Bone-resident mesenchymal stromal cells in healthy ageing and osteoarthritis: enumeration and gene expression analysis in uncultured cells

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

The University of Leeds

Faculty of Medicine and Health

December 2019

The candidate confirms that the work submitted is her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

The work in Chapter 3 and 4 of the thesis have appeared in publication as follows:

The analysis of *in vivo* aging in human bone marrow mesenchymal stromal cells using colony-forming unit-fibroblast assay and the $CD45^{low}CD271^{+}$ phenotype
Stem Cells International, August 2019

Authors: Payal Ganguly, Jehan J El-Jawhari, Agata N Burska, Frederique Ponchel, Peter V Giannoudis and Elena A Jones

I was responsible for: Experimental design, sample collection, performing experiments, analysis of data and manuscript writing.

Work attributed to others: study conception and design (JEJ, ANB, FP, and EAJ); sample collection (PVG), analysis and interpretation of data (JEJ, ANB and EAJ), manuscript preparation and final draft (EAJ), response to reviewers and revisions following peer review (EAJ).

Acknowledgements

I would like to begin with a big thank you to my main supervisor Dr Elena A Jones not only for giving me the opportunity to work on such a globally relevant project but also for guiding, encouraging, criticising, appreciating and understanding my approach at different stages of the project. Apart from being a thoroughly professional and an extremely efficient scientist, I wish to thank Elena for her commendable leadership qualities that has maintained a successful research group of PhDs, postdocs, consultants and experts from across the globe. She is one of those very rare bosses who have the right mix of compassion, strength, vision and motivation and the world desperately needs more of people like her, more leaders like her.

I am very grateful to Leeds Institute of rheumatic and musculoskeletal medicine (LIRMM) for offering me the scholarship and the opportunity to work with people who are best in the field and also with people who will undoubtedly, be the best in the field, in the coming years (contemporary PhD students). A big thank you to University of Leeds (UOL) for being among the most accepting and inviting Universities I have been a part of. UOL pride themselves on their diversity of students and my personal experience will vouch for that. I have met some of the most brilliant minds of the future pursuing a PhD in subjects varying from literature to molecular medicine, from childhood studies to chemical engineering, from water law to plant genetics and many, many more. May this diversity of knowledge keep growing every year!

I would like to extend a big thank you to my postdoctoral supervisors Dr Jehan J El-Jawhari and Dr Agata N. Burska for their unending patience during my laboratory work, analysis and writing phase and for supporting me through and through during the difficult phases of the PhD. Jehan also played a significant role in my understanding of flow cytometry and Agata's inputs were vital for the gene expression and the novel type 1 interferon based chapter. None of this work would have been possible without Professor Peter V. Giannoudis, his patients and the team of surgeons and nurses in Leeds General Infirmary (LGI).

Next, I want to thank the entire MSC research group for being my 4th, but easily, the best research group I have worked with till date. Dr Dennis McGonagle, Dr Thomas Baboolal, Dr Richard Cuthbert, Dr Ala Altaie, Dr Weichao Rachel Zhai, Dr Clara Sanjurjo-Rodriguez and Dr Charlie Bridgewood, Dr Yasser-El-Sherbiny for constructive criticism during lab meetings. My colleagues (and friends) Dr Heather Owston, Dr Dragos Ilas for Mr Tobias Russell, Miss Hannah Rowe, Dr Daniel Perez Witzke, Dr

Thomas Scambler, Dr. Devon Smith, Mr Samuel Lara Reyna, Mr Jonathan Holbrook, Mr Georgi Georgiev and Dr.Katrina Moisely. Dr Heledd Haf Jarosz-Griffith, Dr Emily Caseley, Miss Zoe Wigston, Miss Chen Xia-Dong, Miss Rujiraporn Pitaksalee, Mrs Rekha Parmar and Dr Chi Wong for being very friendly and helpful and co-workers. Miss Priyanka Dutta for her encouragement and warmth and Miss Shelly Pathak, Miss Tejal Bhatt and Mrs Rekha Parmar for all the times they have pampered me, introduced me to the lovely members of their families and have been there to listen and counsel me through difficult situations.

I wish to thank my colleagues from the haematology department for encouragement, guidance, support and help at every stage needed. Dr Sophie Stephenson, Dr Amel Saadi, Dr Marjorie Davison, Dr Katie Holmes, Dr Rebecca Ross, Dr Adewonoula Alase, Dr Mario Cocco, Dr Claire Mckinley, Dr Ruth Kennedy, Dr Jorge Jimenez Lucias, Miss Naomi Gibson, Miss April Baral, Miss Bethany Kuszlewicz, Miss Emma Adams, Mr Nate Lee, Mr Fraser Thomas, Miss Nicole McDermott and Mr Kieran Walker, it has been an absolute pleasure knowing them. I want to convey a huge thank you to the flow cytometry facility in-charge Miss Liz Strszynski and Dr Adam Craig Davison, Dr Claire Taylor for very patiently going through the intense process of using a new equipment and guiding Dr Raechel Barlow for carrying out the enormous task of a laboratory manager for a busy floor as ours. Mrs Angela Greenbank for being very patient and always helping us out with reimbursement forms, meeting room bookings and for always greeting everyone with a bright smile.

Outside of work, I owe a generous thank you to Prof Claire Chambers not only for her insights on PhD life in general but also for introducing the benefits of Zumba in my life. It helped me keep sane through the entire PhD process and today, I cannot imagine my life without it. She has also played a vital role in talking me through the difficult times of the PhD. I would like to thank Dr Kathleen Wright, Dr Amrisha Pandey, Dr Lana Lai and Dr Rohit Thakur for their constant inspiration and motivation. Miss Kamla for her warmth, monthly supply of hand-made desserts and for introducing me to so many people, whom I now call friends. Miss Ambika Kapoor, Miss Richa Yeshevekar, and Miss Arththi Sathnanthar for all the regular catch-ups. Dr Kanhu Charan Pattnayak, his wife Lipsa and their daughter Samvidha for keeping me in their prayers and for looking out for me during the PhD. Dr Wei Goh and Mr Joey Mark Diaz, Dr Gnosi Michiladou and Miss Shuang Wang for their friendship.

Apart from the friends at University, I owe a generous thank you to all my house mates over the last 4 years. Miss Fay, Miss Marta Baltruszewicz, Miss Yixin Ma, Miss Holly

Ingram and Mr. Dominic Flower , Miss Lorienne-Doyle Maw, Miss Aysha Panter and Miss Sophie Roche for being very considerate housemates. It is going to be very hard to find housemates like all the people mentioned above.

Miss Shelly Pathak for keeping up with nearly every phase of my PhD, for introducing me to her lovely family, offering help of every possible kind and for ensuring I had a home away from home in Leeds, especially during the festive seasons. I am very grateful for her friendship. Dr Akhil Krishnan, Miss Manasi Inamdar, Miss Nanda Rohra, Mr Saurabh Patil and Miss Tejal Pant for the continuous flow of care and positivity, for the warmth and love, for ensuring that we caught up every month, for being the source of laughter and for being a guardian angel since 2014, respectively, even from 4,528 miles away! Dr Susmita Basak for her unconditional support and Miss Anusha Sundaram for being in touch nearly every week in good and bad times.

Finally, my family back in Mumbai without whom this would have in no way, been possible. My father, Mr Ramkrishna Ganguly for believing in me even when I did not, for being a constant source of motivation, inspiration, critique and for giving my sister and me the best life lessons on hard work and not giving up. My mother, Mrs Sumana Ganguly for her never ending strength, resilience, and unconditional love. Her dedication for dance inspires anyone and everyone who knows her and I cannot thank both my parents enough for putting up with me for nearly 3 decades now. Last, but not the least, a very big thank you to my younger sister, Miss Bitan Ganguly for being the light of my life, my strength during my weakness and my dose of good thoughts when situations were not the best. Her ability to comprehend situations at her tender age and her passion towards things she believes in, have always pleasantly surprised me. I miss and love her the most.

Abstract

Ageing considerably reduces the quality of life with osteoarthritis (OA) being the most common age-related degenerative disorder. Whilst majority investigations have focussed on cartilage changes in OA, damage in the bone preceding cartilage damage, is increasingly being acknowledged. Bone and cartilage arise from progenitor cells called mesenchymal stromal cells (MSCs) resident in the bone marrow (BM). It was hypothesised that changes in BM MSCs contribute to OA development. It was aimed to investigate changes in BM MSCs in healthy ageing and then explore if these changes were aggravated in OA using flow cytometry for the $CD45^{low}CD271^{+}$ phenotype and gene expression.

Minimally-expanded/uncultured MSCs were used to avoid effects of *in vitro* ageing due to culture expansion. BM aspirates from 51 healthy donors (19-89 years old) were processed for MSC quantification using colony forming unit fibroblast (CFU-F) assay. For OA investigations, MSCs from donors with hip OA (56-83 years old) were recruited. Gene expression of MSC multipotentiality genes, genes associated with cell senescence and type 1 interferon (IFN1) pathway genes was compared between $CD45^{low}CD271^{+}$ MSCs and donor-matched control $CD45^{+}CD271^{-}$ haematopoietic lineage cells (HLCs).

MSC numbers declined with advancing age as measured by both assays but a more prominent decline was noted using CFU-F assay. Colony size and integrated density significantly reduced in old donor MSCs indicating age-related decline in MSC proliferation. When cultured in media with old donor serum, young and old donor MSCs displayed lower proliferation. *IL6* expression from old donors displayed 4-fold increase in both MSCs and HLCs. IFN1 genes displayed strikingly high expression but no age-related changes in MSCs. In OA, number of genes displayed significant differences including *LepR*, *CXCL12* and *IL6* as compared to healthy old donor MSCs. Surface marker expression of CD106 and CD295 were found to decline significantly in MSCs and a similar trend for CD295 decline was observed in HLCs. Age-related increase in *IL6* expression was aggravated more notably in MSCs.

In summary, age-related decline was observed in MSC number and proliferative capacity while gene and surface marker expression displayed non-significant differences as compared to donor-matched HLCs. These data indicate that BM MSCs are potentially more resistant to ageing stimuli *in vivo* compared to other BM resident hematopoietic lineage cells.

List of abbreviations

Abbreviation	Full form
2D	Two dimensional
3R	Replacement, reduction and refinement
7-AAD	7-aminoactinomycin D
A-Fib	Anti-Fibroblast microbeads
AGM	Aorta gonad mesonephores
ALP	Alkaline phosphatase
AP2	Adipocyte protein 2
ATM	Ataxia telangiectasia mutated kinase
BID	BH3 interacting domain
BM	Bone marrow
BMA	Bone marrow aspirate
BMD	Bone mineral density
BT	Bone thickness
BV	Bone volume
C.elegans	*Caenorhabditis elegans*
CAR	CXCL12 abundant reticular
CASP1	*Caspase 1*
CCND2	*Cyclin D2*
CD	Cluster of differentiation
cDNA	Complementary DNA
CFU-F	Colony forming unit-fibroblasts
COX2	Cyclooxygenase 2
Ct	Cycle threshold
Cu/Zn SOD or SOD1	Copper/Zinc Superoxide dismuatse
Cx43/GJ1	Connexin43/ gap junction protein alpha 1
CXCL12	*CXC motif chemokine ligand 12*
DDR	DNA damage response
DMEM	Dulbecco's modified Eagle's medium
DMP1	*Dentin matric acidic phosphoprotein 1*
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTPs	Deoxyribonucleotide triphosphates
DXA	Dual energy X-ray absorptiometry
ECM	Extracellular matrix
EC-SOD or SOD3	Extracellular Superoxide dismutase
EDTA	Ethylenediamintetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ES	Embryonic stem
FABP	Fatty acid binding protein
FABP4	Fatty acid binding protein 4
FACS	Fluorescent activated cell sorting
FCS	Foetal calf serum
FH	Femoral head

FITC	Fluorescein isothiocyanate
FM	Freezing medium
Fzd	Frizzled proteins
GCT	Global clinical trial
GDB	Global burden of diseases
GJIC	Gap junction intercellular communication
Gx	Glutathioneperoxidasecatalase
HLA	human leukocyte antigen
HLCs	Hematopoietic lineage cells
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
HSCs	Hematopoietic stem cells
IC	Iliac crest
ICD	International classification of disease
ID	Integrated Density
IFC	Integrated fluid circuits
IFN1	Type 1 interferon
IFNA1	*Interferon alpha 1*
IFNAR1	*Interferon alpha receptor 1*
IFNAR2	*Interferon alpha receptor 2*
IFNB1	*Interferon beta 1*
IGF	Insulin growth factor
IL6	*Interleukin6*
IL7	*Interleukin7*
IL8	*Interleukin8*
iPSCs	Induced pluripotent stem cells
IRF3	*IFN regulatory factor 3*
IRF9	*IFN regulatory factor 9*
IRG	Interferon regulated genes
ISCT	International society for cellular therapy
ISG20	*Interferon stimulating gene 20*
ISGF3	Interferon stimulated gene factor 3
ISRE	Interferon stimulated response elements
IVIVC	*In vitro in vivo* correlation
JAK1	Janus kinases 1
K/L	Kellgren-Lawrence
Kl	*Klotho*
LAIR1	*Leukocyte-associated immunoglobulin-like receptor 1*
LepR/CD295	Leptin receptor
LNGFR	Low affinity nerve growth factor
MACS	Magnetic activated cell sorting
MAPK	Mitogen activated protein kinase
MCAM/CD146	Melanoma Cell Adhesion Molecule
MEPE	Matrix extracellular phosphogylcoprotein
MFI	Median fluorescence intensity
MMPS	Matrix metallopeptidases
Mn-SOD or SOD2	Manganese-Superoxide dismutase
MRI	Magnetic resonance imaging
MSCA1	Mesenchymal stem cell antigen 1

MSCs	Mesenchymal stromal cells
MTCH2	Mitochondrial homolog carrier 2
NC	Nucleated cells
NH_4Cl	Ammonium Chloride
NK	Natural killer
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
ONS	Office of national statistics
OPG	Osteoprotegerin
OS	Old serum
PBS	Phosphate buffer saline
PDGFRα	Platelet derived growth factor receptor alpha
PDGFRβ	Platelet derived growth factor receptor beta
PHEX	*Phosphate regulating neutral endopeptidase X-linked*
PPAR-γ	Peroxisome proliferator activated receptor-gamma
QCT	Quantitative computed tomography
QOL	Quality of life
qPCR	Quantitative polymerase chain reaction
RANK	Receptor activator of nuclear factor kB
RANKL	Receptor activator of nuclear factor kB ligand
RKOA	Radiographic knee osteoarthritis
RNA	Ribonucleic Acid
RNAse	Ribonuclease
RNF213	*Ring finger protein 213*
ROS	Reactive oxygen species
RT	Room temperature
Rt	Reverse transcription
RUNX2	Runt related transcription factor 2
SAMP6	Senescence accelerated mice –P6
SASP	Senescence associated secretory phenotype
SCE	Stem cell exhaustion
SFRP1	*Secreted frizzled related protein 1*
Sirt6	*Sirtuin6*
SM	StemMACS
SOD	Superoxide dismutase
SOST	*Sclerostin*
SOX2	*Sex determining region Y- box 2*
SPARC	*Secreted protein acidic and rich in cysteine*
STAT1	Signal transducer and activator of transcription 1
STAT2	Signal transducer and activator of transcription 2
STING	*Stimulator of interferon genes*
TBHP	Tert-butylhydrogenperoxide
TF	Transcription factors
THR	Total hip replacement
TM	Thawing medium
Tp53	*Tumour protein 53*
TRAP	Tartrate resistant acid phosphatase
TYK2	Tyrosine kinase 2

UK	United Kingdom
UN	United nations
VCAM-1/CD106	Vascular cell adhesion molecule-1
WHO	World health organisation
YS	Young serum

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Chapter 1 Introduction

1.1 Ageing

Ageing in simple terms, is the process of growing old. This process however has been found to be a result of complex changes within an individual. Growing old has increasingly been associated with decline in strength, loss of physical fitness and increased vulnerability to diseases, all of which eventually lead to a gradual deterioration in the quality of life (QOL) (1). Diet, lifestyle, physical activities, heredity, epigenetics and environment, all of these factors are believed to contribute to this decline in QOL (2, 3). World Health Organisation (WHO) reported in 2018 that the "proportion of the world's population over 60 years old will nearly double from 12% to 22%" within a span of three decades (4). The report also outlines that by 2020, the number of people aged over 60 will outnumber children below the age of 5, suggesting a huge shift in the global demographics. The United Nations (UN) estimates over 3 billion people will be over the age of 60 by 2100, globally (Figure 1.1) (5).

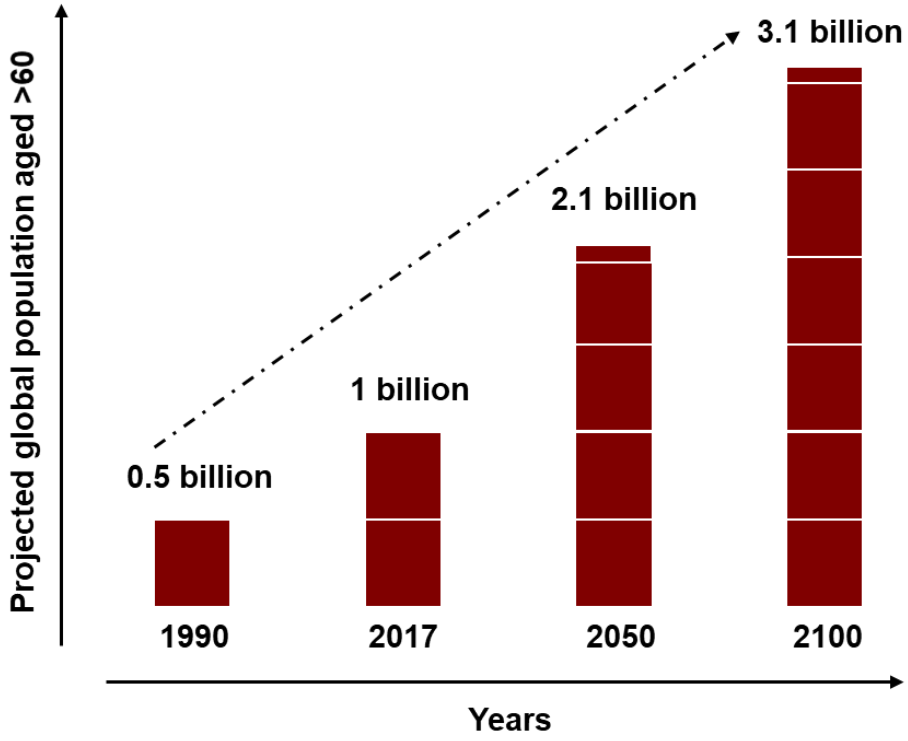

Figure 1.1 Projections of global ageing

Increase in number of people aged over 60 over the next few decades until 2100, adapted from the UN 2017 study (5)

In mid-2014, the United Kingdom (UK) was identified as having an 'ageing population' when the average population age exceeded 40 years old (6). Age UK also points out that 37% of the carers for old people are themselves old and over 72% of them have reported pain or discomfort while providing care (7). The office of national statistics (ONS) predicted in 2017 that by 2039, nearly 74 million of UK population will be over the age of 60 (8). The office for budget responsibility predicts that the public spending between 2019/20 and 2064/65 will increase by £79 billion to meet the needs of the ageing population.

The fact that increasing age has been associated with increased frailty (an elevated risk of decline in health and function (6)) has led to an increased interest in understanding the mechanisms of age-related changes in the past few decades. In 2013, a pioneering paper 'the hallmarks of ageing' was published summarising nine major age-related changes at the cellular and sub-cellular levels. These were grouped into main three themes: cellular senescence, genetic instability and stem cell exhaustion (9). In combination, all these factors caused an increase in individual's vulnerability towards diseases, loss of control over daily activities and overall increased time period in recovering from physical injury.

Considering that increasing age has been linked with several types of cancers, cardiovascular and musculoskeletal diseases, loss in cognitive functions and neuro-degenerative diseases, healthcare experts have introduced a concept of 'healthy ageing' (10), which WHO experts have defined as 'the process of developing and maintaining the functional ability that enables well-being in older age' (1). In other words, even though ageing has been associated with a number of diseases, it is not a disease by itself. It is the natural progression of one's life. Hence, to understand any age-related disease, it is vital to first understand the age-related changes in 'healthy ageing'.

1.1.1 Theories of ageing

In biological terms, ageing is a complex process of gradual build-up of numerous molecular and cellular damages over time. To better understand the process, a number of theories have been postulated over the years to explain different aspects of age-related differences.

The theory of stem cell exhaustion (SCE) outlines the role of stem cells that drive tissue regeneration over an individual's lifetime. It suggests that there is an age-related decline in the number and functionality of the stem cell pool within our bodies that

eventually leads to a reduction in body's ability to regenerate tissues or heal when damaged by injury or a disease (9, 11). Stem cells are undifferentiated cells that have the potential to differentiate either into any lineage (have total potency or are 'totipotent') or into multiple lineages ('multipotent'). Most adult stem cells are multipotent stem cells (12). Stem cells can be of different origin depending upon the tissue source and species they belong to. They can also be categorised as embryonic, if sourced from an embryo (ES cells); adult, if sourced from an adult (adult stem cells) or can also be induced by reprogramming from mature adult cells to an ES-like state (induced pluripotent stem cells, iPSCs) (13). SCE theory of ageing pertains to age-related changes in adult stem cells.

An age-related decline in the proliferation and regeneration capacities of stem cells has been established in the last decade (14) and a number of factors have been proposed as contributors. These include deoxyribonucleic acid (DNA) damage and, accumulation of toxic metabolites (harmful free radicals) in stem cells themselves and a notable loss in the balance between damage and regeneration signals in the surrounding microenvironment. Interestingly, one of the main current debates revolves around the preferential roles of intrinsic (intracellular) or extrinsic (extracellular) factors on the number and basic functions of stem cells. Research focusing on the impact of the extrinsic factors within in the stem cell niches (specific anatomies and microenvironments within which the stem cells reside) has gained an increased interest owing to parabiosys experiments showing that stem cells in old mice can potentially be reversed to be as functional as stem cells in young mice, using systemic factors from young mice (15, 16). However, this concept dates back to 1913 when Carrel performed experiments to understand ageing of organs with the aim of life extension (17). The concept has now gained importance potentially due to an increasing ageing population, worldwide.

Damage to the DNA of stem cells is one of intrinsic factors that have been proposed to lead to SCE. The theory of DNA damage response (DDR) is among the oldest theories aimed at understanding age-related changes at the sub-cellular levels (9, 18, 19). It refers to the accumulation of damage in the DNA over time. The structure and integrity of DNA continuously gets challenged from both intrinsic factors (for example, replication errors and mutations) and extrinsic factors (for example, background radiation, cellular microenvironment and reactive oxygen species or ROS), which play the role of causative agents of DDR (9). In young people, all the cellular damage/debris is automatically eliminated by our body by means of autophagy (engulfment of damaged cells and organelles by authophagosomes followed by their degradation by

lysosomes (20)), which in older age is shown to becomes less efficient (21). Telomere attrition, irreversible arrest in cell cycle (senescence), inadequate response to cellular stress are also key factors contributing to DNA damage (22). Interestingly, ROS acts a major source of formation of DDR which brings us to the next theory of ageing.

The ROS or the free radical theory was first proposed in 1955 by Harman where he suggested that accumulation of cellular damage along with declining integrity of cellular components lead to oxidative stress owing to impaired metabolic cycles in old age (23). The free radical theory of ageing suggests that damages at the cellular and sub-cellular levels are caused by ROS. ROS are generated continuously during regular metabolism but are scavenged due to the presence of naturally occurring anti-oxidant molecules within our bodies (24). The theory outlines that the accumulation of ROS increases with age and overwhelms the ability of protective anti-oxidant molecules to scavenge ROS, thus increasing cellular damage and eventually leading to inability of cells to regenerate. However, the exact mechanisms that govern age-related changes in humans due to ROS are still unknown. Until now, levels of free radicals and anti-oxidants have been used to estimate the balance between ROS levels and the potential anti-oxidant scavengers of ROS but only in culture expanded stem cells (25, 26).

Recently, ROS, DNA damage and SCE theories have been combined leading to various studies investigating age-related changes in hematopoietic stem cells (HSCs) within the bone marrow (BM) niche (27, 28). In independent studies, type 1 interferon (IFN1) was found to play a role in the clearing of senescent cells (29, 30) and IFN1 pathway was found to potentially be the link between DNA damage, ROS and ageing of HSCs (31). Bone and joint cells are produced and maintained by another type of BM-resident adult stem cells (mesenchymal stem/stromal cells (MSCs)) where they co-exist with HSCs in the same microenvironment. However, the roles of various factors like ROS and IFN1, on these stem cells in healthy ageing and age-related musculoskeletal conditions, remain unknown and require further investigation.

1.1.2 Age-related conditions – clinical need to understand healthy ageing

Age-related conditions are a global health burden and combating them is now the driving force for many scientists and economists alike. Section 1.1.1 above outlined the theories of ageing and potential causes of the age-related damaging effects. In this section, some age-related conditions will be discussed in more depth, primarily to highlight the debilitating impact they have on the suffering individual's QOL. In fact, I

believe, the co-existence of the numerous theories about the process of ageing, in reality, indicates a gap in knowledge on the complexity of ageing and its link to age-related diseases. However, there is a possibility that in fact, all the theories are complementary and/or collectively responsible for the different levels of damages (subcellular, cellular and organismal).

There is growing body of evidence that connects different diseases to the process of growing old. For example, accumulation of damage over time, via ROS acting at the sub cellular and cellular levels have been found to be common between healthy ageing and many disease conditions including cancer (18, 32, 33), cardiovascular diseases (34-36), neurodegenerative disease (37-39), as well as diseases of the musculoskeletal system (40-42). Figure 1.2 shows how the known factors for age-related changes can transition to distinct disease conditions, but the main drivers for this transition remain unclear.

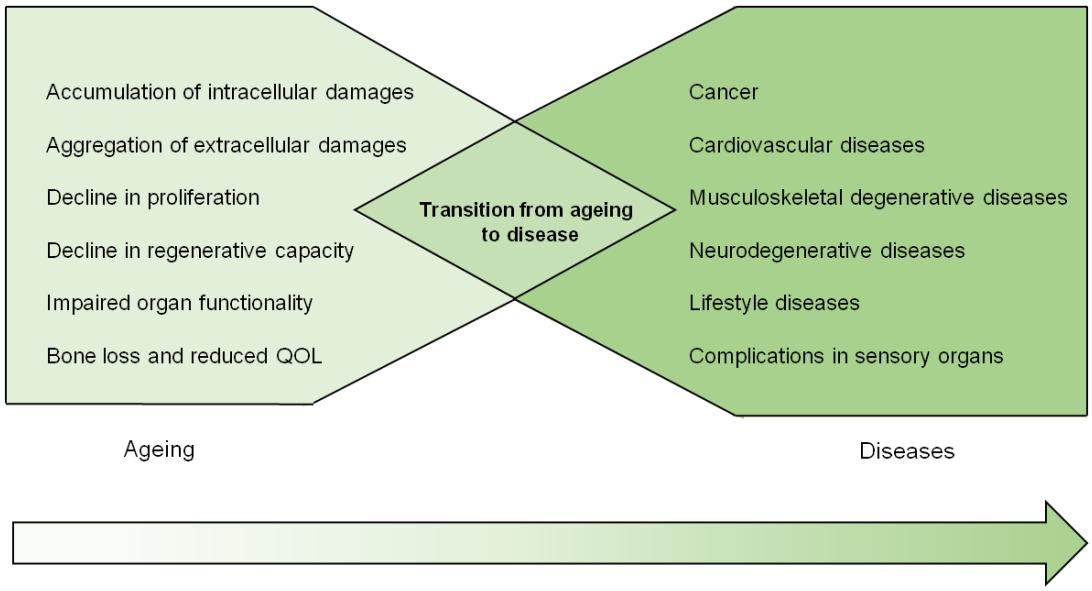

Figure 1.2 Unclear transition from ageing to diseases

Shared features of ageing and age-related diseases with an unclear path towards disease transition (adapted from (43))

Old age has been linked with many diseases along with the reduction in the QOL of older people. Thus, there are scientists who not only consider ageing to be a degenerative disease (44), but also propose that ageing must officially be classified as a disease by the International classification of disease (ICD) (43, 45). The ICD is the classification used by WHO for epidemiology studies and health care management globally, the 11th revision being the most updated version (ICD-11). It is a tool that aids the monitoring of occurrence of diseases to better understand the healthcare condition in countries and among populations. Ageing was not classified as separate disease in

ICD-11 and rightly so, as the concept of ‘ageing is a disease’ is controversial owing to the fact that it is a natural progression of every living being.

In fact, some scientists believe that we begin to ‘age’ right from our conception, even before birth. The process is simply broken down into phases of birth, growth, maturation, adulthood and old age. This idea has been further promoted by Allison *et. al* who found that pregnant rodents that were treated with anti-oxidants, had off-springs that grew old slower than those whose mothers were not given any anti-oxidants (46), suggesting that ageing begins from the moment we are conceived. While advanced age leads to considerable frailty, not every old person is frail. These variations are due to the different environmental factors, genetic make-up and life styles that individuals lead.

Among the diseases associated with old age, diseases of the musculo-skeletal system impacting the bones and joints may be considered the most incapacitating diseases worldwide. The WHO reports that osteoarthritis (OA) is the most common degenerative disease in older population (47). While the disease has primarily been described as the degeneration of cartilage with negative impact on the other joint tissues, recent evidence suggests that the damage to bone plays a key role in the disease progression (48). The disease has largely been identified by degenerating cartilage, stiffness and pain. Increasingly along with these, subchondral bone sclerosis, bone marrow lesions, increase in bone volume but low mineralisation due abnormal bone remodelling (explained in section 1.1.4) have gained recognition as hallmarks of the disease (49, 50), even though the debate as to if these hallmarks are the causes or the effects, are still active.

1.1.3 Bone and bone marrow

The skeletal system consists of bones, cartilage, tendons, ligaments and connective tissues. It forms the framework of our bodies and provides us with basic structure and support. Apart from protecting our internal organs, it is also responsible for our locomotion in daily lives. Bones in particular perform mechanical functions along with serving as the reservoir for the hematopoietic marrow and a reserve for calcium. At this given point of time there are 16,334 active clinical trials globally that are aimed to restore bone and bone related conditions (51).

Structurally, the bone contains inorganic mineral depot and organic extracellular matrix, lipids and water. The organic components include abundant quantities of collagen, proteoglycans, glycolipids and different types of cells. The inorganic component of the

bone is majorly hydroxyapatite ($Ca_{10}(PO_4)6(OH_2)$) (52). The composition involving both, the organic and inorganic components enable the bone to have both, mechanical strength as well as flexibility for movement. The inside of the bone is a very dynamic environment with various types of cells residing and interacting with one another. The bone marrow (BM) within the bone is also where stem cells reside, making it one of the most important organs in the body.

The BM exists within the trabecular spaces in the cancellous bones (such as the iliac crest or IC) and in the medullary cavities of the long (cortical) bones. It is contained by the exterior (cortex) of the bone and includes a complex mesh of blood vessels, cells and fatty tissue. This micro-environment within the bone forms the 'niche', where BM stem cells reside (53). As mentioned, the BM houses two types of adult stem cells and their progenies: HSCs and MSCs. HSCs originate in the aorta-gonad-mesonephors (AGM) region in the embryo and migrate to the liver and then to BM (54-56), where they reside and self-renew for the rest of the adult life. BM HSCs give rise to progenitor cells of both lymphoid as well as myeloid lineages. Lymphoid progenitor cells further give rise to natural killer (NK) cells, T lymphocytes and B lymphocytes. Myeloid progenitor cells on the other hand, give rise to monocytes/macrophages, eosinophils, basophils, neutrophils, erythrocytes and platelets.

HSCs residing in their BM niche replenish billions of cells every day while maintaining themselves over decades, a process known as haematopoiesis (57). The BM or the stem cell niche is the microenvironment within which these stem cells reside. The niche is known to provide the appropriate conditions, growth factors, temperature, oxygen levels to enhance and maintain the cells and their functions within the BM, making it an important factor for stem cell formation, function and survival (56). Within the BM or the stem cell niche, MSCs also exist which originate from the mesoderm and the neural crest, which is a transient embryonic tissue (58).

It has been reported that neural crest cells would migrate to different locations through the blood stream including the BM where their arrival would coincide with the arrival of HSCs. They continued to be there until adulthood and supported HSCs. There have also been reports of these neural crest cells travelling through developing nerves rather than via bloodstream. What is definitely known is that regardless of their embryonic origins, BM resident MSCs can give rise to bone, fat and cartilage lineages. For MSCs specifically, the BM niche can be divided into endosteal and perivascular based on the anatomical location within the microenvironment. Endosteal niche is located around the bone lining in proximity with bone formation cells (osteoblasts), whereas the

perivascular niche is located near the sinusoidal vessel within the BM cavity (59, 60). In mice, MSCs have been also topographically linked to CXC motif chemokine 12 (CXCL12) abundant reticular (CAR) cells that form a network of stromal cells connecting endosteal and perivascular niches. MSCs residing at the endosteal niche are most likely to be directly involved in the process of bone remodelling (58).

1.1.4 Bone remodelling and age-related bone loss

To maintain the integrity of the bone, about 10% of the bone is believed to be remodelled each year (61). The process of bone remodelling involves a balance between old bone removal and new bone formation. However, with increasing age, the rate of new bone formation has been found to fall behind the rate of old bone removal leading to the eventual decline in bone formation and reduced functionalities of the bone (62). The mechanisms underlying bone remodelling have been extensively studied and have revealed the involvement of the *RANKL* (Receptor activator of nuclear factor kB ligand)/*OPG* (Osteoprotegerin) pathway (63).

The cells directly involved in bone remodelling process are osteoblasts (the bone forming cells), osteoclasts (the bone resorption cells) and osteocytes, which are fully mature bone cells embedded within the mineralised matrix (64). Osteoblasts are derived from MSCs and are the effector cells largely known for their ability to produce bone matrix by the secretion of alkaline phosphatase, type 1 collagen, proteoglycans, osteocalcin and osteopontin. They are short-lived and exist and function in clusters along the bone surface. Some of the osteoblasts mature over time and get embedded within the matrix of the bone to eventually become osteocytes (64). Over the years, osteoblasts have been extensively studied in relation to their contribution to age-related bone loss and age-related bone diseases (65, 66).

Osteocytes are the most abundant cells embedded deep inside the bone matrix and their half-life is estimated to be around 25 years (67). Owing to their deep seated location (Figure 1.3), isolation and investigation of these cells pose technical challenges. Thus, most of the osteocyte investigations in humans have largely been based on histology of stained and fixed specimens or using gene expression for osteocyte-specific transcripts. Osteocytes are considered to be the final differentiated cells of the osteoblast lineage that eventually perish as a result of apoptosis/necrosis or autophagy (68). By gene expression, osteocytes have classically been identified by the expression of sclerostin (*SOST*), matrix extracellular phosphogylcoprotein (*MEPE*), dentin matric acidic phosphoprotein 1 (*DMP1*) and phosphate regulating neutral endopeptidase X-linked (*PHEX*) (69). Osteocytes have also been acknowledged as

‘mechanostat’ of the bone that sense and respond to mechanical stress by sending signals to osteoblasts and osteoclasts, as well as MSCs, on the bone surface and modulate bone remodelling (70).

Osteoclasts are the cells that are responsible for the process of resorption of bone. These are multinucleated cells that are derived from the mononuclear progenitors of the myeloid lineage from HSCs, that also give rise to macrophages. Discovered back in 1837, they possess a unique ‘ruffled border’ that is formed by complex finger-like projections from the cytoplasmic membrane, which isolate area under the cells to release high concentration of acid to dissolve bone mineral (71). The acidic environment is achieved by the transfer of H^+ and Cl^- ions across the borders of the ruffles to ensure the dissolution of the non-organic component of the bone to bring about bone resorption (72). They are typically identified due to the presence of tartrate resistant acid phosphatase (TRAP) by immunohistochemistry (71).

Dysregulation of osteoclast activity can lead to both increased bone mass (if there is reduced development or function of osteoclasts) or decreased bone mass (in case of their increased development or function). For the purpose of bone remodelling, the progenitors of osteoclasts are recruited to the surface of the bone, where they mature to form a multinucleated osteoclast, then perform resorption functions and eventually die via apoptosis (73).

The process of bone remodelling is brought about by a number of soluble factors that recruit the above mentioned cells for executing the process. The key players of this process are RANKL, RANK and OPG. RANKL is a protein produced by the osteoblasts as well as MSCs, which binds to its receptor RANK on the surface of osteoclast progenitors. The binding of RANKL to RANK is essential for the formation and function of osteoclasts (71). The formed osteoclasts then begin to resorb the old bone leaving behind a portion of the bone pit known as ‘howship’s lacunae’ exposed. Osteoclasts eventually undergo apoptosis to end resorption (74). Osteoblasts, then form new bone in the bone pit left behind by the osteoclasts for bone formation. OPG, the natural decoy receptor for RANKL is also expressed by osteoblasts and MSCs (69). When OPG binds to RANKL, the latter is no longer available for binding to RANK on osteoclast progenitors and therefore it stops the formation of any further osteoclasts (Figure 1.3).

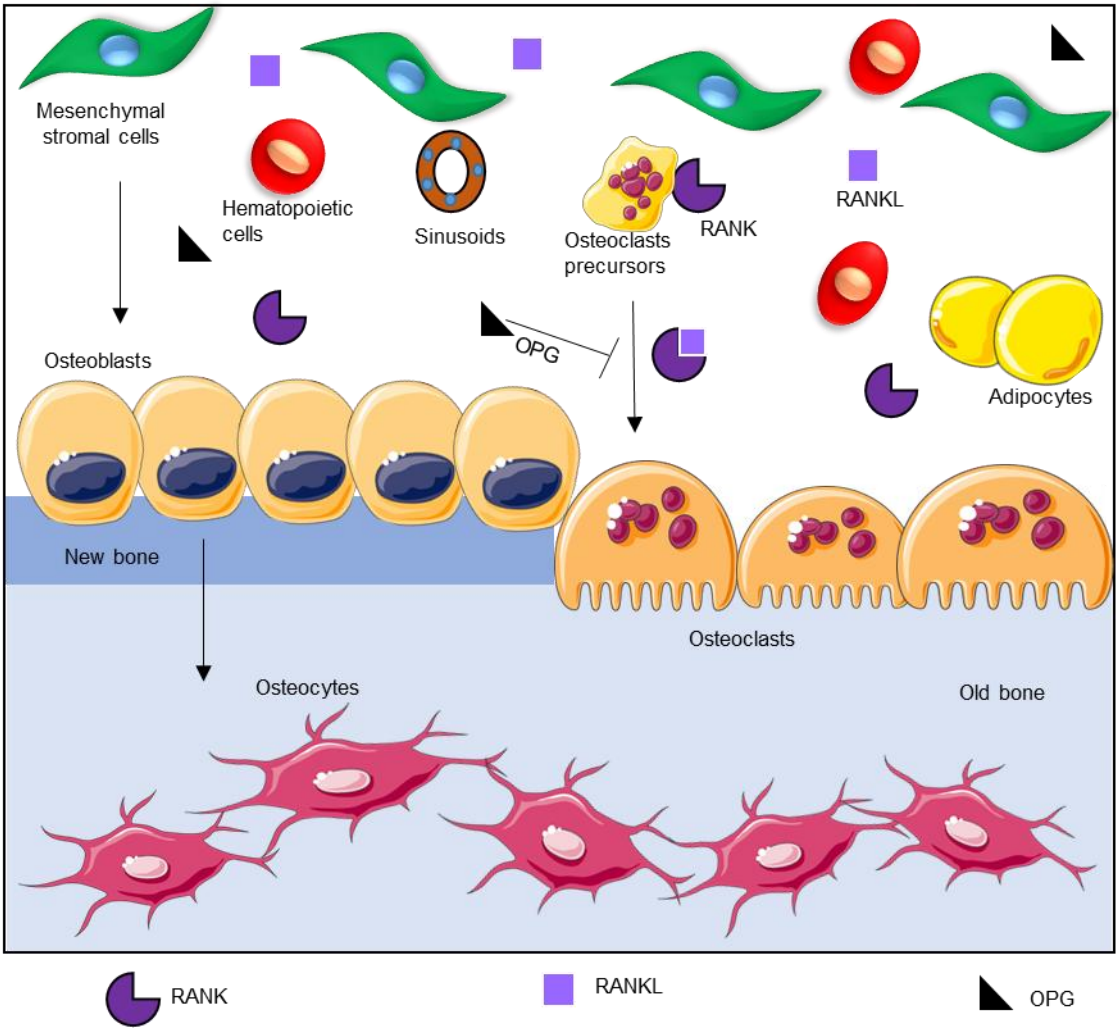

Figure 1.3 Bone remodelling

Figure indicating the different cells involved in the process of bone remodelling. Green spindle shaped cells are MSCs that develop into osteoblasts (shown in orange cells with blue nucleus) that are associated with bone formation. Osteoclast precursors give rise to osteoclasts (shown in orange multinucleated cells with ruffled borders) that are involved with bone resorption. RANK-RANKL binding brings about the process of osteoclast formation which will be stopped if OPG (Decoy receptor of RANKL) binds to RANK instead (adapted from (75))

Bone-remodelling process is therefore tightly regulated and balanced. However, with increasing age, this balance appears to be lost and the rate of bone resorption seems to exceed the rate of new bone formation leading to age-related bone loss (65). Why bone formation rate is reduced in old age remains unclear but it can be speculated that it can be due to a decrease in the number or function of osteoblast progenitors, the MSCs.

Age-related bone loss comprises of a gradual decline in bone mass and strength in both, men and women. Clinically, it is seen as an increase in non-traumatic, low energy

fractures in people of older age, especially over the age of 60. Bone loss can be measured as lower bone mineral density (BMD), bone volume (BV) and bone thickness (BT) on quantitative computed tomography (QCT) and in dual energy X-ray absorptiometry (DXA) scans. Of note, age-related bone loss is higher in women after menopause as compared to age-matched men (76, 77). It is also believed that women begin to lose bone earlier than men and at a greater rate as compared to men, potentially due to the role of sex hormones (78, 79).

The age-related bone loss has also been observed parallel to an increase in the adipose content within the BM. In fact, age-related shift from bone formation to fat formation is potentially the best known age-related change in the BM associated with bone loss (80). With the knowledge that bone is a very complex and dynamic organ, it can be said that these age-related changes in bone are potentially a combination of changes in many cells inside the bone, as well as soluble factors they produce. The reduction of growth factors like insulin growth factor (IGF) which is an important regulator of osteoblast proliferation and differentiation in the bone matrix in older age suggests that the BM microenvironment too plays a part in age-related bone loss (81). The bone cells directly involved in the bone remodelling process are mature and fully-differentiated cells. However, the progenitors of osteoblasts (MSCs) are interestingly, also the progenitors of the fat cells within the BM. Thus, a better understanding of age-related changes in BM MSCs is essential for uncovering the cellular mechanisms of age-related bone loss and their contribution to age-related skeletal diseases such as OA.

1.2 MSCs

1.2.1 Definition, characterisation and $CD45^{-}CD271^{+}$ phenotype

Mesenchymal stromal cells primarily originate from the mesodermal lineage in the embryo and in the neural crest as outlined in section 1.1.3. MSCs are plastic adherent, give rise to bone, fat and cartilage tissues (tri-lineage differentiation potential) and are positive for cell surface cluster of differentiation (CD) markers of CD73, CD90 and CD105 (82). They were first discovered by Friedenstein et. al in 1970s when he found a population of cells within the BM that was highly proliferative, adherent to plastic and formed colonies of fibroblasts when seeded at low density (83). Hence, due to the growth of these cells as colonies of fibroblasts, they came to be known initially as colony forming unit-fibroblasts (CFU-F). The CFU-F assay remains to date, the most accepted method for MSC quantification. Since their discovery, MSCs gained a lot of interest due to their tri-lineage differentiation potential as well as immune-modulating

abilities (84). MSCs have been subsequently isolated from other tissue sources including bone marrow, adipose tissue, synovial tissue and fluid and umbilical cord (85, 86), but these MSCs remain outside the scope of this thesis.

As of now there are globally over 1150 clinical trials that utilise MSCs for applications varying from the regeneration of the liver (GCT no: 0101062750) to the treatment of broncho-pulmonary dysplasia (GCT no:0103683935) (51). Among all the potential sources of MSCs, BM remains the most preferred choice for *in vitro* expansion and further application in clinical settings. This is possibly because the BM is the first and most reliable described MSC source, even though the frequency of MSCs in the BM is very low and ranges between 0.01-0.001% of total nucleated cells (87). Thus they usually need to be enriched and cultured *in vitro* for separating them from other cells and in order to obtain sufficient number of MSCs for clinical applications.

In spite of the growing interest in MSCs, there remain discrepancies in the definition of these cells. The source of the MSCs, the technique used for their isolation, laboratory conditions and many other factors add to this discrepancy. As lack of uniformity can give different results for the same experiments in different laboratories or clinical trials, there is a need to identify key factors to help define MSCs better.

In 2006, the International society for cellular therapy (ISCT) outlined three minimal criteria for defining MSCs in culture. First, MSCs should adhere to plastic, second they should be positive for the expression of CD73, CD90 and CD105 and not expressing CD45 (pan–leukocyte marker), CD34 (common HSC marker), CD14, CD11b, CD79a, CD19 and human leukocyte antigen (HLA) class II. The final criteria for MSCs is their tri-lineage differentiation capacity *in vitro*. For these differentiation assays, plastic adherent cells are grown in culture in medium containing factors for osteogenic, adipogenic and chondrogenic differentiation for 2-3 weeks. For osteogenic differentiation, the level of the enzyme alkaline phosphatase (ALP) found in high quantities in bone and Von Kossa or alizarin red stains indicative of calcium deposition from developing osteoblasts is quantified (82). For adipogenic assay, the level of oil red stain (that stains fat) is measured and for chondrogenic assay, a pellet is formed which is then weighed and the amount of glycosaminoglycans (GAGs, indicative of chondrocytes) is quantified (88).

While the criteria specified by ISCT have significantly contributed to ensuring the uniformity for defining MSCs, there are many factors that were not clearly addressed by the ISCT. To begin with, the first criteria of defining MSCs is the ability of the cell to

adhere to plastic. MSCs are not the only plastic adherent cells – fibroblasts, epithelial cells, endothelial cells and many others - are also adherent to plastic. The next criteria includes the expression of CD73, CD90 and CD105. Even though the ISCT panel has long been used for the characterisation of MSCs (82, 89) and they (CD73, CD90 and CD105) remain largely stable through *in vitro* cultures, it has often been criticised due to the lack of their cell specificity and due to their presence on other types of cells. For example, CD73 and CD105 were found to be expressed by dermal fibroblasts (90, 91) and in umbilical vein endothelial cells (92, 93). CD90 too has been found to be expressed on many other cell types in the BM, hence paving way for the need of a more suitable BM MSC marker. CD90 (Thy 1) is known to mediate cell to cell adhesion and is involved in the adhesion of monocytes and leucocytes to endothelial cells and fibroblasts (94). Also, it is a recognised marker for embryonic stem cells (95). Thus, simply combining the first two criteria, the test for ensuring that cells are MSC is unreliable. While some of these markers have been known to decline with *in vitro* culture expansion (96, 97), the investigations of age-related differences of these surface markers have not provided any conclusive results.

The final criterion is MSC tri-lineage differentiation ability, *in vitro*. While this criterion focusses on the differentiating ability *in vitro*, it critically misses out on the quantitative evaluation of native or uncultured MSC. Also, culturing MSCs have been shown to alter their properties, even at passage zero (P0, cultured but not yet passaged). If the criteria have been assigned to cells that adhere to plastic, it automatically means that they do not define native MSC that are freshly obtained and are uncultured. Changes in cell size, granularity and morphology (98, 99), surface marker expression (97) and gene expression (99) have been shown to alter as a result of *in vitro* culturing. These changes have been referred to MSC '*in vitro* ageing' or ageing induced by culturing cells in non-physiological conditions (100), and are further discusses below in section 1.2.2.

Schneider and Mitsui were first to investigate '*in vitro* ageing' using cultured fibroblasts from young and old donors (101). They found significant difference between fibroblasts from young and old donors with respect to cell proliferation, *in vitro* life span and senescence. They concluded that using cultured cells from young and old donors is a suitable model for investigating age-related changes in cells prior to culture. While the cells used were cultured therefore not devoid of manipulation, their study indicated that *in vitro* culture expansion sensitises cells to two-dimensional (2D) conditions therefore early stages of culture expansion better represent the state of cells *in vivo* compared to late stages.

Owing to lack of clarity in defining uncultured BM MSCs using ISCT criteria, several attempts have been made to investigate surface markers that reliably define them and can be used for their isolation prior to culturing. Mesenchymal stem cell antigen 1 (MSCA-1) and CD271 have been proposed to be positive specific markers of MSCs (94, 102). Historically, Stro-1 was the first surface marker found to be specific for BM MSCs (103). However, Stro-1 was found to be cross reactive with erythroblasts (103) and thus it was used to enrich/identify MSCs in combination with other molecules such as CD106 and CD146 (104) or with platelet derived growth factor receptors alpha and beta (PDGFRα and β) (105, 106) .

CD271 or Low-affinity nerve growth factor receptor (LNGFR) belongs to the tumour necrosis factor receptor super family and has been used as a marker for BM MSCs by several independent research groups in the last two decades. CD271 is also known by the name of p75 neurotrophin receptor and was among the first receptors of its family to be characterised. It has also been suggested to be involved with the functions of cell survival and is suggested to be present in abundance during the growth phase (107). The phenotype $CD45^{low}CD271^{+}$ has been shown to provide best specificity for BM MSCs compared to other candidate markers or their combinations (108, 109). In contrast to CD73, CD105 or CD90, CD271 is absent on fibroblasts indicating its least cross-reactivity with non-MSC adherent cells.

The $CD45^{low}CD271^{+}$ phenotype has also shown to fulfil all the three ISCT criteria needed to define an MSC, that is, the *in vivo* expression of CD73, CD90 and CD105, plastic adhesion and formation of colonies, as well as tri-lineage differentiation following minimal cultivation (110, 111). CD271 has thus become a marker of choice for phenotyping BM MSCs. However, using CD45 as a negative marker or using other known markers like CD106, CD146 along with CD271 provides better characterisation of BM MSCs (112).

1.2.2 Age-related changes in MSCs

Age-related changes in cultured cells are measured using various techniques as large numbers of cells are available for investigation. Quantification of cell numbers (96, 113), telomere length (114), cell proliferative capacity (115), senescence (116), resistance to oxidative stress (117), metabolism (118), alterations in differentiation potential (80, 119) are some of the methods that have commonly been employed for studying age-related changes in cultured MSCs. As seen from the literature, these

investigations have been most commonly performed using cultured MSCs from young and old human donors. However, the effect of passaging on cells' behaviour have not been commonly considered.

Amongst animal models, *Caenorhabditis Elegans* or *C.Elegans* (phylum nematode, round worms with simple/non existing organ systems) has been widely studied owing to its primitive organ system, ease of use and short life-span which enables investigation of age-related changes in the organism during the whole course of its life span (120). However, to understand ageing in humans better, physiologically advanced animals like mice, naked mole rats, rhesus macques (primates) as well as dogs and cats have been used as models (121). Studies like these have immensely contributed to our understanding of age-related differences. Nevertheless, due to ethical considerations, similar experiments cannot be performed in healthy, functional humans. Thus, investigations involving age-related differences in young and old donors remain limited to use of (majorly) culture expanded and rarely, uncultured cells. Next sections will outline the main considerations for the study of age-related changes in human BM MSCs using cultured and uncultured cells.

1.2.2.1 MSC ageing *in vitro* (passage dependent)

As mentioned, age-related changes in human BM MSCs have been historically studied using culture expanded MSCs. However, it hasn't been commonly considered that all primary cultured and plastic adherent cells 'age' *in vitro* as a result of rapid cell division (98, 101). As MSCs are plastic adherent cells, their *in vitro* ageing on tissue culture flasks and similar consumables is relatively well understood and has been studied using proliferation, migration and differentiation assays (98, 122), as well as telomere length (114), senescence assays (99) and gene transcripts measurements (99). A decline in the proliferative capacities of serially-passaged BM MSCs has been observed in various studies (114, 123) along with a change in the expression of certain surface markers with *in vitro* ageing (97). CD106 and CD146 have been associated with young or aged MSCs based on culture expansion *in vitro*. CD106 has been shown to either decline in expression (124), increase in expression (125) or to have random oscillations (123) with *in vitro* passaging. CD146 has been shown to decline in serial passages with culture expansion (124). CD295 has been shown to be higher in cells expanded in culture along with higher levels of Annexin-V indicating increased apoptosis in MSCs of later passages (126). Morphologically, MSCs have been reported to increase in size, appear more flattened and granulated when expanded *in vitro.* Mauney et al. found about 10-fold increase in the size when they compared cells in late

stages with cells in the initial stages of culture expansion (127) along with an increase in cell granularity (99) indicative of *in vitro* ageing and replicative senescence.

Cells in the later passages are known to show greater positivity for senescence, indicated by assays like SA-β-gal (128, 129). Senescence is generally defined as an irreversible arrest of cell proliferation (130) and over the years various methods have been used to identify senescent MSCs in culture (131). Apart from all of the differences mentioned above, the shift towards adipogenic differentiation from osteogenic differentiation has also been shown when MSCs were cultured *in vitro*. Bonab et al. in 2006 have shown that when donor MSCs are cultured over time *in vitro*, then both, their osteogenic and adipogenic potential decline after 5-8 passages (98).

This clearly suggests that *in vitro* differentiation studies are more indicative of replicative senescence that has accumulated over time rather than resembling the actual differentiation capacity of MSCs *in vivo* towards any lineage. The compromised ability of MSCs in later passages to undergo osteogenic differentiation was also recently shown by Yang et al. by gene expression (123) and by other researchers using *in vitro* differentiation assays (132, 133). Even though there are reports that have shown a decline in *in vitro* adipogenic potential with increasing MSC culture (99), the presence of a 'fatty marrow' with advancing age (134) suggests toward an increasing adipogenic differentiation of BM MSCs *in vivo*.

1.2.2.2 MSC ageing *in vitro*: donor age dependent

Summarised evidence from literature outlined in the previous section were performed using BM MSCs from early and late passages. The loss of surface some markers or an increase in cell size that have been observed during this exaggerated ageing process, lead to a hypothesis that similar changes may occur in older donor MSCs *in vivo*.

This section focuses on studies that were performed on BM MSCs from young and old human donors at the same passage, in order to mimic their age-related changes *in vivo,* a little better. A number of studies observed a decline in osteogenic potential (135, 136) and telomere lengths in MSCs from older donors compared to younger donors from the same 'culture age' (114). With respect to surface markers, an up-regulation CD264 (137), CD106 (138) and CD295 (126) has been reported from cultured cells of older donors when compared to cultured cells of young donors. Increased production of ROS along with an increase in the proportion of senescent cells was also observed in cultured cells of older donors as opposed to the cultured cells from younger donors (96).

Stenderup et al. examined the various aspects of MSC ageing using cultured cells from young (18-29 years old) and (68-81 years old) donors and found an age-related decline in the life span of MSCs from older donors (129). Another age-related difference observed when MSCs were cultured was the decline in number of MSCs with advancing age, potentially indicating that higher proportion of MSCs in older donors were senescent (139). These findings suggested that there might be an age-related decline in the number of MSCs *in vivo* potentially due to increased ROS levels, which should also be quantified in uncultured cells.

1.2.2.3 MSC ageing *in vivo*: animal models

Our understanding of age-related changes in uncultured MSCs majorly comes from animal studies. A number of species have been used to understand broader ageing processes *in vivo* using *Caenorhabditis elegans* (or C.elegans due to short life-span) (140), rodents, naked mole rats and primates (121). Investigations specifically regarding the ageing of MSCs have most commonly been restricted to rodents owing to feasibility and ease of handling (141). Among these, the shift from osteogenic to adipogenic differentiation was shown in the 1990s in senescence-accelerated mice–P6 (SAMP6) by Takahashi et al. (142). These mice developed osteoporosis within a few months after birth suggesting that MSC senescence could lead to the imbalance between bone and fat differentiation.

In earlier studies using cultured MSCs, BM MSCs of old C57BL/6 mice (20-26 months old) were found to produce higher amount of fat compared to younger (6-8 months old) mice (143). BM MSCs from the old female Wistar rats (56 weeks) had increased senescence and apoptosis, reduction in proliferative capacity and a decline in bone markers as compared to young (3 weeks) rats (144). Interestingly, the authors did not find any increase in MSC adipogenesis in the old rats when compared to young rats. They found lower number of CFU-Fs in old rats, which was also observed by Katsara and co-workers in adult mice (>3 months) in comparison to young (<1 month) mice (145) and by Josephson and colleagues in old (52 weeks) mice compared to young (12 weeks) and old (146). Studies investigating BM MSCs from young (4 months) and old (15 months) Sprague Dawley rats, found lower tri-differentiation potential in old rat BM MSCs compared to BM MSCs from young rats. The study also observed an age-related decline in the presence of Connexin43 (Cx43) in BM MSCs, (147) which is the most abundant gap junction for intercellular interaction within the bone (148).

In another extensive study, rat BM MSCs were culture expanded to passage 100 and various experiments were carried out to understand the link between *in vitro* ageing and chronological ageing of these animals. BM MSCs from old (12 months) Sprague Dawley rats were compared to young (3 weeks) rats (149) and the results showed that the MSC migration potential and anti-oxidant capacity were the only parameters that decline in both, *in vitro* and chronological ageing. The osteogenic differentiation potential, cell morphology, senescence markers and the expression of genes associated with MSC differentiation showed significant changes only in *in vitro* ageing. Authors concluded that chronological ageing and *in vitro* MSC ageing are distinct processes (149). However, based on accumulated evidence from all presented animal studies, it is possible to hypothesise that *in vitro* MSC ageing (passage dependent) represents an exaggerated ageing process, some features of which (for example, a loss of some surface markers) can be detected in MSCs *in vivo*. Animal studies have also indicated towards a decline in the number and proliferative capacity of BM MSCs, mainly in rodents.

1.3 Osteoarthritis

1.3.1 Epidemiology

Osteoarthritis (OA) is among the most incapacitating degenerative diseases in the world. In the United States, it is the most common joint disorder and 10% of men and 13% of women over the age of 60 suffer from OA (150). It is also cited as the most common rheumatic condition with a huge impact on population and demographics, worldwide (151). Of the 291 conditions examined in the global burden of diseases (GBD) study, hip and knee OA are ranked the 11th major contributor, just after diabetes and falls (151). Incidentally, it is also the most common reason for total hip and knee replacement (152).

The global cost of OA has been estimated to be very high. Chen et al., described total costs as direct costs (medication, surgery), indirect costs (loss of productivity, absence from work) and intangible losses (suffering, decreased QOL and depression). In 2012 in the UK alone, just the direct OA costs were recorded as 168 million for drug therapies and 872 million for joint replacement surgeries (153). The direct and indirect costs of other studies in America and Europe have also revealed considerable amount of cost incurred due to OA. As for intangible loss, the level of anxiety, depression and loss in personal morale along with decline in QOL is unimaginable (154). And, this impacts not only the patient suffering from OA, but also close family members and the loved ones of the patient.

Considering the global burden OA imposes, it has been classified under the diseases of the musculoskeletal system in four further subparts in the ICD by the WHO. The four major categories of OA in the ICD-11 include OA of the hip, OA of the knee, OA of the wrist and hand and OA of other joints. Each of these categories are then further subdivided on the basis of the cause/origin. These are primary, post traumatic, secondary and unspecified causes.

Unfortunately, by the time a patient is diagnosed with the disease, the condition has usually progressed considerably. This means, that by the time, the patient has come to the doctor with complaints of pain and stiffness, chances are, the patient will certainly be given pain relief drugs for pain management and suggested lifestyle changes with immediate effect. The other outcome is that the patient will need surgery. Tissue engineering applications have seen an increased interest owing to the possibility of minimal invasion for joint repair and many biomaterial scaffolds are now available in the market (155); however, the attempts are far from becoming the first choice of therapy on a global scale. The diamond concept (156) of tissue regeneration requiring a scaffold, cells, growth factors and mechanical strength of a given tissue engineering platform often faces challenges to be translated from ‘bench to bed’ successfully (157).

1.3.2 Current understanding of OA

OA is a degenerative disease that may have several underlying causes. Pain in joints is the key symptom for medical attention. WHO’s scientific group on rheumatic diseases estimates that 10% of the world’s population aged 60 years or older have significant clinical problems that could be attributed to OA (158).

Commonly recognised by stiffness and pain in joints, on pain persistence, radiological examination involves measuring joint space narrowing and the presence of osteophytes., thinner cartilage lining and inflammation in the area of pain. While there are different techniques for imaging in OA, the technique of magnetic resonance imaging (MRI) has become a very useful method owing to its ability to evaluate the pathology in structures that are not easily visible in traditional radiographies (159). Due to these observations, initially, OA was approached as the disease of the cartilage. However, it has been recognised as a degenerative disease of the joint owing to the involvement of the cartilage, bone, synovium as well as meniscus and ligaments (160).

In a study in 2008 by Quintana and Azkarte, the authors found the prevalence of hip and knee OA in older population (161). Obesity (162, 163), genetics (164), mechanical

stress (165), physical trauma and age (166), all have been associated with the progression of the disease. While it is understood that over 45% of men and 55% of women over the age of 60 are likely to face OA, it is also established that factors like diet, lifestyle, physical exercise, genetics and environment also play an important role. A number of common factors have been suggested to link old age and OA. These include loss of normal bone structure and increased stiffness in joints (167), oxidative stress due to ROS (168), cellular senescence (169) and reduced QOL (170) outlining that OA is complex and multifactorial. However, majority of these links have been found using cultured chondrocytes and investigations using bone cells remain minimal. Considering OA is multifactorial and the number of links found between ageing and OA, one may hypothesise that these age-related factors are potentially exacerbated in OA patients (Figure 1.4).

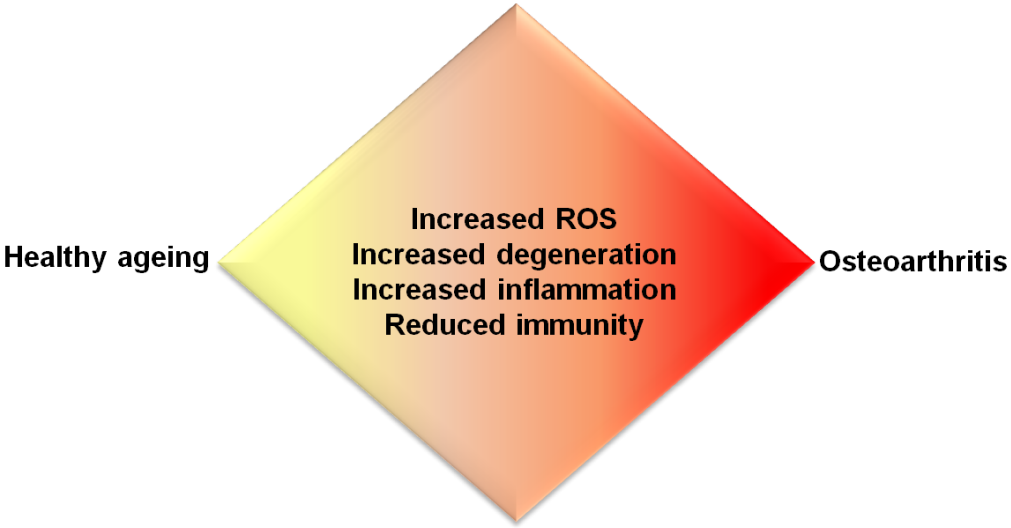

Figure 1.4 Age-related factors that can potentially contribute to OA

The most common way to define OA radiographically is by the use of Kellgren-Lawrence (K/L) radiographic scoring method that defines OA ranging from grades 0-4 depending on the level of severity, presence of osteophytes and joint deformity in general. While a school of thought believes that it is the secretion of pro-inflammatory factors that leads to the degradation of the ECM in cartilage and eventually leads to bone remodelling in OA; another school of thought supports the hypothesis that subchondral bone remodelling and joint inflammation precedes the articular degeneration in OA (171). Another fact supporting our lack of understanding of OA pathophysiology is the presence of over a million scientific publications on 'Osteoarthritis' in google scholar and the disease still being a major health care burden

across the globe with pain management and surgery as the last medical interventions available to patients.

Due to the fact that OA is a multi-factorial disease involving articular cartilage, the subchondral bone as well as the synovium and synovial fluid, the exact mechanisms of the degradative events up to now have been difficult to dissect. Largely, the development of OA may be segregated as ageing dependent processes and ageing independent processes. Ageing dependent processes include the wear and tear mechanisms, such as age-related changes in cartilage matrix, loss of viable chondrocytes due to apoptosis and increase in age-related chondrocyte senescence. As mentioned, age-related process within OA subchondral bone, including MSCs however, remain poorly understood (172). Ageing independent processes include the processes of mechanical load and physical injury, genetic and environmental factors along with stress on lifestyle and obesity which impact individuals, irrespective of their age.

Current approaches for treatment of OA include pharmacological approaches using medication for pain relief like non-steroidal anti-inflammatory drugs (NSAIDs) and others (173) and non-pharmacological approaches. These include surgeries, physical alternatives like orthosis, insoles and foot wear (174), changes in lifestyle (175), use of physiotherapy (176) and an increase in the use of tissue engineering with MSCs (155) for the treatment of OA. Lifestyle changes have also been shown to prevent and potentially reduce the occurrence of OA (177). OA of the hip is the leading cause of the hip replacements in the elderly (178) and with projections of an ageing population worldwide, the incidence of hip replacement in only expected to increase. Thus to investigate the factors in ageing that may be aggravated in OA, samples of hip OA patients were investigated in this thesis.

This project first aimed at investigating changes in number, gene and surface marker expression of BM MSCs in healthy ageing. The same parameters would then be examined in MSCs from donors with hip OA to appreciate any trends in healthy ageing that would be further aggravated in OA.

1.4 Hypotheses and objectives

Hypotheses:

1) There is an age-related decline in the number of BM MSCs in humans.

2) There are age-related differences in the levels of expression of multipotentiality genes, genes associated with senescence and IFN1 pathway in uncultured old donor MSCs compared to young donor MSCs.
3) These age-related differences are further aggravated in uncultured MSCs of patients with primary OA, a major age-related musculoskeletal condition.

Objectives:

1) To quantify the number of BM MSCs across the broad age range (19-89 years old) and between young (19-40 years old), intermediate group (41-59 years old) and old (60-89 years old) donors, using the CFU-F assay and the $CD45^{low}CD271^{+}$ phenotype.
2) To investigate the level of expression multipotentiality genes, genes associated with senescence and IFN1 pathway in uncultured BM MSCs ($CD45^{low}CD271^{+}$) from young (19-40 years old) and old (60-89 years old) donors.
3) To investigate the level of expression of multipotentiality genes, genes associated with senescence and IFN1 pathway in uncultured BM MSCs ($CD45^{low}CD271^{+}$) in patients with primary hip OA.

Chapter 2 Materials and methods

The methods described in this chapter were performed in experiments presented in more than one results chapter. The methods specific to a result chapter have been described in the respective methods section of that chapter. List of reagents, consumables, equipments, softwares used, prepared solutions are listed in Appendix 2 Table 1, 2, 3, 4, 5 respectively.

2.1 Age groups distribution

The term ageing or defining at what age someone can be called old is more than often, subjective. While many references focussed on ageing refer to 'old' as the age of 60 and above (Figure 1.1) globally (5), skeletal ageing become noticeable around the fifth decade of life (62, 96). In both cases, there are individuals, whose biological age (defined as how young their bodies are, how physically active their organs are) do not match their chronological age (actual age in years) (179). Thus to examine age-related changes in BM MSCs in human adults, in this project, three age groups were investigated: young (19-40 years old); intermediate age group (41-59 years old) and old (60-89 years old).

In order to investigate and understand differences in old donors relative to young, this PhD included donors beginning from adulthood (>18 years old). Paediatric donors (<18 years old) were excluded from this study as they are in early stages of growth which includes elongation of bones, sexual development and reaching skeletal maturity (180). Considering this, an obvious difference would be expected in factors like the number of MSCs and gene expression of osteogenic differentiations in old (>60 years old) donors when compared to young, eventually leading to a bias. While an adult over the age of 18 is considered to have attained skeletal maturity, the age of 40 has been suggested to start displaying a decline in BMD (62). Incidence of fractures and bone associated conditions become prevalent around the age of 60 and thus 60 and above was chosen as the cut-off for old donors (1, 181).

Based on the evidence presented above, it can be proposed that in order to understand the process of ageing in BM MSCs that is relevant to their *in vivo* biological ageing, MSCs from human donors must be used prior to any culture expansion. This will avoid the changes in the cells induced artificially through passaging and cultivation-induced replicative senescence. When cultivation cannot be avoided (such as in CFU-F assay), it should be reduced to a minimum. Based on these considerations, uncultured

or minimally-cultured MSCs will be used in this thesis for the purpose of investigating age-related changes in human BM MSCs *in vivo*.

2.2 Ethical approval

Ethical approval for the collection of human tissue samples was obtained from Yorkshire and Humberside National Research Ethics Committee (ethics reference 06/Q1206/127 for bone marrow aspirate (BMA) collection, 14/YH/0087 for femoral head (FH) collection and 04/Q1206/107 for the collection of blood from healthy volunteers (Appendix 5). Participants' details and sample distribution across different study arms are presented in separate tables outlined in Appendix 1.

2.3 Donor selection and sample processing

The age range for the three groups were as follows: 19-40 years old were referred to as the young age group, 41-59 years old were indicated as donors of the intermediate age, and 60-89 years old were referred to as the old age group. The ages of recruited OA donors were between 56-83 years old.

Human BM aspirates (BMA) were collected in 4ml Ethylenediaminetetraacetic acid (EDTA) tubes from the iliac crest (IC) of patients undergoing surgeries for fracture fixation or for removal of metal work in Leeds General Infirmary, Major Trauma Unit. The patients were reported as otherwise healthy. BMA was collected from n=58 donors; 30 males and 28 females with the age range of 19 to 89 years old, median age of 47.5 years old. BMA median volume was 8 ml (range 4-24ml).

In the laboratory, BMA was first passed through a sterile nylon mesh 70μm cell strainer to exclude small clots, if any. The total aspirate volume was recorded and collected in a 50ml sterile falcon tube. Enumeration of MSCs was performed using CFU-F assay explained in Chapter 3 (Appendix 1, Table 1) and flow cytometry (Appendix 1, Table 2) on fresh samples (n=51 and n=32, respectively) followed by experiment for testing resistance to oxidative stress (Appendix 1 Table 2) the next day. The remaining sample was then processed for freezing (section 2.3) for testing MSC proliferation in the presence of human serum (Appendix 1, Table 1) explained in Chapter 3 or for use in cell sorting followed by gene expression (Appendix 1, Tables 2 and 3).

FHs from OA donors (Appendix 1, Table 4) was collected, processed and frozen by Dragos Ilas according to the published methods (182, 183). In brief, frozen cells from

the femoral head were defrosted, stained and then processed for cell sorting as explained in section 2.3 and 2.5 below.

2.4 Cell freezing and defrosting

For freezing of the nucleated cells (NCs), BMA was treated with ammonium chloride (NH_4Cl) solution (BMA: NH_4Cl=1:8, v/v) for 10 minutes at room temperature (RT) for the lysis of erythrocytes and then centrifuged at 650g for 5 minutes at RT to pellet NCs. The supernatant was discarded and the cell pellet was washed twice with PBS to remove any remaining traces of NH_4Cl. The pellet was then re-suspended in 5-10ml of Dulbecco's minimum essential medium (DMEM) supplemented with 10% Fetal calf serum (FCS).

The number of NCs were counted using a haemocytometer by mixing 10μl of cell suspension with 10 μl of trypan blue dye. At least 2 large squares with 16 grids each were counted and the average count was multiplied by 10^4, the dilution factor for trypan blue and the total volume of cell suspension (for example, $56x10^4$ x 2 (dilution factor for trypan blue) x 5ml (total volume of suspension)). The cells were centrifuged again at 650g for 5 minutes at RT. The supernatant was discarded and the cells were re-suspended in the required volume of freezing medium (FM) (Appendix 2, Table 5). 10-$20x10^6$ cells were frozen in each cryovial in 1ml of FM, labelled and first stored in -80°C freezer for at least 24 hours followed by transfer into liquid nitrogen for future use.

To revive frozen cells for future experiments, vials were defrosted in water bath (37°C) and the cell suspension was added to 15ml falcon tubes. Thawing medium (TM) was prepared by adding 20μl DNAse to 20ml of complete medium to prevent accumulation of cells into clumps due to released DNA from damaged cells. 5ml TM was added for every ml of defrosted cell suspension drop by drop. Cells were pelleted at 600g for 5 minutes at RT to remove DMSO and were slowly re-suspended in TM up to desired volume. For any study using these cells, if a clump was observed at any given point of time, the cell suspension was filtered using 70μm cell strainer to obtain a homogenous cell suspension.

2.5 MSC Enrichment using Anti-Fibroblast microbeads

MSCs are a rare population in the BMA (87, 108) and the MSC enrichment step is needed prior to fluorescent activated cell sorting (FACS) to pre-enrich MSC thus saving time and cost of cell sorting. Enrichment of MSCs was performed by three different methods in the first year of this project including Low-affinity nerve growth factor or

(LNGFR) beads and RosetteSep cocktail, and owing to no differences in the proportions and absolute numbers of enriched MSCs obtained, the most feasible method of using Anti-Fibroblast (A-Fib) microbeads was chosen for future experiments. A-Fib beads are magnetically conjugated to anti-D7-Fib antibody allowing MSCs as D7-Fib positive cells (108) to be retained in the column during separation and hence isolating them from D7-Fib negative cells.

For this, defrosted NCs were pelleted at 650g for 5 minutes at RT and were re-suspended in 500μl to 1ml of MACS buffer (Appendix 2, Table 5) to achieve average concentration of $1x10^7$ cells/ml as per the manufacturer's instructions. 40μl of A-Fib beads per 10^7 cells was added to the cell suspension, mixed well and incubated at RT for 30 minutes. The cell-bead suspension was next washed with 1-2ml of MACS buffer and centrifuged at 650g for 5 minutes at RT. The supernatant was carefully removed and the cells were re-suspended in1.5-2ml of MACS buffer. The cell suspension was passed through a cell strainer to ensure that there were no clumps.

MACS column was positioned in multi-stand with the MACS Separators attached, these are powerful magnets and MACS columns amplify the magnetic field by nearly 10,000 folds. MACS buffer alone was first run through the column to prime the column before adding the cell suspension carefully. The fraction of cells retained in the column included the magnetically labelled cells while the cells leaving the column (negative fraction) did not contain the magnetic beads. The column was washed with 2-4ml of MACS buffer to wash out residual negative cells and the negative fraction was collected in a 15ml sterile tube.

Once the negative fraction had been collected, the column was removed from the magnetic field and placed on top of a separate 15ml sterile tube. 2-3 ml of MACS buffer was added into the column and the bead-labelled cells (positive fraction) were collected by firmly pushing the plunger into the column.

2.6 Flow cytometry and Fluorescence activated cell sorting (FACS)

2.6.1 Principle of flow cytometry

Flow cytometry is the technique that uses fluid dynamics (flow) to study and quantify or measure (metry) the different properties of cells (cyto) within a heterogeneous cell population (184). It has five key components that include the sample, fluidics, optics, detectors and output. The core of the instrument is the fluidic system consisting of the

flow chamber with sheath fluid and pressurised lines, both of which are responsible for the transportation of the particles, or in this thesis, cells (185). Excitation optics include lenses which help to direct and focus the other segment of the excitation optics, the lasers. The lasers produce light using high voltage electricity. The point at which the stream of cells interacts with the laser is known as the interrogation point. It is also the point where the optics detect the light scattering (186). At the interrogation point, the light emitted by cells is collected by the collection lens and passed through dichoric mirrors (mirrors with separate reflection/transmission property at two different wavelengths) and filters that ensure the wavelengths of specific lights to be detected by their respective detectors.

The light that travels along the path of the laser is in the forward direction and is assembled by collecting lens as ‘forwards scatter’ or FSC. FSC is indicative of the cell size or surface area and is the result of diffraction of light. The light then travels to the photodiodes which is responsible for the conversion of light into electronic signals to be recorded by the computer system (185). When light comes in contact with cells and refracts or reflects and is collected at 90 degrees to the laser, the scattering pattern is called ‘side scatter’ or SSC. SSC is indicative of granularity and the internal complexity of cells. (185). The light signals captured by both photo diodes and photomultiplier are converted to electronic currents which travel to the amplifier to be converted into voltage pulse. This analogue measure is further amplified by linear and logarithmic amplifiers, converted into digital signals by digital converters which may then be used to produce histograms or plots for data representation (185).

2.6.2 FACS

This is where the use of fluorescent labelling comes in, which combines the functionality of the flow cytometers with fluorescent labelled antibodies used to stain cells. These florescence labelled antibodies can bind to specific cell types and help in distinguishing different types of cells from a heterogeneous mixture of cells. The distance between the absorption and emission maxima (peak) in the spectra of a fluorochrome is known as ‘Stoke’s shift’. The higher the Stoke’s shift, the greater is the separation between the absorbance and the emission of a given fluorochrome (187).

The total photons being absorbed by a fluorochrome are associated with the excitation wavelength. Thus, while FITC is known to absorb light at 400-550 nm, its highest absorbance is usually at 490nm at which it absorbs more photons ensuring that the emission will be more intense (187). Using the fluorochromes and the principle of dynamic light scattering, some flow cytometers can segregate labelled cells from a

heterogeneous cell population. This is referred to as cell sorting. When fluorochrome labelled cells pass the laser beam, they are excited by the correct wavelength of the laser light. After excitation, the emitted light is directed towards emission filters allowing for the detection of multiple fluorochromes emitted by a cell.

The fluorochromes used are usually for cell surface staining or for intracellular staining. Selection of the appropriate fluorochromes for a given study is essential and the panel of fluorochromes to be used is dependent upon the aims of the study. In this thesis, different fluorochromes were used to identify and enumerate MSCs using the $CD45^{low}CD271^{+}$ phenotype (Table 2.1). The same phenotype formed the base of cell sorting into two separate populations of MSCs ($CD45^{low}CD271^{+}$) and HLCs ($CD45^{+}CD271^{-}$). Studies investigating levels of potential age-related surface markers and for detecting oxidative stress in young and old donor uncultured BM MSCs were performed using fluorescent antibodies and dyes listed in table 2.1.

2.6.3 Sample preparation and cell staining for cell sorting

Vial containing FH digest was defrosted in TM, cells were counted and distributed in 10 tubes to be used for cell sorter instrument settings (8 tubes) and cell sorting (2 tubes). Owing to significantly smaller numbers of MSCs in BMA compared to FH digests (182), FH cells were used for setting of the machine for unstained cells, isotype controls and spectral compensation. These procedures were performed by the technical staff of the Flow Cytometry and Imaging Facility in St.James's University Hospital. Cell sorting was usually performed using two BMA samples (from one young and one old donor) on a single day, in addition to a FH sample.

For sorting, the enriched cells from BM samples were counted and distributed equally into 2 tubes, one to be stained with CD271 PEVio770, CD45 V450, CD106 PE and CD295 APC combination and another one for CD271 PEVio770, CD45 V450, CD146 PE and Cx43 APC (Table 2.1). Violet laser (405 nm excitation) was used for the V450 fluorochrome, blue laser (488 nm excitation) was used for PE, PECy7 and PEVio770 and PerCP fluorochromes and the red laser (633 or 640 nm excitation) was used for APC as indicated below in table 2.1.

Average cell numbers for isotype controls were $3x10^5$ while for tubes with cells for sorting, contained up to $3x10^6$ cells. Both, FH and BM cells were centrifuged to form a pellet. The supernatant was discarded and the cells were re-suspended in 100μl of FACS buffer. The cells were then treated with Fc block for 10minutes to prevent non-specific binding.

The cells were subsequently incubated with the antibodies in dark for 15 minutes. After staining, the cells were washed in FACS buffer, re-suspended in 500μl of the same buffer and passed through the filtered-cap flow cytometry tubes to remove the presence of any cellular clumps. Finally, 10μl 7-aminoactinomycin D (7-AAD) was added to all tubes containing cells (except unstained) to distinguish between live and dead cells and run through the FACS machine. The list of antibodies and their volumes, isotype control antibodies and their volumes used is summarized in Table 2.1.

Table 2.1 List of antibodies, isotype controls and their volumes used for various flow cytometry investigations

Experiment	Cells	Antibodies /dye	Instrument	Excitation (nm)	Laser used	Emission (nm)	Isotype control	Vol (μl)
Enumeration*	MSCs	CD271 APC	LSR II	650	633	660	IgG1	20
	MSCs	CD45 PECy7		496	488	785	IgG1	20
	MSCs	7-AAD PerCP		482	488	647	NA	10
ROS production*	MSCs	CD271 PEVio770	LSR II	488	488	775	IgG1	20
	MSCs	CD45 V450		404	405	448	IgG1	20
	MSCs	CellROX FITC		508	488	525	NA	2
	MSCs	Sytox APC		640	633	658	NA	2
FACS*,#	MSCs and HLCs	CD271 PEVio770	BD Cell sorter	488	488	775	IgG1	20
	MSCs and HLCs	CD45 V450		404	405	448	IgG1	20
	MSCs and HLCs	CD106 PE		565	488	575	IgG1	20
	MSCs and HLCs	CD295 APC		650	640	660	IgG2a	10
	MSCs and HLCs	CD146 PE		565	488	575	IgG1	10
	MSCs and HLCs	Cx43 APC		650	640	660	IgG2b	10
	MSCs and HLCs	7-AAD PerCP		482	488	647	NA	10

MSC: $CD45^{low}CD271^{+}$ cells, HLC: $CD45^{+}CD271^{-}$ cells, *data presented in Chapter 4, #data presented in Chapter 6

2.6.4 FACS and sample collection

Once instrument settings were adjusted, stained samples were sorted to collect $CD45^{low}CD271^{+}$ cells (MSCs) and $CD45^{+}CD271^{low}$ cells (HLCs) which would be the control population for the population of interest (MSCs) (188). While sorting, data was simultaneously acquired for the selected surface markers of interest – CD106/Vascular Cell Adhesion Molecule (VCAM-1), CD295/Leptin receptor (LepR), CD146/ Melanoma Cell Adhesion Molecule (MCAM) and Connexin43/ gap junction- (Cx43/GJA-1). Sorted cells were collected into 1.5ml eppendorf tubes containing 350µl Buffer RL (Appendix 2, Table 1) that has guanidinium salts for lysing cells and exposing the genomic contents (189). The salts also ensure the inhibition of RNAse to prevent the degradation of RNA into smaller components. The eppendorfs were frozen at -80°C until further use.

2.6.5 FACS data analysis

Data from FACS was analysed using FlowJo software (version 10). The gating strategy and the histograms for each of the surface markers are shown in the respective chapters. First, the raw files were loaded on to the workspace and each tube within a given experiment was analysed in detail. Dot plot display was chosen to better visualise the cells of interest. 7-AAD negative cells were gated as live cells. Using this live cell gate, the cells with the phenotypes $CD45^{low}CD271^{+}$ (MSC) and $CD45^{+}CD271^{low}$ (HLC) were identified. On each of these cell phenotypes, the expression of surface markers (CD106, CD295, Cx43 and CD146) was analysed using respective histogram displays. MFI was recorded for each of the surface markers for MSCs and HLCs for all samples. The MFI values were then compared and analysed for any differences in relation to donor age or the presence of OA, as described in Chapter 4 and Chapter 6, respectively.

2.7 Gene expression

Genes are the fundamental unit of heredity and information about a person at the genetic level. Measurement of the expression of genes have long been used to identify differentially expressed genes in diseases or other anomalies. To understand if there are any differentially expressed genes in old donor MSCs as compared to young donor MSCs, or in OA, gene expression experiments were carried out using sorted $CD45^{low}CD271^{+}$ MSCs and $CD45^{high}CD271^{-}$ HLCs as the control.

2.7.1 Principle

Polymerase chain reaction (PCR) may be defined as a chain reaction that occurs to increase the final product exponentially with the help of an enzyme polymerase. Thus the reaction enables the production of billions of copies of DNA from a single copy of DNA. The essential components needed for the production of billions of copies from a single reaction include the enzyme, primers, DNA template and nucleotides. Initially, the technique was developed for DNA but was soon applied to RNA as well. However, the technique is qualitative, consumes time for the preparation of gels using hazardous chemicals, is limited in terms of number of samples and allows the detection of the amplified product only after the end of all the cycles.

Quantitative PCR (qPCR) technique is built on the same basic principles of PCR but has several advantages. Not only is the method quantitative but the reaction can be quantified at every step of amplification. This is achieved by the use of fluorescence labelled probes (in this thesis, TaqMan probes). The fluorescent labelled probes have a fluorophore that is not active in the beginning of the reaction as it is attached with a quencher. As the reaction proceeds, the fluorochrome on the 5' end and the quencher on the 3' end of the TaqMan assay (in this thesis FAM-MGB respectively) are separated by taq DNA polymerase. This separation activates the fluorochrome which is now fluorescent ensuring the amplification dependent increase in the fluorescence is tracked and measured by the amplification curve. The sample preparation does not involve the assembly of gels using hazardous materials and allows a large number of sample reactions at the same time (generally up to 386 samples). These factors make qPCR a preferred technique to quantify gene expression over normal PCR.

2.7.2 Fluidigm and Integrated fluid circuits (IFCs)

Due to the population of interest MSCs being a rare population, it was vital to use a method for gene expression that would allow high quality and consistent outputs even with input of sample with low cDNA concentration. Gene expression analysis in sorted MSCs and HLCs were performed using integrated fluid circuits (IFC) chip technology which is based on the principles of microfluidics. These devices are based on the principle of qPCR but allow further miniaturization and integration of liquid handling components on a single chip. Such microfluidic devices utilise technology that combines networks of channels (fluid lines of extremely small dimension (10^{-6}m)), chambers (holding extremely small volumes of liquids (10^{-9}l)) and Nanoflex valves (that can withstand very high pressure (175mmHg)) in a single chip (190). IFC chips allow reactions with small quantities of cDNA and TaqMan primers carrying out numerous PCR reactions (for example up to >9000 reactions for IFC96*96) depending on the size

of the chip, all at once. These chips are aimed at high throughput data collection and analysis of biological samples with low concentration of starting materials.

Two types of IFC chips were used in this thesis based on the number of genes investigated. The Flex Six™ chip (Section 4.2) was used for performing gene expression in Chapter 4 which investigated 12 genes, owing to the 12 segments in each of the 6 compartments of the chip. For investigation of genes in Chapter 5, 2 of 48.48 chips (Section 5.2) were used to examine 96 genes. At one time in one 48.48 chip, 2,304 reactions were carried out. The difference between the two chips used lies in the number of assays and samples that may be run and the final volume of reaction mixtures. The concept, principle and application remains the same. A simple flowchart (Figure 2.1) shows the steps that were followed from the sample preparation to the gene expression data generation, irrespective of the chip used.

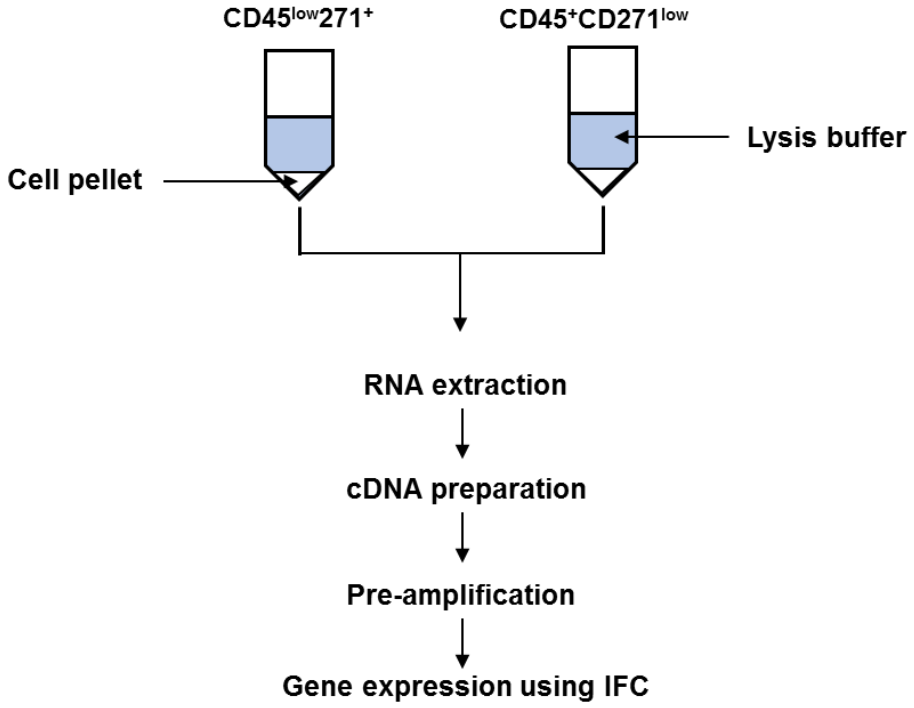

Figure 2.1 Flow-diagram indicating the steps followed for obtaining gene expression data throughout the thesis

2.7.3 Sample preparation

As indicated in section 2.3, frozen NCs from young and old donor cohorts (Appendix 1, Tables 2 and 3) were defrosted and then subjected to cell sorting based on $CD45^{low}CD271^{+}$ and $CD45^{+}CD271^{-}$ phenotypes for MSCs and HLCs, respectively. HLC fraction included BMA cells of the hematopoietic lineage and served as a control to study specificity of the selected panel of genes to MSCs. Cell counts were recorded for

each population fraction. The cells were collected in the RL buffer (lysis buffer) in an eppendorf and the cell lysates were then kept at -80 until further use.

2.7.4 RNA extraction

To minimise the degradation of RNA, the working bench was wiped with RNAse away. RNA extraction was performed using Norgen single cells RNA isolation kit (Appendix 2, Table 1) which uses the column technique for better yield owing to the low number of cells in the fraction of interest (range: 647–22,229 with a median of 4,694 $CD45^{low}CD271^{+}$ MSC). The technique essentially utilises spin column chromatography using resins as the separation matrix which ensures the purification of RNA without the use of phenol or chloroform (189).

Frozen cell lysates were defrosted on ice and 200μl of 100% ethanol was added to each lysate for every 350μl of the sample volume. The mix of cell lysate and ethanol was then gently mixed by vortexing for 10 seconds. The mix was added to a column contained inside a collection tube and centrifuged for 2 minutes at a speed 3,500g at 15°C. This ensures the binding of the RNA to the resin column while proteins are eliminated in the flow-through liquid (189). The flow-through was discarded and the column was washed with 400μl of wash solution by centrifugation for 1 minute at 14,000g to ensure the removal of any remaining impurities.

Genomic DNA digestion ensures the removal of any DNA contamination that was not eliminated in the previous steps and could lead to false positives and lower detection sensitivity in samples. It was performed by adding 100μl of DNAse I digestion mix (DNAse I and DNAse I reaction buffer) to the column containing the RNA and centrifuged for 1 minute at 14,000g ensuring the entire volume had passed through the column. The flow-through was added back onto the column and the column was incubated for 15 minutes at RT. Following incubation, the column was washed twice with 400μl of wash solution by centrifuging for 1 minute at maximum speed. The flow-through was discarded and the column was centrifuged twice to thoroughly dry the resin.

The dry column was placed in a new elution tube and 12μl of elution solution was carefully added (drop-wise) to the centre of the column and incubated for 2-3 minutes to allow the hydration of RNA attached to the column. The column was centrifuged for 1 minute at 200g followed by 1 minute at 14,000g to ensure that the elution solution had passed through pulling the purified RNA along with it. The column was then discarded and the concentration of the eluted RNA was measured using Nanodrop.

Nanodrop measures the amount of a variety of molecules including nucleic acids based on the principle that different molecules can absorb lights at different wavelengths (spectrophotometry). Proteins were measured at 280nm, nucleic acids (sample of interest) at 260nm and organic compounds at 230mn. The eluted RNA was quantified by recording the generated concentration of the RNA in ng/μl by the spectrophotometer. The purity of the RNA samples was checked using the 260/230 ratio indicative of presence of impurities belonging to organic compounds for values below 2 and the 260/280 ratio indicative of protein impurities in the RNA sample below 1.8. The extracted and quantified RNA was then placed in -80°C until further use.

2.7.5 Reverse transcription (Rt) for cDNA preparation

Rt is needed to convert single stranded RNA to cDNA. The Rt component mix was prepared by mixing 14μl of Rt master mix and 28μl of nuclease free water. Rt master mix contains all the components required to prepare cDNA which include deoxyribonucleotide triphosphates (dNTPs), mix of oligo-dTs and random primers, RNAse inhibitor and reverse transcriptase along with buffers. The dNTPs act as the building blocks for the new strand being built, the primers direct the synthesis of the strand, RNAse inhibitor prevents the degradation of RNA in the process and reverse transcriptase is the enzyme that is needed for Rt for cDNA preparation. 3μl of the Rt mix was added to each well in a 96-well plate followed by 2μl RNA sample to each well making the total volume 5μl. The plate was sealed, mixed by vortexing, centrifuged and placed in the thermocycler for the reverse transcription using following the settings: 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C and then hold at 4°C, for approximately 50 minutes. The produced cDNA (5μl) was then stored at -20°C for future use.

2.7.6 Pre-amplification

As BM MSCs are a rare population, the number of cells ($CD45^{low}CD271^{+}$) obtained after cell sorting were relatively low (section 2.6.4) and so was the concentration of the RNA from these cells (5.6-32.5 ng/μl with a median of 11.55ng/μl). Limited quantities of RNA and cDNA often restrict gene expression analysis, ultimately generating no results (191). Thus to ensure detectable levels of starting material (cDNA), pre-amplification (PA) step was needed. This is basically a qPCR with limited cycles before the actual qPCR begins (192). The limited number of cycles (usually 10-18) ensure the amplification of cDNA without significant bias. PA allows for amplification for up to 96 target genes and a pool of primers is prepared form the same gene expression assays that are to be used for the qPCR (191). PA is an established method and is increasingly being used for similar work (193).

TaqMan assays used in different study arms are indicated in respective chapters. For PA, pooled TaqMan assay mix was prepared by mixing 2μl of each TaqMan assay (indicated in respective chapters) and the final volume was made up to 200μl using nuclease-free water. Next, 1μl of PA master mix was added to 1.25μl of the prepared pooled TaqMan assay, 1.5μl of nuclease-free water and 1.25μl of cDNA prepared (section 2.6.5). Total volume of 5μl was transferred to individual wells in a 96-well plate in a pre-determined sequence. The plate was carefully sealed and the samples were pre-amplified on the thermocycler using 18-cycle protocol (2 minutes at 95°C, 15 seconds at 95°C, 4 minutes at 60°C and then hold at 4°C). The sample were then prepared for qPCR in Fluidigm. 18-cycle PA is the recommended number of cycles for samples with a concentration range of 5-20ng of RNA (193). The PA samples were diluted with 15μl Tris-EDTA (TE) buffer and then loaded on the IFC chip for qPCR on the same day.

2.7.7 Sample run

qPCR was performed using Fluidigm Flexsix™ IFC and 48.48 IFC. Flex Six™ chip has 6 independent compartments. Each compartment has 12 assay and 12 sample inlets. Each compartment can be run independently as a separate experimental run or simultaneously. Before the first run on the chip, the complete volume (150μl) control line fluid was carefully added to the accumulator region only, pushing past the spring for the purpose of priming the chip. The 48.48 chip is a single-use chip and needs to primed using the control fluid just as in the Flex Six™ chip. For both the chips during priming, the barrier plugs were kept on and the chip was placed in their respective priming instrument and the prime script was run for 15 minutes.

After priming, the chip was removed from the machine and the barrier plugs of the compartments intended for use were removed. For the assays, 2μl of each TaqMan assay and 2μl of assay loading buffer were mixed and 3μl from this mix was added to the 12 wells in the assay compartment in a pre-determined sequence. Similarly, the sample mix was prepared by adding 28μl of TaqMan universal master mix, 2.8μl of sample loading buffer and 1.8μl of pre-amplified cDNA, and 3μl of the sample mix was added to each well in the sample side of the compartment. The volumes used for 48.48 chip are indicated in Chapter 5, section 5.2.

2.7.8 Data collection, analysis and presentation

The qPCR run was performed on the BioMark™ HD System with the 'Data collection' software and GE Flex Six^{TM} Standard cycling protocol (35-40 cycles) for approximately 60 minutes cycles. Owing to the large number of reactions that can occur simultaneously within extremely small volume (10^{-9} l) in a single run, it surpasses the other quantitative assays for qPCR (193). After completion of the run, IFC was removed from the Biomark and the post run script was run for 5 minutes on IFC Controller HX to relax the valves. This allows the unused compartments to be used in further experiments within a span of 3 months. 'Biomark Real-Time PCR Analysis' software was used for the analysis of the data. First, the cycle threshold (Ct values) were generated for each gene using the software for analysis and the data was exported to Microsoft excel for further calculations. The data were then normalised with *hypoxanthine-guanine phosphoribosyl transferase 1* (*HPRT1*) as the housekeeping gene to generate the ΔCt values for each gene using the formula [Ct $_{\text{target gene}}$ - Ct $_{\text{housekeeping gene}}$]. Finally, the ΔCt values were converted to 'relative expression' for each gene using the formula [$2^{-\Delta Ct}$] and the values were compared between young and old donor cohorts, or in OA cells and age-matched controls, in both MSC and HLC populations.

Cluster and Treeview softwares were used to generate clusters of gene expression for the samples and observe any trends between the expression of genes in $CD45^{low}CD271^{+}$ MSCs and $CD45^{high}CD271^{-}$ HLCs. The softwares were also used to get an overview of gene expression data distribution across the entire donor cohort. Grey squares in clusters represent data that was below detection. Comparative investigations between young and old donors or old donors and OA patients were analysed using GraphPad Prism software (version7.0a).

2.8 Data presentation and Statistical analysis

Various data representation techniques were used for displaying the results obtained. Cluster analysis was used to observe any differences in gene expression across the entire donor cohorts. Dot plots were used to indicate individual values, frequency distribution curves were used to display the data frequency across a range. Bar graphs were used to indicate the results of a subgroup, histograms were used to indicate MFI generated using flow cytometry.

Statistical analysis and graphs preparation were performed using GraphPad Prism software. The distribution of the data was assessed using the Shapiro-Wilk and Kolmogorov-Smirnov tests for normality. As the data were found to be non-parametric,

and unpaired, Mann-Whitney U test was used to compare data between young and old donor groups, or between MSC and HLC fractions. The tests used for each experiment are specified in the respective chapters. For all data, p value of <0.05 was considered significant (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001).

Chapter 3 Proliferative capacity of human BM MSC from using colony the forming unit-fibroblast (CFU-F) assay

3.1 Introduction

As stated in the general introduction (section 1.2.1), BM aspirate from the iliac crest (IC) is a reliable source for MSCs but they exist as a rare population with a frequency between 0.01-0.001% of nucleated cells (87, 108). CFU-F assay has been the oldest method for counting the number of MSCs ever since its discovery by Friedenstein and colleagues (194). MSCs are enumerated by counting the number of single cell derived colonies that are formed on petri dishes based on first, their ability of adhering to plastic and second, their ability to proliferate into a complete colony. Each colony is representative of a single MSC and it takes up to two weeks before the colonies can be visualised. In spite of it being a time consuming method, it is still performed today and remains the most feasible way to enumerate MSCs based on plastic adhesion. Flow cytometry based methods and phenotypes used for enumeration of MSCs will be discussed in Chapter 4. The plastic adherent cells that are not proliferative, either do not form a colony or will form a colony with very few cells. To ensure that a single MSC derived colony that was counted as a colony was a true representation of its proliferative capacity, a group of at least 50 cells was considered a colony in this project (195). Thus, each colony from the CFU-F assay represented a single MSC.

3.1.1 Previous studies investigating age-related changes in number of human BM MSCs based on CFU-F assay

Several groups have performed CFU-F experiments to examine the age-related changes in number of MSCs. These experiments have yielded inconclusive results with some of them showing a decline (96, 114, 132) while some not showing any age-related difference (113, 196, 197) in the number of colonies. This can be due to the lack of uniformity in laboratory protocols used for the CFU-F assay. Table 3.1 lists investigations in the number of MSCs as observed in cultured cells and in uncultured cells. From Table 3.1, it can be seen that many of the studies for investigating the age-related changes in number of MSCs using CFU-F assay have been performed on cultured cells. When grown in cultures, *in vitro* ageing sets in within the MSCs (98, 114), as stated in section 1.2.2, which is not representative of uncultured cells *in vivo* (100). Thus, the actual biological age-related difference in number of native MSCs requires further elucidation.

Table 3.1 List of previous studies investigating age-related change in the number of BM MSCs counted by CFU-F assay

MSC source	Age (years)	Isolation	Media	Colony definition	CFU-F	Ref
Trabecular BM	14-87; no groups	PA	α-MEM+ 10%FCS	> 1mm in diameter	No change	(113)
BM from iliac crest	Y: 22-44; O: 66-74	DC	α-MEM+ 10%FCS	>16 cells	No change	(197)
BM from iliac crest	Y: 0-18; O: 59-75	DC	DMEM+ 10%FCS	ND	Decline	(114)
BM from iliac crest	Y: 19-40; O: >40	DC	DMEM+ 10%FCS	>50 cells	Decline	(96)
BM	Y: 6m-16y; O: 29-76	PA	α-MEM+ 20%FCS	ND	NS decline	(198)
BMA	1-52; no groups	DC	DMEM+ 20%FCS	>50 cells	No change	(199)

BM: Bone marrow, BMA: Bone marrow aspirate, O: Old, Y: Young, PA: Plastic adhesion, DC: Density centrifugation α-MEM: Alpha – Minimum essential medium, DMEM: Dulbecco's minimum essential medium, FCS: Foetal calf serum, ND: Not defined, NS: Non significant

When uncultured cells have been used, different methods in extraction of BMA, further processing of the aspirate for MSC isolation, and using different media, culture conditions as well as colony definition and scoring criteria can all explain the variation in the results (Table 3.1).

3.1.2 Previous studies investigating age-related changes in colony area and density in human BM MSCs based on CFU-F assay

While CFU-F assay has commonly been used to count MSCs, few studies have also analysed the colony area and fewer studies have evaluated the colony density as a measurement of proliferative capacity of MSCs. Ultimately, the number of studies correlating colony area and density to donor age are sparse (listed below in Table 3.2). Colony area measures how big a colony is and larger colony areas have been associated with younger donor samples (132). However, the variations in the methods adapted in independent laboratories and donor variation, make it difficult to come to a consensus with respect to age-related changes in colony area.

Even though colony area measures how big the colony is, it does not take into account the density of cells within a colony. The denser a colony, the more the number of cells that will be closely packed within a colony, indicating higher proliferative rates of colony initiating MSCs. As per my knowledge, there has been only one study that has mentioned colony density in relation to donor age in uncultured BM MSCs (Table 3.2). The study used uncultured cells that observed no change in colony density (200) but they did not present the data for the same.

Quantification of colony size has either been performed by comparing diameters of the colonies or by comparing the area of colonies in millimetre square (mm^2) or related units (132, 201). Quantification of density of a colony is relatively more challenging considering the variation in sizes of the colonies, the patterns of cells in which they may overlap making it difficult to calculate the density. This is potentially why, only 2 studies have mentioned density of colonies in their studies and only one of them reported qualitative observations (Table 3.2). This indicates that a study of BM MSC colony area and density, using a large cohort of donors, is needed.

Table 3.2 List of previous studies investigating age-related changes in colony area and density of human BM MSCs

MSC source	Age (years)	Cultured	Colony size criteria	Change in colony area	Change in colony density	Ref
Trabecular BM	14-87; no groups	UC	NR	Decline	NC (no data)	(113)
Vertebral spine BM	Y: 3-36; O: 41-70	UC (cadavers)	Y-10.23mm^2; O- 8.23 mm^2	Decline	NR	(132)
BM from iliac crest	Y: 22-44; O: 66-74	UC	Y- 7.2mm^2; O- 6.1 mm^2	NC	NR	(197)

BM: Bone marrow, Y: Young, O: Old, UC: uncultured, NR: Not reported, NC: No change

3.1.3 Significance of microenvironment in MSC proliferation

As mentioned previously (in section 3.1.2) CFU-F assay can not only directly measure the number of MSCs from a donor, but it also gives information about the proliferative capacity of these MSCs. MSCs from BMA *in vivo* exist within a complex matrix composed of other types of cells and various other soluble and extracellular matrix factors forming its own micro-environment (Figure 1.3). Stenderup et al. investigated the *in vivo* bone forming capacity of human MSCs obtained from 5 young (24-30 years old) and 5 old (71-81 years old) donors in immuno-deficient mice. Interestingly, they found that the bone forming capacity was maintained even in the MSCs obtained from old donors (202). This could be due to the fact that mouse microenvironment was able to boost the function of human MSCs from old donors.

Microenvironment includes various factors that guide the cells to their fates. Factors like extracellular matrix (ECM), other non-cellular components like soluble proteins, growth factors present in the serum have all known to influence the direction of adult stem cells. Presence of growth factors, conditioned media from older donors (139) as well as serum from older donors (203) have been shown to have an inhibitory effect on culture of MSCs as compared to the effect of growth factors, conditioned media and serum from young donors. These investigations suggested that the microenvironment of old donors also contribute to age-related effects in the cells of older donors. These components play an important role in MSC fate decisions with respect to proliferation, differentiation, migration as well as molecular interactions with other cells. However, the investigation of how these factors influence the area or density of an MSC colony

and thus the proliferative capacity of MSCs within the CFU-F assay, remains unexplored.

Autologous serum flows from blood vessels to the BM where MSCs reside, therefore *in vivo* MSCs from older donors are continuously exposed to 'old donor' serum and vice versa for young donors. Keeping this in mind, several studies have investigated the effect of autologous serum on the proliferative capacity of MSCs, but this has been performed only on culture expanded MSCs (203, 204). Furthermore, such studies have been performed for various aims primarily, finding a human alternative to foetal calf serum for supporting MSC expansion, differentiation and proliferation (205, 206). Only limited studies have used human serum aimed at investigating age-related changes (203), and none in CFU-F assays from uncultured BM MSCs.

This chapter aimed at investigating the age-related changes in the number of BM MSCs using the CFU-F assay. The same assay would be used to investigate the proliferative capacity of BM MSCs from young and old donors by further dissecting the CFU-F assay. The proliferative capacities of BM MSCs from young and old donors would also be investigated when exposed to young and old serum to understand the role of microenvironment (serum) in MSc proliferation.

3.1.4 Hypotheses and objectives

Hypotheses:

1. The number of MSCs measured by CFU-F assay declines with donor age.
2. The proliferative capacity of MSCs measured by colony area and density declines with donor age.
3. The proliferative capacity of MSCs measured by colony area and density is lower when exposed to serum from older donors.
4. Subjecting old donor MSCs to media supplemented with young donor serum will increase their proliferative capacity.

Objectives:

1. To enumerate BM MSCs by CFU-F assay.
2. To investigate the proliferative capacity of MSCs using CFU-F assay.
3. To test the proliferative capacity of MSCs grown in media supplemented with young and old human serum.
4. To compare the proliferative capacity of old donor MSCs grown in young and old human serum.

3.2 Methods

3.2.1 Donor selection

BMA was collected from donors undergoing surgeries for fracture fixation or for removal of metal work in Leeds General Infirmary, Major Trauma Unit in 4ml EDTA tubes as outlined in section 2.2.2. Donors were reported as otherwise healthy and were aged between 19-89 years old (median age=48) with n=27 males and n=24 females with a total of n=51 donors.

3.2.2 Enumeration of MSCs using CFU-F assay

CFU-F assay was performed as previously optimised in our laboratory (111, 207). To perform CFU-F assay, 80μl of freshly collected and filtered BMA was added into duplicate 60mm petri dishes containing 4ml of StemMACS (SM) MSC expansion medium. It was later adapted to 200μl of BMA in duplicate 100mm petri dishes containing 10ml of SM medium to obtain more colonies per dish. Following BMA and SM medium mixing, dishes were placed in the incubator in 37°C and 5%CO_2 and maintained in culture for two weeks. After 48 hours, the dishes were gently washed twice with 10ml of Phosphate buffer saline (PBS) using sterile stripette and 10ml of fresh SM medium was added. Half medium change was performed for these dishes twice a week and the dishes were checked for the growth of colonies under the inverted microscope. On the 14th day, the medium was aspirated completely and the cells were fixed for an hour using 10ml of 3.7% formaldehyde prepared previously (Appendix 2). The dishes were then stained with methylene blue stain for one hour and washed carefully to visualize the colonies. The stained dishes were allowed to air dry, then scanned using the Infinity scanner at 1200dpi resolution. Images of the cells within the colonies were captured using microscope equipped with the Infinity Analyze software. A colony was defined as consisting of at least 50 cells (207). The colonies in each duplicate dishes were manually counted (Figure 3.1) and CFU-F/ml was calculated according to the formula ((1000μl x average number of colonies from 2 dishes)/200μl) and then averaged.

Figure 3.1 illustrates duplicate CFU-F dishes with colonies grown from BM aspirates from representative donors from each age group as outlined in section 1.2.3. ImageJ software was used to investigate the colony area and density which are together indicative of the proliferative capacity of MSCs (201, 208).

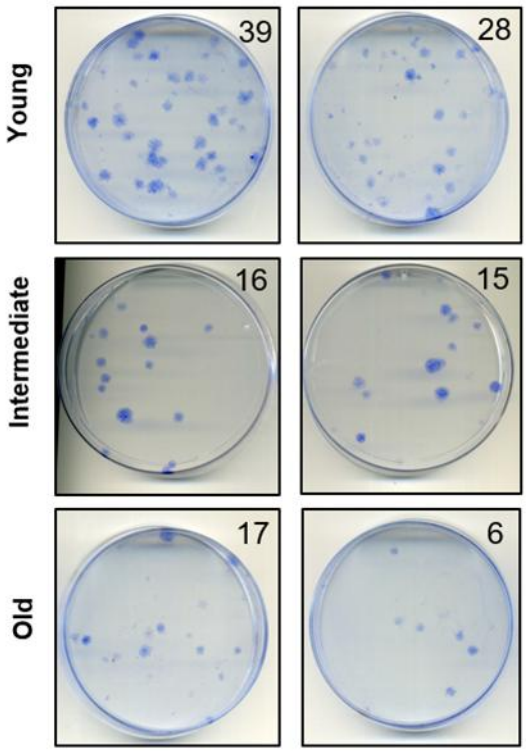

Figure 3.1 Duplicate dishes containing colonies from each age group.

The dishes in the figure illustrate colonies after staining with methylene blue on day 14. The numbers on the top right of every panel indicate the number of colonies counted in each dish.

3.2.3 Colony area analysis

To investigate age-related differences in MSC colony area, dishes were chosen from the young and old donor groups. Initially, CFU-F dishes from 10 young and 10 old donors were selected for analysis using ImageJ. Visually, colonies of various sizes were observed in CFU-F dishes from both young and old donors (Figure 3.1), and there was no clear evidence for separate groups of large or small colonies, as categorised by Gothard et al. (201).

The images of scanned dishes were converted to 'grey-scale' to be recognized by ImageJ software and then measurements for colony area was performed. By default, ImageJ measures area in $(pixel)^2$. The measurement was then calibrated for $millimeter^2$ (mm^2) and the software generated an outline for each of the colonies. To avoid the influence of the background disturbances and to minimize software error (for example, by counting two close colonies as one), the colonies on the edges of the dish and two or more colonies that almost merged as one, were given an outline manually. This enabled the measurement of colony areas for each individual colony. Details about the samples, on which colony area analysis was performed can be found in Table 1, Appendix 1.

Upon initial analysis of colony area, it was observed that the data for colony area was not normally distributed (Figure 3.2). In some donors, dishes contained numerous colonies including very large colonies (for example; donors: 31/F and 62/M). It was decided that selection of CFU-F dishes with low number of colonies would potentially miss out on these rare large colonies leading to a biased analysis of colony areas. Thus, dishes with a minimum of 25 colonies were chosen for the final analysis of colony area and density (Figure 3.2).

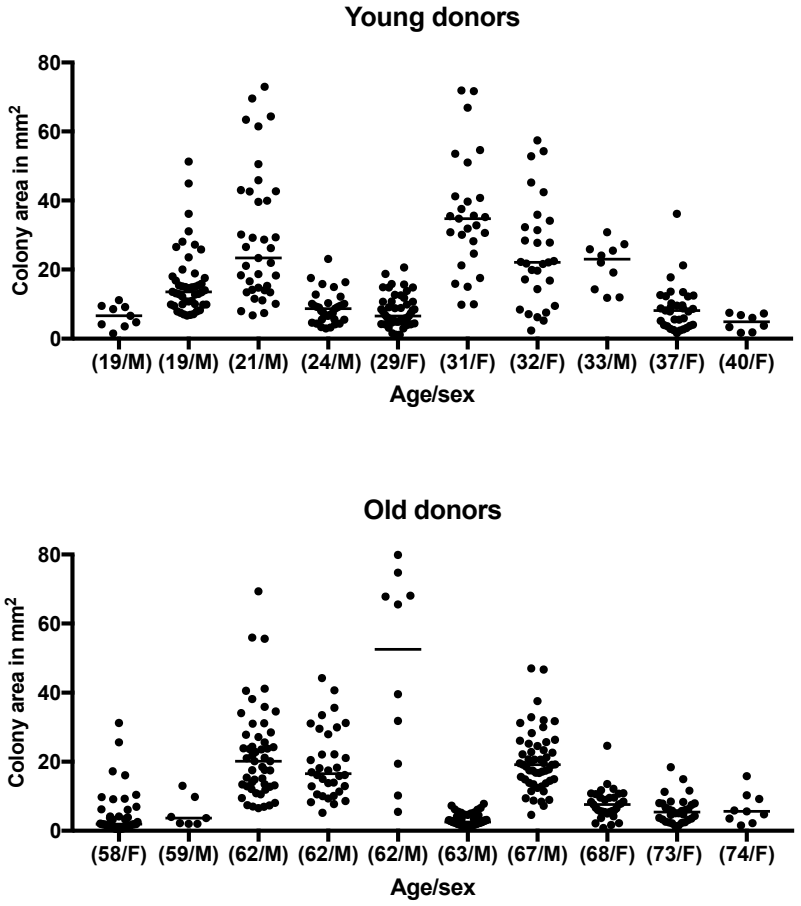

Figure 3.2 Initial studies to evaluate difference in colony area in 10 young and old donors.

Dots indicate individual colonies and horizontal lines indicate median values from young donors (top panel) and old donors (bottom panel). Age/Sex of the donors is indicated within brackets.

The final colony area studies were performed on dishes from n=7 young and n=7 old donors that had generated 25-60 colonies per dish. The results were analysed for young and old donor groups using different approaches as discussed in section 3.3.2.

3.2.4 Colony integrated density (ID) analysis

The data for colony ID was generated along with the data for colony area for all colonies from the same 7 young and 7 old donors, for which the colony area had been calculated. The ID was measured by the software (area * mean grey value) for each of the colonies. The value of ID was always generated by the software, irrespective of the outline for areas done automatically or manually. The values for colony area and ID were generated simultaneously. Once the data for colony area and ID were recorded, the data were compared between young and old donor groups.

3.2.5 Serum collection and preparation of medium with human serum

To generate human serum, 30 ml of blood was collected in 6 ml serum clot activator tubes from 4 young and 4 old healthy donors after obtaining their consent. The tubes contain silica micro particles coated along the inside of the tubes that activate clotting of blood. Once the blood was collected, the tubes were agitated for 5 seconds to allow the homogenous mixing of the blood with the coated silica micro particles. The mix was allowed to stand for 30 minutes and then centrifuged at 2000g for 15 minutes. This allowed the separation of the serum from the cells which was seen as a clear bilayer. The serum from the upper segment of the separated mix was collected carefully to avoid extraction of cells. However, if a small quantity of cells was extracted due to the proximity of the two liquids at the separating junction, the mix was further centrifuged in 1.5ml eppendorfs to ensure complete separation of serum from the cells. This was done for blood from each donor. Subsequently sera from four young donors (equal volumes) were pooled together to generate stocks of 'young' serum (YS). Similarly, 'old' serum (OS) was also prepared and both YS and OS were then aliquoted in 1ml vials and frozen at -20^{o}. Media containing either 10%YS or 10%OS were prepared fresh using DMEM as the basal media and supplemented with 1% Penicillin-Streptomycin for each experiment. Optimisation experiments were performed using serum from a single young donor for the ease of availability of young healthy donors.

3.2.6 Experimental design for investigating MSC colony formation in human serum

The aim of these experiments was to investigate how young and old donor MSCs responded to young and old donor microenvironment (represented by YS and OS), in comparison to SM, as well as how their colony numbers, areas and densities were affected when grown in 'old' human serum (OS) compared to 'young' human serum

(YS). These experiments were performed using frozen cells (for freezing and thawing, refer to section 2.2) from 6 and 4 donors from 'young' and 'old' groups, respectively (Appendix 1, Table 1).

3.2.6.1 Medium optimisation

To test the effect of 'young' and 'old' human serum on MSCs, it was important to ensure the right controls for the experiments. The first medium chosen as a positive control was SM medium that is industrially optimised for isolation, adhesion and growth of MSCs from BMA. However, the serum in SM is foetal calf serum (FCS) but it does not specify the type of basal media used.

As the aim of this experiment was to test the effect of human serum on the colony-forming capacity of BM MSCs, it was important to have a positive control with human serum supplement added to a known basal media. Mesencult supplement or Mcult is a human serum supplement that is optimized for isolation of MSCs *in vitro*. Thus, it was chosen as another positive control added at a concentration of 10% in basal Dulbecco's minimum essential medium (DMEM). DMEM with standard FCS (not optimised for MSCs growth) was used as negative control. All the media were supplemented with respective serum and 1% penicillin/streptomycin antibiotic.

3.2.6.2 Experimental design for control media evaluation

Frozen NCs (predicted to generate approximately 25-30 colonies based on the initial CFU-F assays, (Figure 3.2) were defrosted at 37°C in thawing medium (TM, Appendix 2). The cell suspension was centrifuged to remove the DMSO from the suspension and was re-suspended in TM. The cells were then equally divided into 12 60-mm dishes (3 for each of the medium of choice, Figure 3.3) containing 4 ml SM for an initial 48-hour attachment period.

Subsequently, the media was removed, the non-adherent cells were washed with PBS and complete media changes were performed as follows: 3 dishes were supplemented with 4 ml SM (control), 3 other dishes with 4 ml DMEM/10%YS, 3 other dishes with DMEM+10%Mcult and 3 final dishes with 4ml of DMEM/10%FCS. The dishes were then placed at 37°C and 5%CO_2 for 2 weeks, and half medium changes were performed using the respective media twice a week. On day 14, all media were aspirated and for each condition, two dishes were stained with methylene blue to visualise the colonies, while the adherent cells from the third dish were lifted off using trypsin (Appendix 2) and counted. The stained dishes were allowed to air dry and then analysed for colony area and density, as described in the above section.The steps

involved in selection of appropriate donors and the criteria in testing the effect of MSC colony formation in human serum are shown in a flowchart format in Figure 3.3 followed by the representation of medium change for all conditions in Figure 3.4.

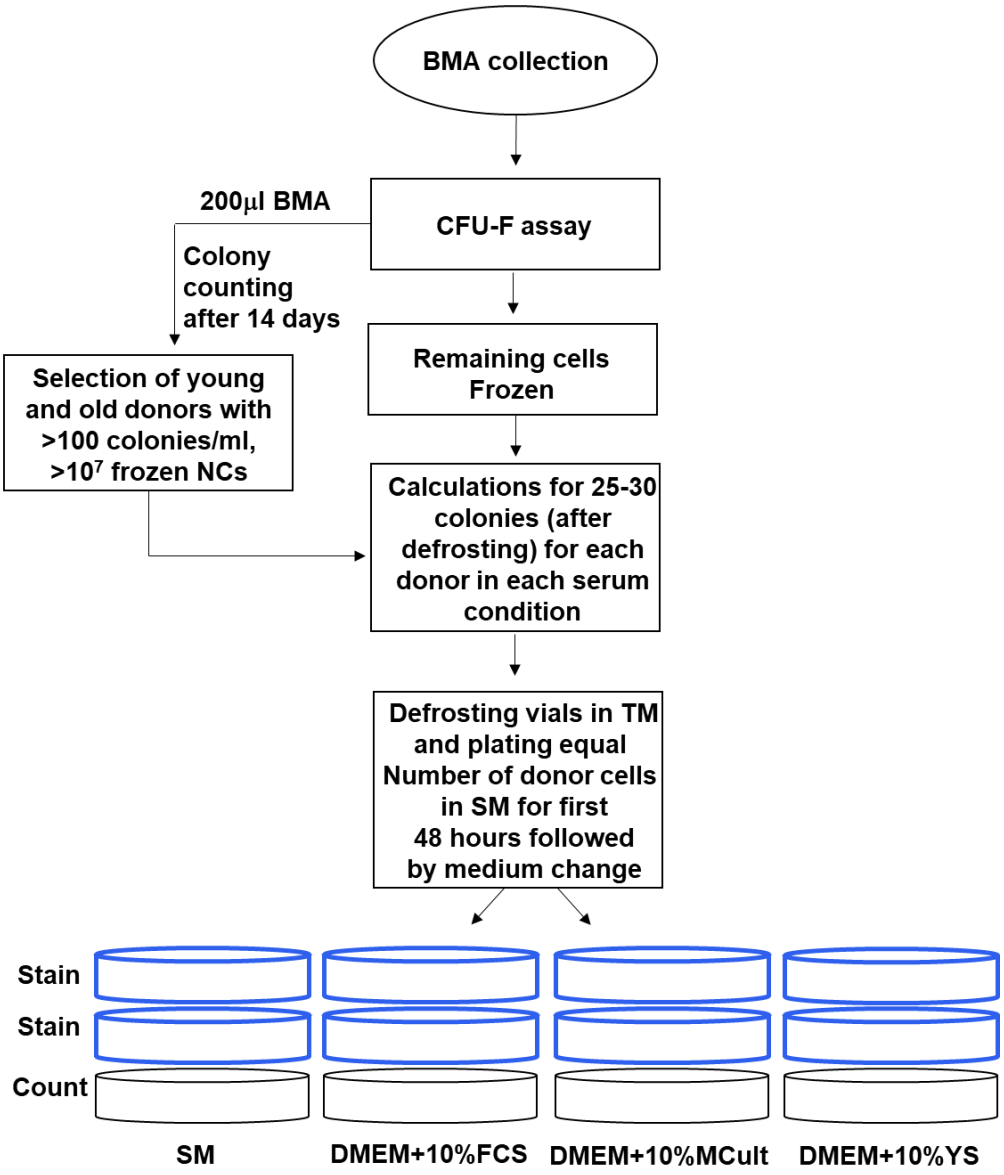

Figure 3.3 Flowchart demonstrating the steps and crucial parameters involved in the selection of donors for testing MSC colony formation in human serum.

Steps have been written as a flow chart to simplify the complexity of factors that were involved in executing the plan of the experiment with human serum. NCs: nucleated cells, TM: thawing medium, SM: StemMACS media, DMEM: Dulbecco's minimum essential medium, FCS: Foetal calf serum, MCult: Mesencult supplement, YS: young serum

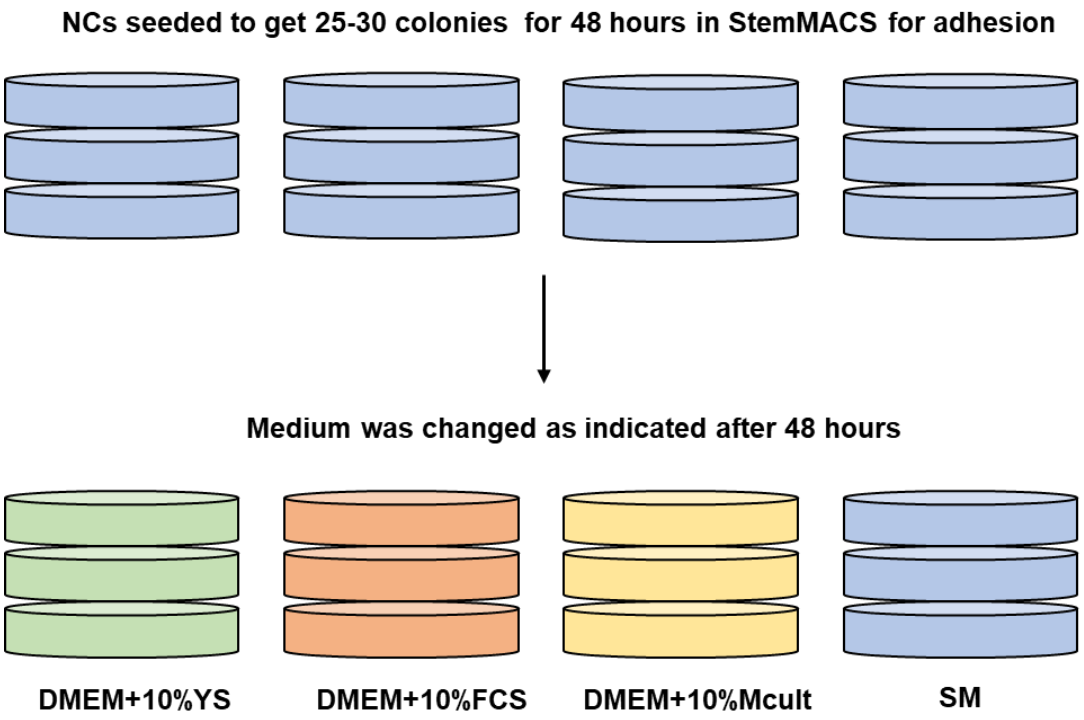

Figure 3.4 Optimisation of controls for testing the proliferation of MSCs in human serum

Equal number of defrosted MSCs were plated in 60mm petri dishes for 48 hours in SM medium to ensure uniform adhesion of MSCs in all dishes. After 48 hours, SM medium was aspirated and the corresponding medium was added (shown in the coloured panel). Half medium change was peformed twice in a week and on day 14, 2 dishes from each medium condition were stained and one was trypsinised for obtaining cell counts.

3.2.6.3 Final experimental design

The final experimental design included pooled YS and OS from n=4 donors from each group. As the optimisation experiments were performed from a single young donor, serum for final experiments were pooled to avoid bias. Observation from results from optimisation experiments (discussed further and shown in Figure 3.10) lead to finalisation of 3 serum conditions for the experiments shown in Figure 3.5. This included the use of SM, DMEM+10%YS (pooled) and DMEM+10%OS (pooled).

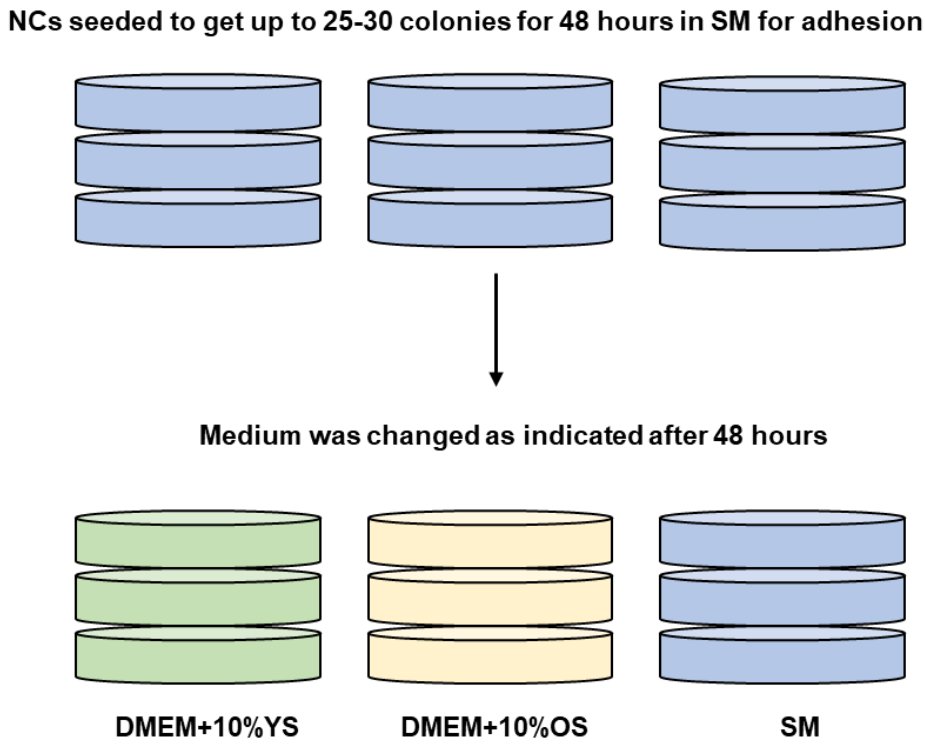

Figure 3.5 Optimised conditions and medium controls for testing MSC colony-forming capacity in human serum.

3.2.7 Statistical tests

Statistical analysis and graphics were performed using GraphPad Prism software (version 7.0a). The normal distribution of the data was assessed using the Shapiro-Wilk and Kolmogorov-Smirnov tests for normality. As no data were found to be normally distributed, Mann-Whitney test and Kruskal-Wallis test with Dunn's correction for multiple comparisons were used to compare two and three groups, respectively. Spearman test was used to analyse correlations. The results were considered significant at p value of <0.05.

3.3 Results

3.3.1 Age-related changes in number of CFU-F

As mentioned in section 3.2.2, the colonies were visualised after staining the cells with methylene blue solution on day 14. Initial observation included scanning the petri dishes for images and manual counting of the colonies. There was no age-related pattern observed in terms of colony size in general when colonies were counted

indicating different types of colonies existed even within the duplicate dishes of a single donor (Figure 3.1).

Once all the colonies were visualised and counted, the data for n=51 donors (n=27 males, n=24 females), was compiled and then correlated with donor age (Figure 3.6A). There was a significant age-related decline (p=0.0153) in the number of colonies. While there were donors with a relatively higher number of colonies among the older donors, an overall decline in the number of colonies (indicative of MSCs) was evident from the figure.

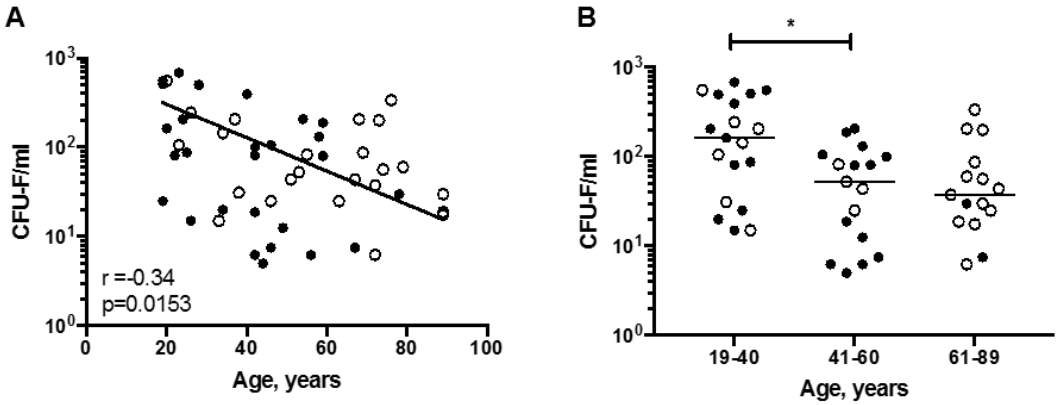

Figure 3.6 Age-related changes in number of CFU-F

(A) Age-related change in the number of CFU-F per ml of BMA across entire donor age range (n=51) and **(B)** in between age groups. Each dot indicates individual donor, black dots represent male donors and empty circles represent female donors. The black line on the left indicates the slope and the on the right, the median values. Spearman non-parametric test was performed for A and Kruskal-wallis test with Dunn's correction was performed for B, *p<0.05.

When the number of colonies was segregated based on the donor age groups (Figure 3.6B), a decline of over 3-folds (p=0.0245) was found between young donors (median=163 CFU-F/ml) and donors of intermediate age group (median=53 CFU-F/ml). When young and old donors (median=38 CFU-F/ml) were compared, a decline of over 4-folds (p=0.0512) was observed. It must be noted that while the overall median was the lowest in the older donors, the decline was the steepest between young donors and donors of intermediate age group. This indicted towards the considerable decline in the number of MSCs in the donors who are over the age of 40. The decline in the number of colonies continued after 60s as well, however, some of the donors over the age of 60 were also observed to have relatively large number of CFU-Fs suggesting young biological age in terms of MSCs despite belonging to the old chronological age group.

To understand if there were age-related changes with respect to gender, male and female donors were split and analysed separately for age-related correlations (Appendix 3, supplementary Figure 3.1A and C). There was a significant decline found in males (p=0.0319; r=-0.41) and a non-significant decline in females (p=0.128; r=-0.32). To understand the variation in each gender, they were further split into three age groups (Appendix 3, supplementary Figure 3.1B and D). The trend for decline in males and females was found to be similar to 3.6A above. However, owing to the limited number of donors in old males and intermediate group females, it was decided to keep the focus on age-related changes without segregating data for males and females further in the thesis.

3.3.2 Age-related changes in colony area

Until now, data from this chapter has established that there is significant decline in the number of colonies (indicative of number of MSCs) with increased donor age. While counting colonies and analysing them, colonies of different sizes were observed (Figure 3.7A). From literature, it was known that each colony is indicative of a single MSC and that colony area and colony morphology has the potential to indicate the proliferative capacity of MSCs (201, 208). However, none of these studies had measured the colony area on uncultured MSCs in relation to age ranging from 20-89 years old or quantified colony density. While the general idea related to the decline in sizes of MSC colonies has been linked with lower MSC proliferation in aged donors (132, 144), investigations on age-related differences in colony density have not been performed.

CFU-F dishes from 14 donors (n=7 young and n=7 old) containing a minimum of 25 colonies each were scanned. The scanned images of the dishes were analysed for colony area as described in section 3.2.3 and all colonies from each age group were combined to increase the statistical power of the analysis. The comparison of colony area between all colonies from young donors (n=321) and all colonies from old donors (n=266) suggested that the area of colonies in old donors (median=10.3 mm^2) was significantly 10% lower (p=0.0289) than the area of colonies in young donors (median=11.2 mm^2) (Figure 3.7 B). However, it was not clear if the decline in the colony area was due to a predominant loss of the larger colonies or due to a shift from large to small colonies in old donors.

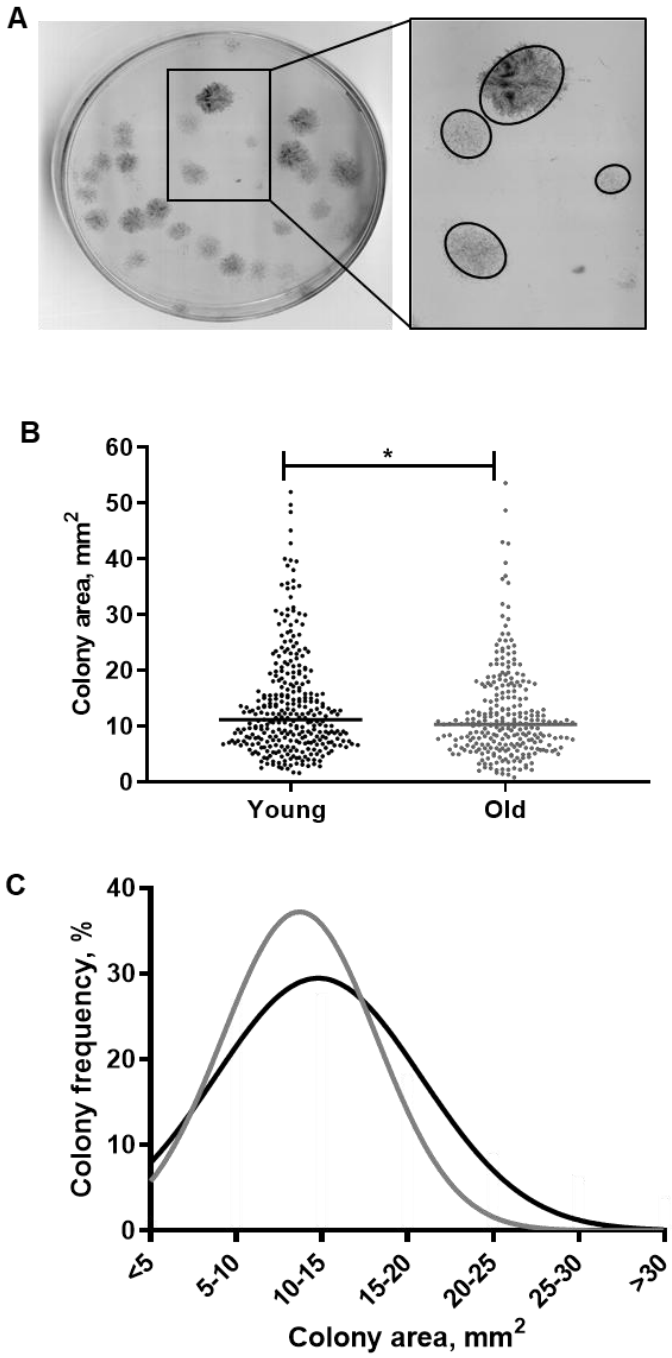

Figure 3.7 Age-related changes in colony area

(A) Presence of different sizes of colonies from a single donor CFU-F dish **(B)** Comparison of colony areas between all colonies from 7 young (321 colonies) and 7 old (266 colonies) donors. Each dot indicates a single colony with median values indicated in horizontal lines **(C)** Frequency distribution of colony area of young donors (black curve) and old donors (grey curve). **p*<0.05, Mann-Whitney U test.

To investigate if there is a loss of bigger colonies in old donors, a frequency distribution curve was generated for the colony areas of both young and old donor MSCs (Figure 3.7C). The range of colonies was set in gaps (referred to as 'bin centres' in GraphPad

Prism) of 5mm^2 in order to analyse the differences in sizes as small as 5mm^2. The frequency distribution of colony areas was next performed using GraphPad Prism software by generating a non-linear fit line based on modal values of pre-defined bin widths of 5 mm^2. This analysis revealed unimodal colony area distributions in both young and old cohorts, however, a prominent shift towards smaller colonies was observed in the old group. The frequency distribution in the figure shows a tendency of young donors to have larger colony areas whereas, the data from old donors indicate a shift towards smaller colonies with colony areas less than 10 mm^2. To segregate the colonies as 'large' or 'small', the median of all 587 colonies from both groups of donors (median=10.64 mm^2) was used as the cut off limit.

3.3.3 Age-related changes in colony integrated density

Colony density data was collected along with the colony area by ImageJ software and was measured in integrated density (ID) units. It was calculated as a product of the area and the mean grey value of a colony. The high mean grey value reflects the closeness of the cells that are stained within the colony, while zero grey value indicates un-stained empty space, that is, space with no cells. The greater the contact between the cells within a colony, the greater would be the mean grey value of the colony.

It was hypothesised that the colonies with the highest IDs would be the biggest and the most dense (indicating the most highly proliferative MSCs) categorised by Oreffo and colleagues in 2013 as 'large dense colonies' (201). Conversely, the colonies with the lowest IDs would be equivalent to the 'small sparse' colonies (208), indicating the least proliferative MSCs. The first comparison was performed between the ID of all colonies from young donors (n=321) and all colonies from old donors (n=266) (Figure 3.8A). The comparison indicated a significantly 32% lower (p<0.0001) ID of colonies in old donors (median=1116 units) as compared to the ID of colonies in young donors (median=1643 units). The next comparison was performed between all the small colonies (area less than median area of 10.64 mm^2 described in the section above) from both young and old donors with all the large colonies from both young and old donors (Figure 3.8B). The data indicated a significant drop (p<0.0001) of over 3-folds in the ID of small colonies (median=777 units) when compared to the ID of large colonies (median=2661 units). As the formula for ID was (area*mean grey value), both, the contact between the cells in a colony and the area of the colony are directly proportional to the actual ID calculated. The fact that the larger colonies generated higher values for ID than small colonies, it was therefore expected and indicated that such segregation may provide further insights on the proliferation potentials of MSCs from Y and O donors.

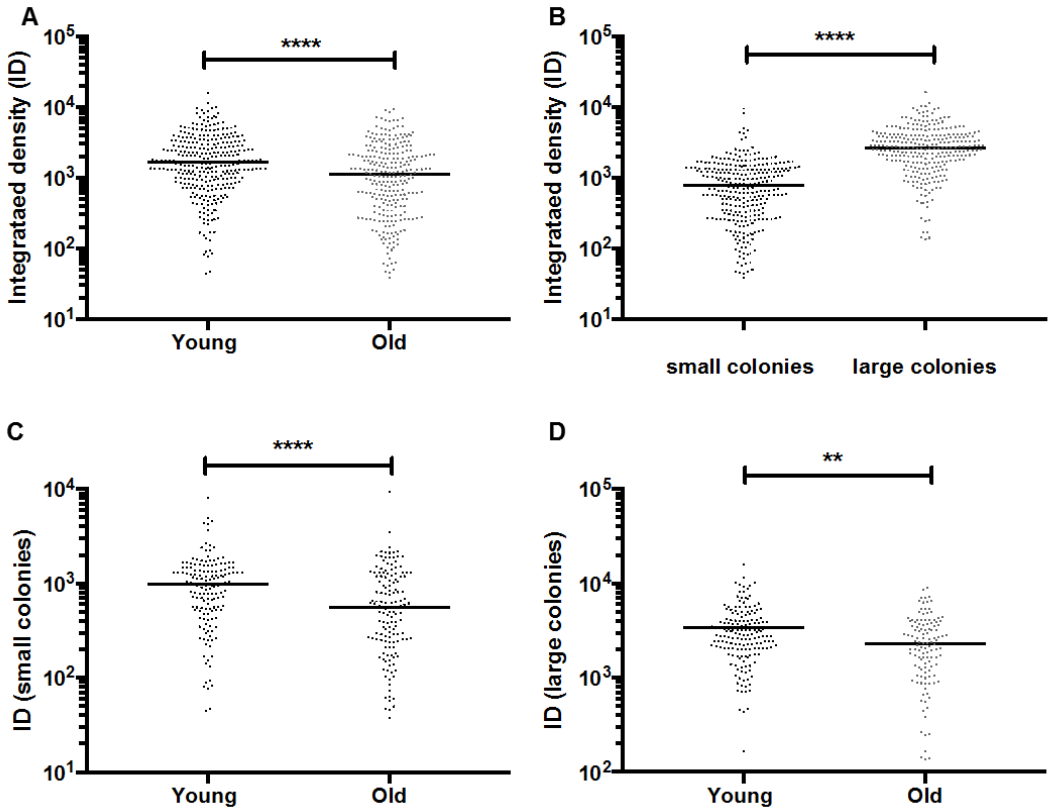

Figure 3.8 Age-related changes in colony ID

(A) Comparison of colony ID of all colonies from young and old donors **(B)** Comparison of all small and large colonies from both age groups based on median area **(C)** Comparison of ID of small colonies in young and old donors **(D)** Comparison of ID of large colonies in young and old donors. Black dots represent young donors (n=7) and grey dots indicate old donors (n=7). Median values in all the figures is represented by the black horizontal line.**p<0.01 and ****p<0.0001, Mann-Whitney U test

To further understand if the ID of each colony type (large or small) had similar age-related trends, the colony ID data for small colonies was first compared between young and old donors (Figure 3.8C). The ID of small colonies from old donors (median=563 units) was significantly lower (p<0.0001) than the ID of colonies from young donors (median=981 units). Similar comparison was applied to large colonies for the two age groups (Figure 3.8D) and a similar trend of lower ID (p=0.0029) was observed in old donors (median=2325 units) as compared to the ID of large colonies in young donors (median=2850 units). The difference in ID was however greater in the small colonies (1.7-folds) as compared to large colonies (1.2-folds). This suggested that there were proportionally more small sparse colonies in old donors, rather than less large dense colonies. Irrespective of the colony size, if they were from young donors, they had higher colony area and ID indicating their higher potential to proliferate as compared to colonies from old donors.

3.3.4 Proliferation of MSCs in human serum conditions

3.3.4.1 Optimisation experiments

The first step of testing the hypothesis that the proliferative capacity of MSCs is further reduced in 'aged' microenvironment was to ensure that the right controls were being used for comparison. Section 3.2.6 outlines the choices of medium used and the rationale behind these choices. To identify the best control(s), young donors with more than 100 CFU-Fs/ml from fresh BMA and with over 10^7 frozen cells (Figure 3.3) were selected to generate approximately 25-30 colonies in 12 dishes each (3 dishes for each of the four different media). Young serum (YS - from 1 young male) was used to optimise the controls as it was more practicable and also because the volume of old serum (OS) was limited owing to rarity of the donors in this age group and so it was saved for the main experiment. Once the young donor cells were added to the dishes and the medium was changed as shown in Figure 3.4, after 14 days, dishes were stained and the number of colonies were counted.

Figure 3.9 shows the results of the optimisation experiment where the dishes containing cells in DMEM+10%FCS on top panel and in DMEM+10%Mcult (bottom panel) did not have visible colonies. While there were groups or clusters of cells seen in both – DMEM+10%FCS and DMEM+10%Mcult, they had less than 50 cells to be considered as a colony for colony analysis. This was repeated with two other donors and the observation for both DMEM with FCS or Mcult was consistent throughout the three experiments. The cell count in dishes containing either DMEM with FCS or with Mcult also consistently revealed fewer or no cells at all (range 0-2 colonies per dish as compared to 30-60 colonies per dish in SM).

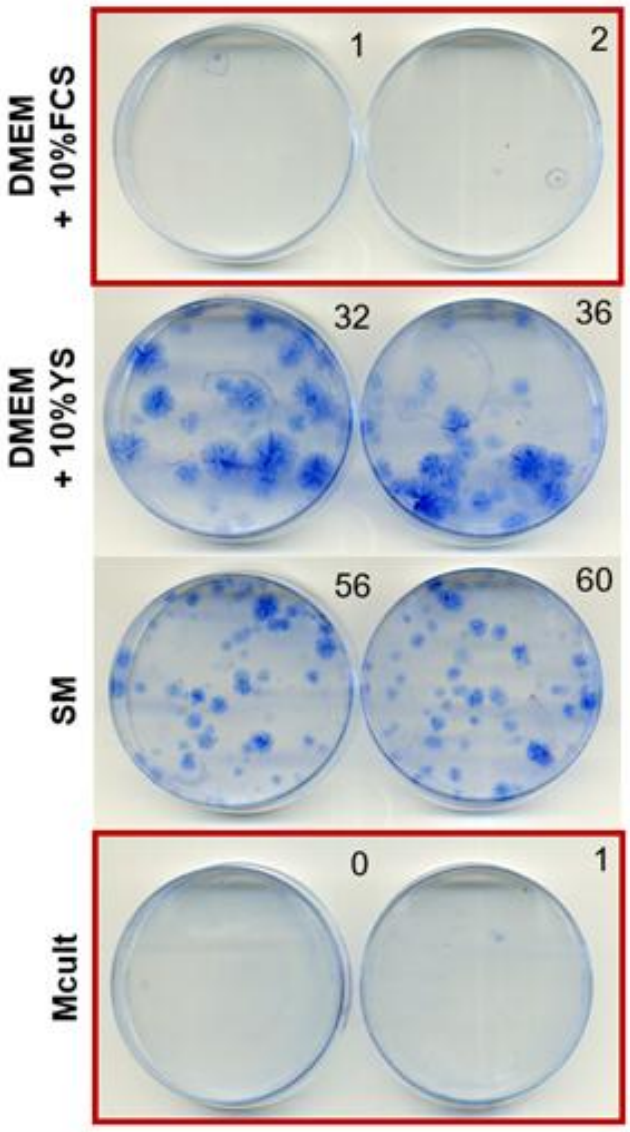

Figure 3.9 Selection of control media

Top and bottom panel (outlined in red) medium conditions did not generate colonies resulting in their elimiation and finalising SM as the positive control. Numbers on the top right corner of every dish indicate the number of colonies in each dish.

Colonies were expected in SM as it is commercially optimised for the adhesion, growth and proliferation of cultured and uncultured MSCs *in vitro* and was shown to generate colonies in previous experiments (section 3.2.1). Number of colonies counted in SM were greater than those in DMEM+10%YS. However, colonies in the latter appeared larger in size as compared to the colonies grown in SM. Mcult is also optimised for adhesion, growth and proliferation of MSCs but only for cultured cells. This could explain the absence of colonies in this experiment with uncultured cells. DMEM+10%FCS has previously been used to grow MSCs and compare with human

serum (206). However, the lot used for this experiment was not specifically selected for uncultured MSC growth. This can explain the lack of colonies from uncultured BMA.

Based on these observations, only SM was chosen as the positive control for subsequent experiments. It can be argued that DMEM+10%FCS could have been still kept as negative control. However, keeping in mind that the number of MSCs declined in older donors (section 3.3.1) and the number of frozen cells required to set up these experiments (9 dishes x 25-60 colonies per dish), it was decided to finalise the experimental set up on 3 sets of conditions: human young serum, human old serum and SM and not perform negative controls.

3.3.4.2 Proliferation of MSCs in media supplemented with human serum

The aims of following experiments were to test if there was a decline in the most proliferative MSCs when grown in media containing old serum and also to evaluate the rejuvenation potential of growing old donor MSCs in young serum as compared to growing in old serum.

As the number of old donors was limited and so were their number of MSCs in most cases, the old donors with 30 CFU-Fs/ml and above were considered for this experiment. Additionally, all of the frozen cells from the old donors were plated as described in section 3.2.5. Altogether, the colony formation from NCs from n=6 young donors and n=9 old donors was evaluated. As expected, seeding old samples that had less than 100 CFU-F/ml and less than 10^7 frozen NCs did not yield any colonies and thus could not be considered for further analysis. The results of the donors that generated colonies have been presented and discussed in detail in the sections below.

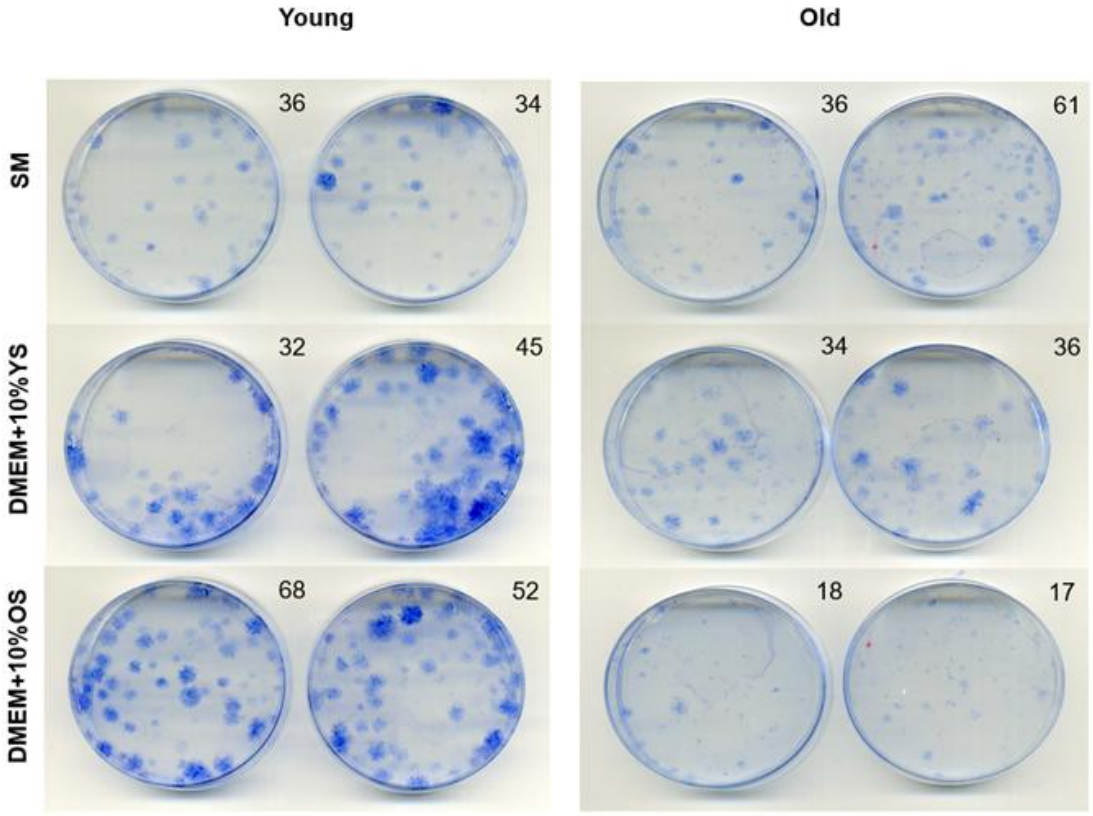

Figure 3.10 Representative donors colonies in all 3 conditions

Top panel shows the scanned image of the stained dishes following culture in SM medium followed by media supplemented with 10%YS in the centre panel and in media supplemented with 10%OS in the bottom panel. Left side indicate representative dishes from a young donor and the right side indicate dishes from an old donor. The numbers on the top right corner of the dishes indicate the number of colonies in each dish.

Figure 3.10 (left side) shows colonies obtained from young donor following culture in SM, media supplemented with 10%YS and media supplemented with 10%OS. In SM (top panel), 36 and 34 colonies were observed. Comparing these colonies with those obtained in media containing human serum (10%YS or 10%OS), they were fewer in number as well as appeared smaller in size. Colonies from young donor MSCs grown in media supplemented with YS appeared merged due to larger colonies in close proximity. Colonies of varying sizes were observed in all 3 conditions.

Figure 3.10 (right side) shows colonies obtained from old donor following culture in SM, media supplemented with 10%YS and media supplemented with 10%OS. In SM (top panel), 36 and 61 colonies were observed. Comparing these colonies with those obtained in media containing human serum (10%YS or 10%OS), they appeared smaller in size. However, colonies in SM were more than the number of colonies obtained from human serum, especially in media supplemented with OS (bottom panel). Colonies grown in media supplemented with YS appeared larger than colonies obtained from other 2 conditions. Colonies of varying sizes were observed in all 3 conditions.

Visually, Figure 3.10 suggests that the colonies are generally bigger in size in media containing human serum as compared to SM. While the difference between the number of colonies or their sizes was not strikingly evident between YS and OS, the general observation of larger colonies seen by the naked eye remained constant for all young donors (n=6) in YS as compared to SM (Figure 3.10).

Using SM as the positive control, all the data for colony area from young and old donor MSCs grown in media supplemented with 10%YS or 10%OS were compared to SM. To confirm if the colonies grown in media supplemented with YS were actually denser than those grown in SM or in media supplemented with OS, the ID of all the colonies were also quantified. Due to the observation that SM generated good number of colonies but smaller in size and media in YS demonstrated otherwise, one of the 3 dishes from each condition was trypsinised and counted to complement the ID data.

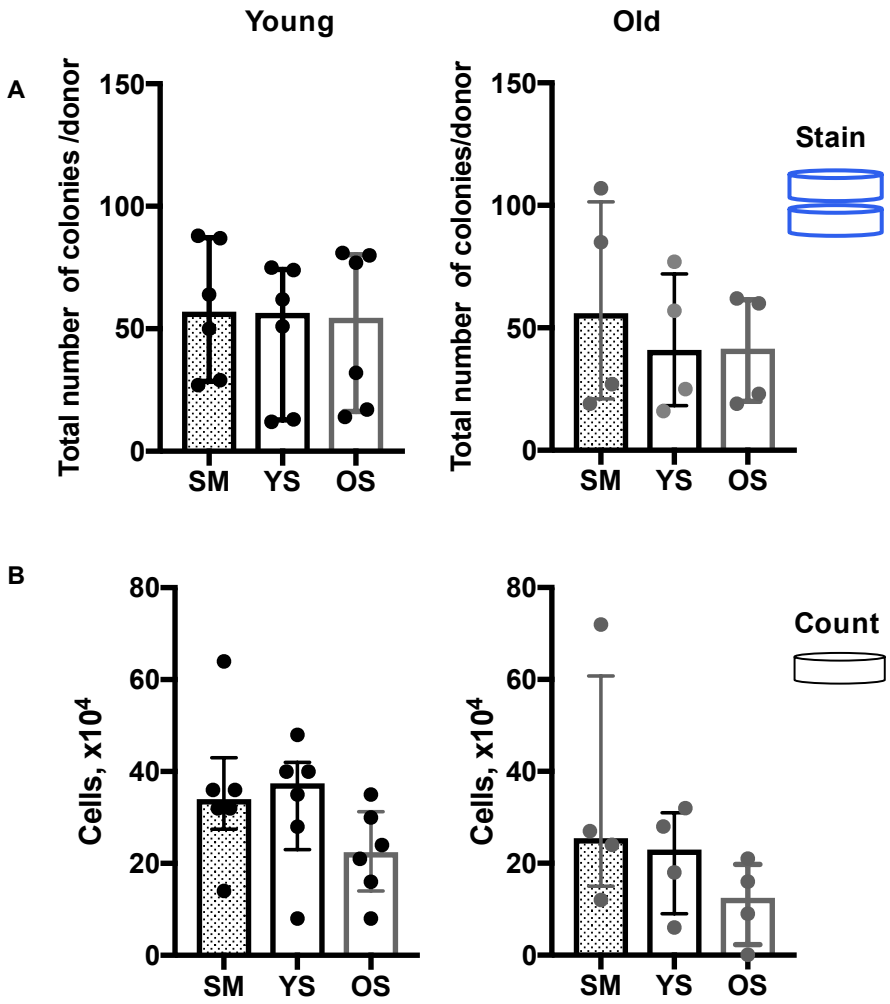

Figure 3.11 Colony and cell counts of young and old donor MSCs seeded in dishes with SM, media supplemented with YS and media supplemented with OS

(A) Number of CFU-Fs as counted from stained dishes in young donors (left) and old donors (right) grown in SM, media supplemented with YS and media supplemented with OS **(B)** Cell counts from the third dish in young donors (left) and old donors (right) grown in SM, media supplemented with YS and media supplemented with OS. Bars indicate median and error bars indicate interquartile range. n=6 young donors and n=4 old donors.

Figure 3.11 represents the colony-forming capacity of young and old donor MSCs in SM, media supplemented with YS and media supplemented with OS. The data in Figure 3.11A shows no significant difference in the number of colonies between young and old donors grown in any of the 3 conditions. However, while the total number of colonies remained similar in SM, a non-significant decline in old donor MSCs was observed in both, media supplemented with YS and media supplemented with OS human serum conditions.

The cells counted from the third dish (Figure 3.11 B) showed interesting additional trends. First, a trend for decline in the number of cells in media supplemented with OS was observed in both young and old donor MSCs compared to their growth in SM and YS conditions, in spite of plating equal number of cells in all conditions for each donor. This suggested that there were colonies with fewer cells in OS conditions when compared to SM and YS conditions. This was further tested by evaluating the colony areas and IDs using methods described in section 3.2.3 and 3.2.4.

Figure 3.12 shows the comparison between colony areas of young and old donor MSCs in the 3 conditions, along with the frequency distribution of the colony areas. Figure 3.12 A, left panel represents the colony areas of all colonies from young donors grown in SM, media supplemented with YS and in media supplemented with OS. For young donors, YS conditions had produced the largest colonies with median area of 11.89mm^2. This was significantly higher than observed for the colonies grown in OS conditions (median area=11.08mm^2, p=0.0062) or SM conditions (median area=8.42mm^2, p<0.0001). Interestingly, the colonies grown in OS were also significantly larger than those grown in SM (p=0.0016).

Frequency distribution of colonies from young donor MSCs (Figure 3.12 A, right panel) grown in SM (dashed line) peaked in between 5-10 and 10-15 mm^2 area boundaries indicating that most frequent colonies in SM were more within this boundary. Frequency distributions of colonies from young donors MSCs grown in media supplemented with YS or OS did not show much difference, consistent with their median values being very close, as mentioned above. Both the curves appeared to have similar range of large and small colonies indicated by the nearly overlapping pattern of the black and grey line. As mentioned, the frequency distribution curve of the colonies from the same donors grown in SM appeared to have a strong shift towards the colonies of smaller size.

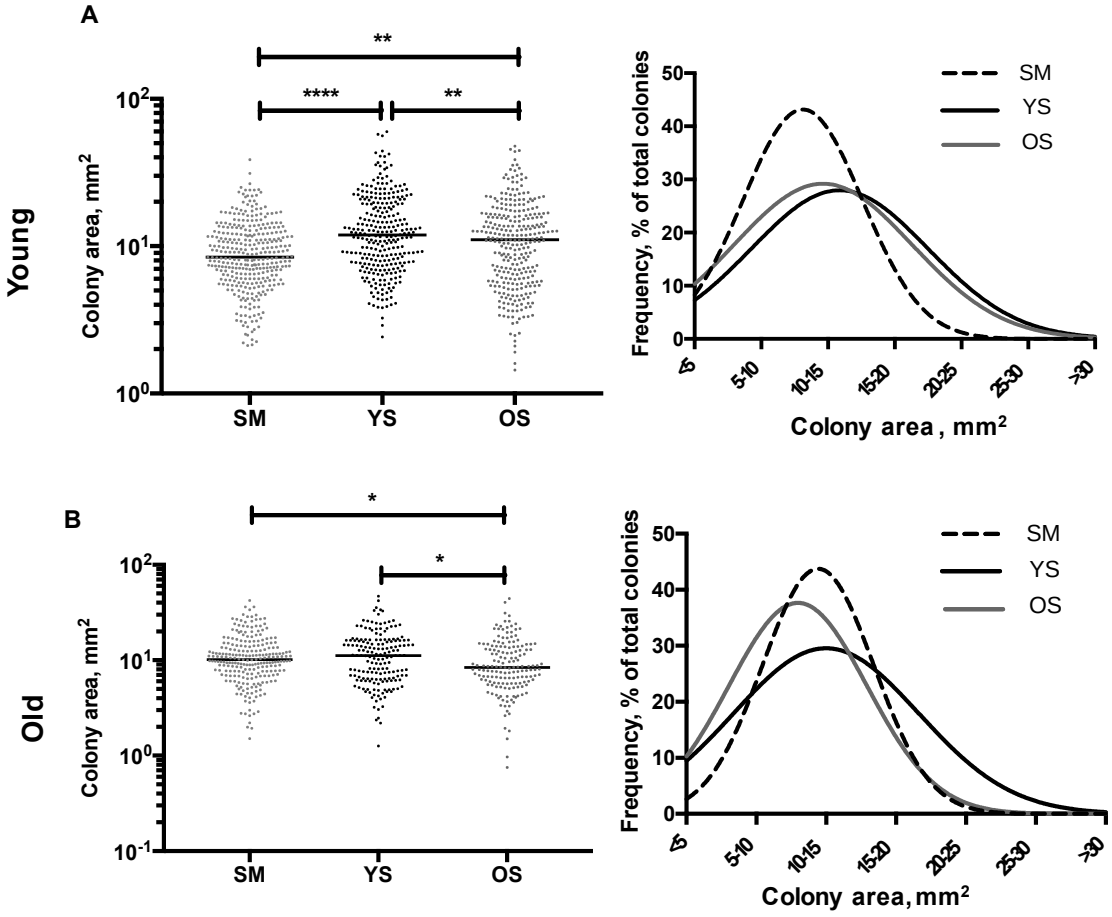

Figure 3.12 The analysis of colony area in SM, media supplemented with YS and media supplemented with OS

(A) Comparison of colony areas in young donors (n=6, left) in SM (n=345 colonies), in media supplemented with YS (n=286 colonies) and in media supplemented with OS (n=301 colonies). Frequency distribution of colony area (right) based on bin centres of 5 mm^2 of colonies from young donors grown in SM (dashed line), media supplemented with YS (solid line) and media supplemented with OS (dotted line) **(B)** Comparison of colony areas in old donors (n=4, left) in SM (n=241 colonies), in media supplemented with YS (n=175 colonies) and in media supplemented with OS (n=165 colonies). Frequency distribution of colony area (right) based on bin centres of 5 mm^2 of colonies from young donors grown in SM (dashed line), media supplemented with YS (solid line) and media supplemented with OS (dotted line). Median is indicated by black horizontal line. Data was considered significant when *p<0.05, **p<0.01, ****p<0.0001, Kruskal-Wallis test with Dunn's correction.

Figure 3.12 B represents the colony areas of all colonies from old donors grown in SM, media supplemented with YS and in media supplemented with OS. For old donors too, YS conditions had produced the largest colonies with median area of 11.19 mm^2. This was significantly higher than for the colonies grown in OS conditions (median area=8.38 mm^2, p=0.0141). While there was no difference found between the colony area of colonies grown in SM (median area=10.14 mm^2) and colonies grown in media supplemented with YS, median area of colonies in SM was significantly larger than the

colonies grown in media supplemented with OS (p=0.0215). Thus, in old donor MSCs, colonies grown in OS showed the lowest colony areas.

Frequency distribution of colonies from old donors in SM (dashed line) once again peaked in near 10-15 mm^2 area indicating that most of the colonies in SM were relatively small in size. However, very different from results in young donors, colonies from old donors in media supplemented with OS shifted further towards the left indicating the presence of colonies smaller in size than those grown in SM (Figure 3.12B, right panel). As expected, colonies grown in media supplemented with YS demonstrated a prominent shift to the right (larger colonies), almost as seen in young donors (Figure 3.12A, right panel).

Overall, this section showed that in young donor MSCs, colonies grown in SM are significantly smaller in size, while those grown in media supplemented with YS or OS do not show any differences in size indicating that OS does not have a detrimental effect on the growth of young donor MSCs. However, old donor MSCs grown in media supplemented with OS generated the colonies of the smallest size. Very interestingly, the same old donor MSCs, when grown in media supplemented with YS, formed bigger colonies. The frequency of colonies grown in media supplemented with YS generally showed a shift towards larger colonies in MSCs from both young and old donors. These data indicated that YS supplementation had a positive effect on the colony size from old donor MSCs.

3.3.4.3 Age-related differences in colony ID in MSCs grown in SM, media supplemented with YS and media supplemented with OS

Next, to understand the age-related differences in density of the colonies, the IDs of the colonies were then analysed for MSCs grown in all the 3 conditions. Colonies from young donors and old donors were segregated as small or large based on the median area of all colonies (colonies grown in media supplemented with YS and with OS) from young as well as old donors (9.4 mm^2). Colony area evaluation in human serum suggested an age-related decline when MSCs were exposed to OS and this was observed for young donor MSCs as well as for old donor MSCs. Next, the data acquired for colony ID from ImageJ was analysed for small and large colonies, first in young donors and then in old donors. It was hypothesised that a similar trend for decline in colony ID would be seen in OS conditions, and particularly for old donor MSCs as compared to when they were grown in media supplemented with YS. All

colonies were segregated into small and large based on the median area of all colonies (grown in all conditions and from young as well as old donors, 9.4 mm^2).

Figure 3.13A represents colony IDs for small colonies from young donors grown in SM, media supplemented with YS and media supplemented with OS. Small colonies grown in media supplemented with YS had the highest IDs (median=1110 units) which was significantly higher than the IDs of colonies grown in SM (median=937 units, *p*=0.0208) or media supplemented with OS (median=887 units, *p*=0.0003). Even though the IDs of colonies grown in media supplemented with OS were the lowest, it was not statistically significantly lower than IDs of colonies in SM.

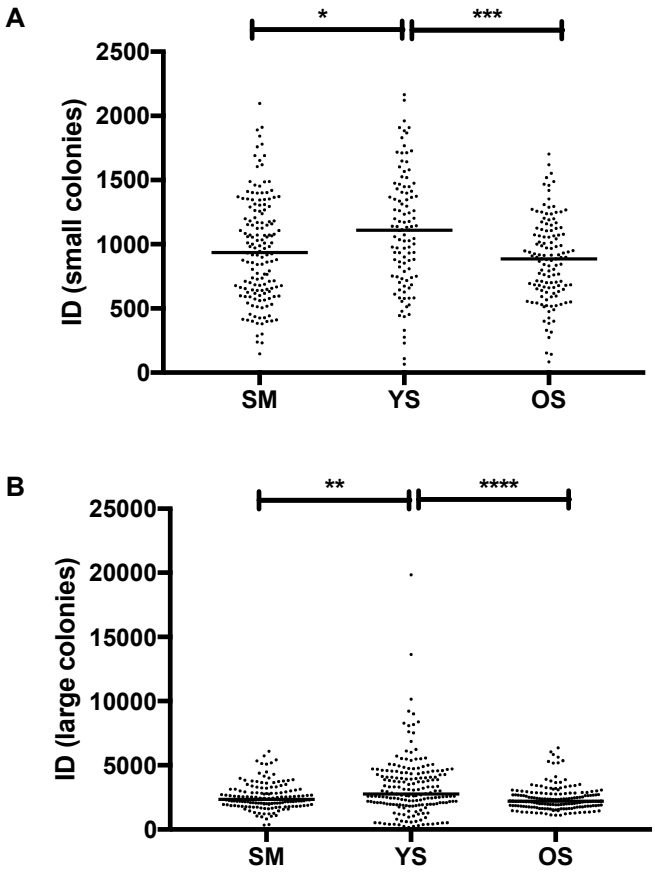

Figure 3.13 ID of colonies from young donors MSCs grown in SM, media supplemented with YS and media supplemented with OS

(A) ID of small colonies in SM (n= 156 colonies), in media supplemented with YS (n=108 colonies) and in media supplemented with OS (n=129 colonies) **(B)** ID of large colonies in SM (n=153 colonies) in media supplemented with YS (n=191 colonies) and in media supplemented with OS (n=172 colonies). Each dot represents a single colony and the median is indicated by black horizontal line across the data set. **p*<0.05, ***p*<0.01, ****p*<0.001 and *****p*<0.0001, , Kruskal-Wallis test with Dunn's correction.

Similar trend was observed for the large colonies from young donors (Figure 3.13 B) showing a significantly higher ID in colonies grown in YS (median=2598 units) as compared to the colonies grown in SM (median=2047 units, p=0.0080) or in media supplemented with OS (median=2200 units, p<0.0001). Altogether, the data presented in Figure 3.13 indicated that in young donors, OS conditions induced a significant decline in the ID of colonies as compared to those grown in SM and YS. This was true for both, small as well as large colonies. This implied that proliferation of MSCs was the lowest when grown in media supplemented with OS and the highest when grown in media supplemented with YS. Using colony ID as an indication of MSC proliferation, it can be concluded that growing MSCs in OS conditions reduced MSC proliferation capacity, and this was observed for both, large and small areas.

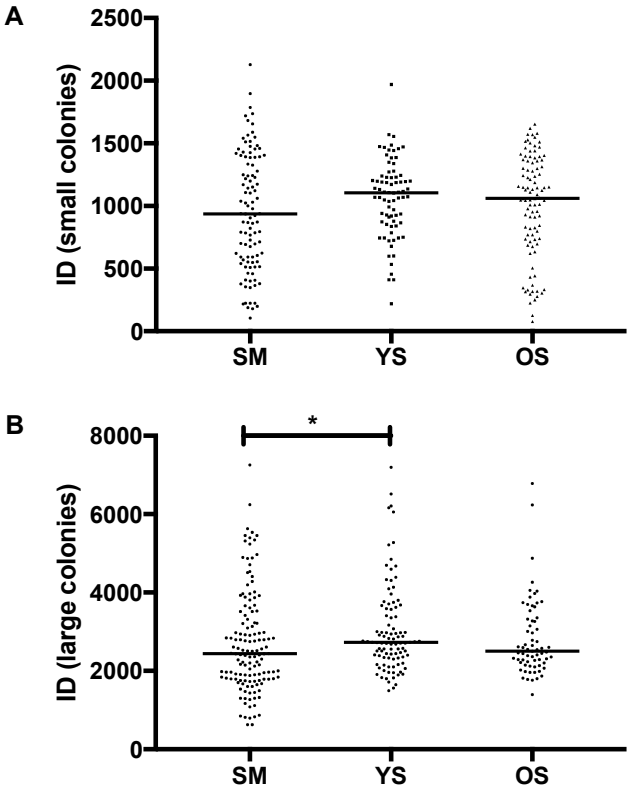

Figure 3.14 ID of colonies from old donors MSCs grown in SM, media supplemented with YS and media supplemented with OS

(A) ID of small colonies in SM (n=103 colonies), in media supplemented with YS (n=76 colonies) and in media supplemented with OS (n=98 colonies). **(B)** ID of large colonies in SM (n=138 colonies) in media supplemented with YS (n=99 colonies) and in media supplemented with OS (n=67 colonies). Each dot represents a single colony and the median is indicated by black horizontal line across the data set. p<0.05*, , Kruskal-Wallis test with Dunn's correction.

Small colonies from old donors did not show any significant difference between the ID of colonies grown in SM, YS or OS (Figure 3.14A). However, the IDs remained lower in OS conditions (median=1062 units) compared to YS conditions (median=1105 units) while being the lowest in SM (median=937 units).

For the large colonies grown from old donor MSCs (Figure 3.14B), the IDs of colonies grown in media supplemented with YS (median=2729 units) were significantly higher than the IDs of colonies grown in SM (median=2441 units p=0.0170) and non-significantly higher that the IDs of colonies grown in OS (median=2501 units). This indicated that, while media supplemented with YS encouraged the proliferation of MSCs from old donors, the effects were small and statistical significance was not achieved. ID analysis of colonies from both young and old donors confirmed that growing MSCs in aged serum induced a decline in the overall proliferation capacity of MSCs (Figure 3.13 and 3.14). The data also indicated that proliferation capacity of MSCs in old donors could be slightly improved when grown in media supplemented with YS as compared OS (Figure 3.14B). The trends for a change were the same for large and small colonies suggesting that YS or OS factors were equally affecting all colony types and were therefore not specific to small or large colonies.

3.4 Discussion

Literature regarding age-related differences in BM MSCs with respect to their numbers has been controversial (Table 3.1). Furthermore, while the number of CFU-Fs have been evaluated for counting MSCs, the size of the colonies has been discussed less often and their density, rarely. In this thesis, it was hypothesised that the number of MSCs in the BM shows an age-related decline. Additionally, the colony size and colony density measured were also hypothesized to show an age-related decline with the loss of the most proliferative MSCs that in this assay, formed the largest and densest colonies. Furthermore, it was hypothesised that MSC 'niche', modelled here with the use of human serum, would additionally impact on MSC colony formation, leading to smaller and less dense colonies when grown in old donor serum.

The data presented in this chapter showed a significant age-related decline in the number of MSCs quantified by CFU-F assay, confirming our hypothesis and indicating an age-related decline in BM MSCs *in vivo*. Furthermore, the MSCs from older donors had significantly lower proliferative capacity than younger donor MSCs as indicated by colony area and colony ID analysis. The potential of human serum to support proliferation of MSCs was clearly demonstrated in the presented experiments with

human serum and confirmed previous studies with culture expanded MSCs (206, 209). The colony area and colony ID results from human serum experiments indicated that 'aged' serum (OS) provided conditions for lower proliferative capacity of MSCs, from both young and old donors. Finally, colony area and ID results indicated a possibility for using young serum to potentially rejuvenate MSCs from old donors, by making CFU-F colonies significantly bigger and slightly more dense.

CFU-F assay has been performed by many groups to investigate the change in the numbers of MSCs. However, the techniques of cell isolation, media used and other factors have varied in different laboratories (Table 3.1). Fewer groups (including our group) have performed the CFU-F assay on fresh BMA to detect the age-related changes in number of MSCs. The results of the current study indicated a significant age-related decline in the number of MSCs (Figure 3.6A), a 4-fold decline in MSC numbers between the young donors (163 colonies/ml BMA) and old donors (38 colonies/ml BMA) using CFU-F assay (Figure 3.6B). These data in principle support previous data by Stolzing et al. (96) where they have shown a significant 4-fold decline in MSC numbers between paediatric (0-20 years old, median of 100 colonies/ml) and adult donors (21-40 years old, median of 25 colonies/ml) by CFU-F assay. However, the groups used in these two studies were different, spanning an age range of 5-55 years old in the Stolzing et al., study and 19-89 years old in the present thesis. The numerical difference in the number of colonies in the common 40-60 years old age group in both the studies could be due to the fact that Stolzing et al., plated 5×10^4 cells/well for CFU-F assay while fresh BMA was used for the current study. Even though the trend of age-related decline in the number of MSCs is common in both the studies, there is notable difference in the age range of donor age used, CFU-F plating method and in the number of donors recruited in the studies. Stolzing *et. al* showed a prominent drop in MSCs after the completion of skeletal growth which is also observed in the present study.

D'Ippolito et al., performed similar studies and found a significant decline in the number of colonies (66.2 ± 9.6 per 10^6 cells) in young (3-36 years old) donors as compared to old (41-70 years old) donors (14.7 ± 2.6 per 10^6 cells) (132). However, they used vertebral BM and counted CFU-ALP (alkaline-phosphatase positive), which could explain the variation in the number of colonies. Muschler et al., performed age correlations studies on iliac crest BMA and also counted CFU-ALP, and found a significant age-related decline in the number of CFU-ALP for both the genders. They also found a decline in the number of CFU-ALP per 10^6 NCs, sharper in females from 13 to 27 in female age groups as compared to 17 to 38 in the male age groups (210).

Using a smaller cohort of patients, similar trend was recently observed by our group with a significant decline in the number of colonies in females as compared to a non-significant decline in males, across the whole age range between 22 and 80 years old (111). Both these studies were in contrast to the gender related data obtained in this project. However, owing to the lower number of female donors in the young and intermediate groups and lower number of males in the old donor groups (Appendix 3, supplementary Figure 3.1), no firm conclusions can be made in terms of age-related declines in the number of MSCs in separate genders. An equal number of donors from both the genders in each age group (minimum n=10) will be required for a firmer conclusion.

The present results indicating a decline in the number of MSCs is in contrast to work done previously by Justessen et al., where they found no significant difference in the number of MSCs in normal conditions with respect to both, age and gender (196). They found a significant difference in the MSC colony number with age only in those treated with osteogenic medium (4+/-4 colonies of seeded 100,000cells/cm^2 NCs for young donors aged 18-42 and 2+/-4 of seeded NCs for old donors aged 66-78). This study also used density centrifugation for erythrocyte removal, scored colonies with 16 or more cells in healthy donors and in osteoporosis patients, in contrast to this study where NH_4Cl was used for erythrocyte lysis, colonies with only more than 50 cells were scored and only healthy donors were recruited.

Jing Li and team used uncultured cells from BM of donors aged 1-52 years old and found no age-related decline in the number of colonies. In contrast, this study analysed uncultured BM MSCs from adults ranging from 19-89 years old and categorised donors in 3 age groups. It used direct BM plating technique which prevented the loss of cells that occurs in centrifugation. It also used a media that was standardised for growing uncultured BM MSCs along with ensuring that a colony always had >50 cells. Overall, in this study, a significant age-related decline was observed in the number of colonies obtained from uncultured BM MSCs with advancing age and a 4-fold decline in old donors (>60 years old) when compared to young donors (<40 years old). Importantly, the decline in the number of MSCs was notable from the age of 40 years old and not 60 years old as expected. While the numerical value was the lowest in old donors, the decline was the steepest in the intermediate age group suggesting the skeletal ageing *in vivo* begins in the fifth decade of life. The other important observation was the presence of old donors with a relatively higher number of MSCs as compared to the others in the same age group, which was comparable to MSC numbers obtained from young donors. This suggested that *in vivo*, these old donors (with high number of

MSCs) potentially had a younger 'biological age' in comparison to others with the same chronological age (same age group).

Colony area and colony ID were analysed next to evaluate the age-related differences in the proliferative capacity of MSCs. Age-related changes in proliferative capacities of MSCs have been studied by different methods using cell proliferation assays, cumulative population doublings calculations and population doubling time in cultured MSCs. Some have shown an age-related decline in MSC proliferation (211) and others, not shown a difference (197). As mentioned in this thesis Introduction (section 1.2.2), such studies are limited by the fact that cultured MSCs undergo passage-dependent *in vitro* ageing process that can over exaggerate or negate any age-related changes in MSCs *in vivo*. On the other hand, the studies that have investigated MSC proliferation capacity prior to passaging are very limited. While they have measured colony area and the cellular contact between the cells in the colony (201), these factors have not been studied in relation to age-related changes. Therefore, the data from this study on any differences in colony sizes and IDs in relation to donor age are entirely novel.

Gothard et al., proposed a segregation of BM MSC colonies into large (>2.5mm diameter) and small (< 2.5mm diameter) based on median diameter chosen form the entire diameter range observed. They also segregated colonies as dense or sparse based on cellular contact confluency within a colony. High cell contact (>80%) was defined as dense and low cell contact (<80%) was defined as sparse colony. In the present study, different sizes of colonies were observed, which did not indicate a clear visual segregation by size or density, and generated a unimodal distribution following frequency distribution analysis by the GraphPad software (Figure 3.7A). To make a fair comparison with the Gothard et al. study, in this study, the colonies were segregated based on the median area from all the colonies (10.64mm^2) obtained from ImageJ software analysis which was equivalent to average diameter of 3.76mm. The diameter was larger than the average colony diameter described in Gothard et al. study and the area was similar to colony areas presented in D'Ippolito study (10.23mm^2 for young donors, 8.64 mm^2 for old donors (132)).

Even though colony area measures the size of the colony indicating the spread of the cells, it does not measure the density of the cells within a colony. A colony can be large and dense or large and sparse as defined by Gothard et al., 2013. A large dense colony has more cells that are in close contact with each other indicating high proliferation and a small sparse colony will have fewer cells that are comparatively less compact in their cellular contact. A large dense colony will thus be derived from an

MSC with high proliferative capacity and a small sparse colony will originate from the least proliferative or pre-senescent MSCs (208). Hence the area of a colony alone is not a true indicator of the proliferative capacity of the MSCs and the colony ID, which is a combined measure of area and density, is thus an improved measure of the proliferative capacity of the MSCs. As listed in Table 3.2, only 2 studies have mentioned colony density in their studies. While one of them found no age-related difference in their research using uncultured cells (200) (data was not shown), the more recent study using cultured cells by Travis et al., in 2017 observed an age-related decline in the colony density visually (no quantification) (139).

ImageJ analysis of colony area and ID in this study helped to generate numerical ID values for all the colonies. The median values obtained from the analysis of colony area measured for all the colonies were used to categorize the colonies as large or small and then compare their ID in young and old donors. The results indicated a significantly higher ID of all colonies in young donors as compared to old donors (p<0.0001) (Figure 3.8A). Small and large colonies were observed in both, young and old donors but young donor colonies always had higher ID irrespective of the size of the colony (Figure 3.8C and D). The decline in IDs was more prominent in small colonies than in large colonies suggesting that old donor MSCs generate proportionally more small sparse colonies than proportionally less large dense colonies. This was opposite to the original hypothesis of fewer large dense colonies originating from most proliferative cells in old donor BM. These are important new data that highly-proliferative MSC remain to be present in old donor BM.

The presented data confirms the concept of decrease in the rate of MSC proliferation with increase in donor age in uncultured cells (212). The analysis of colony area and density has been previously done only by Gothard et al., (201), but they did not investigate these parameters on a large number of colonies with respect to donor age. Furthermore, no CFU-F study, as per my knowledge, has dissected the CFU-F assay at the single colony level to understand the age-related changes in colony area and ID making data from this chapter novel, in comparison to others to study age-related changes in proliferation of minimally cultured individual MSCs. Once the age-related decline in colony area and ID were established, next it was hypothesised that colony area and ID of MSCs when exposed to old donor serum (OS) will show an age-related decline as compared to media supplemented with YS. The other hypothesis with exciting potential applications was to test if the presence of young microenvironment (media supplemented with YS) could reduce the loss of proliferative capacity seen in old donor MSCs. *In vitro*, MSCs are grown in rather artificial conditions that differ from

their native conditions *in vivo* (100). To mirror the biological conditions of old donors, media supplemented with old and young donor serum was used instead of standard media supplemented with FCS. Preliminary experiments established optimal conditions and controls for these investigations, and established that human serum supported colony formation from MSCs better than negative control FCS (Figure 3.9).

In this study, a decline in proliferative capacity of MSCs from both young and old donors was observed when they were grown in media supplemented with OS. Using both colony area and colony ID as parameters to measure MSC proliferation at single-colony level, the data suggested that the use of OS negatively impacted the proliferative capacity of MSCs as seen by the presence of lower frequency of large colonies and higher frequency of small colonies in Figure 3.13 and 3.14. This was also shown by the low colony ID in young donors when grown in OS in Figure 3.13. This can be explained by the increase of ROS in cells as shown in a rat model (117) and by the presence pro-inflammatory factors (213) in OS that inhibit proliferation and eventually lead to MSC senescence. Increased levels of pro-inflammatory cytokines and SASP have been associated with increased age in human donors (214-216). Similar investigation in serum from human donors used for culturing young and old donor MSCs in this thesis, will help to explain the loss of proliferative capacity of MSCs from old donors. It will also provide evidence for the impact of aged microenvironment (SASP released from aged cells in medium containing human serum) *in vivo*.

Abdullah and colleagues studied the effect of aged serum on hMSC-tert cell line and found that the gene expression of MSC multipotentiality markers in old serum was significantly lower (p<0.01) than the cells grown in young serum emphasising the impact of aged microenvironment on MSC-like cells (203). Josephson and colleagues performed experiments in mice and found a significant decline in the number of colonies from young (12 weeks) animal grown in serum from middle-aged (52 weeks) animals. They found an increase of SASP in MSCs grown in serum from middle aged animals (146). Quantification of pro-inflammatory cytokines and SASP molecules (like IL6, IL8, TNF, Tp53) from the media of old donor MSCs would further explain the loss of proliferative capacity of MSCs in OS. Additionally, proliferation of old donor MSCs grown in media supplemented with YS could be only slightly improved (limited rejuvenation). Human serum was used to reflect physiological conditions *in vivo* and indicated towards limited rejuvenation of old donor MSCs in YS. Serum is the fluid that remains after removal of all the clotting factors. The use of other human supplements like PL (platelet lysate) could potentially display stronger rejuvenation, owing to the presence of clotting factors (217). PL is a cell free lysate prepared by disruption of

platelets by freeze/thawing. Investigations using PL have shown that the osteogenic differentiation of MSCs grown in young donor PL was more pronounced as compared to their differentiation when grown in PL from old donors (217, 218).

The concept of MSC rejuvenation with culture in YS was not as pronounced in the present experiments, but positive trends were observed. The colony area measurements of old donor MSCs showed a trend for significantly bigger colonies when grown in media supplemented with YS as compared to OS (Figure 3.12B). Also a trend for higher ID of colonies from old donors grown in YS as compared to OS was found (Figure 3.14B). The fact that colony area was increased more than colony ID can be explained by the fact that YS increased cell migration out of the colony more than cell proliferation, this can be also useful for MSC function *in vivo*. This hypothesis can be further tested by using holographic microscopy by monitoring of cellular movement in real time (219). Growing old donor cells on extracellular matrix derived from younger donors as shown previously (139, 220), could also demonstrate noteworthy conclusions with possible clinical applications of rejuvenation. Overall, the present data indicate that exposing old donor MSCs to supplements (serum, PL, extracellular matrix– collagen fibres and proteins) from young donors can help aged MSC not lose their proliferative capacity any further, and possibly improve their migration capacity. Treatment of uncultured MSCs with anti-oxidants prior to clinical use (46), use of senolytic drugs that target senescent cells using MSC ageing surface markers (221) along with use of supplements to provide a 'young' microenvironment could also aid rejuvenation and delay the process of ageing.

In conclusion, this chapter has shown a significant age-related decline in the number of BM MSCs in older donors compared to young resolving the existing controversy. Colonies from the CFU-F assays showed a significant age-related decline in their area and ID. For the first time, colony sizes and densities were quantified (measured in ID) along with colony area alone and correlated with donor age, and showed an age-related decline in the proliferative capacity of individual MSCs from old donors. The proliferative capacity of young and old donor MSCs were also investigated in more physiological conditions with human serum to mirror *in vivo* conditions in which MSCs reside. OS negatively impacted the proliferative capacity of young and old donor MSCs and exposing old donor cells to YS stopped further loss in their proliferative capacity. These data, for the first time, suggest the importance of the microenvironment (MSC niche) in their functions and showing that both intrinsic and extrinsic factors should be considered in MSC ageing studies.

Chapter 4 Investigating age-related changes in human BM MSC using the $CD45^{low}CD271^{+}$ phenotype

In the previous chapter, age-related changes in number and proliferative capacity of BM MSCs were investigated using the classical colony-forming assay. The results showed a significant decrease in MSC numbers across the whole age range (19-89 years old) (section 3.3.1), as well as their reduced proliferative capacity measured by colonies' integrated density (section 3.3.3). Reduced proliferative capacity was also observed in MSCs when they were grown in media supplemented with old donor serum as compared to media supplemented with young donor serum. This chapter will explore any potential changes in BM MSC gene expression with a focus on the molecules involved in MSC multipotentiality. For this, uncultured BM MSCs were sorted based on the $CD45^{low}CD271^{+}$ phenotype, as previously described (109, 188, 222) and gene expression in young (19-40 years old) and old (60-89 years old) donor MSCs were investigated and compared.

4.1 Introduction

The identification and characterisation of uncultured BM MSCs has been previously performed using flow cytometry and a number of surface markers including Stro-1 (133, 197), SSEA-4 (139, 223), MSCA-1 (224) and others, outlined in section 1.2.1. Over the years, new markers such as CD140a (225), CD140b (226), CD146 (227), and CD295 (228) have also been proposed, sometimes as single markers but usually in combination with ISCT panel markers (105, 229) for ensuring appropriate identification of BM MSCs.

CD271 or Low affinity nerve growth factor (LNGFR) was proposed as a very specific BM MSC marker in 2002 (108, 230). The combination of CD45 (pan-hematopoietic lineage cell marker) and CD271 was later proven to be most useful for the identification of BM MSCs, as it provided the highest resolution and the least cross-reactivity with other BM cells. Since then, $CD45^{low}CD271^{+}$ phenotype has been used by many independent laboratories where CD271 was consistently found to be present in BM MSCs making it a preferred choice for studying uncultured BM MSCs (109, 231, 232). In one study, Torales and colleagues carried out flow cytometry and compared the percentage of cells that expressed CD73, CD90, and CD105, and were negative for CD45 and CD34, with the percentage of cells that were $CD271^{+}$ (233). They found that while the traditional ISCT panel showed an average 0.54% of mononuclear cells as putative MSCs, $CD271^{+}$ alone was highly specific and 0.53% of BM cells expressed CD271. Nevertheless, the work from our laboratory (234), as well as others (109)

showed that the addition of CD45 as a 'negative' gating marker is necessary for a clearer MSC identification, and the removal of $CD271^{low}$ 'passenger' cells that appear to be of a hematopoietic origin (233).

Considering that $CD45^{low}CD271^{+}$ cell phenotype has been extensively used for identification of BM MSCs (section 1.2.1), this phenotype was chosen to be used in this chapter to further investigate age-related changes in the numbers and the gene expression in native BM MSCs without any culture manipulations. As shown in the previous chapter, the age-related decline in the number of BM MSCs quantified using the CFU-F assay was significant (Section 3.3.1), however, the CFU-F assay remains culture-dependent. It was thus logical to explore whether the same decline would be observed using flow cytometry, with no culturing steps involved, and to evaluate the results obtained by both methods. This is of particular importance as the studies that have investigated age-related changes in number of BM MSCs using flow cytometry methods remain limited (Table 4.1).

Table 4.1 includes studies that have investigated the age-related changes in number or percentage of BM MSCs using flow cytometry. While most of the studies (4 out of 6) have indicated a decline in the number of BM MSCs with increasing age, a number of variations can be observed. This includes disparities in grouping methods of 'young' and 'old', additionally, differences in cell isolation techniques and the markers used to identify BM MSCs were most notable. Interestingly, large donor variation is another factor that must be considered, especially when dealing with uncultured cells (235, 236).

Table 4.1 Previous studies examining age-related changes in the number of BM MSCs measured using flow cytometry

Source of MSCs	Age groups	Markers used	Age related change	Ref
BM from iliac crest	Y: 22-44; O: 66-74	STRO-1	No change	(197)
Femoral head BM	Y:<50; I:50-65; O:>65	STRO-1	No change	(237)
BM	13-80; Y:<45	Multiple	Decline	(97)
Femoral neck bone	Y:28-31; O:80-97	$CD271^{+}$ $SSEA\text{-}4^{+}$	Decline	(223)
BMA	22-80; no groups	$CD45^{low}$ $CD271^{high}$	Decline (F only)	(111)
Iliac crest bone graft	24-89; Y:<50; O:>50	$CD45^{low}$ $CD271^{high}$	Decline	(146)

BM: bone marrow, BMA: bone marrow aspirate, Y: young, I: intermediate, O: old, F: females

4.1.1 Candidate MSC multipotentiality genes

As mentioned in section 1.2.2, the adipogenic bias of aged BM MSCs is among the most established age-related changes described to date. Table 4.2 lists previous studies that have investigated the shift from osteogenic to adipogenic differentiation in MSCs in aged donors. Of importance, all these investigations have been performed using cultured MSCs. The disruption in bone formation has been suggested to be due to the impaired ability of MSCs towards osteogenic differentiation in older donors. This loss in bone formation is compensated by excessive adipocyte formation. Different pathways and molecules have been suggested to be responsible for this shift, however, the exact mechanism is not yet completely understood. Of note, bone homeostasis is not only a result of bone formation, but also of bone resorption (section 1.1.4). In one study, an age-related increase in the expression of a bone resorption molecule *RANKL* in pre-adipocytes along with an increase in osteoclastogenesis, was observed within the BM (238). In part age-related bone loss is brought about by disruption of bone resorption, described in section 1.1.4.

Apart from the shift towards adipogenic differentiation and bone remodelling, the other factor that is potentially affected by older age in MSCs, is their communication with other BM cells with the use of trophic factors including chemokines. CXCL12 (C-X-C motif chemokine 12) is one such chemokine known to be critical to maintain the HSC pool within the BM (239, 240) as well as being involved in MSC migration and apoptosis (241). CXCL12 is regulated by Cx43 and Cx45 (242), where Cx43 is the most common gap junction within the bone (148). However, age-related changes in CXCL12 production by native BM MSCs remain unexplored. In terms of direct cellular interactions, connexins are intercellular communication molecules and as an entire family of molecules, have been suggested to have an age-related decline in many tissues with a potential link to human ageing and cancer (243). Both of these molecules will be discussed further in 4.1.1.5.

As BM MSCs are very rare cells (87), the numbers of purified cells are not sufficient to perform standard differentiation assays, which normally use a range of $2x10^5$-$2x10^6$ cells/ assay (108). Therefore, gene expression in $CD45^{low}CD271^+$ cells was studied as the first step to explore their differentiation and cell communication potentials.

4.1.1.1 Osteogenic-lineage transcripts

RUNX2 (Runt related transcription factor 2) is the master regulator of bone formation and belongs to RUNX family of transcription factors (TFs). All molecules of this family

share a common runt domain. *RUNX2* is the earliest indicator of osteogenic differentiation and is also known to tightly regulate chondrocyte hypertrophy and vascular invasion of developing skeletons. In MSCs, levels of *RUNX2* expression have been mainly investigated to detect their osteogenic differentiation capacity and MSC commitment towards osteo-progenitor lineage (244). With respect to age-related changes, overexpression of *RUNX2* has been suggested to contribute to bone resorption in osteoblastic lineage cells by increasing *RANKL* expression (245). However, when narrowing to MSCs from human donors, investigations are sparse. In older donors, the expression of *RUNX2* has either been observed to decrease or have no change in cultured MSCs providing no substantial evidence for age-related differences in uncultured MSCs (Table 4.2). Nevertheless, it could be hypothesised that it may be reduced based on the idea of 'MSC adipogenic bias' with advancing age.

Table 4.2 Previous studies investigating age-related differences in osteogenic, adipogenic and bone remodelling molecules in culture expanded MSCs

Function	Source of cells/ MSCs	Age groups	Genes tested	Age related changes	Reference
Osteogenic differentiation	BM from humans	17-90; Y:<50; O:>50	*RUNX2*	Decreased	(133)
	BMA from IC in humans	Y:<50; A:50-65 ; O:>65	*RUNX2, OC, ALP, OPN*	No changes in *RUNX2*	(246)
	Bone graft from IC	Y:18-49; O:>50	*RUNX2, OC, ALP, OPN*	No changes in *RUNX2*	(247)
Adipogenic differentiation	BMA from IC in humans	13-80; no group	*LPL, PPAR-γ*	No changes in *PPAR-γ*	(97)
	Human ACL	Y:17-27; O:69-79	*LPL, PPAR-γ*	No changes in *PPAR-γ*	(248)
	BM from iliac spine	Y: 18-42; O: 66-78	*PPAR-γ*	No changes	(196)
	Bone from IC	7-78; no group	Leptin receptor CD295	Increased	(126)*
Bone remodelling	BM from humans	Y:<50; O:>55	*RANKL, OPG*	*RANKL* increased, *OPG* decreased	(249)
	BM from humans	Y:<55; O:>55	*RANKL*	*RANKL* increased in females	(250)

BM: Bone marrow, IC: Iliac crest, Y: young O: old, A:adult, *: investigated at protein level

SPARC (Secreted protein acidic and rich in cysteine) or more commonly known as '*Osteonectin*' is a non-structural matricellular glycoprotein in the bone that binds calcium. It is secreted by osteoblasts and pre-osteoblasts during bone formation and is needed for bone calcification (251). Both, over expression (252) and selective deletion of *SPARC* (253) have been associated with impaired motility, suggesting that its normal functioning is vital for bone growth and development. With respect to MSCs, the precursors of osteoblasts, it is not surprising that MSCs too express certain levels of *SPARC*, shown previously by our group (188). Its role in the functions outlined above suggests that it plays an important part in the MSC transition towards the osteogenic lineage. It is thus expected with increasing age, as the fat content increases with a compromise in bone formation, the levels of *SPARC* potentially decline. However, the number of studies investigating this also remain limited, with no study to date investigating any changes in its expression in uncultured MSCs. Interestingly, our previous study using cultured MSCs have shown that *SPARC* expression significantly increased in late-passage, pre-senescent MSCs (222) therefore the present evidence on the association of with MSC ageing or senescence remains controversial.

SFRP1 (Secreted frizzled related protein 1) is a member of the SFRP family of molecules containing cysteine rich glycoproteins, homologous to the putative Wnt (wingless integrated)-binding site of the frizzled proteins (fzd receptors). It acts as a soluble modulator of the Wnt signalling pathway. Wnt signalling pathway is a major pathway involved in skeletal development, bone mass regulation and is often regarded integral for osteogenesis (254). *SFRP1* is well known as a Wnt antagonist that acts by directly binding to Wnt proteins, making it a target for therapeutic approaches for bone remodelling and repair. *SFRP1* has been extensively explored in bone and bone-forming cells (254). The deletion of *SFRP1* reduced osteoblast and osteocyte apoptosis *in vivo* and proliferation and differentiation capacity of osteoblasts *in vitro* in mice (255). It has also been reported to block osteoblast induced osteoclastogenesis by inhibiting *RANKL* dependant osteoclast formation (256). *SFRP1* was also found to suppress Wnt signalling in an immortalised osteoblast cell line (257). Overall, previous studies indicate that *SFRP1* inhibits osteoblast formation and their coupling with osteoclasts. Based on these findings, *SFRP1* inhibitors were developed as potential therapies with the aim of increasing bone formation (258). While their potency has been tested *in vitro, in vivo* efficacy in appropriate animal models or in human cells are yet to be examined.

The effect of age on *SFRP1* expression has not been extensively studied to date. Rauner and co-workers investigated the level of expression of *SFRP1* in the osteoblasts of young (6w), adult (6mo) and old (18mo) male C57BL/6J mice and found a significant age-related decline (259). Considering the above stated findings and the fact that the gene has been found on osteoblastic lineage, it can be hypothesised that MSCs, expression of *SFRP1* could show an age-related decline. Indeed, this was observed in our previous study where *SFRP1* displayed an age-related decline in BM $CD45^{low}CD271^{+}$, cells but this was observed in paediatric donors compared to adult donors (188).

Owing to their involvement in MSC osteogenic differentiation, these three transcripts were selected for investigating age-related differences in uncultured MSCs in relation to their osteogenic differentiation potential.

4.1.1.2 Adipogenic-lineage transcripts

An increase in the transcripts associated with adipogenesis have been observed in MSCs aged *in vitro* (section 1.2.2) or in aged animals *in vivo* (80, 143, 260). Among many transcripts, an increase in *PPAR-γ* (Peroxisome proliferator activated receptor-gamma) *and FABP4* (fatty acid binding protein 4) expression has been associated with the adipogenic differentiation of MSCs (261). *LepR* (Leptin receptor) plays an important role in bone-fat balance (262, 263) and its expression was shown to decline in uncultured cells by our group previously (188). However their donor range included paediatric samples and the oldest donor was 72 years old. Age-related changes in adults and in donors aged above 75, with respect to expression of these transcripts in uncultured MSCs is yet to be explored.

PPAR-γ gene encodes a molecule termed the PPAR-γ receptor. There are two different isoforms of the protein, named PPAR-γ1 and PPAR-γ2 that exist due to alternative splicing of the gene. While PPAR-γ1 is expressed in various cell types including osteoblasts, PPAR-γ2 is restricted to adipocytes including those within the BM (264). The receptor is found in abundance not only in adipose cells but also in pre-adipogenic cells, endothelial cells and vascular smooth cells (265). Owing to its requirement in adipose tissue formation, PPAR-γ is considered a key regulator of adipogenic differentiation. PPAR-γ in MSCs can be both, anti-osteogenic and pro-adipogenic, therefore it can play the role of osteoblastic/adipogenic 'switch' (266). It has thus been described to show 'tremendous potential in novel strategies for bone tissue engineering and clinical applications' (267). As mentioned above, there is published evidence of increased expression of *PPAR-γ* in cultured MSCs from aged

donors as well as in cells aged *in vitro* through culture expansion (143). Based on previous literature , it was hypothesised that there would be an age-related increase in the expression of PPAR-γ in native BM MSCs from older donors.

Leptin receptor (LepR) is another transcript for which there is an increasing body of evidence on its role in the bone-fat balance (262, 263). *LepR* gene encodes for the surface receptor for leptin, a hormone secreted by adipocytes and known to regulate body weight and metabolism (268, 269). To understand the gene encoding for leptin receptor, it is worth taking a step back to understand the role of leptin.

Leptin is an adipokine predominantly produced by adipose cells and is known to contribute to energy balance by acting on hunger/satiety centres located in the hypothalamus (270, 271). Its level increases with weight gain and decreases with reduced weight (272). The adipokine has also been shown to be secreted by skeletal muscles and bone cells and is an established growth factor for muscle and bone in early life (268). The receptor for the leptin adipokine (CD295) has been shown to be present in musculo-skeletal tissues (263). Initially, it was thought that leptin hormone contributed to age–related decline in bone mass via the hypothalamus and the beta-adrenergic receptors (273). However, more recent studies suggest that the hormone may have a more direct role on bone lineage cells including MSCs (274). However, the role of CD295, the leptin receptor in MSC differentiation to bone is not very well understood. With respect to ageing, Laschober and colleagues showed an age-related increase in the level of expression of LepR at the protein level (CD295) in BM MSCs (126). Their study also indicated that the CD295 positive BM MSCs in old donor MSCs were more apoptotic than young donor MSCs, quantified using Annexin-V staining. On the other hand, our group has previously shown an age-related decline of *LepR* gene expression in $CD45^{low}CD271^{+}$ cells in a small number of donors (n=8) (188). We therefore hypothesised an age-related difference in the transcript of this gene in native BM MSCs from old donors.

FABP4 belongs to the FABP (fatty acid binding protein) family of transport proteins that are responsible for the transfer of fat or lipophilic molecules across the cellular membranes and is a well-established marker for differentiated adipocytes (275). It was first detected in adipose tissues and mature adipocytes and has also been termed as adipocyte P2 (ap2) due to similarity with myelin P2 protein (276), which is also a part of the FABP family. It is highly expressed during adipogenic differentiation of MSCs and is transcriptionally controlled by *PPAR-*γ agonists, fatty acids and insulin. With respect to MSCs, *FABP4* has been detected in high levels in MSCs expanded in culture over a

period of 21 days undergoing adipogenic differentiation in parallel to the expression of PPAR-γ (277). Based on these findings, an age-related increase in its expression in uncultured BM MSCs was hypothesised.

4.1.1.3 Oxidative stress in MSCs and anti-oxidant enzyme *SOD3*

Presence of reactive oxygen species (ROS) has been suggested as one of the potential drivers of the adipogenic bias of MSCs (278, 279). ROS are by-products of metabolism inside a cell, mitochondria being the major site of ROS production. Oxidative stress is the consequence of an excessive ROS production and limited activity of antioxidant enzymes to balance the negative impacts of ROS. A decline in the anti-oxidant enzymes with increasing age leads to reduced capacity of scavenging of ROS (280). Excessive ROS causes accumulation of DNA damage leading to cellular senescence in ageing and associated diseases (281). This ultimately drives the cell onto the apoptosis path and is believed to be one of the major causes of age-related differences in MSCs.

During metabolism, ROS intermediate molecules like superoxide and peroxides are formed as a part of cell metabolism. These intermediate ROS products are scavenged by anti-oxidants enzymes like superoxide dismutase (SOD) and glutathione peroxidase catalase (Gx) to prevent the interaction of ROS with lipid membranes and proteins within the body (282). SOD converts superoxide ion to hydrogen peroxide, which is further converted into water and oxygen molecules under catalase and glutathione peroxidase activities (282).

Anti-oxidants thus play an important role in critically maintaining the appropriate ROS levels. SOD or superoxidedismuatse form the first line of defence in scavenging the superoxide molecule (283). 'Dismutation' by definition is a redox reaction that involves the formation of two products of different oxidation states from a single molecule of intermediate oxidation state. Superoxide molecule (O_2^-) has a negative charge which causes it to react with lipids and proteins nearby causing damage. SOD thus gets its name from the function of carrying out 'dismutation' of superoxide molecule to hydrogen peroxide (H_2O_2). H_2O_2 further is scavenged by Gx into water and oxygen molecule. SOD enzymes include SOD1, SOD2 or SOD3. SOD1, also known as Copper/Zinc SOD (Cu/Zn SOD) is mainly located in the cytosol of the cells. SOD2 is also known Manganese-SOD (Mn-SOD) is majorly located in the mitochondria of cells. As mitochondria is the major site of ATP production, SOD2 has been investigated in a large number of studies to understand the impact of this anti-oxidant enzyme in oxidative stress (283).

SOD3 is also known as extracellular SOD (EC-SOD) and gets its name from its location. SOD3 has been shown to be secreted by MSCs (284), while SOD2 and SOD1 do not have any such cellular specificity. Due to its location, it is the only SOD that scavenges ROS in the extracellular compartment of cells and catalyses the dismutation of superoxide molecule. Within the BM, MSCs exist within a hypoxic BM niche with oxygen concentrations as low as 2-8% O_2 (285). Culture expansion in artificial conditions automatically expose these cells to a 'hyperoxic' condition but it is usually referred to as 'normoxic' or normal oxygen concentration due to the widespread practice of *in vitro* expansion. An increase in the oxidative stress in MSCs has often been suggested as a potential cause for the senescence and their adipogenic bias *in vitro* (278, 286).

Mohd Ali and colleagues compared the proliferative capacities of BM MSCs from young (<30) and old (>60) donors in hypoxic (5% O_2) and normoxic (20% O_2) conditions. They found that MSCs from older donors demonstrated reduced proliferative and differentiation abilities along with increase in cellular senescence. Interestingly, they found that MSCs cultured under hypoxic conditions in both age groups were able to demonstrate enhanced self-renewal and proliferative capacity (287). Another study investigated the changes in *SOD3* at the mRNA and protein level during differentiation of cultured BM MSCs. The study found significantly higher levels of *SOD3* during adipogenesis, lower levels during chondrogenesis but no difference during osteogenesis (288).

Considering that increased adipogenic differentiation bias is also age-related (section 1.1.4) and that increase in oxidative stress is among the oldest theories of ageing (section 1.1.1), it can be said that SOD3 may be affected in old donors. However, more clarity in how it alters in uncultured BM MSCs in old donors is required. It may be hypothesised that the level of ROS in old donor MSCs will be greater and the levels of anti-oxidant enzyme *SOD3* in old donor MSCs will be lower than in young donor MSCs.

4.1.1.4 Transcripts associated with bone remodelling

Bone remodelling is a tightly regulated mechanism, which under normal conditions perfectly balances the rate of bone resorption by osteoclasts and the bone formation by osteoblasts. The bone remodelling cascade and the different cells involved was described in detail in section 1.1.4.

RANKL (Receptor activator of nuclear factor kappa beta ligand) is expressed by osteoblasts and binds to its target RANK that is expressed by osteoclast progenitors (64, 74). RANKL is a type II membrane protein from the tumour necrosis factor (TNF) super family. It is a homotrimeric protein and exists in both, soluble and membrane form. However, the soluble form is known to have lesser ability to form osteoclasts (289). MSCs have been known to contribute towards both, bone resorption by expression of *RANKL* and bone formation as precursors of bone forming cells (290). While culture expanded MSCs are able to express *RANKL* and have been shown to contribute to osteoclastogenesis (291), its expression in MSCs in relation to *in vitro* ageing remains controversial (Table 4.2) and in uncultured MSC, unexplored.

RANK (Receptor activator of nuclear factor kappa beta) is the receptor for its ligand, the *RANKL* and is also designated *TNFRSF11A. RANK* is expressed on the surface of osteoclast precursors and *RANKL* binding to *RANK* initiates the process of osteoclast formation. It is expressed on osteoclast precursors (292) which upon maturation form bone resorbing osteoclasts. However, osteoclast formation takes place only after the activation of TNF associated receptor factor 6 (TRAF6) signalling (293). This then mediates the MAPK/NF-Kb pathway which eventually brings about osteoclastogenesis.

OPG (Osteoprotegerin or Tumour necrosis factor super family member 11B/ *TNFSFR11B*) is expressed widely by a number of cells and is the natural decoy/inhibitor of *RANKL*. If *OPG* (instead of *RANKL*) binds to *RANK,* then osteoclast formation stops and bone resorption does not take place. It is highly expressed in tissues including osteoblasts, pre-osteoblasts and other cells of the bone lineage in the BM (71). It also belongs to the TNF receptor superfamily but is different from the other members as a secreted protein that does not contain a trans-membrane domain (294).

An age-related increase in *RANKL* and decrease in *OPG* in MSCs was hypothesised, potentially contributing to increased bone resorption rates *in vivo*, accounting for the age-related bone loss in old age.

4.1.1.5 Transcripts associated with cellular interactions

CXCL12 (Chemokine motif 12 also known as SDF-1 or stromal derived factor-1) is a major chemokine facilitating the chemotaxis of HSCs, MSCs and other BM cells (295). It is a member of a large family of chemokines that are structurally related. It binds to its receptor CXCR4 on target cells and is known for its role in the maintenance of the hematopoietic compartment of the BM niche (239, 240) and in chemotaxis of MSCs (296). In the BM, *CXCL12* is expressed by osteoblasts and endothelial cells as well as

stromal cells lining the endosteal surface (297). Most HSCs within the BM are usually in contact with cells expressing high amounts of *CXCL12* which have been named *CXCL12*-abundant reticular cells (or CAR cells) in mice (239). *CXCL12* is key to maintain the healthy pool of HSCs. The counterpart of CAR cells in human BM are likely to be adventitial reticular cells, topographically overlapping with $CD45^{low}CD271^{+}$ MSCs (298).

In addition to its support for hematopoietic cell activities, such as their retention and egression from the BM, *CXCL12-CXCR4* signalling axis has also been implicated in the processes related to osteoclast recruitment, chemotaxis and bone resorption (299). There are also reports describing the impact of *CXCL12-CXCR4* signalling in metastases of breast cancer, lung cancer and some hematopoietic malignancies to the bone (300).

Investigation in culture expanded MSCs to understand any differences with the advancement of *in vitro* ageing, to the best of my knowledge, does not yet exist. The information regarding the levels of its expression before culturing MSCs, also remain limited. Our group found very high levels of *CXCL12* expression in uncultured $CD45^{-}$ $CD271^{+}$ BM MSCs as compared to $CD45^{+}CD271^{-}$ HLCs but there was no age-related differences observed with 8 donors. As red (hematopoietic) marrow is replaced by yellow (fatty) marrow as a function of age, it was hypothesised that there could potentially be an age-related decrease in the levels of *CXCL12* in uncultured BM MSCs from aged individuals.

Among the connexins, *Connexin43 (Cx43*) is the most abundant gap junction protein in bone cells, mainly associated with the intercellular interaction between osteocytes and osteoblasts (148). It is essential for intercellular interaction, normal bone formation and for the viability of osteocytes (301). As the main gap junction in mature bone-lineage cells, it is therefore an important molecule to investigate with respect to *in vivo* MSCs. In the BM, MSCs interact with each other forming an intricate net or scaffold upon which other BM cells including HSCs reside, these networks are facilitated by *Cx43* gap junctions. The gap junctions also regulate the transcription of *CXCL12* in a cell contact dependant manner (242).

Previous studies investigating age-related differences in the expression of *Cx43* have found a significant decline in native $CD45^{low}CD271^{+}$ BM MSCs (188). Studies in human derived cultured expanded MSCs to investigate changes with *in vitro* ageing do not exist. With respect to animal studies, Donahue's group have investigated the

significance of *Cx43* in bone formation and skeletal homeostasis in rats. They have found no age-related differences in *Cx43* gene expression in cultured BM MSCs (302). Although the data remains controversial, these studies present *Cx43* a potential molecule affected by age in native BM MSCs (303).

At the protein level, Cx43 or gap junction protein alpha 1 (GJA1) is an intercellular membrane spanning channel that mediates direct exchange of small molecules, ions, nucleotides between neighbouring cells. The role of *Cx43* in skeletal metabolism and homeostasis is now well established as gap junction intercellular communication (GJIC) (304). In relation to the bone, a growing body of evidence exists on the importance of Cx43 in modulating bone cells to respond to mechanical signals and growth factors to enable bone healing post fracture (148, 305). Absence or reduced *Cx43* function have been associated with reduced levels of osteogenic genes (306) and reduced level of osteoclast formation leading to decreased bone resorption (307). These findings have established that *Cx43* is vital for growth, development and maintenance of healthy bone health. Owing to the various important roles played by this molecule in the BM, *Cx43* was chosen to be tested at both, the gene and protein levels and was hypothesised to display an age-related decline in BM MSCs in this project.

4.1.2 Candidate age-specific surface makers

The knowledge of age-specific markers for uncultured BM MSCs remains limited, especially when investigating BM MSCs from young and aged human donors. It has been shown in various studies that BM MSCs from younger donors have good therapeutic potential. However, the use of BM MSCs from older donors for clinical applications usually requires further in depth investigation. Identifying a candidate surface marker that could differentiate young from old donor MSCs could be next considered as a marker to indicate the ageing status of native MSCs before further investigations.

The previous chapter showed that there is a decline in the proliferative capacity of BM MSCs from old donors, irrespective of whether they formed large or small colonies. Identification of a marker that could indicate low-proliferative (aged) MSCs, would be very useful for cell sorting to remove these poorly proliferative cells before clinical and therapeutic applications of MSCs.

4.1.2.1 CD106

CD106 also known as vascular cell adhesion molecule (VCAM-1) is a cytokine-inducible cell surface protein capable of mediating adhesion and immunomodulation (308, 309). It is a member of the immunoglobulin family and is expressed on BM MSCs (Table 4.3). However, the level of expression in human BM MSCs has shown considerable experimental variation, whereby decreasing expression (182), increasing expression (125) and random alternations (123) have been shown with *in vitro* culture expansion.

Thus the information on what happens to the expression of CD106 with *in vitro* ageing remains controversial. Cellular adhesion, T cell activation and recruitment of lymphocytes at the site inflammation is known to be mediated by CD106. CD106 also plays an important role in MSC mediated immune-suppression (310). Considering that MSC display their immune-regulatory properties using cell-to-cell adhesion (311) and a general decline in the immune-suppression potential of BM MSCs with age (303), it was hypothesised that the expression of CD106 would display an age-related decline.

4.1.1.6 CD146

CD146 or Melanoma cell adhesion molecule (MCAM) is a 113kDa cell adhesion molecule which was originally described as an endothelial cell marker (312). It is also expressed in a variety of cells including lymphocytes (313). With respect to BM, CD146 identifies the population of the perivascular MSCs (314). A 2011 study by Tormin et al., showed that native BM MSCs with both phenotypes $CD45^{low}CD271^{+}CD146^{+}$ and $CD45^{-}CD271^{+}CD146^{-}$ formed comparable number of colonies in the CFU-F assay and generated similar cultures *in vitro* with similar cell morphology, FACS and gene expression profiles (227). In terms of their location, $CD45^{low}CD271^{+}$146+ cells were found in the perivascular region whereas $CD45^{low}CD271^{+}146^{-}$ cells were present as bone lining cells.

With respect to age-related changes, a few studies listed in Table 4.3, have shown down regulation of the marker in culture. Previous work from our laboratory has shown a decline during *in vitro* MSC ageing in the expression of CD146, as well as CD106 (182). Siegel and co-workers found an age-related decline in the geometric mean of CD146 expressing BM MSCs. However, no data on the expression of CD146 in uncultured BM MSCs in relation to donor age is available. Based on previous evidence of decline in expression of CD146 in culture expanded BM MSCs (Table 4.3), it was hypothesised that CD146 displays an age-related decrease in the expression in old donors.

The aim of this chapter was to investigate any age-related changes in the number of uncultured BM MSCs using the $CD45^{low}CD271^{+}$ phenotype in flow cytometry. The same phenotype would be used to segregate MSCs ($CD45^{low}CD271^{+}$) and HLCs ($CD45^{+}CD271^{-}$) cells and investigate any age-related changes in multipotential gene and surface marker expression.

Table 4.3 Previous studies investigating candidate age-specific surface markers

Surface marker	Source of MSCs	Age groups	Passage/ donor age dependant	Isolation	Medium	Passage/ Age-related change	Ref
CD106	BMA	2-61	Passage dependant	Density centrifugation	NH expansion medium	Decreased	(182)
	IC	T:0-20; YA:20-40; AA:40-60; O:>60	Donor age dependant	Plastic adhesion	MEM +20%FCS	Increased	(138)
	BMA	13-80; Y:<45; I:45-65; O:>65	Donor age dependant	Density centrifugation	Mesencult	Decreased	(97)
CD146	BMA	2-61	Passage dependant	Density centrifugation	NH expansion medium	Decreased	(182)
	BMA	13-80; Y:<45; I:45-65; O:>65	Donor age dependant	Density centrifugation	Mesencult	Decreased	(97)
	BM	NA	Passage dependant	Plastic adhesion	DMEM+ 10%FCS	Decreased	(123)

BMA: Bone marrow aspirate, IC: Iliac crest, T: teenage, YA: young adults, AA: ageing adults, I: intermediate, O:old, NA: not applicable

4.1.2 Hypotheses and Objectives

Hypotheses:

1. The number of $CD45^{low}CD271^{+}$ uncultured BM MSCs demonstrates an age-related decline.
2. The transcripts for osteogenic and adipogenic differentiation measured in $CD45^{low}CD271^{+}$ BM MSCs demonstrate an age-related decline and increase, respectively.
3. The level of ROS in $CD45^{low}CD271^{+}$ BM MSC increases and the expression of anti-oxidant enzyme SOD3 decreases with increasing age.
4. The transcripts for bone remodelling measured in $CD45^{low}CD271^{+}$ BM MSCs demonstrate an age-related bias for bone resorption over bone formation.
5. The transcripts related to cellular interactions measured in $CD45^{low}CD271^{+}$ BM MSCs demonstrate an age-related decline.
6. The candidate surface markers measured by flow cytometry show age-related decrease.

Objectives:

1. To enumerate uncultured BM MSCs by flow cytometry using the $CD45^{low}CD271^{+}$ phenotype across a broad donor age range.
2. To measure transcripts associated with MSC osteogenic (*RUNX2, SPARC, SFRP1*) and adipogenic differentiation (*PPAR-γ, FABP4, LepR*) in $CD45^{low}CD271^{+}$ BM MSCs in young and old donor groups.
3. To quantify ROS and to examine the expression of anti-oxidant enzyme SOD3 in $CD45^{low}CD271^{+}$ BM MSCs in young and old donors.
4. To test the transcripts associated with bone remodelling (*RANK, RANKL, OPG*) in $CD45^{low}CD271^{+}$ BM MSCs in young and old donors.
5. To test transcripts associated with cellular interactions (*CXCL12, Cx43*) in $CD45^{low}CD271^{+}$ BM MSCs in young and old donors.
6. To investigate the expression of surface markers of CD106, CD295, Cx43 and CD146 in $CD45^{low}CD271^{+}$ BM MSCs in young and old donors.

4.2 Methods

4.2.1 Donor selection

Fresh BMA from n=30 donors was used to perform enumeration of BM MSCs using the $CD45^{low}CD271^{+}$ phenotype. Frozen BMA from a total of n=20 donors (10 young and 10 old donors) were used for gene expression and surface marker expression experiments. Donors used in this chapter were n=10 young donors (19-40 years old, median age=27 years old) and n=10 old donors (59-89 years old, median age=68 years old). Samples were defrosted as outlined in section 2.3 of the thesis and the exclusion criteria remained the same as described previously in section 3.2.1

4.2.2 Native MSC enumeration by flow cytometry

Flow cytometry was used as the method is automated, can be performed with minimal manipulation and without any culture expansion. For native BM MSC enumeration, 100μl of fresh BMA was placed in a FACS tube and incubated for 15 minutes at RT with the following antibodies: 20μl CD271-allophycocyanine (APC) and 10μl CD45-phycoerythrin (PE)-cyanine dye 7 (Cy7); and 10μl of 7-Aminoactinomycin D (7-AAD) was added to distinguish between live and dead cells. After staining, erythrocytes were lysed by using 500μl of ammonium chloride (NH_4Cl) solution (Appendix 2, Table 2 Buffers) per the tube for 3-5 minutes at 37ºC. Finally, 50μl of Count bright absolute counting beads were added at RT after vortexing. The cell and bead suspension (total volume 700μl) was run using the Beckton Dickinson (BD) LSRII 4 laser flow cytometer.

Unstained and single antibody stained samples were used to optimize the cytometer voltage settings and spectral compensation. Isotype controls for the antibodies IgG1 APC and PE-Cy7 (Appendix 2, Table 2) were used to set the gating strategy on the population of interest (MSCs). A minimum of 100 MSCs ($CD45^{low}CD271^{+}$ cells) were collected for each sample. The acquisition time was dependent on the cellularity of the sample. Data analysis and gating strategies were optimized based on the method described previously by Cuthbert *et. al* (207) and also shown in figure 4.1.

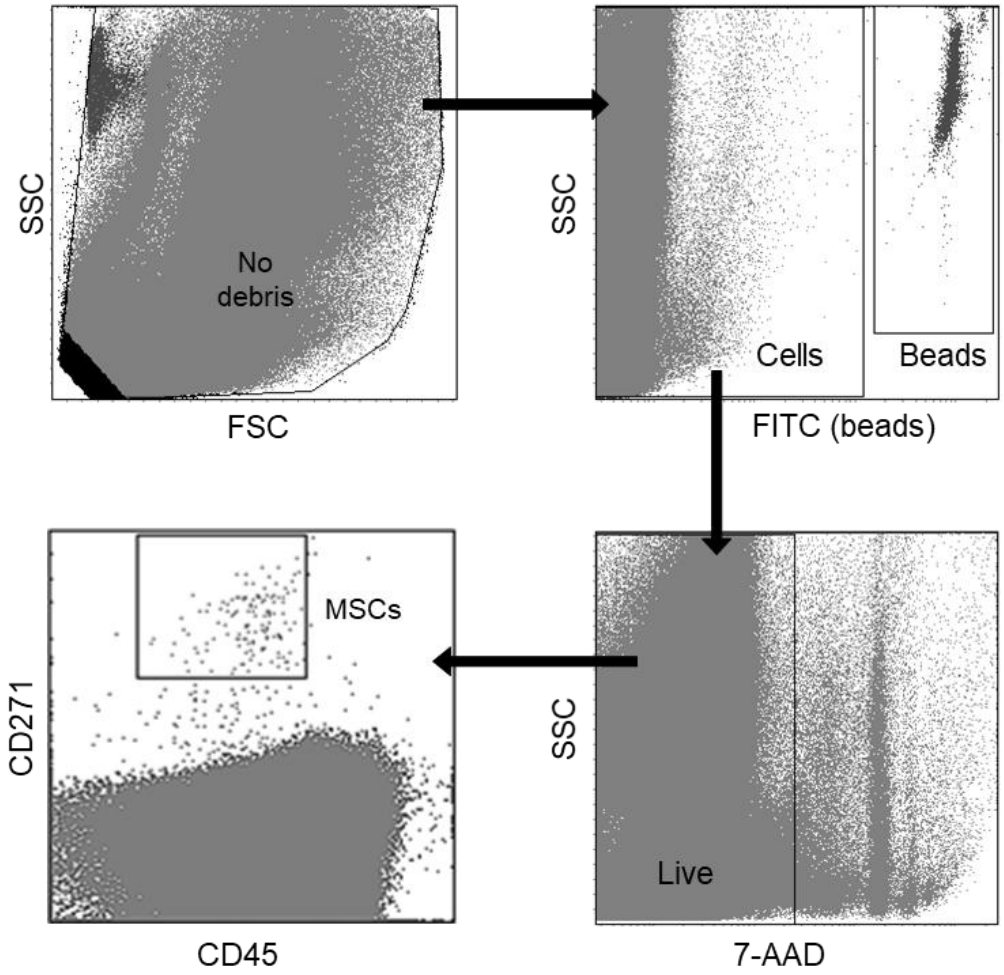

Figure 4.1 Uncultured BM MSC enumeration by flow cytometry using $CD45^{low}CD271^{+}$ phenotype

Gating strategy for enumeration of $CD45^{low}CD271^{+}$ MSCs. Forward and side scatter plot was used to eliminate debris followed by separation of counting beads and cells based on granularity and fluorescence. Then live cells were identified based on 7-AAD negativity from where $CD45^{low}CD271^{+}$ were identified as MSCs. Figure indicates representative donor.

The analysis of the flow cytometry data was performed using the FACS Diva software. Cell debris were eliminated based on forward and side scatter and the counting beads were distinguished based on their granularity and fluorescence. The dead cells were excluded by the uptake of 7-AAD dye. The cell population of interest (MSCs) were identified as $CD45^{low}CD271^{+}$ and the number of MSCs per μl of BMA was calculated using the bead manufacturer's formula [number of live MSC events/number of beads events x number of beads per 50μl/ total sample volume]. The number of MSCs per μl was multiplied by 700 to get the total number of MSCs in 100μl of BMA which was then multiplied by 10 to get the number of MSCs per ml of BMA.

4.2.3 Sample preparation and quantification of ROS in uncultured MSCs

BMA samples from young and old donors were collected as mentioned in section 2.2. RBCs were lysed using NH_4Cl and $2x10^6$ NCs were added to each of the flow tubes. While one tube was not exposed to any chemical, the other tube was exposed to 8mM tert-butylhydrogenperoxide (TBHP) for the generation of ROS under extreme oxidative stress as per optimised protocol (315). Both tubes were then incubated for an hour at 37°C and 5% CO_2. Cells that were not exposed to TBHP served to measure 'basal' levels of ROS in the MSCs.

After incubation, the cells were washed with 2ml 1X PBS and centrifuged at 400g for 5 minutes. The supernatant was discarded and the cells were re-suspended with 100µl of medium containing DMEM+10%FBS. The cells were stained with 20µl CD45 V450 and 20µl of CD271 PE-Vio770 and 2µl of CellROX (FITC) for staining MSCs at the same time for 15 minutes. To identify ROS, cells were stained with CellROX which is a non-fluorescent dye under reduced state but exhibits strong fluorescence upon oxidation localized within the cell. Exposure to TBHP causes additional formation of ROS resulting in further oxidation which was detected by the dye and measured by flow cytometry. The entire process has been summarized in Figure 4.2 below. After 15 minutes of incubation, the cells were washed with PBS for 5 minutes at 400g. The supernatant was discarded and the cells were re-suspended with 100µL of medium containing DMEM+10%FBS. Finally, 2µl of Sytox (APC), dead cell dye was added to distinguish live cells from the dead cells and after 15 minutes of incubation, the sample was quantified for measurement of ROS using flow cytometry.

MFI for CellROX that was indicative of the amount of ROS in the sample was recorded and compared between pre and post stimulation with TBHP. The values were then compared between young and old donors for age-related differences.

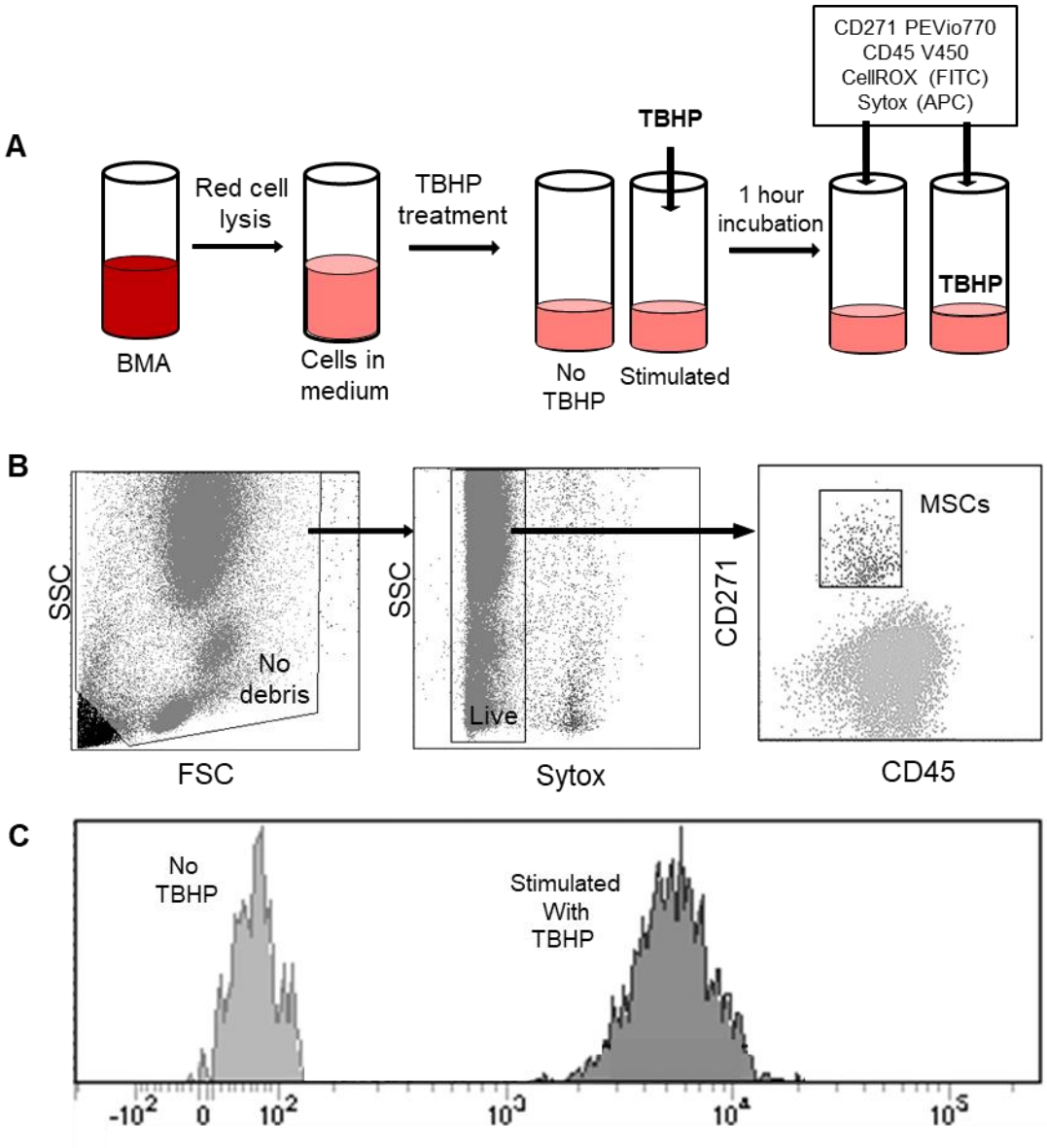

Figure 4.2 Steps involved in the quantification of ROS in $CD45^{low}CD271^{+}$ BM MSCs

(A) BMA was treated with ammonium chloride to eliminate RBCs. This was followed by treatment of cell suspension with tert-butylhydrogenperoxide (TBHP) or no treatment (basal) for an hour in incubator. The cells were then washed, stained with antibodies and quantified using flow cytometry. **(B)** Gating strategy for measuring ROS in $CD45^{low}CD271^{+}$ MSCs, recently published by Jawhari et al*.,* (315). Forward and side scatter plot was used to eliminate debris (left panel) followed by identification of live cells using Sytox negativity (middle panel). Live cells were identified as $CD45^{low}CD271^{+}$ MSCs **(C)** Histogram representation of MFI indicating ROS in $CD45^{low}CD271^{+}$ MSCs. Figure is indicative of representative donor.

4.2.4 Cell sorting

The technique for cell sorting has been described in section 2.4. In brief, frozen nucleated BM cells from both donor groups with at least $40x10^6$ frozen cells were defrosted using TM (section 2.3). The cell suspension was then centrifuged at 400g for 5 minutes for the removal of DMSO. The supernatant was discarded and the cells were re-suspended in 5 ml of the TM. The MSCs were then enriched using Anti-fibroblast microbeads that positively select for cells with D7-fib receptor to enrich the percentage of $CD45^{low}CD271^+$ MSC by positive selection, as previously developed in our laboratory (108) and described in section 2.4.

After enrichment, the cells were distributed in 10 tubes for staining. For isotype controls tubes, a minimum of $2x10^5$ cells were used for each antibody and for cell sorting, up to $3x10^6$ cells were used to collect sufficient number of MSCs. The duration of cell sorting from setting up the spectral compensation and up to the completion of cell sorting for 3 samples varied from a minimum of 180 minutes up to a maximum of 270 minutes depending upon the cellularity of the samples. As a rare population in the BM, the number of $CD45^{low}CD271^+$ cells collected after sorting ranged from a minimum of 999 cells to a maximum of 15,000 cells depending on donor age, technical challenges during defrosting and cell loss due to enrichment. Simultaneously, a maximum of 70,000 cells of the control population of $CD45^+CD271^-$ HLCs were also collected. Their collection was usually accomplished within 45-60 minutes owing to the relatively larger proportion of these cells within the BMA. The number of HLCs collected was thus considerably higher than the number of MSCs collected.

Analysis of flow cytometry data was performed using FlowJo software. Unstained and single antibody stained tubes were used as controls. Gating strategy as described by Cuthbert et al., (207) was followed to record the number of cells and MFI in a selected gate as shown in Figure 4.11. The recorded values of MFI for each surface markers were plotted as a dot plot for young donors (black) and old donors (grey) using GraphPad Prism with median values to illustrate and compare any difference in the MFI.

4.2.5 Gene expression between young and old donors

The general method of qPCR has been described in section 2.5. The experiments were performed in integrated fluid circuit Flex Six^{TM} chip (Fluidigm) which has 2 partitions with 6 compartments each (Figure 4.2). One side of the chip is for placing the TaqMan assays (assays inlet) and the other side is for samples' cDNA (samples inlet). Each compartment can hold up to 12 samples/assays. The compartments are covered using

barrier plugs to prevent any external contamination before being used (Figure 4.3, top panel). The accumulator is the region where the control fluid is added that eventually enables the mobilisation of the assay and the corresponding sample to their reaction depot (Figure 4.3, bottom panel) based on the principles of microfluidic technology.

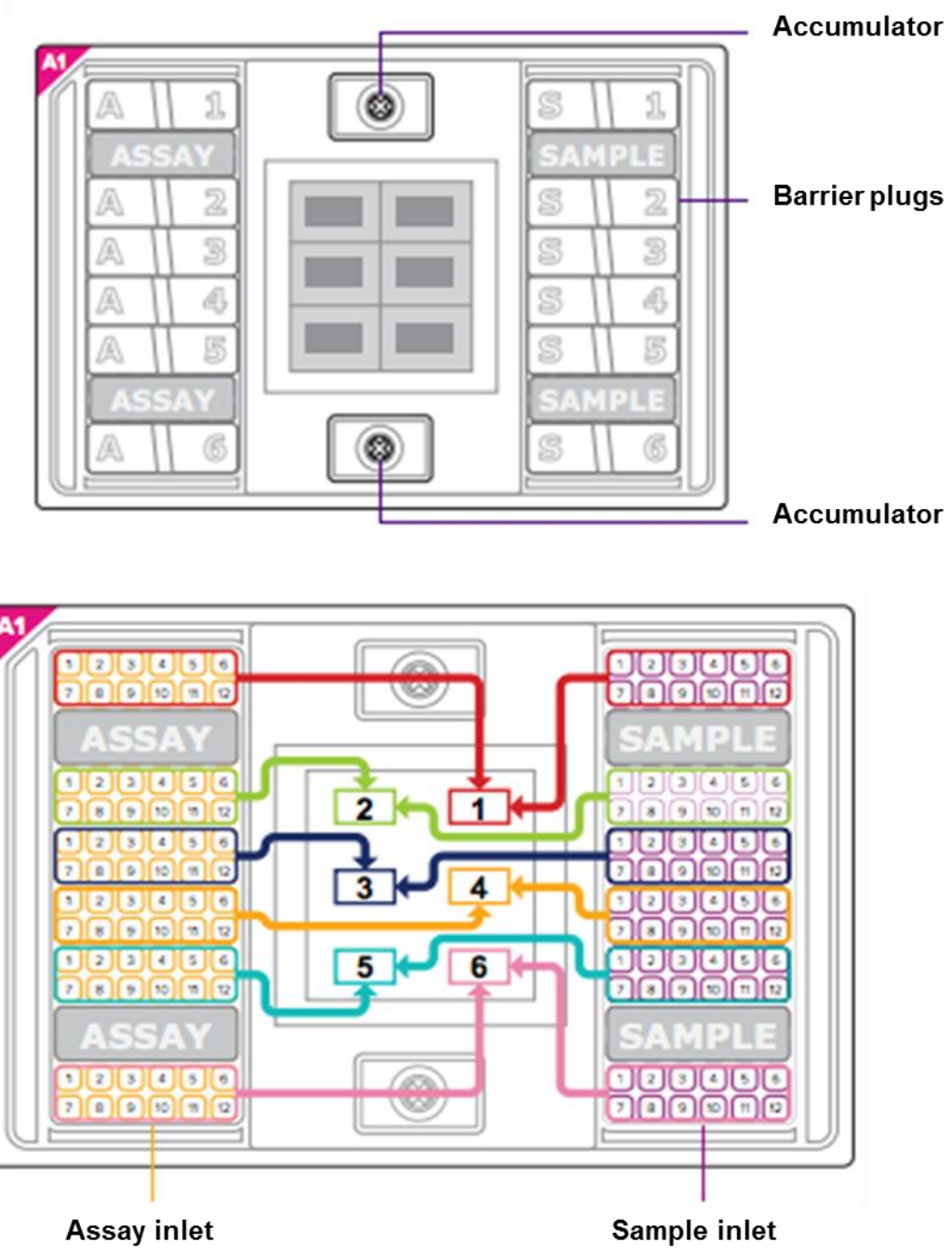

Figure 4.3 Flex Six™ chip showing its different segments as described in the text and shown in manufacturer's protocol

The volumes of reagents, assays and samples used have been described in section 2.5. The TaqMan probes for genes presented in this chapter are shown in Table 4.4

below. This technique was chosen over traditional RT-PCR owing to the lower volume of sample and low concentrations of RNA needed for the experiment. As mentioned earlier, $CD45^{low}CD271^{+}$ is a rare population in the BM and numbers of cells obtained after cell sorting were as low as 999 cells. The collection and elution of RNA was performed to ensure minimal dilution of the RNA content and at the same time to get sufficient RNA quantity for performing experiments. The guidelines provided by the manufacturers were followed in order to carry out the experiment.

For analysis, Fluidigm software was used to set the Ct values and the expression of genes were exported as a Microsoft excel file. In the excel file, first the data was normalised with *HPRT1* as the housekeeping gene to generate the ΔCt values for each gene using the formula [$Ct_{\text{target gene}}$ - $Ct_{\text{housekeeping gene}}$]. Finally, the ΔCt values were converted to 'relative expression' for each gene using the formula [$2^{-\Delta Ct}$] and the values were compared between young and old donor cohorts. To observe trends across the whole donor cohort, cluster analysis was performed using Cluster and Tree view softwares. The software generated a cluster in red (+3 units) indicating genes and samples with highest expression and green (-3 units) indicating genes and samples with comparatively lower expression. Relative expression values closer to the centre of the defined range (-3 to +3) were depicted in black and the values that were below detection level were depicted in grey (shown further below in Figure 4.6).

Table 4.4 List of TaqMan probes used for gene expression study in this chapter

Gene symbol	Genes (full name)	Assay
RUNX2	Runt related transcription factor 2	Hs00231692_m1
SPARC	Secreted protein acidic and rich in cysteine/ Osteonectin	Hs00277762_m1
SFRP1	Secreted frizzled related protein 1	Hs00610060_m1
PPAR-γ	Peroxisome proliferator activated receptor - gamma	Hs01115513_m1
FABP4	Fatty acid binding protein 4	Hs00609791_m1
LepR	Leptin Receptor, encoding CD295 protein	Hs00174492_m1
SOD3	Superoxide dismutase 3	Hs04973910_s1
TFNSF11A/RANK	Receptor activator of nuclear factor kappa-B	Hs00921372_m1
TNFSF11/RANKL	Receptor activator of nuclear factor kappa-B Ligand	Hs01092186_m1
TNFRSF11B/OPG	Osteoprotegerin	Hs00900360_m1
Cx43	Connexin43	Hs00748445_s1
CXCL12	C-X-C motif chemokine 12	Hs00171022_m1
HPRT1	Hypoxanthine phosphoribosyl transferase (housekeeping)	Hs99999909_m1

4.2.6 Statistical analysis

The normal distribution of the data was assessed using the Shapiro-Wilk and Kolmogorov-Smirnov tests for normality. Cluster analysis was used to observe any differences in gene expression across the entire donor cohort. Statistical analysis and graphics were performed using GraphPad Prism software (version 7.0a). As no data were found to be normally distributed, Kruskal-Wallis test with Dunn's correction was used for multiple comparisons, Wilcoxon rank test was performed for paired data and Mann-Whitney test was performed for unpaired data. Spearman test was used to analyse correlations. The results were considered significant at *p* value of <0.05.

4.3 Results

4.3.1 Age-related changes in the number of $CD45^{low}CD271^{+}$ cells

Using the $CD45^{low}CD271^{+}$ phenotype for native BM MSCs, their numbers were quantified for n=30 (10 young, 11 intermediate age, 9 old) donors. Once data collection was completed, the numbers of MSCs were correlated with the age of the donors. The correlation showed an age-related decline in the number of MSCs. However, the decline was non-significant (*p*=0.21).

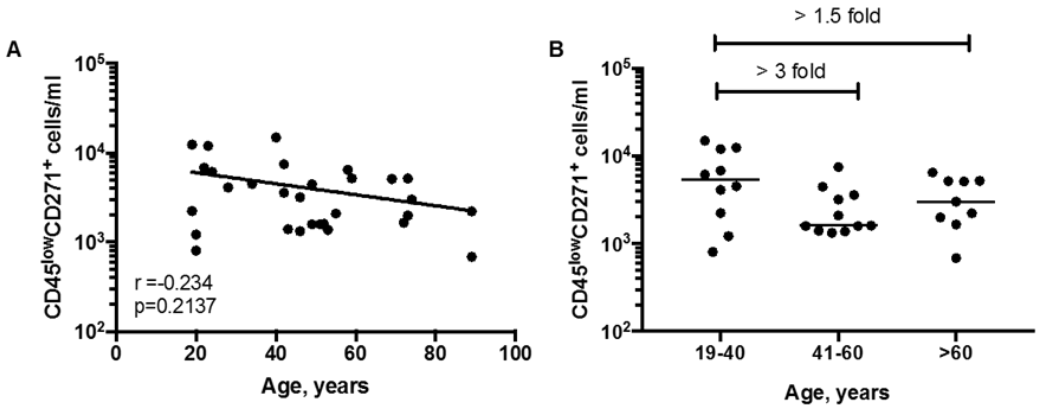

Figure 4.4 Enumeration of MSCs by flow cytometry

(A) Age-related correlation of number of MSCs ($CD45^{low}CD271^{+}$ cells) measured by flow cytometry. (**B)** Age-related change in number of MSCs between the age groups. Data were compared using Kruskal-Wallis with Dunn's correction. Each dot represents an individual donor. Black line in A indicates slope and in B indicate median values for number of cells per milliliter of BM

When the same data was divided into age groups (Figure 4.4B, right panel), a steeper decline was observed in the number of cells per ml of aspirate in intermediate age group (median=1326) as compared to the young donor group (median=5339) than in

the donors of the old age group (median=3006) compared to the young donor group. This was in agreement with the CFU-F findings where the number of MSCs showed a considerable decline commencing form the 5th decade of life. The trend was similar as seen in the number of MSCs detected by the CFU-F assay (as shown previously on Figure 3.4) but the changes in the number of MSCs measured by flow cytometry were found to be non-significant. This could be due to the fact that not all CD45^{low}CD271^{+} cells identified by flow cytometry may attach to plastic and proliferate into a colony of more than 50 cells.

To find how many cells identified by CD45^{low}CD271^{+} phenotype could potentially form a colony, the number of MSCs counted by CFU-F assay and by CD45^{low}CD271^{+} phenotype in donor matched samples were compared (Table 4.5). Across the whole age range, the ratio CD45^{low}CD271^{+}/CFU-F suggested that 1 in every 38 CD45^{low}CD271^{+} cell (median value) detected by flow cytometry would form a colony in our laboratory conditions. However, the ratio of CD45^{low}CD271^{+}/CFU-F was found to be 1 in 26 in young donors, 1 in 27 in the donors of intermediate age and 1 in 73 in old donors (Table 4.5).

Table 4.5 Comparison of number of MSC by CFU-F and flow cytometry

Parameters	19-40 years old	41-60 years old	61-89 years old	Median
CFU-F (colonies/ml, medians)	206 (n=19)	48 (n=18)	41 (n=15)	81
CD45^{low}CD271^{+} (cells/ml, medians)	5339 (n=10)	1326 (n=11)	3006 (n=9)	3094
Ratio (CD45^{low}CD271^{+}/ CFU-F, medians)	25.92	27.63	73.32	38.20

This suggested that the number of colony forming MSCs within CD45^{low}CD271^{+} cell phenotype declines with age. This supported data on colony IDs presented in section 3.3.3 indicating that MSC proliferative capacity declines with age. The next step was to investigate any age-related changes in the transcripts chosen to reflect their multipotential functions.

4.3.2 MSC specificity of the selected genes

Prior to the investigation of any age-related changes in uncultured BM MSCs, the MSC specificity of the chosen transcripts was tested by comparing their expression with

$CD45^{+}CD271^{-}$ HLCs. The list of genes included transcripts for osteogenic differentiation (*RUNX2, SPARC, SFRP-1*), adipogenic differentiation and bone-fat balance (*FABP4, PPAR-γ* and *LepR*), bone remodelling (*RANK, RANKL and OPG*), and cell communication (*CXCL12* and *Cx43*). All of the transcripts were tested for their level of expression in both MSCs and HLCs and a high level of specificity for MSCs was observed for all the molecules in both young and old donors combined, as shown in the cluster analysis in Figure 4.5.

The cluster analysis clearly grouped all the MSCs on the left hand side and the donor matched HLCs on the right hand side (Figure 4.5). Interestingly, within each of the populations of MSCs and HLCs, there was no segregation associated with the donor age. Both, young and old donors were placed randomly by the algorithm of the cluster analysis within the cell subset specific clusters. The grey boxes indicate samples that were below detection. In the figure below, it can be seen that *RANK* was expressed in very few MSCs in MSCs while *OPG, SFRP1* and *SOD3* and were almost absent in MSCs. The fold changes and *p* values indicating the specificity of the individual transcripts for MSCs are shown in Table 4.6.

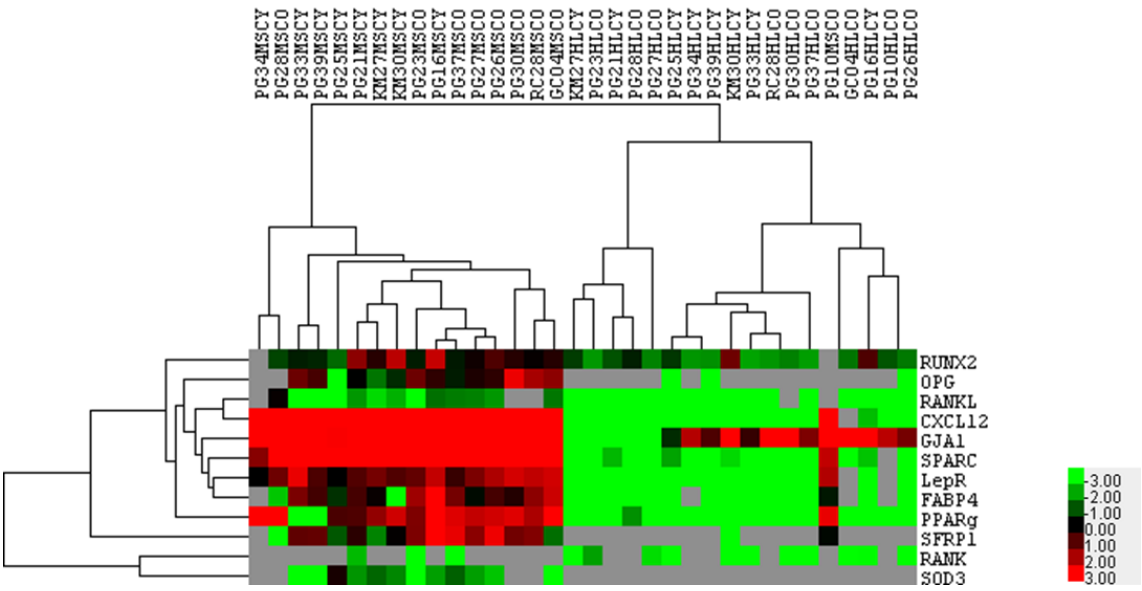

Figure 4.5 Cluster analysis of multipotentiality transcripts in MSCs and HLCs

MSCs form the left side of the cluster and HLCs form the right side of the cluster. Grey squares indicate values that were below detection and black areas indicate values that were closest to the middle of the range (-3 to +3). Dendogram on the top indicate donors and the genes investigated are shown to the right of the cluster.

Table 4.6 Median fold increase in the expression of multipotentiality genes in MSCs compared to HLCs

Transcript	Young donors		Old donors	
	Fold difference	***p* value**	**Fold difference**	***p* value**
RUNX2	2.5	0.0541	3.2	0.0002
SPARC	139.5	0.0002	557	<0.0001
SFRP-1	ND in HLCs	NA	ND in HLCs	NA
PPAR-γ	55.2	0.0019	154.6	<0.0001
FABP4	269.2	0.0012	122.1	<0.0001
LepR	245.3	0.0002	2050	0.0002
SOD3	ND in HLCs	NA	ND in HLCs	NA
RANKL	103.8	0.0003	24.2	0.0047
RANK	1.6	NS	10.8	NS
OPG	ND in HLCs	NA	ND in HLCs	NA
Cx43	21.3	0.0047	11.3	0.0005
CXCL12	63428	0.0002	56182.2	<0.0001

Gene expression levels were measured relative to *HPRT1*. MSCs: Mesenchymal stromal cells, HLCs: Hematopoietic lineage cells, ND: not detected, NA: Not Applicable, NS: Non significant, Mann-Whitney U test

4.3.3 Age-related differences in osteogenic and adipogenic genes in MSCs

After establishing MSC specificity of the selected transcripts, the next investigation involved comparing the transcripts levels reflecting multipotentiality of MSCs between young (19-40 years old) and old (59-89 years old) age groups. The median fold differences for each of the transcripts between young and old groups are shown in Table 4.7 below. A minimum of n=8 donors in young and old group were tested for each transcript.

Table 4.7 Median expression levels and fold differences in selected genes in $CD45^{low}CD271^{+}$MSCs and $CD45^{+}CD271^{-}$HLCs between young and old donor groups

Population	Transcript	Medians in YD	Medians in OD	Fold difference (O/Y)	*p* value
MSCs	*RUNX2*	1.40	1.10	0.79	NS
	SPARC	17.41	20.51	1.18	NS
	SFRP1	1.41	2.76	1.96	NS
	PPAR-γ	2.60	6.20	2.38	NS
	FABP4	1.71	1.76	1.03	NS
	LepR	1.93	4.12	2.13	NS
	SOD3	0.58	0.29	0.50	NS
	RANKL	0.16	0.35	2.19	NS
	OPG	0.99	2.43	2.45	NS
	RANK	1.36	0.76	0.56	NS
	CXCL12	1051.00	1554.00	1.48	NS
	Cx43	36.64	31.54	0.86	NS
HLCs	*RUNX2*	0.56	0.34	0.61	NS
	SPARC	0.12	0.03	0.25	0.0281
	SFRP1	ND	ND	NA	NA
	FABP4	0.006	0.014	2.33	NS
	PPAR-γ	0.05	0.04	0.85	NS
	LepR	0.0080	0.002	0.25	NS
	SOD3	ND	ND	NA	NA
	RANKL	0.0016	0.0146	9.13	0.0047
	OPG	ND	ND	NA	NA
	RANK	0.10	0.07	0.71	NS
	CXCL12	0.02	0.03	1.69	NS
	Cx43	1.72	2.78	1.62	NS

Gene expression levels were measured relative to *HPRT1*. MSCs: Mesenchymal stromal cells, HLCs: Hematopoietic lineage cells, YD: Young donors, OD: Old donors, ND: not detected, NA: Not Applicable, NS: Non significant; Mann-Whitney U test

Relative expression of *RUNX2, SPARC* and *SFRP-1* in MSCs showed no statistical difference between young and old donor groups (Figure 4.6). However, while the expression of *RUNX2* and *SPARC* remained largely the same based on the median values, *SFRP-1* was expressed nearly 2-fold higher in old donors MSCs compared to young donors MSCs (Table 4.7). Taken together, transcripts for osteogenic

differentiation in BM MSCs from old donors did not show significant differences in their expression compared to young donor MSCs.

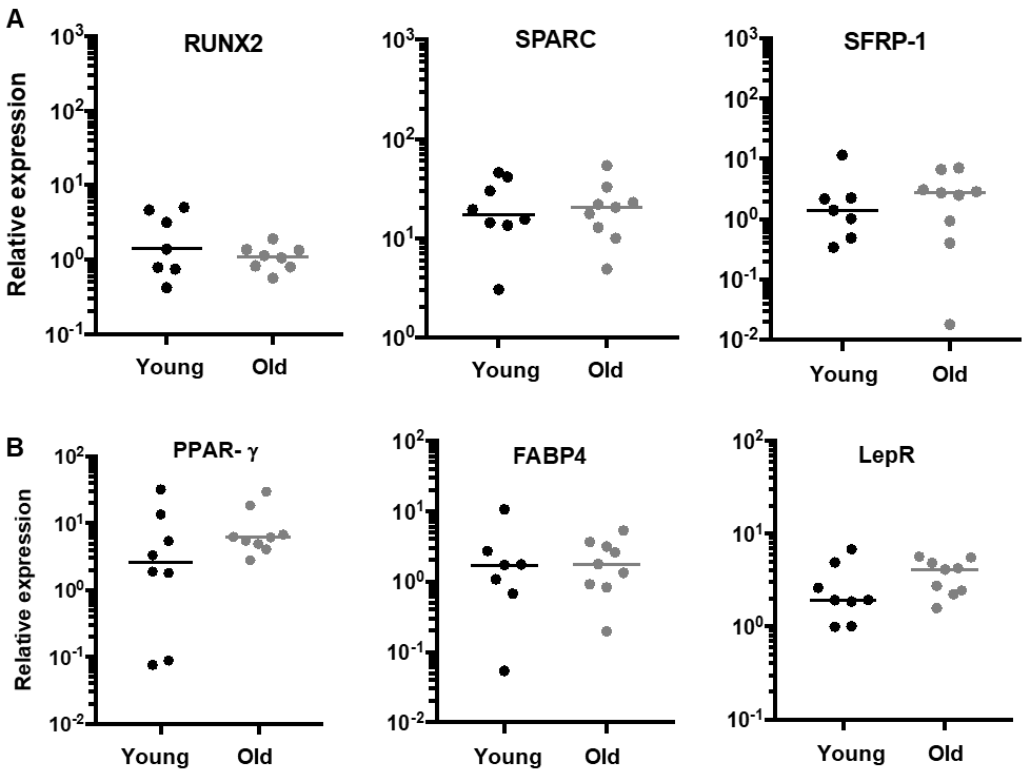

Figure 4.6 Expression of osteogenic and adipogenic transcripts in MSCs from young and old donors

(A) Expression of *RUNX2*, *SPARC* and *SFRP-1* in MSCs. **(B)** Expression of *PPAR-γ*, *FABP4* and *LepR* in MSCs. Young and old donors are indicated in black and grey dots, respectively. Horizontal lines indicate median values.

For the selected adipogenic transcripts, *PPAR-γ* showed 2-fold higher expression in old donors but *FABP4* did not show any age-related differences (Figure 4.6 and Table 4.7). The level of *LepR* also was 2-fold higher in old donors however the differences failed to reach statistical significance (Figure 4.6 and Table 4.7). Overall, data for selected adipogenic molecules in uncultured BM MSCs indicated a potential trend for increase in some transcripts which merits further investigations in a larger cohort of donors. Interestingly, small increases in *PPARγ* and *LepR* were not mirrored by similar increases in *FABP4*.

4.3.4 Resistance to oxidative stress

Resistance to MSC to oxidative stress was measured by quantification of basal intracellular ROS in $CD45^{low}CD271^{+}$ cells by flow cytometry as well as ROS generated by exposing MSCs to TBHP using methods previously established in our group (315). It was hypothesised that the levels of basal ROS and ROS generated using TBHP would display an age-related increase in MSCs. To potentially explain any differences in ROS, the expression of the anti-oxidant enzyme SOD3 was also quantified by qPCR. *SOD3* expression levels were expected to display an age-related decline that could explain the increase in ROS with advancing age.

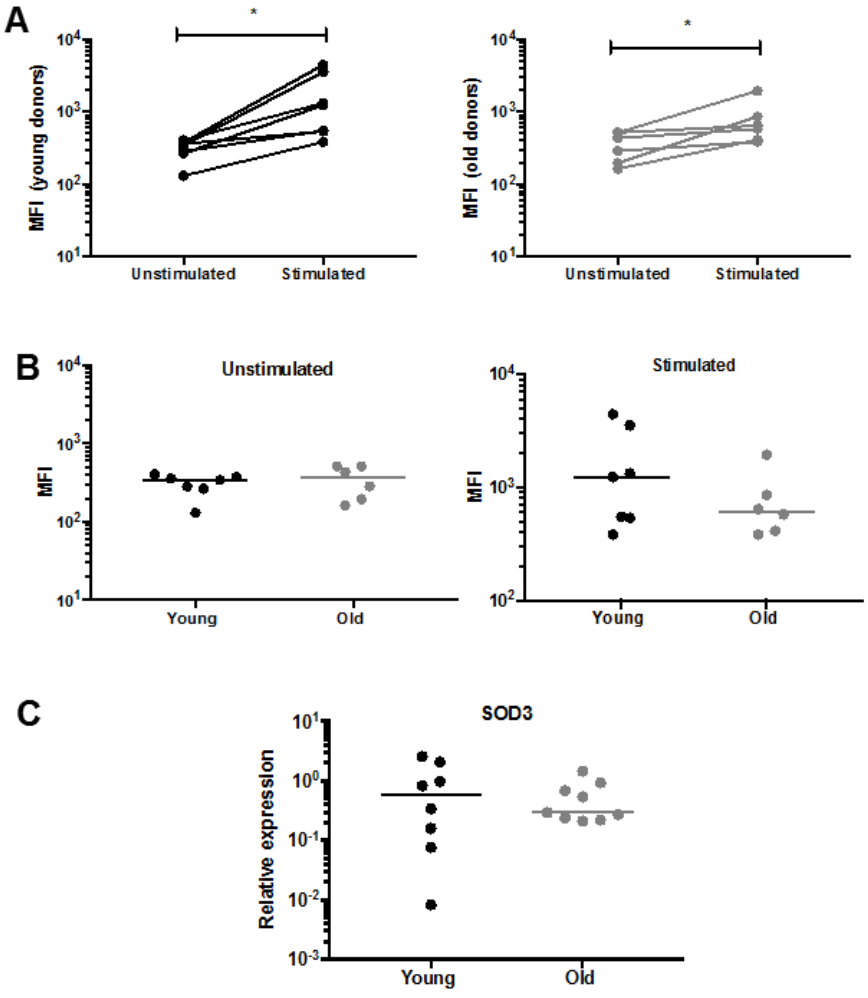

Figure 4.7 Measurement of ROS and anti-oxidant enzyme SOD3 gene in MSCs from young and old donors

(A) Increase in ROS before and after stimulation with TBHP measured by CellROX median fluorescent intensities (MFI) in young donors (left) and old donors (right), Wilcoxon rank sum test. **(B)** Comparison of ROS levels between young and old donors before stimulation with TBHP (left) and after stimulation (right). **(C)** Comparison of expression of anti-oxidant enzyme *SOD3* in young and old donors by gene expression. Young and old donors are indicated in black and grey dots, respectively. Wilcoxon rank sum test, *$p<0.05$.

Comparing stimulated and unstimulated levels of ROS in young and old donor MSCs (Figure 4.7A) showed a significant increase in stimulated state indicating that the exposure to TBHP led to the production of more free oxygen radicals that were detected as higher levels of CellROX. After showing that ROS can be measured by this method in MSCs before and after stimulation, basal levels of ROS were first compared between young and old donors. The basal levels of ROS is indicative of the inherent mitochondrial activity in cells and showed no difference in old donor MSCs compared to young donor MSCs (Figure 4.7B, left panel). When the level of ROS was quantified in the stimulated state, indicating a cells ability to combat extreme oxidative stress, there was similarly no statistically significant difference (Figure 4.7B, right). However, there was a 2-fold decline in the level of stimulated ROS when old donor MSCs were compared to young donor MSCs, based on median values (Figure 4.7B, right panel) and the expected increase in ROs was not observed.

SOD3 the anti-oxidant enzyme is the first line of defence in presence of harmful ROS and is known to be specifically secreted by MSCs. To investigate if this gene declined with advancing age, its expression was compared between young and old donor MSCs using qPCR. Here again, the difference was not statistically significant but the level of SOD3 transcript was 2-fold lower in old donor MSCs (Figure 4.7C).

4.3.5 Age-related differences in bone remodelling and cellular interaction genes in MSCs

The transcripts associated with bone remodelling also showed no statistically significant differences between young and old donor MSCs. As seen from the cluster analysis (Figure 4.5) *RANK* expression was specific for HLCs and barely detected in MSCs. This was expected, as *RANK* is expressed primarily on T cells (section 1.1.4) (63, 316). In contrast to *RANK*, MSCs from old donors showed an over 2-fold increase in the expression of both, *RANKL* and *OPG*, as compared to young donors (Table 4.6). Of note, *RANKL* was detected in only 6 out of the 8 donors MSCs and *OPG* was detected in 7 out of 8 donors MSCs.

Overall, even though statistical significance was not achieved, there was a trend indicating an age-related increase in *RANKL* and *OPG*, in uncultured BM MSCs from old donors (Figure 4.8).

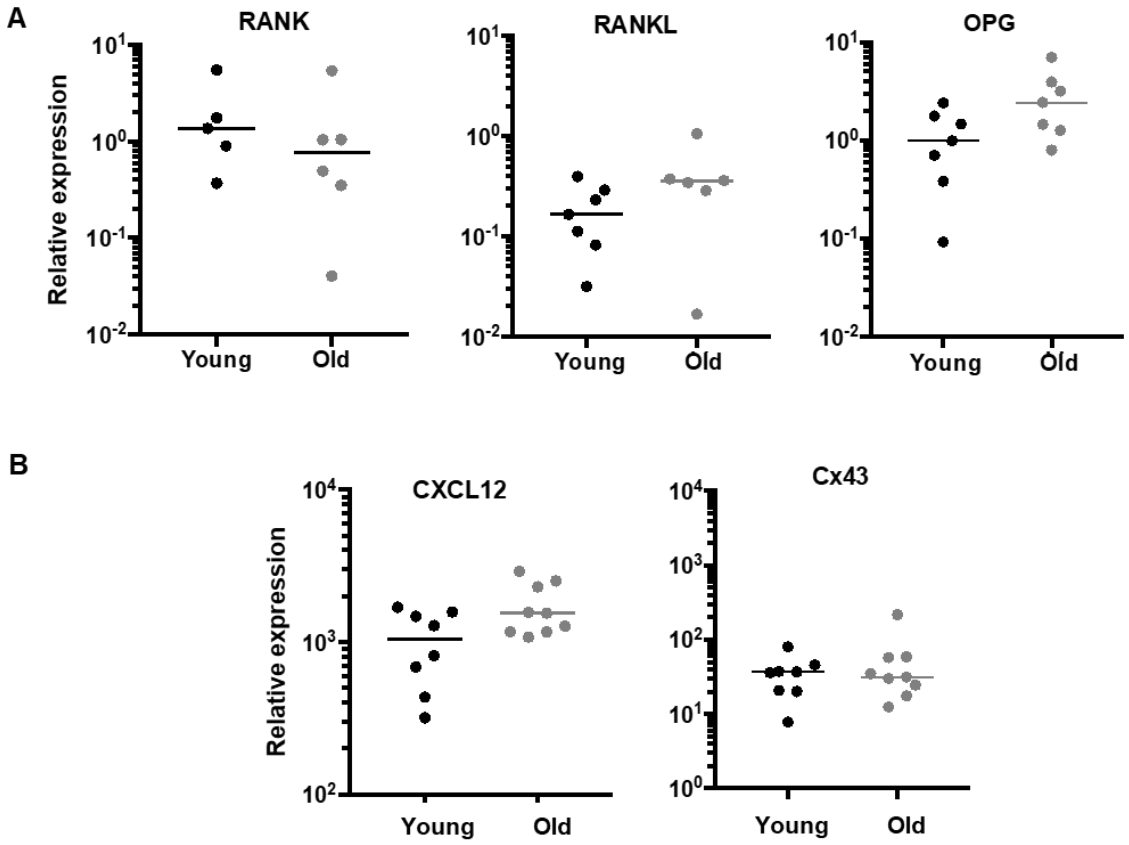

Figure 4.8 Expression of transcripts associated with bone remodelling and cellular interactions in MSCs from young and old donors

(A) Expression of *RANK*, *RANKL* and *OPG* **(B)** Expression of *CXCL12* and *Cx43*. Young and old donors are shown in black are grey dots, respectively. Horizontal lines across the data indicate median values.

The transcripts associated with cellular interactions were next investigated. *CXCL12* is involved in chemotaxis of cells within the BM (section 4.1.1.5), and its expression level was among the highest in $CD45^{low}CD271^{+}$ MSCs in comparison to other transcripts as seen in the cluster (Figure 4.5) suggesting its presence in high quantities in uncultured BM MSCs. No difference in the level of its expression was found between young and old donors (Figure 4.8B, left panel). *Cx43* is involved in intercellular interactions in cells within BM (section 4.1.1.5) and also showed no age-related differences in its gene expression levels in $CD45^{low}CD271^{+}$ MSCs.

4.3.6 Age-related differences in selected gene transcripts in HLCs

The HLCs were sorted as $CD45^{+}CD271^{-}$cells and included the lymphoid and myeloid cell lineages (188, 222). In this project, donor matched $CD45^{+}CD271^{-}$ cells served as a control group for uncultured BM MSCs. As shown in Figure 4.5, the specificity of the

chosen transcripts was very high for MSCs, with the exception of *RANK*. However, with prior knowledge, that most of these transcripts are also expressed by other cells (even if at the low levels, section 4.1.1) their gene expression was next investigated in HLCs and compared between young and old donor groups.

For osteogenic transcripts, while *RUNX2* and *SPARC* were detected in low levels in HLCs, the expression of *SFRP1* was below detection in HLCs further illustrating selectivity of these genes for MSCs. There was 1.6-fold age-related decline in the expression of *RUNX2*, however not statistically significant, and *SPARC* (4-fold, p=0.0281) in older donors HLCs (Figure 4.9A)

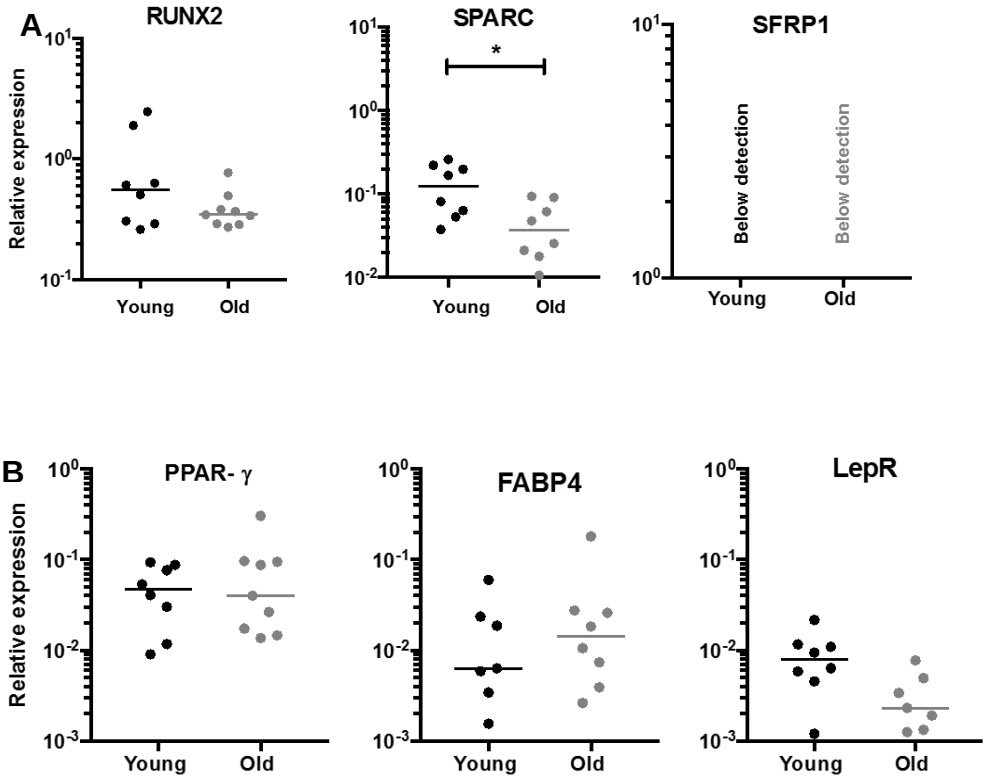

Figure 4.9 Expression of genes associated with osteogenic and adipogenic differentiation in HLCs from young and old donors

(A) Expression of *RUNX2, SPARC* and *SFRP1* **(B)** expression of *PPAR-γ, FABP4* and *LepR*. Horizontal lines across the data indicate medians. Young and old donors are shown in black are grey dots, respectively. p< 0.05*, Mann-Whitney U test.

The transcripts related to adipogenic differentiation and bone-fat balance were next analysed. All three transcripts were detected in higher levels in MSCs compared to donor matched HLCs, as seen in cluster (Figure 4.5). *PPAR-γ* did not show any age-related trends in the HLCs. *FABP4* exhibited a slight trend towards an age-related

increase, whereas *LepR* displayed considerable 4-fold age-related decline in HLCs, which narrowly missed statistical significance (Table 4.6, p=0.0541).

Bone remodelling transcripts were next scrutinised (Figure 4.10A). *RANKL* showed a significant 9-fold increase (p=0.0047) in old donor HLCs. As mentioned, *OPG* was not detected in HLCs (confirming it's high-level specificity for MSCs). The last group of genes to be investigated in HLCs were those involved in cellular interactions. Both the genes, *CXCL12* and *Cx43* were detected in young and old donor HLCs but showed no difference in expression (Figure 4.10B).

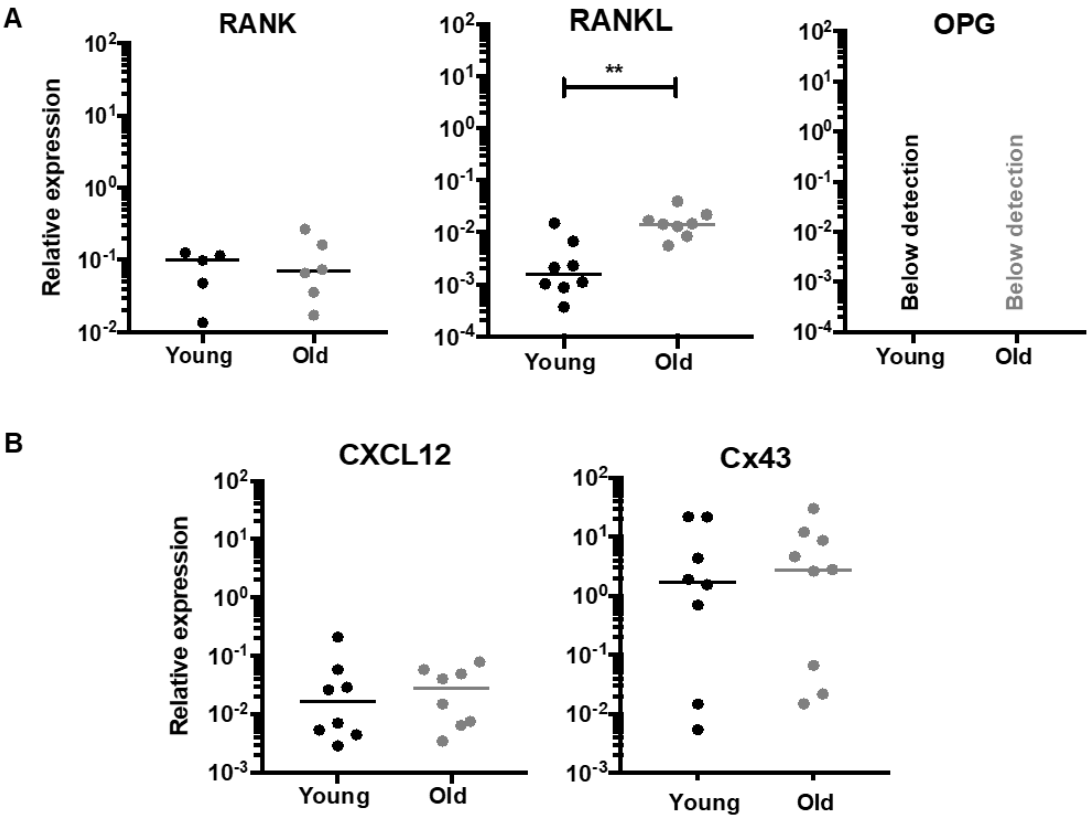

Figure 4.10 Expression of transcripts associated with bone remodelling and cellular interactions in HLCs from young and old donors

(A) Expression of *RANK, RANKL* and *OPG*. **(B)** Expression of *CXCL12* and *Cx43*. Horizontal lines across the data indicate medians. Young and old donors are shown in black are grey dots, respectively. $p < 0.01$**, Mann-Whitney U test.

Similar to the observations in MSCs, the expression of all transcripts in HLCs exhibited large donor variation within the same age group. Altogether, there were no statistically significant differences observed in transcript expression levels in young and old donor HLCs for most transcripts, with the exception of *SPARC* which decreased and *RANKL* which increased in old donors. This was in contrast to MSCs, in which only a trend for increase in *PPAR-γ, LepR, RANKL* and *OPG* was found in older donors.

Two of these transcripts were also investigated at the protein level (LepR/CD295 and Cx43) along with other potential MSC age-related surface molecules CD106 and CD146 (section 4.1.2). The next section includes the results obtained from the investigation of these surface markers in uncultured BM MSCs and control HLCs.

4.3.7 Expression of the selected surface markers on MSCs

Frozen-thawed nucleated cells from young and old donors (Appendix 1, Table 2) were processed for native MSC isolation and characterisation using the $CD45^{low}CD271^{+}$ phenotype. As a control population, $CD45^{+}CD271^{-}$ cell fraction (HLCs) that contained the lymphoid and myeloid lineage cells was also analysed (188). First, the specificity of the selected surface markers for MSCs ($CD45^{low}CD271^{+}$ cells) was tested. For the measurement of the surface marker expression the median fluorescent intensity (MFI) was used. Representative histograms from each donor group are shown in Figure 4.11.

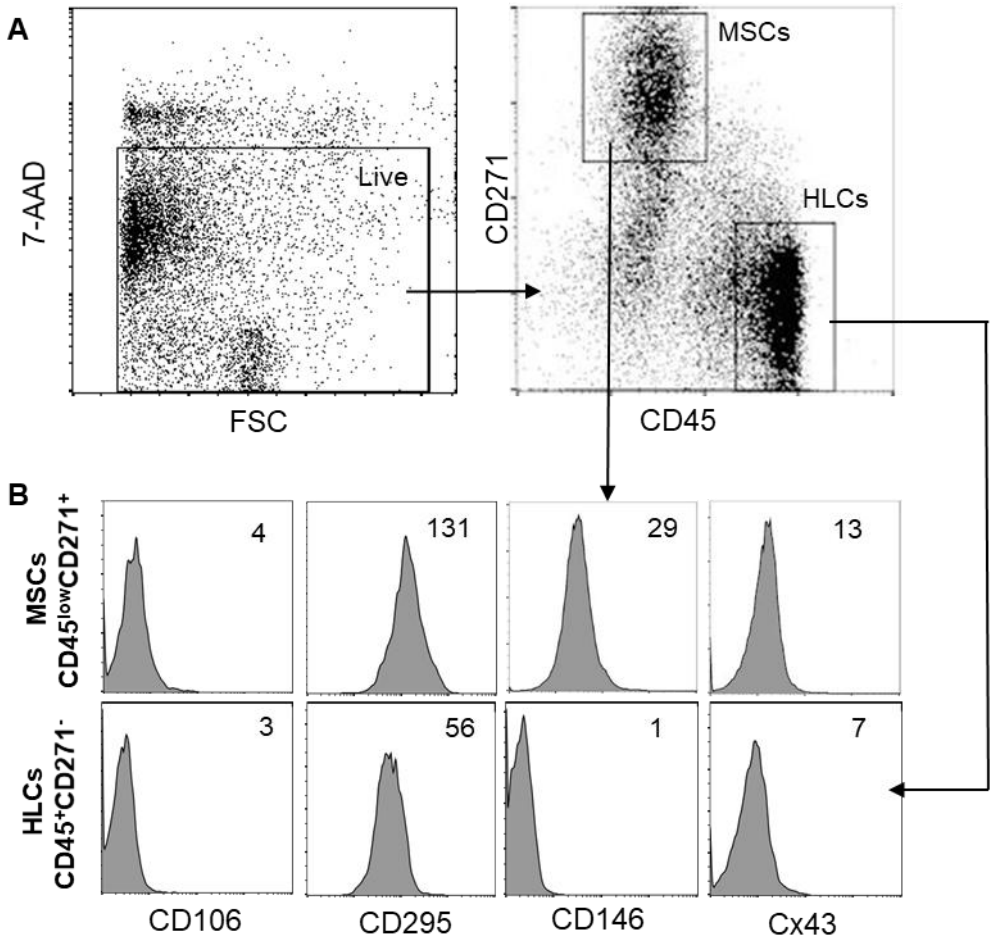

Figure 4.11 Quantification of surface marker expression in BM MSCs and in HLCs

(A) Gating strategy for identification of live cells based on 7-AAD negativity followed by CD271 and CD45 gating for the selection of populations of interest (MSC and HLCs) **(B)** Histograms for individual surface markers in MSCs and HLCs from representative donor. Numbers in the top-right corner indicate MFI.

The MFI of each surface marker was first compared between MSCs and HLCs. Figure 4.11 below shows the expression of each surface molecule in donor matched MSCs and HLCs from all young and old donors, combined.

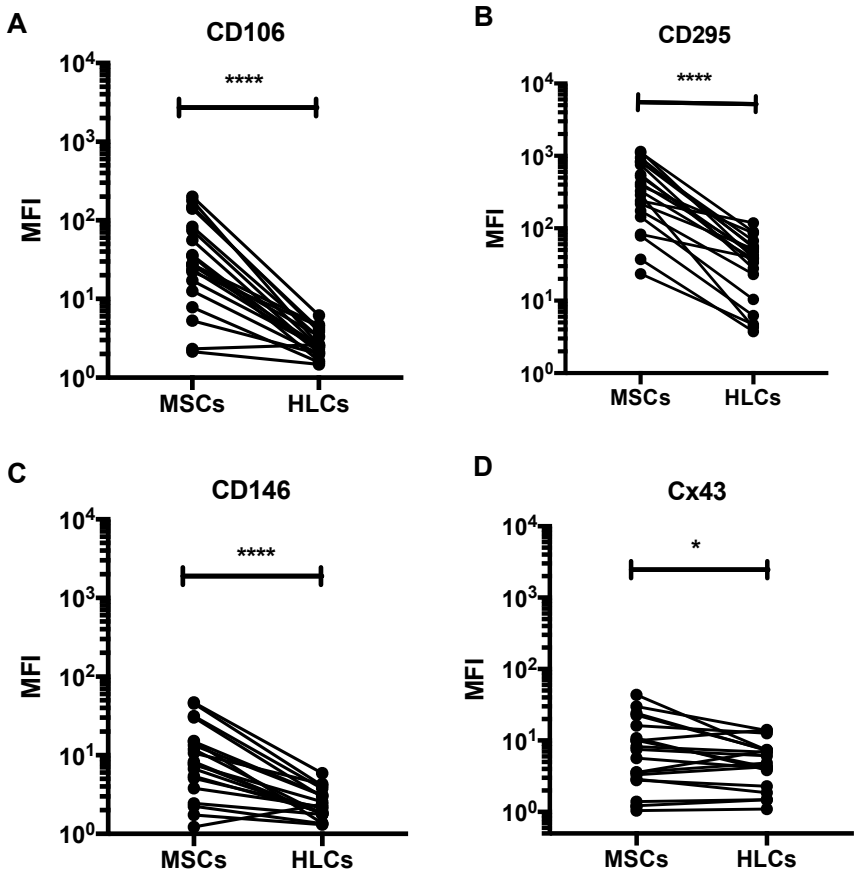

Figure 4.12 Expression of candidate age-specific surface markers on MSCs and HLCs from all donors combined

(A) Expression of CD106 **(B)** Expression of CD295 **(C)** Expression of CD146 **(D)** Expression of Cx43. Each panel shows the expression of the respective marker in MSCs and HLCs in donor-matched cells. Each dot represents an individual donor. **p*<0.05 and *****p*<0.0001, Wilcoxon rank sum test

All surface markers were expressed in significantly higher levels in MSCs compared to HLCs from all donors. The differences in CD106 (*p*<0.0001), CD295 (*p*<0.0001) and CD146 (*p*<0.0001) were highly significant indicating very high specificity for MSCs. CD106 and CD295 showed the highest specificity in terms of fold differences (10-fold each, Figure 4.12A and B), followed by CD146 (nearly 4-fold, Figure 4.12C). While Cx43 showed the lowest fold differences between expression in MSCs and HLCs (less than 2-fold) and the *p* value (*p*=0.0266), but remained significantly specific for MSCs, nevertheless (Figure 4.12D).

The expression of CD295 in MSCs was the highest (ranging between 10^1 and 10^3 MFI) while the expression of CD146 and Cx43 were the lowest, both of which were below 10^2 MFI. All antibodies were used at the manufacturer's recommended concentrations. CD295 and Cx43 specificity at the protein level was also reflected at

the gene level (Figure 4.5 and Figure 4.11, Table 4.6) suggesting consistency in techniques used and results obtained.

4.3.8 Age-related differences in selected surface markers in MSCs

Expression of all four surface markers was then compared between young and old donors, in MSCs and then HLCs separately. No difference in the expression levels in any of the markers in MSCs was found between young and old donors (Figure 4.13). Large donor variation was observed in the expression of these markers within each donor group.

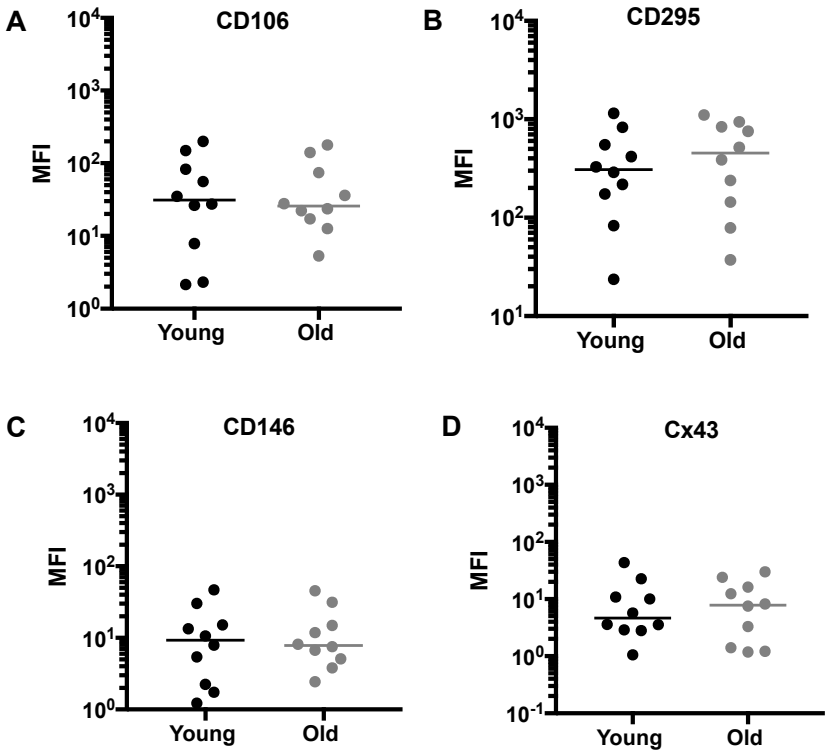

Figure 4.13 Expression of candidate age-related surface markers in MSCs from young and old donors

(A) Expression of CD106 **(B)** Expression of CD295 **(C)** Expression of CD146 **(D)** Expression of Cx43. Young and old donors are represented by black and grey dots, respectively. Each dot represents an individual donor. Horizontal lines across the data represent median values.

Neither of the markers demonstrated any trends when compared between young and old donor MSCs suggesting no age-related difference in the expression of these markers in uncultured BM MSC. The expression of these markers were then investigated in young and old donor HLCs.

4.3.9 Age-related differences in selected surface markers in HLCs

Expression of the four surface markers in HLCs also displayed no age-related difference in their expression as shown in figure 4.14 below.

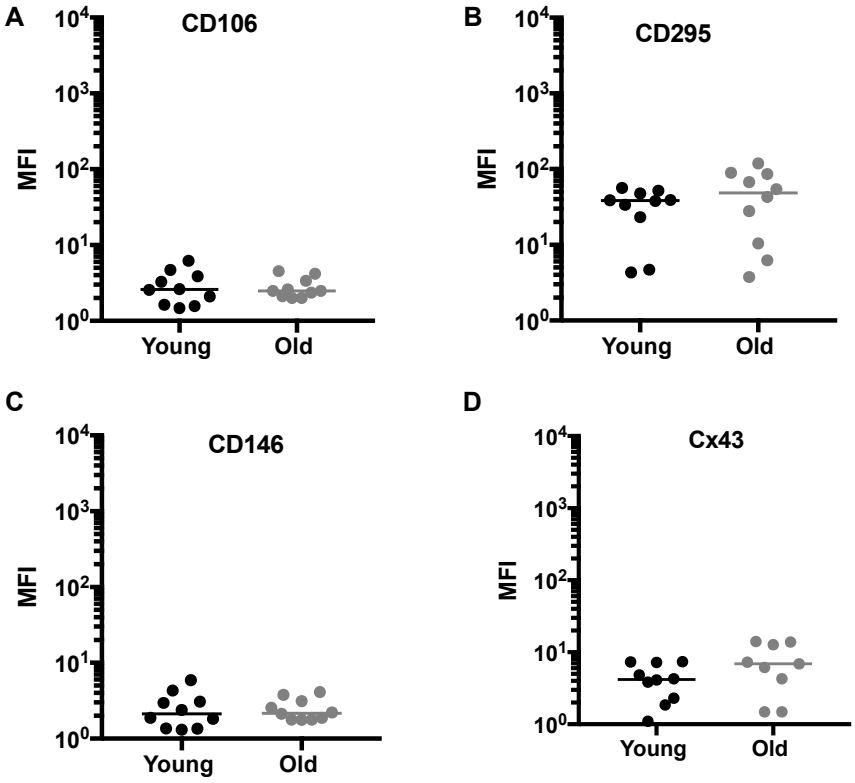

Figure 4.14 Expression of candidate age-related surface markers in MSCs from young and old donors

(A) Expression of CD106 **(B)** Expression of CD295 **(C)** Expression of CD146 **(D)** Expression of Cx43. Young and old donors are represented by black and grey dots, respectively. Each dot represents an individual donor. Horizontal lines across the data represent median values.

Even in HLCs, the surface markers showed no differences in the level of expression in young and old donors. Overall, section 4.3.7 to 4.3.9 displayed significantly higher expression of surface markers in MSCs than in HLCs. With respect to age-related differences, the expression of these age-specific markers displayed no age-related differences or trends in either of the cell types (MSCs and in HLCs).

4.4 Discussion

In this chapter, an age-related decline in the number of $CD45^{low}CD271^{+}$ native BM MSCs using flow cytometry was established confirming the results from previous chapter based on the CFU-F assay. Gene expression of all the selected molecules (apart from *RANK*) showed very high specificity for MSCs as compared to HLCs. The osteogenic transcripts' expression showed no age-related trends in MSCs. Adipogenic transcripts also did not reach significance between young and old groups but displayed a non-significant 2-fold increase in the expression *LepR* and *PPAR-*γ in the old donor group. Interestingly, unlike in the MSCs, *LepR* displayed a significant 2-fold decrease in the HLCs. Quantification of ROS and anti-oxidant enzyme *SOD3* gene expression also did not show any statistically significant difference in old donors, however, the level of *SOD3* detected in old donors as well as the stimulated level of ROS detected in old donors, declined by 2-folds. Transcripts for cellular interactions also did not show any age-related changes in the MSCs or the HLCs. Similarly, surface marker expression exhibited no difference between young and old donor groups and each surface marker was significantly higher-expressed on MSCs compared to HLCs.

Flow cytometry data revealed a trend for native BM MSC to decline with age. However, the decline was not as significant as indicated by the CFU-F assay. Flow cytometry analysis depends upon the gating strategy which can be subjective. However, the analysis and gating strategy used in this study were the same as previously published by our group (111, 234). As shown in previous studies (111, 207), not every $CD45^{low}CD271^{+}$ cell forms a colony in standard CFU-F conditions, and consistent with these publications, flow cytometry data revealed higher number of MSCs per ml of BMA compared to CFU-F assay. The age-related decline was lower as measured by flow cytometry possibly because it identifies all the $CD45^{low}CD271^{+}$ cells as MSCs, irrespective of their ability to proliferate into a colony from a single cell; while a colony having less than 50 cells was not scored as a colony in the CFU-F assay. Overall, the data on BM MSC enumeration by flow cytometry in this study confirmed their age-related decline as measured by CFU-F assay. This has not been shown before when other surface markers for uncultured MSCs were used (237).

Use of $CD45^{low}CD271^{+}$ cells for enumerating MSCs has been established on fresh BM cells before and used by our group (111, 207) and the data was compared to the total colony area showing a positive correlation. The studies however did not look into age-related differences in MSC number by both CFU-F and flow cytometry. Recently, our group also measured $CD45^{low}CD271^{+}$ cells by flow cytometry and CFU-F assay and observed an age-related decline in females but not in males by both assays (111).

However, the study was aimed at comparison of two flow cytometry techniques for evaluation of $CD45^{low}CD271^{+}$ MSCs and was not specifically focussed on age- or gender-related differences. Furthermore, it used a smaller cohort of donors ranging from 22-80 years old. In agreement with data presented in this chapter, recently, Josephson and co-workers also showed a decline in $CD45^{low}CD271^{+}$ cells by flow cytometry when compared across the entire age range (24-89 years old) as well as between young (<50 years) and old (>50) donors (146). However, their group definitions were different and they were not able to show the bigger drop in MSCs occurring from early 40s rather than later in life (from 60s).

A group of transcripts was next chosen to explore if various functions in MSCs may be affected by donor age. Many of the molecules have been previously shown to be specific for MSC by our group (188). In fact, an age-related decline in *LepR and SFRP1* was observed in our previous study using a cohort of patients with ages ranging from 2 to 74 years old. This was not seen in this study with uncultured BM MSCs, most likely due to different age-range of this study that did not include paediatric donors, as in the former.

In this project, there were no or non-significant age-related trends in the transcripts related to osteogenic and adipogenic differentiation. Based on previous studies, the general consensus has been that there is an age-related decline in the osteogenic potential of MSCs paralleled to an increase in their adipogenic potential (Table 4.2). Studies that supported this concept have been based on *in vivo* assays by examining trabecular bone volume, bone mineral density (BMD), total bone volume and marrow adipocyte content. These parameters have been discussed in the introduction in section 1.1.4. These studies provide robust *in vivo* data supporting the process of 'age-related bone loss' and increased bias towards marrow adipogenicity, and formed the foundation of our initial hypothesis for reduced osteogenic and increased adipogenic commitment of uncultured BM MSCs. As these cells were not expanded in culture, they better represent the *in vivo* MSCs in BM, making the data novel and reliable.

However, *in vitro* data relating to both donor-related and passage-related MSC ageing in terms of their osteogenesis remained controversial. Yang and co-workers in 2018 compared the differentiation potential of BM MSCs between passage 4 and passage 8 (123). They tested the differentiation potential using both, *in vitro* differentiation assay and by using gene expression of classic osteogenic and adipogenic markers (*RUNX2* and *PPAR-γ,* respectively). They found that cells from passage 8 were able to maintain the adipogenic potential, but the osteogenic potential was higher in passage 4 MSCs. It

was concluded that the process of adipogenic differentiation is 'better preserved' in BM MSCs than osteogenic differentiation and the growth medium had a strong impact on their differentiation potential, further highlighting the limitation of using cultured MSCs in MSC ageing studies. The present data using a limited number of transcripts have not provided strong evidence for any bias in MSCs, however interesting trends in *PPAR-γ* in particular were observed that supported our original hypothesis. Similar investigation of osteogenic and adipogenic molecules at the protein level using with more donors would help to understand the age-related shift towards adipogenic differentiation in bone *in vivo.*

Another molecule that plays a role in osteogenic-adipogenic balance in the BM is *LepR* or CD295. In this project, LepR was tested at both the transcript and protein levels. By both techniques, LepR showed a trend for a two-fold increase in MSCs from old donors. Before discussing the biological relevance of this data, it is important to note that CD295 was proven to be the most specific of the all tested molecules for human $CD45^{low}CD271^{+}$ MSCs (10-fold higher in MFI compared to control HLCs). Yue and colleagues published a paper on LepR+ BM MSCs in mice showing that *LepR* is not needed for the maintenance of MSCs but acts on MSCs to inhibit osteogenesis and promote adipogenesis (262). They also found that the mice fed with a high fat diet not only had increased levels of plasma Leptin, but their bone-lineage progenitors formed significantly lower CFU-Fs in comparison to the mice that were not fed the high fat diet. This indicated that high serum Leptin may directly inhibit MSC colony formation ability. This was the first study that had connected intake of high fat diet with the impact of LepR+ cells on the regeneration ability of these cells. However, as only adult mice (6mo) were used, no correlations with donor age could be made in this study.

In 2009, Laschober and co-workers studied the expression of CD295 by flow cytometry in cultured BM MSC obtained from donors aged between 7-78 years old without any age grouping. Their findings suggested that CD295 was up-regulated in the 'dying cells' marked by Annexin V (marker for cell death by apoptosis) and that it was found to be higher in old donor BM MSC cultures. They suggested that CD295 is an indicator/marker for apoptotic cells (which are higher in old donor cultures) in BM MSCs (126).

The data from the present study showed a tendency of both, the receptor expression and the gene encoding for the receptor to increase in BM MSCs in older donors, but the differences failed to reach statistical significance. A similar trend was observed for the adipogenic molecule *PPAR-γ.* Should this be statistically confirmed in a larger

cohort study, CD295 expression should be further tested for predicting the adipogenic fate of BM MSCs *in vivo*. Even though the change was not significant, it must be noted that these experiments were performed with nearly no laboratory manipulation of donor MSCs precluding any artefacts due to *in vitro* culture conditions and thus better reflecting MSC age-related changes *in vivo*. The present findings are interesting and complement previous studies showing that its ligand, the hormone Leptin itself, shows an age-related increase in its blood levels (317). This may lead to an overall enhancement of Leptin signalling in MSCs and negatively affect their colony-formation potential.

Knowledge in the increased adipogenic bias of BM MSCs *in vivo* and its potential link to age-related changes of BM MSCs is vital for developing new treatment for diabetes, obesity, osteoarthritis and osteoporosis, the risks of which are much higher in the aged population. The risk of obesity is higher for new-borns that are born to obese mothers and the MSCs from these babies show a greater potential to become adipogenic (318). While the exact mechanism for MSC fate decisions remains to be discovered, literature from past and present projects shed new light on the topic. Future investigations should include obese and non-obese donor cohorts, which quantify both circulating Leptin levels along with *LepR* from BM MSCs at the gene and protein level. These future studies will help to dissect the age-related and diet/obesity-related changes in bone homeostasis due to Leptin and its impact of age-related skeletal disease like osteoarthritis.

As previously stated (section 4.1.1.3), several studies have reported increased ROS during adipogenic differentiation of MSCs as compared to osteogenic differentiation. Accumulation of ROS is also one of the main features of aged cells (281). Based on these considerations, this project investigated the levels of ROS and the expression of anti-oxidant enzyme SOD3 in BM MSCs in young and old donors. *SOD3* transcript was highly specific to MSCs and was not detected in HLCs (Table 4.6). Therefore we expected an age-related increase in the level of ROS in old donors, parallel to a decrease in *SOD3*.

Even though the results were not statistically significant, the decline in the induced levels of ROS in old donor MSCs as compared to young donor MSCs, was unexpected. A 2-fold decline was observed in the expression of *SOD3* in old donor MSCs but it did not reach statistical significance. Recently, Agrahari et al*.,* found that MSCs engineered to overexpress SOD3 were better adapted for survival under serum starved conditions, possibly due to enhanced autophagy (319). Culture expanded

MSCs from old donors (96) as well as long-term cultured BM MSCs have shown to possess lower levels of anti-oxidants such as SODs, as well as increased vulnerability to oxidative stress (320, 321). However, measurement of active ROS and anti-oxidant enzyme expression in uncultured BM MSCs has not been carried out before, making the data from this chapter very novel.

Overall, with respect to the idea that MSCs display an age-related bias towards adipogenic differentiation and there is increased ROS in older donors, this chapter suggests no or only subtle differences in uncultured BM MSCs. While larger donor group numbers could potentially display statistically significant results, the data suggests that at the transcript level, BM MSCs are not necessarily or strongly biased towards adipogenic differentiation. An additional sample set previously treated in hypoxic conditions or with anti-oxidant before inducing oxidative stress in BM MSCs would help explain the decline in both ROS levels and *SOD3* as seen this project. Additionally, exploring other anti-oxidant molecules like *Gx, SOD1* and *SOD2* at gene and protein levels would also help in understanding age-related differences in dealing with oxidative stress.

In regards to bone remodelling transcripts, subtle non-significant increases in both *RANKL* and *OPG* in old donors were found. Principally, increased levels of *RANKL* suggest increased bone resorption, therefore the obtained data are contradictory but remain interesting in relation to BM MSC adipogenesis. The idea that increased BM adipogenesis and bone remodelling are inter-related and impact each other is not completely new. The evidence for this has been furnished in Takeshita *et. al* who suggested that the increased BM adipogenesis is linked to the age-related increased expression of *RANKL* (238). They used uncultured whole BM cells from mice and isolated stromal cells and macrophages. They found increased osteoclastogenesis in adipogenic marrow cultures from aged mice, indicating increased bone resorption and adipogenic bias *in vivo*. They also found an increase in the expression of *PPAR-γ* and other adipogenesis related markers with a 3-fold age-related increase in the level of *RANKL* in whole BM cells. Investigating macrophages from human donors will provide with more comprehensive knowledge about age-related differences in bone remodelling within the BM.

Apart from testing this on uncultured cells, they also used murine BM stromal cell line ST2. The baseline expression of *RANKL* in this cell line was low and the level of *OPG* expression was very high. When the cell line was subjected to adipogenic differentiation, the level of *RANKL* increased and *OPG* decreased, completely

reversing their expression levels. While this study confirmed the age-related adipogenic bias and increased bone resorption in C57BL/6 mice aged 2 months (young) and 2 years (old), animal studies are often criticised for the lack of IVIVC (i*n vitro in vivo* correlation) with humans. This is because organ systems and genetic makeup of animals are not identical to humans. In the presented study, the *RANKL* increase in old donor MSCs was expected, but *OPG* increase was unexpected highlighting such differences between animals and humans.

The transcripts associated with cellular interaction have also shown no age-related changes in the present study. Recent examination by Gomariz and co-workers, suggested that CAR and sinusoidal endothelial cells within the BM compartment of mice remain mostly unaltered and show no age-related difference when inspected for quantitative and structural analysis (322). While they did note an insignificant age-related decline in the number of CARs in old mice (20-24 months old) compared to young (8-12 weeks old) mice, the difference was minor suggesting that *CXCL12* abundant (CAR) cells remain largely preserved in old age. Interestingly, they defined CAR cells by the CD295+ phenotype, which confirms similar cell identity to MSCs as identified in the present study. While there were a good number of studies outlining the CXCL12-CXCR4 signalling and its potential role in BM homeostasis in mice (239, 240), it is difficult to find many studies aimed at investigating age-related changes in this chemokine in cultured or uncultured human BM MSCs. Against our original hypothesis, the data presented in this chapter displayed no age-related decline in the expression of *CXCL12*. Considering the high levels at which *CXCL12* is expressed, detection and quantification of the transcript in large number of donors should not be a hurdle and will be interesting as the present data indicate a very subtle age-related increase of *CXCL12* expression in BM MSCs.

With respect to *Cx43*, the most abundant connexin within bone, no age-related differences were found in this study both at transcript and protein levels. Kar et al., showed age-related decline in mouse osteocytes (323) and more recently, Davis and colleagues have suggested that microRNA21 (miR21) is potentially responsible for this osteocyte *Cx43*-mediated age-related bone loss (305). *Cx43* in osteoblasts has been shown to play an important role in bone remodelling by controlling both bone resorption and bone formation (324) and another study suggested that *Cx43* in bone development acts in coordination with *RUNX2* (325). All of the aforementioned studies were performed on differentiated cells, suggesting that techniques for detecting age-related changes in uncultured progenitors are often not attempted and need validation in humans.

Cx43 has been also implicated in controlling the damaging effects of ROS within the stem cell niche. Grayson et al., observed a significant increase in the *Cx43* immunocytochemistry staining in human BM MSCs cultured in hypoxic conditions. They suggested that *Cx43* potentially contributes to maintaining MSC proliferation and 'stemness' (326). On a similar line of thought, Ishikawa et al have shown the protective role of *Cx43* for HSCs within the BM of mice. They suggested that *Cx43* mediates the transfer of ROS from HSCs to the stromal cells within the BM thus protecting HSCs from damage. Similarly, Kar and colleagues had shown that *Cx43* contributes to the protection of osteocytes against oxidative stress (323).

Based on these studies, our proposed hypothesis was for an age-related decline in Cx43 in uncultured BM MSCs. However, the investigation at both, transcript and protein level did not reveal any age-related differences. Examination of the molecule using a different technique may be needed to further strengthen our conclusion of no age-related differences. Owing to the presence of *Cx43* at cellular junctions, immunostaining techniques or the use of confocal microscopy that has labelled Cx43 at cellular junctions would be a useful tool to investigate and quantify the intensities of stains between young and old donor MSCs.

Investigations of multipotentiality genes in HLCs displayed a significant decline in *SPARC* and a significant increase in *RANKL* in old donors. SPARC is also known to be an immunomodulatory molecule and thus it is not unexpected to observe an age-related decline in *SPARC* in HLCs (316). *RANKL*, as mentioned earlier is expressed on T cells (316). An increase in *RANKL* suggests increased bone resorption. Higher bone resorption with advancing age is expected owing to overall age-related bone loss that has been established in literature (62, 81). The data presented in this chapter with respect to gene expression in HLCs was thus expected and supported the decline in immunomodulatory properties and increasing bone resorption observed with advancing age.

With regards to surface markers, CD106 and CD146 were chosen based on previous evidence of their altered expression in *in vitro* aged MSCs (Table 4.3). For CD106, the results were far from conclusive, with all the studies reported very high donor variation, even within the same age groups (123). Liu *et. al* used human BM MSC cell lines and found significant downregulation of CD106, at both transcript and protein levels, when cells were expanded *in vitro* (327). In contrast, Laschober and co-workers found an upregulation of CD106 at both transcript and protein levels in donors aged over 60

years old (138). They also found that CD106 was highly expressed in MSCs when exposed to inflammatory cytokines TNF-α or IFN-γ (138). Other studies have linked CD106 in MSCs with various immune and inflammation related changes (311, 328), suggesting a potential link between CD106 and inflamm-ageing within BM niches. Finally, Siegel and co-workers found a significant age-related decline in CD106 across a broad age range (20-80 years old) (97).

Since the literature pertaining to cultured MSCs remained contradictory, it was interesting to investigate CD106 expression in uncultured MSCs. The current study showed no age-related difference in the level of CD106 expression in uncultured $CD45^{low}CD271^{+}$ BM MSCs. In agreement with previous studies, this study too found the highest donor variation in the expression of CD106 compared to other markers. In future, investigations in larger number of donors and at the protein level will further increase our understanding.

Similar to CD106, CD146 expression on $CD45^{low}CD271^{+}$ MSCs in the present study also showed no age-related differences. This is in contrast to cultured MSC data, which commonly showed passage- or donor-age related decline. For example, Siegel *et. al* found a donor age-related decline in CD146 expression in BM MSCs at passage 1 (97). More recently, Yang and co-workers examined CD146 expression in culture expanded BM MSCs between passages 4 and 8 and found a passage-related decline in its level expression (123). In contrast, Hagmann and co-workers did not observe any age-related change in the expression of CD146 in culture expanded MSCs (329). Only one study looked at the CD146 MSC expression *in vivo* and found that the $CD271^{+}CD146^{+}$ cells were reduced in frequency in old donors (>55 years old) compared to young donors (19-55 years old) (229).

In conclusion, the present data on CD106 and CD146 suggests that using culture-expanded MSC data for the search of MSC 'ageing' marker *in vivo* is unlikely to be very fruitful. Other methods, for example using cell surface proteomic approach (330) and combining histological staining with flow cytometry for quantifying cell surface target proteins present on senescent MSCs (331) are more likely to provide interesting data (131).

Chapter 5 Expression of type 1 interferon (IFN1) and senescence genes in human BM MSCs

The previous chapter described a non-significant age-related decline in number of BM MSCs measured using the $CD45^{low}CD271^{+}$ phenotype and also examined differences between young and old donor MSCs in level of ROS, anti-oxidant enzyme SOD3 transcript, and selected multipotentiality genes and surface markers. Investigation of SOD3 in MSCs from old donors interestingly revealed a two-fold decline but differences were not statistically significant. Similarly, some trends were observed in the expression of *PPAR-γ, LepR, RANKL and OPG* (over 2-fold increase in older donors), however they too did not reach statistical significance. Unlike MSCs, HLCs displayed a 4-fold decline in osteogenic gene *SPARC* and 10-fold increase in bone remodelling gene *RANKL* in old donors, both of which were statistically significant. This indicated towards the fact that age-related gene expression differences for multipotentiality and possibly other genes could be more pronounced in HLCs as compared to MSCs.

Age-related changes in HSCs and their lineages are better understood owing to the larger number of investigations performed as compared to MSCs. Among these age-related changes, the decrease in lymphoid cells and increase in myeloid cells (myeloid skewing), loss in proliferation and increased DNA-damage in old donors have been well documented (332-334). The theories explaining these differences in aged HSCs are the same that describe MSC ageing (DNA damage, ROS accumulation, increased senescence and others, as explained in section 1.1.1). More recently, the IFN1 pathway has attracted an increasing interest as a potential link between DNA-damage response (DDR), oxidative stress, senescence and ageing in HSCs (335, 336).

As mentioned, HSCs are the progenitors for HLCs which eventually give rise to lymphoid and myeloid lineages. Considering that HSCs give rise to HLCs and that these cells share the same BM niche with MSCs, this chapter aims at investigating new exploratory genes emerging from the HSC 'ageing' field and including those associated with senescence and the IFN1 pathway, in MSCs as well as in HLCs. As in the previous chapter, this chapter also explores any age-related changes in the expression of these genes in uncultured cells and compares young donor and old donor cohorts. Finally, the chapter also aimed at inspecting if age-related changes were more pronounced in HLCs compared to MSCs, as observed in the previous chapter.

5.1 Introduction

Due to the relatively higher number of studies in HSCs, age-related differences in this cell type have been explored to a greater extent than for MSCs. Previous investigations in HSCs, have revealed an age-related increase in the myeloid lineage cells along with a decline in HSC proliferative capacity (337). The tendency to produce less lymphoid lineage cells and more myeloid lineage cells is known as 'myeloid skewing' and potentially contributes to age-related increase in vulnerability to infections (332). Interestingly, the hypothesised mechanisms of these changes that include DNA damage, stem cell exhaustion and the effects of ROS (28, 338) are no different from the hypothesised mechanisms of age-related differences in MSCs that were described in Chapter 1 Section 1.1.1 in theories of ageing. Considering that both MSCs and HSCs reside within the same BM niche (section 1.1.3), it is possible that these damaging factors act on both cell populations (339).

5.1.1 Senescence, SASP and age-related bone loss

Residing within the same niche, MSCs are known to support activities of HSCs including their myeloid and erythroid differentiation and early lymphopoiesis (340). MSCs reside together with other cells in a regulated BM niche that helps maintain the functioning of the microenvironment (Chapter 1, section 1.1.4). MSCs are also known to directly and indirectly modulate and control T cell responses (341) and to inhibit the proliferation and differentiation of B cells (342, 343).

With advancing age and accumulation of ROS and DNA damage in ageing cells, an increasing number of BM cells become senescent (344). Senescence is the state of irreversible growth arrest and is a regulated response to cellular insults like ROS and DNA damage (345). Senescent cells have been identified by various methods including an increase in SA-β-gal, Prelamin A, cell surface lipids like Lipofuscin, Tp53, critically short telomeres as well as differences in gene methylation patterns (131). Tp53 is a transcription factor well known for its function in cell-cycle, DNA-repair, and inducing cell growth as well as apoptosis (346, 347). While the presence of Tp53 is vital for maintenance of cells including BM MSCs (348), excessive levels of the transcription factor are known to be detrimental to healthy ageing (346). Aligned with this, our group found an age-dependant increase in the levels of Tp53 quantified by flow cytometry in culture expanded BM MSCs (96). Considering the aforementioned knowledge, Tp53 was hypothesised to be expressed in higher quantities in old donor cells as compared to young donor cells.

Along with increase in ROS and DNA damage, senescence is also characterised by the senescence associated secretory phenotype (SASP) (130), which includes the release of pro-inflammatory cytokines (like IL6, IL8) and metalloproteinases (MMPs) from senescent cells (345), and can then induce senescence and inflammatory responses in neighbouring cells (131). Senescent MSCs release higher amount of SASP, which reduces the multifunctionality of MSCs (80, 349) as well as accelerates the rate of ageing in younger cells (350). Interestingly, the level of serum IL6 has more than often been found to be higher in older donors as well as in donors with age-related diseases (214, 351, 352) and is an accepted marker of SASP and inflamm-ageing. Inflamm-ageing is defined as inflammation associated with increasing age and age-associated diseases and is explained further below (215, 353). With respect to MSCs, over-expression of IL6 has been shown to disrupt their role in maintaining HSC functionality and homeostasis (354). Thus in this project, the expression of *IL6* was hypothesised to display an age-related increase in both cell populations.

IL7 is essential for B cell development, survival and proliferation (355) and has long been known to be secreted in high quantities by BM MSCs (356). While it is not classified as a pro-inflammatory marker, its role in supporting B cell development in the BM and thus contributing to BM niche homeostasis makes it an important cytokine to investigate. In culture expanded BM cells, IL7 activity was found to drop dramatically in old mice (357) while in synovial fluid of old human donors, the level of IL7 was found to increase (358). Thus, in spite of knowing that IL7 is majorly produced by BM MSCs and its role in B cell formation and development, what happens to its levels in relation to advancing age remains unclear. Knowing that B cells functionalities decline with increasing age even though the change in number remains debateable (359), it was hypothesised that *IL7* expression will show an age-related decline in both cell types.

Along with IL6, IL8 too has been identified as an indicator of SASP (360, 361). While the number of investigations in relation to ageing are relatively fewer for IL8 compared to IL6, it is a known pro-inflammatory marker associated with high stress levels, is increased proportionately with age in urine samples (362) and in cancer patients (363). In BM MSCs co-cultured with colorectal cancer cells, IL8 production was found to enhance the proliferation tumour angiogenesis (364). Thus in this project, the expression of IL8 was hypothesised to be higher in old donor cells as compared to young donor cells.

Within the bone, increase in SASP has been shown to correlate with advancing age in myeloid as well as in osteoblastic cells of aged mice (24 months) when compared to

young mice (6 months) (365). Increased senescence, adipogenic shift of MSC differentiation, increased oxidative stress are all associated with age-related factors giving rise to increased levels of inflammation, which is now referred to as 'inflamm-ageing' (353). Inflamm-ageing conceptually postulates that increasing age is associated with an intrinsic decline of the proliferation potential of immune cells, their increased senescence and DNA damage. This is accompanied with the reduction of protective extrinsic mechanisms within the cellular microenvironment that aim at removal of senescent cells and accumulated damage, that is, autophagy (366). As mentioned in section 1.1.1, autophagy is the body's internal mechanism for the removal of senescent cells and damaged cell organelles by lysosome degradation. This is known to prevent the accumulation of DNA damage in the young and healthy (21). However, with the passage of time and increasing age, efficiency of autophagy declines. This means that the body's inherent capacity to identify and eliminate senescent cells/damaged organelles, also declines (21).

As autophagy declines, the number of senescent cells increase and so does the accumulation of damaged cells and organelles. These senescent cells release pro-inflammatory cytokines and growth factors (SASP), that ultimately contribute to inflamm-ageing (366). Expectedly, the understanding of senescence, autophagy, inflamm-ageing and their contribution in age-related diseases, makes senescence a promising target for anti-ageing strategies (366). These strategies may use seno-therapeutics aimed at removal of senescent cells by killing senescent cells (senolytics), or by modulating SASP (senomorphics) or by the clearance of senescent cells mediated by the body's immune system (seno-inflammation) (367). While senescence and SASP can indicate 'ageing' when they increase in body, anti-ageing genes also play an equally important role in maintaining 'youthful' characteristics of cells (368).

Sirt6 is an anti-ageing gene (369) recognised for regulating DNA breaks and damage repair (370) and its over-expression was found to expand the lifespan of male mice (371). In human dermal fibroblasts, an age-related decline in the expression of *Sirt6* was observed along with higher resistance towards reprogramming. However, the addition of *Sirt6* in old donors dermal fibroblasts (>50 years old) was found to enhance the reprogramming efficiency (372). In relation to MSCs, *Sirt6* has been shown to regulate osteogenic differentiation of BM MSCs in rats (373) and to protect embryonic MSCs from oxidative stress characterised by dysregulated redox metabolism in *Sirt6*$^{-/-}$ human MSC cell lines (374). Another study in 2016 found that knocking out *Sirt6* gene accelerates the process of senescence in human BM MSCs (375). From all of the

aforementioned observations, the expression of *Sirt6* was hypothesised to show an age-related decline in both cell populations.

Investigations with another anti-ageing gene *Klotho* (*Kl*) have shown that its overexpression leads to extended life-span (376), while its mutations lead to decreased lifespan in mice (377) and promoted the process of ageing by damaging telomerase activity (378). The Klotho protein (379) was found to be inversely correlated with increasing age in the serum of human donors (380). Considering these findings, it is not surprising that genetically modified BM MSCs expressing *Kl* has been patented aimed at cell based therapies for many age-related diseases in humans (381). Interestingly, the potential mechanism of *Kl* to reduce senescence and extend life-span has been thought to be via the Tp53/p21 pathway (382). With this collective knowledge, it was hypothesised that there would be a decline in the expression of *Kl* in cells form older donors as compared to young donors.

5.1.2 Type 1 Interferons (IFN1)

IFNs are a group of cytokines that are known to elicit pleotropic biological effects such as immuno-modulation and cell differentiation (383). They were initially recognised for their interference with viral replication, however other broad range of properties beyond their antiviral actions have been recognised recently in various cell types including fibroblasts, dendritic cells and HSCs (384). In stem cells in particular, they have been known to play a role in differentiation, immune-modulation, and their anti-proliferative potentials (385). IFNs may be activated due the presence of extrinsic factors like virus/infection or due the presence of intrinsic stress factors leading to DNA damage.

In health, the levels of type 1 IFNs and IFN responses are fine-tuned to maintain the immune homeostasis between protection against viruses (386) and its toxic effects, which are widely associated with pathological conditions such as autoimmune diseases (i.e. Systemic lupus erythematosus or SLE) (387), chronic infection (388) and cancers (389). IFN1 binds to its receptor that is heterodimeric cell surface receptor, consisting of two transmembrane spanning subunits IFNAR1 and IFNAR2. Most cells types are known to express the receptor (390) including fibroblasts, dendritic cells, and peripheral blood cells including monocytes (391).

The canonical signalling pathway involves IFN1 production and binding to the IFNAR. The receptor is phosphorylated and triggers downstream signal transduction cascade via activation of Janus kinases 1 (JAK) and non-receptor tyrosine kinase 2 (TYK2), which then induces phosphorylation of transcription factors STAT1 and

STAT2. Phosphorylated STAT1 and STAT2 dimerise, dissociate from IFNAR2 and form a trimolecular complex with IFN regulatory factor 9 (IRF9) known as Interferon stimulated gene factor 3 (ISGF3) (392, 393). This complex then translocates to the nucleus where it binds to DNA sequence motif known as IFN stimulated response elements (ISRE) to eventually activate the transcription of a wide range of IFN regulated genes, also known as the IRGs, in an autocrine and paracrine way (Figure 5.1, top panel).

Recently, a study from our group examined the immune-modulating property of MSCs from cancellous bone fragments on CD3/CD28- stimulated CD4+ T cells. It found a 'dose dependant suppression of CD4 T-cell proliferation' along with an increase in the levels of TGFβ in co-cultures (316). It also identified a list of candidate genes that potentially were responsible for eliciting MSC immunomodulation. Interestingly, many transcripts belonged to the IFN1 responsive family of genes, raising interest in understanding age-related differences in the expression of these genes in uncultured BM MSCs and HLCs, and their potential link to inflamm-ageing.

In an early study in 1984, the levels of IFN1 secreted from mononuclear cells were shown to decline in donors aged over 50 (394). In contrast, study in 2011 described DDR-induced expression of IFN genes after double stranded DNA breaks were introduced in HeLa cell lines (395). An intracellular molecule *STING* was increasingly becoming the core molecule mediating IFN expression induced by DDR (396). In health, DNA remains localised to the nucleus or the mitochondria. Thus, the presence of the DNA in the cytoplasm of the cell is expected to trigger DDR. In 2013, cGAS was found to detect the presence of the DNA in the cytoplasm and undergo conformational change. This conformational change in cGAS could now convert ATPs to the second messenger cyclic GMP-AMP (cGAMP) (397) which is a high affinity ligand to the adaptor protein stimulator of IFN genes (*STING)* (398). STING translocates from the endoplasmic reticulum to the golgi bodies which activates IFN regulatory factor 3 (IRF3) which leads to the production of IFN1 (399).

Based on this evidence, the cGAS-cGAMP-STING pathway has been reported to be the link between inflammation-based DDR, senescence as well as cancer (399). Considering that the presence of extrinsic/intrinsic stress factors illicit IFN1 response via STING, cGAS-STING-IFN1 pathway has been proposed as one of the pathways for autophagy (400-402). As mentioned, there has been an increased interest in unravelling the links between DDR, ROS, cell senescence and IFN1 signalling in HSC (30, 403), that share the same niche with the MSCs, giving a strong rationale to study

these molecules in MSCs (Figure 5.1, bottom panel). Furthermore, the connection between DNA damage and the ATM-BID-MTCH2 pathway has been recently proposed to mediate the negative impact of ROS in HSCs (31). In HSCs from a mouse model deficient for Mixed Lineage Leukemia 5 or MLL5), BID (BH3 interacting domain) regulated mitochondrial ROS in DNA damage pathway downstream IFN1 signalling. Another protein - MTCH2 (mitochondrial carrier homolog 2) - protected cells from oxidative stress-induced death, and BID which is the precursor of MTCH2 in the ATM (ataxia telangiectasia mutated kinase)-BID pathway, worked as a MTCH2 antagonist (404).

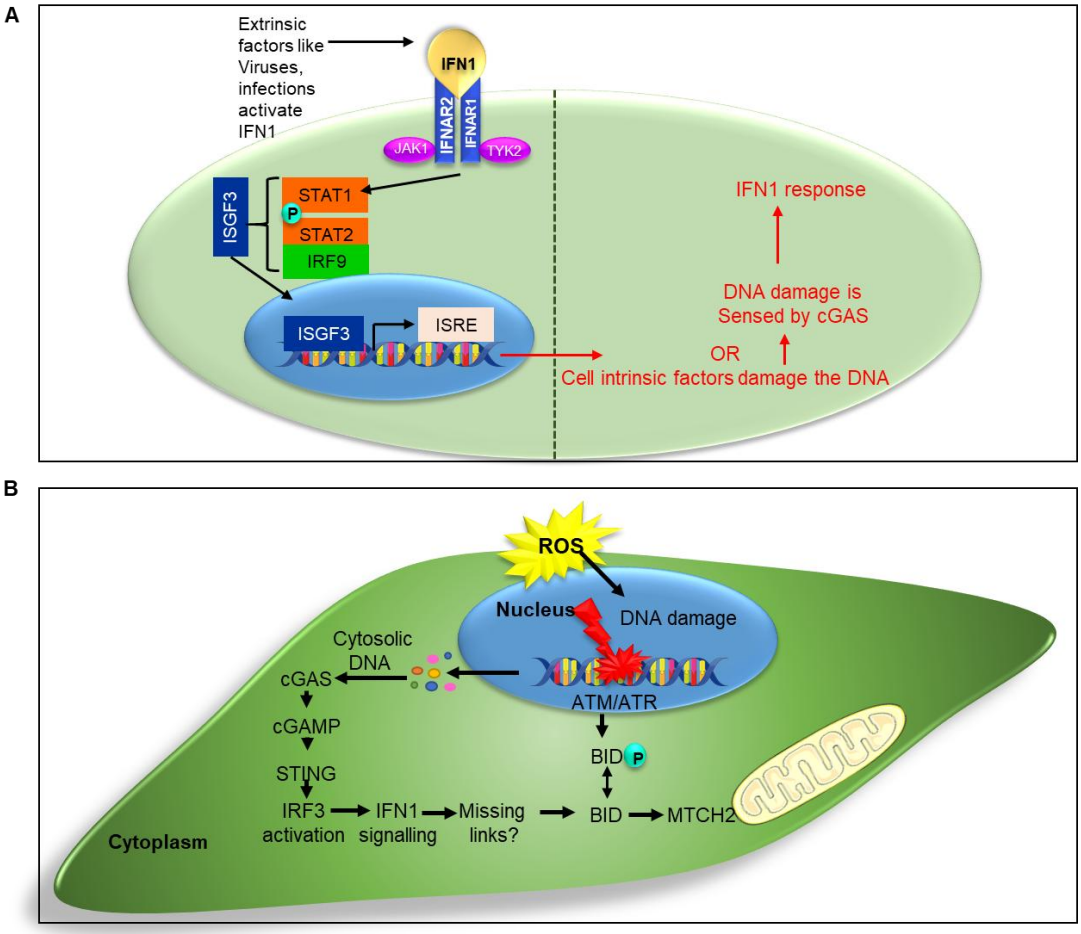

Figure 5.1 IFN1 signal activation and signalling pathway associated with ROS and intrinsic factors (adapted from (393) and (31))

(A) Left: General IFN1 signalling pathway activation by extrinsic factors. IFN1 binds to its receptor and conformational changes begin the cascade of phosphorylation of STAT1 and STAT2 to form ISGF3 that ultimately leads to IFN response elements in the nucleus. Right: IFN1 may also be activated by intrinsic factors like cytoplasmic DNA written in red. **(B)** Intrinsic factors leading to IFN1 response in MSCs: Intrinsic ROS causes DNA damage which leaks out of nucleus and becomes cytosolic DNA. This hypothetically activates IFN1 signalling, potentially leading to mitochondrial ROS.

Under low level of DNA damage, ATM through BID phosphorylation, reduces the level of BID and consequently, the level of ROS in HSCs. However, during DNA damage; loss of BID phosphorylation directs it toward the mitochondria and results in massive ROS production by interacting with MTCH2 (403). To date these investigations have not been performed on human cells. Moreover, the presence of ATM-BID-MTCH2 pathway molecules in MSCs has not been explored before. Because HSCs and MSCs coexist in BM niche, this pathway may be potentially affected in MSCs exposed to oxidative stress and DNA damage (both previously associated with age-related changes).

The role of IFNs and related signalling pathways in the complex and dynamic interplay between MSCs and HLCs in the BM niche remains unexplored. Taking the work from our group that recently found the expression of many IRGs in MSCs, forward along with some more exploratory transcripts related to senescence and anti-ageing, this chapter examined age-related changes in these transcripts in MSCs and HLCs. It was expected that DNA damage and senescence associated genes could potentially be higher in old donor cells, and possibly more in HLCs than in MSCs. List of analysed transcripts is shown in Table 5.1. Furthermore, considering that IFN1 signalling has been more commonly linked to innate immune cells, which are derived from HSCs, it was hypothesised that genes associated with IFN1 pathway would be expressed in higher levels in HLCs as compared to MSCs. Moreover, senescence and SASP genes were hypothesised to increase in older donors in MSCs and in HLCs. Age-related changes in anti-ageing genes, IFN receptors as well as IFN-associated genes would also be expected to be more pronounced in HLCs as compared to MSCs.

This chapter aimed at exploring if uncultured BM MSCs expressed any IFN1 responsive genes in comparison to HLCs along with the expression of senescence associated genes. The chapter also aimed at investigating any age-related changes in these genes in both MSCs and in HLCs.

5.1.3 Hypothesis and objectives

The following hypotheses and aims were postulated for this chapter

Hypotheses:

1. Senescence and SASP associated genes display an increased expression in old donor cells, with greater increase in BM HLCs than in BM MSCs. Anti-ageing genes display the opposite trend.
2. Both BM MSCs and HLCs express the exploratory genes from the IFN1 responsive family of genes, but their expression is higher in HLCs, including selected molecules linking IFN pathway with DNA damage, ROS and senescence.
3. These exploratory transcripts would show age-related decline in cells from older donors, with possibly higher declines in HLCs as compared to BM MSCs.

Objectives:

1. To quantify the expression of senescence, SASP and anti-ageing genes in uncultured BM $CD45^{low}CD271^{+}$ MSCs and $CD45^{+}CD271^{-}$ HLCs from young and old donors.
2. To compare the expression of exploratory genes linking IFN pathway with DNA damage, ROS and senescence in uncultured BM MSCs and HLCs.
3. To investigate the age-related changes in these exploratory genes in uncultured BM MSCs and HLCs.

5.2 Methods

5.2.1 Donor selection

A total of n=12 donor BM cells, 6 young (19-40 years old, median age=27 years old) and 6 old (59-89 years old, median age=68 years old) were used for investigation in this chapter. The description of donor samples used in this chapter have been outlined in Appendix 1, Table 3.

5.2.2 Gene expression for exploratory transcripts analysis

The general method has been described in section 2.5. Briefly, frozen nucleated cells from BM of donors were defrosted (Section 2.3), enriched using anti-fibroblast beads (Section 2.4) and then sorted to collect $CD45^{low}$ $CD271^{+}$ MSCs and $CD45^{+}CD271^{-}$ HLCs and frozen in lysis buffer at -80ºC for further use. Next, these cell lysates were defrosted and the RNA was extracted (Section 2.6.4). The RNA was then reverse transcribed to cDNA (Section 2.6.5), pre-amplified for 18 cycles (Section 2.6.6) and

then qPCR was performed on BioMark™ (Section 2.6.7) using 48.48 dynamic array (Figure 5.2). The principle of these reactions are the same as those described in Section 2.6, but this chip accommodates up to 48 candidate genes.

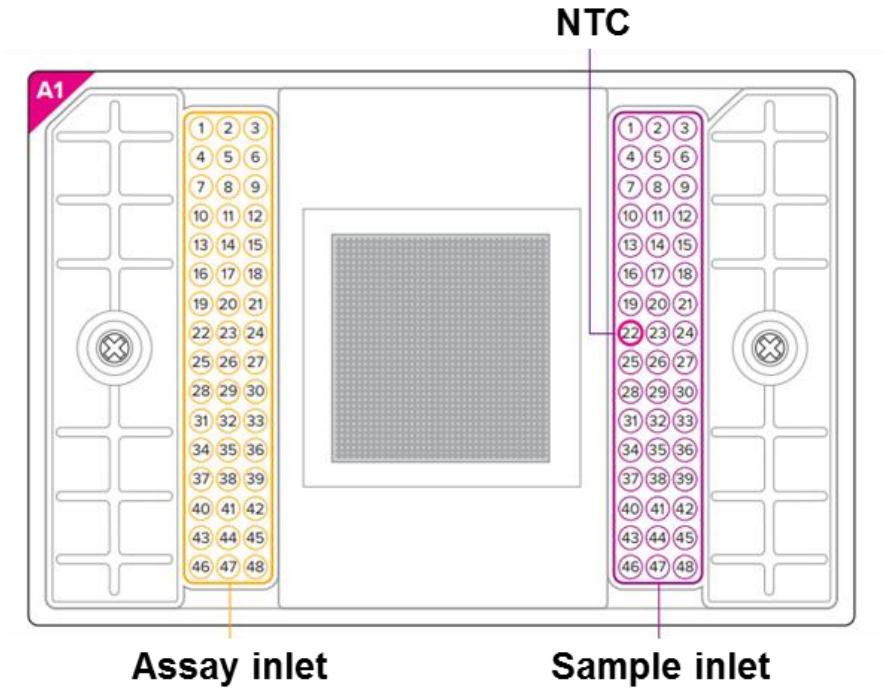

Figure 5.2 48.48 gene chip outlay

The quantification was performed during analysis wherein the Ct values for each gene of interest were normalised to Ct value of *HPRT1* as the housekeeping gene to generate the ΔCt values for each gene using the formula [$Ct_{\text{target gene}}$- $Ct_{\text{housekeeping gene}}$]. Finally, the ΔCt values were converted to 'relative expression' for each gene using the formula [$2^{-\Delta Ct}$] and the values were compared between young and old donors for both BM cell populations (MSCs and HLCs).

5.2.3 Statistical analysis

Cluster analysis was used to observe any differences in gene expression across the entire donor cohort. Statistical analysis and graphics were performed using GraphPad Prism software (version 7.0a). The normal distribution of the data was assessed using the Shapiro-Wilk and Kolmogorov-Smirnov tests for normality. As data were found to be not normally distributed, Mann-Whitney U test was performed for unmatched data. The results were considered significant at *p* value of <0.05.

5.3 Results

5.3.1 The expression of all exploratory transcripts in BM MSCs and HLCs

The expression of transcripts for type 1 IFNs, IFN Receptors and IRGs, as well as genes associated with IFN1>BID>ROS pathway and senescence, was investigated in sorted uncultured BM MSCs and HLCs, based on the $CD45^{low}$ $CD271^{+}$ and $CD45^{+}CD271^{-}$ phenotypes, as described in the previous chapter for the whole donor cohort. Cluster analysis of gene expression data revealed the presence of two distinct clusters for MSCs and HLCs, with the exception of one sample (Figure 5.3). High variation in gene expression between the donors was noted, but surprisingly, an overall pattern of higher expression (more red colour) in s compared to HLCs was found. The higher expressed IRGs in MSCs relative to MSCs included *IFITM1, IFITM3*, *IFI27, SERPING* and IRGs with higher expression in HLCs included *RGS1*, *IL8* and BID (Figure 5.3).

The statistical analysis including the fold differences in the expression of gene shown in figures are shown in Table 5.1. The median values and differences between expression in MSCs and HLCs of the complete list of genes is shown in Appendix 4, Table 5.1.

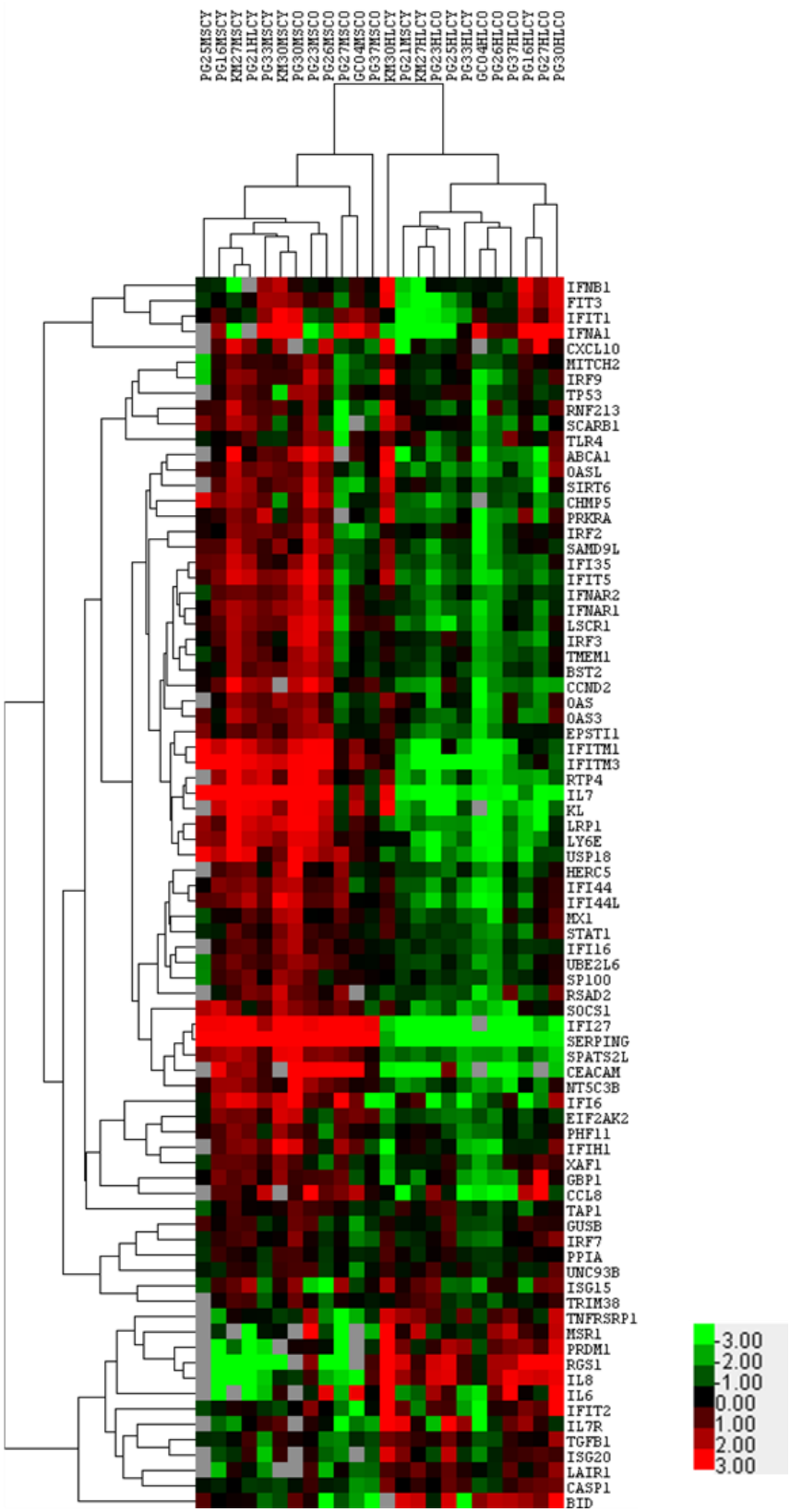

Figure 5.3 Expression of exploratory genes in MSCs and HLCs

Expression of *IFNA1, IFNB1*, *IFN receptors,* IRGs, genes associated with IFN1>BID>ROS pathway, senescence and other exploratory genes grouped by cluster as MSCs (left) and HLCs (right). Colour key in shown at the bottom right of the figure. Grey squares indicate gene expression values that were below detection.

5.3.1.1 Analysis of expression of senescence and anti-ageing associated genes in MSCs and HLCs

For the whole donor cohort, analysis of transcripts associated with senescence and anti-ageing showed that *Tp53* and *Sirt6* displayed no statistically significant differences (p<0.05 for both genes) between MSCs and HLCs, although *Sirt6* had slightly higher expression in MSCs as compared to HLCs (2.86-fold). However, the expression of *Kl* was significantly (25-fold) higher in MSCs (p=0.0083) as compared to HLCs (Figure 5.4). In combination, the pattern of expression for all three genes indicated a possibility for stronger anti-ageing defence mechanisms in MSCs compared to HLCs.

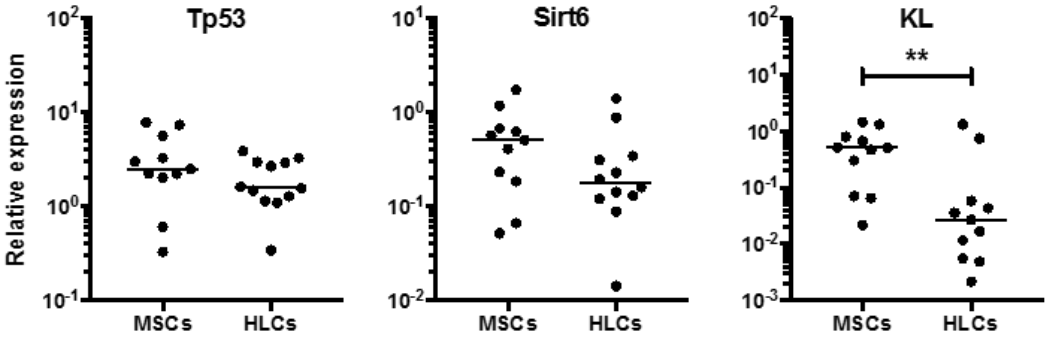

Figure 5.4 Expression of senescence and anti-ageing genes in MSCs and HLCs

Expression of *Tp53*, *Sirt6* and *Kl* in donors in MSCs and HLCs. Horizontal line across data set indicates median values. p<0.01**, Mann-Whitney U test.

5.3.1.2 Analysis of expression of SASP-associated genes in MSCs and HLCs

Two out of three SASP cytokine genes were also differentially expressed between MSCs and HLCs. Cytokine *IL6* showed no difference in expression between the two cell populations (Figure 5.5). As expected, *IL7* displayed significantly higher expression in MSCs (p=0.0005) in comparison to HLCs, and *IL8* displayed higher expression in HLCs (p=0.0022) as compared to MSCs.

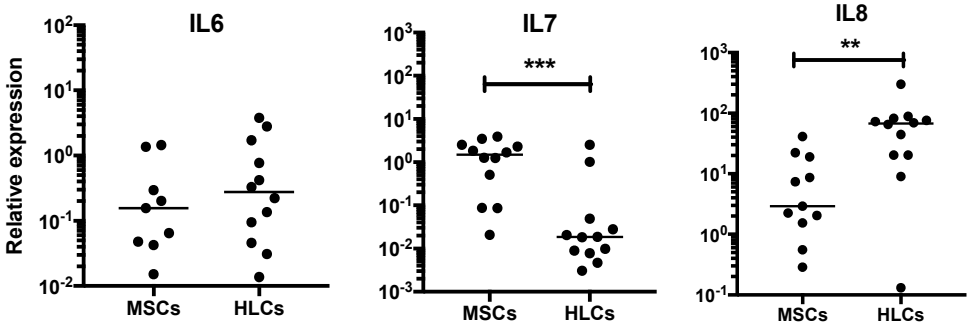

Figure 5.5 Expression of SASP-associated cytokines in MSCs and HLCs

Expression of *IL6, IL7* and *IL8* in MSCs and HLCs. Horizontal line across data set indicates median values. p<0.01** and p<0.001***, Mann-Whitney U test.

5.3.1.3 Expression of IFN1, IFN Receptors and IRGs in MSCs and HLCs

Expression of *IFNA1* and *IFNB1* did not show differences in their expression levels between MSCs and HLCs (Figure 5.6A). Very interestingly, the levels of expression of *IFNAR1* and *IFNAR2* showed a significantly higher expression in MSCs (p=0.0018 and p=0.0332, respectively) compared to HLCs (Figure 5.6B), which was unexpected, given their expression was historically linked to immune-lineage cells.

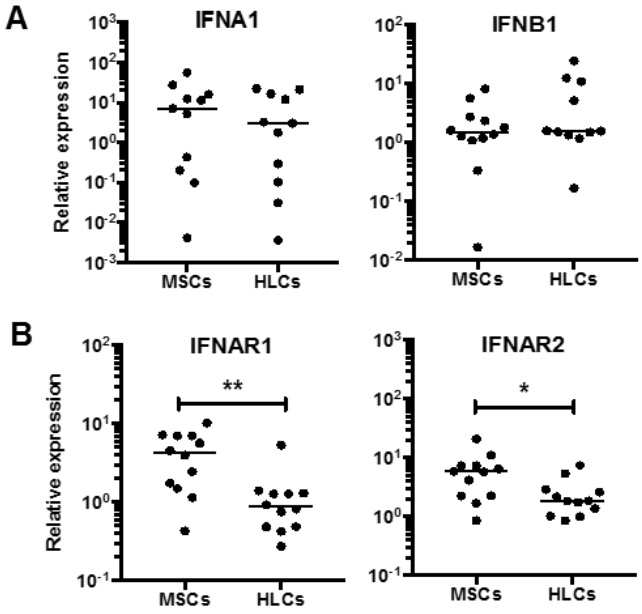

Figure 5.6 Expression of IFNA, IFNB and IFNA receptor genes in MSCs and in HLCs

(A) Expression of *IFNA1* and *IFNB1* in MSCs and HLCs. **(B)** Expression of *IFNAR1* and *IFNAR2* in MSCs and HLCs. Horizontal line across data set indicates median values. p<0.05* and p<0.01**, Mann-Whitney U test.

Among the IRGs, many of them including *USP12, IFITM1* and *IFITM3*, displayed higher expression levels in MSCs (Figure 5.7). Similarly, there were genes including *PRDM1, LAIR* and *CASP1* that were significantly higher expressed in HLCs (Figure 5.8). Overall, and as indicated by the cluster analysis shown in Figure 5.3, a higher proportion of the genes displayed significantly higher expression in MSCs than in HLCs. This was unexpected, given IFN1 gene expression was historically linked to the immune-lineage cells.

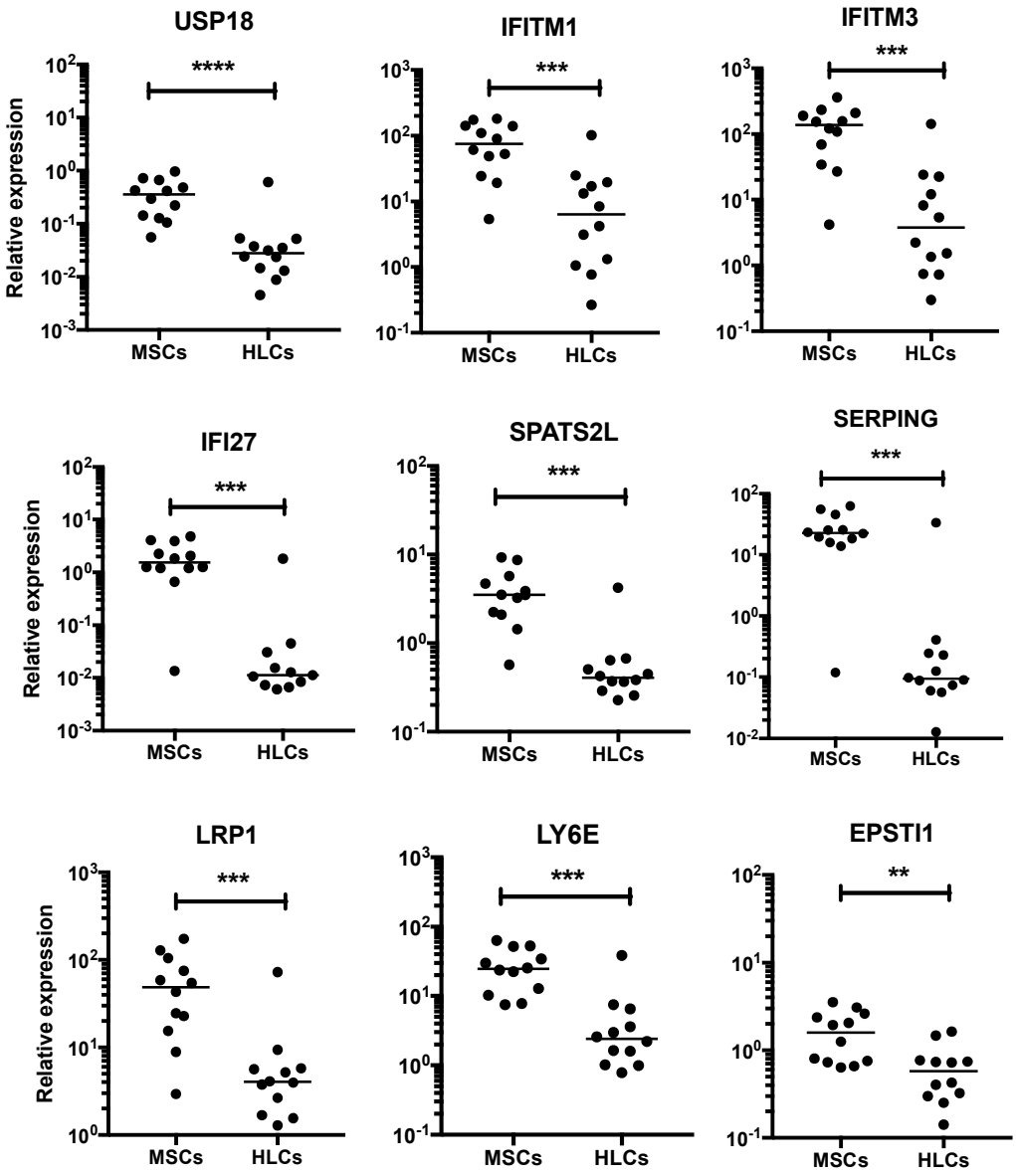

Figure 5.7 Genes displaying significantly higher expression in MSCs than in HLCs

Expression of most significantly differentially expressed genes in MSCs over HLCs. Horizontal line across data set indicates median values. $p<0.01$**, $p<0.001$*** and $p<0.0001$****, Mann-Whitney U test.

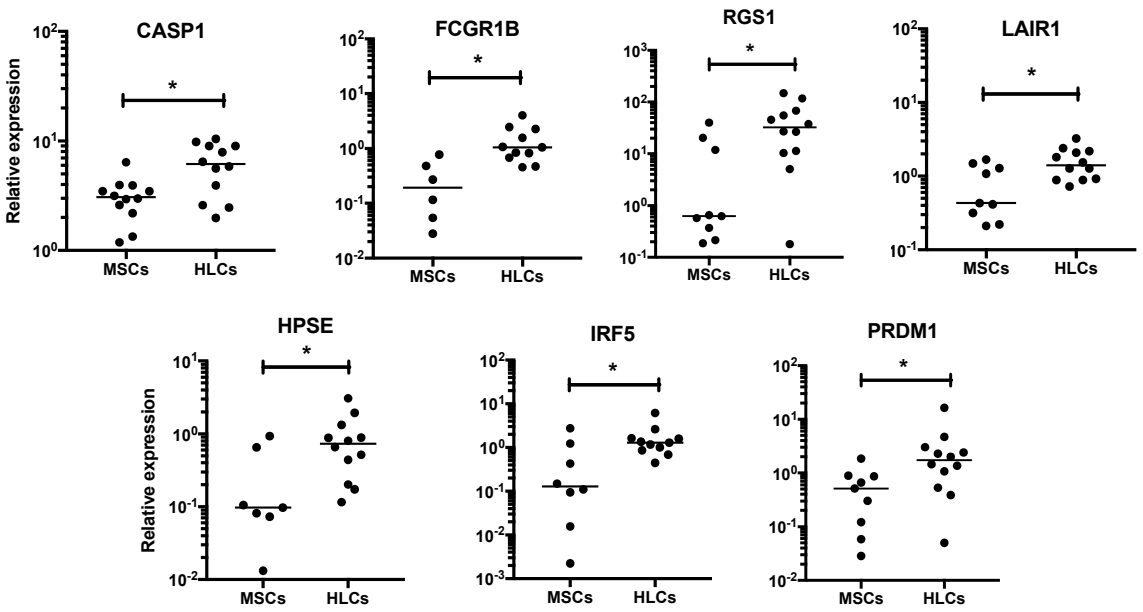

Figure 5.8 Genes displaying significantly higher expression in HLCs than in MSCs

Expression of most significantly differentially expressed genes in HLCs over MSCs. Horizontal line across data set indicates median values. $p<0.05$*, Mann-Whitney U test.

5.3.1.4 Analysis of expression of genes involved in IFN1>BID>ROS pathway

As discussed in the introduction to this chapter, damage to the DNA due to external or internal factors is known to activate IFN1 signalling via *STING, IRF3* and *STAT1* through *IFNA* receptors. This has eventually been shown to mobilize *BID*, which exerts its expression via *MTCH2,* which is the mitochondrial receptor for *BID*. Mobilisation of *BID* has been shown to lead to accumulation of mitochondrial ROS leading to functional defects and senescence in the HSCs. Because HSCs give rise to HLCs and MSCs coexist with HSCs in the BM niche, it was thus in the interest of this project to investigate whether this pathway is also engaged in MSCs.

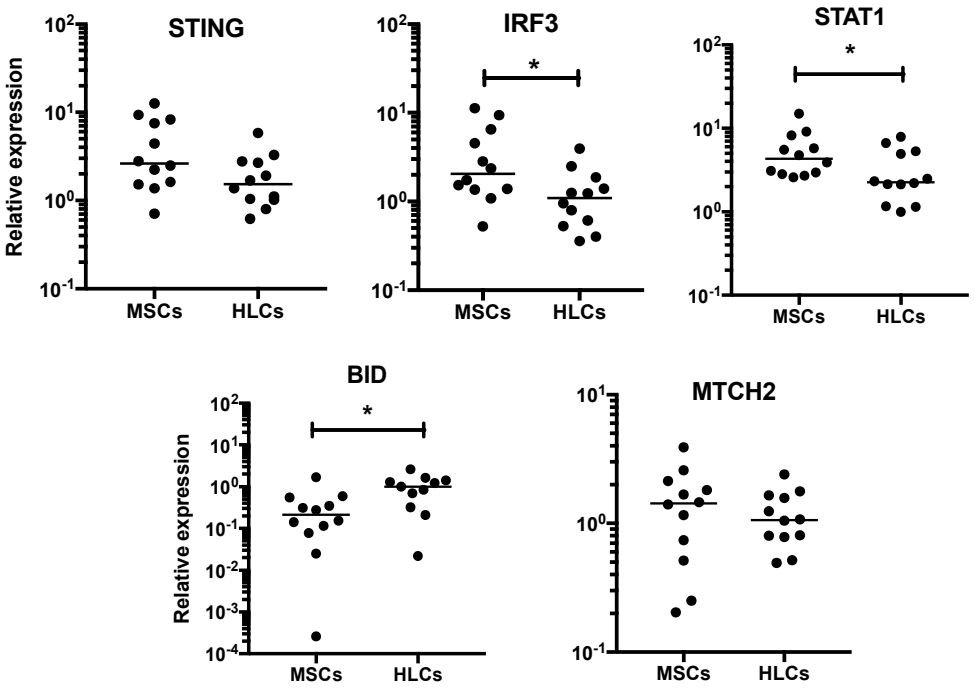

Figure 5.9 Expression of genes associated with IFN1>BID>ROS pathway in MSCs and HLCs

Expression of *STING, IRF3* and *STAT1* (top panel), *BID* and *MTCH2* (bottom panel) in each group. Horizontal line indicates median values. $p<0.05$*, Mann-Whitney U test.

Both *IFNAR1* and *IFNAR2*, displayed higher expression levels in MSCs as shown in figure 5.6B. *STING* showed no significant difference in expression between MSCs and HLCs although a trend for higher levels in MSCs was noted (Table 5.1). *IRF3* and *STAT1* displayed higher expression levels in MSCs as compared to HLCs (p=0.0205 and p=0.0242 respectively, Figure 5.9, top panel). *BID* displayed significantly higher (p=0.0188) expression in HLCs as compared to MSCs while *MTCH2* showed no differences (Figure 5.9, bottom panel).

Table 5.1 Medians of expression of genes shown in Figures 5.4 to 5.6 and 5.9 in MSCs, HLCs, fold differences and their *p* values

Function	Gene	Median (MSCs)	Median (HLCs)	Fold difference (MSCs/HLCs)	*p* value
IFN1 and receptors	*IFNA1*	6.97	3.01	2.32	NS
	IFNB1	1.50	1.59	0.95	NS
	IFNAR1	4.28	0.88	4.86	0.0018
	IFNAR2	5.82	1.84	3.16	0.0332
IFN1-BID-MTCH2 pathway	*STING*	2.64	1.54	1.72	0.0684
	IRF3	2.051	1.90	1.08	0.0242
	STAT1	4.33	2.26	1.92	0.0205
	BID	0.21	1	0.21	0.0188
	MTCH2	1.43	1.06	1.35	NS
Anti-ageing and senescence	*Kl*	0.51	0.02	25.50	0.0083
	Sirt6	0.50	0.17	2.86	NS
	Tp53	2.5	1.59	1.57	NS
SASP	*IL6*	0.15	0.27	0.56	NS
	IL7	1.48	0.016	93.73	0.0005
	IL8	2.92	67.21	0.04	0.0022

NS: Non-significant, Gene expression is normalised to *HPRT1*, Mann-Whitney U test.

Overall, this gene expression pattern potentially indicated a higher level of constitutive IFN1 signalling in MSCs, as well as their possession of the necessary molecular machinery to combat oxidative damage via IFN1>BID>ROS pathway.

5.3.2 Age-related differences in IFN1 signalling pathway related genes

The median values for each gene shown in the figures below in young and old donors, the fold difference with advancing age and their *p* values are indicated in Table 5.2 and Table 5.3. Similar tables for the all the exploratory genes are shown in Appendix 4, Table 5.2 and 5.3 for age-related differences in MSCs and in HLCs, respectively

5.3.2.1 Age-related differences in the expression of senescence and anti-ageing associated genes in MSCs and HLCs

Senescence-associated gene *Tp53* and anti-ageing genes of *Kl* and *Sirt6* did not show any differences between young and old donor MSCs (Figure 5.10A). In HLCs, the expression of all of these genes declined in old donors (Figure 5.10B) with *Tp53* showing a statistically significant decline (p=0.0043). At least in relation to anti-ageing genes, these data indicated a possibility for stronger ‘ageing-resistance’ molecular mechanisms in MSCs compared to HLCs.

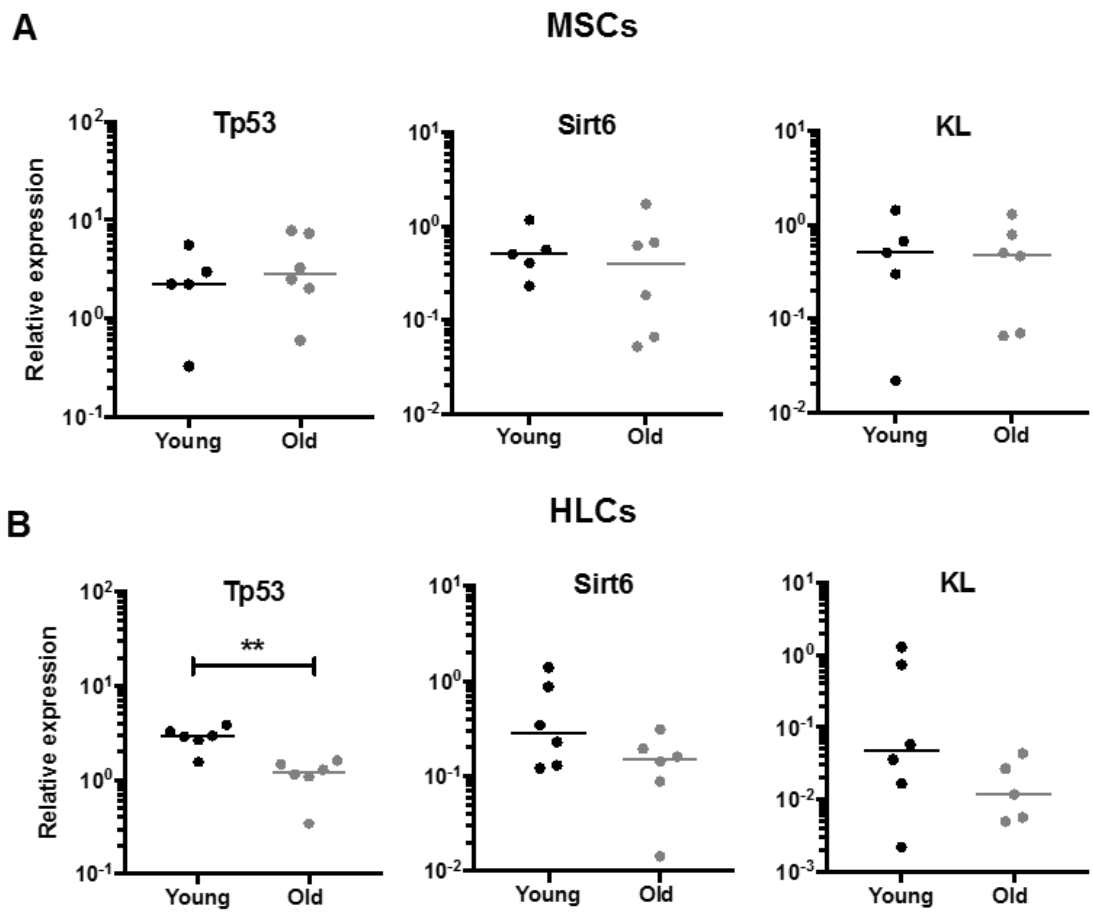

Figure 5.10 Expression of senescence and anti-ageing genes

(A) Expression of *Tp53, Sirt6* and *Kl* in young and old donors MSCs **(B)** Expression of *Tp53*, *Sirt6* and *Kl* in young and old donor HLCs. Young and old donors are indicated in black and grey dots, respectively. Horizontal line across data set indicates median values. $p<0.01$**, Mann-Whitney U test.

5.3.2.2 Age-related differences in SASP-associated cytokine transcripts in MSCs and HLCs

IL6 transcript levels were found to be 4-fold higher in old donor MSCs as compared to MSCs from young donors however the differences failed to reach statistical significance. *IL7* and *IL8* did not show any difference between young and old donor MSCs either (Figure 5.11A). In HLCs, *IL6* transcript was similarly nearly 4-fold higher in from old donors as compared to young donors however the differences did not reach statistical significance. *IL7* showed a slight decline and *IL8* did not show any difference between young and old donor HLCs (Figure 5.11B).

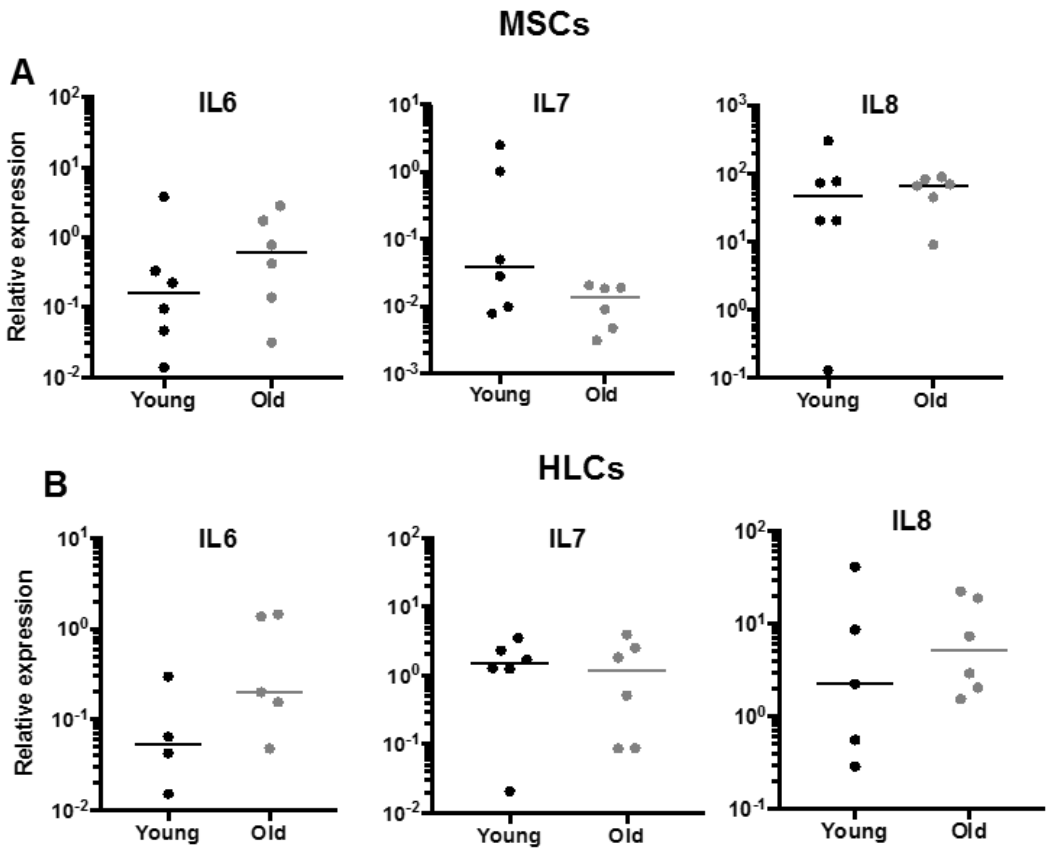

Figure 5.11 Expression of cytokines

(A) Expression of *IL6, IL7* and *IL8* in young and old donor MSCs. **(B)** Expression of *IL6, IL7* and *IL8* in young and old donor HLCs. Young and old donors are indicated in black and grey dots, respectively. Horizontal line across data set indicates median values.

5.3.2.3 Age-related differences in IFN1, IFN Receptors and IRGs in MSCs and HLCs

Cluster analysis between young and old donor MSCs revealed no patterns for differences in type 1 IFN, IFN receptors, IRGs and cytokines. Similarly, investigation of differences between young and old donor HLCs using cluster analysis also revealed no particular trends.

IFNA, IFNB and *IFNAR* transcript levels showed no significant differences or particular trends between young and old donors MSCs (Figure 5.12A). However, in HLCs, some differences were significant. *IFNA1* transcript levels displayed a trend for over 2-fold increase in old donors HLCs compared to young donors. *IFNB1* showed no differences between young and old donor HLCs (Figure 5.12B). *IFNAR1* and *IFNAR2* transcript levels showed declines in their expression in old donor HLCs, which was statistically significant for *IFNAR2* (p=0.041, Figure 5.9B).

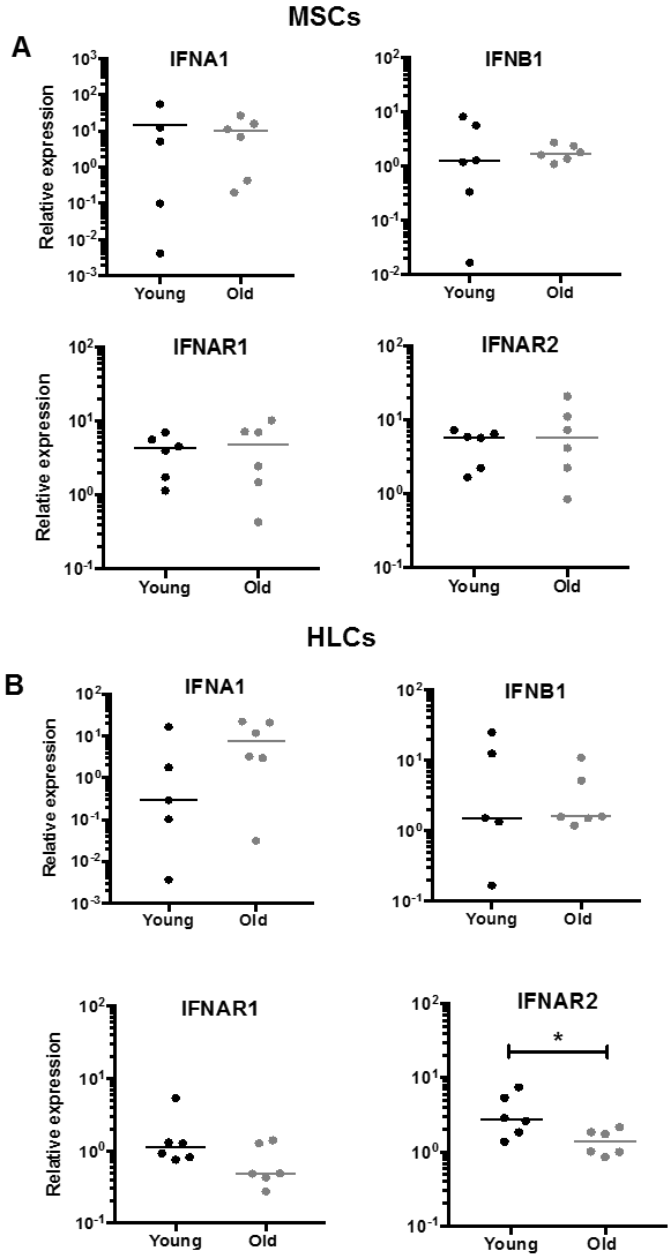

Figure 5.12 Expression of IFNA, IFNB and IFNA receptor genes

(A) Expression of *IFNA1* and *IFNB1* (top panel) and *IFNAR1* and IFNR2 (bottom panel) in young and old donor MSCs **(B)** Expression of *IFNA1*and *IFNB1* (top panel) and *IFNAR1* and *IFNAR2* (bottom panel) in young and old donor HLCs. Young and old donors are indicated in black and grey dots, respectively. Horizontal line across data set indicates median values. $p<0.05$*, Mann-Whitney U test.

Out of all the exploratory IRGs, *RNF213* was the only gene that showed statistically significant lower expression in MSCs from old donors as compared to young donors ($p=0.041$, Figure 5.13A). In the HLCs, more IRGs displayed significant decline in old donors (Figure 5.13B) and Appendix 4, Table 2. This potentially indicted reduced IFN1 signalling in old donor HLCs, which wasn't the case for old donor MSCs.

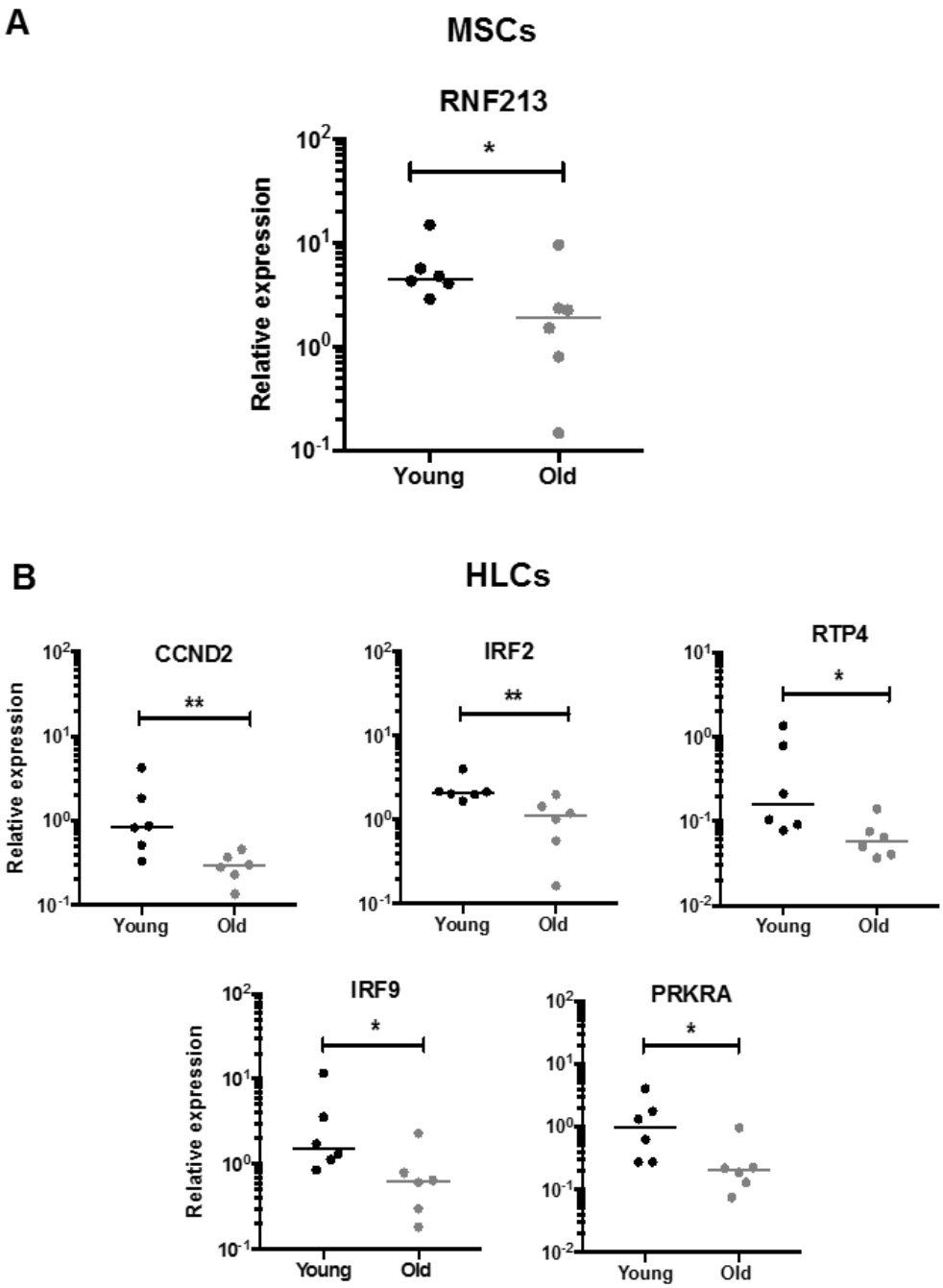

Figure 5.13 Expression of genes displaying statistical age-related differences

(A) Expression of differentially expressed genes in MSCs in young and old donors and **(B)** Expression of differentially expressed genes in in HLCs in young and old donors. Young and old donors are indicated in black and grey dots, respectively. Horizontal line across data set indicates median values. $p<0.05$* and $p<0.01$**, Mann-Whitney U test.

5.3.2.4 Age-related differences in genes associated with IFN1>BID>ROS pathway

The analysis of genes associated with the IFN1>BID>ROS pathway also revealed interesting findings. Again, investigation in MSCs did not show any significant age-related differences, although *STAT1, IRF3* and *STING* showed a trend for an increase in MSCs from old donors. BID expression demonstrated a non-significant, nearly 3-fold decline in old donor MSCs (Figure 5.14A).

Interestingly, investigation of expression of the same genes in young and old donor HLCs revealed different results. While *STAT1* expression showed no difference, *IRF3* displayed a significant decline in old donors HLCs (p=0.033) (Figure 5.14B). Significant declines in old donor HLCs were also seen for *MTCH2* and *STING* expression (p=0.0152 and p=0.041, respectively) while *BID* did not show any age-related differences.

Altogether, investigation of IFN1 family of genes, SASP cytokine genes and other genes linking IFN pathway and cell senescence did not reveal any significant differences between young and old donor MSCs, except for *RNF213* gene, which is an IRG with yet unknown function. While expression of these genes in MSCs did not show any age-related differences, the same genes in HLCs revealed interesting expression patterns. In particular, *IFNAR2,* many IRGs and genes linking IFN1 pathway with ROS and cell senescence displayed a decrease in their expression in old donor HLCs. Altogether, these data indicated a potential reduction in constitutive IFN1 signalling in old donor HLCs but not in old donor MSCs.

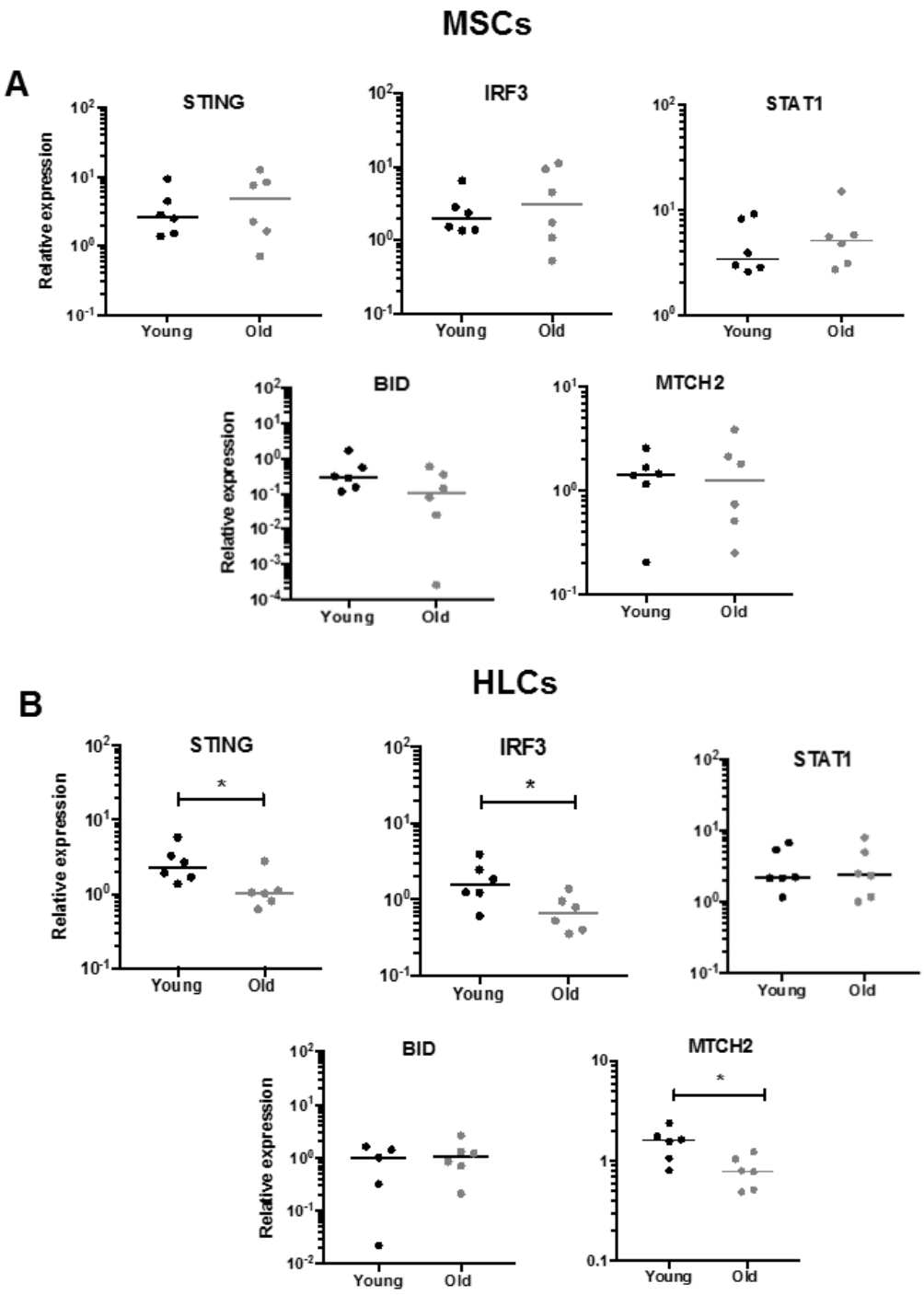

Figure 5.14 Expression of genes associated with IFN1>BID>ROS pathway

(A) Expression of *STING*, *IRF3*, *STAT1*,*BID* and *MTCH2* in young and old donor MSCs **(B)** Expression of *STING*, *IRF3*, *STAT1*,*BID* and *MTCH2* in young and old donor MSCs. Young and old donors are indicated in black and grey dots, respectively. Horizontal line across data set indicates median values. $p<0.05$*, Mann-Whitney U test

Table 5.2 Exploratory genes, age-related fold differences and their *p* values in MSCs

Gene	Medians (young)	Medians (old)	Age-related difference*	*p* value
IFNA1	5.136	9.15	1.78	NS
IFNB1	1.247	1.725	1.38	NS
IFNAR1	4.278	4.757	1.11	NS
IFNAR2	5.82	5.76	0.99	NS
STING	2.63	4.88	1.86	NS
IRF3	1.946	3.13	1.61	NS
STAT1	3.42	5.16	1.51	NS
BID	0.3	0.11	0.37	NS
MTCH2	1.43	1.27	0.89	NS
Kl	0.51	0.48	0.94	NS
Sirt6	0.506	0.406	0.80	NS
Tp53	2.25	2.88	1.28	NS
IL6	0.05	0.2	4.00	NS
IL7	1.481	1.169	0.79	NS
IL8	2.24	5.14	2.29	NS

*Age-related difference calculated by median expression in old donor/median in young donor. NS: Non-significant, Gene expression is normalised to *HPRT1.* Mann-Whitney U test

Table 5.3 Exploratory genes, age-related fold differences and their *p* values in HLCs

Genes	Median (young)	Median (old)	Age-related difference*	*p* value
IFNA1	0.295	7.443	25.23	NS
IFNB1	1.53	1.591	1.04	NS
IFNAR1	1.1	0.48	0.44	NS
IFNAR2	2.75	1.38	0.50	0.041
STING	2.293	1.033	0.45	0.026
IRF3	1.561	0.66	0.42	0.0411
STAT1	2.17	2.4	1.11	NS
BID	1	1.03	1.03	NS
MTCH2	1.61	0.79	0.49	0.0152
Kl	0.04	0.011	0.28	NS
Sirt6	0.28	0.15	0.54	NS
Tp53	2.93	1.21	0.41	0.0043
IL6	0.16	0.6	3.75	NS
IL7	0.038	0.013	0.34	NS
IL8	46.47	67.21	1.45	NS

*Age-related difference calculated by median expression in old donor/median in young donor. NS: Non-significant, Gene expression is normalised to *HPRT1.* Mann-Whitney U test.

5.4 Discussion

This chapter presents novel data with respect to the presence and expression of genes associated with senescence, SASP and anti-ageing genes in uncultured BM MSCs. There were no differences observed in their expression between young and old donor MSCs but all their expression declined in HLCs with *Tp53* displaying a significant decline in expression. In SASP, while *IL6* showed a non-significant increase in old donors in both MSCs as well as in HLCs*, IL7* and *IL8* showed no particular differences in old donors in both, MSCs as well as in HLCs

With respect to a the exploratory IFN1 family of genes, a number of novel findings were observed. Firstly, a large number of genes were found to be differentially expressed. Second, there were more highly expressed genes in MSCs over HLCs rather than the other way around, which was unexpected. This indicated towards the potential immune-related functions of BM MSC via IFN1 pathway as indicated by cancellous bone MSCs previously (316), that will require further explorations. Interestingly, *IFNA1, IFNB1* and the *IFNA* receptors, were expressed significantly higher in MSCs as compared to HLCs. Genes involved in the IFN1>BID>ROS pathway (403) also revealed interesting trends with *STAT1* and *IRF3* indicating greater expression in MSCs, while *BID* displayed higher expression in HLCs as compared to MSCs and this supported the hypothesis of this chapter (31). Among the senescence associated and anti-ageing genes, *Kl* displayed higher expression in MSCs in comparison to HLCs. Finally, in cytokines, *IL7* displayed higher expression in MSCs as compared to HLCs and *IL8* displayed otherwise.

When compared for age-related differences; *IFNA1, IFNB1* and *IFNA* receptors showed no differences in MSCs, but *IFNAR2* displayed a significant decline in HLCs from old donors. The only IRG that showed an age-related difference in MSCs was *RNF213*, while a number of IRGs were found to display a decline in their expression in HLCs. Analysis of genes involved in the IFN1>BID>ROS pathway also showed no changes in expression between young and old donors in MSCs, while in HLCs, *STAT1, IRF3* and *MTCH2* displayed a significant decline in expression between young and old donors.

A recent study investigating the effect of retrotransposon (RT) in human fibroblasts found that the RT in human (long-interspersed element-1 or L1) becomes transcriptionally depressed in senescent cells which activates IFN1 response (336). The senescent cells were prepared by culturing them *in vitro* until their proliferation ceased. They found a high percentage (68%, 57 out of all the 84 tested genes) to be

highly upregulated in senescent cells which were IRG or associated with the IFN1 signalling.

Over 30 of these genes were also tested in this thesis but in relation to age in uncultured BM MSCs and did no observe any age-related grouping in cluster analysis. Also, while other studies found a number of IFN1 associated genes to be upregulated in replicative senescent cells (336), this thesis found a significant decline in only one of the IFN1 associated genes in MSCs. This could be due to the fact that firstly, the cells described in this thesis were MSCs and not skin fibroblasts and secondly, the MSCs and HLCs used in this thesis were uncultured and did not undergo replicative senescence by *in vitro* culture expansion.

The same study also examined the presence of L1 in aged mice and found a significant increase in IFN1 associated genes. They treated old mice with nucleoside reverse transcriptase inhibitors known to antagonise the effect of L1 reverse transcriptase. In doing so, they observed that not only did the IFN1 associated genes showed a downregulation but the SASP pro-inflammatory state was also alleviated. Their *in vivo* mice study thus linked senescence to ageing in mice. The only SASP gene that was found to increase in this project was *IL6* while *Tp53* and IFN1 associated genes were mainly found to decline in older donors, unlike in the above mentioned study. Similar treatments using nucleoside reverse transcriptase inhibitors in uncultured BM cells in a larger number of donors could shed light on the senescence profile of BM cells in association with IFN1.

In this project MSCs did not display any difference in the level of expression of any of the above genes between young and old donors except RNF213 that showed a significant decline. RNF213 or ring finger protein has also been known as ‘mysterin’ owing to the lack of knowledge curated about this gene. It is known as the causative gene for Moyamoya disease which is a disease of blocked arteries within the brain. The disease is more prevalent in East Asian countries of Japan, Korea and China and is more common in women (405). Not much is known about the gene and more exploratory studies are needed to reveal its function in MSCs, as well as in HLCs.

There were a good number of transcripts with over 2-fold age-related differences in MSCs. For example, pro-inflammatory cytokines *IL6 and IL8* were found to be 4-fold and 2-fold higher in the old donors MSCs as compared to young donors, respectively. Of note, while literature suggests that *IL6* is higher by 2-4 fold in older donors (MSCs), much higher the fold difference is suggested to be in circulating levels of this cytokine

in old donors with chronic inflammation (406). Age-related differences were more prevalent in the HLCs, wherein genes like IFN regulatory factor 2 and 9 (*IRF2* and *IRF9*) and cyclin D2 (*CCND2*) displayed a significant decline in old donor HLCs. Similar decline in IRGs has been observed in the peripheral blood mononuclear cells in older donors (394).

BID and MTCH2 regulate the oxidative metabolic state of HSC and thus manage their oxidative stress, which was discovered not too long ago. The hypothesised mechanism behind it was described by Gross et al., in 2017 where DNA damage in the nucleus lead to accumulation of ROS in mitochondria via the IFN>BID>MTCH2 pathway. HSCs as a population have extensively been studied for understanding their biology and ultimately for use in several cell therapies. Not only did this project confirm the expression of these transcripts but also compared it between young and old donor MSCs. While no age-related differences were observed in MSCs, there was a significant age-related decline in *MTCH2* observed in the HLCs.

An age-related decline in IFN1 signature genes was also observed in activated $CD4^+$ T cells from donors of older age (65-85 years old) as compared to younger donors (20-35 years old) when investigated in cells isolated from peripheral blood mononuclear cells (407). Considering that IFN1 are an integral part of the innate immune system that is responsible for triggering responses in bacterial and viral infections, it may suggest that the decline in IFN1 in old age compromises their ability to respond to these infections. With the knowledge that DDR can activate IFN1 signalling and that DNA damage increases in the older age, it may also explain the reduced ability of recognising this damage in older donors owing to the decline in IFN signature genes. Even though the number of investigation of IFN1 on MSCs remain limited, the existing studies have shown the presence of IRGs in culture expanded BM MSCs. The study also showed an increase in the number and size of colonies when BM MSCs were treated with monoclonal antibody against the IFNAR1 chain of human type 1 (385).

The transcripts associated with anti-ageing and senescence namely *Kl, Sirt6* and *Tp53* showed no age-related differences in uncultured BM MSCs. *Kl* has been a well-known anti-ageing gene, the absence or deletion of which observed ageing-like syndrome in mice. In general, overexpression of *Kl* has been observed to increase lifespan, whereas repressing the transcript has been associated with premature ageing. *Tp53* is crucial for DNA repair and induction of apoptosis and senescence and has previously been shown to be increased in MSCs from old donors (96). Age-related decline in *Tp53* in HLCs potentially indicates decline in autophagy ability of cells.

A study in 2016 aimed at identifying senescent cells within mice bone microenvironment and performed varying investigations in T cells, B cells, myeloid cells, MSCs, osteoblasts as well as in osteocytes of young (6mo) and old (24mo) mice (365). They observed an increase in senescence and SASP profile in old mice. While *P16* increased in all the cell types, *Tp53* was highly expressed in osteocytes and myeloid cells and not in MSCs. However, in this thesis, *Tp53* was found to expressed higher in MSCs as compared to HLCs and declined in older donors in both MSCs and in HLCs which was unexpected as *Tp53* increases in old senescent cells (408).

With respect to studies in humans, De Oliveira found that *Kl* down-regulation induced premature senescence in human fibroblasts and downregulation of *p53* in *Kl* attenuated cells restored normal growth in the cells (382). Overall their study demonstrated that *Kl* regulated cellular senescence via the p53/p21 pathway highlighting on the anti-ageing role of *Kl.* More recently, Zhang et al., found that addition of Kl protein to MSC cell lines, attenuated their osteogenic differentiation ability (409). Another human study examined the serum of healthy volunteers and found that Kl declined with increasing age. Their study had a broad range of donor age (0.1-88 years old) including toddlers as well as old donors. While they did observe a decline in Kl across the entire age range, they segregated their young and old groups age <17 as young donors and over 20 years old as old donors (380). The specificity of the *Kl* in MSCs has never been showed before making the data from this project very novel. As an anti-ageing gene, this transcript could potentially be used to understand ageing *in vivo* in MSCs.

Along with *Kl, Sirt6* is another transcript that is recognised for its anti-ageing potential, the deficiency of which has been associated with age-associated degenerative processes (410). It is known to be expressed in BM MSCs and Sun and colleagues found that while its knockout has resulted in decreased osteogenic differentiation and proliferation, its overexpression increased osteogenesis in mice (373). They also found that Sirt6 was doing so via partial suppression of the NF-kb pathway in old mice. Zhang et al., also found the Sirt6 promoted osteogenic differentiation in mice (411), however, their results contradicted the results of Sun and colleagues, and demonstrated the involvement of the BMP pathway instead. Around the same time, another study that generated MSCs from human embryonic stem cells found that Sirt6 played a protective role against ROS in MSCs (374). They found that cells that were depleted of Sirt6 not only were more susceptible to accelerated degeneration but were also more vulnerable to the negative impacts of ROS. Considering these findings, we expected an age-

related decline in the expression of *Sirt6* in uncultured MSCs but did not observe any differences in this project.

Taken together, the anti-ageing transcripts and transcripts associated with senescence showed significant differences in HLCs, but no difference in MSCs. This was as expected in the hypothesis suggesting the MSCs *in vivo* are relatively more resilient to ROS and DNA damage in comparison to HLCs. Previous investigations of these transcripts in uncultured MSCs is sparse, making data from this thesis novel.

Among the cytokines, *IL6* showed no significant difference in its expression level between MSCs and HLCs. This was unexpected as IL6 has closely been associated with inflammation (351). In contrast, *IL7* exhibited very high expression levels in MSCs and *IL8* displayed higher expression in HLCs. *IL6* has been a well-known indicator of inflamm-ageing and has been reported to be higher in old donors and significantly higher in old donor with inflammation (406). Thus it was no surprise that the expression of *IL6* was found to be higher in old donors in both cell types, MSCs and HLCs. IL6 and IL8 are also associated with SASP and have been shown to increase in MSCs that were DNA damaged (354). However, that study was based on MSCs and HSCs in co-culture conditions. In this thesis, the expression of *IL7* showed no age-related differences in either cell populations while *IL8* displayed a non-significant increase in HLCs.

Considering that both HSCs and MSCs originate within the BM (section 1.3.1), they are bound to impact each other. The protective role of MSCs in preventing HSCs from ROS with the help of *Cx43* has already been discussed in section 4.4. It is a well-known fact that age-related changes of increased adipogenesis in MSCs and shift towards myeloid lineage (myeloid skewing, section 1.3.1) in HSCs occur within the same BM niche. However, as the two belong to different stem cells types, they have often been examined separately. Among the few scientists to have linked the two and acknowledge the relationship, were Kovtonyuk and colleagues in their 2016 study (412). Referring to previous studies, they outlined that adipocytes negatively impacted HSC functions supporting B-cell lymphopoiesis. As adipogenesis increased in old age, there is a high possibility that it enhanced the process of age-related myeloid skewing in old individuals.

In summary, this chapter was the first to quantify the expression level of IFN1 associated and other genes in uncultured BM cells. Interestingly, a large number of genes were found to be highly expressed in MSCs as compared to HLCs and this

finding is novel. Furthermore, a number of genes also displayed age-related differences, more in HLCs than in MSCs, and displayed the potential link to DNA damage, IFN1, aging, senescence and oxidative stress. HLCs appear more vulnerable to age-related damage at cellular level as compared to MSCs *in vivo*. Future work would involve exploring the protein levels and functions of the genes that displayed significantly higher expression in MSCs than in HLCs. Ideally, this would require recruitment of a larger donor cohort and controls including investigation of cells that were exposed to extreme oxidative stress prior to analysis of gene and protein expression. Correlation of this data with DNA damage performed by comet assay and senescence assay from the same donors would provide extremely useful links between IFN1, ageing, ROS, DNA damage and senescence.

Chapter 6 Gene and surface marker expression of human BM MSCs in hip osteoarthritis

6.1 Introduction

Osteoarthritis (OA) is a classic age-related degenerative disease which, some researchers think, is inevitable in old age. It is by far the most common joint disease in the world. It leads to a poor QOL by causing debilitating pain in the joint which in turn impacts gait, all of which reduces the daily functions (100). In spite of considerable variation in the definition of the condition, age has been an underlying factor for most of the cases observed (413). While the frequency of OA incidences have been found to increase with age, it is known that not all old people suffer from the disease (414). Increasing age does however, escalates the chances of someone suffering from OA due to decrease in body immunity and accumulation of damage at the cellular level including MSCs (100).

Section 1.3 of this thesis outlined that the impact of OA is not only confined to the patients but also affects the family members and the economy in general, considering the high rate of occurrence of the disease, globally. While OA may have different aetiology and be caused by various factors like lifestyle, mechanical load on the joints, genetics, environmental factors, physical damage and injury; the management of the disease usually involves the same approach. This indicates our lack of understanding in the disease, especially in terms of its pathophysiology at the *in vivo* cellular level. The other example supporting our lack of understanding of the disease are many different theories of OA development and disease progression, which vary between research scholars and OA clinical experts alike.

Early detection of the disease can significantly prevent and/or alleviate the pain and the poor QOL in patients, before it gets to the point where surgery is the only option available. This outlines the need for an increase in the intensity of basic research and applied science research for early OA diagnostics. The last decade has seen a rise in the idea that OA is not a single disease, but a culmination of a number of progressive conditions. One of these conditions may indeed be the premature ageing of bone resident MSCs, leading to altered bone homeostasis and the inability of the subchondral bone to support cartilage nutrition and shock absorbance properties (415, 416). To better understand these individual contributing factors and their pathologies, a number of OA animal models that mimic different aspects of the disease in humans have been developed (417). Similar to the animal models used for understanding the

ageing of MSCs (or even ageing in general), animal models of OA too are varied in several parameters. A number of models exist, each of which have their own advantages, limitations and their applicability for OA ageing studies is discussed below.

6.1.1 Hip OA

In the last 15 years, the aetiology of hip OA has been proposed as different to knee OA. While a number of factors like sex, obesity, genetics, occupation and local injuries are known to contribute to the progression of OA (418), age has strongly been associated with hip OA displaying a sharp increase of incidence from 0.7% in people aged 40-45 to 14% in people aged over 85 (419). Many of the strategies provided for hip OA management have been derived from those developed for knee OA. This has been due to the higher prevalence of knee OA worldwide and due the fact that knee OA is relatively easier to image, scan and analyse as compared to OA of the hip (420).

Pain and stiffness in the hips are the first symptoms of OA. Diagnosis of hip OA is either by radiographic evidence or by clinical examination or a combination of both. Radiographically, K/L scale (section 1.1.5) is used to determine the extent of the damage in the hip joint. Higher K/L score indicates greater extent of bone and cartilage damage including osteophyte formation, narrowing of joint space and subchondral bone sclerosis (421). In spite of radiographic evidence providing significant information about OA of the hip, it has often been criticised when used by itself for the diagnosis of OA. There is strong evidence in the literature that suggests that not every patient with hip pain has radiographic signs of OA. Also, most elderly patients with higher risk of hip OA did not show its evidence by radiography (422). Another study in 2013 'compared the sensitivity of physical examination with radiographs' in the diagnosis of clinically significant hip OA. Interestingly, they found that physical examination was significantly more accurate in diagnosis of hip OA as compared to radiography (423).

Treatment of OA of the hip does not follow an approach any different from that of OA of another part of the body. Despite of the different theories/hypotheses existing around the progression of the disease, the treatment is fundamentally symptomatic. Use of NSAIDS, pain management and changes in lifestyle is often suggested to the patients (424). These include pharmacological intervention similar to those described earlier in section 1.1.5 along with other medications such as anti-resorptives (425). NSAIDs work by inhibiting cyclooxygenase 2 (COX2) which blocks the production of prostaglandins (PGs) at sites of inflammation and tissue damage (426) while anti-resorptives, as the name suggests, function largely by supressing bone resorption.

Unfortunately, most of the cases of a detected hip OA often result in a much invasive hip replacement surgery. The extent of hip damage is what determines whether or not an adult needs to undergo the hip replacement surgery. The surgically added hip implant usually stays intact for up to 15 years, after which there might be a revision surgery. The revision surgery often has poorer impact on supporting the patient after 5 years of revision as compared to the primary total hip replacement (THR) surgery. THR with metal-on-polyethylene bearing surfaces remain the gold standard for the treatment of end-stage hip OA. It provides good relief from pain along with steady longevity as observed across patients (427).

From the literature presented in the above two sections, it is evident that a better understanding of hip OA pathophysiology, including the 'age component' of the disease, could have a significant socioeconomic impact, and that there is no suitable animal models faithfully replicating its progression in humans. The study of MSCs in hip OA joints, and comparing their characteristics to MSCs from healthy aged individuals may shed new light on the progression of this condition potentially leading to new therapies.

6.1.2 Senescence, SASP, ROS and OA

Senescence is a process of irreversible growth arrest that has been observed in ageing cells, and has been outlined in this thesis in sections 1.2.2 and 5.1.1. Considering OA has been associated with older age, a number of studies have investigated cell senescence in OA (428) largely focussing on OA cartilage (429). The presence of senescent cells and the associated secretory phenotype has been found to be significantly higher in OA cartilage compared to healthy cartilage. The SASP detected in OA has largely been attributed to pro-inflammatory cytokines like IL1, IL6 and IL8, and matrix metalloproteinases (MMPs) (430). The initiating factors are believed to be increased DNA damage (431) due to increased ROS (432, 433) leading to increased inflammatory mediators. The presence of senescence in OA is further confirmed by the development of OA-like condition in mice that were injected with senescent fibroblasts in their knees (434).

Basic research has steadily contributed to our knowledge of the multi-factorial aspects of OA including the role of age-related senescence (428) and inflammation in the disease pathogenesis (435). Even though the pieces of the puzzle of OA and senescence are far from complete, there has been significant increase in our understanding of the two. The fact that both, senescence and OA are multifactorial and need more research at the cellular level, challenge the process of collection of relevant

evidence in spite of the hypothesised potential link between the two. Nevertheless, there is a growing body of evidence connecting the impact of cell senescence to primarily degenerative diseases, like OA.

IL6 has been shown to be expressed in high quantities in the serum of patients with OA (436, 437). The Chingford study (Livshits et al., *2009*) on middle aged British women with radiographic knee OA (RKOA, K/L grade≥2) confirmed that higher circulating levels of IL6 in obese patients were indicators for potential OA in future. They found that first, the circulating levels of IL6 in patients with RKOA were consistently higher than in non-OA donors, which increased proportionally with increase in OA severity, and second, that the levels of IL6 increased with increasing BMI (437). Thus, they suggested that higher BMI and increasing serum levels of IL6 are both predictive biomarkers of RKOA and outlined the need for future research on IL6 in OA. This study importantly highlighted the impact of lifestyle (obesity) as a contributor to the disease.

Obesity results in an increased load on joints along with the secretion of adipogenic factors (like adipokines, adipogenic hormones) that mediate inflammatory responses. Also, the fact that there is a well-known shift from muscle mass and bone formation towards increased adipogenic tissue formation also suggests that obesity contributes to both, inflamm-ageing and OA. Another factor that crops up when discussing adipogenic factors and OA, is the presence of harmful reactive oxygen species (ROS) as data from previous research indicates that ROS may be key contributor to the adipogenic bias of MSCs at the expense of their osteogenic potential (438).

The role of ROS in most diseases has been found to be fundamental, owing to the fact that ROS is a by-product of cellular metabolism. It has been closely linked to a number of diseases occurring in almost every organ in the human body (281). This is expected as every organ is made of functionalised tissues which are basically, groups of highly specialised cells. As it may be expected, ROS has been found to be a key contributor of age-related articular cartilage degradation, which has been a major evidence correlating ageing to OA (439-441). In particular, ROS was suggested to contribute to the loss of proliferative capacities of chondrocytes, senescence of chondrocytes, and increase in the production of inflammatory markers along with an increase in oxidative stress in OA cartilage (441, 442).

Alterations in cartilage proteoglycans and other proteins within the cartilage matrix have been discussed at length in several publications over the last two decades (443, 444). The increase in alterations that eventually lead to the drastic increase in ROS

within cells has been thought to be due to the imbalance of natural anti-oxidants in the body (section 4.1.1.3.) Considering this imbalance, several research groups have assessed ROS damage by quantifying ROS in OA samples or by measuring the anti-oxidant enzymes within the cells (445-447). Theoretically, an increase in the ROS levels in OA (433) is expected to be accompanied by a decrease in the levels of anti-oxidant enzymes (446, 447) that ultimately lead to genomic instability and cell senescence of chondrocytes (448).

Regan and colleagues examined the levels of extracellular SOD (SOD3) in the cartilage of hip OA patients by ELISA and found a 4-fold decline in the levels of the SOD3 in OA donors (445). Similarly, other studies have confirmed the decline in the levels of the different naturally occurring anti-oxidant enzymes in OA, but for all of these studies, the focus has been on cartilage/ chondrocytes (449, 450). Altogether, evidences discussed above suggest that age and lifestyle choices taken together may play an important role in OA pathogenesis. These changes manifest themselves as inflamm-ageing and oxidative stress that lead to cellular senescence, which in itself may be a strong contributor to OA. While current research focuses mostly on chondrocytes, similar processes may also occur in MSCs residing in the underlying subchondral bone.

Results from previous chapter indicated the potential involvement of certain IFN1 transcripts in age-related changes within the BM niche. It was thus worth examining if these IFN1 pathway transcripts have any role in OA pathogenesis, even though there has been no such investigation so far. Interestingly, in Lupus, an auto-inflammatory disease extensively studied for the role of IFN1 in its progression, BM MSCs have been shown to be senescent and express altered IFN1 signature. Based on the outlined literature evidence, it could be hypothesised that not only chondrocytes, but also MSCs from OA patients could possess an increased expression of senescence and SASP associated genes, compared to non-OA old age donors. On the other hand, the expression of anti-ageing genes *Kl* and *Sirt6* could be lower. With respect to IFN1 and associated genes, there are no studies that have investigated them with respect to OA. A number of studies, however, have indicated an association between increased IFN signalling in Lupus with increased MSC senescence in the disease, which may also take place in OA MSCs (387, 391, 451).

6.1.3 Changes in MSC multipotentiality genes in MSCs from OA donors

Previous investigations of age-related changes in OA have focussed on the articular cartilage where the first noticeable physiological alterations of OA have been historically found. Only relatively recently, OA has begun to be acknowledged as a disease of the entire joint including considerable changes in the subchondral bone (452), as well as cartilage (outlined in section 6.1). The limited research performed on primary OA guinea pig model indicated that alterations in microstructures of subchondral trabecular bone pointed towards changes in the bone preceding the visible changes in the cartilage (171). However, investigations performed on human bone/bone cells and consequently, the data available for human bone cells including MSCs remains very limited.

A recent study from our laboratory has established that the numbers of MSCs measured by both CFU-F assay and flow cytometry was significantly higher in OA-affected bone (in bone marrow lesions, BML) as compared to a less affected bone in hip OA patients. Compared to non-BML MSCs, the proliferative capacity of BML-MSCs was lower, and they had altered expression levels of bone remodelling molecules *RANKL* and *OPG* (183). While this was the first study to demonstrate numerical, topographical, gene expression and functional alterations in MSCs from OA hips, these examinations were performed on culture expanded MSCs. Furthermore, no study has been yet performed to compare gene expression profiles of MSCs from OA patients with age-matched healthy individuals.

A subsequent study from our group has shown increased expression of bone lineage-related transcripts *RUNX2, OPG* and *SPARC* in hip OA MSCs compared to healthy bone (69), however while their OA MSC donors were in the age-range of 55-89 years old, their healthy controls ranged from a much younger 38 years old up to 93 years old and not age-matched (section 1.3.1).

This chapter aimed at exploring MSC multipotentiality genes, senescence and anti-ageing genes, IFN1 genes and other exploratory genes previously explored in Chapter 4 and Chapter 5, in uncultured MSCs from OA donors compared to MSCs from a cohort of age-matched healthy old donors. The investigation was also performed to find whether genes that displayed age-related trends in the previous two chapters were further aggravated in OA.

The list of transcripts chosen for investigation in this chapter is presented in Table 6.1 including available literature indicating any alterations in these molecules in OA patients.

Table 6.1 List of genes investigated for study in MSCs from OA donors

Gene	Gene (full name)	Evidence in OA
RUNX2	Runt-related transcription factor 2	(69, 453)
SPARC	Secreted Protein Acidic And Rich in Cysteine	(69)
SFRP1	Secreted frizzled-related protein 1	
PPAR-γ	Peroxisome proliferator activated receptor gamma	(69)
FABP4	Fatty acid binding protein 4	(69)
LepR	Leptin receptor/CD295	(454)
SOD3	Superoxidedismuatse 3	(445)
RANKL	Ligand for Receptor Activator of Nuclear factor-Kappa B	(69)
OPG	Osteoprotegerin	(69)
RANK	Receptor Activator of Nuclear factor-Kappa B	
CXCL12	C-X-C motif chemokine ligand 12	(455, 456)
Cx43	Connexin43	(457)
IFNA1	Interferon alpha 1	
IFNB1	Interferon beta 1	
IFNAR1	Interferon alpha receptor 1	
IFNAR2	Interferon alpha receptor 2	
STING	Stimulator of interferon genes	
IRF3	Interferon regulatory factor 3	
STAT1	Signal transducer and activator or transcription 1	
BID	BH3 interacting domain	
MTCH2	Mitochondrial carrier homolog 2	
Kl	Klotho	(409)
Sirt6	Sirtuin 6	(458, 459)
Tp53	Tumour protein 53	(460)
IL6	Interleukin 6	(437, 461)
IL7	Interleukin 7	
IL8	Interleukin 8	

List of transcripts investigated and discussed in this chapter. The top panel includes MSC multipotentiality genes and the bottom panel includes exploratory genes that were investigated in chapter 5 and will be explored in this chapter.

6.1.4 Changes in age-related surface markers in MSCs from OA donors

Among the age-specific surface markers discussed previously in this project (Chapter 4), CD106 expression levels on OA synovial fibroblasts has been reported to be highly elevated as compared to non OA synovial fibroblasts (462). Schett and colleagues in 2009, examined the level of CD106 in the serum from control and hip OA donors and found significantly higher levels of CD106 in the OA donors (463). Not too long after that, Pulsatelli and colleagues found that the serum levels of VCAM-1/CD106 were

significantly higher in donors with erosive hand OA compared to non OA hands (464). Another study showed that the levels of serum CD106 was an indication of the number of joints affected by OA (465). More recently, a study investigated CD106 in hip OA and found no significant increase in men or women or in the total population with respect to the CD106 levels (466). Serum CD106 could be shed from many different cell types inside the body, and no study has yet explored CD106 expression on uncultured hip OA MSCs or HLCs. Based on this evidence, it was hypothesised that expression of CD106 levels on hip OA MSCs or HLCs would be higher in OA.

The growing evidence connecting obesity to OA in the last two decades has witnessed an increased interest in the hormone Leptin and its potential contribution to OA (467). However, the hormone Leptin has been studied considerably more than its receptor. An increase in both, the levels of the hormone leptin and its receptor CD295 in the synovial fluid (468), subchondral osteoblasts (469) and cartilage of OA patients has been observed, correlating positively with the severity of the disease (454). More recently, Voultaneeho et al., in 2014 and Moqi Yan and colleagues in 2018 have found leptin with its receptor contribute to obesity in OA development (470). Voultaneeho et al., suggested that in an obese person, there is an unbalanced leptin signalling which results in an increased production of pro-inflammatory factors (270). Their work, in some way connects Leptin to OA along with the pro-inflammatory factors which have often been associated with inflamm-ageing. Co-incidentally on similar lines, Stannus and co-workers investigated the ‘association between Leptin, IL6 and radiographic hip OA in older people’ (471). While they did not mention the term ‘inflamm-ageing’ in their article, their data set forms the baseline for future studies to investigate the association between OA, obesity and inflamm-ageing. Based on all the evidence, it was hypothesised that Leptin receptor/CD295 levels in OA MSCs would be increased.

Cx43 was another surface marker that was investigated in this project due to its high prevalence on bone cells and related functionalities (section 4.1.3.3). Apart from being closely associated with intercellular interactions within bone cells, Cx43 has also been identified as a crucial player for cell communication in OA. Andrew and colleagues in 2004, found that the number of gap junctions in synovial biopsies of OA donors were increased (4-fold) as compared to the non OA donors (472). In 2014, Gupta et al., showed that the overexpression of Cx43 in human synovial fibroblast cell lines enhanced the expression of genes associated with inflammation in OA and a decrease in the expression of Cx43 in these cells, reduced the expression of many of the catabolic and inflammatory genes (473). In agreement, Casagrande and colleagues found a significantly higher level of Cx43 in the cartilage of shoulder OA patients

compared to healthy controls. Higher levels of Cx43 at the protein level were also observed in human chondrocytes (457). In another investigation, Shichomura et al., compared the levels of Cx43 in patients with RA, OA and in healthy controls (474). In contrast to the above mentioned studies, they found that Cx43 was 'hardly detected' in patients with OA. Based on this evidence Cx43 was expected to be most likely elevated in OA MSCs.

CD146 or MCAM, as discussed previously (Chapter 4) has been used to define MSCs in a number of human tissues (227, 229). The sparse data available for this marker and how its expression alters with the progression of OA, suggests that CD146 positivity was an indication of late stage OA (475). However, another study did not find any other major differences between OA patients and controls with respect to CD146 expression (476). More recently, another group examined the MSCs from synovial fluid of OA patients and found a significant increase in the number of colonies and colonies of larger diameter from OA donors (477). They also inspected the levels of CD146 in both control and OA MSCs by flow cytometry, but found no difference in its expression. Based on this contradicting evidence, CD146 expression on OA BM MSCs was expected to be similar to age-matched healthy MSCs and display no OA-related differences.

The investigations on cellular senescence in OA remain largely limited to cartilage and chondrocytes and information on bone cells in OA related senescence has a long way to go before catching up with the amount of information available on cartilage. Obesity, DNA damage, senescence, ROS - all of these factors appear to play a key role in OA, therefore any alterations in the signalling molecules connecting these pathways in OA MSCs would be a highly novel finding.

This chapter aimed at investigating the expression of all the genes in Chapter 4 and 5 and the surface markers in MSCs and HLCs from hips of OA donors, in comparison to healthy ageing to explore any trends of healthy ageing that could be further aggravated in OA.

6.1.5 Hypotheses and objectives

The following hypotheses and aims were postulated for this chapter

Hypotheses:

1. The transcripts for BM MSC osteogenic and adipogenic differentiation potential, stromal functions and bone remodelling, in $CD45^{low}CD271^{+}$ MSCs are altered in OA patients compared to age-matched healthy individuals.
2. The transcripts related to senescence and IFN1 pathways in $CD45^{low}CD271^{+}$ MSCs are altered in OA patients, with possible increases in senescence and IFN1 related genes as compared to age-matched healthy individuals.
3. Surface markers CD106, CD295, CD146 and Cx43 are altered in OA patients, with possible increases in CD106 and Cx43 as compared to age-matched healthy individuals.
4. Based on findings from the previous chapter, that HLCs were more susceptible to age-related differences than MSCs, OA-related trends in $CD45^{+}CD271^{-}$ HLCs were expected to be more pronounced compared to MSCs.

Objectives:

1. To quantify the level of transcripts indicating BM MSC osteogenic and adipogenic differentiation potentials, stromal functions and bone remodelling, in MSCs from OA patients by qPCR and compare with old donor MSCs.
2. To quantify the level of transcripts related to senescence and IFN1 pathway in BM MSCs and HLCs from OA patients by qPCR and compare with old donor MSCs.
3. To quantify surface markers CD106, CD295, CD146 and Cx43 in MSCs from OA patients by flow cytometry and compare with old donor MSCs.

6.2 Methods

6.2.1 Donor selection

Appendix 2, Table 4 outlines OA samples used. The donor cohort included 5 males and 8 females ranging from 56-83 years old with a median age of 74. Exclusion criteria included any history of cancers, previous surgery of the hip or any other disorder directly affecting the bone. As these donors were mostly above the age of 60, they were more likely to have primary (non-traumatic) hip OA. The FHs once obtained after surgery, were digested in collagenase for 4 hours and frozen by a colleague (183).

6.2.2 Cell sorting

Frozen vials with up to 10^6 nucleated cells from digested OA FHs were defrosted using TM (section 2.2) and washed with PBS for the elimination of any residual DMSO (section 2.4). Once washed, cells were counted and distributed into FACS tubes and stained (refer section 2.5, Table 2.1). Owing to the large number of MSCs in the FH digest (183), the cells from FHs were used not only for the purpose of OA MSC and HLC sorting, but also for instrument setting for MSCs from BMA.

For this purpose, these cells were used as both unstained cells and cells stained with isotype controls to establish the sorting gates for positively-stained cell populations. In contrast to rare BM MSCs, these cells did not undergo any enrichment (section 2.4) prior to cell sorting. This experimental design helped to preserve rare healthy MSCs for sorting (as none were wasted for instrument settings) and ensured consistency as healthy and OA MSCs were always sorted on the same day. The cells were treated with a blocking buffer for 10 minutes followed by staining with respective antibody or isotype control (section 2.5, Table 2.1).

In the end, 7-AAD was added to the cells and they were then passed through a filter-capped FACS tube to prevent any cellular aggregates to avoid blocking of the flow channel. The instrument settings and the isotype controls were set as explained in section 2.4 and the sorted cells were collected directly into a tube containing lysis buffer. The populations collected included the same two populations of $CD45^{low}CD271^{+}$ (MSCs) and $CD45^{+}CD271^{-}$ (HLCs) as previously described for healthy BM MSCs (section 2.4).The gating strategy and the histograms for single markers are shown in Figure 6.2 below

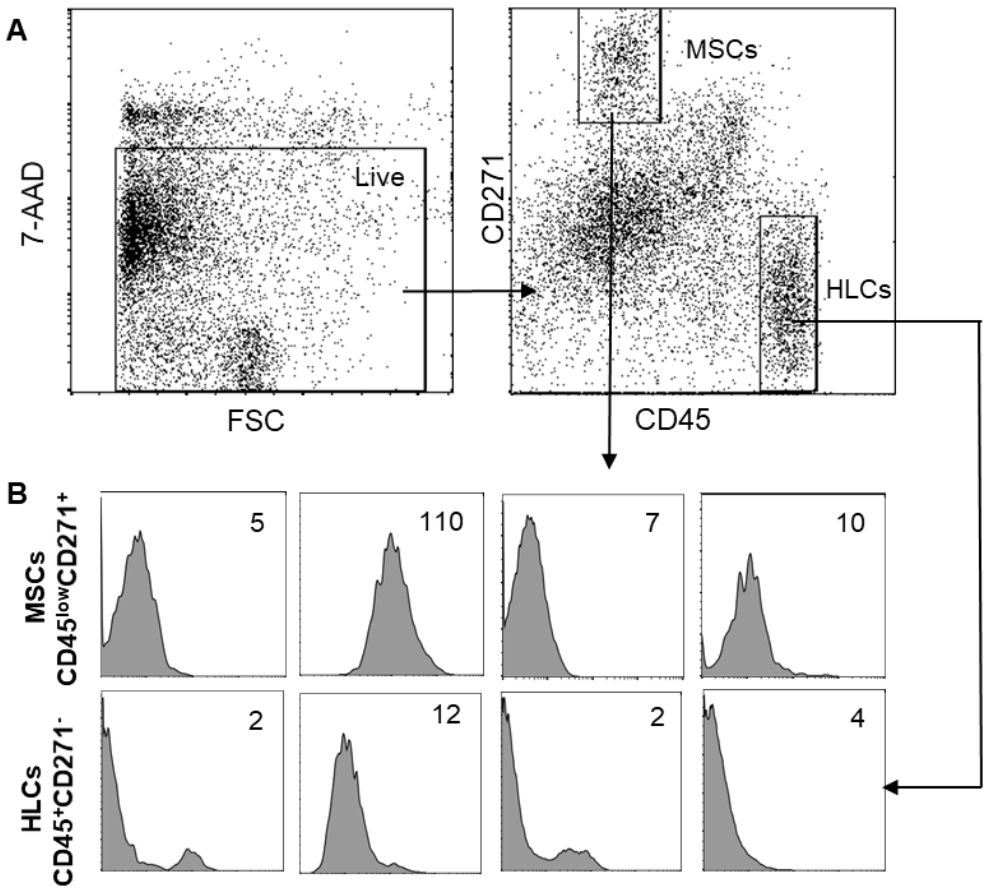

Figure 6.1 Quantification of surface marker expression in OA FH MSCs and HLCs

(A) Gating strategy for identification of live cells based on 7-AAD negativity followed by CD271 and CD45 gating for the selection of populations of interest (MSC and HLCs) **(B)** Histograms for individual surface markers from representative donors in MSCs and HLCs from representative donor. Numbers in the top-right corner indicate MFI.

6.2.3 Gene expression

Transcripts were investigated (Table 6.1) out of which the first 12 were MSC multipotentiality transcripts and the next 9 were associated with senescence and anti-ageing, IFN1 pathway, and ROS. The procedures followed for the quantification of MSC multipotentiality transcripts has previously been described in Section 2.5. In brief, RNA was extracted from cell lysates (obtained from cell sorts) and stored in -80°C until further use. 2µl of RNA was reversely transcribed to cDNA which was stored in -20°C until further use. All samples were then preamplified in the thermocycler using 18 cycles and were stored at −20°C before processing on the BioMark HD. Quantitative PCR was performed using Fluidigm Flex Six™ (MSC multipotentiality genes) and 48.48 IFC (genes associated with senescence and anti-ageing, IFN1 pathway, and ROS) on BioMark HD. Samples and assays were loaded into the reaction chambers of the Flex Six™ using the IFC Controller HX, and then transferred to the BioMark HD for qPCR (95 °C for 10 min; 40 cycles of 95 °C for 15 seconds and 60 °C for

60 sec). The Ct values for genes of interest were normalised to the endogenous control *HPRT1* using the formula [$\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}}$] and relative expression was calculated as $2^{-\Delta Ct}$ and used for statistical analysis.

6.2.4 Statistical analysis

Cluster analysis was used to observe any differences in gene expression across the entire donor cohort. Statistical analysis and graphics were performed using GraphPad Prism software (version 7.0a). The normal distribution of the data was assessed using the Shapiro-Wilk and Kolmogorov-Smirnov tests for normality. As no data were found to be normally distributed, Wilcoxon rank sum test were performed for paired data and Mann-Whitney test was performed for unpaired data. The results were considered significant at p value <0.05.

6.3 Results

The results presented below include evaluation of the MSC multipotentiality gene expression, as first described in chapter 4 (section 4.3) followed by a panel of the exploratory genes, that were covered in chapter 5 (section 5.3). OA patients (median age 74 years old, range 56-83), were compared to the healthy old donor group (median age 68, range 61-89), as described in section 3.3.1. Number of MSCs sorted from control healthy donors ranged from 999 to10,000 cells (median 4,700 cells) and an average 70,000 MSCs were sorted from OA FHs. HLCs from healthy old donors and OA patients were collected from a minimum of 50,000 cells up to a maximum of 70,000 cells.

6.3.1 Differences in the expression of multipotentiality genes between OA MSCs and healthy old donor MSCs

First, the expression of genes indicating MSC multipotentiality was compared in MSCs from clinically diagnosed hip OA patients and healthy old donor MSCs (Figure 6.3, Table 6.1, top panel).

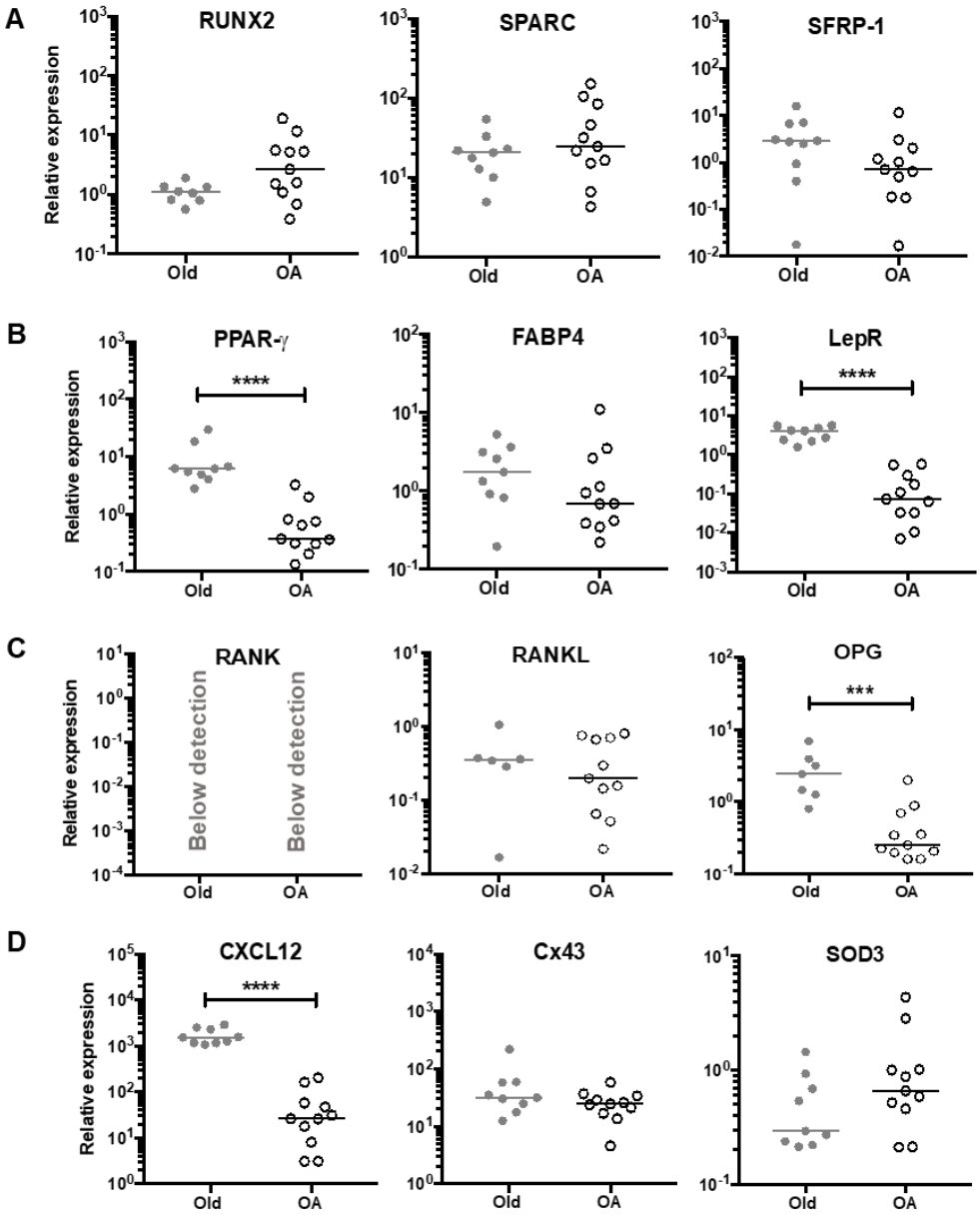

Figure 6.2 Expression of genes associated with MSC multipotential functions in MSCs from healthy old donors and OA patients

(A) Expression of *RUNX2*, *SPARC* and *SFRP-1*, **(B)** Expression of *PPAR-γ*, *FABP4* and *LepR*, **(C)** Expression of *RANK*, *RANKL* and *OPG* **(D)** Expression of *CXCL12*, *Cx43* and *SOD3*. Healthy old donors and OA patients are indicated in grey dots and black empty circles, respectively. Horizontal line across data indicate median values. $p<0.001$*** and $p<0.0001$****, Mann-Whitney U test.

With respect to osteogenic transcripts, there was no significant differences in the level of their expression between OA and old donor MSCs. Expression of *RUNX2* was 2.43-fold higher in OA patients and expression of *SFRP1* was 4-fold lower in OA patients but the differences failed to reach statistical significance ($p=0.062$). Expression of *SPARC* was similar (Figure 6.3A).

Next, the transcripts associated with adipogenic differentiation and bone-fat balance were examined. Both *LepR* and *PPAR-γ* showed significant decline (55-fold, $p<0.0001$ and 16-fold, $p<0.0001$, respectively) in OA MSCs compared to healthy old donor MSCs. *FABP4* showed a tendency of lower expression in OA MSCs (2.5-fold), however, the differences failed to reach statistical significance. Overall, all the three transcripts displayed a decline in OA patients indicating a decline in the formation of adipose tissue in OA patients as compared to healthy old donors (Figure 6.3B).

There was a non-significant decline in the expression of *RANKL* and a significant decline (10-fold) in *OPG* expression in OA patients as compared to healthy old donors (Figure 6.3C) potentially indicating changes in the rate of bone remodelling in OA patients. Among the transcripts associated with MSC stromal function, *Cx43* showed no difference in expression between OA patients and healthy old donors but *CXCL12* was found to be significantly 58-fold lower ($p<0.0001$) in OA patients (Figure 6.3D). This indicated that the stromal functions in MSCs from OA donors were potentially impaired as compared to MSCs from healthy old donors. The expression of *SOD3* was found to increase by 2-fold in OA MSCs as compared to MSCs from healthy old donors. While this was unexpected, future evaluation of this gene along with ROS quantification in FH MSCs would help in gaining better understanding of oxidative stress in uncultured BM MSCs.

Overall, these data potentially indicated a lesser impairment in the osteogenic capacity of OA MSCs, compared to their other functions (such as adipogenesis and stromal support).

6.3.2 Differences in the expression of multipotentiality genes between OA HLCs and healthy old donor HLCs

The investigation of transcripts associated with MSC osteogenic differentiation in HLCs is indicated in Figure 6.4 and the median values for the same is outlined in Table 6.1, bottom panel. No differences were observed in the expression of *RUNX2* and a significant 13-fold increase in the expression of *SPARC* ($p=0.0003$) in OA HLCs compared to healthy old donors HLCs (Figure 6.4A). Adipogenic transcripts *LepR* and *PPAR-γ* did not display any difference in OA HLCs compared to old donors HLCs, but *FABP4* showed significantly 8-fold higher levels in OA HLCs ($p=0.0409$), which is exactly the opposite trend to that observed in the MSCs (Figure 6.4B).

With respect to bone remodelling transcripts, *RANK* and *RANKL* showed no difference in HLC expression levels between healthy old donors and OA patients. OPG was not detected in healthy old donors, therefore it could be reasonably assumed that its expression in OA donors HLCs was elevated (Figure 6.4C).

When the transcripts associated with MSC stromal support were investigated, there was no significant differences in the HLC levels of either *Cx43* or *CXCL12* transcripts between OA patients and healthy old donors. Nevertheless, the expression of *CXCL12* was found to be 2-fold higher in OA HLCs whereas, expression of *Cx43* was 2-fold lower in OA HLCs as compared to HLCs from healthy old donors (Figure 6.4D).

Differences in multifunctionality genes in OA were not aggravated from ageing and thus were only associated with OA. Increase in the expression of *SOD3* in both MSCs and in HLCs in OA was unexpected as OA is often associated with declined SOD3 and increased ROS (446). This suggests need for future work including the detection of ROS from uncultured OA donors alongside the quantification of a larger panel of anti-oxidant enzymes.

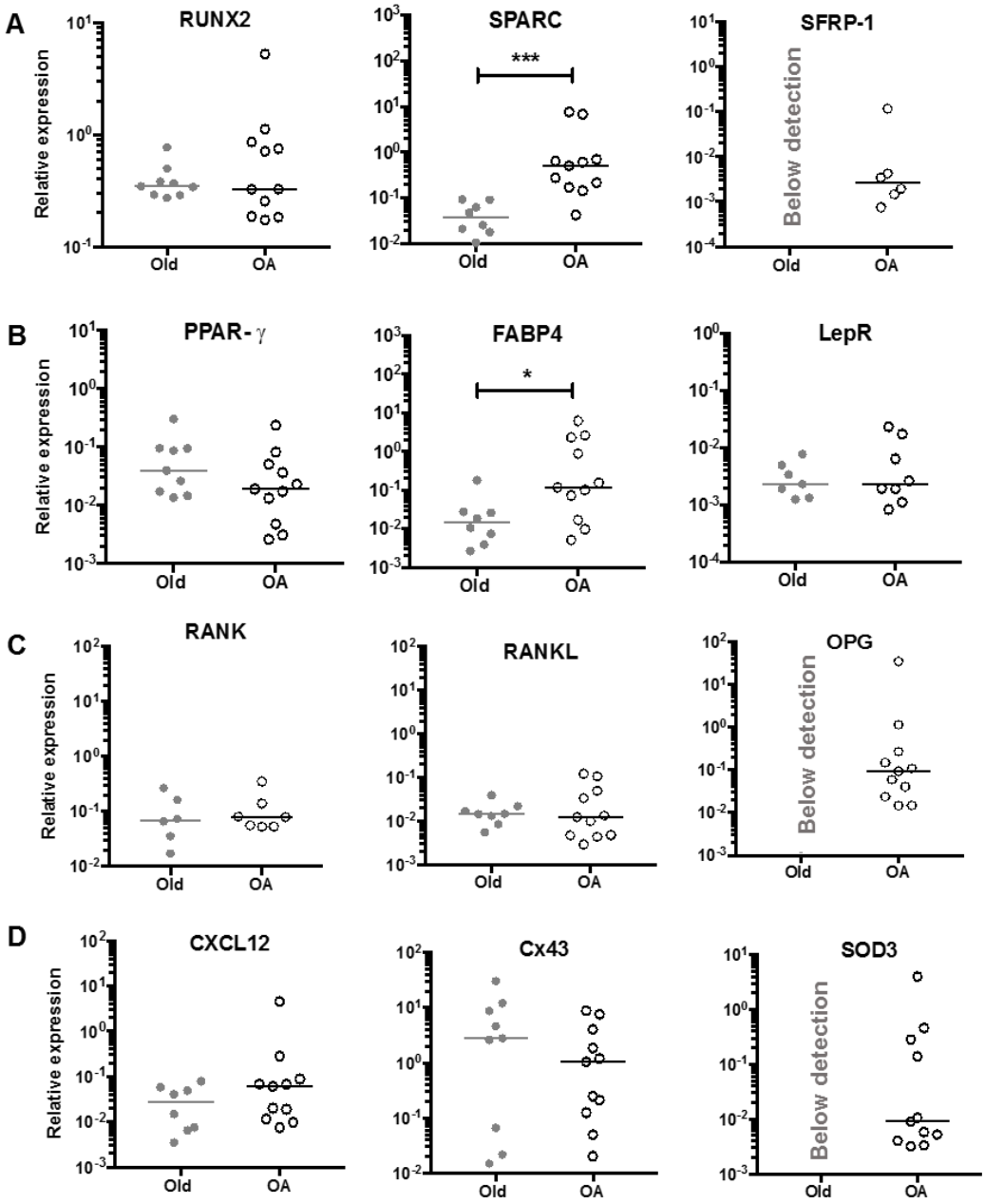

Figure 6.3 Expression of genes associated with MSC multipotential functions in HLCs from healthy old donors and OA patients

(A) Expression of *RUNX2*, *SPARC* and *SFRP-1*, **(B)** Expression of *PPAR-γ*, *FABP4* and *LepR*, **(C)** Expression of *RANK*, *RANKL* and *OPG* **(D)** Expression of *CXCL12*, *Cx43* and *SOD3*. Healthy old donors and OA patients are indicated in grey dots and black empty circles, respectively. Horizontal line across data indicate median values. $p<0.001$* and $p<0.0001$***, Mann-Whitney U test.

Overall, HLCs displayed a different pattern of differences to MSCs between OA patients and healthy old donors. Fold differences and *p* values of all of the above mentioned transcripts in healthy old donors and OA patients are shown in Table 6.1 and 6.2

Table 6.1 Differences in the expression of MSC multipotentiality genes in OA MSCs and HLCs as compared to healthy old donors

Cells	Genes	Medians in Old	Medians in OA	Fold difference*	*p* value
MSCs	*RUNX2*	1.09	2.65	2.43	NS
	SPARC	20.51	24.54	1.20	NS
	SFRP1	2.83	0.71	0.25	NS
	PPAR-γ	6.20	0.37	0.06	<0.0001
	FABP4	1.77	0.69	0.39	NS
	LepR	4.13	0.07	0.02	<0.0001
	SOD3	0.29	0.66	2.28	NS
	RANKL	0.35	0.20	0.56	NS
	OPG	2.44	0.25	0.10	0.0008
	RANK	ND	ND	NA	NA
	CXCL12	1554.00	26.19	0.02	<0.0001
	Cx43	31.55	24.51	0.78	NS
HLCs	*RUNX2*	0.35	0.33	0.95	NS
	SPARC	0.04	0.51	13.86	0.0003
	SFRP1	ND	0.002	NA	NA
	FABP4	0.0145	0.1175	8.12	0.0409
	PPAR-γ	0.04	0.02	0.48	NS
	LepR	0.0023	0.0023	0.99	NS
	SOD3	ND	0.009	NA	NA
	RANKL	0.35	0.19	0.54	NS
	OPG	ND	0.09	NA	NA
	RANK	0.10	0.07	0.71	NS
	CXCL12	0.03	0.06	2.21	NS
	Cx43	2.79	1.05	0.38	NS

Top panel of genes include expression of MSC multipotentiality genes in MSCs and the bottom panel includes expression of HLCs . All expressions are relative to *HPRT1*, Mann-Whitney U test. ***** Medians in OA/ medians in old, NS: non-significant

6.3.3 OA related differences in the expression of senescence, SASP and IFN pathway related genes in MSCs and HLCs

An overall cluster analysis of all exploratory genes revealed some segregation of MSCs and HLCs within FH samples, but not as clear as seen in section 5.3.1, Figure 5.3.

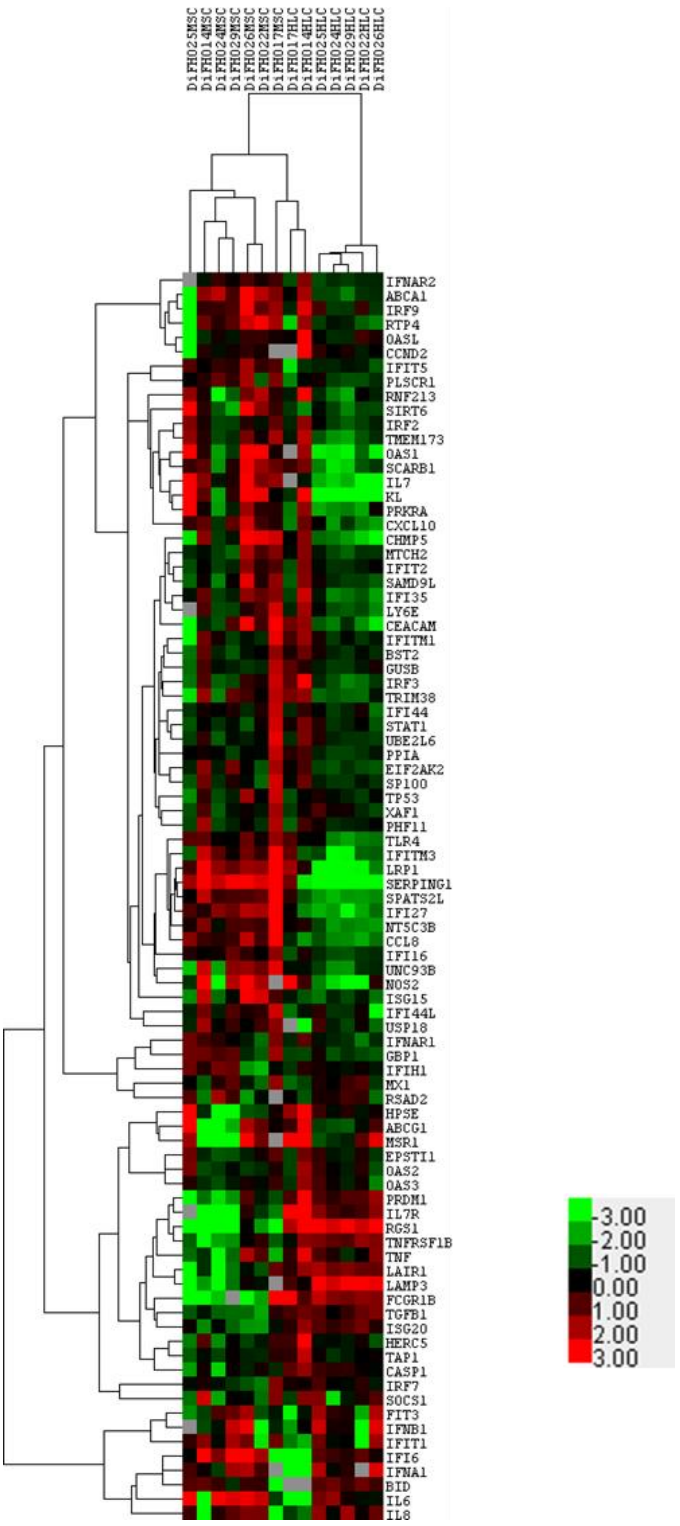

Figure 6.4 Cluster analysis of exploratory genes in OA samples in MSCs and HLCs

Expression of *IFNA1, IFNB1*, *IFN receptors,* IRGs, genes associated with IFN1>BID>ROS pathway, senescence and other exploratory genes grouped by cluster analysis. Dendogram on the top indicate samples and dendogram on the right indicates genes . Colour key in shown at the bottom right of the figure. Grey squares indicate gene expression values that were below detection.

The median values for complete list of genes and differences in old healthy donors and OA patients is presented in Appendix 4, Tables 6.1 and 6.2 for gene expression in MSCs and HLCs, respectively.

With respect to senescence and anti-ageing genes, the following results were obtained. Irrespective of the cell population, the anti-ageing gene *Kl* expression decreased by 4.8-fold in MSCs and by 5.5-fold in HLCs in OA patients as compared to healthy old donors. MSCs from OA patients showed over 3-fold decline in *Tp53* but no change in OA HLCs was observed. The expression of *Sirt6* remained unchanged in both OA MSCs and HLCs. High level of donor variation was observed in the expression of *Kl* in OA patient samples in both, MSCs and in HLCs, and none of these trends were found to be statistically significant (Figure 6.6).

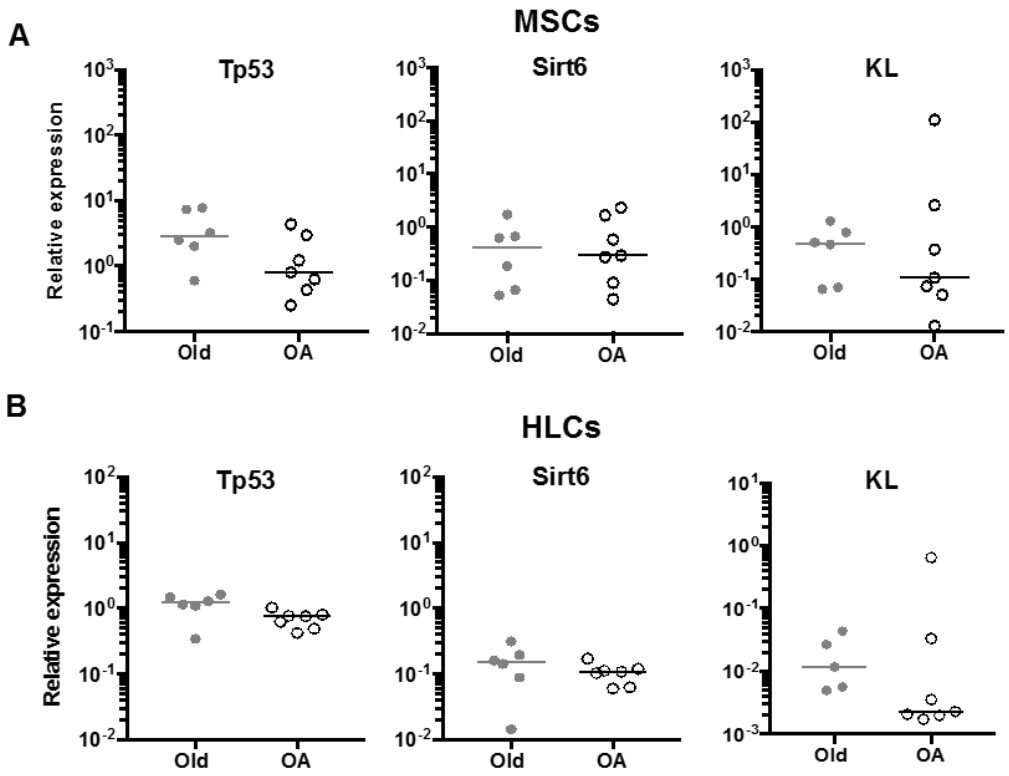

Figure 6.5 Expression of senescence and anti-ageing genes in MSCs and HLCs from healthy old donors and OA patients

(A) Expression of *Tp53, Sirt6* and *Kl* in MSCs. Grey dots indicate old donors and empty circles indicate OA patients. **(B)** Expression of*Tp53*, *Sirt6* and *Kl* in HLCs. Healthy old donors and OA patients are indicated in grey dots and black empty circles, respectively. Horizontal line across data set indicates median values.

When the expression of SASP-associated cytokines was analysed, *IL6* displayed a significant 132-fold increase in OA MSCs (p=0.0101) and also showed a 6-fold

increase in HLCs (not significant), these trends were consistent with its hypothesised changes in OA. *IL7* showed a non-significant 5-fold decline in MSCs and no change in HLCs. *IL8* did not reach statistical significance but showed opposite trends in MSCs and HLCs. While in MSC, it displayed nearly 8-fold increase in OA, in HLCs it had a nearly 4-fold decline (Figure 6.7).

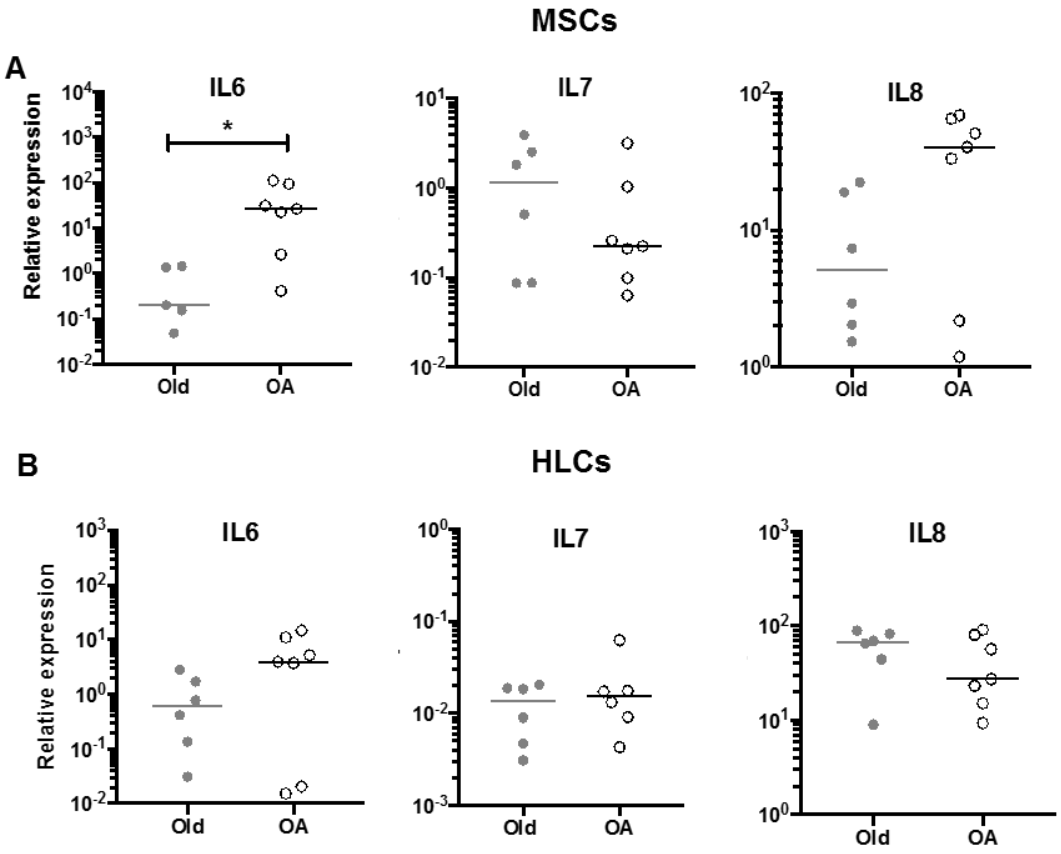

Figure 6.6 Expression of SASP-associated cytokines in MSCs and HLCs from healthy old donors and OA patients

(A) Expression of *IL6, IL7* and *IL8* in MSCs, **(B)** Expression of *IL6, IL7* and *IL8* in HLCs. Healthy old donors and OA patients are indicated in grey dots and black empty circles, respectively. Horizontal line across data set indicates median values. $p<0.05$*, Mann-Whitney U test.

Overall, anti-ageing genes and senescence associated genes did not display any significant OA-related differences even though *Kl* declined in OA patients as displayed in both cell populations. SASP associated cytokine genes displayed significant increase in OA MSCs and a non-significant increase in HLCs.

Table 6.2 Differences in the expression of genes associated with IFN1 pathway, ROS, anti-ageing and senescence, in OA MSCs and HLCs as compared to healthy old donors

Cells	Genes	Medians in Old	Medians in OA	Fold difference*	*p* value
MSCs	*IFNA1*	9.15	2.23	0.24	NS
	IFNB1	1.72	0.27	0.16	NS
	IFNAR1	4.75	1.26	0.27	NS
	IFNAR2	5.76	1.36	0.24	0.041
	STING	4.88	1.25	0.26	NS
	IRF3	3.13	0.85	0.27	0.051
	STAT1	5.16	1.47	0.28	0.0221
	BID	0.11	0.47	4.27	NS
	MTCH2	1.27	0.47	0.37	NS
	Kl	0.48	0.1	0.21	NS
	Sirt6	0.4	0.3	0.75	NS
	Tp53	2.88	0.81	0.28	NS
	IL6	0.2	26.37	131.85	0.0101
	IL7	1.17	0.22	0.19	NS
	IL8	5.14	40.45	7.87	NS
HLCs	*IFNA1*	7.44	1.17	0.16	NS
	IFNB1	1.59	0.39	0.25	NS
	IFNAR1	0.48	0.65	1.35	NS
	IFNAR2	1.38	0.66	0.48	0.035
	STING	1.033	0.457	0.44	NS
	IRF3	0.66	0.43	0.65	NS
	STAT1	2.4	1.57	0.65	NS
	BID	1.032	0.62	0.60	NS
	MTCH2	0.79	0.34	0.43	NS
	Kl	0.011	0.002	0.18	NS
	Sirt6	0.15	0.1	0.67	NS
	Tp53	1.21	0.75	0.62	NS
	IL6	0.59	3.92	6.64	NS
	IL7	0.013	0.015	1.15	NS
	IL8	67.21	27.35	0.41	NS

Top panel of genes include expression of MSC multipotentiality genes in MSCs and the bottom panel includes expression of HLCs . All expressions are relative to *HPRT1*, Mann-Whitney U test. ***** Medians in OA/ medians in old, NS: non-significant.

Differences in *IFNA1*, *IFNB1* and *IFNA* receptors were next analysed for understanding OA associated changes in relation to old age (Figure 6.5). In OA patients, *IFNA1* showed a non-significant decline (4-fold and 6-fold) in MSCs and HLCs, respectively. *IFNB1* showed similar trends with 6- and 4-fold declines in OA MSCs and HLCs,

respectively. *IFNAR1* showed a 3-fold decline in OA MSCs but its expression was unaltered in OA HLCs. *IFNAR2* showed a statistically significant decline in both MSCs (p=0.041) and HLCs (p=0.035) in patients with OA in comparison with old donors.

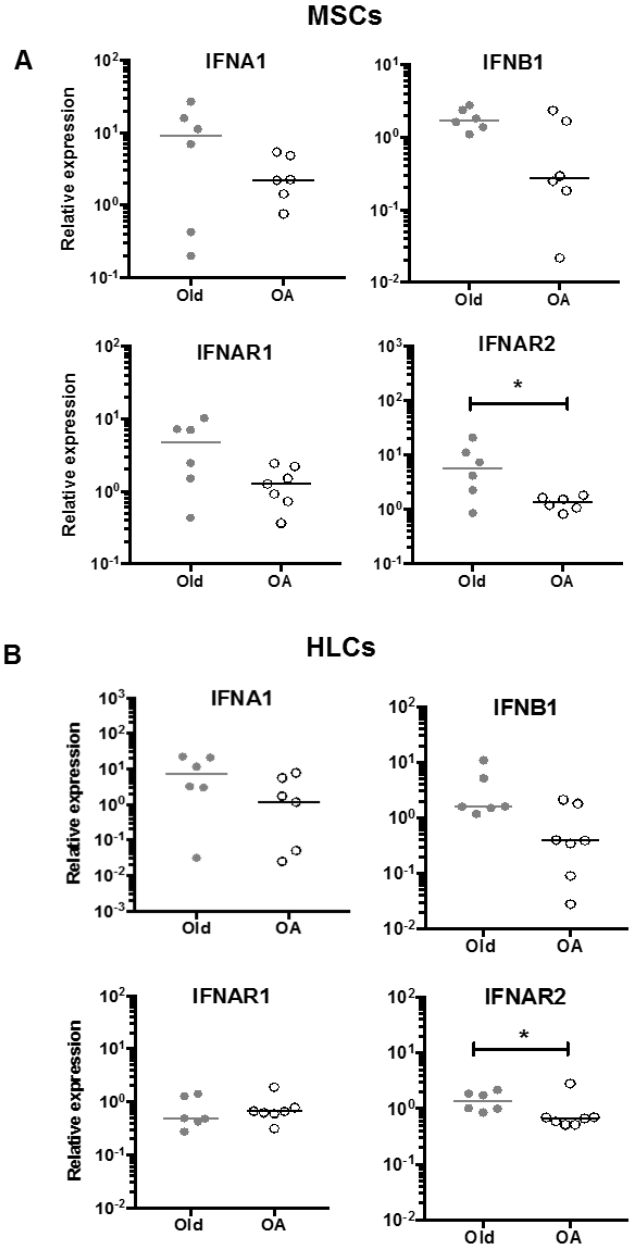

Figure 6.7 Expression of IFNA, IFNB and IFNA receptor genes in MSCs and HLCs from healthy old donors and OA patients

(A) Expression of *IFNA1* and *IFNB1* (top panel) and *IFNAR1* and IFNR2 (bottom panel) in MSCs **(B)** Expression of *IFNA1* and *IFNB1* (top panel) and *IFNAR1* and *IFNAR2* (bottom panel) in HLCs. Healthy old donors and OA patients are indicated in grey dots and black empty circles, respectively. Horizontal line across data set indicates median. p<0.05*, Mann-Whitney U test.

Among the IRGs, a number of genes were found to be differentially expressed in both MSCs and in HLCs in OA patients compared to old donor populations. Figure 6.9

below shows some of the differentially expressed genes in MSCs. While most of the genes declined in OA patients, *ISG20, LAMP3* and *IFI27* were significantly higher in OA patients MSCs. The medians, fold differences and statistical significance (if $p<0.05$) of all the investigated genes are shown in Table 6.2 and Appendix 4.

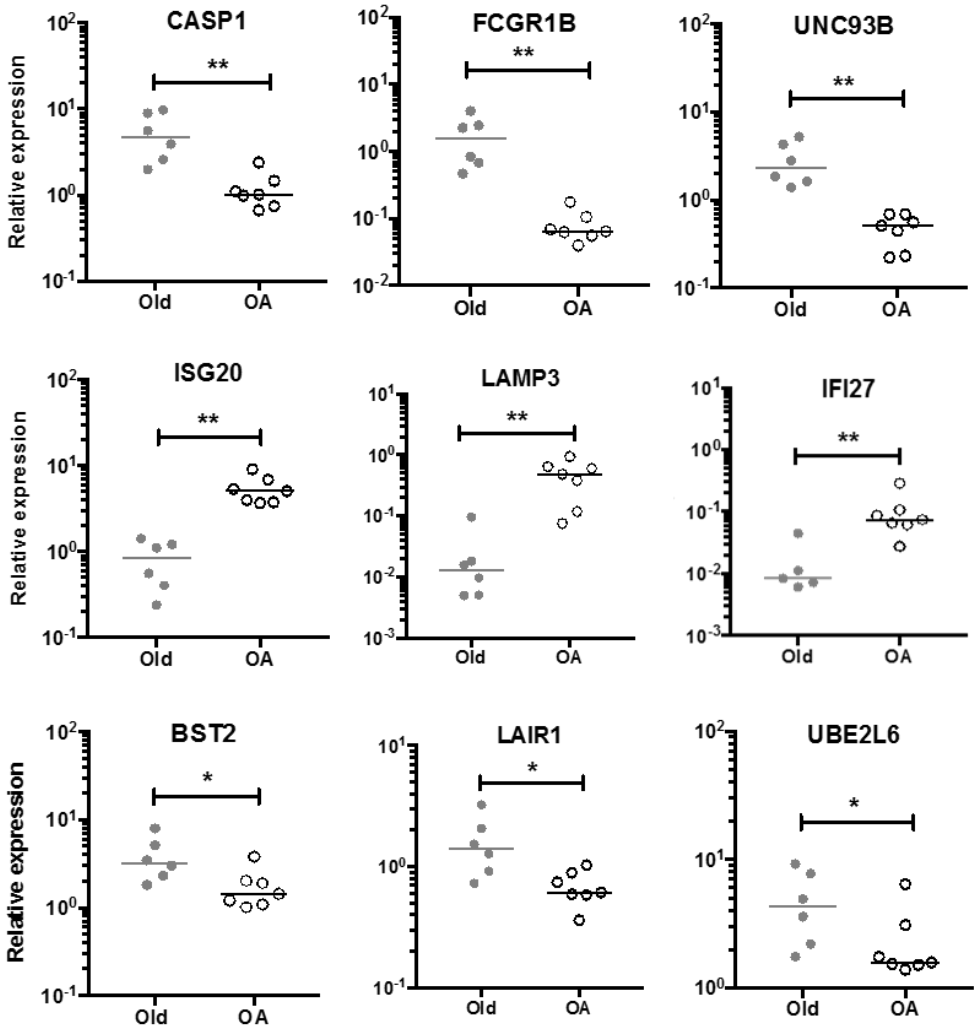

Figure 6.8 Genes expression of IRGs in OA MSCs compared to MSCs from healthy old donors

Examples of differentially expressed IRGs in MSCs from OA donors compared to healthy old donors. Healthy old donors and OA patients are indicated in grey dots and black empty circles, respectively. Horizontal line across data set indicates median. $p<0.05$* and $p<0.01$**, Mann-Whitney U test.

Figure 6.10 displays examples of genes that were differentially expressed in HLCs in OA patients as compared to healthy old donors. Interestingly, *CASP1*, *ISG20* and *LAIR1* were differentially expressed in OA patients irrespective of the population examined (*ISG20* was higher in OA and *CASP1* and *LAIR1* were lower in OA).

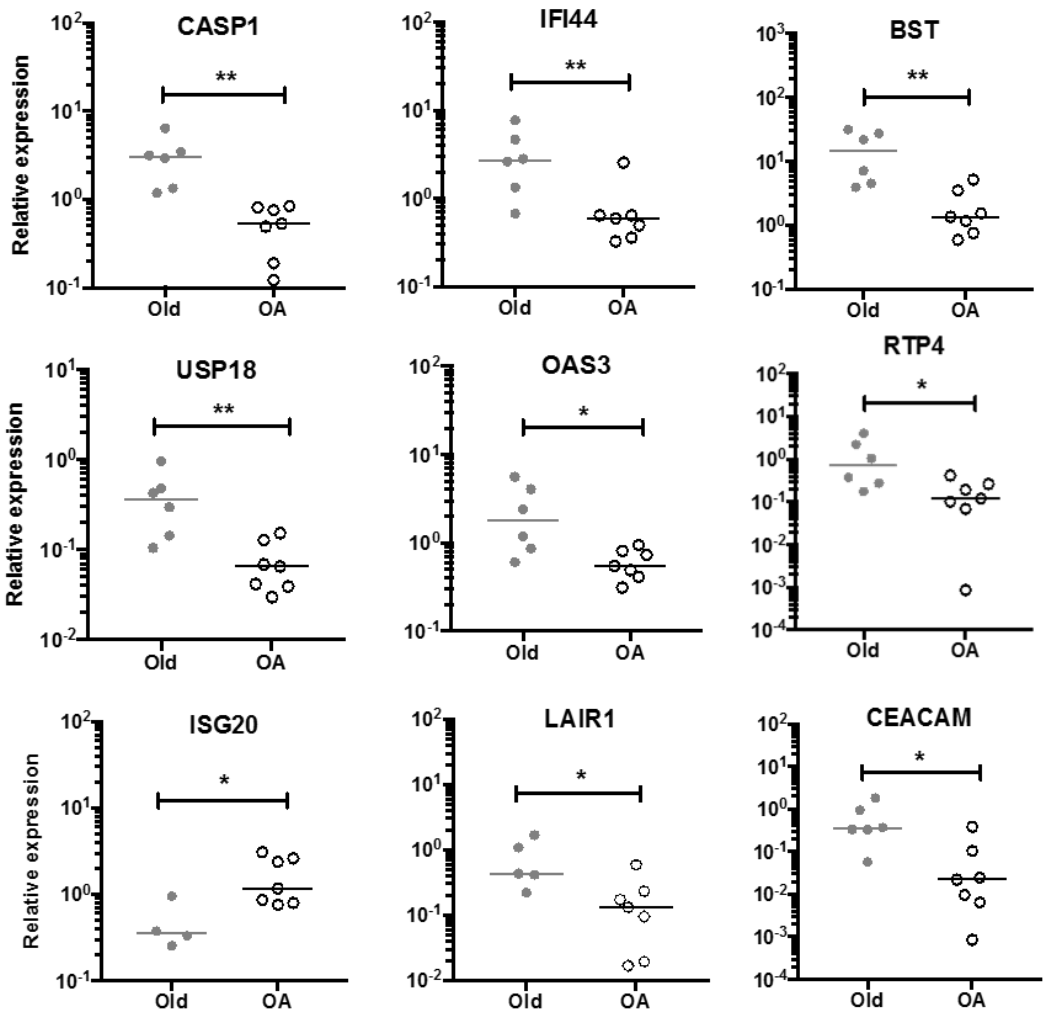

Figure 6.9 Genes expression of IRGs in OA HLCs compared to HLCs from healthy old donors

Examples of differentially expressed IRGs in HLCs from OA donors compared to healthy old donors. Healthy old donors and OA patients are indicated in grey dots and black empty circles, respectively. Horizontal line across data set indicates median. $p<0.05$* and $p<0.01$**, Mann-Whitney U test.

The genes associated with the IFN1>BID>ROS pathway were next investigated for any differences in OA patients as compared to healthy old donors. In MSCs, all the genes except *BID* displayed a decline in OA with *STAT1* showing a significant decline ($p=0.0221$). However, in HLCs, the trends for decline were more subtle, even though a minor decline stayed constant for almost all the genes (Figure 6.11).

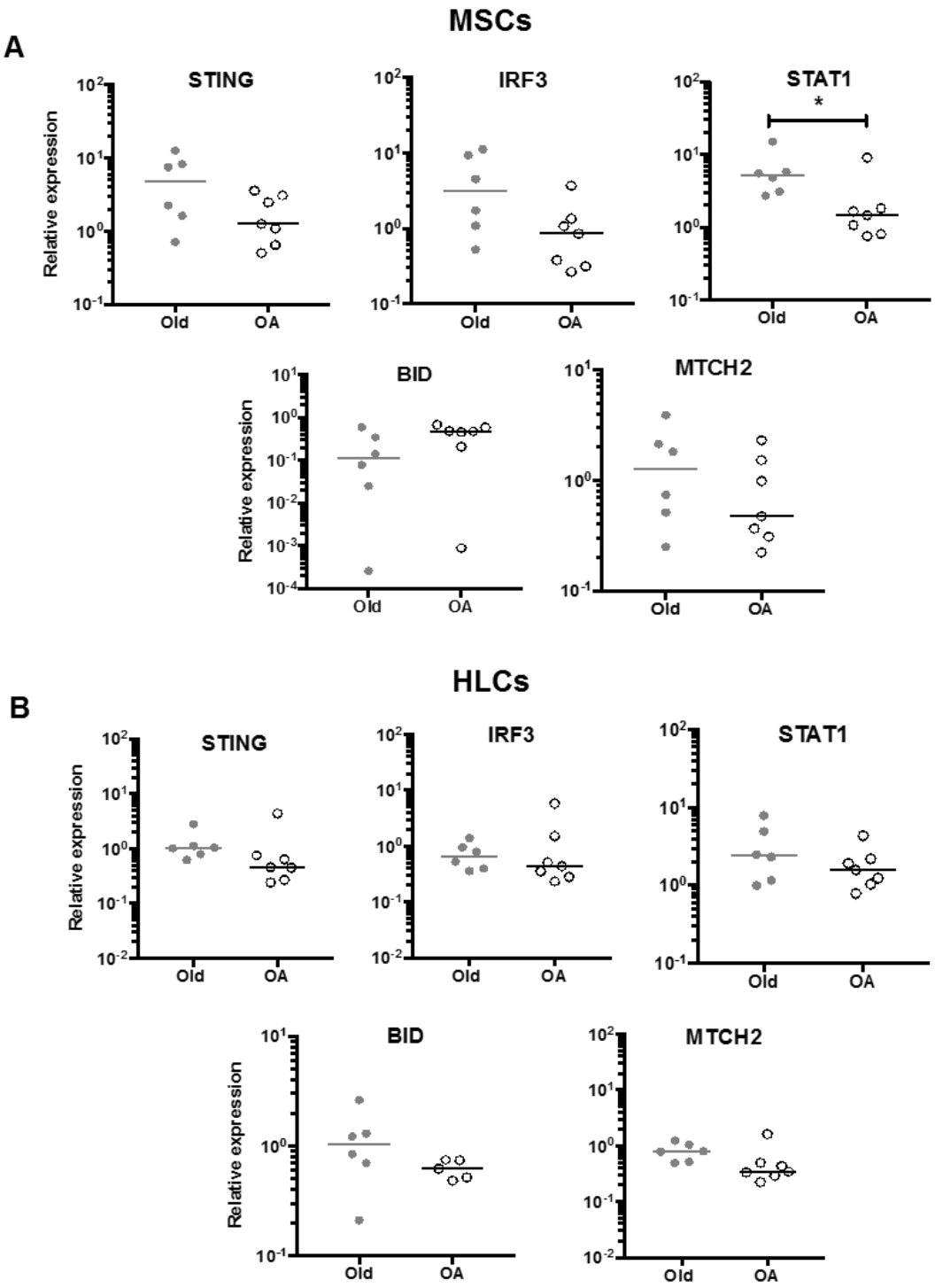

Figure 6.10 Expression of genes associated with IFN1>BID>ROS pathway in MSCs and HLCs from healthy old donors and OA patients

(A) Expression of *STING, STAT1* and *IRF3* (top panel) and *BID, MTCH2* (bottom panel) in MSCs, **(B)** Expression of *STING, STAT1* and *IRF3* (top panel) and *BID*, *MTCH2* (bottom panel) in HLCs. Healthy old donors and OA patients are indicated in grey dots and black empty circles, respectively. Horizontal line across data set indicates median values. $p<0.05$* , Mann-Whitney U test.

In summary, section 6.3.3 investigated differences in exploratory gene expression including anti-ageing and IFN1>BID>ROS pathway genes, anti-ageing, SASP and cytokines, and identified novel molecules that displayed significant differences in their expression in OA MSCs. *IFNAR2* declined significantly in OA MSCs along with a number of IRGs and *STAT1*, a downstream molecule through which IFNs induce the expression of IRGs. As expected, *IL6* increased significantly in OA while anti-ageing gene *Kl* decreased by nearly 5-fold in OA patients. Altogether, these expression patterns indicated a possibility for a reduced IFN1 signalling in OA MSCs, which could further impact on their potency and senescence status.

6.3.4 Biomarkers of age-related OA

In the course of this study, a number of genes that displayed a trend towards age-related differences, were also found to have a similar and aggravated trend in OA. While the expression of some of these genes was found to decline with both increasing age and OA, the others showed an increase in their expression. In MSCs, the statistical significance in these 'aggravated' genes was only observed for *IL6* (Figure 6.12A), while more such genes were found in HLCs. In itself, this indicated that MSCs were more resistant to ageing and OA, compared to HLCs (Figure 6.12B). This section discusses the genes in both cell populations that have displayed distinct trends and statistical significance with respect to age-related differences that were further exacerbated in OA.

The on transcript that displayed a distinct age-related increase followed by a further increase in OA MSCs was *IL6* (p=0.0005), as shown in Figure 6.12A. Genes that displayed a age-related decrease followed by a further decrease in expression in OA HLCs included the anti-ageing gene *Sirt6* (p=0.02), senescence associated *Tp53* (p<0.0001). Interestingly, *IFNAR2* (p=0.0026) also displayed and age and OA-related decrease in HLCs, potentially suggesting an overall decline in the IFN1 cascade with advancing age, that is aggravated in OA. Thus, it was not surprising that the stimulator of IFN genes or *STING* was also found to display an age-and OA-related decline (p=0.008) along with *MTCH2* (p=0.0019) , all shown in Figure 6.12B.

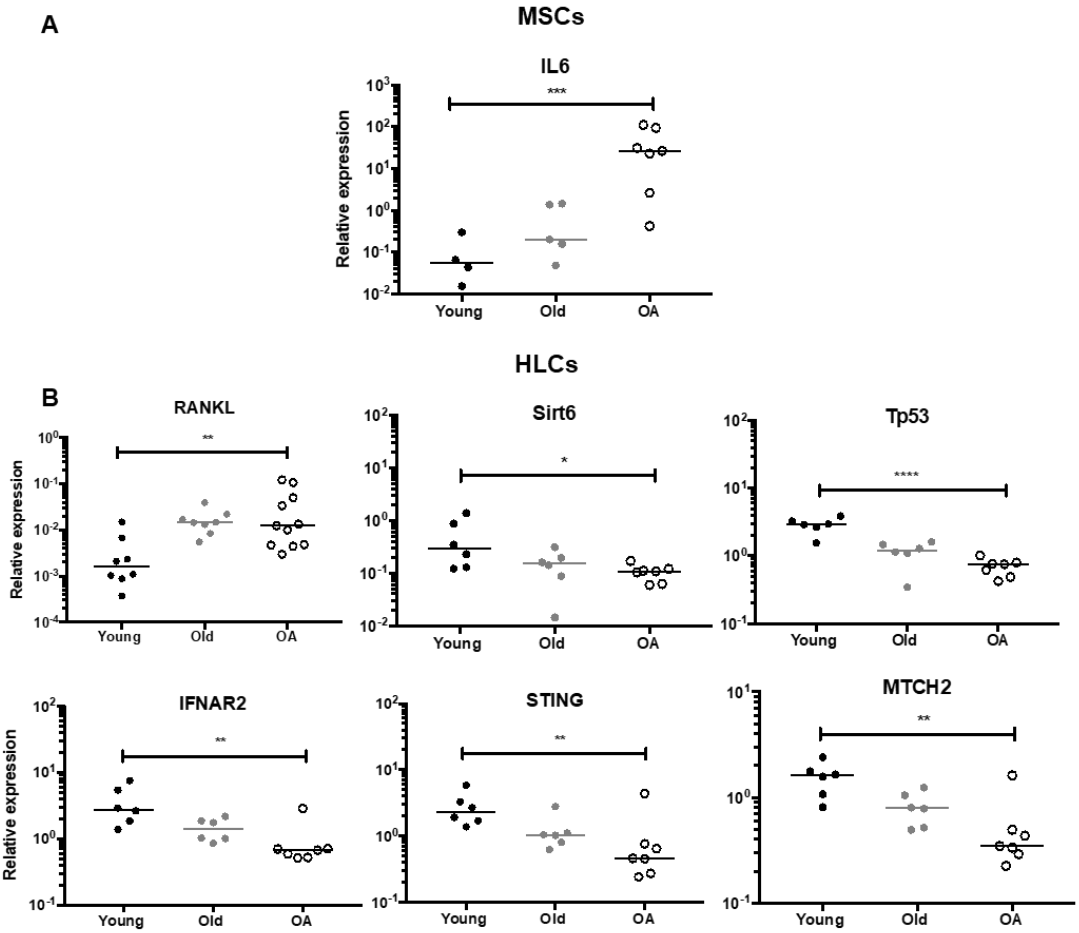

Figure 6.11 Expression of genes that displayed age-related differences that were further exacerbated in OA

(A) Expression of *IL6* in MSCs **(B)** Expression of *RANKL*, *Sirt6, Tp53, IFNAR2, STING* and *MTCH2* in HLCs. Young donors, old donors and OA patients are indicated by black dots, grey dots and empty black circles, respectively. The black line indicates median values. *p<0.05, **p<0.01 and ****p<0.0001, Kruskal-wallis test with Dunn's correction.

Overall, this section outlines the potential markers of age-related OA reflecting the *in vivo* BM niche in uncultured BM MSCs and in HLCs. Further examination of these genes at both gene and protein level in a larger donor cohort would be extremely useful in translating these genes as biomarkers.

6.3.5 Differences in surface marker expression in OA MSCs and HLCs compared to healthy old donors

While none of the surface markers displayed any age-related changes in MSCs (Chapter 4, figure 4.14), CD106 and CD295 showed a significant decrease (4-fold, p=0.0003 and 4-fold, p=0.0043, Figure 6.13A and B, respectively) in their expression in

OA MSCs as compared to old donors MSCs. Expression of CD146 and Cx43 remained unchanged (Figure 6.13C and D). Interestingly, the decline in *LepR* both at the transcript level as well as in the protein level was found to be consistent and statistically significant, suggesting an imbalance between osteogenic and adipogenic differentiation abilities in patients with OA.

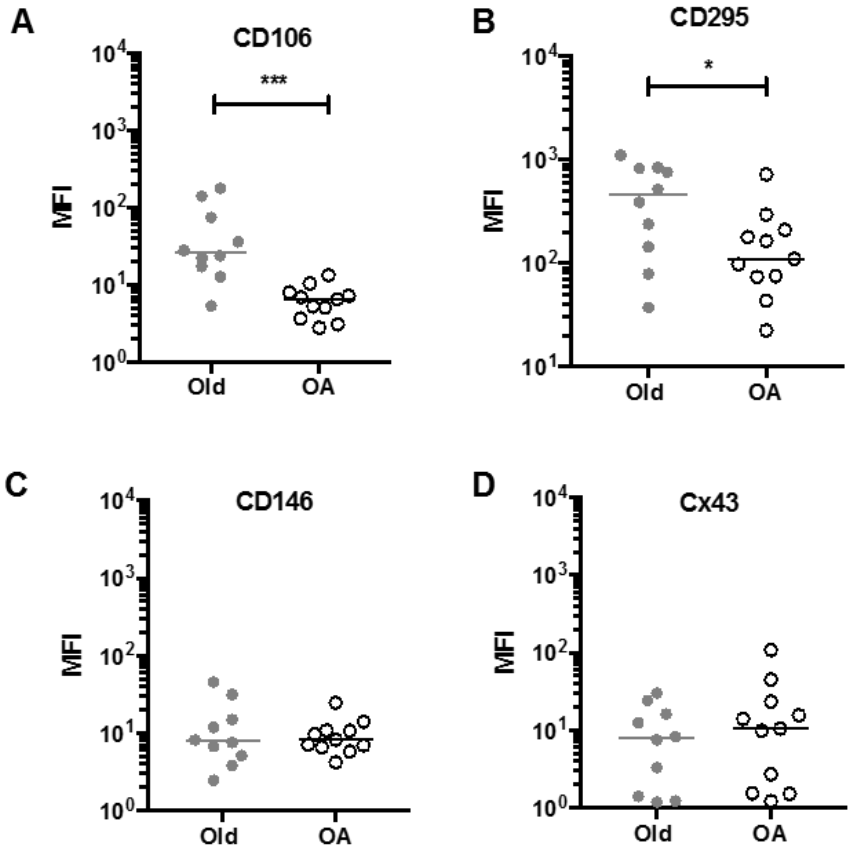

Figure 6.12 Surface marker expression in MSCs from healthy old donors and OA donors

(A) CD106 **(B)** CD295 **(C)** CD146 and (**D)** Cx43 from old healthy donors and OA patients. Healthy old donors and OA patients are indicated in grey dots and black empty circles, respectively. Horizontal line across data indicate median values. *p<0.05 and ***p<0.001, Mann-Whitney U test.

Following comparisons in MSCs, surface marker expression was investigated in HLCs. CD106 and CD146 displayed a trend for increased expression in HLCs (2-fold in each) but the differences failed to reach significance. CD295 and Cx43 on the other hand, displayed a decline in expression in OA patients, but again failed to reach significance (2-fold each) (Figure 6.14). CD295 was found to decline in both MSCs as well as in HLCs.

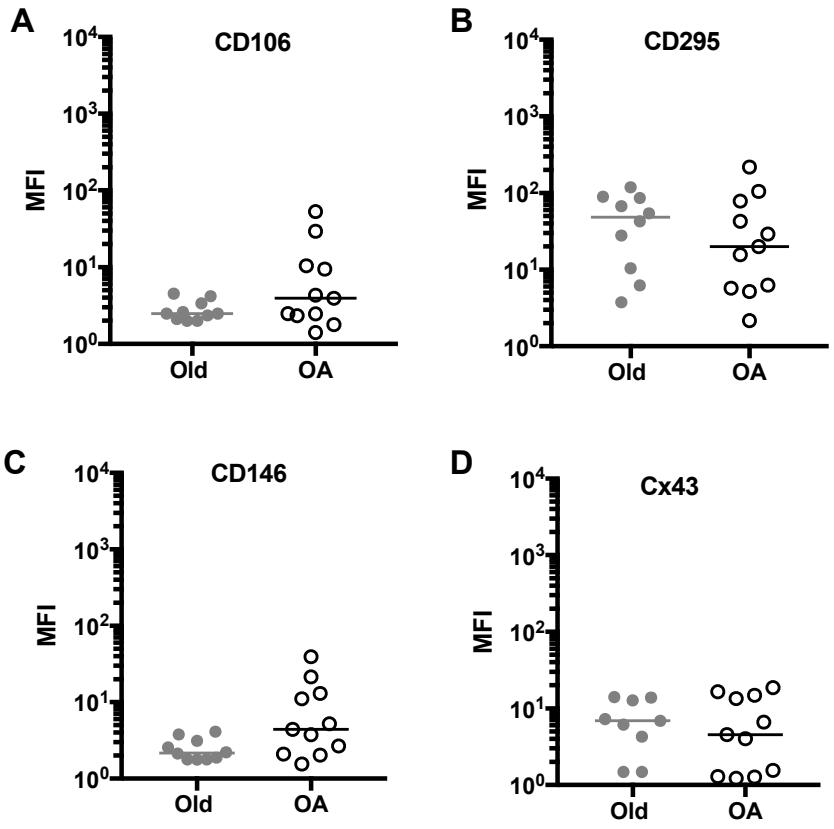

Figure 6.13 Surface marker expression in HLCs from healthy old donors and OA donors

(A) CD106 **(B)** CD295 **(C)** CD146 and **(D)** Cx43 from old healthy donors and OA patients. Healthy old donors and OA patients are indicated in grey dots and black empty circles, respectively. Horizontal line across data indicate median values.

Overall, compared to healthy aged MSCs, MSCs from OA patients displayed a significant decline in *PPAR-γ, LepR, OPG* and *CXCL12* expression. None of these changes could be described as 'aggravated ageing' since all of these genes showed no age-related differences in healthy donor MSCs (section 4.4.3). *LepR* was also investigated at the protein level and a similar significant decline was confirmed in OA patients. This was also unique to OA since no age-related changes in LepR/CD295 were found in healthy old donors (section 4.3.5). Furthermore, like CD295, CD106 was another protein that displayed a very significant decline in MSCs from OA patients, which was likely to be associated with other local or systemic factors contributing to OA since no age-related differences in its expression were found in the previous chapter (section 4.3.5).

In OA HLCs, anti-ageing genes *Sirt6 and Kl*, declined significantly and the expression of IFN-related genes of *IFNAR2, STING, MTCH2* and *IRF3* also declined significantly.

These genes were found to decline in healthy ageing as well. From these findings, it is tempting to speculate that in HLCs, age-related trends particularly in relation to IFN pathway, are further exacerbated in OA. But this is not the case for MSCs. Interestingly, the pro-inflammatory and SASP-associated cytokine *IL6* transcript was found to increase by over 100-fold in OA MSCs and by 7-fold in OA HLCs. *IL6* expression also increased by 4-fold in healthy ageing in both MSCs and HLCs (section 5.3.1). As *IL6* was found to increase in healthy ageing and further aggravated in OA, in both MSCs and HLCs, it would be a good biomarker of age-related OA.

6.4 Discussion

The results from this chapter reveal very interesting alterations in MSCs from OA FH bone compared to healthy aged BM MSCs. Gene expression results indicated a decline in the adipogenic potential of MSCs from OA FHs, no alteration in the levels of osteogenic differentiation transcripts and a significant decline (nearly 10-fold) in the expression of OPG in MSCs from OA patients. A significant decline (60-fold) in the level of *CXCL12* implicated in cell recruitment was also observed. Interestingly, there was no significant change in the transcript encoding an anti-oxidant enzyme *SOD3* in OA MSCs compared to healthy old donors MSCs. The study of exploratory transcripts encoding cytokines revealed an increase in the level of *IL6* transcript in OA, as expected from literature (461, 478). *STAT1* declined in OA patients while no significant changes were observed in ageing and senescence associated genes (*Kl, Sirt6, Tp53*). Interestingly, the anti-ageing gene *Kl* decreased in OA patients by nearly 5-fold but statistical significance wasn't achieved due to high donor variation in its expression levels.

Transcripts associated with osteogenic differentiation potential showed no difference in OA MSCs compared to healthy old donor MSCs. A recent study from our group (Ilas et al., 2019) investigated the level of *RUNX2* in BM MSCS from the FH of OA patients and found a significant increase in the expression of *RUNX2* in MSCs from OA patients as compared to MSCs from healthy donors (69). Even though this was the most relevant study for comparison with the PhD project, the OA patients were not age-matched with healthy donors in the Ilas et al., study. In this project, the genes associated with osteogenic differentiation showed no difference or non-significant 2-fold increase (*RUNX2*) in OA MSCs as compared to healthy old MSCs. Future work with genes associated with osteogenic differentiation in OA must firstly consider the different stages OA and analyse the gene expression separately. Investigating these

molecules at the protein level would further enhance the current understanding of OA progression.

The expression of adipogenic transcripts in MSC population in this project suggested a decline in the fat forming capacity of MSCs from OA donors. This is in agreement with a previous study by Murphy et al. that have shown that the adipogenic potential of MSCs from OA donors is compromised. Interestingly, they also found a decline in the osteogenic potential of MSCs from OA donors which was not what was observed in this project. The variation could be potentially due to the fact that Murphy et al., used cultured cells as opposed to uncultured cells as in this project. Also, while they measured the differentiation potential of MSCs from OA donors *in vitro*, in this project, the cells were uncultured and hence better represented human OA conditions *in vivo*. Interestingly, another study from our group published in 2016 (183) found no defect in the adipogenic differentiation potential of culture-expanded MSCs either. These studies too tested the adipogenic potential of MSCs from hip OA donors *in vitro* under normal culture conditions, further highlighting the need to dissect any changes in OA MSCs prior plastic adhesion and culture. Overall, the expression of adipogenic transcripts were not found to be consistent with the hypothesis that there might be an exaggerated increase in OA, possibly due to other factors like lifestyle and obesity that are involved in age-related OA but were not accounted for in this project.

Recently, a study from our group investigated the expression of MSC multipotentiality transcripts in MSCs from OA FH as compared to the MSCs in healthy donors (69). Interestingly, it showed a significant increase in the expression of *OPG, RUNX2* and *SPARC* in patients with OA as compared to healthy donors. In this PhD project, the trend for *RUNX2* expression was found to be consistent with the previous results from our group, but the level of *SPARC* expression was not. Even though the cells used were also uncultured $CD45^{low}CD271^{+}$ cells, the patient cohorts in Ilas et al., study were not as well age-matched. The Ilas et al., study also used a larger panel of osteogenic transcripts. While expanding the number of transcripts for investigating multipotential functionalities of MSCs would be the way forward, low numbers of $CD45^{low}CD271^{+}$ MSCs in aged donors represent a significant limitation for future work. Using techniques like RNA seq to compare differentially expressed genes in OA as compared to healthy old donors would provide further insight into OA development.

In this project, both *RANKL* and *OPG* displayed a decline in MSCs from OA patients as compared to healthy old donors. While the decline in OPG was significant, the difference in *RANKL* failed to reach statistical significance. *RANKL* and *OPG* are

involved in bone remodelling (discussed in Introduction, section 1.1.4 and in Chapter 4, section 4.1.1) and have been extensively investigated in OA. More relevant to this study, Bitenc Logar and colleagues in 2007 investigated the levels of *RANKL, OPG* in the trabecular bone samples (uncultured) in OA patients (479). They found a significant decline in the levels of *RANKL* in the samples from OA patients compared to those with femoral neck fractures. While they used uncultured samples from donors, they did not isolate/purify any cell types for their experiments and studied mixed population of all the cell types present within the subchondral bone. The most recent study from our laboratory has shown higher *OPG* levels in OA $CD45^{low}CD271^{+}$ MSCs compared to healthy OA MSCs whereas *RANKL* transcript levels were not altered (69). These data differ from the findings from this thesis where *OPG* was significantly lower in OA MSCs. This could be due to the fact that the age of healthy old donors and OA patients in Ilas et al., paper was not as closely age-matched and included healthy old donors in their 40s as compared to this thesis where old donors were strictly over 59 years old.

In another study, Upton and co-workers found a significant increase in the levels of both *RANKL* and OPG obtained from grade 2 OA patients compared to grade 0 OA using K/L system (480). However, Upton and co-workers had used articular cartilage and used IHC for their studies. Kwan et al showed a significant increase in the levels of OPG in OA patients cultured osteoblasts with high levels of prostaglandin E_2 (PGE_2) in comparison with OA patients with lower levels of PGE_2, but not in comparison with healthy donors. In fact, in comparison with healthy donors, the level of *OPG* was lower in both the OA groups but did not show statistical significance. They observed no significant differences in the levels *RANKL*. Considering the MSCs are pre-osteoblasts cells, the results from Kwan et al., study are very comparable to that of the current project. However, they used cultured (P1) cells making the work in this PhD project novel due to the use of uncultured cells for better *in vivo* correlation of the disease progression in humans.

This project also investigated *RANKL* and *OPG* in HLCs which showed no differences in OA patients for *RANKL*. *OPG* was not detected in HLCs from old donors and measurable in OA group suggesting a potential for increase. This project thoroughly investigated transcripts associated with bone remodelling in two pure BM cell populations for understanding bone remodelling processes in OA. This has not been done previously, making this project novel.

Transcripts investigated for MSC stromal functions in this study in MSCs and HLCs from OA patients included *CXCL12* and *Cx43*. In the present study, there was a

significant decline observed in the expression of *CXCL12* in MSCs from OA patients as compared to MSCs from healthy old donors. However, there was no difference observed in the expression of *Cx43* in MSCs between OA patients and old donor groups. In HLCs, their differences observed were not significant. *Cx43* has been shown to be increased in synovial cells OA patients (457) and recently, *Cx43* has been suggested as a therapeutic target to halt OA progression (481). However, both of these studies were performed on cartilage and not on bone.

CXCL12 has been previously investigated in OA owing to its role in chemotaxis and movement of MSCs and other BM cells and its close links with hematopoietic compartment within the BM niche. Previous investigations of *CXCL12* in human OA patients remain limited and debatable. A study of MSCs from synovial joint of OA patients showed that the levels of *CXCL12* were much lower in OA as compared to those without the disease (456). Conversely, another study suggested that *CXCL12* binding to its receptor *CXCR4* contributed to the induction of cartilage degradation in OA (455). Even though a part of the study detected higher levels of *CXCL12* in the OA patients, the model used here was cartilage explants from human OA patients. The accumulation of alternating results complicate the process of finalising the underlying mechanism that brings about the changes in the levels of *CXCL12* in OA. However, with respect to OA, a study in 2017 identified *CXCL12* as an important indicator of knee OA using microarray analysis (482). However, there was no difference in the expression of *CXCL12* in HLCs in this project. Investigating *CXCL12* at the protein level along with the gene, in more donors and comparison in different stages of OA would allow in better understanding of the gene with disease progression.

LepR was also investigated in this thesis, both at the transcript and the protein level. Data from this chapter suggested a significant 55-fold decline in the expression of *LepR* transcript in OA MSCs as compared to healthy old donor MSCs. This trend was consistent with the surface marker expression of *LepR* (CD295) which also displayed a significant decline (3-fold) in MSCs from OA donors. This potentially indicated the impairment of bone-fat balance in OA bone. Interestingly, there was no difference observed in expression of *LepR*, both at the transcript and at the protein level, in the HLCs when compared between OA patients and healthy old donors.

The hormone leptin has been found to have increased expression in the blood, plasma (483) and in the synovial fluid of OA patients and has also been shown to correlate positively with the severity of the disease (468, 484). In other studies, synovial Leptin level has been positively associated with the pain in hip and knee OA in menopausal

women (484) as well shown to be directly proportional to BMI in OA patients (485). A review in 2013 further suggested that the missing link between obesity and OA was the hormone Leptin (270). Interestingly, Leptin was also found to increase in synovial fluid of OA patients and positively correlate with the age of the OA patients (468). Leptin has also been associated with increased levels of bone forming markers in OA patients (486). This could potentially explain the loss of bone-fat balance in OA, whereby Leptin hormone potentially increases and expression of LepR decreases, as seen from data in this chapter. One study reported higher levels of both the hormone leptin and its receptor in OA, but this was investigated in the cartilage OA patients (454).

Considering all of the above literature, it is clear that a lot of work has been performed on the role of Leptin in OA patients consistently reporting elevated levels of Leptin in OA patients. However, most of the studies have been focussed on synovial fluid, cartilage or serum levels. Expression levels of LepR via which the hormone Leptin functions has largely been overlooked and LepR in uncultured MSCs from OA donors has not been studied before. Lower levels of LepR in OA MSCs could potentially be a reaction to the higher levels of leptin in blood, which has already been suggested as a biomarker of the disease. Future work to understand differences in Leptin and its receptor in OA would ideally include blood and BM samples from OA patients to investigate them together in comparison with healthy old donors. Since there was no age-related difference in the expression of CD295 (Chapter4, section 4.3.8), the decline in CD295 observed in this chapter is clearly, specific to OA.

Interestingly, there has been an increase in the number of studies associating OA with ROS and ageing (487). *SOD3* expression in human cartilage is believed to decline in the later stages of OA (445). However, this project found a non-significant 2-fold increase in the level of *SOD3* in OA MSCs as compared to healthy old donor MSCs, which was unexpected. The fact that the present study found minor age-related difference in ROS measurement and that was complemented by a lack of any major difference in *SOD3* in BM MSCs (sections 4.3.3), is in line with the observation of no aggravated differences in *SOD3* in OA MSCs. This supports the notion that levels of ROS and anti-oxidant enzymes quantified in culture expanded MSCs (288) are likely to be in a state of induced oxidative stress and do not mirror the oxidative status of MSCs *in vivo*.

Measurement of ROS alongside antioxidant enzymes genes (like Gx, SODs) in uncultured MSCs has not been performed before. However, the current study did not show any age-related or OA-related differences. Future work should include a larger

panel of number of anti-oxidant enzymes (including all the SODs, Gx, Ascorbic acid) and perform these measurement in larger number of donors. Culture expanded cells are automatically exposed to oxygen concentrations that are significantly higher than in the BM niche *in vivo,* inducing oxidative stress the moment they are cultured in laboratory conditions. This could also explain why data from this project (Section 5.3.1) did not observe any differences in the level of ROS in young and old donor MSCs either.

With respect to the *IFNA1, IFNB1* and *IFNA* receptors, all of these were found to be reduced in OA patients with *IFNAR2* showing significant decline in both MSCs and HLCs. The presence of IFNA receptors has increasingly been associated with chondrocytes, synovial fibroblasts (488) and with activities their like mediating immune responses and operating various signalling networks within the bone (489). How the expression of IFN receptors change with progression of OA is a new field of research, but this can modulate binding with IFNs, and influence the immune signalling by downstream induction of various IRGs. A number of IRGs were found to be differentially expressed in both MSCs and HLCs from OA patients including *LAIR1* (decreased)*, CASP1* (decreased) and *ISG20* (increased). *LAIR1* is known as leukocyte-associated immunoglobulin-like receptor 1 that has previously been proposed as a marker of OA due to significantly higher expression in OA patients (490). In this project however, the expression was found to be significantly lower in both cell types of OA patients. The difference in trend with the aforementioned research could be due to the fact that their source was all cells from the synovial fluid. *CASP1* or caspase1 has also been shown to be upregulated in OA patients when investigated in peripheral blood mononuclear cells (491) or more recently, from synovium (492). However, these studies were based on patients identified with knee OA and not hip OA, and also explored these molecules in immune cells rather than in MSCs. *ISG20* very interestingly, unlike other genes in this chapter, indicated a significant increase in expression (irrespective of cell type) in OA patients. While there is not enough known about the involvement of this gene in OA, data from this project merits further investigation.

Analysis of the results of the genes associated with IFN1>BID>ROS pathway revealed a non-significant decline in nearly all genes, except for *STAT1*, which showed a significant decline in MSCs from OA donors and a non-significant decline in HLCs from OA donors as compared to healthy old donors. A decline in STAT1 was aligned with the reduced expression of IFN receptor explained above. A study that compared *STAT1* in patients with rheumatoid arthritis (RA), OA as well as spondyloarthritis (SpA)

found an overall increase in the level of *STAT1* in all of the 3 types of arthritis from tissue samples at the protein level (493). Another study that compared *STAT1* at the RNA and the protein level, found that *STAT1* was significantly higher in RA as opposed to OA patient synovial tissue samples (494). The opposite trends in this project can be explained by different tissue sources and techniques.

None of the anti-ageing genes or genes associated with senescence showed any significant differences in either of the cell types in OA, with *Tp53* showing a non-significant 3.5-fold decline in MSCs from OA patients. Previously, *Tp53* downregulation has been associated with reduced chondrocyte apoptosis due to strain (460) and another study suggested that miR-34a in the human chondrocytes played a vital role in OA progression via the Sirt1/p53 signalling pathway, making it a potential therapeutic target for OA. *Tp53* has also recently been labelled as one of the 'key genes' to be linked with OA, based on bioinformatics analysis (495).

Not only has *Sirt6* been suggested to prevent cellular senescence in chondrocytes (458), it has also been proposed as a potential therapeutic target for OA (459). *Sirt6* expression in this project showed no difference in OA MSCs or HLCs as compared to healthy old donors. Previous studies have indicated lower expression of *Sirt6* not only in aged mice but also in mice and humans with OA, as compared to healthy controls (496). The study also claimed to protect mice from cartilage damage using Lenti-Sirt6 intra-articular injection and to reduce chondrocyte senescence. Another study showed that inhibition of *Sirt6* from human chondrocytes *in vitro* reduced proliferation, increased senescence, DNA damage and telomere dysfunction (497). While there was no age-related or OA-related difference found in MSCs or HLCs in *Sirt6, Tp53* demonstrated an age-related decline which was further aggravated in OA (section 6.3.4) suggesting its role in ageing, senescence as well as in OA. Exploring Tp53 with p21 and p16 at gene and protein level in larger number of donors would be extremely useful in understanding the role of *Tp53* in age-related diseases.

Kl displayed a trend for decline (5-fold) in both MSCs and in HLCs in OA donors compared to healthy old donors. Previously, *Kl* has been shown to be associated with female Caucasian patients with hand OA wherein they found that a genetic variant in the gene was related to increased vulnerability towards hand OA (498). They also suggested that this association was potentially stemming from bone rather than cartilage. Similarly, another study of *Kl* genetic variants found that this gene was closely related to knee OA in Greek population (499). Each of these investigations recruited over 200 OA patients from whom peripheral blood sample was examined for

the single nucleotide polymorphism in the gene. Very recently, Tilly et al., found that *Kl* expression showed an age-related decline in articular cartilage (500). Although *Kl* expression increased in OA chondrocytes, $KL^{-/-}$ mice did not show any OA-related changes. They suggested that *Kl* potentially modulated pro-inflammatory cytokines in OA cartilage. Like *Tp53*, *Kl* too displayed an age-related decline in expression which was further aggravated in OA, even though the aggravated decline failed to reach statistical significance.

Among the cytokines, *IL6* showed a significant increase in its expression in MSCs and a non-significant increase in HLCs, in OA patients in this project. IL6 is among the most well examined cytokines associated with inflammation and ageing, as shown in discussion of Chapter 5. Thus it was not surprising to observe significantly higher levels of this cytokine transcript in OA. With respect to healthy ageing, there was a 4-fold increase in the expression of *IL6* in both, MSCs and in HLCs. Previous studies have reported elevated levels of IL6 in OA patients, however, most of these studies have used blood/serum samples (501) or synovial fluids (461). Therefore, this was the first study to investigate *IL6* in uncultured MSCs from OA patients and in two cell populations from healthy donors (MSCs and HLCs) simultaneously. IL7 is well-known cytokine to be secreted by BM MSCs (502). With respect to OA, this project found a trend for its decline in OA MSCs and no difference in HLCs. Data on the involvement of IL7 in OA is limited and to the best of my knowledge, has not yet been available on uncultured BM MSCs. Considering that IL7 plays an important role in cell proliferation and maturation of lymphoid cells along with having therapeutic potential as shown in a recent diabetic rat model (503), further investigations on IL7 would be interesting.

Among the surface markers, CD106 and CD295 were found to be significantly lower in OA MSCs in this project. Previously, CD106/VCAM-1 measured using ELISA from blood of OA patients who underwent surgery (n=60) revealed a very significant increase as compared to patients who did not undergo surgery (n=852) (463). Based on the findings that VCAM-1 levels are elevated in severe OA from the previous study, a very recent study investigated the serum levels of VCAM-1 in patients with hip OA (n=100) (466). They found a non-significant 8% increase in the levels of VCAM in patients with hip OA, and concluded that VCAM-1 was not able to distinguish severe OA from age and sex-matched controls. Interestingly, both these studies have tables that indicate BMIs in patients with severe OA to be significantly higher as compared to their control, potentially indicating a disruption in their bone-fat balance. In HLCs, while there was no difference in CD106, the trend for CD295 stayed the same as in MSCs, it displayed a non-significant decline. The different cell source and measuring VCAM-1

released protein as opposed to transcript in this study, explain the opposite results as compared to the findings in this thesis.

Cx43 and CD146 did not show any differences in young and old donors (Section 4.3.4) and neither in MSCs from OA donors. They are expressed on uncultured MSCs and definitely play important functions, however, a larger cohort of donors must be used to understand the differences in the level of expression of Cx43 and CD146 in young and old donor MSCs as well as in OA. One study focussed on the protein interactions of Cx43 with other protein interactors in OA (504). They found over 100 proteins that were closely linked with Cx43. Gene ontology data from their OA donors suggested maximum interactors of Cx43 related to cell adhesion, nucleolus, calmodulin and cytoskeleton from chondrocytes in OA cartilage.

In conclusion, there was a significant decline in adipogenic genes in OA MSCs along with a decline in OPG indicating towards increased bone resorption. *IFNAR2* also declined significantly in OA MSCs which potentially led to the decline in several IRGs in OA. This indicated the role of IFNAR2 as a marker in OA. CD106 and CD295 declined significantly in BM MSCs suggesting a decline in immune-regulatory properties and loss of the bone-fat balance, suggesting these could be potential markers of OA. Senescence associated genes did not show any statistically significant differences but the age-related *Tp53* and S*irt6* expression decline in HLCs was further aggravated in OA. Pro-inflammatory marker *IL6* displayed an age-related increase in both MSCs and in HLCs which was further significantly aggravated in OA. Interestingly, *IFNAR2, MTCH2* and *STING* which is the stimulator of IFN genes, both displayed an age-related decline followed by an exacerbated decline in OA HLCs. This indicates towards potential markers of age-related OA that need further exploration at protein level in a larger cohort of donors.

Chapter 7 Discussion

7.1 Key findings

The aim of this project was to investigate bone resident MSCs in healthy ageing and in OA by means of enumeration and gene expression analysis. In most parts, donor-matched HLCs were used as a control population. A decline in the number and proliferative capacity of MSCs was found in healthy ageing. The genes selected to reflect MSC multifunctionality and selected surface markers did not display any significant differences in old donor MSCs compared to young donor MSCs, but surprisingly, some significant age-related differences, for example in the expression of *RANKL, SPARC* and *LepR* were observed in HLCs. MSCs and HLCs were investigated further for senescence, SASP and IFN1 pathway-related exploratory genes and once again, age-related differences were more evident in HLCs as compared to MSCs. Finally, investigations in OA suggested a significant decline in *CXCL12*, *PPAR*$-\gamma$ and *LepR* expression in MSCs, which for *LepR* was confirmed at the surface protein level by flow cytometry. CD106 also showed a significant decline in MSCs from OA patients. Finally, a number of candidate genes (*SPARC, Kl, IL6,* and *IL8*) were found to display age-related differences in HLCs that were aggravated in OA patients, and *IL6* showed the same trend in MSCs. These candidate genes should be further investigated as potential biomarkers of age-related OA and potentially targeted for future therapeutic approaches aimed at slowing down the progression of the disease.

7.2 Clinical relevance

This project showed a significant age-related decline in the number of BM MSCs measured by the classical CFU-F assay along with a significant decline in the proliferation potential of MSCs from older donors measured by colony area and integrated density (ID) analysis. To understand proliferative capacity of MSCs in relatively more 'natural' conditions, similar experiments were performed in human serum. Growing young donor MSCs in OS conditions resulted in significantly lower colony IDs as compared to when these MSCs were grown in YS conditions. The IDs of colonies from old donor MSCs were significantly higher when grown in YS when compared to OS. This suggested that old donor MSCs could be partially 'rejuvenated' in terms of their proliferation potential when grown in YS, highlighting the importance of cell extrinsic factors in the MSC *in vivo* niche. Thus, Chapter 3 results supported the SCE theory of ageing (section 1.1.1), however, a few of the old donors exhibited MSC numbers that were as good as young donors if not better. Overall, this indicated

towards a general decline in the number and proliferative capacities of MSCs in older population.

However, the presence of old donors with a relatively good number of BM MSCs as compared to the other donors in their cohort, indicated that chronological age is a shared characteristic but biological age is personal and varies from donor to donor. From the clinician point of view, these data suggest that a higher volume of BMA needs to be aspirated from old donors to get a similar 'dose' of MSCs as compared to young donors. And even after a higher 'dose' of MSCs, the MSCs may need further stimulation and exposure to younger microenvironment in order to be used as a successful therapeutic (for fractures and other cell therapies). Rapid quantification of the number of $CD45^{low}$ $CD271^{+}$ cells (111) from the BMA of the donors, MSC fitness and measurement in donor serum predicted by oxidation state (320) along with the level of pro-inflammatory cytokines like *IL6* could help assess the biological age of the donor. This will potentially indicate the biological age of the donor cells to help predict the 'dose' of MSCs needed for a given patient.

Using the $CD45^{low}CD271^{+}$ phenotype to identify MSCs, this project showed a non-significant age-related decline in their numbers. However, this decline has been shown to become significant when data from more donors (n=67) were analysed (505). Adipogenic bias has been suggested as the most accepted feature of 'old' donor MSCs, even though some reports of no changes in their differentiation potential, do exist. While the exact mechanism for this proposed shift remains unknown, presence of ROS in old and damaged cells has often been suggested to be the cause. In this project, no differences in the expression of selected osteogenic- and adipogenic-lineage transcripts were found between young and old donors MSCs. Only some trends in the adipogenic bias were visible in old donor MSCs. In support of with this finding, when ROS and the level of anti-oxidant enzyme SOD3 were measured in MSCs, no significant difference between young and old donors were found. This indicated that scientists must be mindful when reporting on 'age-related' differences in MSCs, considering '*in vitro'* ageing and '*in vivo'* ageing may be driven by different mechanisms.

Transcripts for bone remodelling and stromal functions also did not display any significant age-related differences in MSCs, even though both *RANKL* and *OPG* increased in old donor MSCs. Surface markers also demonstrated no changes in old donor MSCs or in HLCs. Interestingly*, RANKL* and *SPARC* both showed significant declines not in MSCs, but in HLCs indicating that age-related changes in BM MSCs *in*

vivo are probably more subtle in comparison to the age-related changes in HLCs. This finding provided the first evidence that MSCs could be more resistant to age-related damages *in vivo* in comparison to HLCs, even though they may be exposed to the same levels of local cellular and intracellular insults such as ROS and DNA damage.

Taking into account recent research suggesting potential links between DNA damage, senescence, ROS, IFN1 signalling and ageing in HSCs, the question if DNA damage, senescence, ROS and ageing could also be linked to cells of our interest, the MSCs, via IFN-signalling, formed the basis of next set of experiments in Chapter 5. This study is novel (to the best of my knowledge) to investigate a large number of genes on a broad donor age range on uncultured BM MSCs and HLCs using pure cell populations. This chapter revealed very interesting findings. Firstly, while senescence and SASP associated genes displayed trends towards increased damage in old donor MSCs, they did not reach statistical significance. Second, with respect to the expression of IFN1 and related genes in MSC, some of them unexpectedly displayed very high expression in MSCs as compared to HLCs. Next, some novel genes were found to display age-related differences in HLCs. Interestingly, in this investigation too, only one out of 96 genes showed a significant decline in MSCs from older donors, further suggesting that age-related changes are relatively subtle in MSCs *in vivo*. However, genes associated with IFN1>BID>ROS pathway and senescence associated genes displayed significant changes in HLCs. This could be explained by the fact that MSCs are longer living cells and may have developed molecular mechanisms to combat ageing, which is not the case for HLCs that have shorter life-span. It will be interesting to investigate in the future if other long-living bone cells, such as osteocytes, have IFN1>BID>ROS signature similar to MSCs.

Finally, investigations in MSCs from OA donors revealed a significant decline in the expression of adipogenic transcription factor *PPAR-γ* and stromal derived cytokine *CXCL12* in MSCs. The surface marker CD106 also displayed a significant decline in OA donors as compared to healthy old donors and *LepR* showed a significant decline at both gene and protein levels. Many genes associated with IFN1>BID>ROS pathway, including *IFNR2* and *STAT1* were also down-regulated in OA MSCs. Again, OA HLCs demonstrated more profound OA-associated changes, some of which were in the same direction as noted for normal ageing in HLCs. *IL6* was identified as the only gene which displayed an aggravated age-related change in both cell populations in OA. Thus, this investigation essentially paves the way for identifying novel candidate biomarkers for age-related OA.

7.3 The 'bigger picture'

A number of genes investigated in this project have been shown by others to be associated with ageing/OA and/or other age-related diseases. For example, *SPARC* has been established as a key player in obesity and diabetes which are already been considered to be impacted by age (506). Makhluf and colleagues have previously shown an age-related decline in the expression of *OPG* in older OA donors, potentially connecting age with OA (507), however the study remained limited in terms of its patient cohort, given their youngest donor was 38 years old, when the decline in MSCs already take place, as shown in this thesis (Chapter 3) and the bone remodelling dynamics begin to shift (96). Another study on human femoral head BM investigated the levels of *RANKL* and *OPG* in OA patients and found an age-related decline in the levels of *OPG*. They also noticed variation in the levels of *OPG* in the young donors but the old donor cells consistently produced very low levels of *OPG.* The anti-ageing gene *Sirt6* was shown to be significantly reduced in the articular chondrocytes of OA patients that displayed increased cellular senescence as compared to non OA patients (508). Higher levels of IL6 and IL8 was found to be secreted from the synovial of obese OA patients as compared to normal weight OA patients (478).

In this context, my PhD has shown a decline in the expression of *OPG, LepR, PPAR-*γ and *SPARC* in MSCs from OA patients as compared to MSCs from old donors, suggesting a decline in osteogenic as well and adipogenic differentiation. *IFNAR2* declined significantly while *IL6* was found to increase significantly indicative of higher levels of SASP associated with OA MSCs. With respect to age-related differences that were aggravated in OA, the pro-inflammatory cytokine *IL6* displayed a significant increase in MSCs and the anti-ageing genes of *Sirt6* and *Kl* displayed a decline in HLCs.

There has been an enormous rise in the number of publications aimed at the different aspects of OA in the last two decades on the research front which indicates towards the increased needs, availability of funding and thirst for knowledge among the medical and scientific community. However, very few of these publications have been aimed at prevention of the condition, by acting on the aspects of an individual's life that can be controlled (diet, exercise, personal well-being). Healthcare systems globally should aim at early prevention of the disease for which early diagnosis is critical. This can be achieved by enhancing basic research towards early OA biomarkers to deliver more convenient diagnostic tools and better disease-modifying drugs. At the same time, it is equally important to spread awareness and educate people early on about the lifestyle choices that could prevent the disease altogether.

The lack of strong and statistically significant age-related changes in uncultured BM MSCs in this project, as compared to the vast majority of literature outlining significant age-related differences in terms of MSC proliferation potential, ROS production and senescence in cultured MSCs strongly indicate towards two critical issues in experimental approach. First, a stronger emphasis should be directed towards the fact that culture expanded MSCs used in the majority of previous studies undergo *in vitro* ageing which is not the same as the cell ageing *in vivo*. *In vitro* culture forces MSCs to adapt to artificial conditions and enhances proliferation, which has an impact on their differentiation, ROS production and signalling mechanisms combating senescence. Thus, the molecular mechanisms of MSC ageing proposed, based on cultured MSCs are more likely to be applicable only to culture expanded MSCs and do not explain the mechanisms occurring in MSCs *in vivo*.

Second, the concept of biological ageing as opposed to chronological ageing (both *in vivo*) needs to be better defined and considered in every research work that attempts to explore the complexity of ageing. Chronological age refers to the actual age of a donor while the biological age refers to the functionality of the organs (or cells) within the donor. Two or more people with the same chronological age can display difference in the 'fitness' of their organs indicating variation in their 'biological age'. Obese teenagers have been reported to have higher bone age than their peers of same chronological age when quantified using physical examinations (509). DW Belsky and co-workers found that adults of the same age (38 years old) were growing old at different rates described as different 'pace of ageing' quantified by using physical functioning, cognitive functioning and perceptions of well-being (510). They also suggested that investigation of age-related differences in younger (<40 years old) donors may be most useful to identify factors that can be quantified as 'early signs of ageing'. These findings bring our attention back to the various parts of thesis, for example large donor-variations in both 'young' and 'old' donor groups, confirming that the biological age of a person is different to their chronological age and may be determined by diet, exercise, lifestyle and well-being.

Biomedical research for ageing at the cellular level, often tends to investigate age-related differences in phenotypes, genotypes, senescence and other cell functions using chronological ages and *in vitro* assays. With increasing evidence of the impact of extrinsic factors like nutrition, diet and lifestyle impacting an alarmingly large number of people globally, there is a dire need to shift the approach towards considering these factors while designing experiments. While eliminating donors to suit a study design

may not be an option for all, having comprehensive knowledge of the donor's nutrition intake, diet, lifestyle and well-being for at least up to 6 months prior to investigation will help connect the dots better. This is further emphasised by the WHO defining health as the 'state of complete physical, mental and social wellbeing and not merely the absence of a disease' (1). A growing body of evidence shows that good physical and mental health are essential for complete well-being of a person (511, 512), especially in the old age (513). While these factors were not accounted for in this thesis, they could potentially explain the donor variations that were observed across the experiments performed in this project.

Increasingly, more and more online tests are easily available to help determine a person's 'actual age' (i.e. biological age), based on diet, exercise, smoking, drinking, social and mental well-being, in less than 5 minutes (514, 515). Interestingly, the methodologies of these online tests are unclear, there is no known standard or normalisation and they regularly provide very different 'actual age' of the same person who answers questions to different tests. I am personally aged 7, 22 and 38 years old in three different online tests! As more researchers focus on ageing studies and investigation of age-related diseases like OA, the aim must shift towards considering diet, lifestyle and well-being for defining biological age, as well as to consider various scenarios and donor variations in their experimental design to make a study more comprehensive. This will aid better understanding towards early OA diagnosis and eventually sustainable treatment solutions.

7.4 'Comprehensive' research

Even with the advancement of science, technology and our knowledge in healthcare and ageing, most of OA therapies are still aimed at alleviating the symptoms (pain relief for OA) rather than getting to the root of the problem, which is what causes the disease. Basic science plays a crucial role in studying cell behaviour, identifying the molecular pathways and mechanisms for a given condition. For research that may have clinical implications, it is vital that after optimising techniques, the research is encouraged to be performed on uncultured cells or using *in vitro* models (like 3D models using organ-on-a-chip or organoids) that can mimic the conditions *in vivo*. Acknowledgement of the fact that biomedical or healthcare research performed with human samples will inevitably display 'donor variation' in nearly every parameter being tested for any given experiment needs to be at the core of research investigating ageing and associated diseases like OA. Clinical trials may exclude a certain population from their study (for example, obese people) to suit their parameters better,

however, that might not be an option for other researchers that have access to a limited number of human samples.

Thus, having more information about the diet, exercise, lifestyle and well-being state of the donors over at least the last 6 months will help researchers to link various factors that link ageing and OA. Ageing is inevitable and an increase in age-related diseases and disorders make 'age' an important factor in every research. To understand a specific disease or disorder, it is usually approached with a finite focus on the topic. This approach is essential to narrow down and identify molecules, factors and targets for potential therapy for a single disease. When it comes to multi-factorial conditions like ageing and OA, the research plan and experimental design should include a number of variables and parameters to represent much larger donor populations.

For example, to investigate bone age and OA, sample collection should include not only bone samples but also matched sample of peripheral blood from the same donors. It would also include designing experiments to not only quantify a receptor (*LepR*) but also the hormone itself (Leptin) to better understand the signal transduction. The same should apply to IFN 1 type pathway mediators and SASP-associated molecules. To understand early signs of ageing and age-related differences, lifestyle data helping to define donor biological age would also provide essential information about potential causes (and potential targets) of age-related diseases. With multi-factorial conditions like ageing and OA a number of symptoms, parameters, genes and markers may overlap, increasing the complexity of the research work. Having more information will thus help to extrapolate markers or parameters that require further attention.

7.5 Study novelty and contribution to the field

The novelty of this project lies in the use of minimally-manipulated and uncultured human donor cells from a large number of donors and combination of a wide variety of genes. Biomedical research implementing the use of uncultured cells has strong implications and clinical relevance as it better reflects the behaviour and characteristics of cells *in vivo.* Over 50 donor samples were used for examining age-related changes in BM MSC numbers with minimum manipulation. Similar cohort-size investigations have been performed on cultured MSCs (97), but not on uncultured MSCs. This project was also the first of its kind to perform in depth investigation of over 100 genes in two uncultured and purified cell populations co-existing with the BM niche – MSCs and HLCs. Previous studies from our group have investigated 96 or 48 genes, but the cohort sizes were significantly smaller (8 donors (188) and 15 donors (516)), respectively. Also, age-related factors were not considered in these studies.

For the first time, this project evaluated colony sizes and densities to indicate MSC proliferation and heterogeneity in relation to donor age. Previous publications investigated different colony types based on sizes but not in relation to donor age (201, 208) and colony density has never been quantified before. Also for the first time, this project studied IFN1 signature in human MSCs, previous studies have only focussed on HSCs or immune-lineage cells (335, 391, 392). For the first time, this project has shown considerable IRG and IFN1 receptor expression in MSCs, opening up a completely new research area on the role of IFN1 signalling in non-immune cells and MSCs in particular. At this stage, it can be only speculated that MSCs may use IFN1 including their receptors and intracellular machinery to combat accumulating DNA damage and control senescence, future research is needed to further explore these possibilities.

Apart from directing research towards expanding the existing horizon of knowledge on ageing and age-related risks that may eventually lead to OA, this project has also suggested considering both, chronological age and biological age in biomedical research. This project also highlighted the links between general health, ageing and risk of age-related OA at the MSCs level aimed at understanding the roles of MSCs in these processes. Discussions in each chapter outline the need for comprehensive research that must be performed in future to be able to pinpoint to specific factors and targets to better understand age-related OA. Finally, this project also provides first initial data that MSCs may be more resistant to ageing and damaging insults *in vivo*, compared to HLCs, thus making them a good choice for developing autologous cell based therapies.

7.6 Drawbacks and study limitations

Working with a rare population of cells without being able to expand them in culture has certain limitations. The low number of retrieved cells, particularly MSCs from older donors, was always a point of concern with respect to experimental design. As established in the beginning of the thesis, to understand ageing *in vivo*, it is important that the cells being used are not artificially aged *in vitro*. Apart from that, the high degree of donor-to-donor variations was something unexpected but was observed across all the experiments during the course of this PhD. This meant that for many interesting findings, particularly in relation to gene expressions, the noted trends could not be confirmed statistically using the donor numbers available. However these pilot data provided important information on the magnitudes of differences and the variations within the groups, to enable power calculations for a more comprehensive

study. These variations, even with tightly controlled within age groups suggested that chronological age may or may not be represent the biological age of the person. Donor differences within the groups (young or old) could be explained by different donor's BMIs, diet and other lifestyle factors, such information was not available for this study. Due to ethical approvals available, this project did not include matched blood samples, which could have allowed to quantify and compare levels of transcripts in BM cells with the corresponding proteins (like Leptin) in the circulation. Overall, only a limited number of molecules were investigated at both, the transcript and protein level.

This project also did not perform any tests to evaluate and compare MSC differentiation or senescence status between young and old donors, which would be confirmatory to the presented gene expression data. Ideally, donor-matched comparisons could be done between *in vitro* cultured and *in vivo* MSCs. Looking back, quantification of genes and surface markers from the intermediate age group could have also been useful as well as having more information about the donors' about lifestyle, medication and general well-being. As discussed above, lifestyle and well-being are increasingly being associated with age-related diseases and thus, this kind of information would be extremely useful in terms of understanding donors' biological ages.

7.7 Future Directions

With the results obtained, it is clear that a larger cohort of patients is needed. However, with experiments performed in culture expanded MSCs, due to forced oxidative stress conditions, the results are often exaggerated. This can easily lead to scientists going ahead with animal studies and eventually to clinical trials, where over 50% of drugs have been known to fail (517, 518). It is ironical, that while this is the most exciting era for scientific research, biomedical and healthcare based technologies and the potential they hold for the future generation, it is also the era, where over 50% of all new drugs fail by the time they reach phase III clinical trials. It is also ironical, that even though, a human being lives the longest today we could have ever lived before, it also brings along with it the reduced quality of life after the age of 60 in most countries, globally. Not to forget, while there is a considerable increase in the access to different types of cuisines to people everywhere in the global era of networking and travel, 71% of the death every year is actually due to lifestyle disease which includes poor choices of diet and lifestyle (519).

Taking this project forward would include investigation of the genes that have been indicated as potential 'biomarkers' (IL6, anti-ageing genes and IFNA receptors) down to the protein level in larger number of donors including donors of ages across 20-90

(from early adulthood until old age). The last 6-months record about their diet (proportions of proteins, carbohydrates, fats, vitamins, minerals and water intake), lifestyle (drinking, smoking, exercises and medications) and well-being (questionnaires on personal motivation, family-friends-relationships and work life satisfaction) would be maintained. Further, classifying OA donors on the basis of the stage of the disease would be of critical importance. Comparing the levels of these indicative genes and proteins would aid our understanding of changes in these potential biomarkers with disease progression. Techniques like single cell RNA seq and the use of 3D models to mimic bone cells and the microenvironment (520) will further aid in investigations that are representative of changes *in vivo.*

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Appendix 1- List of donor cells used across the different arms of investigation in this thesis

Table 1 Donor details for cells used in Chapter 3

Donors	Sex	Age	CFU-F	Colony area , ID	CFU-F in serum
PGBM002	M	19	Yes		
PGBM003	F	63	Yes		
PGBM004	M	56	Yes		
PGBM005	F	37	Yes	Yes	
PGBM006	F	52	Yes		
HOBM010	F	74	Yes		
PGBM007	F	51	Yes		
HOBM011	M	24	Yes	Yes	
PGBM008	M	28	Yes	Yes	
HOBM012	M	58	Yes		
PGBM009	F	68	Yes	Yes	Yes
PGBM010	F	73	Yes	Yes	
PGBM011	M	42	Yes		
PGBM012	M	40	Yes		
PGBM013	M	42	Yes		
PGBM014	F	34	Yes		Yes
PGBM015	F	20	Yes		Yes
PGBM016	M	19	Yes	Yes	
PGBM017	M	46	Yes		
PGBM018	F	72	Yes		
PGBM019	F	72	Yes		
KMBM020	F	46	Yes		
JAO1	M	20	Yes		
KMBM021	M	19	Yes	Yes	Yes
HOBM013	M	58	Yes		
PGBM020	M	49	Yes		
KMBM027	F	23	Yes		
PGBM021	M	22	Yes		
PGBM022	F	49	Yes		
PGBM023	F	67	Yes		
PGBM024	M	42	Yes		
PGBM025	F	38	Yes		
PGBM026	F	69	Yes		Yes
PGBM027	F	89	Yes		
PGBM028	F	76	Yes	Yes	Yes
KMBM028	M	59	Yes	Yes	

HOBM014	M	44	Yes		
PGBM029	M	34	Yes		
PGBM030	F	89	Yes		
KMBM030	M	25	Yes	Yes	
JAO 25	F	55	Yes	Yes	Yes
PGBM031	F	53	Yes		Yes
PGBM032	M	46	Yes		
PGBM033	M	23	Yes		
JAO039	M	26	Yes		
PGBM034	F	36	Yes		
HOBM018	M	78	Yes		
PGBM036	M	54	Yes	Yes	
PGBM037	M	59	Yes	Yes	
PGBM038	F	64	Yes		
PGBM039	M	29	Yes		
PGBM040	F	37	Yes	Yes	Yes
PGBM041	F	26	Yes		Yes
PGBM042	M	67	Yes		
PGBM043	F	89	Yes		
PGBM044	F	79	Yes		

Age-range:19-89 years old, median: 47.5 years old

Table 2 Donor details for cells used in Chapter 4

Donors	Sex	Age	Enumeration by flow	ROS	Enriched	Cell sorting	MSC genes
PGBM005	F	37	Yes		Yes		
PGBM006	F	52	Yes		Yes		
HOBM010	F	74	Yes				
PGBM007	F	51	Yes				
HOBM011	M	24	Yes				
PGBM008	M	28	Yes		Yes		
PGBM009	F	68	Yes				
PGBM010	F	73	Yes		Yes	Yes	Yes
PGBM011	M	42	Yes				
PGBM012	M	40	Yes				
PGBM013	M	42	Yes				
PGBM014	F	34	Yes				
PGBM015	F	20	Yes				
PGBM016	M	19	Yes		Yes	Yes	Yes
PGBM017	M	46	Yes				
PGBM018	F	72	Yes				
PGBM019	F	72	Yes				
KMBM020	F	46	Yes				
JAO1	M	20	Yes				
KMBM021	M	19	Yes				
HOBM013	M	58	Yes				
PGBM020	M	49	Yes		Yes	Yes	
KMBM027	F	23	Yes		Yes	Yes	Yes
PGBM021	M	22	Yes		Yes	Yes	Yes
PGBM022	F	49	Yes				
PGBM023	F	67	Yes	Yes	Yes	Yes	Yes
PGBM024	M	42	Yes				
PGBM025	F	38	Yes	Yes	Yes	Yes	Yes
PGBM026	F	69	Yes	Yes	Yes	Yes	Yes
PGBM027	F	89	Yes	Yes	Yes	Yes	Yes
PGBM028	F	76	Yes	Yes	Yes	Yes	Yes
KMBM028	M	59	Yes				
HOBM014	M	44	Yes				
PGBM029	M	34	Yes	Yes	Yes	Yes	Yes
PGBM030	F	89		Yes	Yes	Yes	Yes
KMBM030	M	25	Yes	Yes	Yes	Yes	Yes
JAO 25	F	55	Yes				
PGBM031	F	53	Yes				
PGBM033	M	23		Yes	Yes	Yes	Yes
PGBM034	F	36		Yes	Yes	Yes	Yes

PGBM037	M	59	Yes	Yes	Yes	Yes	Yes
PGBM038	F	64			Yes	Yes	Yes
PGBM039	M	29		Yes	Yes	Yes	Yes
PGBM042	M	67	Yes				
PGBM047	M	65		Yes			
RCBM028	M	66			Yes	Yes	Yes
GCBM004	F	61			Yes	Yes	Yes
TRBM029	F	36			Yes	Yes	Yes

Age range: 19-89 years old, median: 47.5 years old

Table 3 Donor details for cells used in Chapter 5

Donors	Sex	Age	Enrichment	Cell sorting	Exploratory genes
PGBM005	F	37	Yes		
PGBM006	F	52	Yes		
PGBM008	M	28	Yes		
PGBM010	F	73	Yes	Yes	Yes
PGBM016	M	19	Yes	Yes	Yes
PGBM020	M	49	Yes	Yes	
KMBM027	F	23	Yes	Yes	Yes
PGBM021	M	22	Yes	Yes	Yes
PGBM023	F	67	Yes	Yes	Yes
PGBM025	F	38	Yes	Yes	Yes
PGBM026	F	69	Yes	Yes	Yes
PGBM027	F	89	Yes	Yes	Yes
PGBM028	F	76	Yes	Yes	
PGBM029	M	34	Yes	Yes	
KMBM030	M	25	Yes	Yes	Yes
PGBM033	M	23	Yes	Yes	Yes
PGBM034	F	36	Yes	Yes	Yes
PGBM037	M	59	Yes	Yes	Yes
PGBM038	F	64	Yes	Yes	Yes
PGBM039	M	29	Yes	Yes	Yes
RCBM028	M	66	Yes	Yes	Yes
GCBM004	F	61	Yes	Yes	Yes
TRBM029	F	36	Yes	Yes	

Age range: 19-89 years old, median: 43.5 years old

Table 4 Donor details for cells used in Chapter 6

Donors	Sex	Age	Cell sorting	MSC genes	Exploratory genes
DiFHOA003	M	74	Yes		
DiFHOA008	F	81	Yes		
DiFHOA014	F	56	Yes	Yes	Yes
DiFHOA017	M	68	Yes	Yes	Yes
DiFHOA018	F	83	Yes	Yes	
DiFHOA019	F	74	Yes	Yes	
DiFHOA021	F	72	Yes	Yes	
DiFHOA022	F	60	Yes	Yes	Yes
DiFHOA024	F	79	Yes	Yes	Yes
DiFHOA025	F	79	Yes	Yes	Yes
DiFHOA026	M	68	Yes	Yes	Yes
DiFHOA027	M	67	Yes	Yes	
DiFHOA029	M	82	Yes	Yes	Yes

Age range: 56-83 years old, median: 74 years old

Appendix 2 – List of reagents, consumables, equipments, softwares, solutions and Taqman probes used throughout the thesis

Table 1 List of reagents used throughout the thesis

Tissue culture	Company
Ammonium Chloride	Stem cells Technologies
Anti-fibroblast beads	MACS Miltenyi Biotec
DMEM	Gibco, Life Technologies
DMSO	Sigma
DNAse	Sigma
EDTA	Sigma
FCS	Thermo Fisher
Formaldehyde	Sigma
MACSelect LNGFR beads	MACS Miltenyi Biotec
Methylene blue	Sigma
PBS	Sigma
Penicillin/Streptomycin	Thermo Fisher
RosetteSep cocktail	Stem Cells Technologies
StemMACS MSC expansion medium	MACS Miltenyi Biotec
Trypan blue	Sigma
Trypsin	Sigma

Flow cytometry/FACS	Company
7-AAD	BD Pharmigen
Blocking buffer	Sigma
BSA	Sigma
CD106 PE	BD Pharmigen
CD146 PE	BD Pharmigen
CD271 APC	MACS Miltenyi Biotec
CD271 PeVio770	MACS Miltenyi Biotec
CD295 APC	BD Pharmigen
CD45 PE-Cy7	BD Pharmigen
CD45 V450	BD Pharmigen
IgG1 PE	BD Pharmigen
IgG2a APC	BD Pharmigen
IgG2b APC	R & D systems
IgG1 PE Vio 770	MACS Miltenyi Biotec
CellROX (FITC)	Thermo Fisher
Connexin43 APC	R & D systems
Countbright absolute counting beads	Life Technologies
FCR	MACS Miltenyi Biotec
TBHP	Thermo Fisher

qPCR	Company
Assay loading buffer	Fluidigm
Elution solution	Norgen Biotek
Nuclease free water	Thermo Fisher
PA master mix	Fluidigm
RL Buffer (lysis buffer)	Norgen Biotek
RNAse away	Thermo Fisher
Rt mastermix	Fluidigm
Sample loading buffer	Fluidigm
Single cell RNA purfication kit	Norgen Biotek
Taqman Universal master mix	Thermo Fisher
Washing solution	Norgen Biotek

Table 2 List of consumables used throughout the thesis

Tissue culture	**Company**
Cell strainer (70μm)	BD Falcon
Cryo vials (1.8ml)	Thermo Scientific
EDTA tubes (4ml)	VACUETTE, Griener Bio-one
Falcon tubes (15 and 50 ml)	Corning
Mr Frosty Freezing container	Thermo Scientific
Petri dishes (60 and 100mm)	Corning
Pipette Tips (10, 200 and 1000μl)	Rainin
Stripettes (5,10 and 25ml)	Corning

Flow cytometry/FACS	**Company**
FACS tubes	Corning
Magnetic columns and plunger	MACS Miltenyi Biotec
Tubes with cell strainers	BD

qPCR	**Company**
48.48 dynamic array IFC	Fluidigm
Collection tubes	Norgen Biotek
Control line fluid	Fluidigm
Eppendorfs (0.5-1.5ml)	Eppendorf
Flex Six™ gene expression IFC	Fluidigm
RNA elution tubes	Norgen Biotek
Single cell RNA spin columns	Norgen Biotek

Table 3 List of equipments used throughout the thesis

Tissue culture	**Company**
Centrifuge	Eppendorf
Class II laminar flow biological safety cabinet	Nuaire
CO2 Incubator	Incusafe
Freezer (-80°C)	New Brunswick Scientific
Haemocytometer	Improved Neubauer, Hawksley
Infinity 1 Camera	Lumenera
MACS separator	MACS Miltenyi Biotec
MACS stand	MACS Miltenyi Biotec
Microscope (Scanner)	Infinity 1, L3200B
Microscope (TC)	Olympus CKX41
Nanodrop Spectrophotometer	Nanodrop
Pipetteboy	Integra
Scanner	Epson perfection 3590 photo
Water bath	Leica

Flow cytometry/FACS	**Company**
Influx 6 way cell sorter	BD Biosciences
LSRII flow cytometer	BD Pharmigen
Pipettes	Gilson

qPCR	**Company**
Biomark HD system	Fluidigm
IFC controller HX	Fluidigm
Thermocycler	Applied biosciences

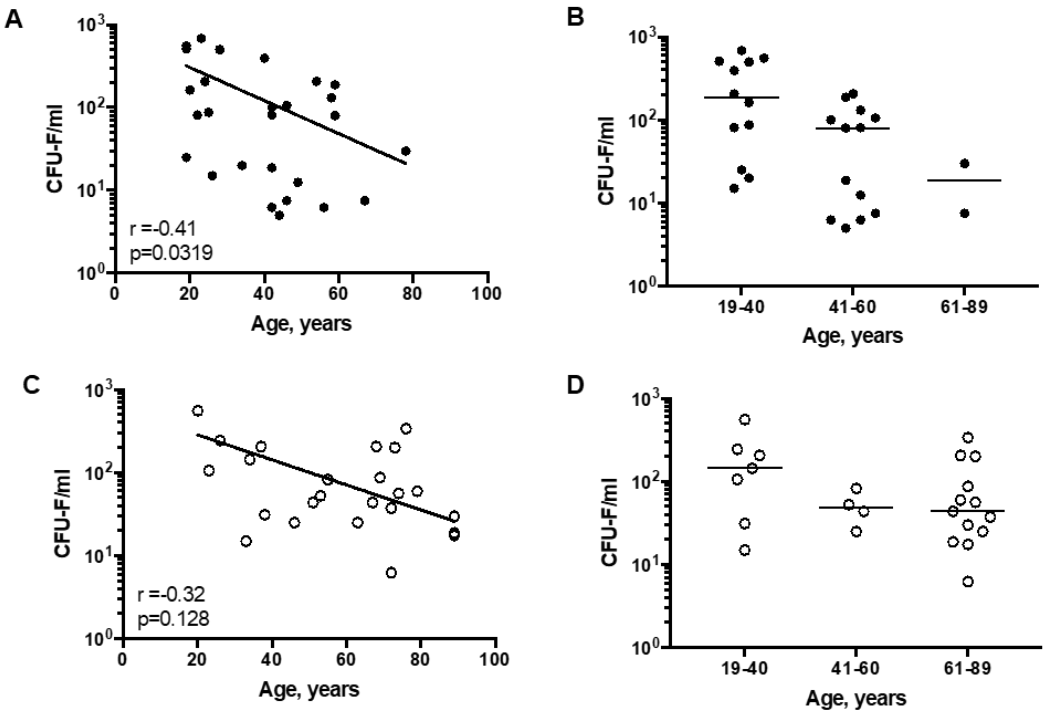
Table 4 List of softwares used throughout the thesis

Softwares	Company
Cluster 3.0	Open source
Data collection software	Biomark
FACS Diva 5.02	BD Biosciences
FlowJo	BD Biosciences
GraphPad Prism (version 7.0a)	GraphPad Software, Inc.
ImageJ	ImageJ
Infinity analyze and capture	Lumenera
Real-time PCR analysis	Biomark
Tree view	Open source

Table 5 List of buffers/reagents prepared and used throughout the thesis

Buffer	Preparation
Ammonium chloride	17.89g NH_4Cl + 2g KCl + 400µl 0.5M EDTA +200ml distilled Water, pH=8.0
FACS buffer	500ml PBS + 0.1%BSA + 0.01%Sodium Azide + 200µl of Na EDTA
Formaldehyde (3.7%)	1ml of 37%formaldehyde + 9 ml PBS
Freezing medium	10%DMSO + 45%FCS + 45%DMEM
MACS buffer	500ml PBS+ 0.1%BSA + EDTA (2ml)
Methylene blue	1%Methylene Blue in10mM borate buffer, pH=8.8
Thawing medium	DMEM+10%FCS + 80µl DNAse for 50ml
Wash buffer (for enrichment)	PBS+ 2%FCS + 2ml EDTA

Appendix 3 – Supplementary Figure 3.1



Age-related changes in the number of CFU-Fs in males (top panel) and females (bottom panel)

(A) Age-related change in the number of CFU-F per ml of BMA across entire donor age range in males and **(B)** in between age groups. **(C)** Age-related change in the number of CFU-F per ml of BMA across entire donor age range in females and **(D)** in between age groups. Each dot indicates individual donor, black dots represent male donors and empty circles represent female donors. The black line on the left indicates the slope and the on the right, the median values. Spearman non-parametric test was performed for A and C and Kruskal-wallis test with Dunn's correction was performed for B and D.

Appendix 4 List of tables indicating expression of exploratory genes from Chapter 5 and 6

All the data was normalised with HPRT1. $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, Mann-Whitney U test. NA: not-applicable, NS: non-significant

Table 5.1 Medians of expression of exploratory genes in all (young and old donors combined) donors compared between MSCs and HLCs

Gene	Median (MSCs)	Median (HLCs)	Fold difference (MSCs/HLCs)	*p* value
ABCA1	1.17	0.24	4.88	0.0249
ABCG1	ND	0.058	NA	NA
AICDA	ND	0.009	NA	NA
BST2	12.06	4.97	2.43	0.0083
CASP1	3.06	6.16	0.50	0.0205
CCL8	0.22	0.072	3.03	0.0591
CCND2	2.36	0.41	5.68	0.0013
CEACAM	0.31	0.007	44.29	0.0021
CHMP5	2.41	0.7	3.44	NS
CXCL10	0.25	0.40	0.61	NS
EPSTI1	1.59	0.57	2.79	0.0045
EIF2AK2	3.81	0.95	3.98	0.0023
FCGR1B	0.19	1.04	0.18	0.0031
GBP1	3.4	1.375	2.47	0.078
GUSB	2.66	3.39	0.78	NS
HERC5	0.36	0.2	1.80	0.0188
HPSE	0.09	0.73	0.13	0.0171
IFI6	3.285	0.438	7.5	0.0145
IFI16	1.66	0.81	2.06	0.0068
IFI27	1.55	0.01	155.10	<0.0001
IFI35	2.68	0.76	3.53	0.0387
IFI44	3.1	1.01	3.07	0.0205
IFI44L	1.50	0.31	4.86	0.008
IFIH1	0.45	0.17	2.65	0.0106
IFIT1	9.13	3.70	2.46	NS
IFIT2	1.53	2.67	0.57	NS
IFIT3	2.69	1.86	1.45	NS
IFIT5	0.63	0.11	5.75	0.0284
IFITM1	75.05	6.33	11.86	0.0005
IFITM3	137.9	3.79	36.39	<0.0001
IFNG	ND	ND	NA	NA
IL7R	0.09	0.18	0.51	NS
IRF2	2.48	1.83	1.36	0.0372
IRF5	0.12	1.28	0.10	0.0157
IRF7	4.73	4.97	0.95	NS
IRF9	1.70	0.99	1.72	NS

ISG15	0.38	0.55	0.69	NS
ISG20	0.39	1.12	0.35	NS
LAIR1	0.43	1.4	0.31	0.0278
LAMP3	ND	ND	NA	NA
LRP1	48.92	4.03	12.14	0.0007
LSCR1	4.12	0.99	4.13	0.0068
LY6E	24.58	2.4	10.24	<0.0001
MSR1	0.11	0.20	0.53	NS
MX1	2.889	1.71	1.69	NS
NT5C3B	0.56	0.16	3.52	0.0018
OAS1	0.61	0.13	4.66	NS
OAS2	1.19	0.67	1.78	0.059
OAS3	2.55	0.7	3.64	0.0121
OASL	1.01	0.22	4.61	0.0284
PHF11	1.79	0.98	1.82	0.0242
PPIA	14.26	13.66	1.04	NS
PRDM1	0.51	1.72	0.30	0.0148
PRDM16	0.18	0.0017	105.29	0.042
PRKRA	0.87	0.27	3.16	NS
RGS1	0.621	32.29	0.02	0.0184
RNF213	3.47	2.64	1.31	NS
RSAD2	0.43	0.13	3.30	0.0169
RTP4	0.96	0.08	12.00	0.0036
SAMD9L	1.67	0.642	2.60	0.0387
SCARB1	0.59	0.349	1.69	NS
SERPING	22	0.09	233.30	0.0003
SIGLEC1	0.13	0.38	0.34	0.0782
SOCS1	2.55	0.46	5.55	0.0003
SP100	6.78	3.86	1.76	0.049
SPATS2L	3.50	0.40	8.66	0.0001
TAP1	2.23	1.64	1.36	NS
TGFB	2.93	5.35	0.55	0.0205
TLR4	1.42	1.44	0.99	NS
TNF	0.17	3.67	0.05	0.013
TNFRSRP1	1.043	2.62	0.40	0.0512
TRIM38	0.793	0.62	1.27	NS
UBE2L6	11.86	4.5	2.64	0.0083
UNC93B	2.08	2.75	0.76	NS
USP18	0.35	0.02	17.50	<0.0001
XAF1	3.93	3.273	1.20	NS

Table 5.2 Medians of expression of exploratory genes between young and old donors in MSCs

Gene	Medians (young)	Medians (old)	Age-related difference (old/young)	*p* value
ABCA1	1.11	2.78	2.51	NS
ABCG1	ND	ND	NA	NA
AICDA	ND	ND	NA	NA
BST2	12.06	14.6	1.21	NS
CASP1	3.23	3.05	0.94	NS
CCL8	0.21	0.26	1.22	NS
CCND2	2.36	3.57	1.52	NS
CEACAM	0.1	0.35	3.50	NS
CHMP5	3.10	1.66	0.54	NS
CXCL10	0.56	0.16	0.29	NS
EPSTI1	1.59	1.69	1.06	NS
EIF2AK2	3.48	3.8	1.09	NS
FCGR1B	0.05	0.27	5.00	NS
GBP1	3.173	3.4	1.07	NS
GUSB	2.98	2.33	0.78	NS
HERC5	0.53	0.34	0.64	NS
HPSE	0.11	0.08	0.80	NS
IFI6	4.36	2.57	0.59	NS
IFI16	1.54	1.76	1.14	NS
IFI27	1.26	2.06	1.63	NS
IFI35	2.68	2.11	0.79	NS
IFI44	3.83	2.71	0.71	NS
IFI44L	1.54	1.42	0.92	NS
IFIH1	0.45	0.43	0.96	NS
IFIT1	7.04	10.06	1.43	NS
IFIT2	1.33	1.68	1.26	NS
IFIT3	1.78	3.4	1.91	NS
IFIT5	0.63	0.55	0.87	NS
IFITM1	75.05	81.38	1.08	NS
IFITM3	139.4	111.9	0.80	NS
IFNG	ND	ND	NA	NA
IL7R	0.22	0.05	0.24	NS
IRF2	2.48	2.23	0.90	NS
IRF5	0.11	0.42	3.91	NS
IRF7	4.73	4.13	0.87	NS
IRF9	1.70	1.35	0.79	NS
ISG15	0.42	0.28	0.67	NS
ISG20	0.51	0.35	0.69	NS
LAIR1	0.8	0.43	0.54	NS
LAMP3	ND	ND	NA	NA

LRP1	50.97	38.69	0.76	NS
LSCR1	4.11	6.41	1.56	NS
LY6E	24.58	21.34	0.87	NS
MSR1	0.12	0.05	0.45	NS
MX1	2.89	2.9	1.00	NS
NT5C3B	0.47	0.67	1.41	NS
OAS1	0.68	0.58	0.85	NS
OAS2	1.52	0.88	0.58	NS
OAS3	2.72	1.79	0.66	NS
OASL	1.09	0.82	0.75	NS
PHF11	2.10	1.76	0.84	NS
PPIA	15.94	12.03	0.75	NS
PRDM1	0.21	0.66	3.14	NS
PRDM16	0.29	0.17	0.6	NS
PRKRA	0.79	1.1	1.39	NS
RGS1	0.62	6.11	9.85	NS
RNF213	4.52	1.88	0.42	0.0411
RSAD2	0.394	0.47	1.19	NS
RTP4	0.96	0.71	0.74	NS
SAMD9L	1.83	0.90	0.50	NS
SCARB1	0.80	0.20	0.25	NS
SERPING	21.95	22.85	1.04	NS
SIGLEC1	0.20	0.13	0.65	NS
SOCS1	3.79	2.13	0.56	NS
SP100	6.75	6.78	1.00	NS
SPATS2L	4.09	3.38	0.83	NS
TAP1	2.11	2.53	1.20	NS
TGFB	3.35	2.59	0.77	NS
TLR4	1.2	2.16	1.80	NS
TNF	1.58	0.155	0.10	NS
TNFRSRP1	1.37	0.76	0.55	NS
TRIM38	0.80	0.73	0.91	NS
UBE2L6	11.26	12.94	1.15	NS
UNC93B	2	2.21	1.105	NS
USP18	0.31	0.36	1.16	NS
XAF1	5.81	3.03	0.52	NS

Table 5.3 Medians of expression of exploratory genes between young and old donors in HLCs

Genes	Median (young)	Median (old)	Age-related difference (old/young)	*p* value
ABCA1	0.53	0.16	0.30	NS
ABCG1	0.07	0.03	0.43	0.0649
AICDA	0.009	0.01	1.11	NS
BST2	6.29	3.24	0.51	NS
CASP1	7.18	4.77	0.66	NS
CCL8	0.09	0.03	0.42	NS
CCND2	0.85	0.291	0.34	NS
CEACAM	0.008	0.003	0.38	0.0667
CHMP5	0.87	0.63	0.72	NS
CXCL10	0.58	0.18	0.31	0.0667
EPSTI1	0.58	0.51	0.88	NS
EIF2AK2	0.80	1.05	1.30	NS
FCGR1B	1.05	1.55	1.48	NS
GBP1	1.67	1.33	0.80	NS
GUSB	4.49	2.57	0.57	NS
HERC5	0.22	0.18	0.82	NS
HPSE	0.73	0.66	0.91	NS
IFI6	0.87	0.76	0.87	NS
IFI16	0.31	0.55	1.77	NS
IFI27	0.014	0.008	0.57	NS
IFI35	0.93	0.50	0.54	0.0649
IFI44	1.01	0.87	0.86	NS
IFI44L	0.31	0.33	1.06	NS
IFIH1	0.19	0.16	0.84	NS
IFIT1	2.17	5.73	2.64	NS
IFIT2	2.51	2.76	1.10	NS
IFIT3	1.76	1.86	1.06	NS
IFIT5	0.12	0.058	0.47	0.0411
IFITM1	18.32	2.22	0.12	NS
IFITM3	7.15	3.45	0.48	NS
IFNG	ND	ND	NA	NA
IL7R	0.49	0.09	0.20	0.026
IRF2	2.09	1.11	0.53	0.0043
IRF5	1.59	1.08	0.68	NS
IRF7	6.37	4.97	0.78	NS
IRF9	1.52	0.62	0.41	0.026
ISG15	0.4	0.6	1.50	NS
ISG20	1.18	0.83	0.70	NS
LAIR1	1.54	1.4	0.91	NS

LAMP3	ND	ND	NA	NA
LRP1	4.65	2.73	0.59	NS
LSCR1	1.29	0.65	0.50	NS
LY6E	2.91	1.8	0.62	NS
MSR1	0.177	0.318	1.80	NS
MX1	1.71	2.13	1.25	NS
NT5C3B	0.23	0.09	0.41	NS
OAS1	0.35	0.05	0.15	NS
OAS2	0.69	0.22	0.32	NS
OAS3	0.9	0.49	0.54	NS
OASL	0.22	0.26	1.18	NS
PHF11	0.77	1.20	1.55	NS
PPIA	16.08	12.35	0.77	NS
PRDM1	2.35	1.4	0.60	NS
PRDM16	0.0012	ND	NA	NA
PRKRA	0.97	0.2	0.21	0.0152
RGS1	28.47	32.29	1.13	NS
RNF213	3.01	1.75	0.58	NS
RSAD2	0.13	0.12	0.92	NS
RTP4	0.15	0.05	0.33	0.0152
SAMD9L	0.86	0.47	0.55	NS
SCARB1	0.431	0.131	0.30	0.0087
SERPING	0.10	0.08	0.80	NS
SIGLEC1	0.38	0.35	0.92	NS
SOCS1	0.72	0.33	0.46	NS
SP100	4.43	3.27	0.74	NS
SPATS2L	0.47	0.37	0.79	NS
TAP1	1.429	1.65	1.15	NS
TGFB	6.42	4.64	0.72	NS
TLR4	1.87	0.92	0.49	NS
TNF	2.98	3.66	1.23	NS
TNFRSRP1	2.69	2.62	0.97	NS
TRIM38	0.58	0.68	1.17	NS
UBE2L6	5.04	4.26	NS	NS
UNC93B	2.9	2.31	0.80	NS
USP18	0.027	0.025	0.93	NS
XAF1	3.212	3.61	1.12	NS

Table 6.1 Medians of expression of exploratory genes between old donors and OA patients in MSCs

Genes	Medians (Old)	Medians (OA)	Difference in OA (OA/Old)	*p* value
ABCA1	2.78	2.88	1.04	NS
ABCG1	0.02	0.21	12.35	NS
AICDA	ND	ND	NA	NA
BST2	14.60	1.36	0.09	0.0047
CASP1	3.04	0.53	0.17	0.0012
CCL8	0.25	0.12	0.48	NS
CCND2	3.57	2.43	0.68	NS
CEACAM	0.35	0.02	0.06	0.0221
CHMP5	1.66	1.78	1.07	NS
CXCL10	0.16	0.63	3.94	NS
EPSTI1	1.69	0.31	0.18	0.035
EIF2AK2	3.81	1.72	0.45	NS
FCGR1B	0.27	0.00	0.01	0.0476
GBP1	3.40	3.30	0.97	NS
GUSB	2.34	0.67	0.29	0.035
HERC5	0.34	0.20	0.59	NS
HPSE	0.08	0.06	0.75	NS
IFI6	2.57	1.45	0.56	NS
IFI16	1.76	1.16	0.66	NS
IFI27	2.15	0.82	0.38	0.014
IFI35	2.11	1.29	0.61	NS
IFI44	2.71	0.60	0.22	0.0047
IFI44L	1.43	0.35	0.25	0.0047
IFIH1	0.42	0.28	0.67	NS
IFIT1	10.06	3.90	0.39	NS
IFIT2	1.68	0.64	0.38	NS
IFIT3	3.40	0.51	0.15	0.014
IFIT5	0.55	0.26	0.47	NS
IFITM1	81.38	8.52	0.10	0.014
IFITM3	111.90	11.60	0.10	0.014
IFNG	ND	ND	NA	NA
IL7R	0.05	0.19	3.66	NS
IRF2	2.23	1.11	0.50	NS
IRF5	0.42	0.01	0.02	NS
IRF7	4.13	2.48	0.60	NS
IRF9	1.35	0.88	0.65	NS
ISG15	0.28	1.56	5.57	NS
ISG20	0.35	1.17	3.34	0.0424
LAIR1	0.43	0.13	0.30	0.0303

LAMP3	ND	ND	NA	NA
LRP1	38.69	8.00	0.21	0.0221
LSCR1	6.41	2.04	0.32	NS
LY6E	21.34	4.07	0.19	0.0152
MSR1	0.06	0.11	2.00	NS
MX1	2.90	1.66	0.57	NS
NT5C3B	0.67	0.37	0.55	NS
OAS1	0.58	0.23	0.39	NS
OAS2	0.88	0.30	0.34	NS
OAS3	1.80	0.54	0.30	0.014
OASL	0.82	0.79	0.96	NS
PHF11	1.76	0.81	0.46	NS
PPIA	12.03	9.65	0.80	NS
PRDM1	0.66	0.71	1.08	NS
PRDM16	0.17	0.11	0.65	NS
PRKRA	1.10	0.82	0.74	NS
RGS1	6.12	0.18	0.03	NS
RNF213	1.88	4.07	2.16	NS
RSAD2	0.47	0.33	0.70	NS
RTP4	0.71	0.12	0.17	0.0221
SAMD9L	0.91	0.58	0.64	NS
SCARB1	0.20	0.24	1.20	NS
SERPING	22.85	8.80	0.39	0.0513
SIGLEC1	0.13	0.01	0.06	NS
SOCS1	2.13	0.76	0.36	NS
SP100	6.78	2.99	0.44	NS
SPATS2L	3.82	2.12	0.55	NS
TAP1	2.53	1.27	0.50	0.0513
TGFB	2.59	2.90	1.12	NS
TLR4	2.16	0.88	0.41	NS
TNF	0.16	0.60	3.88	NS
TNFRSRP1	0.76	0.65	0.86	NS
TRIM38	0.73	0.83	1.14	NS
UBE2L6	12.94	2.08	0.16	0.014
UNC93B	2.21	2.37	1.07	NS
USP18	0.36	0.06	0.17	0.0082
XAF1	3.03	1.51	0.50	NS

Table 6.2 Medians of expression of exploratory genes between old donors and OA patients in HLCs

Genes	Medians (Old)	Medians (OA)	Difference in OA (OA/Old)	*p* value
ABCA1	0.17	0.59	3.51	0.035
ABCG1	0.03	0.17	5.28	0.0012
AICDA	0.01	0.00	0.06	NS
BST2	3.24	1.44	0.44	0.035
CASP1	4.77	1.01	0.21	0.0023
CCL8	0.04	0.02	0.61	NS
CCND2	0.29	2.29	7.90	0.0022
CEACAM	0.00	0.01	2.00	NS
CHMP5	0.60	0.21	0.34	NS
CXCL10	0.19	0.11	0.58	NS
EPSTI1	0.51	0.34	0.67	NS
EIF2AK2	1.05	0.96	0.92	NS
FCGR1B	1.55	0.07	0.04	0.0012
GBP1	1.34	0.85	0.63	NS
GUSB	2.57	0.72	0.28	0.0047
HERC5	0.18	0.22	1.22	NS
HPSE	0.66	0.16	0.24	NS
IFI6	0.55	0.48	0.87	NS
IFI16	0.76	0.56	0.74	NS
IFI27	0.01	0.07	8.75	0.0051
IFI35	0.51	0.29	0.57	NS
IFI44	0.87	0.37	0.43	NS
IFI44L	0.33	0.16	0.48	NS
IFIH1	0.17	0.20	1.20	NS
IFIT1	5.73	1.50	0.26	0.0734
IFIT2	2.76	0.49	0.18	0.0513
IFIT3	1.86	0.76	0.41	NS
IFIT5	0.06	0.13	2.19	NS
IFITM1	2.22	9.11	4.10	0.0734
IFITM3	3.45	2.47	0.72	NS
IFNG	0.07	1.29	17.42	0.0177
IL7R	0.09	2.89	32.11	0.0012
IRF2	1.11	0.65	0.59	NS
IRF5	1.08	0.14	0.13	0.0012
IRF7	4.98	3.19	0.64	NS
IRF9	0.63	0.35	0.56	NS
ISG15	0.59	0.47	0.80	NS
ISG20	0.83	5.08	6.12	0.0012
LAIR1	1.40	0.61	0.44	0.014
LAMP3	0.01	0.49	40.67	0.0023
LRP1	2.73	0.31	0.11	NS
LSCR1	0.65	0.81	1.25	NS
LY6E	1.80	1.40	0.78	NS

MSR1	0.31	0.18	0.58	NS
MX1	2.13	1.68	0.79	NS
NT5C3B	0.09	0.07	0.78	NS
OAS1	0.05	0.02	0.44	NS
OAS2	0.22	0.44	2.00	NS
OAS3	0.49	0.65	1.33	NS
OASL	0.26	0.74	2.85	0.035
PHF11	1.21	0.86	0.71	NS
PPIA	12.35	7.68	0.62	NS
PRDM1	1.40	3.60	2.57	0.0221
PRDM16	ND	ND	NA	NA
PRKRA	0.20	0.21	1.05	NS
RGS1	32.29	21.98	0.68	NS
RNF213	1.75	2.23	1.27	NS
RSAD2	0.12	0.32	2.63	NS
RTP4	0.05	0.03	0.58	NS
SAMD9L	0.47	0.31	0.66	NS
SCARB1	0.13	0.05	0.38	NS
SERPING	0.09	0.09	1.00	NS
SIGLEC1	0.35	0.09	0.25	0.0087
SOCS1	0.33	1.03	3.11	NS
SP100	3.28	2.50	0.76	NS
SPATS2L	0.37	0.32	0.85	NS
TAP1	1.65	1.81	1.10	NS
TGFB	4.64	13.23	2.85	0.014
TLR4	0.92	0.16	0.17	0.0513
TNF	3.66	2.16	0.59	NS
TNFRSRP1	2.62	4.96	1.89	NS
TRIM38	0.68	0.24	0.35	NS
UBE2L6	4.26	1.58	0.37	0.035
UNC93B	2.32	0.51	0.22	0.0012
USP18	0.03	0.03	1.12	NS
XAF1	3.61	1.93	0.53	NS

Appendix 5 Ethics

Dr Ann Morgan
Senior Lecturer and Honorary Consultant
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Clinical Sciences Building
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LS9 7TF

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

Enquiries to Ann Prothero
Ethics Secretary
Direct Line/Ext: 0113 (20) 65652

22 October 2004

Dear Dr Morgan,

Full title of study: Functional characterisation of the genes and proteins involved in the development and severity of autoimmune and (auto)inflammatory diseases
REC reference number: 04/Q1206/107

Thank you for your letter of 08 October 2004, responding to the Committee's request for further information on the above research.

The further information has been considered on behalf of the Committee by the Chairman

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation.

The favourable opinion applies to the following research site:

Site: The Leeds Teaching Hospitals NHS Trust
Principal Investigator: Dr Ann Morgan

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Application dated 15/08/2004
Investigator CV dated 15/08/2004
Protocol for 'Investigation of the molecular mechanisms involved in Tumour Necrosis Factor Receptor associated periodic syndrome (TRAPS) dated 16/08/2004

The Leeds Teaching Hospitals NHS
NHS Trust

Ref: Amy Dickinson

17/07/2014

Research & Development
Leeds Teaching Hospitals NHS Trust
34 Hyde Terrace
Leeds
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Professor Dennis McGonagle
Rheumatology Department
Chapel Allerton Hospital
Chapeltown Road
Leeds
LS7 4SA

Dear Professor Dennis McGonagle

Re: NHS Permission at LTHT for: Collection of joint Mesenchymal Stem Cells by aspiration, biopsy, joint retrieval at arthroplasty or by synovium agitation during arthroscopy
LTHT R&D Number: RR14/11102 (100077/WY)
REC: 14/YH/0087

I confirm that *NHS Permission for research* has been granted for this project at The Leeds Teaching Hospitals NHS Trust (LTHT). NHS Permission is granted based on the information provided in the documents listed below. All amendments (including changes to the research team) must be submitted in accordance with guidance in IRAS. Any change to the status of the project must be notified to the R&D Department.

Permission is granted on the understanding that the study is conducted in accordance with the *Research Governance Framework for Health and Social Care*, ICH GCP (if applicable) and NHS Trust policies and procedures available at http[illegible].

This permission is granted only 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

NHS
Health Research Authority

NRES Committee Yorkshire & The Humber - Leeds East
Yorkshire and Humber REC Office
First Floor, Millside
Mill Pond Lane
Meanwood
Leeds
LS6 4RA

Tel: 0113 3050108
Fax:

12 April 2012

Prof Peter Giannoudis
Consultant
Department of Trauma and Orthopaedics
St James's University Hospital
LS9 7TF

Dear Prof Giannoudis

Study title:	**Biological properties of Mesenchymal Stem Cells in Fracture Healing**
REC reference:	**06/Q1206/127**
Amendment number:	**3/1**
Amendment date:	**11 April 2012**

Thank you for submitting the above amendment, which was received on 12 April 2012. It is noted that this is a modification of an amendment previously rejected by the Committee (our letter of 22nd February 2012 refers).

The modified amendment has been considered on behalf of the Committee by the Vice-Chair.

Ethical opinion

I am pleased to confirm that the Committee has given a favourable ethical opinion of the modified amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved are:

Document	Version	Date
Parental Agreement - Consent Form	1.1	23 March 2012
Participant Consent Form: Patient Consent Form 16 - 17 years old	1.0	23 March 2012
Participant Consent Form: Children Assent Form	1.0	01 February 2012
Participant Information Sheet: Patient Information Sheet 16 - 17 years old	1.0	23 March 2012
Participant Information Sheet: Parental Information Sheet	1.1	23 March 2012
Participant Information Sheet: Children Information Sheet	1.1	23 March 2012
Protocol	6.0	19 December 2011
Modified Amendment		11 April 2012