins in osteogenic differentiation of mesenchymal stem cells.* J Orthop Res, 2007. 25(5): p. 665-77.
22. Marsell, R. and T.A. Einhorn, *The role of endogenous bone morphogenetic*

proteins in normal skeletal repair. Injury, 2009. 40 Suppl 3: p. S4-7.
23. Giannoudis, P.V., E. Jones, and T.A. Einhorn, *Fracture healing and bone repair.* Injury, 2011.
24. Cho, T.J., L.C. Gerstenfeld, and T.A. Einhorn, *Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing.* Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research, 2002. 17(3): p. 513-20.
25. Keramaris, N.C., et al., *Fracture vascularity and bone healing: a systematic review of the role of VEGF.* Injury, 2008. 39 Suppl 2: p. S45-57.
26. Tsiridis, E., N. Upadhyay, and P. Giannoudis, *Molecular aspects of fracture healing: which are the important molecules?* Injury, 2007. 38 Suppl 1: p. S11-25.
27. Gerstenfeld, L.C., et al., *Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation.* J Cell Biochem, 2003. 88(5): p. 873-84.
28. Barnes, G.L., et al., *Growth factor regulation of fracture repair.* Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research, 1999. 14(11): p. 1805-15.
29. Ai-Aql, Z.S., et al., *Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis.* Journal of dental research, 2008. 87(2): p. 107-18.
30. Einhorn, T.A., *The cell and molecular biology of fracture healing.* Clinical orthopaedics and related research, 1998(355 Suppl): p. S7-21.
31. Calori, G.M., et al., *Treatment of long bone non-unions with polytherapy: Indications and clinical results.* Injury, 2011.
32. Ramachandran, M., *Basic orthopaedic sciences : the Stanmore guide*2007, London: Hodder Arnold. x, 304 p.
33. Browner, B.D., *Skeletal trauma : basic science, management, and reconstruction.* 4th ed. ed2009, Philadelphia, Pa. ; [Edinburgh]: Saunders Elsevier. 2 v. (xxv, 2882, 56 p.).
34. Frolke, J.P. and P. Patka, *Definition and classification of fracture non-unions.* Injury, 2007. 38 Suppl 2: p. S19-22.
35. Namkung-Matthai, H., et al., *Osteoporosis influences the early period of fracture healing in a rat osteoporotic model.* Bone, 2001. 28(1): p. 80-6.
36. Marie, P.J., et al., *Osteocalcin and deoxyribonucleic acid synthesis in vitro and histomorphometric indices of bone formation in postmenopausal osteoporosis.* The Journal of clinical endocrinology and metabolism, 1989. 69(2): p. 272-9.
37. Calori, G.M., et al., *Risk factors contributing to fracture non-unions.* Injury, 2007. 38 Suppl 2: p. S11-8.
38. Hayda, R.A., C.T. Brighton, and J.L. Esterhai, Jr., *Pathophysiology of delayed healing.* Clinical orthopaedics and related research, 1998(355 Suppl): p. S31-40.
39. D'Ippolito, G., et al., *Age-related osteogenic potential of mesenchymal stromal stem cells from human vertebral bone marrow.* Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research, 1999. 14(7): p. 1115-22.
40. Stolzing, A., et al., *Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies.* Mechanisms of ageing and development, 2008. 129(3): p. 163-73.
41. Jones, E., et al., *Large-scale extraction and characterization of CD271+ multipotential stromal cells from trabecular bone in health and osteoarthritis: implications for bone regeneration strategies based on uncultured or minimally cultured multipotential stromal cells.* Arthritis and rheumatism, 2010. 62(7): p. 1944-54.
42. Macey, L.R., et al., *Defects of early fracture-healing in experimental diabetes.*

The Journal of bone and joint surgery. American volume, 1989. 71(5): p. 722-33.

43. Sun, Y., et al., *Differentiation of bone marrow-derived mesenchymal stem cells from diabetic patients into insulin-producing cells in vitro.* Chinese medical journal, 2007. 120(9): p. 771-6.
44. Adams, C.I., J.F. Keating, and C.M. Court-Brown, *Cigarette smoking and open tibial fractures.* Injury, 2001. 32(1): p. 61-5.
45. Hoogduijn, M.J., A. Cheng, and P.G. Genever, *Functional nicotinic and muscarinic receptors on mesenchymal stem cells.* Stem cells and development, 2009. 18(1): p. 103-12.
46. Raikin, S.M., et al., *Effect of nicotine on the rate and strength of long bone fracture healing.* Clinical orthopaedics and related research, 1998(353): p. 231-7.
47. Kyro, A., et al., *Are smokers a risk group for delayed healing of tibial shaft fractures?* Annales chirurgiae et gynaecologiae, 1993. 82(4): p. 254-62.
48. Brown, C.W., T.J. Orme, and H.D. Richardson, *The Rate of Pseudoarthrosis (Surgical Nonunion) in Patients Who Are Smokers and Patients Who Are Nonsmokers - a Comparison Study.* Spine, 1986. 11(9): p. 942-943.
49. Wing, K.J., et al., *Stopping nicotine exposure before surgery - The effect on spinal fusion in a rabbit model.* Spine, 2000. 25(1): p. 30-34.
50. Giannoudis, P.V., et al., *Nonunion of the femoral diaphysis. The influence of reaming and non-steroidal anti-inflammatory drugs.* The Journal of bone and joint surgery. British volume, 2000. 82(5): p. 655-8.
51. Pountos, I., et al., *NSAIDS inhibit in vitro MSC chondrogenesis but not osteogenesis: implications for mechanism of bone formation inhibition in man.* Journal of cellular and molecular medicine, 2011. 15(3): p. 525-34.
52. Murnaghan, M., G. Li, and D.R. Marsh, *Nonsteroidal anti-inflammatory drug-induced fracture nonunion: an inhibition of angiogenesis?* The Journal of bone and joint surgery. American volume, 2006. 88 Suppl 3: p. 140-7.
53. Waters, R.V., et al., *Systemic corticosteroids inhibit bone healing in a rabbit ulnar osteotomy model.* Acta orthopaedica Scandinavica, 2000. 71(3): p. 316-21.
54. Boursinos, L.A., et al., *Do steroids, conventional non-steroidal anti-inflammatory drugs and selective Cox-2 inhibitors adversely affect fracture healing?* Journal of musculoskeletal & neuronal interactions, 2009. 9(1): p. 44-52.
55. Adachi, J.D., et al., *Intermittent etidronate therapy to prevent corticosteroid-induced osteoporosis.* The New England journal of medicine, 1997. 337(6): p. 382-7.
56. Wang, G.J., et al., *The effect of core decompression on femoral head blood flow in steroid-induced avascular necrosis of the femoral head.* The Journal of bone and joint surgery. American volume, 1985. 67(1): p. 121-4.
57. Elmali, N., et al., *Fracture healing and bone mass in rats fed on liquid diet containing ethanol.* Alcoholism, clinical and experimental research, 2002. 26(4): p. 509-13.
58. Ronis, M.J., K. Mercer, and J.R. Chen, *Effects of nutrition and alcohol consumption on bone loss.* Current osteoporosis reports, 2011. 9(2): p. 53-9.
59. Laytragoon-Lewin, N., et al., *Direct Effects of Pure Nicotine, Cigarette Smoke Extract, Swedish-type Smokeless Tobacco (Snus) Extract and Ethanol on Human Normal Endothelial Cells and Fibroblasts.* Anticancer research, 2011. 31(5): p. 1527-34.
60. Einhorn, T.A., F. Bonnarens, and A.H. Burstein, *The contributions of dietary protein and mineral to the healing of experimental fractures. A biomechanical study.* The Journal of bone and joint surgery. American volume, 1986. 68(9): p. 1389-95.
61. Karladani, A.H., et al., *The influence of fracture etiology and type on fracture*

healing: a review of 104 consecutive tibial shaft fractures. Archives of orthopaedic and trauma surgery, 2001. 121(6): p. 325-8.

62. Dickson, K., et al., *Delayed unions and nonunions of open tibial fractures. Correlation with arteriography results.* Clinical orthopaedics and related research, 1994(302): p. 189-93.
63. Mehta, M., et al., *Influences of age and mechanical stability on volume, microstructure, and mineralization of the fracture callus during bone healing: is osteoclast activity the key to age-related impaired healing?* Bone, 2010. 47(2): p. 219-28.
64. Claes, L., et al., *Influence of size and stability of the osteotomy gap on the success of fracture healing.* Journal of orthopaedic research : official publication of the Orthopaedic Research Society, 1997. 15(4): p. 577-84.
65. Uhthoff, H.K. and B.A. Rahn, *Healing patterns of metaphyseal fractures.* Clinical orthopaedics and related research, 1981(160): p. 295-303.
66. Court-Brown, C.M. and B. Caesar, *Epidemiology of adult fractures: A review.* Injury, 2006. 37(8): p. 691-7.
67. Court-Brown, C.M. and J. McBirnie, *The epidemiology of tibial fractures.* J Bone Joint Surg Br, 1995. 77(3): p. 417-21.
68. Babis, G.C., et al., *Distal tibial fractures treated with hybrid external fixation.* Injury, 2010. 41(3): p. 253-8.
69. Collinge, C. and R. Protzman, *Outcomes of minimally invasive plate osteosynthesis for metaphyseal distal tibia fractures.* J Orthop Trauma, 2010. 24(1): p. 24-9.
70. Lam, S.W., et al., *Systematic review shows lowered risk of nonunion after reamed nailing in patients with closed tibial shaft fractures.* Injury, 2010.
71. Grebe, A.A., *Fractures of the shaft of the femur.* Am J Surg, 1950. 80(2): p. 161-9.
72. Tzioupis, C. and P.V. Giannoudis, *Prevalence of long-bone non-unions.* Injury, 2007. 38 Suppl 2: p. S3-9.
73. Papakostidis, C., et al., *Femoral biologic plate fixation.* Clinical orthopaedics and related research, 2006. 450: p. 193-202.
74. Alonso, J., W. Geissler, and J.L. Hughes, *External fixation of femoral fractures. Indications and limitations.* Clin Orthop Relat Res, 1989(241): p. 83-8.
75. el Moumni, M., et al., *The incidence of non-union following unreamed intramedullary nailing of femoral shaft fractures.* Injury, 2009. 40(2): p. 205-8.
76. Ekholm, R., et al., *Fractures of the shaft of the humerus. An epidemiological study of 401 fractures.* J Bone Joint Surg Br, 2006. 88(11): p. 1469-73.
77. Sarmiento, A., et al., *Functional bracing for the treatment of fractures of the humeral diaphysis.* J Bone Joint Surg Am, 2000. 82(4): p. 478-86.
78. McKee, M.D., J.G. Seiler, and J.B. Jupiter, *The application of the limited contact dynamic compression plate in the upper extremity: an analysis of 114 consecutive cases.* Injury, 1995. 26(10): p. 661-6.
79. Ramoutar, D.N., et al., *Judet decortication and compression plate fixation of long bone non-union: Is bone graft necessary?* Injury, 2011.
80. Megas, P., *Classification of non-union.* Injury, 2005. 36 Suppl 4: p. S30-7.
81. Geris, L., et al., *Occurrence and treatment of bone atrophic non-unions investigated by an integrative approach.* PLoS computational biology, 2010. 6(9): p. e1000915.
82. Augello, A., T.B. Kurth, and C. De Bari, *Mesenchymal stem cells: a perspective from in vitro cultures to in vivo migration and niches.* European cells & materials, 2010. 20: p. 121-33.
83. Kanakaris, N.K., et al., *Application of BMP-7 to tibial non-unions: a 3-year multicenter experience.* Injury, 2008. 39 Suppl 2: p. S83-90.
84. Jones, E.A., et al., *Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells.* Arthritis Rheum, 2002. 46(12): p. 3349-60.

85. Jones, E.A., et al., *Optimization of a flow cytometry-based protocol for detection and phenotypic characterization of multipotent mesenchymal stromal cells from human bone marrow.* Cytometry B Clin Cytom, 2006. 70(6): p. 391-9.
86. Satija, N.K., et al., *Mesenchymal stem cell-based therapy: a new paradigm in regenerative medicine.* Journal of cellular and molecular medicine, 2009. 13(11-12): p. 4385-402.
87. Hauzeur, J.P. and V. Gangji, *Phases 1-3 clinical trials using adult stem cells in osteonecrosis and nonunion fractures.* Stem cells international, 2010. 2010: p. 410170.
88. Hernigou, P., et al., *Percutaneous autologous bone-marrow grafting for nonunions. Surgical technique.* J Bone Joint Surg Am, 2006. 88 Suppl 1 Pt 2: p. 322-7.
89. Jones, E. and D. McGonagle, *Human bone marrow mesenchymal stem cells in vivo.* Rheumatology (Oxford), 2008. 47(2): p. 126-31.
90. Gao, J., et al., *The dynamic in vivo distribution of bone marrow-derived mesenchymal stem cells after infusion.* Cells Tissues Organs, 2001. 169(1): p. 12-20.
91. Liu, L., et al., *Hypoxia-Inducible Factor-1alpha Is Essential for Hypoxia-Induced Mesenchymal Stem Cell Mobilization into the Peripheral Blood.* Stem cells and development, 2011.
92. Prockop, D.J., *Marrow stromal cells as stem cells for nonhematopoietic tissues.* Science, 1997. 276(5309): p. 71-4.
93. Konstantinov, I.E., *In search of Alexander A. Maximow: the man behind the unitarian theory of hematopoiesis.* Perspectives in biology and medicine, 2000. 43(2): p. 269-76.
94. Chamberlain, G., et al., *Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing.* Stem Cells, 2007. 25(11): p. 2739-49.
95. Friedenstein, A.J., R.K. Chailakhyan, and U.V. Gerasimov, *Bone-Marrow Osteogenic Stem-Cells - Invitro Cultivation and Transplantation in Diffusion-Chambers.* Cell and Tissue Kinetics, 1987. 20(3): p. 263-272.
96. Caplan, A.I., *Mesenchymal stem cells.* Journal of orthopaedic research : official publication of the Orthopaedic Research Society, 1991. 9(5): p. 641-50.
97. Caplan, A.I., *The mesengenic process.* Clin Plast Surg, 1994. 21(3): p. 429-35.
98. Jones, E. and X. Yang, *Mesenchymal stem cells and bone regeneration: Current status.* Injury, 2011. 42(6): p. 562-8.
99. Friedenstein, A.J., et al., *Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues.* Transplantation, 1968. 6(2): p. 230-47.
100. Pittenger, M.F., et al., *Multilineage potential of adult human mesenchymal stem cells.* Science, 1999. 284(5411): p. 143-7.
101. De Bari, C., et al., *Multipotent mesenchymal stem cells from adult human synovial membrane.* Arthritis Rheum, 2001. 44(8): p. 1928-42.
102. English, A., et al., *A comparative assessment of cartilage and joint fat pad as a potential source of cells for autologous therapy development in knee osteoarthritis.* Rheumatology (Oxford), 2007. 46(11): p. 1676-83.
103. Jones, E.A., et al., *Synovial fluid mesenchymal stem cells in health and early osteoarthritis: detection and functional evaluation at the single-cell level.* Arthritis Rheum, 2008. 58(6): p. 1731-40.
104. Majumdar, M.K., et al., *Characterization and functionality of cell surface molecules on human mesenchymal stem cells.* Journal of biomedical science, 2003. 10(2): p. 228-41.
105. Dominici, M., et al., *Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.* Cytotherapy, 2006. 8(4): p. 315-7.

106. Quirici, N., et al., *Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies.* Experimental hematology, 2002. 30(7): p. 783-91.
107. Pountos, I., et al., *The effect of bone morphogenetic protein-2, bone morphogenetic protein-7, parathyroid hormone, and platelet-derived growth factor on the proliferation and osteogenic differentiation of mesenchymal stem cells derived from osteoporotic bone.* Journal of orthopaedic trauma, 2010. 24(9): p. 552-6.
108. Friedenstein, A.J., et al., *Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo.* Transplantation, 1974. 17(4): p. 331-40.
109. Pochampally, R., *Colony forming unit assays for MSCs.* Methods in molecular biology, 2008. 449: p. 83-91.
110. Prockop, D.J., D.G. Phinney, and B.A. Bunnell, *Mesenchymal stem cells : methods and protocols*2008, Totowa: Humana Press. xvi, 192 p.
111. Knoepfler, P.S., *Deconstructing stem cell tumorigenicity: a roadmap to safe regenerative medicine.* Stem cells, 2009. 27(5): p. 1050-6.
112. Horwitz, E.M., et al., *Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta.* Nature medicine, 1999. 5(3): p. 309-13.
113. Quarto, R., et al., *Repair of large bone defects with the use of autologous bone marrow stromal cells.* The New England journal of medicine, 2001. 344(5): p. 385-6.
114. Connolly, J.F., et al., *Autologous marrow injection for delayed unions of the tibia: a preliminary report.* Journal of orthopaedic trauma, 1989. 3(4): p. 276-82.
115. Goel, A., et al., *Percutaneous bone marrow grafting for the treatment of tibial non-union.* Injury, 2005. 36(1): p. 203-6.
116. Hernigou, P., et al., *Percutaneous autologous bone-marrow grafting for nonunions. Influence of the number and concentration of progenitor cells.* J Bone Joint Surg Am, 2005. 87(7): p. 1430-7.
117. Krzymanski, G., M. Kalczak, and W. Wiktor-Jedrzejczak, *The use of bone-marrow-derived fibroblastoid cells and fresh bone marrow in the treatment of bone defects: an experimental study.* International journal of oral and maxillofacial surgery, 1997. 26(1): p. 55-60.
118. Goulet, J.A., et al., *Autogenous iliac crest bone graft. Complications and functional assessment.* Clinical orthopaedics and related research, 1997(339): p. 76-81.
119. Banwart, J.C., M.A. Asher, and R.S. Hassanein, *Iliac crest bone graft harvest donor site morbidity. A statistical evaluation.* Spine, 1995. 20(9): p. 1055-60.
120. Fernyhough, J.C., et al., *Chronic donor site pain complicating bone graft harvesting from the posterior iliac crest for spinal fusion.* Spine, 1992. 17(12): p. 1474-80.
121. Bhargava, R., et al., *Percutaneous autologus bone marrow injection in the treatment of delayed or nonunion.* Indian journal of orthopaedics, 2007. 41(1): p. 67-71.
122. Seebach, C., et al., *Number and proliferative capacity of human mesenchymal stem cells are modulated positively in multiple trauma patients and negatively in atrophic nonunions.* Calcif Tissue Int, 2007. 80(4): p. 294-300.
123. Shen, B., et al., *The role of BMP-7 in chondrogenic and osteogenic differentiation of human bone marrow multipotent mesenchymal stromal cells in vitro.* J Cell Biochem, 2010. 109(2): p. 406-16.
124. Alm, J.J., et al., *Circulating plastic adherent mesenchymal stem cells in aged hip fracture patients.* Journal of orthopaedic research : official publication of the Orthopaedic Research Society, 2010. 28(12): p. 1634-42.
125. Mansilla, E., et al., *Bloodstream cells phenotypically identical to human*

mesenchymal bone marrow stem cells circulate in large amounts under the influence of acute large skin damage: new evidence for their use in regenerative medicine. Transplant Proc, 2006. 38(3): p. 967-9.

126. Risau, W. and I. Flamme, *Vasculogenesis.* Annual review of cell and developmental biology, 1995. 11: p. 73-91.
127. da Silva Meirelles, L., P.C. Chagastelles, and N.B. Nardi, *Mesenchymal stem cells reside in virtually all post-natal organs and tissues.* Journal of cell science, 2006. 119(Pt 11): p. 2204-13.
128. Zvaifler, N.J., et al., *Mesenchymal precursor cells in the blood of normal individuals.* Arthritis research, 2000. 2(6): p. 477-88.
129. Lefevre, S., et al., *Synovial fibroblasts spread rheumatoid arthritis to unaffected joints.* Nature medicine, 2009. 15(12): p. 1414-20.
130. Neumann, E., et al., *Migratory potential of rheumatoid arthritis synovial fibroblasts: Additional perspectives.* Cell cycle, 2010. 9(12).
131. Ojeda-Uribe, M., et al., *Failure to detect spindle-shaped fibroblastoid cell progenitors in PBPC collections.* Acta haematologica, 1993. 90(3): p. 139-43.
132. Hughes, S.P., I.L. Reichert, and I.D. McCarthy, *Biological effects of intramedullary reaming.* The Journal of bone and joint surgery. British volume, 1993. 75(6): p. 845-7.
133. Wozasek, G.E., et al., *Intramedullary pressure changes and fat intravasation during intramedullary nailing: an experimental study in sheep.* The Journal of trauma, 1994. 36(2): p. 202-7.
134. Pape, H.C., et al., *Reamed femoral nailing in sheep: does irrigation and aspiration of intramedullary contents alter the systemic response?* The Journal of bone and joint surgery. American volume, 2005. 87(11): p. 2515-22.
135. Higgins, T.F., V. Casey, and K. Bachus, *Cortical heat generation using an irrigating/aspirating single-pass reaming vs conventional stepwise reaming.* Journal of orthopaedic trauma, 2007. 21(3): p. 192-7.
136. Porter, R.M., et al., *Osteogenic potential of reamer irrigator aspirator (RIA) aspirate collected from patients undergoing hip arthroplasty.* J Orthop Res, 2009. 27(1): p. 42-9.
137. Henrich, D., et al., *RIA reamings and hip aspirate: a comparative evaluation of osteoprogenitor and endothelial progenitor cells.* Injury, 2010. 41 Suppl 2: p. S62-8.
138. Belthur, M.V., et al., *Bone graft harvest using a new intramedullary system.* Clinical orthopaedics and related research, 2008. 466(12): p. 2973-80.
139. Hak, D.J. and J.L. Pittman, *Biological rationale for the intramedullary canal as a source of autograft material.* The Orthopedic clinics of North America, 2010. 41(1): p. 57-61; table of contents.
140. Giannoudis, P.V., C. Tzioupis, and J. Green, *Surgical techniques: how I do it? The Reamer/Irrigator/Aspirator (RIA) system.* Injury, 2009. 40(11): p. 1231-6.
141. Kobbe, P., I.S. Tarkin, and H.C. Pape, *Use of the 'reamer irrigator aspirator' system for non-infected tibial non-union after failed iliac crest grafting.* Injury, 2008. 39(7): p. 796-800.
142. Newman, J.T., et al., *A new minimally invasive technique for large volume bone graft harvest for treatment of fracture nonunions.* Orthopedics, 2008. 31(3): p. 257-61.
143. Miller, M.A., et al., *Autologous bone grafting on steroids: preliminary clinical results. A novel treatment for nonunions and segmental bone defects.* International orthopaedics, 2011. 35(4): p. 599-605.
144. McCall, T.A., et al., *Treatment of large segmental bone defects with reamer-irrigator-aspirator bone graft: technique and case series.* The Orthopedic clinics of North America, 2010. 41(1): p. 63-73; table of contents.
145. Urist, M.R., *Bone: formation by autoinduction.* Science, 1965. 150(698): p. 893-9.

146. White, A.P., et al., *Clinical applications of BMP-7/OP-1 in fractures, nonunions and spinal fusion.* Int Orthop, 2007. 31(6): p. 735-41.
147. Bishop, G.B. and T.A. Einhorn, *Current and future clinical applications of bone morphogenetic proteins in orthopaedic trauma surgery.* Int Orthop, 2007. 31(6): p. 721-7.
148. Dimitriou, R., E. Tsiridis, and P.V. Giannoudis, *Current concepts of molecular aspects of bone healing.* Injury, 2005. 36(12): p. 1392-404.
149. Yasko, A.W., et al., *The healing of segmental bone defects, induced by recombinant human bone morphogenetic protein (rhBMP-2). A radiographic, histological, and biomechanical study in rats.* J Bone Joint Surg Am, 1992. 74(5): p. 659-70.
150. Marden, L.J., et al., *Recombinant human bone morphogenetic protein-2 is superior to demineralized bone matrix in repairing craniotomy defects in rats.* J Biomed Mater Res, 1994. 28(10): p. 1127-38.
151. Makino, T., et al., *Prevention of atrophic nonunion development by recombinant human bone morphogenetic protein-7.* Journal of orthopaedic research : official publication of the Orthopaedic Research Society, 2005. 23(3): p. 632-8.
152. Friedlaender, G.E., et al., *Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions.* J Bone Joint Surg Am, 2001. 83-A Suppl 1(Pt 2): p. S151-8.
153. Bong, M.R., et al., *Osteogenic protein-1 (bone morphogenic protein-7) combined with various adjuncts in the treatment of humeral diaphyseal nonunions.* Bulletin, 2005. 63(1-2): p. 20-3.
154. Moghaddam, A., et al., *Clinical application of BMP 7 in long bone non-unions.* Arch Orthop Trauma Surg, 2010. 130(1): p. 71-6.
155. Guo, X. and X.F. Wang, *Signaling cross-talk between TGF-beta/BMP and other pathways.* Cell research, 2009. 19(1): p. 71-88.
156. Moustakas, A. and C.H. Heldin, *Non-Smad TGF-beta signals.* Journal of cell science, 2005. 118(Pt 16): p. 3573-84.
157. Walsh, D.W., et al., *Extracellular BMP-antagonist regulation in development and disease: tied up in knots.* Trends in cell biology, 2010. 20(5): p. 244-56.
158. Okamoto, M., et al., *Bone morphogenetic proteins in bone stimulate osteoclasts and osteoblasts during bone development.* J Bone Miner Res, 2006. 21(7): p. 1022-33.
159. Connolly, J.F., et al., *Autologous marrow injection as a substitute for operative grafting of tibial nonunions.* Clin Orthop Relat Res, 1991(266): p. 259-70.
160. Jager, M., et al., *Bridging the gap: bone marrow aspiration concentrate reduces autologous bone grafting in osseous defects.* Journal of orthopaedic research : official publication of the Orthopaedic Research Society, 2011. 29(2): p. 173-80.
161. Wenda, K., et al., *Pathogenesis and clinical relevance of bone marrow embolism in medullary nailing--demonstrated by intraoperative echocardiography.* Injury, 1993. 24 Suppl 3: p. S73-81.
162. Kropfl, A., et al., *Intramedullary pressure and bone marrow fat extravasation in reamed and unreamed femoral nailing.* Journal of orthopaedic research : official publication of the Orthopaedic Research Society, 1999. 17(2): p. 261-8.
163. Giannoudis, P.V., et al., *Does mobilisation and transmigration of mesenchymal stem cells occur after trauma?* Injury, 2010. 41(11): p. 1099-102.
164. Wagner, W., et al., *Aging and replicative senescence have related effects on human stem and progenitor cells.* PloS one, 2009. 4(6): p. e5846.
165. Veyrat-Masson, R., et al., *Mesenchymal content of fresh bone marrow: a proposed quality control method for cell therapy.* British journal of haematology, 2007. 139(2): p. 312-20.
166. Castro-Malaspina, H., et al., *Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny.* Blood, 1980. 56(2): p. 289-301.
167. Muraglia, A., R. Cancedda, and R. Quarto, *Clonal mesenchymal progenitors*

from human bone marrow differentiate in vitro according to a hierarchical model. Journal of cell science, 2000. 113 (Pt 7): p. 1161-6.

168. Kuznetsov, S.A., et al., *Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo.* Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research, 1997. 12(9): p. 1335-47.
169. Kwong, F.N., et al., *Altered relative expression of BMPs and BMP inhibitors in cartilaginous areas of human fractures progressing towards nonunion.* Journal of orthopaedic research : official publication of the Orthopaedic Research Society, 2009. 27(6): p. 752-7.
170. Hofmann, A., et al., *Cell viability, osteoblast differentiation, and gene expression are altered in human osteoblasts from hypertrophic fracture non-unions.* Bone, 2008. 42(5): p. 894-906.
171. Gilbert, L., et al., *Expression of the osteoblast differentiation factor RUNX2 (Cbfa1/AML3/Pebp2alpha A) is inhibited by tumor necrosis factor-alpha.* The Journal of biological chemistry, 2002. 277(4): p. 2695-701.
172. Calori, G.M., et al., *Application of rhBMP-7 and platelet-rich plasma in the treatment of long bone non-unions: a prospective randomised clinical study on 120 patients.* Injury, 2008. 39(12): p. 1391-402.
173. O'hEireamhoin, S., J.F. Quinlan, and K.O. Rourke, *The use of bone morphogenetic protein 7 in fracture non-unions.* Orthopaedic Surgery, 2011. 3(1): p. 40-44.
174. Zhi, L., et al., *Synergistic effect of recombinant human bone morphogenic protein-7 and osteogenic differentiation medium on human bone-marrow-derived mesenchymal stem cells in vitro.* International orthopaedics, 2011.
175. Shea, C.M., et al., *BMP treatment of C3H10T1/2 mesenchymal stem cells induces both chondrogenesis and osteogenesis.* J Cell Biochem, 2003. 90(6): p. 1112-27.
176. Lavery, K., et al., *New insights into BMP-7 mediated osteoblastic differentiation of primary human mesenchymal stem cells.* Bone, 2009. 45(1): p. 27-41.
177. Boyan, B.D., et al., *Initial effects of partially purified bone morphogenetic protein on the expression of glycosaminoglycan, collagen, and alkaline phosphatase in nonunion cell cultures.* Clinical orthopaedics and related research, 1992(278): p. 286-304.
178. Baxter, M.A., et al., *Study of telomere length reveals rapid aging of human marrow stromal cells following in vitro expansion.* Stem cells, 2004. 22(5): p. 675-82.
179. Tsuji, K., et al., *Conditional deletion of BMP7 from the limb skeleton does not affect bone formation or fracture repair.* Journal of orthopaedic research : official publication of the Orthopaedic Research Society, 2010. 28(3): p. 384-9.
180. Burastero, G., et al., *The association of human mesenchymal stem cells with BMP-7 improves bone regeneration of critical-size segmental bone defects in athymic rats.* Bone, 2010. 47(1): p. 117-26.
181. Neumann, K., et al., *BMP7 promotes adipogenic but not osteo-/chondrogenic differentiation of adult human bone marrow-derived stem cells in high-density micro-mass culture.* Journal of cellular biochemistry, 2007. 102(3): p. 626-37.
182. Lu, C., et al., *Recombinant human bone morphogenetic protein-7 enhances fracture healing in an ischemic environment.* Journal of orthopaedic research : official publication of the Orthopaedic Research Society, 2010. 28(5): p. 687-96.
183. Kloen, P., et al., *Expression and activation of the BMP-signaling components in human fracture nonunions.* The Journal of bone and joint surgery. American volume, 2002. 84-A(11): p. 1909-18.
184. Dimitriou, R., et al., *The role of inhibitory molecules in fracture healing.* Injury, 2006. 37 Suppl 1: p. S20-9.
185. Eghbali-Fatourechi, G.Z., et al., *Circulating osteoblast-lineage cells in humans.*

N Engl J Med, 2005. 352(19): p. 1959-66.
186. Blazsek, I., J. Chagraoui, and B. Peault, *Ontogenic emergence of the hematon, a morphogenetic stromal unit that supports multipotential hematopoietic progenitors in mouse bone marrow.* Blood, 2000. 96(12): p. 3763-71.
187. Huang, B.K., J.U. Monu, and J. Wandtke, *Pulmonary fat embolism after pelvic and long bone fractures in a trauma patient.* Emerg Radiol, 2009. 16(5): p. 407-9.
188. Kumagai, K., et al., *Circulating cells with osteogenic potential are physiologically mobilized into the fracture healing site in the parabiotic mice model.* Journal of orthopaedic research : official publication of the Orthopaedic Research Society, 2008. 26(2): p. 165-75.
189. Muller, C., et al., *The incidence, pathogenesis, diagnosis, and treatment of fat embolism.* Orthopaedic review, 1994. 23(2): p. 107-17.
190. Galotto, M., et al., *Stromal damage as consequence of high-dose chemo/radiotherapy in bone marrow transplant recipients.* Experimental hematology, 1999. 27(9): p. 1460-1466.

CHAPTER 9

APPENDICES

9.1 APPENDIX 1: ETHICS APPROVALS

The Leeds Teaching Hospitals NHS
NHS Trust

Research & Development Directorate
A/B Corridor, Old Site

The General Infirmary at Leeds
Great George Street
Leeds
West Yorkshire
LS1 3EX

Tel: 0113 392 2878
Fax: 0113 392 6397

www.leedsteachinghospitals.com

25 January 2007

Professor Giannoudis
Consultant Orthopaedic Surgeon
Trauma & Orthopaedic Academic Unit
St. James's University Hospital

Dear Professor Giannoudis

Re: LTHT R&D Approval of Project No OR06/7651 Biological properties of Mesenchymal Stem Cells in Fracture Healing

I write with reference to the above research study. I can now confirm that this study has R&D approval and the study may proceed at The Leeds Teaching Hospitals NHS Trust (LTHT). This organisational level approval is given based on the information provided in the NHS REC Application Form and NHS R&D Application Form.

As principal investigator you have responsibility for the design, management and reporting of the study. In undertaking this research you must comply with the requirements of the *Research Governance Framework for Health and Social Care* which is mandatory for all NHS employees. This document may be accessed on the Department of Health website at http://www.dh.gov.uk/research

R&D approval is therefore given on the understanding that you comply with the requirements of the *Framework* as listed in the attached sheet "Conditions of Approval".

If you have any queries about this approval please do not hesitate to contact the R&D Department on telephone 0113 392 2878.

Indemnity Arrangements

The Leeds Teaching Hospitals NHS Trust participates in the NHS risk pooling scheme administered by the NHS Litigation Authority 'Clinical Negligence Scheme for NHS Trusts' for: (i) medical professional and/or medical malpractice liability; and (ii) general liability. NHS Indemnity for negligent harm is extended to researchers with an employment contract (substantive or honorary) with the Trust. The Trust

only accepts liability for research activity that has been managerially approved by the R&D Department.

The Trust therefore accepts liability for the above research project and extends indemnity for negligent harm to cover you as principal investigator and the researchers listed on the R&D approval form provided that each member of the research team has an employment contract (substantive or honorary) with the Trust. Should there be any changes to the research team please ensure that you inform the R&D Department and that s/he obtains an employment contract with the Trust if required.

Yours sincerely

Dr D R Norfolk
Associate Director of R&D

Approved documents
The documents reviewed and approved are listed as follows

Document	*Version*	*Date of document*
Protocol		19/09/2006
R&D Form		02/09/2006
CMT Approval		22/09/2006
NHS REC Application Form		14/07/2006

Cc: Dr Pountos

The Leeds Teaching Hospitals NHS
NHS Trust

Leeds (East) Research Ethics Committee
Room 5.2, Clinical Sciences Building
St James's University Hospital
Beckett Street
Leeds
LS9 7TF

Tel: 0113 2065652
Fax: 0113 2066772

27 April 2009

Dr Ann Morgan
HEFCE- Clinical Senior Lecturer
Honorary Consultant Rheumatologist
The University of Leeds
St James Hospital
Leeds
LS9 7TF

Dear Dr Morgan

Study title:	**Functional characterisation of the genes and proteins involved in the development and severity of autoimmune and (auto)inflammatory diseases**
REC reference:	**04/Q1206/107**
Amendment number:	**5**
Amendment date:	**26 March 2009**

Thank you for your email of 23 April 2009, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered in correspondence by a sub-committee of the REC.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation, as revised.

Documents approved

The documents reviewed and approved were:

Document	Version	Date
Participant Information Sheet: Healthy Controls	3	26 March 2009
Participant Information Sheet: Disease Controls	3	26 March 2009
Participant Information Sheet: Patients	3	26 March 2009
Notice of Substantial Amendment (non-CTIMPs)		26 March 2009

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

04/Q1206/107:	**Please quote this number on all correspondence**

Yours sincerely

Ann Tunley
Committee Co-ordinator

E-mail: ann.tunley@leedsth.nhs.uk

Copy to: R&D Office for Leeds Teaching Hospitals NHS Trust

9.2 APPENDIX 2: PATIENT INFORMATION SHEETS & CONSENT FORMS

The Leeds Teaching Hospitals NHS
NHS Trust

PATIENT INFORMATION SHEET

Collection of Bone, Bone Marrow & Blood

You are being invited to take part in a RESEARCH study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with friends, relatives, and your GP if you wish. Ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled "medical Research and You". This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from your study doctor.

Thank you for reading this.

1. What is the purpose of the study?
Special cells in the body termed Mesenchymal Stem Cells (MSCs) can make bone, cartilage, bone, muscle, tendon and ligament. MSCs have been found in all tissues to date but just how they work is still not understood. There is interest in the use of MSCs as a way of repairing damaged joints. Our research is aimed at understanding how these cells work in health and in disease.

2. Why have I been chosen?
You suffer from a fractured bone. At surgery we would like to take a small sample of your bone and bone marrow from the fracture site. This tissue is sitting at the fracture site and is normally discarded during the operation when the fracture site is being cleaned and repaired.

Taking bone, bone marrow and blood at the time of surgery will not cause you further discomfort and will not delay healing in any way.

3. Do I have to take part?
It is up to you to decide whether or not to take part. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care that you receive.

4. What will happen to me if I take part?
If you decide to take part you will be asked to sign an informed consent sheet, and you will be given a copy of the information and the signed consent sheet to keep. You will be asked to donate a small amount of bone marrow normally discarded during your operation.

5. What do I have to do?
Apart from donating a small amount of bone, bone marrow and blood for research, there are no other requirements/tests.

It is very important, when giving your medical history to the doctor, that you tell him/her whether or not you are regularly taking any medicine. Taking certain types of medicines means we will not be able to recruit you into the study.

6. What are the side effects of taking part?
There are no side effects. The collection of the bone, bone marrow and blood is of limited quantity.

7. What are the possible benefits of taking part?
You will not benefit directly from taking part in this research and all other aspects of your care will be the same as if you did not take part.

8. What if something goes wrong?
If you are harmed by taking part in this research, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal NHS complaints mechanism may be available to you.

9. Will my taking part in this study be kept confidential?
As soon as the bone, bone marrow and blood are taken from you, all information that identifies you will be removed so that you cannot be recognised.

10. What will happen to the results of the study?
At the end of the study, the results will be written into a scientific paper for publication in a scientific journal.

11. Who is organising the research?
This project is being organized by Doctors of Leeds General Infirmary and Leeds Institute Molecular Medicine under the supervision and support of Leeds University.

12. Who has reviewed this study?
This study has been reviewed by the independent ethics committee called the Leeds (East) ethics committee. This committee is appointed to determine that research studies are ethical and do not impair the rights or well-being of patients. We have received approval by this committee to be able to do this research study.

13. Contact for further information
Please do not hesitate to contact your GP or any other independent person if you need advice. For further information on the study please contact Professor Peter Giannoudis at 0113 3466460.

The Leeds Teaching Hospitals NHS
NHS Trust

PATIENT CONSENT FORM

Collection of Bone, Bone Marrow & Blood

Prof Peter Giannoudis

Patient Name: .....

Patient Identification Number: .....

Please circle as appropriate

1. I have read the patient information sheet for the above study. **Yes/No**

2. I have had the opportunity to ask questions about the study and to discuss it with family and friends if I so wish to. **Yes/No**

3. I understand the purpose of the study, and how I will be involved. **Yes/No**

4. I understand, and accept, that if I take part in the study I may not gain direct personal benefit from it. **Yes/No**

5. I understand that all information collected in the study will be held in confidence and that, if it is presented or published, all my personal details will be removed. **Yes/No**

6. I give permission for responsible individuals from regulatory authorities to have access to my medical notes where it is relevant to my taking part in the research. This is on the understanding that no personal details which might identify me will be presented or published without my permission. **Yes/No**

7. I confirm that I will be taking part in this study of my own free will, and I understand that I am free to withdraw from the study at any time without giving a reason and without affecting my future care or legal rights. **Yes/No**

8. I have spoken to Dr .....

9. I agree to take part in this research study.

PATIENT:
Signed: ..... **Date:** .....

Name (BLOCK CAPITALS): .....

Investigator/Sub-investigator

I have explained the study to the above named participant and he/she has indicated his/her willingness to participate

Signed: ..... **Date:** .....

Name (BLOCK CAPITALS): .....

The Leeds Teaching Hospitals NHS
NHS Trust

PATIENT INFORMATION SHEET

Functional characterisation of the genes and proteins involved in the development and severity of autoimmune and (auto) inflammatory diseases.

PART 1

1. Invitation

You are being invited to take part in a research study. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully, and discuss it with others if you wish.

PART 1 tells you the purpose of this study and what will happen to you if you take part.

PART 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

2. What is the purpose of the study?

Many autoimmune and (auto)inflammatory diseases, such as rheumatoid arthritis, systemic lupus erythematosus, vasculitis and the connective tissue diseases are associated with the presence of specific changes in either an individual's genetic makeup or their immune system. This can lead to alterations in the different components of the immune system or in the proteins that are produced by these genes. We feel these changes may be important for the development of either the disease itself, specific antibodies/ complications or that they may even predispose to or even help us predict more severe disease. We would like to perform some further research to gain a better understanding of their biology and how they may contribute to these various diseases.

3. Why have I been chosen?

You have been chosen because you have a specific autoimmune or inflammatory disease that we are interested in studying further. Alternatively, you may have donated a sample for one of our previous studies and we have already characterised some of your genes. In this study we would like to use your white blood cells to determine if this genetic variant affects the way your cells work. We would also like to store some of your white blood cells and additional components of your blood, such as the proteins and a sample of your DNA and urine. Otherwise, you may be having some fluid drained from one of your joints, an arthroscopy or biopsy to help with the diagnosis of your condition, or as part of your treatment. We would like to be able to use this fluid and/or any surplus tissue samples, which would otherwise be thrown away, for research purposes. These samples will only be used in future studies that continue with this agreed line of research. In order to give us a permanent source of specific genes and proteins we may also like to make some cell-lines from specific proteins or cells from your blood in the laboratory.

4. Do I have to take part?
It is up to you to decide whether or not to take part. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

5. What will happen to me if I take part?

If you decide to take part you will asked to sign an informed consent sheet, and you will be given a copy of the information sheet to keep. You will be asked to donate a sample of blood (up to 50mls), which will be taken at the same time as your routine clinic blood tests. Alternatively, we may ask for a sample of your urine or the ability to use the surplus joint fluid or tissue removed as part of your routine NHS treatment. Taking part will therefore not involve any extra visits to the clinic.

The blood sample will be used for genetic testing, but this is purely for research purposes and you will not be told the results of the tests on your samples. Insurance companies, however, may ask you whether you have previously had genetic tests. Should this situation arise, we advise you to answer "no" in your insurance policy application form. This is because the genetic tests we are doing, are purely for research purposes and have no bearing whatsoever on your current or future insurance policies.

6 What do I have to do?
Apart from donating a small volume of blood for research, there are no other requirements/ tests.

7. What are the side effects of any treatment received when taking part?
You may develop a bruise at the site of the needle but, as stated above, taking part in this study does not involve any additional blood tests.

8. What are the possible benefits of taking part?
You will not benefit directly from taking part in this research, and all other aspects of your care will be the same as if you did not take part.

9. What if there is a problem?
If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your question. **If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.**

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

10. Will my taking part in this study be kept confidential?
Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

11. Contact Details

Please do not hesitate to contact your GP or any other independent person if you need advice. For further information on the study please contact Dr Ann W Morgan or Professor Dennis McGonagle on extension 0113 206 5117.

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

PART 2

12. Will my part in this study be kept confidential?

If you consent to take part in this study, the records obtained while you are in this study as well as related health records will remain strictly confidential at all times. The information will be held securely on paper and electronically at your treating hospital and the University of Leeds, the main team managing this research under the provisions of the 1998 Data Protection Act. Your name will not be passed to anyone else outside the research team or the sponsor, who is not involved in the research.

Information will be transferred from your hospital site to the team at the University of Leeds organizing the research, to enable processing of blood samples, this will be done by mail, however your name will only appear on your consent form and blood sample until it is separated and stored. All other records will have your name removed and will only feature your initials and date of birth.

Your records will be available to people authorised to work on the trial but may also need to be made available to people authorised by the Research Sponsor, which is the organisation responsible for ensuring that the study is carried out correctly. A copy of your consent form may be sent to the Research Sponsor during the course of the study. By signing the consent form you agree to this access for the current study and any further research that may be conducted in relation to it, even if you withdraw from the current study.

The information collected about you may also be shown to authorised people from the UK Regulatory Authority and Independent Ethics Committee; this is to ensure that the study is carried out to the highest possible scientific standards. All will have a duty of confidentiality to you as a research participant.

In line with Good Clinical Practice guidelines, at the end of the study, your data will be securely archived for a minimum of 15 years. Arrangements for confidential destruction will then be made.

20. What will happen to any samples I give?

Once it is sent to the University of Leeds, your blood sample will anonymised and separated into its component parts so that some of your DNA, serum and cells will be stored to form part of our "disease sample repository" so that we can use it in current and future genetic and immunological studies. Any additional studies will be subject to additional independent ethical committee review.

21. Will any Genetic testing be done?

Yes, once anonymised your DNA will be used in genetic testing aimed at finding out which genes are important in both the development and severity of a number of autoimmune and (auto)inflammatory diseases. Your DNA will only be used in future studies which continue the research themes outlined above.

22. What will happen to the results of this research?

The results of the studies using the "disease sample repository" will usually be published in a medical journal or be presented at a scientific conference. The data will be anonymous and none of the patients involved in the study will be identified in any report or publication.

23. Who is organising and funding this research?

This project is partly being funded by grants from the National Institute for Health Research, Arthritis Research Campaign, Research into Ageing and the University of Leeds.

24. Who has reviewed the study?

This study was given favourable ethical opinion for conduct in the NHS by Leeds (East) Research Ethics Committee. This committee is appointed to determine that research studies are ethical and do not impair the rights or well-being of patients. We have received approval by this committee to be able to do this research study.

25. Contact for further information

You are encouraged to ask any questions you wish, before, during or after your treatment. If you have any questions about the study, please speak to your study nurse or doctor, who will be able to provide you with up to date information about the procedure(s) involved. If you wish to read the research on which this study is based, please ask your study nurse or doctor. If you require any further information or have any concerns while taking part in the study please contact one of the following people:

Dr Ann W Morgan or Professor Dennis McGonagle on 0113 2065117

Alternatively if you or your relatives have any questions about this study you may wish to contact your GP or an organisation that is independent of the hospital at which you are being treated:

Arthritis Research Campaign (***arc***) is a registered charity providing information about all aspects of arthritis for patients and their families. They can provide useful booklets. You can contact them on 0870 850 5000, or access their web site at http://www.arc.org.uk

If you decide you would like to take part then please read and sign the consent form. You will be given a copy of this information sheet and the consent form to keep. A copy of the consent form will be filed in your patient notes, one will be filed with the study records and one may be sent to the Research Sponsor.

You can have more time to think this over if you are at all unsure.

Thank you for taking the time to read this information sheet and to consider this study.

The Leeds Teaching Hospitals NHS
NHS Trust

PATIENT CONSENT FORM

Functional characterisation of the genes and proteins involved in the development and severity of autoimmune and (auto) inflammatory diseases.

Patient ID: ..... Initials: ..... Date of Birth: .....

Patient initial each point

1. I confirm that I have read and understand the information sheet dated 26.03.2009 (version 3.0) for the above study, and have had the opportunity to ask questions. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. I agree to take part in the study. _____

2. I understand that my medical records may be looked at by authorised individuals from the Sponsor for the study, the UK Regulatory Authority or the Independent Ethics Committee in order to check that the study is being carried out correctly. I give permission, provided that strict confidentiality is maintained, for these bodies to have access to my medical records for the above study and any further research that may be conducted in relation to it. I also give permission for a copy of my consent form to be sent to the Sponsor for the study. _____

3. I understand that even if I withdraw from the above study, the data and samples collected from me will be used in analysing the results of the study, unless I specifically withdraw consent for this. I understand that my identity will remain anonymous. _____

4. I consent to the storage including electronic, of personal information for the purposes of this study. I understand that any information that could identify me will be kept strictly confidential and that no personal information will be included in the study report or other publication. _____

5. I agree to the samples and cell lines being stored for future research. _____

6. I agree to have genetic tests done on samples for research purposes. _____

Name of the patient	Patient's signature and the date the patient signed the Consent form
Name of the Investigator taking written consent	Investigator's signature and date the Investigator signed the consent form

Original to be retained and filed in the site file. 1 copy to patient, 1 copy to be filed in patient's notes, 1 copy for Sponsor

The Leeds Teaching Hospitals NHS
NHS Trust

PATIENT INFORMATION SHEET (HEALTHY CONTROLS)

Functional characterisation of the genes and proteins involved in the development and severity of autoimmune and (auto) inflammatory diseases.

PART 1

1. Invitation

You are being invited to take part in a research study. Before you decide whether or not to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully, and discuss it with others if you wish.

PART 1 tells you the purpose of this study and what will happen to you if you take part.

PART 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

2. What is the purpose of the study?

Many autoimmune and (auto)inflammatory diseases, such as rheumatoid arthritis, systemic lupus erythematosus, vasculitis and the connective tissue diseases are associated with the presence of specific changes in either an individual's genetic makeup or their immune system. This can lead to alterations in the different components of the immune system or in the proteins that are produced by these genes. We feel these changes may be important for the development of either the disease itself, specific antibodies/ complications or that they may even predispose to or even help us predict more severe disease. We would like to perform some further research to gain a better understanding of their biology and how they may contribute to these various diseases.

3. Why have I been chosen?

In studies of this type we need to compare the genes, proteins and components of the immune system in people with the diseases we are interested in compared to those people of the same age who do not have the disease ("controls"). You have been identified as someone who is unlikely to have one of these conditions and we would like to store some of your blood, such as the white blood cells, proteins and sample of your DNA, and/ or a urine sample to form part of a "control" sample bank. These samples will only be used in future studies that continue with this agreed line of research.

4. Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason.

5. What will happen to me if I take part?
If you decide to take part you will asked to sign an informed consent sheet, and you will be given a copy of the information sheet to keep. You will be asked to donate a sample of blood (up to 50mls). Alternatively, we may ask for a sample of your urine.

The blood sample will be used for genetic testing, but this is purely for research purposes and you will not be told the results of the tests on your samples. Insurance companies, however, may ask you whether you have previously had genetic tests. Should this situation arise, we advise you to answer "no" in your insurance policy application form. This is because the genetic tests we are doing, are purely for research purposes and have no bearing whatsoever on your current or future insurance policies.

6. What do I have to do?
Apart from donating a small volume of blood for research, there are no other requirements/ tests.

7. What are the side effects of any treatment received when taking part?
You may develop a bruise at the site of the needle but, as stated above, taking part in this study does not involve any additional blood tests.

8. What are the possible benefits of taking part?
You will not benefit directly from taking part in this research, and all other aspects of your care will be the same as if you did not take part.

9. What if there is a problem?
If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your question. **If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.**

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

10. Will my taking part in this study be kept confidential?
Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

11. Contact Details
Please do not hesitate to contact your GP or any other independent person if you need advice. For further information on the study please contact Dr Ann W Morgan (0113 3438414) or Dr Dawn Cooper or Dr Sarah Mackie (0113 3438413).

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

PART 2

12. Will my part in this study be kept confidential?

If you consent to take part in this study, the records obtained while you are in this study as well as related health records will remain strictly confidential at all times. The information will be held securely on paper and electronically at your treating hospital and the University of Leeds, the main team managing this research under the provisions of the 1998 Data Protection Act. Your name will not be passed to anyone else outside the research team or the sponsor, who is not involved in the research.

Information will be transferred from your hospital site to the team at the University of Leeds organizing the research, to enable processing of blood samples, this will be done by mail, however your name will only appear on your consent form and blood sample until it is separated and stored. All other records will have your name removed and will only feature your initials and date of birth.

Your records will be available to people authorised to work on the trial but may also need to be made available to people authorised by the Research Sponsor, which is the organisation responsible for ensuring that the study is carried out correctly. A copy of your consent form may be sent to the Research Sponsor during the course of the study. By signing the consent form you agree to this access for the current study and any further research that may be conducted in relation to it, even if you withdraw from the current study.

The information collected about you may also be shown to authorised people from the UK Regulatory Authority and Independent Ethics Committee; this is to ensure that the study is carried out to the highest possible scientific standards. All will have a duty of confidentiality to you as a research participant.

In line with Good Clinical Practice guidelines, at the end of the study, your data will be securely archived for a minimum of 15 years. Arrangements for confidential destruction will then be made.

20. What will happen to any samples I give?

Once it is sent to the University of Leeds, your blood sample will anonymised and separated into its component parts so that some of your DNA, serum and cells will be stored to form part of our "disease sample repository" so that we can use it in current and future genetic and immunological studies. Any additional studies will be subject to additional independent ethical committee review.

21. Will any Genetic testing be done?

Yes, once anonymised your DNA will be used in genetic testing aimed at finding out which genes are important in both the development and severity of a number of autoimmune and (auto)inflammatory diseases. Your DNA will only be used in future studies which continue the research themes outlined above.

22. What will happen to the results of this research?

The results of the studies using the "disease sample repository" will usually be published in a medical journal or be presented at a scientific conference. The data will be anonymous and none of the patients involved in the study will be identified in any report or publication.

23. Who is organising and funding this research?

This project is partly being funded by grants from the National Institute for Health Research, Arthritis Research Campaign, Research into Ageing and the University of Leeds.

24. Who has reviewed the study?

This study was given favourable ethical opinion for conduct in the NHS by Leeds (East) Research Ethics Committee. This committee is appointed to determine that research studies are ethical and do not impair the rights or well-being of patients. We have received approval by this committee to be able to do this research study.

25. Contact for further information

You are encouraged to ask any questions you wish, before, during or after your treatment. If you have any questions about the study, please speak to your study nurse or doctor, who will be able to provide you with up to date information about the procedure(s) involved. If you wish to read the research on which this study is based, please ask your study nurse or doctor. If you require any further information or have any concerns while taking part in the study please contact one of the following people:

Dr Ann W Morgan 0113 2065117

Alternatively if you or your relatives have any questions about this study you may wish to contact your GP or an organisation that is independent of the hospital at which you are being treated:

Arthritis Research Campaign (***arc***) is a registered charity providing information about all aspects of arthritis for patients and their families. They can provide useful booklets. You can contact them on 0870 850 5000, or access their web site at http://www.arc.org.uk

If you decide you would like to take part then please read and sign the consent form. You will be given a copy of this information sheet and the consent form to keep. A copy of the consent form will be filed in your patient notes, one will be filed with the study records and one may be sent to the Research Sponsor.

You can have more time to think this over if you are at all unsure.

Thank you for taking the time to read this information sheet and to consider this study.

The Leeds Teaching Hospitals NHS
NHS Trust

CONSENT FORM

Functional characterisation of the genes and proteins involved in the development and severity of autoimmune and (auto) inflammatory diseases.

Patient ID: ..... Initials: ..... Date of Birth: .....

Patient initial each point

1. I confirm that I have read and understand the information sheet dated 26.03.2009 (version 3.0) for the above study, and have had the opportunity to ask questions. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. I agree to take part in the study. _____

2. I understand that my medical records may be looked at by authorised individuals from the Sponsor for the study, the UK Regulatory Authority or the Independent Ethics Committee in order to check that the study is being carried out correctly. I give permission, provided that strict confidentiality is maintained, for these bodies to have access to my medical records for the above study and any further research that may be conducted in relation to it. I also give permission for a copy of my consent form to be sent to the Sponsor for the study. _____

3. I understand that even if I withdraw from the above study, the data and samples collected from me will be used in analysing the results of the study, unless I specifically withdraw consent for this. I understand that my identity will remain anonymous. _____

4. I consent to the storage including electronic, of personal information for the purposes of this study. I understand that any information that could identify me will be kept strictly confidential and that no personal information will be included in the study report or other publication. _____

5. I agree to the samples and cell lines being stored for future research. _____

6. I agree to have genetic tests done on samples for research purposes. _____

Name of the patient	Patient's signature and the date the patient signed the Consent form
Name of the Investigator taking written consent	Investigator's signature and date the Investigator signed the consent form

Original to be retained and filed in the site file. 1 copy to patient, 1 copy to be filed in patient's notes, 1 copy for Sponsor