Investigating the Role of Insulin-like Growth Factor (IGF) axis in Stem Cell Based Periodontal Bone Regeneration in Osteoporotic Conditions

Arwa Saeed Alghamdi

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Declaration

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

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Abstract

Osteoporosis and periodontal disease share common risk factors and are both prevalent in the elderly population. The use of stem cells has given hope to in various disciplines including periodontal regeneration. This project aims to characterise periodontal ligament stem cells isolated from postmenopausal osteoporotic patients (OP-PDLSCs) and study their osteogenic differentiation along with exploring the potential role of Insulin-like Growth Factor (IGF) axis, which is known to be highly linked to osteogenesis, in this process. OP-PDLSCs (n=3) were studied in comparison to healthy PDLSCs (H-PDLSCs) (n=4). Characterisation for clonogenicity, proliferation rate and surface marker expression was carried out using colony forming unit-fibroblasts, population doubling time and flow cytometry assays respectively. Osteogenic differentiation was assessed using alkaline phosphatase (ALP), and alizarin red (ARS) staining and mineralisation nodules quantification assay. RT-qPCR was used to investigate the gene expression of osteogenic and bone remodelling markers, estrogen receptors (ERs) and IGF axis. ELISA was utilised to study the protein expression of IGF binding protein 4 (IGFBP-4) and its protease (PAPP-A).

Compared to controls, clonogenic and proliferative capacities were lower in the osteoporotic group. Flow cytometry indicated a similar cell surface markers expression for all markers assessed ($CD73^+$, $CD90^+$, $CD105^+$, $CD14^-$, $CD19^-$, $CD45^-$) except for $CD34^-$ and $HLADR^-$, where the expression was slightly higher in the osteoporotic group. ALP and ARS showed lower osteogenic differentiation and mineralisation in OP-PDLSCs. Gene expression showed comparable to lower relative expression levels for *RUNX2, ALPL*, *ColIα1, POSTN*, *OCN*, *RANKL*, *ERs* and higher levels for *OPG* in OP-PDLSCs. Gene expression showed overall lower expression in OP-PDLSCs samples for all IGF axis members except for *IGF-1* (inconsistent), *IGFBP-2*, *IGFBP-4* and *PAPP-A* (higher). Protein levels for IGFBP-4 and PAPP-A were higher in OP-PDLSCs. In conclusion, OP-PDLSCs showed similar phenotypic characteristics to H-PDLSCs with a trend of lower osteogenic differentiation and mineralisation capacities. Further investigations on IGFBP-4 are needed as it could be a target to enhance osteogenic regenerative capacity in postmenopausal OP-PDLSCs.

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ABL	Alveolar bone loss
ACH	Alveolar Crest height
AD	Accumulative days
ADSCs	Adipose derived stem cells
ALP	Alkaline phosphatase
APCs	Antigen presenting cells
APD	Accumulative population doubling
ARS	Alizarin red staining
AT- MSCs	Adipose tissue mesenchymal stem cells
BB	Brilliant blue
BM	Bone marrow
BMD	Bone mineral density
BMP	Bone morphogenic proteins
BMPs	Bone morphogenetic proteins
BMSCs	Bone marrow stem cells
BMUs	Basic multicellular units
BSB	Brilliant stain buffer
BUV	Briliant ultra violet
BV	Brilliant violent
CAL	Clinical attachment loss
cDNA	Complementary deoxyribonucleic acid
CEJ	Cementoenamel junction
CFU-Fs	Colony forming unit fibroblasts
CIITA	Class II transactivator
*Col*Iα*1*	Collagen 1 alpha 1
Ct	Cycle threshold
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPSCs	Dental pulp stem cells
DXA	Dual energy X-ray absorptiometry
ECM	Extracellular matrix

EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
ERs	Estrogen receptors
EVs	Extracellular vesicles
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FMO	Fluorescence minus one
FSC	Forward scatter
FSH	Follicle stimulating hormone
FVS	Fixable viability stain
GBR	Guided bone regeneration
GH	Growth hormone
GHR	Growth hormone receptor
GoI	Gene of interest
GPR30	G protein-coupled estrogen receptor 1
GTR	Guided Tissue regeneration
H-PDLSCs	Healthy periodontal ligament stem/stromal cells
hDPCs	Human dental pulp cells
HKG	Housekeeping gene
HLA	Human leukocyte antigen
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HRP	Horseradish peroxidase
HRT	Hormone replacement therapy
HSC	Hematopoietic stem cells
IFC	Integrated fluid circuits
IFN-ɣ	Interferon gamma
IGF	Insulin-like growth factor
IGF-2R	Insulin-like growth factor receptor II
IGF-1R	Insulin-like growth factor receptor I
IGFBP	Insulin-like growth factor binding proteins

IGFBP-rP	Insulin-like growth factor binding protein related protein
IGFrP	Insulin-like growth factor related protein
IgG2	Immunoglobulin G
IL	Interleukin
IRR	Insulin related receptor
IRS	Insulin receptor substrate
ISCT	International Society of Stem Cell Therapy
L-G	L-glutamine
M-CSF	Macrophage-colony stimulating factor
MAPK	Mitogen-activated protein kinase
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MRONJ	Medications related osteonecrosis of the jaw
MSC	Mesenchymal/ stromal stem cells
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor kappa B
NRT	No reverse transcriptase
NTC	No template control
OCN	Osteocalcin
OP	Osteoporosis/ osteoporotic
OP-PDLSCs	Osteoporotic periodontal ligament stem/stromal cells
OPG	Osteoprotegerin
OSX	Osterix
OVX	Ovariectomised
P	Passage number
P/S	Penicillin/streptomycin
P1NP	Procollagen type 1 N-terminal propeptide
PA	Pre-amplification
PAPP-A	Pregnancy-associated plasma protein A
PBS	Phosphate buffered saline
PD	Population doubling
PDGF	Platelet derived growth factor

PDL	Periodontal ligament
PDLSCs	Periodontal ligament stem/stromal cells
PDT	Population doubling time
PI3-K	Phosphatidylinositol 3-kinase
PMTs	Photomultiplier tubes
POSTN	Periostin
PTFE	Polytetrafluoroethylene
PTH	Parathyroid hormone
RANK	Receptor activator of the nuclear factor kappa-B
RANKL	Receptor activator of the nuclear factor kappa-B ligand
rh	Recombinant human
ROS	Reactive oxygen species
RT	Room temperature
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
RUNX2	Runt related transcription factor 2
SATB2	Special AT-rich sequence binding protein 2
SCAP	Stem Cells from the Apical Papilla
SEM	Scanning electron microscopy
SERMs	Selective estrogen receptor modulators
SHED	Stem Cells exfoliated from Human Deciduous teeth
SSC	Side scatter
TC	Tissue culture
TEM	Transmission electron microscopy
TGF	Transforming growth factor
TIMP	Tissue inhibitors of metalloproteinase
TNF	Tumour necrosis factor
TNSALP	Tissue non-specific alkaline phosphatase
TRACP	Tartrate-resistant acid phosphatase
VEGF	Vascular endothelial growth factor
WHI	Women’s health initiative
WHO	The world health organisation
WJCMSCs	Wharton’s jelly of umbilical cord stem cells
α-MEM	Alpha minimum essential medium

Chapter 1 Literature Review

1.1 General introduction

Osteoporosis (OP) is a bone disease characterised by decreased bone mass as well as microstructural changes that predisposes bone for fragility fracture. The global prevalence of OP is estimated to be 21.7% in the elderly population (Salari et al., 2021) with postmenopausal women being at a higher risk for the disease (Özmen et al., 2024). Periodontal disease is also common with the increase of age, and its prevalence is estimated to be 70% in individuals ≥ 65 years old (Clark et al., 2021). Globally, the number of cases with severe periodontitis was estimated to be 1.1 billion in 70-74 years old patients (Huang et al., 2025).

As a bone disease, OP affects the jawbone and is linked to the aggravation of periodontal disease (Genco and Borgnakke, 2013). In osteoporotic patients, there is some evidence suggesting that OP might have a negative impact on non-surgical periodontal therapies (Gomes-Filho et al., 2013). Dental surgical interventions are considered risk factors for the development of medication related osteoradionecrosis in the jaw for osteoporotic patients on medications such as bisphosphonates (Otto et al., 2015). Although OP is not a contraindication for dental implant placement, osteoporotic patients experience delayed bone healing and their condition might have a potential impact on dental implant osseointegration (Koth et al., 2021).

One of the promising approaches to manage periodontal disease is the use of stem cell-based regenerative therapies (Citterio et al., 2020). According to a recent meta-analysis, stem cell therapy resulted in an improved periodontal regeneration outcomes compared to conventional therapy (Nguyen-Thi et al., 2023). Interestingly, the use of stem cells to enhance osseointegration of dental implants showed positive results in animal models (Sayed et al., 2021). Autologous stem cells remove the need for an external donor and eliminate the immunogenic reaction after implantation (Li et al., 2021). Moreover, cells isolated from oral and dental regions lead to superior results in treating orofacial defects when compared cells isolated from skeletal bone (Akintoye et al., 2006). Literature search revealed little to no published reports studying the potential use of autologous periodontal ligament stem cells in periodontal regeneration in postmenopausal osteoporotic patients. This project focuses on characterising

osteoporotic periodontal stem cells isolated from this patient group and their capacity for osteogenic regeneration. It also investigates the potential role of insulin-like growth factor (IGF) axis members, as a family of proteins that play a role in stem cell differentiation into mineralised tissue (Götz et al., 2006b), in the process. These investigations are eventually aimed at providing customised regenerative periodontal treatment for periodontal disease in postmenopausal osteoporotic patients.

1.2 Overview of osteoporosis

1.2.1 Definition and statistics

The World Health Organisation (WHO) defines OP as a progressive systemic skeletal disease characterised by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture (Liu et al., 2019b, Harvey and Cooper, 2018). Worldwide, OP affects 200 million people with 1 in 3 women and 1 in 5 men above the age of 50 at a higher risk of fragility fracture (Yu and Wang, 2022a). Fractures caused by OP are reported every 3 seconds. (International Osteoporosis Foundation, 2017). Although the prevalence of OP is higher in females, males tend to experience higher rates of mortality fractures (3).

1.2.2 Types

Based on the factors that affect bone metabolism, OP is classified into primary and secondary. Primary OP is further classified into postmenopausal (type I) and senile OP (type II). (Wei et al., 2025). In the postmenopausal phase, women are subject to primary OP since it is linked to estrogen deficiency. During the menopausal transitional period, estrogen levels drop leading to more bone resorption and less bone formation which results in OP (Ji and Yu, 2015). Senile OP affects both men and women and is associated with ageing. Senescent bone marrow mesenchymal stem cells (BMSCs) and increased low grade inflammation have been recognised as contributory factors (Qadir et al., 2020). Secondary OP results from several diseases that affect the bone health and/or the use of certain medications (Tu et al., 2018) (will be discussed section 1.2.4).

1.2.3 Pathophysiology

The disturbance of bone remodelling underpins OP. Bone remodelling is a normal physiological process that involves the replacement of old bone with new bone at the same site within a mature skeleton (Manolagas, 1998). Bone remodelling takes place within bone cavities where temporary anatomical structures, called basic multicellular units (BMUs), are formed (Florencio-Silva et al., 2015). The key cells involved in bone remodelling include: osteoclasts and osteoblasts (Ilas et al., 2017). A BMU that is fully formed consists of osteoclasts at the front end, or bone resorption zone, osteoblasts at the rear end, or bone building zone as well as some connective tissue, blood and nerve supply (Manolagas, 2000).

With age, bone undergoes remodelling by the resorptive action of osteoclasts and the bone deposition of osteoblasts. The mature osteoclasts' lifespan lasts about 12 days and the osteoblasts take about 3 months to replace the resorbed bone. Microscopically, during this process, osteoblasts turn to be largely flatter and wider with a cuboidal shape, some become trapped in the bone as osteocytes, others undergo apoptosis, and those that remain throughout the process become the lining cells that cover the new surface. Osteocytes are considered the most available type of bone cells. They are able to communicate with each other and the new bone cells via canalicular networks (Cosman et al., 2014).

The majority of adult bone mass is accrued at puberty, and by the end of the second decade of life the peak bone mass is reached then maintained for the following decades and eventually starts to drop after the age of 50 (Hereford et al., 2024). Both men and women lose bone mass as they age (Lippuner, 2012). Bone function is highly affected by several hormones and growth factors. Estrogen and testosterone significantly affect bone remodelling with their anti-resorptive action. Moreover, parathyroid hormone (PTH) also increases bone formation by stimulating osteoblast proliferation indirectly through its effects on calcium metabolism (Jilka, 2003). One of the cytokines that has been known to influence the remodelling is receptor activator of the nuclear factor kappa-B ligand (RANKL). Osteoblasts produce RANKL that binds to RANK receptors on osteoclasts resulting in osteoclasts activation leading to bone resorption. In

addition, osteoblasts secrete osteoprotegerin (OPG), also known as osteoclastogenesis inhibitory factor, which blocks RANKL action and is essential for the maintenance of RANK/RANKL/OPG system that contributes to bone homeostasis (McCormick, 2007). The downstream signalling of RANKL/RANK activates NF-κB (nuclear factor kappa B) inducing osteoclastogenesis (Boyce et al., 2010).

Interestingly, estrogen has the ability to augment the production of OPG by osteoblasts which could explain the development of postmenopausal OP as estrogen levels are diminished (Ginaldi et al., 2005). In postmenopausal women, low estrogen levels induce macrophages to produce osteoclastic cytokines thus activating RANK and promoting the activation of osteoclasts (Noirrit-Esclassan et al., 2021). Moreover, in OP, the net balance between osteoblasts and osteoclasts shifts in favour of osteoclasts leading to more bone resorption (Arnett, 2015). Additionally, bone loss resulting from glucocorticoid excess or sex steroid deficiency innately stems from changes in the production of bone cells, by lengthening the lifespan of osteoclasts and shortening the lifespan of osteoblasts (Jilka, 2003). In sex steroid deficiency, bone remodelling increases due to the development of new BMUs or a prolonged lifespan of existing ones (Jilka, 2003). Also, the diminished levels of estrogen lead to elevated osteoclast numbers and that could be attributed to a number of cytokines e.g. interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor (TNF), which are usually suppressed when estrogen levels are normal (Jilka, 1998). With the loss of steroids, osteoclasts erode deeper at the bone tissue than when they are in their normal condition which leads to the removal of the entire of cancellous bone and renders the remaining elements without connection (Manolagas et al., 2002).

1.2.4 Risk factors

Risk factors to OP have been classified into several categories (Lane, 2006). These include; clinical, medical, behavioural, and genetic factors. Clinical factors relate to changes in the physiological parameters in the human body. For example, peak bone mass is defined as the maximum amount of bone a person can attain throughout their life (NOF, 2020). It contributes massively to the bone mass density and is believed that osteoporotic fractures are linked to

low peak bone mass (Cooper and Melton, 1992). In addition to low peak bone mass, ageing, lower levels of serum estradiol and low body weight were found to be risk factors for OP (Cooper and Melton, 1992, Ravn et al., 1999, Cummings et al., 1998). Medical factors relate to certain pathologies (Table 1) and medications use (Table 2).

Table 1: Secondary causes of osteoporosis.

Table adapted from (Yordanov et al., 2025)

Category	Examples
Genetic diseases	Idiopathic juvenile OP, Osteogenesis imperfecta
Endocrine diseases	Hyperthyroidism, Diabetes mellitus, Cushing syndrome, Growth hormone (GH) deficiency
Gastrointestinal diseases	Celiac disease, Inflammatory bowel disease, Hemochromatosis,
Eating disorders	Anorexia neurosa, Bullimia neurosa
Haematological disorders	Multiple myeloma, Systemic mastocytosis, Beta thalassemia major
Renal diseases	Idiopathic hypercalciuria, Chronic kidney disease
Autoimmune diseases	Rheumatoid arthritis, Systemic lupus erythematosus
Vitamin D3	Vitamin D3 deficiency
Infection diseases	Tuberculosis

Table 2 Medications that contribute to osteoporosis.

Table adapted from (Panday et al., 2014, Cosman et al., 2014).

Medication	Indication	Impact on bone
Glucocorticoids	Multiple conditions e.g autoimmune, inflammatory diseases, malegnancies	↓ bone formation ↑ bone resorption
Proton pump inhibitors	↓ stomach acid production	Unknown Suggested mechanism: ↓

		intestinal calcium absorption
Anti-epileptic drugs	Multiple conditions e.g. epilepsy, migraines, psychiatric conditions	Unknown Suggested mechanism: inactivation of vitamin D
Medroxyprogesterone acetate	Oral contraceptive used in the treatment of endometriosis	↓ estrogen level eventually → ↑ bone resorption
Aromatase inhibitors	Adjunctive therapy for estrogen receptors positive breast cancer in postmenopausal women	↓ estrogen formation eventually → bone resorption
Gonadotropin-releasing hormone agonists	Several gynocological conditions e.g. endometriosis, polycystic ovarian syndrome	Block the production of LH and FSH→ ↓ testosterone and estradiol production→ increased bone resorption
Serotonin selective reuptake inhibitors	Several psychological conditions e.g. depression, anxiety	uncertain
Thiazolidinediones	Diabetes type 2	↓ bone formation
Calcineurin inhibitors	Immunosuppression to prevent transplant rejection	Excessive osteoclasts and bone resorption with glucocorticoids
Anticoagulants e.g heparin	Venous thromboembolism	Osteoblast inhibition→ ↓ bone formation ↑ bone resorption

LH; luteinising hormone, FSH; follicle stimulating hormone, ↑; increased, ↓; decreased, →; leading to

Behavioural factors such as cigarette smoking affect calcium absorption in the intestine leading to rapid bone loss and increase in the possibility of hip fracture in older patients (Law and Hackshaw, 1997). In some studies, decreased level of physical activity has been correlated to fracture risk (Nguyen et al., 2000). Additionally, increased alcohol consumption increases the individual's risk for bone loss. In a study on South Korean postmenopausal women, participants with no or higher alcohol consumption showed a 1.7 increased risk for OP when compared to light drinkers (Jang et al., 2017).

Genetic factors can also contribute to OP-induced fractures. The variance of bone density is 46-62% related to heredity (Krall and Dawson-Hughes, 1993). Moreover, biological gender plays an essential role in determining the risk of fracture. When compared to men, women aged 50 years or older have four times higher risk of OP than men (Alswat, 2017). Additionally, race is a major determinant of prevalence of OP based on BMD (Lane, 2006). According to the Third National Health and Nutrition Examination Survey, in white women, OP prevalence was 18% (between 1988-1994) then dropped down to 10% (in 2004-2005). However, in African Americans, the prevalence was lower (6%) and did not change when assessed about 10 years later. In Hispanics, the survey revealed a prevalence of 16% (1988-1994) which declined, insignificantly, to 10% in 2004-2005 (Lane, 2006).

1.2.5 Symptoms

Patients with OP don't initially experience symptoms as they lose bone, hence it is called a 'silent disease'. Unless a sudden strain or a fall that causes a vertebra to collapse or a hip to fracture, for example, patients may not realise they are having OP (NIH, 2019). After the first fracture, one in eight people tend to have their next OP-induced fracture within the first year and two of them will potentially break a bone within the following five years (Juli, 2016).

1.2.6 Diagnosis

The diagnosis of OP can be carried out using different methods to assess bone density at sites with the highest fracture rates. Among those techniques, dual

energy X-ray absorptiometry (DXA) is the most validated method to assess bone mineral density (BMD) at relevant sites such as hip, spine and forearm (World Health Organization, 2007). The WHO developed a diagnostic classification by testing the BMD with DXA, which generates a T-score upon which the classification is made as follows; a T-score ≥-1.0 is normal, T-score between -1.0 and -2.5 indicates osteopenia, T-score ≤ -2.5 indicates OP and a T-score of ≤ -2.5 with fragility fracture indicates a severe form of OP (Lewiecki, 2000).

Several indices can be used to assess changes in the trabecular and cortical microstructure of the jawbone on a panoramic radiograph such as mandibular cortical index, mandibular cortical width and panoramic mandibular index (Koth et al., 2021). Mandibular cortical width provided more accuracy in excluding OP/osteopenia when compared to other oral measures (Calciolari et al., 2015). While these indices can help dentists detect changes in BMD in the jawbone and refer patients for further assessment, they can't solely be used to diagnose OP (Tounta, 2017) . Recent artificial intelligence (AI)-based models showed promising results in detecting OP from panoramic radiographs of postmenopausal women although further studies are needed before considering their clinical application (Fanelli et al., 2025).

1.2.7 Management

1.2.7.1 Nonpharmacological approach

Improvement of bone health can be attained through several measures. These include healthy changes of the individual lifestyle, increasing physical exercise, ensuring the patient is taking the necessary amount of vitamin D and calcium as well as the reduction of smoking and alcohol consumption (Compston et al., 2017).

1.2.7.2 Pharmacological Management

The medications prescribed for OP are categorised into: antiresorptive and anabolic medications. The antiresorptive medications mainly act to reduce bone resorption. Anabolic medications on the other hand act to increase the formation of bone rather than inhibiting the bone resorption. The variation in the prescription

of a specific medication will largely depend on the patient's gender, the degree of fracture risk, the presence of comorbid diseases and / or the use of other medications (Tu et al., 2018).

1.2.7.2.1 Bisphosphonates:

Bisphosphonates (BPs) are the synthetic analogues of the natural pyrophosphate compound (Liu et al., 2019a). It has been suggested that the basic mechanism of action for BPs is that they have high affinity for bone and bind strongly to hydroxyapatite which increases their concentration in the bone especially at areas of high bone remodelling. BPs target osteoclasts inhibiting their differentiation and action, and cause their death by apoptosis (antiresorptive action) (Drake et al., 2008). BPs are classified according to their chemical structure into nitrogen-containing (e.g. alendronate, ibandronate, risedronate, zoledronic acid) and non-nitrogen containing (e.g. etidronate) (AlDhalaan et al., 2020).

BPs share common side effects which include: gastrointestinal disturbances, general body aches, as well as a variety of side effects such as anaemia, electrolyte imbalance, renal impairment and skin reactions (NICE, 2021b). There are reported cases of osteonecrosis of the jaw (will be discussed at section 1.3.6.5) associated with this medication, as well as visual impairments in patients using oral and intravenous BPs (Juli, 2016).

1.2.7.2.2 Denosumab

Denosumab is a monoclonal antibody therapy, which specifically binds to RANKL blocking its interaction with RANK and therefore inhibiting the differentiation, activation and survival of osteoclasts (Dahiya et al., 2015). Denosumab is associated with hypocalcaemia and the risk of developing osteonecrosis of the jaw (NICE, 2021a) similar to BPs.

1.2.7.2.3 Strontium Ranelate

Strontium ranelate (SR) was shown to reduce the risk of hip and vertebral fractures in postmenopausal women with OP through its dual effect as an anti-resorptive and anabolic effects on bone (Reginster et al., 2005). In vitro studies on SR indicated that it has a positive proliferative action on pre-osteoblasts and bone matrix synthesis. In

BMSCs, exposure to SR increased the expression of RUNX2 and osteocalcin in pre-osteoblasts and of bone sialoprotein and osteocalcin in mature osteoblasts (Cesareo et al., 2010). In a recently published animal study, SR was shown to have a moderate capacity to potentially promote bone healing in healthy and OVX rats with calvarial defects (Mardas et al., 2021). Some of the reported side effects include nausea, diarrhea, headaches and venous thromboembolism and should be used with caution in patients with renal impairment (Blake and Fogelman, 2006).

1.2.7.2.4 Hormonal Therapies

Hormone Replacement Therapies (HRT) are therapies designed to replace either estrogen alone and or estrogen with progesterone (Mattson et al., 2002). HRT were commonly used in the last century as they provided a rapid decline in bone resorption in the first few months of therapy. However, due to their association with higher risks of cardiovascular complications and breast cancer, according to the Women's Health Initiative (WHI) trial, their use has declined noticeably between 2002-2010 (Yordanov et al., 2025). However their use in the UK increased between 2010-2021 since WHI suggested that the potential risks with HRT use are related to the time of their use, where risks are less likely to develop if they were used by young postmenopausal women or soon after menopause (Alsugeir et al., 2022). Other hormonal based therapies include selective estrogen receptor modulators (SERMs), testosterone, and PTH analogues (Tu et al., 2018). Raloxifene is an example of SERMs which bind to the estrogen receptor but induce different actions on the tissues than estrogen (Cranney and Adachi, 2005). The side effects of raloxifene are not common, however patients reported cardiovascular complications (Juli, 2016). Testosterone therapy is used to increase BMD in men with low levels of serum testosterone (Liu et al., 2019b). Teriparatide is an example of PTH analogues. It is an anabolic agent which elevates BMD levels by up to 70% reducing the incidence of non-vertebral fractures. Side effects are not common and include nausea, headache, dizziness, temporary hypercalcemia and hypercalciuria. Combination of teriparatide with other anti-osteoporotic medications (e.g. denosumab or zoledronic acid) has produced synergistic effects although not approved or popular (Liu et al., 2019a).

In order to minimise the potential side effects of the pharmacological medications, advanced therapies are now being researched and might provide a promising alternatives to current treatments, which will be discussed in the section below.

1.2.7.3 Advanced therapies for osteoporosis

In an attempt to reduce the side effects of the previously discussed pharmacological therapies and to enhance a bone regenerative treatment for OP, advanced therapies were developed. Regenerative medicine is a promising field that aims to restore normal body structure and function. It utilises stem or progenitor cell transplantation, tissue transplantation and other techniques that stimulate the body's repair process (UKRI, 2021). (Refer to section 1.4.2.1 for stem cell definition, types).

The bone remodelling process and repair depends on local signalling cascades to stimulate the migration of osteoprogenitor cells along with the differentiation, proliferation and extracellular matrix (ECM) production (Nasir et al., 2023). Stem cells, particularly mesenchymal stem cells (MSCs), can enhance bone regeneration by secreting molecules such as insulin-like growth factor (IGF-1), Transforming Growth Factor beta (TGF-β), and vascular endothelial growth factor (VEGF), platelet derived growth factors (PDGFs) and bone morphogenetic proteins (BMPs) . However, the most significant therapeutic effects of stem cells in OP stem from their ability to support the creation of a regenerative environment rather than their ability to differentiate. In other words, the use of MSCs in OP treatment is promising due to the paracrine effects of these cells (Arjmand et al., 2020). In the context of science translation, the use of stem cell transplantation or cell-based therapies in the treatment of bone disorders has been applied on experimental animal models in a variety of bone disorders including OP (Paspaliaris and Kolios, 2019). Although the stem cell therapy approach has been considered in humans lately, published clinical trials on its use to treat humans with OP are limited (Paspaliaris and Kolios, 2019).

In addition to stem cell therapy, the use of Extracellular Vesicles (EVs) to treat OP has gained interest. EVs are particles secreted by cells into the ECM and bodily fluids and are also released into the supernatants of cell cultures *in vitro* (Fang et al., 2024). EVs play a role in intercellular communication, have superior properties compared to MSCs (e.g less immunoreactivity, more specificity) and play a significant role in maintaining homeostasis in the bone microenvironment (Chen et al., 2024b). Although EVs provide a promising future for OP treatment,

vesicles preparation and purification to ensure bioavailability and functional stability is still a challenge (Fang et al., 2024).

1.3 Periodontium and periodontal disease

1.3.1 Anatomy of the periodontium

The periodontium is the tissue attachment apparatus that holds the tooth in the jaw. It is composed of the cementum, periodontal ligament (PDL), alveolar bone and gingiva (Nanci and Bosshardt, 2006). Cementum is avascular mineralised tissue that envelopes the root of teeth and is attached to the alveolar bone via the PDL (Nanci and Bosshardt, 2006). The alveolar bone supports the teeth and undergoes constant bone remodelling (Suchetha et al., 2017). Altogether, these structures function to support the tooth regardless of the variation of mastication forces (Madukwe, 2014). The function of the periodontium is dependent on the integrity of these structures (Nanci, 2007).

Since it is the focus of this project to investigate the effect of OP on periodontal stem cells and their ability to regenerate the periodontium particularly the bone, the PDL structure and function will be expanded in the following section.

1.3.2 Periodontal ligament

During the early stages of tooth development, the PDL originated from the dental follicle and is considered ecto-mesenchymal (Tomokiyo et al., 2018). The PDL is composed of a variety of cells types. On the alveolar side, there are osteoblasts and osteoclasts, within the PDL core; there are fibroblasts, undifferentiated mesenchymal cells, epithelial cell rests of Malassez, neural elements, macrophages and endothelial cells, and at the root surface, it has cementoblasts (Lekic and McCulloch, 1996). The PDL contributes to the formation of the ECM components (largely collagen formation) which contribute to periodontal tissue homeostasis and allow for periodontal tissue regeneration during wound healing (Jönsson et al., 2011). The PDL functions as a shock absorber protecting teeth and alveolar bone from mastication forces whilst providing a sensory input to the mastication system. Along with the gingiva, the PDL acts a barrier against oral

pathogens (de Jong et al., 2017). When this barrier is broken, it can induce the development of periodontal disease (Cho et al., 2021).

1.3.3 Periodontal disease

The term ‘periodontal disease’ describes diseases that impact the tooth supportive structures. It starts with gingivitis, where the gingival tissues are inflamed due to the accummulation of plaque contaning pathogenic bacteria (Kinane et al., 2017). Gingivitis is a reversible condition that, if left untreated, can progress to periodontitis where there is an irreversible distruction of the PDL and alveolar bone and can be assessed by measuring clinical attachment loss (CAL), alveolar bone loss (ABL) and periodontal pocket depth (Hussein et al., 2021).

The clinical definition of periodontitis is that it is an inflmmatory multifactorial disease characterised by progressive destruction of tooth-supprting structures and associated with dysbiotic plaque biofilm. Periodontitis is characterised by three criteria: (1) loss of periodontal supportive structures, which can be assessed clinically through CAL and radiographically through ABL, (2) periodontal pockets and (3) gingival bleeding (Sanz and Tonetti, 2019). The current disease model poses that periodontal disease is multifactorial and is a result of interactions between dysbiotic subgingival biofilm, host immune response, hormonal imbalances and genetics, as well as other risk factors such as tobacco use, ageing, and nuitritional deficienies (Yu and Wang, 2022a).

Worldwide, the prevalence of severe periodontitis is etstimated to be 11% affecting 743 million people (Wang et al., 2025). In high-income countries, the prevalence of periodontitis was estimated to be 47% among the older population (65-74 years) (Nazir et al., 2020). Advanced periodontal disease can lead to bone resorption and loosening of teeth which affect patients’ masticatory function as well as esthetics (Liang et al., 2020)..

1.3.4 Diagnosis

After a thorough assessment of patient’s medical, dental, oral hygiene habits history, clinical assessment of periodontium is assessed using periodontal probes and radiographs to measure certain parameters. Bleeding on probing (BOP) that lasts longer than 10 seconds upon probe retrieval indicates clinically inflamed tissue. Periodontal

pocket depth (PPD) is defined as the distance from the free gingival margin to the base of the gingival/ periodontal pocket. Mild to moderate periodontitis is diagnosed when the PDD is ≤ 6mm. Severe periodontitis is diagnosed when the PDD is > 6mm (Salvi et al., 2023).

1.3.5 Management

The management of periodontial diseases varies based on the severity of the cases. Generally, the management involves ensuring proper oral hygiene measures, scaling and root planning, with some cases requiring the use of antibiotics (whether systemic or local), host modulation therapies or surgical intervention where required (Kwon et al., 2021).

1.3.6 Correlation between osteoporosis and periodontal disease

1.3.6.1 Effect of osteoporosis on the jawbone and the periodontium

Based on the concept that jawbone might be affected as part of the systemic effect of OP on the body's bones, it would be expected that OP could also change the mineral content of the jawbone leading to periodontal disease progression. According to Guiglia et. al's review, the variation of the study designs and assessment tools for periodontal disease and OP makes it difficult to reach a robust conclusion in terms of how one disease impacts the other. A bidirectional relationship has been suggested where the reduced BMD in OP can lead to trabecular bone pattern changes worsening the state of bone resorption caused by the periodontal disease bacteria. On the other hand, further bacterial invasion might result in alteration of bone homeostasis reducing bone density on a local and systematic level impacting the overall BMD (Guiglia et al., 2013). Interestingly, the structure of the alveolar bone (e.g sparse trabeculation) was considered the third best predictor of future extracranial fractures following a history of previous fracture and the use of glucocorticoid medications (Jonasson and Billhult, 2013).

Currently, there is an increasing evidence in the literature correlating systemtic BMD measured at certain skeletal sites to bone density at the jaw (Donos et al., 2023), which has also been linked to reduced estrogen levels rendering the

alveolar bone more susceptible to resorption (Shrivastava, 2024). Other hormones have also been linked to both diseases such as testosterone, progesterone and Follicle Stimulating Hormone (FSH) either by acting on bone or periodontal cells (Zhu et al., 2022).

Furthermore, OP has been specifically correlated with microanatomical changes to the jawbone. This impact might be more evident in the trabecular bone particularly in the interradicular areas of the alveolar bone (Koth et al., 2021) although it varies between genders where females show more spaced trabecular patterns compared to males (Yu and Wang, 2022a). According to Wactawski-Wende et al.(Wactawski-Wende et al., 2005), there was a strong consistent association between the alveolar crest height (ACH), measured between the cementoenamel junction (CEJ) and the most coronal portion of the alveolar bone, and BMD (T-score) in postmenopausal women. They found that the lower the T-score, the more likely it is for the ACH to increase indicating a more severe periodontal disease. In addition, there is a significant correlation between BMD of the skeleton and the mandibular cortical index (Mazumder et al., 2016). Radiographically, the cortex at the mandibular angle was clearly thinner in postmenopausal women (Marya et al., 2015). Concomitantly, Taguchi et al (Taguchi et al., 2007) found that postmenopausal women with thinner mandibular cortical bone were at increased risk for lower vertebral bone mineral densities.

The PDL tissue itself might also affected be by OP. According to Arioka et al. (Arioka et al., 2019), osteoporotic mice showed PDL atrophy along with a reduction in the osteoprogenitor cells. In addition, patients with OP present with thinner PDL and delayed bone repair (Arioka et al., 2019). The impact OP has on PDL and alveolar bone might contribute to teeth loss. For instance, longitudinal studies of postmenopausal women with lower levels of BMD had more tooth loss compared to individuals with normal BMD (Calciolari, 2016) concluding that postmenopausal OP could be a risk factor for periodontitis (Qi et al., 2023). In a study that compared the periodontal status of women with and without osteoporotic fractures, postmenopausal osteoporotic women with fractures have lost more teeth (Martínez-Maestre et al., 2013). In light of the discussion above, the potential correlation between OP and periodontal disease will be discussed in the next section.

1.3.6.2 Effect of osteoporosis on periodontal disease

The suggestion that bone changes associated with OP aggravate periodontal disease (Genco and Borgnakke, 2013) was supported by several publications where OP showed a statically significant negative impact on periodontal disease. For example, in a study by Juluri et al. (Juluri et al., 2015), the periodontal condition in 50 postmenopausal osteoporotic women was compared to an equivalent number of non-osteoporotic women of a similar age group (50-65 years old). CAL was significantly higher in the former group compared to their controls indicating a potential relationship between OP and periodontal disease. However, other parameters such as alveolar bone loss (ABL), pocket depth, and oral hygiene index-simplified were not statically different. In another report by Passos et al. (Passos et al., 2013), postmenopausal women over the age of 50 with a diagnosis OP/ osteopenia were twice as likely to present with periodontitis (assessed by measuring pocket depth, CAL, bleeding on probing (BOP) compared to postmenopausal women with normal BMD, suggesting that OP/ osteopoenia worsened the progress of periodontitis. Similarly, Mohammad et al. (Mohammad et al., 2003), found a statically significant negative correlation between periodontal disease and low BMD in postmenopausal Asian American women regardless of the plaque score. In their report, the osteoporotic group had the highest scores of tooth loss and clinical attachment loss compared to healthy and osteopenic patients. On the contrary, a few reports suggested the lack of a statistically significant association between OP and clinical parameters of periodontitis (Marjanovic et al., 2013, Moeintaghavi et al., 2013). There is a general agreement that low BMD is associated with ABL, however the exact relationship between low peripheral BMD, CAL, and pocket depth remains debatable (Tilotta et al., 2025).

It is apparent that OP and periodontal disease share the feature of excessive bone resorption. In fact, a recent systematic review had suggested that OP and periodontal disease could be a risk factor for each other (Qi et al., 2023). This could potentially be due to the increase in bone resorption-promoting cytokines and diminished estrogen levels resulting in a dysregulated RANK/RANKL/OPG system (Koth et al., 2021). Both diseases share common risk factors (Table 3) even though the exact relationship has not been fully established (Wang and

McCauley, 2016). There have been several suggested mechanisms to explain the correlation between OP and periodontitis. One theory proposes a mechanism by which OP weakens the ability of the periodontal bone to resist infections (Wang and McCauley, 2016). Another suggestion is that in both diseases, a set of hormones that are involved in bone homeostasis, such as estrogen, testosterone, PTH, thyroid hormone and cortisol are dysregulated (Yu and Wang, 2022b). The decline in estrogen associated with menopause leads to degeneration of the PDL, impaired collagen formation and a higher tendency for alveolar bone resorption (Palanisamy, 2025). A third suggested mechanism is the elevation of pro-inflammatory cytokines, such as IL-1, IL-6 and tumour necrosis factor alpha (TNF-α) which occurs in both OP and periodontal disease (Wang and McCauley, 2016). As inflammatory diseases with bone resorption, the enhanced expression of inflammatory markers, especially NFκB, elicits the function of osteoclasts while suppressing the action of osteoblasts (Yu and Wang, 2022a).

Abnormal hormonal changes	• Sex steroid hormones : - Estrogen - Testosterone - Follicle stimulating hormone • Calcitropic hormones - Vit D - PTH - Calcitonin • Circadian rhythm associated hormones - Glucocorticoids - Melatonin • Growth hormone • Thyroid hormone
Metabolic disorders of energy substrates	- Dysregulated glucose metabolism e.g diabetes mellitus - Dysregulated Lipid metabolism e.g hyperlipidemia
Unhealthy Life-style related factors	- Smoking - Excessive alcohol consumption - Psychological stress

Table 3. A list of the shared risk factors between osteoporosis and periodontal disease.

Information source (Zhu et al., 2022).

1.3.6.3 Effect of osteoporosis medications on periodontal disease

Clinical evidence suggests that the use of systemic osteoporotic medications positively affects PDL health. For example, it has been shown that combining HRT (estrogen and/or progesterone) with periodontal treatment improves periodontal health outcomes in osteoporotic patients (Koth et al., 2021), and the use of estrogen replacement therapy improves tooth retention and mobility

(Calciolari, 2016). According to Haas et al. (Haas et al., 2009) , postmenopausal southern Brazilian women who are not using HRT had a greater likelihood of developing of periodontitis (even after the exclusion of confounding factors such as smoking, age, socioeconomic status or dental care habits) compared to postmenopausal women on HRT. Additionally, some clinical studies have reported that the use of BPs leads to improved periodontal parameters, namely CAL, BOP and pocket depth, compared to placebo in patients with chronic periodontitis (Lane et al., 2005). Denosumab has been less extensively studied compared to BPs (Sharma and Reche, 2023), however, since denosumab generally inhibits bone loss (including the jawbone), it might potentially provide a positive impact on periodontal bone loss. There is currently an on-going clinical study registered on the National Health Service (NHS)- Health Research Authority (HRA) website on this particular topic (Culshaw, 2025) and the data are not yet published.

1.3.6.4 Effect of osteoporosis on surgical dental treatments

OP might lead to delayed socket healing after dental extraction, according to a recent systematic review (Só et al., 2021). In a comparative study between healthy and OVX rats, the healing of calvarial critical size defects, using guided bone regeneration, was not statistically significant different, but it showed a trend of less new bone formation and a reduced quality of the formed bone when assessed after one month (Calciolari et al., 2017b). In another animal study comparing periodontal defect healing 3 groups of OVX rats, new bone formation showed the greatest results in the group treated with strontium-incorporated mesoporous bioactive glass (Sr-MBG) scaffolds (46.67%), followed by the group treated with MBG alone (39.33%) and lastly the group where the defect was unfilled (17.5%). The number of tartrate resistant acid phosphatase (TRAP)-positive osteoclasts was reduced in the group treated with Sr-MGB scaffolds suggesting a promising role of strontium in periodontal regeneration (Zhang et al., 2014).

Most studies on surgical dental treatments focused on the impact of OP on dental implants as a tooth replacement modality. The success of dental implant placement might be affected in patients with OP (Donos et al., 2015). Dental implants are artificial replacements for missing teeth roots. They are inserted into

the jawbone to which they attach as well as attaching to the gingival tissue. They act as an anchorage for the prosthetic crown replacement (Ada, 2005). The successful integration between dental implants and bone is known as “osseointegration”. In clinical terms, osseointegration reflects the ability of dental implants to withstand the forces generated during normal oral function (Alghamdi, 2018). Since patients with OP experience delayed bone healing after tooth extraction, it is plausible to consider that osseointegration around dental implants might be negatively affected (Koth et al., 2021). Lower BMD may decrease the success rate of dental implants while insufficient bone volume may increase the complexity and indirectly influence the success of dental implants (Javed et al., 2013, Jacobs, 2003). These factors (BMD and bone volume) reduce with age and other exacerbating factors such as postmenopausal estrogen-deficiency-induced OP (Lotz et al., 2019). Studies agreed on the significant role estrogen deficiency plays in reducing bone-to-implant contact (BIC) and the reduced amount of bone around implants which eventually compromises osseointegration (Dereka et al., 2018). Moreover, the disturbances in bone remodelling in OP contributes to impaired bone healing and osseointegration (Lotz et al., 2019). It has also been hypothesized that the risk associated with implant-osseointegration in OP could be explained by changes in the quality (bone architecture, mineral crystal size) or the quantity of the bone produced during healing (Dao et al., 1993). In a study by Merheb et al.(Merheb et al., 2016), the primary stability of dental implants was tested in osteoporotic patients, and it was found that there was a significant correlation between the density of the local bone (quality) and the stability of implants. Additionally, the lack of bone quantity in osteoporotic patients proposes possible complications during implant placement particularly for maxillary bone. For instance, in patients with thin cortical bone in the in the posterior maxillary ridge, the initial stabilization of implants was challenging (Marchand-Libouban et al., 2013). A procedure known as (sinus lift) in the maxillary bone is used to help support the lack of bone tissue and involves the elevation of maxillary sinus floor (Helmy, 2017). In spite of the preclinical evidence suggesting lower implant-osteoporotic bone osseointegration, further clinical evidence needs to be explored (Donos et al., 2023).

1.3.6.5 Effect of osteoporosis medications on surgical dental treatments

OP and its medications including BPs, the most traditional OP medication (Panait and D'Amelio, 2025), denosumab and raloxifene (in some cases) (Koth et al., 2021), could impact surgical dental treatments. For instance, tooth extraction and dentoalveolar surgeries in patients using BPs create a great concern in the field of oral and maxillofacial surgeries (Lee et al., 2023). Those procedures are considered a risk factor for the development of medications related osteonecrosis of the jaw (MRONJ) (Otto et al., 2015). MRONJ is defined as an exposed area of the maxillofacial region that is necrotic and non-healing for more than 8 weeks in a patient with current or previous history of antiresorptive or antiangiogenic therapy and no history of radiation therapy to the jaws (AlDhalaan et al., 2020). The prevalence of MRONJ varies between countries, type and mode of medication administration. For instance, in osteoporotic patients using oral BPs the prevalence is 0.1-0.2% in the United States, 0.001% in Canada, 0.004% in Scotland and 0.0004% in Germany. In cancer patients treated with subcutaneous denosumab, MRONJ incidence ranges from 0.7-1.9%. In Brazil, the incidence in patients receiving intravenous BPs was 3% (AlRowis et al., 2022). Symptoms of MRONJ may occur spontaneously in the bone or a dental extraction site. They include pain, swelling of the soft tissue, infection due to necrotic bone, teeth loosening and some cases present with ulceration of the soft tissue opposite to the affected site (Sharma et al., 2023) (Figure 1). Associated sinus tract may be found and in severe cases an extraoral fistula might develop (Anil et al., 2013).

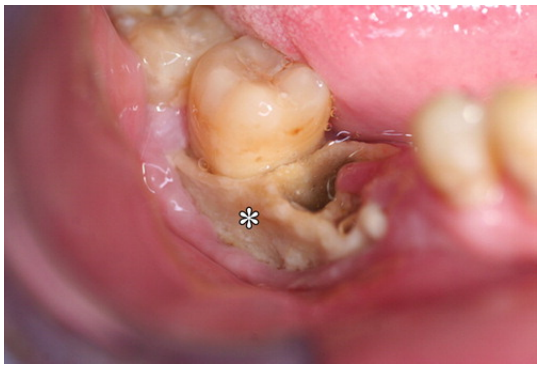


Figure 1: Clinical photograph showing MRONJ.

Image source (Morag et al., 2009).

In addition, BPs can lead to ischemic changes in the extraction site which affect the wound healing (Migliorati et al., 2013b). They also inhibit the endothelial cell function which might lead to tissue necrosis (Smith et al., 2017). Clinically, the healing time in patients using BPs was significantly higher when compared to healthy individuals (Migliorati et al., 2013a). However, it can't be determined whether the impaired healing was caused by BPs or by the OP disease. For example, a study in ovariectomised (OVX) rats conducted to examine the effect of estrogen deficiency on alveolar socket healing, showed that there was a reduced bone content in the sockets post-extraction which subsequently lead to delayed alveolar wound healing and suggests the role of osteoporosis in the delayed healing (Pereira et al., 2007). Osteoporotic patients on BPs should be warned of the possible implant loss and the possibility of poor results of sinus lift surgery in cases of maxillary implant placement (Diz et al., 2013).

Overall, several systemic conditions have been considered as contraindications for the use of dental implants including patients using (intravenous) BPs. The rationale behind these contraindication stems from the fact that osseointegration depends on bone turnover which might be influenced by the use of BPs at the first phase and later by slowing the process of wound healing (Thirunavukarasu et al., 2015). Based on a recent literature review, the variation in the mode of BPs administration is related to the development of MRONJ and hence the possible contraindication for implant treatment (Kawahara et al., 2021). Dental implant treatment for patients on oral BP for the treatment of OP is not contraindicated (Thirunavukarasu et al., 2015). However, patients who use intravenous BP to treat cancer-related conditions are at a higher risk for developing MRONJ with implant placement (Gelazius et al., 2018, Ruggiero et al., 2022) hence, implant treatment is contraindicated in these patients (Thirunavukarasu et al., 2015). According to a recent systematic review of clinical trials (Shibli et al., 2025), implant survival rate was higher than 90% in both osteoporotic and non-osteoporotic patients. However, studies that reported implant failure in the former group demonstrated that it occurred during the first year of implant placement suggesting an issue of osseointegration. Overall, it is advisable that clinicians inform the patients of the potential small risk of acquiring MRONJ upon implant placement and bone regeneration procedures and to take all measures to make the procedure less invasive to promote bone healing (Donos et al., 2023).

1.4 Bone and Periodontal Regeneration

1.4.1 Bone healing in osteoporosis

As discussed previously, an essential part of periodontal structure includes alveolar bone surrounding teeth, which normally undergoes resorption in cases of periodontitis. This section will shed a light on bone healing under osteoporotic conditions drawn from skeletal bone studies. Bone healing involves a series of events; haemostasis, blood clot formation and recruitment of inflammatory cells (Ghiasi et al., 2017). This is followed by a proliferative phase which is composed of fibroplasia and woven bone formation. In fibroplasia, a provisional matrix is formed which is then penetrated by blood vessels, osteoblasts and finger-like projections of woven bone surrounding the blood vessels (Araújo et al., 2015). The last stage of bone healing involves bone remodelling where the immature bone is removed followed by mature bone marrow (BM) and lamellar bone formation (Araújo et al., 2019).

The success of bone healing following fracture depends on the availability of adequate blood supply to ensure the recruitment of inflammatory and mesenchymal cells to the site of fracture. It also depends on the contact between the bony fragments as well as the stability of the fracture site (Tarantino et al., 2011). In addition, bone healing is modulated by the interaction of regulatory factors such as cytokines and hormones (Sanghani-Kerai et al., 2018). Gonadal steroid hormones including estrogen are essential for bone maintenance. Hence in postmenopausal women, bone healing is impaired (Rao and Rao, 2014). Estrogen impacts the mechano-sensitivity of bone cells, possibly via prostaglandins synthesis, when the bone tissue is subject to mechanical strain (Joldersma et al., 2001). Hence, the response to mechanical stimuli might change when estrogen is deficient in postmenopausal women compared to premenopausal women or men. Additionally, in a study by Nikolaou et al., older osteoporotic patients showed a delayed fracture healing (Nikolaou et al., 2009). They attributed that delay to several factors including the jeopardised mechanical strength, decreased number of MSCs in osteoporotic bone as well as the reduction in the release of local growth factors essential for healing e.g bone morphogenetic proteins (BMPs), transforming growth factors (TGFs), IGFs and fibroblast growth factors (FGFs).

In postmenopausal osteoporotic women, bone biopsies showed reduction in the ability of bone formation and was attributed to the impaired proliferation of osteoblastic cells (Marie et al., 1989). Rodríguez et al.'s study on BMSCs isolated from osteoporotic postmenopausal women indicated lower proliferative capacity of these cells alongside a reduced osteogenic differentiation leading to a reduced osteogenesis capacity (Rodríguez et al., 1999). Moreover, in elderly people, changes in the cellular and cytoskeletal features occur during bone healing. The production of collagen, osteocalcin (OCN), alkaline phosphatase (ALP) as well as Runt related transcription factor 2 (RUNX2) are reduced. Also, the number of osteoprogenitor cells along with the number and activity of osteoblasts diminished (Pesce et al., 2009). Moreover, OP entails cellular and biochemical deficiencies that affect bone structure and fracture repair. The decrease in bone thickness and the increase in porosity result in loss of cortical (outer dense) bone and jeopardises its strength. The associated loss of trabecular (inner porous) bone leads to thinning and reduced connection among trabecular plates. The resultant abundantly porous cancellous bone adversely affects fracture fixation in osteoporotic patients (Currey et al., 1996).

Since BM has the common precursor/stem cells for osteoblasts and adipocytes, the formation of adequate osteoblasts requires a balanced relationship in the differentiation pathways for osteoblasts and adipocytes to maintain an adequate bone mass (Wang et al., 2016). In cases of OP, this balance is disrupted which results in negative changes in MSCs function and disturbance in their interaction with their surrounding microenvironment favouring more adipocytic differentiation (Pino et al., 2012). Despite this, a recent review on the correlation between OP and fracture healing indicated that the clinical studies are insufficient to provide evidence on the delayed fracture healing in osteoporotic patients (Chandran et al., 2024).

The following section will discuss periodontal regeneration as a dental application for bone healing.

1.4.2 Current periodontal regenerative approaches and periodontal tissue engineering

Chronic periodontal disease and tooth loss can lead to bone loss in the jaw. In some patients requiring implant placement, there is not enough bone to support that. Therefore, the regeneration of lost alveolar bone is necessary to allow implant osseointegration(Pranathi et al., 2024). First, the “gold standard” bone grafting technique includes the use of autogenous bone grafts (Sakkas et al., 2017). It has been found that the use of orofacial bone to treat orofacial defects leads to more successful results when compared to grafting from non-orofacial sites which indicates site-specific differences in graft integration (Akintoye et al., 2006). Autograft is usually taken from the same intraoral quadrant at which the grafting is planned although it yields limited graft volume (Reynolds et al., 2010). Second, several types of allogenic bone grafts are used and include freeze-dried bone allograft and demineralised freeze-dried bone allografts (Liu et al., 2019c). Third, xenografts e.g. Bio-Oss® has been shown to produce positive effects on bone augmentation (Sheikh et al., 2017). Fourth, alloplastic materials have been synthesised e.g. tricalcium phosphate, hydroxyapatite and bioactive glass (Liu et al., 2019c) and are usually combined with other methods such as guided bone regeneration (GBR) (Jimi et al., 2012). GBR stimulates the growth of new bone in areas of damage and can be used in combination with guided tissue regeneration (GTR) to build the soft tissue in the mouth (Sahrmann et al., 2011). Both GBR and GTR are surgical techniques used to regenerate alveolar bone and tissue surrounding teeth respectively (Pellegrini et al., 2013). Examples of the types of the membranes used in such techniques include non-resorbable membranes e.g. polytetrafluoroethylene (PTFE), absorbable membranes made of natural materials e.g. collagen and chitosan, and third generation membranes which can gradually release bone-healing substances to supplement the process (Marian et al., 2024).

The success of periodontal regeneration relies on several factors. Some of which are patient-related e.g. systemic health, status of smoking, some are site-related e.g. defect morphology, tooth mobility, wound stability, biofilm level or residual BOP and some are surgical-related e.g. flap design, type of biomaterials used and surgeon’s skills (Aslan and Rasperini, 2025, Cortellini and Tonetti, 2015). According to Nibali et al’s. systematic review (Nibali et al., 2020), the treatment

of choice for deep intra-bony defects is resorbable enamel matrix derivatives EMD or resorbable GTR. In cases of wider defects, DBBM is recommended. Papilla preservation techniques should be applied in cases of sot tissue management to achieve successful results.

Even though the above-mentioned technologies can result in alveolar bone and soft tissue regeneration, they are considered clinically unpredictable, and their results are not necessarily satisfactory, as of yet (Chen and Jin, 2010). Some common complications involved in such techniques include the disintegration of the membrane subjecting the tissue to infection which eventually jeopardises the tissue regeneration process (Gao et al., 2024). Also, clinical trials showed that the use of the newly evolved biomaterials is controversial and the process of restoring the structure and function of periodontal tissue still comprises a clinical challenge (Bartold et al., 2016). In osteoporotic patients, there is some evidence suggesting that OP might have a negative impact on non-surgical periodontal therapies (Gomes-Filho et al., 2013). While most of the published literature discusses how OP is related to periodontitis, based on the discussion above (1.3.6.4), it can be estimated that OP might hinder the healing process for periodontal surgical interventions.

To overcome the drawbacks of the current regenerative modalities, engineering for periodontal tissues has emerged as an alternative to regenerate the lost structure and function. For periodontal regenerative therapies to succeed, they require the presence of (1) stem cells that can differentiate into mature phenotype (osteoblasts, cementoblasts and fibroblasts), (2) cell signalling molecules (e.g. growth factors) to modulate the cellular differentiation and tissue formation (3) a three dimensional scaffold (e.g. collagen, bone minerals) to support the process (Han et al., 2014). The next section will focus on the use of stem cells and growth factors.

1.4.2.1 Stem cells

Stem cell therapies have recently received a remarkable amount of attention as a promising tool for regenerative medicine for the treatment of a variety of conditions including OP (Chen et al., 2024b) and periodontal disease (Citterio et

al., 2020). Stem cells are defined as clonogenic cells that have the capacity of cell renewal and multi-lineage differentiation (Weissman, 2000) and can be classified according to their differentiation potential into totipotent, pluripotent, multipotent and unipotent stem cells. Totipotent stem cells are cells that can differentiate into a complete organism or any of its tissues or cell types e.g. the zygote (Condic, 2014). Pluripotent stem cells are cells that can give rise to all body cell types e.g. embryonic stem cells. Multipotent stem cells are cells which have the capacity to differentiate into different cell types within the same lineage and include haematopoietic stem cells (HSCs) and MSCs (Jaenisch and Young, 2008). The last type is unipotent stem cells where cells differentiate into one single cell type (Singh et al., 2016). Adult stem cells can either be multipotent or unipotent (Dulak et al., 2015). Stem cells, particularly MSCs, can enhance bone regeneration by secreting cytokines and growth factors such as VEGF, IGF-1, IL-6 and TGF-β (Arjmand et al., 2020). VEGF initiates angiogenesis in the bone regeneration site (Hu and Olsen, 2016). IGF-1 plays a role in the proliferation of osteoblasts while IL-6 regulates bone resorption by osteoblast. TGF-β regulates the process of bone formation and resorption (Tanaka et al., 1996).

In addition to the BM, adult stem cells can be isolated from a variety of sources including oral and dental tissues such as the PDL, dental pulp, tooth germ, dental follicle, apical papilla, gingiva, oral mucosa and periosteum (Chalisserry et al., 2017) (Figure 2).

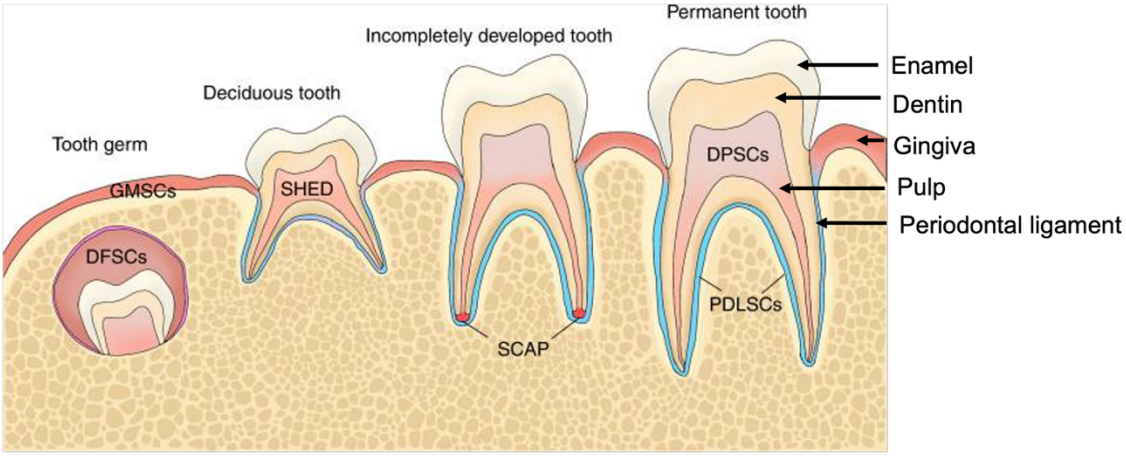


Figure 2. Types and sources of dental stem cells.

GMSCs: gingival mesenchymal stem cells, DFSCs: dental follicle stem cells, SHED: stem cells from human exfoliated deciduous teeth, SCAP: stem cells from the apical papilla, DPSCs: dental pulp stem cells, PDLSCs: periodontal ligament stem cells. Figure Adapted from (Li et al., 2022b).

1.4.2.2 Bone marrow stem cells in bone regeneration; an osteoporotic lens

Bone marrow mesenchymal stem cells (BMSCs) have been considered the gold standard source of stem cells although harvesting them requires invasive procedures (Kern et al., 2006). BMSCs have the capacity to differentiate into osteogenic, chondrogenic and adipogenic lineages (Egusa et al., 2005). However, the number and differentiation potential of stem cells isolated from the marrow decreases with age (Kern et al., 2006). With ageing, BMSCs shift their differentiation from osteogenic to adipogenic lineage which could affect osteoblast formation and bone remodelling and hence be involved in the pathogenesis of OP and osteoarthritis (Ganguly et al., 2017b). Additionally, in postmenopausal women with estrogen deficiency, the multipotency and osteogenic differentiation capacity of BMSCs is reduced while the formation of osteoclasts is enhanced which leads to defective bone formation in OP (Wu et al., 2018). Moreover, BMSCs isolated from postmenopausal women showed 50% reduction in collagen I formation and higher gelatinolytic activity under differentiation conditions with higher adipogenic capacity (Rodríguez et al., 2000). In addition, osteoporotic MSCs secrete decreased amounts of TGF-β and show reduced ability to produce and maintain type I collagen which supports the differentiation pathways towards adipogenesis (Pino et al., 2012).

In a randomised controlled clinical study by Kaigler et al., MSCs isolated from patients' BMs were used to treat localised craniofacial defects and showed promising results (Kaigler et al., 2013). However, other evidence suggested that the use of craniofacial stem cells presents a better source for craniofacial regeneration (Matsubara et al., 2005). The variation in the main findings between these two studies could be a result of their different study designs; the former being a clinical trial and the latter being a combination of in vitro expansion of alveolar BMSCs that were expnded and later transplanted into immunocompromised mice. In a study that compared human iliac BMSCs with orofacial MSCs, it was found that the latter had a higher capacity to proliferate and to differentiate into osteogenic lineage, potentially due to the site from which BMSCs were isolated (Akintoye et al., 2006). When compared to BMSCs derived from appendicular bone, craniofacial MSCs showed greater autophagy and anti-apoptotic capacities with higher pluripotent protein expression (Akintoye et al., 2006). In addition, in a study by Matsubara et al. (Matsubara et al., 2005), alveolar BMSCs have shown high osteogenic potential both *in vitro* and *in vivo* and poor adipogenic differentiation when induced to differentiate. Additionally, alveolar BMSCs can be isolated with minimal pain to the patient which makes them a favourable source of stem cells in bone regeneration (Matsubara et al., 2005). However, their features might change in case of OP. In a study that investigated the alveolar BMSCs from OVX rats, it was shown that the cells showed reduced cellular proliferation rate, colony formation efficacy, stemness and osteogenic differentiation with reduced anti-ageing capacity (Xu et al., 2016).

1.4.2.3 PDL stem cells based periodontal regeneration

The periodontal ligament is an accessible source of stem cells and contains a variety of multipotent stem cells that can be expanded *in vitro* (Seo et al., 2004). Periodontal ligament stem cells (PDLSCs) are a group of cells isolated from the periodontal ligament. They have the capacity to differentiate into fibroblasts, cementoblasts and osteoblasts (Iwayama et al., 2022) as well as their ability to synthesize collagen (Huang et al., 2024). The main aim of periodontal regeneration using stem cells is to restore the structure and function of the tooth attachment apparatus including the alveolar bone, gingiva, PDL and cementum (Zhu and Liang, 2015). PDLSCs express the following MSC cell surface markers

including CD13, CD29, CD44, CD73, CD90, CD105, CD106 and CD166, and lack the expression of HSC markers e.g. CD34, CD45, CD133, endothelial cell markers e.g. CD31 and CD144, monocytes markers e.g CD14 and markers for B cells e.g CD79, CD19 and HLADR (Song et al., 2015). In addition, PDLSCs were found to induce immunosuppressive effects on T and B lymphocytes (Yi et al., 2022) which may reduce the risks associated with their allogenic transplantation (Wada et al., 2009).

There are several factors that affect the PDLSCs properties. First is the tissue origin. For instance, deciduous PDLSCs show more proliferation and differentiation potential than PDLSCs isolated from permanent teeth (Ji et al., 2013, Silvério et al., 2010). Another factor that affects the properties of PDLSCs is the donor's age. Aged donors' samples were found to produce PDLSCs with less regenerative capacity when compared to young donors (Zheng et al., 2009). In a study by Li et al. (Li et al., 2020), PDLSCs isolated from older individuals (35-50 years old) showed less multi-lineage differentiation potential, decreased cell survival and reduced immunosuppression abilities when compared to younger PDLSCs (19-20 years old). Additionally, the health status plays a role in PDLSCs differentiation capacity. PDLSCs isolated from an osteoporotic rat model deposited fewer calcium deposits under osteogenic conditions when compared to their normal counterparts and was explained by the deficiency of expression of estrogen receptors (Zhang et al., 2011). Moreover, the *in vitro* culture medium as well as the growth factors used to study PDLSCs features play an important role in the understanding of their properties. In a study by Jung et al. (Jung et al., 2013), it was found that PDLSCs cultured in alpha minimum essential medium (α-MEM) exhibited a stronger osteogenic differentiation potential and proliferated more than the cells cultured in Dulbecco's modified eagle medium (DMEM). Also, PDLSCs may require a variety of growth factors at different stages to enhance their proliferation (Zhu and Liang, 2015).

Among dental stem cells, PDLSCs can be accessible from periodontal tissues during dental scaling and root planning (Trubiani et al., 2019). PDLSCs exhibit higher osteogenic differentiation potential when compared to dental pulp stem cells as well (Abd. Rahman and Azwa, 2025) and gingival cells (Somerman et al., 1988). In a recent systematic review and meta-analysis, Zhang et al. concluded

that the use of stem cells in periodontal regeneration enhanced periodontal regeneration in patients with periodontitis showing improved periodontal pockets depth, CAL, and bone defect depths although they suggest the need for further randomised clinical trials (Zhang et al., 2022b). Nonetheless, data regarding the characteristics PDLSCs in osteoporotic patients is largely unknown.

1.4.2.4 Signalling molecules

Growth factors modulate the communication between the cells and their surrounding microenvironment via autocrine and paracrine effects (Chen et al., 2010). Growth factors bind to their cell receptors and contribute to the cell fate and the tissue regeneration. Hence, they are of essential importance in tissue engineering (Tayalia and Mooney, 2009). They can switch on certain molecular pathways to control cell proliferation, differentiation and migration (Vishwakarma et al., 2015) as well as regulating the immune function (Shin et al., 2015).

Growth factors are naturally occurring and play a major role in several tissues including the periodontium (Raja et al., 2009). The periodontium contains a plethora of cytokines and growth factors essential for tissue regeneration including FGFs, platelet derived growth factors (PDGFs), TGFs, epidermal growth factor (EGF) and IGFs (Suchetha et al., 2015). In addition to its anti-apoptotic effect on the periodontium, maintenance of stemness and immunomodulation, (Bashir, 2021) IGF-1 has experimentally shown a great promise for promoting periodontal regeneration (Werner and Katz, 2004). The following section will expand on the IGF system and its role in bone, periodontium and OP.

1.5 Insulin-like Growth Factor (IGF)

IGFs were first discovered by Salmon and Daughaday in 1957 (Salmon and Daughaday, 1957). They are small peptide growth factors and are highly homologous to insulin (Lakka Klement et al., 2013). The IGF axis is composed of two main ligands (IGF-1, IGF-2), two main receptors (IGF-1 receptor [IGF-1R], IGF-2 receptor [IGF-2R], high affinity IGF binding proteins (IGFBPs 1-6) (Kawai and Rosen, 2012b) and low affinity binding proteins or IGFBP-related proteins (IGFBP-rP 7-12) (Allard and Duan, 2018). Other members include insulin (as a

ligand), insulin-receptors (IR), and the acid labile subunit (ALS) (Le Roith, 2003b). Refer to (Figure 3) for an illustration of the main components of IGF system.

Since IGF ligands are normally bound to IGFBPs in tissues and circulation, IGFBP proteinases are necessary to release IGF ligands before they can bind to their receptors (Poreba and Durzynska, 2020). These enzymes are broadly categorised into serine proteinases, metalloproteinases (of which are pappalysins) and cathepsins (Clay Bunn and Fowlkes, 2003). Pregnancy-associated plasma protein-A (PAPP-A) and PAPP-A2 are members of pappalysins family which cleave IGFBPs increasing the bioavailability of IGF-1 ligand (Nimptsch et al., 2024). PAPP-A has been identified as the primary IGFBP-4 protease (Hjortebjerg, 2018), but has also been reported to cleave IGFBP-2 and IGFBP-5 (Qin et al., 2006).

The IGF family of proteins has been researched extensively due its essential role in the normal physiology as well as a variety of pathological conditions including growth disorders (Ron, 2007), diabetes, cancer and conditions related to nutritional abnormalities (Le Roith, 2003b). The following section will focus mainly on IGF-1, IGF-2, IGF-1R, IGF-2R, IGFBPs (1-6), IGFBP-rP 7 and PAPP-A.

1.5.1 IGF Ligands

The two ligands IGF-1 and IGF-2 are expressed in several tissues and act mainly via IGF-1R to enhance cellular functions such as cellular proliferation, survival, differentiation, migration as well as metabolic functions (Denley et al., 2005). In prenatal life, the synthesis and regulation of IGF ligands is not well understood. It is mostly suggested that the synthesis is regulated by paracrine factors such as oxygen tension, nutritional status, biochemistry, growth factors, ECM and endocrine factors. In postnatal life, growth hormone (GH) regulates the levels of IGF-1 which induces its expression and release by the liver (Youssef et al., 2017). In rodents, IGF-1 is considered an adult peptide while IGF-2 is mainly expressed during foetal development. In humans, the expression of these ligands is different as IGF-1 and IGF-2 are secreted throughout life which potentially indicates their different physiological roles (Rosenfeld and Hwa, 2009). It has been reported that IGFs play a role in the coupling of bone which is the process through which bone

formation and resorption are linked (Hayden et al., 1995). Hayden et al. (Hayden et al., 1995), discussed the potential mechanisms through which IGFs regulate the process of coupling e.g IGFs stored in bone can act as an autocrine and/or paracrine agent influencing the action of both osteoclasts and osteoblasts during bone resorption and the resorbed cavity re-fill. They also discussed how ageing influences the process of coupling through the reduction of GH which also reduces IGF-1 level eventually impacting the rate of bone resorption to formation.

1.5.1.1 IGF-1

IGF-1 circulates the body as a 70-residue single polypeptide chain with four domains (A-D). Most tissues express IGF-2 however, it is mainly derived from the liver (Delafontaine et al., 2004). The main factors that regulate hepatic IGF-1 are GH, insulin and nutrition (J.E and I, 2012). There is a correlation between IGF-1 and GH. GH performs its actions by binding to the growth hormone receptor (GHR) which is found on most cells' surfaces (Dehkhoda et al., 2018). In humans, GHRs are expressed in the liver, adipose tissue, kidney, heart, intestine, pancreas, lung, cartilage and skeletal muscle (Ballesteros et al., 2000). GH acts either directly, via the GHR, or indirectly, via inducing IGF-1 (Yakar et al., 1999). Increased BMD during puberty is correlated to sex steroids and the GH/IGF axis. On a cellular level, GH and IGF-1 stimulate the proliferation and differentiation of MSCs into osteoblasts via inhibiting adipogenic gene expression (Dixit et al., 2021).

Examples of other factors that regulate hepatic IGF-1 include prostaglandin E2, PTH, angiotensin II, thyroid stimulating hormone and estrogen (Le Roith, 2003a). Moreover, it has been found that IGF-1 is an important protein for the development of the embryo (Baker et al., 1993). IGF-1 null mice (IGF-1$^{-/-}$) are born with more than 60% weight reduction compared to the wild type, and more than 95% of the IGF-1 null young mice (IGF-1$^{-/-}$) die during the perinatal period (Powell-Braxton et al., 1993). In mice with IGF-1 deficiency, growth retardation occurs and the bones formed are smaller compared to the wild type mice (Bikle et al., 2001). On a cellular level, according to Kostenuik et al. (Kostenuik et al., 1999), IGF-1 stimulated the proliferation and differentiation of BM osteoprogenitor cells. IGF-1 has also shown stimulatory effect for matrix bone formation (Hock et

al., 1988). IGF-1 supports the osteoblast-osteoclast interaction to promote the process of osteoclastogenesis (Wang et al., 2006b) and promotes osteoblast survival through inhibiting apoptosis (Hill et al., 1997).

1.5.1.2 IGF-2

IGF-2 consists of 72 amino acids. Similar to IGF-1, IGF-2 has (A-D) domains (Rosen and Niu, 2008). IGF-2 is found in all rodents and human foetal tissues. The levels of IGF-2 decrease in postnatal life in rodents but remain high in human serum and tissues especially in the brain tissue (Shoshana et al., 2018). The physiological role of this protein still needs to be explored (Holly et al., 2019). However, it exerts its effects in virtually all the tissues (Cianfarani, 2012). For example, in a study on murine placenta, it was reported that IGF-2 promotes growth since the deletion of its placental promoter reduces the nutrient transfer capacity of the placenta which affects foetal growth (Sibley et al., 2004). In humans, IGF-2 has been linked to metabolic and cardiovascular disease risk (Cianfarani, 2012). It has been also reported that IGF-2 contributes to maintaining the stemness of a variety of stem cell types e.g. neural and intestinal tissue (Blyth et al., 2020). On a tissue level, IGF-2 enhances cell growth and survival. It particularly promotes chondroblast differentiation and survival as well as regulating the timing of that process (Blyth et al., 2020). Similar to IGF-1, IGF-2 is regulated by IGFBPs particularly IGFBP-6 which binds to IGF-2 with high affinity inhibiting its action (Bach, 2016).

1.5.2 IGF Receptors

IGF-1 exerts its effects mainly through IGF-1R (Powell-Braxton et al., 1993). IGF-1R is a member of the insulin receptor family which also consists of IR and insulin related receptor (IRR). These receptors function as homodimers which are disulfide-linked, and they are also able to work as heterodimer hybrids (Lawrence et al., 2007). IGF-1R is expressed across different cells and tissues (Werner et al., 2008), and its structure is similar to that of IR (Hale and Coward, 2013). The receptor is composed of two extracellular α subunits and two transmembrane β-subunits (Laviola et al., 2007a) (Figure 3).

- α-subunits: it consists of two 130-135 kDa chains. The α-subunits are extracellular and represent the ligand binding sites (Ruben et al., 2009).
- β-subunit: the structure consists of two 90-95-kDa chains (Ruben et al., 2009). It involves a short extracellular domain which contributes the binding of the α-subunit, a transmembrane domain and an intracellular domain where the tyrosine kinase activity takes place (Janssen, 2020).

IGF-2R is considered of less importance for the stimulation of growth but plays an important role in regulating the activities of IGF-1 and IGF-2. It has a different structure than IGF-1 receptor being a single chain protein. IGF-2R binds to IGF-2 with 100 times greater affinity than IGF-1 (Clemmons et al., 2020, Duan et al., 2010). Knockout of IGF-2R gene leads to foetal overgrowth perinatal mortality (Duan et al., 2010). Refer to (Figure 3) for an illustration of the IGF system receptors.

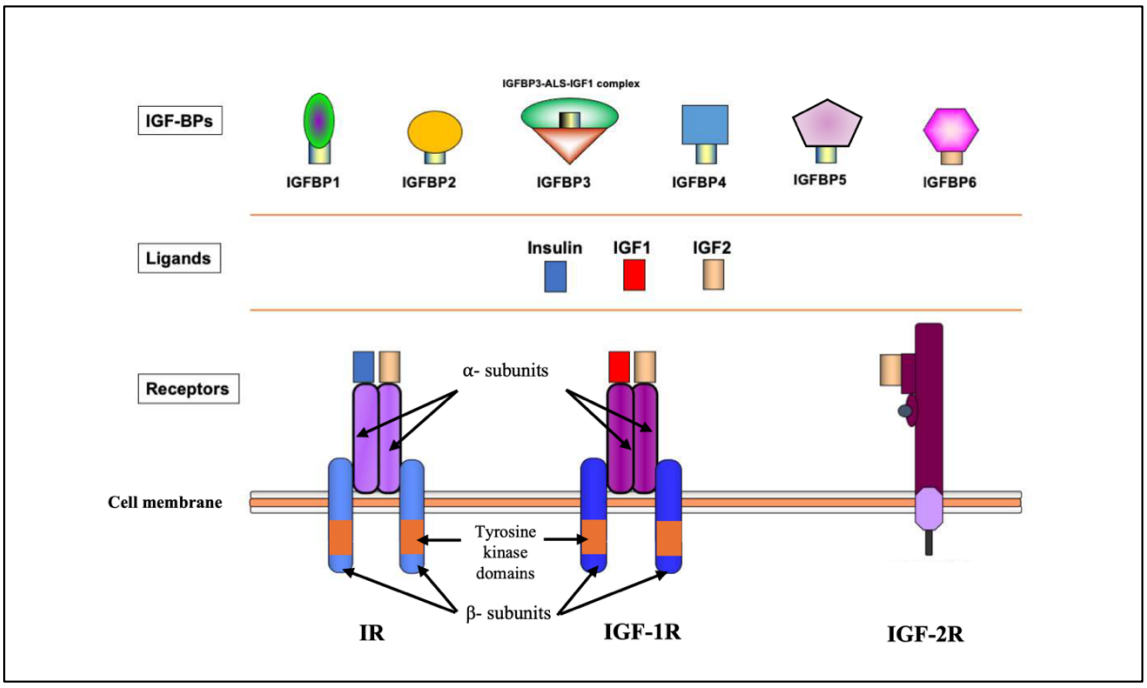


Figure 3. Main components of the IGF system.

This figure shows the different components of the IGF system including IGF ligands, receptors and the six main binding proteins. IGF-1R and Insulin receptor (IR) share a similar structure of two extracellular α-subunits and two transmembrane β-subunits with tyrosine kinase domains. IGF-2R has a different structure of a single chain protein. There is a cross-reactivity between ligands and receptors. Of note is the IGFBP-3-ALS-IGF-1 complex which protects IGF-1 from degradation. Figure Adapted from (Werner, 2023).

1.5.3 IGF Signalling Pathway

The signalling of both IGF-1 and IGF-2 is mediated by IGF-1R although IGF-1R binds to IGF-2 with low affinity (Adams et al., 2004). The tyrosine kinase domains of IGF-1R and IR are essential for their induced signalling. The same tyrosine residues that are phosphorylated by IR signalling are also phosphorylated by the binding of IGF-1 (Le Roith, 2003a). The activated IGF-1R then activates certain substrates i.e insulin receptor substrate (IRS)-1, IRS-2 and homology collagen proteins (Shc) (Laviola et al., 2007a), which then activates two downstream main pathways; phosphatidylinositol 3-kinase (PI3-K)- Akt/ mammalian target of rapamycin (mTOR) pathway, and Ras-mitogen-activated protein kinase-Extracellular signal-regulated kinase (RAS/ERK) pathway (He et al., 2022). The former pathway inhibits apoptosis and activates protein synthesis (Werner, 2023). When the second pathway is activated, mitogenic outcomes are enhanced (He et al., 2022). Generally, IGFs and insulin share common signalling pathways but differ in outcomes, as insulin contributes to metabolic activities while IGFs act to mediate cell fate (Fumihiko and Shin-Ichiro, 2018). Refer to (Figure 4) for a schematic representation of the IGF ligands signalling pathways.

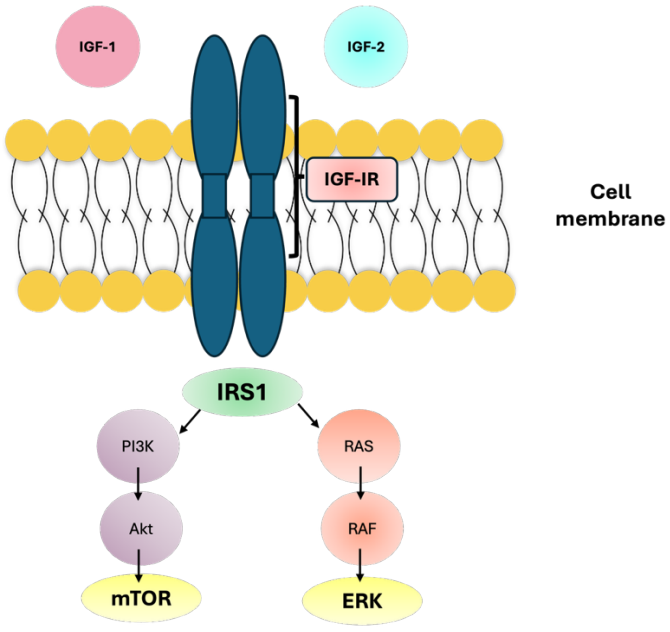


Figure 4: Intracellular signalling pathways for IGFs .

Upon ligand binding to IGF-1R, IGF-1R is activated which then activates insulin receptor substrate 1 (IRS1) which activates either of two downstream signalling pathways: PI3K/Akt/mTOR pathway which inhibits apoptosis or RAS/RAF/ERK pathway for mitogenesis. Figure Adapted from (Werner, 2023).

1.5.4 General Function

IGFs have a similar function to insulin such as elevating the glucose metabolism in fat, increasing the formation of protein, glycogen and lipid. They also contribute to the increase of glucose transport as well as the inhibition of lipolysis (Clemmons, 2012). IGFs perform these functions with less potency compared to insulin (Maki, 2010). In the liver, both IGF ligands were found to play a role in hepatic regeneration (Hoffmann et al., 2020). Moreover, IGF-1, along with GH and sex hormones, contributes to normal bone growth and development (Bouillon and Prodonova, 2000). IGF-1 is also important in longitudinal bone growth, maturity of the skeleton and acquiring and maintaining bone mass during growth and adulthood (Giustina et al., 2008). There is a positive correlation between BMD and serum IGF-1 level in both men and women. It has been reported that lower levels of serum IGF-1 is associated with bone fractures in women (Locatelli and Bianchi, 2014). The basic effect of IGF-1 is explained by the studying of mice deficient in IGF-1 as they show skeletal deformities, delayed mineralisation, reduction of chondrocyte proliferation and increased apoptosis (Laviola et al., 2008). On a cellular level, IGF-1 signalling has been shown to promote osteoblast survival, proliferation and differentiation (Bikle et al., 2015). IGFs also increase the production of collagen and are integrated in the matrix as well (Perrini et al., 2010). IGF-1, along with GH, produces anabolic effects on bone through supporting bone mass acquisition and maintenance (Rosen and Bilezikian, 2001). IGF-1 has been reported to support the process of osteoclastogenesis by maintaining the balance between osteoblasts and osteoclasts (Wang et al., 2006a). Other functions of IGFs have been reported in the development of the kidney, central nervous system, the cardiovascular system, the immune system as well as the development of the embryo (J.E and I, 2012).

1.5.5 IGF binding proteins

Structurally, IGFBPs have three main domains; N-terminal region, were IGFs bind, the C-terminal region, which facilitates the interaction of IGFBPs with other proteins, and a variable linker domain, which is involved in posttranslational regulation (Ruan et al., 2024). The bone matrix holds an abundant amount of inactive IGF-1 and IGF-2 bound to IGFBPs. They play an essential role in bone homeostasis and circulate in elevated concentrations in the vascular system,

which is rich in the bone structure, allowing bone cells to be exposed to these proteins (Masanobu and Clifford, 2012).

In addition to their regulatory function in the circulation, IGFBPs regulate IGFs expression at the tissue level (Rosen and Niu, 2008). The majority of IGF-1 (75%) exists as a ternary complex with IGFBP-3 and ALS, while the remainder of IGF-1 circulates bound to other IGFBPs (Mohan and Kesavan, 2012). Furthermore, IGFBPs affect the bioactivity of IGF-1. Generally, the effect of IGFBPs on IGF-1 signalling depends on their molar ratio. Primarily, IGFBPs act as inhibitors of IGF-1 function (Kawai and Rosen, 2012a). However, IGFBPs can enhance the action of IGFs by preventing their degradation (Rosen and Niu, 2008).

IGFBP-1 is a 30 kDa peptide that is produced mainly in the liver. The production of hepatic IGFBP-1 is regulated by insulin and substrate availability (Rosen and Niu, 2008). IGFBP-1 acts to inhibit the binding of IGF to its receptor and hence inhibiting the metabolic actions of IGF (Lee et al., 1993). IGFBP-2 is a 31 kDa protein. Its concentration in plasma depends on age being higher in infancy and older individuals (van den Beld et al., 2019). IGFBP-2 is considered the major IGF binding protein in cerebrospinal fluid. Mainly, it inhibits biological actions mediated via IGF protein (Collett-Solberg and Cohen, 2000). IGFBP-3 is a 43 kDa glycosylated peptide (Rosen and Niu, 2008). It is the main binding protein and binds to IGF-1 and ALS and performs an antagonistic effect on IGF-1 signalling (Masanobu and Clifford, 2012). This complex sequesters IGFs and allow them to have a longer half-life (Rosen and Niu, 2008). Regulation of the serum IGFBP-3 is provided mainly by the GH (Martin and Baxter, 1988). Extrahepatic synthesis of IGFBP-3 is regulated by paracrine and autocrine factors. The action of IGFBP-3 on IGF-1 on a cellular level could be both stimulatory or inhibitory based on the cell type and the physiological environment (Baxter, 2023). Several IGFBP-3 proteases exist extra- or intravascularly and are regulated by several autocrine and paracrine factors (Rosen and Niu, 2008). IGFBP-4 is one of the most prevalent binding proteins secreted by bone cells. Like most IGFBPs, it inhibits IGF-1 signalling *in vitro* (Kawai and Rosen, 2012b). On the contrary, IGFBP-5 is considered a potential agonist for IGF-1 function as well as being a storage protein (Kawai and Rosen, 2012b). IGFBP-5 promotes cell growth and bone remodelling (Han et al., 2017). IGFBP-6 differs from the

other IGFBPs by having three disulphide bonds and lacking the cysteine-rich motif (Bach, 2015). The levels of IGFBP-6 increase with ageing and vary between males and females being higher in the former (Van Doorn et al., 1999). The main function of IGFBP-6 is to inhibit the actions of IGF-2. It was found that IGFBP-6 inhibited the IGF-2-induced proliferation, migration, differentiation and survival but showed little to no effect on IGF-1 induced actions, partly due to its lower binding affinity (Bach, 2015). IGFBP-7 will be discussed here due to its relevance to bone metabolism. As an IGFrP, IGFBP-7 has a low affinity binding to IGF compared to conventional IGFBPs. IGFBP-7 is involved in cellular differentiation (Walker et al., 2006), cell growth (Nousbeck et al., 2010), senescence and apoptosis (Wajapeyee et al., 2008). It has also been shown to downregulate RANKL-induced osteoporosis and enhance osteogenic differentiation *in vitro* and *in vivo* (Ye et al., 2020).

Overall, IGFBPs are regulated by several systemic agents including PTH, vitamin D3, glucocorticoids, estrogen, retinoic acid and IGFBP proteases (Lindsey and Mohan, 2016).

1.5.6 IGF axis and bone regeneration

As discussed, IGFs are available in the skeleton abundantly. They are stored in an inactive form bound to IGFBPs in the bone matrix (Kawai and Rosen, 2012b). During bone remodelling, initial bone resorption by osteoclasts releases matrix proteins and calcium. IGF-1 is released as well and is thought to be involved in attracting osteoblast precursors to the endosteal surfaces (Kawai and Rosen, 2012b). IGF-1 has also been found to stimulate osteoblasts differentiation (Xian et al., 2012a) as well as enhancing the function of mature osteoblasts (Zhao et al., 2000). IGFs have also been reported to stimulate osteoclast activity (Shoshana et al., 2018).

The first evidence that IGF-1 is involved in bone biology was demonstrated in a study where the IGF-1 gene was lacking in the experimental mice (Liu et al., 1993). More than 80% of the IGF deficient mice died perinatally and the few mice that lived to adulthood showed signs of postnatal growth retardation. In another study of IGF-1 null mice, they exhibited a distinct skeletal phenotype. The cortical

bone was reduced by 24% and the length of their femur bones was shortened. This phenotype was explained by a defect in their osteoclastogenesis (Wang et al., 2006a). A reduced number of osteoclasts was also evident in co-cultures of IGF-1 null mice osteoblasts (Wang et al., 2006a). Moreover, in osteoblasts isolated from the BM of IGF-1 null mice, the expression of RANKL was impaired and the expression of RANK and macrophage colony stimulating factor in long bones was reduced (Bikle et al., 2001). In IGF-1R deficient mice, there was a delay in the calcification of skeleton, evident growth retardation, organ hypoplasia, and they all died postnatally due to respiratory dysfunction (Kawai and Rosen, 2012b).

In the maxillofacial region, IGF-1 resulted in osteoblast proliferation which enhanced the bone formation in the mandibular condyle (Iikubo et al., 2012). This was demonstrated in a study by Kojima et al. (Kojima et al., 2008), where the administration of exogenous IGF-1 resulted in elongation of mandibular alveolar bone and the ascending ramus as well as buccolingual expansion of the dental arch in an acromegaly rat model. In addition, in a study that tested the effect of human IGF-1 on extraction socket healing in rats, it was shown that IGF-1 increased the volume of the newly formed bone and reduced the alveolar height loss post dental extraction (Kumasaka et al., 2015). Interestingly, conditioned media from several bone sites were collected for IGF axis expression. It was found that IGF-2 was the highest and IGFBP-3 was the lowest in mandibular cells. It was postulated that the higher expression of some IGF axis components was to prevent the loss of bone at those sites (Malpe et al., 1997).

During the process of bone remodelling, IGF-1 is secreted by the bone matrix to stimulate the formation of osteoblasts by MSCs. This maintains the structure and the mass of bone which were reported to be downregulated in the pre-osteoblastic cells of IGF-1 receptor knockout mice (Xian et al., 2012b). It was also shown that MSCs proliferate in response to IGF-1 in serum deprived conditions (McCarty et al., 2009) signifying the role of IGF-1 in MSCs proliferation. In addition, MSCs that were isolated from different tissues such as the BM express and secrete IGF-1 *in vitro*. IGF-1 is also involved in regulating osteoblast differentiation (Bikle et al., 2015) among other factors such as TGF-βs, FGFs (Bosetti et al., 2007) and BMPs (Yamaguchi et al., 2008). When rat BMSCs were

transfected with human IGF-1, the cells showed enhanced proliferation and spontaneous differentiation into osteogenic and adipogenic lineages (Hu et al., 2008). IGF-1 has been reported to upregulate the expression of osteogenic differentiation markers including RUNX2 and ALP in human MSCs (Koch et al., 2005). IGF-1 also plays a role in mineralisation. In a study by Reible et al. (Reible et al., 2017), human BMSCs showed an increase in calcium deposition assessed by alizarin red staining after IGF-1 continuous stimulation in osteogenic conditions at high concentrations (6400 ng/mL) compared to lower (100 ng/mL) and medium (800 ng/mL) concentrations. This was also similar to the calcium deposition levels under BMP-7 treatment.

In regards to IGFBPs, IGFBP-1, 2, 4, and 6 generally inhibit growth in IGF-1 stimulated cellular proliferation (Hoeflich et al., 2007), while IGFBP-3 and IGFBP-5 can act as stimulators of IGF-1 actions (Ruan et al., 2024). IGFBP-1 showed low expression levels at primary human osteoblasts under insulin and glucocorticoids regulation although the physiological correlation on bone has not been established (Beattie et al., 2018). IGFBP-2 has shown a negative effect on the proliferation induced by IGF-1, collagen and bone formation. In human participants, the level of serum IGFBP-2 was inversely related to bone mass accretion (Kawai and Rosen, 2012b). It was reported that IGFBP-2 increased with ageing and its levels were conversely associated with BMD in male and female subjects (Amin et al., 2004). In the presence of ECM, the proliferation of osteoblasts can be influenced by IGFBP-2/IGF-2 (Khosla et al., 1998), which proposes potential therapeutic options particularly in the dental field (Hoeflich et al., 2007). Moreover, IGFBP-3 can have a negative effect on the function of IGF-1 signalling. In mice over-expressing IGFBP-3, the level of osteoclasts increased, the proliferative action of osteoblasts was impaired and bone formation was inhibited (Silha et al., 2003). In transgenic mice expressing high levels of IGFBP-4, it was shown that in the bone microenvironment, IGFBP-4 reduced the formation of cancellous bone, bone turnover and impaired postnatal skeletal and somatic growth (Zhang et al., 2003). Interestingly, when PAPP-A cleaves IGFBP-4, it induces an anabolic effect on bone by freeing IGF-1 from its binding to IGFBP-4 (Beattie et al., 2018) (Figure 5). Transgenic mice overexpressing PAPP-A show increased bone formation (Qin et al., 2006) while PAPP-A-null mice show low bone turnover (Tanner et al., 2008), decreased bone strength and delay in

fracture healing (Miller et al., 2007). In transgenic IGFBP-5 mice under the control of the OCN promoter, the volume and formation of bone were reduced (Devlin et al., 2002). However, the osteoclast number did not change in adult transgenic mice overexpressing IGFBP-5 (Devlin et al., 2002). On the contrary, Kanatani et al. (Kanatani et al., 2000), found the IGFBP-5 stimulated osteoclast formation and its bone resorption activity. In contrast to other IGFBPs, IGFBP-6 promoted mitosis and anti-apoptosis in osteoblastic sarcoma cell lines (Schmid et al., 1999).

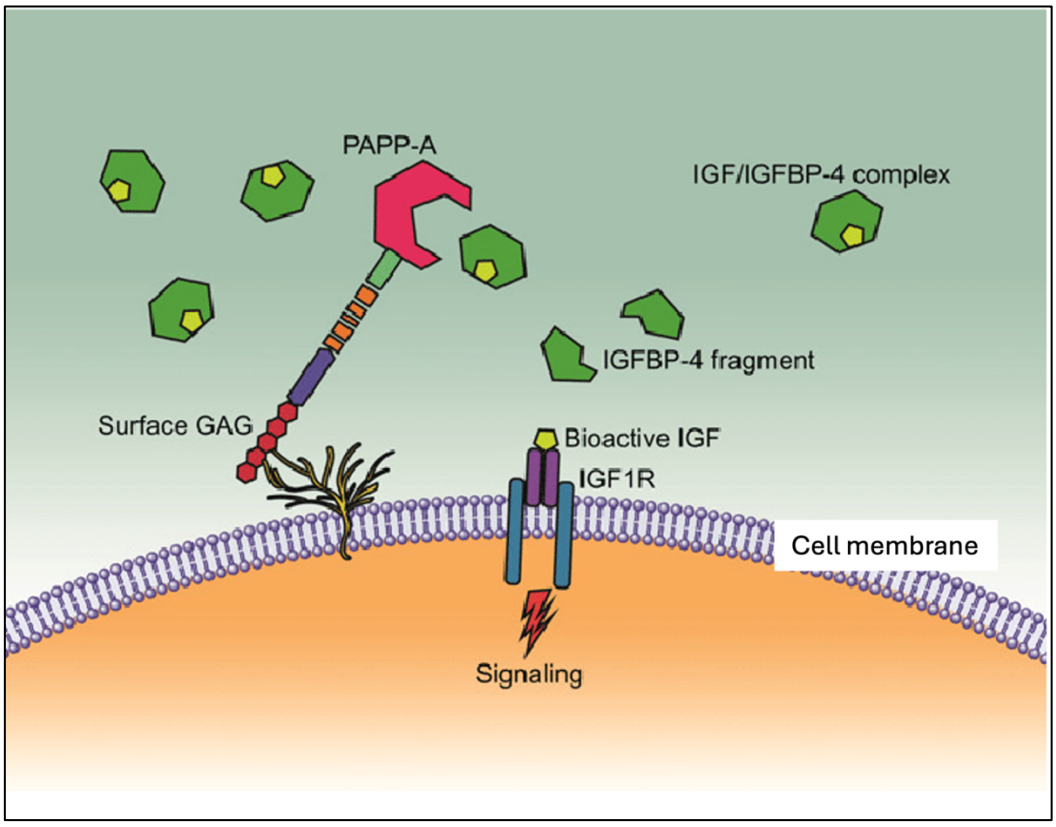


Figure 5. The effect of PAPP-A on IGF signalling.

PAPP-A binds to cell surface glycosaminoglycans (GAGs) and act to release IGF-1 (yellow pentagon) from its binding to IGFBP-4 (green hexagon) hence PAPP-A acts as IGFBP-4 protease. This frees up IGF-1 ligand allowing its binding to IGF-1R and the initiation of its intracellular signalling. Figure adapted from (Conover and Oxvig, 2016).

1.5.7 IGF axis and periodontal regeneration

Several studies have been conducted to investigate the possible effect of the IGF axis in PDLSCs in healthy/ inflamed conditions. In a study by Han et al.

(Han et al., 2017), the application of recombinant human IGFBP-5 on PDLSCs in an inflammatory environment increased the proliferation rate, the osteogenic and odontogenic differentiation. Also, the injection of recombinant human IGFBP-5 induced periodontal regeneration in a periodontitis model. In addition, IGFBP-5 was expressed in PDLSCs at higher levels than non-dental tissues, and it was suggested that it might act as a potential mediator for osteogenic differentiation. IGFBP-5 also promoted ALP activity and mineralisation (Liu et al., 2015). Moreover, Ochiai et al. (Ochiai et al., 2012), suggested that IGF-1 could be used as a promoter for bone regeneration in cases of prolonged inflammatory conditions of PDLSCs. Additionally, IGF-1 was found to be chemotactic for PDL cells and has enhanced protein synthesis during periodontal regeneration (Matsuda et al., 1992). In a study by Howell et al. (Howell et al., 1997), patients with periodontal bony defects treated with 150 µg/ml of combined recombinant human PDGF-BB and recombinant human IGF-1 (rh-IGF-1) in a gel vehicle gained 42.3% defect fill versus 18.5% in the control group. In a study by Sant'Ana et al. (Sant'Ana et al., 2007), the combination of TGF-β1, PDGF and IGF-1 enhanced mitogenesis of PDL cells *in vitro* which suggested their possible role in periodontal regeneration. In an animal model, the local sustained release of IGF-1 from dextran- and gelatin-derived biomaterials, enhanced the periodontal regeneration (Chen et al., 2006). Interestingly, IGF-1 derivative has been used for the treatment of implant surfaces. The combination of IGF-1 with PDGF contributed to the initial bone repair around implants (Stefani et al., 2000). Implant treatment with recombinant human bone morphogenetic protein 2 (rhBMP-2) could enhance implant osseointegration in synergy with rhbFGF and rhIGF-1 (Lan et al., 2006).

1.6 Clinical relevance:

It is the hope that through the understanding of the characteristics of PDLSCs isolated from postmenopausal osteoporotic female patients that patient-specific techniques can be created to aid with the process of periodontal bone regeneration in situ particularly in relation to the osteogenic capacity of those cells. Also, due to the role of IGF axis in bone formation, investigating the potential role of the IGF axis might provide insights into its contribution to the process of periodontal bone regeneration, which could potentially be beneficial with developing therapeutic targets to members of the IGF axis. The efforts presented

in this body of work aim to eventually improve the periodontal conditions in patients with chronic periodontitis through the utilisation of stem-cell based techniques.

In conclusion, this chapter addressed some of the relevant background knowledge available in the literature in regards to OP and its interaction with periodontal disease along with the impact of its medications on dental surgical treatments. It also provided an overview on stem cells as a tool for regenerative dentistry. The IGF axis was also discussed as an essential family of growth factors for osteogenesis and how closely related they are to BMD and OP.
However, based on database search and apart from some animal-based work (Arioka et al., 2019), there were no reports that discussed the characteristics of periodontal stem cells isolated from postmenopausal osteoporotic patients or their potential use as an autologous source of stem cells in periodontal bone regeneration. The literature also provided limited information on the expression of IGF axis members in this type of cells under postmenopausal osteoporotic conditions. Hence, as will be mentioned in Chapter 2, this will be the focus of this project.

Chapter 2 Aims and Objectives

2.1 Aim of the project

Comparing the phenotypic and the osteogenic differentiation capacity of PDLSCs isolated from postmenopausal osteoporotic patients and healthy individuals with a special interest in the characterisation of the IGF axis

2.2 Objectives

- To characterise stem cell populations of PDLSCs isolated from healthy and postmenopausal osteoporotic patients.
- To compare the osteogenic differentiation potential of PDLSCs isolated from healthy and postmenopausal osteoporotic patients.
- To compare the gene and protein expressions of IGF axis molecules in PDLSCs isolated from healthy and postmenopausal osteoporotic patients, under basal and osteogenic conditions.

Chapter 3 Materials and Methods

3.1 Materials

3.1.1 General tissue culture materials

Table 4. General tissue culture materials: List of Consumables

Material	Product number	Manufacturer
6-well plates	3516	Corning Incorporated Costar
BRAND® pipette (pastettes)	Z331759	Sigma Aldrich
Cell scraper	541070	Greiner Bio-one (GBO)
Cell strainer	542070	Greiner Bio-one (GBO)
CryoPure Tubes (2mL)	72.380	SARSTEDT AG & Co. KG
Falcon tubes (15 mL)	430790	Corning incorporated- costar
Falcon Tubes (50 mL)	430828	Corning incorporated- costar
Filter unit (0.2 μm)	SLGP033RS	Merck Millipore Ltd
Microtubes (size 0.5 mL)	72.699	SARSTEDT AG&Co.KG
Microtubes (size 1.5 mL)	72.690	SARSTEDT AG&Co.KG
Needle	AN 2138R1	TERUMO
Parafilm	GC 781	Appleton Woods
Pasteur pipettes	612-1702	VWR
Pipette tips (size 10/20μl)	S1120-3810	TipOne® (starlab)
Pipette tips (size 1000μl)	S1122-1730	TipOne® (starlab)
Pipette tips (size 20μl)	S1120-1810	TipOne® (starlab)
Pipette tips (size 200μl)	S1120-9710	TipOne® (starlab)
Scalpel (size 11)	0503	Swann-Morton®
Stripettes (10mL)	4101	Corning incorporated- costar
Stripettes (25mL)	4489	Corning incorporated- costar
Stripettes (50mL)	501	Corning incorporated- costar
Stripettes (5mL)	4487	Corning incorporated- costar
Syringe (10mL)	307736	BD
Tissue culture flask T175 cm2	431080	Corning

Tissue culture flask T25 cm2	430693	Corning
Tissue culture flask T75 cm2	430641	Corning
Tweezer	930229-1EA	Sigma Aldrich

Table 5. General tissue culture materials: List of Reagents

Reagent	Product number	Manufacturer
0.25% Trypsin-EDTA solution	T4049	SIGMA
4% Formaldehyde	J60401.AP	ThermoFisher Scientific
Alpha MEM with sodium pyrovate	M4526-500ML	Sigma Aldrich
Collagenase I (lyophilized)	17100-017	Gibco
DiMethyl SulfOxide (DMSO)	276855	SIGMA
Dispase II	4942078001	Sigma Aldrich
Fetal Bovine Serum (FBS)	A5256701	Gibco
L-Glutamine	G7513	SIGMA
Penicillin- Streptomycin (Pen/Strept)	P4458	SIGMA
Phosphate Buffered Saline	21.040-CV	Corning
Trypan blue (0.4% solution)	T8154-100ML	Scientific Laboratory Supplies (SLS)

3.1.2 Colony forming units- fibroblasts (CFU-Fs)

Table 6. Materials used for CFU-Fs assay

Material	Product number	Manufacturer
100mm TC dishes	430167	Corning
Loffler's methylene blue solution (4.2g/L)	1.01287.0100	Merck KGaA

3.1.3 Population doubling time (PDT)

Table 7. Materials used for PDT assay

Material	Product number	Manufacturer
Haemocytometer	88.748	Weber England
Tissue culture flask T25 cm2	430693	Corning
Trypan blue (0.4% solution)	T8154-100ML	Scientific Laboratory Supplies (SLS)

3.1.4 Flow cytometry

Table 8. List of the antibody panel used in flow cytometry

Antibody-fluorophore	Clone	Product number	Manufacturer
Mouse Anti-Human CD73-PE	AD2	561014	BD
Mouse Anti-Human CD90-FITC	5E10	561969	BD
Mouse Anti-Human CD105-BV421	266	566265	BD
Mouse Anti-Human CD14-BV510	MPHIP9	563079	BD
Mouse Anti-Human CD19-APC	HIB19	561742	BD
Mouse Anti-Human CD34-BB700	581	745835	BD
Mouse Anti-Human CD45-BV650	HI30	563717	BD
Mouse Anti-Human HLA-DR-BUV395	G46-6	565972	BD

Table 9. Other materials used for flow cytometry

Material	Product number	Manufacturer
Bovine serum albumin	A9418-5G	Sigma Aldrich
Brilliant stain buffer	563794	BD
FACS tubes	352052	FALCON
Fc block	564219	BD
Fixable viability stain	565388	BD
Fixation buffer (Cytofix)	554655	BD
Sodium azide	S2002 -25G	Sigma Aldrich
Vaccum filteration (0.2 μm)	430758	Avantor by VWR

3.1.5 Alkaline phosphatase (ALP) staining

Table 10. Materials used for ALP staining

Material	Product number	Manufacturer
Ethanol, Absolute, Molecular Biology Grade, 500ml	16606002	Fisher Chemical
Fast blue RR salt capsules	FBS25-10 CAP	Sigma-Aldrich
Naphthol AS-MX phosphate alkaline solution	855-20ML	Sigma-Aldrich

3.1.6 Alizarin red staining (ARS)

Table 11. Materials used for ARS

Material	Product #	Manufacturer
96-well plates opaque-walled transparent-bottom	60052182	Perkin Elmer
Alizarin Red Staining and Quantification kit	8678	ScienCell
Cell scraper	541070	Greiner Bio-one (GBO)

3.1.7 Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Table 12. List of Taqman® probes used for RT-qPCR

Gene	Assay ID
ALPL	Hs01029144_m1
BGLAP (OCN)	Hs01587814_g1
Collα-I	Hs00164004_m1
Estrogen receptor alpha	Hs00174860_m1
Estrogen receptor beta	Hs01100359_m1
GPR30	Hs01922715_s1
HPRT1	Hs02800695_m1
IGF-1	Hs01547656_m1
IGF-1R	Hs00609566_m1
IGF-2	Hs04188276_m1
IGF-2R	Hs00974474_m1
IGFBP-1	Hs00236877_m1
IGFBP-2	Hs01040719_m1
IGFBP-3	Hs00181211_m1
IGFBP-4	Hs01057900_m1
IGFBP-5	Hs00181213_m1
IGFBP-6	Hs00181853_m1
IGFBP-7	Hs00266026_m1
MMP1	Hs00899658_m1
MMP13	Hs00942589_m1
MMP2	Hs01548728_m1
MMP9	Hs00957562_m1
PAPP-A	Hs01032307_m1
POSTN	Hs01566750_m1
RUNX2	Hs01047973_m1
STC1	Hs00174970_m1
STC2	Hs01063215_m1
TIMP2	Hs01091319_m1
TIMP3	Hs00927214_m1
TNFRSF11B (OPG)	Hs00900360_m1

TNFSF11 (RANKL)	Hs00243522_m1
TNFSF11a (RANK)	Hs00921372_m1

Table 13. List of materials used for RT-qPCR experiment

Material	Product number	Manufacturer
1X TE buffer	12090015	ThermoFisher
2-Mercaptoethanol	M3148-100ML	Sigma Aldrich
20X GE Sample Loading Reagent	85000735	Standard BioTools (Fluidigm®)
2X Assay Loading Reagent (1.5mL)	85000736	Standard BioTools (Fluidigm®))
2X Taqman Universal Master Mix	4304437	Appied Biosystems
Adhesive PCR Plate sealers	AB0558	ThermoFisher
High capacity RNA to cDNA kit	4387406	Applied Biosystems
IFC chip	BMK-M-48.48	Standard BioTools (Fluidigm®)
IFC syringe	89000020	Standard BioTools (Fluidigm®)
PCR® 96-well plate	000-THER-FA1	ELKay
Pre-Amp master mix	100-5744	Appied Biosystems
RNA free microtubes (size 0.2 mL)	TFI0201	Bio-RAD
RNA free microtubes (size 2 mL)	AM12475	ThermoFischer Scientific
RNase free water	10977-035	Invitrogen
RNase-free Dnase set	79254	Qiagen
RNase zap	AM9780	ThermoFischer Scientific
RNeasy® mini kit (RNA extraction)	74104	Qiagen

3.1.8 Enzyme linked immunosorbent assay (ELISA)

Table 14. Materials used for ELISA

Item	Product number	Manufacturer
IGFBP4 kit	DY804	R&D Systems (Biotechne)
Ancillary kit 3	DY009B	R&D Systems (Biotechne)
PAPPA kit (Human Pappalysin-1)	DY2487-05	R&D Systems (Biotechne)
Ancillary kit 2	DY008B	R&D Systems (Biotechne)
Normal Goat Serum (Reagent Additive 1)	DY005	R&D Systems (Biotechne)
ELISA plate sealers	DY992	R&D Systems (Biotechne)

Table 15. List of equipment used in the different project experiments

Equipment:	Manufacturer	Use
Aspirator	BVC central	General
Biomark	Fluidigm	RT-qPCR
Centrifuge	eppendorf	General
CO_2 Incubator	SANYO MCOZOAIC	General
CytoFlex LX	Beckman Coulter	FC
Fluidigm MX IFC controller	Fluidigm	RT-qPCR
Fume hood	Clean Air Limited	General
Glass slides	Thermo Scientific	General
Haemocytometer	Weber England	General
Laboratory Scale	OHAUS	General
Light Microscope	ZEIZZ (Axiovert 25)	General
Light Microscope	OLYMPUS (CKX41)	General
Scanner	Epson	CFU-F/ ALP/ ARS
Microscope	Zeiss (Axiovert)	General
Microscope (pics)	Zeiss (Axio)	General

Mr Frosty Freezing container	Thermo Fisher	General
NanoDrop 2000	ThermoScientific	RT-qPCR
Pipette controller	HIRSCHMANN/ VWR/ Accumax	General
Pipette sizes P2μl, P10μl, P20μl, P100μl, P200μl, P1000μl	Gilson	General
Plate reader (spectrophotometre)	ThermoScientific (Varioskan Lux)	ARS/ ELISA
ThermoCycler	Techne	RT-qPCR
Thermocycler (Nexus Cycler)	Applied Biosystems	RT-qPCR
Tissue Culture Hood	CAS (Contained Air Solutions)	General
Vortex	MixiMatic Jencons	General
Water bath	Grant	General
Weighing Boat	LevGo	General

3.2 Methods

3.2.1 Sample collection

The isolation of healthy and osteoporotic PDLSCs was approved by the Dental Research Ethics Committee (DREC ref: 040221/AA/317). Refer to (Appendix A) for a copy of the ethical approval.

Inclusion criteria for test and control samples:

The inclusion criteria for the osteoporosis (OP) patients:

1- Postmenopausal women
2- Age: 55-80 years old
3- A confirmed diagnosis of OP using DXA scan
4- Patients are free from diseases and medications that might influence periodontal health or bone metabolism
5- Teeth collected are sound impacted molars with no periodontal disease

The inclusion criteria for the control participants:

1. Healthy i.e. no history of OP, hormonal treatment, osteoporotic medications use or any other medication that affects bone metabolism or periodontal health
2. Age: 55-80 years old

Teeth collected are sound molars with no periodontal disease

Due to the limited availability of samples, the inclusion criteria were modified to include sound/ reasonably sound (detectable signs of enamel demeniralisation)/ carious teeth with no clinical signs of periodontal involvement for the osteoporotic donors, based on the clinical judgement of the clinician. Also, all osteoporotic patients were using OP medications. For the healthy donors, age-matching was not attainable, therefore the samples were collected from permanent molars of healthy donors from outside the proposed age range, which is expected to mask the impact of osteoporosis (i.e not being able to distinguish whether the changes in the results are due to age difference or osteoporosis).

Table 16 and Table 17 present donors' and samples' information both healthy and osteoporotic respectively.

Samples used in this project were collected from three healthy and three osteoporotic donors (biological replicates) who had extracted molar teeth as part

of an elective/ therapeutic treatment plan respectively. The number of the teeth from which PDLSCs were isolated was n=4 for H-PDLSCs (one donor provided 2 samples from 2 different molars) and n=3 for OP-PDLSCs. Periodontal tissue was isolated from molars with healthy periodontal tissues, as assessed by the extracting clinician. For the healthy group, the extracted molars were sound/ reasonably sound while the osteoporotic group provided carious molars.

Healthy Donors:

Donor code	Received as	Age (Years)	Biological gender	Tooth type	Tooth condition
H-PDLSC donor 1	Freshly extracted tooth	32	Female	2nd molar	Reasonably sound
H-PDLSC donor 2	Passaged cells (P4)	18	Male	3rd molar	NA
H-PDLSC donor 3a	Freshly extracted tooth	13	Female	1st molar	Sound
H-PDLSC donor 3b	Freshly extracted tooth	13		1st molar	Reasonably sound

Table 16. Healthy donors information and samples' details.

NA; not applicable, sound; no detectable lesions, reasonably sound; detectable signs of enamel caries

Osteoporotic Donors:

Donor code	Received as	Age	Biological Gender	Tooth type	Tooth condition	Relevant medical History
OP-PDLSC donor 1	Freshly extracted tooth	85	Female	1st molar	Carious	- Smoking History: none - Alcohol use: less than 5 units/ week - History of hysterectomy - A confirmed diagnosis of OP for 3 years - Medications: Alendronic acid, Steroid treatment, Hormone Replacement Therapy (HRT)
OP-PDLSC donor 2	Freshly extracted tooth	77	Female	1st molar	Carious	- Smoking History: Yes - Alcohol use: less than 5 units/ week - A confirmed diagnosis of OP (10 years) - Medications: Denosumab, HRT, Aspirin, Losartan, Calcichew, Atorvastatin, Lansoprazole
OP-PDLSC donor 3	Freshly extracted tooth	52	Female	2nd molar	Restored	- Smoking: Yes (10-20 cigarettes/ day) - Alcohol use: 5-15 units/ week - A confirmed diagnosis of OP (18 months) - Medications: Alendronic acid, propranol

Table 17. Osteoporotic donors' information and samples' details.

NA; not applicable, sound; no detectable lesion, reasonably sound; detectable signs of enamel demineralisation

3.2.2 Isolation of periodontal stromal/stem cells

Once teeth were received from the clinical department, they were washed twice with phosphate buffered saline (PBS), then soaked in PBS supplemented with 10% Penicillin/Streptomycin (P/S) for 20 minutes. PDL tissue was then carefully extracted, within a maximum of 48 hours, using a sterile surgical scalpel (size 11). The tissue was isolated from the mid root region to prevent contamination with gingival junctional epithelium coronally or periapical tissues, blood vessels and nerve supply apically. The harvested tissues were then manually minced using the scalpel and digested using a mixture of 3 mg/mL collagenase type I and 4mg/mL dispase type II both dissolved in PBS and filter-sterilised through 0.2 μm filter unit. The tissue fragments in the enzymatic mixture were incubated at 37°C for 1 hour and gently agitated every 15 minutes. After 1 hour, the digestion was arrested using 3 mL of expansion/ basal media consisting of (α-MEM) supplemented with 20% foetal bovine serum (FBS), 1% P/S and 1% L-glutamine (L-G) and a cell pellet was obtained by centrifugation at 290 xg for 5 minutes. After spinning, the supernatant was discarded, and cell pellet was resuspended in the expansion media (5mL). The cell suspension was passed through a 70 μm cell strainer to generate a single cell suspension, and then cells were cultured in T25 cm^2 tissue culture flasks and incubated at 37°C and 5% CO_2. The flasks were inspected for cell attachment, morphology, growth and presence or absence of infection, and the media was changed after 48 hours then once a week thereafter, until the cells were 80-90% confluent before expanding the culture.

3.2.3 Cell culture and expansion

Once the cells were ready for passaging (~80% confluency to avoid cellular contact inhibition), the media was aspirated, and cells were washed in PBS prior to adding pre-warmed 0.25% trypsin-EDTA to the attached cell monolayer and incubating the cells at 37°C for 5 minutes to allow for cellular detachment. The flask was examined under an inverted microscope to ensure the cells have detached. Next, to neutralise the trypsin action, 10% of the total trypsin volume of the expansion media was added. The cell/trypsin/media mixture was collected into a suitable falcon tube size (depending on the total volume of cell suspension) and centrifuged at 290 xg for 5 minutes. Once the cycle was completed, the

supernatant was aspirated, keeping the formed pellet intact, then the cells were resuspended in expansion media after which they were counted and plated into T75 cm^2 or T175 cm^2 flasks depending on the experiment design (with an average plating density of ~$2x10^6$ and $5x10^6$ cells for T75 cm^2 and T175 cm^2 flasks, respectively). In osteogenic experiments, cell culture was expanded in osteogenic media composed of expansion/basal media supplemented with 50µg/mL L-ascorbic acid and 10nM dexamethasone.

3.2.4 Cell counting

To ensure consistent cell seeding density across experiments, cells were counted at each passage. For this, 30 µl of the cell suspension was withdrawn and mixed with 30 µl of 0.4% trypan blue solution. A total of 10 µl of the mixture was added into the haemocytometer chamber and covered with a glass slide. Using a tally counter, cells were counted in 4 squares of (16-grid squares) in the haemocytometer under the light microscope. Only the cells that looked rounded or polygonal with stained periphery and clear body were counted. The total cell count per 1 ml was calculated using the following equation:

$$\frac{\text{number of counted cells}}{4} \times 2\ (\text{dilution factor}) \times 10^4 = \text{cell number / ml}$$

3.2.5 Cells cryopreservation

To preserve cells for future experiments, cells were stored at -80°C freezers. In order to obtain this, similar steps of the cell expansion were followed but instead of plating the cells into flasks, 10% (v/v) of Dimethyl Sulfoxide (DMSO) was added to the cell suspension, then the suspension was distributed in 1.8 mL cryovials. Cryovials were then labelled and placed in Mr Frosty container to allow for gradual decrease of temperature during freezing (-1°C/ minute) which was then kept at a -80°C freezer for 24 hours before transferring the cryovials into freezing boxes.

For the materials used in PDLSCs isolation, cell culturing, counting and cryopreservation, refer to Table 4, Table 5, and Table 15 in the materials section.

3.2.6 Colony forming units fibroblasts (CFU-Fs) Assay

One of the features that Friedestein and his colleagues identified as MSC precursors from bone marrow explants was their ability to form adherent colonies of cells that resemble fibroblasts known as Colony Forming Units fibroblasts (CFU-Fs) (Gronthos et al., 2003). This technique was used in this project to assess the potential of stromal cells to proliferate and form colonies at a given passage. H-PDLSCs (n=4) and OP-PDLSCs (n=3) were cultured in T75 cm^2 flasks. Once the cells reached 80% confluency, cells were detached, and the total cell count was determined. For plating density optimisation, PDLSCs between passages (P2-P4) were plated on 100mm dishes at the following densities $5x10^3$, $2x10^3$, $1x10^3$, $5x10^2$ cells/ dish in duplicates. Based on the colonies' distribution, morphology, and the percentage of proliferative cells (which was calculated by dividing the number of colonies by the number of plated cells) the optimal density was selected as $5x10^2$ cells/ dish. After 48 hours, the debris was removed by PBS washing and the media was changed. The dishes were incubated for 14 days at 37 °C with media change twice per week. After 14 days in culture, the media was removed, and the dishes were washed twice with PBS. Next, the formed colonies were fixed with 4% formaldehyde solution (3ml) at room temperature (RT) for 20 minutes. After that, the formaldehyde was discarded and 4ml of Löffler's methylene blue solution (4.2g/L) was added to the dishes and incubated for 20-30 minutes to stain the colonies. Once the staining time was complete, the dishes were washed with distilled water to remove any excess stain. The dishes were left to dry overnight before counting the colonies. A colony was defined as a group of cells ranging from 50-300 cells. The colonies were counted manually and visualised under the microscope for confirmation. CFU-Fs dishes were scanned at 800 dpi using a generic Epson scanner. For details on the materials used, refer to Table 6.

3.2.7 Population doubling time (PDT) Assay

When cells are plated, they enter a "lag phase" which is essential for cells to re-build their cytoskeleton and secrete extracellular matrix. The cells then enter a "log phase" where they proliferate exponentially and double in number. This time is known as population doubling time (PDT). The cells later reach a "stationary phase" where their growth ceases (Assanga, 2013) (Figure 6). The PDT

experiment was used to assess the long-term proliferation rate of cells, in the log phase, and to estimate the time needed by the cells to double in number.

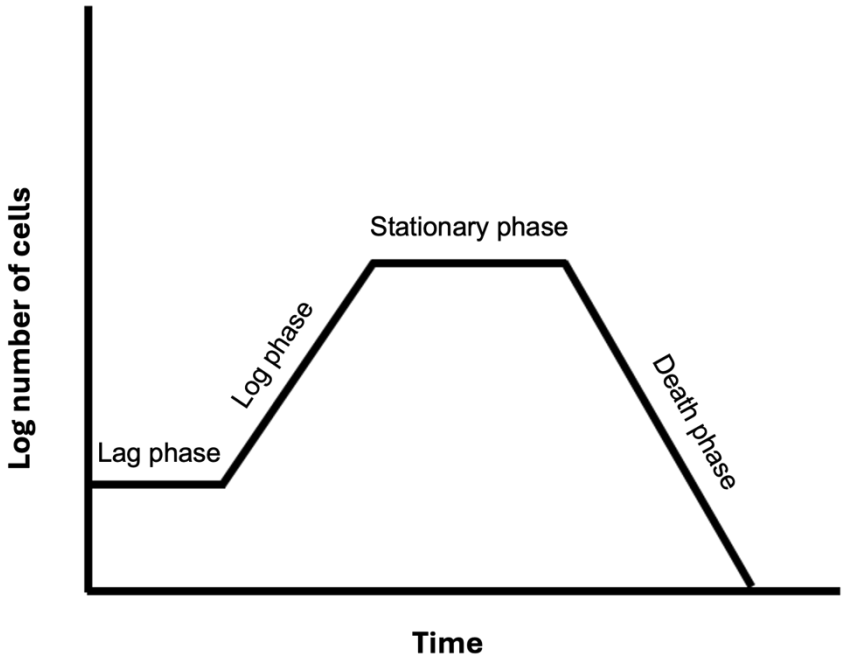


Figure 6. Phases of the cell cycle
The different phases of the cell cycle starting with a lag phase where cells adjust to the new environment and build their cytoskeleton, followed by log (growth) phase, then the stationary (plateau phase) where cells cease growth and terminating with the death phase where cell death where the number of alive cells decreases. Graph adapted from (Kumar, 2016).

In practical terms, doubling time is the average time between successful cell divisions calculated from the cell numbers produced at the trypsinisation step in relation to the number of seeded cells and plotted over time. H-PDLSCs (n=4) and OP-PDLSCs (n=3) were plated at $1x10^5$ cells per T25 cm^2 flask. The cells were cultured in the expansion media starting at the earliest passage available (P2-P4) then for consecutive passages until culture reached senescence or up to 2 months (whichever happens first). For every passage, the flasks were incubated in 37°C incubator with media change once a week. Once the cells reached 80% confluency, they were detached and counted. The cell counts were recorded in an excel sheet (version 16.99) along with the corresponding passage numbers, number of seeded and counted cells as well as dates of seeding and counting

For every passage, the population doubling (PD) and PDT were calculated as follows:

PD= Log (trypsinised cell count/ seeded cell count) ^{2}

PDT= accumulative days of all passages/ accumulative population doubling of all passages for each donor.

Accumulative days between passages were counted by adding the total days required for cells to be 80% confluent for each passage sequentially. Similarly, accumulative population doubling was counted by adding the sum of PD of all passages. Refer to Table 7 for PDT materials specification.

3.2.8 Flow Cytometry

3.2.8.1 Principle

Flow cytometry is an essential technique that allows the assessment of individual cells in a cell population. It can provide information on the expression of stem cell markers with fluorescent light scatter properties (Ullas and Sinclair, 2024). Two of the physical cell features typically assessed using flow cytometry are forward scatter (FSC) and side scatter (SSC). FSC indicates the relative cell size while the SSC indicates the granularity or complexity of the cell (McKinnon, 2018a).

The aim of the flow cytometric analysis reported here was to identify the expression profile of mesenchymal stem cell surface markers for OP-PDLSCs and compare them to the healthy controls (H-PDLSCs).

Cells were labelled using fluorescent conjugated antibodies which contain flourochromes (fluorescent dyes to label the cells) and the analysis was carried out using a flow cytometer. Flow cytometers are mainly composed of fluidics, optics, electronic systems and a computer. Fluidics are made of sheath fluid that direct the liquid-containing sample to a light source. Optics are formed of excitation optics (lasers), which focus the light on cells and excite the flourophores, and collection optics (photomultiplier tubes (PMTs)), which generate the light signals that are later converted by the electronic system into

digital data that can be read by a computer (McKinnon, 2018b) (Adan et al., 2017).

The practical steps of the flow cytometry experiments will be discussed next. Below is an overall figure summarising the experimental process (Figure 7).

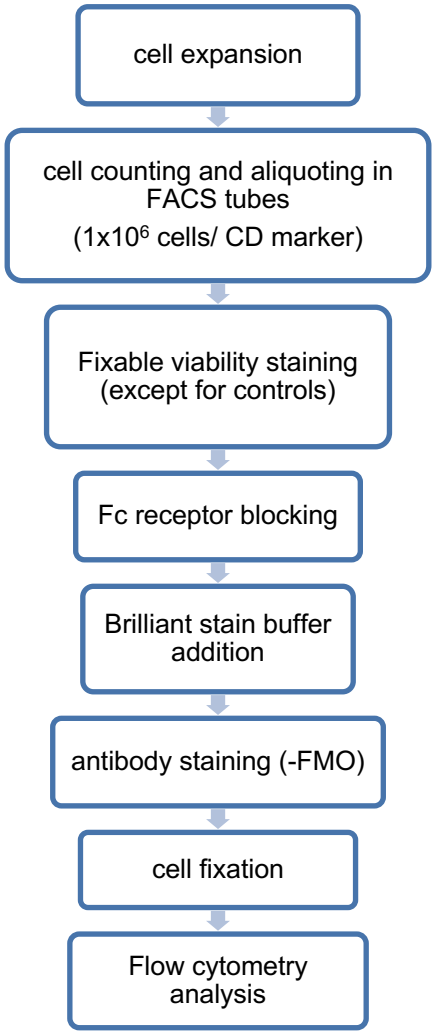


Figure 7. A flow chart of flow cytometry experiment steps.

FACS: Fluorescence activated cell sorting, FMO: Fluorescence minus one

3.2.8.2 Experiment controls

Before starting the flow cytometry experiment, several controls were considered to ensure rigor results including controls for autofluorescence, background

staining and spectral overlap. Autofluorescence was assessed by using an unstained sample to exclude fluorescence inherent to the samples without the addition of any antibodies. Background staining was minimised using a compensation file which was created using single beads (an experiment conducted by a colleague using the same antibody panel (Hussein, 2023)) that were stained with individual antibodies (with different wavelength flurophores) to ensure each “colour” is detected in its designated PMT. Spectral overlap was excluded between emission spectra using mathematical calculations generated by the software. Briefly, the fluorophores used have different emission spectra, some of which have wavelengths that are approximate to each other which could result in signalling at a detector that the fluorophore was not assigned to originally, leading to signalling overlap (known as spectral overlap). Compensation ensures precision and specificity of fluorescence quantification because it subtracts the spectral overlap and only reports the fluorescence generated by the excitation of the main fluorophore (associated with a single anti-CD marker) per detector channel.

When the cell surface expression of multiple CD markers is being assessed in one sample, FMO (fluorescence minus one) controls are recommended to be used (Hulspas et al., 2009a) rather than isotype controls. FMO controls contain all the antibodies used to stain the sample with one antibody being omitted in turn (McKinnon, 2018c). FMOs act as negative controls and are used to set the threshold gates (to determine the marginal error of staining as 2%), the gate will then be applied to the events from the sample stained with the full panel of antibodies to determine the percentage of markers expression (Drescher et al., 2021). On the contrary, isotype controls are not able to account for a spectral overlap or more importantly for the possible artefactual effects due to the interference of multiple types of antibody reagents with one another (Maecker and Trotter, 2006) and are more relevant in experiments when a few antibodies are used where the main interest is to exclude background staining. When using complex panels of antibodies FMO controls are argued to be the most suitable (Hulspas et al., 2009b).

The next sections will discuss the practical steps of the flow cytometry experiment.

3.2.8.3 Sample preparation

Cultures from H-PDLSCs (n=4) and OP-PDLSCs (n=3) were expanded to passage 6 in T175 cm^2 flasks. Once the culture reached 80% confluency, cells were harvested using trypsin, washed twice with PBS using centrifuge set at 290 xg for 5 minutes then resuspended in PBS. Cell suspension was then passed through a cell strainer to ensure its uniformity and to remove any cell clumps that might block the flow cytometer. Cells were then counted and an amount of $1x10^6$ cells/tube were collected to be stained for each of the flow cytometry antibody panel. Information for the used antibody panel is presented in the materials section (Table 8), and all other flow cytometry-specific materials are listed in Table 9.

Cells were stained using fixable viability stain (FVS) reconstituted in cell culture-grade DMSO with 1µl of FVS added to 1ml of cell suspension. The mixture was incubated for 10-15 minutes at RT and protected from light. Cells were then washed twice using FACS buffer (PBS supplemented with 0.5% Bovine Serum Albumin, 0.05% sodium azide). Fc block (5µl) was then diluted in FACS buffer (45µl) (1:50), added to cells to minimise non-specific binding, and incubated for 10 minutes at RT. After the incubation, brilliant stain buffer (BSB) was added (50 µl/ antibody tube) at 4°C in the dark to prevent cross-reactivity of the dyes. This was required for Briliant Ultra Violet (BUV), Brilliant Violent (BV) and Brilliant Blue (BB) dyes. Figure 8 indicates the practical steps followed before staining. Cells were then stained with antibodies using volumes and dilutions recommended by manufacturer and have been titrated (Hussein et al., 2024) excluding the marker of interest in the FMO tube (Table 18), all sample tubes were incubated on ice in the dark for 45 minutes. Cells were later washed twice with FACS buffer (1ml/ tube) and centrifuged at 290 xg for 5 minutes set at 4°C. The same centrifuge setting was followed for the washes to follow.

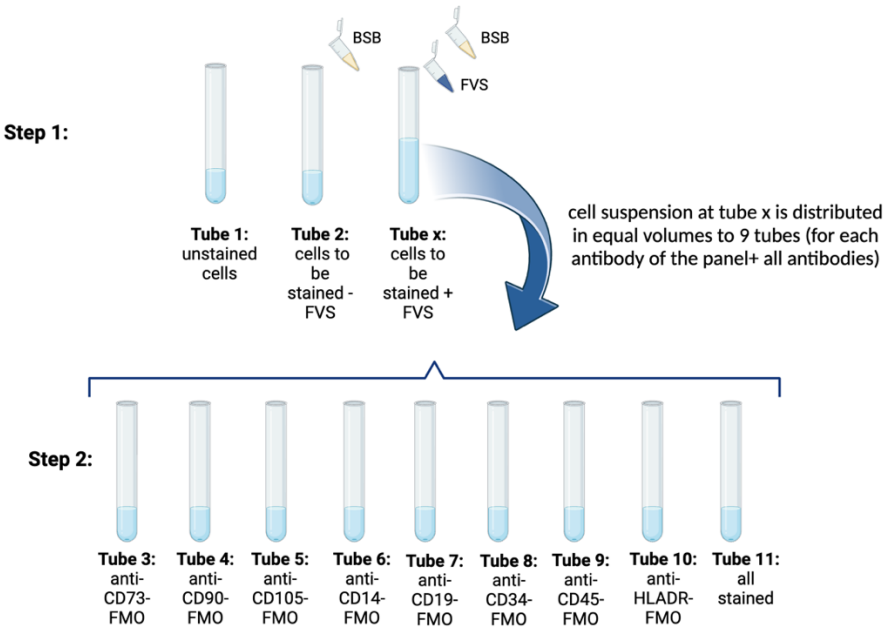


Figure 8. An illustration of the stepwise process used for flow cytometry sample preparation.

BSB; brilliant stain buffer, FVS; fixable viability stain, FMO; fluorescence minus one. Graph was created using BioRender.com

Cells were then fixed using Cytofix™ to preserve the staining using 250 µl of fixation buffer per sample tube. Tubes were incubated at 4°C in the dark for 15 minutes. After incubation, 1ml of FACS buffer was used to wash the cells. Cells were later resuspended at 500 µl of FACS buffer per tube. Tubes were stored in the dark at 4°C until analysis within 5 days. Analysis was carried out within a maximum of 2 days of fixation to avoid getting weaker fluorescing signals.

3.2.8.4 Data analysis

For flow cytometry analysis, data were collected on Cytoflex LS flow cytometer with 40,000 events collected per sample file and analysed using CytExpert software (version 2.4.0.28). Detailed data analysis will be discussed in Chapter 4, results section 4.2.3.

Tube #	**Volume/ tube**	**Cells**	**FVS ↓**	**Anti-CD73 ↓**	**Anti-CD90 ↓**	**Anti-CD105 ↓**	**Anti-CD14 ↓**	**Anti-CD19 ↓**	**Anti-CD34 ↓**	**Anti-CD45 ↓**	**Anti-HLADR ↓**
1		Unstained	X	X	X	X	X	X	X	X	X
2	0.5 µl	FVS- FMO	X	CD73	CD90	CD105	CD14	CD19	CD34	CD45	HLADR
3	10 µl	CD73- FMO	FVS	X	CD90	CD105	CD14	CD19	CD34	CD45	HLADR
4	0.5 µl	CD90- FMO	FVS	CD73	X	CD105	CD14	CD19	CD34	CD45	HLADR
5	5 µl	CD105- FMO	FVS	CD73	CD90	X	CD14	CD19	CD34	CD45	HLADR
6	5 µl	CD14- FMO	FVS	CD73	CD90	CD105	X	CD19	CD34	CD45	HLADR
7	20 µl	CD19- FMO	FVS	CD73	CD90	CD105	CD14	X	CD34	CD45	HLADR
8	0.5 µl	CD34- FMO	FVS	CD73	CD90	CD105	CD14	CD19	X	CD45	HLADR
9	2.5 µl	CD45- FMO	FVS	CD73	CD90	CD105	CD14	CD19	CD34	X	HLADR
10	5 µl	HLA-DR	FVS	CD73	CD90	CD105	CD14	CD19	CD34	CD45	X
11		All stained	FVS	CD73	CD90	CD105	CD14	CD19	CD34	CD45	HLADR

Table 18. flow cytometry antibody panel in the staining sequence with the volumes of each staining reagent used.

3.2.9 Alkaline phosphatase staining (ALP)

Alkaline Phosphatase (ALP) staining is commonly used to detect the early and mid-differentiation steps of stem cells into osteoblasts (Trivedi et al., 2020). This technique relies on the presence of a substrate (naphthol) to detect the ALP activity within the sample (Ruan et al., 2006). H-PDLSCs (n=4x3 technical replicates) and OP-PDLSCs (n=3x 3 technical replicates) were plated on 6-well plates at a cell density of $2x10^5$ cells/well. Half of each plate (3 wells) contained cells that were cultured in basal media while in the other 3 wells, cells were treated with osteogenic media.

The experiment was carried out at 3 time points: 2, 3 and 4 weeks. Cell culture was stopped at each time point, washed twice with PBS then fixed using 95% ethanol for 30 minutes. When staining was planned later, plates were stored at 4-8°C with regular replenishing of the 95% ethanol. After fixation, cells were washed twice with deionised water before being stained. Cells were then stained using 3mL of the Fast Blue dye mixture. The dye was prepared by dissolving 1 Fast blue RR salt capsule in 48ml of deionised water at 37°C while protected from light, and once the capsule was dissolved, 2ml of Naphthol AS-MX Phosphate (ALP substrate) was added. Samples were incubated in the dark at RT for 30 minutes then washed twice with deionised water. The plates were scanned using a generic Epson scanner at 800 dpi. Microscopic images were captured using Infinity Analyser Software (release 6.5). Table 10 provides information on the materials used and Figure 9 shows the experiment design.

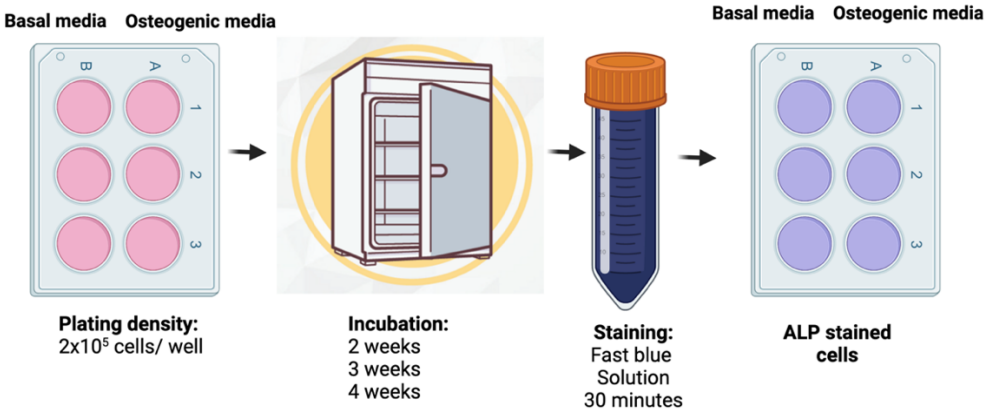


Figure 9. ALP staining experiment design.

Figure created using BioRender.com

3.2.10 Alizarin Red Staining (ARS)

Alizarin Red Staining (ARS) has typically been used detect the deposition of calcium by cultured cells (Gregory et al., 2004). The stain contains an anthraquinone derivative which can react with calcium cations forming a visible complex that is red/ orange in colour which can be later extracted and quantified using a colorimetric assay (Bernar et al., 2022).

3.2.10.1 Staining

These experiments aimed at detecting the mineralisation deposition as an indication of mature osteoblastic differentiation of the OP-PDLSCs compared to the healthy controls. H-PDLSCs (n=4x 3 technical replicates) and OP-PDLSCs (n=3x 3 technical replicates) were plated on 6-well plates with a density of $2x10^5$ cells/well. The experiment was carried out with the same design and incubation periods as ALP staining. Upon the completion of each time point, cell cultures were ceased. This assay was carried out according to manufacturer's instructions (ScienCell™). Briefly the cells were washed three times with PBS then fixed using 4% formaldehyde for 45 minutes at RT with gentle shaking. The fixative was then removed, and the cells were washed three times with deionised water. Cells were then stained with 1 mL of 40 mM of ARS per well, then incubated in the dark at RT with gentle shaking for 20-30 minutes. Then the dye was removed, and cells were washed with deionised water 5 times. The plates were tilted for 2 minutes to remove excess water and stored at -20°C before quantification.

3.2.10.2 Quantification assay

Quantification was carried out as per manufacturer's instructions. To extract the stain, samples in 6-well culture plates were incubated with 10% acetic acid (800 µl/ well) for 20 minutes at RT with gentle shaking. Then, cells were detached from the plate using a cell scraper then collected into 1.5mL microtubes, vortexed for 30 seconds, sealed with parafilm, to avoid evaporation, and heated at 85°C in a water bath for 10 minutes. Samples were then incubated on ice for 5 minutes before centrifugation at 20,000 xg for 15 minutes. Five hundred microliters (500 µl) of the supernatant from each microtube was transferred into new tubes and 200 µl of 10% ammonium hydroxide was added to the samples to neutralise the

acidic solution. One hundred and fifty microliters (150µl) of the samples were then aliquoted in triplicate into a 96-well plate (opaque-walled, transparent-bottom Perkin Elmer). ARS standards prepared using 4mM ARS stain, diluted with standard dilution solution at 2,1,0.5, 0.25, 0.125, 0.0635, 0.0313 mM and aliquoted in the plate in triplicates. The absorbance at 405nm was measured using SkanIt software (version 6.1.1) on the plate reader (ThermoScientific (Varioskan Lux)). Table 11 indicates specifications of the materials used and Figure 10 summarises the ARS experiment and minerals quantification steps.

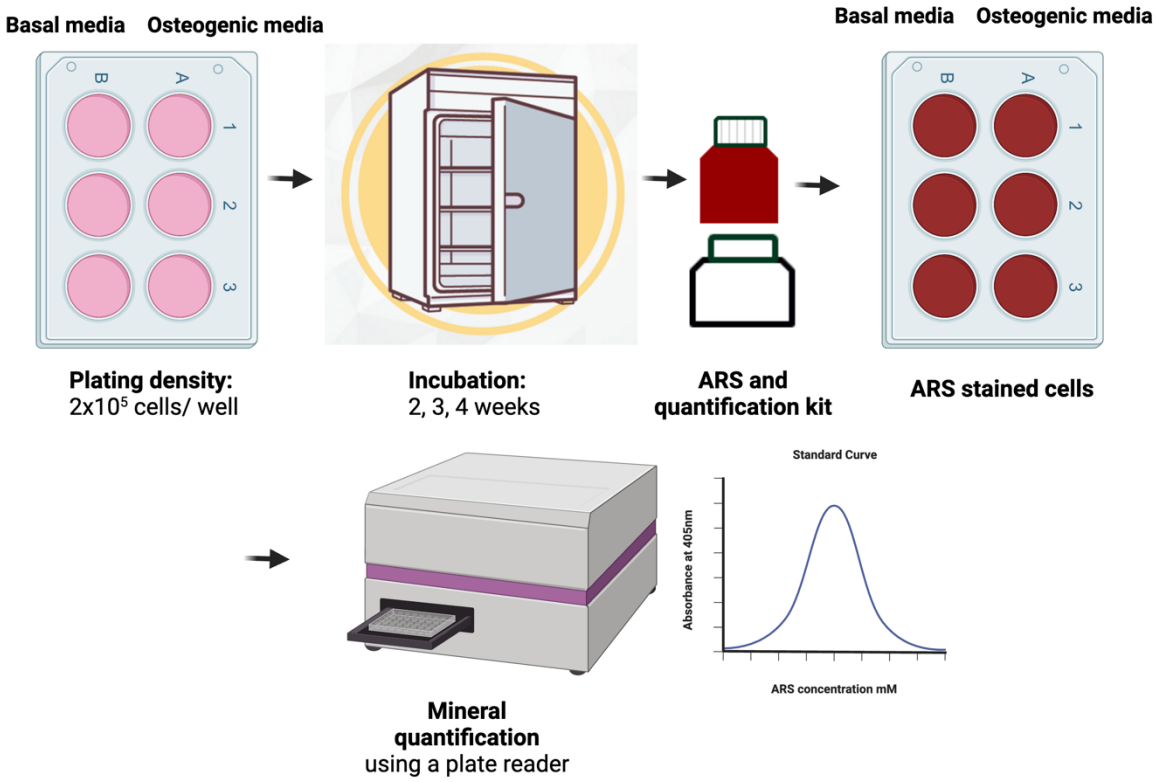


Figure 10. Alizarin red staining and mineral quantification assay steps.
Figure created using BioRender.com

3.2.11 Gene expression assays using reverse transcription quantitative polymerase chain reaction

The study of gene expression using Polymerase Chain Reaction (PCR) is essential to understand the molecular mechanisms for health and disease. In the context of this project, Reverse Transcription quantitative PCR (RT-qPCR) was used as a tool to characterise OP-PDLSCs and their osteogenic differentiation potential at sequential time points in comparison to healthy controls. Reverse transcription is the process by which RNA molecules are converted into complementary DNA (cDNA) using a reverse transcriptase enzyme to be ultimately amplified by PCR (Mo et al., 2012). RT-qPCR was used in this project to study the expression of osteogenic genes, bone remodelling genes, estrogen receptors, and IGF axis members.

3.2.11.1 Principle

PCR is an enzymatic reaction that allows the amplification of small specifically selected DNA fragments from a pool of DNA. These short DNA sequences are primers attached to specific gene sequences and are amplified to reach a detectable level. The reaction requires nucleotides, primers, templates (the sample) and DNA polymerase. A minute amount of sample is usually required to be amplified using PCR, making it a sensitive technique (Garibyan and Avashia, 2013).

PCR uses repeated rounds of the same sequential reaction steps:

- Denaturation stage at high temperatures (90-95°C) to separate the double stranded DNA template into single strands (Zhang et al., 2019).
- The annealing phase at (52-58°C) permits the primers (initiators of sequence extension) to attach to complementary strands of the DNA template (Zhang et al., 2019).
- The extension stage (68-72°C) at these temperatures DNA polymerase extends the newly formed strand in a 5’ to 3’ direction using complimentary base pairs from the reaction solution to the ones in the template (Wages Jr, 2005).

Generally, the PCR reaction generates DNA copies (amplicons) from a template exponentially. In the exponential phase, the reagents are plenty and products are exactly doubled at each cycle. This is followed by the linear phase where the reaction continues but at a slower rate since the reagents have been utilised (Biosystems, 2010). Lastly is the plateau phase where the reaction ceases either due to inhibitors within the template, limited reagents or the accumulation of pyrophosphate molecules (Arya et al., 2005).

The data generated by the PCR reaction is quantifiable owing to the use of fluorescently labelled probes (Taqman® probes for this project). These probes are dual-labelled oligonucleotides fluorophores containing a reporter at the 5' end of the probe and a quencher at the 3' end. With the action of the 5' Taq DNA polymerase, the bond between the quencher and the reporter is broken and the signal fluorescing by the reporter is then measured allowing the reaction to be quantified (Tajadini et al., 2014). For the list of probes used in this project, refer to Table 12.

In this project, Integrated Fluid Circuits (IFC) chips were used to study the gene expression of the samples.

3.2.11.2 Integrated Fluid Circuits (IFC) chips

IFC chips were designed by Fluidigm® (currently known as Standard Biotools™) as nanofluidic biochips that enhance PCR technology (Ramakrishnan et al., 2013b). Among the advantages of this chip is its ability to analyse multiple genes from multiple samples at once (Tozaki et al., 2020).

IFC chips are available in different sizes, the one used in this project is the dynamic array 48.48 chip which has 48 inlets for the samples and 48 inlets for assays rendering it the capacity to produce 2304 reactions (Wang et al., 2009). On either side of the chip, there are 2 check valves through which the control fluid line is injected. The central square utilises the microfluidics technology and contains fluid lines, NanoFlex™ valves and chambers. The NanofFlex™ valves are made of elastomeric materials and controls the fluid flow under high pressure as they deflect during the PCR reaction creating a tight seal (Ramakrishnan et al., 2013a). The mixture of samples, assays and reagents inside the chambers is controlled via microscopic gates eliminating pipetting errors and the possibility of contamination (BioTools, 2024). (Figure 11)

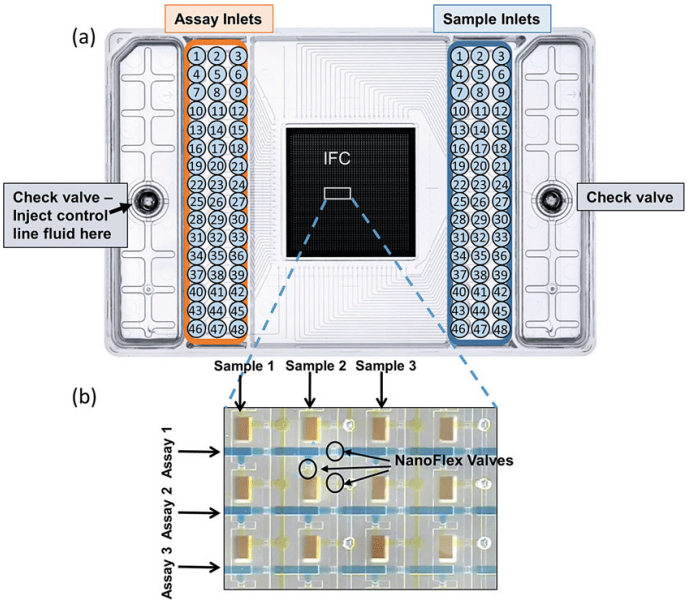


Figure 11. IFC chip.

a) components of the chip including 48 assay inlets, 48 sample inlets and 2 check valves. b) magnification of a section of the microfluidic chambers at which the samples, assay and reagents are mixed and where the control of the fluid flow is governed by the nanoflex valve. Figure source (Larsen et al., 2020).

3.2.11.3 Method:

For RT-qPCR materials specifications, refer to Table 13. The following flowchart (Figure 12) summarises the RT-qPCR experimental protocol.

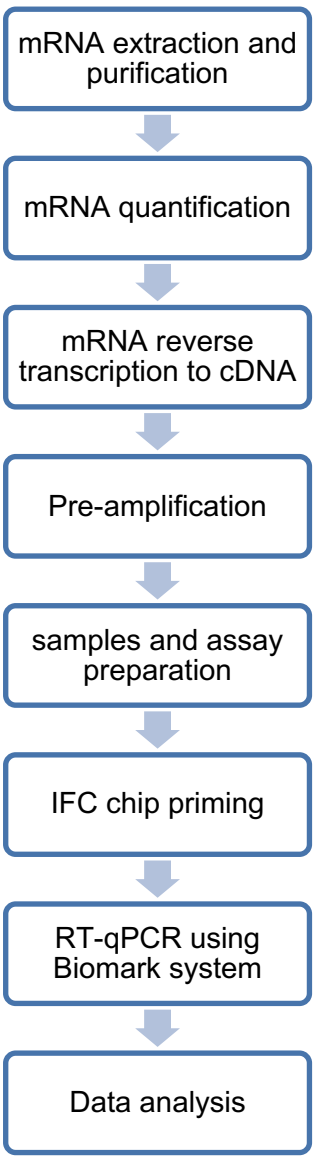


Figure 12: Summary of RT-qPCR steps.

3.2.11.3.1 mRNA extraction

The aim of this step was to extract and purify mRNA from cells to assess the expression of genes of interest. This was carried out using RNeasy® mini kit (Qiagen) according to the manufacturer's instructions. This kit utilises binding properties of silica-based membrane in the spin-column as well as the spinning speed to extract mRNA. To avoid RNase contamination, the workstation was first cleaned with RNaseZap™ (ThermoFoscher Scientific) and nuclease-free water and microtubes used for all steps to reduce RNase contamination. Cells were harvested using trypsin as previously described (3.2.3), and were washed with twice with

PBS at 290 xg for 5 minutes. Following removal of the supernatant, the pellet was transferred into 1.5mL microtube.

A mixture of 1mL RLT buffer and 10μl β-mercaptoethanol was prepared of which 350μl was added to the cell pellet. An equal volume (350μl) of 70% molecular grade ethanol (diluted with nuclease-free water) was added and the lysate was mixed well by pipetting. Seven hundred microliters (700μl) of the mixture was transferred to an RNeasy® Mini spin column placed in a 2mL collection tube and centrifuged at 8000 xg for 15 seconds and the flow-through was discarded.

For efficient removal of genomic DNA, RNase-free DNase set (Qiagen) was used. A volume of 350μl of buffer RW1 was added to the spin column, centrifuged for 15 seconds at 8000 xg and the flow-through discarded. Next, the DNase I incubation mix was prepared by mixing 10μl of DNase I stock solution with 70μl of buffer RDD. This was added to the spin column and incubated at RT for 15 minutes.

After that, buffer RW1 (350μl) was added to the spin column, centrifuged for 15 seconds at 8000 xg and the flow-through was discarded.

3.2.11.3.2 mRNA purification

Using the same centrifugation settings, 500μl of buffer RPE was added and spun twice for 15 seconds then 2 minutes respectively, and the flow-through was discarded. To dry the membrane, the spin column was centrifuged at full speed for 1 minute. Thirty microliters (30μl) of nuclease-free water was added to the column and the purified mRNA was eluted into a new 1.5mL microtube by centrifugation for 1 minute at 8000 xg. The mRNA was placed on ice to ensure optimal stability before measuring the concentration and purity of mRNA.

3.2.11.3.3 mRNA quantification:

A 2μl aliquot of each of the eluted mRNA samples was used to assess concentration (ng/mL) using a Nanodrop 2000 spectrophotometer. The purity was assessed using the 260/280 ratio of absorbance, with an optimal range considered to be between 1.8- 2.0.

In cases where samples' 260/280 ratio was not optimal, the kit's manufacturer recommended following the same protocol while ensuring that RLT buffer is warmed to 37°C for 5-7 minutes to remove any precipitation of salts. The buffer is then allowed to cool to RT for 10 minutes. Buffer RLT mixture with β-mercaptoethanol was to be added to cells then warmed up for 1 minute to allow β-mercaptoethanol action in breaking down the RNase. It was also recommended at the end of the process to add 1 or 2 additional buffer RPE washes then adding 500µl of absolute molecular grade ethanol, centrifugation for 15 seconds at 8000xgthen allowing the ethanol to evaporate before proceeding with the mRNA extraction.

3.2.11.3.4 Reverse transcription to complementary DNA (cDNA)

After the extraction of mRNA, samples were converted into cDNA, which provides a DNA template for the amplification and is more stable for further analysis of the samples using q-PCR. This is known as reverse transcription and the reaction was performed using High-Capacity RNA-to-cDNA™ kit (Applied Biosystems) as per the protocol supplied. The kit was thawed on ice, after which the reverse transcription (RT) mix was prepared on ice as follows: a total of 20µl was prepared by adding 10µl of the 2X RT buffer mix, 1µl of the 20X RT enzyme, up-to 2 µg of the purified mRNA (a maximum of 9 µl of a sample using nuclease free water) is added to the reaction mix. The RT mix was then placed in a thermocycler for 60 minutes at 37°C, for 5 minutes at 95°C and finally the reaction was held at 4°C. The cDNA samples were stored at -20°C until further analysis through RT-qPCR.

3.2.11.3.5 Pre-amplification

To increase the cDNA quantity to be analysed, pre-amplification (PA) was carried out. Before proceeding with PA, experiment controls were prepared. No Template Control (NTC) served to detect contamination with nucleic acids and was prepared by adding 10µl of 2X RT buffer mix, 1µl of 20X RT enzyme mix, and 9µl of nuclease-free water. In addition, No Reverse Transcriptase (NRT) control was used to detect any contamination of mRNA samples with DNA. NRT was prepared by adding 10µl of 2X RT buffer mix, an mRNA sample, and nuclease-free water (the amounts of mRNA sample and water used were according to the manufacturer's instructions discussed in the reverse transcription section 3.2.11.3.4).

For PA, first, the pooled Taqman® assay mix (mix A) was prepared by adding 2µl of each Taqman® probe (Table 12) with 104µl of 1xTE buffer (to make up a total volume of 200 µl). After that, mix B was prepared by adding 53µl of PreAmp master mix (1µl/well), and 79.5µl of nuclease-free water (1.5µl/ well) with 66.25µl of mix A (1.25µl/ well). A longitudinal half of a 96-well PCR plate (48 wells) on a plate holder was used to prepare the samples for PA. In each well, 3.75µl of mix B was aliquoted along with 1.25µl of each cDNA sample and controls (a total volume of 5µl / well). The plate was properly sealed with an adhesive plate sealer to avoid evaporation of the samples under high temperatures. The plate was then inserted in a thermocycler (Nexus cycler) and Fluidigm-PA protocol was applied. The protocol included initial denaturation of the plate samples at 95°C for 2 minutes, followed by a cycle of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 4 minutes. The cycle was repeated 14 times. The plate was cooled down and held at 4°C. Once the samples were ready, 20µl of 1xTE buffer was added to each well to dilute the cDNA. The plate was stored at -20°C for RT-qPCR analysis later.

3.2.11.3.6 RT-qPCR

After amplifying the cDNA samples, they are now ready for processing of the genetic sequence of the genes of interest and house-keeping gene using IFC chips.

A. Samples and assays preparation:

To prepare the samples and the assays, 2x 96-well PCR plates were used: one for the assays and one for the samples. In the assay-plate, 3µl of 2x assay loading reagent was added per well along with 3µl of each Taqman® probe. In the samples-plate, 3µl of 2x Taqman® universal master mix , 0.3µl of 20x GE sample loading reagent and 2.7µl of the pre-amplified diluted cDNA and controls were added in each well. The contents of each well were well-mixed by pipetting.

B. Chip priming and mixing:

Fluidgim® 48.48 dynamic array IFC chip (Fluidgim®) was used. Two Fluidigm® syringes (0.3ml of control line fluid/ syringe) were injected at either side of the chip valves in order to activate it. The chip was then inserted into a Fluidigm® MX loader to prime the chip for 12 minutes. Then, 5µl were added from the prepared samples and assay plates in their designated inlets on the chip. The aliquoting was carried out within 60 minutes of chip priming. The chip was then inserted again into the MX loader to mix the samples, assays and reagents for 60 minutes.

C. RTq-PCR using Biomark

After mixing the reagents and samples in the chip, it was inserted into the Biomark™ HD (Fluidigm®) system for gene expression analysis and data collection. The protocol followed was GE 48.48 Standard v1 which ran 40 PCR cycles for approximately 60 minutes. Refer to (Table 13) and (Table 15) for specifications on the materials and equipment used.

3.2.11.3.7 Data analysis:

Data generated by Biomark™ HD system were analysed using Fluidigm® Real-Time PCR Software (version 4.7.1) which provided information on Ct

(Cycle Threshold) of each gene. Data that were flagged by the software were individually assessed for the level of expression in relation to the threshold and the Ct curve presentation (the presence of the exponential, linear and the plateau phases of PCR). Data that didn't match the optimal expression of a PCR curve were excluded.

Next, using Microsoft Excel (version 16.99), ∆Ct values were calculated by normalising Ct values of each gene of interest (GoI) to the CT values of HPRT1, the Housekeeping gene (HKG), using the following equation:

$$\Delta Ct = Ct\ (GoI) - Ct\ \ (HKG)$$

Then the relative expression was calculated using $2^{-\Delta Ct}$ equation. Data were later exported to GraphPad Prism (version 10.2.3) where graphs were plotted comparing the relative change in gene expression between the osteoporotic and healthy groups at different time points (2, 3, 4 weeks) and culture conditions (basal and osteogenic) separately. Data were lastly assessed for statistical difference.

3.2.12 Enzyme Linked Immunosorbent Assay (ELISA)

3.2.12.1 Principle:

Enzyme Linked Immunosorbent Assay (ELISA) is an immunological technique that is commonly used in research and clinical settings. It is based on the interaction between an antigen (target protein in the sample) and a monoclonal antibody, selected to specifically recognise it. The amount of antigen secreted can be quantified via a colorimetric reaction between an added substrate and the enzyme linked to the antibody (Hayrapetyan et al., 2023). In this project the most common ELISA technique, “sandwich ELISA”, was used. It is a technique where a small volume of the sample (which contains the antigen) is added to an antibody that is bound to a solid phase (e.g. well-plate base) to allow for the antigen to capture the antibody. This step is followed by washing then adding an enzyme linked antibody forming a complex where the antigen is sandwiched between the two antibodies used (Alhajj M, 2023). A substrate is later added to the enzyme to reveal its activity generating a colour. The presence of the colour indicates the presence of the protein being investigated and vice versa (Aydin, 2015). The quantification of the colour produced is carried out using a plate reader and comparison against concentration standards (Sakamoto et al., 2018).

3.2.12.2 Method:

Cells from H-PDLSCs (n=4 x 3 technical replicates) and OP-PDLSCs (n=3x 3 technical replicates) were cultured under basal and osteogenic conditions in 6-well plates for 2, 3 and 4 weeks at P6. Conditioned medium (3mL) was collected at each time and stored as 250 µL aliquots. The level of both IGFBP-4 protein and its protease (PAPP-A) were analysed. IGFBP-4 and PAPP-A DuoSets with their compatible ancillary kits (R&D Systems (Biotechne)) were used according to manufacturer’s instructions. Refer to Table 14 for kits details.

The protocol was carried out over 2 days. On the first day, the supplied 96-well plates were coated with mouse anti-human capture antibodies (100 µL/well) diluted with the coating buffer at (1:180) for IGFBP-4, (1:120) for PAPP-A, and incubated overnight at RT. On the following day, the excess capture antibody was removed using 1X wash buffer 3 times (300 µL/well). To prevent non-specific

binding to the antibodies, the plate was blocked using reagent diluent diluted with 1X PBS (1:5) for IGFBP-4, and (1:10) for PAPP-A (300 µL/well) for a minimum of 1 hour at RT, after which the plate was washed using 1X wash buffer 3 times. After preparing the plate, the serially diluted standards (1:2), ranging from 32ng/mL to 0.5ng/mL for IGFBP-4 and from 50ng/mL to 0.781ng/mL for PAPP-A, and the samples were added. In the optimisation experiment, the following dilutions were used: undiluted, 1:2, 1:10, 1:20, 1:50. After optimisation and based on the range of the concentration's readings in comparison to the standards, 1:50 dilution of samples was used for IGFBP-4 experiments while undiluted samples were used for PAPP-A. Standards and samples were diluted using a reagent diluent and added as 100 µL/well then incubated for 2 hours at RT. Plates were then washed using 1X wash buffer 3 times (300 µL/well) to remove unbound antigens. Biotinylated goat anti-human IGFBP-4 detection antibody was diluted with 2% heat inactivated normal goat serum to (100ng/mL) while biotinylated goat anti-human PAPP-A detection antibody was diluted with 1x reagent diluent to 200ng/mL. Diluted detection antibody was then added (100 µL/well) and the plate was covered with an adhesive strip and incubated for 2 hours at RT after which the excess detection antibody was removed by washes with 1x wash buffer 3 times (300 µL/well). Diluted streptavidin-HRP (Horseradish Peroxidase), (1:200) for IGFBP-4 and a (1:40) dilution for PAPP-A, was then added and the plate was covered and incubated in the dark for 20 minutes at RT, after which the plate was washed 3 times with 1x wash buffer. After that, the substrate solution was added (100 µL/well) and as soon as the gradient difference in the colour between standards appeared (an average of 5 minutes for IGFBP-4, and 9 minutes for PAPP-A), the reaction was stopped using a stop solution (50 µL/well) with gentle tapping of the plate to ensure a thorough mixing.

The optical density of the standards and the samples was measured at 450nm and at 540nm using a Varioskan Lux plate reader (ThermoFisher Scientific) and analysed with SkanIt RE software (version 6.1.1). The values obtained from readings at 540 nm were subtracted from values at 450 nm to correct for optical imperfections in the plate. The duplicated readings of standards and samples were averaged then the blank value was subtracted from all samples' values. An online tool called GainData® (arigos' ELISA Calculator) was used to generate a 4PL curve and to calculate the concentration of the samples.

https://www.arigobio.com/elisa-analysis

Figure 13 shows a summary of ELISA steps with a visual analysis of the ELISA sandwich technique.

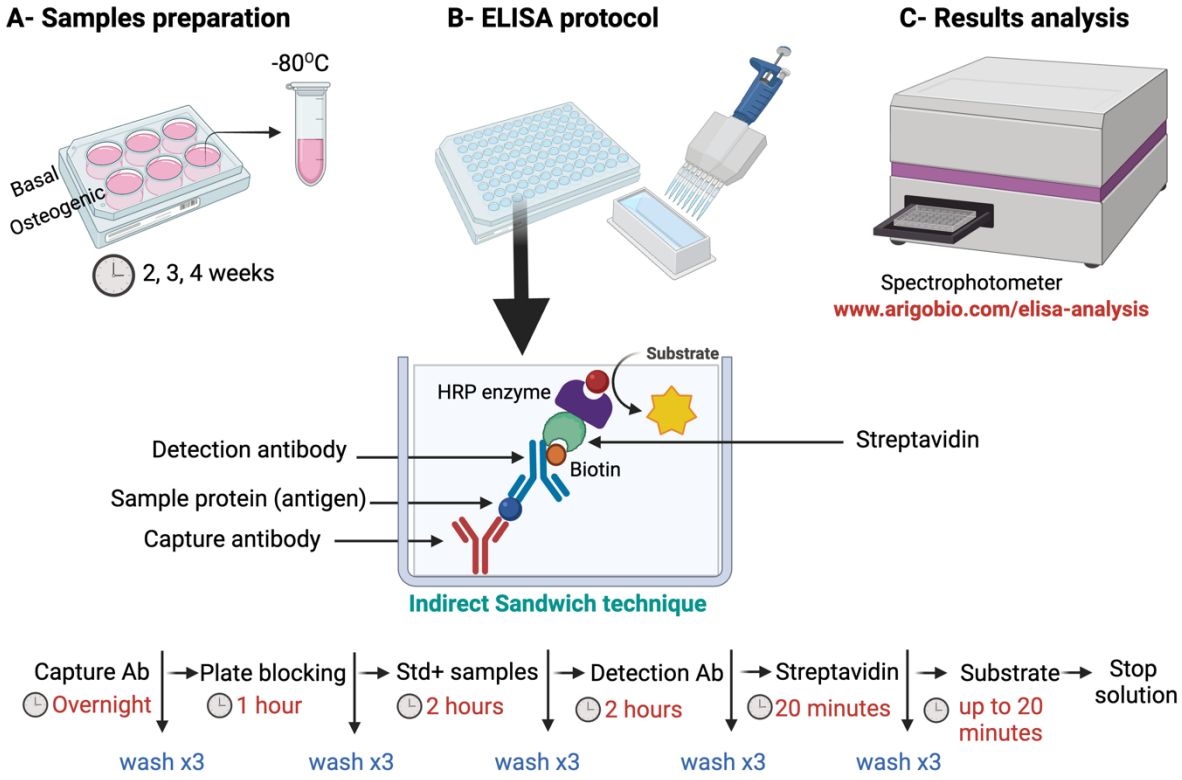


Figure 13. A summary of ELISA steps

Illustration for the sandwich ELISA technique used to quantify levels of secreted IGFBP-4 and PAPP-A in conditioned tissue culture media. Figure created using BioRender.com

Below is a schematic summary of the methods used for this project classified according to the main objective they were designed to study (Figure 14).

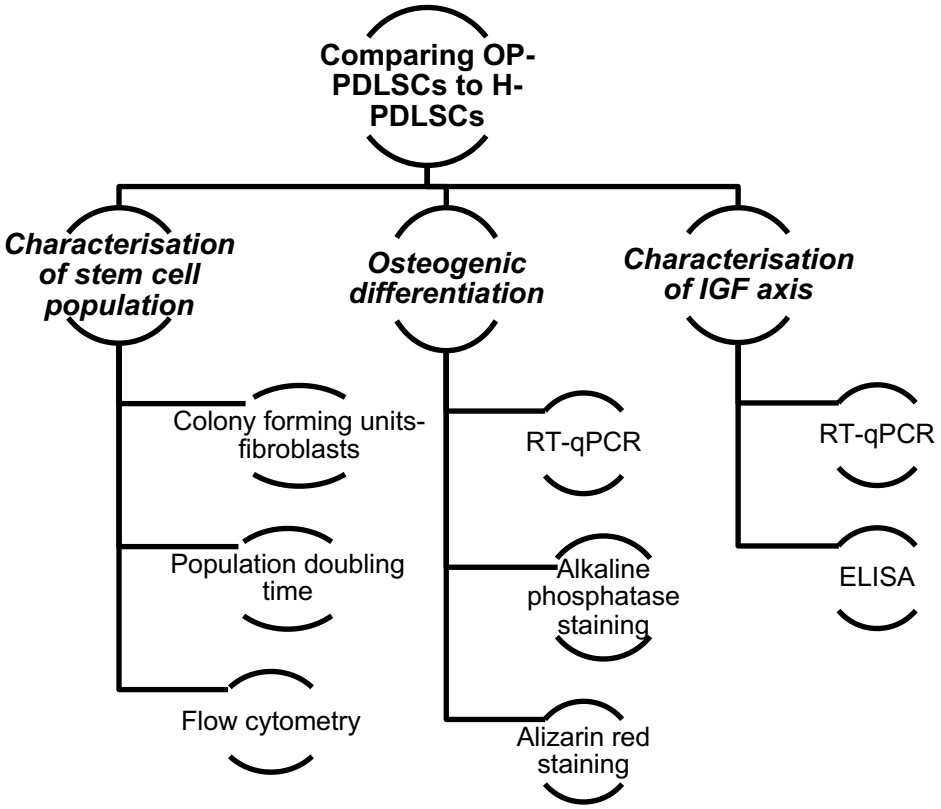


Figure 14. a summary of the main methods used for this project

3.2.12.3 Statistical Analysis:

GraphPad Prism (version 9.5.1) was used to analyse the results. Normality was first assessed using Shapiro-Wilk test. Normally distributed data were compared using unpaired t-test while nonparametric distribution was assessed using Mann-Whitney test. For CFU-F, PDT and flow cytometry, the means of each group (healthy vs osteoporotic) were compared. In experiments where samples were treated with 2 different culture conditions (i.e basal and osteogenic) at 3 time points (2, 3 and 4 weeks), namely ARS, RT-qPCR and ELISA, the comparison was carried out between the groups for each time point and culture condition separately to assess the potential impact of OP (alone as one variable) using the same statistical analysis methods. All data were presented as means and standard deviations. Statistical significance was identified when P values were less than 0.05.

Chapter 4 Characterisation of Periodontal Ligament Stem Cells Isolated from Osteoporotic Patients

4.1 Introduction:

Stem cell characterisation allows the understanding of cellular behaviour and potentially provides an insight into the molecular features shaping the stem cell therapy. In the context of this project, periodontal and bone regeneration is more relevant in older population since the prevalence of periodontal disease increases with age (Tomokiyo et al., 2018). As discussed in (Chapter 1), studies that characterised periodontal ligament stem (purified)/stromal (heterogenous) cells isolated from osteoporotic humans (OP-PDLSCs) are lacking, and hence this will be the focus here. This chapter aims at comparing the colony formation ability, proliferative capacity and expression of MSC surface marker antigens in OP-PDLSCs versus healthy PDLSCs (H-PDLSCs). The colony formation and self-renewal capacity was confirmed using CFU-Fs assay (Gronthos et al., 2003, Taylor and Clegg, 2011). Population doubling time (PDT) assay was used to assess the long-term proliferation of OP-PDLSCs (Greenwood et al., 2004). Moreover, the expression of surface markers antigens was assessed using flow cytometry (Ullas and Sinclair, 2024). Clinically, it is essential to understand the self-renewal and growth capacity of OP-PDLSCs since this helps understand their potential to regenerate the lost tissue (Poliwoda et al., 2022). Additionally, understanding the stemness of OP-PDLSCs in relation to other MSCs will be vital in designing customised stem cell based oral and dental therapies for osteoporotic patients.

4.2 Results:

4.2.1 Colony formation:

H-PDLSCs (n=4) and OP-PDLSCs (n=3) passages (P2-P4) were plated in culture dishes at a density of $5x10^2$ cells/dish and were allowed to grow under basal conditions for 14 days after which colonies were stained with

methylene blue dye and counted (detailed method in Chapter 3, section 3.2.6). The average number of CFU-Fs for H-PDLSCs was 75 ± 18 colonies compared to 44 ± 22 in the OP-PDLSCs group. Despite the lower numbers in OP-PDLSCs, there was no statistically significant difference between the two groups (Figure 15) using unpaired t-test.

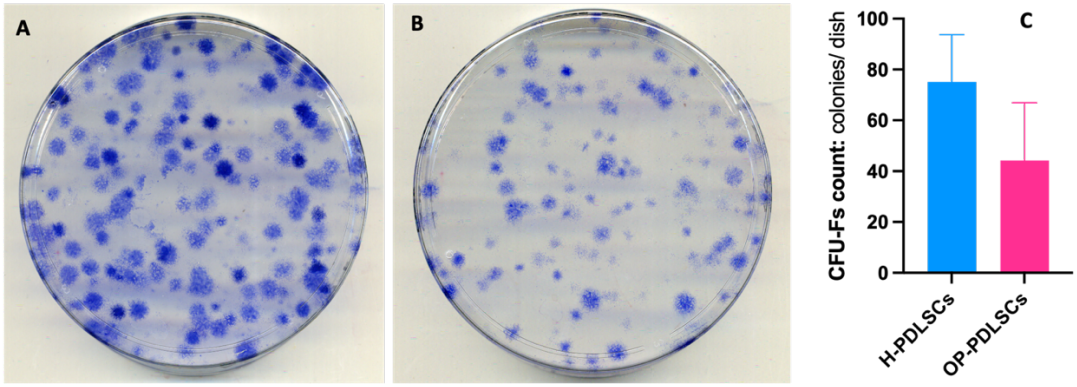


Figure 15. CFU-Fs for H-PDLSCs versus OP-PDLSCs (representative donors).

Image of CFU-Fs for H-PDLSCs (A) and OP-PDLSCs (B). Quantitative analysis of CFU-F plotted graphically as means ± SD (C).

4.2.2 Proliferation capacity:

PDT experiments were carried out to study the growth rate of OP-PDLSCs compared to healthy controls. For each donor, cells were plated at $1x10^5$ cells/ $T25cm^2$ flask, and once 80% confluent, cells were detached, counted and re-seeded. Population doubling (PD), accumulative days (AD), accumulative population doubling (APD) and population doubling time (PDT) were calculated as described previously in the methods (Chapter 3, section 3.2.7). These data were collected until the cells ceased growing or for up 2 months (whichever occurs first) from as early as P2 (where possible) up to P16 for some donors. Table 19 shows the means and SD for H- PDLSCs (n=4) and OP-PDLSCs (n=3) as well as the data of PD, AD, APD and PDT for the individual passage numbers P4-P9. The passage number range was selected because it was common among all donors from both groups.

The average PDT was calculated for each group from all donors. Data was presented as means ±SD. PDT was 1.6 ± 0.12 days for H-PDLSCs and 2.3 ±

0.36 days for OP-PDLSCs indicating that the osteoporotic group had a slower proliferation rate. Figure 16 shows the PDT (in days) for OP-PDLSCs compared to H- PDLSCs. The line representing the osteoporotic samples had a steeper slope compared to the healthy samples indicating that with subculturing (i.e increased passage number) OP- PDLSCs required a longer time to proliferate, particularly at later passages. Statistical analysis was carried out using unpaired t-test and indicated no statistical significance.

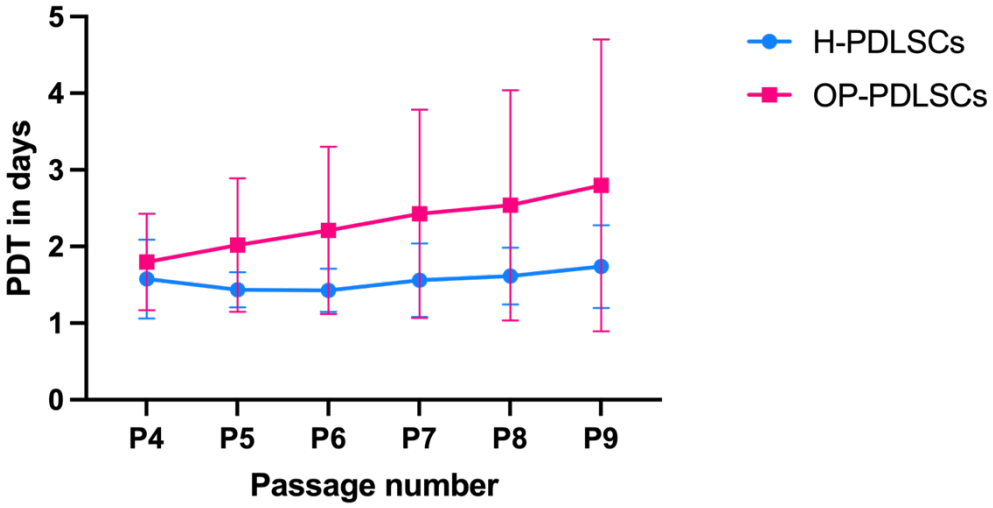


Figure 16. PDT for H-PDLSCs versus OP-PDLSCs donors for individual passage numbers.

This figure shows the PDT of OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) presented as means ± SD for each passage (P4-P9).

	H-PDLSCs				OP-PDLSCs				P value for PDT
	PD	AD	APD	PDT	PD	AD	APD	PDT	
P4	2.74 ± 1.01	8.25 ± 3.4	6.03 ± 3.5	1.58 ± 0.51	2.27± 0.55	13 ± 4	7.34± 1.37	1.80± 0.63	0.63
P5	2.40 ± 0.33	11.50 ± 3.32	8.43 ± 3.52	1.43 ± 0.23	2.29± 0.98	20 ± 3.46	10.55± 2.27	2.02± 0.87	0.24
P6	3.22 ± 0.91	15.75 ± 3.3	11.65 ± 4.3	1.43 ± 0.28	1.70± 0.62	25 ± 5.29	12.25± 2.88	2.21± 1.09	0.23
P7	2.97 ± 1.54	20.75 ± 2.87	14.62 ± 5.83	1.56 ± 0.48	1.67± 0.95	30.33 ± 6.81	13.92± 3.82	2.43± 1.36	0.28
P8	3.04 ± 1.72	26.50 ± 5.26	17.67 ± 7.22	1.61 ± 0.37	1.78± 0.05	36 ± 10.58	15.70± 3.86	2.54± 1.5	0.28
P9	2.46 ± 1.93	31.50 ± 6.4	20.12 ± 9.15	1.74 ± 0.54	1.69± 1.16	42.33 ± 13.8	17.39± 5	2.80± 1.9	0.33

Table 19. PD, AD, APD and PDT for H-PDLSCs versus OP-PDLSCs.

This table shows the means and SD for population doubling (PD), accumulative Days (AD), accumulative population doublings (APDs), and population doubling time (PDT) for H-DLSC (n=4) and OP-PDLSCs (n=3), passages (P4-9). PDT was calculated as an average of all passages for all donors from both groups and statistically compared and considered significant when p value was less than 0.05.

4.2.3 Expression of MSCs surface markers:

4.2.3.1 Expression of MSC surface markers of osteoporotic and healthy PDLSCs cultures:

The expression of surface markers typically used to define MSCs was assessed on the PDLSC cultures and compared between those derived from osteoporotic and healthy control donors. Cells from both groups, (n=4 for H-PDLSCs) and (n=3 for OP-PDLSCs), were cultured at P6 under basal conditions until they reached ~80% confluency. They were then stained with antibodies to assess the expression of MSC surface markers, which could be expected to be expressed, and non-MSCs surface markers, which would not be expected to be expressed. Table 8 includes the antibody panel used. Details on culture expansion and staining were discussed in Chapter 3 (3.2.8).

Before commencing the analysis of surface markers expression, a gate was set based upon the forward scatter (FSC) and side scatter (SSC) properties, which indicate the physical properties of healthy stem cells, and by excluding debris. This was followed by gating to exclude dead cells as indicated by strong positive FVS780 fluorescence generated by uptake of fixable viability stain (FVS) by dead cells (Figure 17). These gating steps ensure that only live cells were taken forward and included in subsequent analysis steps involving surface marker expression.

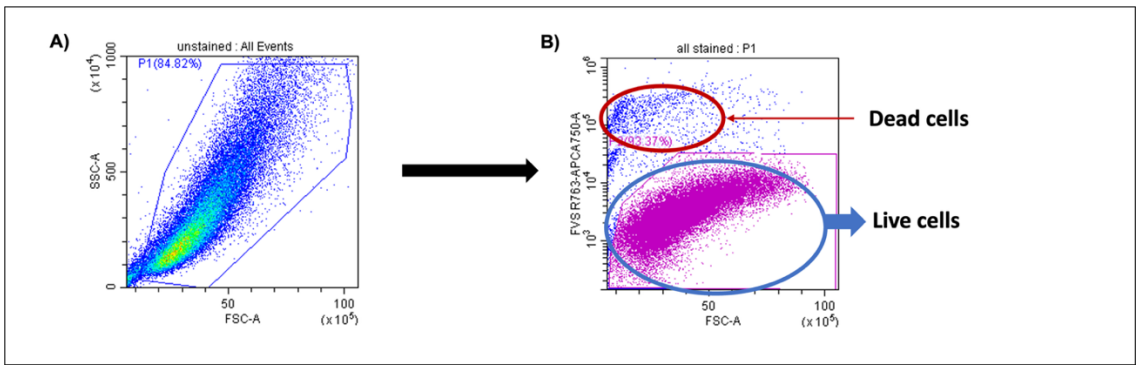


Figure 17. Gating for forward (FSC) and side scatter (SSC) and the selection of live cells before further flow cytometry analysis (representative donor).

Dot plot (A) shows the gating strategy based on FSC and SCC to exclude debris, which was followed by the selection of live cells by the exclusion of dead cells which stain brightly with FVS due to non-intact cell membranes (B).

As explained previously (Chapter 3, section 3.2.8.2), FMO was used as a negative control. A gate was set on each FMO sample by excluding 2% of its population (as a margin of error) (Figure 18. A) and applying the same gate threshold on the sample which was stained for all antibodies (Figure 18. B), the percentage of expression was thus determined. The percentage of expression indicates the proportion of cells expressing a specific marker of interest. An overlay histogram with data from the FMO and all- stained samples was created to assess the shift (Figure 18. C). Expression of any individual marker was identified by the shift on the X axis (when plotting fluorescence due to antibody specific for that marker) in the all-stained sample (green curve) compared to its FMO (red curve) (Figure 18. C top row). Lack of, or minimal shift of the all-stained sample indicated a lack of/ minimal expression, such as that expected for a negative marker (Figure 18. C bottom row). Of note is the term 'positive marker' is used to indicate a marker that is known to be expressed on PDLSCs while the term 'negative marker' indicates a marker that is known to identify other lineages of cells and is not expected to be expressed on PDLSCs.

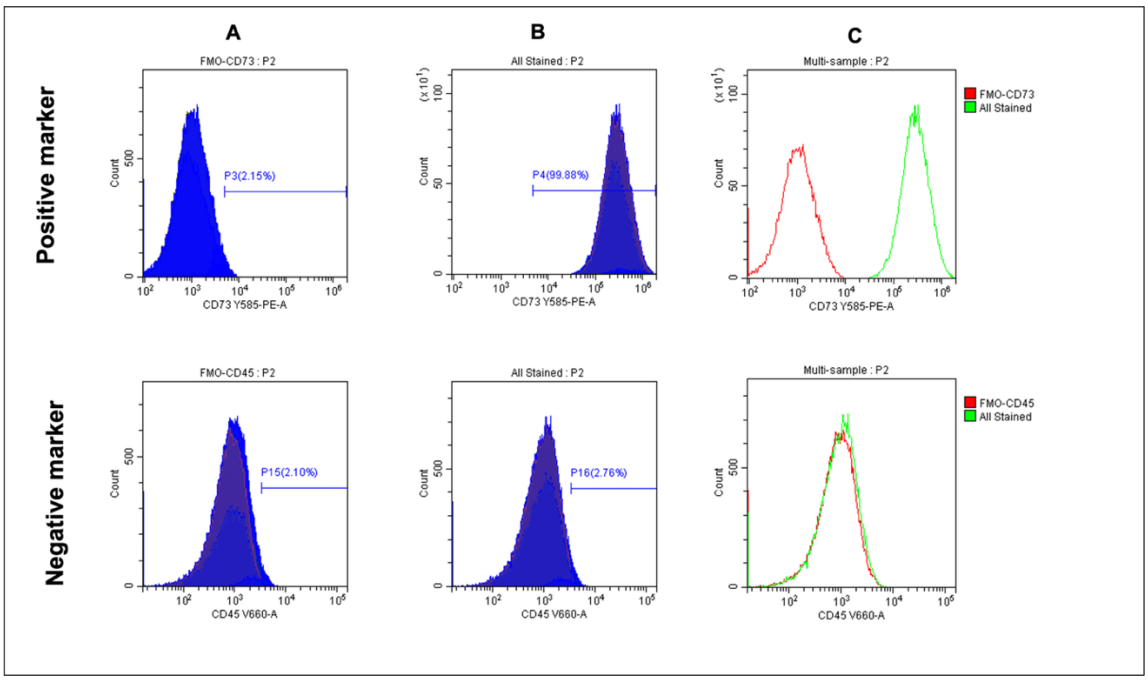


Figure 18. Identification of the percentage of live cells fluorescing for a specific marker (representative donor and markers).

Top row: a positive marker (e.g. CD73), bottom row: a negative marker (e.g. CD45). P2 indicates the channel at which selected cells were live and met the FSC and SCC criteria.

A) Setting the cut-off point (margin of error) at 2% on the FMO of the marker of interest.

B) Using the same gate set on the previous step, the amount of shift is detected on the all-stained sample (e.g. 99.88% for the positive marker and 2.76% for the negative marker in the presented histogram graphs).

C) Overlay histogram showing fluorescence data from both the FMO sample (red line) as well as the marker of interest (green line). Of note is the amount of shift on the X axis; a considerable shift for the positive marker (top row) and the absence of noticeable shift for the negative marker (bottom row).

The same gating strategy was applied on each marker for both groups to calculate the proportion of cells expressing the CD marker of interest. Histograms for all donors were presented individually in Figures 19-26 along with bar charts comparing the markers' mean percentage of expression for all donors. Statistical analysis for all samples was carried out using Mann-Whitney test indicating no statistically significant difference between donor groups.

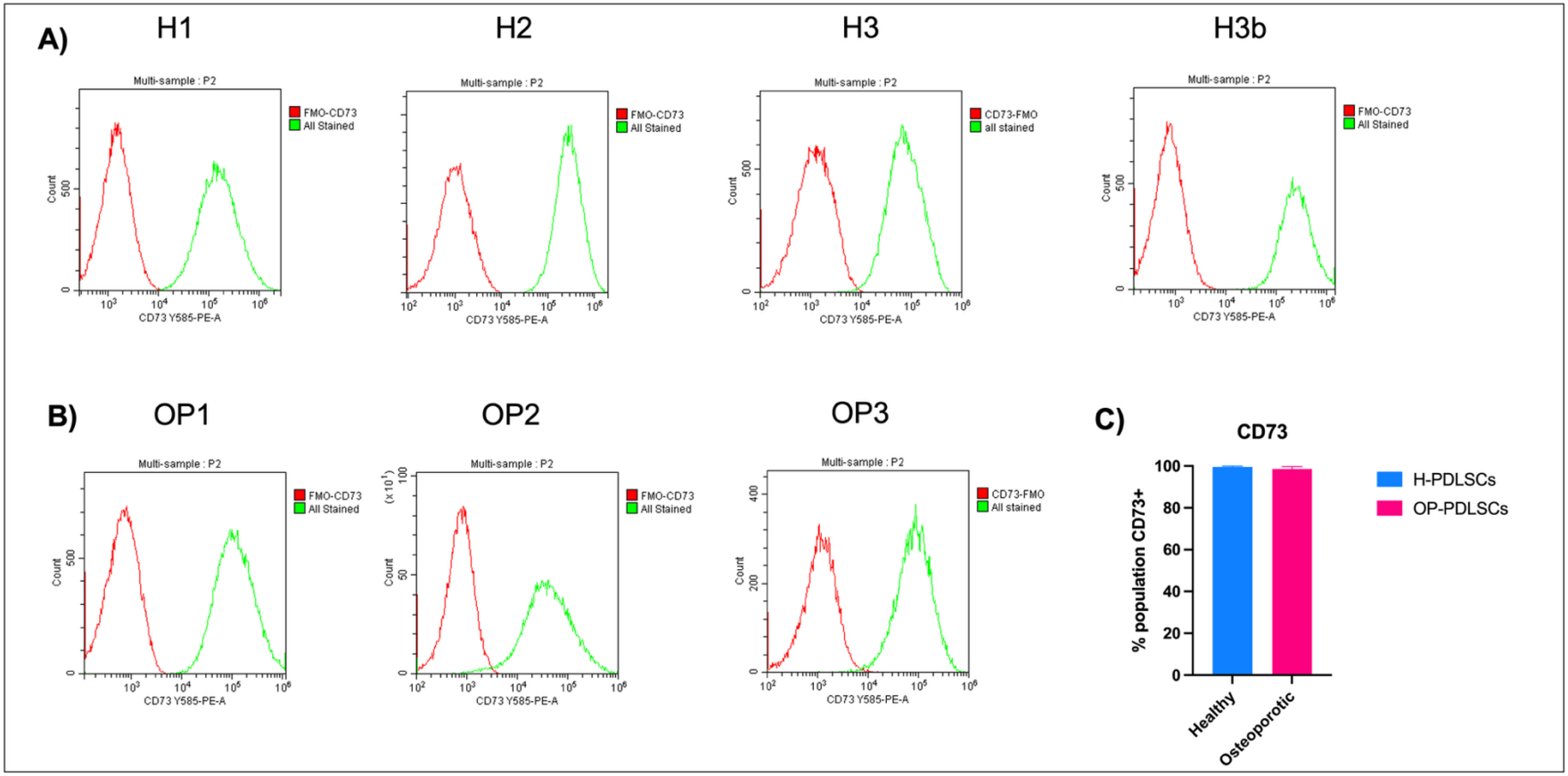


Figure 19. Proportion of cells in healthy and osteoporotic PDLSC cultures that express CD73, indicated by histogram overlay data.

Data presented for H-PDLSCs n=4 (H1,2,3,3b) (A) and OP-PDLSC n=3 (OP1,2,3) (B) under basal conditions. Red lines indicate FMO (anti-CD73 absent) baseline fluorescence, while the green line indicates the “all-stained sample” which includes the anti-CD73 antibody. The shift of the green line indicates increased fluorescence due to binding of the specific fluorphore conjugated antibody to the marker of interest being expressed on the cells. The bar chart (C) compares the mean percentage of CD73 expression for all donors from both groups ±SD.

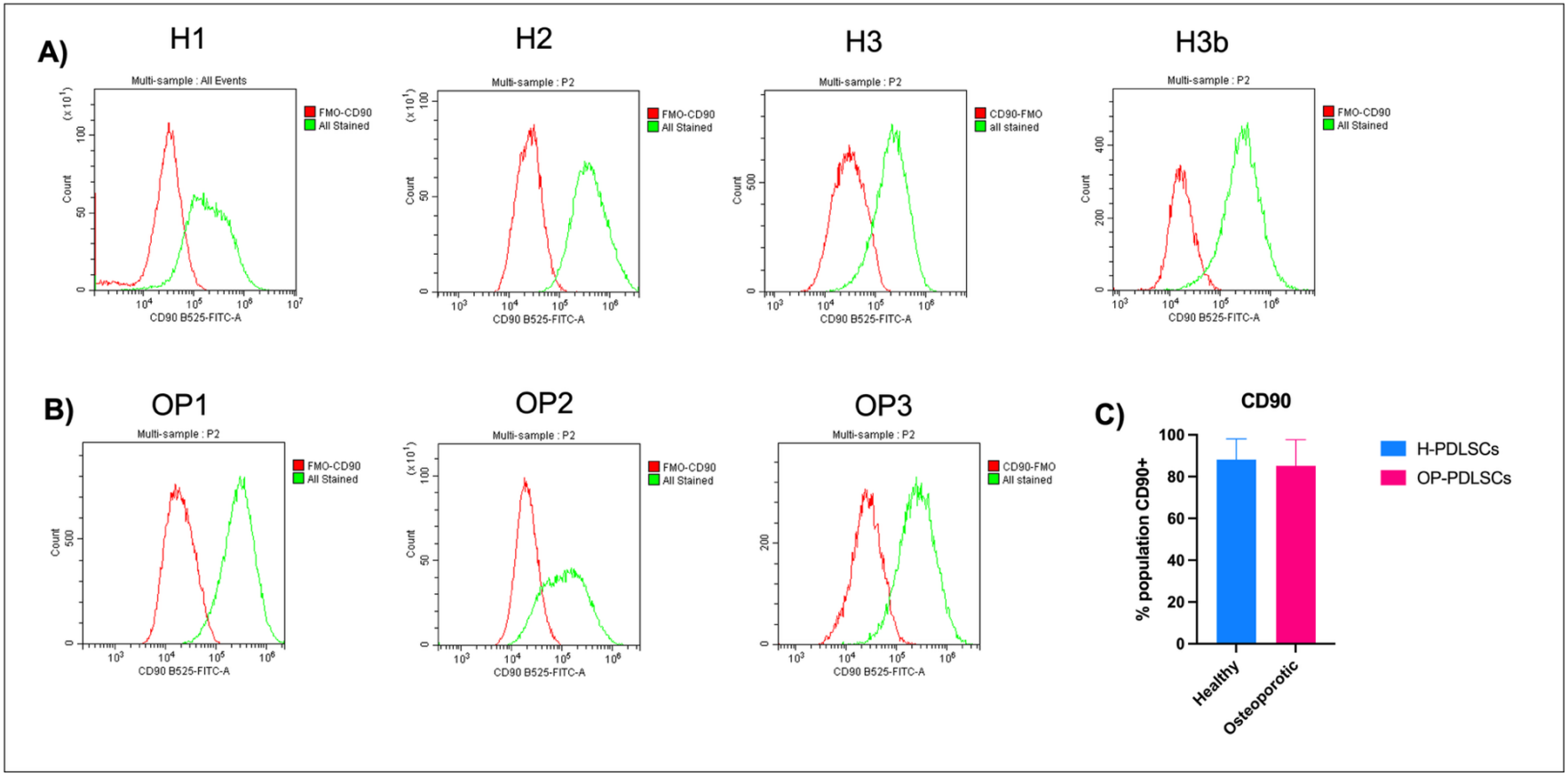


Figure 20. Proportion of cells in healthy and osteoporotic PDLSC cultures that express CD90, indicated by histogram overlay data.

Data presented for H-PDLSCs n=4 (H1,2,3,3b) (A) and OP-PDLSC n=3 (OP1,2,3) (B) under basal conditions. Red lines indicate FMO (anti-CD90 absent) baseline fluorescence, while the green line indicates the “all-stained sample” which includes the anti-CD90 antibody. The shift of the green line indicates increased fluorescence due to binding of the specific fluorphore conjugated antibody to the marker of interest being expressed on the cells. The bar chart (C) compares the mean percentage of CD90 expression for all donors from both groups ±SD.

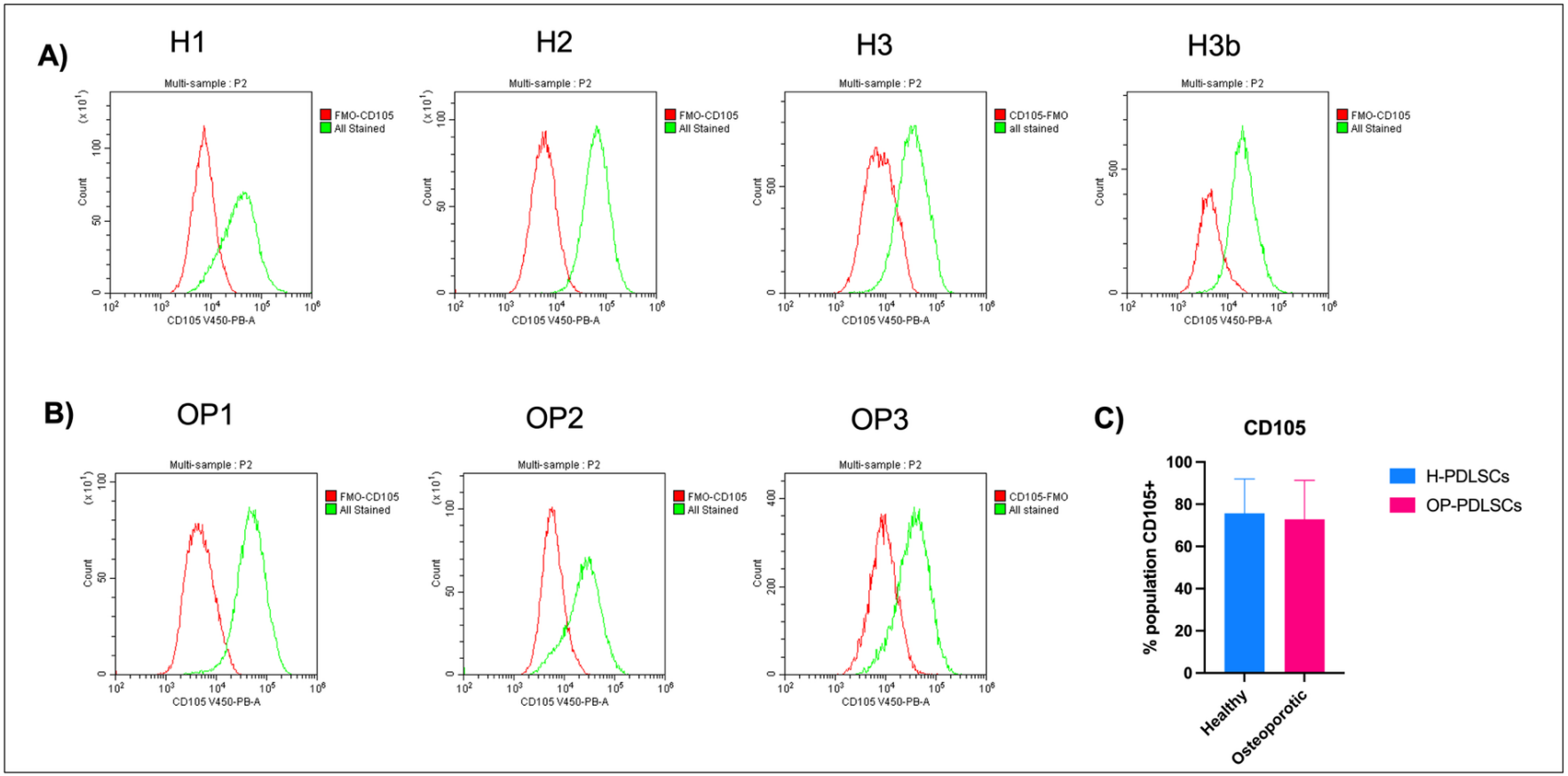


Figure 21. Proportion of cells in healthy and osteoporotic PDLSC cultures that express CD105, indicated by histogram overlay data.

Data presented for H-PDLSCs n=4 (H1,2,3,3b) (A) and OP-PDLSC n=3 (OP1,2,3) (B) under basal conditions. Red lines indicate FMO (anti-CD105 absent) baseline fluorescence, while the green line indicates the “all-stained sample” which includes the anti-CD105 antibody. The shift of the green line indicates increased fluorescence due to binding of the specific fluorphore conjugated antibody to the marker of interest being expressed on the cells. The bar chart (C) compares the mean percentage of CD105 expression for all donors from both groups ±SD.

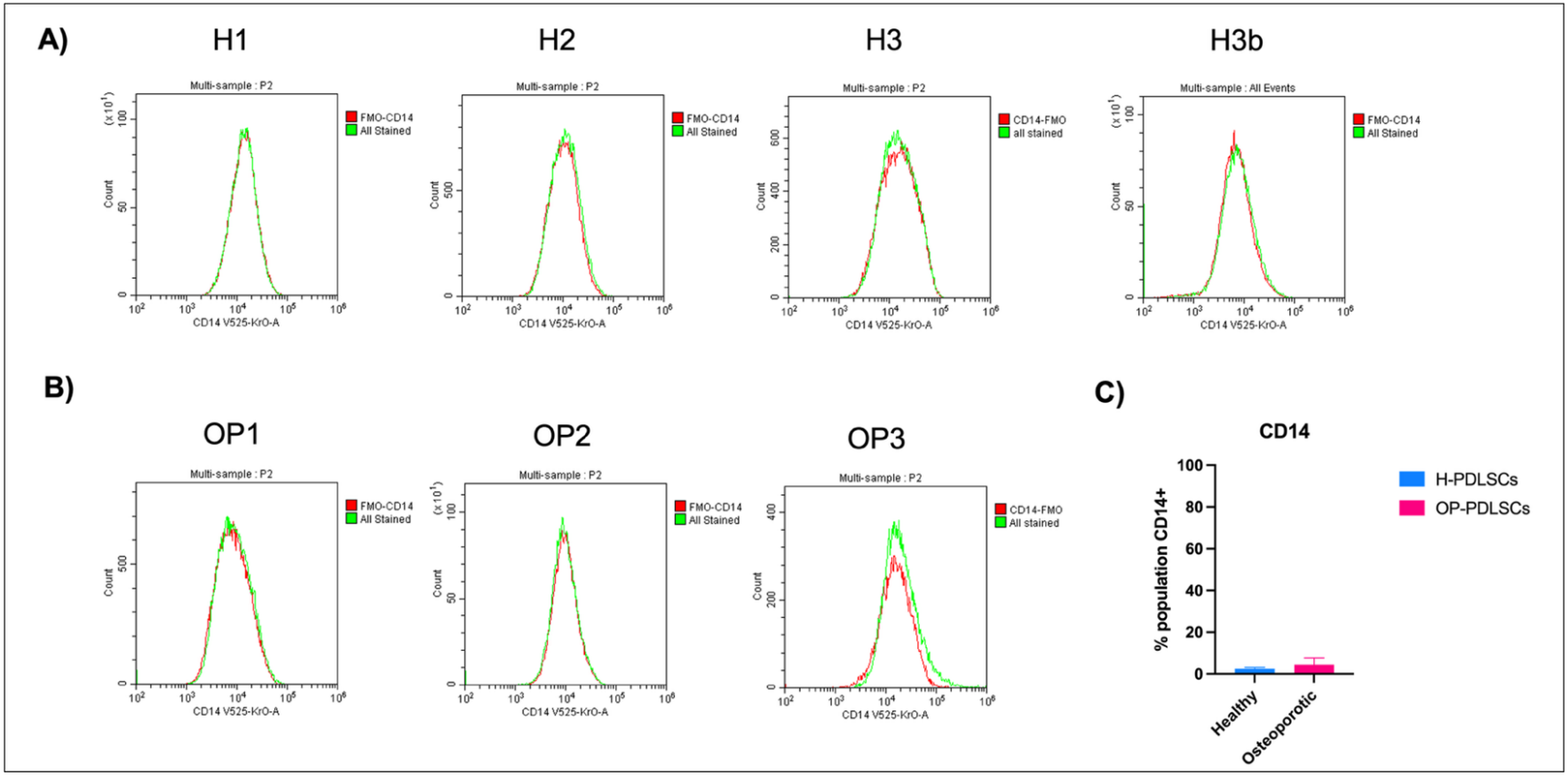


Figure 22. Proportion of cells in healthy and osteoporotic PDLSC cultures that express CD14, indicated by histogram overlay data.

Data presented for H-PDLSCs n=4 (H1,2,3,3b) (A) and OP-PDLSC n=3 (OP1,2,3) (B) under basal conditions. Red lines indicate FMO (anti-CD14 absent) baseline fluorescence, while the green line indicates the “all-stained sample” which includes the anti-CD14 antibody. Lack of forward shifting (overlapping of graph lines) indicates lack of expression. The bar chart (C) compares the mean percentage of CD14 (minimal) expression for all donors from both groups ±SD.

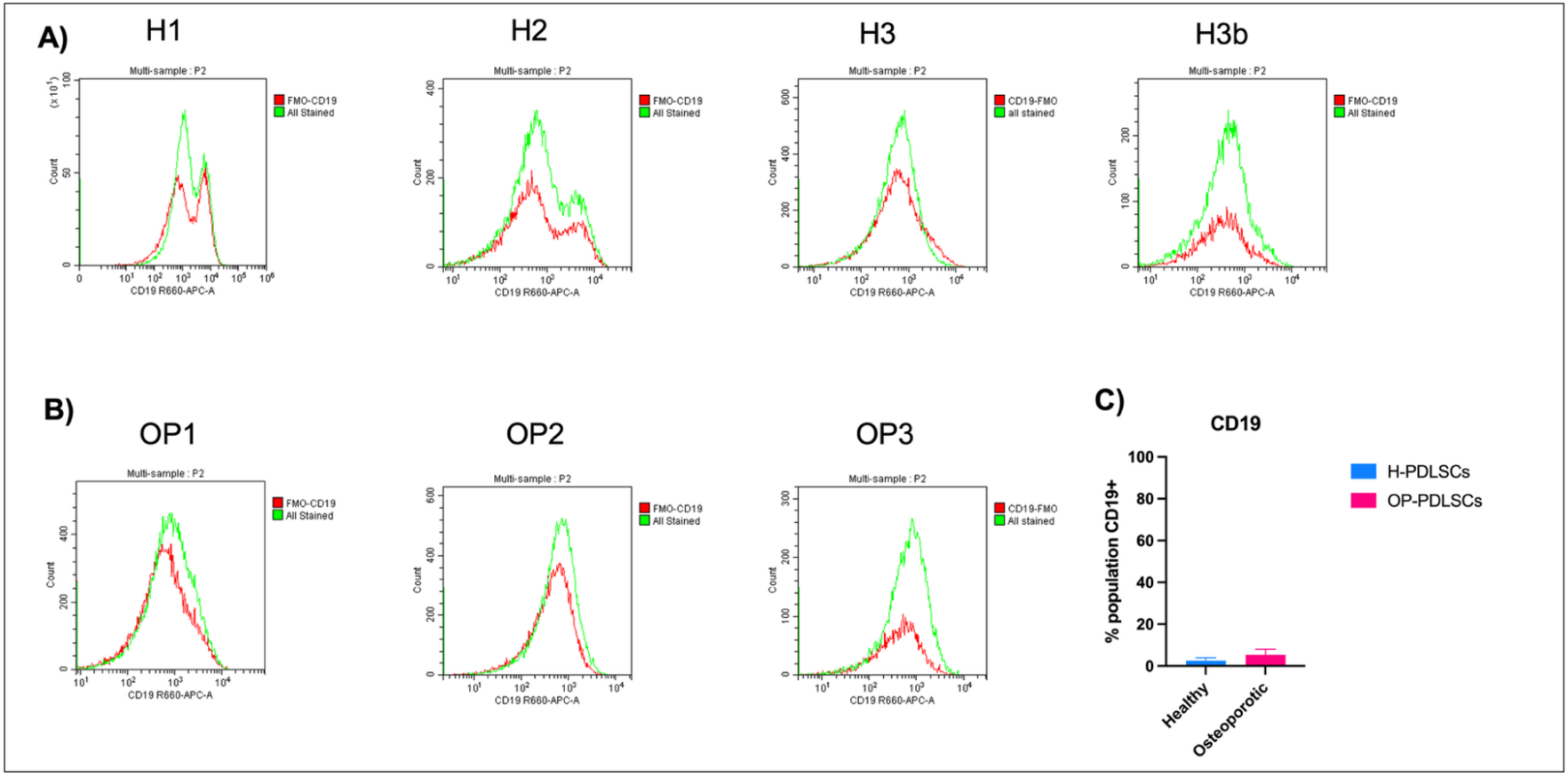


Figure 23. Proportion of cells in healthy and osteoporotic PDLSC cultures that express CD19, indicated by histogram overlay data.

Data presented for H-PDLSCs n=4 (H1,2,3,3b) (A) and OP-PDLSC n=3 (OP1,2,3) (B) under basal conditions. Red lines indicate FMO (anti-CD19 absent) baseline fluorescence, while the green line indicates the “all-stained sample” which includes the anti-CD19 antibody. Lack of forward shifting (overlapping of graph lines) indicates lack of expression. Of note is the shoulder presentation at H1 and H2 indicating heterogenous population in culture. The bar chart (C) compares the mean percentage of CD19 (minimal) expression for all donors from both groups ±SD.

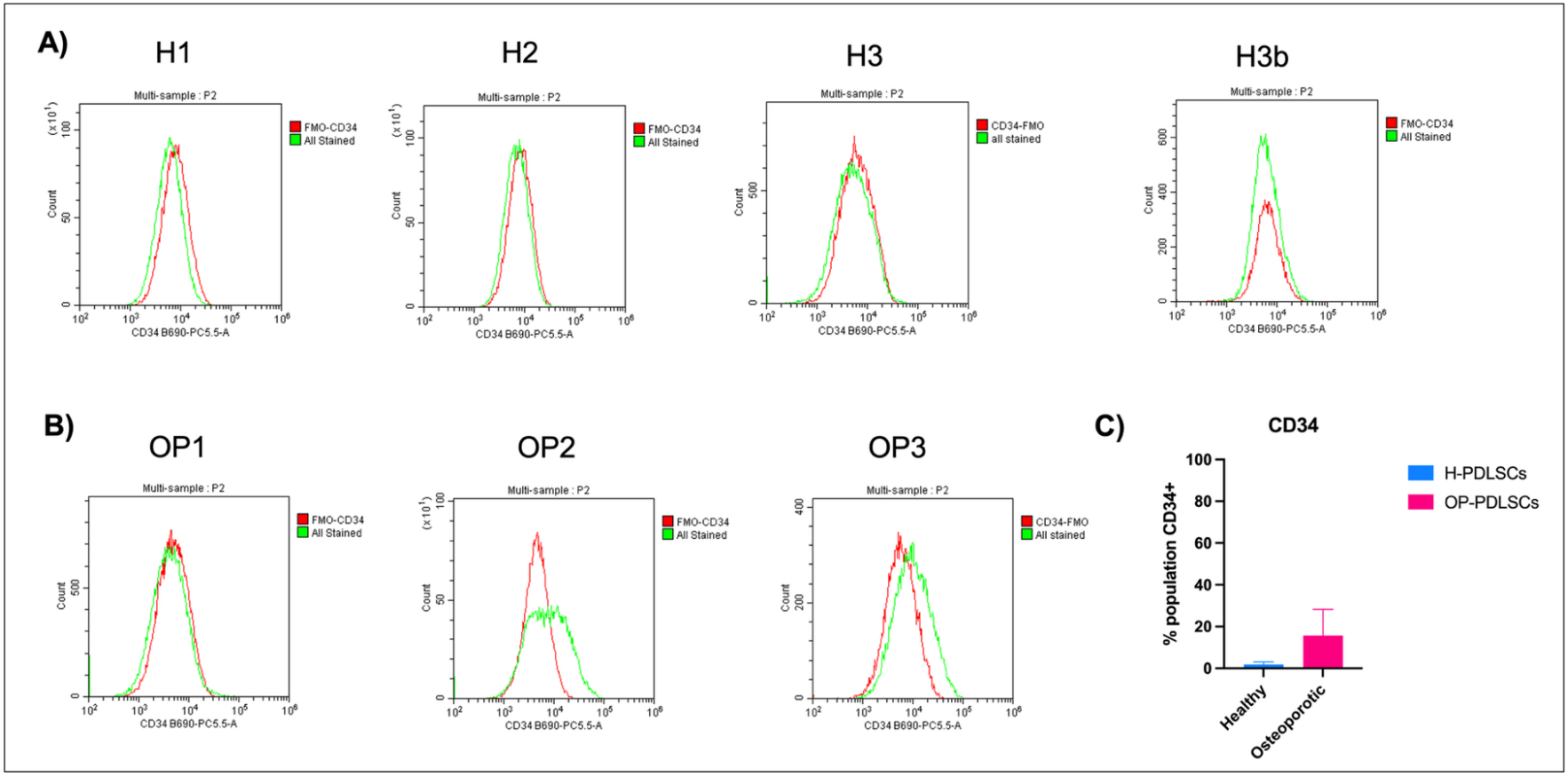


Figure 24. Proportion of cells in healthy and osteoporotic PDLSC cultures that express CD34, indicated by histogram overlay data.

Data presented for H-PDLSCs n=4 (H1,2,3,3b) (A) and OP-PDLSC n=3 (OP1,2,3) (B) under basal conditions. Red lines indicate FMO (anti-CD34 absent) baseline fluorescence, while the green line indicates the “all-stained sample” which includes the anti- CD34 antibody. Lack of forward shifting (overlapping of graph lines) indicates lack of expression although there is a noticeable fluorescence for cultures OP2 and OP3. The bar chart (C) compares the mean percentage of CD34 (minimal) expression for all donors from both groups ±SD.

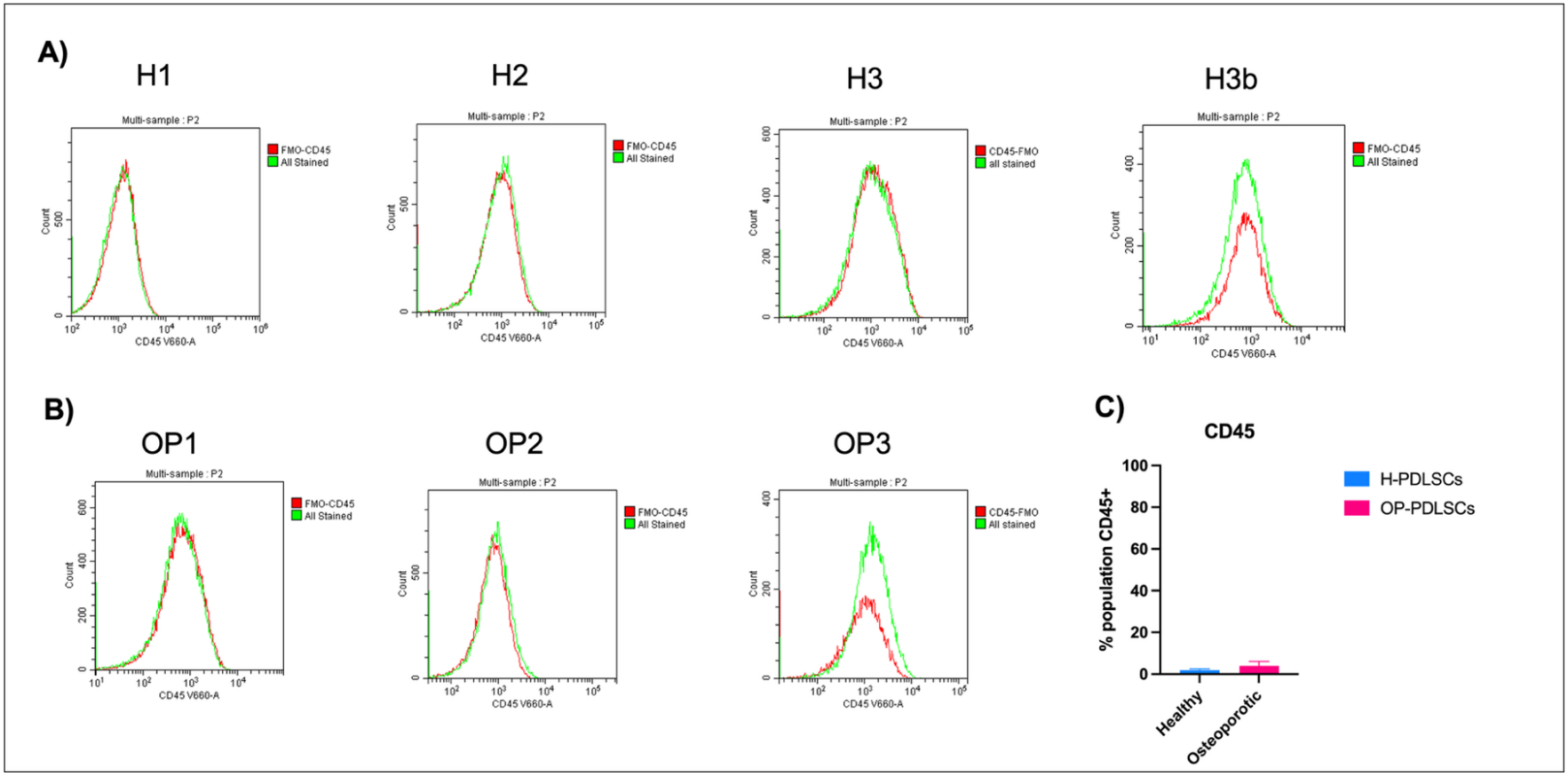


Figure 25. Proportion of cells in healthy and osteoporotic PDLSC cultures that express CD45, indicated by histogram overlay data.

Data presented for H-PDLSCs n=4 (H1,2,3,3b) (A) and OP-PDLSC n=3 (OP1,2,3) (B) under basal conditions. Red lines indicate FMO (anti-CD45 absent) baseline fluorescence, while the green line indicates the “all-stained sample” which includes the anti- CD45 antibody. Lack of forward shifting (overlapping of graph lines) indicates lack of expression. The bar chart (C) compares the mean percentage of CD45 (minimal) expression for all donors from both groups ±SD.

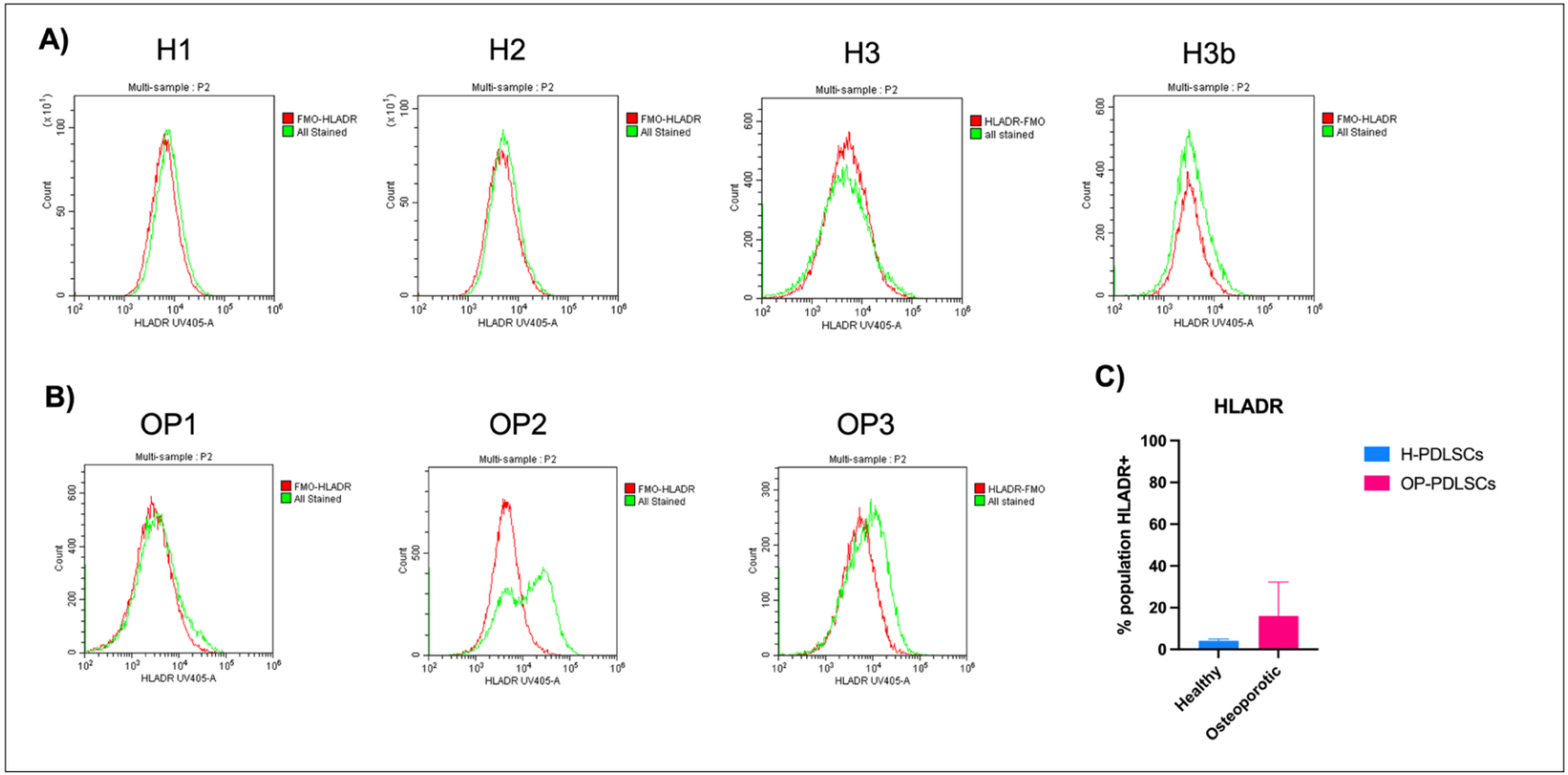


Figure 26. Proportion of cells in healthy and osteoporotic PDLSC cultures that express HLADR, indicated by histogram overlay data.

Data presented for H-PDLSCs n=4 (H1,2,3,3b) (A) and OP-PDLSC n=3 (OP1,2,3) (B) under basal conditions. Red lines indicate FMO (anti-HLADR absent) baseline fluorescence, while the green line indicates the “all-stained sample” which includes the anti- HLADR antibody. Lack of forward shifting (overlapping of graph lines) indicates lack of expression. There is a marked fluorescence for culture OP2 and a slight shift for OP3. The bar chart (C) compares the mean percentage of HLADR (minimal) expression for all donors from both groups ±SD.

In both study groups, the positive MSC marker with the highest expression level was CD73 (mean 99.5±0.4% for H-PDLSCs, 98.5±0.9% for OP-PDLSCs) followed by CD90 (mean 88.18±9.8% for H-PDLSCs, 85.1±10.2% for OP-PDLSCs) then CD105 (mean 75.6±16.3 for H-PDLSCs, 72.9±15.1% for OP-PDLSCs). Overall, all healthy samples showed higher expression level of the positive (MSC) markers with no statistically significant difference compared to the osteoporotic samples using Mann-Whitney test. As for the negative markers, CD45 showed the lowest expression level in both groups (mean 1.8±0.6% for H-PDLSCs, 3.9±1.7% for OP-PDLSCs) whilst HLADR was the marker with the highest expression (mean 4.14±0.8% for H-PDLSCs, 16.1±13.2% for OP-PDLSCs). The proportion of cells expressing the rest of the negative markers varied as follows: CD14 (mean 2.5±0.6% for H-PDLSCs compared to 4.4±2.7% for OP-PDLSCs), CD19 (mean 2.6±1.3% for H-PDLSCs versus 5.4±2.1% for OP-PDLSCs) and CD34 (mean 1.9±1.1% for H-PDLSCs compared to 15.74±10.2% for OP-PDLSCs). Similar to the positive markers, the overall proportion of the negative markers was less in the healthy group compared to the osteoporotic group with no statistically significant difference using Mann-Whitney test. Data are summarised in (Table 20). Figure 27 shows the median fluorescence intensity (MFI) values, which represent the strength of fluorescence signal indicating the expression of more of the protein marker on the cell surfaces.

Whilst no statistical significance was found between the groups, it is worth noting that the expression of CD34 was evidently higher in samples provided by osteoporotic donors 2 and 3 (27.5% and 17.01%,) respectively compared to donor 1 (2.71%), and higher HLADR level for OP donor 2 (34.76%) compared to osteoporotic donor 1 (5.85%) and donor 3 (7.57%) (Figure 28).

Marker	Type	Healthy donors (mean ± SD)%	OP donors (mean ± SD)%	P value
CD73	MSC (+ve)	99.5±0.4	98.5±0.9	0.2
CD90	MSC (+ve)	88.18±9.8	85.1±10.2	0.9
CD105	MSC (+ve)	75.6±16.3	72.9±15.1	0.9
CD14	MSC (-ve)	2.5±0.6	4.4±2.7	0.6
CD19	MSC (-ve)	2.6±1.3	5.4±2.1	0.2
CD34	MSC (-ve)	1.91±1.1	15.74±10.2	0.1
CD45	MSC (-ve)	1.8±0.6	3.9±1.7	0.1
HLADR	MSC (-ve)	4.14±0.8	16.1±13.2	0.06

Table 20. Phenotypic MSC surface marker expression percentage.

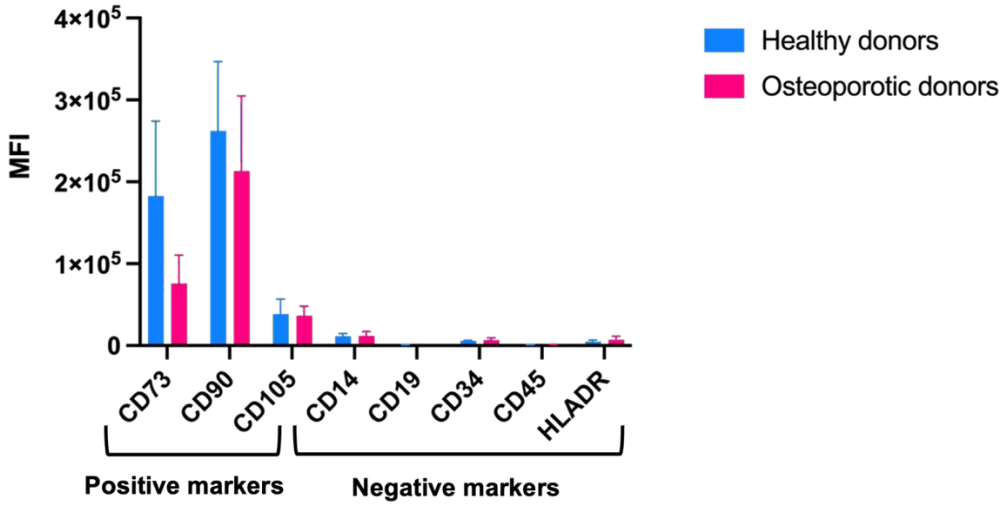


Figure 27. Median Fluorescence Intensity (MFI).

The median fluorescence intensity of the staining for each of the CD markers. Fluorescence from biological replicates ±SD; H- PDLSCs: n=4, OP-PDLSCs: n=3. Fluorescence intensity relates to the amount of marker that is being expressed on cells.

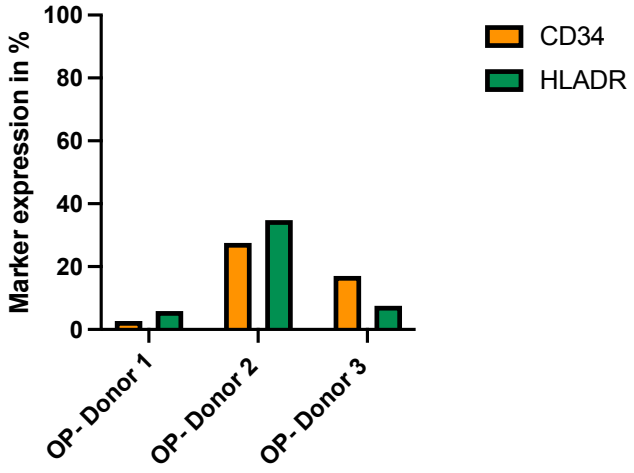


Figure 28. The variation in the expression of CD34 and HLADR across the osteoporotic donors' PDLSCs.

Bar chart indicates the proportion of the total population of cells that express CD34 and HLADR for the OP donors (n=3). Of note is the variation of markers expression across the donors.

4.2.3.2 The proportion of MSC population in osteoporotic and healthy PDLSCs

To calculate the proportion of MSC markers expressed within each cell culture, the percentage of live cells with the expected expression pattern for all positive and negative stem cell markers was calculated. This was carried out using a sequential gating strategy (Figure 29). First, cells were selected by gating to meet the physical (FSC and SSC) characteristics (step 1), then live cells were selected using FVS (step 2). These cells were then assessed against the defining criteria of stem cells i.e. cells that are $CD90^+$ and $CD73^+$ (step 3), $CD105^+$ and $CD14^-$ (step 4), $CD45^-$ and $CD19^-$ (step 5) and $CD34^-$ and $HLADR^-$ (step 6). A comparison of the total stem cell percentage for H-PDLSCs (mean 47.42± 16.03%) versus OP-PDLSCs (mean 46.61±13.24%) was plotted (Figure 30). Mann-Whitney test indicated no significant difference between the 2 groups. MSCs proportions for each individual donor are presented in (Table 21). The position of the gating thresholds of what is defined as a positive or a negative marker was determined by the position of the FMO staining in the same way that thresholds were set in the previous section (4.2.3.1).

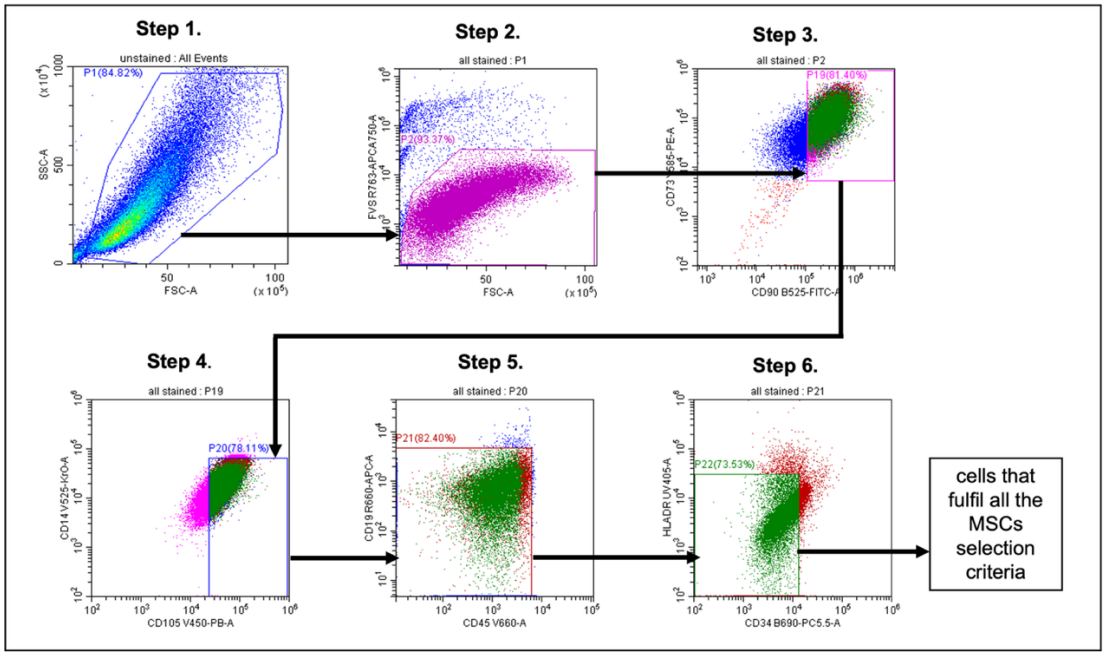


Figure 29. Sequential analysis of flow cytometry antibody panel to calculate percentage of live cells that have MSC cell phenotype (representative donor).

Sequential gating for $CD73^+$ / $CD90^+$ / $CD105^+$ / $CD14^-$ / $CD45^-$ / $CD19^-$ / $CD34^-$ / $HLADR^-$. At each step, the gating thresholds were those set previously based on the appropriate FMO control. Step 1. Exclusion of debris by Forward scatter (FSC) and Side Scatter (SSC) gate. Step 2. Exclusion of dead cells by fixable viability stain gate. Step 3. Selection of live cells that are $CD73^+$ and $CD90^+$. Step 4. Further selection of cells which are $CD14^-$ and $CD105^+$. Step 5. Further selection of cells which are $CD19^-$ and $CD45^-$. Step 6. Further selection of cells that are $HLADR^-$ and CD34- . Events within the rectangular gate (dark green) at step 6 shows the total proportion of cells that fulfil the stem cell criteria.

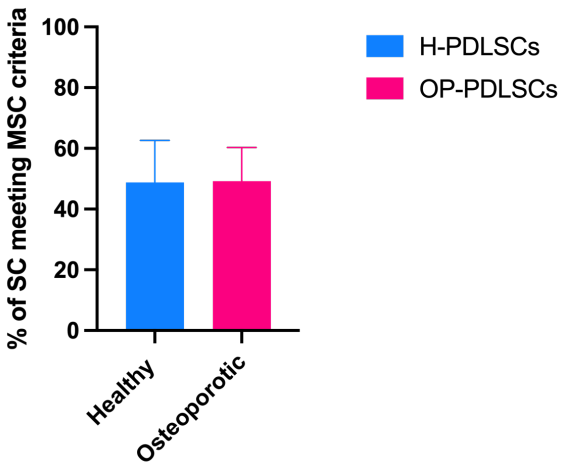


Figure 30. Percentage of cells matching the defining criteria of MSCs ($CD73^+$, $CD90^+$, $CD105^+$, $CD14^-$, $CD34^-$, $CD19^-$, $CD45^-$ and $HLADR^-$).

Mean values of biological replicates ± SD from healthy PDL tissue (n=4) compared to osteoporotic PDL tissue (n=3). The total percentage was calculated by dividing the number of events in the final gate (which has cells fulfilling all stem cell criteria) by the total number of events in the live cells gate.

Donor	MSC proportion
Healthy donor 1	59%
Healthy donor 2	62.6%
Healthy donor 3	38.53%
Healthy donor 3b	29.41%
OP donor 1	60.27%
OP donor 1	49.18%
OP donor 1	30.37%

Table 21. The proportion of cells matching MSCs criteria for individual donors.

4.3 Discussion:

The overall data presented in this chapter indicate that PDLSCs isolated from OP donors have the capacity to generate colonies, proliferate for extended periods and express the tested MSCs surface markers. The proliferative capacity of those cells showed a trend of less proliferation than the control (H-PDLSCs). However, the phenotypic characterisation was similar, with the exception CD34 and HLADR markers which will be discussed later in this section. There is scant literature regarding the characterisation of dental stem cells isolated from OP patients and hence the results will be discussed in relation to reports involving BMSCs isolated from osteoporotic patients and/ or similar work carried out in osteoporotic ovariectomised (OVX) animal models which simulates postmenopausal osteoporotic conditions.

CFU-Fs assay has gained importance in stem cell research since cells within these colonies possess high proliferation and differentiation capacities, as indicated in BMSCs research (Oreffo et al., 1998). Despite the lack of statistical significance, the data presented here showed fewer CFU-Fs colonies were generated in the osteoporotic group versus healthy controls. This is in line with Wu et al. study, where OP-BMSCs isolated from OVX rats showed fewer CFU-Fs colonies compared to the control group (Wu et al., 2018). Additionally, similar to the findings in this project, Čamernik et al. (Čamernik et al., 2020) investigated CFU-F% of human OP-BMSCs compared to healthy controls and reported a lack of significant difference between the 2 groups. On the contrary, results from the Stenderup et al. study (Stenderup et al., 2001) of $STRO1^+$ (purified) BMSCs isolated from osteoporotic patients revealed a greater CFU-Fs colony count in OP-BMSCs (129±11) compared to healthy old (99±19) and young healthy individuals (87±12) but with no statistically significant difference among the groups. The variation in results could be attributed to the process of sorting and purification of stem cells before studying in contrast to the samples used in this project (heterogenous PDLSCs) as well as the different cell origins (bone marrow vs PDL). The variation could also be attributed to the difference in age and medical history of the participants.

In this project, PDT results indicated that OP-PDLSCs had a slightly slower proliferation rate in comparison to H-PDLSCs. This resembled OP-BMSCs

isolated from postmenopausal when compared to healthy BMSCs up to 10 days of culture in a study by Rodríguez et al. (Rodríguez et al., 1999). Although the information drawn from their study on OP-BMSCs is similar to OP-PDLSCs, the technique used was different (growth curve rather than PDT) and the passage numbers used were not specified. Defining the passage number is critical since it gives an indication of the proliferative capacity in relation to the cellular age *in vitro*. In contrast to the findings presented here, PDLSCs isolated from molars of OVX rats showed a higher proliferative rate when compared to controls, which was justified as a compensatory mechanism for the loss of estrogen (Zhang et al., 2011). Although their data were based on samples from PDLSCs, the cells used were isolated from PDL tissue of a different species, and the proliferation was assessed using MTT over a period of 13 days compared to PDT over 2 months in this study.

It is apparent from the data presented here that PDT increases with the progress of passage number (*in vitro* ageing) possibly due to the increase of the β-galactosidase enzyme (a senescence marker) (Ganguly et al., 2017a), which was observed to rise with the increase of population doublings (PD). Another possible explanation for the increased PDT in this study is the difference in donors' age in the osteoporotic patients versus the healthy group. In a study by Li et al. (Li et al., 2020), proliferation of adults PDLSCs (age range: 35-50 years) was compared to young PDLSCs (age range 19- 20 years) over a 7-day period. The proliferation rate was slower in the adult group. A direct relationship between age and proliferation rate could not be drawn for OP-PDLSCs due to the lack of data from age-matched healthy group which is a limitation of this study.

The flow cytometry experiments were carried out to study the immunophenotypic characteristics of OP-PDLSCs compared to H-PDLSCs. Flow cytometry is ideal for that purpose, and the strength of this technique is derived from its ability to perform highly complex and quantitative analysis for individual cells in a heterogenous population (Donnenberg et al., 2013). The stem cell surface marker panel selected for this project was based on the ISCT criteria for MSCs where CD73, CD90, CD105 are expressed (≧95%

expression) and where CD14, CD19, CD34, CD45 and HLADR are either lacking expression or are minimally expressed (≦ 2% expression) (Dominici et al., 2006). The flow cytometry findings for H-PDLSCs in this project agreed with previously published reports (Abedian et al., 2020, Abe et al., 2022, Kadkhoda et al., 2016, Tamaki et al., 2013) characterising human H-PDLSCs. In the cited studies, CD73 and CD90 and CD105 were expressed in high proportions and CD14, CD34, CD45 showed minimal expression. On the other hand, data from OP-PDLSCs studied here expressed MSCs markers (namely $CD73^+$ (mean 98.5±0.9%), $CD90^+$ (mean 85.1±10.2%), $CD105^+$ (mean 72.9±15.1%)) and showed minimal expression of non-MSC markers ($CD14^-$ (mean 4.4±2.7%, $CD19^-$ (5.4±2.1%), and $CD45^-$ (3.9±1.7)).

In a study by Čamernik et al. (Čamernik et al., 2020), BMSCs were isolated from the femoral neck fracture site of healthy and osteoporotic patients (average age 80.6 years) and were assessed for MSC markers compared to osteoarthritic BMSCs and muscle-derived MSCs. Freshly isolated OP-BMSCs were tested for CD14/CD19/CD45 which were expressed in less than 2% of the population whereas culture-expanded OP-BMSCs expressed CD73, CD90 in high proportions (>95%) and CD105 (around 70%) which is in line with the findings in this report for OP-PDLSCs. The difference with the values presented in this study could be attributed to the source of cells examined (BM compared to PDL), the media used to expand the cultures (MSCs media versus α-MEM) or inherent donors variations. In agreement with Čamernik et al.'s report, the expression of CD105 in this project was considerably less in both groups (mean 75.6±16.3 for H-PDLSCs and 72.9±15.1 for OP-PDLSCs) compared to the values suggested by ISCT. Another report by Garna et al. (Garna et al., 2020), revealed different degrees of expression of CD105 (86.4%± 10.9, 11.7%± 2.1 and 95.6% ± 3.8%) for human PDLSCs, dental pulp stem cells (DPSCs) and BMSCs respectively. In an additional paper published by Ponnaiyan et al (Ponnaiyan and Jegadeesan, 2014), analysis of phenotypic marker expression was carried out between DPSCs and BMSCs isolated from human tissues. $CD105^+$ population from the isolated cells was significantly less (34.54 ± 1.91%) on the surface of dental cells compared to (83.14 ± 1.94%) on bone marrow cells. They attributed this lower proportion of expression to the role CD105 plays in haematopoiesis.

Whether the variation between the expression of markers is a factor inherent to the different sources of cells (pulp versus PDL versus BM), passage number (*in vitro* age),or due to variation between donors, requires increasing the number of samples included in the study which was beyond the scope of this project.

Intriguingly, the proportion of cells expressing CD34 was higher in two of the OP-PDLSCs, compared to other negative markers, (i.e CD34= 27.5%, 17% for donors 2 and 3 respectively). CD34 plays a role in hematopoietic stem cells (HSCs) cyto-adhesion and regulates their cellular proliferation and differentiation. It is also involved in trafficking stem cells into niches within the bone marrow (Viswanathan et al., 2017). There is a debate in the literature as to whether CD34 is a positive marker for MSC and that its expression *in vitro* is not indicative of its status *in vivo* (Viswanathan et al., 2017, Sidney et al., 2014, Lin et al., 2013, Lin et al., 2012). However, there are reported MSCs that show an expression for CD34. In a study where human BMSCs, adipose tissue MSCs (AT- MSCs) and Wharton's jelly MSCs were compared using flow cytometry to detect several MSC markers, AT-MSCs showed a higher proportion of CD34 (10±2.7%) compared to other MSCs which had a lower levels (≦2%). This was attributed to the variation of the tissue sources used (Petrenko et al., 2020). Relative to this study, it might be possible to suggest that OP-PDLSCs were differentiating toward an adipose tissue lineage (similar to OP-BMSCs (Hu et al., 2018)). However, more rigorous experiments to confirm this theory need to be performed. Examples of other tissues that are $CD34^{+}$ include hair follicle associated pluripotent stem cells, which was indicative of their relatively undifferentiated state (Hoffman and Amoh, 2018), embryonic fibroblasts, epithelial progenitor cells, keratinocyte stem cells and satellite cells of skeletal muscles (Viswanathan et al., 2017).

Furthermore, the acquisition of $CD34^{+}$ status in MSCs might be a result of subculturing (Sidney et al., 2014, Lin et al., 2012). It has been reported that Goodell et. al (Goodell et al., 1996) had isolated a side subpopulation of murine whole BMSCs, using Hoechst 33342 (a fluorescent nuclear dye), which when tested were found to be CD34 negative. Upon *in vitro* expansion for 5 weeks, the cells then became CD34 positive (Viswanathan et al., 2017). In addition, in their review, Lin et al. (Lin et al., 2012), have discussed the origin of using CD34 as a positive marker for MSCs was established based on data from MSCs that

were adherent to plastic, not necessarily residing in the bone marrow, which might not be indicative of MSCs true nature. According to their review, the presence of HSC displaying $CD34^-$ phenotype has also been confirmed, which suggests the questionability of using CD34 as a definite negative marker for MSCs. In order to confirm whether higher $CD34^+$ in OP-PDLSCs is a result of subculturing, the expression in cultured cells needs to be compared with freshly isolated cells or FACS purified OP-PDLSCs which was not attainable in this project because the cell yield on the day of isolation (P0) was not sufficient for the flow cytometry (as well as for other experiments).

Similar to CD34, the proportion of HLADR was evidently higher in osteoporotic donor 2 (34.76%). This shows a greater variation in the expression of these two markers among the osteoporotic donors (potentially indicating they might be less suitable for characterisation in this type of cells). HLA (human leukocyte antigen), also known as major histocompatibility complex class II (MHC-II), aids the immune system in identifying foreign antigens (Choo, 2007). HLADR is heterodimeric, composed of a heavy alpha chain and a light beta chain which is highly polymorphic at the peptide binding site. It is largely expressed on antigen presenting cells (APCs) i.e. macrophages, monocytes, dendritic cells and B cells (Liu et al., 2023). It is possible that the observation of higher HLADR levels could be a result of inflammation (OP is an inflammatory disease) and/ or ageing (Hughes et al., 2020, Lv et al., 2014). Inflammation has been linked to ageing (a term known as inflamm-ageing (Ferrucci and Fabbri, 2018)) due to the observation of higher levels of inflammatory markers e.g. IL-6, TNF-α and IL-1. These markers activate osteoclasts and hence a causal relationship between age-related OP and systemic inflammation in elder individuals has been suggested (Ginaldi et al., 2005). It could be possible that OP-PDLSCs used in this project release interferon gamma (IFN-ɣ) into the culture media which might then activate the class II trans-activator (CIITA) protein, an essential regulator for MHC II expression (Kuçi et al., 2024), leading to a higher expression of HLADR. It has been suggested that IFN-ɣ is majorly involved in the etiology of OP (Li et al., 2024). Vasandan et al (Vasandan et al., 2014), compared the inflammatory response of PDLSCs and DPSCs extracted from the same donor upon priming with IFN- ɣ and TNF-α. Cells were isolated from extracted third molars (donors

age 17-28 years). Interestingly, when PDLSCs were treated with IFN-ɣ for 72 hours, HLADR expression was enhanced. The proportion of cells that expressed HLADR post stimulation was not specified in their paper, but the graphs indicate a massive shift in fluorescence compared to controls. These data suggest the need for further investigation to assess the supernatant of culture media of OP-PDLSCs to detect possibly secreted inflammatory markers.

Another possible explanation for the variation in the marker expression in this project could be attributed to the heterogenous nature of the cultures since OP-PDLSCs were not purified. PDL tissue contains cell types other than stem cells including fibroblasts, osteoclasts, osteoblasts, epithelial cell rests of Malassez, macrophages and monocytes (Nanci and Bosshardt, 2006), which might have inherent variations in markers expression and have responded differently to the growth factors supplied in the culture media. Cells within the culture could also have differentiated at a different rate and some of them might have committed to a specific lineage. Furthermore, it is possible that PDLSCs used in this project could have existed in different niches within the PDL tissues of the donors. Cell niche could play a role in the different cellular behaviour under both healthy and disease conditions (Pérez et al., 2018). Specifically, the microenvironment in OP is characterised by cellular dysfunction and abnormal cytokine secretion as well as changes in pH and oxygen level (hypoxia) which ultimately impacts cell growth and function (Luo et al., 2023). Conclusively, relying solely on the MSCs surface markers to decide the stemness of the isolated cells is inconclusive as the other parameters (e.g. trilineage differentiation) need to be assessed. Additionally, there could be variation between donors which would require a larger sample to confirm.

From a technical standpoint, it is less likely that any increase in fluorescence seen in the HLADR and CD34 stained samples was a result of background staining since an Fc block reagent was used throughout the process and had successfully reduced the background staining when all other markers were assessed from the same batch of cells. The Fc block used is designed to prevent the non-specific binding of the Fc portion of the antibody reagents to

the cells, most commonly this occurs via Fc receptors of which there are multiple forms expressed on a range of cell types. Also, all the antibodies used in this project share the same isotype class, which excludes the lack of specificity for the reagent. Furthermore, further analysis was carried out to confirm whether the cells that are $CD34^+$ and $HLADR^+$ fulfil the criteria for all other MSC markers, to identify if it is only the expression of these markers that is an apparent anomaly. Data were re-analysed using a “back-gating” strategy. This involved starting with gating on all $CD34^+$ live cells and then subsequently investigating this $CD34^+$ populations expression of all the other positive and negative markers used. Back-gating was applied to cells considerably exceeding the cut-off point using the same technique applied during the sequential analysis performed with the other donors from both groups (Appendix B). It revealed that for OP donor 2 (Figure 61), 42% of $CD34^+$ cells and 43.5% of $HLADR^+$ cells fulfil all other criteria for stem cell definition which was also applicable for 31.2% of $CD34^+$ cells for OP donor 3 (Figure 62). These values suggest $CD34^+$ and $HLADR^+$ cells could be considered part of PDLSCs population.

When the total MSC proportion percentage was calculated in OP-PDLSCs compared to H-PLDSCs, it revealed almost similar proportions with no statistically significant difference (46.61±13.24% vs 47.42±16.03% respectively). Using a similar method, the identification of the stem cell population using $CD73^+/CD90^+/CD105^+/CD14^-/CD19^-/CD34^-/CD45^-/HLADR^-$ criteria was carried out for BMSCs isolated from osteoarthritic knee joint of diabetic and non- diabetic patients (94.02±1.5% vs 82.88±7.28% respectively) and revealed no statical significant difference between the groups (Hussein et al., 2024). However, the stem cell population results were considerably higher than the values calculated for PDLSCs in this project. In the previously mentioned Čamernik et al.study (Čamernik et al., 2020), calculation of BMSCs was based on the exclusion of $CD45^-/CD34^-/CD14^-/CD19^-$ cells for control and osteoporotic samples and had also showed no significant difference between the groups (mean ~90% for controls and ~75% for osteoporotic donors).

For the data presented here, it is important to note that the calculation of PDLSCs in the cell cultures was created by the inclusion of ISCT suggested MSCs positive markers and the exclusion of negative markers. If a different

panel for OP-PDLSCs was established (e.g. excluding/ redefining values for CD105, CD34 and HLADR) then the percentages of MSC population would vary accordingly. The overall results indicate an inclination of periodontal stem cells toward expressing a different MSC phenotype than BMSCs which raises the question of whether a different set of dental MSC phenotype panel (than what is known for BMSCs) needs to be considered. Whether that theory is valid requires further research to include a larger sample size to be able to widely assess the expression of MSC markers in this tissue type.

Chapter 5 Osteogenic Differentiation of Osteoporotic Periodontal Stem Cells

5.1 Introduction:

Osteogenic differentiation is a process by which mesenchymal stromal/stem cells differentiate to osteoblasts. It is an essential process during bone development, repair and remodelling (Smith and Eliseev, 2021). In the oral and maxillofacial region, bone defects caused by trauma, diseases (e.g. periodontal disease), or surgical treatments can result in functional impairment as well as esthetic and psychological challenges (Fujii et al., 2023). Using bone regenerative modalities for treatment have been promising, and PDLSCs have been proven effective in the repair of periodontal defects through their periodontal regenerative capacities (Jin et al., 2022, Iwayama et al., 2022). Also, systemic disease can affect the ability of stem cells to regenerate bone, for example, in osteoporosis (OP), BMSCs exhibit a reduced capacity to differentiate into osteoblasts and become more inclined to differentiate into adipocytes (Hu et al., 2018). However, there is limited literature on how OP impacts the differentiation of PDLSCs, hence it is essential to investigate this to enhance our understanding of the disease mechanism and its effect on the progress of periodontal disease and alveolar bone regeneration in this group of patients. Furthermore, this can inform the design of regenerative therapies that aim to improve the clinical outcomes for osteoporotic patients. This chapter will investigate the effect of OP on the expression of some of the key osteogenic markers (*RUNX2, ALPL*, *Col*$I\alpha1$, *POSTN, OCN*), as well as bone remodelling markers of the *RANK*, *RANKL*, *OPG* pathway, in comparison to their expression in healthy individuals.

5.2 Results:

5.2.1 Comparing alkaline phosphatase (ALP) staining in OP-PDLSCs to H-PDLSCs:

ALP staining (methods explained in Chapter 3, section 3.2.9) was carried out to assess initial osteoblastic differentiation. When compared to H-PDLSCs, the qualitative assessment of ALP staining for OP-PDLSCs revealed less intense staining across all time points (2, 3 and 4 weeks) and culturing conditions (basal

and osteogenic). With the progress of culture, there was a slight increase in staining after 4 weeks in OP-PDLSCs under osteogenic conditions, however, still much less staining compared to H-PDLSCs (Figure 31).

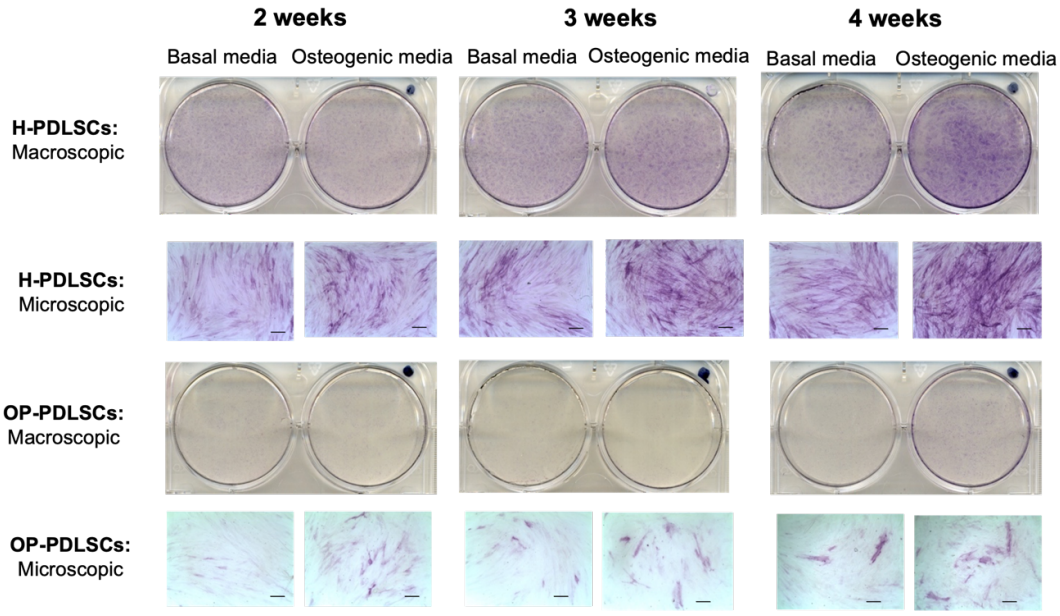


Figure 31. Comparing ALP staining for OP-PDLSCs versus H-PDLSCs (representative donors)

Macroscopic and microscopic images of ALP staining in H-PDLSCs and OP-PDLSCs cultured under basal and osteogenic conditions at 2, 3 and 4 weeks. Scale bar=100μm.

5.2.2 Comparing alizarin red staining (ARS) and mineralisation nodules quantification in OP-PDLSCs to H-PDLSCs:

The capacity of healthy and osteoporotic PDLSCs to deposit minerals was assessed using ARS and ARS quantification (method details in section 3.2.10). Qualitative assessment of the stain indicated unnoticeable response from the OP-PDLSCs to osteogenic differentiation media when compared to basal media (Figure 32. A). Microscopic assessment showed fewer and smaller mineralisation nodules deposited by OP-PDLSCs compared to H-PDLSCs under both culture conditions and time points.

Additionally, mineral quantification for OP-PDLSCs under basal and osteogenic conditions after 3 and 4 weeks revealed lesser amount of mineral deposition than that of H-PDLSCs (Figure 32. B). The mean mineral concentration for OP-

PDLSCs (n=3) remained the same across all 3 time points under osteogenic conditions (0.01±0.004 mM), indicating lack of response from the OP-PDLSCs to osteogenic induction. Whereas, for H-PDLSCs (n=4) under osteogenic conditions, the mean mineral concentration increased with time: for example, the mean was 0.014±0.012mM after 2 weeks and 0.04±0.04 mM after both 3 weeks and 4 weeks.

To study the effect of OP on mineralisation statistically, the analysis was carried out between OP-PDLSCs and H-PDLSCs under the same conditions and time points using unpaired t-test or Mann-Whitney test (depending on normality) and indicated no significant difference. However, there was a trend of lower mineralisation for OP-PDLSCs especially after 3 and 4 weeks.

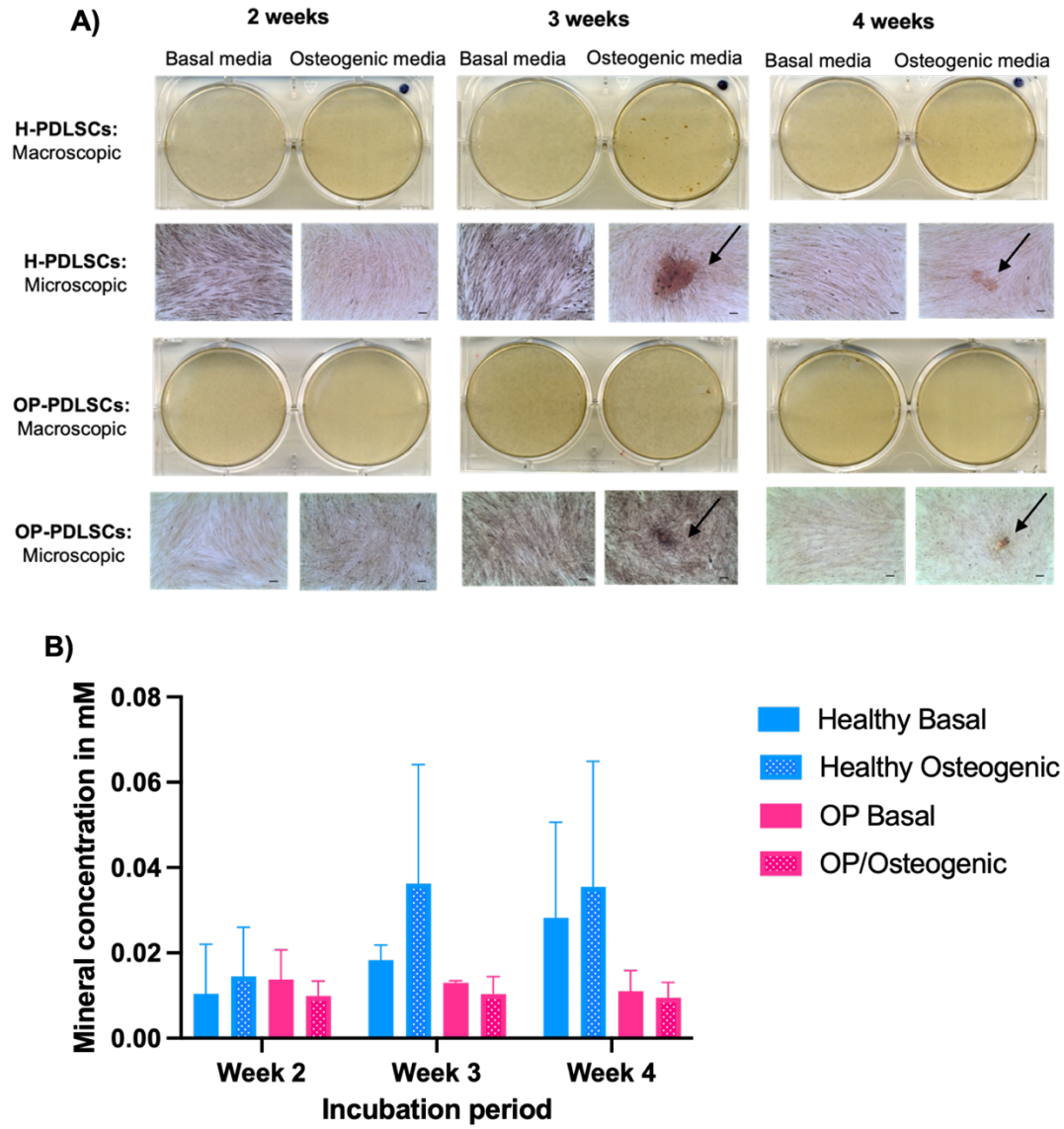


Figure 32. Comparing ARS and quantification for OP-PSLSCs vs H-PDLSCs.

Macroscopic and microscopic assessment of ARS for H-PDLSCs (n=4) and OP-PDLSCs (n=3) under basal and osteogenic conditions after 2, 3 and 4 weeks with arrows indicating mineralization nodules (representative donors) (A) Scale bar= 100μm. Quantification for ARS for donors from OP-PDLSCs (n=3) and H-PDLSCs (n=4) (B). Data presented as means ± SD.

5.2.3 Comparing relative gene expression of osteogenic/ osteogenesis-promoting marker genes in OP-PDLSCs to H-PDLSCs:

The following results represent the relative change in gene expression values using $2^{-\Delta Ct}$ equation where genes of interest were normalised to *HPRT1* house-keeping gene (the experiment internal control). Experiments were carried out using H-PDLSCs (n=4) and OP-PDLSCs (n=3) cultured in basal and osteogenic conditions for 2, 3 and 4 weeks. Details on RT-qPCR method is discussed in (section 3.2.11).

Statistical analysis was carried out using unpaired t-test or Mann-Whitney test depending on the data distribution for each time point and culture condition individually. The following results will focus on comparing the expression in OP-PDLSCs to H-PDLSCs at the three time points and culture condition. It will also focus on the variation on the expression of genes in OP-PDLSCs over the culture period.

5.2.3.1 RUNX2

Under basal conditions, relative gene expression for *RUNX2* in OP-PDLSCs was lower at 2 and 4 weeks compared to healthy controls, with comparable levels at week 3. Levels increased at 3 weeks then lowered at 4 weeks compared to 2 weeks in OP-PDLSCs (Figure 33. A).

Under osteogenic conditions, expression of *RUNX2* was slightly lower in OP-PDLSCs compared to H-PDLSCs with a close to consistent levels over the culture period in OP-PDLSCs (Figure 33 .B).

There was no statistically significant difference between healthy and osteoporotic groups at the different time points and culture conditions assessed.

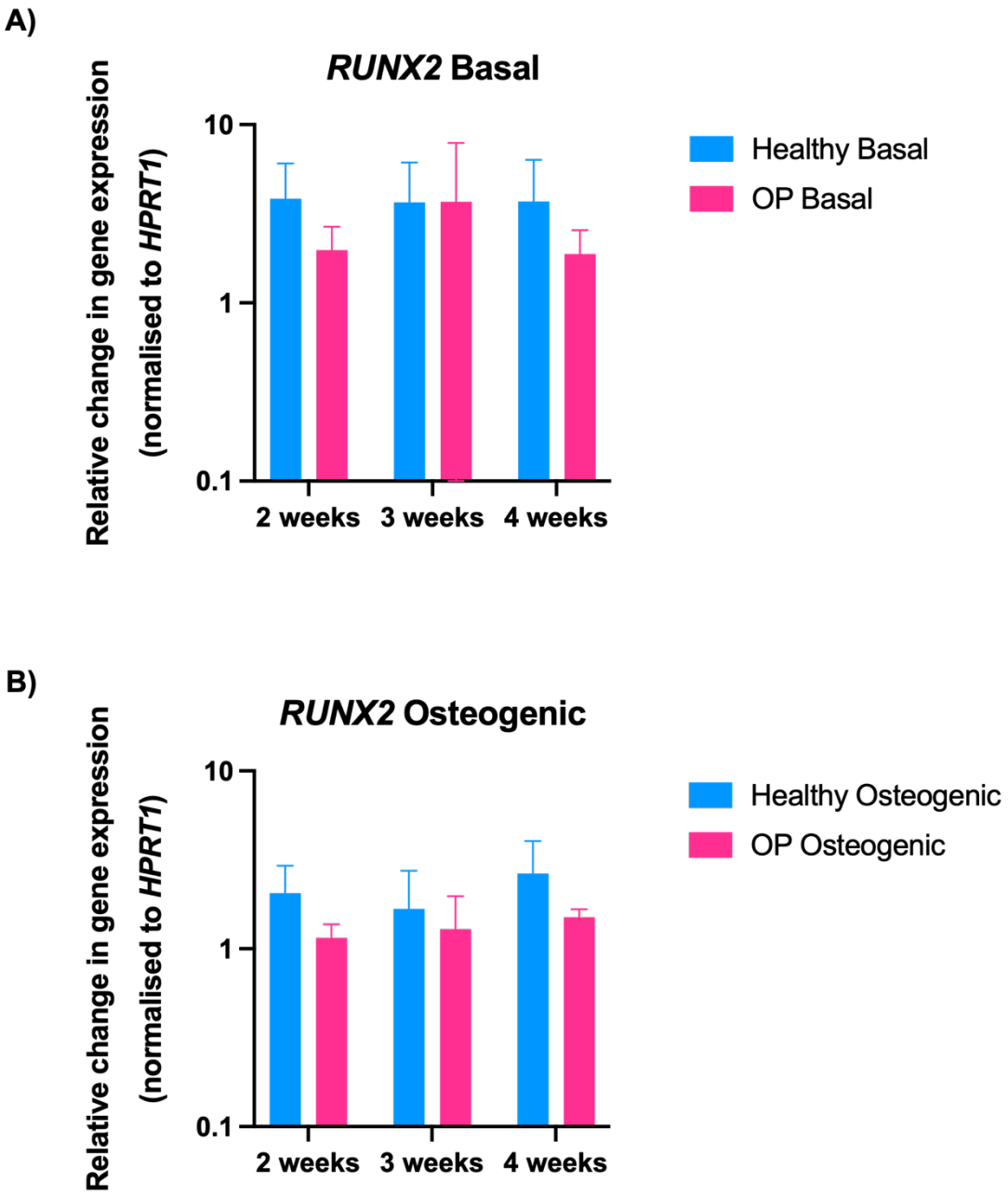


Figure 33. Relative *RUNX2* gene expression for OP-PDLSCs compared to H-PDLSCs

The relative expression of *RUNX2* normalised to *HPRT1* gene. Values plotted for OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic (B) conditions at 2, 3 and 4 weeks. Data presented as means± SD.

5.2.3.2 ALPL

Under basal conditions, relative *ALPL* gene expression in the OP-PDLSCs was comparable to healthy controls at 2 weeks, slightly higher at 3 weeks and slightly lower at 4 weeks. The expression of *ALPL* levels in OP-PDLSCs remained stable with the progress of culture with a slight decrease at 4 weeks (Figure 34. A).

Under osteogenic conditions, relative gene expression levels were slightly higher at 2 weeks, slightly lower at 3 weeks in OP-PDLSCs compared to H-PDLSCs, while the levels were comparable at 4 weeks between the groups. Over the culture period, levels in OP-PDLSCs were similar at 3 weeks, slightly higher at 4 weeks compared to 2 weeks (Figure 34. B).

There was no statistically significant difference between healthy and osteoporotic groups at all time points and culture conditions.

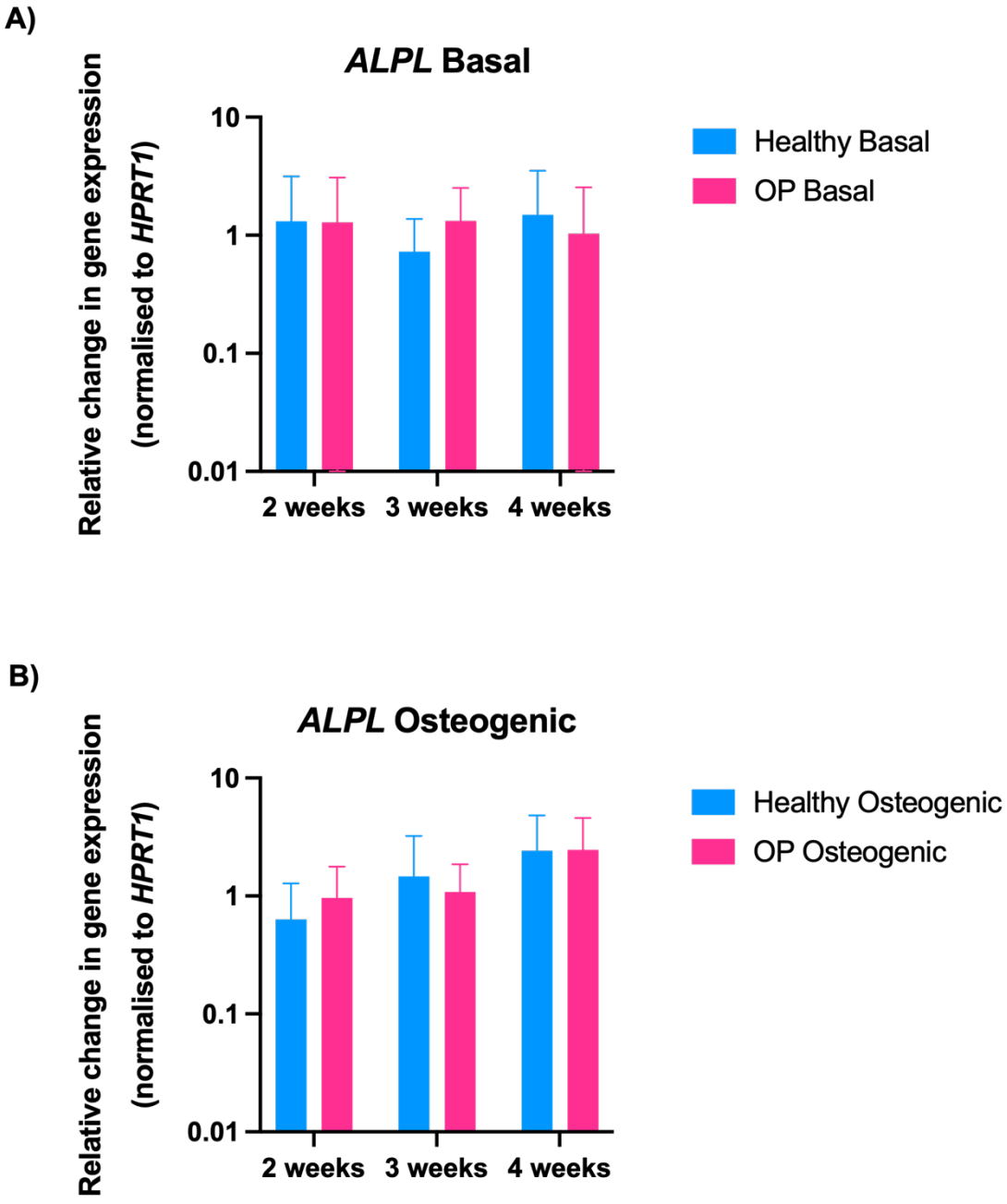


Figure 34. Relative *ALPL* gene expression for OP-PDLSCs compared to H-PDLSCs.

The relative expression of *ALPL* normalised to *HPRT1* gene. Values plotted for OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

5.2.3.3 Collα1

Under basal conditions, *Collα1* expression levels were lower in OP-PDLSCs compared to healthy controls. Levels were close to consistent in OP-PDLSCs with the progress of culture (Figure 35. A).

Under osteogenic conditions, *Collα1* expression levels for OP-PDLSCs were slightly higher at 2 weeks, considerably lower at 3 weeks and lower at 4 weeks in OP-PDLSCs compared to H-PDLSCs. Gene expression levels decreased at 3 weeks then elevated to a similar level at 4 weeks compared to 2 weeks in OP-PDLSCs (Figure 35. B).

There was no statistically significant difference between healthy and osteoporotic groups when the gene expression was assessed at all time points and culture conditions.

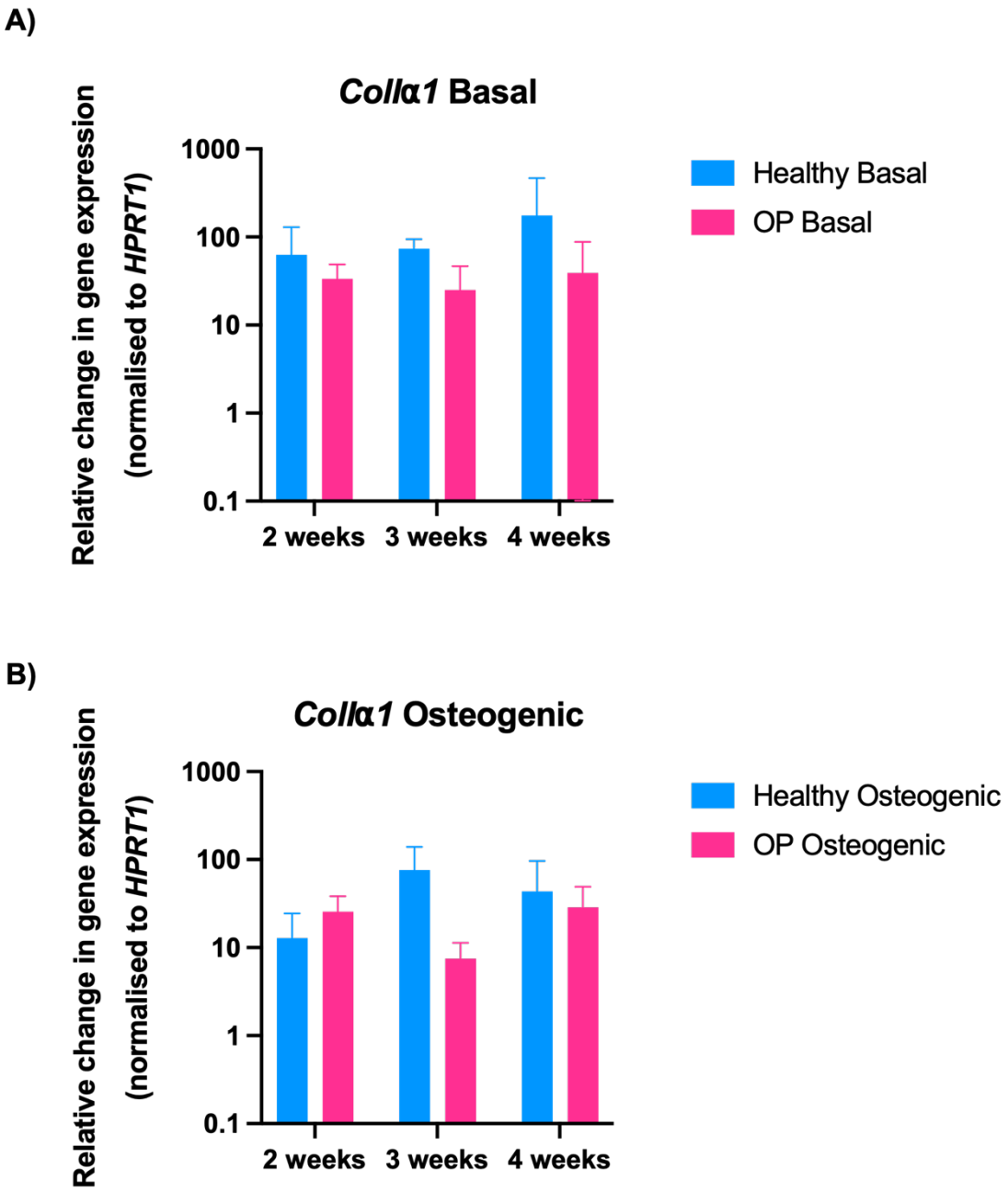


Figure 35. Relative *Collα1* gene expression for OP-PDLSCs compared to H-PDLSCs

The relative expression of *Collα1* normalised to *HPRT1* gene. Values plotted for OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal and osteogenic conditions at 2, 3 and 4 weeks. Data presented as means± SD.

5.2.3.4 POSTN

Under basal conditions, *POSTN* expression levels in OP-PDLSCs were lower at 2 and 4 weeks and slightly higher at 3 weeks compared to H-PDLSCs. Levels elevated at 3 weeks then slightly decreased at 4 weeks in OP-PDLSCs with the progress of culture (Figure 36. A).

Under osteogenic conditions, in OP-PDLSCs, *POSTN* showed a trend of consistently lower expression compared to healthy controls but remained relatively the same with the progress of culture within the osteoporotic group (Figure 36. B). A statistically significant difference was found under osteogenic conditions at 4 weeks, using unpaired t-test, between OP-PDLSCs and H-PDLSCs ($p=0.02$).

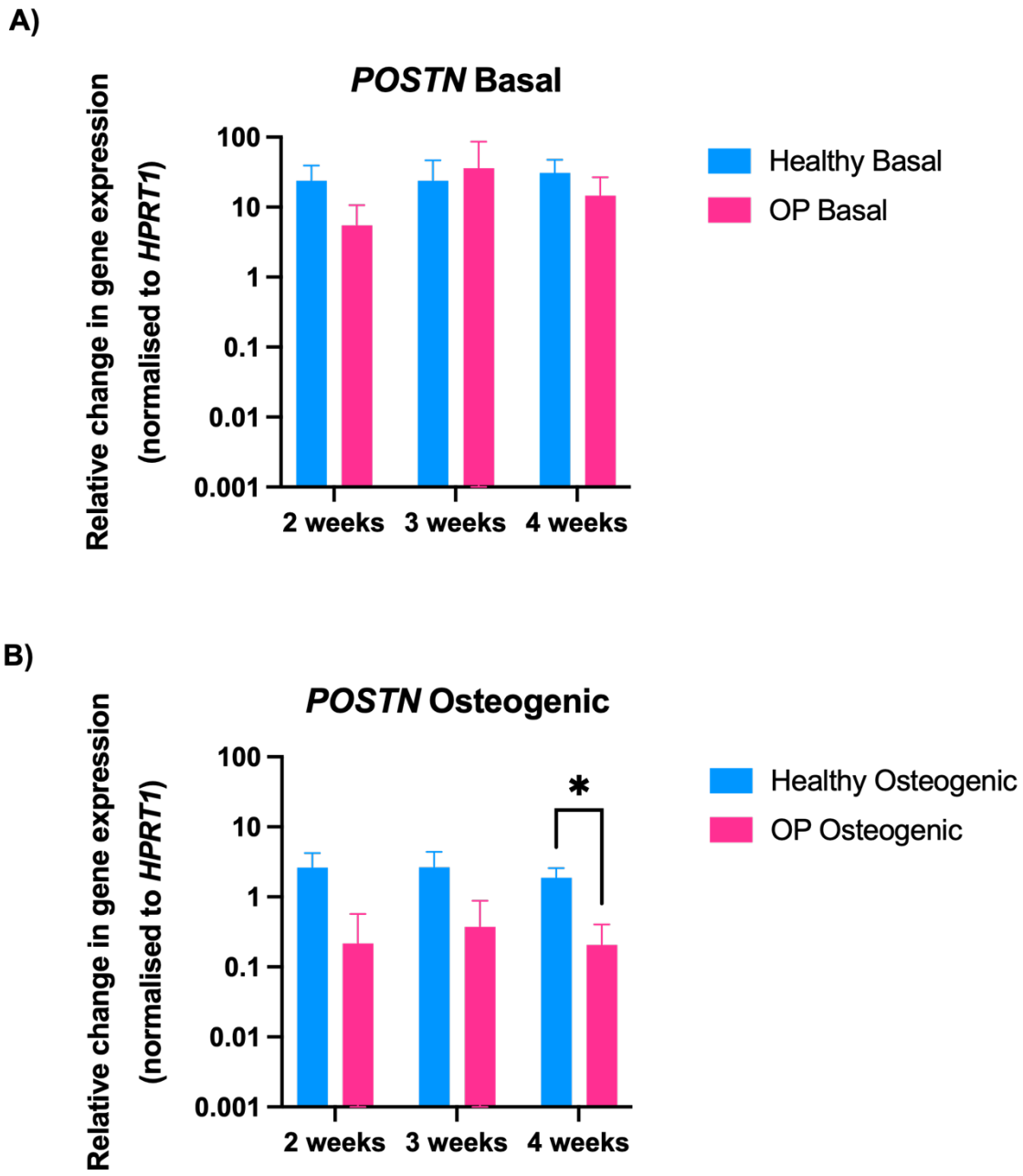


Figure 36. Relative *POSTN* gene expression for OP-PDLSCs compared to H-PDLSCs

The relative expression of *POSTN* normalised to *HPRT1* gene. Values plotted for OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic (B) conditions at 2, 3 and 4 weeks. Data presented as means± SD. The asterisk * indicates a statistical significant difference ($P < 0.05$).

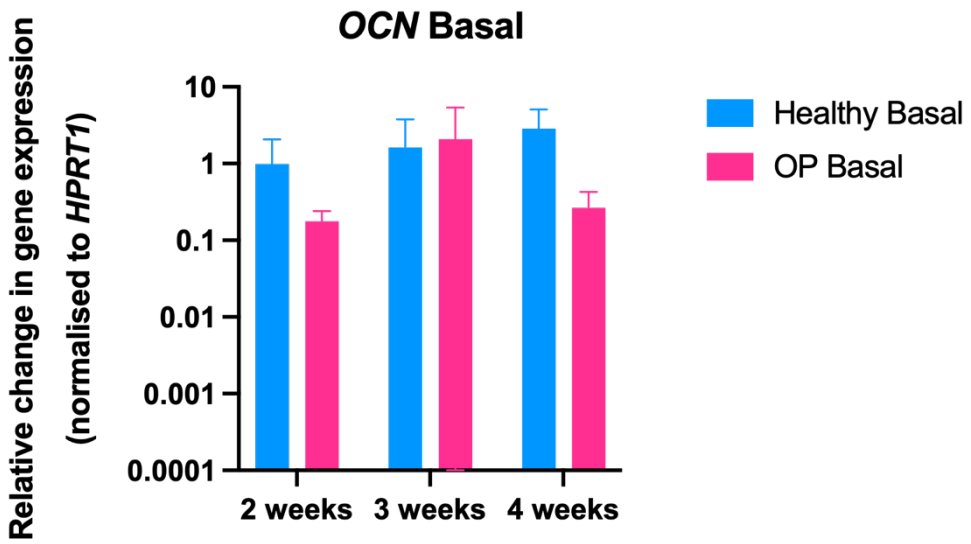
5.2.3.5 OCN

Under basal conditions, gene expression for *OCN* in OP-PDLSCs was lower at 2 and 4 weeks compared to healthy controls, with comparable levels at week 3. Levels elevated at 3 weeks then dropped to a similar level to 2 weeks at the 4th week in OP-PDLSCs with the progress of culture (Figure 37. A).

Under osteogenic conditions, expression levels for *OCN* were slightly lower in OP-PDLSCs compared to H-PDLSCs. With the progress of culture, expression values for *OCN* remained the same in the osteoporotic group (Figure 37. B).

There was no statistically significant difference between healthy and osteoporotic groups when gene expression was assessed at all time points and culture conditions.

A)



B)

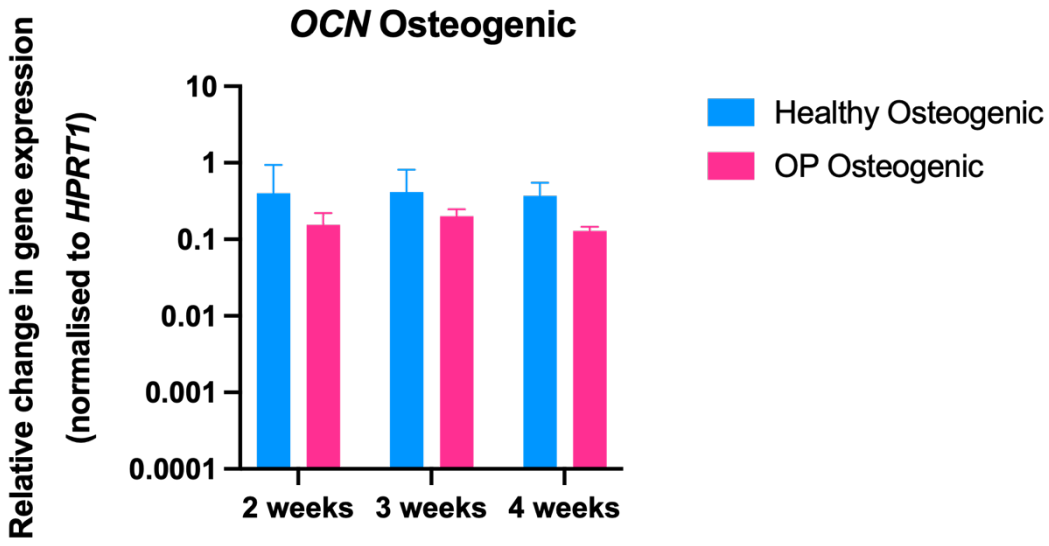


Figure 37. Relative *OCN* gene expression for OP-PDLSCs compared to H-PDLSCs.

The relative expression of *OCN* normalised to *HPRT1* gene. Values plotted for OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic (B) conditions at 2, 3 and 4 weeks. Data presented as means± SD.

5.2.4 Relative gene expression of bone remodelling genes in OP-PDLSCs compared to H-PDLSCs:

5.2.4.1 RANK

Under basal conditions, relative expression levels of *RANK* were comparable between the cell groups, and they remained close to constant in OP-PDLSCs with the progress of culture (Figure 38. A). The expression of only one of the 3 osteoporotic donors was detectable at 4 weeks.

Under osteogenic conditions, the expression pattern showed a trend of higher expression levels in OP-PDLSCs compared to healthy controls at all time points. An increase in *RANK* expression levels was observed at 3 and 4 weeks compared to 2 weeks in OP-PDLSCs (Figure 38. B).

There was no statistically significant difference between the expression in OP-PDLSCs and H-PDLSCs at all time points and culture conditions. However, there was a notable trend of higher expression in OP-PDLSCs versus H-PDLSCs at all time points when cells were cultured in osteogenic media.

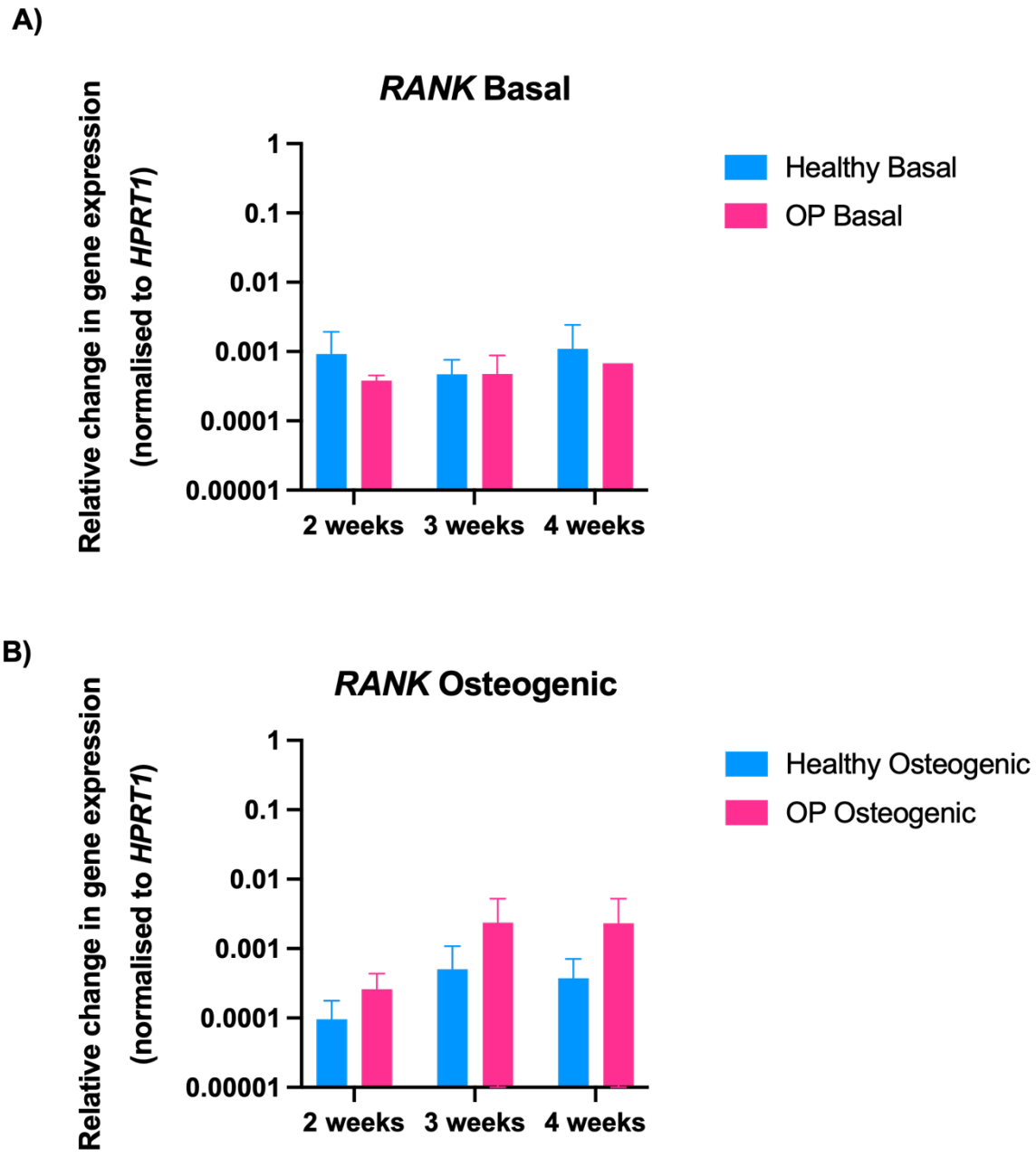


Figure 38. Relative *RANK* gene expression for OP-PDLSCs compared to H-PDLSCs

The relative expression of *RANK* normalised to *HPRT1* gene. Values plotted for OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal and osteogenic conditions at 2, 3 and 4 weeks. Data presented as means± SD.

5.2.4.2 RANKL

RANKL expression was very low in both healthy and osteoporotic PDLSCs, with undetectable levels in the majority of the samples at 3 weeks (in H-PDLSCs under basal conditions and in OP-PDLSCs under osteogenic conditions). Under basal conditions, the expression values for *RANKL* in OP-PDLSCs were lower than those in the healthy controls with the most evident decrease at 2 and 4 weeks (Figure 39. A). Similarly, under osteogenic conditions, the levels of *RANKL* expression were lower in the OP group compared to H-PDLSCs at all time points (Figure 39. B).

There was no statistically significant difference between the expression in both cell groups under basal or osteogenic conditions and under the different time points. Nonetheless, there was a trend of lower *RANKL* expression in OP-PDLSCs when compared to H-PDLSCs under all conditions and time points.

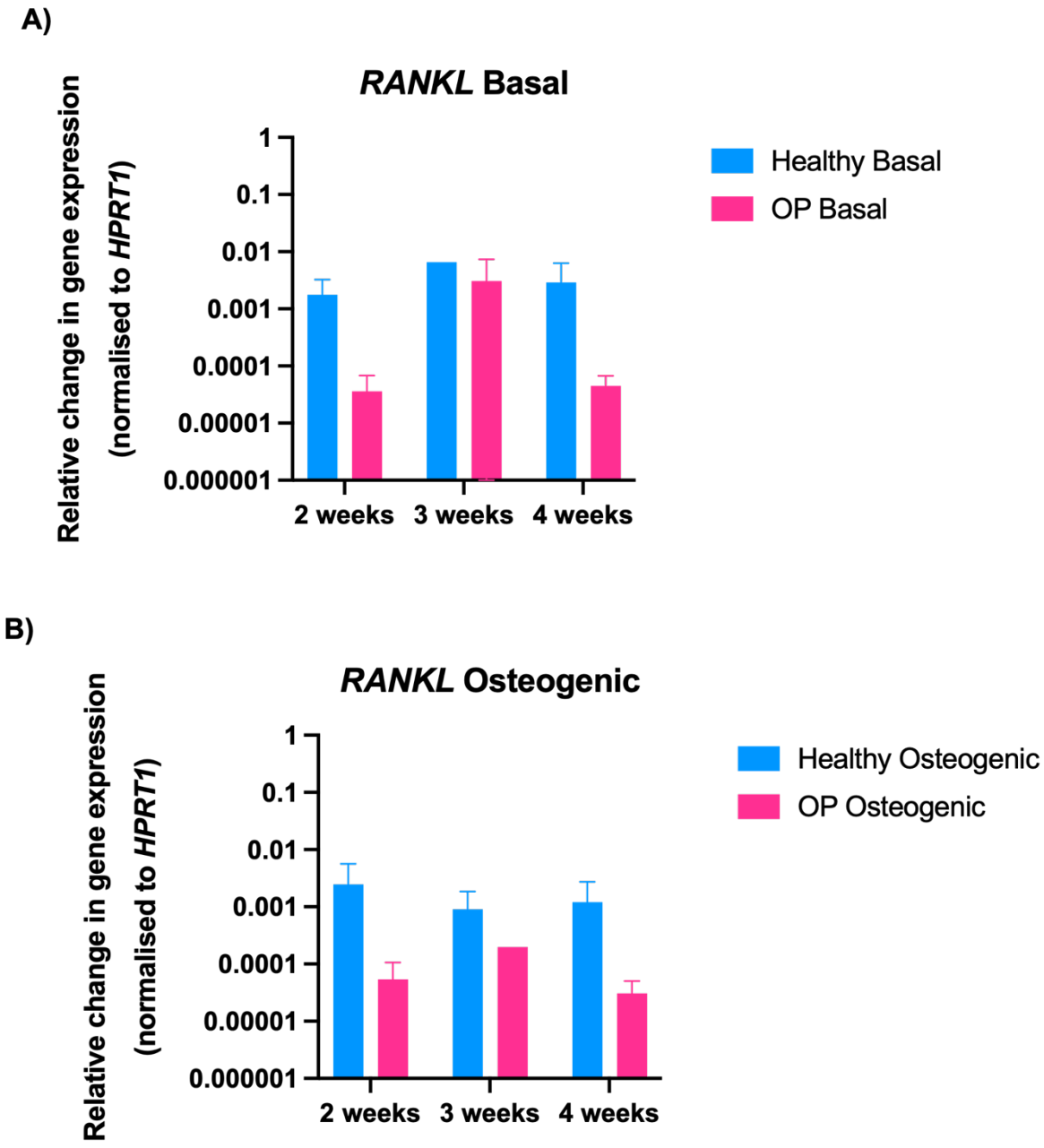


Figure 39. Relative *RANKL* gene expression for OP-PDLSCs compared to H-PDLSCs

The relative expression of *RANKL* normalised to *HPRT1* gene. Values plotted for OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal and osteogenic conditions at 2, 3 and 4 weeks. Data presented as means± SD.

5.2.4.3 OPG

Under both culture conditions, relative expression levels of *OPG* were higher in OP-PDLSCs group compared to H-PDLSCs at all time points with constant levels of gene expression in OP-PDLSCs with the progress of culture (Figure 40. A) (basal) and (Figure 40. B) (osteogenic).

Statistical analysis to compare the level of *OPG* expression in OP-PDLSCs vs H-PDLSCs was carried out using unpaired t-test. The difference was significant under basal conditions at 2 weeks ($p=0.02$) and under osteogenic conditions at 3 weeks ($p=0.04$) in OP-PDLSCs compared to H-PDLSCs.

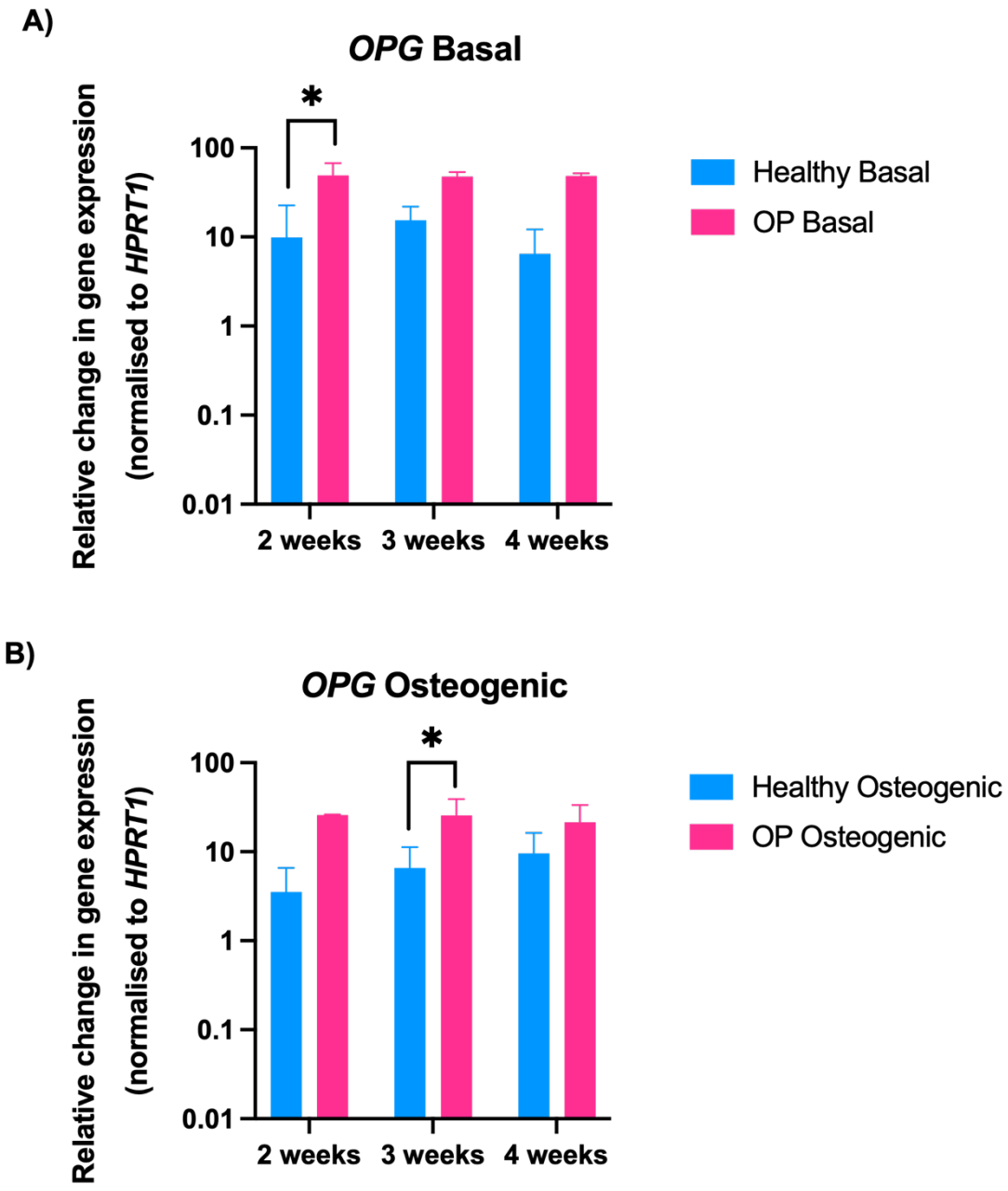


Figure 40. Relative *OPG* gene expression for OP-PDLSCs compared to H-PDLSCs

The relative expression of *OPG* normalised to *HPRT1* gene. Values plotted for OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic (B) conditions at 2, 3 and 4 weeks. Data presented as means± SD. The asterisk * indicates a significant difference ($P < 0.05$).

5.2.4.4 *RANKL/OPG* ratio

Under both culture conditions, *RANKL* to *OPG* ratio was surprisingly lower in OP-PDLSCs compared to H-PDLSCs at all time points (Figure 41. A) (basal) and (Figure 41. B) (osteogenic). This gives an indication that OP-PDLSCs may possibly have lower osteoclastic support activity compared to healthy controls.

There was no statistically significant difference between the *RANKL/OPG* ratio in OP-PDLSCs and H-PDLSCs at all time points and culture conditions. However, there was a notable trend of lower ratio in OP-PDLSCs versus H-PDLSCs.

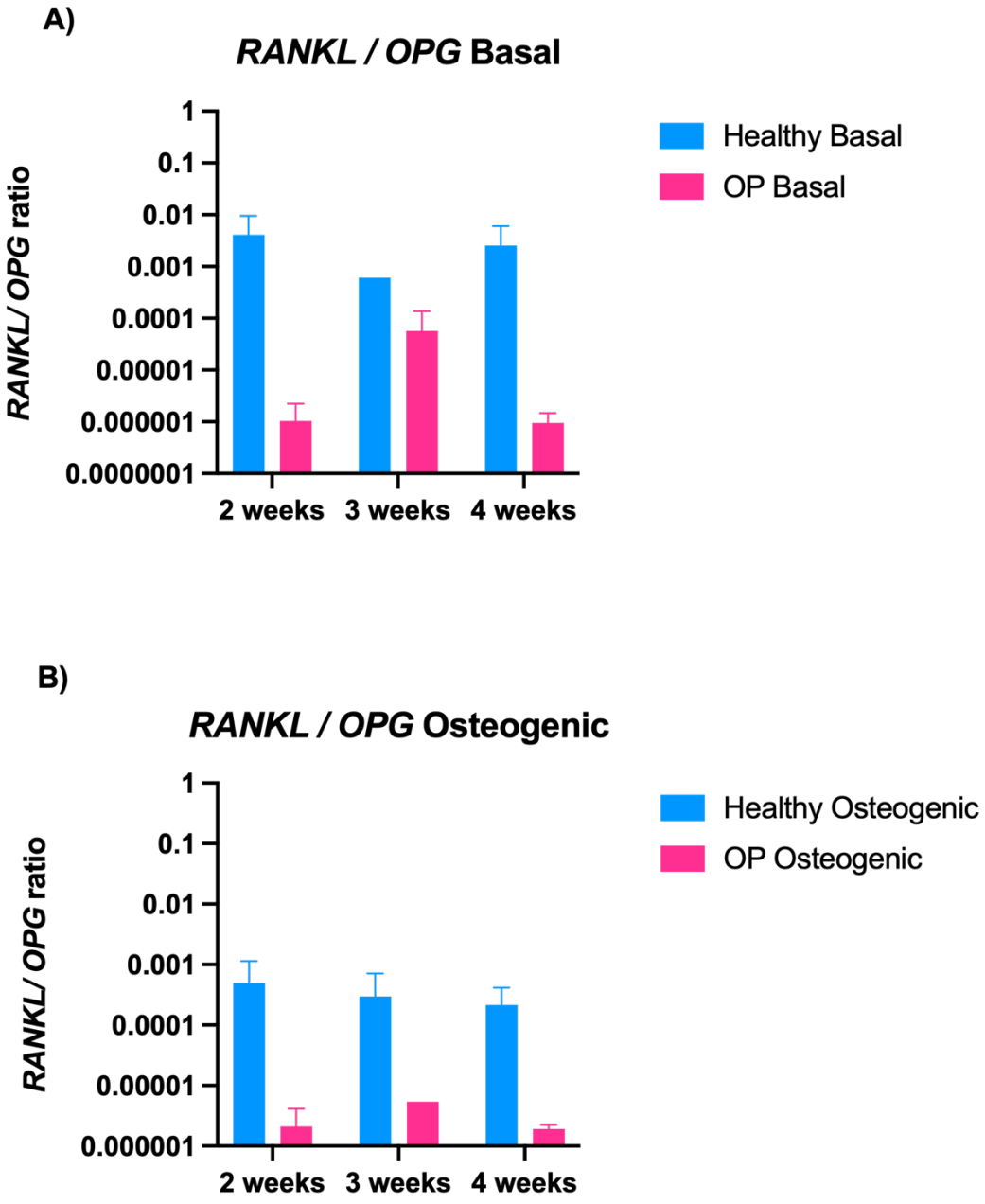


Figure 41. Relative expression values for *RANKL/OPG* ratio for OP-PDLSCs compared to H-PDLSCs

The relative expression values of *RANKL to OPG ratios* calculated by dividing each gene expression value (*RANKL*) in one group with the corresponding gene (*OPG*) value at the same time point in the same group. Values plotted for OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic (B) conditions at 2, 3 and 4 weeks. Data presented as means± SD.

5.3 Discussion:

This chapter presented data related to osteogenic differentiation of OP-PDLSCs compared to healthy controls (H-PDLSCs). ALP staining assay indicated a slightly lower osteoblastic differentiation tendecny, and, similarly, ARS quantification assay revealed a trend of a slightly lower mineralisation capacity in OP-PDLSCs compared to H-PDLSCs. Gene expression studies showed comparable to lower relative expression levels for *RUNX2, ALPL*, *Col*$I\alpha$*1, POSTN*, *OCN*, ERs and *RANK* (Basal) in OP-PDLSCs. However, higher levels for *RANK* (osteogenic) and *OPG* were reported in OP-PDLSCs in comparison to H-PDLSCs. Although other variables have been tested (i.e. time and culture media), the following section will focus on the potential effect of OP on the capacity of PDLSCs for osteogenic differentiation. Also, since there is very little or no literature regarding osteogenic differentiation of PDLSCs isolated from postmenopausal osteoporotic human patients, some comparisons in the discussion below will be based on findings from OVX animal models or BMSCs studies.

ALP enzyme assessed in the cells used in this study is an isoform of alkaline phosphatases known as tissue non-specific alkaline phosphatase (TNSALP). This enzyme is ubiquitous and plays essential roles in the mineralisation of hard tissue (Vimalraj, 2020). Additionally, ALP is a marker for osteoblast differentiation (Liu et al., 2021b) and contributes to the process of hydroxyapatite formation by providing phosphate ions and hydrolysing pyrophosphate (an inhibitor of bone matrix formation) (Štefková et al., 2015).

ALP staining was less intense in the osteoporotic group compared to H-PDLSCs. In a study conducted by Rodríguez et al. on human OP-BMSCs, quantifiable ALP activity under osteogenic conditions declined over a period of (4-16 days) compared to healthy controls (Rodríguez et al., 1999), which matches OP-PDLSCs findings within earlier time frames. A similar finding was also reported by Liu et al., where BMSCs from OVX rats showed less intense staining compared to sham rats after 7 days of osteogenic induction (Liu et al., 2021a). Moreover, although the staining was reduced in the OP-PDLSCs, there was a late increase in the ALP in the same group after 4 weeks. This could potentially indicate a delayed initiation of osteoblast differentiation. Since this assay is not

quantifiable, it was challenging to determine the exact difference in ALP activity between healthy and osteoporotic PDLSCs.

ARS calcium quantification results from this study showed a trend of reduced ability of OP-PDLSCs to mineralise in comparison to H-PDLSCs. Furthermore, osteoporotic samples showed no detectable response to osteogenic media in terms of mineral production throughout the 3 time points. This result was supported by a recent study where BMSCs extracted from OVX mice mandibles showed less mineral concentration in the osteoporotic samples compared to controls after 21 days of osteogenic induction (Cao et al., 2023).

Both ALP and AR staining results suggest a reduced osteogenic differentiation and mineralisation capacities of OP-PDLSCs. Since ALP is secreted by osteoblasts to increase the levels of inorganic phosphate and reduce pyrophosphate (Vimalraj, 2020), it seems that the decrease in ALP could have inhibited mineralised nodule formation by osteoporotic cells compared to healthy controls. Although cytochemical stains showed less mineralisation potential by OP-PDLSCs, it was necessary to investigate the changes occurring on a gene level for some of the key bone formation and remodelling genes, which will be discussed below.

Osteogenic and osteoclastogenic markers gene expression was compared in both healthy and OP-PDLSCs using RT-qPCR. *RUNX2*, *ALPL*, *Col*$I\alpha1$ and *POSTN* are early markers for osteoblast differentiation (Narayanan et al., 2019, Zainal Ariffin et al., 2022, Merle and Garnero, 2012) while *OCN* is a late osteoblastic marker (Huang et al., 2007). *RUNX2* also regulates several osteoblastic markers including *ALPL*, *Col*$I\alpha1$ and *OCN* (Bruderer et al., 2014, Thiagarajan et al., 2017).

RUNX2 is an essential transcription factor for osteoblast differentiation, deposition of matrix and mineralisation (Thiagarajan et al., 2017). It belongs to a family of transcription factors (RUNT) which modulates several cell processes including the development of multiple cell lineages and their differentiation (Tarkkonen et al., 2017). Reduced levels of *RUNX2* are highly associated with postmenopausal OP and breast cancer (Kim et al., 2020). The silencing of *RUNX2* gene by methylation may occur with the inhibition of ovulation leading to

reduced gene transcription, decreased protein translation and higher potential for OP (Yalaev et al., 2024). Hence, it is considered a target for OP treatment (Komori, 2022). The relative expression of *RUNX2* in OP-PDLSCs was lower compared to H-PDLSCs under all-time points and culture conditions (except for 3 weeks under basal conditions where the results were comparable). This is consistent with the significantly lower *RUNX2* expression levels observed in BMSCs of OVX rats after 28 days of culture under osteogenic conditions when compared to sham rats (Ren et al., 2020).

TNSALP mentioned earlier is encoded by *ALPL* gene of which mutations have been linked to skeletal diseases such as hypophosphatasia, which is characterised by low bone mass, and can lead to OP (Dong et al., 2024). OP-PDLSCs showed inconsistent *ALPL* expression under both culture conditions to that of healthy controls at all time points. A different trend was reported by Haddouti et al. (Haddouti et al., 2020) in human vertebral OP-BMSCs where comparable *ALPL* levels were reported at 1 and 2 weeks of osteogenic induction compared to healthy cells but were slightly higher at 3 weeks. The lack of consistency might require more samples to reach a clear conclusion. However, data presented in this chapter might be an indication that OP could potentially have a little impact on early stages of cell differentiation. The observed discrepancy with ALP staining compared to gene expression data can be explained by the fact that the former assay indicates ALP enzymatic activity whereas RT-qPCR measures *ALPL* transcript levels.

During bone formation, osteoblasts deposit bone matrix (osteoid) mainly by formation of collagen type I, which is encoded by *ColIα1* and *ColIα2* genes, where hydroxyapatite crystals will later be incorporated to form mineralised bone (Ben Shoham et al., 2016). Only *ColIα1* was investigated in this study. Interestingly, polymorphisms in this gene have been associated with low bone mass density and higher osteoporotic fractures in women (Peris et al., 2000). *ColIα1* relative expression was noticeably lower in osteoporotic cells compared to healthy controls (except for 2 weeks under osteogenic conditions where the expression was very slightly higher in the OP group). In OVX rats BMSCs, collagen I expression was reduced at all-time points compared to adipose stem cells, which is in line with the pattern of this gene expression in OP-PDLSCs (Boeloni et al.,

2014). In the Haddouti et al. study mentioned (Haddouti et al., 2020), the expression of *ColIα1* in human vertebral OP-BMSCs was compared to healthy controls at days 1, 3, 7, 14, and 21 after osteogenic induction. Unlike the results assessed here for OP-PDLSCs, the relative expression level for OP-BMSCs was comparable in both groups at all time points. This suggests that BMSCs might not be the optimal type of cells to be compared with PDLSCs due to the different locations and functions they serve in situ, emphasising the novelty of this study.

POSTN is an essential marker for the integrity of periodontal ligament due its role in the cross-linkage and distribution of ECM proteins in the periodontium (Du and Li, 2019). Additionally, *POSTN* is involved in osteoblast differentiation and collagen type I formation and its reduced levels predispose patients to OP (Pickering et al., 2023). Studies on *POSTN*-null mice showed increased osteoclastic activity (Rios et al., 2005). Overall, in this study, *POSTN* levels were reduced in the OP group compared to the healthy one (except for 3 weeks under basal conditions where the levels were slightly higher), and the differences reached a statistical significance at week 4 under osteogenic conditions. This is similar to the findings in BMSCs from OVX rat which showed significantly lower expression of *POSTN* after 7 days of osteogenic induction compared to sham rats (Liu et al., 2021a). The lower levels of this gene could be an indication of reduced periodontal regenerative capacity of OP-PDLSCs (Du and Li, 2019).

OCN is a mineralization-associated bone marker (Ikegame et al., 2019). It is secreted by osteoblasts and is essential to align apatite crystals with the collagen fibres in the bone matrix (Komori, 2022). Similar to *RUNX2*, *OCN* has shown lower expression in OP-DLSCs compared to H-PDLSCs at all time points and culture conditions (except for slightly higher levels at 3 weeks under basal conditions). In a study of alveolar BM osteoblasts derived from female OVX rats, the relative expression of *OCN* was significantly lower after 10 days of culture compared to healthy controls (Vargas-Sánchez et al., 2020) which is in line with the findings of this study.

On a patho-physiological level, bone resorption is governed by the change in balance of the activity of the *RANKL*/*RANK*/*OPG* axis. *RANKL* is secreted by

osteoblasts, fibroblasts, and activated T or B cells (Belibasakis and Bostanci, 2012). Once this ligand binds to its receptor (*RANK*) on pre-osteoclasts, it enhances their differentiation into mature osteoclasts which adhere to bone surface and initiate resorption through the activation of *NFκB1* and related osteoclastogenesis genes (Ono et al., 2020, Crockett et al., 2011). *OPG* acts as a decoy receptor for *RANKL* and prevents its binding to *RANK* and hence reduces the osteoclastic activity on bone (Tobeiha et al., 2020). Therefore, the ratio of *RANKL*/*OPG* indicates which process is likely to take the upperhand; the higher the ratio, the more likley bone resorption is and vice versa (Zhang et al., 2022a). In this study, *RANK* gene showed higher levels in the osteoporotic samples under osteogenic conditions indicating an upregulation of this receptor. However, interestingly, *RANKL* levels trended lower while *OPG* levels trended higher in OP-PDLSCs (statistically significant at 2 weeks under basal and 3 weeks under osteogenic conditions) compared to healthy controls suggestive of potentially lower *RANKL*/*OPG* ratio for the OP group. This is contrary to bone related *in vitro* and clinical reports where increased *RANKL*/*OPG* ratio was observed in OP-BMSCs (Wang et al., 2018) and in patients with low bone mineral density (Azizieh et al., 2019). Since *RANK*/ *RANKL*/ *OPG* pathway is commonly targeted with some anti-resorptive medications (Chen et al., 2021), a possible interpretation for the decreased *RANKL*/*OPG* in OP-PDLSCs could be that all donors were receiving one or more types of anti-osteoporosis medications (Table 17 in Chapter 3). As a bisphosphonate, alendronic acid acts as inhibitors for osteoclasts action (Hedvičáková et al., 2021).HRTs prevent the effects of postmenopausal estrogen deficiency on bone by inhibiting *RANKL* and increasing *OPG* (Zhivodernikov et al., 2023). Similarly, denosumab acts as a monoclonal antibody targeting *RANKL* leading to decreased tendency of bone resorption (Zhang et al., 2020). Intriguingly, denosumab was reported to enhance osteogenic differentiation in alveolar bone MSCs when used in physiological concentrations (Mosch et al., 2019). Whether the reduced level of *RANKL*/*OPG* in OP-PDLSCs is attributed to the effect of OP medications or rather an inherent feature of these cells remains unknown and warrants further exploration using samples from untreated but diagnosed postmenopausal osteoporotic donors.

It is also worth noting that some of samples were below the detection level for RANK (OP group under basal conditions at 4 weeks) and RANKL (healthy group

under basal and OP group under osteogenic conditions, both at 3 weeks) and the detectable values were very low. This variation of gene expression could be due to donors' variation and suggests the need to include more samples in the future and/ or use a more sensitive house-keeping gene and/or RANKL probe. It is also important to mention that some of the results here were compared to findings from animal studies, and while animal studies can give an insight into the different cellular features in the human body, they still do fundamentally vary because of the different species (Bracken, 2009).

In conclusion, results from this chapter indicated lower osteogenic differentiation and mineralisation of OP-PDLSCs compared to H-PDLSCs. Although RT-qPCR helped assess the gene expression levels, it didn't not provide sufficient information regarding PDLSCs behavior, and further studies need to take place to assess the potential translation of those genes into proteins and their role in impacting osteogenesis. Some of these studies will be discussed in the following chapter.

Chapter 6 Expression of Estrogen Receptors in OP-PDLSCs under Osteogenic Conditions

6.1 Introduction:

As discussed previously (2), one of the leading causes of OP in postmenopausal women is reduction of estrogen levels (Ji and Yu, 2015). Estrogen conducts its osteoporotective effects, either directly or indirectly, through the binding to estrogen receptors (ERs) namely ER-α, ER- β, which are nuclear, and G protein coupled receptor (GPR30), which is membrane-bound (Fuentes and Silveyra, 2019). In the periodontium, both nuclear receptors have been detected and shown to stimulate bone formation capacity by periodontal ligament cells through higher expression of ALP, osteocalcin, and mineralised nodules deposition (Shapiro and Freeman, 2014). GPR30 has also been detected in the periodontium and it was demonstrated that its function might be influenced by inflammatory cytokines such as IL-1β *(Luo et al., 2012b)*. This chapter aims to explore the gene expression of these receptors in OP-PDLSCs compared to H-PDLSCs to investigate their potential contribution to the reduced potential of OP-PDLSCs for bone formation.

6.2 Results:

6.2.1 Relative gene expression of estrogen receptor genes in OP-PDLSCs compared to H-PDLSCs:

6.2.1.1 Estrogen Receptor α

Under basal conditions, the relative expression of *ERα* in OP-PDLSCs was comparable at 2 and 3 weeks and slightly lower at 4 weeks compared to H-PDLSCs at all time points (Figure 42. A) while levels were comparable at all time points under osteogenic conditions (Figure 42. B). With the progress of culture, the expression levels in OP-PDLSCs didn't significantly change under both culture conditions.There was no statistically significant difference between healthy and osteoporotic groups at all time points and culture conditions.

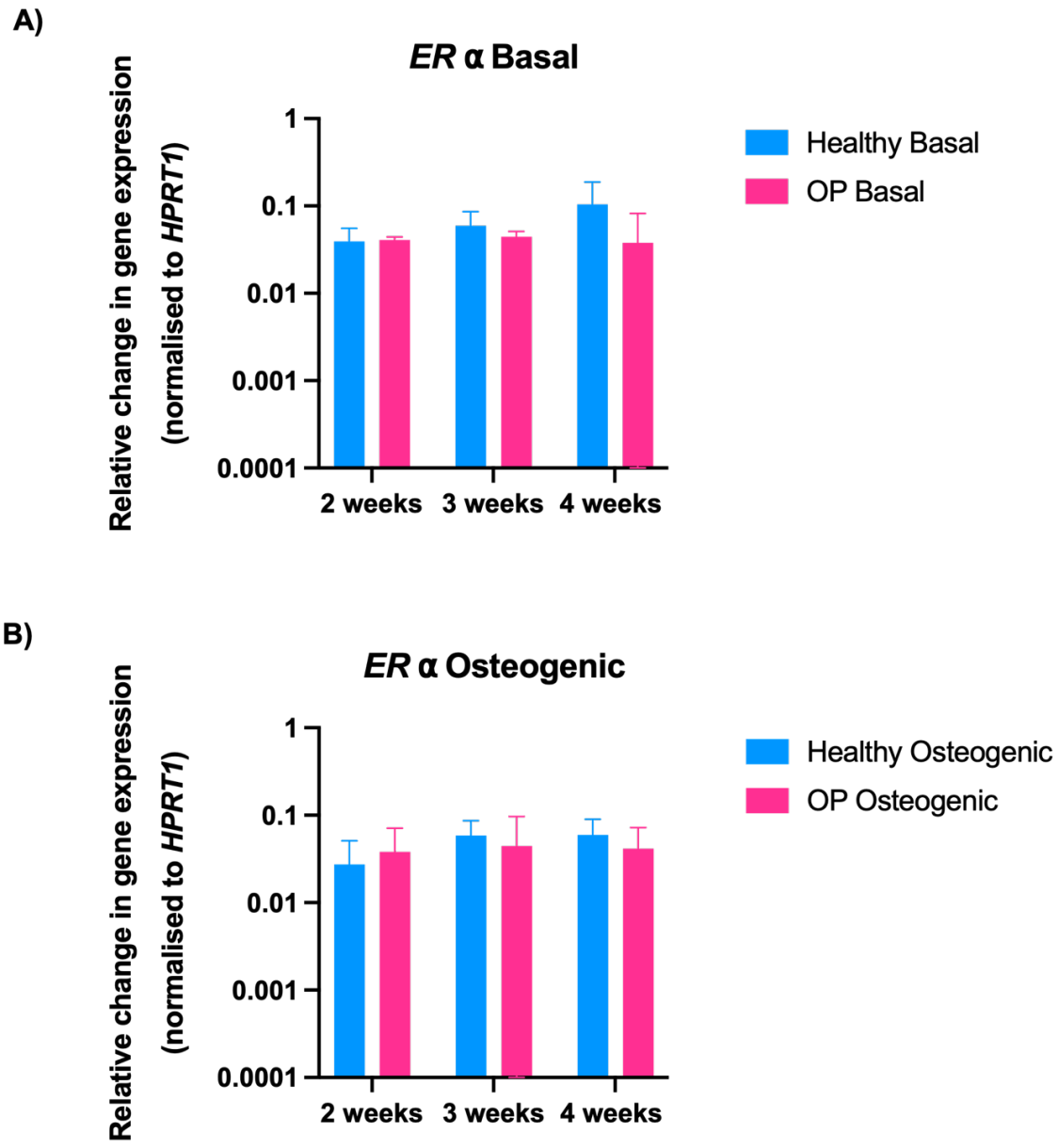


Figure 42. Relative *ERα* gene expression for OP-PDLSCs compared to H-PDLSCs

The relative expression of *ERα* normalised to *HPRT1* gene. Values plotted for OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic (B) conditions at 2, 3 and 4 weeks. Data presented as means± SD.

6.2.1.2 Estrogen Receptor β

Under basal conditions, the relative expression results for *ERβ* were comparable between the two groups with a slightly lower expression in the osteoporotic group at 2 and 4 weeks. Levels remained consistent across culture period in OP-PDLSCs (Figure 43. A).

Under osteogenic conditions, there was a noticeably lower expression at all time points in OP-PDLSCs compared to H-PDLSCs particularly after 3 weeks. As the time increased, compared to 2 weeks, levels of *ERβ* slightly decreased at 3 weeks then increased to a similar level at 4 weeks (Figure 43. B).

Statistical analysis between showed no significant difference between OP-PDLSCs and H-PDLSCs under both culture conditions and at the different time points. However, there was a consistently lower expression trend for OP-PDLSCs under osteogenic conditions compared to H-PDLSCs.

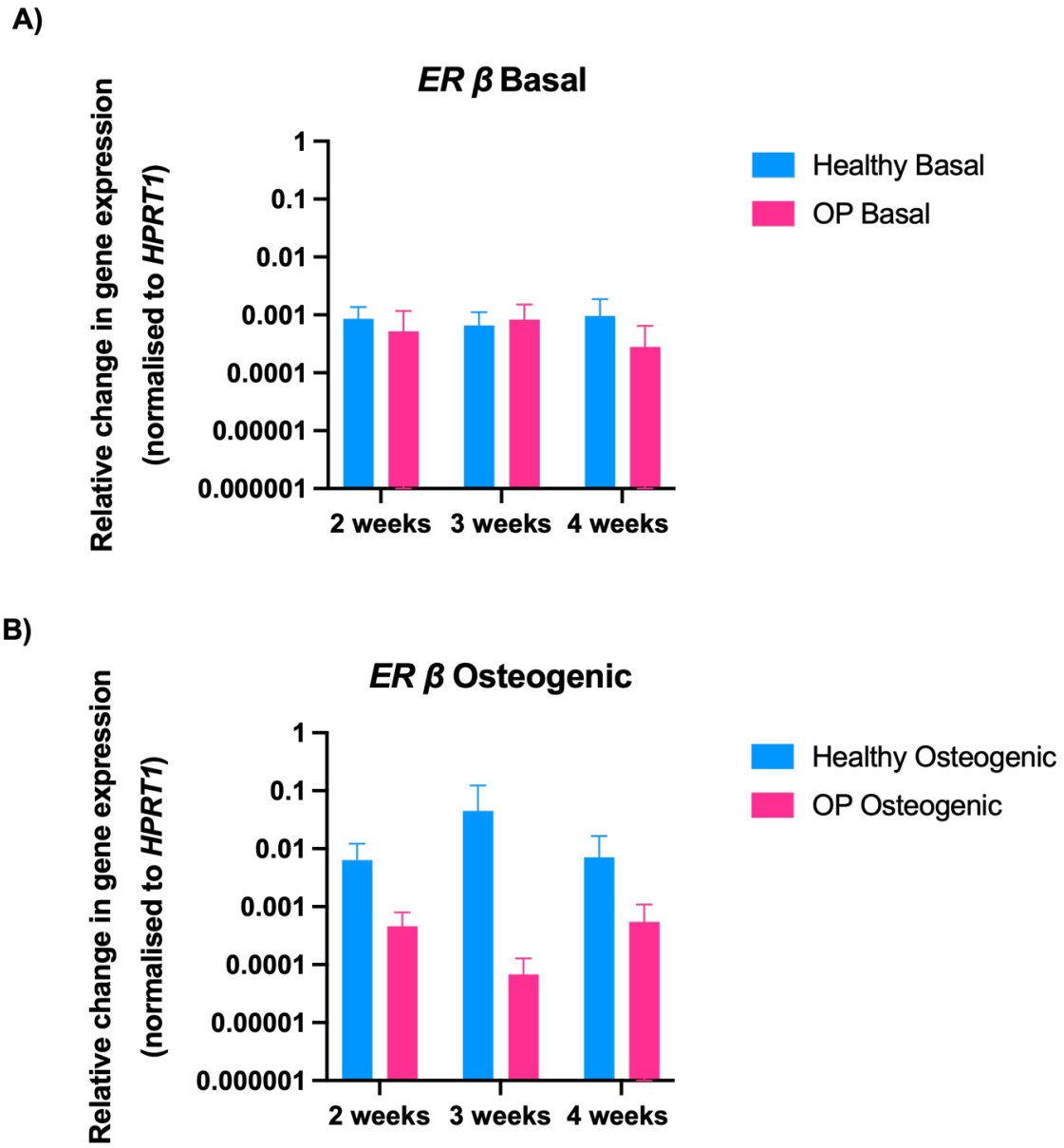


Figure 43. Relative *ERβ* gene expression for OP-PDLSCs compared to H-PDLSCs

The relative expression of *ERβ* normalised to *HPRT1* gene. Values plotted for OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal and osteogenic conditions at 2, 3 and 4 weeks. Data presented as means± SD.

6.2.1.3 GPR30

Under both culture conditions, OP-PDLSCs showed less *GPR30* expression compared to healthy controls at all time points. Over the culture period, values remained almost steady in OP-PDLSCs under basal conditions (Figure 44. A). While under osteogenic conditions, there was an increase in *GPR30* levels in OP-PDLSCs at 3 and 4 weeks compared to 2 weeks (Figure 44.B).

Statistical analysis between OP-PDLSCs and H-PDLSCs revealed no statistically significant difference under either of the culture conditions which was similarly observed when comparing time points within the same groups. Regardless of the lack of statistically significant difference, there was a trend of a notably lower *GPR30* expression in OP-PDLSCs under both culture conditions when compared to healthy controls.

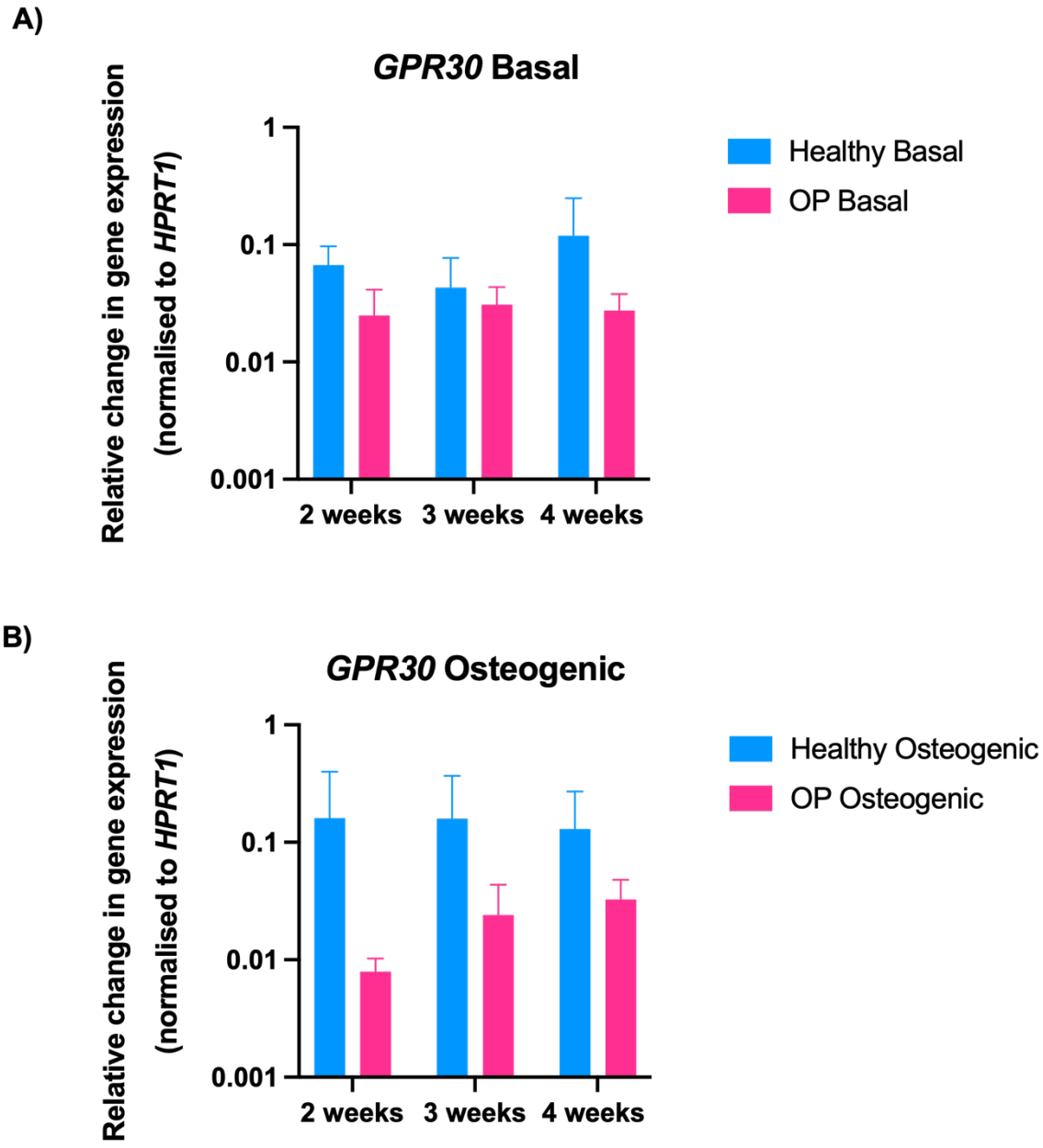


Figure 44. Relative *GPR30* gene expression for OP-PDLSCs compared to H-PDLSCs

The relative expression of *GPR30* normalised to *HPRT1* gene. Values plotted for OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal and osteogenic conditions at 2, 3 and 4 weeks. Data presented as means± SD.

6.3 Discussion:

In addition to already explored osteogenic differentiation markers (Chapter 5), the slightly reduced osteogenic differentiation capacity of OP-PDLSCs could be linked to the reduced estrogen levels in the osteoporotic donors. Results of a recent systematic review on the effect of estrogen deficiency on PDLSCs revealed that the reduction of estrogen level in postmenopuasal women negatively affected the osteogenic differentiation capacity of these cells (Di Naro et al., 2021). When the gene expression of estrogen receptors (ERs) was investigated in this study, OP-PDLSCs showed generally lower *ERβ* (particularly under osteogenic conditions) and *GPR30* expression than that of the healthy controls with similar levels for *ERα* in both groups. There is a controversial reporting in the literature for the expression of nuclear receptors (*ERα* and *ERβ*) in PDL cells (Tang et al., 2008, Cao et al., 2007, Jönsson et al., 2004). However, most reports suggest that estrogen induces osteogenic differentiation (Mamalis et al., 2011, Liang et al., 2008b) and has antiresorptive effects (Liang et al., 2008a) in these cells mainly through *ERβ*, similar to reports from dental pulp stem cells (Alhodhodi et al., 2017). It is important to note that two of the osteoportic donors were receiving Hormone Replacement Therapies (HRTs) before the isolation of PDLSCs, which might have influenced the expression of ERs in their periodontal tissues. Studying the expression of ERs in OP-PDLSCs in response to exogenous estrogen was beyond the scope of this project, so it is challenging to draw a direct correlation from the limited data available.

GPR30 is a transmembrane receptor mainly expressed in the endoplasmic reticulum and has been identified in multiple cells including PDLSCs (Luo et al., 2012a). It has been reported to mediate osteogenic differentiation changes in the periodontium through *GPR30*-PI3K-AKT-mTOR signalling pathway (Zhao et al., 2020). The gene expression of *GPR30* in OP-PDLSCs was lower than that of H-PDLSCs. Since data regarding *GPR30* expression in OP-PDLSCs are scarce, the exact mechanisms through which estrogen induces changes in these cells through this receptor requires further research.

Chapter 7 IGF Axis Expression in OP-PDLSCs under Osteogenic Conditions

7.1 Introduction:

Insulin-like Growth Factor (IGF) axis pathway is essential for several cellular processes including cell proliferation, migration, survival and differentiation (Al-Kharobi et al., 2014). The IGF axis is composed of two ligands (IGF-1 and IGF-2), two receptors (IGF-1R and IGF-2R) and six IGF binding proteins (IGFBPs) (Forbes et al., 2012) along with IGFBP proteases (Hjortebjerg, 2018). IGFBPs modulate the bioavailability of IGF ligands to receptors and hence their biological activity. IGFBPs can be classified according to their affinity as either high affinity (IGFBP 1-6) or low affinity, or IGFBP related proteins (IGFBP-rP 1-10) (Chen et al., 2024a).

In the context of bone, deletion of IGF-1 and IGF-1R in animal studies has resulted in skeletal malformation and low bone mineral density (BMD) (Greere et al., 2023). The observation of low BMD has also been linked to lower levels of IGF-1 in human serum and was associated with osteoporotic fractures (Chen et al., 2017). Additionally, elevated levels of IGFBP-4 have been associated with inhibition of IGF-1 osteogenic actions and this was modulated by IGFBP-4 protease (also known as Pregnancy-Associated Plasma Protein A (PAPP-A)). In a dental context, the IGF axis was proven to play a role in dental stem cells proliferation, differentiation and mineralisation (Bashir, 2021). Furthermore, previous reports have investigated the role of IGF axis members in the osteogenic and odontogenic differentiation of Human Dental Pulp Cells (hDPCs) (Al-Khafaji et al., 2018, Alkharobi et al., 2016) as well as its role in periodontal regeneration (Han et al., 2017, Liu et al., 2015). Nonetheless, the literature lacks reports on the involvement of the IGF axis in the periodontal regeneration of OP-PDLSCs posing a need to explore the potential impact of OP on IGF axis function since reduction of IGF ligands levels has been associated with lower BMD, reduced osteoprogenitors' differentiation in the skeleton (Crane et al., 2013), and potentially lower capacity to regenerate periodontal bone. Hence, this chapter will investigate the relative gene expression of key components of the IGF axis

including IGF ligands, receptors, IGFBPs 1-6, IGFBP-rP1 (also known as IGFBP-7), PAPP-A and its inhibitors (STC1, STC2) in OP-PDLSCs compared to H-PDLSCs. The chapter will also focus on analysing the protein expression for IGFBP-4 along with its protease (PAPP-A) to explore the potential inhibitory role of IGFBP-4 on IGF osteogenic action in OP-PDLSCs.

7.2 Results:

As discussed in (Chapter 3, section 3.2.11), the following section will describe the results of relative gene expression for the IGF axis and related components (normalized to *HPRT1*) under basal and osteogenic conditions at 3 time points (2, 3 and 4 weeks). The focus of this section will be on OP-PDLSCs results in comparison to H-PDLSCs, both at the individual time points and over the whole culture period.

7.2.1 Expression of IGF genes:

7.2.1.1 Expression of IGF ligands:

7.2.1.1.1 *IGF-1*

Under basal conditions, *IGF-1* relative expression was comparable at 2 weeks, and slightly higher at 3 and 4 weeks in OP-PDLSCs compared to healthy controls. There was a gradual increase in the levels of *IGF-1* with the progress of the culture (Figure 45. A).

Under osteogenic conditions, the levels were slightly lower at 2 and 3 weeks and slightly higher at 4 weeks in OP-PDLSCs compared to H-PDLSCs. There was also a gradual increase in the levels of *IGF-1* with the progress of the culture (Figure 45. B).

There was no statistically significant difference between healthy and osteoporotic groups at all time points and culture conditions.

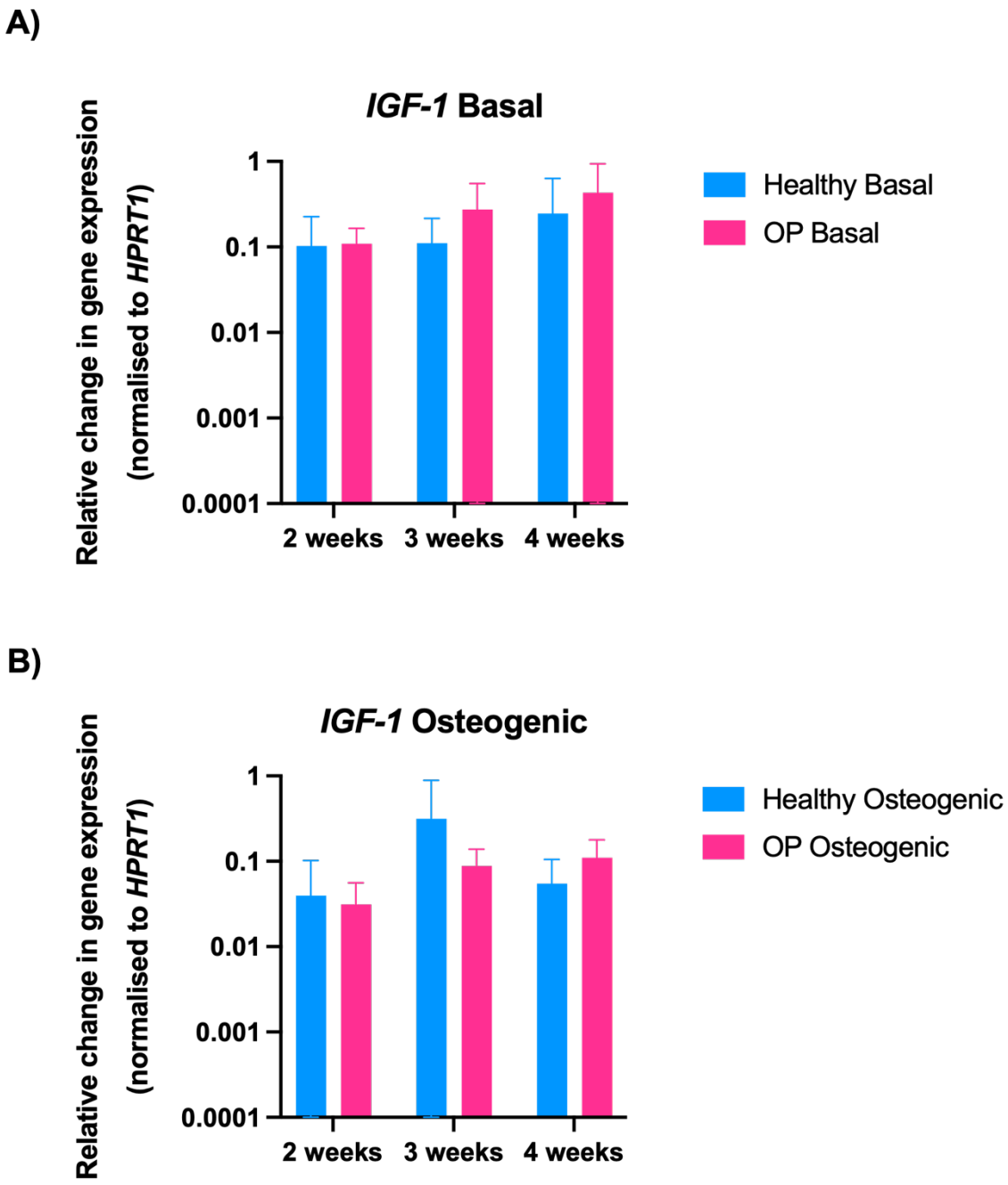


Figure 45. Relative *IGF-1* gene expression in OP-PDLSCs compared to H-PDLSCs.

The relative gene expression levels of *IGF-1* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

7.2.1.1.2 IGF-2

Under both culture conditions (basal and osteogenic), relative expression levels of *IGF-2* showed a trend of lower expression in OP-PDLSCs compared to H-PDLSCs. With the progress of the time in culture , *IGF-2* levels were higher at 3 and 4 weeks in OP-PDLSCs compared to 2 weeks under both culture conditions (Figure 46. A) (basal) and (Figure 46. B) (osteogenic).

Despite the consistent trend of lower IGF-2 expression in the OP-PDLSCs compared to the healthy, there was no statistically significant difference between the 2 cell populations at the different time points and culture conditions.

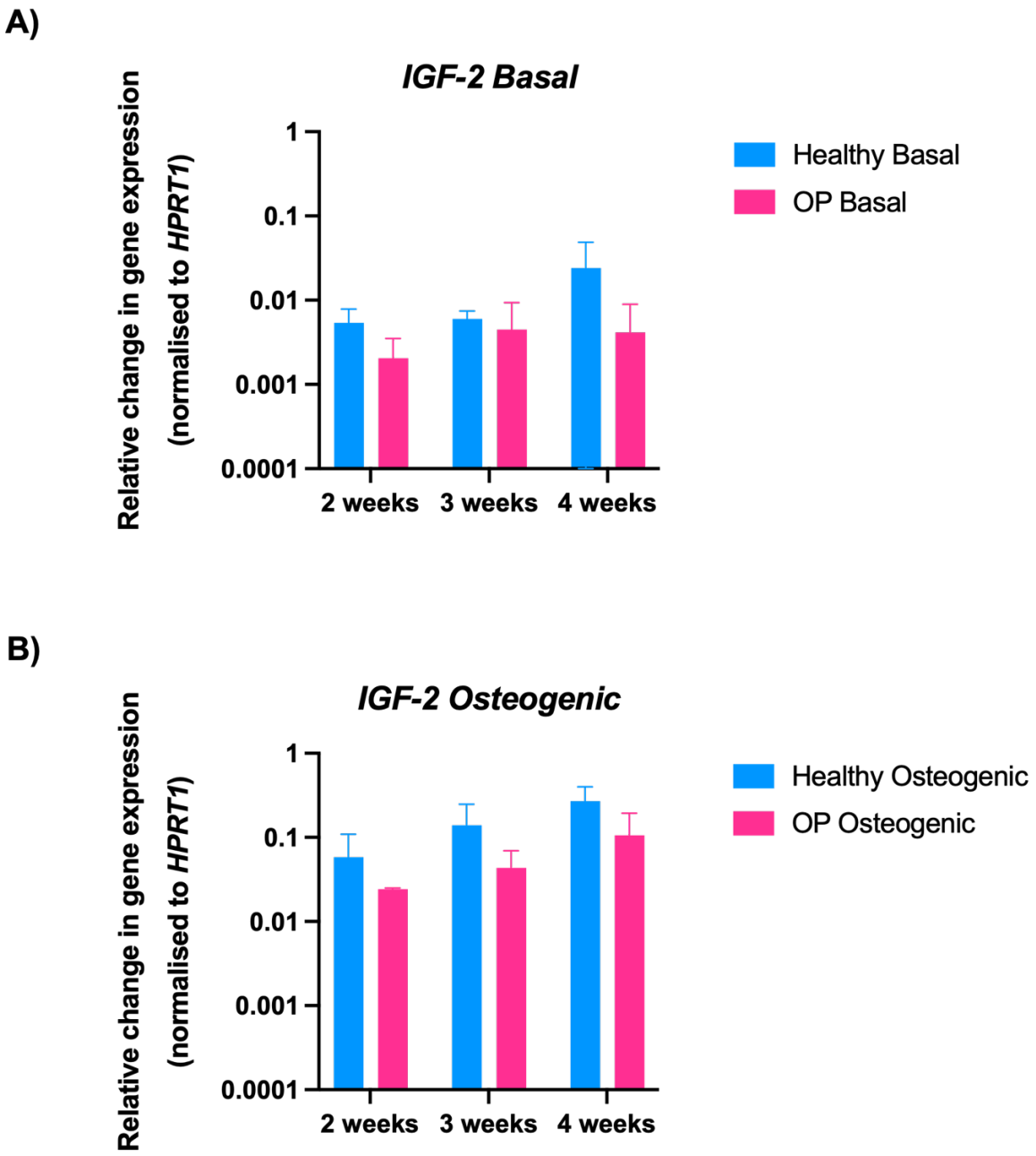


Figure 46. Relative *IGF-2* gene expression for OP-PDLSCs compared to H-PDLSCs.

The relative gene expression levels of IGF-*2* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

7.2.1.2 Expression of IGF receptor genes:

7.2.1.2.1 *IGF-1R*

Under basal conditions, relative expression levels for *IGF-1R* were lower at 2 weeks, slightly higher at 3 weeks then lower at 4 weeks in OP-PDLSCs compared to H-PDLSCs. Over the culture period, levels were slightly higher at 3 weeks then dropped at 4 weeks compared to 2 weeks within the osteoporotic group (Figure 47. A).

Under osteogenic conditions, the expression pattern changed. Levels were slightly higher in OP-PDLSCs at 2 weeks, then evidently dropped at 3 weeks then continued declining at 4 weeks compared to H-PDLSCs. As the culture time increased, *IGF-1R* levels were gradually decreasing within the OP group (Figure 47. B).

No statistically significant differences were observed in the gene expression, between the osteoporotic and healthy groups at all time points and culture conditions.

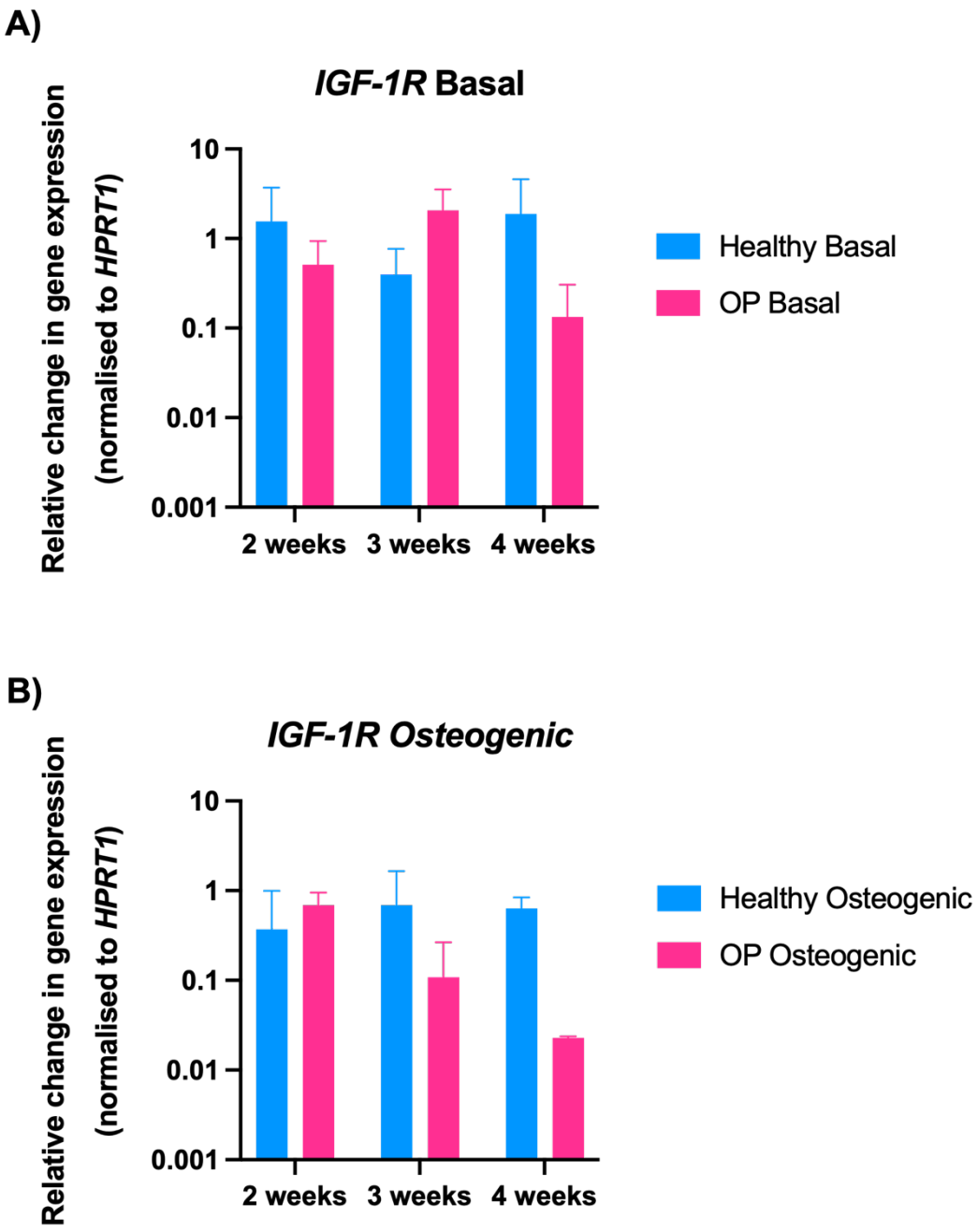


Figure 47. Relative *IGF-1R* gene expression for OP-PDLSCs compared to H-PDLSCs.

The relative change in gene expression of *IGF-1R* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

7.2.1.2.2 *IGF-2R*

Under basal conditions, levels of *IGF-2R* were lower at 2 and 3 weeks in OP-PDLSCs compared to H-PDLSCs then slightly increased to become comparable with H-PDLSCs at 4 weeks. Over the culture period, levels dropped for OP-PDLSCs at 3 weeks then slightly increased at 4 weeks compared to 2 and 3 weeks (Figure 48. A).

Under osteogenic conditions, *IGF-2R* levels were lower in OP-PDLSCs compared to H-PDLSCs at all time points. The gene expression remained somewhat constant throughout all-time points in OP-PDLSCs (Figure 48. B).

There was no statistically significant difference between healthy and osteoporotic groups at all time points and culture conditions.

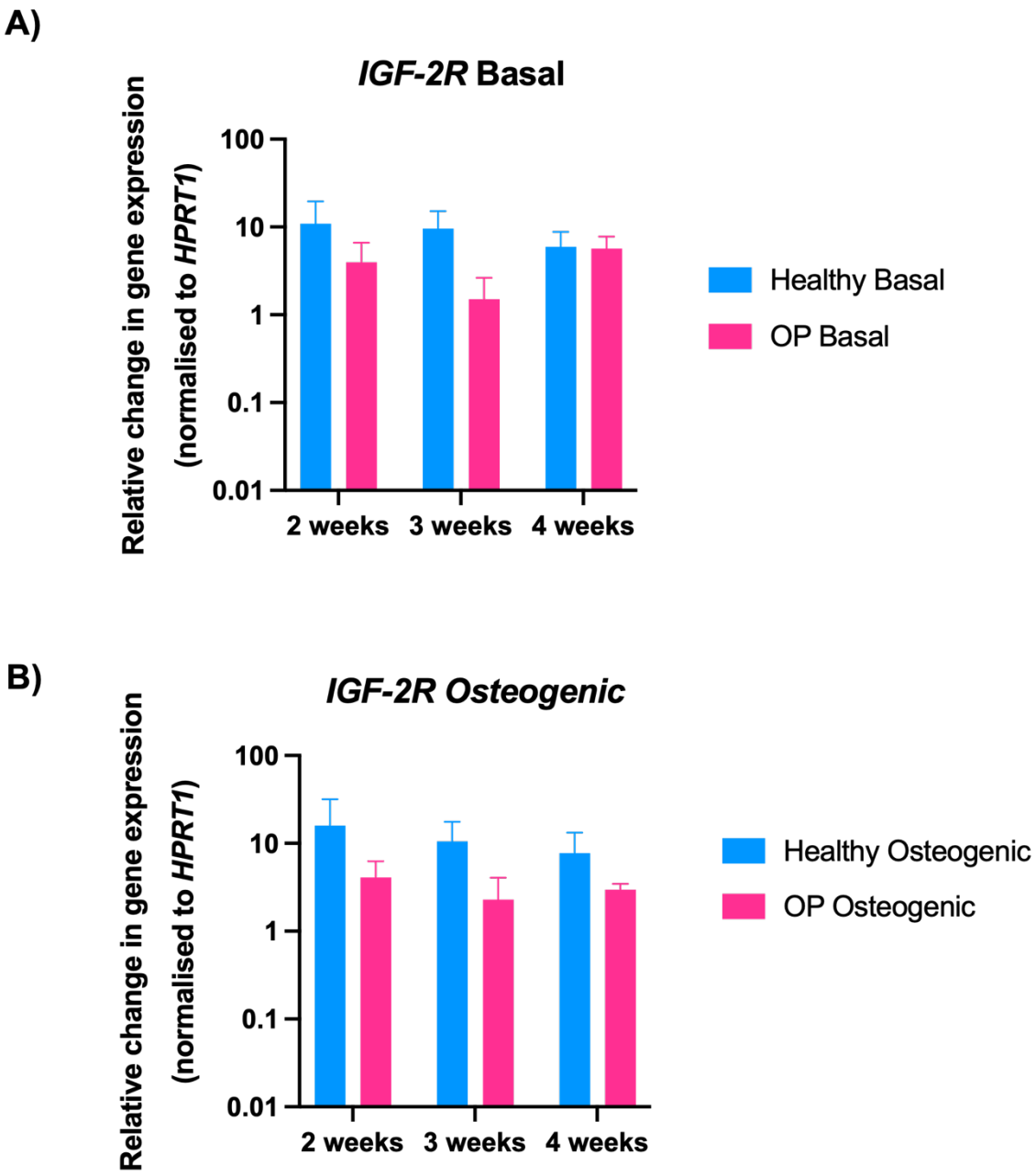


Figure 48. Relative *IGF-2R* gene expression for OP-PDLSCs compared to H-PDLSCs.

The relative change in gene expression levels of *IGF-2R* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

7.2.1.3 Expression of IGF binding proteins genes

7.2.1.3.1 *IGFBP-1*

Under both culture conditions (basal and osteogenic), relative expression levels for *IGFBP-1* were lower at all time points in OP-PDLSCs compared to healthy controls. Levels were comparable over the culture period for OP-PDLSCs (Figure 49. A) (basal) and (Figure 49. B) (osteogenic).

No statistically significant differences were observed in the gene expression, between the osteoporotic and healthy groups at both culture conditions and all time points.

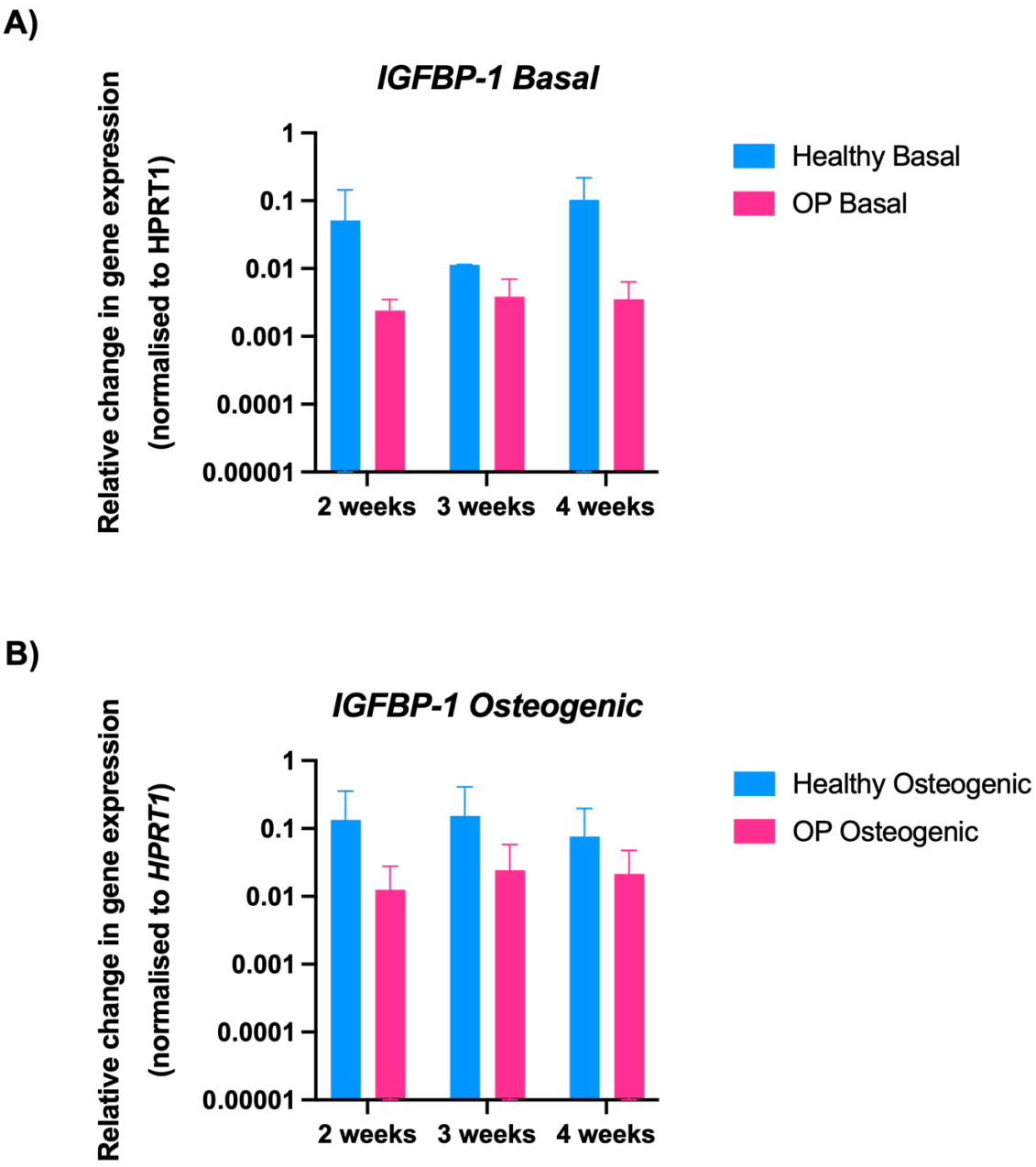


Figure 49. Relative *IGFBP-1* gene expression for OP-PDLSCs compared to H-PDLSCs.

The relative change in gene expression levels of *IGFBP-1* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

7.2.1.3.2 *IGFBP-2*

IGFBP-2 expression was higher under both basal and osteogenic conditions in OP-PDLSCs compared to H-PDLSCs and at all time points except at week 3 under osteogenic conditions, where the expression was comparable in both cell groups. *IGFBP-2* levels increased at 3 weeks then decreased at 4 weeks compared to 2 weeks in OP-PDLSCs under basal conditions (Figure 50. A), while the levels were constant across the time points in OP-PDLSCs under osteogenic conditions (Figure 50. B).

There was no statistically significant difference between healthy and osteoporotic groups when the gene expression was assessed at all time points and culture conditions.

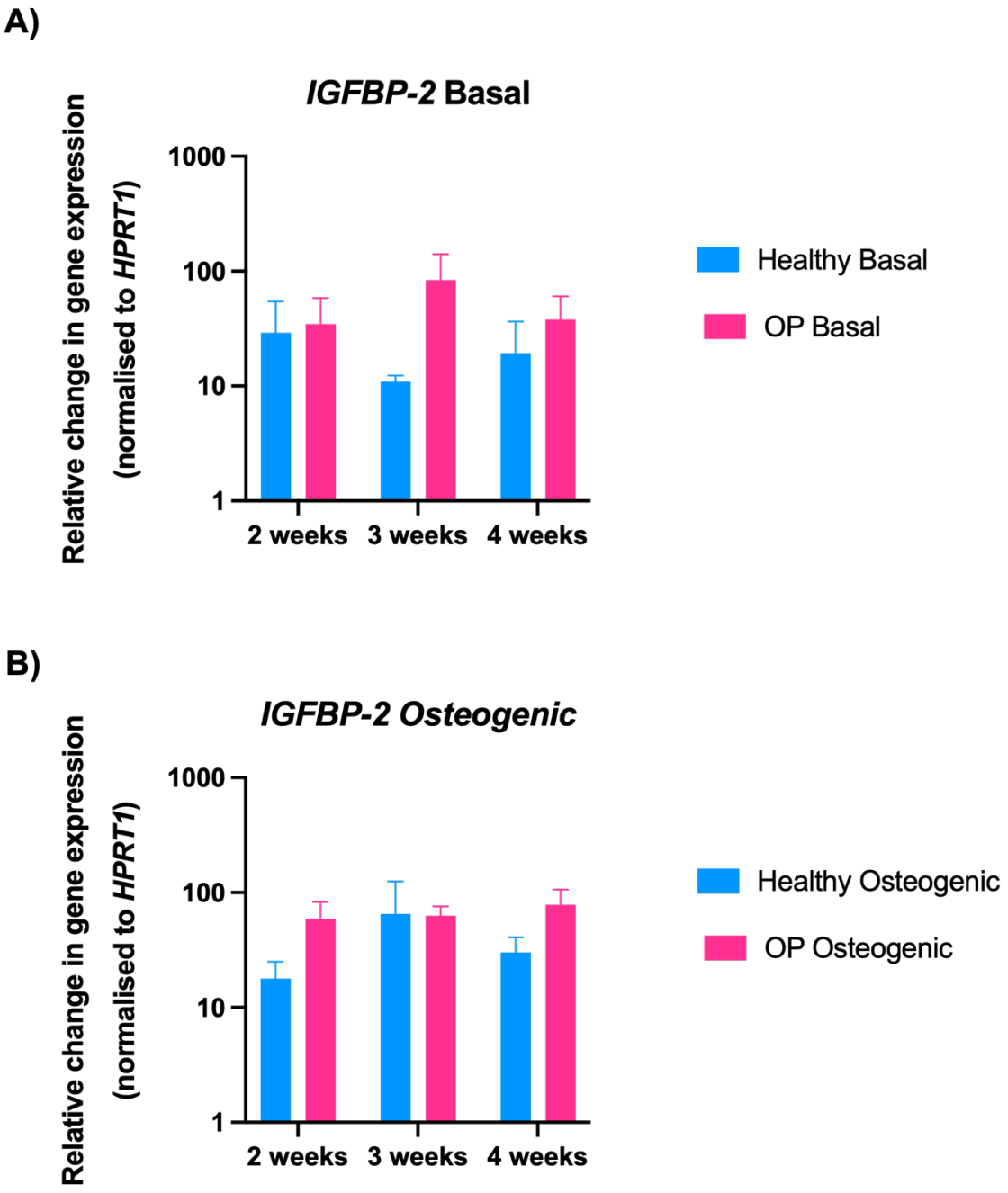


Figure 50. Relative *IGFBP-2* gene expression for OP-PDLSCs compared to H-PDLSCs.

The relative change in gene expression levels of *IGFBP-2* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

7.2.1.3.3 *IGFBP-3*

Under basal conditions, relative expression levels for *IGFBP-3* were comparable between OP-PDLSCs and H-PDLSCs. Also, levels were close to consistent in OP-PDLSCs over the culture period (Figure 51. A).

Under osteogenic conditions, *IGFBP-3* levels were comparable at 2 weeks, and slightly lower in OP-PDLSCs at 3 and 4 weeks. With the progress of the culture, gene levels increased at 3 and 4 weeks compared to 2 weeks in OP-PDLSCs (Figure 51. B).

No statistically significant differences were observed in the gene expression, between the osteoporotic and healthy groups at all time points and both culture conditions.

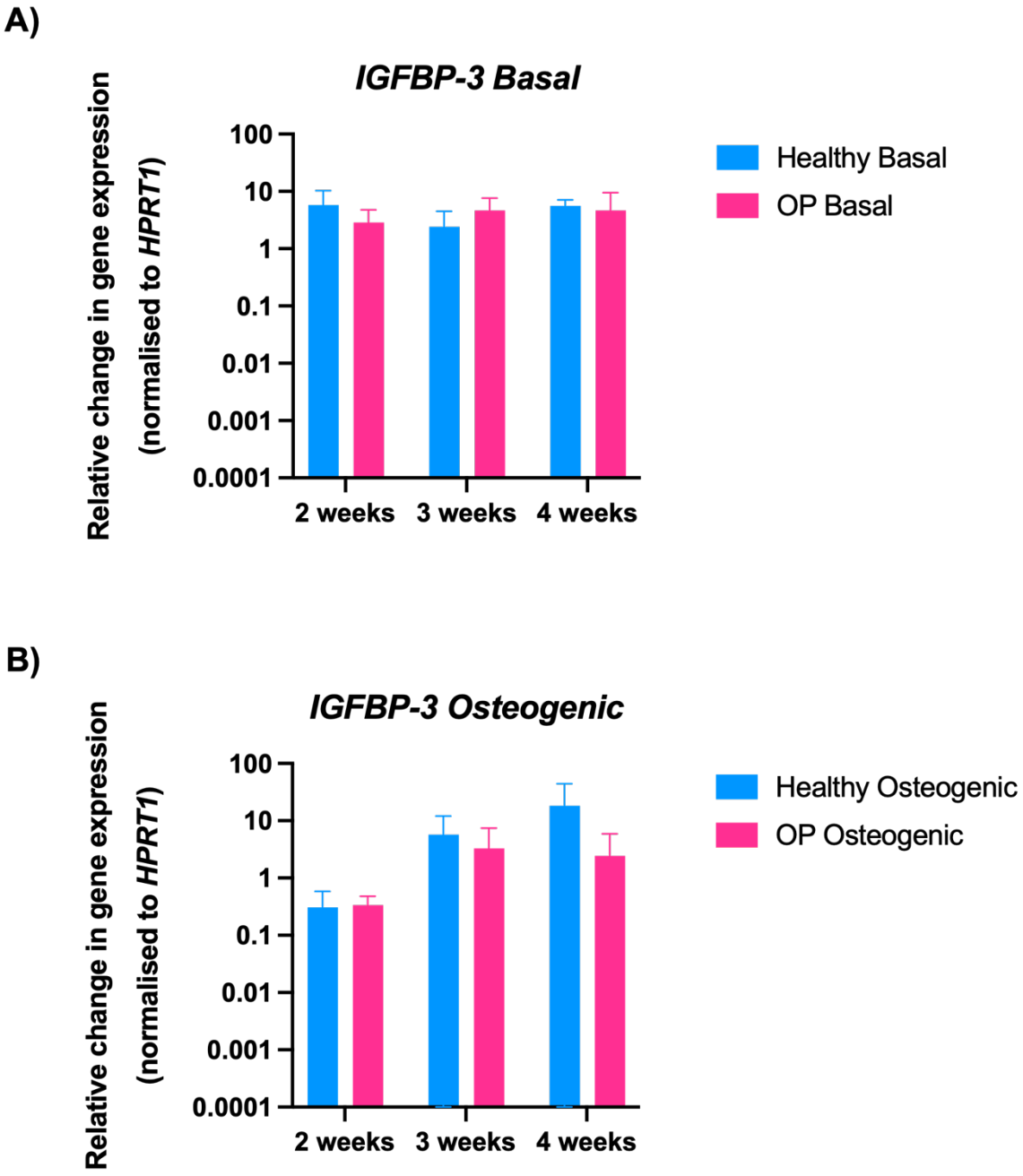


Figure 51. Relative *IGFBP-3* gene expression for OP-PDLSCs compared to H-PDLSCs.

The relative change in gene expression of *IGFBP-3* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

7.2.1.3.4 *IGFBP-4*

Under basal conditions, relative expression levels for *IGFBP-4* were higher at all time points in OP-PDLSCs compared to H-PDLSCs. Gene expression levels slightly increased at 3 weeks then decreased at 4 weeks to a similar level as 2 weeks in OP-PDLSCs compared to H-PDLSCs (Figure 52. A).

Under osteogenic conditions, *IGFBP-4* levels were comparable at 2 and 3 weeks in both cell groups, with a slight increase in OP-PDLSCs at 4 weeks compared to healthy controls. Levels were constant in OP-PDLSCs samples across the 3 culture time points (Figure 52. B).

Although there was a consistent pattern of elevated IGFBP-4 expression in OP-PDLSCs, there was no statistically significant difference between healthy and osteoporotic groups at the different time points and culture conditions.

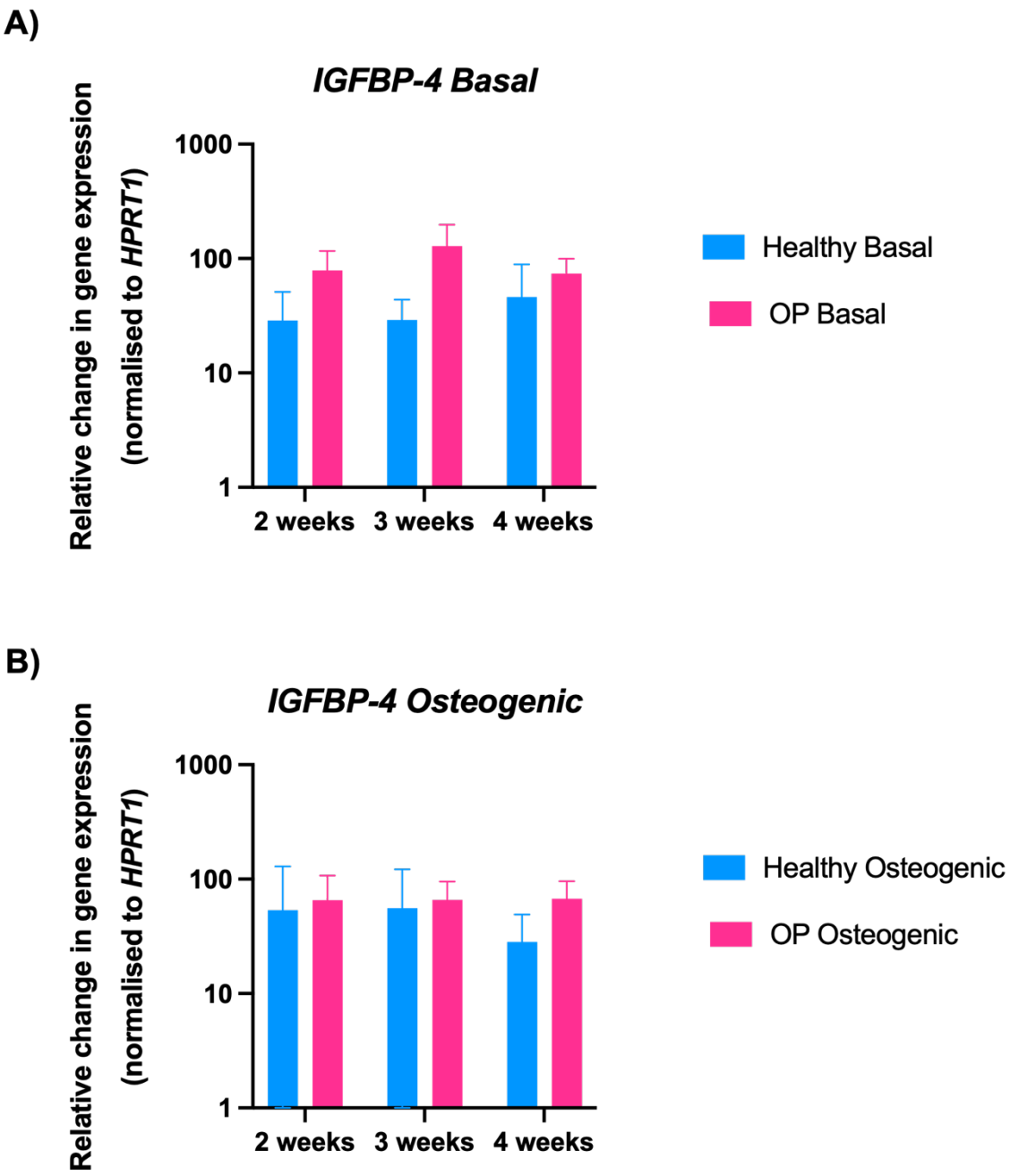


Figure 52. Relative *IGFBP-4* gene expression for OP-PDLSCs compared to H-PDLSCs.

The relative change in gene expression levels of *IGFBP-4* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

7.2.1.3.5 *IGFBP-5*

Under basal conditions, relative expression levels of *IGFBP-5* were lower in OP-PDLSCs at all time points compared to H-PDLSCs. Over the culture period, gene levels were close to consistent in OP-PDLSCs (Figure 53. A).

Under osteogenic conditions, *IGFBP-5* levels were slightly higher at 2 weeks and slightly lower at 4 weeks in OP-PDLSCs compared to H-PDLSCs with comparable levels at 3 weeks between the groups. With the progress of the culture, there was a slight increase at 3 and 4 weeks compared to 2 weeks in OP-PDLSCs (Figure 53. B).

No statistically significant differences were observed in the gene expression, between the osteoporotic and healthy groups when the gene expression was assessed at the different time points and culture conditions.

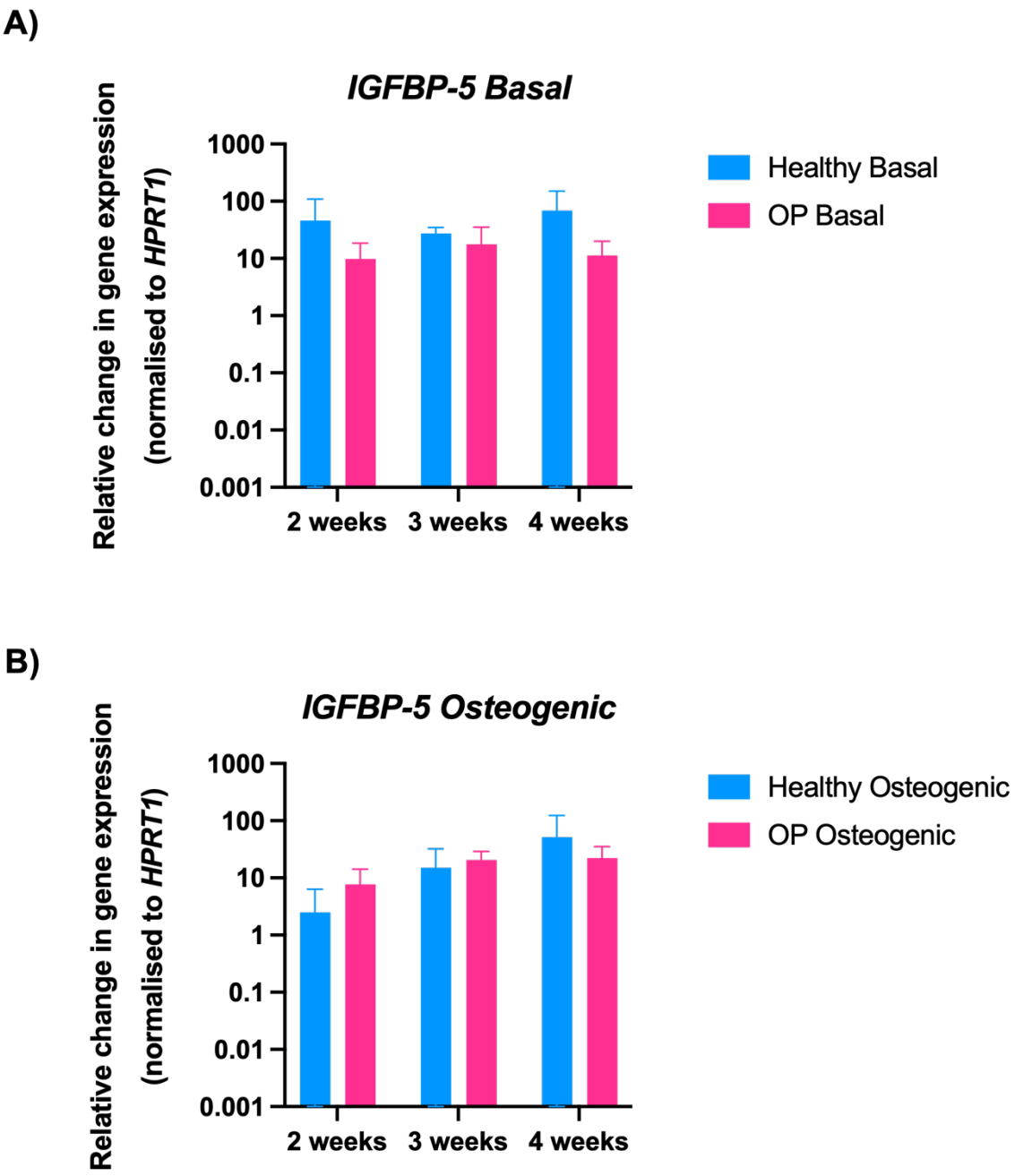


Figure 53. Relative *IGFBP-5* gene expression for OP-PDLSCs compared to H-PDLSCs.

The relative change in gene expression levels of *IGFBP-5* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

7.2.1.3.6 *IGFBP-6*

Under basal conditions, relative expression levels for *IGFBP-6* were comparable at 2 and 3 weeks between the osteoporotic and healthy groups with evidently lower levels at 4 weeks in OP-PDLSCs. There was a decline in the gene levels at 3 and 4 weeks compared to 2 weeks within the OP-PDLSCs group (Figure 54. A).

Under osteogenic conditions, the expression was comparable in both groups at 2 weeks but decreased at 3 and 4 weeks in OP-PDLSCs in comparison to H-PDLSCs. *IGFBP-6* levels were similar at 3 and 4 weeks but lower than 2 weeks in OP-PDLSCs with the progress of the culture (Figure 54. B).

There was no statistically significant difference between healthy and osteoporotic groups when the gene expression was assessed at the different time points and culture conditions.

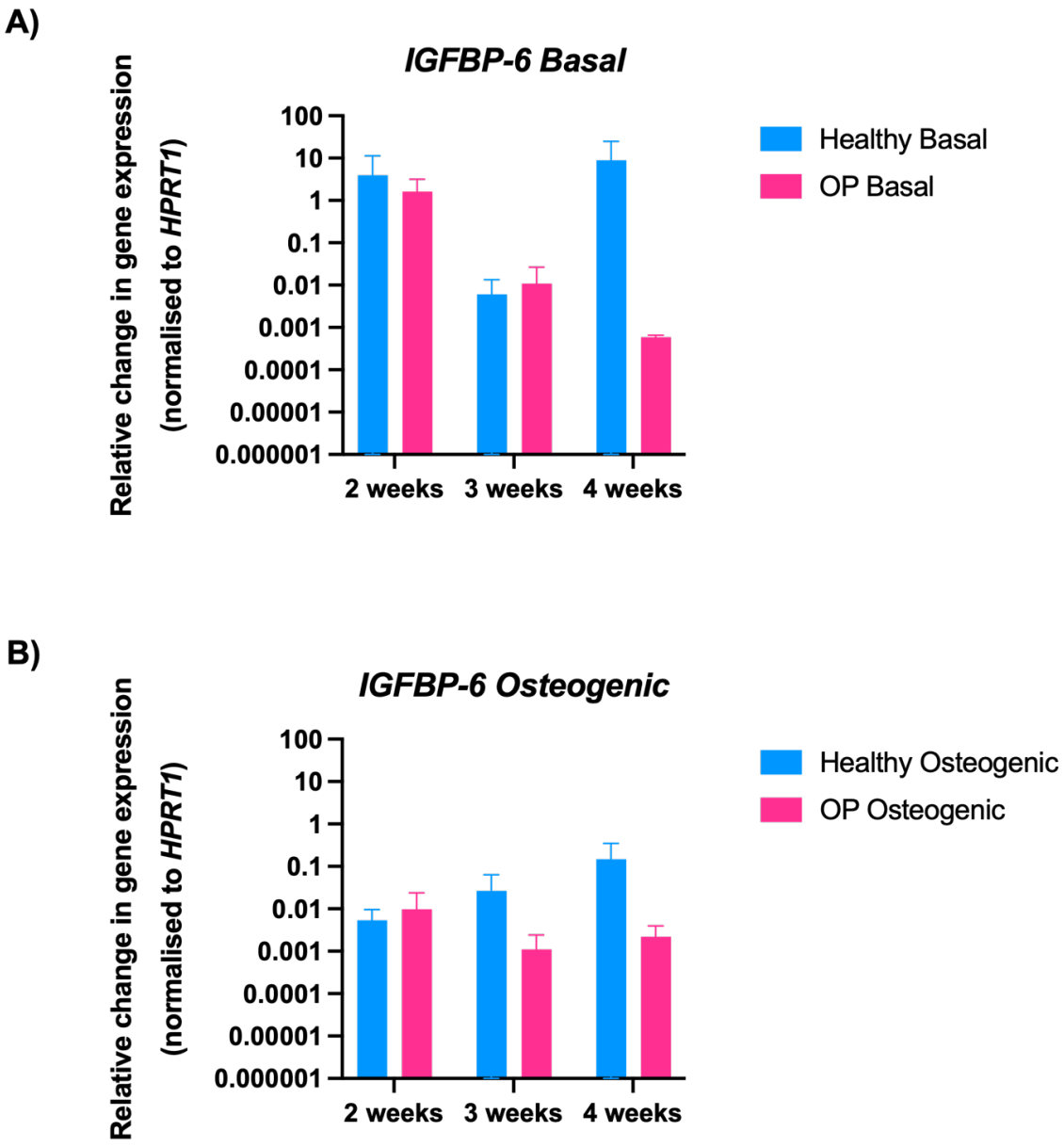


Figure 54. Relative *IGFBP-6* gene expression for OP-PDLSCs compared to H-PDLSCs.

The relative change in gene expression levels of *IGFBP-6* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

7.2.1.3.7 *IGFBP-7*

Under basal conditions, relative expression levels of *IGFBP-7* were higher in OP-PDLSCs compared to H-PDLSCs at all time points. With the progress of the culture, levels were close to consistent in OP-PDLSCs (Figure 55. A).

Under osteogenic conditions, levels were higher at 2 and 3 weeks in OP-PDLSCs compared to H-PDLSCs with comparable levels at 4 weeks between both groups. With the progress of the culture, levels slightly increased at 3 weeks then slightly decreased at 4 weeks in OP-PDLSCs compared to 2 weeks (Figure 55. B). There was a statistically significant difference in the expression of *IGFBP-7* at 3 weeks under osteogenic conditions ($p= 0.04$) using unpaired t-test.

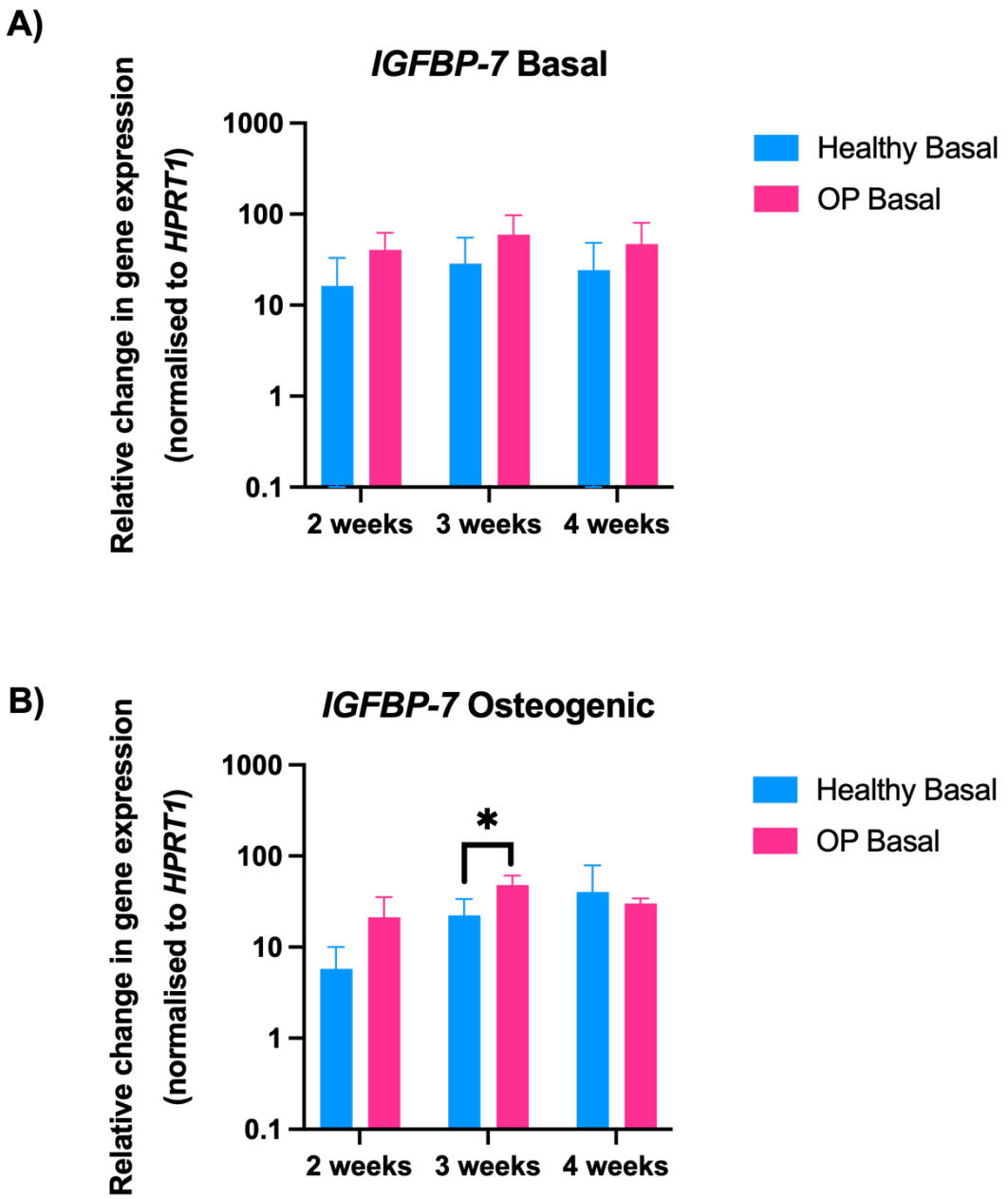


Figure 55. Relative *IGFBP-7* gene expression for OP-PDLSCs compared to H-PDLSCs.

The relative change in gene expression levels of *IGFBP-7* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD. * indicates a statically significant difference ($p < 0.05$).

7.2.2 IGFBP-4 protease gene (*PAPP-A*):

Under both culture conditions (basal and osteogenic), relative expression levels for *PAPP-A* were higher for OP-PDLSCs compared to H-PDLSCs at all time points. The levels were close to consistent with the progress of the culture, (Figure 56. A) (basal) and (Figure 56 .B) (osteogenic).

Under osteogenic conditions at 4 weeks, there was a statistically significant difference between OP-PDLSCs and H-PDLSCs was when the effect of the disease was assessed ($p= 0.03$) using unpaired t test.

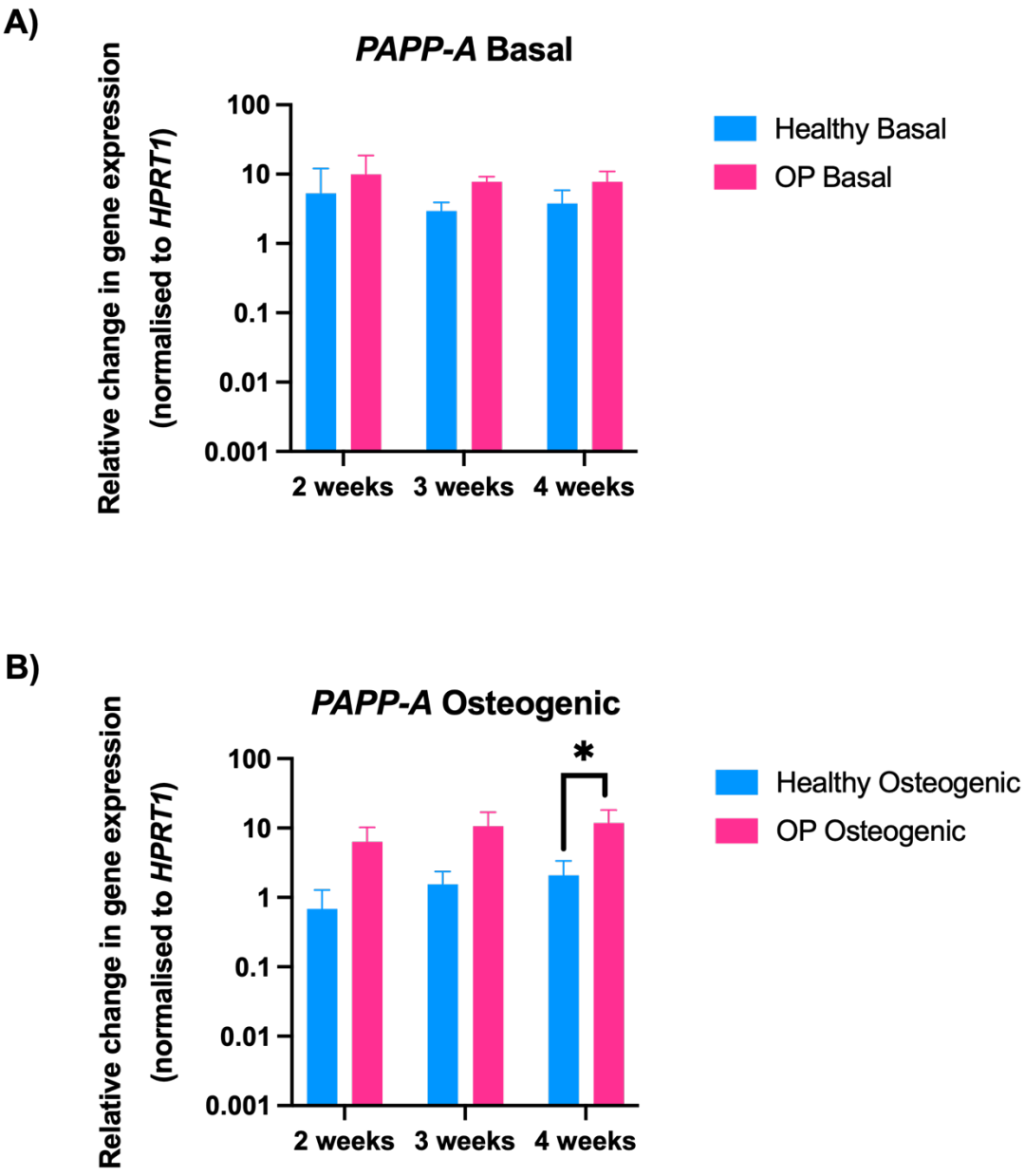


Figure 56. Relative *PAPP-A* gene expression for OP-PDLSCs compared to H-PDLSCs.

The relative change in gene expression levels of *PAPP-A* normalised to *HPRT1* gene. Values plotted for OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD. * indicates a statically significant difference ($p < 0.05$).

7.2.3 Expression of PAPP-A inhibitors:

The gene expression of PAPP-A inhibitors (*STC-1* and *STC-2)* was assessed under both basal and osteogenic cultures at 2, 3 and 4 weeks due to their potential inhibitory role on the activity of PAPP-A and eventually contributory role in elevated IGFBP-4 levels.

7.2.3.1 STC-1

Under basal conditions, relative expression values for *STC-1* were slightly higher in OP-PDLSCs samples compared to healthy controls at all time points. With the progress of the culture, gene levels in OP-PDLSCs slightly increased at 4 weeks compared to earlier time points (2 and 3 weeks) (Figure 57. A).

Under osteogenic conditions, *STC-1* gene expression levels were notably higher in OP-PDLSCs compared to H-PDLSCs. Levels in the osteoporotic group were consistent with the progress of time (Figure 57. B).

Although there was a trend of higher *STC-1* expression in OP-PDLSCs compared to H-PDLSCs, there was no statistically significant difference between the groups when the gene expression was assessed at the different time points and culture conditions.

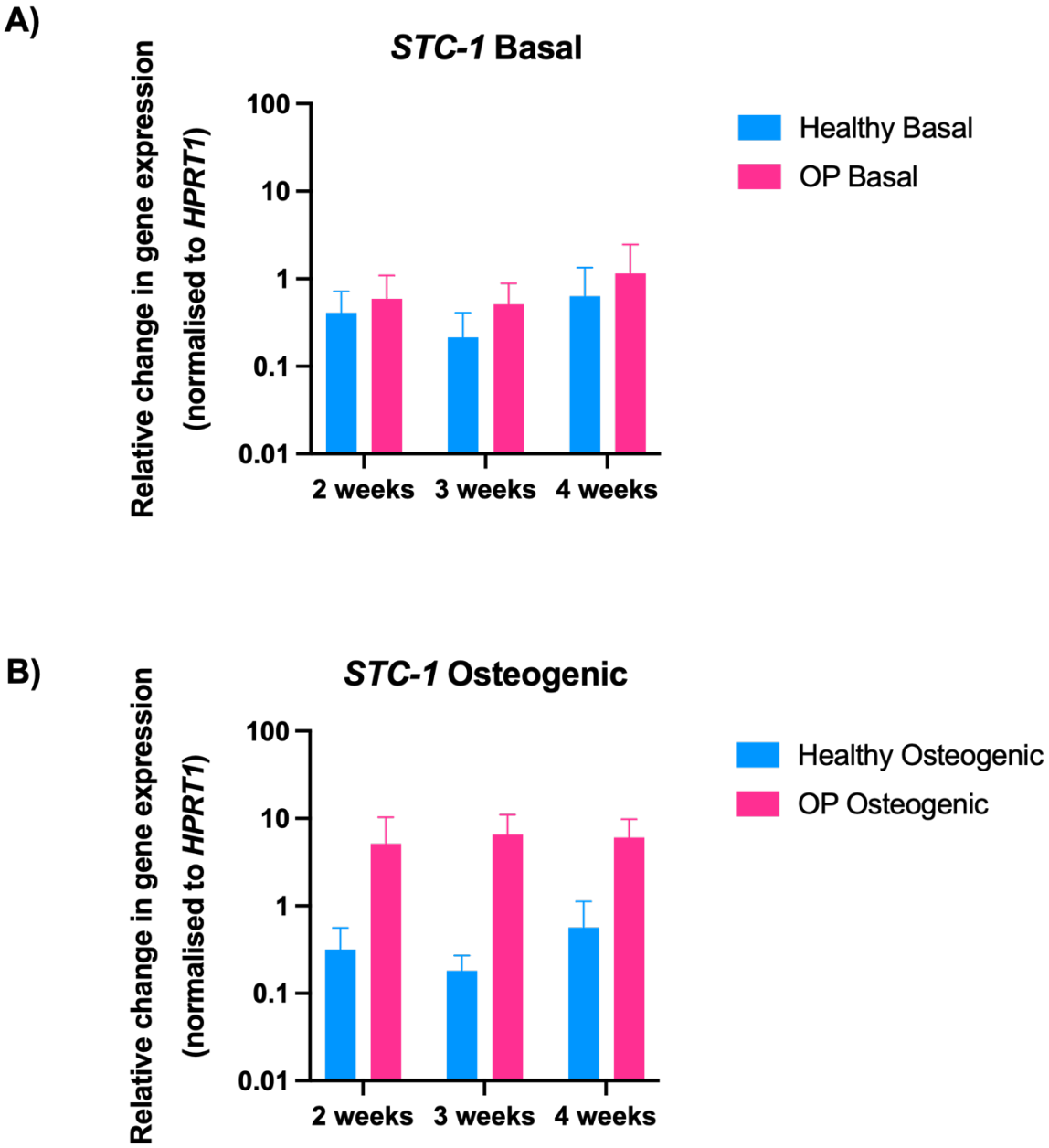


Figure 57. Relative *STC-1* gene expression for OP-PDLSCs compared to H-PDLSCs.

The relative change in gene expression levels of *STC-1* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

7.2.3.2 STC-2

Under basal conditions, relative expression levels for *STC-2* were comparable between the groups. Levels remained close to consistent over the culture period in OP-PDLSCs (Figure 58. A).

Under osteogenic conditions, levels were comparable between the groups at 2 weeks, but slightly lower in OP-PDLSCs at 3 and 4 weeks compared to H-PDLSCs. Levels remained close to consistent across the time points in OP-PDLSCs (Figure 58.B).

No statistically significant differences were observed in the gene expression, between the osteoporotic and healthy groups when the gene expression was assessed at the different experiment time points and culture conditions.

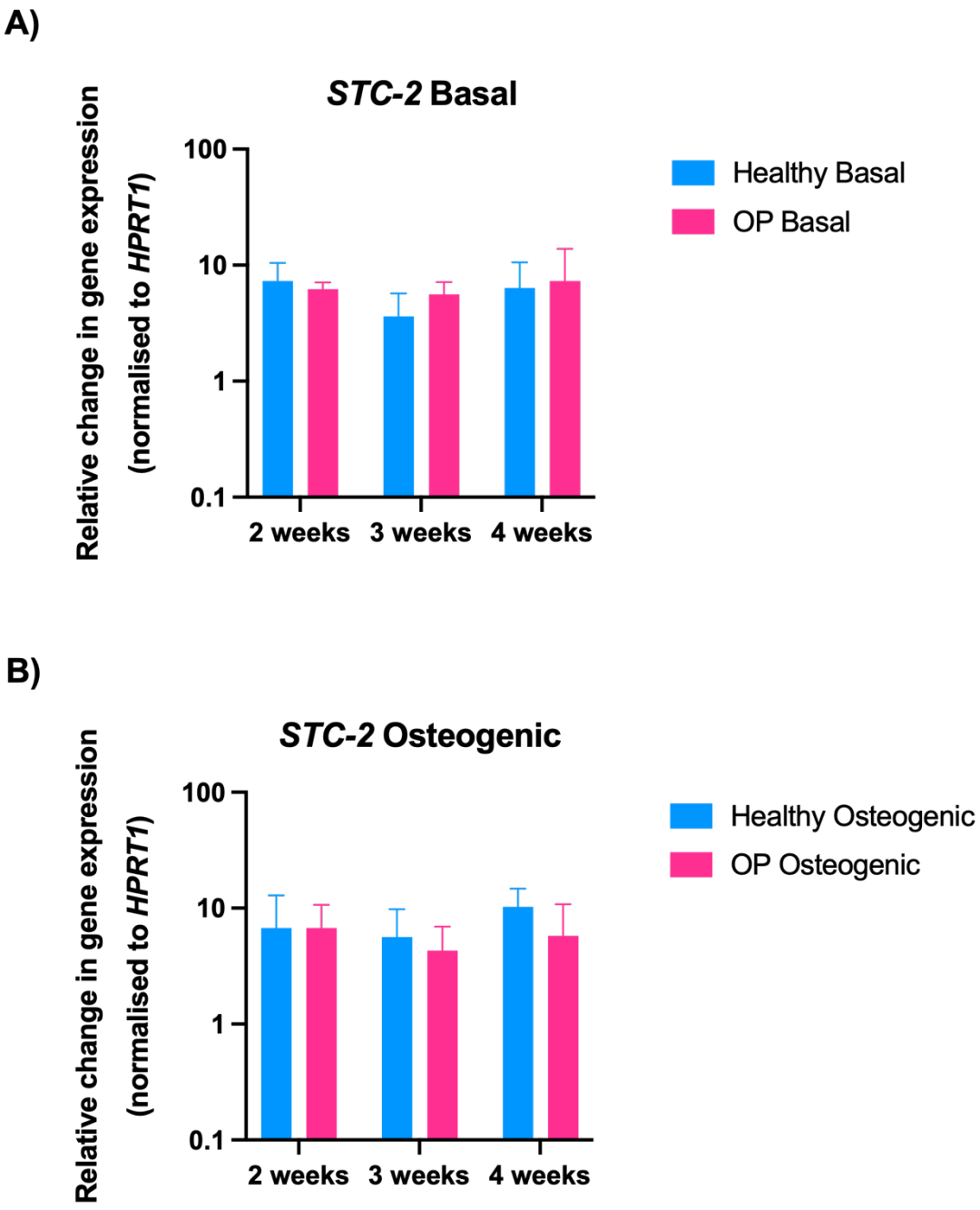


Figure 58. Relative *STC-2* gene expression for OP-PDLSCs compared to H-PDLSCs.

The relative change in gene expression levels of *STC-2* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

7.2.4 Protein Expression:

ELISA was used to investigate the secreted protein levels of IGFBP-4 and its inhibitor PAPP-A in the culture media of OP-PDLSCs and H-PDLSCs at the 3 time points of the experiment (2, 3 and 4 weeks). Detailed method is discussed in section 3.2.12.2. These 2 proteins were particularly investigated to study the potential correlation between the reduced osteogenic differentiation in OP samples and IGFBP-4 (which is known to be inhibitory to IGF-1) and whether that is linked to the variation in the secreted levels of IGFBP-4 protease (PAPP-A).

7.2.4.1 IGFBP-4

Overall, under both culture conditions (basal and osteogenic), IGFBP-4 protein levels in cell culture media were higher in OP-PDLSCs compared to H-PDLSCs (except for 2 weeks under basal conditions where the levels were comparable between the groups). Protein levels were higher at 3 and 4 weeks compared to 2 weeks in the OP group with the progress of culture under both culture conditions (Figure 59. A) (basal) and (Figure 59. B) (osteogenic). It is also worth noting the variation in the expression values across the samples on the graph (longer error bars particularly under basal conditions), indicating variations between the donors.

Although there was no statistically significant difference by impact of the disease, there was a trend of higher IGFBP-4 expression for OP-PDLSCs compared to H-PDLSCs.

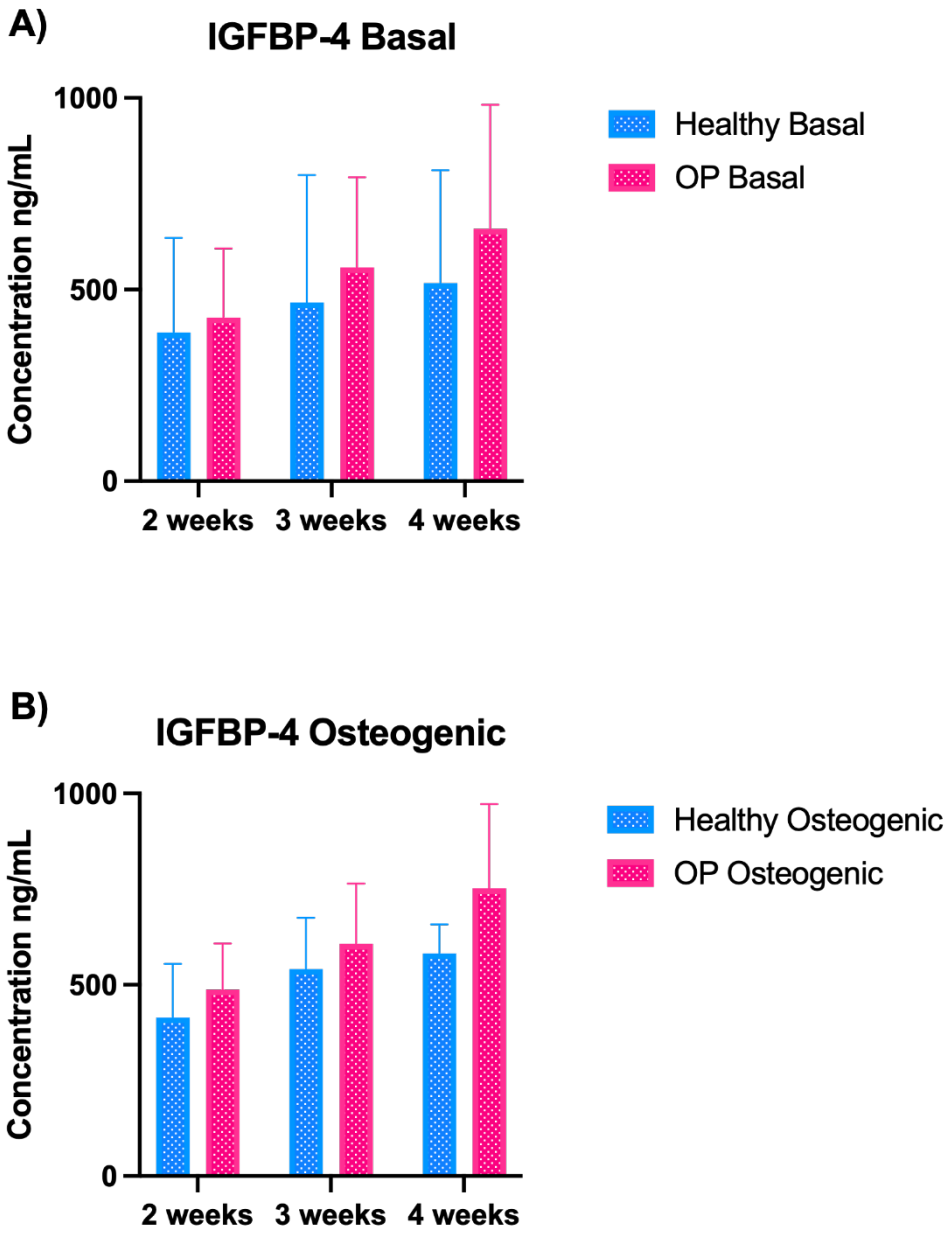


Figure 59. IGFBP-4 protein levels for OP-PDLSCs compared to H-PDLSCs.

This graph shows IGFBP-4 secreted protein levels in tissue culture media investigated using ELISA. Values plotted for OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

7.2.4.2 IGFBP-4 Protease (PAPP-A)

Generally, PAPP-A levels secreted in culture conditions were very low in both OP-PDLSCs and H-PDLSCs (less than 0.2 ng/ml) under both culture conditions and at most time points, which was below the detection range of the kit used (0.78-50ng/mL). PAPP-A protein was undetectable in the culture media alone (both basal and osteogenic) without the samples.

Under both culture conditions, PAPP-A levels were higher in the osteoporotic sample's media compared to H-PDLSCs' at all time points and culture conditions with an increase in the secreted protein values with the progress of culture period. (Figure 60. A) (basal), (Figure 60. B) (osteogenic). Although there was no statistically significant difference between the groups, there was a high degree of variability in the samples' readings particularly for OP-PDLSCs at 4 weeks under osteogenic conditions which had led to a sharp increase in the readings.

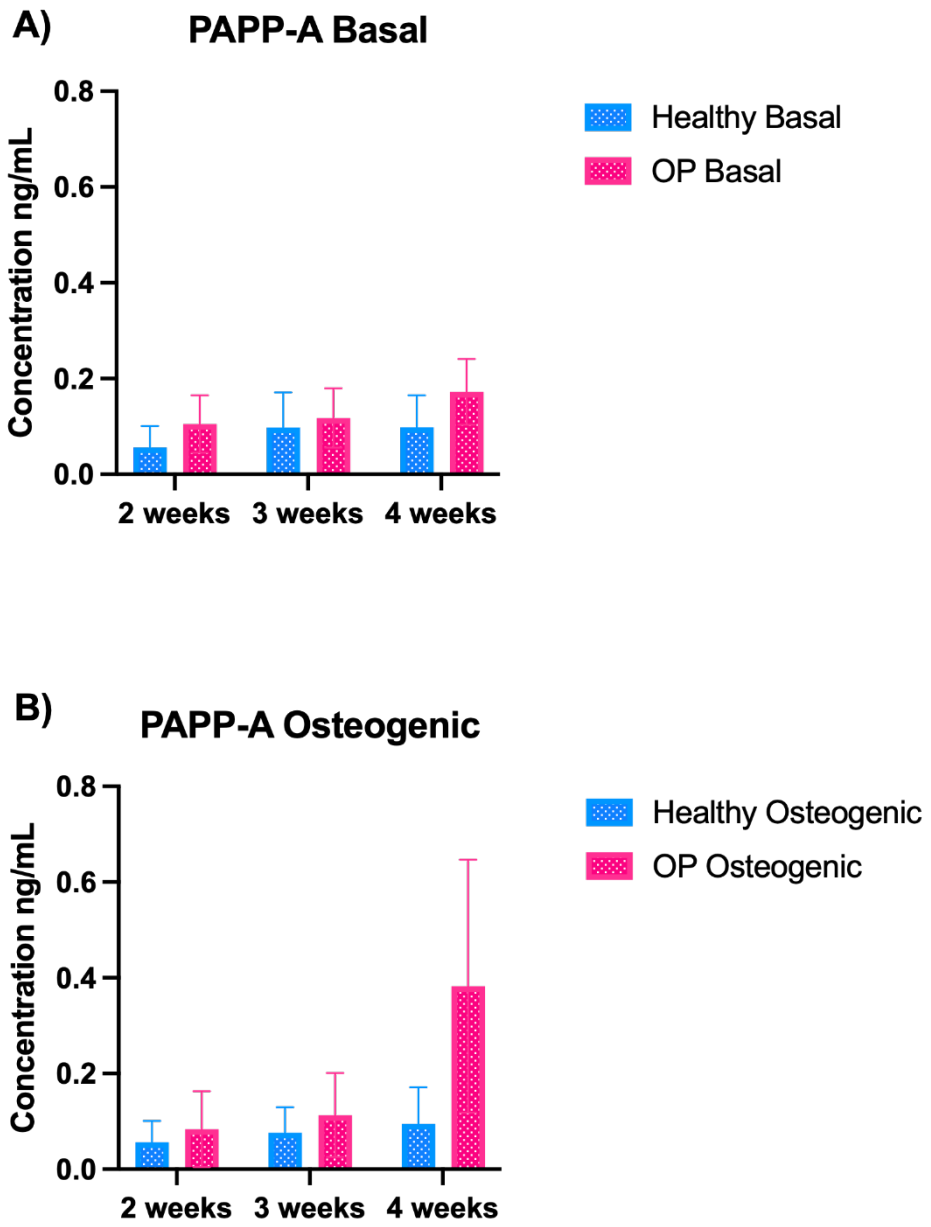


Figure 60. PAPP-A protein levels for OP-PDLSCs compared to H-PDLSCs.

This graph shows PAPP-A secreted protein levels in tissue culture media investigated using ELISA. Values plotted for OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

7.3 Discussion:

PDL cells are considered reservoirs of IGF system components (Götz et al., 2006a), implying the role this axis may play within the periodontium's turnover and regeneration. This chapter presented data for the relative expression of IGF axis genes in OP-PDLSCs. An assessment of the expression pattern for the genes coding for the IGF family members showed an overall lower expression in OP-PDLSCs samples compared to H-PDLSCs for all axis members except for *IGF-1* (variable), *IGFBP-2*, *IGFBP-4* and *IGFBP-7* (higher). The analysis then was focused on the IGFBP-4 inhibitor (PAPP-A) and its inhibitors (*STC-1* and *STC-2*).

The first IGF axis components to be discussed here are IGF ligands. Most published reports discuss IGF ligands levels in osteoporotic patients' sera or studying effect of exogenous addition of IGF axis members in varying concentrations on cells *in vitro*. The approach reported here is novel, therefore relating the results presented directly to this previously published literature is more challenging. The following section will discuss the results presented in this chapter in light of the available reports relating to OP, bone, and PDL-related studies. Where that is not attainable, the discussion will relate to published literature obtained from work with other dental stem cell sources.

In this study, OP-PDLSCs showed variable levels of *IGF-1* in comparison with those obtained from healthy controls. This could indicate that OP has a minimal impact on the expression of IGF-1, though it must be acknowledged that data is presented as an average of 3 donors and donor variability might have played a role in this fluctuating level. In a study by Sakaguchi et al. (Sakaguchi et al., 2017), the addition of a cytokine cocktail containing IGF-1, VEGF-A and TGFβ-1 (to mimic the MSC secretome), promoted periodontal regeneration and angiogenesis in a bifurcation defect in a canine model. Although the study didn't specify the impact of the individual cytokines used, with the support of the published literature (discussed in Chapter 1 section. 1.5), it can be theorised that IGF-1 contributes to periodontal regeneration. In osteoporotic patients, decreased levels of serum IGF-1 has been linked to increased probability of bone fracture and can partially justify the reduction of BMD in postmenopausal women

(Greere et al., 2023). In a clinical study where IGF-1 and IGF-2 were measured in the sera of 50 osteoporotic women, a significant decrease in their levels was reported compared to healthy controls (Boonen et al., 1999). Although no consistent reduction in IGF-1 levels was seen in cultures of cells from osteoporotic patients, these published findings suggest an inverse relationship between IGF-1 and OP which couldn't be concluded for in this study. However, the expression of *IGF-2* gene in OP-PDLSCs showed an overall consistent trend of lower expression compared to H-PDLSCs. Based on a study on the role of IGF-2 on Stem Cells from the Apical Papilla (SCAPs), isolated from incomplete roots of human extracted third molars, the application of 5ng/mL of IGF-2 promoted cellular proliferation, osteogenic/ dentinogenic and neurogenic differentiation (Diao et al., 2020). Whilst these data were derived from healthy SCAPs, one could speculate a similar role of IGF-2 in PDLSCs. Here, the lower levels of *IGF-2* in OP-PDLSCs might have contributed to the reduced proliferation and osteogenic differentiation profile of these cells. Another study compared the osteogenic potential of Stem Cells exfoliated from Human Deciduous teeth (SHED) to human adipose stem cells revealed that higher protein expression of IGF-2 was associated with elevated osteogenic potential, which was assessed by measuring the ARS and ALP activity as well as gene expression of *RUNX2*, *ALP* and *OCN* (Fanganiello et al., 2015). These findings suggest that the reduced *IGF-2* levels in OP-PDLSCs could have contributed to the reduced mineralization.

The second component of the IGF family is IGF receptors. IGF-1 induces its osteogenic actions through binding of IGF-1R receptor (Ma et al., 2016). IGF-1R is a transmembrane receptor with two alpha extracellular subunits and two beta intracellular subunits. The binding of IGF-1 to IGF-1R induces receptor autophosphorylation via tyrosine kinase activity (Kheralla et al., 2010, Blyth et al., 2020). The importance of IGF-1R was indicated by the impairment of osteoblastic differentiation and matrix mineralisation seen in IGF-R1 knockout mice (Ruan et al., 2024). Additionally, IGF-2 can bind to IGF-1R and IGF-2R but binding with the latter does not initiate signal transduction and leads to reduced bioavailability of IGF-2 and its eventual degradation (Ruan et al., 2024). The majority of IGF biological actions such as cellular proliferation, differentiation and survival are driven via IGF-1R (Laviola et al., 2007b). In this study, the relative expression level of *IGF-1R* and *IGF-2R* genes in OP-PDLSCs generally showed a trend of

lower expression compared to H-PDLSCs, which could be directly related to the effect of OP on the expression of these receptors. In a study by Perrini et al. (Perrini et al., 2008), human osteoporotic osteoblasts showed an increase in IGF-1R phosphorylation in basal conditions compared to age-matched controls, while the IGF-1R protein response was blunt when OP osteoblasts were treated with IGF-1. While receptor phosphorylation was not assessed in this study, Perrini et al.'s study contradict the findings presented here for OP-PDLSCs. Moreover, in a study by Parker et al. (Parkar et al., 2001), the expression of several growth factors receptors, including IGF-1R, was compared in normal and healing human periodontal tissues. Gingival and PDL tissues were isolated from extracted premolars as well as samples of regenerated tissue from patients following Guided Tissue Regeneration (GTR). IGF-1R was not detectable in normal PDL tissue and showed a weak to limited expression in the regenerated periodontal tissue potentially suggesting the minimal involvement of this receptor in periodontal regeneration.

The third group of IGF axis to be discussed in relation to OP-PDLSCs are the IGFBPs. IGFBPs are a family of proteins involved in regulating IGF ligands bioavailability in serum and tissues (Dixit et al., 2021, Allard and Duan, 2018). They bind to IGFs with a similar or a higher affinity than IGF-1R (Duan and Allard, 2020) and can either have IGF- enhancing effects or can influence osteoblasts independently (Hoeflich et al., 2007). In tissues, IGFBPs can either inhibit or enhance IGF-1 action by sequestering IGF-1 or releasing it to be available for receptor binding (Perrini et al., 2010). Therefore, changes in IGFBP members can have an evident impact on the action of the ligands (Masanobu and Clifford, 2012).

Published reports have correlated the serum level of IGF system components and the susceptibility to bone fracture in patients with OP (Jehle et al., 2003, Sugimoto et al., 1997, Yamaguchi et al., 2006). In bone related studies, generally, IGFBPs 1, 2, 4 and 6 are known as inhibitors while IGFBP3 and 5 are considered mediators of IGF-1 action (Ruan et al., 2024). However, levels of IGFBPs in osteoblasts isolated from different sites of bone have been inconsistent indicating the IGFBP expression is site-specific in bone (Hoeflich et al., 2007), and could potentially be different among different tissues. In a dental context, members of

the IGFBPs have broadly enhanced actions of dental stem cells in terms of proliferation, osteogenic/ odontogenic differentiation or neurogenic differentiation (e.g. in DPSCs) (Bashir, 2021). However, PDL related studies have mainly focused on the correlation between IGFBP-5 and PDLSCs (Han et al., 2017, Liu et al., 2015) rather than the complex set of IGFBPs. The following section will discuss IGFBPs relative gene expression in their numerical order, however results from IGFBP-4 will be discussed at the end of this section and will be linked it to the findings for its protease and the protease inhibitors.

IGFBP-1 has been reported to have an inhibitory role on IGF-1 action through forming a complex with IGF-1 limiting its binding to IGF-1R (Lindsey and Mohan, 2016). It has also been found that IGFBP-1 promoted RANKL-induced osteoclastogenesis (Wang et al., 2015). According to Jehle et. al (Jehle et al., 2003), serum level of IGFBP-1 in patients with OP was significantly higher than control participants (4.1 fold). Ageing was also associated with an increased level of IGFBP-1, but the mechanism remains unknown (Rosen et al., 1998). Moreover, in human dental pulp cells, relative expression of IGFBP-1 was undetectable under basal conditions (Al-Khafaji et al., 2018), which is not in agreement with the findings from this study. In unstimulated human PDL cells, IGFBP-1 was the least expressed compared to other IGF axis members investigated (Reckenbeil et al., 2017). In this study, relative gene expression analysis in OP-PDLSCs showed a trend of less *IGFBP-1* expression compared to healthy cells, which contradicts the findings from osteoporotic serum studies and published literature from healthy dental cells. This could be justified by the differences in the source of tissues OP-PDLSCs compared to other sources serum (Jehle et al., 2003) or dental pulp (Al-Khafaji et al., 2018)).

Data regarding IGFBP-2 function is conflicting in terms of its regulation to IGF ligands, with reports suggesting its osteogenesis-supportive effects (Beattie et al., 2018) and others conversely claiming its inhibitory role (Masanobu and Clifford, 2012). Relative gene expression levels for *IGFBP-2* in OP-PDLSCs were reported here as generally higher than those in H-PDLSCs. In a study by Sugimoto et al. (Sugimoto et al., 1997), serum levels of IGFBP-2 (and IGF-1 and IGFBP3) were assessed in 165 Japanese postmenopausal women of which 62 had non-traumatic spinal fracture. The study showed that IGFBP-2 had an

inverse relationship with the BMD level regardless of the presence of fracture. They suggested that the elevation of IGFBP-2 levels was age-dependent (rather than OP-dependent). Hence, findings from serum IGFBP-2 expression levels in patients with lower BMD are in line with the findings for *IGFBP-2* gene expression in OP-PDLSCs, and also suggests ageing as a contributing factor (although the comparison was drawn between gene and protein levels). On the contrary, a relevant study by Palermo et al. (Palermo et al., 2004), observed that IGFBP-2 had potentiating effect on ALP expression in rat tibial osteoblasts suggesting a positive correlation between IGFBP-2 and osteogenic differentiation. Similarly, in a study by Alkahrobi et al. (Alkharobi et al., 2016), *IGFBP-2* gene and protein levels in differentiating healthy dental pulp cells under osteogenic conditions were upregulated, potentially indicating its role in enhancing osteogenesis, which is not in agreement with the findings of this study. In other words, this finding contradicts the theory that elevated *IGFBP-2* in OP-PDLSCs contributes to inhibit osteogenic differentiation. Nevertheless, the comparison elements are not identical since the mentioned studies were conducted on animal samples or healthy human DPSCs rather than on osteoporotic periodontal cells.

Analogous to IGFBP-2, published data report that IGFBP-3 showed stimulatory effects while others associated it with a reciprocal inhibitory effect on bone metabolism (Beattie et al., 2018). In this study, *IGFBP-3* expression in PDLSCs samples was overall comparable between OP and healthy groups (except for 3 and 4 weeks under osteogenic conditions). Kveiborg et al. (Kveiborg et al., 2000) carried out a study on an osteblastic cell line isolated from trabecular bone of the iliac crests, and classified to young, middle and old ages cells based on their growth stage *in vitro*, IGFBP-3 levels decreased with ageing by 30% in the gene and 16% in the protein expression. In addition, in a study by Ueland et al. (Ueland et al., 2003), the bone matrix content of trabecular iliac crest samples indicated an age-related increase of IGFBP-3 in postmenopausal women with low BMD with a history of atraumatic fracture. In the context of dental cells, Alkharobi et al.'s study revealed that healthy human dental pulp cells differentiating under osteogenic conditions had lower secreted levels of IGFBP-3 protein (Alkharobi et al., 2016). Upon reflection on the findings from this study and the cited literature, it is apparent that the expression of *IGFBP-3* in OP-PDLSCs differs from that of osteoblasts or healthy dental pulp stem cells.

However, it can be estimated that ageing might influence its gene expression, whether that is clinical ageing, given the samples were extracted from older patients, or *in vitro* ageing, since the used PDLSCs from both groups were sub-cultured until passage 6. It is worth mentioning that it was not attainable to characterise freshly extracted PDL cells at passage 0 since the cell yield was minimal and culture expansion was necessary to carry out the work needed for this project.

The expression of *IGFBP-5* was overall lower in OP-PDLSCs compared to H-PDLSCs (except for 2 and 3 weeks under osteogenic conditions where the expression was slightly higher). Interestingly, most clinical research studying IGFBPs and periodontal regeneration seemed to be focused on IGFBP-5 with an overall agreement on its positive effects. In a study by Han et al. (Han et al., 2017), the reintroduction of exogenous recombinant human IGFBP-5 (rhIGFBP-5) to PDLSCs (with knocked down IGFBP-5) under inflammatory conditions enhanced the cellular migration and osteogenic differentiation. In the same study, rhIGFBP-5 application on periodontal defects *in vivo* showed similar periodontal regenerative results. Similarly, another study by Liu et al. (Liu et al., 2015) also confirmed the positive role of IGFBP-5 in the osteogenic differentiation and reduction of inflammatory markers in human PDLSCs (both healthy and inflamed). They also showed higher levels of IGFBP-5 gene and protein levels in PDLSCs compared to other stem cells studied including BMSCs, adipose-derived stem cells (ADSCs), and Wharton's jelly of umbilical cord stem cells (WJCMSCs). In their study, the lesions created in an *in vivo* model with periodontitis showed more bone deposition after 12 weeks of Flag-IGFBPs WJCMSCs injection compared to controls. In the context of bone, IGFBP-5 is the most abundant protein in bone tissue compared to other IGFBPs. IGFBP-5 can either enhance the action of IGF-1 or inhibit it (Duan and Allard, 2020). IGFBP-5 showed an IGF-dependent mitogenic effect on human osteoblasts while independently, it aided the differentiation of osteoblastic cell lines (Beattie et al., 2018). In Boonen et al.'s study, IGFBP-5 levels in osteoporotic postmenopausal women were significantly lower compared to controls (Boonen et al., 1999). Conversely, in the Ueland et al's report, IGFBP-5 protein levels in trabecular bone matrices of postmenopausal women were elevated. They suggested that IGFBPs contributed to the pathogenesis of postmenopausal OP through regulating the bioavailability of

IGFs (Ueland et al., 2003). Conclusively, the lower expression trend of IGFBP-5 in OP-PDLSCs, in this study, is in agreement with published literature which suggests its supportive role in bone regeneration and hence the lack of it could result in an inverse outcome.

The relative expression of *IGFBP-6* was lower in OP-PDLSCs group compared to healthy controls (except for 2 weeks and 3 weeks under osteogenic and basal conditions respectively where the expression was higher). In bone studies, IGFBP-6 has an inhibitory role on IGF-2 action on cell survival, proliferation migration and differentiation, but due to its lower binding affinity to IGF-1, it has little to no impact on its actions (Bach, 2015). Additionally, IGFBP-6 has been reported to inhibit osteoblasts' differentiation, and this effect can be carried out independent of IGF-1 (Fang et al., 2023). In the Jehle et al. study mentioned, serum IGFBP-6 (among other binding proteins) was tested in patients with OP (males and females with varying OP types) and it indicated increased levels of this binding protein (2.1 fold) compared to controls (Jehle et al., 2003). In human premolar PDLs, the addition of IGF-2 and IGFBP-6 led to reduced proliferation and osteogenic differentiation as assessed by *ALP* and *OCN* gene expression levels. Significant inhibition of *ALP* and *OCN* was noted after day 5 and after day 1 of IGFBP-6 treatment alone (Konermann et al., 2013). Considering this evidence, results for *IGFBP-6* in OP-PDLSCs were contradictory in comparison to the published data. These differences could be explained by the variation of the cells and tissues used (serum vs PDL), a fundamental difference in the experimental design (assessment over a few days compared to up to 4 weeks in this study) or potentially it could denote a unique expression of *IGFBP-6* in this type of cells.

IGFBP-7 is a low affinity binding protein and is secreted by osteoblasts to enhance bone formation, and its secretion is controlled by Parathyroid Hormone (PTH) (Mazziotti et al., 2022). IGFBP-7 has also been reported to reduce osteoclastogenesis (Fang et al., 2023). In this study, *IGFBP-7* relative expression was higher to comparable in OP-PDLSCs samples in relation to H-PDLSCs, with a statistically significant difference under osteogenic conditions at 3 weeks. In a study by Ye et al. (Ye et al., 2020), bone marrow derived murine macrophages were induced to differentiate into osteoclasts using Macrophage-colony

stimulating factor (M-CSF) and RANKL. Treatment with IGFBP-7 reduced RANKL-induced osteoclastogenesis *in vitro* as well as suppressing osteoclastogenesis related markers. *In vivo* experiments of OVX mice in the same study revealed that the addition of IGFBP-7 reduced ovariectomy-induced bone resorption, indicating the potential protective role of this protein. Furthermore, Li et al. (Li et al., 2022a) studied the impact of IGFBP-7 on DPSCs isolated from human impacted third molars and noticed an increased cellular senescence upon knocking IGFBP-7 down. They also noticed an increase of osteogenic differentiation (assessed by ALP and ARS) and a decreased senescence when DPSCs were treated with IGFBP-7. Due to its osteogenesis-supportive role, these data pose the suggestion that *IGFBP-7* might be reduced for OP-PDLSCs. However, the results from this study suggested otherwise, which could also be attributed to the medications used by the participants including alendroic acid, HRT and denosumab, which generally target bone resorption. Although the medications might have had an impact on the expression of the IGF axis members, published literature is deficient regarding how the use of systemic medications might affect the expression of a specific growth factor in OP-PDLSCs.

The last binding protein with most interesting findings, to be discussed in this chapter is IGFBP-4. Relative expression for *IGFBP-4* gene for OP-PDLSCs showed a higher trend of expression than that of healthy controls. Correspondingly, elevated protein levels were also observed in the osteoporotic group. Bone studies have suggested an IGF inhibitory role for IGFBP-4, and that its overexpression led to decreased osteogenesis in osteoblasts (Beattie et al., 2018). In postmenopausal women, the expression of IGFBP-4 has been linked to BMD and physical activity. For instance, in a cross-sectional study of postmenopausal South-Asian women, serum IGFBP-4 readings were positively correlated with age and inversely related to BMD and physical activity (Sittadjody et al., 2012). On the contrary, in a study to assess the inflammatory cytokines in postmenopausal women, serum IGFBP-4 levels were lower in participants with low BMD (Wu et al., 2020). Additionally, in Boonen's study (Boonen et al., 1999), there was no difference in IGFBP-4 serum levels when elderly women with hip fractures were compared to healthy controls. Interestingly, the previous studies displayed a variation of IGFBP-4 expression in human serum OP studies.

Moreover, IGFBP-4 expression was suggested to be impacted by biological gender and estrogen secretion. In Madrias et al.'s study (Maridas et al., 2017), they used IGFBP-4 knockout male and female mice models to study the impact of IGFBP-4 loss on bone. Their results showed increased bone resorption and reduced cortical and trabecular bone in female mice along with microarchitectural changes. These changes were not observed in male counterparts which rather showed minimal increase in trabeculae. They suggested these alterations were caused by the estrogen's modulation of IGFBP-4 action. While results from animal bone studies might not work as a precise basis to draw comparisons, it gives an insight on the potential impact of estrogen deficiency in this study's participants on IGFBP-4 expression. Nevertheless, it is not clear whether osteoporotic participants on HRT would have had different IGFBP-4 expression which requires further independent studies on diagnosed patients who didn't commence medical treatment. Intriguingly, in a study by Al-Khafaji et al. (Al-Khafaji et al., 2018), actions promoted by IGF-1 treatment for osteogenically differentiating DPSCs was diminished by the addition of IGFBP-4 suggesting its inhibitory role in this type of cells. IGFBP-4 was inhibited by structural cleavage and the cleavage site was identified at carboxyl terminal side of methionine 135 producing two fragments of 14kDa and 18kDa (Conover et al., 1995), and PAPP-A was identified as the protease responsible for this cleavage (Beattie et al., 2018). Based on this finding as well as the higher expression of IGFBP-4 on a gene level, it was hypothesised that OP conducts its IGF-1 inhibitory effects via upregulation of IGFBP-4 hence, investigations on its protease (PAPP-A) as well as PAPP-A inhibitors (STC1 and STC2) were carried out.

The relative expression of the *PAPP-A* gene and protein levels were higher in OP-PDLSCs compared to H-PDLSCs. Upregulated *PAPP-A* levels in OP-PDLSCs could indicate an increased transcription and translation of PAPP-A in an attempt to downregulate the elevated IGFBP-4 levels. Although most secreted PAPP-A readings were below the used kits' detection level, they were at evidently lower levels when compared to IGFBP-4 protein readings (average readings below 0.2ng/mL compared to a minimal reading of 380 ng/mL for PAPP-A and IGFBP-4 respectively). Also, it was challenging to conclude whether the lower PAPP-A levels could indicate its reduced function or whether a small amount of this protease is needed to inhibit IGFBP-4 function.

A fundamental study on the role of PAPP-A in bone metabolism was conducted by Phang et al. (Phang et al., 2010). Two transgenic strains of mice were used; one overexpressing PAPP-A in bone while the other expressed a variant of IGFBP-4 that is resistant to proteolysis. They demonstrated an increase in BMD (by 20-25%) in the former group while the latter group showed a 25% decrease in BMD along with growth retardation and impaired skeletal development. They suggested that PAPP-A's anabolic role in bone happens mainly through increasing IGF bioavailability. A similar report by Qin et al. (Qin et al., 2006), indicated that the bone anabolic effects induced by PAPP-A are IGF-dependent. They observed an increase in the concentration of IGF-1 in the culture media of murine osteoblasts upon treatment with recombinant PAPP-A. They also suggested that the positive effects on bone were a result of increased osteoblasts rather than reduced osteoclasts denoting its potential route of action. Additionally, in the absence of PAPP-A, Tanner et al's study (Tanner et al., 2008) showed that trabecular bone volume was decreased in PAPP-A knockout mice (femoral and tibial bone) compared to wild type mice. They also tested procollagen type 1 N-terminal propeptide (P1NP) (bone formation marker) and tartrate-resistant acid phosphatase (TRACP) (bone resorption marker) and found a decrease at the level of the former at 4-6 months old mice and a significant increase of the latter at 2 months. Levels of serum and trabecular IGF-1 were not impacted by abolishing PAPP-A indicating these impacts are IGF-independent which complements the previous studies.

Additionally, in the previously mentioned study by Al-Khafaji et al. (Al-Khafaji et al., 2018), IGF axis components (including IGFBP-4 protease PAPP-A) were assessed in the conditioned media of hDPCs under both basal and osteogenic conditions. It was demonstrated that IGFBP4 activity was modified by enzymatic cleavage. Exogenous IGFBP-4 was incubated in hDPCs conditioned media and proteolyzed from 28kDa to 14kDa fragments indicating endogenous PAPP-A activity by these cells. Moreover, the addition of PAPP-A inhibitory antibody reduced IGFBP-4 proteolysis completely allowing IGFs to bind to cell surface receptors indicating an indirect PAPP-A anabolic effect. The findings from their study indicated that PAPP-A was twice as elevated in osteogenic culture media compared to basal conditions. Additionally, it was found that STC-2 was more

abundant than STC-1 in hDPCs, and was able to form STC-2:PAPP-A complexes, suggesting PAPP-A activity may be regulated by STC-2.

Furthermore, *STC-1* relative expression in OP-PDLSCs showed a higher trend of expression while results from STC-2 were generally comparable. STC-1 and STC-2 were identified as potent inhibitors of PAPP-A where STC-1 has high affinity binding while STC-2 can bind covalently to PAPP-A forming a disulfide bond hence blocking the inhibition of IGFBP-4 without blocking the active binding side (Conover and Oxvig, 2023). The upregulation of *STC-1* in OP might be the cellular attempt to reduce PAPP-A levels relating to its higher affinity in contrast to STC-2. It is challenging to reach conclusions on the interactions of IGFBP4, PAPP-A, STC-1 and STC-2 based on an abstract assessment of the gene expression. Therefore, there is a need for further functional studies to understand the exact interactions and mechanisms involved and how they ultimately impact IGF-1 regulation.

Another set of markers that has been correlated to the function of IGFBPs is their potential inhibition by matrix metalloproteinases (MMPs) and their interactions with MMPs inhibitors, tissue inhibitor metalloproteinases (TIMPs) (Fowlkes et al., 1995, Byun et al., 2000, Kok and Barton, 2021). Relative gene expression data for *MMP1*, *MMP2*, *MMP9*, *MMP13*, *TIMP-2*, *TIMP-3* are presented at Appendix C) (Figures 61-68) but not discussed in this thesis.

In conclusion, it is apparent from the discussion above that the correlation between the different IGFBPs functions is complex and often controversial and inconclusive at times. However, results from this chapter suggest that elevated levels of IGFBP-4 in OP-PDLSCs could be related to the inhibited osteogenic differentiation and mineralisation in those cells making IGFBP-4 a potential target for treatment to enhance the periodontal regeneration.

Chapter 8 General Discussion

Periodontal regeneration using stem cells is a promising technique for the treatment of periodontal disease (Citterio et al., 2020). Using autologous source of stem cells provides less immunogenic reactions as opposed to allogeneic stem cells and grafts (Li et al., 2021). Furthermore, site-specific stem cells provide more enhanced results compared to using stem cells from other tissue sources in the body (e.g. cells from axial or appendicular bone) (Akintoye et al., 2006). Since osteoporotic patients experience periodontal bone and teeth loss, they might require implant placement and benefit from regenerative therapies. The main question this project asks is how different OP-PDLSCs compared to H-PDLSCs, and if OP affects the ability of OP-PDLSCs for osteogenic differentiation and consequently stem cell-based bone regeneration. It further focuses on the potential role of IGF axis components in shaping the osteogenic differentiation capacity of these cells. All these investigations aim to eventually provide an insight to help improve clinical dental therapies for osteoporotic patients. The focus on the IGF axis will potentially give a better understanding of the involvement of this axis in osteogenesis under osteoporotic conditions (Feng and Meng, 2021, Crane et al., 2013) and hence might aid in the regeneration of the periodontal bone. To the best of my knowledge, this is the first body of work that focuses on PDLSCs isolated from human postmenopausal osteoporotic patients.

Results from the characterisation experiments (CFU-Fs and PDT) showed less clonogenic and proliferation capacities by OP-PDLSCs, as discussed in (Chapter 4). This was confirmed in other studies by the same observations in other types of OP-MSCs (Wu et al., 2018, Čamernik et al., 2020, Rodríguez et al., 1999). Additionally, OP-PDLSCs displayed a similar phenotype (fibroblastic cells that express MSCs surface antigen markers) to H-PDLSCs. However, there was an increased expression of CD34 in two osteoporotic donors and an increase in HDLAR expression in one osteoporotic donor, opposing the traditional MSCs expression. The inter-donor variation could have played a role in this expression pattern, or it could be an inherent feature of OP-PDLSCs, due to its different niche and site specificity.

The osteogenic differentiation potential of OP-PDLSCs was explored in (Chapter 5) and indicated a pattern of lower expression of osteogenic genes (*RUNX2, OCN, ColIα1, and POSTN)* with a statistically significant difference for *POSTN* expression between osteoporotic and healthy groups at 4 weeks under osteogenic conditions. There was also a consistent trend of less mineral deposition by those cells (ARS results). This led to the major finding of this study that although OP-PDLSCs displayed a trend of a similar phenotype to H-PDLSCs, their ability to differentiate into osteoblastic lineage and deposit minerals was potentially compromised. These findings need to be interpreted with caution due to the limited sample size and the lack of statistical significance.

Since postmenopausal OP has been linked to diminished estrogen levels (Cheng et al., 2022), the expression of estrogen receptors (ERs) was also investigated. OP-PDLSCs also showed a consistent trend of lower expression of ERs (particularly *ERβ* and *GPR30*) compared to H-PDLSCs. Further studies are needed to be able to draw potential interactions between estrogen levels and OP in those cells.

As discussed in (Chapter 7), the gene expression for IGF ligands and receptors was consistently less in OP-PDLSCs compared to H-PDLSCs under osteogenic conditions (most time points for *IGF-1*, *IGF-1R* and all time points for *IGF-2* and *IGF-2R*). IGFBPs associated with IGF-1 activation, based on prior bone research (Ruan et al., 2024), were either reduced (*IGFBP-3*) or inconsistent (*IGFBP-5*), and those associated with IGF-1 inhibition showed either higher (*IGFBP-2, IGFBP-4, IGFBP-7*) or lower expression (*IGFBP-1 and IGFBP-6*) in OP-PDLSCs compared to controls. This indicates that the expression pattern of IGF axis members was altered in OP-PDLSCs, which might be a target for further investigation and a potential use for therapy.

Based on previously published studies (Beattie et al., 2018, Al-Kharobi et al., 2014), this project theorised that the trend of elevated IGFBP-4 levels could contribute to OP impact on PDLSCs, since IGFBP-4 is associated with inhibition of mineralisation and bone formation (Zhou et al., 2003). This was observed both on the gene and protein levels in this study. PAPP-A as one of the main proteinases that cleave IGFBP-4 was considerably less expressed at the

transcript level and protein levels compared to IGFBP-4, in both OP and healthy samples. This was not in line with the theory that the elevated levels of IGFBP-4 in OP-PDLSCs were merely the result of lower PAPP-A levels. Whether PAPP-A secreted from osteoporotic samples is less efficient in inhibiting IGFBP-4 compared to healthy samples is not clear from the findings of this study. Future studies investigating the response of OP-PDLSCs and H-PDLSCs treated with IGFBP-4 to exogenous PAPP-A, would be needed to address this. Overall, it is important to acknowledge the complexity of studying IGF system, particularly when limited data is available on its impact on PDLSCs under osteoporotic conditions in the literature. In a recently published review, Baxter attempted to find the potential reasons for this complexity particularly for IGFBPs element of the system. He explained the influence of IGFBPs on IGF receptors and ligands by processes such as post-translational modification (extracellularly) or intracellularly through modifications of receptor-dependent cascades activation. He concluded the discussion suggesting the need for further research in this field to understand how the different cellular environments could impact the IGF-dependant functions of IGFBPs (Baxter, 2023).

Ageing has been linked to alterations in stem cell properties (Pérez et al., 2018) including PDLSCs (Li et al., 2020). Ageing is also associated with a reduced capacity of periodontal tissue homeostasis (Lim et al., 2014), hence predisposing stem cells to accumulate DNA damage overtime. This in turn might lead to a reduction of stem cells' number and diminish their responsiveness to external stimuli (Huang et al., 2016). Adult PDLSCs, for example, demonstrated reduced proliferative, multi-lineage differentiation and immunosuppression properties when compared to younger cells (Li et al., 2020, Zhang et al., 2024). Ageing is also associated with an overall decline in IGF-1 potentially related to reduced GH levels (Masanobu and Clifford, 2012, Janssen, 2019). Another relevant hormonal change that occurs with ageing is estrogen deficiency (Sui et al., 2016). In addition to the impact of reduced estrogen on disturbing MSC homeostasis, estrogen deficiency might lead to accumulation of reactive oxygen species (ROS) leading to cellular impairment and potentially apoptosis (Zheng et al., 2019). In this study, all osteoporotic donors were postmenopausal with the age range of (52-85 years) compared to healthy controls of a much younger age range (13-32 years). Despite covering the extremes of age range and menopause, a limitation

of this study is the absence of samples from age matched healthy female controls (free of OP). This was due to the difficulty and challenge of collecting samples with healthy PDL from postmenopausal female donors in general, let alone finding healthy PDL tissues from donors who are of matched age that are free of severe periodontal disease or with relatively healthy PDL.

All the donors who participated in this study had been officially diagnosed with OP using DXA scans and were on regular medications (treatment duration ranging from 18 months to 10 years). Clinical evidence suggests that the use of systemic osteoporotic medications affects PDL health. For example, it has been shown that combining HRT (estrogen and/or progesterone) with periodontal treatment improves periodontal health outcomes in osteoporotic patients (Koth et al., 2021), and the use of estrogen replacement therapy improves tooth retention and mobility (Calciolari, 2016). Additionally, some clinical studies have reported that the use of bisphosphonates (BPs) leads to improved periodontal parameters, namely clinical attachment loss (CAL), bleeding on probing (BOP) and pocket depth (PD), compared to placebo in patients with chronic periodontitis (Lane et al., 2005). Whilst these medications have a relatively rapid half-life in the serum (ranging from 2.7 hrs for HRT (Ginsburg et al., 1998, Sabatino et al., 2014) to 6 hours for BPs (Chapurlat and Delmas, 2006)), they might have a cumulative effect on PDLSCs over the long duration they are used for. For instance, it can take up to 10 years to eliminate BPs from bone since it has a high affinity to hydroxyapatite crystals (Chandran et al., 2024). Based on this, it is apparent that the accumulative impact of regular use of osteoporotic medications on OP-PDLSCs cannot be totally excluded. It is not possible at this stage to decide whether that impact is positive or negative since analysing the individual effect of OP medications on OP-PDLSCs is beyond the scope of this project. It was also almost impossible to isolate samples from osteoporotic patients who had been diagnosed but didn’t start treatment due to the difficulty in liaising between the different clinical services as well as the potential jeopardy to patients’ health if they had to delay therapy.

Additionally, two out of the three osteoporotic donors were smokers while no history of smoking was reported in the healthy donors. Smoking has been negatively linked to periodontal health and regeneration (Kanmaz et al., 2021). In a study by Ng et al., PDLSCs isolated from human smokers showed reduced

proliferation, migration and osteogenic differentiation capacities compared to non-smokers (Ng et al., 2015). In agreement, Jiang et al. (Jiang et al., 2024), noted that the addition of nicotine (10^{-3} – 10^{-8} M for 48hrs) to human PDLSCs negatively impacted cellular proliferation, differentiation and stemness. In addition, tobacco smoking has been significantly associated with periodontitis through its impact on the composition of oral microbiota, modifying immune response and periodontal healing capacity (Leite et al., 2018). Smoking has also been linked to increase in ROS, which limits the regenerative capacities of stem cells (Park et al., 2024) as well as the increase of inflammatory markers such as IL-1β, IL-6 and TNF-α (Radvar et al., 2017). In two of the osteoporotic samples, , the hidden impact of smoking on cellular alterations cannot be purely excluded and hence the negative implications of smoking on studied aspects (stemness, proliferation, osteogenic differentiation) might have negatively impacted in OP-PDLSCs.

Most of the published osteogenic differentiation research utilised the addition of L-ascorbic acid, dexamethasone and β-glycerophosphate to basal growth media. However, in this project the osteogenic media was prepared by adding L-ascorbic acid and dexamethasone to basal media (with the exclusion of β-glycerophosphate). This was used to successfully induce osteogenic differentiation of MSCs by other researchers using a combination of both components and/or each component individually (El-Gendy et al., 2013, Nuttelman et al., 2006, Choi et al., 2008, Alkharobi et al., 2016, Alhodhodi et al., 2017, Cheng et al., 1994). L-ascorbic acid (vitamin C) contributes to collagen matrix synthesis, and it was shown to induce osteogenic differentiation by activating integrin synthesis and cell-matrix interaction (Hadzir et al., 2014). Dexamethasone is a synthetic glucocorticoid. Endogenous glucocorticoids are essential to induce osteogenic differentiation during embryogenesis (Mushtaq et al., 2002). Dexamethasone also contributes to osteogenic differentiation by activating Wnt/β-catenin signalling upon which *RUNX2* expression is dependent (Hamidouche et al., 2008). β-glycerophosphate acts as a source of phosphate aiding in hydroxyapatite mineral formation (Freeman et al., 2016). The addition of β-glycerophosphate to the osteogenic media was excluded from the experiments carried out throughout this project to avoid the generation of false

positive results (Langenbach and Handschel, 2013), i.e. dystrophic mineralisation, which might obscure mineralisation caused by the osteogenic differentiation of PDLSCs in both research groups.

Overall, all results discussed including colony formation, surface phenotype, differentiation and IGF axis expression might have been influenced by the OP-PDLSCs microenvironment. Stem cells exist in tissues in specific microenvironment knows as “stem cell niche” (Mokry and Pisal, 2015) which was shown to influence stem cell properties (Martinez-Agosto et al., 2007). The stem cell niche is not solely the area where the stem cells reside, but it includes other neighbouring cells, vascular cells, peripheral nerves and ECM components (Yi et al., 2022, Mokry and Pisal, 2015). The microenvironment, and therefore the stem cells within, can be impacted by several factors. For instance, inflammation might disturb the stem cell niche owing to disturbances in the immune cell component (Yi et al., 2022). Inflammation associated with carious lesions and periodontal disease might lead to changes in cell proliferation, migration, colony formation and differentiation capacities (Okić-Đorđević et al., 2021). In clinical terms, patients at the age range required for this project tend to extract teeth for dental involvement rather than for impaction, esthetic or orthodontic reasons. Therefore, it was very challenging to find donors who required extraction of sound teeth during the period of this study from this age group. Hence, while the clinical inclusion criteria insisted on ruling out periodontal disease from osteoporotic donors in this project, it was not possible to exclude carious lesions completely. Subsequently, the effect of subclinical inflammation could not be excluded either.

Finally, the project's strength and novelty lie in being the first study to assess this cell population and shed a light on some of the important molecular mechanisms of the disease that affected the function of this cell population in postmenopausal patients. However, one of the faced challenges during designing, executing and writing this study was the lack of literature on periodontal ligament cells population from human donors or PDL tissues to support this work. It is possible that OP impacts jawbone differently from skeletal bone and potentially impacts PDL differently. For instance, healthy BMSCs isolated from the maxilla and mandible show enhanced biological properties (delayed senescence, enhanced osteogenic differentiation) compared to BMSCs isolated from axial bone (Koth et

al., 2021). Additionally, relying on findings from animal studies might not be ideal since animal models do not replicate the exact pathogenesis of OP (Calciolari et al., 2017a). The lack of relevant studies warrants a need for further research in this field to help understand and develop targeted therapies for periodontal regeneration in osteoporotic patients.

8.1 Conclusion:

In conclusion, this project revealed that OP-PDLSCs maintained a similar phenotype comparison to H-PDLSCs with a trend of reduced cellular proliferation. It also indicated the potential negative impact of OP on the osteogenic differentiation and mineralisation capacities of PDLSCs. The variation in the expression of ERs and bone remodelling markers (RANK/ RANKL/ OPG) was also explored. It finally investigated the changes of IGF axis components in OP-PDLSCs under osteoinductive environments, with a focus on IGFBP-4 and its inhibitor suggesting their potential contribution to the reduced osteogenic differentiation and that IGFP-4 could be a potential therapeutic target that requires further studies to be confirmed.

8.2 COVID-19 impact:

Due to the restrictions imposed during the COVID-19 pandemic, which coincided with the start of this project, a few numbers of samples was collected owing to the scarcity of samples that fulfil all the inclusion criteria. Also, while every effort was made to exclude the gender variation by collecting samples from the same gender (female), one out of the three healthy donors was male. This was due to the limited flow of dental patients during the early phase of the project. However, the data obtained from this donor were not massively different from the other healthy female donors.

8.3 Future work:

In the future, it would be valuable to include controls of a similar age group and matched genders. Albeit very challenging, it might also be helpful to collect periodontal tissues from osteoporotic donors who have been diagnosed but did

not commence therapy. Both of these suggestions would help us comprehend the impact of OP on PDLSCs alone without other compounding factors.

Regarding laboratory experiments, it would be interesting to characterise and examine the mineralised crystals deposited by OP-PDLSCs compared to healthy controls using methods such as scanning electron microscopy (SEM), infrared spectroscopy or transmission electron microscopy (TEM) (Rodriguez Lugo et al., 2006)). Furthermore, plating OP-PDLSCs on regenerative scaffolds (either natural or synthetic (Wu et al., 2021)) would also deepen our understanding of the behaviour of the cells in 3D environments. In addition, further functional studies on IGFBP-4 would be beneficial. For example, it might be worth investigating whether the inhibition of IGFBP-4 (through the exogenous application of PAPP-A or the addition of anti-human IGFBP-4 antibody) would change the response of OP-PDLSCs to osteogenic induction. It would also be interesting to compare the effect of OP on stem cells from different niches such as BM-MSCs and dental pulp to help understand the role site specificity might play in the impact of a certain disease on stem cells.

While this project shed a light on the expression of ERs, functional studies on the pathway interactions would give a better understanding for the correlation between estrogen and OP in PDLSCs. On a molecular level, a separate set of experiments could also be carried out to simulate hormonal therapies e.g. investigating whether estrogen treatment of OP-PDLSCs would have an impact on the osteoclastogenesis pathway (RANKL/ RANK/OPG) using RT-qPCR and ELISA (for gene and protein levels, respectively). Another example is experiments to understand the downstream signalling pathway of OP in PDLSCs (e.g. Wnt signalling (Wang et al., 2024)).

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Appendix

Appendix A) Copy of the ethical approval for the project

DREC ref: 040221/AA/317
Study title: Characterisation of Dental Stem Cells Derived from Osteoporotic Patients

Thank you for submitting the above Tissue Bank application to the Dental Research Ethics Committee (DREC). Your application has been reviewed and I am pleased to confirm that is has been approved for 20 teeth in the first instance. Once you have collected 20 teeth from the Bank, please send a request to Julie McDermott quoting the above reference number to apply for a further 20 teeth, and so on.

Please note: Due to the current Covid-19 pandemic, there are fewer teeth coming into the Bank. Please be aware that teeth may not be supplied to you all at once and will be made available to you as and when become available.

If you have any questions, please do not hesitate to contact me.
With best wishes for the success of your study.

For and on behalf of
Professor David Wood
DREC Chair

Appendix B) Back-gating strategy for flow cytometry (markers CD34, HLADR)

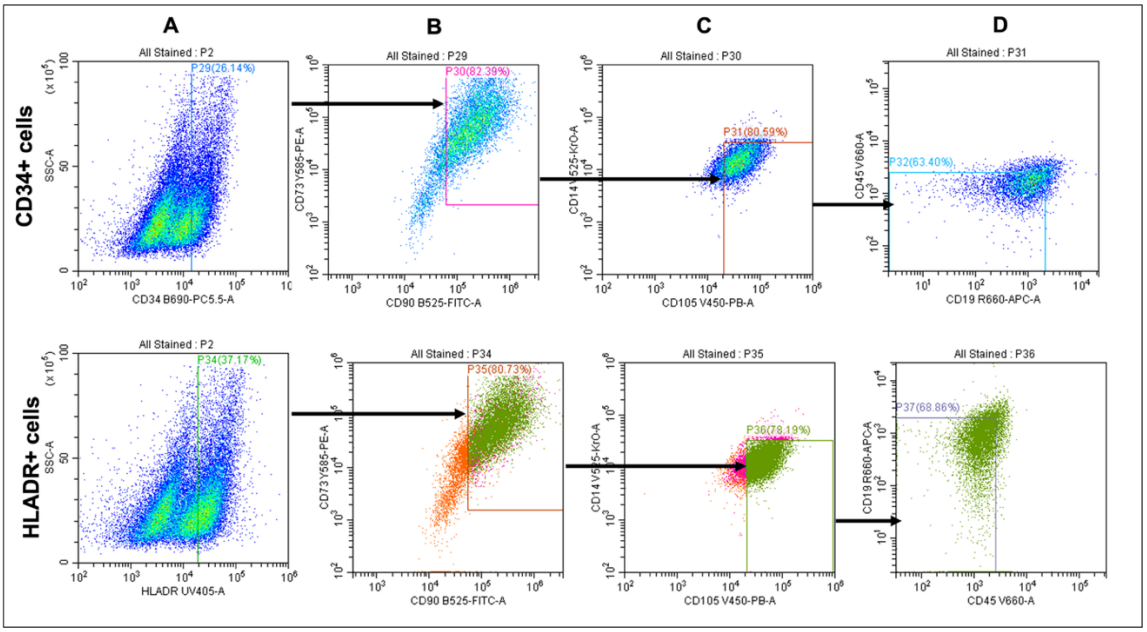

Figure 61. Back-gating of CD34+ and HLADR+ cells (data for OP-PDLSCs isolated from donor 2).

Sequential analysis strategy was carried out to investigate whether $CD34^+$ cells (top row) and $HLADR^+$ cells (bottom row) fulfil other MSC criteria. (A) selection of $CD34^+$ and $HLADR^+$ cells (B) using the gate with the identified population, $CD90^+$ and $CD73^+$ cells were selected. (C) Further selection of CD105+ and $CD14^-$ cells. (D) further selection of $CD19^-$ and $CD45^-$ cells.

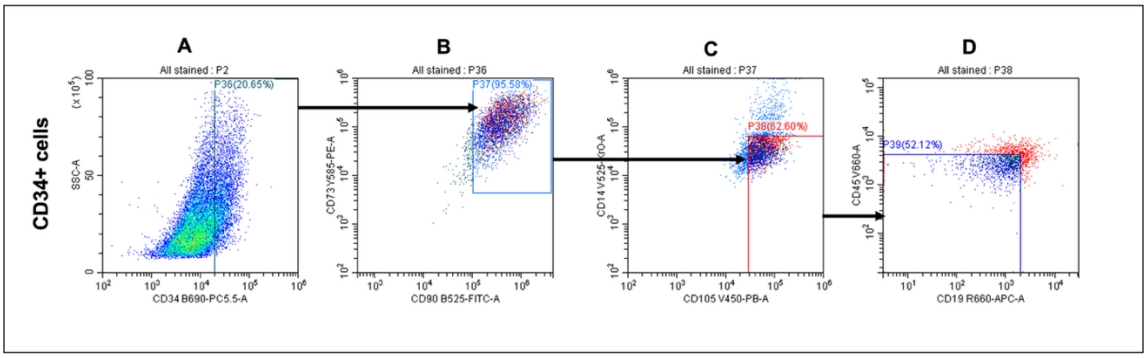

Figure 62. Back-gating of CD34+ cells (data for OP-PDLSCs isolated from donor 3).

Sequential analysis strategy was carried out to investigate whether $CD34^+$ cells fulfil other MSC criteria. (A) selection of $CD34^+$ cells (B) using the gate with the identified population, $CD90^+$ and $CD73^+$ cells were selected. (C) Further selection of $CD105^+$ and $CD14^-$ cells. (D) further selection of $CD19^-$ and $CD45^-$ cells.

Appendix C) Relative changes in gene expression of matrix metalloproteinases (MMPs) and tissue inhibitor metalloproteinases (TIMPs)

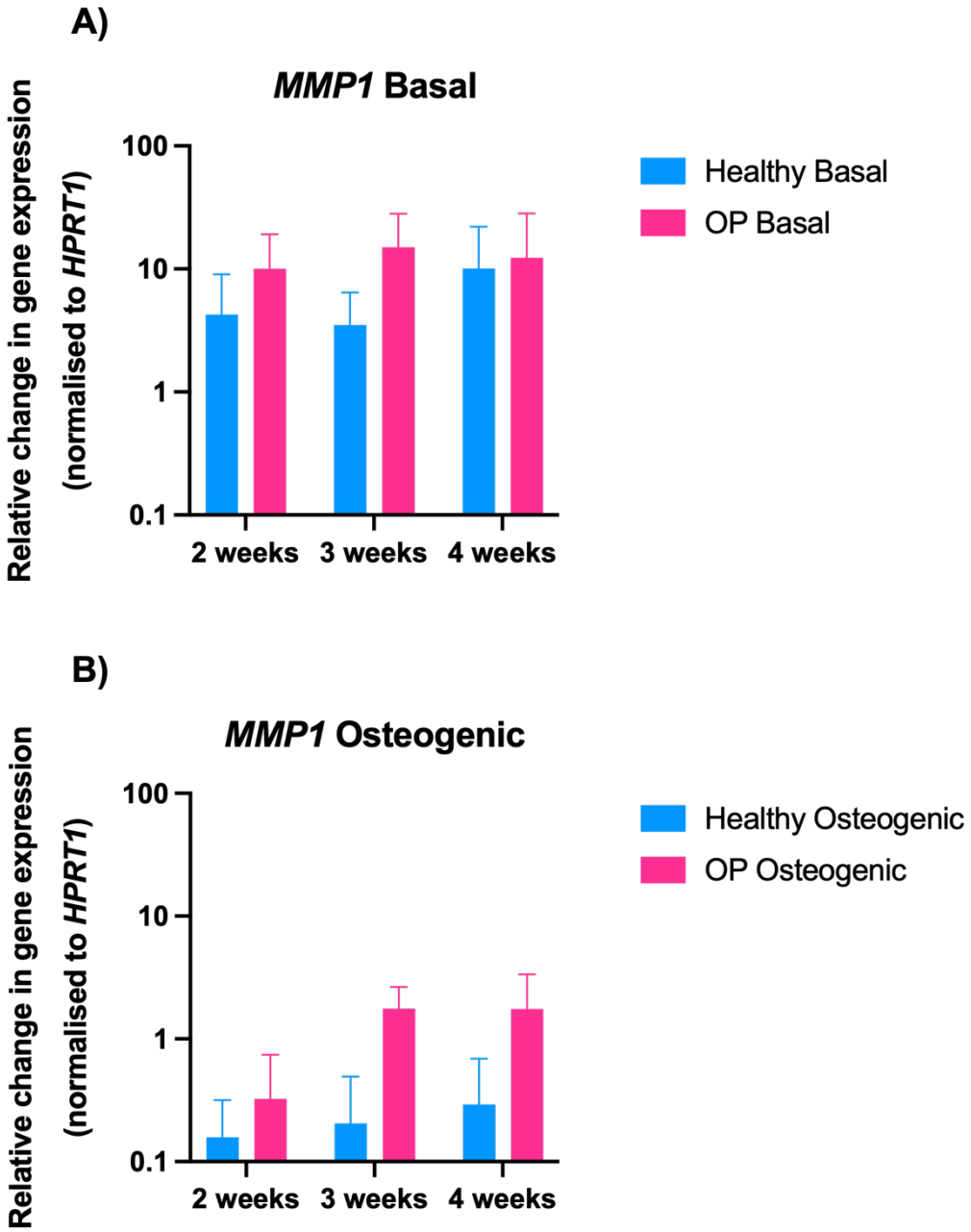

Figure 63. Relative *MMP1* gene expression in OP-PDLSCs compared to H-PDLSCs.

The relative gene expression levels of *MMP1* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

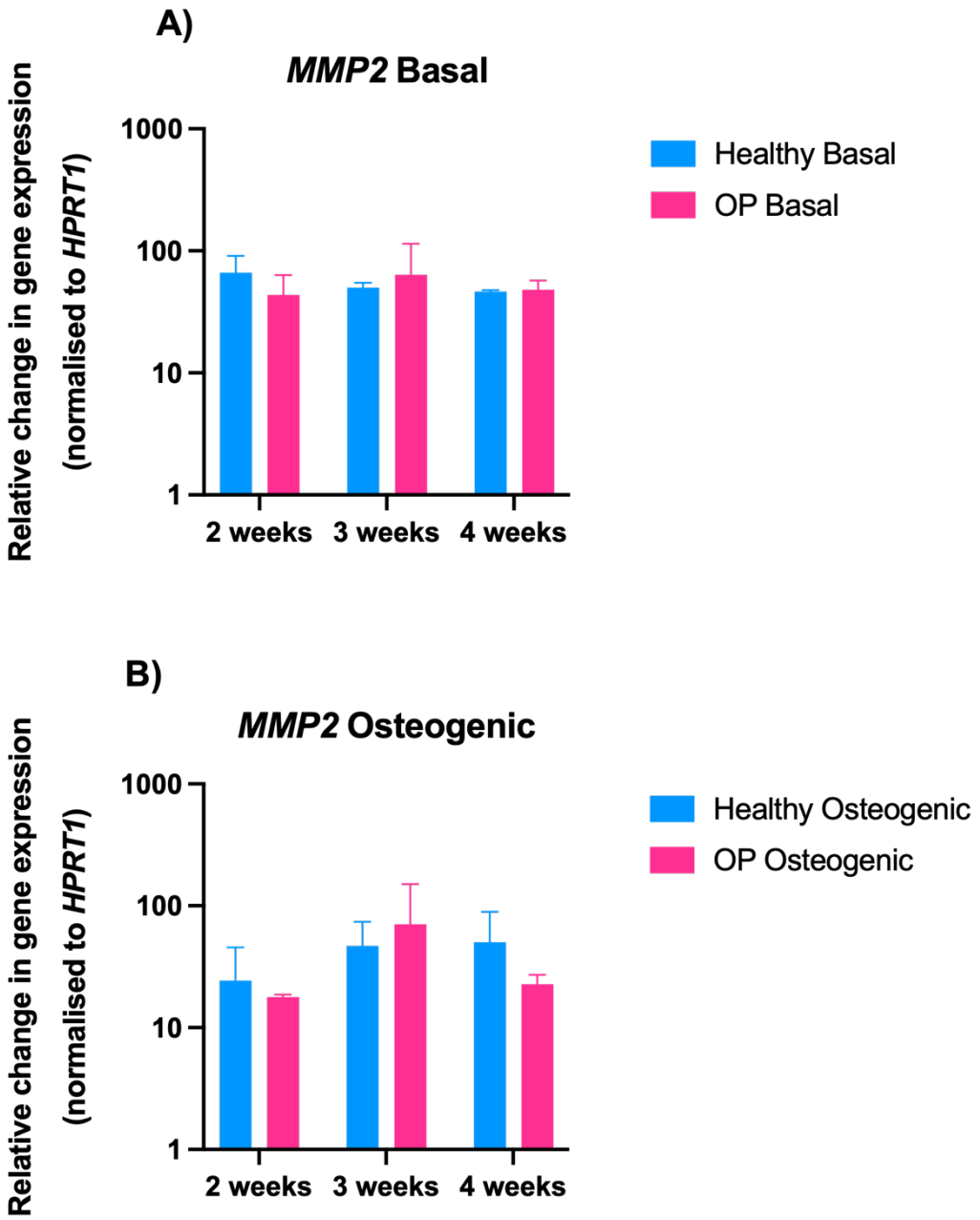

Figure 64. Relative *MMP2* gene expression in OP-PDLSCs compared to H-PDLSCs.

The relative gene expression levels of *MMP2* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

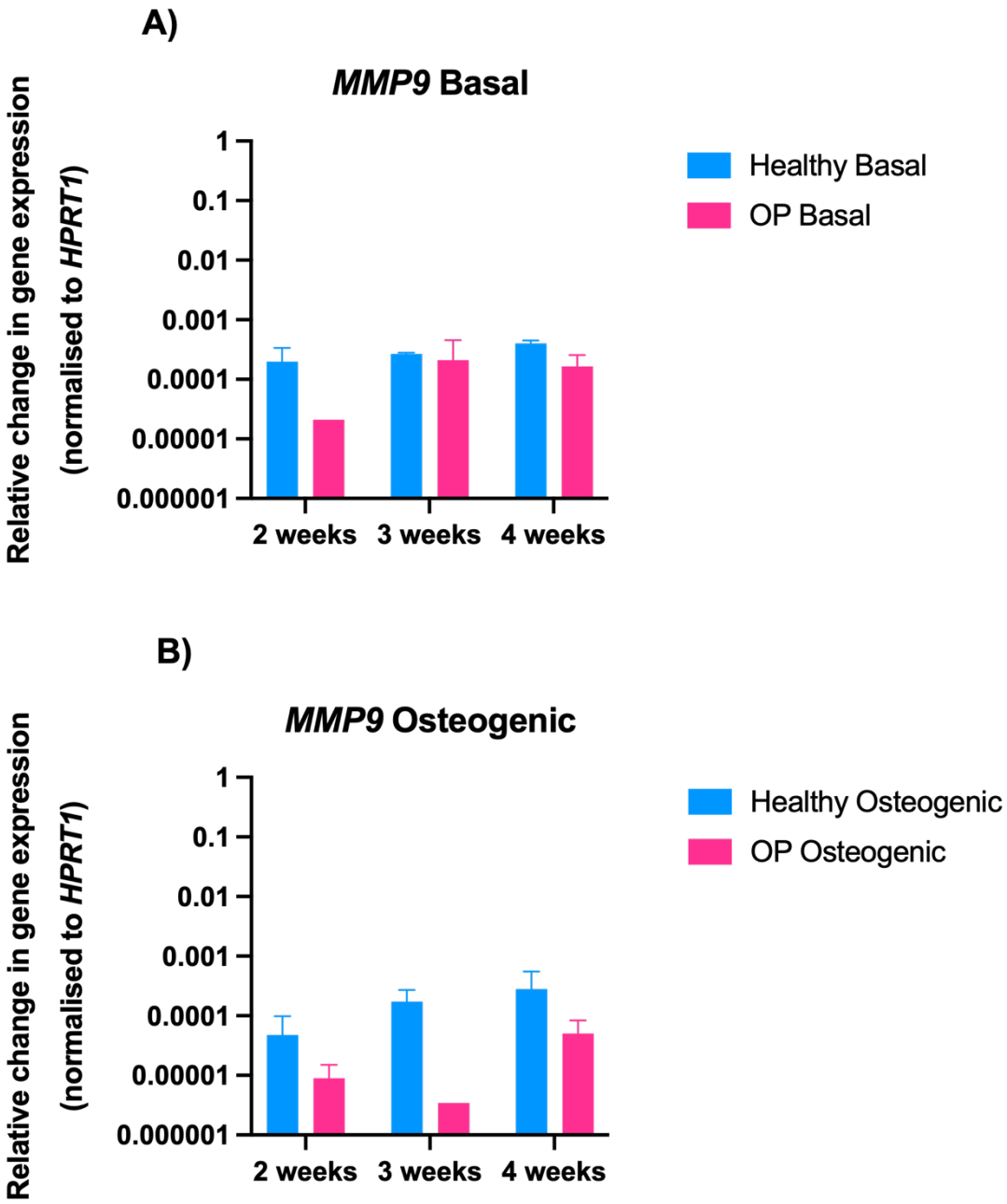

Figure 65. Relative *MMP9* gene expression in OP-PDLSCs compared to H-PDLSCs.

The relative gene expression levels of *MMP9* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

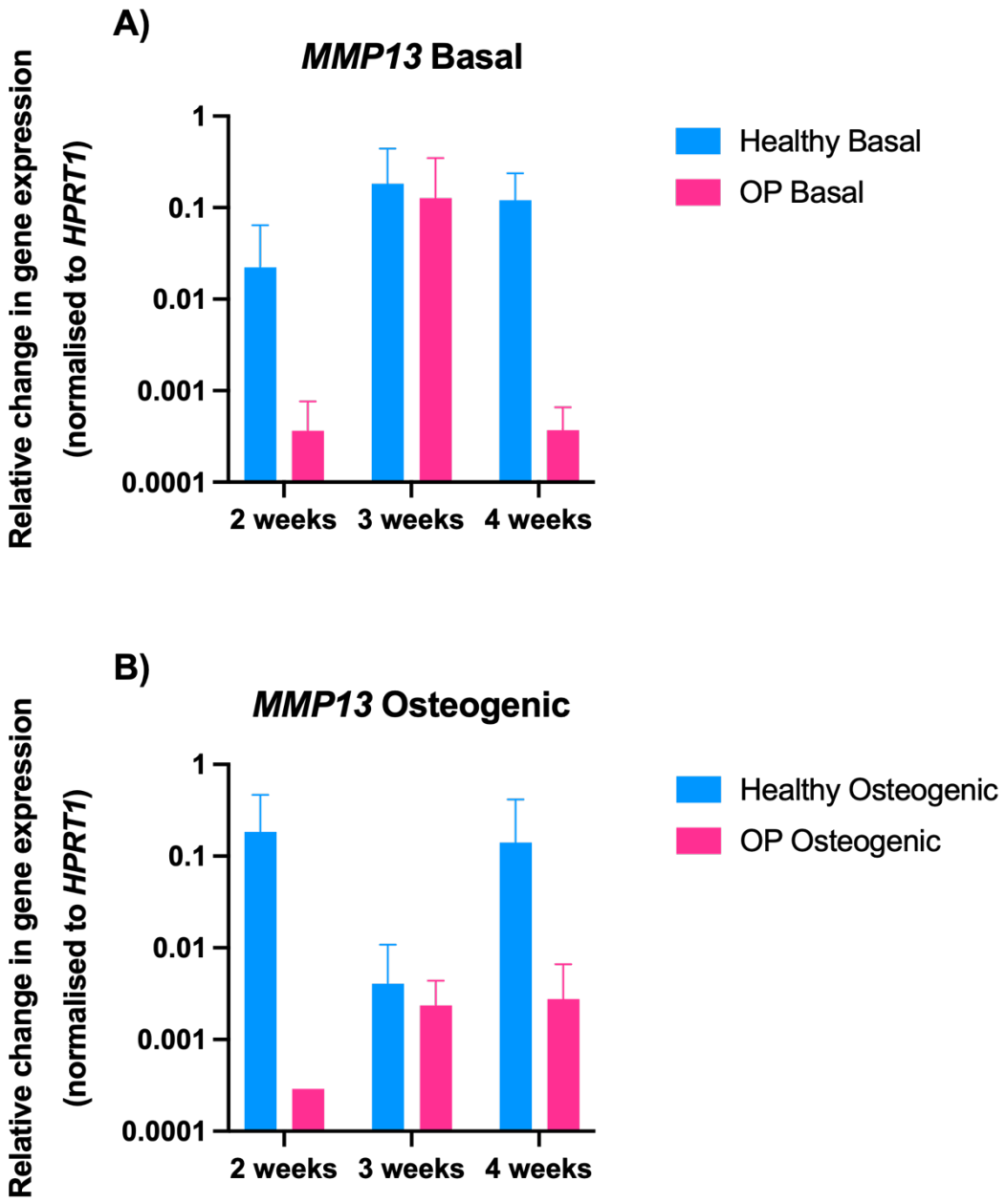

Figure 66. Relative *MMP13* gene expression in OP-PDLSCs compared to H-PDLSCs.

The relative gene expression levels of *MMP13* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

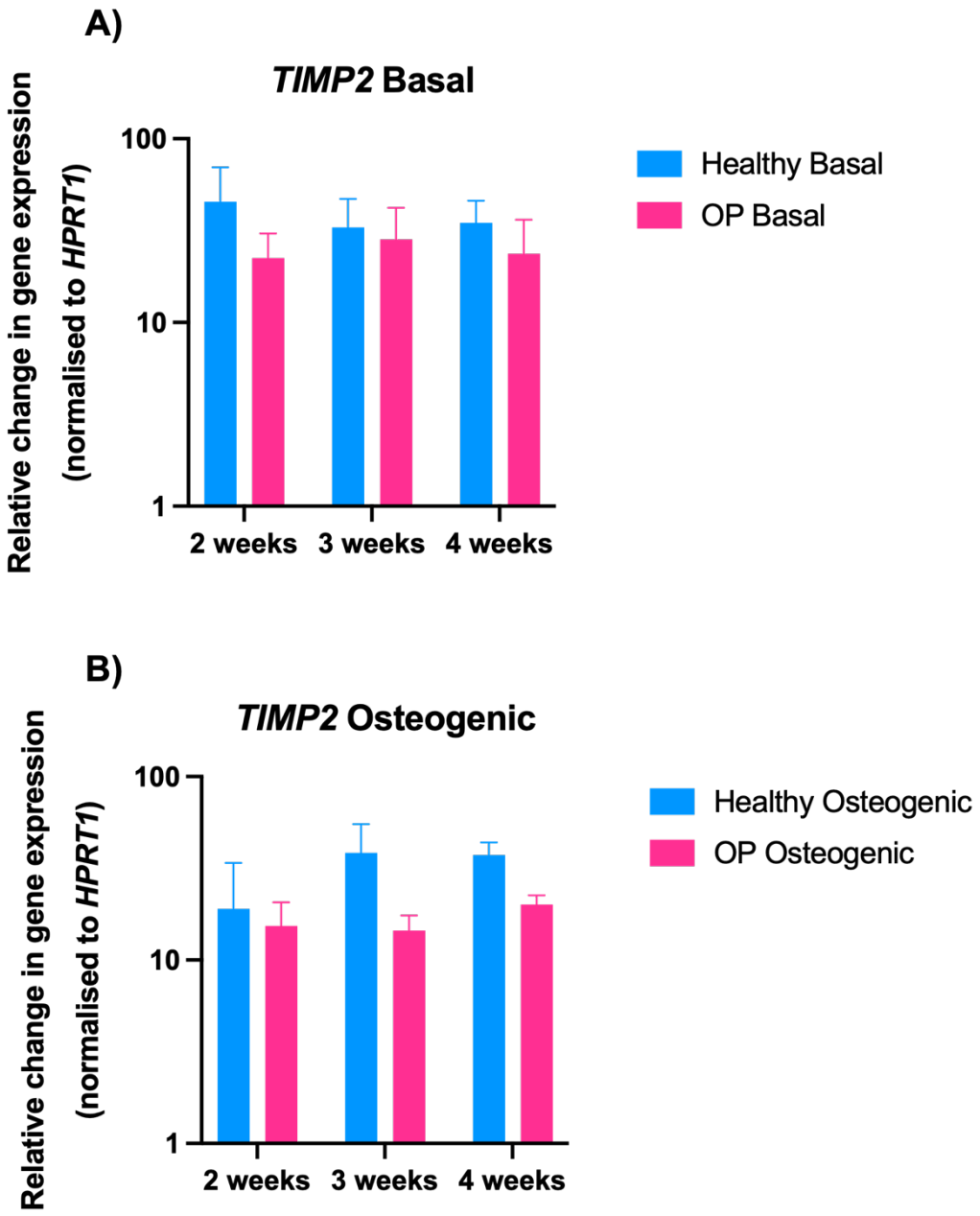

Figure 67. Relative *TIMP2* gene expression in OP-PDLSCs compared to H-PDLSCs.

The relative gene expression levels of *TIMP2* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

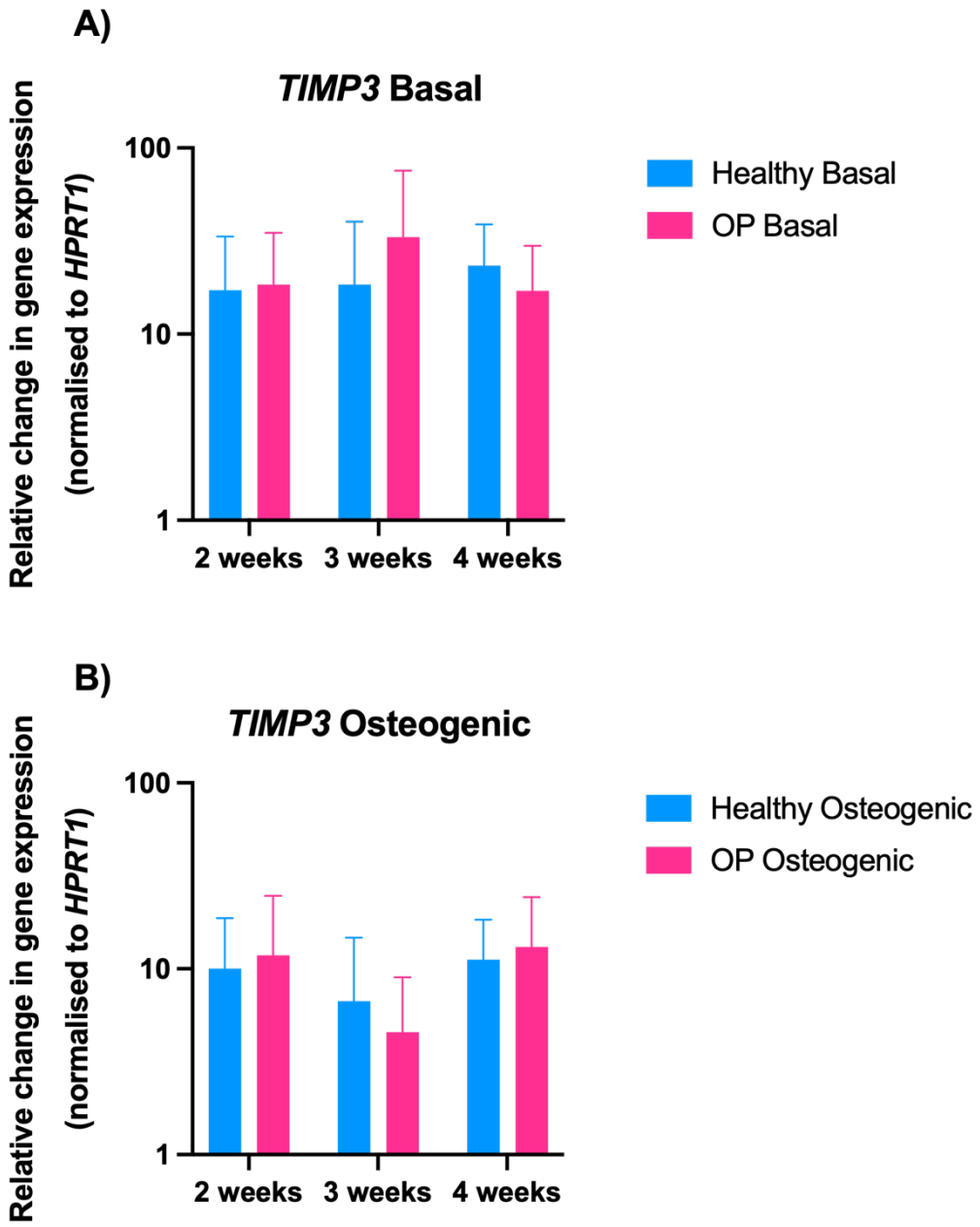

Figure 68. Relative *TIMP3* gene expression in OP-PDLSCs compared to H-PDLSCs.

The relative gene expression levels of *TIMP3* normalised to *HPRT1* gene in OP-PDLSCs (n=3) compared to H-PDLSCs (n=4) under basal (A) and osteogenic conditions (B) at 2, 3 and 4 weeks. Data presented as means± SD.

Appendix D) List of presentations

Poster Presentation:

Arwa Alghamdi, Josie Meade, Tiffany Li, Alasdair McKechnie, Elena Jones, Reem El-Gendy

Primary Characterisation and Osteogenic Differentiation of Periodontal Stem Cells under Osteoporotic Conditions, Tissue and Cell Engineering Society conference (TCES), University of Birmingham, UK (May 2022)

Arwa Alghamdi, Josie Meade, Tiffany Li, Alasdair McKechnie, Elena Jones, Reem El-Gendy

Characterisation of Periodontal Stem Cells Isolated from Osteoporotic Patients, Pan European Region International Association of Dental Research (PER-IADR), France (September 2022)

Oral Presentations:

Arwa Alghamdi, Josie Meade, Tiffany Li, Alasdair McKechnie, Elena Jones, Reem El-Gendy

Investigating the Role of Insulin-like Growth Factor (IGF) Axis in Stem Cell Based Dental Regeneration in Osteoporotic conditions, Online Oral Biology Symposium I, a collaboration between University of Brasilia and University of Leeds (May 2021).

Arwa Alghamdi, Josie Meade, Tiffany Li, Alasdair McKechnie, Elena Jones, Reem El-Gendy

Primary Characterisation and Osteogenic Differentiation of Periodontal Stem Cells under Osteogenic Conditions, Biomaterials and Tissue Engineering group (BiTEG) 21st annual white rose meeting, University of York, UK (December 2022)

Arwa Alghamdi, Josie Meade, Ala Altaie, Tiffany Li, , Alasdair McKechnie, Elena Jones, Reem El-Gendy

Characterisation and Osteogenic Differentiation of Periodontal Stem Cells Isolated from Osteoporotic Patients, The British Society for Oral and Dental Research (BSODR) annual scientific meeting, Queen Mary University of London, United Kingdom (September 2023)

Arwa Alghamdi, Josie Meade, Ala Altaie, Tiffany Li, , Alasdair McKechnie, Elena Jones, Reem El-Gendy

Osteoporotic Periodontal Stem Cells; Is there Hope for Regeneration? Early Career Researcher (ECR) Event, University of Leeds, UK (September 2024)