

Title: Identifying the Optimum Source of Mesenchymal Stem Cells for Use in Knee Surgery

Running Title: MSC Sources for Knee Surgery

Authors:

Benjamin M Davies^{1*}, Sarah JB Snelling¹, Lynn Quek², Osnat Hakimi¹, Hua Ye³

Andrew Carr¹, Andrew J Price¹

1 – Nuffield Department of Orthopaedics, Rheumatology, and Musculoskeletal Sciences,
University of Oxford

2 – Weatherall Institute of Molecular Medicine, University of Oxford

3 – Institute of Biomedical Engineering, Department of Engineering Science, University of
Oxford

***Corresponding Author:**

Nuffield Department of Orthopaedics, Rheumatology, and Musculoskeletal Sciences

Botnar Research Centre

Windmill Road

Oxford, OX3 7HE

Telephone: +44 1865 227644

Fax: +44 1865 737640

Email: Benjamin.davies@ndorms.ox.ac.uk

Author Contributions:

BD, HY, AC, and AP devised the research plan.

BD, SS, LQ, and OH carried out laboratory procedures.

BD analysed the results.

All authors reviewed and approved the final manuscript.

Abstract:

Single sitting procedures where the mononuclear cell fraction is extracted from bone marrow and implanted directly into cartilage and bone defects are becoming more popular as novel treatments for cartilage defects which have, until now had few treatment options. This is on the basis that the mesenchymal stem cells (MSCs) contained within will repair the damaged tissue. This study sought to determine if the femur and tibia could provide equivalent amounts of mesenchymal stem cells, with equivalent viability and proliferative capacity, to that obtained from the gold standard of the pelvis in order to potentially reduce the morbidity associated with these procedures. Bone marrow was extracted from the pelvis, femur, and tibia of human subjects. The mononuclear cell fraction was extracted and cultured in the laboratory. Mesenchymal stem cell populations were assessed using a colony forming unit count. Viability was assessed using a PrestoBlue viability assay. Population doubling number was calculated between the end of passage 0 and passage 3 to determine the proliferative abilities of the different populations. Finally, the cell surface phenotype of the cells was determined by flow cytometry. The results showed that the pelvis was superior to the femur and tibia in terms of the number of stem cells isolated. There was no statistically significant difference in the phenotype of the cells isolated from different locations. This work shows that when undertaking single sitting procedures, the pelvis remains the optimum source for obtaining MSCs, despite the morbidity associated with bone marrow collection from the pelvis.

Keywords:

Knee, cartilage damage, mesenchymal stem cell, single stage procedure, orthopaedic surgery, human, knee stem cells, iliac crest stem cells

1. Introduction:

Musculoskeletal disorders form a large part of the worldwide burden of disease, accounting for 20.8% of all years lived with disability globally¹. Within this category, cartilage disorders are a cause of significant morbidity. Hip and knee osteoarthritis (OA) alone are the 11th greatest cause of disability worldwide² alongside earlier manifestation of cartilage damage such as osteochondral defects which may lead to more widespread cartilage damage³. There are approximately 1 million joint replacement procedures undertaken annually in the USA⁴ and a further million knee arthroscopies⁵. Cartilage defects have been identified in up to 60% of individuals undergoing knee arthroscopy⁶ and a significant number of these would be potentially amenable to some form of treatment.

A variety of different treatments have been devised for cartilage defects. Microfracture, as described by Steadman *et al.*⁷, is the longest established marrow-stimulation technique designed to repair areas of damaged cartilage. However, although microfracture is, both technically and logistically, a relatively simple procedure, it is often of limited effectiveness in the longer term with recurrence of symptoms such as pain after approximately 5 years⁸. Subsequently a number of other techniques, such as autologous chondrocyte implantation (ACI)⁹ and matrix-assisted chondrocyte implantation (MACI)¹⁰, have also been developed to improve on this efficacy. At present, although these more advanced cartilage repair techniques have shown some clinical effectiveness in a number of trials, their cost effectiveness has precluded them gaining widespread adoption. The lack of long-term information to show any superiority of ACI and MACI over microfracture is a further reason for cell based therapies to have failed to gain widespread adoption¹¹.

Against this background of a significant burden of disease with no widespread and effective treatment other than total joint arthroplasty, the use of mesenchymal stem cells (MSCs) has been seen as a potential source of new treatments.

MSCs have become the most utilized form of stem cell for developing cartilage repair treatments due to their lack of ethical concerns¹², relative ease of harvesting from tissues such as bone marrow and adipose tissue, and their ability to differentiate into chondrocytes. A number of systems have been developed to make use of bone marrow to provide a concentrated source of MSCs in point-of-care devices designed for single-stage procedures¹³⁻¹⁵. These devices are designed to take MSC containing tissue (usually bone marrow) and process it in the operating room to concentrate the MSCs into a small volume that can be used immediately in regenerative procedures.

MSCs have been successfully isolated from the bone marrow of a variety of different skeletal locations including the humerus, vertebra, pelvis, femur, tibia, and calcaneus¹⁶⁻¹⁹. However, there is still a lack of information as to the anatomical location that provides the best source of bone marrow to provide a high yield of functional MSCs. There is also variation in the differentiation ability of MSCs, with those obtained from bone marrow being more suitable for differentiation into chondrocytes than those obtained from the other easily accessible source, adipose tissue²⁰. The majority of studies have not measured the MSC yield from sites within the same individual, which is vital for accurate comparisons due to the high level of inter-individual variation²¹. The aim of this study was, therefore, to determine whether the pelvis, femur, or tibia provided the best source of MSCs in terms of both absolute yield and cellular function by carrying out intra-subject comparisons from all three locations.

2. Materials and Methods:

2.1 Subjects

Participants were recruited from individuals due to undergo total knee replacement at a single institution specializing in orthopaedic surgery. Participants were enrolled after obtaining informed consent. The study received ethical approval from a Regional Ethics Committee prior to commencement (12/SC/0484). Exclusion criteria were: a history of rheumatoid arthritis or other inflammatory arthritis, a history of septic arthritis in the limb undergoing surgery, bisphosphonate use, and oral steroid use.

2.2 Sample Collection and Processing

At the time of surgery, which took place under general anaesthetic, bone marrow aspiration was performed using the same technique from (1) the anterior superior iliac spine (through a 0.5 cm incision), (2) the tibia, and (3) the femur (through the surgical wound employed for joint replacement) in each patient recruited. Bone marrow was collected using an 8G x 15 cm Jamshidi® needle (CareFusion, San Diego, USA) and 30 ml syringe. It was then placed in an EDTA blood collection tube to prevent coagulation. A maximum of 10 mls of bone marrow was collected to avoid dilution by peripheral blood²². The marrow was diluted with RPMI media (Life Technologies, Paisley, UK) and then passed through a 70 µm filter (Fisherbrand, Loughborough, UK). A density-based centrifugation method was used to separate the mononuclear cell layer (Lymphoprep, Axis-Shield, Dundee, UK, with centrifugation at 400 g for 25 minutes) which was removed and washed with RPMI media. A cell count was then performed using a Millipore Scepter® cell counter (Merck Millipore, Billerica, MA, USA). Cells were cultured in MSC-specific tissue culture media (MesenPRO RS, Life Technologies, Paisley, UK) at 37°C and 5% CO₂ at an initial seeding density of approximately 1×10^6 cells/cm²^{22,23}. After 48 hours all non-adherent cells were removed. Remaining cells were cultured until the end of passage 3 (P3) to

provide enough cells for further experiments. Cells were plated at a density of $3\text{-}5 \times 10^3$ cells/cm² (in line with manufacturer's recommendations for the culture media used). Media was changed every 2-3 days. Cell counts were carried out with a haemocytometer at each passage (P) to enable doubling numbers to be established. Population doublings were calculated using the following formula, where N is the number of cells at the end of passage 3 and N_0 is the number of cells at the end of passage zero:

$$\text{Number of Population Doublings} = \frac{\log \frac{N}{N_0}}{\log 2}$$

2.3 CFU-F Assessment

A colony forming unit fibroblast (CFU-F) count was carried out in duplicate on an aliquot of the mononuclear cells prior to their being placed in tissue culture. Cells were seeded at a density of 1.0×10^4 per cm². These samples were maintained in 6-well plates in the same culture conditions as those cells undergoing expansion. After 14 days the media was removed and the cells fixed with methanol. Colonies were stained with Giemsa stain (VWR, Lutterworth, UK). Colonies were counted if they were ≥ 1 mm in diameter. To ensure reliability of the manual counting method three independent observers undertook CFU-F counts at 2 separate time points. Intra-class correlation coefficients (ICC) demonstrated a good degree of agreement both between and within observers (ICC 0.90, 95% CI 0.85-0.94 and ICC 0.98, 95% CI 0.97-0.99 respectively).

2.4 Growth Kinetics

At the end of passage 0 (P0), cell viability was assessed using Prestoblue (Life Technologies, Paisley, UK) according to manufacturer's protocol. An aliquot of cells were separated from those lifted at the end of P0 and were seeded at 10 000 cells/well of a 12-well plate and viability assessed at days 1, 7, and 14. Briefly, a 10% solution of PrestoBlue was made up with the MSC media. Cells were incubated in this solution for 1 hour. The solution was then removed and

fluorescence measured against a control that had been placed in a well with no cells.

Fluorescence was measured in triplicate using a FLUOstar OPTIMA platereader (BMG Labtech, Aylesbury, UK) with an excitation wavelength of 535 nm and an emission wavelength of 615 nm.

2.5 Flow Cytometry Assessment of Phenotype

At the end of passage 3 (P3) 500 000 cells were used for flow cytometry using antibodies against CD34, CD45, CD73, CD90, and CD105 (Table 1). This panel of antibodies was selected as no single antibody can successfully identify MSCs. The panel was selected on the basis of previous studies²⁴⁻²⁶ and the recommendations of the International Society for Cellular Therapy position statement²⁷. Compensation beads (BD™ CompBeads) were used to provide negative controls and carry out compensation due to the relatively homogenous nature of the populations being analysed. Samples were analysed on a BD LSRFortessa flow cytometer. Data analysis was carried out in FlowJo 10.0 (TreeStar Inc. Ashland, OR, USA).

Table 1: Flow cytometry antibody characteristics and supplier

Antibody	Target	Supplier
CD34	Cell to cell adhesion factor	Beckman Coulter, High Wycombe, UK
CD45	Protein tyrosine phosphatase, receptor type, C	BioLegend, London, UK
CD73	5'-nucleotidase	BioLegend, London, UK
CD90	Thy-1	eBioscience, San Diego, USA
CD105	Endoglin	BioLegend, London, UK

2.6 Statistics

Statistical analysis was carried out using Prism 6 (GraphPad Software Inc, La Jolla, CA, USA) except for intra-class correlation coefficients which were calculated in STATA 13 (Statacorp, College Station, TX, USA). D'Agostino-Pearson omnibus normality tests were carried out on all

results. Two-way ANOVA was used for comparisons where the samples were normally distributed. Friedman's test was used for non-parametric samples. In situations where missing data prevented the direct comparison of all three groups the Wilcoxon matched-pairs test was used to compare each pair of locations in turn. The level of significance was taken as 0.05. Where applicable error bars and ranges represent standard deviations.

3. Results:

Samples were collected from 10 individuals, with a mean age of 65.7 years ($SD=6.2$ yrs; range 55.9-77.2 yrs). There were six males and four females. The mean body mass index (BMI) was 28.3 ($SD=4.5$; range=19.9-36.5). All individuals had either a total knee replacement or a unicompartamental knee replacement for OA.

The mean volumes of aspirate obtained from the pelvis, femur, and tibia were 10.35 ml ($SD=2.68$), 4.60 ml ($SD=2.05$) and 4.95 ml ($SD=1.50$), respectively which was significantly different ($p < 0.0001$). The mean number of mononuclear cells obtained per ml of aspirate were 1.61×10^6 ($SD=0.95$), 0.72×10^6 ($SD=0.53$), and 0.63×10^6 ($SD=0.35$) from the pelvis, femur, and tibia respectively. This also represented a significant difference ($p=0.0007$).

No significant correlations were identified between amount of aspirate obtained or the number of mononuclear cells per ml and any of the demographic data available, including age.

CFU-F counts showed a significant difference between the three sites in terms of both the CFU-F per 100,000 cells and the CFU-F per ml of bone marrow aspirate, which was calculated by multiplying the CFU-F per 100,000 cells by the initial cell count divided by 100,000. This is shown in Figure 1.

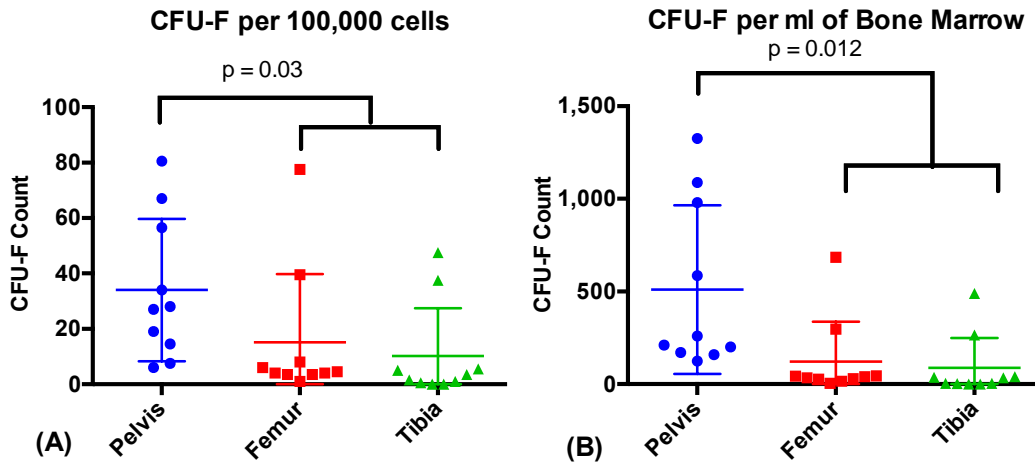


Figure 1: Colony Forming Unit-Fibroblast Counts from the three anatomical locations tested A) Per 100,000 cells and B) Adjusted to per ml of bone marrow. Bars represent mean and standard deviations

Statistical analysis showed that there was a significant difference between the different anatomical locations in both terms of CFU-F per 100,000 cells and per ml (Friedman statistic = 7.2, $p = 0.03$ and Friedman statistic = 78.6, $p = 0.012$ respectively). Two way ANOVA testing demonstrated that the individual had almost as much effect upon yield as the location sampled, although the degree of interaction between the two factors made this difficult to interpret statistically (Individual: $F(9, 10) = 112.3$, $MS = 209897$, $p < 0.0001$, Location: $F(2, 20) = 1127$, $MS = 1.102 \times 10^6$, $p < 0.0001$).

The number of population doublings between the end of P0 and P3 was not significantly different between the different locations (Figure 2). The mean number of days spent in culture between the end of P0 and P3 was 27.8 (SD 20.5). Cell yields at the P3 are shown in Table 2.

Table 2: Cell Counts at the end of P3. These values are adjusted to account for not all cells being transferred from one passage to another.

Sample	Cell Count at end of P3 (x 10 ⁶ unless otherwise stated)		
	Pelvis	Femur	Tibia
1	3.47	1.05	2.70
2	109.40	6.20	41.18
3	2.25		5.40
4	69.58	38.09	11.66
5	24.50	1.00	15.32
6	2.92	0.15	2.30
7	3.90	0.60	
8	101.8 x 10 ¹²	2.15	4.00
9	0.40	41.78	5.40
10	73.40	1.23	

PrestoBlue viability assessment showed some variation in growth curves over the 14-day period of testing but no one location was superior to another in terms of viability (Figure 3).

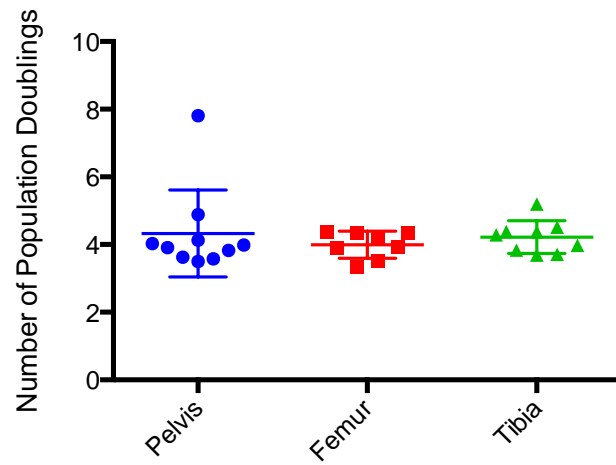


Figure 2: Number of population doublings in aspirates from the three anatomical locations tested between Passage 0 and the end of Passage 3. Bars represent mean and standard deviations.

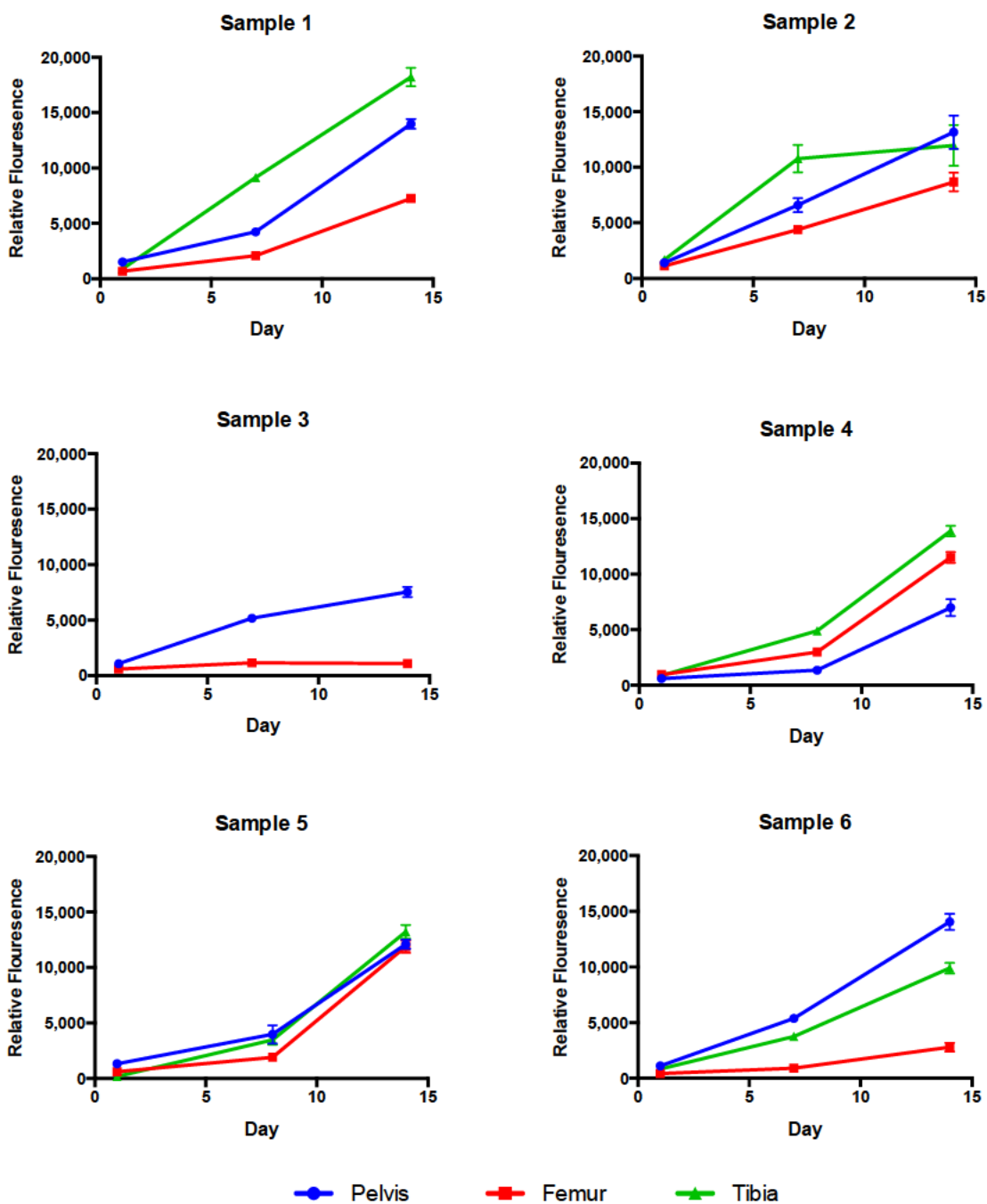


Figure 3: Biomass change in the different anatomical locations monitored using PrestoBlue viability assays. Each graph shows an individual a patient (n=6). Bars represent mean and standard deviations.

Flow cytometry of each sample at the end of P3 demonstrated relatively consistent expression of CD73, CD90, and CD105 in all samples with co-efficients of variation below 10% for all groups

except CD90 pelvic samples where one individual's very low level of expression led to a CoV of 44.02%. CD34 and CD45 expression was more variable as shown in Table 3, with CoVs between 20% and 90%.

Table 3: Expression of individual surface antigens in the different anatomical locations tested (n=6)

Sample		Expression of Surface Antigen (%)				
		CD34	CD45	CD73	CD90	CD105
2	Pelvis	40.9	1.2	95.2	96.5	83.5
	Femur	41.8	2	94.5	96.5	84.8
	Tibia	99.2	42.5	99.5	99.4	90.8
4	Pelvis	85.1	54.1	95.8	3.6	89.1
	Femur	88.5	80.8	96.1	99.3	89.6
	Tibia	86.2	83	96.3	99.6	88.4
5	Pelvis	90	60.7	96.7	99.3	91.2
	Femur	92.9	72.9	94.3	95.2	90.2
	Tibia	86.7	56.3	93.9	98.2	85.6
6	Pelvis	57.9	30.7	92.2	99.5	70.8
	Femur					
	Tibia	86.5	46.1	95.4	99.5	81.3
8	Pelvis	17	13.3	95.2	98.2	84.6
	Femur	29.3	9.5	96.9	95.8	92.3
	Tibia	44.3	12.3	96.7	97.2	94.4
10	Pelvis	89.9	86.7	97.3	98.9	88.4
	Femur	65.8	16.7	99.4	99.5	97.8
	Tibia					

4. Discussion:

The purpose of this study was to establish the optimum source for harvesting MSCs for use in point-of-care MSC treatments of cartilage damage within the knee. This is the first study to compare the pelvis with the two long bone sources within the knee in the same individuals. The proliferation of this type of treatment without the answers to basic questions such as this could potentially undermine the success of otherwise useful treatments. In terms of both the amount of bone marrow that could be easily aspirated and the number of MSCs per 100,000 cells, the pelvis out-performed both the tibia and the femur. The assessment of proliferative ability and cell surface phenotype did not reveal any obvious differences between the different locations. It may therefore be concluded that although the functional abilities of MSCs from the three different locations do not vary, the number of MSCs obtainable from the different locations does.

It should also be noted that whilst these results demonstrated a clear superiority in yield from the pelvis this result is, perhaps, less significant than the demonstration of the large variation in yields between individuals which was not related to age or any other variable. This variation in yields between individuals indicates that any point-of-care treatment may be seriously undermined by the variability in the source tissues. A method of assessing this variability prior to, or at the time of, harvesting will be vital if these treatments are to eliminate this variability and so become able to ensure reliable outcomes.

Flow cytometry analysis did not reveal any significant differences in surface antigen expression between samples from different anatomical locations. There was some variation in expression, especially of CD34 and CD45, when compared with the standard profile²⁷. However, a number of studies in recent years have demonstrated that surface antigen expression in MSCs may be more

variable than previously believed. MSCs have been shown to be CD34 positive in some situations²⁸ and have also been shown to alter their surface antigens during culture²⁹.

Whilst no previous study has looked at these three anatomical locations within individuals to assess both yield and function, the results of this study are in line with similar studies that have sought to try and identify the most appropriate location for sourcing MSCs. Previous work has established the superiority of the pelvis to the distal tibia and calcaneus as a source of MSCs¹⁹. Narbona-Carceles *et al.* have also demonstrated the improved yield of mononuclear cells from the pelvis when compared with the femur and tibia, but did not assess MSC yields in the initial aspirates or quantify differentiation³⁰.

This study did not identify any relationship between the age of the patient and the yield of MSCs. Whilst it is generally accepted that the yield of MSCs does decrease with age³¹ the majority of this decrease occurs in the first two decades of life and the relatively smaller changes at later stages may mean that MSC treatments are just as viable, in terms of yield, in individuals in their 50s as in their 30s. For example, three papers which have demonstrated a decrease with age had a skeletally immature early cohort³²⁻³⁴ as the young comparison group, making it difficult to draw a distinction between different adult groups on the basis of their data. When comparisons of the MSC yield from bone marrow are undertaken in study populations that do not include skeletally immature individuals, it is much more difficult to demonstrate a significant relationship between age and MSC yield. In a group of forty individuals aged from 23 to 80 years Hyer *et al.* found no association between increasing age and declining MSC numbers¹⁹. This mirrors the findings of Povsic *et al.*, who found no association between age and MSCs numbers in a group of 81 individuals with an age range of 18 to 85 years³⁵. Siegel *et al.* have demonstrated no age-related difference in differentiation capacity of bone marrow derived MSCs³⁶, showing that differentiation capacity and the release of trophic factors was related to phenotype instead.

As mentioned previously, the differentiation abilities of MSCs from different tissue types has been shown to vary. Synovial derived MSCs have, for example, been shown to have superior chondrogenic potential compared with bone marrow derived MSCs³⁷, but the difficulty of obtaining MSCs from the synovium makes this source impractical. Studies of the differentiation abilities of adipose derived MSCs have proved equivocal, with studies to both suggest and refute a propensity to differentiate into adipose tissue²⁰.

A small number of studies have compared the functional abilities of bone marrow derived MSCs from different skeletal locations. Murphy *et al.* demonstrated reduced chondrogenic and adipogenic activity in bone marrow MSCs taken from either the femur or tibia when compared to the iliac crest. Their study, however, had a relatively old population with a mean age of 71 who were undergoing total hip or knee replacement. More significantly, either femoral or tibial samples were taken rather than both, and the samples were collected after the joint replacement procedure. The large amount of disruption caused to the architecture of the bone by both the instrumentation used in the procedure and the heat effects of the cement which is typically used in joint replacement procedures make it difficult to determine the usefulness of either of these bones as a source of MSCs in individuals who have yet to undergo such a procedure³⁸. Barbanti Brodano *et al.* have shown improved growth characteristics and osteogenic differentiation abilities in vertebral MSCs when compared with iliac crest MSCs³⁹. Their study did, however, use a variety of different sources, including commercial, for the samples and there was no intra-subject comparison, making it difficult to draw firm conclusions.

It should be remembered that the mechanism by which MSCs are felt to have their beneficial effect is not fully understood. The importance of immune-modulation, paracrine, and cell-to-cell interactions in driving the reparative effect of these treatments, as opposed to direct

differentiation of the implanted cells into new tissue, means that the differentiation ability of these cells may be less important than previously thought^{40,41}.

The lack of evidence of a significant functional advantage of cells from one skeletal location over another, as shown in this study and in the papers discussed above would further support the use of the location providing the greatest yield.

This study was affected by a number of limitations. Most notably it was not possible to obtain sufficient cells to carry out differentiation and flow cytometry on all the samples collected. This is always likely to be an issue when working with primary human cells and demonstrates that samples obtained during single-stage procedures may not always contain sufficient numbers of MSCs to be a viable treatment option. A number of previous studies have also shown that it is not possible to successfully analyse all samples when working with primary cells^{35,42}.

The small number of samples involved in this study may also be viewed as limiting the interpretation of the results obtained. The use of intra-subject comparisons does mitigate this to a degree and the numbers involved compare favourably with other studies that have sought to undertake detailed analysis on primary cells from patients to identify MSCs.

The relatively high age of the cohort of participants may also be criticised as reducing the applicability of the results to a wider population. However, within our cohort there was no relationship between age and MSC yield (Spearman Correlation, $p=0.385$). Additionally, the majority of papers that have investigated the relationship between age and MSC yield in humans have not found any significant relationship and there is no consensus as to the effect of age upon MSC numbers⁴³. Those papers which did show a decline with age included skeletally immature individuals who tended to be in their teenage years which may well have influenced the results³⁴. No previous paper has sought to establish and compare the yield and functional ability of MSCs obtained from the pelvis, tibia and femur during knee arthroplasty. As the knee itself is the

location of the majority of cartilage defects suitable for MSC-based treatments, it was important to establish the best site for MSC harvest while minimizing operative sites and associated morbidities. Given the very high level of inter-subject variability in MSC yields in general, it was vital that any comparison was carried out within subjects. Previous papers have carried out inter-subject comparison only ³⁸, or have not fully characterised the samples obtained in order to assess both the yield and function of harvested cells ³⁰. This paper therefore provides stronger evidence for the use of the pelvis in these types of procedures.

Importantly this work shows that there is a large degree of variability in yield and this may be a key factor that may limit the utility of single-site and step procedures.

Many questions remain unanswered regarding the science underlying the use of MSCs in cartilage treatment. The “dose” of cells that is required for a treatment to be successful has not yet been established, and given the variability in yields between individuals it may well be that treatment failures are due to insufficient cell numbers. The impact of the other components of a mono-nuclear cellular layer, as opposed to a pure population of laboratory produced MSCs has also not been determined.

The importance of recently recognised mechanisms of action of MSCs such as paracrine effects and cell-to-cell interactions⁴⁰ has also not been fully established and whilst some work has been done in this area no formal standards have been set ^{36,44}. The development of new, standardised mechanisms of assessing MSC function with respect to cartilage repair will be an important area for future work. This will allow for easier comparison of treatments in a similar way to the recent International Society for Cellular Therapy guidance with respect to cell therapies for immune disorders⁴⁵.

If treatments involving MSCs are to be developed in an evidence-based manner it is important that these questions are answered, as without them the fundamentals underpinning a variety of

MSC treatments may well be flawed. We have shown that the pelvis provides the best source of MSCs in terms of yield, although there is wide variation in yields between different individuals which does not appear to be related to any particular characteristic. Future work should focus on determining the minimum dose required for these treatments to be effective and on investigating how the yield of MSCs can be assessed in real time to ensure point-of-care treatments are using suitable doses.

Acknowledgements

This work was sponsored by the Oxford Stem Cell Institute, the National Institute for Health Research Musculoskeletal Biomedical Research Unit, and Arthritis Research UK (Grant 20087). We would like to acknowledge Kim Wheway, Bridget Watkins and the staff of the Oxford Musculoskeletal Biobank for their assistance in obtaining samples.

Conflicts of Interest

The authors have no conflicts of interest to declare.

References

1. Global Burden of Disease Study 2013 Collaborators. 2015. Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* 386(9995):743–800.
2. Cross M, Smith E, Hoy D, et al. 2014. The global burden of hip and knee osteoarthritis: estimates from the Global Burden of Disease 2010 study. *Ann Rheum Dis* 73(7):1323–1330.
3. Gelber AC, Hochberg MC, Mead LA, et al. 2000. Joint injury in young adults and risk for subsequent knee and hip osteoarthritis. *Ann. Intern. Med.* 133(5):321–328.

4. Maradit Kremers H, Larson DR, Crowson CS, et al. 2015. Prevalence of Total Hip and Knee Replacement in the United States. *J Bone Joint Surg Am* 97(17):1386–1397.
5. Kim S, Bosque J, Meehan JP, et al. 2011. Increase in outpatient knee arthroscopy in the United States: a comparison of National Surveys of Ambulatory Surgery, 1996 and 2006. *J Bone Joint Surg Am* 93(11):994–1000.
6. Widuchowski W, Widuchowski J, Trzaska T. 2007. Articular cartilage defects: Study of 25,124 knee arthroscopies. *Knee* 14(3):177–182.
7. Steadman JR, Rodkey WG, Singleton SB. 1997. Microfracture technique for full-thickness chondral defects: Technique and clinical results. *Oper Tech Orthop* 7(4):300–304.
8. Goyal D, Keyhani S, Lee EH, Hui JHP. 2013. Evidence-based status of microfracture technique: a systematic review of level I and II studies. *Arthroscopy* 29(9):1579–1588.
9. Brittberg M, Lindahl A, Nilsson A, et al. 1994. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N Engl J Med* 331(14):889–895.
10. Cherubino P, Grassi FA, Bulgheroni P, Ronga M. 2003. Autologous chondrocyte implantation using a bilayer collagen membrane: a preliminary report. *J Orthop Surg (Hong Kong)* 11(1):10–15.
11. Mundi R, Bedi A, Chow L, et al. 2016. Cartilage Restoration of the Knee: A Systematic Review and Meta-analysis of Level 1 Studies. *Am J Sports Med* 44(7):1888–1895.
12. Espinoza N, Peterson M. 2012. How to depolarise the ethical debate over human embryonic stem cell research (and other ethical debates too!). *Journal of Medical Ethics*

38(8):496–500.

13. Cesca Therapeutics. 2016. AutoXpress® (MarrowXpress) - Cesca Therapeutics [Internet].www.cescatherapeutics.com [cited 2016 Aug 25] Available from: <http://cescatherapeutics.com/blood-marrow-processing/mxp-marrowxpress/>.
14. Harvest Terumo BCT. 2016. Harvest Bone Marrow Aspirate Concentrate (BMAC) System [Internet].[harvesttech.com](http://www.harvesttech.com) [cited 2016 Aug 12] Available from: <https://www.harvesttech.com/clinician/clinician-home/harvest-bone-marrow-aspirate-concentrate-bmac-system>.
15. Arteriocyte. 2015. Arteriocyte MAR0Max [Internet].www.arteriocyte.com [cited 2016 Aug 12] Available from: <http://www.arteriocyte.com/mar0maxtrade.html>.
16. Mazzocca AD, McCarthy MBR, Chowaniec DM, et al. 2010. Rapid Isolation of Human Stem Cells (Connective Tissue Progenitor Cells) From the Proximal Humerus During Arthroscopic Rotator Cuff Surgery. *Am J Sports Med* 38(7):1438–1447.
17. Beitzel K, McCarthy MB, Cote MP, et al. 2012. Rapid isolation of human stem cells (connective progenitor cells) from the distal femur during arthroscopic knee surgery. *Arthroscopy* 28(1):74–84.
18. McLain RF, Fleming JE, Boehm CA, Muschler GF. 2005. Aspiration of osteoprogenitor cells for augmenting spinal fusion: comparison of progenitor cell concentrations from the vertebral body and iliac crest. *J Bone Joint Surg Am* 87(12):2655–2661.
19. Hyer CF, Berlet GC, Bussewitz BW, et al. 2013. Quantitative assessment of the yield of osteoblastic connective tissue progenitors in bone marrow aspirate from the iliac crest,

- tibia, and calcaneus. *J Bone Joint Surg Am* 95(14):1312–1316.
20. Strioga M, Viswanathan S, Darinkas A, et al. 2012. Same or not the same? Comparison of adipose tissue-derived versus bone marrow-derived mesenchymal stem and stromal cells. *Stem Cells Dev* 21(14):2724–2752.
 21. Siddappa R, Licht R, van Blitterswijk C, de Boer J. 2007. Donor variation and loss of multipotency during in vitro expansion of human mesenchymal stem cells for bone tissue engineering. *J Orthop Res* 25(8):1029–1041.
 22. Hernigou P, Homma Y, Flouzat Lachaniette CH, et al. 2013. Benefits of small volume and small syringe for bone marrow aspirations of mesenchymal stem cells. *Int Orthop* 37(11):2279–2287.
 23. Kern S, Eichler H, Stoeve J, et al. 2006. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 24(5):1294–1301.
 24. Alvarez-Viejo M, Menendez-Menendez Y, Blanco-Gelaz MA, et al. 2013. Quantifying mesenchymal stem cells in the mononuclear cell fraction of bone marrow samples obtained for cell therapy. *Transplant Proc* 45(1):434–439.
 25. Rozemuller H, Prins H-J, Naaijken B, et al. 2010. Prospective isolation of mesenchymal stem cells from multiple mammalian species using cross-reacting anti-human monoclonal antibodies. *Stem Cells Dev* 19(12):1911–1921.
 26. Chan AKC, Heathman TRJ, Coopman K, Hewitt CJ. 2014. Multiparameter flow cytometry for the characterisation of extracellular markers on human mesenchymal stem cells. *Biotechnol Lett* 36(4):731–741.

27. Dominici M, Le Blanc K, Mueller I, et al. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8(4):315–317.
28. Lin C-S, Ning H, Lin G, Lue TF. 2012. Is CD34 Truly a Negative Marker for Mesenchymal Stem Cells? *Cytotherapy* 14(10):1159–1163.
29. Mark P, Kleinsorge M, Gaebel R, et al. 2013. Human Mesenchymal Stem Cells Display Reduced Expression of CD105 after Culture in Serum-Free Medium. *Stem Cells Int* 2013:698076.
30. Narbona-Carceles J, Vaquero J, B S SS-S, et al. 2014. Bone marrow mesenchymal stem cell aspirates from alternative sources Is the knee as good as the iliac crest? *Injury* 45:S42–S47.
31. Caplan AI. 2007. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J Cell Physiol* 213(2):341–347.
32. Nishida S, Endo N, Yamagiwa H, et al. 1999. Number of osteoprogenitor cells in human bone marrow markedly decreases after skeletal maturation. *J Bone Miner Metab* 17(3):171–177.
33. Baxter MA, Wynn RF, Jowitt SN, et al. 2004. Study of telomere length reveals rapid aging of human marrow stromal cells following in vitro expansion. *Stem Cells* 22(5):675–682.
34. Stolzing A, Jones EA, McGonagle D, Scutt A. 2008. Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. *Mech Ageing Dev* 129(3):163–173.

35. Povsic TJ, Zhou J, Adams SD, et al. 2010. Aging is not associated with bone marrow-resident progenitor cell depletion. *J Gerontol A Biol Sci Med Sci* 65(10):1042–1050.
36. Siegel G, Kluba T, Hermanutz-Klein U, et al. 2013. Phenotype, donor age and gender affect function of human bone marrow-derived mesenchymal stromal cells. *BMC Med* 11(1):146.
37. Jones BA, Pei M. 2012. Synovium-derived stem cells: a tissue-specific stem cell for cartilage engineering and regeneration. *Tissue Eng Part B Rev* 18(4):301–311.
38. Murphy JM, Dixon K, Beck S, et al. 2002. Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. *Arthritis Rheum* 46(3):704–713.
39. Barbanti Brodano G, Terzi S, Trombi L, et al. 2013. Mesenchymal stem cells derived from vertebrae (vMSCs) show best biological properties. *Eur Spine J* 22(S6):979–984.
40. Prockop DJ, Oh JY. 2012. Medical therapies with adult stem/progenitor cells (MSCs): a backward journey from dramatic results in vivo to the cellular and molecular explanations. *J Cell Biochem* 113(5):1460–1469.
41. Bianco P, Cao X, Frenette PS, et al. 2013. The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. *Nat Med* 19(1):35–42.
42. Pierini M, Di Bella C, Dozza B, et al. 2013. The Posterior Iliac Crest Outperforms the Anterior Iliac Crest When Obtaining Mesenchymal Stem Cells from Bone Marrow. *J Bone Joint Surg Am* 95(12):1101–1107.

43. Fossett E, Khan WS, Pastides P, Adesida AB. 2012. The Effects of Ageing on Proliferation Potential, Differentiation Potential and Cell Surface Characterisation of Human Mesenchymal Stem Cells. *Curr Stem Cell Res Ther* 7(4):282–286.
44. François M, Romieu-Mourez R, Li M, Galipeau J. 2012. Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation. *Mol Ther* 20(1):187–195.
45. Galipeau J, Krampera M, Barrett J, et al. 2016. International Society for Cellular Therapy perspective on immune functional assays for mesenchymal stromal cells as potency release criterion for advanced phase clinical trials. *Cytotherapy* 18(2):151–159.