UNIVERSITY OF LIVERPOOL

Circulating microRNAs in Osteoporosis

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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August 2017

Doctor of Philosophy Declaration

I hereby declare that this dissertation is a record of work carried out in the unit of clinical chemistry at the Institute of Ageing and Chronic Disease at the University of Liverpool during the period of March 2013 to August 2017. This work has not been previously submitted to the University or any other institution in application for admission to a degree or other qualification except where otherwise indicated as help which is appropriately acknowledged.

August 2017

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Abdullah Mandourah BSc, MSc

Dedications

This work presented in this thesis is dedicated to the soul of my parents. Without their unconditional love and support, I would not have been able to achieve what I have today. My Allah bless them and give them the highest place in the paradise.

Special thanks go to my wife and kids, to my brothers and sisters and to my friends who believed in me and kept me going through the hard times.

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Last but not least, also a word of thanks to my close friend Ayed Dera, as without him, I would never have a great encouragement and competitive atmosphere during my study. Thank you for always listening, discussing and keeping me confident and happy

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List of Abbreviations

Abbreviation	Description
Δ	Delta
3'UTRs	3' untranslated regions
ALP	Alkaline Phosphatase
AREs	AU-rich elements
AUC	Area Under the Curve
BALP	Bone Alkaline Phosphatase
BMD	Bone Mineral Density
BMM	Bone Marrow Macrophage
BMP	Bone Morphogenic Protein
BMPR2	Bone Morphogenetic Protein Receptor Type 2
BMU	Bone Multicellular Unit
BP	Bisphosphonates
BTM	Bone Turnover Marker
Ca	Calcium
cDNA	complementary deoxyribonucleic acid (DNA)
CGR	Centre of Genomic Research
cKO	Conditional Knockout
CML	Chronic Myeloid Leukaemia
COSHH	Control of Substances Hazardous to Health Regulations
Ct value	cycle threshold value also called quantification cycle (Cq)
CTIMP	Clinical trial of an investigational medicinal product

Abbreviation	Description
CTX	C-terminal crosslinked telopeptide
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's Phosphate Buffered Saline
DPD	Deoxypyridinoline
DXA	Dual-energy X-ray Absorptiometry
EDTA	Ethylene Diamine Tetra Acetic
ESCC	Oesophageal Squamous Cell Carcinoma
EU27	27 countries of the European Union
FGF-23	fibroblast growth factor 23
FN	Femoral Neck
FRAX	Fracture Risk Assessment Tool
GLP-2	glucagon-like peptide 2
hADSC	Human adipose-derived stromal/stem cell
HKG	Housekeeping Gene
hMSC	Human mesenchymal stem cell
HOB	Human osteoblast
HPLC	High-performance liquid chromatography
HR-pQCT	High resolution peripheral quantitative computed tomography
ICTP	Carboxyterminal telopeptide of type I collagen
ID	Identification number
IOF	International osteoporosis foundation
LS	Lumbar Spine

Abbreviation	Description
M-CSF	Macrophage Colony-Stimulating Factor
miRISC	miRNA induced silencing complex
miRNA	MicroRNA
mRNA	messenger RNA
MSC	mesenchymal stem cell
mTOR	mammalian target of rapamycin
NHS	National Health Service
NOPF	Non-Osteoporosis, Female
NOPFP2	Non-Osteoporosis, Female Pool#2
NOPM	Non-Osteoporosis, Male
NOPMP1	Non-Osteoporosis, Male Pool#1
NRES	National Research Ethics Service
NRT	No-Reverse Transcriptase
NTX	-terminal crosslinked telopeptide
OCN	Osteocalcin
OPAF	Osteopenia, Female
OPAFP3	Osteopenia, Female Pool#3
OPF	Osteoporosis, Female
OPFP4	Osteoporosis, Female Pool#4
OPG	Osteoprotegerin
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline

Abbreviation	Description
PCa	prostate cancer
PICP	Procollagen type I C-terminal propeptide
PINP	Procollagen I Intact N-Terminal propeptide
PIS	Participant Information Sheet
PPC	PCR Positive Control
pri-miRNA	primary miRNA transcript
PUVA	psoralen and ultraviolet A
PYD	Pyridinoline
RANK	receptor activator of the NF-κB
RANKL	RANK ligand
RLBUHT	The Royal Liverpool and Broadgreen University Hospitals
rMSCs	rat mesenchymal stem cells
RNA	ribonucleic acid
ROC Curve	Receiver operating characteristic curve
RT	Reverse Transcriptase
RT	Room Temperature
RTC	Reverse Transcription Control
RT-qPCR	Real time -Quantitative Polymerase chain reaction
SD	standard deviation
TH	Total Hip
TRACP-5b	Tartrate resistant acid phosphatase – 5b
UHR	Ultra-High Recovery Microcentrifuge Tube

Abbreviation	Description
Vit D	Vitamin D
wt	wild-type

List of published abstracts/presentation

Abdullah Y. Mandourah, R. Barraclough, R. Van't Hof, L. Ranganath and D. Barraclough. *Circulating microRNAs in Osteoporosis*. Paper in Progress.

Abdullah Y. Mandourah, R. Barraclough, R. Van't Hof, L. Ranganath and D. Barraclough. *Circulating microRNA in metabolic bone diseases-Osteoporosis*. A poster presentation in Annual Bone Research Society Annual Meeting (30 Jun 2016-2 July 2016, Liverpool, UK).

Abdullah Y. Mandourah, R. Barraclough, R. Van't Hof, L. Ranganath and D. Barraclough. *Circulating microRNA in metabolic bone diseases-Osteoporosis*. A poster presentation in Saudi Student Conference (31 January- 1 February 2015, London, UK).

Abstract

Osteoporosis is the most common age-related bone disease. It is clinically symptomless until the first fracture happens and once diagnosed it is often found to be associated with low bone mineral density (LBMD) of T-Score ≤ -2.5. Circulating microRNAs (miRNAs) have been used successfully as promising biomarkers to diagnose and assess the progression of complex diseases such as cancer and cardiovascular diseases, as well as the effectiveness of treatment.

This research aims to identify circulatory miRNAs associated with the progression of osteoporosis in a test group of patients using advanced PCR arrays initially and the identified differentially-expressed miRNAs were validated in individual clinical specimens using RT-qPCR. The potential target genes were analyzed using bioinformatics tools.

Ethical approval was obtained prior to patient recruitment. A total of 161 participants were recruited and assigned to five groups: Non-Osteoporosis control group (T-Score ≥-1), osteopenia (T-Score <-1 and >-2.5 SD), osteopenia with fracture (T-Score <-1 and >-2.5 SD), osteoporosis (T-Score ≤ -2.5 SD) and osteoporosis with fracture (T-Score ≤ -2.5 SD). RNAs were extracted and analyzed from all serum and plasma samples.

A panel of 49 differentially expressed miRNAs (up or down by >3 fold) between osteopenia and osteoporosis patient groups was identified using a miRNA PCR Array. Six miRNAs: miR-215-5p, miR-99a-5p, miR-100, miR-373-5p, miR-4516 and miR-122-5P, were significantly differentially-expressed between osteoporosis and osteopenia patients by initial RT-qPCR screening. Further analysis showed that the levels of circulating miRNAs: hsa-miR-373-5p, hsa-miR-122-5p and hsa-miR-215-5p and plasma hsa-4516 were associated with fragility fracture, and correlated with low bone mineral density. The results suggest that these miRNAs could be potential diagnostic biomarkers for osteoporosis in the future. Potential target genes of these miRNAs were also analyzed using bioinformatics tools. The project demonstrated that circulating miRNAs can be purified from serum and plasma and could be developed as critical diagnostic tools for osteoporosis.

Chapter 1: Introduction

1.1 Bone Biology

1.1.1 Bone Function

Bone plays multifunctional roles as structural connective tissue that provides mechanical support and protection for vital organs, maintaining mineral haemostasis and haematopoiesis (Harada and Rodan, 2003, Taichman, 2005).

Bone also acts as an endocrine system that regulates the mineral haemostasis and energy metabolism as well as responding to other vital tissue systems, such as kidney, parathyroid gland, vascular, adipose tissue and hypothalamus (Fukumoto and Martin, 2009, Confavreux, 2011), by secreting fibroblast growth factor 23 (FGF-23) and osteocalcin (OCN) (Burr and Akkus, 2014). Most of FGF-23 is produced by osteocytes and causes a reduction of renal reabsorption of phosphate and decreases serum level of vitamin D3 (Burr and Akkus, 2014).

1.1.2 Bone Structure

Bones comprises two types of component: cortical bone (85% of bone), which is dense, solid, and surrounds the marrow space, providing mechanical and protective function, and the trabecular bone (15% of bone), which is composed of a spongy network of trabecular plates and rods interspersed in the bone marrow compartment and is involved in metabolic function (Burr and Akkus, 2014).

1.1.3 Bone Composition

Both cortical and trabecular bone mass material contains organic and mineral components. The organic component (Osteoid) accounts for 20-25% of total bone mass and is composed mostly of cross linked type I collagen (90%), the remainder being proteoglycan & non-collagenous proteins such as osteocalcin, osteopontin and

osteonectin. 65% of bone mass is mineral matrix, mainly calcium and phosphorus in the form of insoluble hydroxyapatite, as well as other ions, Mg^{2+}, Na^{+}, K^{+}, Cl^{-}, $HCO3^{-}$ and the remaining 10% is water (Burr and Akkus, 2014). Both collagen(s) and the minerals combine to form a composite material that provides rigidity to the structure and the collagen provides resilience and ductility (Florencio-Silva et al., 2015).

1.1.4 Cells of Bone

Bone contains four cell types: osteoblasts, osteocytes, bone lining cells and osteoclasts (Buckwalter et al., 1996, Downey and Siegel, 2006), (Figure 1-1). Osteoblasts, osteocytes and bone lining cells are derived from mesenchymal stem cells, whereas osteoclasts originate from haemopoietic stem cells (Downey and Siegel, 2006).

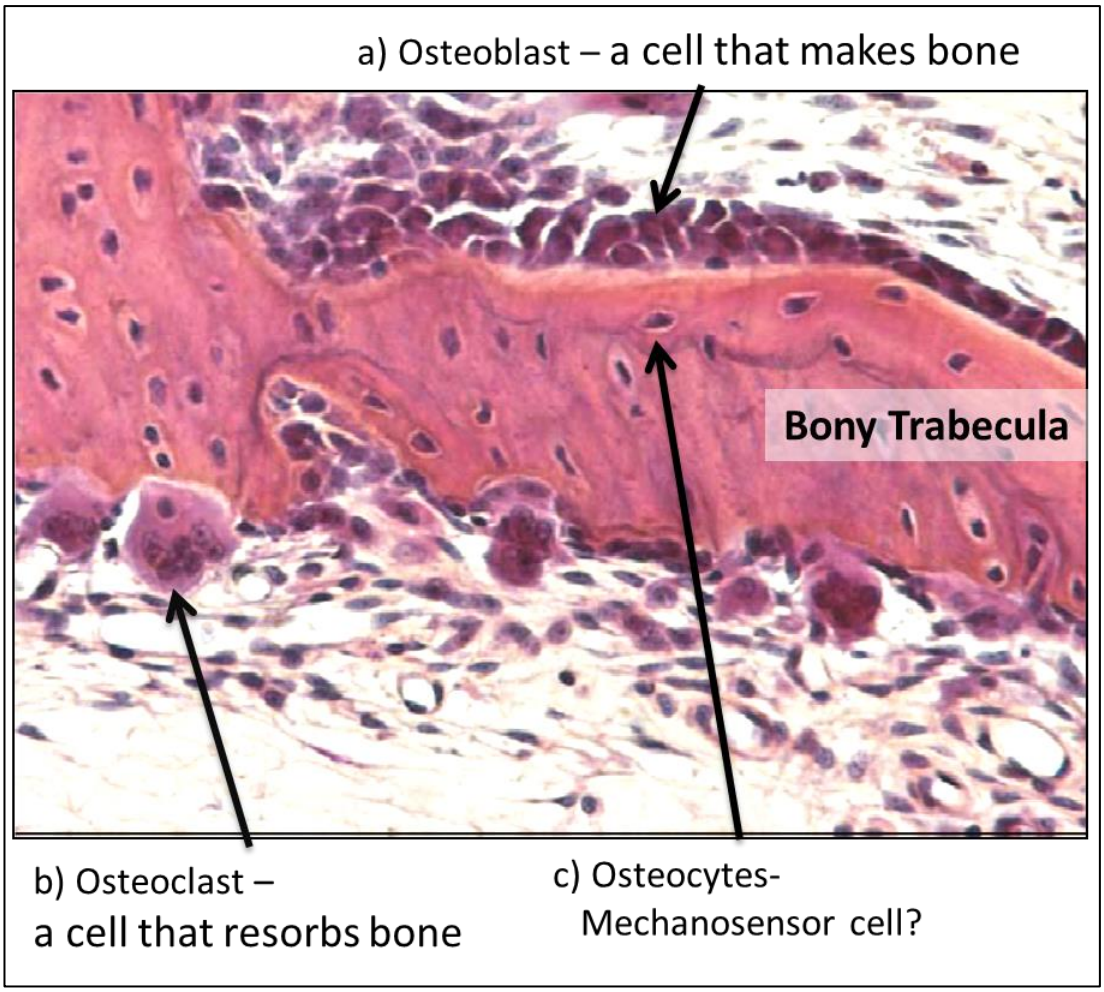


Figure 1-1: Light micrograph of a portions of mouse bone
Bone Cells; HE-stained section showing a portion of a bony trabecula with a) osteoblasts, b) osteoclasts, and c) and osteocytes. *By kind permission of Prof van't Hof* (University of Liverpool).

1.1.4.1 Osteoblasts

Osteoblasts are cuboidal cells that are located along the bone surface, comprising 4–6% of the total resident bone cells, derived from mesenchymal stem cells (MSC) (Capulli et al., 2014). Functionally, the osteoblast is responsible for production of the organic matrix, which is composed of Type 1 Collagen and non-collagenous proteins, including osteocalcin (Ducy et al., 2000, Everts et al., 2002). In addition, osteoblasts, under the influence of parathyroid hormone and local cytokines, release protein

mediators such as Receptor Activator of the NF-κB (RANK) Ligand (RANKL) that activate osteoclasts (Downey and Siegel, 2006, Udagawa et al., 1999).

Eventually, osteoblasts follow one of three developmental pathways: they may remain active osteoblasts on the edge of the bone matrix, they may become surrounded by matrix and become osteocytes, or become relatively inactive and form bone lining cells, referred to as 'resting osteoblasts' (Downey and Siegel, 2006).

1.1.4.2 Osteocytes

Osteocytes are located within lacunae surrounded by mineralized bone matrix and account for 90-95% of the bone cells with a lifespan of up to 25 years (Franz-Odendaal et al., 2006). Osteocytes are derived from osteoblasts by differentiation. As osteoblasts mature, and more matrix is laid down, they become embedded within the bone matrix, and become osteocytes that act both as mechanosensors, responsible for the adaptation of bone to mechanical force, and as endocrine cells that produce hormones (Bonewald and Johnson, 2008, Florencio-Silva et al., 2015). In addition, osteocytes are considered to be the major source of molecules that regulate the generation and activity of osteoclasts, such as the decoy receptor osteoprotegerin (OPG) and the RANKL (Kramer et al., 2010, Xiong and O'Brien, 2012, Bellido, 2014).

1.1.4.3 bone lining cells

Bone lining cells are quiescent flat-shaped osteoblasts covering bone surfaces (Figure 1-1), and thought to have a specific role in linking bone resorption to bone formation (Florencio-Silva et al., 2015). Lining cells in the presence of PTH secrete matrix metalloproteases (MMPs) that remove the osteoid covering of the bone matrix in

preparation for osteoclastic removal of bone (Everts et al., 2002), and possibly later differentiate into bone-forming osteoblasts (Delaisse, 2014, Andersen et al., 2009). Lining cells prevent the direct interaction between osteoclasts and the bone matrix, when bone resorption should not occur, and participate in osteoclast differentiation from mononuclear cells of the hematopoietic stem cell lineage by producing osteoprotegerin (OPG) and the RANKL (Florencio-Silva et al., 2015).

1.1.4.4 Osteoclasts

Osteoclasts are multinucleated cells, that arise from the fusion of their mononuclear precursors in the hematopoietic stem cell lineage (Florencio-Silva et al., 2015). These precursor cells express the cell surface receptors for the macrophage colony-stimulating factor (M-CSF), secreted by osteoprogenitor mesenchymal cells and osteoblasts, and RANKL, secreted by osteoblasts, osteocytes, and stromal cells (Crockett et al., 2011a).

Osteoclast differentiation from its mononuclear precursors is triggered through a cell-cell contact between osteoclast precursor cells with upregulated expression of RANK and its ligand (RANKL) that is expressed by osteoblasts, stromal cells and more predominantly in osteocytes (Nakashima et al., 2011). RANK signalling is tightly regulated by a decoy receptor for RANKL, osteoprotegerin (OPG), that can block osteoclast formation which is produced by osteoblasts and stromal cells (Crockett et al., 2011b) and osteocytes (Bellido, 2014).

1.1.5 Bone Remodeling (Build up/Break down)

The health of bone is maintained through a delicate and continuous balanced process of bone remodelling that takes place throughout life to maintains the structural

integrity and strength of the bone, by removing old or damaged bone and replacing it with new, strong bone (Raisz, 1999).

Remodeling occurs in a temporary anatomic unit of osteoclasts and osteoblasts called a bone multicellular unit (BMU) (Seeman, 2008). This involves the initiation of bone degradation and resorption of bone mineral by osteoclasts (Teitelbaum, 2007). Cytokines are released at the site of bone remodeling by osteoblasts to recruit the osteoclasts from their mononuclear precursors, allowing them to adhere to the bone surface (Teitelbaum, 2007). Osteoclasts release hydrogen ions that create an acidic microenvironment, dissolving the mineralised component, which is degraded by Cathepsin k (Wilson et al., 2009). Subsequently, the reversal phase, the switch from degradation, to synthesis, begins by osteoblast (Andersen et al., 2013) and lining cells (Delaisse, 2014) preparing the bone surface for new osteoblasts, derived from mesenchymal cells, followed by the secretion by osteoblasts of bone matrix proteins, called osteoid, that become mineralized to form new bone (Karsenty et al., 2009). As the osteoblasts continue to differentiate, 5 to 20 % of mature osteoblasts become entombed in the matrix that they generate and subsequently mineralizes, and some are restored on the bone surface, others differentiate into either osteocytes or bone surface lining cells (Bellido, 2014) (Figure 1-2). These delicate interrelationships can become unbalanced and lead to conditions such as osteoporosis.

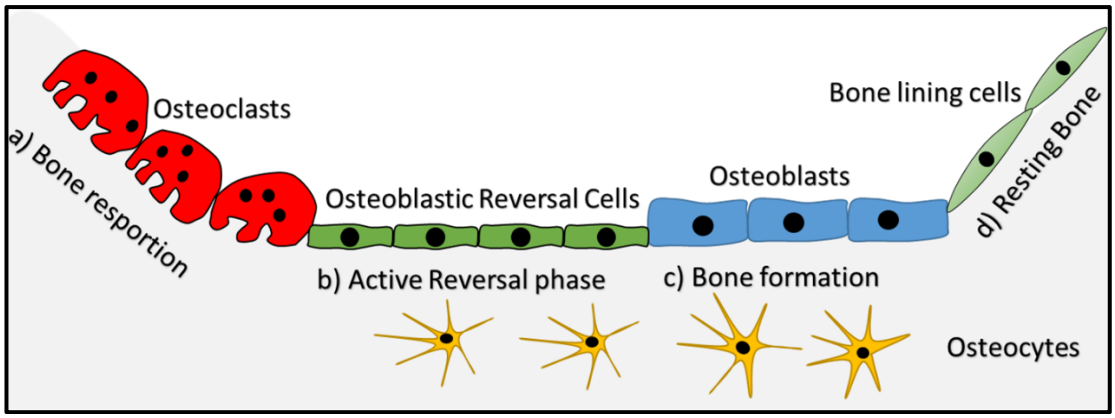


Figure 1-2: Bone remodelling (build up/break down):

a) Resorption: Stimulated osteoclasts erode bone, creating a cavity. **b) Reversal**: osteoblastic reversal cells (osteoblasts and lining cells) prepare bone surface for osteoblasts to begin forming bone. **c) Formation**: Osteoblasts replace resorbed bone and fill the cavity with new bone **d) Resting**: Bone surface rests until a new remodeling cycle begins.

1.2 Osteoporosis

Osteoporosis is the most common bone disease worldwide (Johnell and Kanis, 2006), characterized by unbalanced bone remodeling (Kanis, 1994), low bone mass and altered bone architecture leading to increased susceptibility to bone fragility (Kanis et al., 1997), as shown in Figure 1-3B, reduced mobility and altered quality of life (Svedbom et al., 2013). Osteoporosis occurs when the activity of bone-resorbing osteoclasts surpasses that of bone-forming osteoblast (Bell et al., 1999).

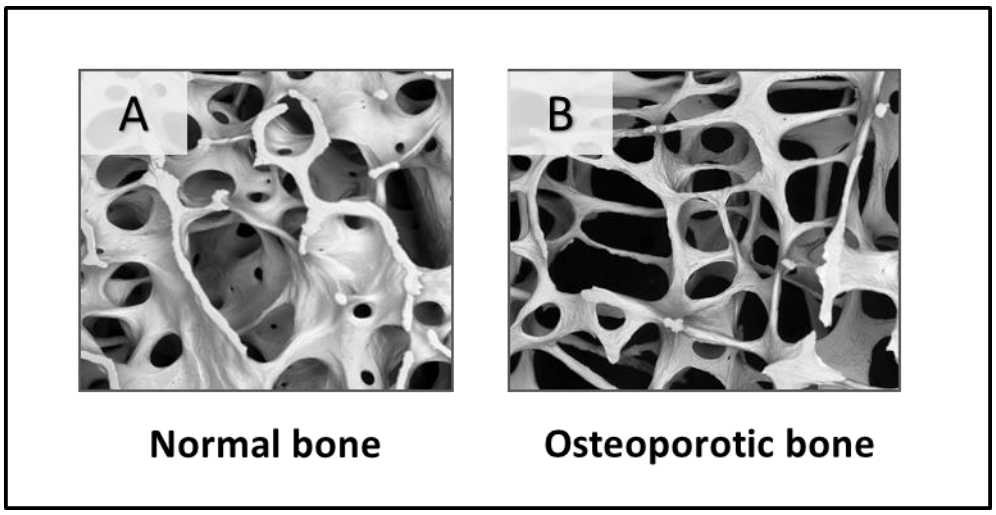


Figure 1-3: Diagrammatic difference between normal bone tissue (trabecular) and Osteoporotic Bone tissue.
Courtesy of Prof Alan Boyde (Queen Mary University of London)

1.2.1 Epidemiology

An overall estimate of incidence of osteoporotic fractures worldwide is more than 8.5 million persons aged over 50 years (Johnell and Kanis, 2006), with approximately 3.5 million in the 27 countries of the European Union (EU27) (Hernlund et al., 2013), and one third (34%) of osteoporotic sufferers are men (Hernlund et al., 2013). The estimated ratio of these cases in the UK alone is about one in two women and one in five men over the age of 50 years, with annual cost to health service of around £3.5 billion of direct cost of treatment and long term fracture care (Svedbom et al., 2013) and the costs are expected increase 25% by year 2025 (Hernlund et al., 2013).

According to a study conducted in 2010, 536,000 new osteoporotic fragility fractures occur each year in the UK, including 79,000 hip fractures, 69,000 forearm fractures, 66,000 vertebral fractures and 322,000 other fractures (pelvis, rib, humerus, tibia, fibula, clavicle, scapula, sternum and other femoral fractures) (Svedbom et al., 2013).

1.2.2 Burden of Osteoporotic Fracture

Nearly 75% of all hip fractures occur in women (Jordan and Cooper, 2002), and around 53% of patients suffering a hip fracture can no longer live independently and 28.7% die within 12 months of the fracture.

Ethnic differences could attribute in bone resistance to fracture. Black, Hispanic and Asian people in the USA have lower fracture rates than Caucasians (Wright et al., 2012, Cauley et al., 2011), and in the UK, black individuals have lower rates of fragility fracture than Caucasians, see (Figure 1-4) (Curtis et al., 2016).

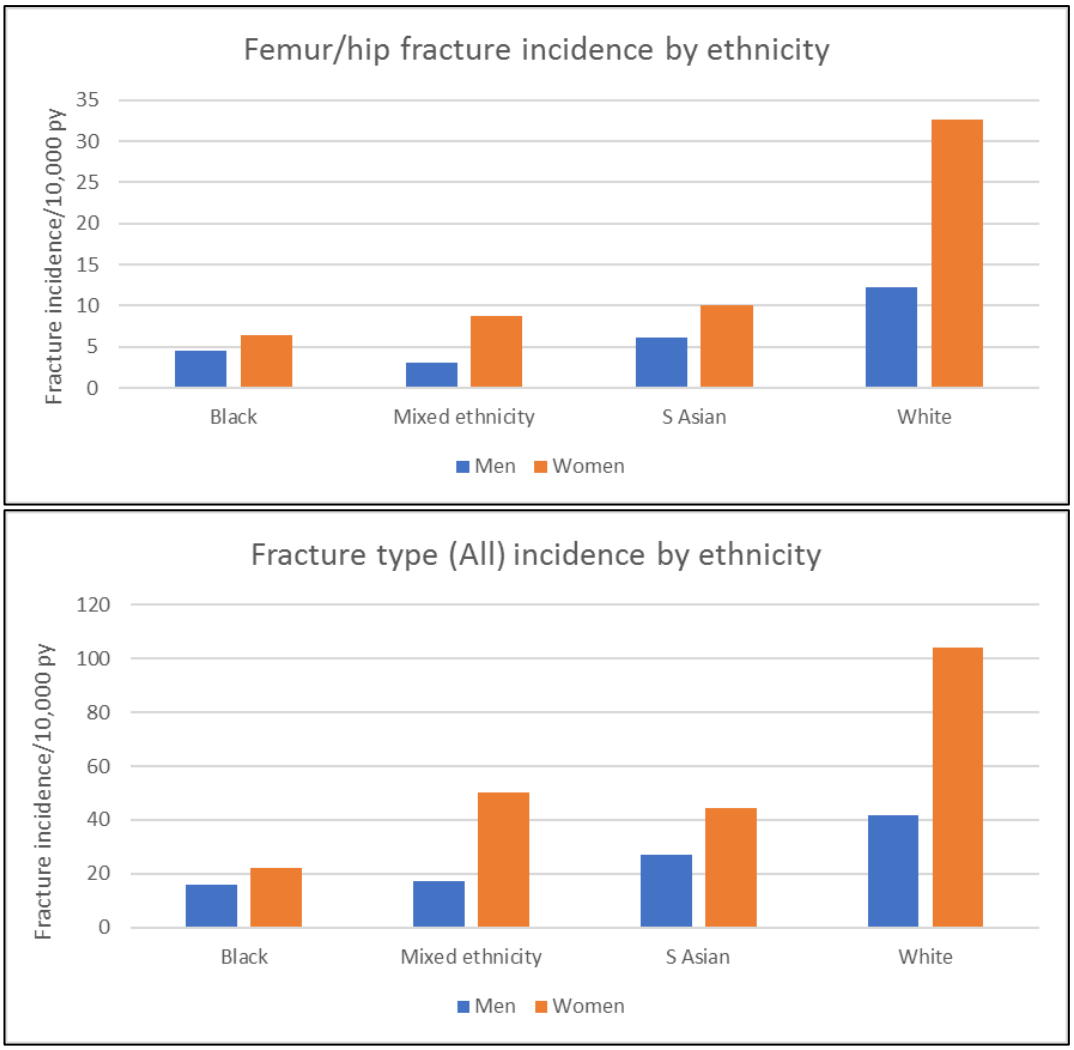


Figure 1-4: Incidence of hip/femur and fragility fractures (all types) by ethnicity in men and women aged over 50 years in the UK.
Chart adopted from (Curtis et al., 2016)

1.2.3 Diagnosis

1.2.3.1 Dual-energy X-ray Absorptiometry (DXA)

The diagnosis of osteoporosis can be made using conventional radiography (X-ray absorptiometry) and by measuring the bone mineral density (BMD), the amount of bone mass per unit area (areal density, g/cm^2) or per unit volume (volumetric density, g/cm^3), using the Dual-energy X-ray (DXA) (Kanis and Johnell, 2005) and high resolution peripheral quantitative computed tomography (HR-pQCT) (Nishiyama et al., 2013). However, DXA is the most common technique used for assessing the risk of osteoporosis (Kanis, 1994).

The WHO study group (Simon and Mack, 2003), based on the DXA diagnostically, categorised women into four general groups. The first group is considered normal if the BMD value is within 1 standard deviation (SD) of the young female adult reference mean (T-score ≥ -1). The second group is osteopenia, bone condition with BMD less than normal and greater than osteoporosis, if the value of the BMD is <-1 and >-2.5 SD. The third group is diagnosed as osteoporosis, if the value of the BMD is T-score ≤ -2.5 SD, and the last group is a severe osteoporosis, if the value of the BMD is ≤ -2.5 SD in the presence of one or more fragility fractures (Kanis, 1994, Simon and Mack, 2003). Current guidelines favour the measurement of BMD at both the femoral neck and at the lumbar spine and patients are defined as having osteoporosis based on the lower of the two T-scores so obtained (Compston et al., 2017).

1.2.3.2 Fracture Risk Assessment

The risk of fracture increases approximately 3-fold for each SD decrease in BMD, but the rise of risk varies according to the patient's age, the site of fracture, the technique used to assess fragility fracture and the outcome of multiple risk factors (Johnell et al., 2005, Compston et al., 2017). This includes the sex of patient, ethnicity, number of previous falls, smoking, disorders associated with osteoporosis such as alcohol intake, use of glucocorticoids, diagnosis of rheumatoid arthritis and disorders strongly associated with osteoporosis such as inflammatory bowel disease, endocrine disorders and diabetes (Compston et al., 2017, Rossini et al., 2016), using established WHO fracture risk assessment prediction tools, such as FRAX®, QFractureScores®, and the Garvan Institute fracture calculator (Das and Crockett, 2013, Sandhu and Hampson, 2011).

1.2.3.3 Bone Turnover Markers

Diagnosis of osteoporosis is made by doing routine blood tests for calcium, phosphorus, non-specific alkaline phosphatase, oestrogen, Vitamin D and parathyroid hormone levels (Jones et al., 1998, Vieth et al., 2003), as well as the biochemical assessment based on bone resportion, such as serum and urine level of type I collagen C- (CTX) and N-telopeptides (NTX) and urine level of pyridinolines (PYD) and deoxypyridinolines (DPD) (Emami et al., 1999, Ohishi et al., 1998), tartrate-resistant acid phosphatase (TRAcP) (Stoffel et al., 2007) and cathepsin K (Karsdal et al., 2005, Holzer et al., 2005), as well as indices for bone formation markers such as bone specific ALP (Bowles et al., 1997), osteocalcin (Veitch et al., 2006, Stoffel et al.,

2007), serum and urine level of type I collagen C- (PICP) (Veitch et al., 2006, Bone, 1992), N-telopeptides (PINP) ((Hanson et al., 1992) (Table 1-1).

Table 1-1: Biochemical Bone Turnover Markers (BTM) commonly used in clinical practice

Table was adapted from (Vasikaran et al., 2011, Sandhu and Hampson, 2011)

Bone Turnover Markers (BTM)	**Analytical body fluid sample**
Bone resorption markers	
Pyridinoline (PYD)	Urine
Deoxypyridinoline (free DPD, total DPD)	Urine
C-terminal crosslinked telopeptide (S-bCTX, CTX)	Urine and serum/plasma
Carboxyterminal telopeptide of type I collagen (ICTP)	Serum
N-terminal crosslinked telopeptide (NTX)	Urine and serum/plasma
Tartrate resistant acid phosphatase – 5b (TRAcP-5b)	Serum
Bone formation markers	
Procollagen type I C-terminal propeptide (PICP)	Serum
Procollagen type I N-terminal propeptide (PINP)	Serum/plasma
Bone Alkaline Phosphatase (Bone ALP)	Serum
Osteocalcin (OC): intact, total, or undercarboxylated	Serum/plasma/Urine

1.2.3.3.1 Biochemical Markers of Bone Resorption

1.2.3.3.1.1 Pyridinoline and deoxypyridinoline

The collagen crosslinks, pyridinoline (PYD) and deoxypyridinoline (DPD) are found in fibril-forming types of collagen (types I, II and III), formed during the extracellular maturation of fibrillar collagens and are released into the circulation as degradation products of mature collagen (Naylor and Eastell, 2012, Seibel, 2005). Crosslinked collagens are proteolytically broken down and the crosslinked components are released into the circulation and the urine (Seibel, 2005). DPD is almost solely found in bone (Naylor and Eastell, 2012), while PYD is found in cartilage (Naylor and Eastell, 2012). Therefore, the ratio of DPD/PYD is higher in bone compared to other tissues. Crosslinked collagens can be measured accurately in urine by high-performance liquid chromatography (HPLC) and free DPD can be measured by ELISA (Kamel et al., 1995, Naylor and Eastell, 2012). In addition, the measurement of urinary crosslinked collagen is not affected by dietary sources, and is currently considered the best index for assessing bone resorption (osteoporosis, Paget's disease of bone, vitamin D deficiency, hyperparathyroidism, corticosteroid therapy, etc.) (Seibel, 2005).

1.2.3.3.1.2 C-telopeptide of type-I collagen (CTX) and N-telopeptide of type-I collagen (NTX)

The carboxy- and amino-terminal (NTX) cross-linked telopeptides (CTX and NTX) are collagen type I peptide fragments, that are released into the blood circulation during the bone resportion process (Seibel, 2005). Serum/plasma CTX was recommended by the International Osteoporosis Foundation (IOF) to be used as a reference marker for bone turnover (Vasikaran et al., 2011). The elevated serum concentration of CTX

has been reported in patients with increased bone resorption, compared to patients taking bisphosphonate antiresorptive therapy (Christgau et al., 2000). However, its contribution to the diagnosis of osteoporosis is low (Mohamed et al., 2014), and the technique is used mainly for therapeutic monitoring.

1.2.3.3.1.3 The Carboxy-terminal Telopeptide of type-I collagen (ICTP)

C-terminal crosslinking telopeptide of type I collagen (ICTP) is separated from collagen by metalloproteinases (MMP-2, -9, -13, or -14) and is measured by Radioimmunoassay (Risteli et al., 1993). The assay has been shown to be sensitive for pathological bone resorption, such as in multiple myeloma and metastatic bone disease (Seibel, 2005). Serum ICTP level has been shown to be elevated after osteoporotic fragility fracture in patients (Takahara et al., 2007).

1.2.3.3.1.4 Enzymes derived from osteoclasts

TRAcP 5b is a member of the acid phosphatase family, that is resistant to inhibition by tartrate and is expressed by osteoclast cells (Halleen et al., 2006). The elevated level of serum TRAP 5b level has been associated with increased fracture risk for up to a decade in elderly women (Ivaska et al., 2010), and found to be a useful therapeutic monitoring marker for anti-resorptive treatment (Nenonen et al., 2005). However, its activity has be found to be increased in patients with bone metastases from cancers in other tissues (Chao et al., 2010).

Cathepsin K, a member of the peptidase C1 protein family, is a proteolytic enzyme that is abundant in osteoclasts (Saftig et al., 1998) that plays a vital role in the degradation of bone type I collagen (Atley et al., 2000) and may participate partially in the disorder of bone remodelling. Circulating serum cathepsin K expression has

been shown to be higher in osteoporosis & Paget's disease patients (Meier et al., 2006), as well as in rheumatoid arthritis compared to healthy subjects (Skoumal et al., 2005). In contrast, there was no significant difference in serum cathepsin K level between four study groups (postmenopausal: healthy women, osteoporosis, osteopenia; premenopausal: healthy women) (Adolf et al., 2012). Therefore, further clinical evaluation is required for this potential biomarker (Naylor and Eastell, 2012).

1.2.3.3.2 Biochemical markers of bone formation

Formation markers, products of osteoblast activity, are expressed during different phases of osteoblast differentiation and extracellular matrix maturation (Naylor and Eastell, 2012), and they are particularly useful for monitoring bone formation therapies and antiresorptive therapies (Eastell et al., 2006, Vasikaran et al., 2011).

1.2.3.3.2.1 Propeptides of type I procollagen

Procollagen type I propeptides are derived from collagen type I, which is the most common collagen type found in mineralized bone (Krieg et al., 1988). The formation of procollagen type I requires the cleavage of propeptides: procollagen type I C-terminal (PICP) and N-terminal (PINP) (Francini et al., 1993). Both propeptides (PICP and PINP) are released into the circulation (Francini et al., 1993) and their concentrations reflect the synthesis rate of collagen type I (Francini et al., 1993). PINP is considered to be the most sensitive marker of bone formation(Naylor and Eastell, 2012); it can be measured by radioimmunoassay (Melkko et al., 1996) and is primarily used for osteoporosis antiresorptive therapeutic monitoring (Eastell et al., 2006). However, the direction of diagnostic interpretation depends on the antiresorptive therapy used. For instance, there was an increased level of serum procollagen type I

propeptides (PINP) in >20% in osteoporosis patients after 3-6 months of teriparatide therapy, which indicated an adequate therapeutic response (Eastell et al., 2006). In contrast, there was a decreased PINP level of up to 70% from baseline after 6 months of therapy in patients taking bisphosphonate therapy (McClung et al., 2005).

1.2.3.3.2.2 Non-collagenous proteins

1.2.3.3.2.2.1 Bone specific Alkaline Phosphatase (BALP)

Alkaline Phosphatases (ALP) are membrane-bound glycoproteins produced by various tissues (liver, bone, intestine, placenta and kidney) (Weiss et al., 1986, Seibel, 2005). Bone ALP (BALP) represent approximately 50% of total ALP activity in adults with normal liver function, whereas up to 90% arises from bone in children and adolescents (Seibel, 2005). BALP are more specific for bone formation than total ALP and is used to assess clinical conditions such as osteoporosis, Paget's disease and chronic kidney disease (Naylor and Eastell, 2012). However, cross-reactivity between liver and bone ALP (up to 20%) could happen in some clinical conditions, such as the case with biliary obstruction (Seibel, 2005).

1.2.3.3.2.2.2 Osteocalcin (OC)

Osteocalcin (OC) is a non-collagen protein that binds to hydroxyapatite deposited in the bone matrix, that is synthesized by osteoblasts and hypertrophic chondrocytes (Seibel, 2005), and is widely accepted as a specific marker of bone osteoblastic activity (Brown et al., 1984). After its release from osteoblasts, much of the osteocalcin is incorporated into the extracellular bone matrix (EBM), but some is released to the circulation, where it can be detected by immunoassay (Seibel, 2005). Decreased level of osteocalcin was observed in postmenopausal osteoporosis and

osteopenia patients after 6 to 12 months of hormone replacement therapy (HRT) based on percent baseline changes in lumbar BMD (Chen et al., 1996) and in patients taking antiresorptive agents (bisphosphonates or hormone replacement therapy) of > 20% from baseline after 3 to 6 months of therapy (Delmas et al., 2000). Indeed, elevated levels of osteocalcin could be released during osteoclastic bone resorption as well as during bone formation (Cloos and Christgau, 2004). However, biological variation has been encountered when different methodologies were used (Seibel et al., 2001).

1.2.3.3.3 Limitations of Current Bone Turnover Marker (BTM) Measurement

The use of BTM as routine markers remains a challenge due to their broad biological and analytical variation, even with identical assays and methods, which may be as much as a 7.3-fold difference (Sandhu and Hampson, 2011). The study was conducted by (Seibel et al., 2001) between 73 laboratories using two serum and two urine pools from several healthy volunteers and tested eight different bone turnover markers: bone-specific alkaline phosphatase (ALP), serum osteocalcin (OC), urinary free and total deoxypyridinoline (DPD), urinary pyridinoline, urinary N-terminal crosslinked telopeptide (NTX), serum C-terminal telopeptide of type I collagen (ICTP) and urinary hydroxyproline (Seibel et al., 2001).

1.2.3.3.3.1 Non-modifiable Preanalytical Variability

There are two main groups of confounding factors for BTMs' pre-analytical variability: Non-modifiable and modifiable factors (Naylor and Eastell, 2012). The non-modifiable factor includes: age, sex and ethnicity/race, geographical location, medication such as glucocorticoids, antiresorptive drugs (bisphosphonates, selective

estrogen receptor modulators [SERMs], hormone replacement therapy [HRT]) and hormonal contraceptives; diseases such as primary hyperparathyroidism, Paget's disease, myeloma, Inflammation (RA), chronic kidney disease and liver disease (Naylor and Eastell, 2012).

1.2.3.3.3.2 Modifiable Preanalytical Variability

The modifiable factors include circadian rhythms, which are more noticeable for bone turnover markers than those of bone formation. For instance, mean concentrations of Beta-CTX were higher 66% in samples obtained in the early hours of the morning (0130 and 0430) than in samples obtained in the late morning and afternoon (1100 and 1500) (Wichers et al., 1999). Further modifiable factors include seasonal changes, stage of menstrual cycle of the subject, level of exercise and finally meal intake are also confounding factors for the biological variability. It has been noticed that patients have reduced serum CTX-I levels (20%) 1 hour after breakfast, attributed to the production of the gut hormone, glucagon-like peptide 2 (GLP-2) (Henriksen et al., 2003).

In conclusion, the existing biomarkers are unsatisfactory in many respects; they are not useful diagnostic tool for osteoporosis (Sandhu and Hampson, 2011, Vasikaran and Chubb, 2016) and their best-established clinical use is in monitoring treatment efficacy and compliance (Sandhu and Hampson, 2011). In addition, variation of reported levels of BTM has been encountered between clinical laboratories using identical assays and methods (Seibel et al., 2001), and a very abnormal BTM value may be a clue to the presence of bone pathology other than uncomplicated osteoporosis (Vasikaran and Chubb, 2016).

1.2.4 RNA

A key step in the production of any protein is transcription of Ribonucleic acid (RNA) and then its translation to peptides, however, RNA is no established to have a number of essential roles in a number of biological processes such as coding, decoding, regulation, and expression of genes (Alberts et al., 2015).

The most well characterised forms of RNA are single stranded messenger RNA, transfer RNA and ribosomal RNA (mRNA, tRNA, rRNA). mRNA carries the code for protein structure from the DNA to ribosomes where it can be used to produce proteins, tRNA decodes the message in mRNA by matching amino acids to the mRNA code, and ribosomal RNA (rRNA) form the structure of the ribosome (Alberts et al., 2015).

Recently, new types of protein non-coding small silencing RNAs that guided complex and diverse organisation of gene regulation in plants and animals were identified (Ghildiyal and Zamore, 2009), such as microRNAs (miRNAs), small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs).

MiRNAs that were discovered have wide and far reaching biological regulatory actions (He and Hannon, 2004) and, as we will see may provide a new source of diagnostically useful biomarkers for bone diseases (Hackl et al., 2016, Lian et al., 2012, van Wijnen et al., 2013).

1.2.4.1 MicroRNAs

MicroRNAs (miRNAs) are endogenous non-coding RNA molecules of small size, approximately 21-23 nucleotides, that are involved in diverse signalling pathways,

including control of cellular developmental timing, proliferation, differentiation and apoptosis (Bartel, 2004, Ambros, 2004).

miRNAs post-transcriptionally down regulate the expression of up to 60% of human protein-coding genes, either by translation inhibition of target messenger RNA (mRNA) (Li et al., 2013) or promoting mRNA degradation and/or directly interfering with protein translation (Friedman et al., 2009, Bartel, 2009) (Figure 1-5 (Guay and Regazzi, 2013).

However, there is evidence indicating that miRNAs can also activate translation (Vasudevan et al., 2007), through the AU-rich elements (AREs) binding protein, HuR, and its related family of ELAV proteins and conserved miRNA target sites in mRNA 3' untranslated regions (3'UTRs). They may also induce positive transcriptional regulation of mRNAs, directly or indirectly, in conjunction with associated effector proteins (microribonucleoproteins, microRNPs) (Vasudevan, 2012, Valinezhad Orang et al., 2014), by targeting sites in gene promoter regions (Place et al., 2008).

Notably, a single miRNA can regulate many different protein coding genes and a single gene can be targeted by a group of miRNAs, providing fine-tuning of a single mRNA target expression (Doench and Sharp, 2004, Lewis et al., 2003).

A miRNA is produced from a stem-loop RNA precursor that is generated from an independent transcriptional unit or from an intron of a genes that encodes a protein (Rodriguez et al., 2004), transcribed by either RNA polymerase II (Lee et al., 2004) or RNA polymerase III (Cai et al., 2004, Borchert et al., 2006) into a primary miRNA transcript (pri-miRNA), which is then processed by the endonuclease Drosha into an approximately 70nt hairpin structure (precursor miRNA) (Lee et al., 2003, Cullen,

2004). This is then exported to the cytoplasm via an Exportin‑5-dependent process (Guay and Regazzi, 2013, Ruby et al., 2007).

The resulting short hairpin RNA (precursor miRNA) undergoes cleavage by Dicer and gives rise to a short mature miRNA-duplex (Winter et al., 2009) and generates imperfect miRNA duplexes consisting of a guide strand (miRNA) and passenger strand miRNA, termed miRNA* (Cifuentes et al., 2010). After strand separation, either the 5' (*miRNA-5p*) or 3' (*miRNA-3p*) region of the miRNA becomes selectively incorporated into the miRNA induced silencing complex (miRISC) (Bartel, 2009) and guides translational repression by pairing with specific partially complementary 3'-UTR regulatory elements of the target mRNAs (Ambros, 2001).

Alternatively, the miRNA can be released by the cell with the mature miRNA bound to RNA-binding proteins such as Argonaute-2 (Arroyo et al., 2011) or to lipoproteins (Vickers et al., 2011). The other strand miRNA*, is degraded. Or else, miRNAs can be loaded in microvesicles (Kosaka et al., 2010) formed by plasma membrane blebbing or in exosomes (Taylor and Gercel-Taylor, 2008, Valadi et al., 2007) that are released into the extracellular space upon exocytic fusion of multivesicular bodies with the plasma membrane (Guay and Regazzi, 2013).

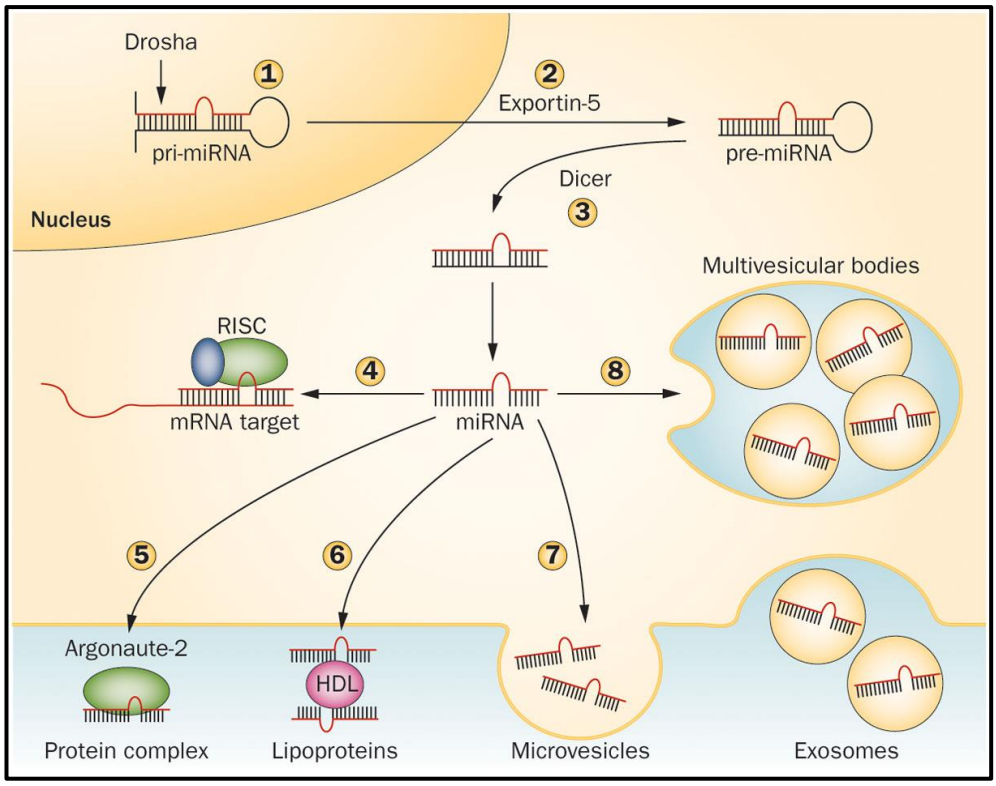


Figure 1-5: miRNA Biogenesis.
(1) Ribonuclease III Drosha enzyme cleaves the Pri-miRNA into Pre-miRNA. (2) The pre-miRNA is transported into the cytoplasm by Exportin 5. (3) It further cleaved by Dicer enzyme yielding 21-23 nucleotide duplexes. (4) One strand can associate to the RNA Induced Splicing Complex (RISC) and guide translational repression of target mRNAs or (5) Released by the cell and the mature miRNA binds to RNA-binding proteins such as Argonaute-2 or (6) to lipoproteins. (7) Alternatively, the miRNA can be loaded in microvesicles formed by plasma membrane blebbing or in (8) into exosomes that are released in the extracellular space upon exocytic fusion of multivesicular bodies with the plasma membrane. This figure was taken from (Guay and Regazzi, 2013).

1.2.4.1.1 Role of miRNA in skeletal development and disease

Many reports have highlighted the involvement of miRNAs as key regulators of osteoblast-mediated bone development, homeostasis (Lian et al., 2012, Sera and Zur Nieden, 2017) and bone remodelling (van Wijnen et al., 2013, Lian et al., 2012) (Table 1-2). Furthermore, it has been shown that cytokines and hormones that are involved in bone development and bone remodeling, such as bone morphogenic protein (BMP) (Salazar et al., 2016), RANKL (Kagiya and Nakamura, 2013) and estrogen (Sugatani and Hruska, 2013, An et al., 2013), have impact in miRNA expression.

1.2.4.1.2 Circulating miRNA as Osteoporosis Biomarkers

Studies on circulating miRNAs in patients with bone diseases have only recently been reported (Hackl et al., 2016) (Table 1-2). Some of the pilot studies were restricted to circulating monocytes, as these are the cells that can differentiate into osteoclasts and secrete osteoclastogenic factors (Cao et al., 2014, Wang et al., 2012c), while some studies were conducted on serum/plasma samples of postmenopausal women with low bone mineral density (BMD) compared to postmenopausal women with normal BMD (Li et al., 2014, Yavropoulou et al., 2017) and on osteoporotic fractured patients compared to control groups (Panach et al., 2015, Seeliger et al., 2014, Weilner et al., 2015). In addition, one study was conducted on whole blood of postmenopausal women with low BMD compared to postmenopausal women with normal BMD (Meng et al., 2015).

Table 1-2: Overview of studies on miRNA expression in blood circulation and bone tissues in the context of human osteoporosis

miRNAs	Specie (s)	Target gene(s)	Endogenous miRNA expression	miRNA function	Related disease(s)	References
133a-3p	Mouse C2C12 mesenchymal cells Human monocytes and plasma	Runx2, CXCL11, CXCR3 and SLC39A1	Upregulated in monocytes and plasma of osteoporosis patients	Inhibits osteogenesis	Osteoporosis	(Mencia Castano et al., 2016, Wang et al., 2012c, Li et al., 2014, Li et al., 2008)
let-7g-5p	Human adipose-derived stromal/stem cells (hADSCs) human mesenchymal stem cells (hMSCs)	HMGA2 Not mentioned	Downregulated in the serum of patients with osteoporosis.	Promotes osteogenic differentiation	Osteoporosis	(Wei et al., 2014, Weilner et al., 2015)
miR-100-5p	Human adipose-derived mesenchymal stem cells (hASCs)	BMPR2	Upregulated in the serum of osteoporotic fractured patients	Inhibits osteogenic differentiation	Osteoporosis	(Zeng et al., 2012a, Seeliger et al., 2014)
miR-10a-5p	Human Unrestricted somatic stem	Not mentioned	Upregulated in the serum of patient with osteoporosis	Upregulated during osteogenic differentiation	Osteoporosis	(Trompeter et al., 2013,

miRNAs	Specie (s)	Target gene(s)	Endogenous miRNA expression	miRNA function	Related disease(s)	References
	cells (USSC, SA5/73 and SA8/25) and hMSCs					Weilner et al., 2015)
miR-10b-5p	human hMSCs	Not mentioned	Upregulated in the serum of osteoporotic fractured patients	Upregulated during osteogenic differentiation	Osteoporosis	(Weilner et al., 2015)
miR-122-5p	Mouse bone mesenchymal stem cells (MSCs) Human serum	Tbr1 Not mentioned	Upregulated in patients with osteoporotic fracture	Downregulate the expression of bone mesenchymal stem cells	Osteoporosis	(Yang et al., 2017, Seeliger et al., 2014, Panach et al., 2015)
miR-124-3p	Mouse BMMs Human serum	NFATc1 Not mentioned	Upregulated in the serum of patients with osteoporotic fracture	Inhibits osteoclast differentiation	Osteoporosis	(Lee et al., 2013, Seeliger et al., 2014, Yavropoulou et al., 2017)
miR-125a	human CD14+ peripheral blood mononuclear cell (PBMC)	TRAF6	Dramatically downregulated during osteoclastogenesis	Inhibit osteoclastogenesis	May be involved in metabolic disease	(Cheng et al., 2013, Guo et al., 2014)
miR-125b-5p	mouse MSCs Human	Lin-28 Not mentioned	Upregulated in the serum of patients with osteoporosis	Inhibits osteogenic differentiation	Osteoporosis	(Mizuno et al., 2008, Seeliger et al., 2014,

miRNAs	Specie (s)	Target gene(s)	Endogenous miRNA expression	miRNA function	Related disease(s)	References
						Panach et al., 2015)
miR-130b-3p	Human (Osteosarcoma cell line)	NKD2	Upregulated in the whole blood of patients with osteoporosis	targets NKD2 and regulates the Wnt signalling to promote proliferation and inhibit apoptosis of OS cells	Osteosarcoma	(Li et al., 2016c)
miR-133a	Human PBMCs	Not mentioned	Upregulated in the monocytes of low BMD postmenopausal women	Not mentioned	Postmenopausal osteoporosis	(Wang et al., 2012c)
miR-133b	Mouse C2C12 MSCs Human serum	Runx2 Not mentioned	upregulated in the plasma of patients with osteoporosis, in contrast it shown to be downregulated in the serum of patients with osteoporosis by Weilner et al 2015	Inhibits osteogenic differentiation via Runx2	Osteoporosis	(Li et al., 2008, Weilner et al., 2015, Li et al., 2014)
miR-143-3p	Mouse C2C12 MSCs Human serum	MyoD, MyoG, myf5, and MyHC Not mentioned	Upregulated in the serum of patients with osteoporosis	Upregulated during myoblast differentiation	Osteoporosis	(Panach et al., 2015, Du et al., 2016)

miRNAs	Specie (s)	Target gene(s)	Endogenous miRNA expression	miRNA function	Related disease(s)	References
miR-148a	Human CD14+ PBMCs, mouse C57BL/6 ovariectomized	MAFB	Upregulated during osteoclast differentiation Unregulated in serum of osteoporotic fractured patients Upregulated in plasma of osteoporosis women	Promote osteoclastogenesis	Osteoporosis, contributes to low BMD in lupus patients	(Seeliger et al., 2014, Cheng et al., 2013, Liu et al., 2015, Bedene et al., 2016)
miR-151a-3p	Human whole blood	Not mentioned	Upregulated in the whole blood of patients with osteoporosis	Not mentioned	Osteoporosis	(Meng et al., 2015)
miR-155	mouse RAW 264.7 Cells, mouse with Dicer deficiency in osteoclasts (cKO mutant), C57BL/6, IFNb and IFNAR1 deficient mice	SOCS1, MITF, SHIP	RANKL treatment suppress miR-155 levels in BMMs from Dicer-deficient mice	Suppress osteoclast differentiation	Osteoclast-mediated diseases	(Mizoguchi et al., 2010, Mann et al., 2010, Zhang et al., 2012)

miRNAs	Specie (s)	Target gene(s)	Endogenous miRNA expression	miRNA function	Related disease(s)	References
miR-194-5p	Human whole blood	Not mentioned	Upregulated in the whole blood of patients with osteoporosis	Not determined	Osteoporosis	(Meng et al., 2015)
miR-210-3p	Human serum and bone metastasis tissue	NFKB1	Upregulated in bone metastasis Downregulated in the serum of osteoporotic fractured patients	activation of NF-κB signalling pathway	Prostate Cancer Bone metastasis Osteoporosis	(Panach et al., 2015, Ren et al., 2017)
miR-21-5p	wild-type (wt), ERα deficient, C57BL/6J, DGCR8-floxed (DGCR8F/F) and MiR-21 floxed (miR-21f/f) mice human serum	FasL & PDCD4 Not mentioned	Upregulated in RANKL-induced osteoclastogenesis Promotes osteogenic differentiation and impairs adipogenic differentiation Downregulated in the serum of Low BMD compared to control	Enhances osteoclast differentiation & Inhibits osteoclast apoptosis	Postmenopausal osteoporosis	(Sugatani et al., 2011, Sugatani and Hruska, 2013) (Trohatou et al., 2014, Seeliger et al., 2014, Yavropoulou et al., 2017)
miR-223	mouse RAW264.7 cells human rheumatoid arthritis (RA)	Not mentioned NFIA	Downregulated in osteoclast differentiation Downregulated in osteoclast differentiation	Overexpression completely blocks osteoclast formation Inhibits osteoclast differentiation	Might be related to bone metabolic disorders Bone destruction in rheumatoid arthritis	(Kagiya and Nakamura, 2013, Sugatani and Hruska, 2007)

miRNAs	Specie (s)	Target gene(s)	Endogenous miRNA expression	miRNA function	Related disease(s)	References
	synovium and PBMC					(Shibuya et al., 2013)
miR-22-3p	Human Unrestricted somatic stem cells (USSC) and serum	Not mentioned	Upregulated in the serum of patients with osteoporosis	Upregulated during osteogenic differentiation	Osteoporosis	(Trompeter et al., 2013, Weilner et al., 2015)
miR-23a-3p	Mouse preosteoblast Cell line MC3T3-E1 Human serum	SATB2	Upregulated in the serum of osteoporotic fractured patients	Inhibits osteogenic differentiation via SATB2	Osteoporosis	(Hassan et al., 2010, Seeliger et al., 2014)
miR-24-3p	Mouse preosteoblast Cell line MC3T3-E1 Human serum	SATB2 Not mentioned	Upregulated in patients with osteoporotic fracture	Inhibits osteogenic differentiation via SATB2	Osteoporosis	(Hassan et al., 2010, Seeliger et al., 2014)
miR-29	Mouse monocytic cell line RAW264.7	CDC42, SRGAP2, NFIA, CD93, CALCR	Increased during osteoclast differentiation	miR-29 family member that sustains migration and commitment of precursors to osteoclastogenesis	Possibly related to increased osteoclast formation with aging	(Franceschetti et al., 2013)
miR-29a	Rat bone tissue	Not mentioned	Reduced expression in glucocorticoid-induced bone loss	Inhibits GC-induced osteoclast differentiation	Glucocorticoid-induced bone loss	(Wang et al., 2013)

miRNAs	Specie (s)	Target gene(s)	Endogenous miRNA expression	miRNA function	Related disease(s)	References
miR-29b	human PBMCs	c-Fos, MMP2	Downregulated in RANKL induced osteoclastogenesis	Inhibits osteoclast differentiation	Multiple myeloma-related bone disease	(Rossi et al., 2013)
miR-31	mouse bone marrow-derived macrophages (BMM)	RhoA	Highly upregulated during osteoclast development upon RANKL stimulation	promote ring-shaped mature osteoclasts formation, attributed to cytoskeleton organization	Not mentioned	(Mizoguchi et al., 2013)
miR-328-3p	Human serum	Not mentioned	Downregulated in the serum of patients with osteoporotic fracture	Not mentioned	Osteoporosis	(Weilner et al., 2015)
miR-34a-5p	Mouse 34a-Tie2-Tg cultures Human bone tissues and serum	Tgif2 Not mentioned	Downregulated during osteoclast differentiation	blocks osteoporosis and bone metastasis by inhibiting osteoclastogenesis and Tgif2	Osteoporosis, osteosarcoma	(Krzeszinski et al., 2014) (Wang et al., 2015c, Panach et al., 2015)
miR-378	Mouse RAW264.7 and MOCP-5 cells	Not mentioned	Upregulated in osteoclast differentiation	Not mentioned	Osteolytic bone metastasis	(Kagiya and Nakamura, 2013, Ell et al., 2013)
miR-422a	human monocytes	CBL, CD226, IGF1, PAG1, TOB2	Upregulated with marginal significance in the low BMD	Not mentioned	Osteoporosis	(Cao et al., 2014)

miRNAs	Specie (s)	Target gene(s)	Endogenous miRNA expression	miRNA function	Related disease(s)	References
			postmenopausal women			
miR-503	human PBMCs	RANK	Markedly reduced in postmenopausal osteoporosis women	Inhibits RANKL-induced osteoclast differentiation	osteoporosis	(Chen et al., 2014a)
miR-590-5p	Human mesenchymal stem cells (HMSCs) and whole blood	APC	Upregulated in the whole blood of patients with osteoporosis	Upregulated during osteogenic differentiation	Osteoporosis	(Wu et al., 2016, Meng et al., 2015)
miR-660*	Human whole blood	Not mentioned	Upregulated in postmenopausal osteoporosis women	Not mentioned	osteoporosis	(Meng et al., 2015)
miR-93	Mouse C57BL/6 cells Human serum	Sp7 Not mentioned	Upregulated in the serum of osteoporotic fractured patients	Attenuates osteoblast mineralization	Osteoporosis	(Yang et al., 2012, Seeliger et al., 2014)

*Not validated

1.2.4.2 miRNAs as biomarkers.

miRNAs can pass into the blood or other body fluids with potential paracrine or endocrine functions and can be monitored as biomarkers for a multitude of disease states (Mitchell et al., 2008, Gennari et al., 2016) and could be promising novels biomarkers for bone diseases diagnosis (van Wijnen et al., 2013, Gilad et al., 2008, Hackl et al., 2016, Ji et al., 2016). They could be reliable as far as their efficiency (specificity & sensitivity) and cost effectiveness, and could surpass the current diagnostic biomarkers.

Discovery of differentially-expressed miRNAs associated with osteoporosis and/or osteoporotic fractures, by quantitative real time (RT-qRT) PCR could be of benefit to the diagnosis of osteoporosis in the future, due to their availability and stability in serum (Mitchell et al., 2008, Kroh et al., 2010, Cortez and Calin, 2009) and other body fluids (Kosaka et al., 2010). Indeed, they fulfil the essential criteria of a valuable biomarker: measurability, reliability and cost effectiveness (Morrow and de Lemos, 2007), and have potential to serve as non-invasive biomarkers for bone diseases (Hackl et al., 2016).

1.3 Aims of the Research

The aim of the research project is to identify changes in the levels of circulating miRNA that might be associated with osteoporosis in human patients and to assess the potential benefit to the diagnosis of osteoporosis in clinical specimens.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Blood Collection and Sample Processing Materials

No.	Item	Cat#	Source
1.	SARSTEDT S-Monovette® 7.5mL. Serum Tube (Clotting Activator)	01.1601.001	STARLAB Ltd, UK
2.	SARSTEDT S-Monovette® 9mL K3E, Potassium EDTA	02.1066.001	STARLAB Ltd, UK
3.	Ficoll-Paque Premium 1.077g/mL	17-5442-02	GE Healthcare Life Sciences, UK
4.	Greiner Bio-One 10 mL Leucosep™ tube, sterile for 3-8 mL blood	163290	Greiner bio-one Ltd, UK
5.	Filter Tip (Sterile), 0.5-10µL	02-707-442	Fisher Scientific Ltd, UK
6.	Filter Tip (Sterile), 0.5-10µL	02-707-442	Fisher Scientific Ltd, UK
7.	Filter Tip (Sterile), 2-20µL	02-707-432	Fisher Scientific Ltd, UK
8.	Filter Tip (Sterile), 100-1000µL	02-707-404	Fisher Scientific Ltd, UK
9.	1.5mL Ultra High Recovery (UHR) Microcentrifuge Tube	E1415-2600	STARLAB Ltd, UK
10.	2.0mL Ultra High Recovery (UHR) Microcentrifuge Tube	I1420-2600	STARLAB Ltd, UK
11.	STARLAB 15 mL Polypropylene Centrifuge Tubes	E1415-0200	STARLAB Ltd, UK
12.	STARLAB 50 mL Polypropylene Centrifuge Tubes	E1450-0200	STARLAB Ltd, UK

2.1.2 Molecular Biology Materials.

No.	Item	Cat#	Source
1.	RNaseZap® Solution	AM9782	Life Technologies Ltd, UK
2.	Trizol LS Reagent	10296028	Life Technologies Ltd, UK
3.	Chloroform [Sigma] (>99.5% Purity, 119.38 g/mol (mW))	C2432-500mL	Sigma-Aldrich Company Ltd., UK
4.	UltraPure™ Glycogen (20 µg/µL in RNase-free water)	10814010	Life Technologies Ltd, UK
5.	Ethyl alcohol, Pure ≥95% [Sigma]	E7148-500mL	Sigma-Aldrich Company Ltd., UK

No.	Item	Cat#	Source
6.	5nM Sysn-cel-miR-39' reagent	MSY0000010	QIAGEN Ltd, UK.
7.	miRNeasy Mini Kit	217004	QIAGEN Ltd, UK.
8.	miScript II RT Kit	218161	QIAGEN Ltd, UK.
9.	miScript SYBR Green PCR Kit	218073	QIAGEN Ltd, UK.
10.	Hs_RNU6-2_11 (RNU6-6P) miScript Primer Assay	MS00033740	QIAGEN Ltd, UK.
11.	Hs_SNORD96A_11 miScript Primer Assay	MS00033733	QIAGEN Ltd, UK.
12.	miScript miRNA PCR Array Human Serum & Plasma 384HC	MIHS-3106Z	QIAGEN Ltd, UK.
13.	Multiply® µStrip 0.2 mL, white, strip of 8 without lid, 120 strips in the minigrip bag	72.985.092	STARLAB Ltd, UK
14.	Multiply® µStripPro strip of 8 with attached flat lid, transparent	72.991.103	STARLAB Ltd, UK

2.2 Documents and Forms

No.	Item	Ref No.
1.	North West-Greater Manchester West National Research Ethics Service (NRES) Committee	11/NW/0593
2.	Royal Liverpool and Broadgreen University Hospitals NHS trust	4195/UOL 000760
3.	The Royal Liverpool and Broadgreen University Hospitals NHS Trust Approval Letter for Non-CTIMP Studies.	15/EE/0051 23/06/2015
4.	Letter of invitation to participant [Letter to patient - Molecular Regulators in Osteoporosis].	Ver. 1.2, 25/08/2015
5.	Participant Information Sheet (PIS) for the Patient Molecular- Regulators in Osteoporosis.	Ver. 1.2, 25/02/2015
6.	Participant Information Sheet for Healthy Volunteer-Molecular Regulators in Osteoporosis.	Ver. 1.1. 25/02/2015
7.	Participant Consent Form	Ver. 1.2. 25/02/2015
8.	Osteoporosis Patient Medical Form	
9.	Osteoporosis Clinical Specimen Form	
10.	Healthy Control Clinical Specimen Form	

2.3 Methods

This study has been carried out under ethical approval from the England Health Research Authority National Research Ethics Service Committee, North West-Greater Manchester West [REC reference: 11/NW/0593] and East of England-Essex [REC reference 15/EE/0051].

Informed consent was obtained for all participants prior to sample collection. Clinical samples were obtained from participants including local healthy volunteers and patients who were referred to the Royal Liverpool Broadgreen University Hospital NHS Trust based in Liverpool who either presented at the Bone clinic or at the Department of Radiology for a bone mineral density (BMD) scan.

Briefly, all participants, including patients and healthy volunteers (non-osteoporosis), were invited to participate in the research study and had been given participant information sheet (PIS) '*Patient Information-Molecular Regulators in Osteoporosis*' to read (see Appendix 8.3). The aim of the research, patient selection criteria, what will happen if they like to take part, what will happen to the collected blood sample, and the ethical issues related to the confidentiality of information obtained from the patient medical file had been highlighted, (see Appendix 8.3). They had an opportunity to read and ask any questions or discuss any concerns related to this study. Upon their approval, patient consent form was read to them, and each patient gave their own agreement and signed the form before having their blood sample taken (Figure 2-1). The form consists of three parts; one copy was handed to the patient; another copy was placed in the patient's medical file and the third copy was kept in the university file as part of ethical approval documentation. The

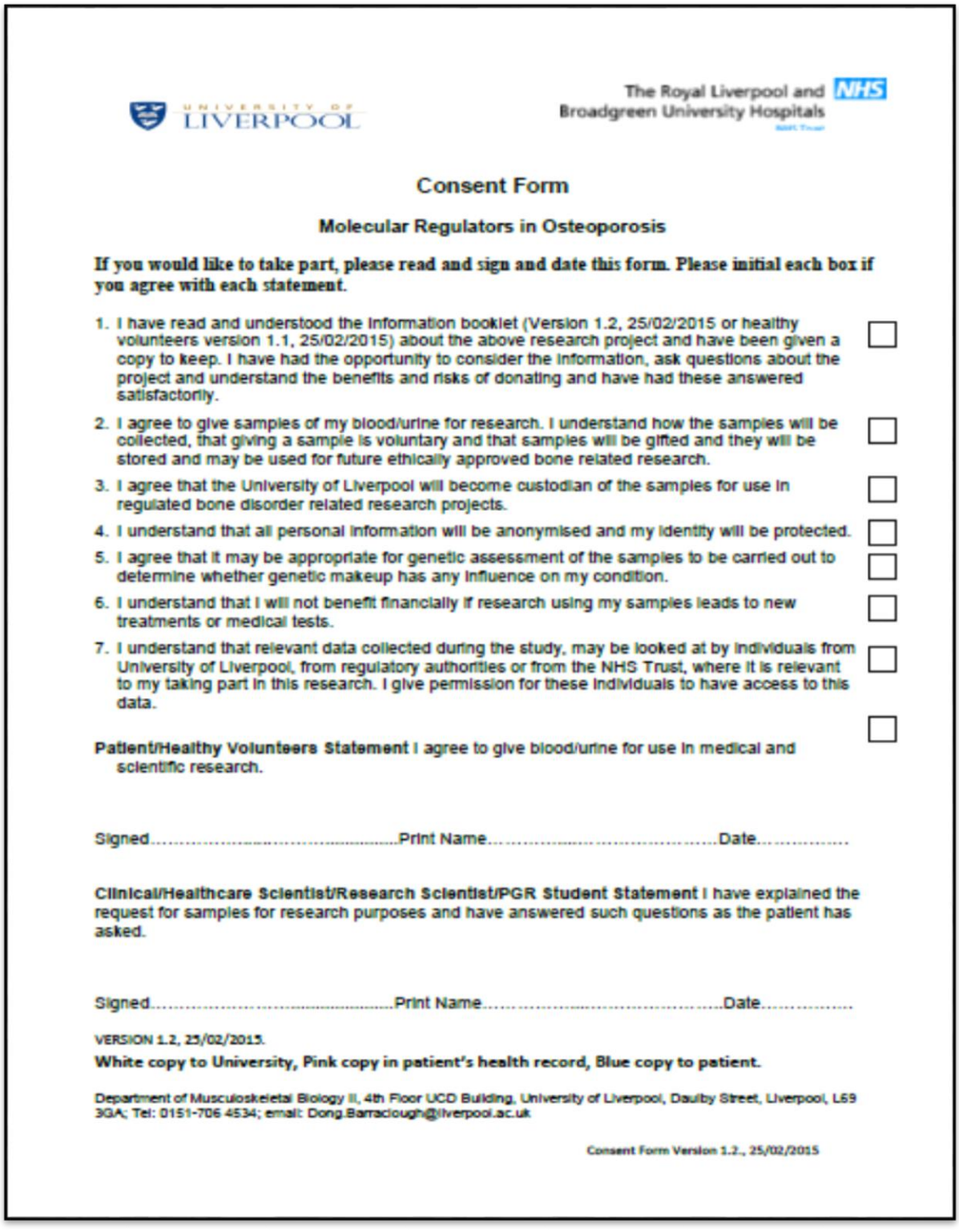
'Osteoporosis Patient' medical form was completed by a medical doctor. The form consists of patient demographic information, site of fracture, BMD T-score, frequency of fracture, medication, risk factors and any special notes (Figure 2-2).

An anonymised identification number was created for all participants, and their age and gender were recorded in the 'Osteoporosis Patient' Medical record file as shown in Table 2-1.

Table 2-1: Sample of 'Osteoporosis Patient' Medical Record File

For details refer to appendix 8.4

No.	Date of Clinic	Sample ID YYMMDD OP##	DOB	Age	Sex F/M	T Score Lumbar Spine (LS)	T Score Femoral Neck (FN)	T score Total IP (TH)	Number of Fractures	Position of Fracture Wrist [W] Vertebrae [V] Hip [H] Rib [R] Ankle [A Other [O]	Date of last Fracture	Medication History Calcium Vitamin D Bisphosphonates Prodelos Denosumab Others	Risk Factors	Type OP	Notes



UNIVERSITY OF LIVERPOOL

The Royal Liverpool and Broadgreen University Hospitals NHS

Consent Form

Molecular Regulators in Osteoporosis

If you would like to take part, please read and sign and date this form. Please initial each box if you agree with each statement.

1. I have read and understood the information booklet (Version 1.2, 25/02/2015 or healthy volunteers version 1.1, 25/02/2015) about the above research project and have been given a copy to keep. I have had the opportunity to consider the information, ask questions about the project and understand the benefits and risks of donating and have had these answered satisfactorily. ☐
2. I agree to give samples of my blood/urine for research. I understand how the samples will be collected, that giving a sample is voluntary and that samples will be gifted and they will be stored and may be used for future ethically approved bone related research. ☐
3. I agree that the University of Liverpool will become custodian of the samples for use in regulated bone disorder related research projects. ☐
4. I understand that all personal information will be anonymised and my identity will be protected. ☐
5. I agree that it may be appropriate for genetic assessment of the samples to be carried out to determine whether genetic makeup has any influence on my condition. ☐
6. I understand that I will not benefit financially if research using my samples leads to new treatments or medical tests. ☐
7. I understand that relevant data collected during the study, may be looked at by individuals from University of Liverpool, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to this data. ☐

Patient/Healthy Volunteers Statement I agree to give blood/urine for use in medical and scientific research. ☐

Signed……Print Name……Date……

Clinical/Healthcare Scientist/Research Scientist/PGR Student Statement I have explained the request for samples for research purposes and have answered such questions as the patient has asked.

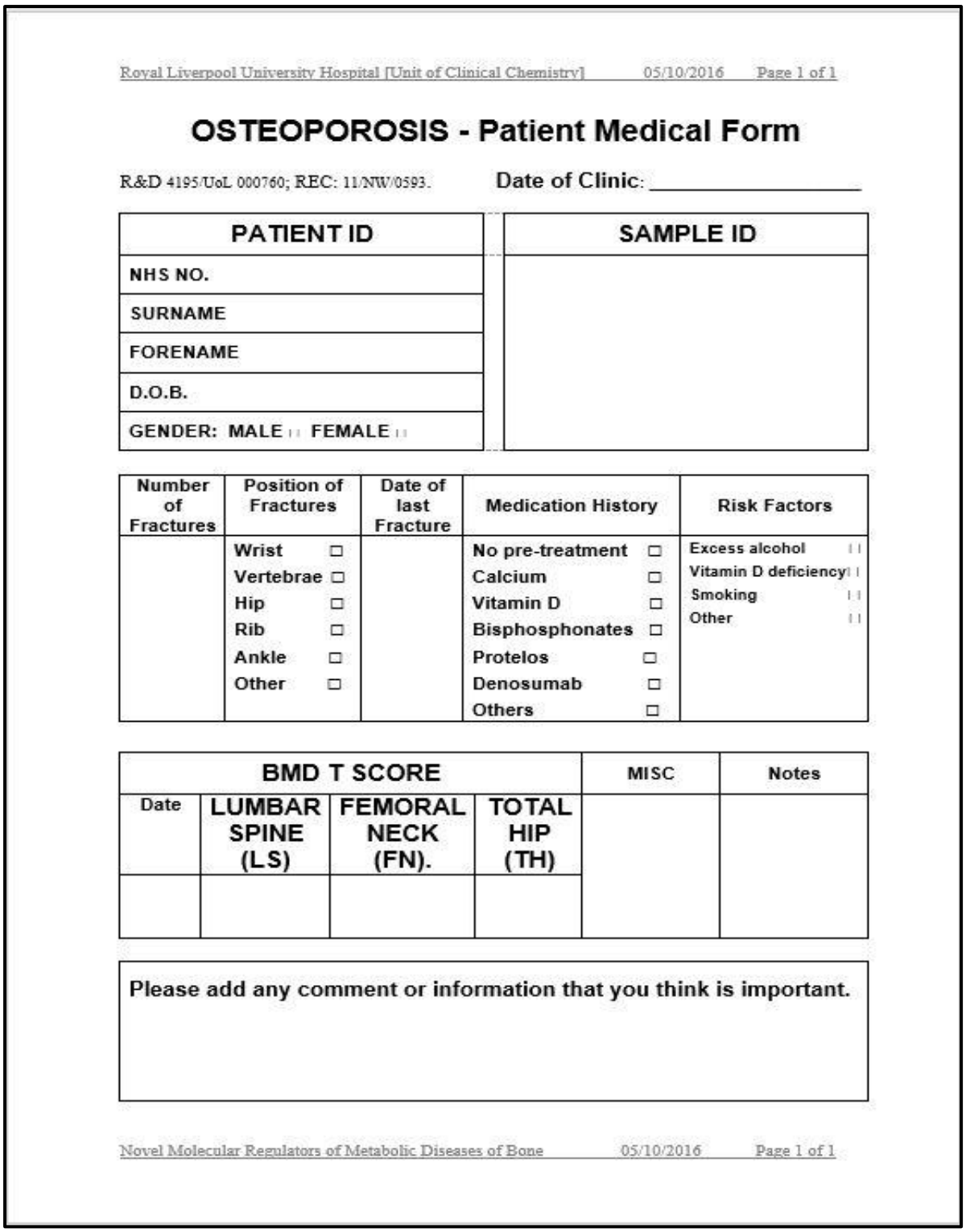
Signed……Print Name……Date……

VERSION 1.2, 25/02/2015.

White copy to University, Pink copy in patient's health record, Blue copy to patient.

Department of Musculoskeletal Biology II, 4th Floor UCD Building, University of Liverpool, Daulby Street, Liverpool, L69 3GA; Tel: 0151-706 4534; email: Dong.Barraclough@liverpool.ac.uk

Figure 2-1: Consent Form



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OSTEOPOROSIS - Patient Medical Form

R&D 4195/UoL 000760; REC: 11/NW/0593. Date of Clinic: _____

PATIENT ID	SAMPLE ID
NHS NO.	
SURNAME	
FORENAME	
D.O.B.	
GENDER: MALE ☐ FEMALE ☐	

Number of Fractures	Position of Fractures	Date of last Fracture	Medication History	Risk Factors
	Wrist ☐ Vertebrae ☐ Hip ☐ Rib ☐ Ankle ☐ Other ☐		No pre-treatment ☐ Calcium ☐ Vitamin D ☐ Bisphosphonates ☐ Protelos ☐ Denosumab ☐ Others ☐	Excess alcohol ☐ Vitamin D deficiency ☐ Smoking ☐ Other ☐

BMD T SCORE				MISC	Notes
Date	LUMBAR SPINE (LS)	FEMORAL NECK (FN).	TOTAL HIP (TH)		

Please add any comment or information that you think is important.

Novel Molecular Regulators of Metabolic Diseases of Bone 05/10/2016 Page 1 of 1

Figure 2-2:Osteoporosis Patient Medical Form

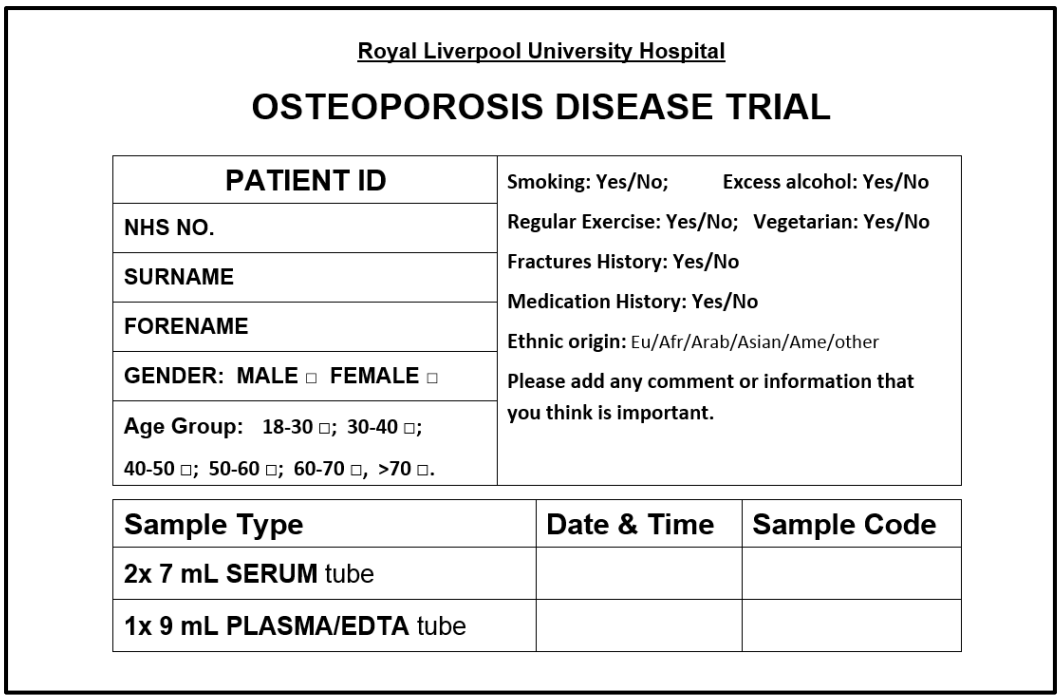
2.4 Sample Collection and Preparation

Blood samples were drawn by qualified phlebotomists in the blood collection area at the RLBUHT following standard Safety Protocol. Samples of blood (approximately 25mL) for isolation of serum and plasma were obtained, using two 7.5mL S-Monovette® with (Clotting Activator) and one 9mL S-Monovette® K3E, Potassium EDTA tubes (Blood Collection and Sample Processing Materials 2.1.1). Samples were inverted gently for approximately 10 times, and kept in an upright position at room temperature for 0.5 to 2hrs. Collection's date and time were documented in the collected tubes and on the patient's medical form (Figure 2-3).

Each participant's specimens were given a unique and anonymised alphanumeric identification number (ID) composed of six digits made up from the year, month and day of collection, followed by two letters, either OP for osteoporosis or HC for healthy volunteer, two digits from the participant serial number. Finally, the 'S' alphabet for serum or 'PE' for plasma/EDTA and sample number. Additionally, a unique aliquot letter, a, b, c, d, …etc., were used when there were multiple similar samples blood components aliquots as shown in example below.

Example:	130709OP01 S1a (YYMMDDXX## A#n)

Patients clinical information and sample ID were documented in the 'Osteoporosis Clinical Specimen form' (See below).



Royal Liverpool University Hospital

OSTEOPOROSIS DISEASE TRIAL

PATIENT ID	Smoking: Yes/No; Excess alcohol: Yes/No Regular Exercise: Yes/No; Vegetarian: Yes/No Fractures History: Yes/No Medication History: Yes/No Ethnic origin: Eu/Afr/Arab/Asian/Ame/other Please add any comment or information that you think is important.
NHS NO.	
SURNAME	
FORENAME	
GENDER: MALE □ FEMALE □	
Age Group: 18-30 □; 30-40 □; 40-50 □; 50-60 □; 60-70 □, >70 □.	

Sample Type	Date & Time	Sample Code
2x 7 mL SERUM tube		
1x 9 mL PLASMA/EDTA tube		

Figure 2-3: Osteoporosis Clinical Specimen Form

2.4.1 Serum and Plasma Preparation

Serum and plasma were obtained from the blood specimens by centrifuging the collected samples at 2,500 x g (Thermo Heraeus Megafuge 16R centrifuge) for 30 minutes at room temperature. The supernatant yields of approximately 3-3.5mL were initially transferred into three new sterile 1.5 mL Micro tubes (Blood Collection and Sample Processing Materials2.1.1). A further centrifugation step for these aliquots was carried out at 14,000 x g (Thermo IEC Microcentrifuge CL 17 R) for 30 minutes at 4°C, to maintain RNA stability and to remove additional cellular and protein debris. Approximately 1.2mL of the supernatants were then transferred into a new RNAse free Micro tubes. The final yield obtained from the collected blood samples was 2-3 aliquots of approximately 1.2mL each for both serum and plasma samples. Specimens were then stored in a -80°C freezer for future analysis.

2.4.2 Isolation of Peripheral Blood Mononuclear Cells [PBMC]

Peripheral blood mononuclear cells (PBMCs) were isolated from 1:2 diluted EDTA packed red blood cells containing the buffy coat (see Figure 2-4) with Dulbecco's Phosphate Buffered Saline (DPBS) (Blood Collection and Sample Processing Materials 2.1.1) in 15 mL Polypropylene Centrifuge Tubes.

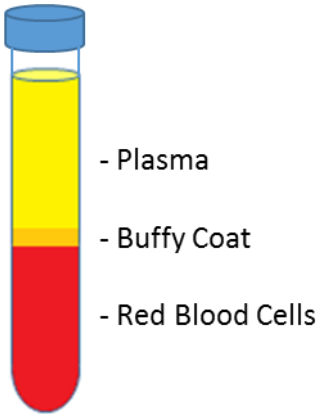


Figure 2-4: EDTA Blood Components.

Initially, 3mL of Ficoll-premium (1.077g/mL) solution was added into the bottom of a Greiner Bio-One 10 mL Leucosep™ tube and centrifuged at room temperature for 1 minute at 900 x g, so the separation medium is now located below the porous barrier. Approximately 6 mL of the PBS-diluted [1:2] EDTA blood samples were carefully pipetted down the side of the Leucosep™ tube held at 45 degrees and the tube was centrifuged at 900 x g for 30 minutes at room temperature (Thermo TX-400 Swinging Bucket Rotor and Thermo Heraeus Megafuge 16R), and slowed without the brake to prevent swirling of the samples. The interface layer (buffy coat) that contained the PBMC was collected into a sterile 15 mL polypropylene centrifuge tubes, and washed with PBS at room temperature, (see Figure 2-5). Cell pellets were suspended in 10mL of cold Ammonium-Chloride-Potassium Lysing Buffer, incubated at 4°C for 1 minute, centrifuged at 2,500 x g for 5 minutes at 4°C. Following two washing steps,

each using 10 mL PBS, centrifugation at 2,500 x g at room temperature for 5 minutes, the cell pellets were resuspended with 0.5mL PBS, transferred into 1.5 mL Micro tubes, centrifuged at 14,000 x g at 4°C for 5 minutes to form a tight cell pellet. Cell pellets were stored frozen at -80°C to await further analysis.

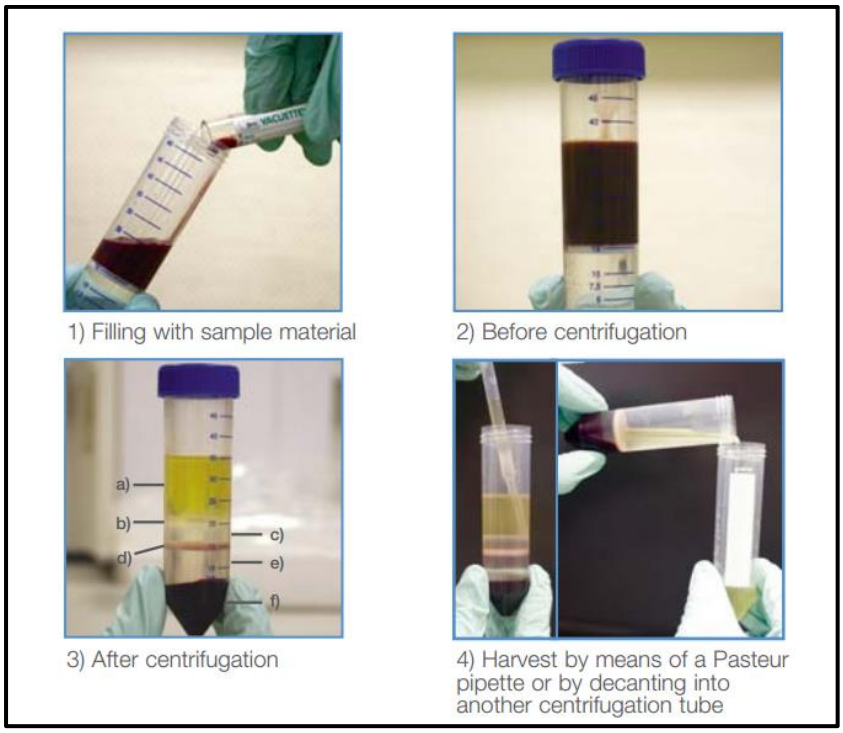


Figure 2-5: PBMC isolation using Ficoll-premium (1.077g/mL density gradient) solution.
1) Pour the approximately 6mL PBS-diluted EDTA blood sample into 10mL Leucosep™ tube. 2) Centrifugate at room temperature for 30 minutes at 900 x g using a swinging bucket rotor. Switch off brakes of the centrifuge. 3) After centrifugation, the sequence of layers occurs as follows a) PBS-diluted plasma, b) interface layer (consisting of lymphocytes / PBMCs), c) Ficoll-premium (1.077g/mL) separation medium, d) porous barrier, e) Ficoll-premium (1.077g/mL) separation medium and f) pellet (erythrocytes and granulocytes). 4) Harvest the interface layer containing PBMCs by a Pasteur pipette into new a sterile 15 mL polypropylene centrifuge tube. Cited from greinerbio one Catalogue 2017.

2.4.3 Sample Storage

Prior to being frozen for storage at -80°C, all aliquots of serum, plasma and PBMC cell pellets were labelled, anonymously, with their unique identification numbers, their specimen types, date of processing.

2.5 RNA Extraction

2.5.1 Isolation and purification of RNA from serum and plasma

Isolation of small RNA was achieved by using a combination of TRIzol® LS reagent and a QIAGEN miRNeasy Mini Kit. Generally, 400 µL aliquots of serum or plasma was added into a 2.0mL Ultra High Recovery (UHR) microcentrifuge tube containing 1200 µL TRIzol® LS reagent. The tube was vortexed at the highest speed (Vortex-Genies2) for 30-60 seconds and incubated at room temperature for 15-30 minutes. Then 2.4 µL of glycogen (10 mg/mL)) were added to improve the yield of RNA and 5 µL of 5nM synthetic miRNA-39 from *Caenorhabditis elegans* (Sysn-cel-miR-39) (QIAGEN) was added as a spike-in control. After adding 320 µL chloroform (99.5%), the mixture was vortexed vigorously for 30 seconds, incubated on ice for 10 minutes and centrifuged for 30 minutes at 14,000 x g at 4°C (Thermo IEC Microcentrifuge). The clear upper aqueous solution containing RNA was transferred into a sterile 2.0mL microcentrifuge tube. RNA was precipitated by adding 1.5 volume of 100% ethanol and vortexed at medium speed. Purification of extracted total RNA was performed using miRNeasy columns (QIAGEN) according to the manufacturer's recommendation. Briefly, up to 700 µL of the sample was pipetted into an miRNeasy Mini spin column in a 2 mL collection tube, centrifuged at ≥ 8000 x g for 15 seconds at room temperature and the flow-through was discarded. The step was repeated for the remaining ethanol's precipitated sample. Followed by two washing steps using 700 µL of RNeasy RWT and 500 µL x2 RPE Buffers. RNA was eluted

from the Mini spin column using 30µL of RNase-free water, followed by 50µL for a second elution. The resulting purified RNAs were stored frozen at -80^{o}C until use.

2.5.2 RNA Concentration and Purity

RNA concentrations were measured initially using a Thermo Scientific NanoDrop™ 2000 spectrophotometer. The measurement of RNA concentration was in ng/µL, and the ratio of absorbance at 260nm/280nm was used to assess the purity of nucleic acids (DNA or RNA) from either protein or phenol contaminant, and at 260/230 to measure the nucleic acid purity from residual phenol, residual guanidine from the Trizol reagent or glycogen used for precipitation of the RNA.

The assessment of RNA quality and concentration was also carried out in the Centre of Genomic Research (CGR), University of Liverpool using Agilent Eukaryote Total RNA Pico chip kit and an Agilent 2100 analyser. 2.0 µL of RNA fraction (>8ng/µL) were sent to the Centre of Genomic Research (CGR), University of Liverpool for RNA quality check.

2.6 Real-Time Quantitative PCR (RT-qPCR)

2.6.1 RNA Reverse Transcription

RNA reverse transcription was carried out on total RNA isolated from serum and plasma samples. 100 ng of purified RNA was mixed with 4 µL of 5x QIAGEN miScript HiSpec Buffer, 2 µL of 10x QIAGEN miScript Nucleics Mix (dNTPs, rATP, oligo-dT primers, and an internal synthetic RNA control [miRTC]) and 2 µL of QIAGEN miScript Reverse Transcriptase Mix (poly(A) polymerase & reverse transcriptase) (Table 2-2). Reverse Transcriptase (RT) was replaced by RNase-free water for the no-RT control preparation. The RNA reverse transcription reaction mixtures, of final volumes of 20 µL, were incubated in a Bio-Rad T100 thermal cycler (Chapter 2: Materials and Methods **Error! Reference source not found.**) at

37°C for 60 minutes, then at 95°C for 5 minutes to inactivate the reverse transcriptase enzyme and held at 4 °C. The resulting reverse transcription products were stored frozen at -20 °C and were used as a template for RT-qPCR amplification.

Table 2-2: Reverse transcription reaction components

Component	**RT-master mix (1x)**	**No-RT control**
1. RNase-free water	µL Variable	µL Variable
2. 5x miScript HiSpec Buffer	4 µL	4 µL
3. 10x miScript Nucleics Mix	2 µL	2 µL
4. Total RNA containing miRNA	µL Variable (100ng)	µL Variable (100ng)
5. miScript Reverse Transcriptase Mix	2 µL	no
Total Volume	20 µL	20 µL

2.6.2 Real time -Quantitative Polymerase chain reaction (RT-qPCR)

RT-qPCR was performed by adding 1µL of 1:3 diluted RNA reverse transcription products to 5µL of 2x QIAGEN QuantiTect SYBR Green PCR Master Mix (HotStarTaq® DNA Polymerase, QuantiTect SYBR Green PCR Buffer, dNTP mix, including dUTP, SYBR Green I, ROX™ passive reference dye and 5 mM $MgCl_2$), 1µL of 10x miScript Universal Primer (reverse primer), and 1 µL of 10x Primer Mix (forward miScript primer, Table 2-3) with a final volume of 10µL. The RT-qPCR mixtures were incubated in 96 well plates at 95°C for 15 min as per manufacturer's recommendation, to activate the DNA polymerase, followed by 45 cycles of the following 3 steps: denaturation at 94°C for 15s, annealing at 55°C for 30s and extension at 70°C for 30s, followed by, melting steps of 95°C for 10s, 65°C for 60s and 97°C for 1s Continuous Mode), and final cooling step of 37°C for 30s. All RT-qPCR reactions were performed in duplicate and a No-Reverse Transcriptase (NRT) control was included. RT-qPCR amplification was performed

using a Roche Lightcycler 96® real-time PCR system. All primers used for RT-qPCR in this study were from QIAGEN as shown in Table 2-3.

Table 2-3: A list of miScript miRNA Primers used for RT-qPCR

NO.	miRNA	miRBase miRNA database/Forward Primer Sequence	QIAGEN miScript Catalog
1.	hsa-miR-1193	MIMAT0015049: 5'GGGAUGGUAGACCGGUGACGUGC	MS00020265
2.	hsa-miR-1281	MIMAT0005939: 5'UCGCCUCCUCCUCUCCC	MS00014455
3.	hsa-miR-3923	MIMAT0018198: 5'AACUAGUAAUGUUGGAUUAGGG	MS00023611
4.	hsa-miR-485-5p	MIMAT0002175: 5'AGAGGCUGGCCGUGAUGAAUUC	MS00006972
5.	hsa-miR-4258	MIMAT0016879: 5'CCCCGCCACCGCCUUGG	MS00021175
6.	hsa-miR-4274	MIMAT0005828: 5'CACUGUAGGUGAUGGUGAGAGUGGGCA	MS00014105
7.	hsa-miR-3911	MIMAT0018185: 5'UGUGUGGAUCCUGGAGGAGGCA	MS00023527
8.	hsa-miR-1290	MIMAT0005880: 5'UGGAUUUUUGGAUCAGGGA	MS00014518
9.	hsa-miR-143-3p	MIMAT0000435: 5'UGAGAUGAAGCACUGUAGCUC	MS00003514
10.	hsa-miR-4516	MIMAT0019053: 5'GGGAGAAGGGUCGGGGC	MS00037555
11.	hsa-miR-4306	MIMAT0016858: 5'UGGAGAGAAAGGCAGUA	MS00021511
12.	hsa-miR-548e-3p	MIMAT0005874: 5'AAAAACUGAGACUACUUUUGCA	MS00014735
13.	hsa-miR-100-5p	MIMAT0000098: 5'AACCCGUAGAUCCGAACUUGUG	MS00003388
14.	hsa-miR-122-5p	MIMAT0000421: 5'UGGAGUGUGACAAUGGUGUUUG	MS00003416
15.	hsa-miR-215-5p	MIMAT0000272: 5'AUGACCUAUGAAUUGACAGAC	MS00003829
16.	hsa-miR-548d-5p	MIMAT0004812: 5'AAAAGUAAUUGUGGUUUUUGCC	MS00010136
17.	hsa-miR-450a-5p	MIMAT0001545: 5'UUUUGCGAUGUGUUCCUAAUAU	MS00006937
18.	hsa-miR-373-5p	MIMAT0000725: 5'ACUCAAAAUGGGGGCGCUUUCC	MS00006867

NO.	miRNA	miRBase miRNA database/Forward Primer Sequence	QIAGEN miScript Catalog
19.	hsa-miR-375	MIMAT0000728: 5'UUUGUUCGUUCGGCUCGCGUGA	MS00004088
20.	hsa-miR-99a-5p	MIMAT0000097: 5'AACCCGUAGAUCCGAUCUUGUG	MS00003374
21.	hsa-miR-145-3p	MIMAT0004601: 5'GGAUUCCUGGAAAUACUGUUCU	MS00008708
22.	hsa-miR-206	MIMAT0000462: 5'UGGAAUGUAAGGAAGUGUGUGG	MS00003787
23.	hsa-SNORD96A	CCTGGTGATG ACAGATGGCA TTGTCAGCCA ATCCCCAAGT GGGAGTGAGG ACATGTCCTG CAATTCTGAA GG	MS00033733
24.	hsa-RNU6-6P	GTGCTCGCTT CGGCAGCACA TATACTAAAA TTGGAACGAT ACAGAGAAGA TTAGCATGGC	MS00033740

Data was analysed using Roche Light cycler 96® software which enables the comparison of the signal obtained for the target miRNA of interest with that of a reference control gene. Relative quantities of miRNA were calculated using the $\Delta\Delta$Ct methods after normalization to the control. Both hsa-SNORD96A (homo sapiens small nucleolar RNA, C/D box 96A) and hsa-RNU6-6P (homo sapiens small nuclear 6, pseudogene) were used as RT-qPCR normalization control. The expression levels of miRNA between the osteopenia, osteoporosis and non-osteoporosis control groups was calculated using the $\Delta\Delta$Ct equation (Equation 2-2) as well as the fold change ($2^{-\Delta\Delta CT}$) for each miRNA (Equation 2-3) (Bustin et al., 2009). Since Ct values greater than 35 were considered to be below the detection level of the reaction, (indicated as N/A, not detected), all miRNA RT-qPCR reactions with Ct > 35 were included in the analysis as undetected and assigned the Ct values of 35.

Equation 2-1: Pathway-focused gene ΔCt

$$\Delta Ct = (Ct\ Gene\ of\ Interest) - (Ct\ Average\ of\ Housekeeping\ Gene)$$

Equation 2-2: Gene Expression across two groups ΔΔCt

$$\Delta\Delta Ct = \Delta Ct(Target\ Group) - \Delta Ct\ (Control\ Group)$$

Equation 2-3: Fold Change Expression

$$Fold\ Change = 2^{-(\Delta\Delta Ct)}$$

2.7 miRNAs Array Analysis

The expression level of miRNA in four pooled serum groups including a pool of 6 non-osteoporotic male samples (NOPMP1), a pool of 4 non-osteoporotic female samples (NOPFP1), a pool of 8 osteopenia female samples (OPAFP3) and a pool of 9 osteoporosis female samples (OPFP4) (Table 2-4) were profiled using Human Serum & Plasma miRNA PCR Array MIHS-3106Z (QIAGEN), which represented 370 mature miRNAs and six reference genes, including SNORD61, SNORD68, SNORD72, SNORD95. SNORD96A and RNU6-6P and two RNA and PCR quality controls including Reverse Transcription control [miRTC] and positive PCR Control [PPC]).

Each pool contained 50 ng of purified RNA from individual serum samples. RT-qPCR based miRNA array analysis were carried out by QIAGEN.

Table 2-4: Serum RNA Pools for QIAGEN miRNA PCR Array

No.	Category	ID No (no. of Samples)	Total RNA (ng)
1.	non-osteoporotic male pool 1 (NOPMP1)	NOPMP1 (6)	300
2.	non-osteoporotic female Pool 2 (NOPFP2)	NOPFP2 (4)	400
3.	Osteopenia, Female Pool 3 (OPAFP3)	OPAFP3 (8)	400
4.	Osteoporosis, Female, Pool 4 (OPFP4)	OPFP4 (9)	450

Briefly, miRNAs were converted to cDNA by miScript Reverse Transcription (RT) and pre-amplified using QIAGEN preamplification mix and QIAGEN miScript PreAMP PCR kit. The amplified cDNA was combined with QuantiTect SYBR green PCR master mix and mixtures were aliquoted across miScript PCR array Plate (See Figure 2-6). Levels of miRNAs were determined via real time PCR (RT-PCR). All reported Ct value greater than 30 or as N/A (not detected) were reported as 30 and considered not significant and the Ct values were excluded (as described in 2.6.2).

Data were normalized to the average of the two control small RNA molecules, SNORD96A and RNU6-6P, using the fold change ($2^{-\Delta\Delta CT}$) method (refer to 2.6.2).

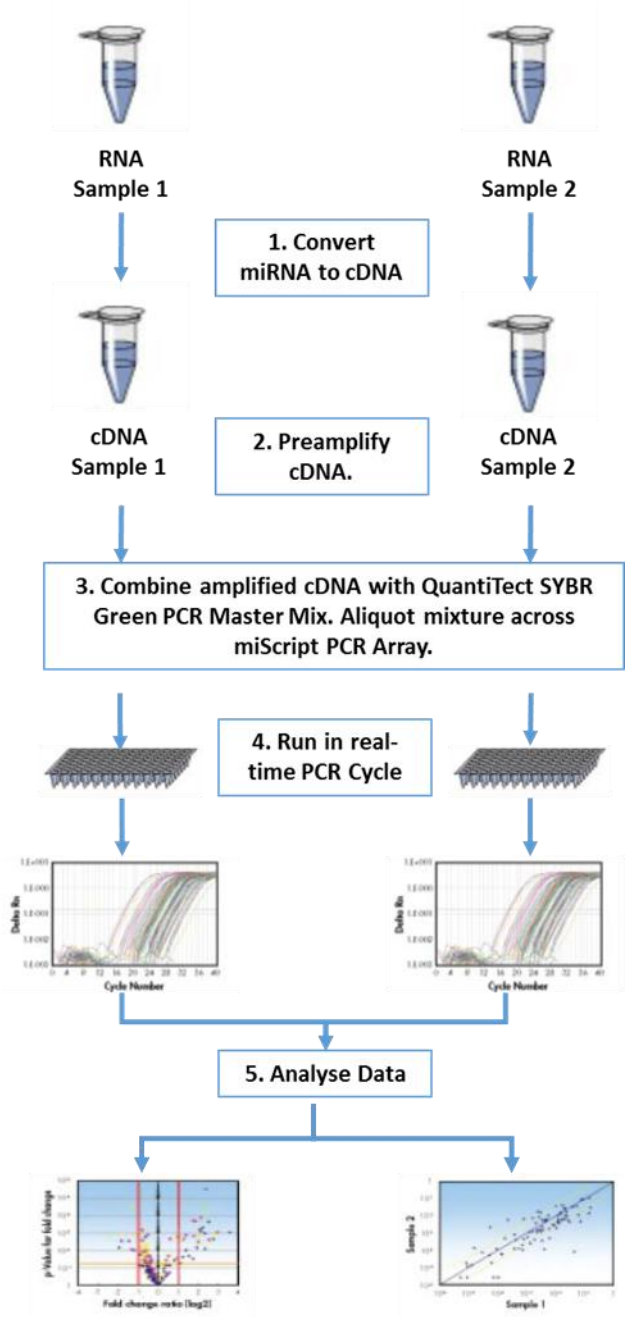


Figure 2-6: RT-qPCR Array Protocol.
Cited from QIAGEN miRNesy mini kit Handbook.

2.8 Bioinformatics Data Analysis

To calculate the fold change in miRNA levels between serum RNA pools, data analysis web-based portal software (http://www.sabiosciences.com/mirnaArrayDataAnalysis.php) from QIAGEN was used.

miRWalk 2.0 (Dweep and Gretz, 2015) was used for predicted miRNA gene targets. Gene ontology annotation (Carbon et al., 2009, The Gene Ontology, 2015), Enrichr database (Kuleshov et al., 2016) and DAVID Bioinformatics Resources ver. 6.8: Oct. 2016 (Huang da et al., 2009) were used for gene functional annotation. InteractiVenn: web-based Venn diagram tool (Heberle et al., 2015) was used to cluster genes associated with miRNA and osteoporosis disease. String software (Szklarczyk et al., 2017) was used to generate protein to protein signalling interaction diagrams.

2.9 Statistical Analyses

A variety of statistical tools have been used to measure the numerical analytical data, including IBM SPSS Statistic 22.0 (UK Head Office IBM United Kingdom Limited PO Box 41, North Harbour Portsmouth Hampshire, PO6 3AU) and GraphPad Prism version 7 (GraphPad Software, Inc. 7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA).

RT-PCR 2^-ΔCt results were transformed to logarithmic data in an Excel spreadsheet, and analysed in GraphPad Prism by One-way ANOVA with post hoc 'Bonferroni's multiple comparisons test' and by Mann Whitney test (two-tail) statistical tests. Comparisons were made between non-osteoporotic, osteopenia and osteoporotic groups to determine any significance of differentially expressed results between these groups. Results were displayed as 'Box-and-Whisker' plots with the 25th, middle and 75th percentiles, and minimum to maximum ranges (error bars).

SPSS Ver. 22.0 was used to calculate the AUC (ROC) diagnostic value of miRNAs. The cut-off points with the highest sensitivity and specificity were determined. The minimum level of significance was $p<0.05$.

Chapter 3: Identification of Differentially Expressed Circulating miRNAs in Osteoporosis

3.1 Introduction

Osteoporosis is an age related skeletal disorder, caused by a relative increase of bone resorption over bone formation, is characterised by decreased bone mass and bone strength predisposing to fracture (Kanis et al., 2013). Osteoporosis is normally non-symptomatic until a fracture occurs.

Current diagnosis for osteoporosis is mainly based on bone mineral density (BMD) using Dual-Energy-X-ray (DEXA) scanning, the assessment of clinical risk factors using WHO Fracture Risk Assessment Tool (FRAX) and different bone turnover markers (BTM)(Silva and Bilezikian, 2011).

Despite the fact that the risk of fracture increases with decreasing levels of BMD, many patients with osteoporosis do not go on to have a fracture and that most fracture in the general population occur in patients without osteoporosis (Das and Crockett, 2013, Marshall et al., 1996, Richards et al., 2012). Even with the use of current bone turnover markers (BTMs) such as serum Procollagen I Intact N-Terminal propeptide (s-PINP), a bone formation marker or C-terminal collagen crosslinks (s-CTX), a bone resorption marker(Bauer et al., 2012), as a diagnostic tool remains a challenge, due to their analytical and biological variability (Sandhu and Hampson, 2011), inadequate quality control and lack of reference population databases (Bauer et al., 2012).

Circulating miRNAs (miRNA) which are small non-coding RNAs of 18–22 nucleotides (Rodriguez et al., 2004), that control gene expression and participate in many pathological disorders could be used as novel biomarkers to assess the health status and progression of disease (Guay and Regazzi, 2013).

Several studies have shown that miRNAs play a significant role in skeletal development where Dicer enzyme found to be an essential component for biogenesis of miRNAs in which dicer was knocked down resulted in altered chondrocyte proliferation and differentiation in Col2-:Dicer$^{fl/fl}$ transgenic mice compared to wildtype mice (Kobayashi et al., 2008), delayed perinatal osteoblast differentiation and a dramatic increase in postnatal bone mass in targeted deletion of Dicer in mature osteoblasts (Dicer$^{\Delta oc/\Delta oc}$) mice compared to conditional deletion of Dicer (Dicer$^{c/c}$) control mice (Gaur et al., 2010), and reduced bone remodelling in mice with Dicer deficiency in osteoclasts (cKO mutant) compared to wildtype mice (Mizoguchi et al., 2010).

Although a few pilot studies have shown miRNAs in blood samples could be associated with osteoporotic fractures in very small numbers of patients (Panach et al., 2015, Seeliger et al., 2014, Weilner et al., 2015), or in low bone mineral density (BMD) patients versus normal BMD control (Li et al., 2014), more clinical research is needed to investigate whether circulating miRNA could be used as a routine diagnostic application in osteoporosis.

The aim of the research is to identify differentially-expressed circulating miRNA as potential osteoporotic biomarkers in clinical specimens using micro RNA PCR array.

3.2 Results

After obtaining ethical approval and patient consent, blood samples were drawn from osteoporotic (study), and non-osteoporosis (control) subjects. Patients were categorised into four osteoporotic groups, based on their BMD T-score and fragility fracture history (Materials & Methods 2.3.1). Group A for osteopenia patients without fracture (BMD T score <-1.0 and >-2.5), group B for osteoporosis patients (T score ≤ -2.5), group C for osteoporosis with fracture (BMD T score ≤-2.5) and finally group D for osteopenia patients with fragility fracture (T score <-1.0 & >-2.5).

A total number of sixty-one osteoporotic and non-osteoporotic participants were recruited (Table 3-1). Twenty-five percent of the recruits were healthy non-osteoporotic adults (7 male & 8 Female), however BMD's T-Score (T-S) were not available for this group. Twenty-four percent were osteopenia (with and without fracture) with average BMD T-Score of -2.0 ± 0.3 and the remaining 51% were osteoporosis patients (average BMD T-Score = -3.2 ± 0.7). There was no significant difference in mean age between the four osteoporotic study groups (Figure 3-1). Indeed, 89% of the Osteoporotic participants were females (Figure 3-2).

Table 3-1: Total number of Participants recruited between 2012-2013 (Total n=61)
Participants recruited between 2012-2013 had no BMD (g/cm^2) records

Medical Diagnosis	Sex	Avg. Age ±SD (n)	Avg. T-Score Lumbar Spine (L2-L4) ±SD
Non-Osteoporotic	F	33.8 ± 9.9 (n= 8)	No data
	M	27.9 ± 4.9 (n= 7)	
	T	**31 ± 8.3 (n= 15)**	
Osteopenia	**F**	**73 ± 5 (n= 8)**	**-1.8 ± 0.5**
Osteopenia with Fracture	F	62.3 ± 8.4 (n= 6)	-1.7 ± 0.7
	M	86 (n= 1)	-1 ±
	T	**65.7 ± 11.8 (n= 7)**	**-1.6 ± 0.7**
Osteoporosis	F	64.1 ± 11.9 (n= 10)	-2.9 ± 1
	M	68.5 ± 21.9 (n= 2)	-3 ± 1.3
	T	**64.8 ± 12.8 (n= 12)**	**-2.9 ± 1**
Osteoporosis with Fracture	F	69.5 ± 11.5 (n= 17)	-2.8 ± 1.1
	M	57.5 ± 19.1 (n= 2)	-3.4 ± 0.2
	T	**68.3 ± 12.3 (n= 19)**	**-2.9 ± 1**
Grand Total	**61**		

F= Female, M= Male and T= Total

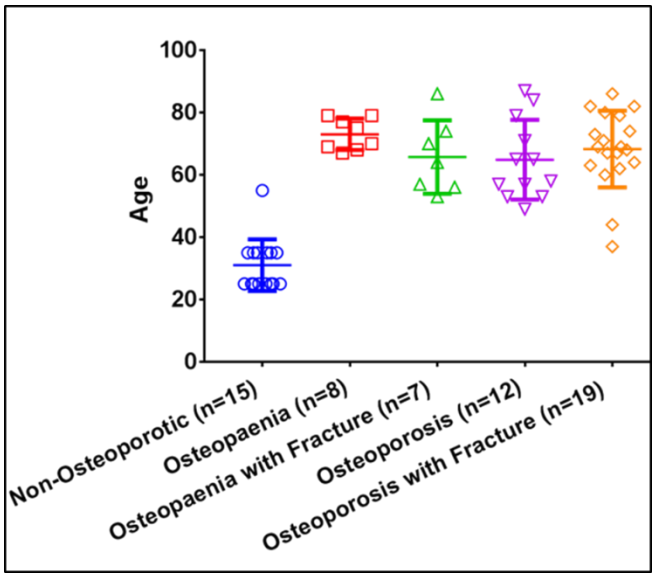


Figure 3-1: Ages of patients between experimental groups.
Plot shows average age between non-Osteoporosis, osteopenia, osteopenia with fracture, osteoporosis, and osteoporosis with fracture. There was significant age difference (*P<0.0001) between the low bone mineral density (BMD) groups (osteopenia and osteoporosis) and the non-osteoporosis group.

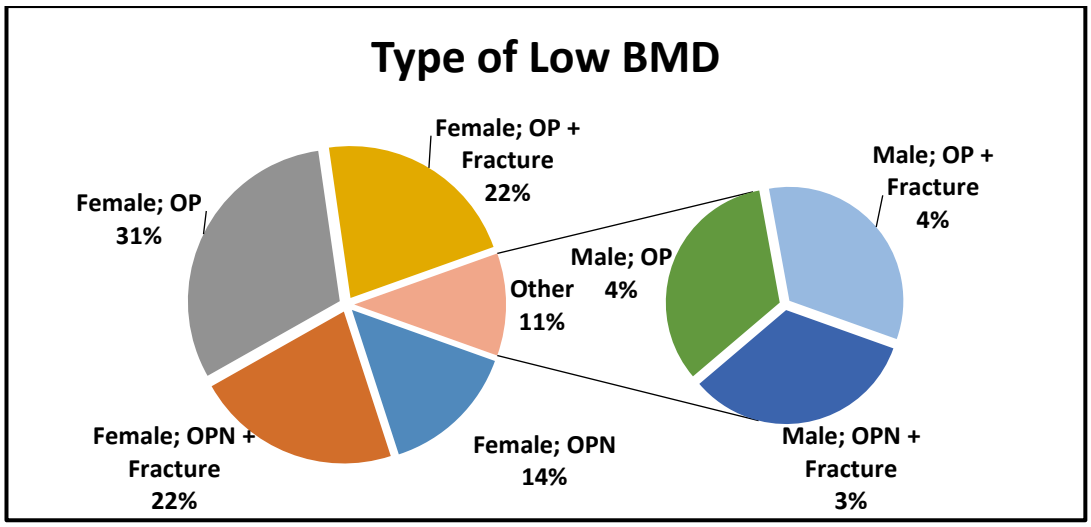


Figure 3-2: Type of Low BMD Patients Recruited in 2012-2013.

The left side of the figure represents the overall percentages of participants, while the right side represents the male subjects. OPN=Osteopenia, OP=Osteoporosis.

The majority of female patients (n=10) who were >60 years had severe osteoporosis with fracture. However, in the case of the osteopenia with fracture group the majority of females (n=6) was between 40 and 60 years (Figure 3-3).

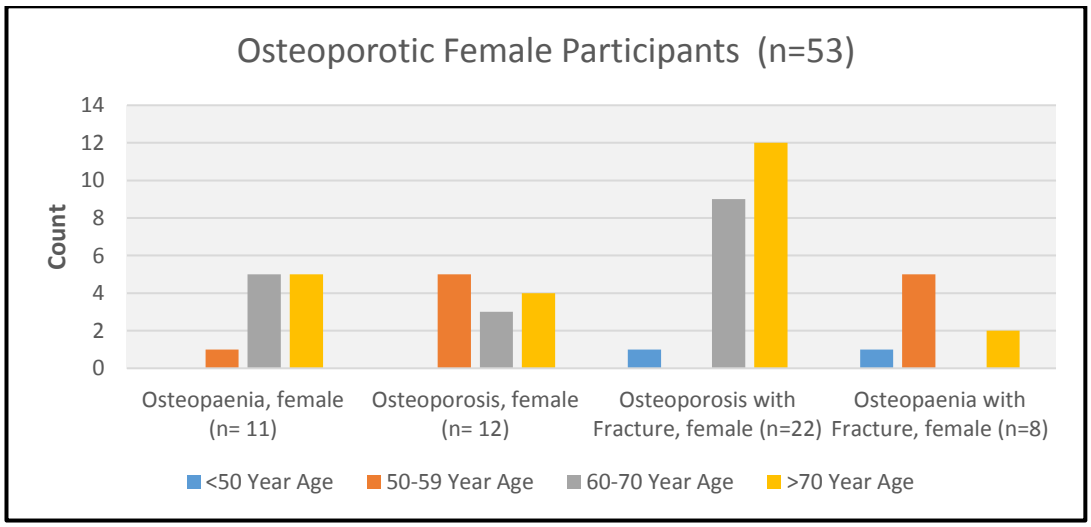


Figure 3-3: Number of Osteopenia and Osteoporosis Female Participants based on diagnostic category & age.

Majority of Osteopenia, female was of age 60-70 years (n=5, Grey) and >70 year (n=5, Yellow). Osteoporosis, female (n=12) with variable number of 50-59 year (n=5, Orange), 60-70 year (n=3, Grey) and > 70 year (n=4, Yellow). Majority of Osteoporosis with fracture, female (n=22) were of age: 60-70 year, n=9, grey & > 70 years, n=12, yellow. While majority of Osteopenia with fracture female (n=8) were of age 50-59 years (n=5). One of each osteoporosis with fracture and osteopenia with fracture female patients were of age < 50 years.

3.2.1 Quality of miRNA Extracted from Serum and Plasma Samples

3.2.1.1 miRNA Quality Control

RNAs extracted from participants' serum and plasma samples were checked for purity and concentration using a NanoDrop™ 2000 spectrophotometer (Materials and Methods 2.5.1.).

RNAs were extracted from forty-three serum and thirty-one plasma samples with average RNA yields of: 1029.5 ± 642.2 ng and 1273.7 ± 589.3 ng, respectively. In fact, the RNAs yield in plasma were relatively higher than those extracted from serum samples with lesser variability (Table 3-2). While, both serum and plasma samples have relatively similar RNA 260/280 ratio with mean value of 1.33 ± 0.12 and 1.4 ± 0.13, and RNA 260/230 ratio of RNA 260/230 ratio with mean value of 0.23 ± 0.04 and 0.28 ± 0.08, respectively (Table 3-2).

Table 3-2: Summary of quality check of RNA isolated from clinical samples

Specimen Type	No. of Samples	RNA 260/280 Optical Density Ratio Mean ± SD	RNA 260/230 Optical Density Ratio Mean ± SD	Total RNA (ng) Mean ± SD
Serum	43	1.33 ± 0.12	0.23 ± 0.04	1029.5 ± 642.2
Plasma	31	1.4 ± 0.13	0.28 ± 0.08	1273.7 ± 589.3

Additional RNA quality assessment was performed using a Eukaryote Total RNA Pico chip on Agilent 2100 bioanalyzer (Materials and Methods 2.5.2.). The results were analysed by the 2100 Expert Software and visual output confirmed that the isolated total small RNA (including miRNA) bands peaked between 25-200nt (Figure 3-4, A-E). Total serum RNA concentration for non-osteoporosis (control) was of 915 pg/µL with highest fluorescence band peaked of 20 [FU] between 25-200 nucleotide [nt] (Figure

3-4, A), 972 pg/µL for osteopenia (Figure 3-4, B), 1,068 pg/µL for osteopenia with fracture (Figure 3-4, C), 1,006 pg/µL for osteoporosis (Figure 3-4, D) and 1,505 pg/µL for osteoporosis with fracture.

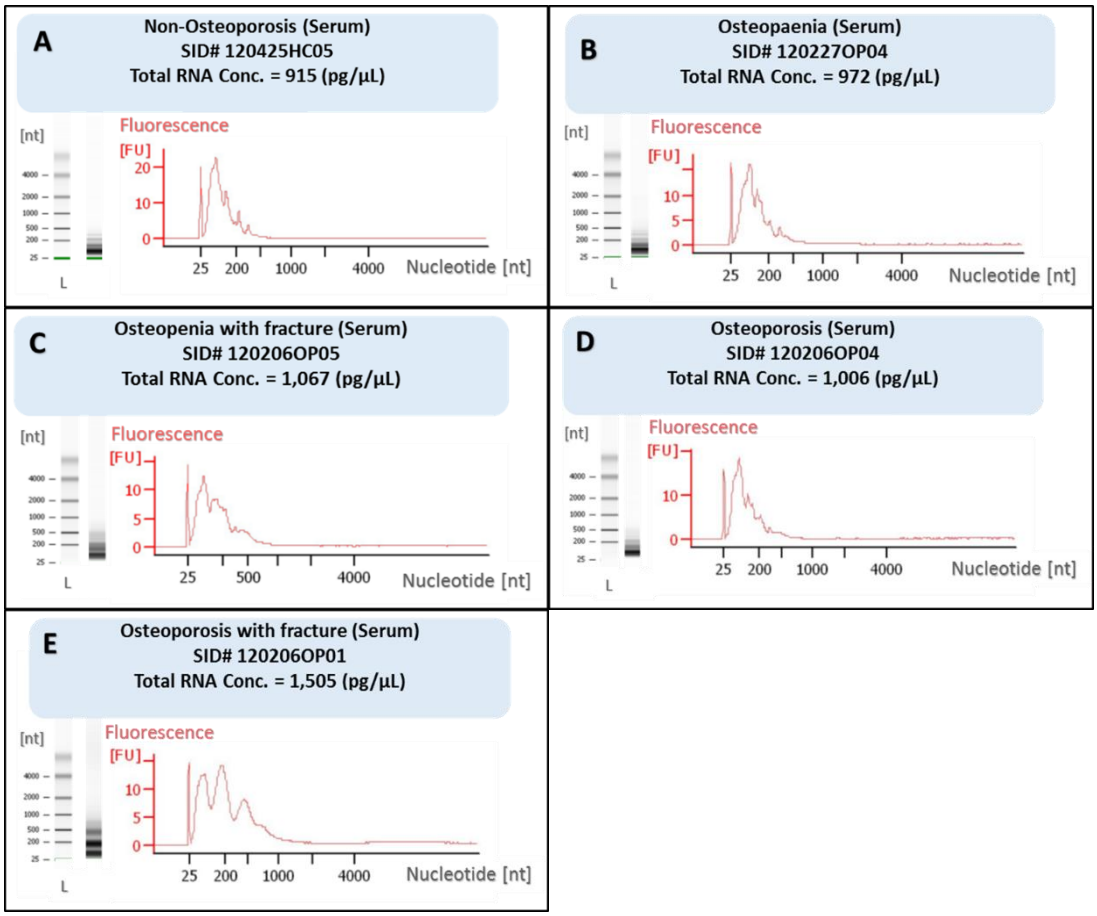


Figure 3-4: Sample of quality checks of total RNA analysis by Agilent 2100 Bioanalyser

The gel electropherogram images and fluorescence plots show the total RNA pattern performed on serum RNA samples from groups of Non-Osteoporosis (control), SID# 120425HC05(A), Osteopenia, SID# 120227OP04 (B), Osteopenia with Fracture, SID# 120206OP05 (C), Osteoporosis, SID# 120206OP04 (D) and Osteoporosis with Fracture, SID# 120206OP01 (E). FU= Fluorescence, RIN= RNA Integrity Number, Small RNA fractions (including miRNAs) are between 25-200 nucleotide (nt). The gel electropherogram images and fluorescence plots were generated using the 2100 Expert Software.

3.2.1.2 Establishment of RNA Pools for RNA Array Analysis

For cost-efficient sampling strategy, the first step toward the identification of circulating miRNA expression in osteoporosis patients was by the PCR array screening of 372 circulating miRNAs in four serum RNA pools: Non-Osteoporosis Male Pool1 (NOPMP1, 6 samples), Non-Osteoporosis Female Pool2 (NOPFP2, 4 samples), Osteopenia Female Pool3 (OPAFP3, 8 samples) and Osteoporosis Female Pool4 (OPFP4, 9 samples) (Materials and Methods 2.7.) (Table 3-3).

Table 3-3: Serum RNA Pool Concentration for QIAGEN miRNA PCR Array

Samples	Sample ID	RNA-conc. (ng/µL)	Volume [µL] (50ng/ Sample)	Total RNA [ng]/Pool
Non-Osteoporosis, MalePool 1 [NOPMP1]	120425HC04 S1a	9.8	5.1	
	121212HC02 S1a	12.5	4.0	
	121212HC03 S1a	14.0	3.6	
	130718HC02 S1a	14.8	3.4	
	130930HC02 S1b	12.6	4.0	
	131001HC01 S1a	11.2	4.5	
NOPMP1, Total	6		24.5 µL	300 ng
Non-Osteoporosis, FemalePool 2 [NOPFP2] *	120425HC01 S1a	42.5	2.4	
	120425HC08 S1a	11.1	9.0	
	120425HC14 S1a	18.7	5.3	
	130718HC01 S1a	15.2	6.6	
NOPFP2, Total*	4*		23.3 µL	400 ng*
Osteopenia, Female Pool 3 [OPAFP3]	120312OP01 S1a	11.22	4.5	
	120315OP04 S1a	13.6	3.7	
	120320OP01 S1a	29.69	1.7	
	120416OP01 S1a	27.4	1.8	
	120416OP02 S1a	18.4	2.7	
	120417OP02 S1a	21.9	2.3	

Samples	Sample ID	RNA-conc. (ng/µL)	Volume [µL] (50ng/ Sample)	Total RNA [ng]/Pool
	120508OP02 S1a	18.5	2.7	
	121030OP01 S1a	22.8	2.2	
OPAFP3, Total	8		21.5 µL	400 ng
Osteoporosis, Female Pool 4 [OPFP4]	130718HC01 S1a	5.0	10.0	
	120312OP01 S1a	8.4	6.0	
	120315OP04 S1a	15.8	3.2	
	120320OP01 S1a	4.7	10.6	
	120416OP01 S1a	9.0	5.6	
	120416OP02 S1a	10.0	5.0	
	120417OP02 S1a	9.48	5.3	
	120508OP02 S1a	24.2	2.1	
	121030OP01 S1a	10.2	4.9	
OPFP4, Total	9		52.6 µL	450 ng

*(100ng/Sample)

RNA pools were checked for RNA quality and yields (Materials and Methods 2.7.). The RNA purity at 260nm/280nm was > 1.4 and the concentration between 167 to 473 ng/pool, which is reasonably acceptable for serum or plasma extracted samples (Table 3-4). In addition, miRNA concentration and ratio analysis to the small RNA was carried out using Agilent 2100 expert software (Figure 3-5). Individual serum miRNAs pool concentration was between 390 and 515 pg/µL (67%-80%) (Table 3-4), with miRNA intense peak at 35 nt as shown in the electropherogram (Figure 3-5).

Table 3-4: Four Serum RNA Pools concentration and integrity by Nanodrop™ 2000 spectrophotometer, and miRNA concentration by Agilent 2100 Bioanalyser.

Sample ID	Name	RNA Conc. (ng/pool)	RNA Optical Density 260/280 Ratio	RNA Optical Density 260/230 Ratio	miRNA%	miRNA conc. (pg/µL)
NOPMP1	Non-Osteoporosis, Male	363.3	1.45	0.13	80%	514.9
NOPFP2	Non-Osteoporosis, Female	169.6	1.56	0.09	78%	517.8
OPAFP3	Osteopenia, Female	447.8	1.45	0.25	82%	964.2
OPFP4	Osteoporosis, Female	472.5	1.72	0.17	67%	391.8

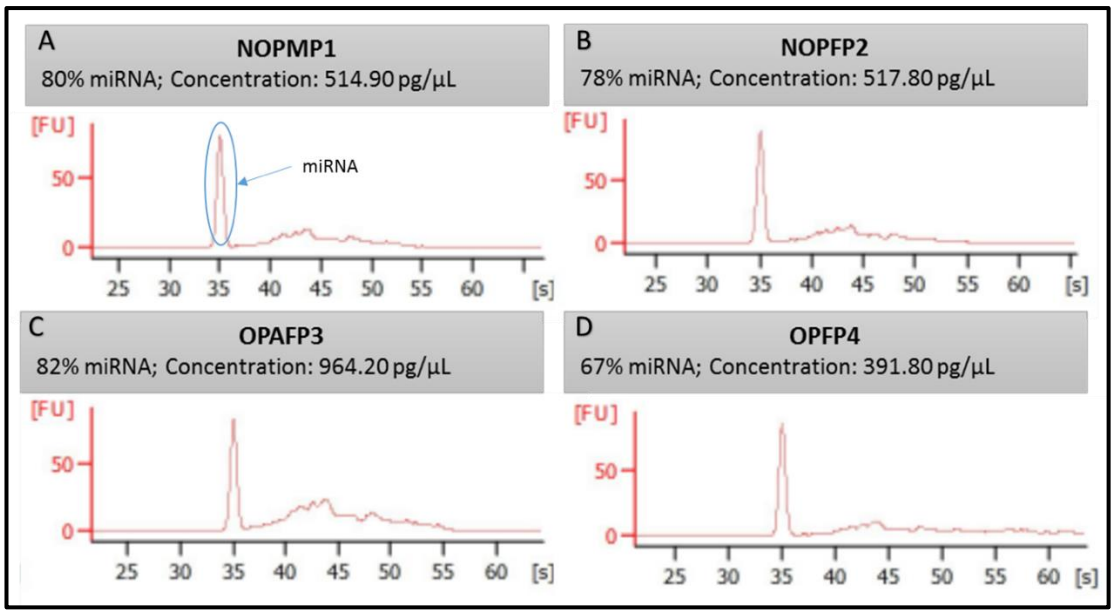


Figure 3-5: Electropherograms showing RNA Quality Concentration & Integrity for four Serum RNA Pools.

NOPMP1= Non-osteoporosis, male Pool1 (A), NOPFP2 = Non-osteoporosis female Pool2 (B), OPAFP3 = Osteopenia, female, Pool3 (C) and OPFP4 = Osteoporosis, female, Pool4(D) with miRNA peak displayed at 35nt. Analysis done using Agilent 2100 Bioanalyser.

3.2.2 miRNA PCR Array Data Analysis

Extracted serum-RNAs pools were reverse transcribed to cDNA. RT-qPCR was performed in a single mode run (Materials and Methods 2.7.). Ct data for miRNA, was generated by qPCR, and delivered as an Excel Workbook (Ct_Values.xls) (Materials and Methods 2.8.). The efficiency of the polymerase chain reaction and the reverse transcription reaction, was determined by using positive PCR controls and reverse transcription control (Materials and Methods 2.7.). The difference between the average Ct reverse transcription control (RTC) and the average PCR positive control (PPC) was less than zero (Table 3-5). Therefore, all samples had successful PCR and efficient reverse transcription reactions.

Table 3-5: Quality Check of PCR Array Data

Array	NOPFP1	OPAFP3	OPFP4	NOPMP2
Average Ct (PPC)	18.54	18.54	18.53	18.53
ST DEV Ct (PPC)	0.02	0.02	0.11	0.06
Average Ct (RTC)	16.4	16.53	16.3	16.81
ST DEV Ct (RTC)	0.03	0.04	0.07	0.1
ΔCt (AVG RTC - AVG PPC)	-2.14	-2.01	-2.23	-1.72
RT Efficiency	Pass	Pass	Pass	Pass

Criteria: If ΔCt (AVG RTC - AVG PPC) <= 0, RT Efficiency reports 'Pass'; otherwise, RT Efficiency reports 'Inquiry'. **PPC**= PCR Positive Control, **RTC** = Reverse Transcription Control, **RT** = Reverse Transcription, **NOPFP1** = Non-Osteoporosis, Female Pool1, **OPAFP3** = Osteopenia, Female Pool3, **OPFP4** = Osteoporosis, Female Pool4 and **NOPMP2** = Non-osteoporosis, Male Pool2.

3.2.2.1 Ct Values Data Distribution Assessment

A preliminary evaluation study for the Ct value showed that out of 384 miRNAs and PCR controls, approximately 48% of the distributed Ct values were below 25, 46% between 25-30, and the remaining 5% had late Ct amplification (30-35) or were undetectable (Table 3-6).

Table 3-6: Distribution of Ct Values

Ct Range	NOPFP1 Distribution of Ct Values (%)	NOPMP2 Distribution of Ct Values (%)	OPAFP3 Distribution of Ct Values (%)	OPFP4 Distribution of Ct Values (%)
<25	188 (48.96%)	180 (46.88%)	201 (52.34%)	170 (44.27%)
25-30	174 (45.31%)	184 (47.92%)	161 (41.93%)	195 (50.78%)
30-35	22 (5.73%)	20 (5.21%)	22 (5.73%)	19 (4.95%)
Absent Calls	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)

NOPF = Non-Osteoporosis, Female Pool1 (Control), NOPMP2 = Non-Osteoporosis, Male Pool2 (Control), OPAFP3= Osteopenia, Female Pool3 andOPFP4=Osteoporosis, Female Pool4 and.

3.2.2.2 Establishment of PCR Array Threshold Cycle Cut off

The threshold Ct cut off value for the preamplified miRNAs was set up to 30 cycles. Those miRNAs with Ct values greater than 30 or displayed as (N/A) were reported as 30 and considered as not expressed.

Out of 372 tested miRNAs, there were 21 targets (hsa-miR-184, hsa-miR-372-3p, hsa-miR-19b-1-5p, hsa-miR-1286, hsa-miR-138-1-3p, hsa-miR-3655, hsa-miR-373-3p, hsa-miR-605-5p, hsa-miR-412-3p, hsa-miR-4267, hsa-miR-133a-3p, hsa-miR-4258, hsa-miR-208a-3p, hsa-miR-9-5p, hsa-miR-3689e, hsa-miR-4524a-3p, hsa-miR-2355-5p, hsa-miR-136-5p, hsa-miR-196b-3p, hsa-miR-1277-3p and hsa-miR-2467-3p) that were not expressed and were excluded from the study. The remaining, 185 (48%) gave valid Ct value of <25 and 178 (46%) with Ct value between 25-30

3.2.2.3 Selection of Normalization/Housekeeping Gene

To select the best normalization control for the miRNA expression in the serum, fourteen miRNAs and small nucleolar RNAs (snoRNAs) with no significant Ct value mean difference (≤ 2 cycle) between the four serum RNA pools were identified. These are 7 miRNAs (hsa-miR-152-3p, hsa-miR-335-5p, hsa-miR-29b-3p, hsa-miR-185-5p, hsa-miR-24-3p, hsa-miR-20b-5p and hsa-miR-20a-5p), and six PCR normalization/housekeeping genes (SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A and RNU6-2) as well as one Spike-in control (cel-miR-39) as shown in (Figure 3-6).

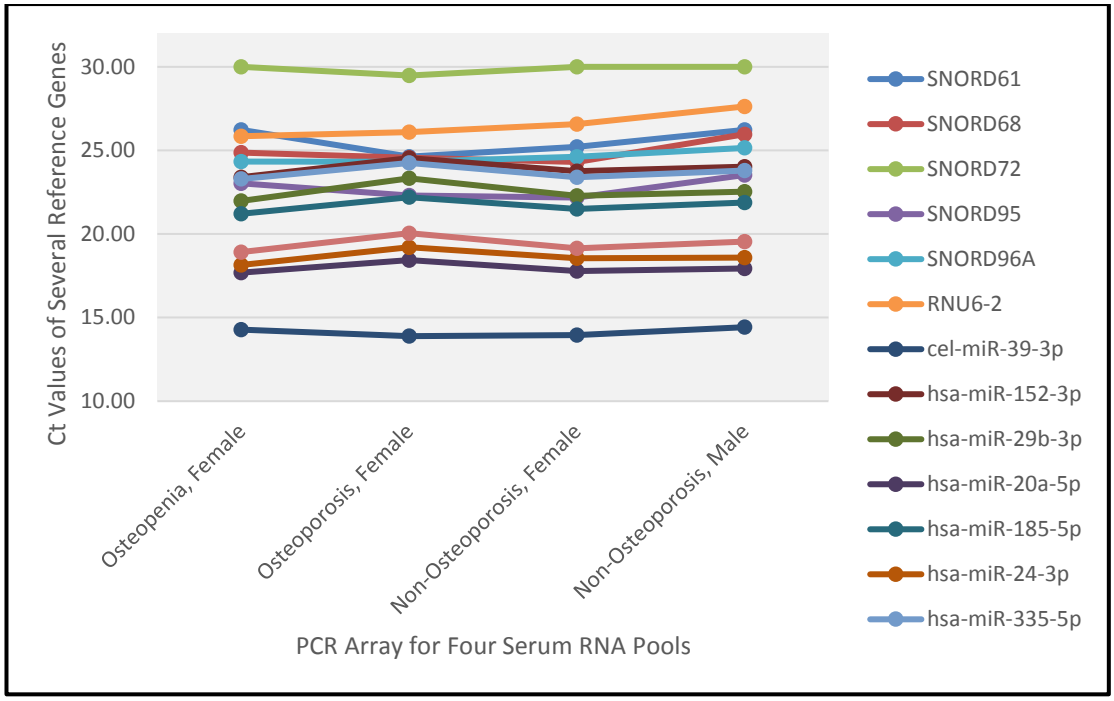


Figure 3-6: PCR array's Ct values result from four serum RNA Pools for 14 Genes. Circulating serum of: 7 miRNAs; hsa-miR-152-3p, hsa-miR-19b-3p, hsa-miR-20a-5p, hsa-miR-185-5p, hsa-miR-24-3p, hsa-miR-335-5p, hsa-miR-20b-5p, 1 spike-in Control, cel-miR-39 and 6 small non-coding RNA (normalization Controls); SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A & RNU6-2. Dots denote the Ct value of each miRNA per group.

Following identification of the RNAs to be used for normalization, the relative expression ($2^{-\Delta Ct}$) of miRNA of interest between Osteoporosis and Osteopenia female groups were measured using different combination of normalization controls as listed in (Table 3-7): two housekeeping genes (HKGs), SNORD96A and RNU6-2; three HKGs, SNORD68, SNORD96A and RNU6-2; four HKGs, SNORD68, SNORD95, SNORD96A and RNU6-2; 6 HKGs, SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A and RNU6-2+ Cel-miR-39 and a set of 7 miRNAs-control group, hsa-miR-152-3p, hsa-miR-29b-3p, hsa-miR-20a-5p, hsa-miR-185-5p, hsa-miR-24-3p, hsa-miR-335-5p, hsa-miR-20b-5p and hsa-miR-18a-5p. miRNAs relative expression ($2^{-\Delta Ct}$) of ≥ 2-fold change upregulation are displayed in red colour, while those downregulated by ≤ -2-fold change are displayed in blue (Table 3-7).

Table 3-7: miRNA Fold Change Expression between Osteoporosis and Osteopenia female groups using different combination of normalization controls.

No.	Mature ID	2HKGs	3HKGs	4HKGs	6HKGs+ Cel-miR-39	miRNAs-Control Group
1	hsa-miR-3923	**4.1**	**3.7**	**3.3**	**2.8**	**7.8**
2	hsa-miR-4258	**4.0**	**3.6**	**3.2**	**2.7**	**7.6**
3	hsa-miR-485-5p	**2.4**	**2.2**	**1.9**	**1.6**	**4.5**
4	hsa-miR-196b-3p	**2.8**	**2.5**	**2.2**	**1.9**	**5.3**
5	hsa-miR-1193	**2.4**	**2.1**	1.9	1.6	**4.5**
6	hsa-miR-1281	**2.3**	**2.1**	1.9	1.6	**4.4**
7	hsa-miR-4274	**2.3**	**2.0**	1.8	1.5	**4.3**
8	hsa-miR-100-5p	**-32.1**	**-35.4**	**-40.0**	**-47.9**	**-16.9**
9	hsa-miR-4516	**-9.1**	**-10.0**	**-11.3**	**-13.6**	**-4.8**
10	hsa-miR-145-3p	**-8.8**	**-9.7**	**-10.9**	**-13.1**	**-4.6**
11	hsa-miR-4306	**-7.5**	**-8.3**	**-9.4**	**-11.3**	**-4.0**
12	hsa-miR-548e-3p	**-6.4**	**-7.0**	**-8.0**	**-9.5**	**-3.4**
13	hsa-miR-206	**-6.3**	**-7.0**	**-7.9**	**-9.5**	**-3.3**
14	hsa-miR-215-5p	**-5.8**	**-6.4**	**-7.2**	**-8.6**	**-3.1**
15	hsa-miR-122-5p	**-5.7**	**-6.3**	**-7.1**	**-8.5**	**-3.0**
16	hsa-miR-3911	**-5.2**	**-5.7**	**-6.4**	**-7.7**	**-2.7**
17	hsa-miR-548d-5p	**-4.7**	**-5.2**	**-5.8**	**-7.0**	**-2.5**
18	hsa-miR-373-5p	**-4.5**	**-4.9**	**-5.6**	**-6.7**	**-2.4**
19	hsa-miR-99a-5p	**-4.4**	**-4.9**	**-5.5**	**-6.6**	**-2.3**
20	hsa-miR-450a-5p	**-4.2**	**-4.6**	**-5.2**	**-6.2**	**-2.2**
21	hsa-miR-143-3p	**-4.0**	**-4.5**	**-5.0**	**-6.0**	**-2.1**
22	hsa-miR-375	**-3.9**	**-4.3**	**-4.9**	**-5.8**	**-2.1**

2HKGs: Two housekeeping genes (SNORD96A and RNU6-2)**, 3HKGS;** SNORD68, SNORD96A and RNU6-2**, 4HKGs;** SNORD68, SNORD95, SNORD96A and RNU6-2, **6HKGs;** SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A and RNU6-2**, Spike-in**

control: cel-miR-39 **and miRNAs-Control Group;** hsa-miR-152-3p, hsa-miR-29b-3p, hsa-miR-20a-5p, hsa-miR-185-5p, hsa-miR-24-3p, hsa-miR-335-5p, hsa-miR-20b-5p and hsa-miR-18a-5p**. Red =** ≥ 2-fold change upregulated**; blue =** ≤ -2-fold change Downregulated; **black** = <2 fold-change >-2(no significant up-/down-regulation).

Two PCR control genes; SNORD96A and RNU6-2 had Ct mean value of 24.6 ± 0.4 and

25.6 ± 0.6 respectively, and the combined Ct means value are 25±0.5 (Figure 3-7).

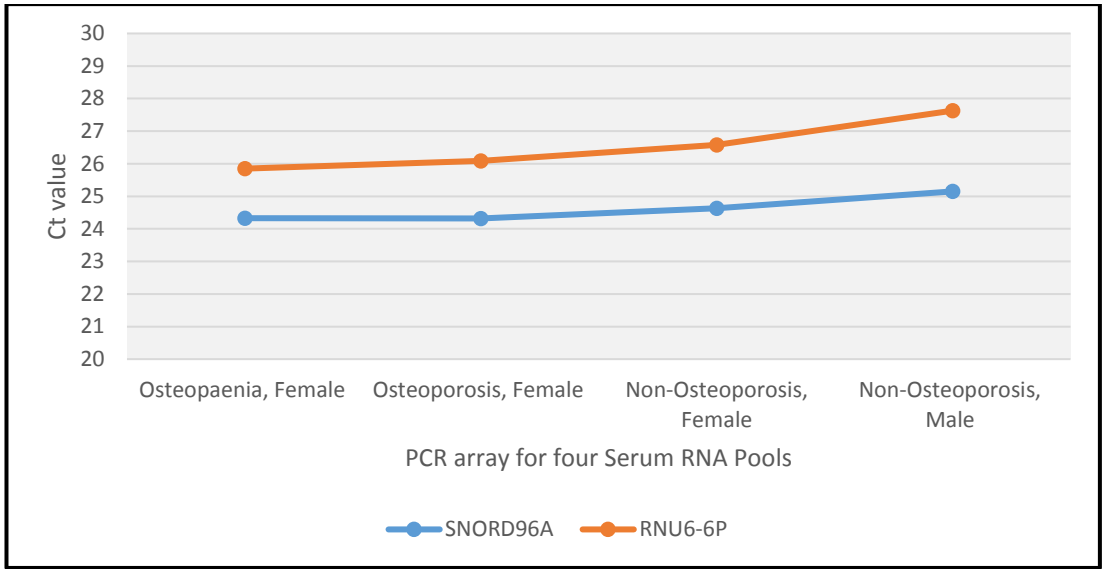


Figure 3-7: PCR array's Ct values result from four serum RNA Pools for SNORD96A & RNU6-2 (normalization housekeeping genes).
Dots denote the Ct value of each miRNA per group.

These two genes were selected as normalization controls for miRNA PCR fold change

($2^{-\Delta\Delta CT}$) expression based on their consistent Ct reading among the control and study

groups (Table 3-8), as well as acceptable average delta Ct expression ($2^{-\Delta Ct}$).

Table 3-8: SNORD96A & RNU6-2 (Housekeeping/Normalization Control) Ct value expression in four serum RNA Pools.

Gene Symbol	Normalization Control	NOPMP1 (Control)	NOFPF2 (Control)	OPAFP3 (Group 1)	OPFP4 (Group 2)
SNORD96A	☑	25.15	24.63	24.33	24.32
RNU6-6P	☑	27.63	26.58	25.85	26.09
Mean		26.39	25.61	25.09	25.20

NOPMP1 = Non-Osteoporosis, Male Pool1 (Control), **NOPFP2** = Non-Osteoporosis, Female Pool2 (Control), **OPAFP3**= Osteopenia, Female Pool3 and **OPFP4**=Osteoporosis, Female Pool4.

3.2.2.4 Identify Differentially Expressed miRNAs from miRNA array

3.2.2.4.1 Identify Differentially Expressed miRNAs between Non-Osteoporosis Male and Non-Osteoporosis Female using miRNA PCR array

A differential miRNAs expression analysis between non-osteoporosis male and female serum RNA pools was performed using QIAGEN miRNA PCR Array Data Analysis Web Portal software (Materials and Methods 2.8.). When the data was normalised using two normalising genes; SNORD96A and RNU6-2, out of 372 circulating miRNAs 91 (24%) were upregulated (≥2-fold change) in the non-osteoporosis male group, including hsa-miR-3120-3p (8.9-fold change) and hsa-miR-34c-3p (6.9-fold change). Four miRNAs were downregulated (2.4-fold change) including hsa-miR-328-3p and hsa-miR-122-5p (Figure 3-8).

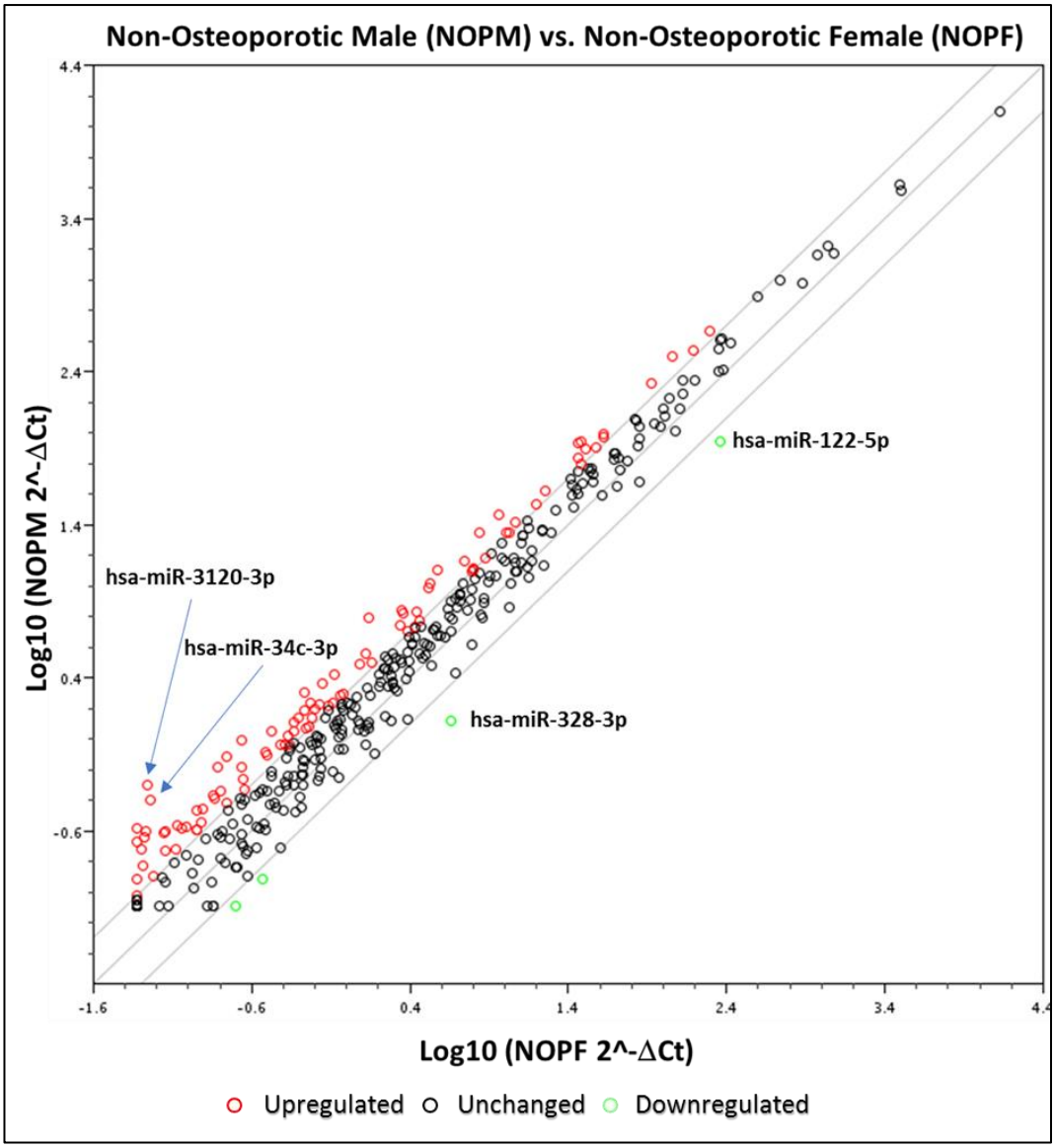


Figure 3-8 Scatter Plot of miRNA expression (normalized groups) between Non-Osteoporosis, Male group (NOPM) vs Non-Osteoporosis, Female group (NOPF).
Log10 transformed data of all miRNAs expression ($2^{-\Delta Ct}$) in the miScript PCR Array were taken to construct this scatter plot. The middle line indicates relative fold change ($2^{-\Delta Ct}$) of 1. The left and right lines indicate the fold change in gene expression threshold, which was defined as 2-fold. miRNAs upregulated with ≥ 2-fold change are depicted in Red circle and those downregulated with ≤ -2-fold change are in Green circle by miScript PCR array.

Listed in Table 3-9 summarises the most differentially expressed miRNAs in the non-osteoporosis, male group compared to non-osteoporosis, female group, eight miRNAs: were significantly upregulated by ≥ 4-fold change (highlighted in Red) and

three miRNAs were downregulated by ≤-2.4-fold change (highlighted in Blue): hsa-miR-328-3p, hsa-miR-122-5p and hsa-let-7g-3p.

The sex of participants could have an influence on the dysregulation expression of circulating miRNAs (Dai and Ahmed, 2014, Guo et al., 2017, Ameling et al., 2015). Therefore, the non-osteoporosis male control group was excluded from further study.

Table 3-9: Differentially-expressed miRNA between Male and Female Non-Osteoporosis groups, using the QIAGEN Human Serum & Plasma miRNA PCR Array.
Red = ≥ 2-fold change upregulated; **blue =** ≤ -2-fold change Downregulated

No.	Mature ID	Fold Regulation
1.	hsa-miR-3120-3p	**8.9**
2.	hsa-miR-34c-3p	**6.9**
3.	hsa-miR-15a-3p	**4.6**
4.	hsa-miR-485-3p	**4.5**
5.	hsa-miR-1-3p	**4.5**
6.	hsa-miR-139-3p	**4.3**
7.	hsa-miR-138-1-3p	**4.3**
8.	hsa-miR-206	**4.3**
9.	hsa-miR-328-3p	**-3.5**
10.	hsa-miR-122-5p	**-2.6**
11.	hsa-let-7g-3p	**-2.4**

3.2.2.4.2 Identify Differentially Expressed miRNAs Between Osteopenia, Female and Non-Osteoporosis, Female using miRNA PCR array

Twenty nine miRNAs (8%) were dysregulated in osteopenia, female pool compared to non-osteoporosis female group. 7 miRNAs (2%) were upregulated by (≥2.0 fold change) including hsa-miR-3120-3p (≥3.1 fold change) and hsa-miR-4516 (≥2.7 fold change (Figure 3-9), and 22 (6%) were downregulated (≤-2.0 fold change) including

some which exhibited large differential expression, namely, hsa-miR-3622a-5p (≤-4.3 fold change) and hsa-miR-122-5p (≤-4.2 fold change).

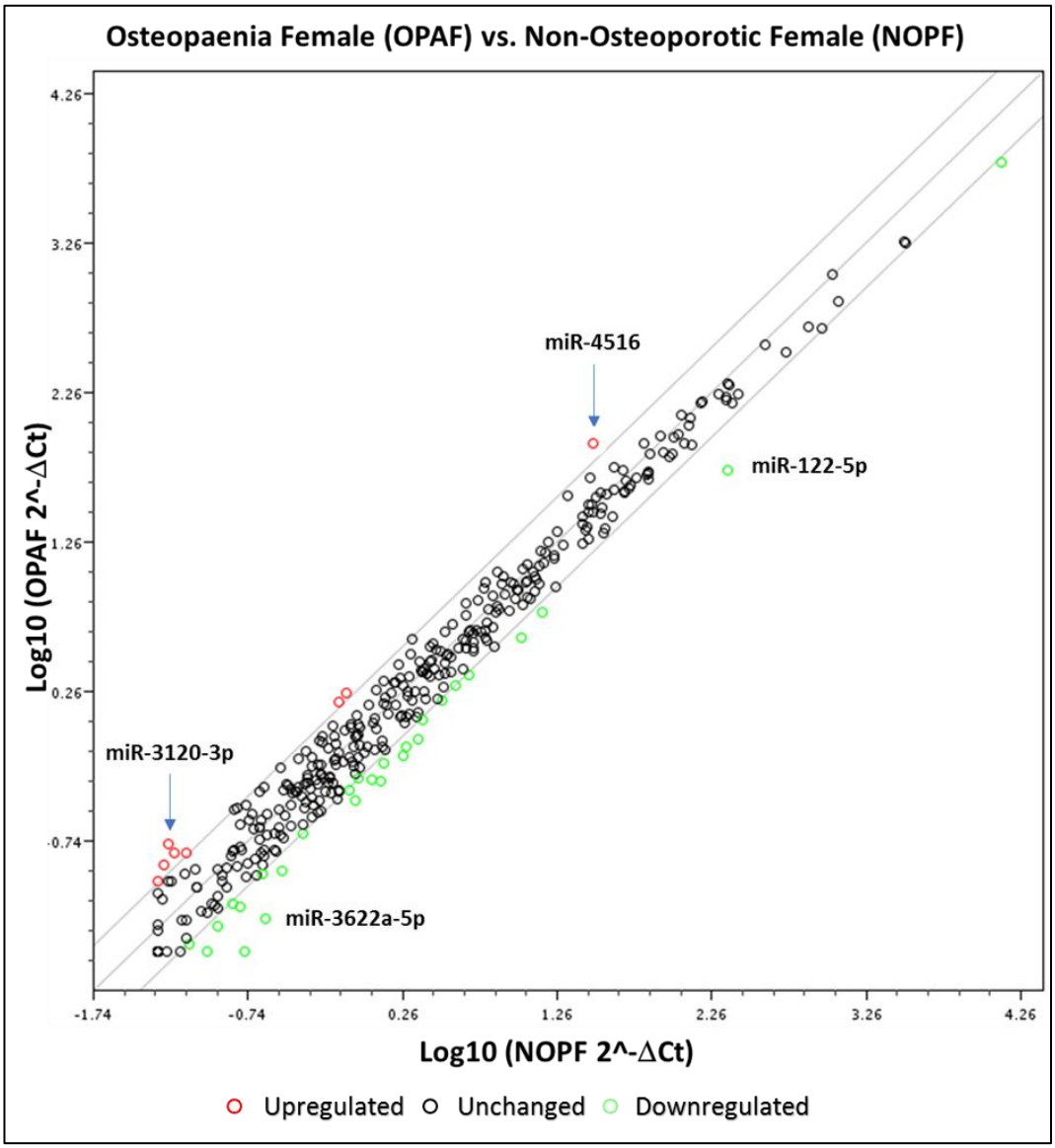


Figure 3-9: Scatter Plot of miRNA expression (normalized groups) between Osteopenia, female group (OPAF) vs Non-Osteoporotic, female group (NOPF).
Log10 transformed data of all miRNAs expression ($2^{-\Delta Ct}$) in the miScript PCR Array were taken to construct this scatter plot. The middle line indicates relative fold change ($2^{-\Delta Ct}$) of 1. The left and right lines indicate the fold change in gene expression threshold, which was defined as 2-fold. miRNAs upregulated with >2-fold change are depicted in Red circle and those downregulated with < -2-fold change are in Green circle.

Listed in Table 3-10 are the most differentially expressed miRNAs in osteopenia, female group compared to the non-osteoporosis female group. five were upregulated (> 2.0-fold change) (highlighted in red) and eight miRNAs were downregulated by <-2.5-fold change (highlighted in blue).

Table 3-10: Differentially-expressed miRNA in Osteopenia, Female Group Compared with Non-Osteoporosis, Female Group using the QIAGEN Human Serum & Plasma miRNA PCR Array.

No.	Mature ID	Fold Regulation
1.	hsa-miR-3120-3p	**3.1**
2.	hsa-miR-4516	**2.7**
3.	hsa-miR-542-5p	**2.5**
4.	hsa-miR-21-3p	**2.4**
5.	hsa-miR-3651	**2.3**
6.	hsa-miR-3622a-5p	**-4.3**
7.	hsa-miR-122-5p	**-4.2**
8.	hsa-miR-4732-3p	**-2.7**
9.	hsa-miR-1976	**-2.7**
10.	hsa-miR-203a-3p	**-2.6**
11.	hsa-miR-1281	**-2.6**
12.	hsa-miR-32-5p	**-2.5**
13.	hsa-miR-1913	**-2.5**

Red = ≥ 2-fold change upregulated**; blue =** ≤ -2-fold change Downregulated

3.2.2.4.3 Identify Differentially Expressed miRNAs between Osteoporosis and Non-Osteoporosis using miRNA PCR array

Sixty miRNAs (16%) were downregulated (≤ -2-fold change) in the osteoporosis group compared to non-osteoporotic, female group, including hsa-miR-122-5p (-24-fold change), hsa-miR-100-5p (-38-fold change), hsa-miR-215-5p (-10-fold change) and hsa-miR-3911 (-8-fold change). Two miRNAs were upregulated (≥ 2-fold change) (hsa-miR-21-3 and hsa-miR-1231) as shown in (Figure 3-10).

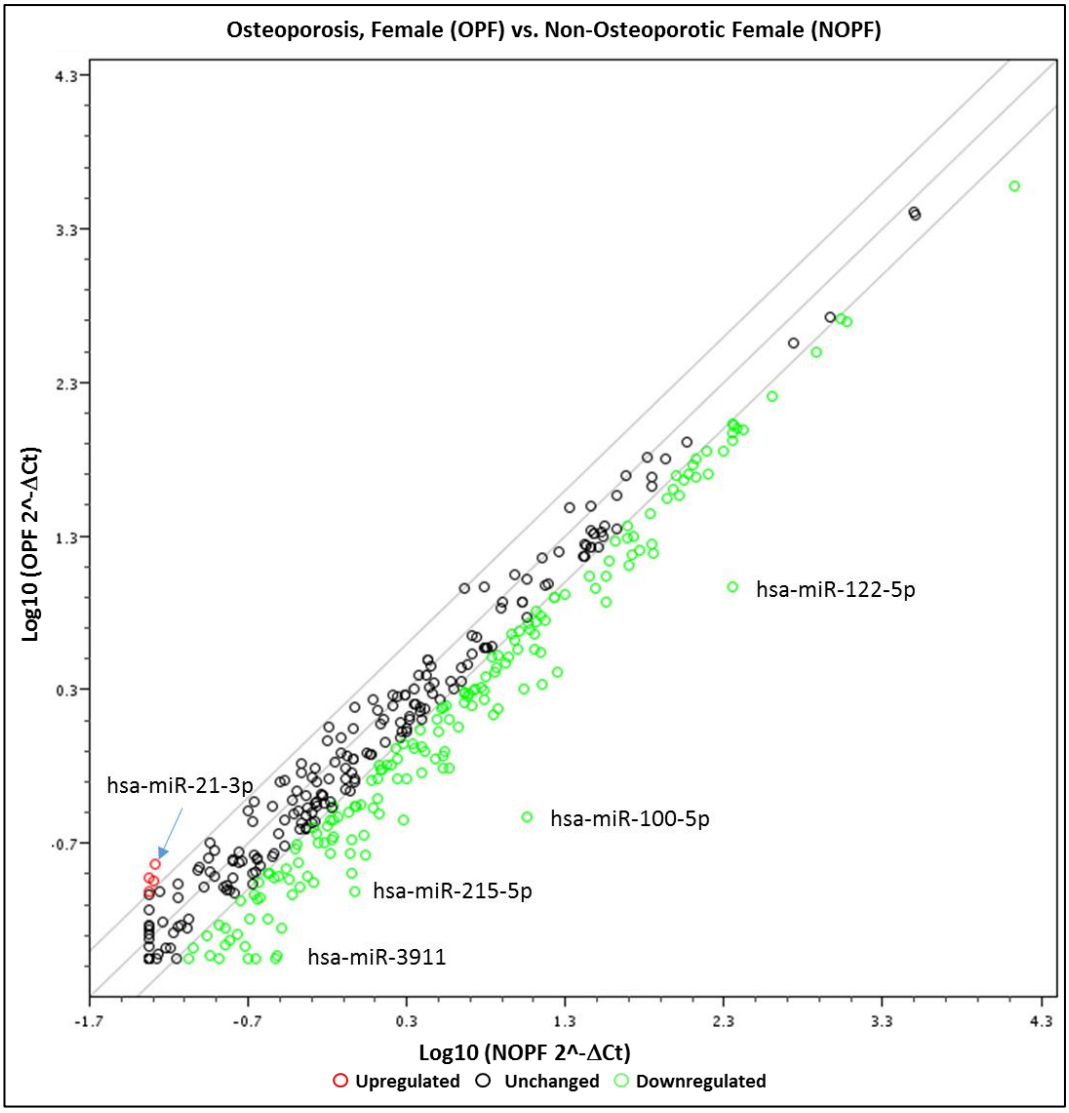


Figure 3-10: Scatter Plot of miRNA expression (normalized groups) between Osteoporosis, female group (OPF) vs Non-Osteoporotic, female group (NOPF).
Log10 transformed data of all miRNAs expression ($2^{-\Delta Ct}$) in the miScript PCR Array were taken to construct this scatter plot. The middle line indicates relative fold change ($2^{-\Delta Ct}$) of 1. The left and right lines indicate the fold change in gene expression threshold, which was defined as 2-fold. miRNAs upregulated with >2-fold change are depicted in Red circle and those downregulated with < -2-fold change are in Green circle.

Listed in Table 3-11 are the most differentially expressed miRNAs in the osteoporosis group compared to non-Osteoporosis, Female group. Two miRNAs: hsa-miR-21-3p and hsa-miR-1231 were significantly upregulated by > 2-fold change (highlighted in red) and sixteen miRNAs were downregulated by <-2-fold change (highlighted in blue): hsa-miR-100-5p, hsa-miR-122-5p, hsa-miR-215-5p, hsa-miR-3911, hsa-miR-

1290, hsa-miR-32-5p, hsa-miR-194-5p, hsa-miR-145-3p, hsa-let-7a-3p, hsa-miR-4306, hsa-miR-10b-5p, hsa-miR-365b-3p, hsa-miR-200b-3p and hsa-miR-99a-5p.

Table 3-11: Differentially-expressed miRNA in Osteoporosis, Female Group Compared with Non-Osteoporosis, Female Group using the QIAGEN Human Serum & Plasma miRNA PCR Array.

Red = ≥ 2-fold change upregulated**; blue =** ≤ -2-fold change Downregulated

No.	Mature ID	Fold Change
1.	hsa-miR-21-3p	**2.8**
2.	hsa-miR-1231	**2.3**
3.	hsa-miR-100-5p	**-38.3**
4.	hsa-miR-122-5p	**-24.1**
5.	hsa-miR-215-5p	**-9.8**
6.	hsa-miR-3911	**-8.3**
7.	hsa-miR-1290	**-6.9**
8.	hsa-miR-32-5p	**-6.8**
9.	hsa-miR-194-5p	**-6.8**
10.	hsa-miR-192-5p	**-6.7**
11.	hsa-miR-145-3p	**-6.5**
12.	hsa-let-7a-3p	**-6.1**
13.	hsa-miR-4306	**-5.8**
14.	hsa-miR-10b-5p	**-5.5**
15.	hsa-miR-365b-3p	**-5.4**
16.	hsa-miR-200b-3p	**-5.1**
17.	hsa-miR-16-2-3p	**-5.1**
18.	hsa-miR-99a-5p	**-5.0**

3.2.2.4.4 Identify differentially expressed miRNAs Between Osteoporosis and Osteopenia using miRNA PCR array

45 (12%) circulating miRNAs were downregulated (≤ -2-fold change) in the osteoporosis group compared to the osteopenia group (Figure 3-11), and 8 (2%) were upregulated (≥ 2-fold change).

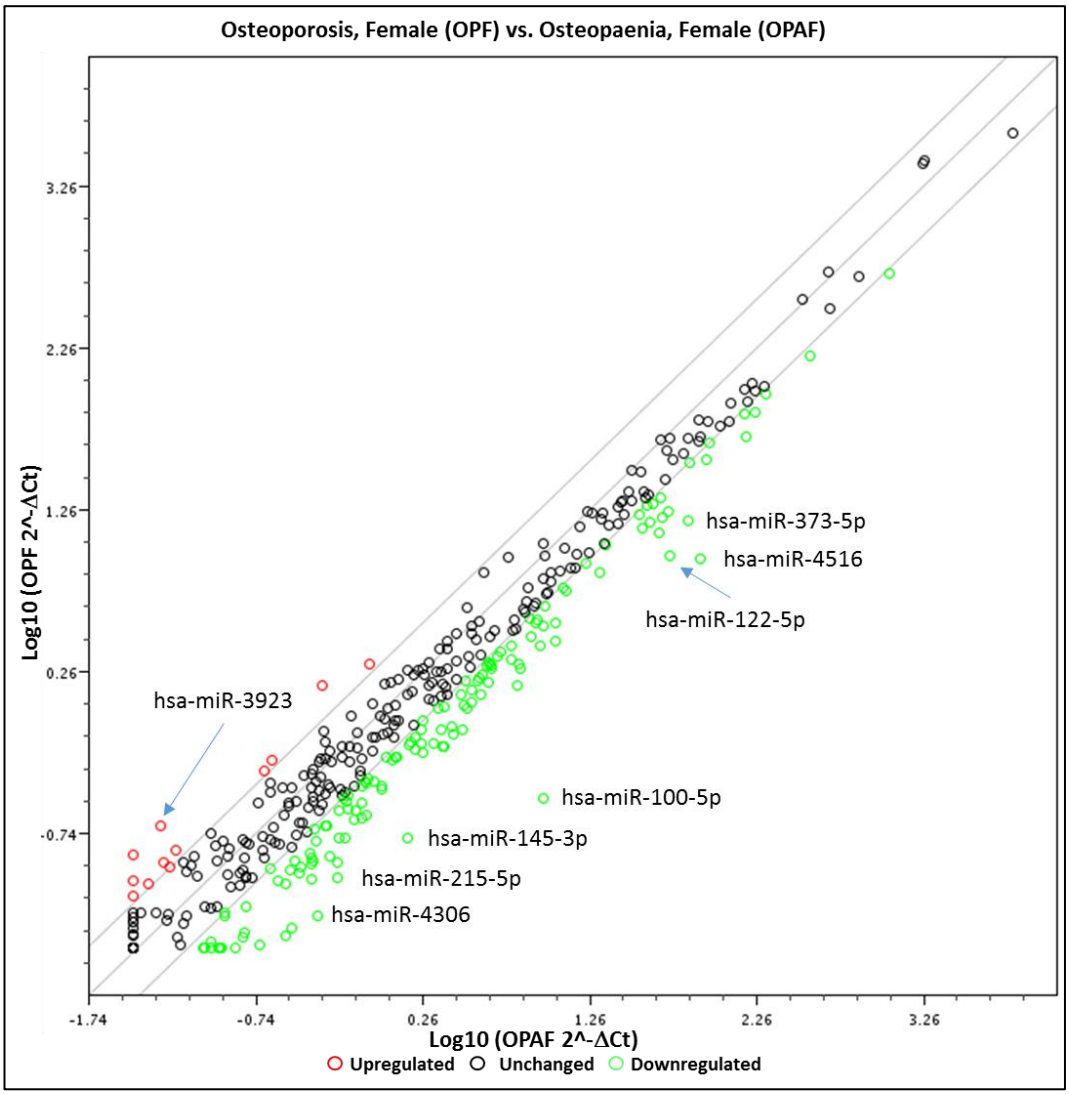


Figure 3-11: Scatter Plot of miRNA expression between Osteoporosis, female group (OPF) vs Osteopenia, Female group (OPAF).

Log10 transformed data of all miRNAs expression ($2^{-\Delta Ct}$) in the miScript PCR Array were taken to construct this scatter plot. The middle line indicates relative fold change ($2^{-\Delta Ct}$) of 1. The left and right lines indicate the fold change in gene expression threshold, which was defined as 2-fold. miRNAs upregulated with ≥ 2-fold change are depicted in Red circle and those downregulated with ≤ -2-fold change are in Green circle by miScript PCR array.

Among them, 24 miRNAs were significantly expressed between osteoporosis and osteopenia groups: 8 were upregulated: hsa-miR-1193, hsa-miR-1281, hsa-miR-3923, hsa-miR-485-5p, hsa-miR-4274, hsa-miR-4258, SNORD61 and hsa-miR-196b-3p (≥ 2-fold, Red in display) and 16 were downregulated: hsa-miR-100-5p, hsa-miR-4516, hsa-miR-145-3p, hsa-miR-4306, hsa-miR-548e-3p, hsa-miR-206, hsa-miR-215-5p, hsa-miR-122-5p, hsa-miR-3911, hsa-miR-548d-5p, hsa-miR-373-5p, hsa-miR-99a-5p, hsa-miR-375, hsa-miR-450a-5p and hsa-miR-143-3p (≤ -3-fold change, Blue in display) as shown in (Table 3-12) were selected for further individual RT-qPCR screening in the low BMD (Osteoporosis, & Osteopenia) and normal BMD (non-osteoporosis) subjects.

Table 3-12: Differentially-expressed miRNA in Osteoporosis, Female Group Compared with Osteopenia, Female Group using the QIAGEN Human Serum & Plasma miRNA PCR Array.

Red = ≥ 2-fold change upregulated**; blue =** ≤ -2-fold change Downregulated

No.	Mature ID	Fold Change
1.	hsa-miR-3923	**4.1**
2.	hsa-miR-4258	**4.0**
3.	SNORD61	**3.3**
4.	hsa-miR-196b-3p	**2.8**
5.	hsa-miR-485-5p	**2.8**
6.	hsa-miR-1193	**2.4**
7.	hsa-miR-2467-3p	**2.4**
8.	hsa-miR-1281	**2.3**
9.	hsa-miR-4274	**2.3**
10.	hsa-miR-100-5p	**-32.1**
11.	hsa-miR-4516	**-9.1**
12.	hsa-miR-145-3p	**-8.8**
13.	hsa-miR-4306	**-7.5**
14.	hsa-miR-548e-3p	**-6.4**
15.	hsa-miR-206	**-6.3**
16.	hsa-miR-215-5p	**-5.8**
17.	hsa-miR-122-5p	**-5.7**

No.	Mature ID	Fold Change
18.	hsa-miR-3911	**-5.2**
19.	hsa-miR-548d-5p	**-4.7**
20.	hsa-miR-373-5p	**-4.5**
21.	hsa-miR-99a-5p	**-4.4**
22.	hsa-miR-375	**-3.9**
23.	hsa-miR-450a-5p	**-4.2**
24.	hsa-miR-143-3p	**-4.0**
25.	hsa-miR-1290	**-3.6**

3.2.3 RT-qPCR initial screening

The expression level of twenty-four miRNAs identified by PCR array were screened on individual serum samples using RT-qPCR. 100ng RNA purified from individual serum samples including osteopenia and osteoporosis were reverse transcribed first, then RT-qPCR was performed in duplicate using QIAGEN miScript miRNA primers (Materials and Methods 2.6.2., Table 2-2), and obtained Ct values from each RT-qPCR were analysed using two-way ANOVA followed by Bonferroni' multiple comparisons test (Materials and Methods 2.9).

3.2.3.1 Establish optimized condition for RT-qPCR

Firstly, to get the appropriate cDNA concentration for individual miRNA expression, the optimisation process started on circulating serum hsa-miR-100-5p, which was the most significantly downregulated miRNA in the miRNA PCR array (≤-32 fold change), on three sets of cDNA diluted samples: 1:10 (10ng), 1:5 (50ng) and 1:3 (33ng) as shown in Figure 3-12.

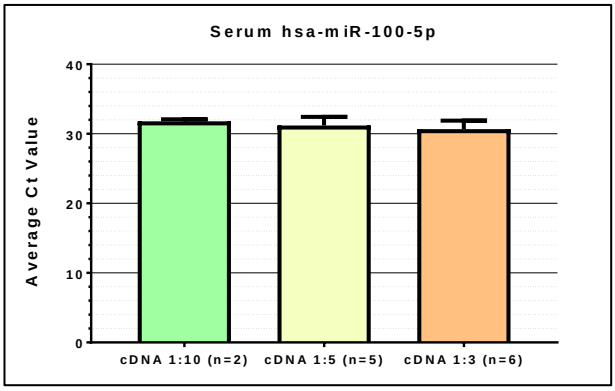


Figure 3-12: Validation of hsa-miR-100-5p Ct value expression between cDNA samples diluted 1:10, 1:5 and 1:3.
miRNA expression of hsa-miRNA-100-5p diluted 1:10 (Green, Osteopenia n=2), diluted 1:5 (Yellow, osteopenia n=1 and osteoporosis n= 4) and diluted 1:3 (Orange, osteopenia n=2 and osteoporosis=4).

miRNA RT-qPCR Ct mean value for hsa-miR-100-5p (diluted 1:10 and 1:5 and 1:3) were ≥ 32 Ct. (Table 3-13). Out of three Osteoporosis with fracture one had a Ct value ≥40 in both dilutions (1:5 and 1:3), which indicate late RNA amplification or test was not detected (Figure 3-12).

The average miRNA RT-qPCR Ct mean for has-miR-100-5p (cDNA diluted 1:3) were lower than has-miR-100-5p (cDNA diluted 1:5) of average Ct mean difference of -1.4 (Table 3-13), but not when compared with has-miR-100-5p (cDNA diluted 1:10).

Table 3-13: miRNA RT-qPCR Ct mean value for circulating serum hsa-miR-100-5p in cDNA diluted 1:10, 1:5 and 1:3 samples.

100ng cDNA of osteopenia (with/without fracture) and, osteoporosis (with/without fracture) were diluted 1:10 (10ng), 1:5 (20ng) and 1:3 (33ng).

Clinical Category	**Specimen type (Total no.)**	**has-miR-100-5p**		
		(cDNA diluted 1:10) Ct Mean ± SD	(cDNA diluted 1:5) Ct Mean ± SD	(cDNA diluted 1:3) Ct Mean ± SD
Osteopenia without fracture	Serum (n=1)	32 ± 0.1	32.8± 0.1	32.4 ± 0.1
Osteopenia with fracture	Serum (n=1)	31.6 ± 0.2		30.7 ± 0.2
Osteoporosis without fracture	Serum (n=1)		31 ± 0.2	29.7 ± 0.1
Severe osteoporosis with fracture	Serum (n=3)		34.7 ± 5.9	33.6 ± 4.7
Total Average		**31.8 ± 0.2**	**33.6 ± 4.9**	**32.2 ± 3.7**

Although, there were no significant expression differences between the three cDNA dilutions: cDNA diluted 1:5 (10ng), cDNA diluted 1:5 (20ng) and diluted 1:3 (33ng) was measured, we decided to use the later one as a dilution protocol for the rest of the study.

3.2.3.2 RT-qPCR screen

The next step was validating the most down/up-regulated miRNAs identified by PCR array, including serum hsa-miR-100-5p (≤-32 fold change), hsa-miR-4516 (-9-fold) and hsa-miR-3923 (4.1-fold) in four LBMD study groups: Osteopenia, Osteopenia with fracture, Osteoporosis and Osteoporosis with fracture.

The Ct mean value for the circulating serum has-miR-100-5p and has-miR-4516 of the four LBMD subgroups (Figure 3-14) were ranged from 30.9 to 31.6 and 25.6 to 26, respectively (Table 3-15), with insignificant Ct mean difference between the four LBMD subgroups (Table 3-14). Whereas, the earliest Ct amplification for hsa-miR-3923 was ≥34 Ct, which indicate late or no RNA amplification (Table 3-15). Therefore, hsa-miR-3923 was excluded from any further studies, while hsa-miR-100-5p and hsa-miR-4516 was kept for further studies.

The following step was the validation of additional two downregulated miRNAs (by PCR array): hsa-miR-122-5p (-5.7-fold) and hsa-miR-373-5p (-4.5-fold) between LBMD subgroups: Osteopenia (n=8), Osteopenia with fracture (n=7), Osteoporosis (n=17) and Osteoporosis with fracture (n= 14) (Figure 3-14).

The Ct value expressions for hsa-miR-122-5p and hsa-miR-373-5p of the four LBMD study groups (Figure 3-14) were ranged from 25.8 to 27.6 and 32 to 32.7 Ct, respectively (Figure 3-14 and Table 3-15). Hsa-miR-122-5p Ct mean difference between Osteoporosis with fracture vs. Osteopenia without fracture was up to 2 Ct, while for hsa-miR-373-5p there were insignificant Ct mean difference between the four LBMD subgroups (Table 3-14). Both miRNAs were kept for further study due to their significant expression by PCR array.

Furthermore, four downregulated circulating serum miRNAs by PCR array: hsa-miR-145-3p (-8.8 fold by PCR array), hsa-miR-206 (-6.3-fold change), hsa-miR-4258 (4-fold change) and hsa-miR-4306 (-7.5-fold change) were validated for the Ct expression and ranged from 29 to 31.2, 30.8 to 34, 32.5 to 35.8 and 30.9 to 34.1 Ct, respectively (Figure 3-14) (Table 3-15). But, there were no significant Ct mean difference of these four miRNAs between the four LBMD groups. Therefore, these miRNAs were excluded from the study.

Due to limited sample volume and high cost of testing materials, fifteen miRNAs were initially validated in only two LBMD individuals: Osteopenia (SID# 120315OP04) versus Osteoporosis (SID# 120320OP03) (Figure 3-13). Out of these, five miRNAs were upregulated in Osteoporosis by PCR array: hsa-miR-196b-3p (2.8-fold), hsa-miR-485-5p (2.8-fold), hsa-miR-1193 (2.4-fold), hsa-miR-2467-3p (2.4-fold) and hsa-miR-4274 (2.3-fold), and the rest were downregulated: hsa-miR-548e-3p (-6.4-fold), hsa-miR-215-5p (-5.8-fold), hsa-miR-548d-5p (-4.7-fold), hsa-miR-99a-5p (-4.4-fold), hsa-miR-450a-5p (-4.2-fold), hsa-miR-375 (-3.9-fold), hsa-miR-1290 (-3.6-fold), hsa-miR-143-3p (-4 fold), hsa-miR-1281 (2.3-fold) and hsa-miR-3911 (-5.2-fold).

The earliest Ct amplification for 4 miRNAs: hsa-miR-196b-3p, hsa-miR-2467-3p, hsa-miR-3911 and hsa-miR-485-5p were ≥ 35 Ct (Figure 3-13 and Table 3-15), and were excluded from any future study. The Ct value for hsa-miR-375 in both osteopenia and osteoporosis subjects was of 22.5 ± 0.1 Ct, with non-significant mean difference of -0.15, and were kept for further study as a normalisation control.

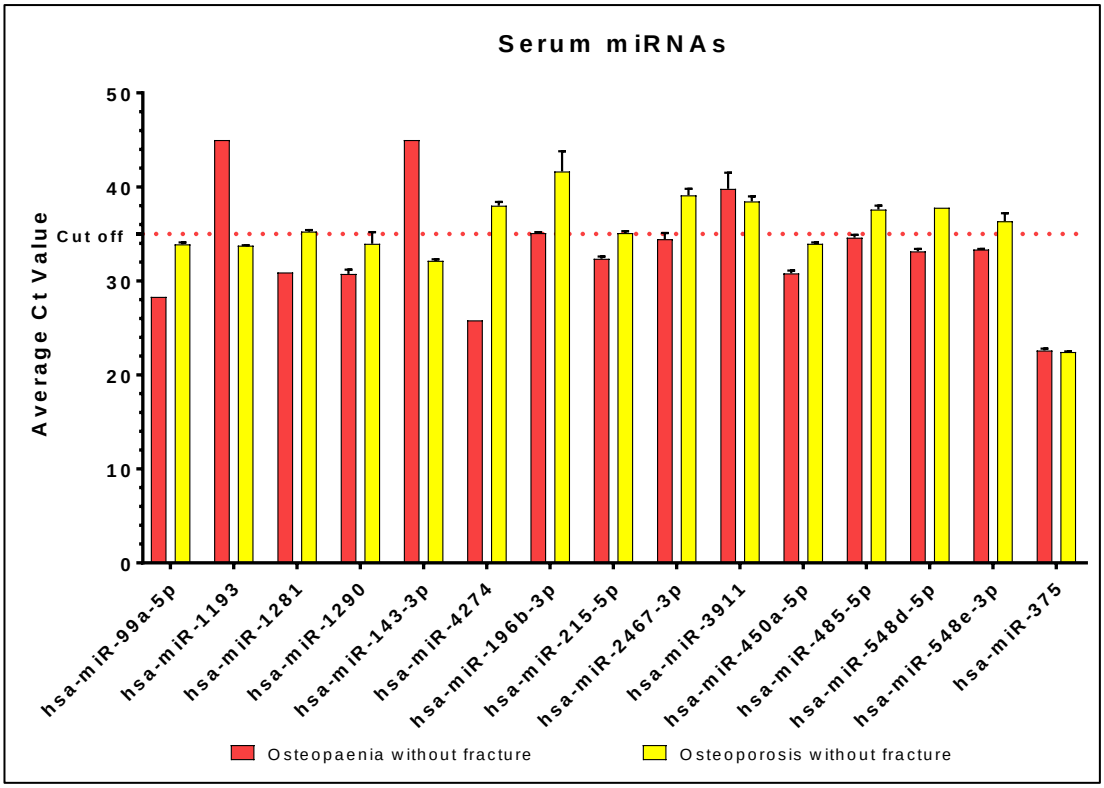


Figure 3-13: Ct expression for 15 circulating serum miRNAs between two LBMD Subjects:

Osteopenia without fracture and Osteoporosis without fracture for 15 Circulating serum miRNAs: hsa-miR-99a-5p (28.3 to 33.9), hsa-miR-1193 (33.8 to 45), hsa-miR-1281 (30.9 to 35.3), hsa-miR-1290 (30.8 to 34), hsa-miR-143-3p (32.2 to 45), hsa-miR-4274 (25.8 to 38), hsa-miR-196b-3p (35.1 to 41.7), hsa-miR-215-5p (32.4 to 35.1), hsa-miR-2467-3p (34.5 to 39.1), hsa-miR-3911 (38.5 to 39.8), hsa-miR-450a-5p (30.8 to 34), hsa-miR-485-5p (34.6 to 37.6), hsa-miR-548d-5p (33.2 to 37.8), hsa-miR-548e-3p (33.4 to 36.4) and hsa-miR-375 (22.5 to 22.6). Each bar represents the mean ±SD of duplicate run.

Subsequently, hsa-miR-1193, hsa-miR-1281, hsa-miR-1290, hsa-miR-143-3p, hsa-miR-215-5p, hsa-miR-375, hsa-miR-4274, and hsa-miR-99a-5p were further tested on four LBMD subjects and showed no significant Ct mean diff between the four groups (Table 3-15). Hsa-miR-215-5p and hsa-miR-99a-5p were kept for future study (Figure 3-14.

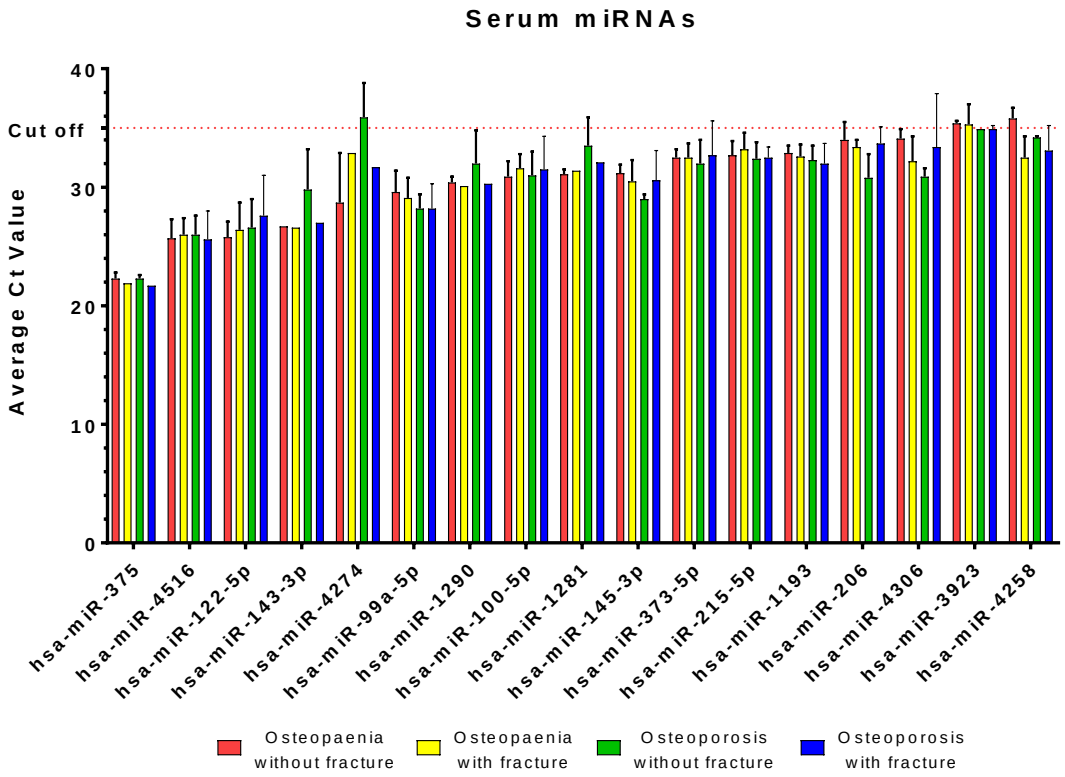


Figure 3-14: Ct expression for 17 circulating serum miRNAs between four LBMD Subjects:

Osteopenia without fracture, Osteopenia with fracture, Osteoporosis without fracture and Osteoporosis with fracture. hsa-miR-375 (Ct= 21.7 to 22.3), hsa-miR-4516 (Ct= 25.6 to 26), hsa-miR-122-5p (25.8 to 27.6), hsa-miR-143-3p (26.6 to 29.8), hsa-miR-4274 (28.7 to 35.9), hsa-miR-99a-5p (28.2 to 29.6), hsa-miR-1290 (30.1 to 32), hsa-miR-100-5p (30.9 to 31.6), hsa-miR-1281 (31.1 to 33.5), hsa-miR-145-3p (29 to 31.2), hsa-miR-373-5p (32 to 32.7), hsa-miR-215-5p (32.4 to 33.2), hsa-miR-1193 (32 to 32.9), hsa-miR-206 (30.8 to 34), hsa-miR-4306 (30.9 to 34.1), hsa-miR-3923 (34.9 to 35.4), and hsa-miR-4258 (32.5 to 35.8) . Each bar represents the mean and ±SD of duplicate run.

Table 3-14: Ct mean difference of circulating serum miRNAs between four LBMD groups

Two way Anova with Bonferroni's multiple comparison test for the Ct mean difference between four LBMD groups: Osteopenia, Osteopenia with fracture, Osteoporosis and Osteoporosis with fracture showed no significant P value.

miRNA	Test details	Mean Diff.
hsa-miR-100-5p	Osteopenia with fracture (n= 7) vs. Osteopenia without fracture (n= 8)	0.7
	Osteoporosis without fracture (n= 17) vs. Osteopenia without fracture (n= 8)	0.1
	Osteoporosis with fracture (n= 14) vs. Osteopenia without fracture (n= 8)	0.6
	Osteoporosis without fracture (n= 17) vs. Osteopenia with fracture (n= 7)	-0.6
	Osteoporosis with fracture (n= 14) vs. Osteopenia with fracture (n= 7)	-0.1
	Osteoporosis with fracture (n= 14) vs. Osteoporosis without fracture (n= 17)	0.5
hsa-miR-4516	Osteopenia with fracture (n= 7) vs. Osteopenia without fracture (n= 8)	0.3
	Osteoporosis without fracture (n= 16) vs. Osteopenia without fracture (n= 8)	0.3
	Osteoporosis with fracture (n= 14) vs. Osteopenia without fracture (n= 8)	-0.1
	Osteoporosis without fracture (n= 16) vs. Osteopenia with fracture (n= 7)	0
	Osteoporosis with fracture (n= 14) vs. Osteopenia with fracture (n= 7)	-0.4
	Osteoporosis with fracture (n= 14) vs. Osteoporosis without fracture (n= 16)	-0.4
hsa-miR-122-5p	Osteopenia with fracture (n= 7) vs. Osteopenia without fracture (n= 8)	0.6
	Osteoporosis without fracture (n= 16) vs. Osteopenia without fracture (n= 8)	0.8
	Osteoporosis with fracture (n= 14) vs. Osteopenia without fracture (n= 8)	1.8
	Osteoporosis without fracture (n= 16) vs. Osteopenia with fracture (n= 7)	0.2
	Osteoporosis with fracture (n= 14) vs. Osteopenia with fracture (n= 7)	1.2

miRNA	Test details	Mean Diff.
	Osteoporosis with fracture (n= 14) vs. Osteoporosis without fracture (n= 16)	1
hsa-miR-373-5p	Osteopenia with fracture (n= 7) vs. Osteopenia without fracture (n= 7)	0
	Osteoporosis without fracture (n= 17) vs. Osteopenia without fracture (n= 7)	-0.5
	Osteoporosis with fracture (n= 14) vs. Osteopenia without fracture (n= 7)	0.2
	Osteoporosis without fracture (n= 17) vs. Osteopenia with fracture (n= 7)	-0.5
	Osteoporosis with fracture (n= 14) vs. Osteopenia with fracture (n= 7)	0.2
	Osteoporosis with fracture (n= 14) vs. Osteoporosis without fracture (n= 17)	0.7
hsa-miR-215-5p	Osteopenia with fracture (n= 5) vs. Osteopenia without fracture (n= 6)	0.5
	Osteoporosis without fracture (n= 12) vs. Osteopenia without fracture (n= 6)	-0.3
	Osteoporosis with fracture (n= 8) vs. Osteopenia without fracture (n= 6)	-0.2
	Osteoporosis without fracture (n= 12) vs. Osteopenia with fracture (n= 5)	-0.8
	Osteoporosis with fracture (n= 8) vs. Osteopenia with fracture (n= 5)	-0.7
	Osteoporosis with fracture (n= 8) vs. Osteoporosis without fracture (n= 12)	0.1
hsa-miR-99a-5p	Osteopenia with fracture (n= 4) vs. Osteopenia without fracture (n= 5)	-0.5
	Osteoporosis without fracture (n= 11) vs. Osteopenia without fracture (n= 5)	-1.4
	Osteoporosis with fracture (n= 7) vs. Osteopenia without fracture (n= 5)	-1.4
	Osteoporosis without fracture (n= 11) vs. Osteopenia with fracture (n= 4)	-0.9
	Osteoporosis with fracture (n= 7) vs. Osteopenia with fracture (n= 4)	-0.9
	Osteoporosis with fracture (n= 7) vs. Osteoporosis without fracture (n= 11)	0

In summary, the outcomes from RT-qPCR screening process was the selection of 6 miRNAs: hsa-miR-4516 (26Ct ± 2), hsa-miR-122-5p (27Ct ± 3), hsa-miR-99a-5p (29Ct ± 2), hsa-miR-373-5p (32Ct ± 2), hsa-miR-215-5p (33Ct ± 2) and hsa-miR-100-5p (31Ct ± 2) with earliest miRNA expression of 26 Ct and Ct value mean difference between the osteopenia and osteoporosis group >2 Ct (Table 3-15).

Table 3-15: Summary Table for 24 circulating serum miRNAs Average delta Ct value in clinical samples

miRNA	Osteopenia Ct Mean ± SD (N)	Osteopenia with fracture Ct Mean ± SD (N)	Osteoporosis Ct Mean ± SD (N)	Osteoporosis with fracture Mean Ct ± SD (N)
hsa-miR-100-5p	30.9 ± 1.3 (n=8)	31.6 ± 1.2 (n=7)	31 ± 2 (n=17)	31.5 ± 2.8 (n=14)
hsa-miR-1193	32.9 ± 0.6 (n=6)	32.6 ± 1 (n=5)	32.3 ± 1.2 (n=12)	32 ± 1.7 (n=8)
hsa-miR-122-5p	25.8 ± 1.3 (n=8)	26.4 ± 2.3 (n=7)	26.6 ± 2.4 (n=16)	27.6 ± 3.4 (n=14)
hsa-miR-1281	31.1 ± 0.4 (n=2)	31.4 ± 0 (n=1)	33.5 ± 2.4 (n=2)	32.1 ± 0 (n=1)
hsa-miR-1290	30.4 ± 0.5 (n=2)	30.1 ± 0 (n=1)	32 ± 2.8 (n=2)	30.3 ± 0 (n=1)
hsa-miR-143-3p	26.7 ± 0 (n=2)	26.6 ± 0 (n=1)	29.8 ± 3.4 (n=2)	27 ± 0 (n=1)
hsa-miR-145-3p	31.2 ± 0.7 (n=4)	30.5 ± 1.8 (n=4)	29 ± 0.4 (n=4)	30.6 ± 2.5 (n=4)
hsa-miR-196b-3p	35.1 ± 0.2 (n=1)		41.6 ± 3 (n=1)	
hsa-miR-206	34 ± 1.5 (n=4)	33.4 ± 0.6 (n=4)	30.8 ± 2 (n=4)	33.7 ± 1.4 (n=4)
hsa-miR-215-5p	32.7 ± 1.2 (n=6)	33.2 ± 1.4 (n=5)	32.4 ± 1.4 (n=12)	32.5 ± 0.9 (n=8)
hsa-miR-2467-3p	34.5 ± 0.9 (n=1)		39.1 ± 1 (n=1)	
hsa-miR-373-5p	32.5 ± 0.7 (n=7)	32.5 ± 1.2 (n=7)	32 ± 2 (n=17)	32.7 ± 2.9 (n=14)
hsa-miR-375	22.3 ± 0.5 (n=2)	21.9 ± 0 (n=1)	22.3 ± 0.3 (n=2)	21.7 ± 0 (n=1)

miRNA	Osteopenia Ct Mean ± SD (N)	Osteopenia with fracture Ct Mean ± SD (N)	Osteoporosis Ct Mean ± SD (N)	Osteoporosis with fracture Mean Ct ± SD (N)
hsa-miR-3911	39.8 ± 2.4 (n=1)		38.4 ± 0.8 (n=1)	
hsa-miR-3923	35.4 ± 0.2 (n=2)	35.3 ± 1.7 (n=2)	34.9 ± 0 (n=1)	34.9 ± 0.3 (n=3)
hsa-miR-4258	35.8 ± 0.9 (n=4)	32.5 ± 1.8 (n=4)	34.2 ± 0.1 (n=4)	33.1 ± 2.1 (n=4)
hsa-miR-4274	28.7 ± 4.2 (n=2)	32.9 ± 0 (n=1)	35.9 ± 2.9 (n=2)	31.7 ± 0 (n=1)
hsa-miR-4306	34.1 ± 0.8 (n=4)	32.2 ± 2.1 (n=4)	30.9 ± 0.7 (n=4)	33.4 ± 4.5 (n=4)
hsa-miR-450a-5p	30.8 ± 0.4 (n=1)		33.9 ± 0.2 (n=1)	
hsa-miR-4516	25.7 ± 1.6 (n=8)	26 ± 1.4 (n=7)	26 ± 1.6 (n=16)	25.6 ± 2.4 (n=14)
hsa-miR-485-5p	34.6 ± 0.4 (n=1)		37.6 ± 0.6 (n=1)	
hsa-miR-548d-5p	33.1 ± 0 (n=1)		37.8 ± 0 (n=1)	
hsa-miR-548e-3p	33.4 ± 0.1 (n=1)		36.4 ± 1.2 (n=1)	
hsa-miR-99a-5p	29.6 ± 1.8 (n=5)	29.1 ± 1.7 (n=4)	28.2 ± 1.2 (n=11)	28.2 ± 2.1 (n=7)

3.3 Discussion

This study was designed to identify circulatory miRNAs associated with the progression of osteoporosis in a test group of patients using PCR arrays. Preliminary screening for circulating serum miRNAs associated with osteoporosis using miRNA PCR array that were capable of detecting more than 300 circulating serum/plasma miRNAs per set showed significant changes in the levels of twenty-four miRNAs in osteoporotic female patients. Wherein, six miRNAs: hsa-miR-4516, hsa-miR-99a-5p, hsa-miR-373-5p, hsa-miR-215-5p, hsa-miR-100-5p and hsa-miR-122-5p were remarkably downregulated by > 2-fold in the osteoporosis patient group compared to the osteopenia group.

The study set out to compare the level of secreted miRNAs in certain patterns of low bone mass with respect to those from preferably age matched non-osteoporosis controls. The levels of 6 miRNAs, hsa-miR-4516, hsa-miR-99a-5p, hsa-miR-373-5p, hsa-miR-215-5p, hsa-miR-100-5p and hsa-miR-122-5p, in clinical sample pools were screened using RT-qPCR. The results showed that these 6 miRNAs might be associated with osteoporosis. Changes in the levels of some of the miRNAs identified in the present study have previously been associated with normal and abnormal bone development. For example, MiR-99a has been found to regulate early chondrogenic differentiation of rat mesenchymal stem cells by targeting the BMPR2 gene. (Zhou et al., 2016), and to inhibit tumour cell proliferation via targeting of TNFAIP8 in osteosarcoma cells. (Xing and Ren, 2016).

miRNA-100 overexpression was found to inhibits bone morphogenetic protein-(BMP)-induced osteoblast differentiation by targeting BMPR2 (Zeng et al., 2012b),

and by decreasing Smad1 protein levels (Fu et al., 2016). miR-100, and miR-125b were reported to be upregulated in osteoporotic fractured patients' serum and bone tissues compared to non-osteoporotic controls. (Seeliger et al., 2014).

miR-122, which is abundantly detected on hepatocytes (Laterza et al., 2013), was found to be upregulated in the serum of patients with osteoporotic fractures compared to non-osteoporotic controls (Seeliger et al., 2014, Panach et al., 2015), and was found to be under-expressed in osteosarcoma tissues (Xiao et al., 2015). However, no effect has been reported in the context of bone metabolism (Weilner et al., 2015).

The levels of miR-4516, miR-215 and MiR-373 in serum/plasma have been found to be associated with various pathological processes. For example, miR-215 was suggested to be a potential prognostic biomarker in colon cancer (Karaayvaz et al., 2011), and could contribute to progression of kidney cancer metastasis through different biological processes (White et al., 2011). Plasma miR-215 was downregulated in Chronic Myeloid Leukaemia (CML) patients undergoing successful discontinuation of Imatinib therapy (Ohyashiki et al., 2016).

MiR-373 functions as an oncogene and targets YOD1 gene in cervical cancer (Wang et al., 2015b), and promotes migration and invasion in human oesophageal squamous cell carcinoma (ESCC) (Liu et al., 2016). However, there is, so far, no published report linking these circulating miRNAs, miR-4516, miR-215 and MiR-373, with osteoporosis. Thus, further analysis will be necessary.

In conclusion: a number of differentially-expressed miRNAs between osteoporosis and osteopenia groups have been identified using miRNA PCR arrays. After initial RT-qPCR screening, 6 out of 24 miRNAs might be possible candidates associated with osteoporosis. Therefore, the next step was to investigate these 6 miRNAs in individual clinical serum and plasma samples, to assess their potential diagnostic value for osteoporosis. These experiments will be discussed in the next chapter.

Chapter 4 Potential Circulating miRNAs as Diagnostic Biomarkers

4.1 Introduction

In the previous chapter, 6 miRNAs were found to be differentially-expressed in serum samples from osteoporosis patients. Interestingly, the expression changes of the six circulating miRNAs in serum/plasma have been found to be associated with various pathological processes. For example, circulating hsa-miR-4516 was found to be upregulated in plasma samples of HIV-associated neurocognitive disorder compared to a control group (Asahchop et al., 2016). miR-4516 was also reported to be associated with prostate cancer (Bell et al., 2015), cardiovascular (Zhang et al., 2016) and skin diseases (Chowdhari and Saini, 2014). However, there is no published report indicating miR-4516 associating with osteoporosis.

MiR-373 has diverse functions in cancer (Wei et al., 2015). Circulating serum hsa-miR-373 was significantly upregulated with the progression of breast cancer disease (Eichelser et al., 2013), and in epithelial ovarian cancer patients (Meng et al., 2016) compared to healthy women. Regarding bone related diseases, miR-373 was significantly downregulated in osteoarthritis patients compared to normal controls (Iliopoulos et al., 2008). However, until now, there has been no published data showing that miR373 is involved in the development of osteoporosis.

Circulating miR-215 was found to be significantly upregulated in patients with both Barrett's esophagus or columnar-lined esophagus compared to esophagitis (Cabibi et al., 2016), as well as, in chronic hepatitis and hepatocellular carcinoma compared to a control group (Zhang et al., 2014). However, there is no evidence to show that miR215 is associated with osteoporosis.

Circulating miR-122-5p has been found to be upregulated in the plasma of patients with hepatitis B viral infection compared to healthy controls (Zhang et al., 2010), in patients with biliary atresia, an idiopathic neonatal liver disease, compared to age matched healthy controls (Peng et al., 2016) and in the serum of patients with severe acute viral hepatitis with coagulopathy, as compared to patients without coagulopathy (Weseslindtner et al., 2016). In the matter of skeletal diseases, hsa-miR-122-5p was found to be dysregulated in osteosarcoma cells compared to normal tissue (Xiao et al., 2015), over-expressed in serum of patients with osteoporotic fractures compared to non-osteoporotic (Seeliger et al., 2014) and osteoarthritis sufferers (Panach et al., 2015), with significant clinical discrimination value of > 0.7 during validation (Panach et al., 2015).

miR-100 upregulation was found to inhibit osteogenic differentiation by targeting bone morphogenetic protein receptor type II (BMPR2) (Zeng et al., 2012b) and Smad1 protein (Fu et al., 2016). MiR-100 was significantly upregulated in the serum and bone tissue of osteoporotic patients (Seeliger et al., 2014).

MiR-99a-5p was found to play vital role in early chondrogenic differentiation in rat (Zhou et al., 2016), was shown to be downregulated in osteosarcoma tissue relative to noncancerous bone tissues (Zhao et al., 2016, Gougelet et al., 2011). Circulating plasma miR-99a was downregulated in acute myocardial infarction patients compared to healthy controls (Yang et al., 2016) and in endometrioid endometrial cancer compared to healthy controls (Torres et al., 2012). In contrast, circulating serum miR-99a was shown to be upregulated in HBV infected patients compared to healthy controls (Li et al., 2010).

The purpose of this Chapter is therefore to investigate the association of the panel of circulating miRNAs from Chapter 3 (hsa-miR-4516, hsa-miR-373-5p, hsa-miR-122-5p, hsa-miR-215-5p, hsa-miR-99a-5p and hsa-miR-100-5p) with osteoporosis to verify whether these circulating serum/plasma miRNAs could be used in future as molecular biomarkers.

To this end, Real-Time quantitative PCR (RT-qPCR) experiments were performed on clinical serum and plasma samples. The relationship between miRNA expression changes and BMD index was also examined to investigate their combined association with osteoporosis.

4.2 Results

Additional ethical approval for the additional clinical specimens needed was obtained from the RLBUHT [Ref No: 15/EE/0051], prior to participants' recruitment [Materials and Methods 2.3.1.]. Clinical samples were obtained from one hundred and sixty-one participants after informed consent.

According to the Bone Mineral Density (BMD) T-Score and the clinical findings, samples were classified into 5 groups: Non-osteoporosis control (BMD *T*-Score >-1, n= 30), Osteopenia (-2.5 ≤ BMD T-Score ≤ -1, n= 63), Osteopenia with fracture (-2.5 ≤ BMD T-Score ≤ -1, n= 15), Osteoporosis (BMD T-Score ≤ -2.5, n= 34) and Osteoporosis with fracture (BMD T-Score ≤ -2.5, n= 19) (Table 4-1). Among those patients with low bone mass (BMD *T*-score < -1), 97 patients were without fractures and 34 patients were with fractures. 81% of participants were female. The average age of healthy non-osteoporosis control participants was 45 years ± SD 20. The average age of osteopenia and osteoporosis patients was 66 years ± SD 10.7 and 68 years ± SD12.6, respectively. 18 non-osteoporotic participants were under 40 years old without a history of bone fracture. In addition, four Low Bone Mineral Density (LBMD) Osteopenia (n=2), Osteoporosis (n=1) and Osteoporosis with fracture (n=1) were under 40 years of age (Table 4-1).

Table 4-1: Summary of characteristics of clinical samples

Clinical Category	**No. of Participants**	**Sex [Female/ Male]**	**Age Average ±SD**	**BMD Average ±SD (g/cm^2)**	**T-Score (Lumbar Spine (L2-L4)**
Non-osteoporosis control	30	20/10	45 ± 20	1 ± 0.1	0.6 ± 1.4
Osteopenia	63	53/10	65 ± 10.3	0.8 ± 0.1	-1.2 ± 0.9
Osteopenia with fracture	15	13/2	66 ± 11.2	0.9 ± 0.1	-1.1 ± 1.1
Osteoporosis	34	28/6	67 ± 13	0.7 ± 0.1	-2.8 ± 0.9
Osteoporosis with fracture	19	17/2	68 ± 12.3	No data	-2.9 ± 1
Total	**161**		**62 ± 13.4**	**0.9 ± 0.1**	**-1.5 ± 1.1**

4.2.1 miRNA quality and yields

miRNAs were extracted from 161 serum and 142 plasma samples using TRIzol® LS reagent and QIAGEN miRNeasy Mini Kit (Materials and Methods 2.5), and were checked for purity and concentration using a NanoDrop™ 2000 spectrophotometer (Material & Method 2.5.1), with average RNA yields of 1437.1 ± 680.8 ng and 1297 ± 607 ng, respectively (Table 4-2). Both extracted serum and plasma RNA samples have relatively similar RNA concentration (ranging between 700 to 2000 ng/mL), and 260/280 and 260/230 RNA ratio (Table 4-2). High quality RNAs purified from the 161 serum and 142 plasma samples were used for RT-qPCR analysis to determine the levels of the six miRNAs, hsa-miR-4516, hsa-miR-122-5p, hsa-miR-99a-5p, hsa-miR-100-5p, hsa-miR-373-5p and hsa-miR-215-5p, as described in the next section.

Table 4-2: Summary of quality check of RNA isolated from clinical samples

Specimen Type	**No. of Samples**	**RNA 260/280 Ratio Mean ± SD**	**RNA 260/230 Ratio Mean ± SD**	**Total RNA Yield (ng) Mean ± SD**
Serum	161	1.3 ± 0.05	0.3 ± 0.01	1437.1 ± 680.8
Plasma	142*	1.2 ± 0.04	0.4 ± 0.16	1297 ± 607

*No plasma sample were obtained from 19 participants.

4.2.2 The levels of miRNA in clinical samples using RT-qPCR

100 ng of purified RNAs from each serum or plasma sample, in the osteopenia (with and without fracture), osteoporosis (with and without fracture) and non-osteoporosis groups was reverse transcribed to generate first strand cDNA. RT-qPCR was performed using diluted first strand cDNA at a ratio of 1:3 in duplicate for each sample as described in Material & Methods, 2.6. The Ct value for each sample obtained from each RT-qPCR run was normalised using two small RNA molecules, SNORD96A and RNU6-2. The relative levels of miRNAs in serum or plasma samples were calculated using the $2^{-\Delta Ct}$ methods (Materials and Methods, 3.2.3.3).

The average levels of miRNA in serum and plasma value (log10 [$2^{-\Delta Ct}$]) for the five study groups: non-osteoporotic, osteopenia (with/without fracture) and osteoporosis (with/without fracture) are listed in Appendix 8.5. Hsa-miR-215-5p and hsa-miR-99a-5p were tested on 161 serum samples, but only 128 and 134 participants' plasma samples, respectively and hsa-miR-373-5p was tested on 157 serum and 136 plasma samples. The reduced number of samples was due to an insufficiency of extracted serum/plasma RNA samples for some patients. All other miRNAs were tested on the full number of 161 serum and 142 plasma samples.

4.2.3 Identify miRNAs associated with age

All members of the panel of miRNA from Chapter 3 were tested for a relationship between expression and patient age. Only three miRNAs showed such a relationship, namely hsa-miR-4516, hsa-miR-373-5p and hsa-miR-100-5p. Hsa-miR-4516, hsa-miR-373-5p showed significant association with increasing age in serum samples (p =< 0.0001 and 0.023, respectively), but not in plasma samples (p= 0.41 and 0.44, respectively) (Figure 4-1 and Figure 4-2), whereas hsa-miR-100-5p significantly associated with age in plasma, but not in serum samples (p = 0.0128 and 0.576, respectively) (Figure 4-3).

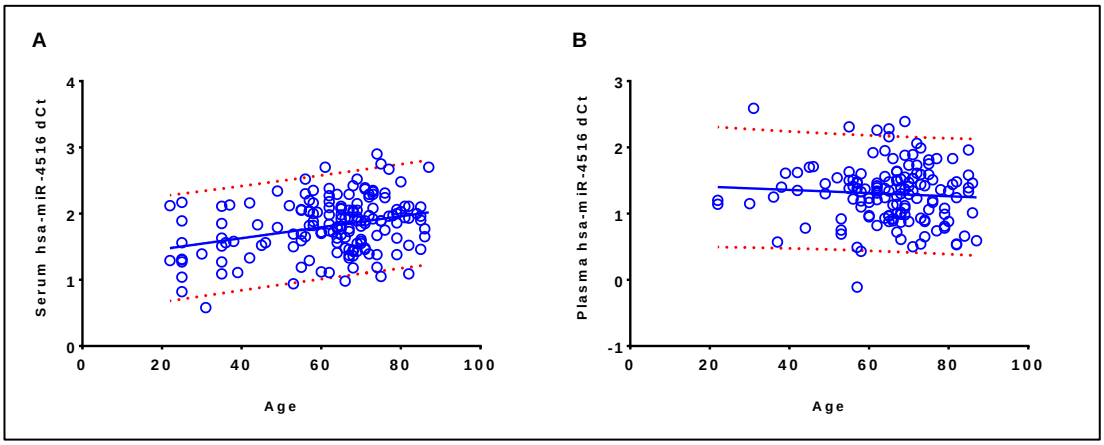


Figure 4-1: The level of circulating hsa-miR-4516 associated with age
Panel A: The levels of hsa-miR-4516 in serum is significantly associated with age, (p (two-tailed) < 0.0001, Pearson r = 0.32 and 95% CI 0.17. to 0.45). Panel B, the levels of hsa-miR-4516 in plasma were not associated with age (p = 0.4133, Pearson r= 0.069 and 95% CI -0.23 to 0.097). Blue line= regression line and Red line= 95% prediction band.

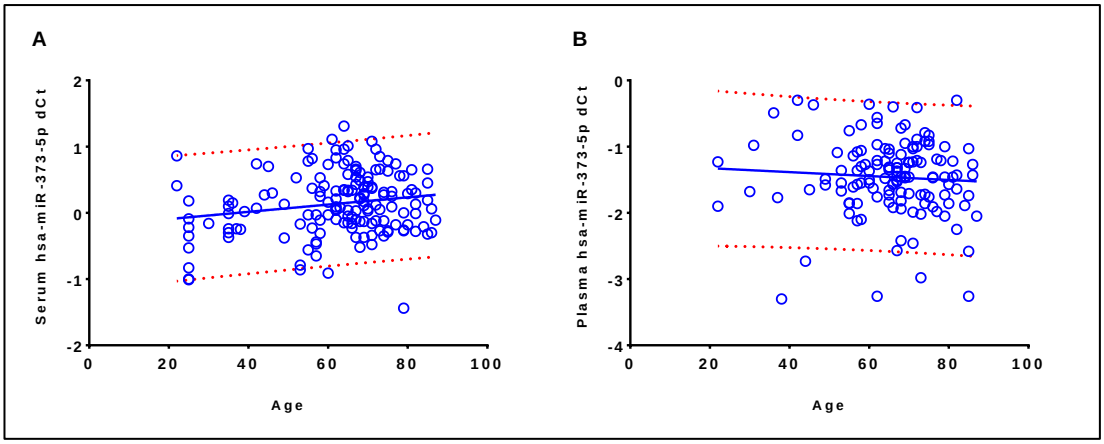


Figure 4-2: The level of circulating hsa-miR-373-5p associated with Age.
Panel A, the levels of hsa-miR-373-5p in serum samples significantly increased with increasing age (*p* (two-tailed) = 0.023, Pearson r = 0.18 and 95% CI 0.03 to 0.33). Panel B: The levels of hsa-miR-373-5p in plasma samples were not associated with increasing age (*p* (two-tailed) = 0.4396, Pearson r= -0.067 and 95% CI -0.23 to 0.1). Blue line= regression line and Red line= 95% prediction band.

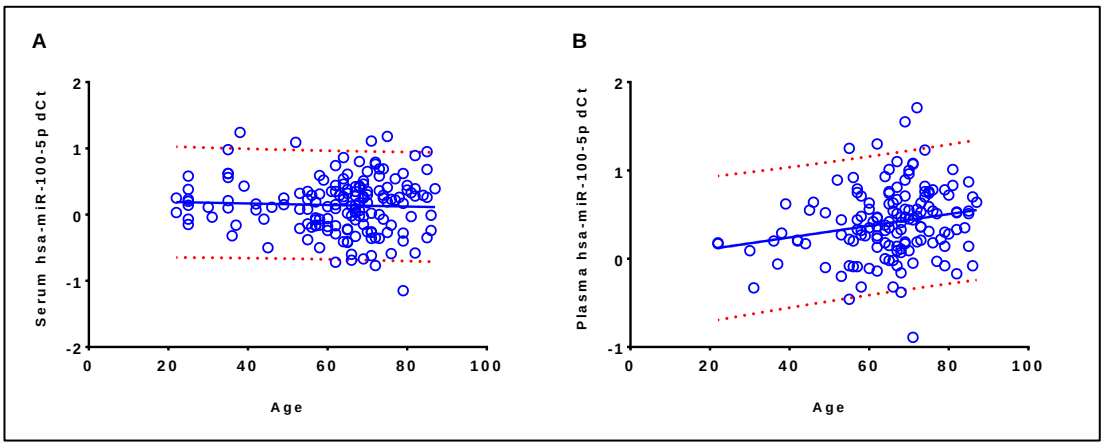


Figure 4-3: The levels of circulating hsa-miR-100-5p associated with age
Panel A, the levels of hsa-miR-100-5p in serum samples was not associated with participants' age, (*p* (two-tailed) = 0.58, Pearson r=-0.044 and 95% CI =-0.2 to 0.11). Panel B, the levels of hsa-miR-100-5p in plasma samples were significantly increased. with participants 'age, (*p* (two-tailed) = 0.0128, Pearson r = 0.21 and 95% CI 0.05 to 0.36). Blue line= regression line and Red line= 95% prediction band.

These results suggest a possible relationship with age for these three sample, although it is not possible to explain the differences between serum and plasma samples. Since participant's age was a confounding factor in the expression of some of the circulating miRNAs (4.2.4). Therefore, those participants under 40 years old were not included in the following investigation.

4.2.4 Correlation of the levels of miRNA in Plasma Versus Serum

In order to verify if the levels of circulating miRNA in blood are affected by the specimen type, a correlation study for the logarithmically (Log10) transformed miRNA qPCR $2^{\wedge-\Delta Ct}$ data was performed between serum and plasma samples for participants who are over 40 years of age (n=134). Pearson coefficient correlation analysis showed that the levels of both miRNAs, hsa-miR-122-5p and hsa-miR-4516 in serum correlate with their levels in plasma samples (*p* (two-tailed) < 0.0001, and 0.0019, respectively, Figure A & B). Thus, the levels of both miRNAs, hsa-miR-122-5p and hsa-miR-4516 are not interfered by the sample types. Therefore, either serum or plasma sample type is suitable for the detection of these miRNAs using RT-qPCR.

The level of hsa-miR-100-5p was much lower in plasma compared to that in serum samples as shown in (Figure 4-4, C), (*p* (two-tailed) = 0.0029, Pearson r =-0.26 and 95%CI -0.41 to -0.09). The result indicates that the levels of has-miR100-5p is proportionally decreased in plasma compared to serum samples.

The levels of hsa-miR-99a-5p, hsa-miR-373-5p and hsa-miR-215-5p in plasma samples were not significant different to those in serum samples. Either serum or plasma samples are suitable to detect the level of these three miRNAs.

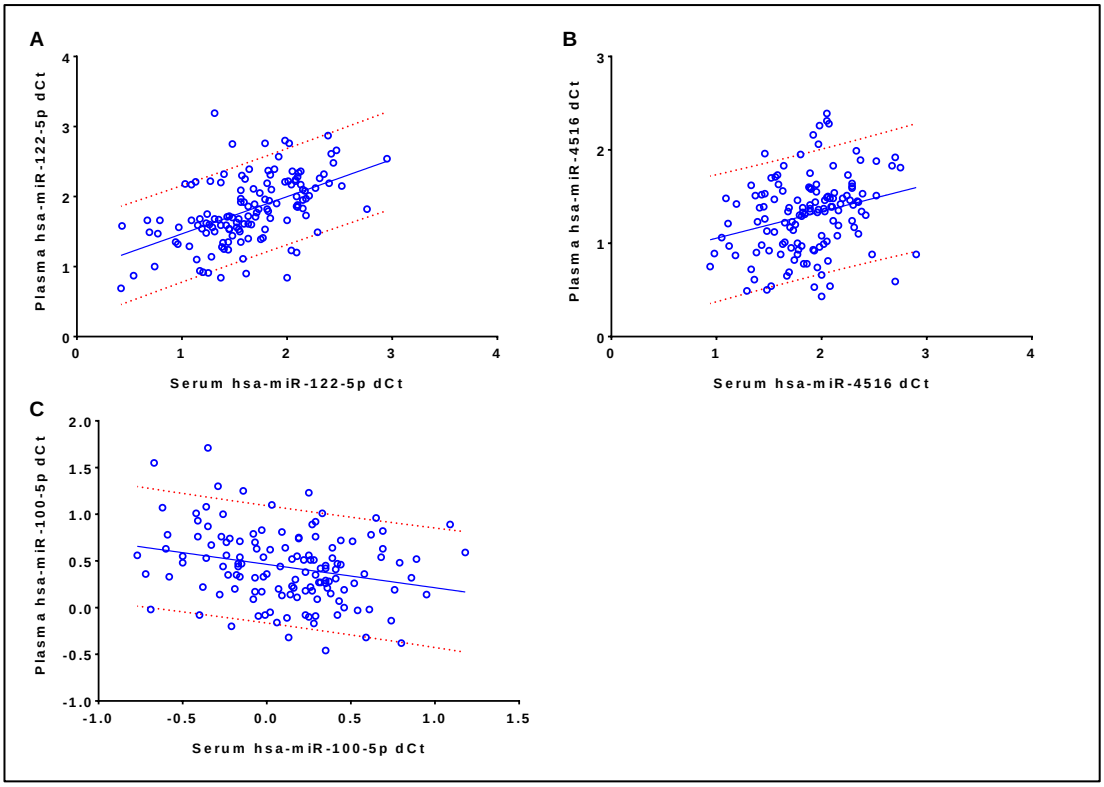


Figure 4-4: Correlation of circulating miRNA expression between serum and plasma samples (n=134)

Panel A: The levels of miR122-5p show strong correlation between plasma and serum samples (p (two-tailed) < 0.0001, Pearson r =0.52). Panel B: The levels of hsa-miR-4516 show strong correlation between serum and plasma samples (*p* (two-tailed) = 0.0019, Pearson r =0.27. Panel C: the levels of hsa-miR-100-5p show significant reverse correlation between plasma and serum samples (*p* (two-tailed) = 0.0029, Pearson r =-0.26). Blue line= regression line and Red line= 95% prediction band.

4.2.5 Differentially expressed circulating miRNAs associated with osteoporosis patients

Six miRNAs were validated on 139 serum samples and 134 plasma samples using RT-qPCR. One-way ANOVA analysis, with 'Benferroni's multiple comparison test' analysis and 'Box-and-Whisker' plots were used to identify differentially-expressed miRNAs associated with osteoporosis patients (Materials and Methods 2.9). The results show that has-miR-373-5p is differentially-expressed between non-osteoporotic control, osteopenia and osteoporosis patients as shown in Figure 4-5A.

The levels of hsa-miR-373-5p in serum are lower in osteopenia and osteoporosis groups compared to the non-osteoporosis control group (ANOVA $p = 0.0003$) (Figure 4-5-A). There was significant down-regulation of hsa-miR-373-5p in osteopenia (with & without fracture) and in osteoporosis (with & without fracture) compared to the non-osteoporosis control group ($p = 0.048$ and 0.001, respectively, Bonferroni's correction) (Table 4-3). The levels of hsa-miR-373 were significantly lower in osteoporosis (with & without fracture) compared to the osteopenia, group ($p= 0.021$). In contrast, there were no significant differences in the levels of hsa-miR-373-5p in plasma from the same groups of patients (ANOVA $p = 0.42$) (Figure 4-5). These results show that significantly reduced levels of hsa-miR-373-5p in serum, but not plasma, were associated with the presence of osteopenia and osteoporosis.

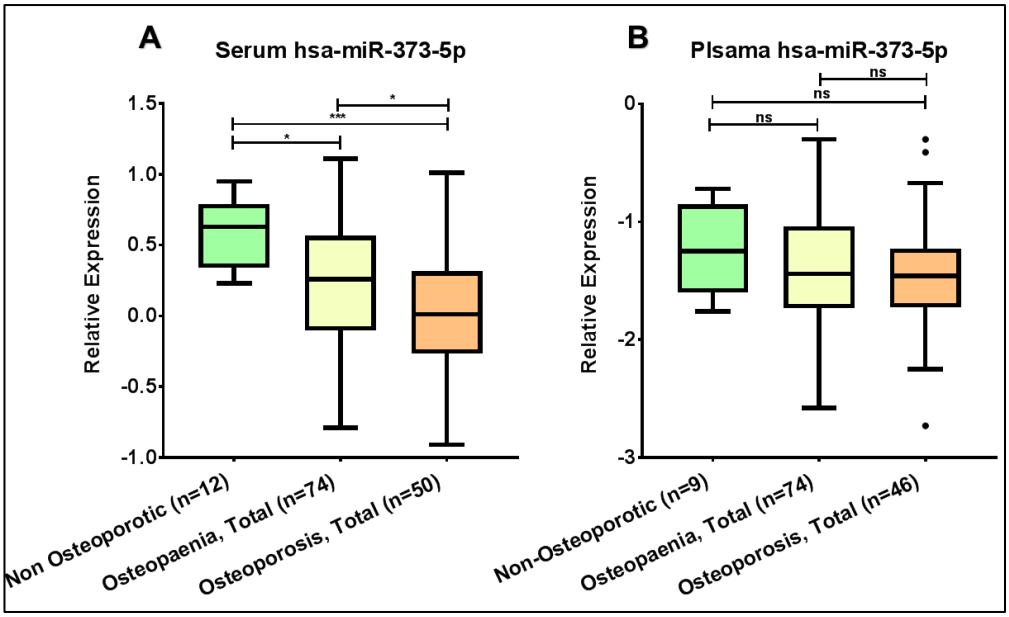


Figure 4-5: Differentially expressed has-miR-373-5p associated with Osteoporosis. Box plots show the 25th, 50th, and 75th percentiles and minimum to maximum ranges of the levels of hsa-miR-373-5p in serum and plasma among non-osteoporosis, osteopenia and osteoporosis groups. Panel A shows a statistically significant correlation of serum hsa-miR-373-5p among the osteoporosis patients and the osteopenia patients, compared to the non-osteoporotic control (n=12), (ANOVA $p< 0.0003$). Panel B shows that the level of hsa-miR-373-5p in plasma samples was not significantly different among non-osteoporotic control, osteopenia patients, and osteoporosis patients, ($p=0.4$). ns = not significant, $*p < 0.05$ and $***p < 0.001$. The comparison indicated by Bonferroni's multiple comparisons test (One-way ANOVA).

Table 4-3: One-way ANOVA multiple comparisons test for hsa-miR-373-5p expression level between Non-Osteoporosis, Osteopenia, and Osteoporosis.

Data analysed	ANOVA *p* Value	Bonferroni's multiple comparisons test	Mean Diff. (95.00% CI of diff.)	Bonferroni's *p* Value
Serum hsa-miR-373-5p	0.0003	Osteopenia, (n=74) vs. Non-Osteoporotic (n=12)	-0.32 (-0.641 to -0.002)	0.048
		Osteoporosis, (n=50) vs. Non-Osteoporotic (n=12)	-0.53 (-0.859 to -0.198)	0.001
		Osteoporosis, (n=50) vs. Osteopenia, (n=74)	-0.21 (-0.39 to -0.024)	0.021

The level of hsa-miR-122-5p in serum was significantly down-regulated in the osteoporosis patients compared to non-osteoporosis control and osteopenia, groups (ANOVA *p*= 0.002) (Figure 4-6), (Bonferroni's p= 0.008 and 0.021), respectively (Table 4-4). The level of hsa-miR-122-5p in plasma samples was not significantly changed in osteoporosis patients or osteopenia patients compared to control groups (ANOVA *p* value= 0.21) (Figure 4-6B). The results show that serum, but not plasma, hsa-miR-122-5p might be down-regulated in the development of osteoporosis.

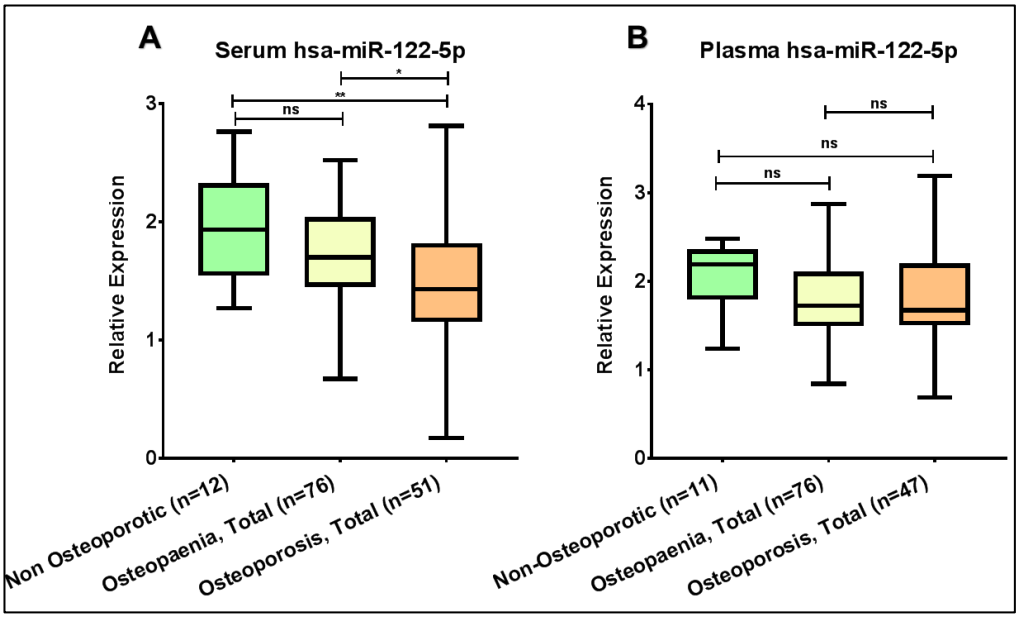


Figure 4-6: Differentially expressed hsa-miR-122-5p associated with Osteoporosis. Box Plot shows the 25th, 50th, and 75th percentiles and minimum to maximum ranges of the levels of hsa-miR-122-5p among Non-osteoporosis, Osteopenia and Osteoporosis groups. Panel A: box plots show a statistically significant correlation of hsa-miR-122-5p among the osteoporosis patients (ANOVA *p* = 0.002) compared to the non-Osteoporotic and Osteopenia, *p* (Bonferroni's)= 0.008 and *p* (Bonferroni's)= 0.021, respectively. Panel B, box plots show the level of hsa-miR-122-5p in plasma samples was not significant among non-osteoporotic control (n=11), osteopenia patients and osteoporosis patients. **p* <0.05, ***p* <0.01 and ns = not significant. The comparison indicated by Bonferroni's multiple comparisons test (One-way ANOVA).

Table 4-4: One-way ANOVA multiple comparisons test for hsa-miR-122-5p expression level between Non-Osteoporosis, Osteopenia and Osteoporosis.

Data analysed	ANOVA *p* Value	Bonferroni's multiple comparisons test	Mean Diff. (95.00% CI of diff.)	Bonferroni's *p* Value
Serum hsa-miR-122-5p	0.002	Osteopenia (n=76) vs. Non-Osteoporotic (n=12)	-0.23 (-0.577 to 0.123)	0.356
		Osteoporosis (n=51) vs. Non-Osteoporotic (n=12)	-0.46 (-0.818 to -0.096)	0.008
		Osteoporosis (n=51) vs. Osteopenia (n=76)	-0.23 (-0.434 to -0.026)	0.021

The level of hsa-miR-215-5p in serum in Osteoporosis (Figure 4-7A), but not in osteopenia patients, was significantly lower compared to the non-osteoporotic control group (ANOVA *p* = 0.014, p (Bonferroni's) = 0.013, Table 4-5). There were no significant differences in levels of hsa-miR-215-5p in plasma samples between the three groups (ANOVA p value= 0.42, Figure 4-7B). The results show that hsa-miR-215-5p is differentially-expressed in serum, and is associated with Osteoporosis, but not osteopenia.

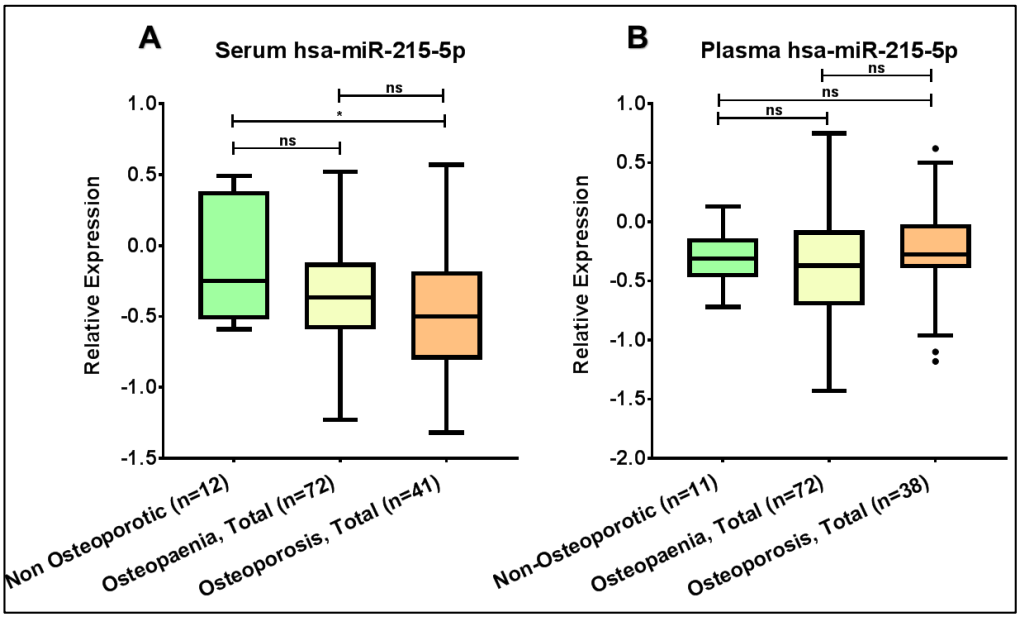


Figure 4-7: Differentially expressed hsa-miR-215-5p associated with Osteoporosis. Box Plot shows the 25th, 50th, and 75th percentiles and minimum to maximum ranges of the levels of hsa-miR-215-5p among non-osteoporosis, osteopenia and osteoporosis groups. Panel A shows a statistically significant correlation of hsa-miR-

215-5p among the osteoporosis patients (ANOVA p = 0.014) compared to the non-Osteoporotic p (Bonferroni's)= 0.013. Panel B shows that the level of hsa-miR-215-5p in plasma samples was not significantly different among non-osteoporotic control, osteopenia patients and osteoporosis patients. ns = not significant and *p <0.05. The comparison indicated by Bonferroni's multiple comparisons test (One-way ANOVA).

Table 4-5: One-way ANOVA multiple comparisons test for hsa-miR-215-5p expression level between Non-Osteoporosis, Osteopenia and Osteoporosis

Data analysed	**ANOVA *p* Value**	**Bonferroni's multiple comparisons test**	**Mean Diff. (95.00% CI of diff.)**	**Bonferroni's *p* Value**
Serum hsa-miR-215-5p	0.014	Osteopenia (n=72) vs. Non-Osteoporotic (n=12)	-0.23 (-0.508 to 0.048)	0.139
		Osteoporosis (n=41) vs. Non-Osteoporotic (n=12)	-0.35 (-0.642 to -0.059)	0.013
		Osteoporosis (n=41) vs. Osteopenia(n=72)	-0.12 (-0.295 to 0.054)	0.292

The levels of circulating hsa-miR-4516 in plasma was significantly lower in the osteoporosis patients compared to both non-osteoporotic and osteopenia groups (ANOVA *p*= 0.009, Figure 4-8B), (Benferroni's p = 0.025, and 0.048 respectively, (Table 4-6). The levels of has-miR-4516 did not show difference in serum samples (ANOVA P value= 0.15) (Figure 4-8A). The results suggest that hsa-miR-4516 might be associated with the development of osteoporosis.

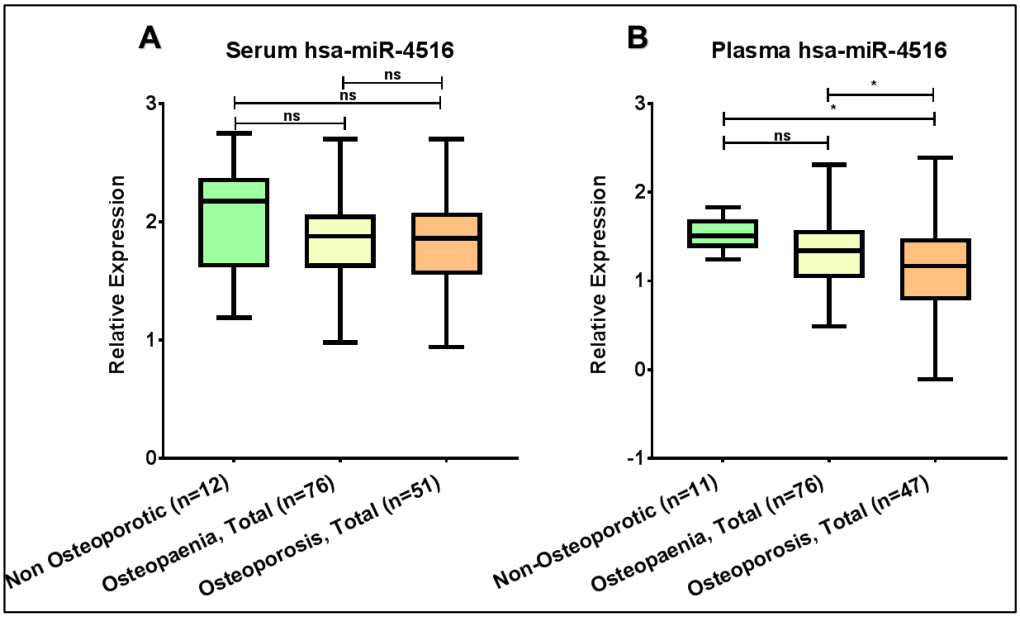


Figure 4-8: Differentially expressed hsa-miR-4516 associated with Osteoporosis. Box Plot show the 25th, 50th, and 75th percentiles and minimum to maximum ranges of the levels of hsa-miR-4516 among non-osteoporosis, osteopenia and osteoporosis groups. Panel A: box plots show the level of hsa-miR-4516 in serum samples was not significant among non-osteoporotic control, osteopenia patients and osteoporosis patients. Panel B, box plots show a statistically significant correlation of hsa-miR-4516 among the osteoporosis patients (ANOVA *p*= 0.009) compared to the non-Osteoporotic and osteopenic, *p* (Bonferroni's)= 0.025 and *p* (Bonferroni's)= 0.048. ns = not significant and **p* <0.05. The comparison indicated by Bonferroni's multiple comparisons test (One-way ANOVA).

Table 4-6: One-way ANOVA multiple comparisons test for hsa-miR-4516 expression level between Non-Osteoporosis, Osteopenia and Osteoporosis.

Data analysed	ANOVA *p* Value	Bonferroni's multiple comparisons test	Mean Diff. (95.00% CI of diff.)	Bonferroni's *p* Value
Plasma hsa-miR-4516	0.009	Osteopenia (n=76) vs. Non-Osteoporotic (n=11)	-0.19 (-0.514 to 0.142)	0.516
		Osteoporosis (n=47) vs. Non-Osteoporotic (n=11)	-0.38 (-0.716 to -0.035)	0.025
		Osteoporosis (n=47) vs. Osteopenia, Total (n=76)	-0.19 (-0.379 to -0.001)	0.048

4.2.6 Identify circulating miRNAs associated with osteoporotic fracture

To find out whether the decrease in bone mineral density associated with or without fragility fracture has an influence on the miRNA levels in serum, osteopenia and osteoporosis participants (>40-year age) were categorised into four subgroups: osteopenia without fracture (n=61), osteopenia with fracture (n=15), osteoporosis without fracture (n=33) and osteoporosis with fracture (n=18) (Materials and Methods 2.3.1 & 2.3.2) and compared with a non-osteoporotic control group (n=12; >40-year age).

hsa-miR-373-5p. The results show that the levels of hsa-miR-373-5p in serum were lower in the osteoporosis patients with fragility fracture compared to the non-osteoporotic control group, osteopenia group (ANOVA $p < 0.0001$) (Figure 4-9A). The levels of hsa-miR-373-5p in serum samples were lower in osteopenia with fracture and osteoporosis without fracture compared to non-osteoporotic (*p* (Bonferroni's) =0.045 and 0.033, respectively, Table 4-7), and even much lower in the osteoporosis patients with fracture compared to Osteopenia and Osteoporosis without fracture groups (*p* (Bonferroni's) = 0.0004 and 0.05, respectively, Table 4-7).

The levels of hsa-miR-373-5p in plasma samples were not significantly different between these groups confirming the results in (Figure 4-9B). The results indicate that the decreased levels of hsa-miR-373-5p in serum might be associated with osteopaenic and osteoporotic fracture.

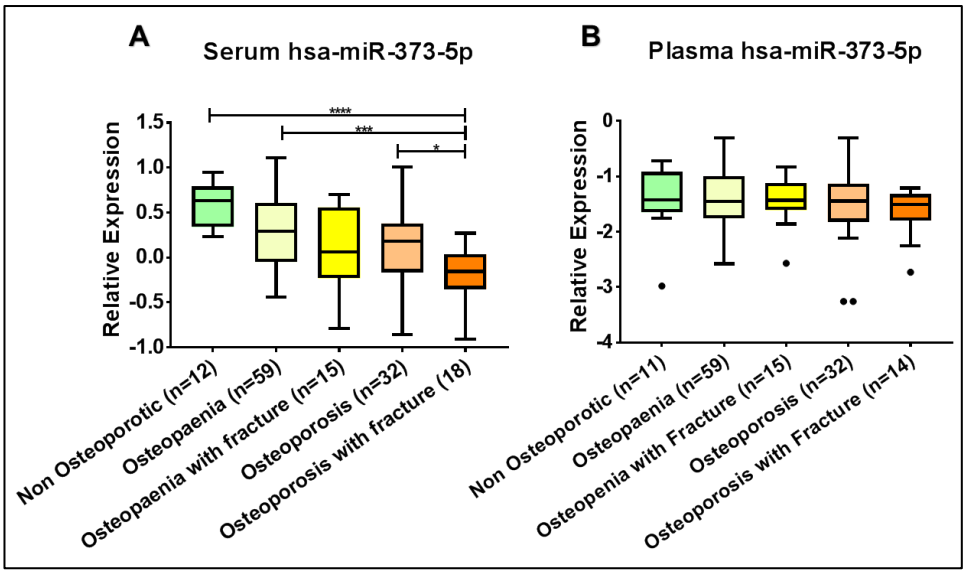


Figure 4-9: Identification of hsa-miR-373-5p associated with osteoporosis.
Panel A: Box pots show that the levels of hsa-miR-373-5p in serum were associated with osteoporosis patients with fracture (ANOVA p<0.0001) compared to non-osteoporotic, osteopenia and osteoporosis (p (Bonferroni's) <0.0001, 0.0004 and 0.05, respectively). Panel B: Box plots show that the levels of hsa-miR-373-5p in plasma were not associated with osteoporosis patients with fracture (ANOVA p=0.59). *p <0.05, ***p <0.001 and ****p <0.0001. The comparison indicated by Bonferroni's multiple comparisons test (One-way ANOVA).

Table 4-7: One-way ANOVA multiple comparisons test for hsa-miR-373-5p expression level between Non-Osteoporosis, Osteopenia, Osteopenia with Fracture, Osteoporosis and Osteoporosis with Fracture

Data analysed	ANOVA *p* Value	Bonferroni's multiple comparisons test	Mean Diff. (95.00% CI of diff.)	Bonferroni's *p* Value
Serum hsa-miR-373-5p	<0.0001	Osteopenia without fracture (n=59) vs. Non-Osteoporotic (n=12)	-0.29 (-0.659 to 0.082)	0.281
		Osteopenia with fracture (n=15) vs. Non-Osteoporotic (n=12)	-0.45 (-0.901 to -0.005)	0.045
		Osteoporosis without fracture (n=32) vs. Non-Osteoporotic (n=12)	-0.41 (-0.808 to -0.019)	0.033

Data analysed	ANOVA *p* Value	Bonferroni's multiple comparisons test	Mean Diff. (95.00% CI of diff.)	Bonferroni's *p* Value
		Osteoporosis with fracture (18) vs. Non-Osteoporotic (n=12)	-0.76 (-1.201 to -0.317)	<0.0001
		Osteopenia with fracture (n=15) vs. Osteopenia without fracture (n=59)	-0.16 (-0.491 to 0.161)	>0.99
		Osteoporosis without fracture (n=32) vs. Osteopenia without fracture (n=59)	-0.13 (-0.373 to 0.123)	>0.99
		Osteoporosis with fracture (18) vs. Osteopenia without fracture (n=59)	-0.47 (-0.789 to -0.153)	0.0004
		Osteoporosis without fracture (n=32) vs. Osteopenia with fracture (n=15)	0.04 (-0.313 to 0.393)	>0.99
		Osteoporosis with fracture (18) vs. Osteopenia with fracture (n=15)	-0.31 (-0.711 to 0.1)	0.332
		Osteoporosis with fracture (18) vs. Osteoporosis without fracture(n=32)	-0.35 (-0.691 to 0)	0.05

hsa-miR-99a-5p. The levels of hsa-miR-99a-5p in serum samples were significantly higher in the osteoporosis patients with fracture compared to the osteopenia and osteoporosis patients (ANOVA p = 0.0002) (Figure 4-10A), (p (Bonferroni's) <0.0001 and 0.0007, respectively (Table 4-8). But the levels of hsa-miR-99a-5p in plasma samples were not significantly difference compared to the non-osteoporotic control group, ANOVA *p*= 0.3, Figure 4-10B) and (p (Bonferroni's= 0.237) (Table 4-8). The results show that the increasing level of hsa-miR-99a-5p in serum might associate with the osteoporotic fracture.

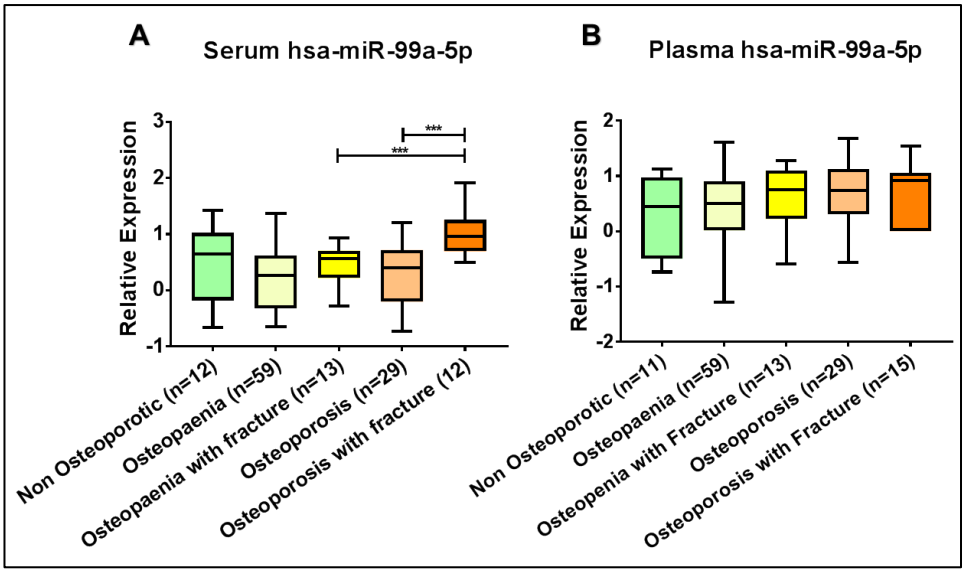


Figure 4-10: Identification of hsa-miR-99a-5p associated with osteoporosis.
Panel A: Box pots show that the levels of hsa-miR-99a-5p in serum were associated with osteoporosis patients with fracture (ANOVA *p*= 0.0002) compared to osteopenia and osteoporosis (p (Bonferroni's) < 0.0001 and 0.0007, respectively). Panel B: Box plots show that the levels of hsa-miR-99a-5p in plasma were not associated with osteoporosis patients with fracture (ANOVA p=0.31). ****p* <0.001. The comparison indicated by Bonferroni's multiple comparisons test (One-way ANOVA).

Table 4-8: One-way ANOVA multiple comparisons test for hsa-miR-99a-5p expression level between Non-Osteoporosis, Osteopenia, Osteopenia with Fracture, Osteoporosis and Osteoporosis with Fracture

Data analysed	ANOVA *p* Value	Bonferroni's multiple comparisons test	Mean Diff. (95.00% CI of diff.)	Bonferroni's *p* Value
Serum hsa-miR-99a-5p	0.0002	Osteopenia without fracture (n=59) vs. Non-Osteoporotic (n=12)	-0.29 (-0.78 to 0.21)	0.969
		Osteopenia with fracture (n=13) vs. Non-Osteoporotic (n=12)	-0.05 (-0.67 to 0.58)	>0.99
		Osteoporosis without fracture (n=29) vs. Non-Osteoporotic (n=12)	-0.26 (-0.79 to 0.28)	>0.99
		Osteoporosis with fracture (12) vs. Non-Osteoporotic (n=12)	0.51 (-0.13 to 1.15)	0.237
		Osteopenia with fracture (n=13) vs. Osteopenia without fracture (n=59)	0.24 (-0.24 to 0.72)	>0.99

Data analysed	ANOVA *p* Value	Bonferroni's multiple comparisons test	Mean Diff. (95.00% CI of diff.)	Bonferroni's *p* Value
		Osteoporosis without fracture (n=29) vs. Osteopenia without fracture (n=59)	0.03 (-0.32 to 0.38)	>0.99
		Osteoporosis with fracture (12) vs. Osteopenia without fracture (n=59)	0.8 (0.31 to 1.29)	<0.0001
		Osteoporosis without fracture (n=29) vs. Osteopenia with fracture (n=13)	-0.21 (-0.73 to 0.31)	>0.99
		Osteoporosis with fracture (12) vs. Osteopenia with fracture (n=13)	0.56 (-0.07 to 1.18)	0.121
		Osteoporosis with fracture (12) vs. Osteoporosis without fracture (n=29)	0.77 (0.23 to 1.30)	0.0007

hsa-miR-122-5p. The levels of hsa-miR-122-5p in serum were significantly lower in the osteoporosis patients with fracture compared to the non-osteoporotic and osteoporosis without fracture patients (ANOVA p= 0.03, (Figure 4-11A), (*p* (Bonferroni's) = 0.049 and 0.048, respectively (Table 4-9). However, the levels of hsa-miR-122-5p in plasma did not show significant difference between these groups (Figure 4-11B). The results indicate that the decreasing levels of hsa-miR-122-5p in serum might is statistically be associated with the osteoporotic fracture.

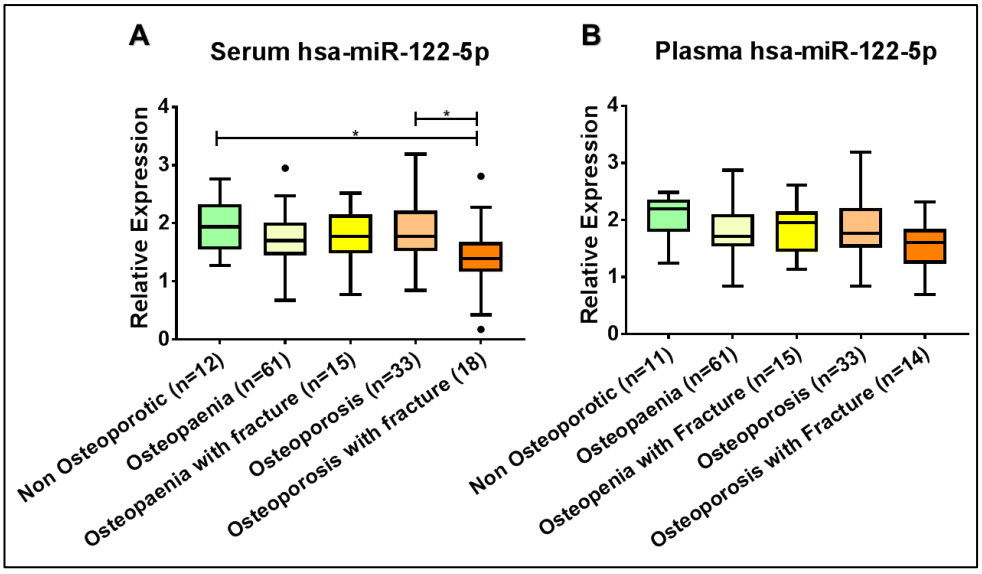


Figure 4-11: Identification of hsa-miR-122-5p associated with osteoporosis.
Panel A: Box pots show that the levels of hsa-miR-122-5p in serum were associated with osteoporosis patients with fracture (ANOVA *p*= 0.03) compared to non-osteoporotic and osteoporosis (p (Bonferroni's)= 0.049, and 0.048, respectively). Panel B: Box plots show that the levels of hsa-miR-122-5p in plasma were not associated with osteoporosis patients with fracture (ANOVA *p*=0.228). **p* <0.05. The comparison indicated by Bonferroni's multiple comparisons test (One-way ANOVA).

Table 4-9: One-way ANOVA multiple comparisons test for hsa-miR-122-5p expression level between Non-Osteoporosis, Osteopenia, Osteopenia with Fracture, Osteoporosis and Osteoporosis with Fracture

Data analysed	ANOVA *p* Value	Bonferroni's multiple comparisons test	Mean Diff. (95.00% CI of diff.)	Bonferroni's *p* Value
Serum hsa-miR-122-5p	0.0299	Osteopenia (n=61) vs. Non-Osteoporotic (n=12)	-0.22 (-0.65 to 0.21)	>0.99
		Osteopenia with fracture (n=15) vs. Non-Osteoporotic (n=12)	-0.16 (-0.69 to 0.36)	>0.99
		Osteoporosis without fracture (n=33) vs. Non-Osteoporotic (n=12)	-0.11 (-0.57 to 0.35)	>0.99
		Osteoporosis with fracture (18) vs. Non-Osteoporotic (n=12)	-0.51 (-1.02 to 0)	0.049
		Osteopenia with fracture (n=15) vs. Osteopenia without fracture (n=61)	0.06 (-0.33 to 0.45)	>0.99
		Osteoporosis without fracture (n=33) vs. Osteopenia without fracture (n=61)	0.11 (-0.18 to 0.41)	>0.99

Data analysed	ANOVA *p* Value	Bonferroni's multiple comparisons test	Mean Diff. (95.00% CI of diff.)	Bonferroni's *p* Value
		Osteoporosis with fracture (18) vs. Osteopenia without fracture (n=61)	-0.29 (-0.65 to 0.08)	0.266
		Osteoporosis without fracture (n=33) vs. Osteopenia with fracture (n=15)	0.06 (-0.37 to 0.48)	>0.99
		Osteoporosis with fracture (18) vs. Osteopenia with fracture (n=15)	-0.34 (-0.82 to 0.13)	0.41
		Osteoporosis with fracture (18) vs. Osteoporosis without fracture (n=33)	-0.4 (-0.8 to 0)	0.048

hsa-miR-4516. The levels of hsa-miR-4516 in plasma were significantly lower in the osteoporosis with fracture patients compared to non-osteoporosis and osteopenia without fracture (ANOVA *p* value= 0.0002, Figure 4-12B), *p* (Benferroni's)= 0.004 and 0.002, respectively) (Table 4-10). Furthermore, the level hsa-miR-4516 in plasma were also lower in osteopenia with fracture compared to non-osteoporosis and osteopenia without fracture groups with insignificant p (Benferroni's) of 0.06 and 0.06, respectively) (Table 4-10). In contrast, the levels of hsa-miR-4516 in serum did not show significant differences between groups (Figure 4-12A). The results indicate that plasma hsa-miR-4516 might associate with osteoporotic fracture.

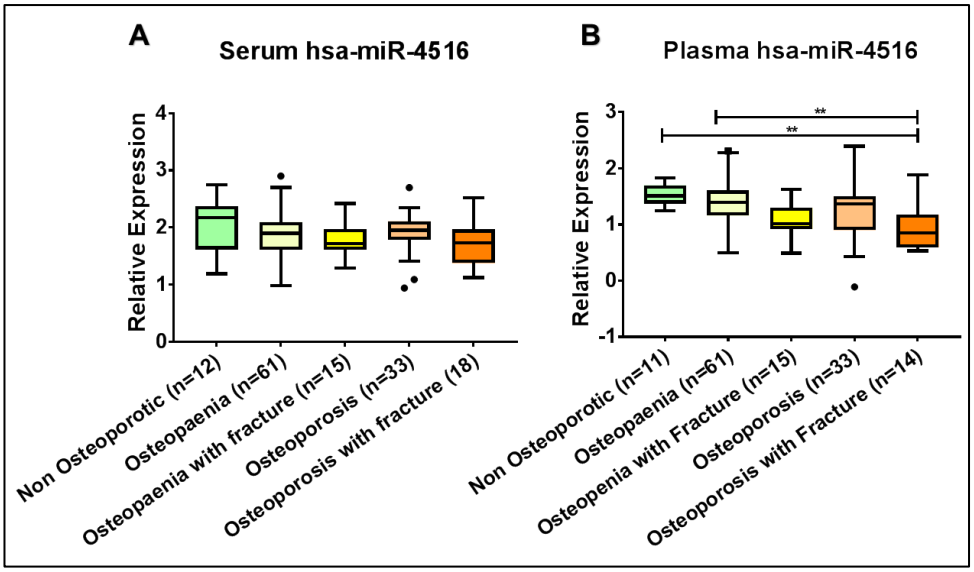


Figure 4-12: Identification of hsa-miR-4516 associated with osteoporosis.
Panel A: Box plots show that the levels of hsa-miR-4516 in serum were not associated with osteoporosis patients with fracture (ANOVA *p*=0.140). Panel B: Box plots show that the levels of hsa-miR-4516 in plasma were associated with osteoporosis patients with fracture (ANOVA *p*= 0.0002) compared to non-osteoporotic and osteopenia (*p* (Bonferroni's)= 0.004 and 0.002, respectively. ***p* <0.01. The comparison indicated by Bonferroni's multiple comparisons test (One-way ANOVA).

Table 4-10: One-way ANOVA multiple comparisons test for hsa-miR-4516 expression level between Non-Osteoporosis, Osteopenia, Osteopenia with Fracture, Osteoporosis and Osteoporosis with Fracture

Data analysed	ANOVA *p* Value	Bonferroni's multiple comparisons test	Mean Diff. (95.00% CI of diff.)	Bonferroni's *p* Value
Plasma hsa-miR-4516	0.0002	Osteopenia without fracture (n=61) vs. Non-Osteoporotic (n=11)	-0.12 (-0.5 to 0.25)	>0.99
		Osteopenia with Fracture (n=15) vs. Non-Osteoporotic (n=11)	-0.44 (-0.9 to 0.01)	0.062
		Osteoporosis without fracture (n=33) vs. Non-Osteoporotic (n=11)	-0.29 (-0.69 to 0.11)	0.43
		Osteoporosis with Fracture (n=14) vs. Non-Osteoporotic (n=11)	-0.59 (-1.05 to -0.12)	0.004
		Osteopenia with Fracture (n=15) vs. Osteopenia without fracture (n=61)	-0.32 (-0.65 to 0.01)	0.062
		Osteoporosis without fracture (n=33) vs.	-0.16 (-0.41 to 0.08)	0.609

Data analysed	**ANOVA *p* Value**	**Bonferroni's multiple comparisons test**	**Mean Diff. (95.00% CI of diff.)**	**Bonferroni's *p* Value**
		Osteopenia without fracture (n=61)		
		Osteoporosis with Fracture (n=14) vs. Osteopenia without fracture (n=61)	-0.46 (-0.8 to -0.12)	0.002
		Osteoporosis without fracture (n=33) vs. Osteopenia with Fracture (n=15)	0.16 (-0.2 to 0.52)	>0.99
		Osteoporosis with Fracture (n=14) vs. Osteopenia with Fracture (n=15)	-0.14 (-0.57 to 0.29)	>0.99
		Osteoporosis with Fracture (n=14) vs. Osteoporosis without fracture (n=33)	-0.3 (-0.67 to 0.07)	0.212

hsa-miR-215-5p and hsa-miR-100-5p. The levels of circulating miRNAs, hsa-miR-215-5p and hsa-miR-100-5p in clinical samples did not show significant difference among the five groups: Non-Osteoporosis, Osteopenia, Osteopenia without fracture, Osteoporosis and Osteoporosis without fracture.

4.2.7 miRNA Expression in Low Bone Mineral Density (BMD) Fractured Individuals

To find out if the fragility fracture rather than the Bone Mineral Density (BMD) correlates with the miRNA expression, participants' serum and plasma miRNA data were categorised into three main groups: non-osteoporotic (control group) (T Score > -1.0), Low Bone Mineral Density (BMD) without fracture (T Score ≤ -1.0) and low BMD with fracture (T Score ≤ -1.0). One-way ANOVA analysis followed by 'Bonferroni's multiple comparison test' were used as before. The results show that four miRNAs, hsa-miR-373-5p, hsa-miR-99a-5p, hsa-miR-215-5p and hsa-miR-4516 were strongly associated with patients' low BMD and fracture.

The levels of hsa-miR-373-5p in serum were significantly lower in the low BMD with fracture group compared to the non-osteoporosis controls and the low BMD without fracture patients (ANOVA p <0.0001, Figure 4-13A) (Bonferroni's p of <0.0001 and 0.0030, respectively). The levels of hsa-miR-373-5p in plasma were not significant different in the low BMD with fracture patients compared to the non-osteoporosis and the low BMD without fracture patients (ANOVA p= 0.64, Figure 4-13B). The results indicate that the decreasing levels of hsa-miR-373-5p in serum might be associated with low BMD and fragility fracture in these patients.

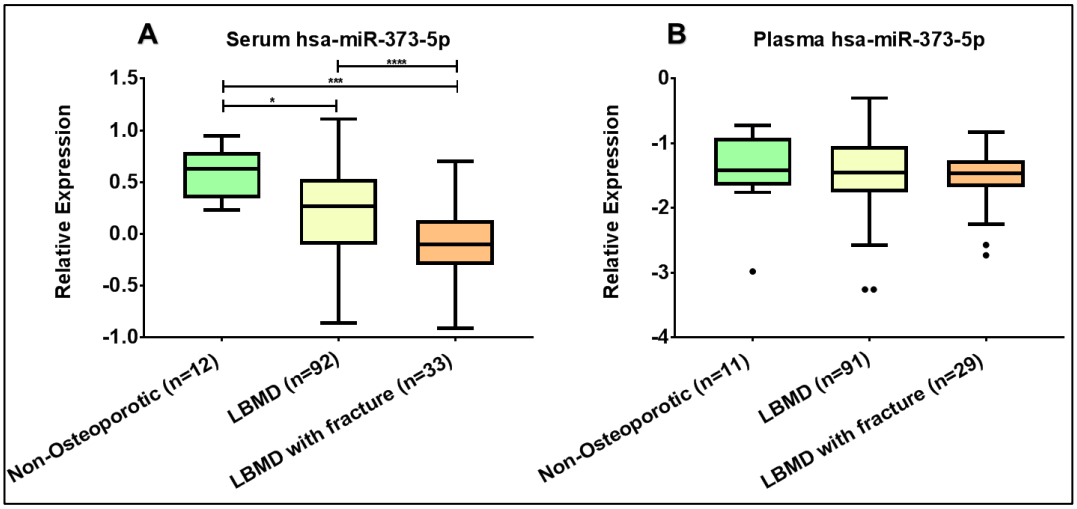


Figure 4-13: Identification of hsa-miR-373-5p associated with Low Bone Mineral Density with Fracture.

Panel A: Box plots show that the levels of hsa-miR-373-5p in serum were significantly lower in the low BMD with fracture patients compared to the non-osteoporosis and Low BMD without fracture patients (ANOVA p <0.0001). Panel B: Box plots show the levels of hsa-miR-373-5p in plasma were not significant different in the low BMD with fracture patients compared to the non-osteoporosis and Low BMD without fracture patients (ANOVA p=0.64). *p <0.05 and ***p <0.001. The comparison indicated by Bonferroni's multiple comparisons test (One-way ANOVA).

The levels of hsa-miR-99a-5p in serum were significantly higher in the low BMD with fracture patients compared to the low BMD without fracture patients, (ANOVA p <0.0001, Figure 4-14A). The levels of hsa-miR-99a-5p in plasma samples was not significant difference between these groups (ANOVA p= 0.26, Figure 4-13B). The results indicate that the increasing levels of hsa-miR-99a-5p in serum might associated with the fragility fracture in the low BMD with fracture patients.

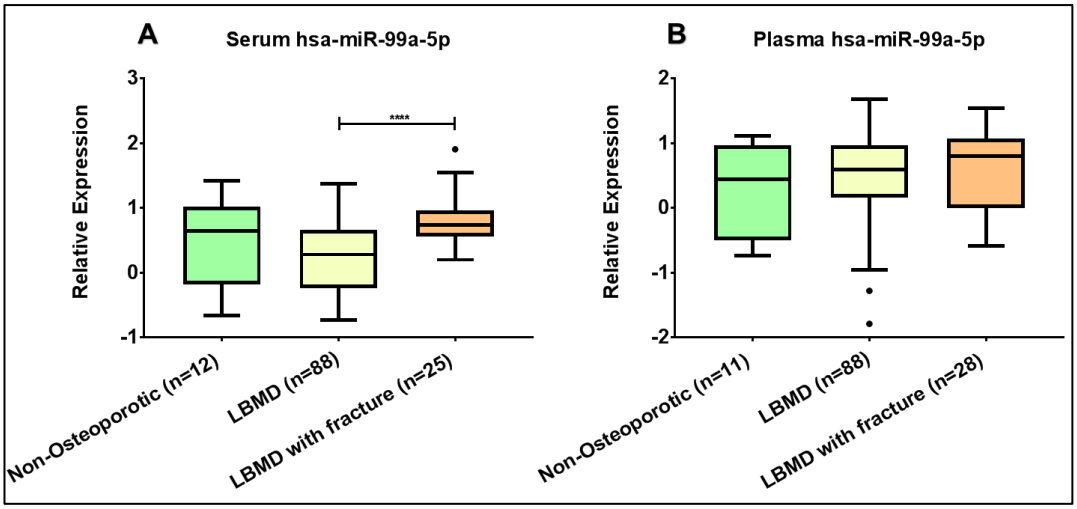


Figure 4-14: Identification of hsa-miR-99a-5p associated with Low Bone Mineral Density with Fracture.

Panel A: Box plots show the levels of hsa-miR-99a-5p in serum were significant lower in the low BMD with fracture patients compared to Low BMD without fracture patients (ANOVA p <0.0001). Panel B: Box plots show the levels of hsa-miR-99a-5p in plasma were not significant different in the low BMD with fracture patients compared to the non-osteoporosis and Low BMD without fracture patients (ANOVA p= 0.26). ***p <0.001. The comparison indicated by Bonferroni's multiple comparisons test (One-way ANOVA).

The levels of hsa-miR-215-5p in serum samples were lower in the low BMD with fracture patients compared to non-osteoporosis controls (ANOVA p = 0.021, Figure 4-15A). However, the levels of hsa-miR-215-5p in plasma samples were not significant among the three group (p =0.768, Figure 4-15B).

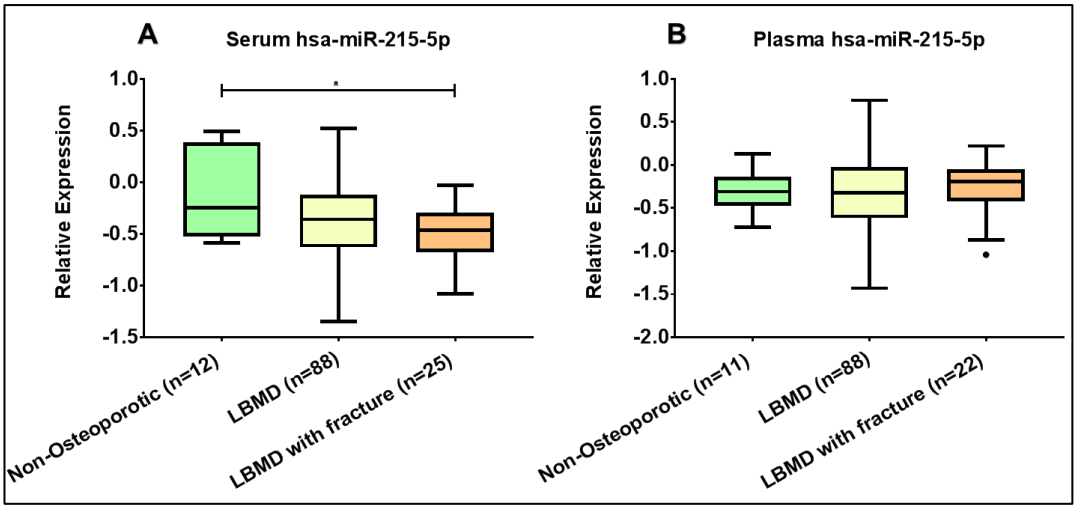


Figure 4-15: Identification of hsa-miR-215-5p associated with Low Bone Mineral Density with Fracture.

Panel A: Box plots show the levels of hsa-miR-215-5p in serum were significant lower in the low BMD with fracture patients compared to the non-osteoporosis (ANOVA *p* =0.021). Panel B: Box plots show the levels of hsa-miR-215-5p in plasma were not significant different in the low BMD with fracture patients compared to the non-osteoporosis and Low BMD without fracture patients (ANOVA *p*=0.77). **p* <0.05. The comparison indicated by Bonferroni's multiple comparisons test (One-way ANOVA).

The levels of hsa-miR-4516 in serum and plasma samples were much lower in the low BMD with fracture patients compared to the non-osteoporosis controls and the low BMD without fracture patients (ANOVA *p*= 0.03 and <0.001, respectively), (Figure 4-16A & B), Bonferroni's *p*= 0.036 and 0.0015, respectively. In addition, the levels of hsa-miR-4516 in plasma samples were significantly lower in the low BMD with fracture patients compared to LBMD without fracture patients (Bonferroni' *p*= 0.0005). The results suggest that decreased levels of hsa-miR-4516 in clinical samples might be associated with osteoporotic fragility fracture in these patients.

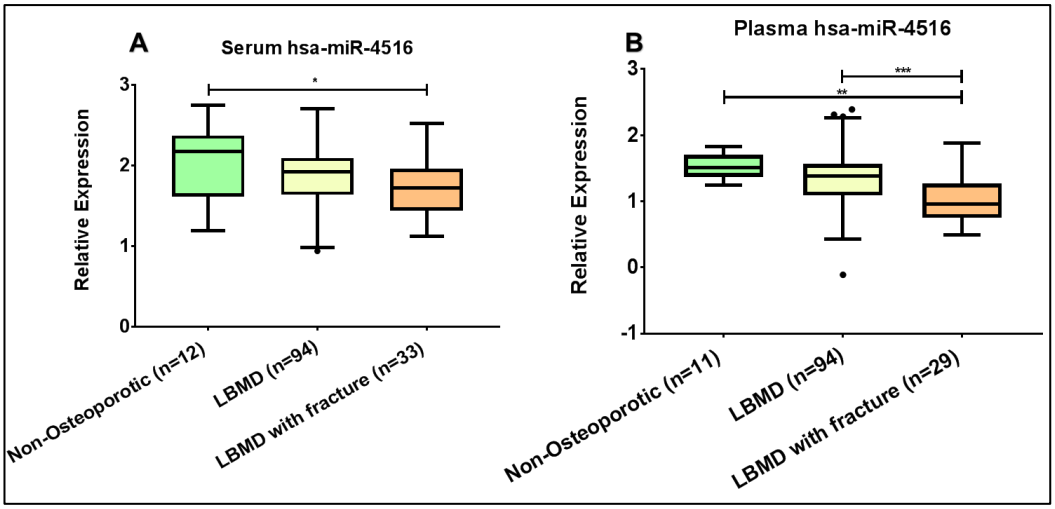


Figure 4-16: Identification of hsa-miR-4516 associated with Low Bone Mineral Density with Fracture.

Panel A: Box plots show the levels of hsa-miR-4516 in serum were significant lower in the low BMD with fracture patients compared to the non-osteoporosis (ANOVA *p* =0.033). Panel B: Box plots show the levels of hsa-miR-4516 in plasma were lower in the low BMD with fracture patients compared to the non-osteoporosis and Low BMD without fracture patients (ANOVA *p*=0.0001). **p* <0.05, ***p* <0.01 and ****p* <0.001. The comparison indicated by Bonferroni's multiple comparisons test (One-way ANOVA).

4.2.8 Identify miRNAs from the panel that are associated with lumbar spine T-Score

Identification of miRNAs associated with the bone mineral density (BMD) Lumbar spine (LS) T-score were conducted to verify if there is a significant association between the up-/down- regulation of these miRNAs with lumbar spine T-score.

The increased levels of hsa-miR-373-5p and hsa-miR-4516 in serum and plasma samples were significantly associated with the lumbar spine (L2-L4) T-score as shown in (Figure 4-17A & B) and (Figure 4-18A & B). Pearson coefficient correlation analysis showed that hsa-miR-373-5p in serum and plasma are strongly associated with lumbar spine (L2-4) T-score (*p*= 0.0002 and 0.0051, respectively) (Figure 4-17A & B). The levels of hsa-miR-4516 in serum or plasma samples were significantly associated with lumbar spine (L2-L4) T-score (*p* = 0.031 and 0.0002, respectively) (Figure 4-18A & B).

The levels of both hsa-miR-122-5p and hsa-miR-215-5p in serum were also significantly increased with the lumbar spine (L2-L4) T-score (p = 0.0083 and 0.0435, respectively) (Figure 4-19, A & Figure 4-20, A). Nevertheless, the levels of hsa-miR-122-5p and hsa-miR-215-5p in plasma samples were not significant associated with lumbar spine (L2-L4) (Figure 4-19B and Figure 4-20B).

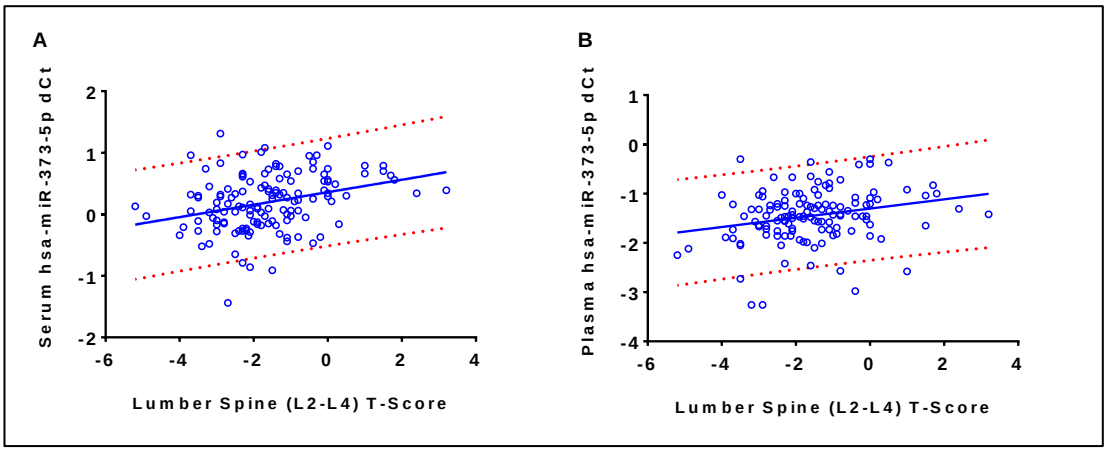


Figure 4-17: Correlation of the levels of hsa-miR-373-5p in clinical samples with the lumbar spine (L2-L4) T-Score.

Panel A: The levels of hsa-miR-373-5p in serum significantly increased with increasing lumbar spine (L2-L4) T-score of the subject (p (two-tailed) = 0.0002). Panel B: The levels of hsa-miR-373-5p in plasma samples significantly increased with increasing lumbar spine (L2-L4) of the subject (p (two-tailed) = 0.0051). Blue line= regression line and Red line= 95% prediction band.

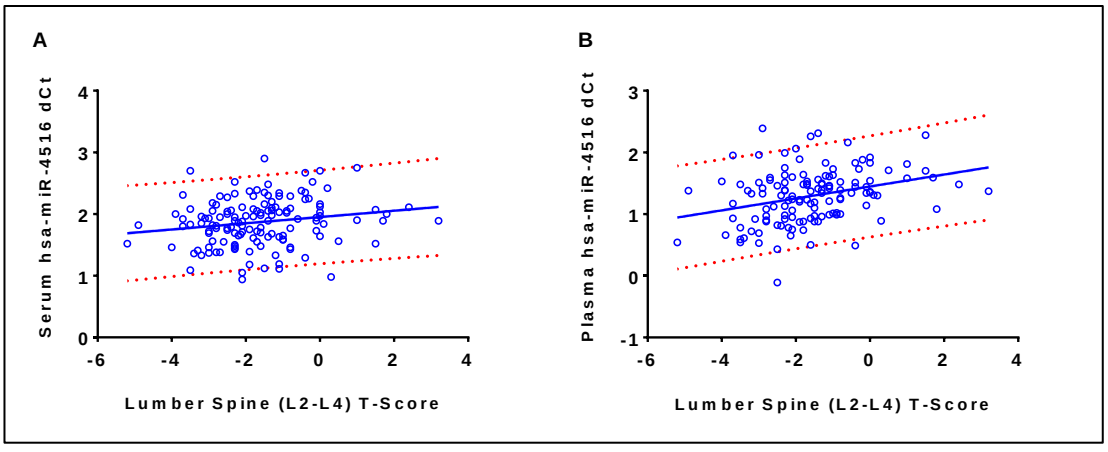


Figure 4-18: Correlation of the levels of hsa-miR-4516 in clinical samples with the lumbar spine (L2-L4) T-Score.

Panel A: The levels of hsa-miR-4516 in serum significantly increased with increasing lumbar spine (L2-L4) of the subject (p (two-tailed) = 0.031). Panel B: The levels of hsa-miR-4516 in plasma samples significantly increased with increasing lumbar spine (L2-

L4) of the subject (*p* (two-tailed) = 0.0002). Blue line= regression line and Red line= 95% prediction band.

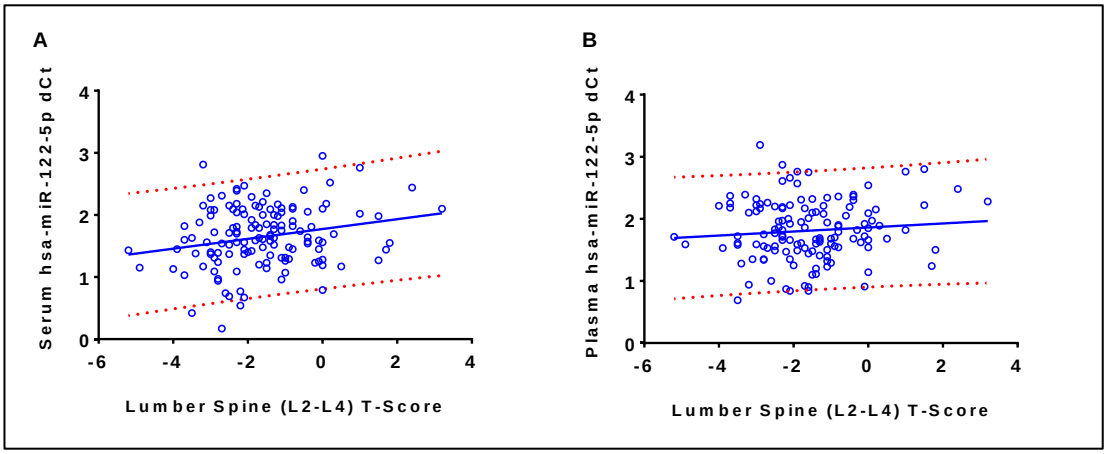


Figure 4-19: Correlation of the levels of hsa-miR-122-5p in clinical samples with the lumbar spine (L2-L4) T-Score.

Panel A: The levels of hsa-miR-122-5p in serum significantly increased with increasing lumbar spine (L2-L4) of the subject (*p* (two-tailed) = 0.008). Panel B: The levels of hsa-miR-122-5p in plasma samples was not significant associated with the lumbar spine (L2-L4) of the subject (*p* (two-tailed) = 0.28). Blue line= regression line and Red line= 95% prediction band.

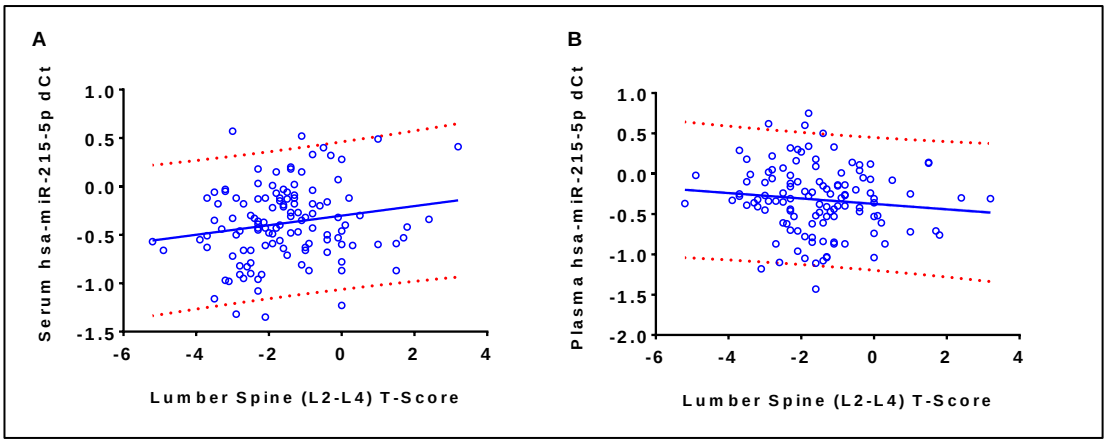


Figure 4-20: Correlation of the levels of hsa-miR-215-5p in clinical samples with the lumbar spine (L2-L4) T-Score.

Panel A: The levels of hsa-miR-215-5p in serum significantly increased with increasing lumbar spine (L2-L4) of the subject (*p* (two-tailed) = 0.043). Panel B: The levels of hsa-miR-215-5p in plasma samples was not significant associated with lumbar spine (L2-L4) of the subject (*p* (two-tailed) = 0.21). Blue line= regression line and Red line= 95% prediction band.

4.2.9 Correlation Among Three Serum miRNAs Expression

To verify if combining the downregulation of the three serum miRNAs, hsa-miR-373-5p, hsa-miR-122-5p and hsa-miR-215-5p, has a significant clinical empowerment on the diagnosis of osteoporosis, a correlation study for the mixed level of these three miRNAs was performed.

All the three circulating serum miRNAs have relatively similar expression among the three main groups: non-osteoporosis control and osteopenia (with/without fracture) and Osteoporosis (with/without fracture) groups, between hsa-miR-373-5p and hsa-miR-122-5p with *p* value of 0.021 (Pearson's r= 0.2), between hsa-miR-373-5p and hsa-miR-215-5p *with p* value of 0.01 (Pearson's r= 0.23) and between hsa-miR-122-5p and hsa-miR-215-5p with p value <0.0001 (Pearson's r= 0.23). However, there were no distinct up/or down regulation populations of the three main study groups: Non-Osteoporosis (colour= green) Osteopenia (colour= yellow/blue/or brown) and Osteoporosis groups (colour= olive green/navy blue/or dark brown) (Figure 4-21).

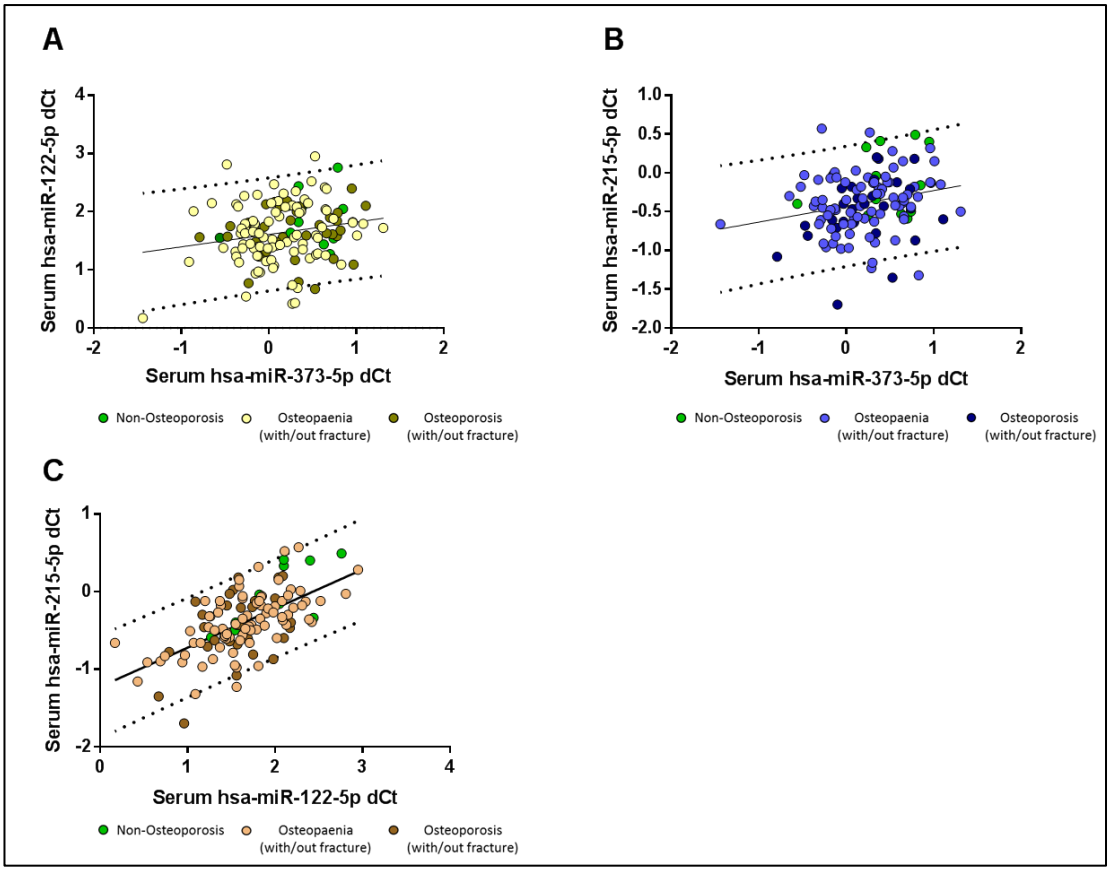


Figure 4-21: Correlation of miRNA expression between three circulating serum miRNAs

Pearson coefficient correlation analysis showed that the expression level of the three circulating serum correlates to each other: hsa-miR-373-5p vs. hsa-miR-122-5p with p value =0.021 (A) hsa-miR-373-5p vs. hsa-miR-215-5p with p value =0.011 (B) and hsa-miR-122-5p vs. hsa-miR-215-5p with p value <0.0001. Colour: Green for Non-osteoporosis, Yellow/blue/or Brown= Osteopenia (with/out fracture) and Olive green/navy blue/or dark Brown = Osteoporosis (with/out fracture).

4.2.10 Diagnostic Value of miRNAs for osteoporosis

The assessment of potential diagnostic value of the four miRNAs in serum and plasma for osteoporosis was performed using a receiver operating characteristic (ROC) curve. The associated area under the curve (AUC) for the relative expression of these miRNAs (hsa-miR-373-5p, hsa-miR-122-5p, hsa-miR-215-5p and hsa-miR-4516) was used to confirm the diagnostic value of each miRNA (Table 4-11).

There are acceptable diagnostic values for the levels of hsa-miR-373-5p in serum and the levels of hsa-miR-4516 in plasma for osteoporosis patients (AUC= of 0.717, *p* =0.013) and (AUC= 0.727, *p* =0.023), respectively) (Figure 4-22A & B). This indicates that both miRNAs have the potential to be used as a diagnostic marker for osteoporosis.

The AUC discrimination accuracy for serum for the remaining two miRNAs: hsa-miR-215-5p and hsa-miR-122-5p were low (0. 6≤ AUC <0.7) as shown (Table 4-11). AUC was 0.67 for both serum hsa-miR-215-5p and hsa-miR-122-5p (P value=0.056 and 0.058, respectively) (Figure 4-22C & D).

Table 4-11: Diagnostic value of serum and plasma miRNAs for Osteoporosis

Data analysed	**Specimen**	**AUC (95% CI)**	***p* value**
hsa-miR-373-5p	Serum	0.717 (0.568 to 0.865)	0.013
hsa-miR-4516	Plasma	0.727 (0.613 to 0.841)	0.023
hsa-miR-215-5p	Serum	0.668 (0.507 to 0.829)	0.056
hsa-miR-122-5p	Serum	0.666 (0.512 to 0.820)	0.058

p value <0.05 is significant.

The sensitivity and specificity associated with the optimal cut-off points for the 4 circulating miRNAs in clinical samples from osteoporosis patients are listed in Table 4-12. The sensitivity and specificity for hsa-miR-373-5p is 66.1% and 75.0%, and 68.8% and 66.7% for hsa-miR-4516. While the remaining miRNAs had variable sensitivity with low specificity of 58.3% for the optimal cut off point.

Table 4-12: Sensitivity and Specificity of the miRNAs in clinical samples from osteoporosis patients

miRNA	Specimen Type	Sensitivity (%)	Specificity (%)
hsa-miR-373-5p	Serum	66.1%	75.0%
hsa-miR-4516	Plasma	68.8%	66.7%
hsa-miR-122-5p	Serum	66.1%	58.3%
hsa-miR-215-5p	Serum	56.6%	58.3%

Data were evaluated via a cut-off point which jointly maximises both sensitivity and specificity. If the cut-off point is raised, there are fewer false positives but more false negatives the test is highly specific but not very sensitive.

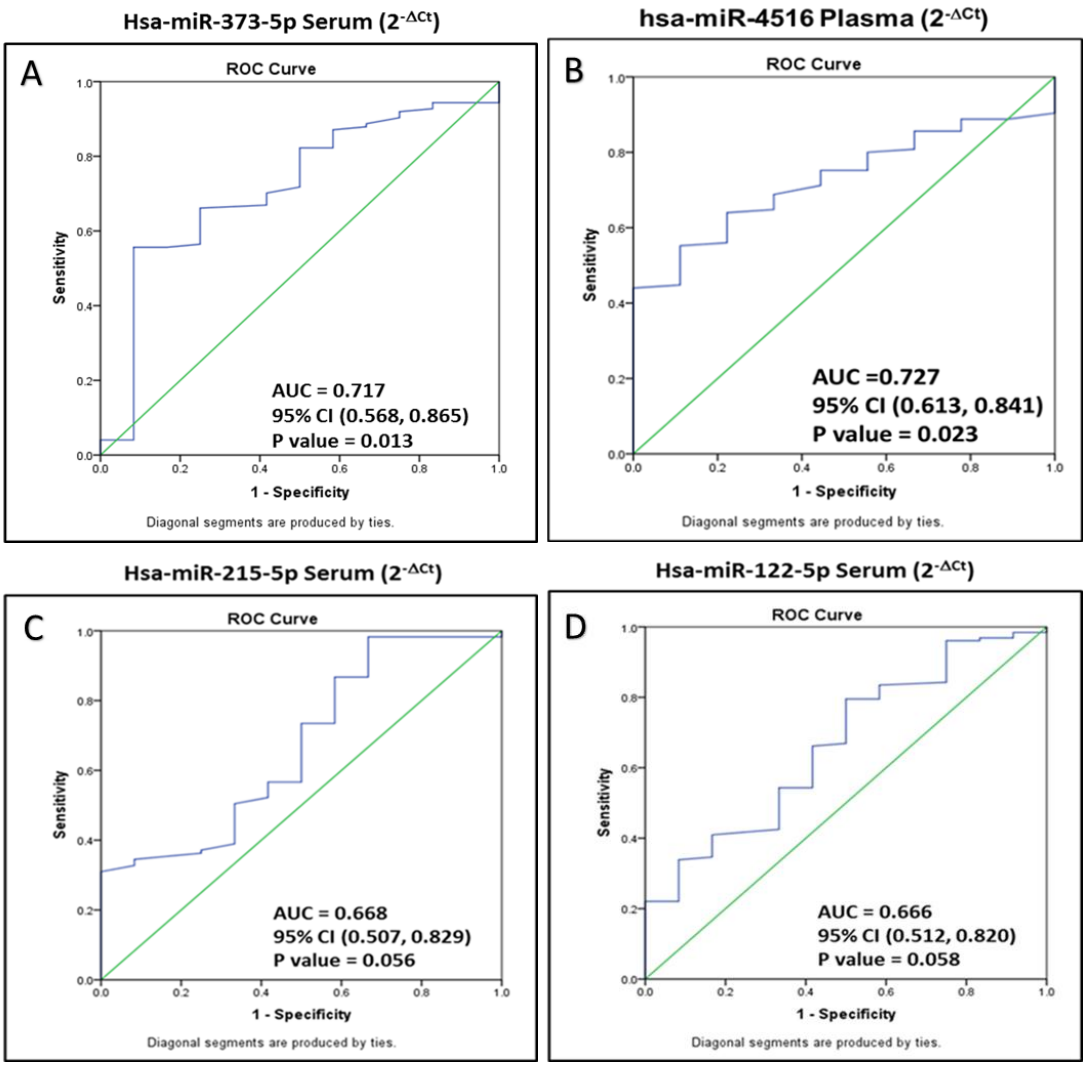


Figure 4-22: Diagnostic value of circulating miRNAs for osteoporosis.
ROC curves show acceptable AUC for hsa-miR-373-5p in serum samples (A) and hsa-miR-4516 in plasma samples (B) from osteoporosis patients, [AUC= 0.717 and 0.727] and *p* (two-tailed) = 0.013 and 0.023, respectively. ROC curves show fair AUC for hsa-miR-215-5p (C) and hsa-miR-122-5p (D) in serum samples from osteoporosis patients [AUC= 0.668 and 0.666] and *p* (two-tailed) = 0.056 and 0.058, respectively. AUC = Area Under the Curve. a fair AUC for hsa-miR-122-5p in serum samples from osteoporosis patients [AUC= 0.666,95% CI = 0.512 to 0.820, p = 0.058]. CI = Confidence interval. AUC <0.6 fail (discrimination no better than chance), 0. 6≤ AUC <0.7 low accuracy, 0.7 ≤ AUC < 0.9 moderate accuracy and AUC ≥ 0.9 high accuracy (Akobeng, 2007).

AUC discrimination was performed for the combined level of the three miRNAs: hsa-miR-373-5p, hsa-miR-122-5p and hsa-miR-122-5p in serum and hsa-miR-4516 in plasma of osteoporosis patients to find out if the combined analytical data have a

more powerful AUC diagnostic value (Figure 4-23). The ROC curve for these four miRNAs together for osteoporosis patients showed an improved AUC value of 0.774 (*p* value=0.007).

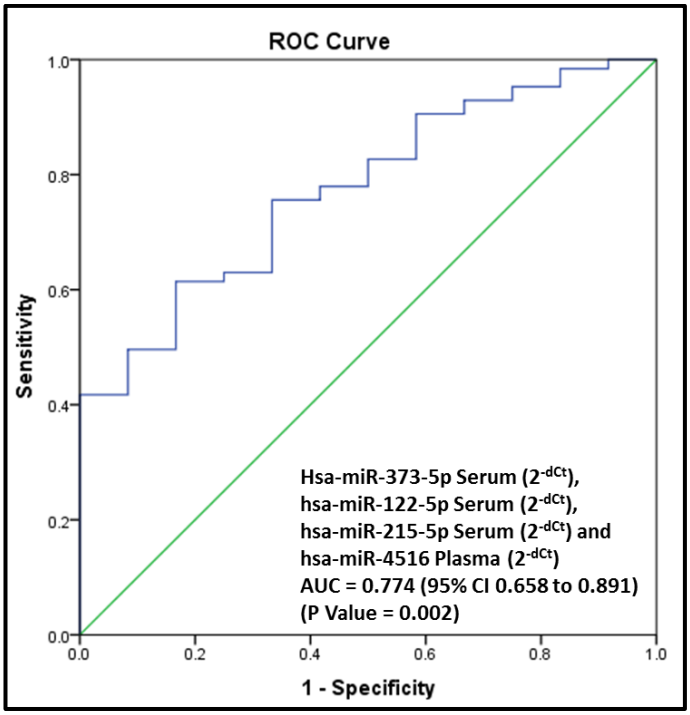


Figure 4-23: Diagnostic value of four combined circulating miRNAs for osteoporosis. ROC curve show an acceptable AUC for the three miRNAs: hsa-miR-373-5p, hsa-miR-122-5p and hsa-miR-215-5p in serum and hsa-miR-4516 in plasma samples from osteoporosis patients [AUC= 0.774 and *p*=0.002]. AUC = Area Under Cover. AUC <0.06 fail (discrimination no better than chance), 0. 6≤ AUC <0.7 low accuracy, 0.7 ≤ AUC < 0.9 moderate accuracy and AUC ≥ 0.9 high accuracy.

4.2.11 Diagnostic Value Assessment Based on Serum/Plasma miRNA Expression and T-Score

The potential diagnostic value of the significantly regulated miRNAs with the association of Lumbar Spine T-Score (LS2-LS4) record was performed using a receiver operating characteristic (ROC) curve. The relative expression of the four circulating miRNAs: hsa-miR-373-5p, hsa-miR-122-5p, hsa-miR-215-5p and hsa-miR-4516 from the participants' serum and plasma samples were logarithmically (Log10) transformed, and the associated area under the curve (AUC) was used to identify any diagnostic value of each miRNA.

Hsa-miR-373-5p, hsa-miR-215-5p and hsa-miR-122-5p associated with a Lumbar Spine (LS) T-Score in the serum of osteoporosis patients have an AUC of 0.921, 0.940 and 0.933, respectively with (*p* (two-tailed) <0.001) (Figure 4-24A, C & D). Hsa-miR-4516 in plasma with the association of LS T-score have an AUC of 0.918 (*p* (two-tailed) < 0.001) (Figure 4-24B). This indicates that these miRNAs: hsa-miR-373-5p, miR-215-5p, hsa-miR-122-5p and hsa-miR-4516 have an improved diagnostic AUC accuracy when associated with BMD T-score.

The sensitivity and specificity associated with the optimal cut-off points related to the four circulating miRNAs are listed in Table 4-13. Circulating serum hsa-miR-215-5p has the highest sensitivity and specificity for the optimal cut off point of 86.6% and 83.3% respectively, and sensitivity of 89.9% and specificity of 83.3%. for serum hsa-miR-373-5p. Both hsa-miR-4516 in plasma and hsa-miR-122-5p in serum of osteoporosis patients have an improved sensitivity of 85% and specificity of 83.3% when associated with BMD T-Score.

Table 4-13: Sensitivity and Specificity of the Regulated miRNAs in the serum and plasma of Osteoporotic and Non-Osteoporotic Participants associated with Lumbar Spine (LS2-4) T-score.

miRNA	Specimen Type	Sensitivity (%)	Specificity (%)
hsa-miR-215-5p	Serum	89.80%	83.30%
hsa-miR-373-5p	Serum	86.60%	83.30%
hsa-miR-4516	Plasma	85.00%	83.30%
hsa-miR-122-5p	Serum	85.80%	83.30%

Data were evaluated via a cut-off point which jointly maximises both sensitivity and specificity.

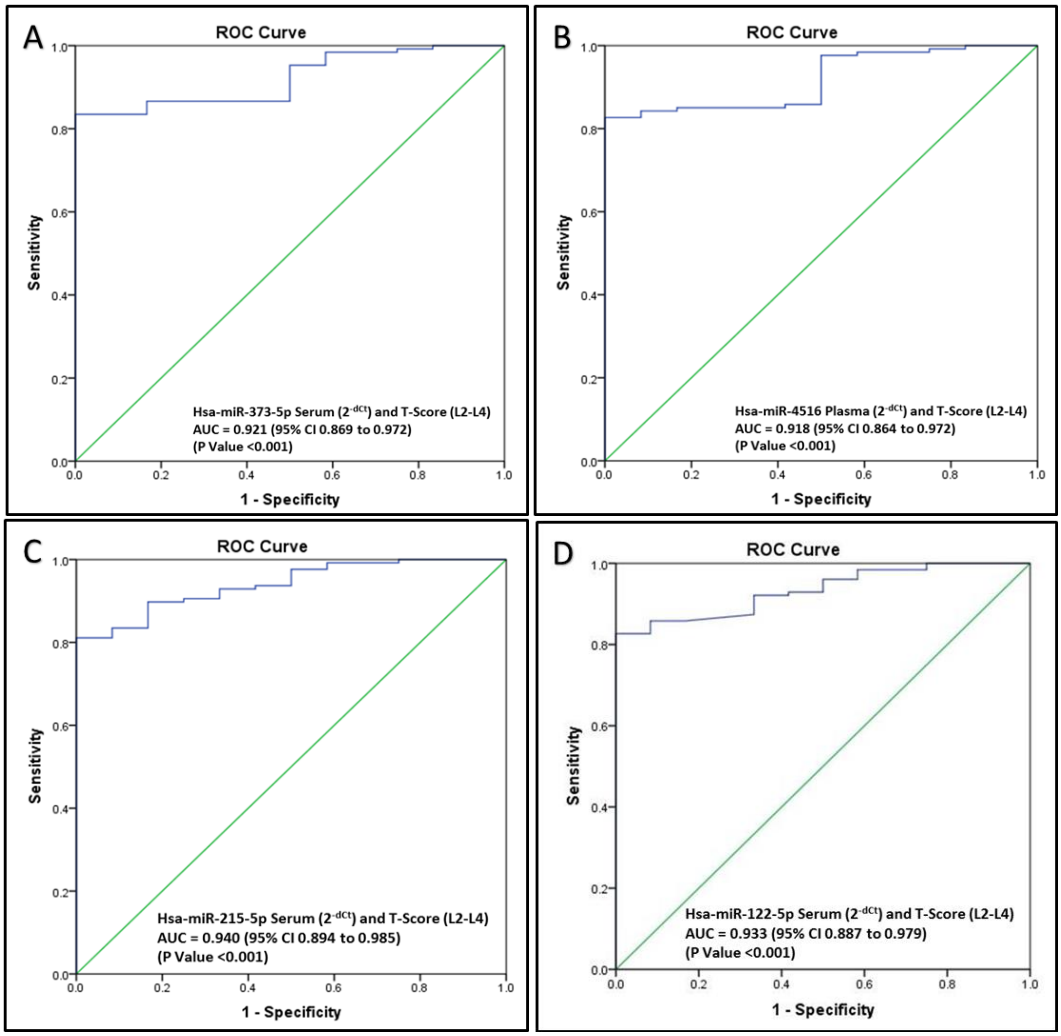


Figure 4-24: Diagnostic value of circulating miRNAs with Lumbar Spine T-Score for osteoporosis.

ROC curve show acceptable AUC for hsa-miR-373-5p, hsa-miR-215-5p and hsa-miR-122-5p in serum (Panel A, C and D, respectively) and hsa-miR-4516 in plasma (Panel B) with a Lumbar Spine (LS2-LS4) T-Score from osteoporosis patients [AUC= 0.921, 0.94, 0.933 and 0.918, with *p* value< 0.001]. AUC = Area Under Cover. AUC <0.06 fail (discrimination no better than chance), 0. 6≤ AUC <0.7 low accuracy, 0.7 ≤ AUC < 0.9 moderate accuracy and AUC ≥ 0.9 high accuracy.

4.3 Discussion

In this study, circulating miRNAs in osteoporotic fractured and non-fractured patients' sera and plasma were compared to age matched non-osteoporotic controls. Three circulating serum miRNAs: hsa-miR-373-5p, hsa-miR-122-5p and hsa-miR-215-5p and plasma hsa-4516 were significantly downregulated in the osteoporosis patients. Indeed, the downregulation of these circulating miRNAs were consistent with the progression of the osteoporotic fragility fracture, and their expressions level correlate with the BMD Lumbar Spine T-score. Therefore, downregulation of these miRNAs could contribute in the progression of osteoporosis. ROC analysis showed that serum hsa-miR-373-5p and plasma hsa-miR-4516 miRNAs were potent diagnostic value in discriminating osteoporotic fractured patients from non-osteoporosis control group, and could be pursued to validate the results on a separate cohort of patients. On the other hand, hsa-miR-99a-5p was significantly upregulated in Low BMD with fracture compared to LBMD without fracture, but it did not significantly correlate with the non-osteoporotic control group. Indeed, both miRNAs: hsa-miR-99a-5p and hsa-miR-100-5p showed no overall significant dysregulation expression in osteoporosis patients.

Interestingly, the downregulation of hsa-miR-373-5p in the cartilage of osteoarthritis patients compared to normal control (Iliopoulos et al., 2008) was shown to correlate with our finding in the osteoporosis subjects. So far, no published data relating the biological function of mir-373-5p in any bone related diseases has been reported.

The miRNA hsa-miR-122-5p has been shown in the present study to be associated with osteoporosis (Seeliger et al., 2014, Panach et al., 2015). Contrary to our study,

hsa-miR-122-5p was shown to be upregulated in osteoporotic fractured (hip fracture) serum and plasma samples compared to osteoarthritic group (Panach et al., 2015, Seeliger et al., 2014). In addition, miR-122 was significantly downregulated in osteosarcoma cell lines compared to normal fibroblast cell lines (Xiao et al., 2015). However, our findings showed that there was a significant downregulation of circulating serum miR-122-5p in osteoporotic individuals, which was consistent with the preliminary miRNA PCR array testing.

As far as for miR-4516 and miR-215-5p, even though, hsa-miR-215-5p was found by others to be decreased in osteosarcoma patients (Wang et al., 2015c), up to date there have been no published data correlating the regulation of these circulating miRNAs with osteoporosis disease. Therefore, this study is the first to highlight the correlation between these circulating miRNAs and osteoporosis.

miR-100 overexpression was observed previously in the serum and bone tissue of osteoporotic fractured patients (Seeliger et al., 2014), and under-expressed in osteosarcoma tumour samples (Maire et al., 2011, Huang et al., 2014). While, it was not differentially expressed in our study, and there is no clear evidence about why this variation happened.

MiR-99a has been found by others to be up-regulated in the osteosarcoma cell line MG-63 compared to human osteoblast (HOB) cell lines (Hu et al., 2012), and its overexpression regulates early chondrogenic differentiation of rat mesenchymal stem cells (rMSCs) by reducing the levels of bone morphogenetic protein (BMP) receptor type 2 (BMPR2) (Zhou et al., 2016). Whereas, it was found to be downregulated in bone tissue of osteosarcoma patients with increased level of its

target gene mammalian target of rapamycin (mTOR) (Zhao et al., 2016). Our findings, showed that serum miR-99a was significantly up-regulated in the serum of osteoporotic fractured participants, suggesting that this up-regulation is mainly due to the fragility fracture associated with LBMD**.**

Our observations showed that the expression of hsa-miR-373-5p, hsa-miR-215-5p and hsa-miR-99a-5p in plasma did not match with serum among the study groups, and there was a significantly decreased level of has-miR100-5p in plasma compared to the serum. In contrast, relatively similar expression of hsa-miR-122-5p and hsa-miR-4516 between serum and plasma was observed. This poor correlation could be due to the release of additional RNA from platelets during the coagulation process (Wang et al., 2012b) or could be due interfering substances, such as the collection tube components and test sample additives (Dimeski, 2008). However, it is not possible presently to identify the cause of this analytical discrepancy.

In conclusion three circulating serum miRNAs: hsa-miR-373-5p, hsa-miR-122-5p and hsa-miR-215-5p, as well as one plasma miRNA (miR-4516) were significantly downregulated in osteoporosis patients. Both hsa-miR-373-5p and hsa-miR-4516 have an AUC of moderate accuracy of 0.72 and 0.73, respectively. The dysregulation of these miRNAs in LBMD patients positively correlates with their BMD (LS) T-Score. While, there was a significant upregulation of serum miR-99a in osteoporotic fractured subjects, the expression of circulating serum and plasma hsa-miR-99a did not correlate with the individuals' BMD (LS) T-Score.

The next step will focus on the potential target genes that are post-transcriptionally regulated be these 6 circulating miRNAs and their role in normal bone haemostasis and in osteoporosis.

Chapter 5 miRNA Predicted Gene targets in Osteoporosis

5.1 Introduction

miRNAs play an important role as post-transcriptional regulators for up to 60% of human protein-coding genes (Friedman et al., 2009). They also play critical roles in bone cell growth and development (Bakhshandeh et al., 2012, Eguchi et al., 2013), bone remodelling processes (Lian et al., 2012) and there is a correlation of miRNAs dysfunction with bone diseases (van Wijnen et al., 2013).

Following the validation process for the identification of novel miRNAs associated with osteoporosis described in Chapter 4, four circulating miRNAs (hsa-miR-373-5p, hsa-miR-122-5p, hsa-miR-215-5p and hsa-miR-4516) were found to be differentially-expressed in clinical samples (serum or plasma) in the osteoporosis patients compared to the osteopenia patients and non-osteoporotic controls. The results suggest that these four differently-expressed miRNAs in clinical samples might associate with osteoporosis. Changes in serum/ plasma miRNAs may reflect underlying changes in cells associated with bone fragility. It is important to identify putative gene targets for theses 4 miRNAs that might be potentially involved in osteoporosis.

The aim of this Chapter is to identify potential miRNA gene targets that are involved in bone remodeling and osteoporosis, using computational prediction algorithms, and understanding their biological functions in osteoporosis using bioinformatic analysis.

miRNA gene targets were predicted by identifying mRNAs with conserved pairing to the 5' region of the miRNA, known as the miRNA seed region, by searching for perfect Watson-Crick complementarity over conserved 6–8mer seed matches to miRNA seed

region (Lewis et al., 2003). The identification was achieved using the miRWalk 2.0 database (Dweep and Gretz, 2015) that uses four putative target algorithms (miRWalk, miRanda, RNA22 and Targetscan) (Materials & Methods 2.8).

In addition, String, version 10.5 database was used to identify direct (physical) and indirect (predicted) protein-protein interaction (Szklarczyk et al., 2017), between these target gene products that are involved in bone remodeling and bone diseases.

5.2 Results

5.2.1 Characteristics of miRNAs and functions

Initially, using literature databases, associations of changes in circulating hsa-miR-373-5p, hsa-miR-122-5p, hsa-miR-215-5p and hsa-miR-4516 with human disease states were reviewed. Circulating serum miR-373 level has been shown to be increased in breast cancer patients compared to healthy controls (Chen et al., 2013, Eichelser et al., 2014, Muller et al., 2014), in epithelial ovarian cancer patients compared to healthy control (Meng, Muller et al. 2016) and in testicular germ cell cancer patients compared to healthy control (Syring et al., 2015). In contrast, circulating serum miR-373 level has been shown to be decreased in pancreatic cancer patients (PC) compared to healthy control (Hua et al., 2017) and reduced in hypertrophic cardiomyopathy (HCM) patients with diffuse myocardial fibrosis compared to healthy volunteers (Fang et al., 2015), Table 5-1).

Circulating serum miR-122 has been shown to be increased in non-alcoholic fatty liver disease (NAFLD) patients compared to NAFLD with hepatocellular carcinoma (HCC) (Akuta et al., 2016), and in chronic hepatitis C (HCV)-related hepatocellular carcinoma patients compared to healthy control (El-Garem et al., 2014). In addition,

circulating serum miR-122 level was also Increased in ST-Elevation Myocardial Infarction patients (STEMI) compared to non-STEMI controls (Cortez-Dias et al., 2016). Decreased level of circulating serum miR-122 has been observed in gastric cancer with distant metastasis (GC/DM) patients compared to healthy controls (Chen et al., 2014b) and in patients with congenital Huntington's disease compared to healthy controls (Diez-Planelles et al., 2016), Table 5-1).

The tissue levels of miR-215-5p miRNA have been reported to be increased in Barrett's esophagus tissues compared to normal control (Cabibi et al., 2016), in gastric cancer compared to adjacent non-tumour tissues (Deng et al., 2014, Li et al., 2016a, Xu and Fan, 2015), in primary gliomas compared to neoplastic brain tissues (Tong et al., 2015) and in chronic hepatitis and hepatocellular carcinoma compared to healthy control tissues (Zhang et al., 2014). In contrast, miR-215 expression has been found to be decreased in colon cancer compared to adjacent normal tissues (Chen et al., 2016, Karaayvaz et al., 2011), in nephroblastomas, embryonal renal neoplasms, compared to nephrogenic rests (NRs), Wilms' tumour (WT) precursors, tissues (Senanayake et al., 2012), in metastatic clear cell renal cell carcinoma (ccRCC) compared to adjacent normal tissues (Khella et al., 2013), in metastatic RCC compared to primary RCC (White et al., 2011), in epithelial ovarian cancer (EOC) compared to non-EOC (other ovarian tumours or ovarian cystadenomas) control tissues, in non-small cell lung cancer compared to non-cancerous lung tissues (Hou et al., 2015) and in breast cancer compared to adjacent normal tissues (Zhou et al., 2014), Table 5-1).

MiR-4516 level has been found to be increased in non-invasive follicular thyroid neoplasms with papillary-like nuclear features compared to follicular adenomas (Borrelli et al., 2017), in the human keratinocyte line, HaCaT cells, with psoralen and ultraviolet A (PUVA) treatment compared to HaCaT cells without PUVA treatment (Chowdhari and Saini, 2014), and in salvage radiation therapy after radical prostatectomy compared to no-recurrence post-salvage radiation therapy (Bell et al., 2015). Circulating serum miR-4516 has been found to be increased in individuals living in a city with moderate air pollution compared to individuals living in a tourist city (Li et al., 2016b). In addition, circulating plasma miR-4516 level has been found to be increased in HIV-associated neurocognitive disorder (HAND) compared to HIV without HAND (Asahchop et al., 2016), Table 5-1).

However, although it is clear that the four selected miRNAs exhibit changed level in disease states, there are so-far no published reports of circulating miR-373, miR-215 and miR-4516 associated with osteoporosis.

The next step was to bioinformatically identify biological processes associated with the target genes of hsa-miR-373-5p, hsa-miR-215-5p, hsa-miR-122-5p and hsa-miR-4516. A search of the miRWalk database revealed several genes predicted as targets for each of the four identified miRNAs in the serum from osteoporotic patients. The analysis of these genes using the Enrichr database (Kuleshov et al., 2016) shows biological involvement of these miRNAs in osteoporosis (Figure 5-1).

Table 5-1: Overview of studies on miRNA expression in blood circulation and body tissues in the context of human pathogenesis described in the text.

miRNA	Source	Disease	Specimen type	Expression level	Reference
miR-373	Human	breast ductal carcinoma patients with lymph node metastasis (n= 35) vs. ductal carcinoma patients without lymph node metastasis (n= 25) and healthy female donors (n= 10)	Plasma	Increased	(Chen et al., 2013)
	Human	breast cancer patients (M0, n= 120) vs. patients with overt metastasis (M1, n= 32) healthy women (n= 40)	Serum	Increased	(Eichelser et al., 2013)
	Human	invasive breast cancer (n= 168) vs. benign breast diseases (n= 19) and healthy women (n= 28)	Serum	Increased	(Eichelser et al., 2014)
	Human	epithelial ovarian cancer (EOC, n= 60) vs. benign ovarian diseases (n= 20) and healthy women (n= 32)	Serum	Increased	(Meng et al., 2016)
	Human	HER2-postive breast cancer after neoadjuvant therapy (n= 127) vs. HER2-postive breast cancer before therapy (n= 127) and healthy controls (n= 19)	Serum	Increased	(Muller et al., 2014)

miRNA	Source	Disease	Specimen type	Expression level	Reference
	Human	patients with testicular germ cell cancer (n= 30) vs. healthy subjects (n= 18)	serum	Increased	(Syring et al., 2015)
	Human	hypertrophic cardiomyopathy (HCM) with diffuse myocardial fibrosis (T1< 470 ms, n= 28) vs. HCM (T1 ≥ 470 ms, n= 27)	Plasma	Decreased	(Fang et al., 2015)
	Human	pancreatic cancer (PC, n= 130) vs. benign pancreatic tumor (n= 30), chronic pancreatitis (n= 20) and healthy volunteers (n= 50)	Serum	Decreased	(Hua et al., 2017)
miR-122	human	Non-alcoholic fatty liver disease (NAFLD) patients without hepatocellular carcinoma (HCC) (n=278) vs. NAFLD/HCC patients (n=27)	Serum	Increased	(Akuta et al., 2016)
	human	gastric cancer with distant metastasis (GC/DM, n=12) vs. gastric cancer with no distant metastasis (GC/NDM, n=12) and healthy controls (HCs)	Plasma	Decreased	(Chen et al., 2014b)
	human	HBV/HCV dual infection patients (n=76) vs. HCV and HBV mono-infection (n=105 and 39, respectively)	Serum	Increased	(Cheng et al., 2015)

miRNA	Source	Disease	Specimen type	Expression level	Reference
	human	ST-Elevation Myocardial Infarction (STEMI, n= 142) vs. non-STEMI controls (n= 18)	Serum	Increased	(Cortez-Dias et al., 2016)
	human	ST-Elevation Myocardial Infarction (STEMI, n= 33) vs. healthy donors (n= 17)	Plasma	decreased	(D'Alessandra et al., 2010)
	human	Huntington's disease (HD, n= 15) vs. Control (n= 7)	Plasma	Decreased	(Diez-Planelles et al., 2016)
	human	chronic hepatitis C(HCV)-related hepatocellular carcinoma (HCC, n= 90) vs. healthy control (n=10)	Serum	Increased	(El-Garem et al., 2014)
	Human	End-stage renal disease patients (ESRD, n= 17) vs. healthy control (n= 22)	Plasma	decreased	(Rivoli et al., 2016)
	Human	neoadjuvant chemotherapy (NAC) for breast cancer (n=22) vs. primary breast cancer without chemotherapy treatment (n= 12)	Plasma	Increased	(Freres et al., 2015)
miR-215	Human Bone tissue and cell lines: U-2 OS, SJSA-1, HT-29 bone cells and A549 cells	Stage II colon cancer tissue vs.	Colon tissue	decreased	Braun, C. J., et al. (2008).

miRNA	Source	Disease	Specimen type	Expression level	Reference
	Human	Barrett's esophagus (BE, n=30) patients vs. normal control (n=30)	Esophageal mucosal tissue biopsies and serum	Increased	(Cabibi et al., 2016)
	Human	Colon cancer specimens (n=44) vs. adjacent normal tissues (n=44)	Colon tissue	Decreased	(Chen et al., 2016)
	Human	Gastric tumor specimens (n= 51) vs. and adjacent normal tissues (n= 51)	Tissue	Increased	(Deng et al., 2014)
	Human	Epithelial ovarian cancer (EOC, n= 85) vs. non-EOC (other ovarian tumors or ovarian cystadenomas) control (n= 63)	Tissue	Decreased	(Ge et al., 2016)
	Human	non-small cell lung cancer (NSCLC, n= 115) vs. adjacent non-cancerous lung tissues (n= 115)	Tissue	Decreased	(Hou et al., 2015)
	Human	colon cancer (n= 34) vs. normal control (n= 34)	Tissue	Decreased	(Karaayvaz et al., 2011)
	Human	metastatic clear cell renal cell carcinoma (ccRCC, formalin-fixed n= 61)	Tissue	Decreased	(Khella et al., 2013)
	Human	gastric cancer (GC, n=77) vs. adjacent non-tumor tissues (n= 77)	Tissue	Increased	(Li et al., 2016a)

miRNA	Source	Disease	Specimen type	Expression level	Reference
	Human	Nephroblastomas, embryonal renal neoplasms vs. nephrogenic rests (NRs)	Tissue	Decreased	(Senanayake et al., 2012)
	Human	primary gliomas (n= 179) vs. non-neoplastic brain tissues (n= 10)	Tissue	Increased	(Tong et al., 2015)
	Human	osteosarcoma (n= 80) vs. adjacent noncancerous bone tissues (n= 80)	Tissue	Decreased	(Wang et al., 2015c)
	Human	acute myeloid leukemia (n= 113) vs. healthy control (n= 25)	white blood cell (WBC)	Decreased	(Wang et al., 2016)
	Human	Metastatic Renal cell carcinoma (RCC, n= 40) vs. primary RCC (n= 40)	Tissue	Decreased	(White et al., 2011)
	Human	Gastric cancer (n= 38) vs. adjacent non-malignant tissues (n= 38)	Tissue	Increased	(Xu and Fan, 2015)
	Human	Chronic hepatitis (n= 118) and Hepatocellular carcinoma (HCC, n= 95) vs. healthy control (n= 127)	Serum	Increased	(Zhang et al., 2014)
	Human	breast cancer vs. adjacent non-malignant tissues	Tissue	Decreased	(Zhou et al., 2014)
MiR-4516	Human	salvage radiation therapy after radical prostatectomy (n=43) vs. no recurrence post-salvage radiation therapy	Tissue	Increased	(Bell et al., 2015)

miRNA	Source	Disease	Specimen type	Expression level	Reference
	Human	HIV-associated neurocognitive disorder (HAND, n=22) vs. HIV without HAND (non-HAND, n= 25)	Plasma	Increased	(Asahchop et al., 2016)
	Human	Non-invasive follicular thyroid neoplasms with papillary-like nuclear features' (NIFTPs, n=19) vs. follicular adenomas (n= 18) and infiltrative follicular variant of papillary thyroid carcinomas (n= 17)	Tissue	Increased	(Borrelli et al., 2017)
	Human	HaCaT cells with PUVA treatment vs. HaCaT cells without PUVA treatment	cultured human keratinocytes (HaCaT cells)	Increased	(Chowdhari and Saini, 2014)
	Human	individuals living in a city with moderate air pollution (n= 120) vs. individuals living in a tourist city (n= 120)	Serum	Increased	(Li et al., 2016b)
	Human	salt-sensitive (SS, N = 3) and inverse salt-sensitive (ISS, N = 3) vs. salt-resistant (SR, N = 4)	Urine	Increased in SS and decreased in ISS	(Gildea et al., 2013)
	Human	Amyotrophic Lateral Sclerosis (ALS) (n= 48) vs. healthy control (n= 47)	Plasma	reference genes	(Takahashi et al., 2015)
	Human	coronary artery disease (CAD, n= 141) vs. healthy control (n= 141)	Plasma	reference genes	(Zhang et al., 2016)

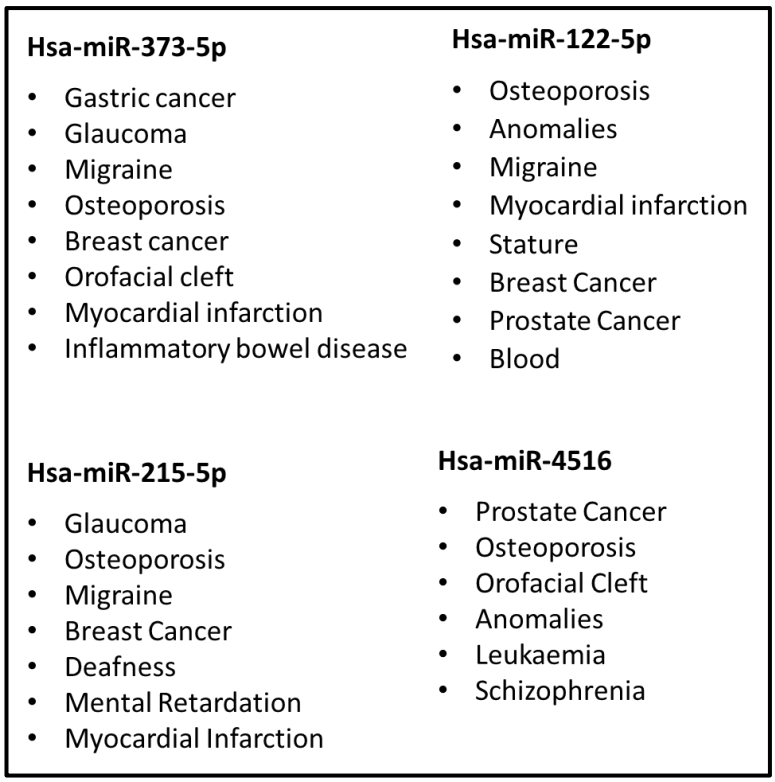


Figure 5-1: In silico identification of disease associations of genes regulated by hsa-miRNAs 373-5p, 122-5p, 215-5p and 4516.
Disease associations of genes regulated by hsa-miR-373-5p, hsa-miR-122-5p, hsa-miR-215-5p, and hsa-miR-4516 were identified from OMIM disease annotations using the Enrichr database (Kuleshov et al., 2016).

5.2.2 Osteoporosis-related genes and miRNAs:

In this Section, the osteoporosis associated genes regulated by hsa-miR-373-5p, hsa-miR-122-5p, hsa-miR-215-5p and hsa-miR-4516 were identified. To achieve this, initially, eighty-nine genes associated with osteoporosis were identified using the search term 'Osteoporosis [DOID: 11476]' from the miRWalk2.0 database. The 3' untranslated regions of the mRNAs encoded by these 89 genes were checked for target sequences for hsa-miR-373-5p, hsa-miR-122-5p, hsa-miR-215-5p and hsa-miR-4516, using 4 different algorithms (miRWalk, miRanda, RNA22 and Targetscan), within the miRWalk 2.0 database. Any genes containing miRNA target sequences

within the 3'-UTR regions of their mRNAs predicted by at least 2 of the 4 Osteoporosis-related genes were recorded.

5.2.2.1 Osteoporosis-related genes and miR-373-5p

For hsa-miR-373-5p, a total of 9171 predicted gene targets were identified. Out of the 89 osteoporosis genes, fifty-two genes contained the hsa-miR-373-5p predicted target mRNA binding site within the 3'UTR region (Figure 5-2) with 29 of these predicted by two or more algorithms (Table 5-2).

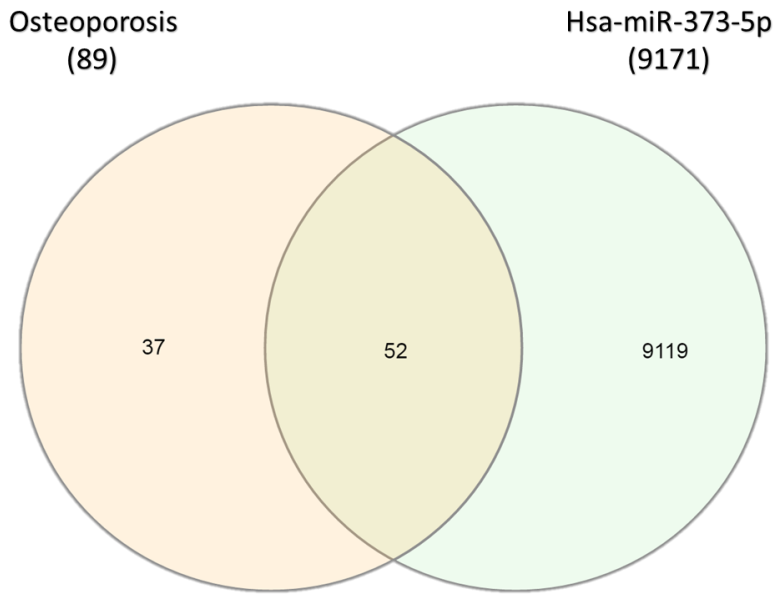


Figure 5-2: Venn diagram denoting the overlap between osteoporosis-related genes and hsa-miR-373-5p putative gene targets.
The 52 osteoporosis-related genes which contained has-miR-373-5p target sites according to at least one target algorithm were : *ABCB1*, AHSG, *ALDH7A1*, ALOX15, *ANKH*, *AR*, ARHGEF3, BMP2, *BMP2K*, *CA8*, CALCR, CD40, *CD44*, *CD47*, *CNR1*, *CNR2*, *COL1A1*, CST3, *CYP19A1*, *CYP1B1*, CYP27B1, *CYP3A4*, DCAF13, DKK1, *ESR1*, ESR2, *FSHB*, HGF, *IGF1*, *IGF1R*, IL1A, *IL1B*, *IRS2*, *LRP6*, *MAPK1*, *MTHFR*, *PON1*, *PTHLH*, PTK2B, QPCT, *RUNX2*, *SOST*, *SPARC*, *STAT1*, *THBS1*, THSD4, *THSD7A*, TNFRSF11B, *TRAF6*, *TWIST1*, *UGT2B17*, *VPS13B*. Venn Diagram was generated using InteractiVenn Software (Heberle et al., 2015).

The 29 genes are: *ANKH*, *CYP1B1*, *IL1B*, *LRP6*, *STAT1*, *UGT2B17*, *BMP2K*, *CA8*, *CD44*, *CD47*, *CNR1*, *CNR2*, *ESR1*, *MAPK1*, *MTHFR*, *PON1*, *SOST*, *SPARC*, *TRAF6*, *VPS13B*, *ABCB1*, *ALDH7A1*, *AR*, *COL1A1*, *FSHB*, *IGF1R*, *IRS2*, *THBS1* and *THSD7A*.

Table 5-2: Putative osteoporosis-related genes containing hsa-miR-373-5p target sequences within the 3'-UTR regions of their mRNAs

Predicted by at least 2 of the 4 different algorithms from the miRWalk2.0's database. 0= not identified 1 = identified.

Gene	Description	miRWalk	miRanda	RNA22	Targetscan	Sum
ANKH	*ANKH* inorganic pyrophosphate transport regulator	1	1	1	1	4
CYP1B1	cytochrome P450 family 1 subfamily B member 1	1	1	1	1	4
IL1B	interleukin 1 beta	1	1	1	1	4
LRP6	LDL receptor related protein 6	1	1	1	1	4
STAT1	signal transducer and activator of transcription 1	1	1	1	1	4
UGT2B17	UDP glucuronosyltransferase family 2 member B17	1	1	1	1	4
BMP2K	BMP2 inducible kinase	0	1	1	1	3
CA8	carbonic anhydrase 8	1	1	0	1	3
CD44	*CD44* molecule (Indian blood group)	1	1	0	1	3
CD47	*CD47* molecule	1	1	0	1	3
CNR1	cannabinoid receptor 1	1	1	0	1	3
CNR2	cannabinoid receptor 2	1	1	0	1	3
ESR1	estrogen receptor 1	1	1	0	1	3
MAPK1	mitogen-activated protein kinase 1	1	0	1	1	3
MTHFR	methylenetetrahydrofolate reductase	1	1	0	1	3
PON1	paraoxonase 1	1	1	0	1	3
SOST	sclerostin	0	1	1	1	3
SPARC	secreted protein acidic and cysteine rich	1	1	0	1	3
TRAF6	TNF receptor associated factor 6	1	1	0	1	3
VPS13B	vacuolar protein sorting 13 homolog B	1	1	0	1	3
ABCB1	ATP binding cassette subfamily B member 1	1	0	1	0	2
ALDH7A1	aldehyde dehydrogenase 7 family member A1	1	0	0	1	2
AR	androgen receptor	1	0	1	0	2
COL1A1	collagen type I alpha 1 chain	0	1	0	1	2
FSHB	follicle stimulating hormone beta subunit	0	1	0	1	2
IGF1R	insulin like growth factor 1 receptor	1	0	0	1	2

Gene	Description	miRWalk	miRanda	RNA22	Targetscan	Sum
IRS2	insulin receptor substrate 2	1	0	1	0	2
THBS1	thrombospondin 1	1	0	0	1	2
THSD7A	thrombospondin type 1 domain containing 7A	0	1	0	1	2
Total	**29**					

5.2.2.2 Osteoporosis-related genes and hsa-miR-122-5p

For hsa-miR-122-5p, a total of 10891 genes contained predicted target sequence within the 3'-UTR regions of their mRNAs (miRWalk, miRanda, RNA22 and Targetscan). Sixty out of the 89 putative osteoporosis-related genes were identified as targets for hsa-miR-122-5p (Figure 5-3).

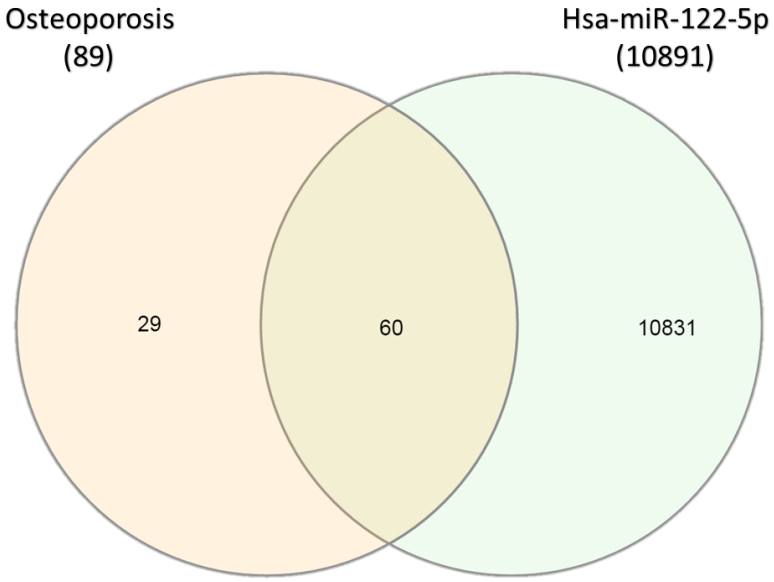


Figure 5-3: Venn diagram denoting the overlap between osteoporosis-related genes and hsa-miR-122-5p putative gene targets.

The 60 osteoporosis-related genes which contained has-miR-122-5p target sites according to at least one target algorithm were: *ALDH7A1*, ALOX15, *ALPL*, *ANKH*, *AR*, ARHGEF3, *BMP2K*, *CA10*, CALCR, CD40, *CD44*, *CD47*, *CNR1*, *CNR2*, *COL1A1*, CTSK, *CYP19A1*, CYP1A1, *CYP1B1*, *CYP3A4*, DKK1, *ESR1*, FGFBP1, *FSHB*, *GH1*, HGF, ICAM1, *IGF1*, *IGF1R*, IL15, *IL1B*, IL6, *INSL3*, *IRS2*, KIT, *LRP6*, *MAPK1*, *MTHFR*, PDLIM4, *PLOD1*, *PTHLH*, PTN, QPCT, *RUNX2*, RXFP2, *SLC22A11*, *SOST*, *SPARC*, *STAT1*, *THBS1*, THSD4,

TNF, TNFRSF11A, *TNFRSF1B*, *TRAF6*, *TSC22D3*, *TWIST1*, *VDR*, *VPS13B*, WNK4. Venn Diagram was generated using InteractiVenn Software

Out of the 60 genes associated with osteoporosis, 21 genes (*TNFRSF1B*, *ALPL*, *ANKH*, *BMP2K*, *ESR1*, *IGF1R*, *RUNX2*, *AR*, *CA10*, *CD44*, *CNR2*, *CYP3A4*, *FSHB*, *GH1*, *INSL3*, *LRP6*, *MAPK1*, *PTHLH*, *SPARC*, *TSC22D3* and *VDR*) were predicted by at least two of the four algorithms (Table 5-3).

Table 5-3: Putative osteoporosis-related genes containing hsa-miR-122-5p target sequences within the 3'-UTR regions of their mRNAs

Predicted by at least 2 of the 4 different algorithms from the miRWalk2.0's database. 0= not identified 1 = identified.

Gene	Description	miRWalk	miRanda	RNA22	Targetscan	Sum
TNFRSF 1B	TNF receptor superfamily member 1B	1	1	1	1	4
ALPL	alkaline phosphatase, liver/bone/kidney	1	1	0	1	3
ANKH	*ANKH* inorganic pyrophosphate transport regulator	1	0	1	1	3
BMP2K	BMP2 inducible kinase	1	0	1	1	3
ESR1	estrogen receptor 1	0	1	1	1	3
IGF1R	insulin like growth factor 1 receptor	1	1	0	1	3
RUNX2	runt related transcription factor 2	1	1	0	1	3
AR	androgen receptor	1	0	0	0	2
CA10	carbonic anhydrase 10	1	0	0	1	2
CD44	*CD44* molecule (Indian blood group)	1	0	0	1	2
CNR2	cannabinoid receptor 2	1	0	0	1	2
CYP3A4	cytochrome P450 family 3 subfamily A member 4	1	0	0	1	2
FSHB	follicle stimulating hormone beta subunit	1	0	1	0	2
GH1	growth hormone 1	1	0	0	1	2
INSL3	insulin like 3	1	0	0	1	2
LRP6	LDL receptor related protein 6	1	0	1	0	2
MAPK1	mitogen-activated protein kinase 1	1	0	1	0	2
PTHLH	parathyroid hormone like hormone	1	1	0	1	2
SPARC	secreted protein acidic and cysteine rich	1	0	1	0	2
TSC22D 3	TSC22 domain family member 3	1	0	0	1	2

Gene	Description	miRWalk	miRanda	RNA22	Targetscan	Sum
VDR	vitamin D (1,25- dihydroxyvitamin D3) receptor	1	0	0	1	2
Total	**21**					

5.2.2.3 Osteoporosis related genes and Hsa-miR-215-5p

4762 genes contain hsa-miR-215-5p target sequences within the 3'-UTR regions of their mRNAs predicted by one of the 4 algorithms (miRWalk, miRanda, RNA22 and/or Targetscan). 33 out of the 89 osteoporosis-related genes were putative targets for hsa-miR-215-5p (Figure 5-4).

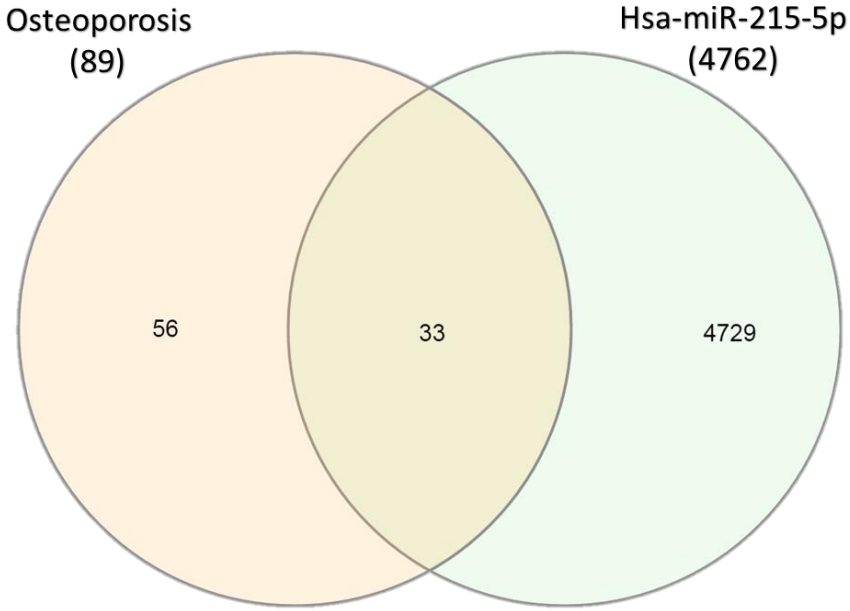


Figure 5-4: Venn diagram denoting the overlap between osteoporosis-related genes and hsa-miR-215-5p putative gene targets.
The 33 osteoporosis-related genes which contained has-miR-215-5p target sites according to at least one target algorithm were: *ALDH7A1*, *ANKH*, *AR*, ARHGEF3, *BMP2K*, *CA10*, *CA8*, *CD47*, CTSK, *CYP19A1*, *CYP1B1*, *CYP3A4*, *ESR1*, FGFBP1, *FSHB*, HSD11B1, ICAM1, *IGF1*, *IGF1R*, IL15, IL1A, IL6, *IRS2*, ITGA1, *MAPK1*, *PLOD1*, *PTHLH*, *RUNX2*, *SPARC*, *THSD7A*, *TRAF6*, *TWIST1*, *VDR*. Venn Diagram was generated using InteractiVenn Software.

Of these, 33 osteoporosis genes that were predicted targets for hsa-miR-215-5p, 9 genes, *MAPK1*, *SPARC*, *CD47*, *ESR1*, *TRAF6*, *BMP2K*, *CYP1B1*, *IGF1* and *VDR* were identified by at least 2 of the 4 algorithms (miRWalk, miRanda, RNA22 and Targetscan) (Table 5-4).

Table 5-4: Putative osteoporosis-related genes containing hsa-miR-215-5p target sequences within the 3'-UTR regions of their mRNAs

Predicted by at least 2 of the 4 different algorithms from the miRWalk2.0's database. 0= not identified 1 = identified.

Gene	description	miRWalk	miRanda	RNA22	Targetscan	Sum
MAPK1	mitogen-activated protein kinase 1	1	1	1	1	4
SPARC	secreted protein acidic and cysteine rich	1	1	1	1	4
CD47	*CD47* molecule	1	1	0	1	3
ESR1	estrogen receptor 1	1	1	0	1	3
TRAF6	TNF receptor associated factor 6	1	1	0	1	3
BMP2K	BMP2 inducible kinase	0	1	0	1	2
CYP1B1	cytochrome P450 family 1 subfamily B member 1	1	0	1	0	2
IGF1	insulin like growth factor 1	0	1	0	1	2
VDR	vitamin D (1,25- dihydroxyvitamin D3) receptor	1	0	0	1	2
Total	**9**					

5.2.2.4 Osteoporosis-related genes and Hsa-miR-4516

7641 putative genes contain hsa-miR-4516 target sequences within the 3'-UTR regions of their mRNAs predicted by one of the 4 algorithms (miRWalk, miRanda, RNA22 and Targetscan). 47 osteoporosis-related genes were identified as targets for hsa-miR-4516 (Figure 5-5).

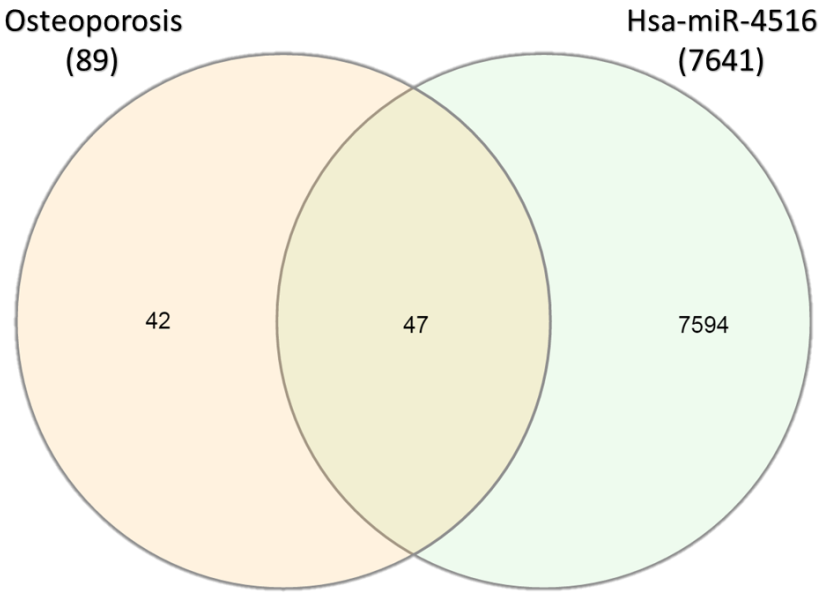


Figure 5-5: Venn diagram denoting the overlap between osteoporosis-related genes and hsa-miR-4516 putative gene targets.

The 47 osteoporosis-related genes which contained has-miR-4516 target sites according to at least one target algorithm were: *ALDH7A1*, *ALPL*, *ANKH*, *AR*, ARHGEF3, BGLAP, BMP2, *BMP2K*, *CA10*, CALCR, *CD44*, *CD47*, *CNR1*, *COL1A1*, CTSK, *CYP17A1*, *CYP19A1*, CYP1A1, CYP27B1, *FSHB*, *IGF1*, *IGF1R*, *INSL3*, ITGA1, KIT, *LRP6*, *LTF*, *MAPK1*, *MAPK3*, *MTHFR*, *PLOD1*, *PON1*, *PTHLH*, PTK2B, *RUNX2*, *SLC22A11*, *SPARC*, *STAT1*, *THBS1*, THSD4, *THSD7A*, TNFRSF11A, *TNFRSF1B*, *TRAF6*, *TSC22D3*, *VDR* and *VPS13B*. Venn Diagram was generated using InteractiVenn Software.

Of the 47 predicted gene targets associated with osteoporosis, twenty-one genes (*AR*, *CYP17A1*, *CYP19A1*, *IGF1R*, KIT, *LTF*, *MAPK3*, *PLOD1*, *RUNX2*, *SLC22A11*, *SPARC*, *THBS1*, *TRAF6*, *VDR*, *BMP2K*, *CD47*, *CNR1*, *FSHB*, *MTHFR*, *PTHLH* and *TSC22D3*) were identified by at least 2 of the 4 algorithms (miRWalk, miRanda, RNA22 and Targetscan) (Table 5-5).

Table 5-5: Putative osteoporosis-related genes containing hsa-miR-4516 target sequences within the 3'-UTR regions of their mRNAs

Predicted by at least 2 of the 4 different algorithms from the miRWalk2.0's database. 0= not identified 1 = identified.

Gene	Description	miRWalk	miRanda	RNA22	Targetscan	Sum
AR	androgen receptor	1	1	0	1	3
CYP17A1	cytochrome P450 family 17 subfamily A member 1	1	1	0	1	3
CYP19A1	cytochrome P450 family 19 subfamily A member 1	1	1	0	1	3
IGF1R	insulin like growth factor 1 receptor	1	1	0	1	3
KIT	KIT proto-oncogene receptor tyrosine kinase	1	1	0	1	3
LTF	lactotransferrin	1	1	0	1	3
MAPK3	mitogen-activated protein kinase 3	1	1	0	1	3
PLOD1	procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	1	1	0	1	3
RUNX2	runt related transcription factor 2	1	1	0	1	3
SLC22A11	solute carrier family 22 member 11	1	1	0	1	3
SPARC	secreted protein acidic and cysteine rich	1	1	0	1	3
THBS1	thrombospondin 1	1	1	0	1	3
TRAF6	TNF receptor associated factor 6	1	1	0	1	3
VDR	vitamin D (1,25- dihydroxyvitamin D3) receptor	1	1	0	1	3
BMP2K	BMP2 inducible kinase	1	0	0	1	2
CD47	*CD47* molecule	0	1	0	1	2
CNR1	cannabinoid receptor 1	1	0	0	1	2
FSHB	follicle stimulating hormone beta subunit	1	0	0	1	2
MTHFR	methylenetetrahydrofolate reductase	1	0	0	1	2
PTHLH	parathyroid hormone like hormone	1	0	0	1	2
TSC22D3	TSC22 domain family member 3	0	1	0	1	2
Total	**21**					

5.2.2.5 Common Osteoporosis-related genes among the four miRNAs

Two genes, BMP2 inducible kinase (*BMP2K*) and secreted protein acidic and cysteine rich (*SPARC*) contained the recognition sequences for all 4 miRNAs, hsa-miR-122-5p, hsa-miR-215-5p, hsa-miR-373-5p and hsa-miR-4516. A total of eight genes contained the recognition sequences for three of the miRNAs. Androgen receptor *(AR)*, insulin like growth factor 1 receptor (*IGF1R*) and follicle stimulating hormone beta subunit (*FSHB*) contained hsa-miR-122-5p, hsa-miR-373-5p and hsa-miR-4516 recognition sequences. CD47 molecule (*CD47*) and TNF receptor associated factor 6 (*TRAF6*) contained hsa-miR-215-5p, hsa-miR-373-5p and hsa-miR-4516 recognition sequences. Estrogen receptor 1 (*ESR1*) and mitogen-activated protein kinase 1 (*MAPK1*) contained hsa-miR-373-5p, hsa-miR-122-5p and hsa-miR-215-5p recognition sequences. Finally, vitamin D (1,25- dihydroxy vitamin D3) receptor (*VDR*) contained recognition sequences for hsa-miR-122-5p, hsa-miR-215-5p and hsa-miR-4516 (Table 5-6).

Two miRNAs: hsa-miR-122-5p and hsa-miR-373-5p, shared four gene targets: ANKH inorganic pyrophosphate transport regulator (*ANKH*), CD44 molecule (Indian blood group, *CD44*), cannabinoid receptor 2 (*CNR2*) and LDL receptor related protein 6 (*LRP6*). While, hsa-miR-122-5p and hsa-miR-4516 shared three genes: parathyroid hormone like hormone (*PTHLH*), runt related transcription factor 2 (*RUNX2*) and TSC22 domain family member 3 (*TSC22D3*). Furthermore, hsa-miR-373-5p and hsa-miR-4516 shared another three genes: cannabinoid receptor 1 (*CNR1*), methylenetetrahydrofolate reductase (*MTHFR*) and thrombospondin 1 (*THBS1*). Likewise, cytochrome P450 family 1 subfamily B member 1 (*CYP1B1*) was found to be a putative gene for hsa-miR-373-5p and hsa-miR-215-5p (Table 5-6).

Moreover, twelve genes were unique targets for hsa-miR-373-5p: ATP binding cassette subfamily B member 1 (*ABCB1*), aldehyde dehydrogenase 7 family member A1 (*ALDH7A1*), carbonic anhydrase 8 *(CA8*), collagen type I alpha 1 chain (*COL1A1*), interleukin 1 beta (*IL1B*), insulin receptor substrate 2 (*IRS2*), paraoxonase 1 (*PON1*), sclerostin (*SOST*), signal transducer and activator of transcription 1 (*STAT1*), thrombospondin type 1 domain containing 7A (*THSD7A*), UDP glucuronosyltransferase family 2 member B17 (*UGT2B17*) *and* vacuolar protein sorting 13 homolog (*BVPS13B*), seven genes for hsa-miR-4516: cytochrome P450 family 17 subfamily A member 1 (*CYP17A1*), cytochrome P450 family 19 subfamily A member 1 (*CYP19A1*), *KIT proto-oncogene receptor tyrosine kinase* (*KIT*), *lactotransferrin* (*LTF*), mitogen-activated protein kinase 3 (*MAPK3*), procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (*PLOD1*) and solute carrier family 22 member 11 (*SLC22A11*), 6 genes for hsa-miR-122-5p: alkaline phosphatase, liver/bone/kidney (*ALPL*), carbonic anhydrase 10 (*CA10*), cytochrome P450 family 3 subfamily A member 4 (*CYP3A4*), growth hormone 1 (*GH1*), insulin like 3 (*INSL3*) and TNF receptor superfamily member 1B (*TNFRSF1B*). While, insulin like growth factor 1 (*IGF1)* (≥ two algorithms) was uniquely identified as predicted binding site for hsa-miR-215-5p only (Table 5-6).

Table 5-6: Osteoporosis-related genes and circulating miRNAs.
This table show that some genes were targeted by more than one miRNA.

Gene	Description	hsa-miR-122-5p	hsa-miR-215-5p	hsa-miR-373-5p	hsa-miR-4516	miRNA Total
BMP2K	BMP2 inducible kinase	X	X	X	X	4
SPARC	secreted protein acidic and cysteine rich	X	X	X	X	4
AR	androgen receptor	X		X	X	3
IGF1R	insulin like growth factor 1 receptor	X		X	X	3
FSHB	follicle stimulating hormone beta subunit	X		X	X	3
CD47	*CD47* molecule		X	X	X	3
TRAF6	TNF receptor associated factor 6		X	X	X	3
ESR1	estrogen receptor 1	X	X	X		3
MAPK1	mitogen-activated protein kinase 1	X	X	X		3
VDR	vitamin D (1,25- dihydroxy vitamin D3) receptor	X	X		X	3
ANKH	*ANKH* inorganic pyrophosphate transport regulator	X		X		2
CD44	*CD44* molecule (Indian blood group)	X		X		2
CNR2	cannabinoid receptor 2	X		X		2
LRP6	LDL receptor related protein 6	X		X		2
PTHLH	parathyroid hormone like hormone	X			X	2
RUNX2	runt related transcription factor 2	X			X	2
TSC22D3	TSC22 domain family member 3	X			X	2
CNR1	cannabinoid receptor 1			X	X	2
MTHFR	methylenetetrahydrofolate reductase			X	X	2
THBS1	thrombospondin 1			X	X	2
CYP1B1	cytochrome P450 family 1 subfamily B member 1		X	X		2
ALPL	alkaline phosphatase, liver/bone/kidney	X				1
CA10	carbonic anhydrase 10	X				1
CYP3A4	cytochrome P450 family 3 subfamily A member 4	X				1
GH1	growth hormone 1	X				1
INSL3	insulin like 3	X				1
TNFRSF1B	TNF receptor superfamily member 1B	X				1
IGF1	insulin like growth factor 1		X			1
ABCB1	ATP binding cassette subfamily B member 1			X		1

Gene	Description	hsa-miR-122-5p	hsa-miR-215-5p	hsa-miR-373-5p	hsa-miR-4516	miRNA Total
ALDH7A1	aldehyde dehydrogenase 7 family member A1			X		1
CA8	carbonic anhydrase 8			X		1
COL1A1	collagen type I alpha 1 chain			X		1
IL1B	interleukin 1 beta			X		1
IRS2	insulin receptor substrate 2			X		1
PON1	paraoxonase 1			X		1
SOST	sclerostin			X		1
STAT1	signal transducer and activator of transcription 1			X		1
THSD7A	thrombospondin type 1 domain containing 7A			X		1
UGT2B17	UDP glucuronosyltransferase family 2 member B17			X		1
VPS13B	vacuolar protein sorting 13 homolog B			X		1
CYP17A1	cytochrome P450 family 17 subfamily A member 1				X	1
CYP19A1	cytochrome P450 family 19 subfamily A member 1				X	1
KIT	KIT proto-oncogene receptor tyrosine kinase				X	1
LTF	lactotransferrin				X	1
MAPK3	mitogen-activated protein kinase 3				X	1
PLOD1	procollagen-lysine,2-oxoglutarate 5-dioxygenase 1				X	1
SLC22A11	solute carrier family 22 member 11				X	1
Total	**47**	**21**	**9**	**29**	**21**	

5.2.3 Protein–protein interaction networks within miRNA osteoporosis-related target mRNAs.

Search Tool for the Retrieval of Interacting Genes (String, version 10.5) (Szklarczyk et al., 2015) was used to investigate the biological interactions between the 47 miRNA predicted osteoporosis–associated gene targets shown in Table 5-6 (5.2.2.5), and 40 of these genes were shown to be interrelated with at least one other gene (Figure 5-6) (Table 5-7).

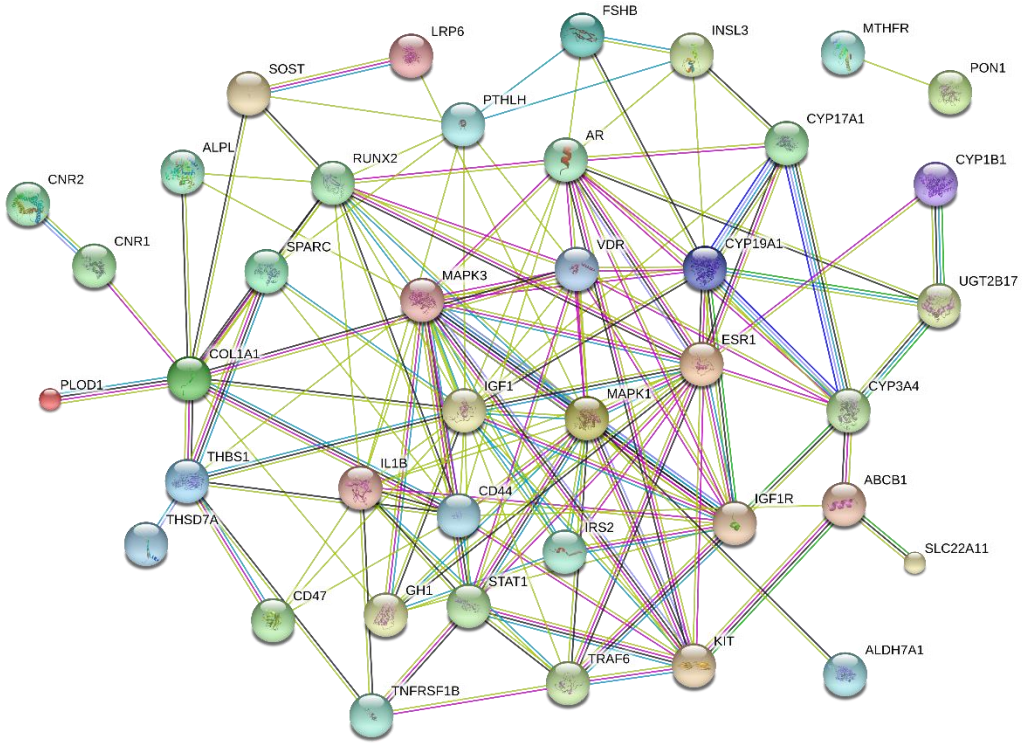
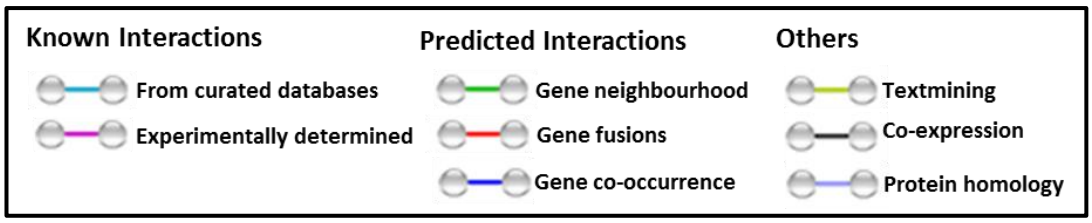


Figure 5-6: Protein-Protein Interaction Network for putative miRNA Gene target (n=40).

Total of 40 predicted gene targets: *ABCB1, ALDH7A1, ALPL, AR, CD44, CD47, CNR1, CNR2, COL1A1, CYP17A1, CYP19A1, CYP1B1, CYP3A4, ESR1, FSHB, GH1, IGF1, IGF1R, IL1B, INSL3, IRS2, KIT, LRP6, MAPK1, MAPK3, MTHFR, PLOD1, PON1, PTHLH, RUNX2, SLC22A11, SOST, SPARC, STAT1, THBS1, THSD7A, TNFRSF1B, TRAF6, UGT2B17* and *VDR* for miRNAs: hsa-miR-373-5p, hsa-miR-122-5p, hsa-miR-215-5p and hsa-miR-4516 were identified. The nodes and edges represent the proteins (genes) and interactions, respectively. Image was generated using String version 10.5 Protein-Protein interaction network (Szklarczyk et al., 2015).



Interestingly, insulin like growth factor 1 (*IGF1*) was the node with the highest number of interacting genes (n= 20), which means that interactions must pass through this node to reach other portions of the network; there were 17 interacting genes for the nodes, estrogen receptor 1 (*ESR1*) and mitogen-activated protein kinase 3 (*MAPK1), 16 for* MAPK*3*, then 14 for the androgen receptor (*AR*) node and 13 for both *CD44* and runt related transcription factor 2 (*RUNX2*). In addition, 12 genes interacted with insulin like growth factor 1 receptor (*IGF1R*) and interleukin 1 beta (*IL1B*), respectively and 11 for collagen type I alpha 1 chain (*COL1A1*) and cytochrome P450 family 19 subfamily A member 1 (*CYP19A1*), respectively. Likewise, 9 genes interacted with KIT proto-oncogene receptor tyrosine kinase (*KIT*), as well as for signal transducer and activator of transcription 1 (*STAT1*). In the same fashion, eight genes were interrelated with cytochrome P450 family 3 subfamily A member 4 (*CYP3A4)*, parathyroid hormone like hormone (*PTHLH*), thrombospondin 1 (*THBS1*) and TNF receptor associated factor 6 (*TRAF6*), seven for growth hormone 1 (*GH1*), then 6 genes for cytochrome P450 family 17 subfamily A member 1 (*CYP17A1*), insulin receptor substrate 2 (*IRS2*) and secreted protein acidic and cysteine rich (*SPARC*), insulin like 3 (*INSL3*, n=5); ATP binding cassette subfamily B member 1 (*ABCB1*), *CD47*, follicle stimulating hormone beta subunit (*FSHB*), sclerostin (*SOST*), *TNFRSF1B* and *UGT2B17* (n= 4, each), and finally, 2 for each of cannabinoid receptor

1 (*CNR1*), cytochrome P450 family 1 subfamily B member 1 (*CYP1B1*) and LDL receptor related protein 6 (*LRP6*) (Table 5-7).

Table 5-7: Protein-Protein Interaction for four miRNAs predicted gene target.
Data was generated using String protein-protein interaction network software (Ver.10) (Szklarczyk et al., 2015).

Gene (node)	*Interrelated Genes*	No. of Genes
IGF1	*AR, CD44, CD47, COL1A1, CYP17A1, CYP19A1, ESR1, GH1, IGF1R, IL1B, IRS2, KIT, MAPK1, MAPK3, PTHLH, RUNX2, SPARC, STAT1, THBS1* and *VDR*	20
ESR1	*AR, CD44, CYP17A1, CYP19A1, CYP1B1, CYP3A4, GH1, IGF1, IGF1R, IL1B, IRS2, KIT, MAPK1, MAPK3, RUNX2, STAT1* and *TRAF6*	17
MAPK3	*ALPL, AR, CD44, CYP19A1, ESR1, GH1, IGF1, IGF1R, IL1B, IRS2, KIT, MAPK1, RUNX2, STAT1, THBS1, TRAF6* and *VDR*	17
MAPK1	*ALDH7A1, AR, CD44, ESR1, GH1, IGF1, IGF1R, IL1B, IRS2, KIT, LRP6, MAPK3, RUNX2, STAT1, TRAF6* and *VDR*	16
AR	*CD44, CYP17A1, CYP19A1, CYP3A4, ESR1, FSHB, IGF1, IGF1R, INSL3, KIT, MAPK1, MAPK3, RUNX2* and *UGT2B17*	14
CD44	*ABCB1, AR, CD47, COL1A1, ESR1, IGF1, IL1B, KIT, MAPK1, MAPK3, RUNX2, SPARC* and *THBS1*	13
RUNX2	*ALPL, AR, CD44, COL1A1, ESR1, IGF1, IL1B, MAPK1, MAPK3, PTHLH, SOST, SPARC* and *VDR*	13
IGF1R	*AR, CYP19A1, CYP3A4, ESR1, GH1, IGF1, IL1B, IRS2, MAPK1, MAPK3, TRAF6* and *VDR*	12
IL1B	*CD44, CD47, ESR1, IGF1, IGF1R, MAPK1, MAPK3, PTHLH, RUNX2, STAT1, TNFRSF1B* and *TRAF6*	12
COL1A1	*ALPL, CD44, CNR1, GH1, IGF1, PLOD1, RUNX2, SOST, SPARC, THBS1* and *VDR*	11
CYP19A1	*AR, CYP17A1, CYP3A4, ESR1, FSHB, IGF1, IGF1R, INSL3, MAPK3, UGT2B17* and *VDR*	11
VDR	*COL1A1, CYP19A1, CYP3A4, IGF1, IGF1R, MAPK1, MAPK3, PTHLH, RUNX2 and STAT1*	10
KIT	*ABCB1, AR, CD44, ESR1, IGF1, MAPK1, MAPK3, STAT1* and *TRAF6*	9
STAT1	*ESR1, IGF1, IL1B, KIT, MAPK1, MAPK3, TNFRSF1B, TRAF6* and *VDR*	9
CYP3A4	*ABCB1, AR, CYP17A1, CYP19A1, ESR1, IGF1R, UGT2B17* and *VDR*	8
PTHLH	*FSHB, IGF1, IL1B, INSL3, RUNX2, SOST, SPARC* and *VDR*	8
THBS1	*CD44, CD47, COL1A1, IGF1, MAPK3, SPARC, THSD7A* and *TNFRSF1B*	8
TRAF6	*ESR1, IGF1R, IL1B, KIT, MAPK1, MAPK3, STAT1* and *TNFRSF1B*	8

Gene (node)	***Interrelated Genes***	No. of Genes
GH1	*COL1A1, ESR1, IGF1, IGF1R, IRS2, MAPK1* and *MAPK3*	7
CYP17A1	*AR, CYP19A1, CYP3A4, ESR1, IGF1* and *INSL3*	6
IRS2	*ESR1, GH1, IGF1, IGF1R, MAPK1* and *MAPK3*	6
SPARC	*CD44, COL1A1, IGF1, PTHLH, RUNX2* and *THBS1*	6
INSL3	*AR, CYP17A1, CYP19A1, FSHB* and *PTHLH*	5
ABCB1	*CD44, CYP3A4, KIT* and *SLC22A11*	4
CD47	*CD44, IGF1, IL1B* and *THBS1*	4
FSHB	*AR, CYP19A1, INSL3* and *PTHLH*	4
SOST	*COL1A1, LRP6, PTHLH* and *RUNX2*	4
TNFRSF1B	*IL1B, STAT1, THBS1* and *TRAF6*	4
UGT2B17	*AR, CYP19A1, CYP1B1* and *CYP3A4*	4
ALPL	*COL1A1, MAPK3* and *RUNX2*	3
CNR1	*CNR2* and *COL1A1*	2
CYP1B1	*ESR1* and *UGT2B17*	2
LRP6	*MAPK1* and *SOST*	2
ALDH7A1	*MAPK1*	1
CNR2	*CNR1*	1
MTHFR	*PON1*	1
PLOD1	*COL1A1*	1
PON1	*MTHFR*	1
SLC22A11	*ABCB1*	1
THSD7A	*THBS1*	1

5.2.4 Signalling Pathways

5.2.4.1 Bone remodelling

Out of the 40 osteoporosis-related genes with target sequences for miRNAs hsa-miR-373-5p, hsa-miR-122-5p, hsa-miR-215-5p and hsa-miR-4516 related by the String network (refer to 5.2.3), Eleven genes were involved in skeletal system development (GO:0001501) as shown in Figure 5-7.

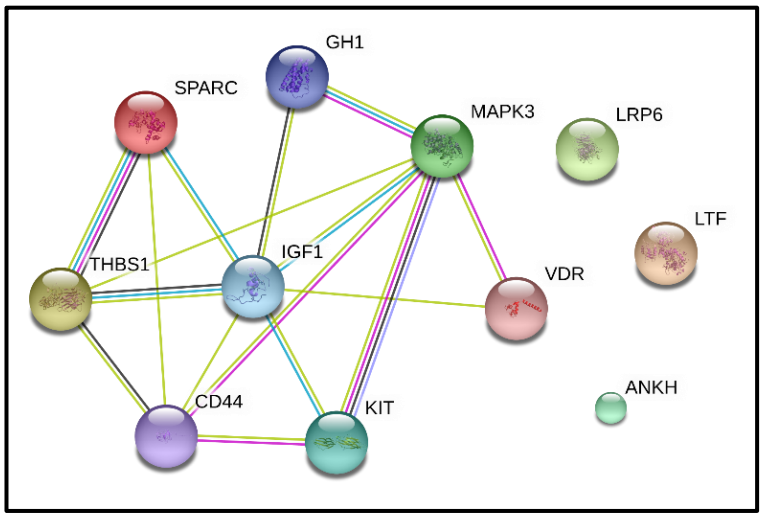


Figure 5-7: Protein-Protein Interaction Network for putative miRNA Gene target involved in skeletal system development.
11 predicted gene targets: *ANKH, CD44, GH1, IGF1, KIT, LRP6, LTF, MAPK3, SPARC, THBS1* and *VDR* were identified as being associated with skeletal system development. The nodes and edges represent the proteins (genes) and interactions, respectively. Image was generated using String version 10.5 Protein-Protein interaction network.

Ten genes were involved in bone development (GO:0060348), seven genes in the ossification process (GO:0001503) *COL1A1, IGF1, LTF, PTHLH, SOST, SPARC* and *TRAF6*, with six genes involved in ossification regulation (GO:0030278) *ANKH*, *BMP2K*, *IGF1*, *LTF*, *RUNX2* and *SOST* and six genes: *ALPL, COL1A1, LTF, PTHLH, RUNX2*

and THBS1 in bone morphogenesis (GO:0060349) as well as in skeletal system morphogenesis (GO:0048705), (Figure 5-8).

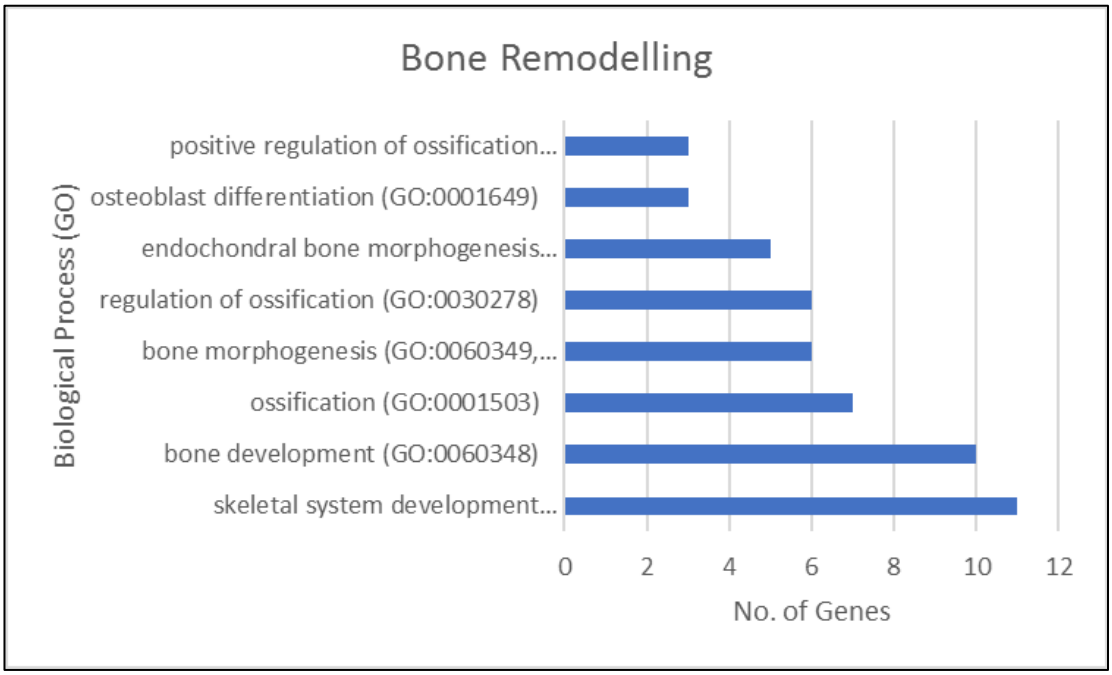


Figure 5-8: Numbers of Gene from the 40 gene String network s involved in Bone Remodelling.

The most common genes involved in bone development, morphogenesis and osteoblast differentiation are: *ALPL*, *COL1A1*, *IGF1*, *LTF*, *PTHLH*, *RUNX2* and *THBS1*. In contrast, the most common genes involved in bone remodeling signalling pathway and osteoclast differentiation were *FSHB*, *SOST* and *TRAF6*. Indeed, *TRAF6* shown to be involved in bone resorption (GO:0045453), osteoclast differentiation (GO:0030316) and positive regulation of osteoclast differentiation (GO:0045672), while *SOST* was involved in negative regulation of BMP signalling pathway (GO:0030514) and in the negative regulation of ossification (GO:0030279) (Table 5-6).

Table 5-8: Genes involved in Bone Remodelling and their Biological Process (GO id)

Genes	Biological Process (GO ID)
ANKH, CD44, GH1, IGF1, KIT, LRP6, LTF, MAPK3, SPARC, THBS1 and ***VDR***	skeletal system development (GO:0001501)
ALPL, COL1A1, GH1, IGF1, KIT, LTF, PTHLH, RUNX2, SPARC and ***THBS1***	bone development (GO:0060348)
COL1A1, IGF1, LTF, PTHLH, SOST, SPARC and ***TRAF6***	ossification (GO:0001503)
ALPL, COL1A1, LTF, PTHLH, RUNX2 and ***THBS1***	bone morphogenesis (GO:0060349, GO:0048705)
ANKH, BMP2K, IGF1, LTF, RUNX2 and ***SOST***	regulation of ossification (GO:0030278)
ALPL, COL1A1, PTHLH, RUNX2 and ***THBS1***	endochondral bone morphogenesis (GO:0060350, GO:0001958)
ALPL, COL1A1 and ***PTHLH***	osteoblast differentiation (GO:0001649)
IGF1, LTF and ***RUNX2***	positive regulation of ossification (GO:0045778) and osteoblast differentiation (GO:0045669)
MAPK3 and ***RUNX2***	BMP signalling pathway (GO:0030509)
GH1 and ***IGF1***	bone maturation (GO:0070977)
COL1A1 and ***THBS1***	cartilage development involved in endochondral bone morphogenesis (GO:0060351)
COL1A1 and ***CYP1B1***	collagen fibril organization (GO:0030199)
PTHLH and ***RUNX2***	osteoblast development (GO:0002076)
MAPK1 and ***MAPK3***	regulation of cytoskeleton organization (GO:0051493)
TRAF6	bone resorption (GO:0045453), bone remodeling (GO: 0046849), osteoclast differentiation (GO: 0030316) and positive regulation of osteoclast differentiation (GO: 0045672)
COL1A1	bone trabecula formation (GO:0060346) and intramembranous ossification (GO:0001957)
SOST	negative regulation of ossification (GO:0030279) and BMP signalling pathway (GO:0030514)
FSHB	positive regulation of bone resorption (GO:0045780)

5.2.4.2 Wnt Receptor Signalling Pathway

Wnt signalling pathways are a group of signal transduction pathways that participate in the regulation of cell differentiation, proliferation, and apoptosis (Huelsken and Birchmeier, 2001), and, through these mechanisms play a key role in bone remodeling and disease (Harada and Rodan, 2003, Baron and Kneissel, 2013). Three genes, *CD44*, *LRP6* and *SOST* are involved in regulation of the Wnt signalling pathway (GO:0030111) and regulation of canonical Wnt signalling pathway (GO:0060828). *COL1A1* shown to involved in positive regulation of Wnt signalling pathway (GO:0030177), while *SOST* is involved in the negative regulation of Wnt signalling pathway (GO:0030178) negative regulation of canonical Wnt signalling pathway (GO:0090090). *LRP6* was involved in Wnt Signalling Pathway, including the positive and negative regulation of the canonical Wnt signalling pathways, while *SOST* was involved in the negative regulation of canonical Wnt signalling pathway.

Table 5-9: Genes involved in Wnt Signalling Pathway and their Biological Process (GO)

Genes	Biological Process (GO ID)
CD44*, *LRP6* and *SOST	Wnt signalling pathway (GO:0016055)
COL1A1*, *LRP6* and *SOST	regulation of Wnt (GO:0030111) and canonical Wnt (GO:0060828) signalling pathways
COL1A1* and *LRP6	positive regulation of Wnt signalling pathway (GO:0030177)
LRP6* and *SOST	negative regulation of Wnt and canonical Wnt (GO:0090090) signalling pathways
COL1A1* and *LRP6	positive regulation of canonical Wnt signalling pathway (GO:0090263)
LRP6	canonical Wnt signalling pathway (GO:0060070) involved in regulation of cell proliferation (GO:0044340)

5.2.4.3 Insulin, insulin-like growth factor and growth hormone Regulation

Insulin-like growth factors (IGFs): *IGF1* and IGF2 that are produced by bone cells regulate specific osteoblastic and osteoclastic functions (Rosen et al., 1994). In total, 17 genes from the 40 genes in the String network were involved in the 'Insulin & Insulin Like Growth Factor regulation' signalling pathways. Eleven genes were involved in cellular response to growth factor stimulus (GO:0071363). Five genes, *GH1*, *IRS2*, *MAPK1*, *MAPK3* and *STAT1,* were involved in the growth hormone receptor signalling pathway (GO:0060396). 4 genes, *IGF1R*, *IRS2*, *MAPK1* and *MAPK3,* were involved in the insulin receptor signalling pathway (GO:0008286), cellular response to insulin stimulus (GO:0032869) and response to insulin (GO:0032868) (Figure 5-9).

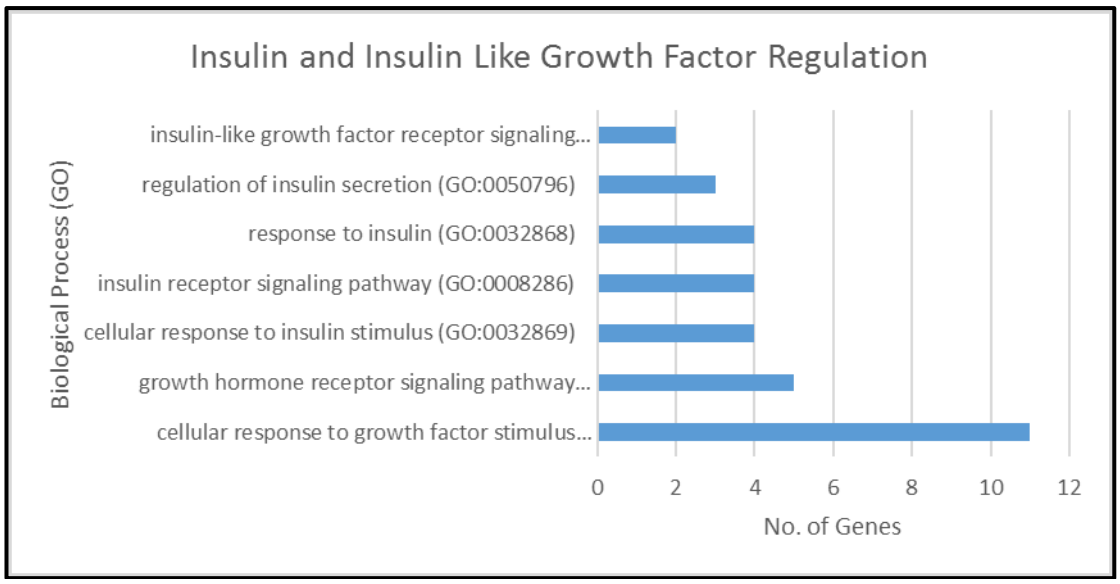


Figure 5-9: Numbers of genes from the 40 member String network involved in Insulin & Insulin Like Growth Factor regulation

In addition, *CNR1*, *IL1B* and *IRS2* were involved in regulation of insulin secretion (GO:0050796), while *IGF1* and *IGF1R* were involved in insulin-like growth factor receptor signalling pathway (GO:0048009).

Table 5-10: Genes involved in the insulin and insulin like growth factor signalling pathways with their Biological Process (GO id)

Genes	Biological Process (GO ID)
CD44, COL1A1, FSHB, IRS2, KIT, MAPK1, MAPK3, RUNX2, SPARC, TNFRSF1B and ***TRAF6***	cellular response to growth factor stimulus (GO:0071363)
GH1, IRS2, MAPK1, MAPK3 and ***STAT1***	growth hormone receptor signalling pathway (GO:0060396)
IGF1R, IRS2, MAPK1 and ***MAPK3***	insulin receptor signalling pathway (GO:0008286), response to insulin (GO:0032868) and cellular response to insulin stimulus (GO:0032869).
CNR1, IL1B and ***IRS2***	regulation of insulin secretion (GO:0050796)
IGF1 and ***IGF1R***	insulin-like growth factor receptor signalling pathway (GO:0048009)

5.2.4.4 MAPK Activity

Mitogen-activated protein kinases (MAPKs) are part of intracellular signalling pathways that play an essential role in cellular proliferation, differentiation (Aouadi

et al., 2006), immune responses (Dong et al., 2002) and apoptosis regulation (Wada and Penninger, 2004). MAPKs are essential regulators of RANKL-mediated osteoclastogenesis that contribute to bone loss (Ge et al., 2007, Boyle et al., 2014). Ten genes were involved in positive regulation of MAPK cascade (GO:0043410), wherein 8 genes were involved in regulation of MAPK cascade (GO:0043408). Seven genes: *GH1, IL1B, KIT, MAPK1, MAPK3, THBS1* and *TRAF6* were involved in positive regulation of MAP kinase activity (GO:0043406) and regulation of MAP kinase activity (GO:0043405). Furthermore, six genes: *IL1B*, KIT, *MAPK1*, *MAPK3*, *THBS1* and *TRAF6* were involved in activation of MAPK activity (GO:0000187) (Figure 5-10).

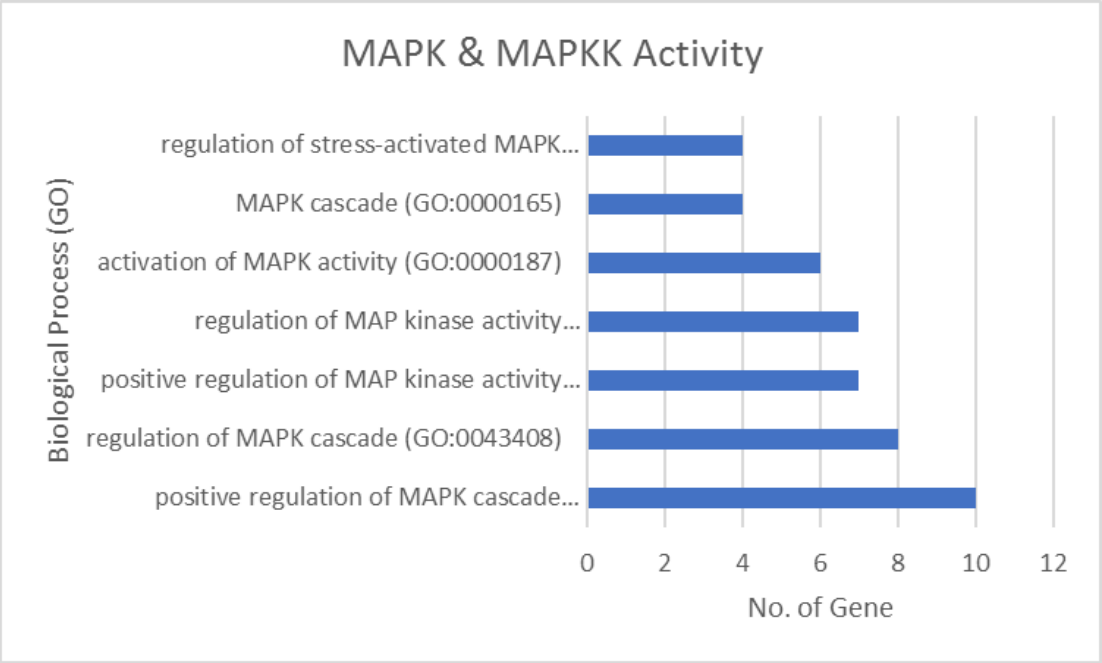


Figure 5-10: Numbers of genes from the 40 member String network involved in MAPK and MAPKK activity

Seven genes *CD44*, *GH1*, KIT, *MAPK1*, *MAPK3*, *THBS1* and *TRAF6* were associated with positive regulation of MAP kinase activity and involved in the regulation of MAPK cascade. Additionally, both MAPKs, *MAPK1* and *MAPK3,* were involved in the activation of MAPKK activity (GO:0000186) and MAPK import into the nucleus (GO:0000189). In contrast, *IL1B*, *IGF1* and *IGF1R* are involved in both, the negative

regulation of MAPK cascade (GO:0043409) and positive regulation of MAPK cascade (GO:0043410) (Table 5-11).

Table 5-11: Genes involved in MAPK activity with their Biological Process (GO id)

Genes	Biological Process (GO)
CD44, GH1, IGF1, IGF1R, IL1B, KIT, MAPK1, MAPK3, THBS1* and *TRAF6	positive regulation of MAPK cascade (GO:0043410)
CD44, GH1, IL1B, KIT, MAPK1, MAPK3, THBS1* and *TRAF6	regulation of MAPK cascade (GO:0043408)
GH1, IL1B, KIT, MAPK1, MAPK3, THBS1* and *TRAF6	Positive regulation of MAP kinase activity (GO:0043405 GO:0043406)
IL1B, KIT, MAPK1, MAPK3, THBS1* and *TRAF6	activation of MAPK activity (GO:0000187)
IGF1, IL1B, MAPK3* and *TRAF6	MAPK cascade (GO:0000165)
IGF1R, MAPK1, MAPK3* and *TRAF6	regulation of stress-activated MAPK cascade (GO:0032872)
IGF1, IGF1R* and *IL1B	negative regulation of MAPK cascade (GO:0043409)
MAPK1, MAPK3* and *TRAF6	stress-activated MAPK cascade (GO:0051403)
MAPK1* and *MAPK3	activation of MAPKK activity (GO:0000186) and MAPK import into nucleus (GO:0000189)
IL1B	negative regulation of MAP kinase activity (GO:0043407)
TRAF6	positive regulation of stress-activated MAPK cascade (GO:0032874)

5.2.4.5 Steroid hormones associated with osteoporosis

Gonadal steroid hormones play a vital role in maintaining lifetime bone mass in men, women, and children (Harman et al., 2001). Out of 40 predicted osteoporosis-related gene targets (refer to 5.2.3), 17 genes were associated with the hormonal regulation of osteoporosis, wherein, ten : *ALPL*, *AR*, *COL1A1*, *ESR1*, *GH1*, *IL1B*, *MAPK1*, *SPARC*, *THBS1* and *VDR* were involved in the response to steroid hormone (GO:0048545), 7 genes (*CYP17A1*, *CYP1B1*, *CYP3A4*, *ESR1*, *FSHB*, *IL1B* and *UGT2B17*) were involved in steroid metabolic process (GO:0008202), and 5 genes: *COL1A1*, *ESR1*, *GH1*, *IL1B* and

MAPK1 were involved in response to estrogen (GO:0043627) (Figure 5-11) (Table 5-12).

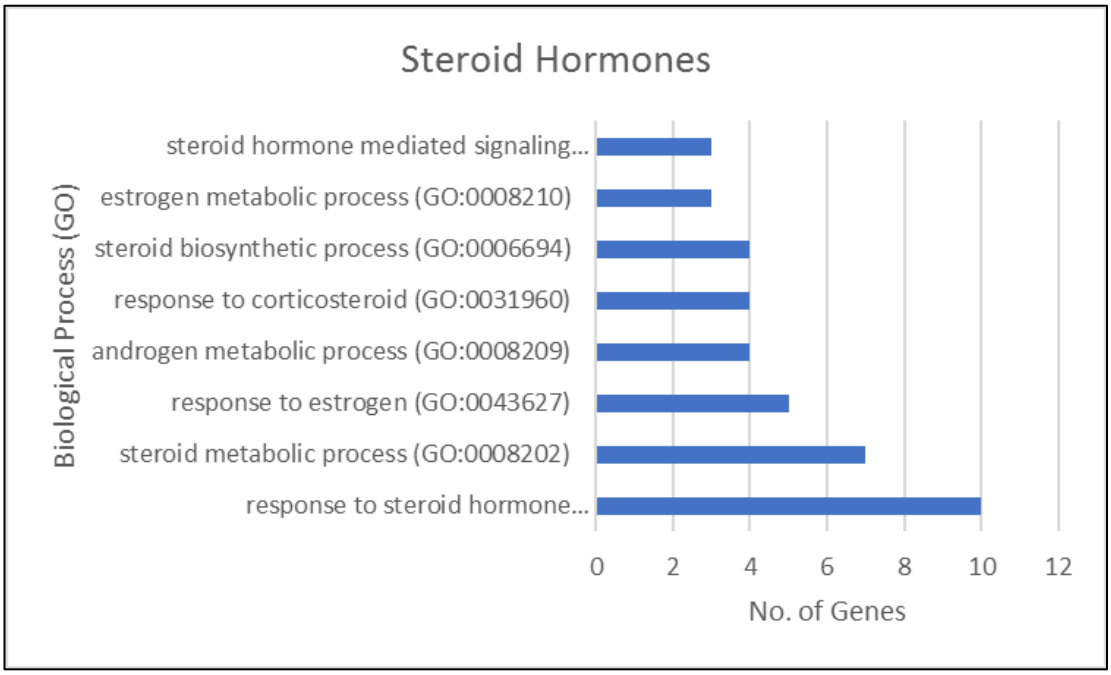


Figure 5-11: Numbers of Genes from the 40 gene network involved in Steroid hormone regulation.

ESR1 and *IL1B* were involved in the response to steroid hormone (GO:0048545), steroid metabolic process (GO:0008202) and response to estrogen (GO:0043627). In addition, *ESR1*, *AR* and *VDR* were involved in steroid hormone mediated signalling pathways (GO:0043401).

Table 5-12: Osteoporosis-related genes involved in steroid hormone activity and their Biological Process (GO id)

Genes	Biological Process (GO)
ALPL, AR, COL1A1, ESR1, GH1, IL1B, MAPK1, SPARC, THBS1* and *VDR	Response to steroid hormone (GO:0048545)
CYP17A1, CYP1B1, CYP3A4, ESR1, FSHB, IL1B* and *UGT2B17	steroid metabolic process (GO:0008202)
COL1A1, ESR1, GH1, IL1B* and *MAPK1	response to estrogen (GO:0043627)
CYP17A1, CYP19A1, CYP3A4* and *ESR1	androgen metabolic process (GO:0008209)

Genes	Biological Process (GO)
ALPL, COL1A1, IL1B* and *SPARC	response to corticosteroid (GO:0031960)
CYP17A1, CYP19A1, CYP3A4* and *FSHB	steroid biosynthetic process (GO:0006694)
CYP19A1, CYP1B1* and *IL1B	estrogen metabolic process (GO:0008210)
AR, ESR1* and *VDR	steroid hormone mediated signalling pathway (GO:0043401)
CYP17A1* and *FSHB	progesterone metabolic process (GO:0042448)
AR	androgen receptor signalling pathway (GO:0030521)
IGF1	negative regulation of androgen receptor signalling pathway (GO:0060766)
THBS1	response to progesterone (GO:0032570)
CYP3A4	steroid catabolic process (GO:0006706)

5.2.4.6 Apoptosis Process

Apoptosis is a genetically programmed cell death (Kerr et al., 1972) that is involved in bone remodeling (Xing and Boyce, 2005). Sixteen genes from the 40 genes in the String network were shown to be involved in regulation of the apoptotic process (GO:0042981), eleven genes were involved in negative regulation of the apoptotic process (GO:0043066), eight genes were involved in apoptotic process (GO:0006915) and 6 genes were involved in the positive regulation of apoptotic process (GO:0043065) (Figure 5-12).

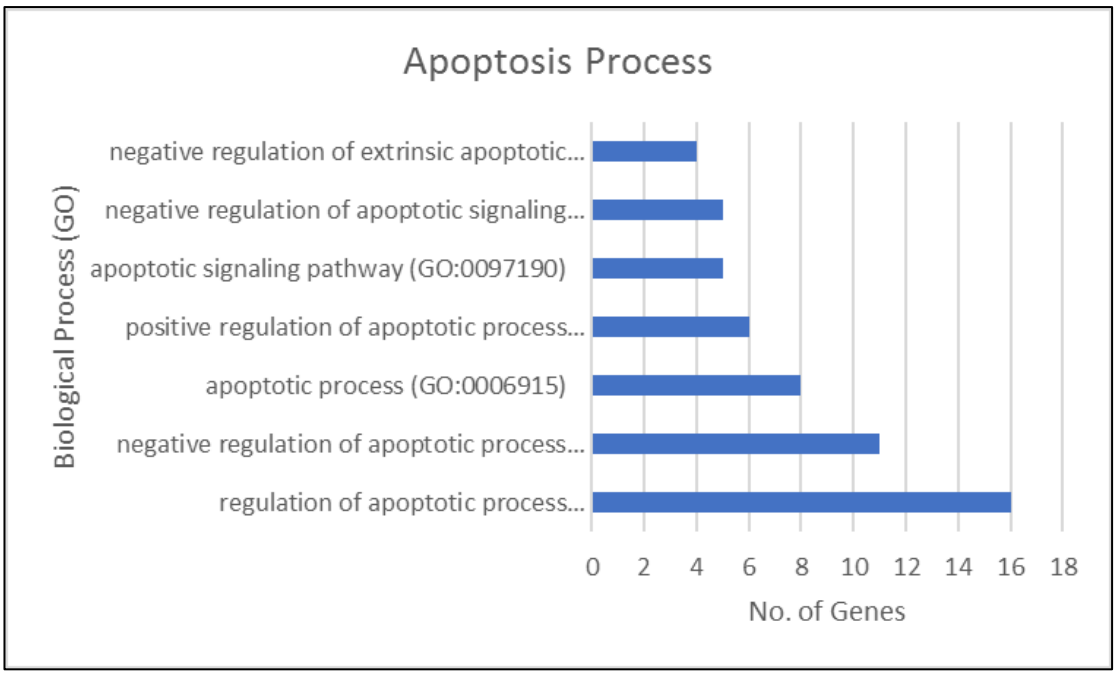


Figure 5-12: Number of genes from the 40 gene network involved in the apoptotic process.

Eight genes: *AR*, *CD44*, *IGF1*, *INSL3*, *IRS2*, *LRP6*, *LTF* and *TSC22D3* were shown to be mainly associated with the negative regulation of the apoptotic process. In contrast, three genes: *CNR1*, *CYP1B1* and *VDR* were shown to be involved mainly in the positive regulation of the apoptotic process (GO:0043065). However, *IL1B*, *THBS1* and *LRP6* were shown to be involved in both the positive and negative regulation of the apoptotic process.

Table 5-13: Genes involved in Apoptosis Process in their Biological Process (GO id)

Genes	Biological Process (GO)
AR, *CD44*, *CNR1*, *CYP1B1*, *ESR1*, *IGF1*, *IL1B*, *INSL3*, *IRS2*, *LRP6*, LTF, *STAT1*, *THBS1*, *TRAF6*, *TSC22D3* and *VDR*	regulation of apoptotic process (GO:0042981)
AR, *CD44*, *IGF1*, *IL1B*, *INSL3*, *IRS2*, *LRP6*, *LTF*, *THBS1*, *TRAF6* and *TSC22D3*	negative regulation of apoptotic process (GO:0043066)
CD44*, *CYP1B1*, *IGF1*, *MAPK1*, *MAPK3*, *STAT1*, *THBS1*, *TNFRSF1B* and *TRAF6	apoptotic process (GO:0006915)
CNR1*, *CYP1B1*, *IL1B*, *THBS1*, *TRAF6* and *VDR	positive regulation of apoptotic process (GO:0043065)
CYP1B1*, *IGF1*, *IL1B*, *TNFRSF1B* and *TRAF6	apoptotic signalling pathway (GO:0097190)
AR, *CD44*, *IGF1*, *IL1B* and *THBS1*	negative regulation of apoptotic signalling pathway (GO:2001234)
AR, *IGF1*, *IL1B* and *THBS1*	negative regulation of extrinsic apoptotic signalling pathway (GO:2001237)
IGF1*, *IL1B* and *TNFRSF1B	extrinsic apoptotic signalling pathway (GO:0097191)
IGF1*, *IGF1R* and *LRP6	negative regulation of muscle cell apoptotic process (GO:0010656)
CD44* and *THBS1	negative regulation of cysteine-type endopeptidase activity involved in apoptotic process (GO:0043154)
IGF1* and *LRP6	negative regulation of smooth muscle cell apoptotic process (GO:0034392)

5.2.5 Role of miRNAs in Bone Remodeling

List of genes that are involved in bone remodeling and in bone pathogenesis 'Osteoporosis' were identified (refer to 5.2.4.1). Sclerostin (*SOST*) a Wnt antagonist glycoprotein secreted by osteocytes (van Bezooijen et al., 2004) has been found to be a predicted target for circulating serum hsa-miR-373-5p (refer to 0). Binding of Sclerostin to its receptors, low-density lipoprotein-related proteins 5/6 (*LRP5/6* receptors), inhibits the Wnt signalling pathway in human embryonic kidney A293T cells and mouse osteoblastic MC3T3 cells (Li et al., 2005, Ellies et al., 2006), and eventually inhibits osteoblast formation (Poole et al., 2005). *LRP6,* which is expressed in osteoblastic cell (Li et al., 2005) has been found to be a predicted target for both hsa-miR-373-5p and hsa-miR-122-5p (Figure 5-13).

Estrogens and androgen are sex steroid hormones that play an essential role in bone development and remodeling, and deficiency of these hormones increases bone remodelling with resorption rate exceeding formation leading to osteoporosis (Cauley, 2015). Estrogen activity is mediated by binding to *ESR1* and *ESR2* (Fitzpatrick, 2006), while the activity of androgens is mediated by binding to the androgen receptor (*AR*) (Beato and Klug, 2000). Both, estrogen receptor 1 (*ESR1*) and androgen receptor (*AR*) that are expressed in osteoblasts, osteoclasts and their progenitor cells (Zallone, 2006, Manolagas et al., 2013, Pederson et al., 1999) are predicted target for hsa-miR-373-5p and hsa-miR-122-5p. Therefore, hsa-miR-122-5p and hsa-miR-373-5p could possibly downregulate *ESR1* and *AR* expression leading to impairment of estrogen/ESR1 and androgen/AR signalling pathways, leading to increased bone turnover and resorption rate (Figure 5-13).

Hsa-miR-373-5p, hsa-miR-215-5p and hsa-miR-4516 were predicted to target *CD47* and tumour necrosis factor receptor-associated factor 6 (*TRAF6*) (Figure 5-13). *CD47* is a membrane protein belonging to the superfamily of immunoglobulins that have been shown to be expressed in all hematopoietic cells, including macrophages (Brown et al., 1990), and *TRAF6* a transduction factor for RANK binding to RANKL, that is expressed in a wide range of tissues including monocytes (Lamothe et al., 2007) and is involved in the activation of downstream signalling pathways, such as nuclear factor of activated T-cells 1 (NFATc1), nuclear factor-kappa B (NF-κB), microphthalmia transcription factor (MITF) and c-Fos which are responsible for osteoclast differentiation (Kim and Kim, 2014).

BMP2 inducible kinase (*BMP2K*) (Salazar et al., 2016) and secreted protein acidic and cysteine rich (*SPARC*) (Delany et al., 2000) are involved in bone development and osteoblast differentiation. In addition, hsa-miR-4516 was predicted to target *MAPK3* (Ge et al., 2007) and *SPARC* genes (Figure 5-13). Therefore, dysregulation of *MAPK1*, *MAPK3*, *BMP2K* and *SPARC* expression by these miRNAs could lead to high bone turnover.

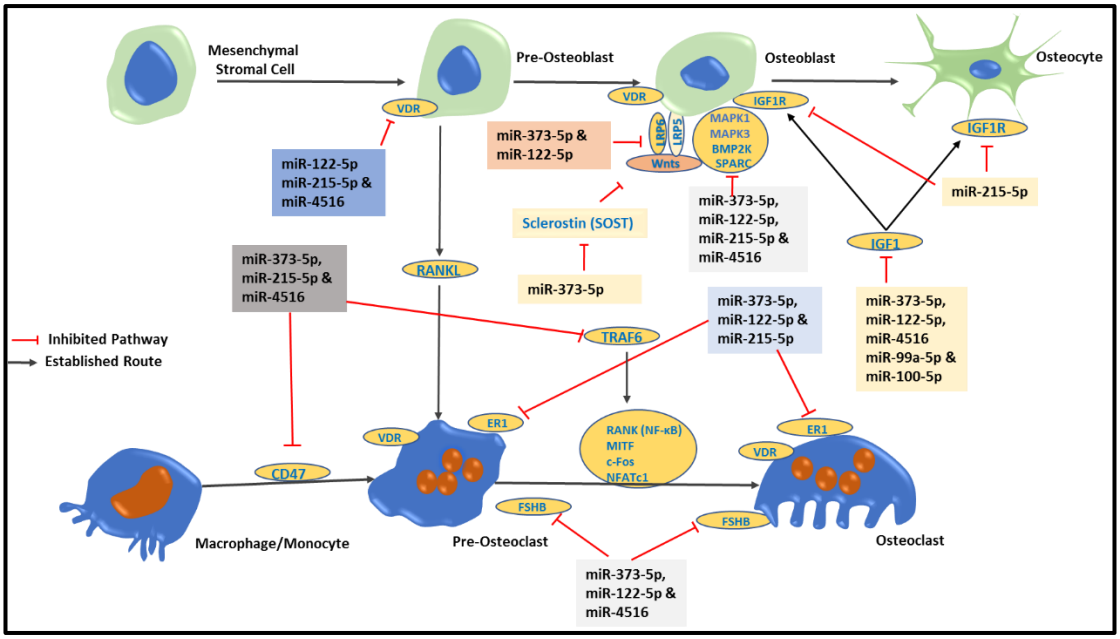


Figure 5-13: Role of miRNAs in Bone Remodeling.

Summary key molecules and functions, refer to Appendix 8.7.

5.3 Discussion

In this chapter, the study set out to assess the post-transcriptional effects of miRNAs on genes/mRNAs that are involved in osteoporosis.

Forty seven protein coding genes were identified as targets for circulating serum/plasma miRNAs: hsa-miR-373-5p, hsa-miR-122-5p, and hsa-miR-215-5p and hsa-miR-4516 by 2 or more miRNA predicted target algorithms. Some of these genes are involved in osteoblastogenesis, while some others are involved in osteoclastogenesis according to gene ontology databases. Up to 60% of the predicted genes were targets for hsa-miR-373-5p, including Sclerostin *SOST* gene, 44% for both hsa-miR-122-5p and hsa-miR-4516, and 19% for hsa-miR-215-5p. Interestingly, BMP2 inducible kinase (*BMP2K*) and secreted protein acidic and cysteine rich (*SPARC*) were the most common gene target for the four circulating miRNAs (hsa-miR-122-5p, hsa-miR-215-5p, hsa-miR-373-5p and hsa-miR-4516) and nine genes (AR, *IGF1R*, *CD47*, *ESR1*, *FSHB*, *MAPK1*, *SPARC*, *TRAF6* and *VDR*) were targets among four circulating miRNAs: hsa-miR-373-5p, hsa-miR-122-5p, hsa-miR-215-5p and hsa-miR-4516.

Sclerostin (*SOST*), a Wnt antagonist glycoprotein secreted by osteocytes (Winkler et al., 2003, van Bezooijen et al., 2004) was shown to be a target for the most significantly downregulated circulating serum miRNA, hsa-miR-373-5p in osteoporotic fractured patients. Reduced bone mineral density was noticed in heterogenous $LRP6^{+}/LRP6^{-}$ mutant mice compared to wild type mice (Holmen et al., 2004). *LRP6* was found to be a common target gene for both hsa-mir-373-5p and hsa-mir-122-5p. therefore, hsa-miR-373-5p and miR-122-5p might have a vital role in bone haemostasis by downregulating *SOST* expression and inhibiting its binding to

LDL receptor-related protein 5 (LRP5) and *LRP6* and halting its negative canonical Wnt signalling pathway (Semenov et al., 2005, Li et al., 2005).

Tumour necrosis factor receptor-associated factor 6 (*TRAF6*) and *CD47* were found to be putative target proteins for hsa-miR-373-5p, hsa-miR-215-5p and hsa-miR-4516. Both *CD47* and *TRAF6* have been shown to be involved in promoting cell fusions involving mono-nucleated pre-osteoclasts (Kim and Kim, 2014) and the activation of downstream signalling pathways, which are responsible for osteoclast differentiation (Moller et al., 2017, Han et al., 2000). This might suggest that, downregulation of hsa-miR-373-5p, hsa-miR-215-5p and hsa-miR-4516 in LBMD subjects could promote the overexpression of *CD47* and *TRAF6* leading to increased bone turnover.

ESR1 was a predicted target gene for hsa-miR-373-5p, hsa-miR-122-5p and hsa-miR-215-5p. AR was a predicted target gene for hsa-miR-373-5p, hsa-miR-122-5p and hsa-miR-4516. Others have shown the deletion of *ESR1* in mature osteoblast and osteocytes in mice increased osteoblastic apoptosis, while global deletion of *AR* in male mice result in high bone turnover and increased resorption of bone (Manolagas et al., 2013). In addition, *ESR1* was found to be downregulated in the peripheral blood cells (PBCs) of postmenopausal female osteopenia and osteoporosis patients compared to a postmenopausal, non-osteoporotic control group (Chou et al., 2016).

Follicle stimulating hormone beta subunit (*FSHB*) a pituitary glycoprotein hormone that stimulates the development of ovarian follicles and spermatogenesis in the reproductive organs was also a putative target for hsa-miR-373-5p, hsa-miR-122-5p, and hsa-miR-4516. *FSHB* has been found to be expressed in osteoclasts and their

precursors, but not osteoblasts (Martin and Gaddy, 2006). Increased level of circulating serum FSH in osteoporosis postmenopausal woman compared to postmenopausal woman control group was observed (Wang et al., 2015a). Indeed, all the three binding receptors, *FSHB*, *ESR* and *AR,* are putative target proteins for hsa-miR-373-5p and hsa-miR-122-5p. Their overexpression could be enhanced by the downregulation of hsa-miR-373-5p and hsa-miR-122-5p. However, the dysregulation mechanism of these genes (*FSHB*, *ESR* and *AR*) associated with bone remodelling is not yet clear.

In the String analysis, insulin like growth factor 1 (*IGF1*) was the node with the highest number of interacting genes. Insulin like growth factor 1 (*IGF1*) is the most abundant growth factor in the skeleton, that is crucial for normal bone development and for bone remodeling (Rosen and Donahue, 1998, Rosen, 2004), and has been found to be significantly decreased in women with low bone mineral density (LBMD), compared to normal (Liu et al., 2008). *IGF1* binding to its receptor *IGF1R* has been shown to stimulate signalling pathway of the osteoblastic differentiation (Perrini et al., 2008, Cornish et al., 2004). Conditional *IGF1* knockout mice have an overall decreased bone formation compared to control groups (Govoni et al., 2007), while *IGF1R*-knockout mice have reduced bone mass compared to wild type control (Xian et al., 2012). In human hepatocellular carcinoma (HCC) cell lines (HepG2, Hep3B, and SK-Hep-1) (Bai et al., 2009) and breast cancer (BC) cell lines (MCF-7, T47d, MDA-MB-231 and BT549 cells), *IGF1R* was a target gene for miR-122-5p (Wang et al., 2012a), as well as a putative target protein for hsa-miR-373-5p and hsa-miR-4516-5p. While *IGF1* was found to be putative target gene for hsa-miR-215-5p. Therefore, downregulation of *IGF1* and/or its binding receptor *IGF1R* by these miRNAs could

contributes to the progression of bone turnover leading to osteoporosis. However, the mechanism by which the downregulation of circulating miRNAs involved in the dysregulation of *IGF1* and *IGF1R* still unknown.

BMP2K and *SPARC* have been shown to be putative targets for all four miRNAs identified in this study, hsa-miR-373-5p, hsa-miR-122-5p, hsa-miR-215-5p and hsa-miR-4516. Bone morphogenetic protein2 (*BMP2*) plays a key role in skeletal development and patterning (Kearns et al., 2001). Expression of BMP2-inducible kinase (*BMP2K*), a transmembrane cell surface receptor with serine/threonine protein kinase activity is increased during BMP-2 induced differentiation (Kearns et al., 2001). Both, BMP2-inducible kinase (*BMP2K*) and secreted protein acidic and cysteine rich (*SPARC*) are essential for skeletal differentiation and survival (Salazar et al., 2016, Kearns et al., 2001, Delany et al., 2000, Li et al., 2009). *BMP2K* expression has been found to be upregulated during osteoblast differentiation (Kearns et al., 2001), while decreased levels of *SPARC* in osteonectin-null mice were associated with a decreased level of bone remodeling leading to profound osteopenia (Delany et al., 2000), and mutation of *SPARC* gene was associated with idiopathic osteoporosis in men, compared to age matched control group (Delany et al., 2008). Again, the correlation between the downregulated circulating miRNAs, hsa-miR-373-5p, hsa-miR-122-5p, hsa-miR-215-5p and hsa-miR-4516 and gene expression of *BMP2K* and *SPARC* in osteoporosis patients is unknown.

Likewise, Mitogen-activated protein kinase 1 (*MAPK1*) is a putative target for hsa-miR-373-5p, hsa-miR-122-5p and hsa-miR-215-5p, while *MAPK3* is a putative target for hsa-miR-4516. Both *MAPK1* and *MAPK3* are expressed in osteoblasts, that act in

a signalling cascade involved in the regulation of bone mass via the control of osteoblast differentiation (Ge et al., 2007, Greenblatt et al., 2010, Zou et al., 2011). However, the dysregulation expression of *MAPK1* and *MAPK3* associated with the downregulation of miR-373-5p, hsa-miR-122-5p, hsa-miR-215-5p and hsa-miR-4516 needs to be verified.

The present study showed that, *VDR* is a predicted target for hsa-miR-122-5p, hsa-miR-215-5p and hsa-miR-4516. *VDR* is widely distributed in bone cells, chondrocytes, osteoblasts, osteocytes, and osteoclasts (Haussler et al., 1998, Bikle, 2012). The biologically active metabolite of Vitamin D (1,25-Dihydroxyvitamin D3) and its receptor Vitamin D receptor (*VDR*), together play a vital role in calcium and phosphate homeostasis, and skeletal metabolism, and are considered to be important candidate genes of osteoporosis (Haussler et al., 1998).

In summary, in this Chapter, a relationship between osteoporosis-related genes and the four miRNAs, hsa-miR-373-5p, hsa-miR-122-5p, hsa-miR-215-5p and hsa-miR-4516, has been clearly demonstrated. However, for some target genes, the effects are opposite to those expected from the known action of intracellular miRNAs. Others have shown that the downregulation of circulating miRNAs is not necessarily positively correlated with their cellular expression (Waters et al., 2012). Taken together the findings in this Chapter, of the downregulation of circulating miRNAs, hsa-miR-373-5p, hsa-miR-122-5p, hsa-miR-215-5p and hsa-miR-4516 and their associations with osteoporosis-related predicted gene targets might suggest that circulating miRNAs could have dual function as negative and positive post-transcriptional regulators for protein coding genes.

Chapter 6. Final Discussion and Future Work

6.1 Discussion

Osteoporosis is a silent disease which usually becomes apparent only after a bone fracture has occurred. Currently the diagnosis of osteoporosis is primarily based on the measurement of bone mineral density (BMD) using DXA scan (Kanis, 1994) and the assessment of fragility fracture's risk factors (Johnell et al., 2005). However, both tests have limitation in regard to osteoporotic fragility fracture diagnosis when contributing factors such as diabetic osteopathy (Schwartz et al., 2011), aortic calcification and soft-tissue calcification (Unnanuntana et al., 2010) are present. Furthermore, Changes in BMD occur slowly, and it may take one to two years to detect any differences after osteoporosis treatment (Bonnick and Shulman, 2006).

In addition, the measurement of bone turnover markers (BTMs) in patients' blood and/or urine samples has been used for monitoring treatment and assessment of fracture risk (Naylor and Eastell, 2012), but the level of BTM markers in bone diseases, such as osteoporosis, are affected by preanalytical (Naylor and Eastell, 2012) and analytical variabilities (Garnero, 2017), as well as the lack of established normal reference population and standardized quality control (Bauer et al., 2012).

Therefore, due to their diagnostic and therapeutic monitoring limitations, there is a demand for reliable non-invasive and cost efficient diagnostic biomarkers for osteoporosis. miRNAs are molecular regulators and play an important role in bone formation and resorption processes (van Wijnen et al., 2013). Up- or down-expressed miRNAs are involved in different physiological and pathological conditions (Velu et al., 2012), including bone-related diseases (Hackl et al., 2016). Indeed, circulating miRNAs show remarkable stability in body fluids under different storage conditions

(Mitchell et al., 2008) and extreme conditions of repeated freeze and thaw cycle and exposure low and high pH solutions (Chen et al., 2008). Moreover, miRNAs can be detected using conventional RT-qPCR assays that are specific, sensitive and reproducible.

In this study, 49 differentially expressed miRNAs between osteopenia and osteoporosis patient groups were identified, initially using miRNA PCR arrays. Six miRNAs, miR-215-5p, miR-99a-5p, miR-100, miR-373-5p, miR-4516 and miR-122-5P, were significantly differentially-expressed between osteoporosis and osteopenia patients by initial RT-qPCR screening. The levels of four of these miRNAs, hsa-miR-373-5p, hsa-miR-122-5p and hsa-miR-215-5p and hsa-4516 were associated with osteoporosis patients, compared to non-osteoporotic participants.

Noticeably, miRNAs, hsa-miR-373-5p, hsa-miR-122-5p and hsa-miR-215-5p were detectable in serum samples, but miR-4516 was detectible in plasma samples, suggesting that the clinical specimen sample type seems to influence the detection of the miRNAs. The discrepancy of miRNA expression between serum and plasma could be due to the association of miRNA released from platelets (Wang et al., 2012b) or due to the presence of interfering substances that might be associated with collection tube additives (Dimeski, 2008). However, the cause of this analytical discrepancy still unknown.

An important finding in this study was that hsa-miR-373-5p, hsa-miR-122-5p, hsa-miR-215-5p and hsa-4516 were significantly downregulated in the osteoporosis patients compared to non-osteoporotic participants. Further analysis showed that the levels of these circulating miRNAs were associated with fragility fracture and

correlated with the low bone mineral density in osteoporosis patients. Both serum hsa-miR-373-5p and plasma hsa-miR-4516 in have acceptable diagnostic values of AUC= 0.72 and 0.73 respectively in osteoporosis patients.

Computational analysis for predicting targets for these 4 miRNAs has identified 48 osteoporosis associated genes including sclerostin and low density lipoprotein receptor/related protein 6, TNF receptor-associated factor 6 and CD47, estrogen, androgen and follicle stimulating hormone β-subunit, insulin-like growth factor and its receptor, bone morphogenetic protein and secreted protein acidic and cysteine rich, mitogen activated protein kinases 1 and 3 and the vitamin D receptor. These genes play critical roles in bone related signalling pathways. Thus, miRNAs hsa-miR-373-5p, hsa-miR-122-5p, hsa-miR-215-5p and hsa-4516 and the predicted target genes are potentially valuable biomarkers for the detection of osteoporosis.

6.2 Future Work

The present study has identified 4 miRNAs as potential markers of osteoporosis. The next step will be to replicate the present findings in a clinical trial with a larger number of samples from age matched control and study groups using interval samples for a set period of time. As well, correlates miRNA expression findings with current biomarkers in use, to put the preanalytical variations such as life style, diet, health condition and circadian rhythm as well as analytical variabilities into account (Hackl et al., 2016), to test the diagnostic value, which, if successful, could lead to a multi-centre National or international clinical trial. However, taking blood is an invasive technique and it would be advantageous to be able to detect the miRNAs in body fluids. Testing of circulating miRNAs in osteoporosis patients' urine sample

could make sampling easier and is possible because of the stability of miRNAs in body fluids (Mall et al., 2013).

In order to experimentally test the function of the miRNAs identified in this project, cultured cell systems could be established, in which the miRNAs are individually knocked out/knocked in, and the effect of the reduced miRNA level on the expression of the relevant osteoporosis-related genes identified on the present project will be directly tested. Particular attention will be paid to the relevant signalling pathways identified in the present project.

References

ADOLF, D., WEX, T., JAHN, O., RIEBAU, C., HALANGK, W., KLOSE, S., WESTPHAL, S., AMTHAUER, H., WINCKLER, S. & PIATEK, S. 2012. Serum cathepsin K levels are not suitable to differentiate women with chronic bone disorders such as osteopenia and osteoporosis from healthy pre- and postmenopausal women. *Maturitas,* 71**,** 169-72.

AKOBENG, A. K. 2007. Understanding diagnostic tests 3: Receiver operating characteristic curves. *Acta Paediatr,* 96**,** 644-7.

AKUTA, N., KAWAMURA, Y., SUZUKI, F., SAITOH, S., ARASE, Y., KUNIMOTO, H., SORIN, Y., FUJIYAMA, S., SEZAKI, H., HOSAKA, T., KOBAYASHI, M., SUZUKI, Y., KOBAYASHI, M., IKEDA, K. & KUMADA, H. 2016. Impact of circulating miR-122 for histological features and hepatocellular carcinoma of nonalcoholic fatty liver disease in Japan. *Hepatol Int,* 10**,** 647-56.

ALBERTS, B., JOHNSON, A., LEWIS, J., MORGAN, D., RAFF, M., ROBERTS, K. & WALTER, P. 2015. How Cells Read the Genome: From DNA to Protein. *Molecular Biology of the Cell, Sixth Edition***,** 299-368.

AMBROS, V. 2001. microRNAs: tiny regulators with great potential. *Cell,* 107**,** 823-6.

AMBROS, V. 2004. The functions of animal microRNAs. *Nature,* 431**,** 350-5.

AMELING, S., KACPROWSKI, T., CHILUKOTI, R. K., MALSCH, C., LIEBSCHER, V., SUHRE, K., PIETZNER, M., FRIEDRICH, N., HOMUTH, G., HAMMER, E. & VÖLKER, U. 2015. Associations of circulating plasma microRNAs with age, body mass index and sex in a population-based study. *BMC Medical Genomics,* 8**,** 61.

AN, J. H., OHN, J. H., SONG, J. A., YANG, J. Y., PARK, H., CHOI, H. J., KIM, S. W., KIM, S. Y., PARK, W. Y. & SHIN, C. S. 2013. Changes of microRNA profile and microRNA-mRNA regulatory network in bones of ovariectomized mice. *J Bone Miner Res, 3, 644-56*.

ANDERSEN, T. L., ABDELGAWAD, M. E., KRISTENSEN, H. B., HAUGE, E. M., ROLIGHED, L., BOLLERSLEV, J., KJAERSGAARD-ANDERSEN, P. & DELAISSE, J. M. 2013. Understanding coupling between bone resorption and formation: are reversal cells the missing link? *Am J Pathol,* 183**,** 235-46.

ANDERSEN, T. L., SONDERGAARD, T. E., SKORZYNSKA, K. E., DAGNAES-HANSEN, F., PLESNER, T. L., HAUGE, E. M., PLESNER, T. & DELAISSE, J. M. 2009. A physical mechanism for coupling bone resorption and formation in adult human bone. *Am J Pathol,* 174**,** 239-47.

AOUADI, M., BINETRUY, B., CARON, L., LE MARCHAND-BRUSTEL, Y. & BOST, F. 2006. Role of MAPKs in development and differentiation: lessons from knockout mice. *Biochimie,* 88**,** 1091-8.

ARROYO, J. D., CHEVILLET, J. R., KROH, E. M., RUF, I. K., PRITCHARD, C. C., GIBSON, D. F., MITCHELL, P. S., BENNETT, C. F., POGOSOVA-AGADJANYAN, E. L., STIREWALT, D. L., TAIT, J. F. & TEWARI, M. 2011. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci U S A,* 108**,** 5003-8.

ASAHCHOP, E. L., AKINWUMI, S. M., BRANTON, W. G., FUJIWARA, E., GILL, M. J. & POWER, C. 2016. Plasma microRNA profiling predicts HIV-associated neurocognitive disorder. *Aids,* 30**,** 2021-31.

ATLEY, L. M., MORT, J. S., LALUMIERE, M. & EYRE, D. R. 2000. Proteolysis of human bone collagen by cathepsin K: characterization of the cleavage sites generating by cross-linked N-telopeptide neoepitope. *Bone,* 26**,** 241-7.

BAI, S., NASSER, M. W., WANG, B., HSU, S. H., DATTA, J., KUTAY, H., YADAV, A., NUOVO, G., KUMAR, P. & GHOSHAL, K. 2009. MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. *J Biol Chem,* 284**,** 32015-27.

BAKHSHANDEH, B., SOLEIMANI, M., HAFIZI, M., PAYLAKHI, S. H. & GHAEMI, N. 2012. MicroRNA signature associated with osteogenic lineage commitment. *Mol Biol Rep,* 39.

BARON, R. & KNEISSEL, M. 2013. WNT signaling in bone homeostasis and disease: from human mutations to treatments. *Nat Med,* 19**,** 179-92.

BARTEL, D. P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell,* 116**,** 281-97.

BARTEL, D. P. 2009. MicroRNAs: target recognition and regulatory functions. *Cell,* 136**,** 215-33.

BAUER, D., KREGE, J., LANE, N., LEARY, E., LIBANATI, C., MILLER, P., MYERS, G., SILVERMAN, S., VESPER, H. W., LEE, D., PAYETTE, M. & RANDALL, S. 2012. National Bone Health Alliance Bone Turnover Marker Project: current practices and the need for US harmonization, standardization, and common reference ranges. *Osteoporos Int,* 23**,** 2425-33.

BEATO, M. & KLUG, J. 2000. Steroid hormone receptors: an update. *Hum Reprod Update,* 6**,** 225-36.

BEDENE, A., MENCEJ BEDRAC, S., JESE, L., MARC, J., VRTACNIK, P., PREZELJ, J., KOCJAN, T., KRANJC, T. & OSTANEK, B. 2016. MiR-148a the epigenetic regulator of bone homeostasis is increased in plasma of osteoporotic postmenopausal women. *Wien Klin Wochenschr,* 128**,** 519-26.

BELL, E. H., KIRSTE, S., FLEMING, J. L., STEGMAIER, P., DRENDEL, V., MO, X., LING, S., FABIAN, D., MANRING, I., JILG, C. A., SCHULTZE-SEEMANN, W., MCNULTY, M., ZYNGER, D. L., MARTIN, D., WHITE, J., WERNER, M., GROSU, A. L. & CHAKRAVARTI, A. 2015. A novel miRNA-based predictive model for biochemical failure following post-prostatectomy salvage radiation therapy. *PLoS One,* 10**,** e0118745.

BELL, K. L., LOVERIDGE, N., POWER, J., RUSHTON, N. & REEVE, J. 1999. Intracapsular hip fracture: increased cortical remodeling in the thinned and porous anterior region of the femoral neck. *Osteoporos Int,* 10**,** 248-57.

BELLIDO, T. 2014. Osteocyte-driven bone remodeling. *Calcif Tissue Int,* 94**,** 25-34.

BIKLE, D. D. 2012. Vitamin D and Bone. *Current osteoporosis reports,* 10**,** 151-9.

BONE, H. G. 1992. The future of osteoporosis diagnosis and therapy. *Ann Ital Med Int,* 7**,** 166S-170S.

BONEWALD, L. F. & JOHNSON, M. L. 2008. Osteocytes, mechanosensing and Wnt signaling. *Bone,* 42**,** 606-15.

BONNICK, S. L. & SHULMAN, L. 2006. Monitoring osteoporosis therapy: bone mineral density, bone turnover markers, or both? *Am J Med,* 119**,** S25-31.

BORCHERT, G. M., LANIER, W. & DAVIDSON, B. L. 2006. RNA polymerase III transcribes human microRNAs. *Nat Struct Mol Biol,* 13**,** 1097-101.

BORRELLI, N., DENARO, M., UGOLINI, C., POMA, A. M., MICCOLI, M., VITTI, P., MICCOLI, P. & BASOLO, F. 2017. miRNA expression profiling of 'noninvasive follicular thyroid neoplasms with papillary-like nuclear features' compared

with adenomas and infiltrative follicular variants of papillary thyroid carcinomas. *Mod Pathol,* 30**,** 39-51.

BOWLES, S. A., KURDY, N., DAVIS, A. M. & FRANCE, M. W. 1997. Changes in serum bone-specific alkaline phosphatase following tibial fracture. *Ann Clin Biochem,* 34 (Pt 6)**,** 690-1.

BOYLE, D. L., HAMMAKER, D., EDGAR, M., ZAISS, M. M., TEUFEL, S., DAVID, J. P., SCHETT, G. & FIRESTEIN, G. S. 2014. Differential roles of MAPK kinases MKK3 and MKK6 in osteoclastogenesis and bone loss. *PLoS One,* 9**,** e84818.

BROWN, E., HOOPER, L., HO, T. & GRESHAM, H. 1990. Integrin-associated protein: a 50-kD plasma membrane antigen physically and functionally associated with integrins. *The Journal of Cell Biology,* 111**,** 2785.

BROWN, J. P., DELMAS, P. D., MALAVAL, L., EDOUARD, C., CHAPUY, M. C. & MEUNIER, P. J. 1984. Serum bone Gla-protein: a specific marker for bone formation in postmenopausal osteoporosis. *Lancet,* 1**,** 1091-3.

BUCKWALTER, J. A., GLIMCHER, M. J., COOPER, R. R. & RECKER, R. 1996. Bone biology. I: Structure, blood supply, cells, matrix, and mineralization. *Instr Course Lect,* 45**,** 371-86.

BURR, D. B. & AKKUS, O. 2014. Chapter 1 - Bone Morphology and Organization. *In:* BURR, D. B. & ALLEN, M. R. (eds.) *Basic and Applied Bone Biology.* San Diego: Academic Press, 3-25.

BUSTIN, S. A., BENES, V., GARSON, J. A., HELLEMANS, J., HUGGETT, J., KUBISTA, M., MUELLER, R., NOLAN, T., PFAFFL, M. W., SHIPLEY, G. L., VANDESOMPELE, J. & WITTWER, C. T. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem,* 55**,** 611-22.

CABIBI, D., CARUSO, S., BAZAN, V., CASTIGLIA, M., BRONTE, G., INGRAO, S., FANALE, D., CANGEMI, A., CALO, V., LISTI, A., INCORVAIA, L., GALVANO, A., PANTUSO, G., FIORENTINO, E., CASTORINA, S. & RUSSO, A. 2016. Analysis of tissue and circulating microRNA expression during metaplastic transformation of the esophagus. *Oncotarget,* 7**,** 47821-30.

CAI, X., HAGEDORN, C. H. & CULLEN, B. R. 2004. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA,* 10**,** 1957-66.

CAO, Z., MOORE, B. T., WANG, Y., PENG, X. H., LAPPE, J. M., RECKER, R. R. & XIAO, P. 2014. MiR-422a as a potential cellular microRNA biomarker for postmenopausal osteoporosis. *PLoS One,* 9**,** e97098.

CAPULLI, M., PAONE, R. & RUCCI, N. 2014. Osteoblast and osteocyte: Games without frontiers. *Archives of Biochemistry and Biophysics,* 561**,** 3-12.

CARBON, S., IRELAND, A., MUNGALL, C. J., SHU, S., MARSHALL, B., LEWIS, S., THE AMI, G. O. H. & THE WEB PRESENCE WORKING, G. 2009. AmiGO: online access to ontology and annotation data. *Bioinformatics,* 25**,** 288-9.

CAULEY, J. A. 2015. Estrogen and bone health in men and women. *Steroids,* 99**,** 11-5.

CAULEY, J. A., EL-HAJJ FULEIHAN, G., ARABI, A., FUJIWARA, S., RAGI-EIS, S., CALDERON, A., CHIONH, S. B., CHEN, Z., CURTIS, J. R., DANIELSON, M. E., HANLEY, D. A., KROGER, H., KUNG, A. W. C., LESNYAK, O., NIEVES, J., PLUSKIEWICZ, W., EL RASSI, R., SILVERMAN, S., SCHOTT, A.-M., RIZZOLI, R. & LUCKEY, M. 2011. Official Positions for FRAX® Clinical Regarding International Differences. *Journal of Clinical Densitometry,* 14**,** 240-62.

CHAO, T.-Y., WU, Y.-Y. & JANCKILA, A. J. 2010. Tartrate-resistant acid phosphatase isoform 5b (TRACP 5b) as a serum maker for cancer with bone metastasis. *Clinica Chimica Acta,* 411**,** 1553-64.

CHEN, C., CHENG, P., XIE, H., ZHOU, H. D., WU, X. P., LIAO, E. Y. & LUO, X. H. 2014a. MiR-503 regulates osteoclastogenesis via targeting RANK. *J Bone Miner Res,* 29**,** 338-47.

CHEN, J. T., HOSODA, K., HASUMI, K., OGATA, E. & SHIRAKI, M. 1996. Serum N-terminal osteocalcin is a good indicator for estimating responders to hormone replacement therapy in postmenopausal women. *J Bone Miner Res,* 11**,** 1784-92.

CHEN, Q., GE, X., ZHANG, Y., XIA, H., YUAN, D., TANG, Q., CHEN, L., PANG, X., LENG, W. & BI, F. 2014b. Plasma miR-122 and miR-192 as potential novel biomarkers for the early detection of distant metastasis of gastric cancer. *Oncol Rep,* 31**,** 1863-70.

CHEN, W., CAI, F., ZHANG, B., BAREKATI, Z. & ZHONG, X. Y. 2013. The level of circulating miRNA-10b and miRNA-373 in detecting lymph node metastasis of breast cancer: potential biomarkers. *Tumour Biol,* 34**,** 455-62.

CHEN, X., BA, Y., MA, L., CAI, X., YIN, Y., WANG, K., GUO, J., ZHANG, Y., CHEN, J., GUO, X., LI, Q., LI, X., WANG, W., ZHANG, Y., WANG, J., JIANG, X., XIANG, Y., XU, C., ZHENG, P., ZHANG, J., LI, R., ZHANG, H., SHANG, X., GONG, T., NING, G., WANG, J., ZEN, K., ZHANG, J. & ZHANG, C. Y. 2008. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res,* 18**,** 997-1006.

CHEN, Z., HAN, S., HUANG, W., WU, J., LIU, Y., CAI, S., HE, Y., WU, S. & SONG, W. 2016. MicroRNA-215 suppresses cell proliferation, migration and invasion of colon cancer by repressing Yin-Yang 1. *Biochem Biophys Res Commun,* 479**,** 482-488.

CHENG, H. R., KAO, J. H., WU, H. L., TSENG, T. C., LIU, C. H., YANG, H. C., SU, T. H., CHEN, P. J., CHEN, D. S. & LIU, C. J. 2015. Clinical significance of circulating miR-122 in patients with dual chronic hepatitis B and C virus infection. *Hepatol Int,* 9**,** 35-42.

CHENG, P., CHEN, C., HE, H. B., HU, R., ZHOU, H. D., XIE, H., ZHU, W., DAI, R. C., WU, X. P., LIAO, E. Y. & LUO, X. H. 2013. miR-148a regulates osteoclastogenesis by targeting V-maf musculoaponeurotic fibrosarcoma oncogene homolog B. *J Bone Miner Res,* 28**,** 1180-90.

CHOU, C. W., CHIANG, T. I., CHANG, I. C., HUANG, C. H. & CHENG, Y. W. 2016. Expression levels of estrogen receptor alpha mRNA in peripheral blood cells are an independent biomarker for postmenopausal osteoporosis. *BBA Clin,* 5**,** 124-9.

CHOWDHARI, S. & SAINI, N. 2014. hsa-miR-4516 mediated downregulation of STAT3/CDK6/UBE2N plays a role in PUVA induced apoptosis in keratinocytes. *J Cell Physiol,* 229**,** 1630-8.

CHRISTGAU, S., BITSCH-JENSEN, O., HANOVER BJARNASON, N., GAMWELL HENRIKSEN, E., QVIST, P., ALEXANDERSEN, P. & BANG HENRIKSEN, D. 2000. Serum CrossLaps for monitoring the response in individuals undergoing antiresorptive therapy. *Bone,* 26**,** 505-11.

CIFUENTES, D., XUE, H., TAYLOR, D. W., PATNODE, H., MISHIMA, Y., CHELOUFI, S., MA, E., MANE, S., HANNON, G. J., LAWSON, N. D., WOLFE, S. A. & GIRALDEZ, A. J. 2010. A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. *Science,* 328**,** 1694-8.

CLOOS, P. A. & CHRISTGAU, S. 2004. Characterization of aged osteocalcin fragments derived from bone resorption. *Clin Lab,* 50**,** 585-98.

COMPSTON, J., COOPER, A., COOPER, C., GITTOES, N., GREGSON, C., HARVEY, N., HOPE, S., KANIS, J. A., MCCLOSKEY, E. V., POOLE, K. E. S., REID, D. M., SELBY, P., THOMPSON, F., THURSTON, A., VINE, N. & NATIONAL OSTEOPOROSIS GUIDELINE, G. 2017. UK clinical guideline for the prevention and treatment of osteoporosis. *Arch Osteoporos,* 12**,** 43.

CONFAVREUX, C. B. 2011. Bone: from a reservoir of minerals to a regulator of energy metabolism. *Kidney Int Suppl***,** S14-9.

CORNISH, J., GREY, A., CALLON, K. E., NAOT, D., HILL, B. L., LIN, C. Q., BALCHIN, L. M. & REID, I. R. 2004. Shared pathways of osteoblast mitogenesis induced by amylin, adrenomedullin, and IGF-1. *Biochem Biophys Res Commun,* 318**,** 240-6.

CORTEZ-DIAS, N., COSTA, M. C., CARRILHO-FERREIRA, P., SILVA, D., JORGE, C., CALISTO, C., PESSOA, T., ROBALO MARTINS, S., DE SOUSA, J. C., DA SILVA, P. C., FIUZA, M., DIOGO, A. N., PINTO, F. J. & ENGUITA, F. J. 2016. Circulating miR-122-5p/miR-133b Ratio Is a Specific Early Prognostic Biomarker in Acute Myocardial Infarction. *Circ J,* 80**,** 2183-91.

CORTEZ, M. A. & CALIN, G. A. 2009. MicroRNA identification in plasma and serum: a new tool to diagnose and monitor diseases. *Expert Opin Biol Ther,* 9**,** 703-711.

CROCKETT, J. C., MELLIS, D. J., SCOTT, D. I. & HELFRICH, M. H. 2011a. New knowledge on critical osteoclast formation and activation pathways from study of rare genetic diseases of osteoclasts: focus on the RANK/RANKL axis. *Osteoporosis International,* 22**,** 1-20.

CROCKETT, J. C., ROGERS, M. J., COXON, F. P., HOCKING, L. J. & HELFRICH, M. H. 2011b. Bone remodelling at a glance. *Journal of Cell Science,* 124**,** 991-8.

CULLEN, B. R. 2004. Transcription and processing of human microRNA precursors. *Mol Cell,* 16**,** 861-5.

CURTIS, E. M., VAN DER VELDE, R., MOON, R. J., VAN DEN BERGH, J. P. W., GEUSENS, P., DE VRIES, F., VAN STAA, T. P., COOPER, C. & HARVEY, N. C. 2016. Epidemiology of fractures in the United Kingdom 1988–2012: Variation with age, sex, geography, ethnicity and socioeconomic status. *Bone,* 87**,** 19-26.

D'ALESSANDRA, Y., DEVANNA, P., LIMANA, F., STRAINO, S., DI CARLO, A., BRAMBILLA, P. G., RUBINO, M., CARENA, M. C., SPAZZAFUMO, L., DE SIMONE, M., MICHELI, B., BIGLIOLI, P., ACHILLI, F., MARTELLI, F., MAGGIOLINI, S., MARENZI, G., POMPILIO, G. & CAPOGROSSI, M. C. 2010. Circulating microRNAs are new and sensitive biomarkers of myocardial infarction. *Eur Heart J,* 31**,** 2765-73.

DAI, R. & AHMED, S. A. 2014. Sexual dimorphism of miRNA expression: a new perspective in understanding the sex bias of autoimmune diseases. *Ther Clin Risk Manag,* 10**,** 151-63.

DAS, S. & CROCKETT, J. C. 2013. Osteoporosis - a current view of pharmacological prevention and treatment. *Drug Des Devel Ther,* 7**,** 435-48.

DELAISSE, J.-M. 2014. The reversal phase of the bone-remodeling cycle: cellular prerequisites for coupling resorption and formation. *BoneKEy Rep,* 3.

DELANY, A. M., AMLING, M., PRIEMEL, M., HOWE, C., BARON, R. & CANALIS, E. 2000. Osteopenia and decreased bone formation in osteonectin-deficient mice. *J Clin Invest,* 105**,** 915-23.

DELANY, A. M., MCMAHON, D. J., POWELL, J. S., GREENBERG, D. A. & KURLAND, E. S. 2008. Osteonectin/SPARC polymorphisms in Caucasian men with idiopathic osteoporosis. *Osteoporos Int,* 19**,** 969-78.

DELMAS, P. D., EASTELL, R., GARNERO, P., SEIBEL, M. J., STEPAN, J. & COMMITTEE OF SCIENTIFIC ADVISORS OF THE INTERNATIONAL OSTEOPOROSIS, F. 2000. The use of biochemical markers of bone turnover in osteoporosis. Committee of Scientific Advisors of the International Osteoporosis Foundation. *Osteoporos Int,* 11 Suppl 6**,** S2-17.

DENG, Y., HUANG, Z., XU, Y., JIN, J., ZHUO, W., ZHANG, C., ZHANG, X., SHEN, M., YAN, X., WANG, L., WANG, X., KANG, Y., SI, J. & ZHOU, T. 2014. MiR-215 modulates gastric cancer cell proliferation by targeting RB1. *Cancer Lett,* 342**,** 27-35.

DIEZ-PLANELLES, C., SANCHEZ-LOZANO, P., CRESPO, M. C., GIL-ZAMORANO, J., RIBACOBA, R., GONZALEZ, N., SUAREZ, E., MARTINEZ-DESCALS, A., MARTINEZ-CAMBLOR, P., ALVAREZ, V., MARTIN-HERNANDEZ, R., HUERTA-RUIZ, I., GONZALEZ-GARCIA, I., COSGAYA, J. M., VISIOLI, F., DAVALOS, A., IGLESIAS-GUTIERREZ, E. & TOMAS-ZAPICO, C. 2016. Circulating microRNAs in Huntington's disease: Emerging mediators in metabolic impairment. *Pharmacol Res,* 108**,** 102-10.

DIMESKI, G. 2008. Interference Testing. *The Clinical Biochemist Reviews,* 29**,** S43-S48.

DOENCH, J. G. & SHARP, P. A. 2004. Specificity of microRNA target selection in translational repression. *Genes Dev,* 18**,** 504-11.

DONG, C., DAVIS, R. J. & FLAVELL, R. A. 2002. MAP kinases in the immune response. *Annu Rev Immunol,* 20**,** 55-72.

DOWNEY, P. A. & SIEGEL, M. I. 2006. Bone Biology and the Clinical Implications for Osteoporosis. *Physical Therapy,* 86**,** 77-91.

DU, J., ZHANG, Y., SHEN, L., LUO, J., LEI, H., ZHANG, P., PU, Q., LIU, Y., SHUAI, S., LI, Q., LI, X., ZHANG, S. & ZHU, L. 2016. Effect of miR-143-3p on C2C12 myoblast differentiation. *Biosci Biotechnol Biochem,* 80**,** 706-11.

DUCY, P., SCHINKE, T. & KARSENTY, G. 2000. The Osteoblast: A Sophisticated Fibroblast under Central Surveillance. *Science,* 289**,** 1501-04.

DWEEP, H. & GRETZ, N. 2015. miRWalk2.0: a comprehensive atlas of microRNA-target interactions. *Nat Methods,* 12**,** 697.

EASTELL, R., KREGE, J. H., CHEN, P., GLASS, E. V. & REGINSTER, J. Y. 2006. Development of an algorithm for using PINP to monitor treatment of patients with teriparatide. *Curr Med Res Opin,* 22**,** 61-6.

EGUCHI, T., WATANABE, K., HARA, E. S., ONO, M., KUBOKI, T. & CALDERWOOD, S. K. 2013. OstemiR: a novel panel of microRNA biomarkers in osteoblastic and osteocytic differentiation from mesencymal stem cells. *PLoS One,* 8**,** e58796.

EICHELSER, C., FLESCH-JANYS, D., CHANG-CLAUDE, J., PANTEL, K. & SCHWARZENBACH, H. 2013. Deregulated serum concentrations of circulating cell-free microRNAs miR-17, miR-34a, miR-155, and miR-373 in human breast cancer development and progression. *Clin Chem,* 59**,** 1489-96.

EICHELSER, C., STUCKRATH, I., MULLER, V., MILDE-LANGOSCH, K., WIKMAN, H., PANTEL, K. & SCHWARZENBACH, H. 2014. Increased serum levels of circulating exosomal microRNA-373 in receptor-negative breast cancer patients. *Oncotarget,* 5**,** 9650-63.

EL-GAREM, H., AMMER, A., SHEHAB, H., SHAKER, O., ANWER, M., EL-AKEL, W. & OMAR, H. 2014. Circulating microRNA, miR-122 and miR-221 signature in Egyptian patients with chronic hepatitis C related hepatocellular carcinoma. *World J Hepatol,* 6**,** 818-24.

ELL, B., MERCATALI, L., IBRAHIM, T., CAMPBELL, N., SCHWARZENBACH, H., PANTEL, K., AMADORI, D. & KANG, Y. 2013. Tumor-induced osteoclast miRNA changes as regulators and biomarkers of osteolytic bone metastasis. *Cancer Cell,* 24**,** 542-56.

ELLIES, D. L., VIVIANO, B., MCCARTHY, J., REY, J. P., ITASAKI, N., SAUNDERS, S. & KRUMLAUF, R. 2006. Bone density ligand, Sclerostin, directly interacts with LRP5 but not LRP5G171V to modulate Wnt activity. *J Bone Miner Res,* 21**,** 1738-49.

EMAMI, A., LARSSON, A., PETREN-MALLMIN, M. & LARSSON, S. 1999. Serum bone markers after intramedullary fixed tibial fractures. *Clin Orthop Relat Res***,** 220-9.

EVERTS, V., DELAISSE, J. M., KORPER, W., JANSEN, D. C., TIGCHELAAR-GUTTER, W., SAFTIG, P. & BEERTSEN, W. 2002. The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation. *J Bone Miner Res,* 17**,** 77-90.

FANG, L., ELLIMS, A. H., MOORE, X. L., WHITE, D. A., TAYLOR, A. J., CHIN-DUSTING, J. & DART, A. M. 2015. Circulating microRNAs as biomarkers for diffuse myocardial fibrosis in patients with hypertrophic cardiomyopathy. *J Transl Med,* 13**,** 314.

FITZPATRICK, L. A. 2006. Estrogen therapy for postmenopausal osteoporosis. *Arq Bras Endocrinol Metabol,* 50**,** 705-19.

FLORENCIO-SILVA, R., SASSO, G. R., SASSO-CERRI, E., SIMOES, M. J. & CERRI, P. S. 2015. Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells. *Biomed Res Int,* 2015**,** 421746.

FRANCESCHETTI, T., KESSLER, C. B., LEE, S. K. & DELANY, A. M. 2013. miR-29 promotes murine osteoclastogenesis by regulating osteoclast commitment and migration. *J Biol Chem,* 288**,** 33347-60.

FRANCINI, G., GONNELLI, S., PETRIOLI, R., BRUNI, S., MARSILI, S., AQUINO, A. & CAMPOREALE, A. 1993. Procollagen type I carboxy-terminal propeptide as a marker of osteoblastic bone metastases. *Cancer Epidemiol Biomarkers Prev,* 2**,** 125-9.

FRANZ-ODENDAAL, T. A., HALL, B. K. & WITTEN, P. E. 2006. Buried alive: how osteoblasts become osteocytes. *Dev Dyn,* 235**,** 176-90.

FRERES, P., JOSSE, C., BOVY, N., BOUKERROUCHA, M., STRUMAN, I., BOURS, V. & JERUSALEM, G. 2015. Neoadjuvant Chemotherapy in Breast Cancer Patients Induces miR-34a and miR-122 Expression. *J Cell Physiol,* 230**,** 473-81.

FRIEDMAN, R. C., FARH, K. K., BURGE, C. B. & BARTEL, D. P. 2009. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res,* 19**,** 92-105.

FU, H. L., PAN, H. X., ZHAO, B., DONG, B. C., SHAO, L., FU, G. S., WANG, Q. & LI, M. 2016. MicroRNA-100 inhibits bone morphogenetic protein-induced osteoblast differentiation by targeting Smad1. *European Review for Medical and Pharmacological Sciences,* 20**,** 3911-3919.

FUKUMOTO, S. & MARTIN, T. J. 2009. Bone as an endocrine organ. *Trends Endocrinol Metab,* 20**,** 230-6.

GARNERO, P. 2017. The Utility of Biomarkers in Osteoporosis Management. *Molecular Diagnosis & Therapy***,** 1-18.

GAUR, T., HUSSAIN, S., MUDHASANI, R., PARULKAR, I., COLBY, J. L., FREDERICK, D., KREAM, B. E., VAN WIJNEN, A. J., STEIN, J. L., STEIN, G. S., JONES, S. N. & LIAN, J. B. 2010. Dicer inactivation in osteoprogenitor cells compromises fetal survival and bone formation, while excision in differentiated osteoblasts increases bone mass in the adult mouse. *Dev Biol,* 340**,** 10-21.

GE, C., XIAO, G., JIANG, D. & FRANCESCHI, R. T. 2007. Critical role of the extracellular signal-regulated kinase-MAPK pathway in osteoblast differentiation and skeletal development. *J Cell Biol,* 176.

GE, G., ZHANG, W., NIU, L., YAN, Y., REN, Y. & ZOU, Y. 2016. miR-215 functions as a tumor suppressor in epithelial ovarian cancer through regulation of the X-chromosome-linked inhibitor of apoptosis. *Oncol Rep,* 35**,** 1816-22.

GENNARI, L., BIANCIARDI, S. & MERLOTTI, D. 2016. MicroRNAs in bone diseases. *Osteoporosis International***,** 1-23.

GHILDIYAL, M. & ZAMORE, P. D. 2009. Small silencing RNAs: an expanding universe. *Nat Rev Genet,* 10**,** 94-108.

GILAD, S., MEIRI, E., YOGEV, Y., BENJAMIN, S., LEBANONY, D., YERUSHALMI, N., BENJAMIN, H., KUSHNIR, M., CHOLAKH, H., MELAMED, N., BENTWICH, Z., HOD, M., GOREN, Y. & CHAJUT, A. 2008. Serum microRNAs are promising novel biomarkers. *PLoS One,* 3**,** e3148.

GILDEA, J. J., CARLSON, J. M., SCHOEFFEL, C. D., CAREY, R. M. & FELDER, R. A. 2013. Urinary exosome miRNome analysis and its applications to salt sensitivity of blood pressure. *Clin Biochem,* 46**,** 1131-4.

GOUGELET, A., PISSALOUX, D., BESSE, A., PEREZ, J., DUC, A., DUTOUR, A., BLAY, J. Y. & ALBERTI, L. 2011. Micro-RNA profiles in osteosarcoma as a predictive tool for ifosfamide response. *Int J Cancer,* 129**,** 680-90.

GOVONI, K. E., WERGEDAL, J. E., FLORIN, L., ANGEL, P., BAYLINK, D. J. & MOHAN, S. 2007. Conditional deletion of insulin-like growth factor-I in collagen type 1alpha2-expressing cells results in postnatal lethality and a dramatic reduction in bone accretion. *Endocrinology,* 148**,** 5706-15.

GREENBLATT, M. B., SHIM, J. H., ZOU, W., SITARA, D., SCHWEITZER, M., HU, D., LOTINUN, S., SANO, Y., BARON, R., PARK, J. M., ARTHUR, S., XIE, M., SCHNEIDER, M. D., ZHAI, B., GYGI, S., DAVIS, R. & GLIMCHER, L. H. 2010. The p38 MAPK pathway is essential for skeletogenesis and bone homeostasis in mice. *J Clin Invest,* 120**,** 2457-73.

GUAY, C. & REGAZZI, R. 2013. Circulating microRNAs as novel biomarkers for diabetes mellitus. *Nat Rev Endocrinol,* 9**,** 513-21.

GUO, L., ZHANG, Q., MA, X., WANG, J. & LIANG, T. 2017. miRNA and mRNA expression analysis reveals potential sex-biased miRNA expression. 7**,** 39812.

GUO, L. J., LIAO, L., YANG, L., LI, Y. & JIANG, T. J. 2014. MiR-125a TNF receptor-associated factor 6 to inhibit osteoclastogenesis. *Exp Cell Res,* 321**,** 142-52.

HACKL, M., HEILMEIER, U., WEILNER, S. & GRILLARI, J. 2016. Circulating microRNAs as novel biomarkers for bone diseases - Complex signatures for multifactorial diseases? *Molecular and Cellular Endocrinology,* 432**,** 93-105.

HALLEEN, J. M., TIITINEN, S. L., YLIPAHKALA, H., FAGERLUND, K. M. & VAANANEN, H. K. 2006. Tartrate-resistant acid phosphatase 5b (TRACP 5b) as a marker of bone resorption. *Clin Lab,* 52**,** 499-509.

HAN, X., STERLING, H., CHEN, Y., SAGINARIO, C., BROWN, E. J., FRAZIER, W. A., LINDBERG, F. P. & VIGNERY, A. 2000. CD47, a ligand for the macrophage fusion receptor, participates in macrophage multinucleation. *J Biol Chem,* 275**,** 37984-92.

HANSON, D. A., WEIS, M. A., BOLLEN, A. M., MASLAN, S. L., SINGER, F. R. & EYRE, D. R. 1992. A specific immunoassay for monitoring human bone resorption: quantitation of type I collagen cross-linked N-telopeptides in urine. *J Bone Miner Res,* 7**,** 1251-8.

HARADA, S. & RODAN, G. A. 2003. Control of osteoblast function and regulation of bone mass. *Nature,* 423**,** 349-55.

HARMAN, S. M., METTER, E. J., TOBIN, J. D., PEARSON, J. & BLACKMAN, M. R. 2001. Longitudinal effects of aging on serum total and free testosterone levels in healthy men. Baltimore Longitudinal Study of Aging. *J Clin Endocrinol Metab,* 86**,** 724-31.

HASSAN, M. Q., GORDON, J. A., BELOTI, M. M., CROCE, C. M., VAN WIJNEN, A. J., STEIN, J. L., STEIN, G. S. & LIAN, J. B. 2010. A network connecting Runx2, SATB2, and the miR-23a~27a~24-2 cluster regulates the osteoblast differentiation program. *Proc Natl Acad Sci U S A,* 107**,** 19879-84.

HAUSSLER, M. R., WHITFIELD, G. K., HAUSSLER, C. A., HSIEH, J. C., THOMPSON, P. D., SELZNICK, S. H., DOMINGUEZ, C. E. & JURUTKA, P. W. 1998. The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. *J Bone Miner Res,* 13**,** 325-49.

HE, L. & HANNON, G. J. 2004. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet,* 5**,** 522-31.

HEBERLE, H., MEIRELLES, G. V., DA SILVA, F. R., TELLES, G. P. & MINGHIM, R. 2015. InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams. *BMC Bioinformatics,* 16**,** 169.

HENRIKSEN, D. B., ALEXANDERSEN, P., BJARNASON, N. H., VILSBOLL, T., HARTMANN, B., HENRIKSEN, E. E., BYRJALSEN, I., KRARUP, T., HOLST, J. J. & CHRISTIANSEN, C. 2003. Role of gastrointestinal hormones in postprandial reduction of bone resorption. *J Bone Miner Res,* 18**,** 2180-9.

HERNLUND, E., SVEDBOM, A., IVERGARD, M., COMPSTON, J., COOPER, C., STENMARK, J., MCCLOSKEY, E. V., JONSSON, B. & KANIS, J. A. 2013. Osteoporosis in the European Union: medical management, epidemiology and economic burden. A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA). *Arch Osteoporos,* 8**,** 136.

HOLMEN, S. L., GIAMBERNARDI, T. A., ZYLSTRA, C. R., BUCKNER-BERGHUIS, B. D., RESAU, J. H., HESS, J. F., GLATT, V., BOUXSEIN, M. L., AI, M., WARMAN, M. L.

& WILLIAMS, B. O. 2004. Decreased BMD and limb deformities in mice carrying mutations in both Lrp5 and Lrp6. *J Bone Miner Res,* 19**,** 2033-40.

HOLZER, G., NOSKE, H., LANG, T., HOLZER, L. & WILLINGER, U. 2005. Soluble cathepsin K: a novel marker for the prediction of nontraumatic fractures? *J Lab Clin Med,* 146**,** 13-7.

HOU, Y., ZHEN, J., XU, X., ZHEN, K., ZHU, B., PAN, R. & ZHAO, C. 2015. miR-215 functions as a tumor suppressor and directly targets ZEB2 in human non-small cell lung cancer. *Oncol Lett,* 10**,** 1985-92.

HU, H., ZHANG, Y., CAI, X. H., HUANG, J. F. & CAI, L. 2012. Changes in microRNA expression in the MG-63 osteosarcoma cell line compared with osteoblasts. *Oncol Lett,* 4**,** 1037-42.

HUA, Y., CHEN, H., WANG, L., WANG, F., WANG, P., NING, Z., LI, Y., LIU, L., CHEN, Z. & MENG, Z. 2017. Low serum miR-373 predicts poor prognosis in patients with pancreatic cancer. *Cancer Biomark,* 20**,** 95-100.

HUANG DA, W., SHERMAN, B. T. & LEMPICKI, R. A. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc,* 4**,** 44-57.

HUANG, J. H., GAO, K. D., LIN, J. & WANG, Q. G. 2014. MicroRNA-100 inhibits osteosarcoma cell proliferation by targeting Cyr61. *Tumor Biology,* 35**,** 1095-1100.

HUELSKEN, J. & BIRCHMEIER, W. 2001. New aspects of Wnt signaling pathways in higher vertebrates. *Current Opinion in Genetics & Development,* 11**,** 547-553.

ILIOPOULOS, D., MALIZOS, K. N., OIKONOMOU, P. & TSEZOU, A. 2008. Integrative microRNA and proteomic approaches identify novel osteoarthritis genes and their collaborative metabolic and inflammatory networks. *PLoS One,* 3**,** e3740.

IVASKA, K. K., GERDHEM, P., VAANANEN, H. K., AKESSON, K. & OBRANT, K. J. 2010. Bone turnover markers and prediction of fracture: a prospective follow-up study of 1040 elderly women for a mean of 9 years. *J Bone Miner Res,* 25**,** 393-403.

JI, X., CHEN, X. & YU, X. 2016. MicroRNAs in Osteoclastogenesis and Function: Potential Therapeutic Targets for Osteoporosis. *Int J Mol Sci,* 17**,** 349.

JOHNELL, O. & KANIS, J. A. 2006. An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. *Osteoporos Int,* 17**,** 1726-33.

JOHNELL, O., KANIS, J. A., ODEN, A., JOHANSSON, H., DE LAET, C., DELMAS, P., EISMAN, J. A., FUJIWARA, S., KROGER, H., MELLSTROM, D., MEUNIER, P. J., MELTON, L. J., 3RD, O'NEILL, T., POLS, H., REEVE, J., SILMAN, A. & TENENHOUSE, A. 2005. Predictive value of BMD for hip and other fractures. *J Bone Miner Res,* 20**,** 1185-94.

JONES, G., STRUGNELL, S. A. & DELUCA, H. F. 1998. Current understanding of the molecular actions of vitamin D. *Physiol Rev,* 78**,** 1193-231.

JORDAN, K. M. & COOPER, C. 2002. Epidemiology of osteoporosis. *Best Pract Res Clin Rheumatol,* 16**,** 795-806.

KAGIYA, T. & NAKAMURA, S. 2013. Expression profiling of microRNAs in RAW264.7 cells treated with a combination of tumor necrosis factor alpha and RANKL during osteoclast differentiation. *J Periodontal Res,* 48**,** 373-85.

KAMEL, S., BRAZIER, M., NERI, V., PICARD, C., SAMSON, L., DESMET, G. & SEBERT, J. L. 1995. Multiple molecular forms of pyridinolines cross-links excreted in human urine evaluated by chromatographic and immunoassay methods. *J Bone Miner Res,* 10**,** 1385-92.

KANIS, J. A. 1994. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis: synopsis of a WHO report. WHO Study Group. *Osteoporos Int,* 4**,** 368-81.

KANIS, J. A., DELMAS, P., BURCKHARDT, P., COOPER, C. & TORGERSON, D. 1997. Guidelines for diagnosis and management of osteoporosis. The European Foundation for Osteoporosis and Bone Disease. *Osteoporos Int,* 7**,** 390-406.

KANIS, J. A. & JOHNELL, O. 2005. Requirements for DXA for the management of osteoporosis in Europe. *Osteoporos Int,* 16**,** 229-38.

KANIS, J. A., MCCLOSKEY, E. V., JOHANSSON, H., COOPER, C., RIZZOLI, R. & REGINSTER, J. Y. 2013. European guidance for the diagnosis and management of osteoporosis in postmenopausal women. *Osteoporosis International,* 24**,** 23-57.

KARAAYVAZ, M., PAL, T., SONG, B., ZHANG, C., GEORGAKOPOULOS, P., MEHMOOD, S., BURKE, S., SHROYER, K. & JU, J. 2011. Prognostic significance of miR-215 in colon cancer. *Clin Colorectal Cancer,* 10**,** 340-7.

KARSDAL, M. A., HENRIKSEN, K., SORENSEN, M. G., GRAM, J., SCHALLER, S., DZIEGIEL, M. H., HEEGAARD, A. M., CHRISTOPHERSEN, P., MARTIN, T. J., CHRISTIANSEN, C. & BOLLERSLEV, J. 2005. Acidification of the osteoclastic resorption compartment provides insight into the coupling of bone formation to bone resorption. *Am J Pathol,* 166**,** 467-76.

KARSENTY, G., KRONENBERG, H. M. & SETTEMBRE, C. 2009. Genetic control of bone formation. *Annu Rev Cell Dev Biol,* 25**,** 629-48.

KEARNS, A. E., DONOHUE, M. M., SANYAL, B. & DEMAY, M. B. 2001. Cloning and characterization of a novel protein kinase that impairs osteoblast differentiation in vitro. *J Biol Chem,* 276**,** 42213-8.

KERR, J. F., WYLLIE, A. H. & CURRIE, A. R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer,* 26**,** 239-57.

KHELLA, H. W., BAKHET, M., ALLO, G., JEWETT, M. A., GIRGIS, A. H., LATIF, A., GIRGIS, H., VON BOTH, I., BJARNASON, G. A. & YOUSEF, G. M. 2013. miR-192, miR-194 and miR-215: a convergent microRNA network suppressing tumor progression in renal cell carcinoma. *Carcinogenesis,* 34**,** 2231-9.

KIM, J. H. & KIM, N. 2014. Regulation of NFATc1 in Osteoclast Differentiation. *Journal of Bone Metabolism,* 21**,** 233-41.

KOBAYASHI, T., LU, J., COBB, B. S., RODDA, S. J., MCMAHON, A. P., SCHIPANI, E., MERKENSCHLAGER, M. & KRONENBERG, H. M. 2008. Dicer-dependent pathways regulate chondrocyte proliferation and differentiation. *Proc Natl Acad Sci U S A,* 105**,** 1949-54.

KOSAKA, N., IGUCHI, H. & OCHIYA, T. 2010. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. *Cancer Sci,* 101**,** 2087-92.

KRAMER, I., HALLEUX, C., KELLER, H., PEGURRI, M., GOOI, J. H., WEBER, P. B., FENG, J. Q., BONEWALD, L. F. & KNEISSEL, M. 2010. Osteocyte Wnt/β-Catenin

Signaling Is Required for Normal Bone Homeostasis. *Molecular and Cellular Biology,* 30**,** 3071-85.

KRIEG, T., HEIN, R., HATAMOCHI, A. & AUMAILLEY, M. 1988. Molecular and clinical aspects of connective tissue. *Eur J Clin Invest,* 18**,** 105-23.

KROH, E. M., PARKIN, R. K., MITCHELL, P. S. & TEWARI, M. 2010. Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). *Methods,* 50**,** 298-301.

KRZESZINSKI, J. Y., WEI, W., HUYNH, H., JIN, Z., WANG, X., CHANG, T. C., XIE, X. J., HE, L., MANGALA, L. S., LOPEZ-BERESTEIN, G., SOOD, A. K., MENDELL, J. T. & WAN, Y. 2014. miR-34a blocks osteoporosis and bone metastasis by inhibiting osteoclastogenesis and Tgif2. *Nature,* 512**,** 431-5.

KULESHOV, M. V., JONES, M. R., ROUILLARD, A. D., FERNANDEZ, N. F., DUAN, Q., WANG, Z., KOPLEV, S., JENKINS, S. L., JAGODNIK, K. M., LACHMANN, A., MCDERMOTT, M. G., MONTEIRO, C. D., GUNDERSEN, G. W. & MA'AYAN, A. 2016. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res,* 44**,** W90-7.

LAMOTHE, B., WEBSTER, W. K., GOPINATHAN, A., BESSE, A., CAMPOS, A. D. & DARNAY, B. G. 2007. TRAF6 Ubiquitin Ligase is Essential for RANKL Signaling and Osteoclast Differentiation. *Biochemical and biophysical research communications,* 359**,** 1044-49.

LATERZA, O. F., SCOTT, M. G., GARRETT-ENGELE, P. W., KORENBLAT, K. M. & LOCKWOOD, C. M. 2013. Circulating miR-122 as a potential biomarker of liver disease. *Biomark Med,* 7**,** 205-10.

LEE, Y., AHN, C., HAN, J., CHOI, H., KIM, J., YIM, J., LEE, J., PROVOST, P., RADMARK, O., KIM, S. & KIM, V. N. 2003. The nuclear RNase III Drosha initiates microRNA processing. *Nature,* 425**,** 415-9.

LEE, Y., KIM, H. J., PARK, C. K., KIM, Y. G., LEE, H. J., KIM, J. Y. & KIM, H. H. 2013. MicroRNA-124 regulates osteoclast differentiation. *Bone,* 56**,** 383-9.

LEE, Y., KIM, M., HAN, J., YEOM, K. H., LEE, S., BAEK, S. H. & KIM, V. N. 2004. MicroRNA genes are transcribed by RNA polymerase II. *EMBO J,* 23**,** 4051-60.

LEWIS, B. P., SHIH, I. H., JONES-RHOADES, M. W., BARTEL, D. P. & BURGE, C. B. 2003. Prediction of mammalian microRNA targets. *Cell,* 115**,** 787-98.

LI, H., WANG, Z., FU, Q. & ZHANG, J. 2014. Plasma miRNA levels correlate with sensitivity to bone mineral density in postmenopausal osteoporosis patients. *Biomarkers,* 19**,** 553-6.

LI, H., XIE, H., LIU, W., HU, R., HUANG, B., TAN, Y. F., XU, K., SHENG, Z. F., ZHOU, H. D., WU, X. P. & LUO, X. H. 2009. A novel microRNA targeting HDAC5 regulates osteoblast differentiation in mice and contributes to primary osteoporosis in humans. *J Clin Invest,* 119**,** 3666-77.

LI, L. M., HU, Z. B., ZHOU, Z. X., CHEN, X., LIU, F. Y., ZHANG, J. F., SHEN, H. B., ZHANG, C. Y. & ZEN, K. 2010. Serum microRNA Profiles Serve as Novel Biomarkers for HBV Infection and Diagnosis of HBV-Positive Hepatocarcinoma. *Cancer Research,* 70**,** 9798-807.

LI, N., ZHANG, Q. Y., ZOU, J. L., LI, Z. W., TIAN, T. T., DONG, B., LIU, X. J., GE, S., ZHU, Y., GAO, J. & SHEN, L. 2016a. miR-215 promotes malignant progression of gastric cancer by targeting RUNX1. *Oncotarget,* 7**,** 4817-28.

LI, S., LIU, L., ZHUANG, X., YU, Y., LIU, X., CUI, X., JI, L., PAN, Z., CAO, X., MO, B., ZHANG, F., RAIKHEL, N., JIANG, L. & CHEN, X. 2013. microRNAs inhibit the translation of target mRNAs on the endoplasmic reticulum in Arabidopsis. *Cell,* 153**,** 562-574.

LI, X., LV, Y., HAO, J., SUN, H., GAO, N., ZHANG, C., LU, R., WANG, S., YIN, L., PU, Y. & CHEN, R. 2016b. Role of microRNA-4516 involved autophagy associated with exposure to fine particulate matter. *Oncotarget, 7(29):45385-97*.

LI, X., ZHANG, Y., KANG, H., LIU, W., LIU, P., ZHANG, J., HARRIS, S. E. & WU, D. 2005. Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. *J Biol Chem,* 280**,** 19883-7.

LI, Z., HASSAN, M. Q., VOLINIA, S., VAN WIJNEN, A. J., STEIN, J. L., CROCE, C. M., LIAN, J. B. & STEIN, G. S. 2008. A microRNA signature for a BMP2-induced osteoblast lineage commitment program. *Proc Natl Acad Sci U S A,* 105**,** 13906-11.

LI, Z., LI, Y., WANG, N., YANG, L., ZHAO, W. & ZENG, X. 2016c. miR-130b targets NKD2 and regulates the Wnt signaling to promote proliferation and inhibit apoptosis in osteosarcoma cells. *Biochem Biophys Res Commun,* 471**,** 479-85.

LIAN, J. B., STEIN, G. S., VAN WIJNEN, A. J., STEIN, J. L., HASSAN, M. Q., GAUR, T. & ZHANG, Y. 2012. MicroRNA control of bone formation and homeostasis. *Nat Rev Endocrinol,* 8**,** 212-27.

LIU, J., DANG, L., LI, D., LIANG, C., HE, X., WU, H., QIAN, A., YANG, Z., AU, D. W., CHIANG, M. W., ZHANG, B. T., HAN, Q., YUE, K. K., ZHANG, H., LV, C., PAN, X., XU, J., BIAN, Z., SHANG, P., TAN, W., LIANG, Z., GUO, B., LU, A. & ZHANG, G. 2015. A delivery system specifically approaching bone resorption surfaces to facilitate therapeutic modulation of microRNAs in osteoclasts. *Biomaterials,* 52**,** 148-60.

LIU, J. M., ZHAO, H. Y., NING, G., CHEN, Y., ZHANG, L. Z., SUN, L. H., ZHAO, Y. J., XU, M. Y. & CHEN, J. L. 2008. IGF-1 as an early marker for low bone mass or osteoporosis in premenopausal and postmenopausal women. *J Bone Miner Metab,* 26**,** 159-64.

LIU, W. Z., LI, M. K., CHEN, X. M., ZHANG, D. K., WEI, L., ZHANG, Z. C., WANG, S., MENG, L., ZHU, S. & LI, B. C. 2016. MicroRNA-373 promotes migration and invasion in human esophageal squamous cell carcinoma by inhibiting TIMP3 expression. *American Journal of Cancer Research,* 6**,** 1-14.

MAIRE, G., MARTIN, J. W., YOSHIMOTO, M., CHILTON-MACNEILL, S., ZIELENSKA, M. & SQUIRE, J. A. 2011. Analysis of miRNA-gene expression-genomic profiles reveals complex mechanisms of microRNA deregulation in osteosarcoma. *Cancer Genet,* 204**,** 138-46.

MALL, C., ROCKE, D. M., DURBIN-JOHNSON, B. & WEISS, R. H. 2013. Stability of miRNA in human urine supports its biomarker potential. *Biomark Med,* 7**,** 623-31.

MANN, M., BARAD, O., AGAMI, R., GEIGER, B. & HORNSTEIN, E. 2010. miRNA-based mechanism for the commitment of multipotent progenitors to a single cellular fate. *Proc Natl Acad Sci U S A,* 107**,** 15804-9.

MANOLAGAS, S. C., O'BRIEN, C. A. & ALMEIDA, M. 2013. The role of estrogen and androgen receptors in bone health and disease. *Nat Rev Endocrinol,* 9**,** 699-712.

MARSHALL, D., JOHNELL, O. & WEDEL, H. 1996. Meta-analysis of how well measures of bone mineral density predict occurrence of osteoporotic fractures. *BMJ,* 312**,** 1254-9.

MARTIN, T. J. & GADDY, D. 2006. Bone loss goes beyond estrogen. *Nat Med,* 12**,** 612-3.

MCCLUNG, M. R., SAN MARTIN, J., MILLER, P. D., CIVITELLI, R., BANDEIRA, F., OMIZO, M., DONLEY, D. W., DALSKY, G. P. & ERIKSEN, E. F. 2005. Opposite bone remodeling effects of teriparatide and alendronate in increasing bone mass. *Arch Intern Med,* 165**,** 1762-8.

MEIER, C., MEINHARDT, U., GREENFIELD, J. R., DE WINTER, J., NGUYEN, T. V., DUNSTAN, C. R. & SEIBEL, M. J. 2006. Serum cathepsin K concentrations reflect osteoclastic activity in women with postmenopausal osteoporosis and patients with Paget's disease. *Clin Lab,* 52**,** 1-10.

MELKKO, J., KAUPPILA, S., NIEMI, S., RISTELI, L., HAUKIPURO, K., JUKKOLA, A. & RISTELI, J. 1996. Immunoassay for intact amino-terminal propeptide of human type I procollagen. *Clin Chem,* 42**,** 947-54.

MENCIA CASTANO, I., CURTIN, C. M., DUFFY, G. P. & O'BRIEN, F. J. 2016. Next generation bone tissue engineering: non-viral miR-133a inhibition using collagen-nanohydroxyapatite scaffolds rapidly enhances osteogenesis. *Sci Rep,* 6**,** 27941.

MENG, J., ZHANG, D., PAN, N., SUN, N., WANG, Q., FAN, J., ZHOU, P., ZHU, W. & JIANG, L. 2015. Identification of miR-194-5p as a potential biomarker for postmenopausal osteoporosis. *PeerJ,* 3**,** e971.

MENG, X., MULLER, V., MILDE-LANGOSCH, K., TRILLSCH, F., PANTEL, K. & SCHWARZENBACH, H. 2016. Circulating Cell-Free miR-373, miR-200a, miR-200b and miR-200c in Patients with Epithelial Ovarian Cancer. *Adv Exp Med Biol,* 924**,** 3-8.

MITCHELL, P. S., PARKIN, R. K., KROH, E. M., FRITZ, B. R., WYMAN, S. K., POGOSOVA-AGADJANYAN, E. L., PETERSON, A., NOTEBOOM, J., O'BRIANT, K. C., ALLEN, A., LIN, D. W., URBAN, N., DRESCHER, C. W., KNUDSEN, B. S., STIREWALT, D. L., GENTLEMAN, R., VESSELLA, R. L., NELSON, P. S., MARTIN, D. B. & TEWARI, M. 2008. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A,* 105**,** 10513-8.

MIZOGUCHI, F., IZU, Y., HAYATA, T., HEMMI, H., NAKASHIMA, K., NAKAMURA, T., KATO, S., MIYASAKA, N., EZURA, Y. & NODA, M. 2010. Osteoclast-specific Dicer gene deficiency suppresses osteoclastic bone resorption. *J Cell Biochem,* 109**,** 866-75.

MIZOGUCHI, F., MURAKAMI, Y., SAITO, T., MIYASAKA, N. & KOHSAKA, H. 2013. miR-31 controls osteoclast formation and bone resorption by targeting RhoA. *Arthritis Res Ther,* 15**,** R102.

MIZUNO, Y., YAGI, K., TOKUZAWA, Y., KANESAKI-YATSUKA, Y., SUDA, T., KATAGIRI, T., FUKUDA, T., MARUYAMA, M., OKUDA, A., AMEMIYA, T., KONDOH, Y., TASHIRO, H. & OKAZAKI, Y. 2008. miR-125b inhibits osteoblastic differentiation by down-regulation of cell proliferation. *Biochem Biophys Res Commun,* 368**,** 267-72.

MOHAMED, Y., HAIFA, H., DATEL, O., FADOUA, H. N., SMEH, B. H., MAHBOUBA, J., SAOUSSEN, Z., BEJIA, I., MONGI, T., MOHAMED, F. N. & NACEUR, B. 2014. [The

role of biochemical markers of bone turnover in the diagnosis of osteoporosis and predicting fracture risk]. *Tunis Med,* 92**,** 304-10.

MOLLER, A. M., DELAISSE, J. M. & SOE, K. 2017. Osteoclast Fusion: Time-Lapse Reveals Involvement of CD47 and Syncytin-1 at Different Stages of Nuclearity. *J Cell Physiol,* 232**,** 1396-1403.

MORROW, D. A. & DE LEMOS, J. A. 2007. Benchmarks for the assessment of novel cardiovascular biomarkers. *Circulation,* 115**,** 949-52.

MULLER, V., GADE, S., STEINBACH, B., LOIBL, S., VON MINCKWITZ, G., UNTCH, M., SCHWEDLER, K., LUBBE, K., SCHEM, C., FASCHING, P. A., MAU, C., PANTEL, K. & SCHWARZENBACH, H. 2014. Changes in serum levels of miR-21, miR-210, and miR-373 in HER2-positive breast cancer patients undergoing neoadjuvant therapy: a translational research project within the Geparquinto trial. *Breast Cancer Res Treat,* 147**,** 61-8.

NAKASHIMA, T., HAYASHI, M., FUKUNAGA, T., KURATA, K., OH-HORA, M., FENG, J. Q., BONEWALD, L. F., KODAMA, T., WUTZ, A., WAGNER, E. F., PENNINGER, J. M. & TAKAYANAGI, H. 2011. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat Med,* 17**,** 1231-4.

NAYLOR, K. & EASTELL, R. 2012. Bone turnover markers: use in osteoporosis. *Nat Rev Rheumatol,* 8**,** 379-389.

NENONEN, A., CHENG, S., IVASKA, K. K., ALATALO, S. L., LEHTIMAKI, T., SCHMIDT-GAYK, H., UUSI-RASI, K., HEINONEN, A., KANNUS, P., SIEVANEN, H., VUORI, I., VAANANEN, H. K. & HALLEEN, J. M. 2005. Serum TRACP 5b is a useful marker for monitoring alendronate treatment: comparison with other markers of bone turnover. *J Bone Miner Res,* 20**,** 1804-12.

NISHIYAMA, K. K., MACDONALD, H. M., HANLEY, D. A. & BOYD, S. K. 2013. Women with previous fragility fractures can be classified based on bone microarchitecture and finite element analysis measured with HR-pQCT. *Osteoporos Int,* 24**,** 1733-40.

OHISHI, T., TAKAHASHI, M., KUSHIDA, K., HOSHINO, H., TSUCHIKAWA, T., NAITOH, K. & INOUE, T. 1998. Changes of biochemical markers during fracture healing. *Arch Orthop Trauma Surg,* 118**,** 126-30.

OHYASHIKI, K., UMEZU, T., KATAGIRI, S., KOBAYASHI, C., AZUMA, K., TAUCHI, T., OKABE, S., FUKUOKA, Y. & OHYASHIKI, J. H. 2016. Downregulation of Plasma miR-215 in Chronic Myeloid Leukemia Patients with Successful Discontinuation of Imatinib. *International Journal of Molecular Sciences,* 17, 570.

PANACH, L., MIFSUT, D., TARIN, J. J., CANO, A. & GARCIA-PEREZ, M. A. 2015. Serum Circulating MicroRNAs as Biomarkers of Osteoporotic Fracture. *Calcif Tissue Int,* 97**,** 495-505.

PEDERSON, L., KREMER, M., JUDD, J., PASCOE, D., SPELSBERG, T. C., RIGGS, B. L. & OURSLER, M. J. 1999. Androgens regulate bone resorption activity of isolated osteoclasts in vitro. *Proc Natl Acad Sci U S A,* 96**,** 505-10.

PENG, X., YANG, L., LIU, H., PANG, S., CHEN, Y., FU, J., CHEN, Y., WEN, Z., ZHANG, R., ZHU, B., YU, J. & INVERNIZZI, P. 2016. Identification of Circulating MicroRNAs in Biliary Atresia by Next-Generation Sequencing. *J Pediatr Gastroenterol Nutr,* 63**,** 518-23.

PERRINI, S., NATALICCHIO, A., LAVIOLA, L., CIGNARELLI, A., MELCHIORRE, M., DE STEFANO, F., CACCIOPPOLI, C., LEONARDINI, A., MARTEMUCCI, S., BELSANTI, G., MICCOLI, S., CIAMPOLILLO, A., CORRADO, A., CANTATORE, F. P., GIORGINO, R. & GIORGINO, F. 2008. Abnormalities of insulin-like growth factor-I signaling and impaired cell proliferation in osteoblasts from subjects with osteoporosis. *Endocrinology,* 149**,** 1302-13.

PLACE, R. F., LI, L. C., POOKOT, D., NOONAN, E. J. & DAHIYA, R. 2008. MicroRNA-373 induces expression of genes with complementary promoter sequences. *Proc Natl Acad Sci U S A,* 105**,** 1608-13.

POOLE, K. E., VAN BEZOOIJEN, R. L., LOVERIDGE, N., HAMERSMA, H., PAPAPOULOS, S. E., LOWIK, C. W. & REEVE, J. 2005. Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation. *Faseb j,* 19**,** 1842-4.

RAISZ, L. G. 1999. Physiology and pathophysiology of bone remodeling. *Clin Chem,* 45**,** 1353-8.

REN, D., YANG, Q., DAI, Y., GUO, W., DU, H., SONG, L. & PENG, X. 2017. Oncogenic miR-210-3p promotes prostate cancer cell EMT and bone metastasis via NF-kappaB signaling pathway. *Mol Cancer,* 16**,** 117.

RICHARDS, J. B., ZHENG, H. F. & SPECTOR, T. D. 2012. Genetics of osteoporosis from genome-wide association studies: advances and challenges. *Nat Rev Genet,* 13**,** 576-88.

RISTELI, J., ELOMAA, I., NIEMI, S., NOVAMO, A. & RISTELI, L. 1993. Radioimmunoassay for the pyridinoline cross-linked carboxy-terminal telopeptide of type I collagen: a new serum marker of bone collagen degradation. *Clin Chem,* 39**,** 635-40.

RIVOLI, L., VLIEGENTHART, A. D., DE POTTER, C. M., VAN BRAGT, J. J., TZOUMAS, N., GALLACHER, P., FARRAH, T. E., DHAUN, N. & DEAR, J. W. 2016. The effect of renal dysfunction and haemodialysis on circulating liver specific miR-122. *Br J Clin Pharmacol*.

RODRIGUEZ, A., GRIFFITHS-JONES, S., ASHURST, J. L. & BRADLEY, A. 2004. Identification of mammalian microRNA host genes and transcription units. *Genome Res,* 14**,** 1902-10.

ROSEN, C. J. 2004. Insulin-like growth factor I and bone mineral density: experience from animal models and human observational studies. *Best Pract Res Clin Endocrinol Metab,* 18**,** 423-35.

ROSEN, C. J. & DONAHUE, L. R. 1998. Insulin-like growth factors and bone: the osteoporosis connection revisited. *Proc Soc Exp Biol Med,* 219**,** 1-7.

ROSEN, C. J., DONAHUE, L. R. & HUNTER, S. J. 1994. Insulin-like growth factors and bone: the osteoporosis connection. *Proc Soc Exp Biol Med,* 206**,** 83-102.

ROSSI, M., PITARI, M. R., AMODIO, N., DI MARTINO, M. T., CONFORTI, F., LEONE, E., BOTTA, C., PAOLINO, F. M., DEL GIUDICE, T., IULIANO, E., CARAGLIA, M., FERRARINI, M., GIORDANO, A., TAGLIAFERRI, P. & TASSONE, P. 2013. miR-29b negatively regulates human osteoclastic cell differentiation and function: implications for the treatment of multiple myeloma-related bone disease. *J Cell Physiol,* 228**,** 1506-15.

ROSSINI, M., ADAMI, S., BERTOLDO, F., DIACINTI, D., GATTI, D., GIANNINI, S., GIUSTI, A., MALAVOLTA, N., MINISOLA, S., OSELLA, G., PEDRAZZONI, M., SINIGAGLIA,

L., VIAPIANA, O. & ISAIA, G. C. 2016. Guidelines for the diagnosis, prevention and management of osteoporosis. *Reumatismo,* 68**,** 1-39.

RUBY, J. G., JAN, C. H. & BARTEL, D. P. 2007. Intronic microRNA precursors that bypass Drosha processing. *Nature,* 448**,** 83-6.

SAFTIG, P., HUNZIKER, E., WEHMEYER, O., JONES, S., BOYDE, A., ROMMERSKIRCH, W., MORITZ, J. D., SCHU, P. & VON FIGURA, K. 1998. Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. *Proc Natl Acad Sci U S A,* 95**,** 13453-8.

SALAZAR, V. S., GAMER, L. W. & ROSEN, V. 2016. BMP signalling in skeletal development, disease and repair. *Nat Rev Endocrinol,* 12**,** 203-21.

SANDHU, S. K. & HAMPSON, G. 2011. The pathogenesis, diagnosis, investigation and management of osteoporosis. *J Clin Pathol,* 64**,** 1042-50.

SCHWARTZ, A. V., VITTINGHOFF, E., BAUER, D. C., HILLIER, T. A., STROTMEYER, E. S., ENSRUD, K. E., DONALDSON, M. G., CAULEY, J. A., HARRIS, T. B., KOSTER, A., WOMACK, C. R., PALERMO, L., BLACK, D. M., STUDY OF OSTEOPOROTIC FRACTURES RESEARCH, G., OSTEOPOROTIC FRACTURES IN MEN RESEARCH, G., HEALTH, A. & BODY COMPOSITION RESEARCH, G. 2011. Association of BMD and FRAX score with risk of fracture in older adults with type 2 diabetes. *JAMA,* 305**,** 2184-92.

SEELIGER, C., KARPINSKI, K., HAUG, A. T., VESTER, H., SCHMITT, A., BAUER, J. S. & VAN GRIENSVEN, M. 2014. Five freely circulating miRNAs and bone tissue miRNAs are associated with osteoporotic fractures. *J Bone Miner Res,* 29**,** 1718-28.

SEEMAN, E. 2008. Bone quality: the material and structural basis of bone strength. *J Bone Miner Metab,* 26**,** 1-8.

SEIBEL, M. J. 2005. Biochemical markers of bone turnover: part I: biochemistry and variability. *Clin Biochem Rev,* 26**,** 97-122.

SEIBEL, M. J., LANG, M. & GEILENKEUSER, W. J. 2001. Interlaboratory variation of biochemical markers of bone turnover. *Clin Chem,* 47**,** 1443-50.

SEMENOV, M., TAMAI, K. & HE, X. 2005. SOST is a ligand for LRP5/LRP6 and a Wnt signaling inhibitor. *J Biol Chem,* 280**,** 26770-5.

SENANAYAKE, U., DAS, S., VESELY, P., ALZOUGHBI, W., FROHLICH, L. F., CHOWDHURY, P., LEUSCHNER, I., HOEFLER, G. & GUERTL, B. 2012. miR-192, miR-194, miR-215, miR-200c and miR-141 are downregulated and their common target ACVR2B is strongly expressed in renal childhood neoplasms. *Carcinogenesis,* 33**,** 1014-21.

SERA, S. R. & ZUR NIEDEN, N. I. 2017. microRNA Regulation of Skeletal Development. *Curr Osteoporos Rep,* 15**,** 353-66.

SHIBUYA, H., NAKASA, T., ADACHI, N., NAGATA, Y., ISHIKAWA, M., DEIE, M., SUZUKI, O. & OCHI, M. 2013. Overexpression of microRNA-223 in rheumatoid arthritis synovium controls osteoclast differentiation. *Mod Rheumatol,* 23**,** 674-85.

SILVA, B. C. & BILEZIKIAN, J. P. 2011. New approaches to the treatment of osteoporosis. *Annu Rev Med,* 62**,** 307-22.

SIMON, J. A. & MACK, C. J. 2003. Prevention and management of osteoporosis. *Clin Cornerstone,* Suppl 2**,** S5-12.

SKOUMAL, M., HABERHAUER, G., KOLARZ, G., HAWA, G., WOLOSZCZUK, W. & KLINGLER, A. 2005. Serum cathepsin K levels of patients with longstanding

rheumatoid arthritis: correlation with radiological destruction. *Arthritis Res Ther,* 7**,** R65-70.

STOFFEL, K., ENGLER, H., KUSTER, M. & RIESEN, W. 2007. Changes in biochemical markers after lower limb fractures. *Clin Chem,* 53**,** 131-4.

SUGATANI, T. & HRUSKA, K. A. 2007. MicroRNA-223 is a key factor in osteoclast differentiation. *J Cell Biochem,* 101**,** 996-9.

SUGATANI, T. & HRUSKA, K. A. 2013. Down-regulation of miR-21 biogenesis by estrogen action contributes to osteoclastic apoptosis. *J Cell Biochem,* 114**,** 1217-22.

SUGATANI, T., VACHER, J. & HRUSKA, K. A. 2011. A microRNA expression signature of osteoclastogenesis. *Blood,* 117**,** 3648-57.

SVEDBOM, A., HERNLUND, E., IVERGARD, M., COMPSTON, J., COOPER, C., STENMARK, J., MCCLOSKEY, E. V., JONSSON, B., KANIS, J. A. & IOF, E. U. R. P. O. 2013. Osteoporosis in the European Union: a compendium of country-specific reports. *Arch Osteoporos,* 8**,** 137.

SYRING, I., BARTELS, J., HOLDENRIEDER, S., KRISTIANSEN, G., MULLER, S. C. & ELLINGER, J. 2015. Circulating serum miRNA (miR-367-3p, miR-371a-3p, miR-372-3p and miR-373-3p) as biomarkers in patients with testicular germ cell cancer. *J Urol,* 193**,** 331-7.

SZKLARCZYK, D., FRANCESCHINI, A., WYDER, S., FORSLUND, K., HELLER, D., HUERTA-CEPAS, J., SIMONOVIC, M., ROTH, A., SANTOS, A., TSAFOU, K. P., KUHN, M., BORK, P., JENSEN, L. J. & VON MERING, C. 2015. STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res,* 43**,** D447-52.

SZKLARCZYK, D., MORRIS, J. H., COOK, H., KUHN, M., WYDER, S., SIMONOVIC, M., SANTOS, A., DONCHEVA, N. T., ROTH, A., BORK, P., JENSEN, L. J. & VON MERING, C. 2017. The STRING database in 2017: quality-controlled protein–protein association networks, made broadly accessible. *Nucleic Acids Research,* 45**,** D362-D368.

TAICHMAN, R. S. 2005. Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. *Blood,* 105**,** 2631-9.

TAKAHARA, K., KAMIMURA, M., HASHIDATE, H., UCHIYAMA, S. & NAKAGAWA, H. 2007. Change of cross-linked telopeptide of type I collagen (ICTP) and other bone resorption markers in patients with bone fragility fractures. *J Orthop Sci,* 12**,** 219-26.

TAKAHASHI, I., HAMA, Y., MATSUSHIMA, M., HIROTANI, M., KANO, T., HOHZEN, H., YABE, I., UTSUMI, J. & SASAKI, H. 2015. Identification of plasma microRNAs as a biomarker of sporadic Amyotrophic Lateral Sclerosis. *Mol Brain,* 8**,** 67.

TAYLOR, D. D. & GERCEL-TAYLOR, C. 2008. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecologic Oncology,* 110**,** 13-21.

TEITELBAUM, S. L. 2007. Osteoclasts: what do they do and how do they do it? *Am J Pathol,* 170**,** 427-35.

THE GENE ONTOLOGY, C. 2015. Gene Ontology Consortium: going forward. *Nucleic Acids Research,* 43**,** D1049-D1056.

TONG, Y. Q., LIU, B., ZHENG, H. Y., GU, J., LIU, H., LI, F., TAN, B. H., HARTMAN, M., SONG, C. & LI, Y. 2015. MiR-215, an activator of the CTNNBIP1/beta-catenin

pathway, is a marker of poor prognosis in human glioma. *Oncotarget,* 6**,** 25024-33.

TORRES, A., TORRES, K., PESCI, A., CECCARONI, M., PASZKOWSKI, T., CASSANDRINI, P., ZAMBONI, G. & MACIEJEWSKI, R. 2012. Deregulation of miR-100, miR-99a and miR-199b in tissues and plasma coexists with increased expression of mTOR kinase in endometrioid endometrial carcinoma. *BMC Cancer,* 12**,** 369.

TROHATOU, O., ZAGOURA, D., BITSIKA, V., PAPPA, K. I., ANTSAKLIS, A., ANAGNOU, N. P. & ROUBELAKIS, M. G. 2014. Sox2 suppression by miR-21 governs human mesenchymal stem cell properties. *Stem Cells Transl Med,* 3**,** 54-68.

TROMPETER, H. I., DREESEN, J., HERMANN, E., IWANIUK, K. M., HAFNER, M., RENWICK, N., TUSCHL, T. & WERNET, P. 2013. MicroRNAs miR-26a, miR-26b, and miR-29b accelerate osteogenic differentiation of unrestricted somatic stem cells from human cord blood. *BMC Genomics,* 14**,** 111.

UDAGAWA, N., TAKAHASHI, N., JIMI, E., MATSUZAKI, K., TSURUKAI, T., ITOH, K., NAKAGAWA, N., YASUDA, H., GOTO, M., TSUDA, E., HIGASHIO, K., GILLESPIE, M. T., MARTIN, T. J. & SUDA, T. 1999. Osteoblasts/stromal cells stimulate osteoclast activation through expression of osteoclast differentiation factor/RANKL but not macrophage colony-stimulating factor: receptor activator of NF-kappa B ligand. *Bone,* 25**,** 517-23.

UNNANUNTANA, A., GLADNICK, B. P., DONNELLY, E. & LANE, J. M. 2010. The Assessment of Fracture Risk. *The Journal of Bone and Joint Surgery. American volume.,* 92**,** 743-753.

VALADI, H., EKSTROM, K., BOSSIOS, A., SJOSTRAND, M., LEE, J. J. & LOTVALL, J. O. 2007. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol,* 9**,** 654-9.

VALINEZHAD ORANG, A., SAFARALIZADEH, R. & KAZEMZADEH-BAVILI, M. 2014. Mechanisms of miRNA-Mediated Gene Regulation from Common Downregulation to mRNA-Specific Upregulation. *Int J Genomics,* 2014**,** 970607.

VAN BEZOOIJEN, R. L., ROELEN, B. A., VISSER, A., VAN DER WEE-PALS, L., DE WILT, E., KARPERIEN, M., HAMERSMA, H., PAPAPOULOS, S. E., TEN DIJKE, P. & LOWIK, C. W. 2004. Sclerostin is an osteocyte-expressed negative regulator of bone formation, but not a classical BMP antagonist. *J Exp Med,* 199**,** 805-14.

VAN WIJNEN, A. J., VAN DE PEPPEL, J., VAN LEEUWEN, J. P., LIAN, J. B., STEIN, G. S., WESTENDORF, J. J., OURSLER, M. J., IM, H. J., TAIPALEENMAKI, H., HESSE, E., RIESTER, S. & KAKAR, S. 2013. MicroRNA functions in osteogenesis and dysfunctions in osteoporosis. *Curr Osteoporos Rep,* 11**,** 72-82.

VASIKARAN, S., EASTELL, R., BRUYERE, O., FOLDES, A. J., GARNERO, P., GRIESMACHER, A., MCCLUNG, M., MORRIS, H. A., SILVERMAN, S., TRENTI, T., WAHL, D. A., COOPER, C., KANIS, J. A. & GROUP, I.-I. B. M. S. W. 2011. Markers of bone turnover for the prediction of fracture risk and monitoring of osteoporosis treatment: a need for international reference standards. *Osteoporos Int,* 22**,** 391-420.

VASIKARAN, S. D. & CHUBB, S. A. 2016. The use of biochemical markers of bone turnover in the clinical management of primary and secondary osteoporosis. *Endocrine,* 52**,** 222-5.

VASUDEVAN, S. 2012. Posttranscriptional upregulation by microRNAs. *Wiley Interdiscip Rev RNA,* 3**,** 311-30.

VASUDEVAN, S., TONG, Y. & STEITZ, J. A. 2007. Switching from repression to activation: microRNAs can up-regulate translation. *Science,* 318**,** 1931-4.

VEITCH, S. W., FINDLAY, S. C., HAMER, A. J., BLUMSOHN, A., EASTELL, R. & INGLE, B. M. 2006. Changes in bone mass and bone turnover following tibial shaft fracture. *Osteoporos Int,* 17**,** 364-72.

VELU, V. K., RAMESH, R. & SRINIVASAN, A. R. 2012. Circulating MicroRNAs as Biomarkers in Health and Disease. *Journal of Clinical and Diagnostic Research : JCDR,* 6**,** 1791-1795.

VICKERS, K. C., PALMISANO, B. T., SHOUCRI, B. M., SHAMBUREK, R. D. & REMALEY, A. T. 2011. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol,* 13**,** 423-33.

VIETH, R., LADAK, Y. & WALFISH, P. G. 2003. Age-related changes in the 25-hydroxyvitamin D versus parathyroid hormone relationship suggest a different reason why older adults require more vitamin D. *J Clin Endocrinol Metab,* 88**,** 185-91.

WADA, T. & PENNINGER, J. M. 2004. Mitogen-activated protein kinases in apoptosis regulation. *Oncogene,* 23**,** 2838-49.

WANG, B., WANG, H. & YANG, Z. 2012a. MiR-122 inhibits cell proliferation and tumorigenesis of breast cancer by targeting IGF1R. *PLoS One,* 7**,** e47053.

WANG, F. S., CHUANG, P. C., LIN, C. L., CHEN, M. W., KE, H. J., CHANG, Y. H., CHEN, Y. S., WU, S. L. & KO, J. Y. 2013. MicroRNA-29a protects against glucocorticoid-induced bone loss and fragility in rats by orchestrating bone acquisition and resorption. *Arthritis Rheum,* 65**,** 1530-40.

WANG, J., ZHANG, W., YU, C., ZHANG, X., ZHANG, H., GUAN, Q., ZHAO, J. & XU, J. 2015a. Follicle-Stimulating Hormone Increases the Risk of Postmenopausal Osteoporosis by Stimulating Osteoclast Differentiation. *PLoS ONE,* 10**,** e0134986.

WANG, K., YUAN, Y., CHO, J. H., MCCLARTY, S., BAXTER, D. & GALAS, D. J. 2012b. Comparing the MicroRNA spectrum between serum and plasma. *PLoS One,* 7**,** e41561.

WANG, L. Q., ZHANG, Y., YAN, H., LIU, K. J. & ZHANG, S. 2015b. MicroRNA-373 functions as an oncogene and targets YOD1 gene in cervical cancer. *Biochemical and Biophysical Research Communications,* 459**,** 515-520.

WANG, Y., JIA, L. S., YUAN, W., WU, Z., WANG, H. B., XU, T., SUN, J. C., CHENG, K. F. & SHI, J. G. 2015c. Low miR-34a and miR-192 are associated with unfavorable prognosis in patients suffering from osteosarcoma. *Am J Transl Res,* 7**,** 111-9.

WANG, Y., LI, L., MOORE, B. T., PENG, X. H., FANG, X., LAPPE, J. M., RECKER, R. R. & XIAO, P. 2012c. MiR-133a in human circulating monocytes: a potential biomarker associated with postmenopausal osteoporosis. *PLoS One,* 7**,** e34641.

WANG, Y. X., ZHANG, T. J., YANG, D. Q., YAO, D. M., YANG, L., ZHOU, J. D., DENG, Z. Q., MA, J. C., GUO, H., WEN, X. M., LIN, J. & QIAN, J. 2016. Reduced miR-215 expression predicts poor prognosis in patients with acute myeloid leukemia. *Jpn J Clin Oncol,* 46**,** 350-6.

WATERS, P. S., MCDERMOTT, A. M., WALL, D., HENEGHAN, H. M., MILLER, N., NEWELL, J., KERIN, M. J. & DWYER, R. M. 2012. Relationship between circulating and tissue microRNAs in a murine model of breast cancer. *PLoS One,* 7**,** e50459.

WEI, F. R., CAO, C. H., XU, X. Q. & WANG, J. F. 2015. Diverse functions of miR-373 in cancer. *Journal of Translational Medicine,* 13.

WEI, J., LI, H., WANG, S., LI, T., FAN, J., LIANG, X., LI, J., HAN, Q., ZHU, L., FAN, L. & ZHAO, R. C. 2014. let-7 enhances osteogenesis and bone formation while repressing adipogenesis of human stromal/mesenchymal stem cells by regulating HMGA2. *Stem Cells Dev,* 23**,** 1452-63.

WEILNER, S., SKALICKY, S., SALZER, B., KEIDER, V., WAGNER, M., HILDNER, F., GABRIEL, C., DOVJAK, P., PIETSCHMANN, P., GRILLARI-VOGLAUER, R., GRILLARI, J. & HACKL, M. 2015. Differentially circulating miRNAs after recent osteoporotic fractures can influence osteogenic differentiation. *Bone,* 79**,** 43-51.

WEISS, M. J., HENTHORN, P. S., LAFFERTY, M. A., SLAUGHTER, C., RADUCHA, M. & HARRIS, H. 1986. Isolation and characterization of a cDNA encoding a human liver/bone/kidney-type alkaline phosphatase. *Proc Natl Acad Sci U S A,* 83**,** 7182-6.

WESESLINDTNER, L., MACHELEIDT, I., EISCHEID, H., STRASSL, R., HOFER, H., POPOW-KRAUPP, T., DIENES, H. P., HOLZMANN, H. & ODENTHAL, M. 2016. Micro RNAs mir-106a, mir-122 and mir-197 are increased in severe acute viral hepatitis with coagulopathy. *Liver Int,* 36**,** 353-60.

WHITE, N. M., KHELLA, H. W., GRIGULL, J., ADZOVIC, S., YOUSSEF, Y. M., HONEY, R. J., STEWART, R., PACE, K. T., BJARNASON, G. A., JEWETT, M. A., EVANS, A. J., GABRIL, M. & YOUSEF, G. M. 2011. miRNA profiling in metastatic renal cell carcinoma reveals a tumour-suppressor effect for miR-215. *Br J Cancer,* 105**,** 1741-9.

WICHERS, M., SCHMIDT, E., BIDLINGMAIER, F. & KLINGMULLER, D. 1999. Diurnal rhythm of CrossLaps in human serum. *Clin Chem,* 45**,** 1858-60.

WILSON, S. R., PETERS, C., SAFTIG, P. & BROMME, D. 2009. Cathepsin K activity-dependent regulation of osteoclast actin ring formation and bone resorption. *J Biol Chem,* 284**,** 2584-92.

WINKLER, D. G., SUTHERLAND, M. K., GEOGHEGAN, J. C., YU, C., HAYES, T., SKONIER, J. E., SHPEKTOR, D., JONAS, M., KOVACEVICH, B. R., STAEHLING-HAMPTON, K., APPLEBY, M., BRUNKOW, M. E. & LATHAM, J. A. 2003. Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. *Embo j,* 22**,** 6267-76.

WINTER, J., JUNG, S., KELLER, S., GREGORY, R. I. & DIEDERICHS, S. 2009. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol,* 11**,** 228-34.

WRIGHT, N. C., SAAG, K. G., CURTIS, J. R., SMITH, W. K., KILGORE, M. L., MORRISEY, M. A., YUN, H., ZHANG, J. & DELZELL, E. S. 2012. Recent trends in hip fracture rates by race/ethnicity among older US adults. *J Bone Miner Res,* 27**,** 2325-32.

WU, S., LIU, W. & ZHOU, L. 2016. MiR-590-3p regulates osteogenic differentiation of human mesenchymal stem cells by regulating APC gene. *Biochem Biophys Res Commun,* 478**,** 1582-7.

XIAN, L., WU, X., PANG, L., LOU, M., ROSEN, C. J., QIU, T., CRANE, J., FRASSICA, F., ZHANG, L., RODRIGUEZ, J. P., JIA, X., YAKAR, S., XUAN, S., EFSTRATIADIS, A., WAN, M. & CAO, X. 2012. Matrix IGF-1 maintains bone mass by activation of mTOR in mesenchymal stem cells. *Nat Med,* 18**,** 1095-01.

XIAO, F., CHEN, J., LIAN, C., HAN, P. & ZHANG, C. 2015. Tumor necrosis factor-related apoptosis-inducing ligand induces cytotoxicity specific to osteosarcoma by microRNA response elements. *Mol Med Rep,* 11**,** 739-45.

XING, L. & BOYCE, B. F. 2005. Regulation of apoptosis in osteoclasts and osteoblastic cells. *Biochemical and Biophysical Research Communications,* 328**,** 709-720.

XIONG, J. & O'BRIEN, C. A. 2012. Osteocyte RANKL: new insights into the control of bone remodeling. *J Bone Miner Res,* 27**,** 499-505.

XU, Y. J. & FAN, Y. 2015. MiR-215/192 participates in gastric cancer progression. *Clin Transl Oncol,* 17**,** 34-40.

YANG, L., CHENG, P., CHEN, C., HE, H. B., XIE, G. Q., ZHOU, H. D., XIE, H., WU, X. P. & LUO, X. H. 2012. miR-93/Sp7 function loop mediates osteoblast mineralization. *J Bone Miner Res,* 27**,** 1598-606.

YANG, S. Y., WANG, Y. Q., GAO, H. M., WANG, B. & HE, Q. 2016. The clinical value of circulating miR-99a in plasma of patients with acute myocardial infarction. *European Review for Medical and Pharmacological Sciences,* 20**,** 5193-7.

YANG, Y., SHEN, Z., SUN, W., GAO, S., LI, Y. & GUO, Y. 2017. The role of miR-122-5p in negatively regulating T-box brain 1 expression on the differentiation of mouse bone mesenchymal stem cells. *Neuroreport,* 28**,** 367-74.

YAVROPOULOU, M. P., ANASTASILAKIS, A. D., MAKRAS, P., TSALIKAKIS, D. G., GRAMMATIKI, M. & YOVOS, J. G. 2017. Expression of microRNAs that regulate bone turnover in the serum of postmenopausal women with low bone mass and vertebral fractures. *Eur J Endocrinol,* 176**,** 169-76.

ZALLONE, A. 2006. Direct and indirect estrogen actions on osteoblasts and osteoclasts. *Ann N Y Acad Sci,* 1068**,** 173-9.

ZENG, Y., QU, X., LI, H., HUANG, S., WANG, S., XU, Q., LIN, R., HAN, Q., LI, J. & ZHAO, R. C. 2012a. MicroRNA-100 regulates osteogenic differentiation of human adipose-derived mesenchymal stem cells by targeting BMPR2. *FEBS Lett,* 586**,** 2375-81.

ZENG, Y., QU, X. B., LI, H. L., HUANG, S., WANG, S. H., XU, Q. L., LIN, R. Z., HAN, Q., LI, J. & ZHAO, R. C. 2012b. MicroRNA-100 regulates osteogenic differentiation of human adipose-derived mesenchymal stem cells by targeting BMPR2. *Febs Letters,* 586**,** 2375-81.

ZHANG, J., ZHAO, H., CHEN, J., XIA, B., JIN, Y., WEI, W., SHEN, J. & HUANG, Y. 2012. Interferon-beta-induced miR-155 inhibits osteoclast differentiation by targeting SOCS1 and MITF. *FEBS Lett,* 586**,** 3255-62.

ZHANG, Y., JIA, Y., ZHENG, R., GUO, Y., WANG, Y., GUO, H., FEI, M. & SUN, S. 2010. Plasma microRNA-122 as a biomarker for viral-, alcohol-, and chemical-related hepatic diseases. *Clin Chem,* 56**,** 1830-8.

ZHANG, Y., TANG, W., PENG, L., TANG, J. & YUAN, Z. 2016. Identification and validation of microRNAs as endogenous controls for quantitative polymerase chain reaction in plasma for stable coronary artery disease. *Cardiol J,* 23**,** 694-703.

ZHANG, Z. Q., MENG, H., WANG, N., LIANG, L. N., LIU, L. N., LU, S. M. & LUAN, Y. 2014. Serum microRNA 143 and microRNA 215 as potential biomarkers for the diagnosis of chronic hepatitis and hepatocellular carcinoma. *Diagn Pathol,* 9**,** 135.

ZHAO, J., CHEN, F., ZHOU, Q., PAN, W., WANG, X., XU, J., NI, L. & YANG, H. 2016. Aberrant expression of microRNA-99a and its target gene mTOR associated with malignant progression and poor prognosis in patients with osteosarcoma. *Onco Targets Ther,* 9**,** 1589-97.

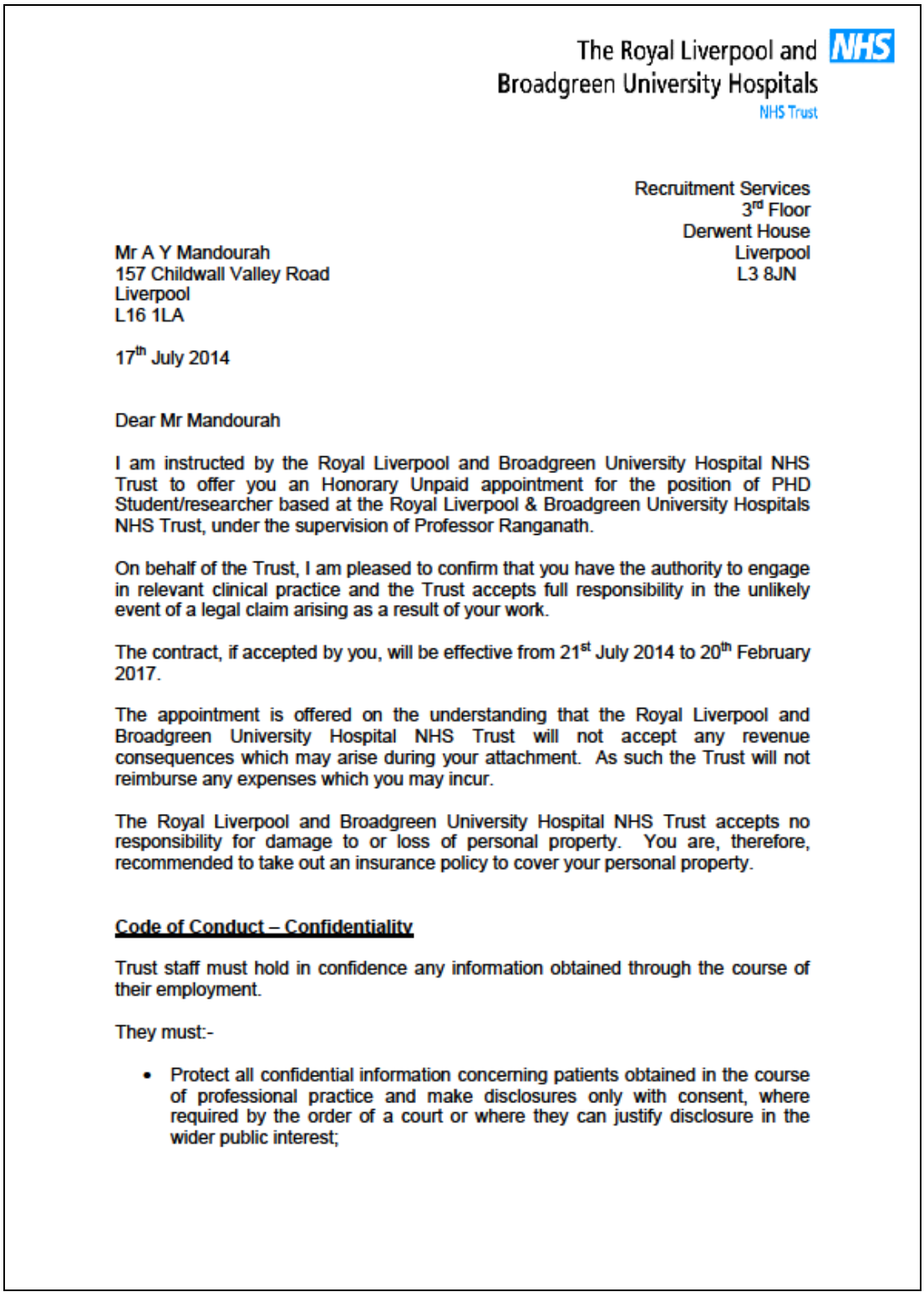
ZHOU, S. W., SU, B. B., ZHOU, Y., FENG, Y. Q., GUO, Y., WANG, Y. X., QI, P. & XU, S. 2014. Aberrant miR-215 expression is associated with clinical outcome in breast cancer patients. *Med Oncol,* 31**,** 259.

ZHOU, X., WANG, J., SUN, H., QI, Y., XU, W., LUO, D., JIN, X., LI, C., CHEN, W., LIN, Z., LI, F., ZHANG, R. & LI, G. 2016. MicroRNA-99a regulates early chondrogenic differentiation of rat mesenchymal stem cells by targeting the BMPR2 gene. *Cell Tissue Res,* 366**,** 143-53.

ZOU, W., GREENBLATT, M. B., SHIM, J. H., KANT, S., ZHAI, B., LOTINUN, S., BRADY, N., HU, D. Z., GYGI, S. P., BARON, R., DAVIS, R. J., JONES, D. & GLIMCHER, L. H. 2011. MLK3 regulates bone development downstream of the faciogenital dysplasia protein FGD1 in mice. *J Clin Invest,* 121**,** 4383-92.

Appendix

8.1 Honorary Contract Letter



The Royal Liverpool and Broadgreen University Hospitals NHS Trust

Recruitment Services
3rd Floor
Derwent House
Liverpool
L3 8JN

Mr A Y Mandourah
157 Childwall Valley Road
Liverpool
L16 1LA

17th July 2014

Dear Mr Mandourah

I am instructed by the Royal Liverpool and Broadgreen University Hospital NHS Trust to offer you an Honorary Unpaid appointment for the position of PHD Student/researcher based at the Royal Liverpool & Broadgreen University Hospitals NHS Trust, under the supervision of Professor Ranganath.

On behalf of the Trust, I am pleased to confirm that you have the authority to engage in relevant clinical practice and the Trust accepts full responsibility in the unlikely event of a legal claim arising as a result of your work.

The contract, if accepted by you, will be effective from 21st July 2014 to 20th February 2017.

The appointment is offered on the understanding that the Royal Liverpool and Broadgreen University Hospital NHS Trust will not accept any revenue consequences which may arise during your attachment. As such the Trust will not reimburse any expenses which you may incur.

The Royal Liverpool and Broadgreen University Hospital NHS Trust accepts no responsibility for damage to or loss of personal property. You are, therefore, recommended to take out an insurance policy to cover your personal property.

Code of Conduct – Confidentiality

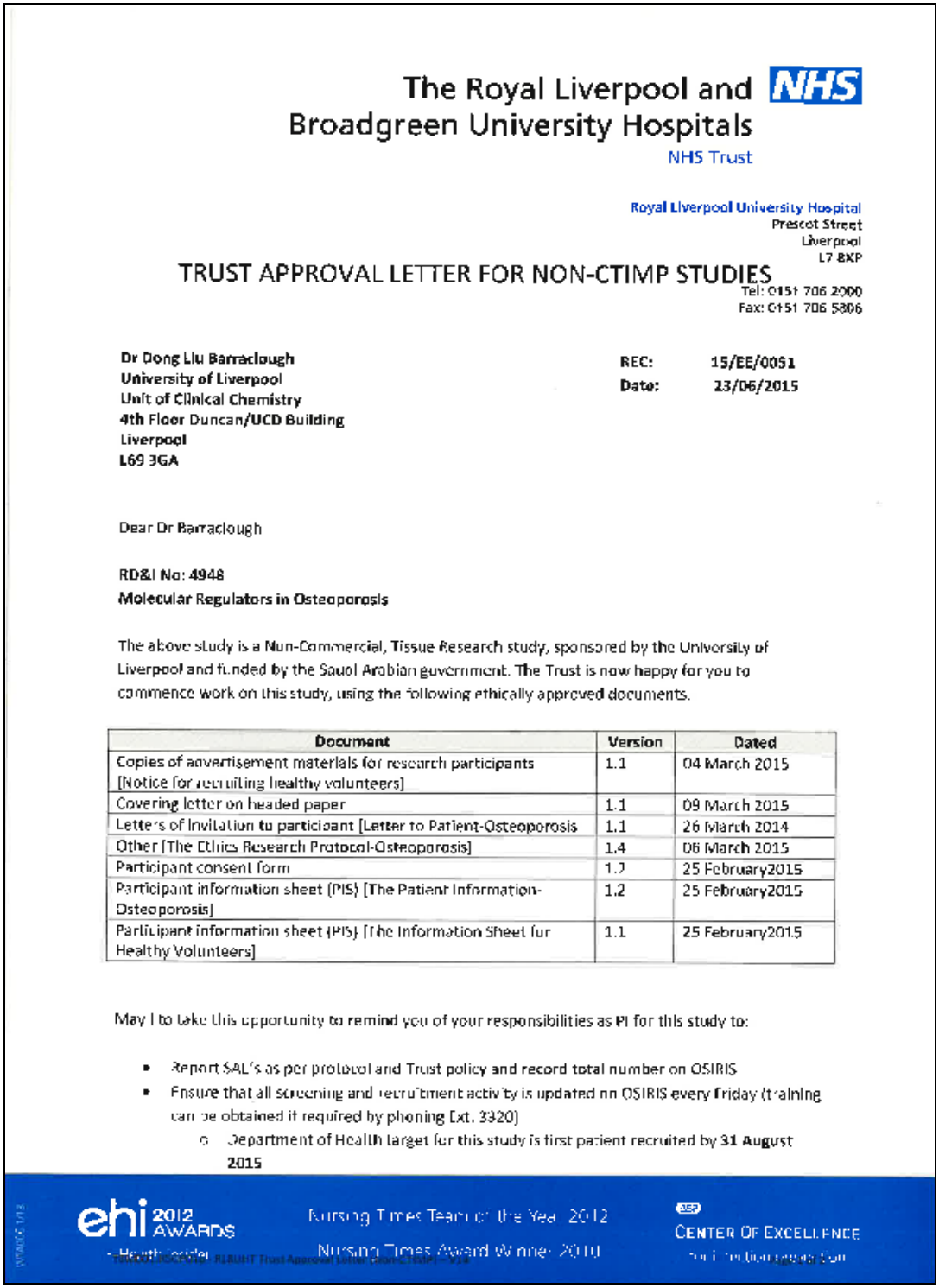
Trust staff must hold in confidence any information obtained through the course of their employment.

They must:-

- Protect all confidential information concerning patients obtained in the course of professional practice and make disclosures only with consent, where required by the order of a court or where they can justify disclosure in the wider public interest;

Figure 8-1: Honorary Contract Letter

8.2 NHS Trust Approval Letter for Non-clinical trial of an investigational medicinal product (Non-CTIMP) Studies



The Royal Liverpool and Broadgreen University Hospitals NHS Trust

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TRUST APPROVAL LETTER FOR NON-CTIMP STUDIES

Dr Dong Liu Barraclough
University of Liverpool
Unit of Clinical Chemistry
4th Floor Duncan/UCD Building
Liverpool
L69 3GA

REC: 15/EE/0051
Date: 23/06/2015

Dear Dr Barraclough

RD&I No: 4948
Molecular Regulators in Osteoporosis

The above study is a Non-Commercial, Tissue Research study, sponsored by the University of Liverpool and funded by the Saudi Arabian government. The Trust is now happy for you to commence work on this study, using the following ethically approved documents.

Document	Version	Dated
Copies of advertisement materials for research participants [Notice for recruiting healthy volunteers]	1.1	04 March 2015
Covering letter on headed paper	1.1	09 March 2015
Letters of invitation to participant [Letter to Patient-Osteoporosis	1.1	26 March 2014
Other [The Ethics Research Protocol-Osteoporosis]	1.4	06 March 2015
Participant consent form	1.1	25 February2015
Participant information sheet (PIS) [The Patient Information-Osteoporosis]	1.2	25 February2015
Participant information sheet (PIS) [The Information Sheet for Healthy Volunteers]	1.1	25 February2015

May I to take this opportunity to remind you of your responsibilities as PI for this study to:

- Report SAE's as per protocol and Trust policy and record total number on OSIRIS
- Ensure that all screening and recruitment activity is updated on OSIRIS every Friday (training can be obtained if required by phoning Ext. 3320)
  - Department of Health target for this study is first patient recruited by 31 August 2015

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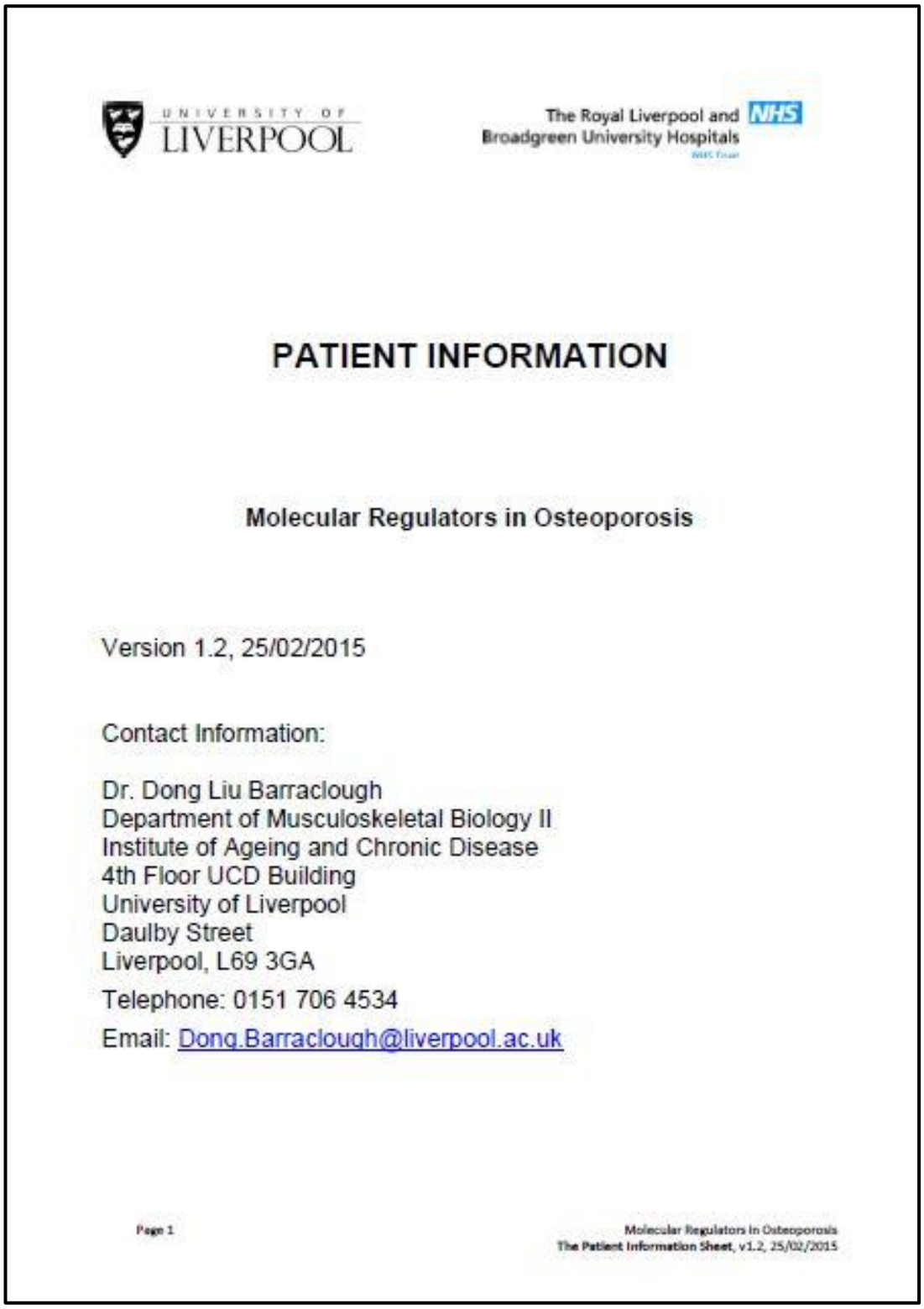
Nursing Times Team of the Year 2012
Nursing Times Award Winner 2010
CENTER OF EXCELLENCE

Figure 8-2: NHS Trust Approval Letter for Non-clinical trial of an investigational medicinal product (Non-CTIMP) Studies.

8.3 Participant Information Sheet



UNIVERSITY OF LIVERPOOL

The Royal Liverpool and Broadgreen University Hospitals NHS

PATIENT INFORMATION

Molecular Regulators in Osteoporosis

Version 1.2, 25/02/2015

Contact Information:

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Institute of Ageing and Chronic Disease
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Daulby Street
Liverpool, L69 3GA
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Email: Dong.Barraclough@liverpool.ac.uk

Figure 8-3: Participant Information Sheet (PIS) - Regulators in Osteoporosis

We would like to invite you to take part in a research study. Before you decide whether to take part, you need to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish.

Our research team, based in the Institute of Ageing and Chronic Disease at the University of Liverpool, has a strong scientific research interest in metabolic bone diseases such as osteoporosis. We wish to analyse blood/urine samples to investigate the causes, development and biological regulators of these diseases. It is hoped that the resulting knowledge will help patients in the future.

Why have you been invited?

You have been invited to participate because you may be at risk of one of the metabolic bone disorders that we are researching.

What will happen if I choose to take part?

We would like your informed consent to take a small sample of blood and/or urine samples. Blood samples will be approximately 20-50 mL, approximately 2-4 tablespoons. Urine samples will be approximately 100 mL, approximately 8 tablespoons. This will extend your outpatient stay by 20-30 minutes.

What are the advantages and disadvantages of taking part?

The results of research carried out using your samples, and those of others, may help in the future discovery of new drugs and treatments for patients suffering from metabolic bone disorders.

There will be no direct benefit to yourself as you will not be identifiable to the research team.

There will be no additional risks if you choose to participate.

When the blood sample is taken, occasionally, this may require an additional entry site to the routine blood sample. On the small number of occasions that this may occur, there is the small chance you may experience some bruising at the site.

In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against the University of Liverpool, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

Do I have to take part?

It is up to you to decide whether or not to allow us to collect a sample of your blood or urine. If you do decide to take part, then you can keep this information booklet and you will be asked to sign an informed consent form. If you decide not to take part, you do not need to give a reason.

Whatever your decision, it will not affect the standard of care that you receive.

What will happen to my blood/urine sample?

All samples will be anonymised, catalogued and stored in the lockable ultra-low temperature -80^{o}C freezer in the designated University laboratory which is locked when not in use. Our research team will be able to use your gift of samples to understand the causes of a bone disease and to improve treatment and care for

patients in the future. All research will be carried out in the lockable designated University clinical medicine research laboratory. Your blood will not be used for transplantation or reproductive cloning. Nor will the blood/urine samples be used for non-medical or non-scientific purposes.

We will use some of blood/urine samples to obtain genetic related material (DNA, RNA and protein) and/or to isolate cells and culture them. [Note: Your genes are made up of DNA which contains information that determines in part the traits, such as eye colour, height or disease risk, which are passed on from parent to child. RNA is made from DNA. RNA has a major role in making proteins. Proteins are the building blocks of your body, cells and organs].

Because we do not perform whole genome studies, there will be no risk of incidental findings. We will only analyse RNA samples to investigate differentially expressed RNA profiles. We will not use DNA, RNA or protein samples or cells for any purpose other than research and the research team will not be able to identify you in any way. You do not need to declare to your insurance company the fact that we have taken a sample of your genetic material. Any part of the sample you donate which has not been used for research will be stored in the licenced Liverpool Tissue Bank at the University of Liverpool and an application for ethical review for future bone related research will be obtained. If no further research ethics application is approved by the NRES, all samples will be disposed in accordance with the Human Tissue Authority's Code of Practice.

What if researchers find new information about my condition?

All blood/urine donations will be anonymous and the researchers will not be able to identify you from the sample.

Will anybody make a profit from my blood/urine samples?

You are asked to donate your blood/urine samples for research as a gift and will not receive a financial reward either now or in the future. The University will not sell your blood/urine samples for profit to any other researcher or organisation. However, your blood/urine samples may be used in a research project that may lead to the development of new drugs or treatments. It will not be possible for you to make a claim for money as you will be waiving all commercial rights relating to the samples you donate. Any drug, treatment or test developed may help all of us in the future.

What will happen to the results of the research study?

Research studies using blood/urine samples may take several years to complete. Results will be published when appropriate in scientific papers and magazines and presented at scientific meetings.

You will not be able to be identified if research using your blood/urine samples is published in any scientific papers.

Will my taking part in this study be kept confidential?

All information that is collected related to your medical condition will be kept strictly confidential. No personal information will be stored with blood/urine samples and you will not be able to be identified.

8.4 Osteoporosis Patients Medical Record

Table 8-1: Osteoporosis Medical Record-without ID 2012-2013

No.	Date of Clinic	Sample ID YYMMDDOP##	DOB	Age	Sex F/M	T Score Lumbar Spine (LS)	T Score Femoral Neck (FN)	T score Total IP (TH)	Number of Fractures	Position of Fracture Wrist [W] Vertebrae [V] Hip [H] Rib [R] Ankle [A Other [O]	Date of last Fracture	Medication History Calcium Vitamin D Bisphosphonates Prodelos Denosumab Others	Risk Factors	Type OP	Notes
1	6/2/12	120206OP01	30/7/75	37	F	-4.0	-2.9		1	H			smoker	C	
2	6/2/12	120206OP02	1/3/43	69	F	-2.8	-2.3		2	R	2005/06	Prodelos	VD	C	
3	6/2/12	120206OP03	3/6/55	57	F	-4.9	-3.5		0			Ca/Vd/Bis		B	
4	6/2/12	120206OP04	15/5/47	65	F	-2.8	-2.8		0			Ca/Vd		B	
5	6/2/12	120206OP05	22/7/26	86	M	-1.0	-1.5		multiple	V		ca/Bis/Preotact		D	
6	27/2/12	120227OP01	15/7/56	56	F	-2.2			2	A+foot		Vd	Vd+smoking	D	
7	27/2/12	120227OP02	13/6/38	74	F	-2.8	-2.1		3	2W+V	>3Y	Ca+BP		C	
8	27/2/12	120227OP03	29/4/41	71	F	-4.0	-2.5					Ca	Coeliac	B	
9	27/2/12	120227OP04	7/8/43	69	F					tib		Ca		A	OPE
10	27/2/12	120227OP05	9/8/29	83	F	-2.8	-3.1					Ca+BP		C	
11	27/2/12	120227OP06	22/12/32	79	F	-1.4	-2.5		1	V	>5Y	Ca+BP		C	
12	28/2/12	120228OP01	2/9/60	52	F	-2.1	-1.2		2	O (shoulder+ elbow)	2011/12	BP/Prodelos		D	SIOP+hx of asthma
13	28/2/12	120228OP02	20/3/50	62	F	-2.5	-2		4	3R+O (pelvis)	2008/11	No pretreat		C	OP spine, OPN hips
14	28/2/12	120228OP03	12/1/45	67	F	-3.0	-1.8		5	W+2V+R+O (foot)		Ca+BP		C	Hx asthma, COPD
15	28/2/12	120228OP04	28/3/51	61	F	-1.5	-2.8		2	W+tib/fib		Ca+BP		C	Previous Raloxifene
16	28/2/12	120228OP05	3/9/32	80	F	-2.2	-3.0		0			Ca/BP		B	
17	5/3/12	120305OP01	31/7/54	58	F	-2.0	-2.0		2	Wx2	2009	No pretreat	FH of OP	D	hysterectomy 45yrs
18	5/3/12	120305OP02	29/7/48	64	F	-0.5	-0.5		0			VD	VitD deficiency	Norm	NBD

No.	Date of Clinic	Sample ID YYMMDDOP##	DOB	Age	Sex F/M	T Score Lumbar Spine (LS)	T Score Femoral Neck (FN)	T score Total IP (TH)	Number of Fractures	Position of Fracture Wrist [W] Vertebrae [V] Hip [H] Rib [R] Ankle [A Other [O]	Date of last Fracture	Medication History Calcium Vitamin D Bisphosphonates Prodelos Denosumab Others	Risk Factors	Type OP	Notes
19	6/3/12	120306OP01	22/6/64	48	F				0			No pretreat			Due scan May 2012 scan elsewhere no results
20	8/3/12	120308OP01	17/3/40	72	M	-3.2			1	W	2006	BP	VitD deficiency	C	
21	8/3/12	120308OP02	17/10/40	71	F				1	H	2005	BP	Rh arthritis	C	
22	8/3/12	120308OP03	4/7/24	88	F				5	V, 2H,2O	2011	Ca, VD, Deno sumab		C	On BP, Prodelos in past, Denosumab starting today
23	12/3/12	120312OP01	21/11/58	53	F	-2.3	-1.8		1	Toe	1999	Ca, BP		A	Oral BP3yrs about to start IV
24	13/3/12	120313OP01	24/9/37	75	F				1	V		Ca		C	Due to start IV Zol
25	13/3/12	120313OP02	16/10/29	82	F	-3.0	-2.1		6	3xW,2xR,1h umerus	2001	Ca, BP		C	Due to start Denosumab
26	15/3/12	120315OP01	3/11/46	65	F	-2.8	-2.7		0			BP		B	Primary Hyper parathyroidism
27	15/3/12	120315OP02	2/6/40	72	F	-1.9	-2.4		2	W,R femur	2010	BP	Smoking	D	
28	15/3/12	120315OP03	25/5/62	50	F	-1.1	-3.8		0			No pretreat	Excess alcohol, VitD deficiency, family history of	B	
29	15/3/12	120315OP04	28/5/32	80	F	-1.1	-2.0		0			VitD,BP	VitD deficiency	A	
30	19/3/12	120319OP01	12/1/55	57	F	-2.5	-1.1		0			Aledronate		B	
31	20/3/12	120320OP01	26/1/42	70	F	-1.8	-1.6		1	femur	2011	Ca,BP		A	nsedronate

Appendix

No.	Date of Clinic	Sample ID YYMMDDOP##	DOB	Age	Sex F/M	T Score Lumbar Spine (LS)	T Score Femoral Neck (FN)	T score Total IP (TH)	Number of Fractures	Position of Fracture Wrist [W] Vertebrae [V] Hip [H] Rib [R] Ankle [A Other [O]	Date of last Fracture	Medication History Calcium Vitamin D Bisphosphonates Prodelos Denosumab Others	Risk Factors	Type OP	Notes
32	20/3/12	120320OP02	9/3/54	58	F	-2.5	-0.6		0			VD,prodelos	VitD deficiency	B	
33	20/3/12	120320OP03	9/6/58	54	F	-3.0			0			Ca,BP		B	OP, Premature menopause
34	22/3/12	120322OP01	20/8/25	87	F	-2.5	-1.8		3	1W, 2x shoulder	2006	BP	Primary hyperpaxa	C	
35	22/3/12	120322OP02	27/10/58	53	M	-2.1	-2.5		0			No pretreat	Excess alcohol, VitD deficiency,	B	
36	22/3/12	120322OP03	29/5/42	70	F	-0.2	-2.6		3	1W, 1V, 1H	2009	BP	Excess alcohol, VitD deficiency, Severe	C	
37	22/3/12	120322OP04	2/3/29	83	F	-0.9	-1.7		3	1W,2xmetertarsal	2010	BP	Polymyalgia rheumatica	D	
38	22/3/12	120322OP05	18/9/32	80	F	-2.4	-1.8		0			BP	Breast cancer	A	
39	22/3/12	120322OP06	14/4/27	85	M	-3.9	-3.2		0			Prodelos	VitD deficiency, CVA	B	
40	10/4/12	120410OP01	9/4/64	48	F	0.9	-1.1		1	W		previous BP, Ca	Excess Alcohol	D	
41	13/4/12	120413OP01	27/6/41	71	F	-2.1	-1.0		0			Ca, VitD	Barrett's oesophagitis	A	Osteopenia, no fractures
42	16/4/12	120416OP01	2/2/43	69	F	-2.3	-1.5		0			Ca		A	
43	16/4/12	120416OP02	23/11/44	67	F	-1.8	-1.1		0			Ca		A	
44	17/4/12	120417OP01	3/1/25	87	F	-3.5	-3.0		0			Ca		B	OP, no treatment
45	17/4/12	120417OP02	14/6/38	74	F	-2.1	-1.1		0			VitD, BP		A	

Appendix

No.	Date of Clinic	Sample ID YYMMDDOP##	DOB	Age	Sex F/M	T Score Lumbar Spine (LS)	T Score Femoral Neck (FN)	T score Total IP (TH)	Number of Fractures	Position of Fracture Wrist [W] Vertebrae [V] Hip [H] Rib [R] Ankle [A Other [O]	Date of last Fracture	Medication History Calcium Vitamin D Bisphosphonates Prodelos Denosumab Others	Risk Factors	Type OP	Notes
46	8/5/12	120508OP01	6/3/55	57	F	-0.4	-1.8		2	Foot, Hand	2006	Ca, VitD	VitD deficiency	D	CTx= 0.26
47	8/5/12	120508OP02	12/2/44	68	F	-0.9	-2.0		0			Ca		A	
48	10/5/12	120510OP01	14/1/54	58	F	-2.1	-1.3		2	Rib, L shoulder	Rib (06) Shoulder (08)	Ca, VitD	Ulcerative Colitis	D	
49	15/10/12	121015OP01	5/12/68	44	M	-3.5	-2.4		1	H	31/05/12	Ca, VitD,	Excess alcohol, VitD deficiency,	C	
50	16/10/12	121016OP01	10/11/45	67	F	-3.2	-1.6		0			Ca		C	Osteopenia history
51	16/10/12	121016OP02	8/3/44	69	F	-3.4	-2.1					Ca, VitD, BP, protelos		C	
52	22/10/12	121022OP01	26/10/30	82	F	-5.2	-3.7		2	shoulder	2009	Ca, VitD, BP,	VitD deficiency	C	
53	30/10/12	121030OP01	30/4/35	78	F	-1.8	-1.7		0			Ca, VitD, BP,	VitD deficiency	A	
54	5/11/12	121105OP1	5/4/39	74	F	-3.5	-1.0					Ca, VitD, BP, protelos	VitD deficiency	C	
55	15/7/13	130715OP01	28/9/49	64	F	-2.9	-2.5		0			VitD, BP, Ibandronate	VitD deficiency	C	
56	15/7/13	130715OP02	18/12/38	75	M				3	V	2011	Ca, VitD, BP			No report for BMD T Score
57	15/7/13	130715OP03	7/5/34	79	F	-2.7		-2.1	multiple	V	2009	Ca, VitD, BP		C	BP was taken >7 years
58	15/7/13	130715OP04	31/7/42	71	F	-3.3	-2.0		0			Ca, VitD, BP		C	
59	15/7/13	130715OP05	17/3/40	73	M	-2.3			1	W	2010	Ca, VitD, BP	VitD deficiency		S. No. 20 for same Patient BP (2002-2012)
60	16/7/13	130716OP01	28/4/50	63	F	-3.0	-1.0					BP	VitD deficiency	C	S. No. 2 for same Patient

No.	Date of Clinic	Sample ID YYMMDDOP##	DOB	Age	Sex F/M	T Score Lumbar Spine (LS)	T Score Femoral Neck (FN)	T score Total IP (TH)	Number of Fractures	Position of Fracture Wrist [W] Vertebrae [V] Hip [H] Rib [R] Ankle [A Other [O]	Date of last Fracture	Medication History Calcium Vitamin D Bisphosphonates Prodelos Denosumab Others	Risk Factors	Type OP	Notes
													Excess Alcohol		BP (2007-2010)
61	17/9/13	130917OP01	12/10/49	64	F	-2.4			1	O				B	Left Foot
62	23/9/13	130923OP01	19/2/40	74	F	-2.1			multiple	V	2006	Ca, BP		B	
63	21/10/13	131021OP01	6/1/50	64	M				1	V	2012		Excess alcohol	A	

Table 8-2: Osteoporosis Medical Record-without ID 2015-2016

#	Date of Clinic	Sample ID	DOB	Age	Sex F/M	Smoking Yes/No	Excess alcohol Yes/No	Regular Exercise Yes/No	Vegetarian Yes/No	Fractures History Yes/No	Number of Fractures	Position of Fracture	Date of last Fracture	Medication History	Ethnic origin	T-Score Lumbar Spine L1	T Score Lumbar Spine (L2-L4)	T Score Femoral Neck	T score Total HIP (TH)	BMD [g/cm2]	Medical Diagnosis	Clinical History
1	03/09/2015	150903HC01		30	M	No	No	Yes	No	No				No	Arab						Healthy	
2	03/09/2015	150903HC02		30-40	M	No	No	Yes	No	No				No	Arab						Healthy	
3	26/10/2015	151026HC01	09/11/1957	58	F	No	No	Yes	No	No				No	Eu	-0.5	-1.5	-1.7		0.773	Osteopenia	Family history of Osteoporosis
4	26/10/2015	151026OP01	03/11/1947	68	F	No	No	Yes	No	Yes	1	W	2007	Ca	Eu	-2	-0.9	-1.7		0.771	Osteopenia /Normal	Osteopenia
5	26/10/2015	151026OP02	27/02/1948	67	F	No	No	No	Yes	Yes				Ca/VitD	Asian /In	-2.2	-1.9	-1.6		0.787	Osteopenia	On regular steroids

Appendix

#	Date of Clinic	Sample ID	DOB	Age	Sex F/M	Smoking Yes/No	Excess alcohol Yes/No	Regular Exercise Yes/No	Vegetarian Yes/No	Fractures History Yes/No	Number of Fractures	Position of Fracture	Date of last Fracture	Medication History	Ethnic origin	T-Score Lumbar Spine L1	T Score Lumbar Spine (L2-L4)	T Score Femoral Neck	T score Total HIP (TH)	BMD [g/cm2]	Medical Diagnosis	Clinical History
6	26/10/2015	151026 OP03	23/03/1950	65	M		No	No	No	No				Steriod	Eu	-1	0.3	-1.6		0.857	Osteopenia /Normal	Osteopenia
7	27/10/2015	151027 OP01	07/01/1949	66	F	No	No	Yes	No	Yes				Yes	Eu	-1.6	-0.8	-1.9		0.75	Osteopenia	Fracture
8	27/10/2015	151027 OP02	07/05/1948	67	F	Yes	Yes	Yes		Yes				Yes	Eu	-1.7	-2.3	-2		0.74	Osteopenia	
9	27/10/2015	151027 OP03	19/05/1960	55	F	No	No	No	No	No				No	Eu	-1.3	-1.4	-0.8		0.886	Osteopenia /Normal	Coeliac disease
10	27/10/2015	151027 OP04	16/07/1960	55	F	Yes	Yes	No	No	No				Yes	Eu	-1.3	-1.3	-1.6		0.784	Osteopenia	
11	27/10/2015	151027 OP05	05/02/1954	61	F	No	No	No	No	No				Yes	Eu	-1.5	-0.5	-0.5		0.923	Normal/Osteopenia	Asthma
12	28/10/2015	151028 OP01	21/01/1951	64	F	No	No	No	No	Yes				Yes	Eu	-1.9	-1.3	-0.7		0.896	Osteopenia /Normal	Fracture
13	28/10/2015	151028 OP02	22/02/1947	68	F	No	No	Yes	No	No				Yes	Eu	0.8	3.2	1.3		1.138	Normal	Falls
14	28/10/2015	151028 HC01	08/02/1977	38	F	Yes	No	No	No	No				No	Eu	-1.3	-0.8	-0.3		0.944	Normal/osteopenia	Low Vitamin D
15	29/10/2015	151029 OP01	31/12/1935	80	F	No	No	No	No	No				Yes, Thymain, HBP	Eu	-1.1	0	-1.6		0.794	Osteopenia /Normal	

Appendix

#	Date of Clinic	Sample ID	DOB	Age	Sex F/M	Smoking Yes/No	Excess alcohol Yes/No	Regular Exercise Yes/No	Vegetarian Yes/No	Fractures History Yes/No	Number of Fractures	Position of Fracture	Date of last Fracture	Medication History	Ethnic origin	T-Score Lumbar Spine L1	T Score Lumbar Spine (L2-L4)	T Score Femoral Neck	T score Total HIP (TH)	BMD [g/cm2]	Medical Diagnosis	Clinical History
16	29/10/2015	151029OP02	09/01/1931	84	M	No	No	No	No	No				Aspirin,Vit D	Eu	-0.9	1	-2.4		0.752	Osteopenia	Osteopenia
17	29/10/2015	151029OP03	11/04/1943	72	M	No	No	No	No	No				Aspirin	Eu	-2.9	-2.3	-2.4		0.754	Osteopenia	Osteoporosis
18	29/10/2015	151029OP04	25/03/1931	84	F	No	No	No	No	No				No	Eu	-3.2	-3.2	-2.5		0.68	Osteoporosis	Falls.
19	29/10/2015	151029OP05	21/08/1977	38	M	No	No	No	No	No				Ca+VitD	Asian	-0.1	-0.1	-1.9		0.819	Osteopenia	Prolonged steroid use
20	02/11/2015	151102OP01	02/04/1952	63	F	No	No	Yes	No	No				YES,multi-Vit for 3-4Y	Eu	-0.8	-0.8	-0.1		0.972	Normal	Osteopenia
21	02/11/2015	151102OP02	29/12/1959	56	F	No	No	Yes	No	Yes		W		Yes,VitD	Eu	-0.2	-1.4	0.5		1.043	Osteopenia	Vitamin D deficiency
22	02/11/2015	151102OP03	06/08/1953	62	F	No	No	No	No	No				Yes, Alendronate	Eu	-2.5	-2.9	-2.4		0.693	Osteoporosis	
23	03/11/2015	151103OP01	19/12/1951	64	F	No	Yes	Yes	No	No				BP+VitD	Eu	-2.8	-3.3	-1.7		0.776	Osteoporosis	Osteoporosis
24	03/11/2015	151103OP02	28/05/1942	73	F	No	No	Yes	No	Yes				Yes, allopurinol for gout		-0.4	-0.4	-0.5		0.925	Normal/osteopenia	
25	03/11/2015	151103OP03	27/05/1930	85	F	No	No	No	Yes	No				Yes,	Eu	-2.5	-3	-1.9		0.749	Osteoporosis	Taking bisphosphonate
26	03/11/2015	151103OP04	18/08/1973	42	F	No	Yes	Yes	No	Yes				No	Eu	-1.6	-1.2	0.7		1.061	Osteopenia	several fractures
27	03/11/2015	151103OP05	04/01/1946	69	F	No	No	Yes	No	No				Yes		-2.6	-1.7	-2.4		0.698	Osteopenia	Early menopause
28	04/11/2015	151104OP01	30/04/1944	71	F	No	No	No	No	No				No	Eu	-2.1	-1.1	-1.3		0.821	Osteopenia	Family history of osteoporosis

Appendix

#	Date of Clinic	Sample ID	DOB	Age	Sex F/M	Smoking Yes/No	Excess alcohol Yes/No	Regular Exercise Yes/No	Vegetarian Yes/No	Fractures History Yes/No	Number of Fractures	Position of Fracture	Date of last Fracture	Medication History	Ethnic origin	T-Score Lumbar Spine L1	T Score Lumbar Spine (L2-L4)	T Score Femoral Neck	T score Total HIP (TH)	BMD [g/cm2]	Medical Diagnosis	Clinical History
29	05/11/2015	151105 OP01	30/06/1945	70	M	No	No	Yes	No	No				drug for high cholesterol		-1.7	-1	-1.6		0.861	Osteopenia.	Taking alendronic acid
30	05/11/2015	151105 OP02	26/12/1940	75	M	No	Yes	Yes	No	Yes				Aspirin, BP	Eu	-0.1	1	-0.4		1.014	Normal	Stress fracture
31	05/11/2015	151105 OP03	14/08/1963	52	F	Yes	Yes	Yes	No	Yes				alendronic acid	Eu	-0.2	0	-1.4		0.816	Osteopenia	Osteopenia
32	05/11/2015	151105 OP04	08/02/1952	63	F	No	No	No	No	No				No	Eu	-1.7	-1.4	-2.1		0.723	Osteopenia	Osteopenia
33	05/11/2015	151105 OP05	01/12/1941	74	M	No	No	Yes	No	No				No	Eu	-1.5	-1.1	-1.3		0.819	Osteopenia	Osteopenia
34	16/11/2015	151116 OP01	24/03/1994	21	F	No	No	No	No	No				No	Eu	-3.4	-3.6	-1.8		0.76	Osteoporosis	Osteoporosis
35	16/11/2015	151116 OP2	10/06/1950	65	F	No	No	Yes	No	No				alendronic acid	Eu	-0.9	-1.3	-2.4		0.686	Osteopenia	Osteoporosis
36	17/11/2015	151117 OP01	26/07/1950	65	F	No	No	No	No	Yes				Calcium	Eu	-2.1	-1.8	-2.5		0.677	Osteoporosis	Osteopenia
37	17/11/2015	151117 OP02	14/10/1955	60	M	No	No	No	No	Yes				No	Eu	-1.1	-1.6	-0.7		0.983	Osteopenia	Osteopenia
38	17/11/2015	151117 OP03	03/11/1947	68	F	No	No	No	No	No				VitD	Eu	-0.8	-0.8	0		0.98	Normal	Osteoporosis
39	17/11/2015	151117 OP04	06/03/1941	74	F	No	No	No	No	Yes				No	Eu	1.1	1.7	-0.8		0.886	Normal	Falls
40	17/11/2015	151117 OP05	21/10/1949	66	M	Yes	No	No	No	Yes				No	Eu	-0.7	0	-1.7		0.855	Osteopenia	
41	18/11/2015	151118 OP01	19/05/1934	81	F	No	No	No	No	Yes				Cal+Alendronic	Eu	-3.9	-3.5	-2.3		0.701	Osteoporosis	Fragility fracture

Appendix

#	Date of Clinic	Sample ID	DOB	Age	Sex F/M	Smoking Yes/No	Excess alcohol Yes/No	Regular Exercise Yes/No	Vegetarian Yes/No	Fractures History Yes/No	Number of Fractures	Position of Fracture	Date of last Fracture	Medication History	Ethnic origin	T-Score Lumbar Spine L1	T Score Lumbar Spine (L2-L4)	T Score Femoral Neck	T score Total HIP (TH)	BMD [g/cm2]	Medical Diagnosis	Clinical History
42	19/11/2015	151119 OP01	03/01/1974	41	F	No	No	Yes	No	No				No	Eu	0.2	0	-1.2		0.834	Normal/Osteopenia	Osteopenia
43	19/11/2015	151119 OP02	20/04/1947	68	F	No	No	Yes	No	No				Yes	Eu	-2.6	-2.6	-1.6		0.791	Osteoporosis	Fracture
44	19/11/2015	151119 OP03	17/03/1970	45	F	No	No	Yes	No	No				Yes	Eu	-0.2	0.5	-1.3		0.824	Osteopenia /Normal	Crohns
45	23/11/2015	151123 OP01	30/05/1980	35	F	No	No	Yes	Yes	Yes				Yes, Ca+VitD	Eu	-0.5	-0.9	-1.1		0.849	Normal/Osteopenia	Osteoporosis
46	23/11/2015	151123 OP02	29/09/1957	58	F	No	No	Yes	No	No				Yes, Ca+VitD	Eu	-1.8	-2.1	-1.5		0.796	Osteopenia	
47	23/11/2015	151123 OP03	28/03/1954	61	F	No	No	Yes	No	No				Yes, BP	Eu	-0.7	-1.1	-1		0.855	Osteopenia	Vit D deficiency
48	23/11/2015	151123 OP04	01/06/1944	71	F	No	No	Yes	No	Yes				Yes, Ca+pain killer	Eu	-2.6	-0.3	-2.5		0.682	Osteoporosis.	Fragility fracture
49	24/11/2015	151124 OP01	21/04/1949	66	F	Yes	No	No	No	Yes				No	Eu	-1.9	-1.7	-1.5		0.8	Osteopenia	Fracture
50	24/11/2015	151124 OP02	08/09/1948	67	F	Yes	No	Yes	No	Yes				Ca+	Eu	-1.2	-0.1	-1.3		0.826	Osteopenia	Rheumatoid arthritis
51	24/11/2015	151124 OP03	29/04/1940	75	F	No	No	Yes	No	No				BP+VitD	Eu	-2.1	-1.9	-2.2		0.719	Osteopenia	
52	25/11/2015	151125 OP01	23/02/1967	48	M	No	No	Yes	No	No				No	Eu	-1.8	-2.4	-1.5		0.88	Osteopenia	Osteopenia
53	26/11/2015	151126 OP01	31/12/1956	59	M	No	No	Yes	No	No				Ca	Eu	-0.8	-1.6	-3.2		0.66	Osteoporosis	Osteoporosis
54	26/11/2015	151126 OP02	20/01/1949	66	F	No	No	Yes	No	Yes				YES	Eu	-0.8	-2.3	-0.6		0.905	Osteopenia	Fractures
55	26/11/2015	151126 OP03	03/01/1947	68	F	No	No	No	No	Yes				Ca	Eu	-3	-2.7	-2.8		0.643	Osteoporosis	Osteopenia

Appendix

#	Date of Clinic	Sample ID	DOB	Age	Sex F/M	Smoking Yes/No	Excess alcohol Yes/No	Regular Exercise Yes/No	Vegetarian Yes/No	Fractures History Yes/No	Number of Fractures	Position of Fracture	Date of last Fracture	Medication History	Ethnic origin	T-Score Lumbar Spine L1	T Score Lumbar Spine (L2-L4)	T Score Femoral Neck	T score Total HIP (TH)	BMD [g/cm2]	Medical Diagnosis	Clinical History
56	30/11/2015	151130OP01	01/01/1934	81	M	No	No	Yes	No	No				No	Eu	0.5	-2.1	-3.1		0.667	Osteoporosis.	Fracture
57	30/11/2015	151130OP02	06/03/1938	77	F	No	No	No	No	Yes				Yes	Eu	-0.1	0	-1.8		0.763	Osteopenia	Fragility fracture
58	30/11/2015	151130OP03	06/07/1947	68	F	No	No	Yes	No	Yes				No	Eu	0.2	0.2	-1.6		0.794	Osteopenia	Fragility fracture
59	01/12/2015	151201OP01	31/03/1954	61	F	No	No	Yes	No	No				VitD+B12	Eu	-0.9	-0.8	-1.5		0.801	Osteopenia	
60	01/12/2015	151201OP02	03/01/1939	76	F	No	No	No	No	No				No	Eu	-0.3	-0.4	-0.5		0.914	Normal	Regular steroids
61	01/12/2015	151201OP03	20/09/1934	81	F	No	No	No	No	No				BP+cholesterol	Eu	-2.4	-1.2	-3.6		0.547	Osteoporosis	Insufficiency fracture
62	01/12/2015	151201OP04	07/06/1954	61	F	No	No	Yes	No	No				Ca+VitD	Eu	-0.3	0.1	-1.1		0.852	Osteopenia/Normal	Family history of osteoporosis
63	03/12/2015	151203OP01	20/08/1959	56	F	No	No	No	No	No				No	Eu	-1.5	-2.3	-2.1		0.726	Osteopenia/Osteoporosis	Family history of osteoporosis
64	03/12/2015	151203OP02	21/11/1944	71	F	No	No	Yes	No	Yes				ALendronic acid+VitD	Eu	-1.3	-0.1	-1.3		0.819	Osteopenia	Osteopenia
65	03/12/2015	151203OP03	12/06/1951	64	F	Yes	No	Yes	No	Yes				Yes	Eu	0.9	2.4	-0.1		0.964	Normal	Fragility fracture
66	14/12/2015	151214OP01	09/09/1957	58	F	No	No	No	No	No				Yes	Eu	-3.6	-2.9	-2.2		0.721	Osteoporosis	Osteoporosis
67	14/12/2015	151214OP02	22/01/1958	57	F	No	No	Yes	No	No				Yes,+VitD-Ca	Afr	-1	-1.5	0.8		1.081	Osteopenia	Family history of osteoporosis
68	15/12/2015	151215OP01	20/09/1949	66	M	Yes	No	No	No	Yes				No	Eu	-0.7	-3.1	-1.3		0.905	Osteoporosis	

Appendix

#	Date of Clinic	Sample ID	DOB	Age	Sex F/M	Smoking Yes/No	Excess alcohol Yes/No	Regular Exercise Yes/No	Vegetarian Yes/No	Fractures History Yes/No	Number of Fractures	Position of Fracture	Date of last Fracture	Medication History	Ethnic origin	T-Score Lumbar Spine L1	T Score Lumbar Spine (L2-L4)	T Score Femoral Neck	T score Total HIP (TH)	BMD [g/cm2]	Medical Diagnosis	Clinical History
69	15/12/2015	151215 OP02	15/02/1956	59	F	No	No	No	No	Yes				BP	Eu	-1.6	-1.7	0.1		0.997	Osteopenia	Fragility fracture
70	15/12/2015	151215 OP03	09/11/1948	67	F	No	No	No	No	No				No	Eu	-1.6	-0.8	-1.1		0.851	Osteopenia	
71	16/12/2015	151216 OP01	19/02/1940	75	F	No	No	Yes	No	Yes				Ca	Eu	-4.3	-3.7	-3		0.617	Osteoporosis	op post Zoledronate
72	16/12/2015	151216 OP02	06/12/1941	74	F	Yes	No	No	No	No				Yes	Eu	-2.5	-1.5	-1.5		0.803	Osteopenia	Rheumatoid arthritis
73	16/12/2015	151216 OP03	02/01/1951	64	F	Yes	No	No	No	No				Ca	Eu	-0.5	1.5	-1.4		0.811	Osteopenia	coelaic disease
74	16/12/2015	151216 OP04	22/04/1951	64	F	No	No	No	No	No				Ca+VitD	Eu	-0.9	-0.6	-1.4		0.816	Osteopenia	Osteopenia
75	21/01/2016	160121 OP01	02/05/1961	55	F	No	No	Yes	Yes	No					Eu	-2.8	-2.3	-1.6		0.789	Osteopenia	Family history of Osteoporosis
76	27/01/2016	160127 OP01	21/05/1994	22	F	No	No	Yes	No	No				VitD	Eu	1	1.6	2.1		1.231	Normal	Oestoporosis/Crohn's disease
77	27/01/2016	160127 OP02	09/04/1955	61	F	No	No	No	No	No				YES	EU	0.6	0	-1.9		0.751	Osteopenia /Normal	Coelaic disease
78	27/01/2016	160127 OP03	01/10/1944	72	F	No	No	Yes	No	No				YES	EU	-1	-1.7	-0.3		0.942	Osteopenia /Normal	Rheumatoid arthritis
79	28/01/2016	160128 OP01	24/05/1935	81	F	No	No	No	No	Yes				VitD	EU	-1.8	-0.1	-1.1		0.846	Osteopenia /Normal	
80	28/01/2016	160128 OP02	03/05/1971	45	F	No	No	No	No	No				Ca	Eu	1.8	1.5	0.4		1.028	Normal	Early menopause
81	28/01/2016	160128 OP03	29/12/1943	73	F	No	No	No	No	Yes				YES	EU	-2.6	-1.9	-1.2		0.842	Osteopenia	Osteoporosis
82	29/01/2016	160129 OP01	30/12/1936	80	M	No	No	No	No	Yes				No	EU	1.5	1.8	-2.4			Osteopenia /with Fractures	Osteoporosis

Appendix

#	Date of Clinic	Sample ID	DOB	Age	Sex F/M	Smoking Yes/No	Excess alcohol Yes/No	Regular Exercise Yes/No	Vegetarian Yes/No	Fractures History Yes/No	Number of Fractures	Position of Fracture	Date of last Fracture	Medication History	Ethnic origin	T-Score Lumbar Spine L1	T Score Lumbar Spine (L2-L4)	T Score Femoral Neck	T score Total HIP (TH)	BMD [g/cm2]	Medical Diagnosis	Clinical History
83	29/01/2016	160129 OP02	28/06/1952	64	F	No	No	No	No	No				VitD+Ca	EU	-4.4	-3.7	-2.6		0.665	Osteoporosis	OP
84	29/01/2016	160129 OP03	07/02/1946	70	F	No	No	Yes	No	No				VitD	Eu	-1.5	-1.4	-2.5		0.684	Osteoporosis/Osteopenia	
85	29/01/2016	160129 OP04	09/07/1945	71	F	No	No	Yes	Yes	No				YES	EU	-1.1	-1.6	-1.9		0.752	Osteopenia	Osteopenia
86	02/02/2016	160202 OP01	24/08/1946	70	F	No	No	Yes	No	Yes				YES	EU	-3.8	-2.9	-1			Osteoporosis	OP
87	02/02/2016	160202 OP02	04/04/1954	62	F	No	No	Yes	No	Yes				No	EU	-1.7	-1.6	-0.2		0.957	Osteopenia /Normal	Osteopenia
88	02/02/2016	160202 OP03	17/09/1960	56	F	No	No	Yes	No	Yes				No	EU	-1.3	-1	-1.4		0.817	Osteopenia /Normal	Early Menopause
89	02/02/2016	160202 OP04	06/11/1930	86	F	No	No	Yes	No	Yes				VITD	EU	-2.2	-1.3	-2		0.738	Osteopenia	Osteoporosis
90	03/02/2016	160203 OP01	10/06/1944	72	F	No	No	Yes	No	No				VITD	EU	-1.1	-2	0		0.978	Osteopenia	Osteoporosis
91	03/02/2016	160203 OP02	26/04/1950	66	F	Yes	No	Yes	No	Yes				VITD	EU	-1.8	-2.3	-1.6		0.79	Osteopenia	Osteopenia
92	03/02/2016	160203 OP03	08/06/1941	75	F	Yes	No	No	No	Yes				CA	EU	-2.3	-2.3	-2.1		0.728	Osteopenia	Osteopenia
93	03/02/2016	160203 OP04	30/06/1945	71	F	No	No	No	No	Yes				YES	EU	-2.4	-1.9	-1.3		0.82	Osteopenia	taking alendronate
94	03/02/2016	160203 OP05	05/11/1942	74	F	Yes	No	No	No	No				YES	EU	-3.4	-3.7	-1.8		0.769	Osteoporosis	Osteopenia
95	08/02/2016	160208 OP01	02/02/1956	60	M	Yes	No	Yes	No	No				No	EU	-3.2	-2.1	-1.6		0.864	Osteopenia /osteoporosis	Vertebral wedging
96	08/02/2016	160208 OP02	11/05/1959	57	F	Yes	No	Yes	No	Yes				Y	EU	-2.5	-1.1	1.1		1.112	Osteopenia	Fragility fracture

Appendix

#	Date of Clinic	Sample ID	DOB	Age	Sex F/M	Smoking Yes/No	Excess alcohol Yes/No	Regular Exercise Yes/No	Vegetarian Yes/No	Fractures History Yes/No	Number of Fractures	Position of Fracture	Date of last Fracture	Medication History	Ethnic origin	T-Score Lumbar Spine L1	T Score Lumbar Spine (L2-L4)	T Score Femoral Neck	T score Total HIP (TH)	BMD [g/cm2]	Medical Diagnosis	Clinical History
97	08/02/2016	160208 OP03	05/11/1939	77	F	No	Yes	Yes	No	No				Y	EU	-1	-0.4	-1		0.856	Normal	Osteopenia
98	09/02/2016	160209 OP01	15/05/1954	62	M	No	No	Yes	No	No				Y	EU	-1.6	-2.5	-2.6		0.726	Osteoporosis	long term steroids
99	09/02/2016	160209 OP02	18/08/1943	73	F	No	No	No	No	Yes				Y	EU	-3	-2.7	-2.4		0.695	Osteoporosis	
100	09/02/2016	160209 OP03	14/03/1959	57	F	No	No	Yes	No	No				Y	EU	-1.3	-1.3	0.8		1.081	Osteopenia	osteopenia

8.5 General Equipment

No.	Item	Cat#	Source
1.	ESCO Class II BS Cabinet AC2-4G1SP Biological safety cabinet class II.	CAB7019	ESCO
2.	Thermo Heraeus Megafuge 16R Centrifuge (Refrigerated)	75004270	Thermo Fisher, UK
3.	Thermo TX-400 Swinging Bucket Rotor, Capacity (4 x 400mL)	75003181	Thermo Fisher, UK
4.	Thermo Round Buckets for TX-400 Rotor (4)	75003655	Thermo Fisher, UK
5.	Thermo Scientific Heraeus Fresco Centrifuge 17R (Refrigerated)	75002420	Thermo Fisher Ltd, UK
6.	Microcentrifuge SCF2	CFH-500-020B	Fisher Scientific UK
7.	Genies2 Vortex mixer	MIX5000	Scientific Laboratory Supplies Ltd
8.	BIO-RAD T100 Thermocycler.	186-1096	Bio-Rad Laboratories Ltd., UK
9.	Roche Lightcycler® 96 Real-Time PCR System.	05815 916 001	Roche Diagnostics Ltd, UK
10.	Roche LightCycler®480 Multi-well Plate 96, white 5 x 10 plates (with sealing foils).	04729692001	Roche Diagnostics Ltd, UK
11.	Thermo Scientific NanoDrop™ 2000 spectrophotometer	ND-2000C	Thermo Fisher Ltd, UK

8.6 Circulating serum and plasma miRNA qPCR 2^-ΔCt data Summary Table

Table 8-3: Circulating serum and plasma miRNA qPCR $2^{\wedge -\Delta Ct}$ data Summary Table

Average $2^{-\Delta Ct}$ (Log10) of 6 circulating serum/plasma miRNAs: hsa-miR-100-5p, hsa-miR-122-5p, hsa-miR-215-5p, hsa-miR-373-5p, hsa-miR-4516 and hsa-miR-99a-5p in Non-Osteoporosis, Osteopenia (with/without fracture) and Osteoporosis (with/without fracture)

Circulating miRNA	**Non-Osteoporotic $2^{-\Delta Ct}$ Mean ± SD (N)**	**Osteopenia $2^{-\Delta Ct}$ Mean ± SD (N)**	**Osteopenia with Fracture $2^{-\Delta Ct}$ Mean ± SD (N)**	**Osteoporosis $2^{-\Delta Ct}$ Mean ± SD (N)**	**Osteoporosis with Fracture $2^{-\Delta Ct}$ Mean ± SD (N)**
hsa-miR-100-5p Serum	3 ± 3.1 (30)	2.2 ± 2.8 (63)	1.5 ± 1 (15)	1.7 ± 1.8 (34)	2.5 ± 3 (19)
hsa-miR-100-5p Plasma	2.7 ± 1.5 (15)	4.9 ± 7.3 (63)	2.2 ± 1.3 (15)	4.5 ± 6.2 (34)	3.2 ± 2.2 (15)
hsa-miR-122-5p Serum	111.7 ± 122.7 (30)	101.5 ± 198 (63)	97.4 ± 96.3 (15)	52.8 ± 52.3 (34)	67.4 ± 146.1 (19)
hsa-miR-122-5p Plasma	128.5 ± 85.6 (15)	131.4 ± 175.7 (63)	101.5 ± 100.6 (15)	130.5 ± 259.1 (34)	57.2 ± 60.6 (15)
hsa-miR-215-5p Serum	0.9 ± 0.8 (29)	0.6 ± 0.5 (61)	0.4 ± 0.3 (13)	0.5 ± 0.5 (30)	0.6 ± 1 (12)
hsa-miR-215-5p Plasma	0.5 ± 0.3 (15)	0.7 ± 0.9 (61)	0.6 ± 0.5 (13)	0.8 ± 0.9 (30)	0.8 ± 0.4 (9)
hsa-miR-373-5p Serum	2.3 ± 2.5 (29)	2.7 ± 2.6 (61)	2 ± 1.7 (15)	2.4 ± 2.7 (33)	1.8 ± 4.6 (19)
hsa-miR-373-5p Plasma	0.1 ± 0.1 (12)	0.1 ± 0.1 (61)	0.1 ± 0 (15)	0.1 ± 0.1 (33)	0 ± 0 (15)
hsa-miR-4516 Serum	101 ± 131.3 (30)	108.1 ± 122.1 (63)	70.4 ± 59.1 (15)	104.7 ± 89.4 (34)	84.8 ± 89.8 (19)
hsa-miR-4516 Plasma	57.6 ± 93.3 (15)	38.4 ± 43.3 (63)	15.6 ± 12.1 (15)	31.2 ± 43.7 (34)	14 ± 19.4 (15)
hsa-miR-99a-5p Serum	9.1 ± 8.5 (29)	4 ± 6.3 (61)	3.8 ± 2.4 (13)	3.4 ± 3.4 (30)	16.8 ± 22.1 (12)

Circulating miRNA	Non-Osteoporotic $2^{-\Delta Ct}$ Mean ± SD (N)	Osteopenia $2^{-\Delta Ct}$ Mean ± SD (N)	Osteopenia with Fracture $2^{-\Delta Ct}$ Mean ± SD (N)	Osteoporosis $2^{-\Delta Ct}$ Mean ± SD (N)	Osteoporosis with Fracture $2^{-\Delta Ct}$ Mean ± SD (N)
hsa-miR-99a-5p Plasma	3.4 ± 4.4 (15)	6 ± 7.6 (61)	7.4 ± 6.4 (13)	8.9 ± 10.2 (30)	8.6 ± 9.2 (15)

Total number of tested samples for: hsa-miR-100-5p (serum n= 161, plasma n= 142), hsa-miR-122-5p (Serum n= 161, plasma n=142), hsa-miR-215-5p (serum n= 145, plasma n= 128), hsa-miR-373-5p (serum n= 157, plasma n= 136), hsa-miR-4516 (serum n= 161, plasma n= 142) and hsa-miR-99a-5p (serum n= 145, plasma n= 134).

8.7 Geneontology

Table 8-4: Geneontology

Geneontology (GO) Evidence Codes; a) **Experimental Evidence codes; IDA**: Inferred from Direct Assay, **IMP**: Inferred from Mutant Phenotype, **IGI**: Inferred from Genetic Interaction, **IEP**: Inferred from Expression Pattern. b) **Computational Analysis evidence codes; ISS**: Inferred from Sequence or structural Similarity, **IBA**: Inferred from Biological aspect of Ancestor. **c) Author Statement evidence codes; TAS**: Traceable Author Statement, **NAS**: Non-traceable Author Statement. d) **Curatorial Statement codes**; **IC**: Inferred by Curator, Automatically-Assigned evidence code; IEA: Inferred from Electronic Annotation**.** e) **Automatically-assigned Evidence Codes; IEA**: Inferred from Electronic Annotation. **Reactome:R-HAS-** is open-source, curated and peer reviewed pathway database (Fabregat et al., 2016). **GO_REF:0000002**= Gene Ontology annotation through association of InterPro records with GO terms. **GO_REF:0000107**= Automatic transfer of experimentally verified manual GO annotation data to orthologs using Ensembl., **GO_REF:0000024**= Manual transfer of experimentally-verified manual GO annotation data to orthologs by curator judgment of sequence similarity. **GO_REF:0000033**= Annotation inferences using phylogenetic trees. **GO_REF:0000037**= Gene Ontology annotation based on manual assignment of UniProtKB keywords in UniProtKB/Swiss-Prot entries.

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
ABCB1	P08183	Multidrug resistance protein 1	ceramide translocation (GO:0099040)	PTHR24221	[IDA; (van Helvoort et al., 1996)]
			ceramide-translocating ATPase activity (GO:0099038)		[IDA; (van Helvoort et al., 1996)]
			G2/M transition of mitotic cell cycle (GO:0000086)		[IDA; (Yamamoto et al., 2009)]
			phosphatidylcholine-translocating ATPase activity (GO:0090554)		[IDA; (van Helvoort et al., 1996)]
			phosphatidylethanolamine-translocating ATPase activity (GO:0090555)		[IDA; (van Helvoort et al., 1996)]
			phospholipid translocation (GO:0045332)		[IDA; (van Helvoort et al., 1996)]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
ALPL	P05186	Alkaline phosphatase, tissue-nonspecific isozyme	cementum mineralization (GO:0071529)	PTHR11596	[IEA; GO REF:0000107]
			endochondral ossification (GO:0001958)		[IEA; GO REF:0000107]
			osteoblast differentiation (GO:0001649)		[IDA; (Foster et al., 2005)]
			response to glucocorticoid (GO:0051384)		[IEA; GO REF:0000107]
			response to vitamin D (GO:0033280)		[IEP; (van Driel et al., 2006)]
			skeletal system development (GO:0001501)		[TAS; (Mornet et al., 1998)]
ANKH	Q9HCJ1	Progressive ankylosis protein homolog	regulation of bone mineralization (GO:0030500)	PTHR28384:SF2	[ISS; GO REF:0000024], [TAS; (Nurnberg et al., 2001)]
			skeletal system development (GO:0001501)		[NAS; (Ho et al., 2000)]
AR	P10275	Androgen receptor	activation of prostate induction by androgen receptor signalling pathway (GO:0060520)	PTHR24084	[IEA; GO REF:0000107]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
			androgen receptor activity (GO:0004882)		[IDA; (Amir et al., 2003, Thomas et al., 2010, Son et al., 2010, Hsu et al., 2014)], [IMP; (Xu et al., 2009)], [NAS; (Fujimoto et al., 1999), [TAS; (Lin et al., 2004)]
			androgen receptor signalling pathway (GO:0030521)		[IDA; (Thomas et al., 2010, Son et al., 2010)]
			cellular response to steroid hormone stimulus (GO:0071383)		[IMP; (Li et al., 2003)]
			epithelial cell differentiation involved in prostate gland development (GO:0060742)		[IEA; GO REF:0000107]
			epithelial cell morphogenesis (GO:0003382)		[IEA; GO REF:0000107]
			lateral sprouting involved in mammary gland duct morphogenesis (GO:0060599)		[IEA; GO REF:0000107]
			morphogenesis of an epithelial fold (GO:0060571)		[IEA; GO REF:0000107]
			positive regulation of cell differentiation (GO:0045597)		[IMP; (Chauhan et al., 2003)]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
			positive regulation of insulin-like growth factor receptor signalling pathway (GO:0043568)		[IEA; GO REF:0000107]
			positive regulation of intracellular estrogen receptor signalling pathway (GO:0033148)		[IEA; GO REF:0000107]
			positive regulation of MAPK cascade (GO:0043410)		[IEA; GO REF:0000107]
			positive regulation of NF-kappaB transcription factor activity (GO:0051092)		[IMP; (Lamb et al., 2011)]
			prostate gland epithelium morphogenesis (GO:0060740)		[IEA; GO REF:0000107]
			tertiary branching involved in mammary gland duct morphogenesis (GO:0060748)		[IEA; GO REF:0000107]
CD44	P16070	CD44 antigen	cartilage development (GO:0051216)	PTHR10225	[IEP; (Nicoll et al., 2002)]
			positive regulation of ERK1 and ERK2 cascade (GO:0070374)		[IDA; (Shi et al., 2006)]
CD47	Q08722	Leukocyte surface antigen CD47	positive regulation of cell proliferation (GO:0008284), positive regulation of cell-cell adhesion (GO:0022409), positive regulation of T cell activation (GO:0050870)	PTHR10613	[IDA; (Piccio et al., 2005)]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
CNR1	P21554	Cannabinoid receptor 1	positive regulation of neuron projection development (GO:0010976)	PTHR22750	[IEA; GO REF:0000107]
			regulation of insulin secretion (GO:0050796)		[IEA; GO REF:0000107]
CNR2	P34972	Cannabinoid receptor 2	negative regulation of nitric-oxide synthase activity (GO:0051001)	PTHR22750	[IEA; GO REF:0000107]
COL1A1	P02452	Collagen alpha-1(I) chain	bone trabecula formation (GO:0060346)	PTHR24023	[IEA; GO REF:0000107]
			cartilage development involved in endochondral bone morphogenesis (GO:0060351)		[IEA; GO REF:0000107]
			cellular response to tumour necrosis factor (GO:0071356)		[IEA; GO REF:0000107]
			cellular response to vitamin E (GO:0071306)		[IEA; GO REF:0000107]
			embryonic skeletal system development (GO:0048706)		[IMP; (Kamoun-Goldrat et al., 2008)]
			endochondral ossification (GO:0001958)		[IEA; GO REF:0000107]
			intramembranous ossification (GO:0001957)		[IEA; GO REF:0000107]
			osteoblast differentiation (GO:0001649)		[IEA; GO REF:0000107]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
			positive regulation of canonical Wnt signalling pathway (GO:0090263)		[IDA; (Medici and Nawshad, 2010)]
			positive regulation of epithelial to mesenchymal transition (GO:0010718)		[IDA; (Medici and Nawshad, 2010)]
			protein transport (GO:0015031)		[IEA; GO REF:0000107]
			response to corticosteroid (GO:0031960)		[IEA; GO REF:0000107]
			skeletal system development (GO:0001501)		[IMP; (Chamberlain et al., 2004, Tsuneyoshi et al., 1991, Cohn et al., 1993)]
			tooth mineralization (GO:0034505)		[IMP; (De Coster et al., 2007)]
CYP17A1	P05093	Steroid 17-alpha-hydroxylase/17,20 lyase	androgen biosynthetic process (GO:0006702)	PTHR24289:SF5	[TAS; Reactome: R-HSA-193048]
			progesterone metabolic process (GO:0042448)		[IDA; (DeVore and Scott, 2012)]
CYP19A1	P11511	Aromatase	androgen catabolic process (GO:0006710)	PTHR24294	[IDA; (Leitner et al., 2015)]
			estrogen biosynthetic process (GO:0006703)		[TAS; Reactome: R-HSA-193144]
CYP1B1	Q16678	Cytochrome P450 1B1	estrogen metabolic process (GO:0008210)	PTHR24299:SF16	[IDA; (Nishida et al., 2013)]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
			negative regulation of NF-kappaB transcription factor activity (GO:0032088)		[ISS; GO REF:0000024]
			retinol metabolic process (GO:0042572)		[IDA; (Choudhary et al., 2004)]
			trabecular meshwork development (GO:0002930)		[ISS; GO REF:0000024]
CYP3A4	P08684	Cytochrome P450 3A4	androgen metabolic process (GO:0008209)	PTHR24292:SF73	[TAS; (Fisher et al., 2009)]
ESR1	P03372	Estrogen receptor	androgen metabolic process (GO:0008209)	PTHR24084	[IEA; GO REF:0000107]
			cellular response to estrogen stimulus (GO:0071391)		[IEA; GO REF:0000107]
			epithelial cell development (GO:0002064)		[IEA; GO REF:0000107]
			epithelial cell proliferation involved in mammary gland duct elongation (GO:0060750)		[IEA; GO REF:0000107]
			estrogen receptor activity (GO:0030284)		[IDA; (Sabbah et al., 1998), [NAS; (Greene et al., 1986), [TAS; (Anandappa et al., 2000)]
			intracellular estrogen receptor signalling pathway (GO:0030520)		[IDA; (Sabbah et al., 1998), [NAS; (Greene et al., 1986)]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
			intracellular steroid hormone receptor signalling pathway (GO:0030518)		[ISS; GO REF:0000024]
			mammary gland branching involved in pregnancy (GO:0060745)		[IEA; GO REF:0000107]
			negative regulation of I-kappaB kinase/NF-kappaB signalling (GO:0043124)		[IDA; (Liu et al., 2005, Stein and Yang, 1995)]
			prostate epithelial cord arborization involved in prostate glandular acinus morphogenesis (GO:0060527)		[IEA; GO REF:0000107]
			prostate epithelial cord elongation (GO:0060523)		[IEA; GO REF:0000107]
			regulation of branching involved in prostate gland morphogenesis (GO:0060687)		[IEA; GO REF:0000107]
			response to estrogen (GO:0043627)		[IDA; (Yahata et al., 2001)]
			RNA polymerase II transcription factor activity, estrogen-activated sequence-specific DNA binding (GO:0038052)		[IDA; (Laganiere et al., 2005), [IGI; (Kim et al., 2008)]
			steroid hormone receptor activity (GO:0003707)		[TAS; (Kahlert et al., 2000)]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
FSHB	P01225	Follitropin subunit beta	positive regulation of bone resorption (GO:0045780)	PTHR11515	[IEA; GO REF:0000107]
			progesterone biosynthetic process (GO:0006701)		[TAS; (Liu et al., 2003)]
			regulation of osteoclast differentiation (GO:0045670)		[IEA; GO REF:0000107]
GH1	P01241	Somatotropin	bone maturation (GO:0070977)	PTHR11417	[IDA; (van Gool et al., 2010)]
			positive regulation of insulin-like growth factor receptor signalling pathway (GO:0043568)		[IDA; (Goddard et al., 1995)]
			positive regulation of MAP kinase activity (GO:0043406)		[TAS; (VanderKuur et al., 1994)]
IGF1	P05019	Insulin-like growth factor I	activation of MAPK activity (GO:0000187)	PTHR11454	[IMP; (Fujita et al., 2012)]
			bone mineralization involved in bone maturation (GO:0035630)		[IDA; (Koch et al., 2005)]
			ERK1 and ERK2 cascade (GO:0070371)		[IMP; (Jeong et al., 2014)]
			negative regulation of oocyte development (GO:0060283)		[IMP; (Velazquez et al., 2011)]
			positive regulation of cell growth involved in cardiac muscle cell development (GO:0061051)		[IDA; (Song et al., 2014)]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
			positive regulation of insulin-like growth factor receptor signalling pathway (GO:0043568)		[IDA; (Imai et al., 2000, Freund et al., 1993)]
			positive regulation of MAPK cascade (GO:0043410)		[IDA; (Munoz et al., 2009)]
			positive regulation of osteoblast differentiation (GO:0045669)		[IDA; (Koch et al., 2005)]
			positive regulation of protein import into nucleus, translocation (GO:0033160)		[IDA; (Munoz et al., 2009)]
			positive regulation of protein secretion (GO:0050714)		[IMP; (Sunic et al., 1998)]
			positive regulation of smooth muscle cell proliferation (GO:0048661)		[IDA; (Imai et al., 2000, Jia et al., 2006)]
			skeletal muscle satellite cell maintenance involved in skeletal muscle regeneration (GO:0014834)		[IDA; (Ates et al., 2007)]
			skeletal system development (GO:0001501)		[TAS; (Semsarian et al., 1999)]
IGF1R	P08069	Insulin-like growth factor 1 receptor	inactivation of MAPKK activity (GO:0051389)	PTHR24416	[IDA; (Galvan et al., 2003)]
			regulation of JNK cascade (GO:0046328)		[IDA; (Galvan et al., 2003)]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
IL1B	P01584	Interleukin-1 beta	activation of MAPK activity (GO:0000187)	PTHR10078	[IDA; (Madge and Pober, 2000)]
			MAPK cascade (GO:0000165)		[IMP; (Wu et al., 2011)]
			negative regulation of adiponectin secretion (GO:0070164)		[ISS; (Lagathu et al., 2006)]
			negative regulation of MAP kinase activity (GO:0043407)		[ISS; (Lagathu et al., 2006)]
			positive regulation of interleukin-6 secretion (GO:2000778)		[IEA; GO REF:0000107]
			positive regulation of JNK cascade (GO:0046330)		[IBA; GO REF:0000033]
			positive regulation of NF-kappaB import into nucleus (GO:0042346)		[IDA; (Al-Sadi et al., 2008)]
			positive regulation of NF-kappaB transcription factor activity (GO:0051092)		[IDA; (Wesche et al., 1999, Jung et al., 2003)]
			positive regulation of protein export from nucleus (GO:0046827)		[NAS; (Luo et al., 2009)]
			regulation of establishment of endothelial barrier (GO:1903140)		[IDA; (Clark et al., 2015)]
			regulation of I-kappaB kinase/NF-kappaB signalling (GO:0043122)		[IDA; (Madge and Pober, 2000)]
			regulation of insulin secretion (GO:0050796)		[IDA; (Corbett et al., 1993)]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
INSL3	P51460	Insulin-like 3	positive regulation of epithelial cell migration (GO:0010634)	PTHR10423	[IDA; (Hampel et al., 2013)]
			positive regulation of wound healing (GO:0090303)		[IDA; (Hampel et al., 2013)]
IRS2	Q9Y4H2	Insulin receptor substrate 2	MAPK cascade (GO:0000165)	PTHR10614	[TAS; Reactome: R-HSA-5673001]
			positive regulation of insulin secretion (GO:0032024)		[ISS; GO REF:0000024]
KIT	P10721	Mast/stem cell growth factor receptor Kit	actin cytoskeleton reorganization (GO:0031532)	PTHR24416	[IDA; (Blume-Jensen et al., 1991)]
			activation of MAPK activity (GO:0000187)		[IDA; (Kim et al., 2011)]
			MAPK cascade (GO:0000165)		[TAS; Reactome: R-HSA-5673001]
			megakaryocyte development (GO:0035855)		[ISS; GO REF:0000024]
			positive regulation of long-term neuronal synaptic plasticity (GO:0048170)		[IEA; GO REF:0000107]
			positive regulation of MAPK cascade (GO:0043410)		[IMP; (Kim et al., 2011)]
			positive regulation of vascular smooth muscle cell differentiation (GO:1905065)		[IDA; (Davis et al., 2009)]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
LRP6	O75581	Low-density lipoprotein receptor-related protein 6	axis elongation involved in somitogenesis (GO:0090245)	PTHR10529	[IBA; GO REF:0000033]
			beta-catenin destruction complex disassembly (GO:1904886)		[TAS; Reactome: R-HSA-4641262]
			canonical Wnt signalling pathway (GO:0060070)		[IDA; (Holmen et al., 2002, Zilberberg et al., 2004, Nam et al., 2006, George et al., 2007, Davidson et al., 2009, Gnad et al., 2010)], [IMP; (Li et al., 2008)], [TAS; (Berwick and Harvey, 2012)]
			canonical Wnt signalling pathway involved in neural crest cell differentiation (GO:0044335)		[IC; (Tamai et al., 2000)]
			canonical Wnt signalling pathway involved in regulation of cell proliferation (GO:0044340)		[IC; (Wang et al., 2004)]
			convergent extension (GO:0060026)		[IBA; GO REF:0000033]
			coreceptor activity involved in canonical Wnt signalling pathway (GO:1904928)		[NAS; (Arenas, 2014)]
			coreceptor activity involved in Wnt signalling pathway (GO:0071936)		[IDA; (Tamai et al., 2000)]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
			midbrain-hindbrain boundary development (GO:0030917)		[IBA; GO REF:0000033]
			negative regulation of canonical Wnt signalling pathway (GO:0090090)		[TAS; Reactome: R-HSA-3772470]
			neural crest cell differentiation (GO:0014033), neural crest formation (GO:0014029)		[IDA; (Tamai et al., 2000)]
			neural tube closure (GO:0001843)		[IBA; GO REF:0000033]
			pericardium morphogenesis (GO:0003344)		[IBA; GO REF:0000033]
			positive regulation of canonical Wnt signalling pathway (GO:0090263)		[IDA; (Semenov et al., 2001, Li et al., 2002, Wang et al., 2004, Piao et al., 2008, Jeong et al., 2010)]
			positive regulation of Wnt signalling pathway involved in dorsal/ventral axis specification (GO:2000055)		[IDA; (Semenov et al., 2005)]
			regulation of canonical Wnt signalling pathway (GO:0060828)		[IGI; (Caruso et al., 2006)]
			trachea cartilage morphogenesis (GO:0060535)		[IBA; GO REF:0000033]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
			Wnt signalling pathway (GO:0016055)		[IDA; (Mao et al., 2001, Li et al., 2005), [IMP; (Tamai et al., 2000, Cruciat et al., 2010)]
			Wnt signalling pathway involved in dorsal/ventral axis specification (GO:0044332)		[IDA; (Tamai et al., 2000)]
			Wnt signalling pathway involved in midbrain dopaminergic neuron differentiation (GO:1904953)		[TAS; (Arenas, 2014)]
			Wnt signalling pathway involved in somitogenesis (GO:0090244)		[IBA; GO REF:0000033]
			Wnt-activated receptor activity (GO:0042813)		[IBA; GO REF:0000033]
MAPK1	P28482	Mitogen-activated protein kinase 1	activation of MAPK activity (GO:0000187)	PTHR24055	[TAS; Reactome: R-HSA-112409]
			cardiac neural crest cell development involved in heart development (GO:0061308)		[IEA; GO REF:0000107]
			ERK1 and ERK2 cascade (GO:0070371)		[IDA; (Pandey et al., 2005)]
			mammary gland epithelial cell proliferation (GO:0033598)		[IEA; GO REF:0000107]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
			MAP kinase activity (GO:0004707)		[IBA; GO REF:0000033], [TAS; Reactome: R-HSA-5654560, -5654562, -5654565, -5654566], [TAS; Reactome: R-HSA-5654560, -5654562, -5654565, -5654566]
			MAPK cascade (GO:0000165)		[TAS; Reactome: R-HSA-5673001]
			MAPK import into nucleus (GO:0000189)		[IEA; GO REF:0000107]
			negative regulation of cell differentiation (GO:0045596)		[IEA; GO REF:0000107]
			regulation of cytoskeleton organization (GO:0051493)		[TAS; (Yao and Seger, 2009)]
			regulation of ossification (GO:0030278)		[IEA; GO REF:0000107]
			regulation of stress-activated MAPK cascade (GO:0032872)		[TAS; (Yao and Seger, 2009)]
			response to estrogen (GO:0043627)		[IEA; GO REF:0000107]
			stress-activated MAPK cascade (GO:0051403)		[IDA; (Ko et al., 2001)]
MAPK3	P27361	Mitogen-activated protein kinase 3	activation of MAPK activity (GO:0000187)	PTHR24055	[TAS; Reactome: R-HSA-112409]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
			cardiac neural crest cell development involved in heart development (GO:0061308)		[IEA; GO REF:0000107]
			cartilage development (GO:0051216)		[IEA; GO REF:0000107]
			ERK1 and ERK2 cascade (GO:0070371)		[IEA; GO REF:0000107]
			MAP kinase activity (GO:0004707)		[IDA; (Zheng and Guan, 1993)], [NAS; (Charest et al., 1993)], [TAS; Reactome: R-HSA-5654560, -5654562, -5654565, -5654566, -73722]
			MAPK cascade (GO:0000165)		[NAS; (Wang et al., 2010)], [TAS; Reactome: R-HSA-5673001]
			MAPK import into nucleus (GO:0000189)		[IEA; GO REF:0000107]
			positive regulation of cytokine secretion involved in immune response (GO:0002741)		[IEA; GO REF:0000107]
			positive regulation of ERK1 and ERK2 cascade (GO:0070374)		[IMP; (Yang et al., 2009)]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
			regulation of cytoskeleton organization (GO:0051493)		[TAS; (Yao and Seger, 2009)]
			regulation of ossification (GO:0030278)		[IEA; GO REF:0000107]
			regulation of stress-activated MAPK cascade (GO:0032872)		[TAS; (Yao and Seger, 2009)]
			stress-activated MAPK cascade (GO:0051403)		[IDA; (Ko et al., 2001)]
MTHFR	P42898	Methylenetetrahydrofolate reductase	response to folic acid (GO:0051593)	PTHR21091:SF136	[IEA; GO REF:0000107]
			response to vitamin B2 (GO:0033274)		[IEA; GO REF:0000107]
PON1	P27169	Serum paraoxonase/arylesterase 1	aromatic compound catabolic process (GO:0019439)	PTHR11799	[IDA; (Draganov et al., 2005)]
			aryldialkylphosphatase activity (GO:0004063)		[IDA; (Harel et al., 2004), 7638166]
			carboxylic acid catabolic process (GO:0046395)		[IDA; (Sorenson et al., 1995)]
			not negative regulation of plasma lipoprotein particle oxidation (GO:0034445)		[IDA; (Teiber et al., 2004)]
			organophosphate catabolic process (GO:0046434)		[IDA; (Sorenson et al., 1995)]
			phosphatidylcholine metabolic process (GO:0046470)		[IDA; (Rosenblat et al., 2005)]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
			positive regulation of binding (GO:0051099)		[IDA; (Rosenblat et al., 2005)]
			positive regulation of cholesterol efflux (GO:0010875)		[IDA; (Rosenblat et al., 2005)]
			positive regulation of transporter activity (GO:0032411)		[IDA; (Rosenblat et al., 2005)]
PTHLH	P12272	Parathyroid hormone-related protein	adenylate cyclase-activating G-protein coupled receptor signalling pathway (GO:0007189)	PTHR17223	[IDA; (Lanske et al., 1998)]
			negative regulation of chondrocyte differentiation (GO:0032331)		[IDA;(Amling et al., 1997)]
			osteoblast development (GO:0002076)		[IBA; GO REF:0000033]
			positive regulation of cAMP biosynthetic process (GO:0030819), regulation of gene expression (GO:0010468)		[IDA; (Amling et al., 1997)]
			skeletal system development (GO:0001501)		[IDA; (Amling et al., 1997)]
RUNX2	Q13950	Runt-related transcription factor 2	chondrocyte development (GO:0002063)	PTHR11950	[IEA; GO REF:0000107]
			chondrocyte differentiation (GO:0002062)		[IBA; GO REF:0000033]
			embryonic cranial skeleton morphogenesis (GO:0048701)		[IEA; GO REF:0000107]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
			endochondral ossification (GO:0001958)		[IEA; GO REF:0000107]
			ossification (GO:0001503)		[TAS; (Backstrom et al., 2002)]
			osteoblast development (GO:0002076)		[IEA; GO REF:0000107]
			osteoblast differentiation (GO:0001649)		[IEP; (Lambertini et al., 2010)], [TAS; (Backstrom et al., 2002)]
			osteoblast fate commitment (GO:0002051)		[IEA; GO REF:0000107]
			positive regulation of chondrocyte differentiation (GO:0032332)		[IEA; GO REF:0000107]
			positive regulation of osteoblast differentiation (GO:0045669)		[IEA; GO REF:0000107]
			regulation of cell differentiation (GO:0045595)		[IBA; GO REF:0000033]
SOST	Q9BQB4	Sclerostin	negative regulation of canonical Wnt signalling pathway (GO:0090090)	PTHR14903	[IDA; (Semenov et al., 2005)], [TAS; Reactome: R-HSA-3772470]
			negative regulation of ossification (GO:0030279)		[NAS; (Balemans et al., 2001, Leupin et al., 2007)]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
			negative regulation of Wnt signalling pathway involved in dorsal/ventral axis specification (GO:2000054)		[IDA; (Semenov et al., 2005)]
			ossification (GO:0001503)		[IEA; GO REF:0000107]
			Wnt signalling pathway (GO:0016055)		[IEA; GO REF:0000037]
SPARC	P09486	SPARC	bone development (GO:0060348)	PTHR13866	[IEA; GO REF:0000107]
			ossification (GO:0001503)		[IEA; GO REF:0000107]
			response to glucocorticoid (GO:0051384)		[IEA; GO REF:0000107]
			response to L-ascorbic acid (GO:0033591)		[IEA; GO REF:0000107]
STAT1	P42224	Signal transducer and activator of transcription 1-alpha/beta	metanephric mesenchymal cell differentiation (GO:0072162)	PTHR11801	[ISS; GO REF:0000024]
			metanephric mesenchymal cell proliferation involved in metanephros development (GO:0072136)		[ISS; GO REF:0000024]
			negative regulation of I-kappaB kinase/NF-kappaB signalling (GO:0043124)		[IMP; (Wang et al., 2000)]
			negative regulation of mesenchymal to epithelial transition involved in metanephros morphogenesis (GO:0003340)		[ISS; GO REF:0000024]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
			negative regulation of metanephric nephron tubule epithelial cell differentiation (GO:0072308)		[ISS; GO REF:0000024]
			positive regulation of smooth muscle cell proliferation (GO:0048661)		[ISS; GO REF:0000024]
			renal tubule development (GO:0061326)		[IMP; (Kim et al., 2010)]
			tumour necrosis factor-mediated signalling pathway (GO:0033209)		[IDA; (Wang et al., 2000)]
THBS1	P07996	Thrombospondin-1	activation of MAPK activity (GO:0000187)	PTHR10199	[IMP; (Staniszewska et al., 2007)]
			cellular response to tumour necrosis factor (GO:0071356)		[IEA; GO REF:0000107]
			positive regulation of smooth muscle cell proliferation (GO:0048661)		[IDA; (Hamakawa et al., 2014)]
			response to progesterone (GO:0032570)		[TAS; (Adams, 1997)]
TNFRSF1B	P20333	Tumour necrosis factor receptor superfamily member 1B	tumour necrosis factor-activated receptor activity (GO:0005031)	PTHR23097:SF161	[IBA; GO REF:0000033]
			tumour necrosis factor-mediated signalling pathway (GO:0033209)		[TAS; Reactome: R-HSA-5668541]
TRAF6	Q9Y4K3	TNF receptor-associated factor 6	activation of MAPK activity (GO:0000187)	PTHR10131	[TAS; Reactome: R-HSA-450302]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
			activation of NF-kappaB-inducing kinase activity (GO:0007250)		[IMP; (Sun et al., 2004)]
			bone resorption (GO:0045453)		[IEA; GO REF:0000107]
			JNK cascade (GO:0007254)		[TAS; Reactome: R-HSA-450321]
			neural tube closure (GO:0001843)		[IEA; GO REF:0000107]
			ossification (GO:0001503)		[IEA; GO REF:0000107]
			osteoclast differentiation (GO:0030316)		[IEA; GO REF:0000107]
			positive regulation of I-kappaB kinase/NF-kappaB signalling (GO:0043123)		[IDA; (Lamothe et al., 2007)], [TAS; Reactome: R-HSA-937039]
			positive regulation of JUN kinase activity (GO:0043507)		[IDA; (Shin et al., 2002)], [NAS; (Lamothe et al., 2007)]
			positive regulation of NF-kappaB transcription factor activity (GO:0051092)		[IDA; (Zapata et al., 2001) (Shin et al., 2002, Wang et al., 2006)], [IMP; (Lee et al., 2002)], [TAS; Reactome: R-HSA-445989]
			positive regulation of osteoclast differentiation (GO:0045672)		[IDA; (Lamothe et al., 2007)]

Gene	UniProtKB ID	Protein name	Biological Process (GO)	PANTHER family	Evidence with Reference
			positive regulation of smooth muscle cell proliferation (GO:0048661)		[IEA; GO REF:0000107]
			regulation of immunoglobulin secretion (GO:0051023)		[IEA; GO REF:0000107]
VDR	P11473	Vitamin D3 receptor	calcitriol receptor activity (GO:0008434)	PTHR24082	[IDA; (Makishima et al., 2002, Kemmis et al., 2006, Hawker et al., 2007)]
			decidualization (GO:0046697)		[IEP; (Vigano et al., 2006)]
			mammary gland branching involved in pregnancy (GO:0060745)		[IEA; GO REF:0000107]
			positive regulation of keratinocyte differentiation (GO:0045618)		[IMP; (Hawker et al., 2007)]
			skeletal system development (GO:0001501)		[IEA; GO REF:0000107]
			steroid hormone mediated signalling pathway (GO:0043401)		[IEA; GO REF:0000002]
			steroid hormone receptor activity (GO:0003707)		[IEA; GO REF:0000002]
			vitamin D receptor signalling pathway (GO:0070561)		[IDA; (Kemmis et al., 2006)]
VPS13B	Q7Z7G8	Vacuolar protein sorting-associated protein 13B	protein transport (GO:0015031)	PTHR12517	[IEA; GO REF:0000037]